Characterization Of Protein Prenyltransferases And Protein Prenylation In Plasmodium Falciparum

2004

Thiago Gaspar DaSilva

University of Central Florida

Find similar works at: https://stars.library.ucf.edu/etd

University of Central Florida Libraries http://library.ucf.edu

Part of the Molecular Biology Commons

STARS Citation

DaSilva, Thiago Gaspar, "Characterization Of Protein Prenyltransferases And Protein Prenylation In Plasmodium Falciparum" (2004). Electronic Theses and Dissertations. 129.

https://stars.library.ucf.edu/etd/129

This Masters Thesis (Open Access) is brought to you for free and open access by STARS. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of STARS. For more information, please contact lee.dotson@ucf.edu.
CHARACTERIZATION OF PROTEIN PRENYLTRANSFERASES AND PROTEIN PRENYLATION IN 
PLASMODIUM FALCIPARUM

by

THIAGO BEZERRA GASPAR CARVALHO DA SILVA
B.S. University of Central Florida, 1999

A thesis submitted in partial fulfillment of the requirements 
for the degree of Master of Science 
in the Department of Molecular Biology and Microbiology 
in the College of Health and Public Affairs 
at the University of Central Florida 
Orlando, Florida

Summer Term
2004
ABSTRACT

Malaria kills at least one million people each year, mostly children — a death every 30 seconds. Almost one half of the world population is at risk from malaria. Antimalarial drugs are the only means for the treatment of about 500 million annual global malaria cases. Because of prevalent drug-resistance it is extremely urgent to identify new drug targets. Many proteins involved in eukaryotic signal transduction and cell cycle progression undergo post-translational lipid modification by a prenyl group. Protein prenyltransferases, which catalyze the post-translational prenyl modification, have been established as a target for anticancer therapy. Research done in our laboratory has demonstrated recently that prenyl modification of proteins could be a novel target for the development of antimalarial drugs.

The goal of this study is to understand the molecular mechanism of protein prenylation in Plasmodium. The key to use of prenyltransferase inhibitors for the pharmacological intervention is a thorough understanding of the in vivo prenylation pathways in the malaria parasite. Knowledge of the physiological functions of the cellular protein substrates of malarial prenyltransferases is an important first step in the elucidation of the mechanism of antimalarial action of inhibitors of protein prenylation.

The research described in this thesis revealed the evidence for the existence of farnesylated and geranylgeranylated malaria parasite proteins. The study shows that the
dynamics of protein prenylation changes with the intraerythrocytic development cycle of the parasite. We detected that prenylated proteins in the 50 kDa range were mostly farnesylated and that the proteins in the 22-25 kDa range were mostly geranylgeranylated. The prenylation of \( P. falciparum \) proteins is inhibited by prenyltransferase inhibitors. We have also demonstrated unique features of protein prenylation in \( P. falciparum \) compared to the human host such as farnesylation of proteins are sensitive to inhibition by geranylgeranyltransferase inhibitors. \( \text{In-silico} \) search of the malarial genome sequence identified potential protein prenyltransferase substrates. One of these substrates is a SNARE protein Ykt6 homologue. The malarial Ykt6 was recombinantly expressed and subjected to \( \text{an in-vitro} \) prenylation assay. We showed that the recombinant Ykt6 was indeed a substrate for the malarial prenyltransferase
To my wife Donna, I love you.
ACKNOWLEDGMENTS

I would like to thank Dr Debopam Chakrabarti for his guidance and encouragement. Thank you to my committee (Dr. Ratna Chakrabarti, Dr. Pappachan E. Kolattukudy) and to Dr Karl Chai for all their support and advice. Thank you to members of the lab present and past: David Moe, Jeffrey Sedita, Dr. Prakash Pendyala, Dr. Valerie Bracchi-Ricard and Timothy Love. I would also like to thank those people who have given me support throughout this process.

I would also like to thank my parents (Kleber and Maria Gaspar), my brothers (Felipe and Kleber Jr), and my wife and child (Donna and Dylan) for their love guidance support and belief. None of this would have been possible without you.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 The Organism</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Life Cycle</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Attachment to Endothelium</td>
<td>11</td>
</tr>
<tr>
<td>1.4 Pathology and Clinical Features</td>
<td>11</td>
</tr>
<tr>
<td>1.5 Antimalarials</td>
<td>13</td>
</tr>
<tr>
<td>1.6 The future</td>
<td>15</td>
</tr>
<tr>
<td>1.7 Targeting Prenyltransferase</td>
<td>16</td>
</tr>
<tr>
<td>1.8 Prenyltransferases</td>
<td>18</td>
</tr>
<tr>
<td>1.9 Inhibitors of Protein Prenylation</td>
<td>23</td>
</tr>
<tr>
<td>1.10 Prenylated Proteins</td>
<td>24</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>27</td>
</tr>
<tr>
<td>2.1 Culture</td>
<td>27</td>
</tr>
</tbody>
</table>
2.2 Synchronization ........................................................................................................................................ 27
2.3 Collection of Parasites ................................................................................................................................ 28
2.4 Hypoxanthine Incorporation Assay ............................................................................................................ 28
2.5 Radiolabeling of P. falciparum Prenylated Proteins ....................................................................................... 29
2.6 Inhibition of in vivo Radiolabeling of P. Falciparum Prenylated Proteins with Peptidomimetics ............................................................................................................................ 30
2.7 Effect of Farnesyltransferase Inhibitors on P. falciparum Protein synthesis ............................................. 30
2.8 Effect of FTI-277 on P. falciparum Maturation ............................................................................................ 31
2.9 Indirect immunofluorescence microscopy ................................................................................................. 32
2.10 Preparation of ammonium sulfate fraction ................................................................................................. 33
2.11 Determination of Protein Concentration .................................................................................................. 34
2.12 Mono-Q Chromatography of P. falciparum protein farnesyltransferase activity ................................. 34
2.13 Assay for Farnesyltransferase Activity ..................................................................................................... 35
2.14 Cloning PfYKT6.2 ...................................................................................................................................... 36
2.15 Expression of Recombinant Protein ......................................................................................................... 38
2.16 Purification of PfYKT6 ............................................................................................................................ 38
2.17 DNA Sequencing ....................................................................................................................................... 39
2.18 In vitro Inhibition of Ftase Activity .......................................................................................................... 40
2.19 SDS PAGE, Gel Fixing and Protein Transfer .......................................................................................... 41
3. RESULTS .................................................................................................................................................... 42
3.1 Stage-Dependent prenylation of P.Falciparum Proteins ............................................................................ 42
3.2 Two-dimensional separation of radiolabeled P. falciparum prenylated proteins ............... 44
3.3 Mode of prenylation of *P. falciparum* Proteins ................................................................. 47
3.4 Inhibition of *P.falciparum* growth by prenyltransferase inhibitors. ................................. 47
3.5 Morphological changes in inhibitor treated parasites. ....................................................... 49
3.6 Immunofluorescence microscopy of inhibitor treated parasites. ........................................ 49
3.7 Inhibition of *invivo* Radiolabeling of P. falciparum Prenylated Proteins ...................... 54
3.8 Effect of prenyltransferase inhibitors on protein synthesis in *P. falciparum* ................. 56
3.9 Identification of potentially prenylated proteins in *P.falciparum* .................................. 58
3.10 Cloning of *PfYKT6* ............................................................................................................ 60
3.11 Test expression of PfYKT6 ................................................................................................. 60
3.12 Induction and Purification of *PfYKT6* ........................................................................... 61
3.13 *In Vitro* prenylation of PfYkt6 ....................................................................................... 61
4. DISCUSSION .......................................................................................................................... 66
LIST OF REFERENCES .................................................................................................................. 72
LIST OF FIGURES

1. Global status of resistance to antimalarial drugs .......................................................... 5
2. The life cycle of Plasmodium Falciparum .................................................................... 10
3. The DOXP/MEP pathway of IPP biosynthesis............................................................. 17
4. Structures along the FTase reaction path ...................................................................... 20
5. Overview of protein prenylation .................................................................................. 22
6. Structural comparison of Ftase inhibitors 25
7. Scintillation Proximation Assay. .................................................................................. 37
8. Stage Dependent prenylation of parasites .................................................................... 43
9. Two-dimentional separation of proteins labeled with \[^{3}H\] farnesol during erythrocytic stages of differention. ...................................................................................................................... 45
10. Two-dimentional separation of proteins labeled with \[^{3}H\] geranylgeraniol during erythrocytic stages of differention ...................................................................................................................... 46
11. Mode of prenylation of P. falciparum proteins ............................................................. 48
12. Inhibition of \[^{3}H\]hypoxanthine incorporation by FTI-277 ........................................ 50
13. Inhibition of \[^{3}H\]hypoxanthine incorporation by GGTI-298 ...................................... 51
14. Inhibition of \[^{3}H\]hypoxanthine incorporation by GGTI-286 ...................................... 52
15. Effect of FTI-277 on the intraerythrocytic maturation of *P. falciparum* .................. 53
16. Localization of prenylated proteins ........................................................................... 55
17. Inhibition of *in vivo* radiolabeling of *P. falciparum* prenylated proteins with peptidomimetics ........................................................................................................................................ 57
18. Effect of prenyltransferase inhibitors on protein synthesis in *P. Falciparum* .......... 59
19. Agarose gel electrophoresis of PfYKT6 PCR product used in cloning ....................... 62
20. Western blot analysis of PfYKT6 test expression .......................................................... 62
21. SDS-PAGE analysis of PfYKT6 TALON purification ................................................... 63
22. *In-Vitro* inhibition of plasmodial protein farnesyltransferase .................................... 65
1. INTRODUCTION

Malaria dates back to the dawn of mankind. Scientists have shown, by studying the parasite's family tree that malaria evolved from a chimp parasite nearly 8 million years ago; about the same time that pre-humans branched off the primate lineage. Researchers have been able to trace the ancestry of *plasmodium falciparum*, the most effective parasite in causing malaria in humans, to a small population that began between 9,500 and 23,000 years ago. Interestingly, this date coincides with the dawn of agriculture, which probably fueled the spread of malaria due to increased population densities and the increased availability of stagnant water in which mosquitoes could breed.

Hypocrites wrote of malaria, some 2,500 years ago, and divided the fever into different types: quotidian (daily), tertian (alternate days) and quartan (every fourth day). Allusions to the role of the mosquito in the transmission of the disease are plentiful throughout history. As far back as 500 BC when Susruta, a Brahmin priest, gave a description of malarial fever that he is said to have attributed to mosquitoes. However, until the late 19th century it was generally believed that malaria (from Italian: mal' aria or bad air) was caused by a poisonous vapor or *miasma* released from swamps. In 1816 Giovanni Rasori of Parma, while suffering from malarial fever in prison, was the first to doubt the "bad air" theory and to designate that a microorganism is responsible for the disease. The major breakthrough to confirm Parma’s hypotheses occurred in 1880 when the French physician, Charles Louis Alphonse Laveran,
discovered the malaria parasite while observing freshly drawn blood from a malaria patient. He noticed that after 15 to 20 minutes in an in vitro fluid state, the protozoa would emit extremely motile flagella. These motile organisms (which now are referred to as gametocytes) were originally called *Oscillaria malariae*. In 1886 Marchiafava and Celli gave the name *Plasmodium* to the genus. In 1887 William Henry Welch named the parasite that Laveran had discovered *Plasmodium falciparum*.

It was during the 1890's that William George MacCallum's research led him to believe that Laveran's flagellated bodies played an important role in the life cycle of the parasite, in particular its extra-human phase. Sir Patrick Manson gave evidence that the parasite was not dependent upon man for its survival. Thus, he paved the way for the theory that the disease was transmitted by a "suctorial insect." In 1897, Ronald Ross, who had been encouraged by Patrick Manson to investigate his hypotheses, used microscopy to detect the parasite in the wall of the stomach as well as in the digestive tract of the mosquito *Anopheles Stephensi*. The Italian Giovanni Battista Grassi demonstrated, in 1898, (with the *Plasmodium vivax*) the capacity of the *Anopheles* to transmit *Plasmodium* to humans.

Malaria was eliminated from the United States and from most of Europe during the first half of the twentieth century through a concerted effort involving changes in agricultural practices, home construction, land use and vector control. The discovery that DDT (dichlorodiphenyltrichloroethane) was an effective insecticide in 1939 led the way to an international eradication program in the 1950s. Problems with DDT, such as cost of spraying, resistance of communities to the spraying, and a high toxicity toward fish led to the interruption
of the program and subsequent survival of malaria as a disease with epidemic proportions. The private sector lost interest in the fight against malaria due to the loss of profit motive as countries affected by the disease are underdeveloped and its citizens cannot afford newly developed drugs.

Thus, for many years, there was little change in mortality and morbidity from malaria. Then in 1957 the first chloroquine-resistant malaria parasites were discovered in Thailand and in South America in 1959 (CDC, 1978). From Southeast Asia and South America, chloroquine resistant strains of malaria spread worldwide. The emergence of DDT-resistant vectors only added to the rapid increase in morbidity. Alternative insecticides such as malathion are not an economically feasible treatment for many malaria endemic countries and recent studies have shown that resistance to this pesticide has also emerged. By 1976, 42 species of *Anopheles* were resistant to one or more insecticides (Shuler, 1985).

As chloroquine-resistant parasites have spread throughout Africa, the antifolate combination of pyrimethamine and sulphadoxine has increasingly become the drug of choice for the treatment of uncomplicated *falciparum* malaria. Malawi was the first African country to make this shift, in 1993, and pyrimethamine-sulphadoxine was designated the first-line drug in Kenya in 1998. In both Southeast Asia and South America, the exceptional pace of selection for resistance to pyrimethamine-sulphadoxine limited its useful therapeutic life (UTL) to about 5 years. In East Africa, pyrimethamine-sulphadoxine has been the first-line drug for less than 5 years, but selection of parasites resistant to pyrimethamine-sulphadoxine is already evident, and the clinical effectiveness of this formulation is decreasing (Sibley, 2001). Figure 1 illustrates a
current geographic distribution of strains resistant to chloroquine and sulphadoxine-pyrimethamine.

Although malaria is an important health problem in some parts of Asia and South America, its main impact is in sub-Saharan Africa where at least 90% of deaths from malaria occur. Recent attempts to tally the global burden of malaria indicate that at least one million deaths occur from malaria each year i.e., a death from malaria every 30 seconds (Snow, 1999). There are approximately 500 million people at risk from malaria in Africa. Beyond the appalling toll on human life, the economic cost of malaria is staggering because of the cost due to the management of the disease. Countries where malaria has reached endemic proportions are among the poorest in the world. Thus, overall economic impact of malaria is likely to be much more substantial than suggested by estimates of direct costs alone since malaria contributes to loss in production output, lack of foreign investment and improper education due to frequent infection in children.

1.1 The Organism

The causative agent of malaria is a parasitic protozoan called *Plasmodium*. Four species of *Plasmodium* are known to infect humans: *falciparum, vivax, ovale, malarium*. The genome of *Plasmodium falciparum* is 22.8 megabases (Mb) long and contains 14 chromosomes ranging in size from 0.643 to 3.29 Mb. To date, it is the most (A + T)-rich genome sequenced with 80.6% A + T. Approximately 5,300 protein-encoding genes were identified (Gardner, 2002).
Figure 1 Global status of resistance to chloroquine and sulphadoxine/ pyrimethamine, the two most widely used antimalarial drugs. Data are from the WHO. [originally from Ridley, 2002]
The mitochondrial genome of *Plasmodium* is small (about 6 kb) and encodes no tRNA’s, which must be imported (Vaidya, 1989). *Plasmodium* contains single rRNA clusters distributed on different chromosomes instead of long tandemly repeated arrays of rRNA genes. The sequence of rRNA gene units vary between units of similar size and the expression of these units is developmentally regulated, resulting in differential expression of rRNA units through development (Li, 1994). About sixty percent of the predicted proteins appear to be novel and have no significant similarity to proteins from other organisms that are in the database. (Gardner, 2002). The apicoplast, a residual plastid located at the apical end of the parasite shared by all organisms in the phylum apicomplexa, has a 35-kb genome (Wilson, 1996). Because of its small size proper function of the apicoplast, as well as the mitochondria, requires enrichment of its proteome by the nucleus (Vaidya, 1989; Waller, 1998).

### 1.2 Life Cycle

The life cycle of *Plasmodium falciparum* is split between two hosts: the *Anopheles* mosquito and humans. Only the female mosquito plays host to *Plasmodium*. When the female mosquito feeds on an infected human, she may ingest sexual stages of the parasite and will become a breeding ground for the parasite. Once ingested, gametocytes will go through gametogenesis and escape from the host erythrocyte. Several factors participate in the induction of gametogenesis, including a drop in temperature, an increase in carbon dioxide, and mosquito metabolites. The sexual forms of the parasite are referred to as microgametes and
macrogametes, male and female respectively. In the mid-gut microgametes become exflagellated and fertilize the macrogamete leading to a zygote. The zygote develops into a motile ookinete, which subsequently becomes an oocyst. Once in the outer wall of the midgut, the oocyst undergoes sporogony: multiple rounds of asexual replication resulting in the production of sporozoites. Once the oocyst ruptures, it releases sporozoites into the hemocoel of the mosquito. The sporozoites eventually invade the salivary gland of the mosquito where they will stay until the mosquito takes another blood meal and subsequently begins the asexual stage of life cycle (Harrison, 1978; Schmidt, 1989).

In humans, infection is initiated when sporozoites are injected with the saliva of a feeding mosquito into the subcutaneous tissue, and less-frequently directly into the bloodstream; from there, sporozoites travel to the liver. Sporozoites pass through several hepatocytes before invasion is followed by parasite development (Mota, 2001).

Inside the hepatocyte, the parasite undergoes an asexual replication known as exoerythrocytic schizogony. The sporozoites will develop into tens of thousands of merozoites, which can each invade an erythrocyte (RBC) once released from the liver. A small proportion of asexual parasites convert to gametocytes that are essential for transmitting the infection to others through female anopheline mosquitoes. The disease in caused by asexual parasites multiplying in RBCs. This process usually takes 48 hours, however some parasites from *Plasmodium vivax* and *Plasmodium ovale* go thorough a dormant period instead of readily entering exoerythrocytic schizogony. These Hypnozoites will move out of dormancy after
several weeks to months (or years) have passed since the primary infection and are responsible for relapses (Krotoski, 1989).

Once a merozoite is released into the bloodstream, it will flow until it binds an RBC. At this point, the merozoite will undergo apical reorientation. Receptors that mediate invasion of the RBC (and liver) are found in the rhoptries, micronemes and dense granules which are contained within the apicoplast (Adams, 1990). These organelles will only release the receptors after contact with the RBC has been made, thus avoiding neutralization by antibodies. The merozoite induces a small depression in the erythrocyte membrane. The area of the RBC to which the merozoite is attached thickens and forms a junction with the plasma membrane of the merozoite. As the merozoite enters the invagination in the erythrocyte surface, the junction—in the form of a circumferential zone of attachment between the erythrocyte and merozoite—moves along the confronted membranes to maintain its position at the orifice of the invagination. This process will continue until the entire merozoite is enveloped by the RBC membrane (Dvorak, 1995).

There are two known families of parasite receptors involved in invasion: the Duffy-binding like proteins (DBL) and the reticulocyte-binding-like (RBL) proteins. These receptors determine the flexibility for invasion by the various Plasmodium species (Adams, 1992; Rayner, 2001). After entering the erythrocyte the parasite undergoes a growth period, followed by asexual replication. This stage of the lifecycle is marked by three microscopically distinct phases: ring, trophozoite and schizont. In the ring stage, the parasite spreads itself into a thin biconcave disc, thicker around its perimeter where the elongated nucleus is present and thinner in
the middle, giving it the appearance of a ring in Giemsa-stained blood smears. The parasite is contained within membrane-lined cavity known as the parasitophorous vacuole (PV). From this vacuole, the parasite feeds on hemoglobin and takes up other nutrients from the plasma through its cytostome. As the ring stage enlarges, the parasite begins to synthesize molecules specific to this stage. Some of these molecules are exported to the RBC membrane, thus modifying its composition.

The ring eventually grows into the more rounded trophozoite stage. This is the most metabolically active stage of the parasites asexual life cycle. The parasite continues to modify the RBC membrane so that it can now adhere to endothelial tissues and increase its permeability to nutrients. The feeding on hemoglobin continues, and the heme products of hemoglobin digestion crystallize into particles of dark pigment, haemozoin, scattered within the food (pigment) vacuole.

The end of the trophozoite phase comes with the onset of nuclear division without cytokinesis. The schizont stage consists of 3 to 5 rounds of nuclear replication followed by a budding process. At the end of the schizont stage, the parasite bursts its host cell and releases merozoits, which are capable of re-entering the asexual stage of the life cycle. The rupture of schizont induces the production of cytokines, particularly tumor necrosis factor (TNF) (Kwiatkowski, 1989). Even though most gametocytes develop from liver schizonts, and prolonged asexual lifecycles decrease the chance of a parasite re-entering the sexual life cycle, changes in environment such as increased parasitemia can induce formation of gametocytes from the asexual erythrocytic stages. Figure 2 illustrates both stages of the malarial life cycle.
Figure 2. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. (Of note, in P. vivax and P. ovale a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony). Merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Some parasites differentiate into sexual erythrocytic stages (gametocytes). Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal. The parasites’ multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes. The zygotes in turn become motile and elongated (ookinetes) which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle. [Originally from http://www.dpd.cdc.gov]
1.3 Attachment to Endothelium

Asexual parasites, in the trophozoite or schizont stage, and gametocytes can adhere to the endothelial tissues. Thus, only the ring stage form of the parasite is found in the circulating blood (Newbold, 1999). This adhesion process involves a mechanism where parasites first tether then roll before becoming firmly attached (Ho, 1999). This process is mediated by an assortment of host proteins, however to this date, firm attachment under flow can be provided by two host receptors: CD36 and chondroitin sulphate A (CSA) (Cooke, 1994). The parasite aids in this process by expressing and exporting a protein to the RBC membrane. This protein — \textit{P. falciparum} erythrocyte membrane protein 1 (PfEMP1), is the only protein responsible for adherence on the infected RBC to a myriad of host receptors. PfEMP1 is highly polymorphic, antigenically variant, and expressed on the erythrocyte surface at electron-dense protrusions termed knobs (Baruch, 1995). PfEMP1 is encoded by the \textit{var} gene. There are 50-150 \textit{var} genes on multiple parasite chromosomes, and some are in clustered arrangements (Su, 1995).

1.4 Pathology and Clinical Features

The parasite feeds on its host intracellular proteins, mainly hemoglobin. The changes in infected RBCs include spherical shape, membrane protuberances and appearance of parasite-expressed protein in RBC membrane. These proteins lead to adhesion of host cell to other infected RBCs (clumping), to uninfected RBCs (resetting) and to endothelial tissue (Chu, 1997).
These malarial aggregates are central to the pathogenesis, resulting in blockage of veins and capillaries as well as inhibiting the clearing of the parasite by the spleen.

Anemia in the first clinical manifestation of malaria and is an inevitable consequence of erythrocyte parasitization and involves the phagocytosis of parasitized and unparasitized cells, dyserythropoiesis (abnormal RBC synthesis) and decrease in erythropoiesis (RBC production) in bone marrow. TNF, induced by schizont rupture, is considered an important aggravating factor in the pathogenesis of anemia (Clark, 1988).

Another early sign of malaria infection is the enlargement and hardening of the spleen and Liver. Blockage of splenic vessels may lead to hemorrhage and the liver assumes a brown, gray or even black color as a result of deposition of malaria pigment. Lactic acidosis (as a result of glucose catabolism by RBC and loss of liver function) renal inflammation, lower blood pressure and tachycardia (lower heart rate) are also common. Other organs affected by the parasite are the GI tract, lungs and placenta.

Cerebral malaria is the most common complication and cause of death in severe *P. falciparum* infection. Other complications include thrombocytopenia (a disorder in which the number of platelets is abnormally low), splenic rupture, dilated hepatic sinusoids containing Kupfer cells and parasitized RBCs, malarial hepatitis and renal failure.
1.5 Antimalarials

Jesuits in Peru discovered the first known natural antimalarial and analgesic, quinine, in the seventeenth Century. Quinine is an alkaloid derived from the bark of the *Cinchona ledgeriana* tree. The use of quinine in the prevention of malaria was only surpassed by its synthetic derivatives quinacrine, primaquine and most notably chloroquine. Quinine, and its synthetic analogs, remains the drug of choice for the treatment of malaria in most parts of the world (Riddley, 1998). Side effects to quinine treatment are commonly referred to as cinchonism and may involve tinnitus (ringing in ear), hearing loss, dizziness, nausea, uneasiness, restlessness and blurring of vision. Chloroquine is thought to exert its antimalarial effect by preventing the polymerization of toxic heme released during proteolysis of hemoglobin in the *Plasmodium* digestive vacuole (Sullivan, 1996).

Another class of chemicals that have shown strong antimalarial activity are commonly referred to as antifolates. Fully reduced folate cofactors are essential for the key one-carbon transfer reactions that are needed for nucleotide biosynthesis and amino-acid metabolism. At present, the most significant antifolate used to treat malaria is the combination of the 2,4-diaminopyrimidine pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR), and sulphadoxine, a sulphonamide that interferes with the action of dihydroopteroate synthase (DHPS), another enzyme in the folate pathway (Ridley, 2002; Takechi, 2001).

Artemisinin (sweet wormwood or `qinghao') is an ancient Chinese herbal remedy used by Chinese herbal medicine practitioners for at least 2000 years. Its antimalarial activity was found
in 1967 when Chinese scientists screened a series of traditional remedies for drug activities. (Meshnick et al., 1991). Incubation of infected RBC’s with artemisinin leads to parasite death through protein alkylation. Artemisinin interacts with intraparasitic heme-iron (derived from the proteolysis of host cell hemoglobin), this interaction breaks the endoperoxide bridge found within artemisinin generating free radicals. There are many theories as to how these free radicals induce parasite death. The most accepted theory is that the artemisinin-derived free radicals appear to damage specific intracellular targets, possibly via alkylation. In the past decade, a general consensus has formed supporting the mechanism (Jefford, 1995; Hong 1994).

A combination of atovaquone and proguanil has been found to be quite effective in treating malaria. It is believed that atovaquone inhibits electron transport and destabilizes the cytochrome bcl complex, causing proton leakage to occur through this site, and that proguanil, through as yet unclear means, enhances this destabilization. This synergy results in a higher proton leakage at lower concentrations of atovaquone (srivastava, 1999)

Resistance to treatment has emerged regardless of what regiment of drugs are used. Plasmodium falciparum drug-resistant malaria originates from chromosome mutations. Impaired chloroquine uptake by the parasite vacuole is a common characteristic of resistant strains, and this phenotype is correlated with mutations of the Pfmdr1, Pfcg2 and Pfcrt genes;

Resistance to antifolates arises due to various point mutations of dihydrofolate reductase (DHFR) or DHPS. The S108N substitution in DHFR is the principal mutation associated with resistance to pyrimethamine or cycloguanil. This mutation does not affect the enzyme’s operation on its natural substrate. Mutations in DHPS (A437G and K540E) account for
resistance to sulphadoxine. Mutations in cytochrome bc1 gene (Y268N) yields atovaquone resistant parasites (Le Bras, 2003).

There is currently no evidence for clinically relevant artemisinin resistance. However, since artemisinin derivatives are being widely used, artemisinin resistance is likely to develop eventually. Artemisinin-resistant strains of *P. falciparum* and *P. yoelii* have been obtained in the laboratory (Inselburg, 1985, Peters and Robinson, 1999).

### 1.6 The future

Malarial research is currently experiencing a renaissance period, which is fueled by the genome sequencing and an increase of funds for malaria research provided by established donors such as the National Institutes for Health, the Wellcome Trust and by new donors such as The Gates Foundation. The Roll Back Malaria (RBM) initiative, a global partnership founded by the World Health Organization (WHO), the United Nations Development Program (UNDP), the United Nations Children's Fund (UNICEF) and the World Bank, has as its goal the reduction of malaria by 50% in the year 2010. To reach this task, a great deal of attention has been aimed at understanding the sub cellular mechanism of the parasite. A thorough understanding of the parasite’s molecular biology will be absolutely essential for the discovery of potential drug targets. Protein prenyltransferases, enzymes required for the post-translational modification of proteins, have become a target for antimalarial development. Furthermore, inhibitors of mammalian protein farnesyltransferase are being intensively developed by a large number of
major pharmaceutical companies because such compounds show profound ability to arrest the growth of transformed cells and cause shrinkage of tumor xenographs in experimental animals. Protein Prenylation is important for the malarial parasite’s growth and differentiation as prenyltransferase inhibitors have shown antimalarial activity (Chakrabarti, 1998; Jomaa, 1999; Okhanda, 2001). Thus, it is possible to target protein farnesyltransferase inhibitors (PFTIs) as anti-parasitic agents. PFTIs also have an advantage over new inhibitors since a wealth of information such as pharmacokinetic, toxicity, and bioavailability data is available from previous studies on mammalian systems. This "piggy back" approach allows rapid development of drugs and may result in inexpensive medicine. This is crucial because malaria is most prevalent in underdeveloped countries.

1.7 Targeting Prenyltransferase

Isoprenoids are compounds formed from isopentenyl pyrophosphate, which contains a five-carbon backbone also known as an isoprene unit. *P. falciparum* has an unconventional method of synthesizing isoprenoids. This method, also found in bacteria, algae and plants, is the DOXP pathway (Gardner, 2002; Jomaa, 1999). The naming substance, 1-deoxy-D-xylulose 5-phosphate, is the condensation product of glyceraldehyde 3-phosphate and pyruvate by DOXP-synthetase. The pathway is shown in figure 3.

The post translational modification process known as prenylation involves two types isoprenoids: the 15-carbon farnesyl pyrophosphate (FPP) and the 20-carbon
Figure 3. The DOXP/MEP pathway of IPP biosynthesis in the plastids of plants. The first five enzymes are known and have been numbered from 1–5. The second enzyme DXR can specifically be blocked by the herbicide fosmidomycin. This pathway also occurs in algae, the malaria parasite P. falciparum and in pathogenic bacteria. [Originally from Lichtenthaler, 2000; p.788]
geranygeranylpyrophosphate (GGPP). Specifically, protein prenylation is the attachment of FPP and GGPP to conserved cysteine residues at or near the C-terminus of acceptor proteins. Prenylation reactions are catalyzed by three different enzymes: farnesyltransferase (Ftase), geranylgeranyltransferase (GGTase) type I, and GGTase type II.

1.8 Prenyltransferases

Protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type-I (GGTase-I) catalyze the addition of farnesyl and geranylgeranyl groups to proteins that have a C-terminal CAAX motif. In this motif, the ‘C’ is a cysteine to which the isoprenoid is covalently linked, the ‘A’ is an aliphatic amino acid, and the ‘X’ residue provides prenylation specificity. In mammalian systems, protein farnesyltransferase (PFT) farnesylates the cysteine residue in carboxyl-terminal containing an X conferring methionine, glutamine, serine, threonine, or cysteine and protein geranylgeranyl transferase-I (PGGT-I) shows specificity for X residues conferring leucine (Moores, 1991). FTase and GGTase I are metalloenzymes composed of an α and a β subunit. Both enzymes require zinc to function whereas GGTase I also requires Magnesium (Reiss, 1992). They share an identical, 48 kDa. α subunit. The β subunit of Ftase and GGTase are 46kDa and 43kDa, respectively (Andres, 1993; Moomaw, 1992; Yokoyama, 1991). Binding of the prenyl group and acceptor protein is mediated by the β subunit whereas the catalytic activity is controlled by the α subunit (Park, 1997; Zhang, 1996).
The secondary structure of the α subunit of FTase is composed of series of helices, which form several helical hairpins. These helical hairpins form a double-layered, right-handed superhelix resulting in a crescent shape that partly envelops the β subunit (Park, 1997). The exact reaction path of PFTase has been elucidated (Long, 2002). The first step in the process involves the binding of the prenyl group substrate to the enzyme. The prenyl group binds to an enzymatic cleft in the β subunit. This cleft has precise dimensions so that if the correct prenyl group becomes attached, its terminal carbon will interact with the bottom of the cleft and the diphosphate group will interact with the zinc ion located atop the cleft. The acceptor protein binds next. It is important to note that the prenyl substrate forms a substantial part of the binding domain for the acceptor protein. During a transitional state the phosphodiester bond is rotated repositioning the α-phosphate and allowing interaction between the C₁ atom of the isoprenoids and the cysteine thiolate of the acceptor protein. The diphosphate group’s negative charges are mediated by tyrosine 300 on the β subunit, lysine 164 on the α subunit and magnesium (in GGTase I, magnesium is replaced by lysine 311 on the β subunit). The zinc and the C–S bond of the cysteine thiolate mediate a nucleophilic attack on C₁ of the isoprenoids. This zinc-catalyzed sulfur alkylation mechanism is likely to be the same mechanism used by all protein prenyltransferases. The substrate, prenylated protein, is only released when fresh FPP substrate binds to Ftase (Long, 2002; Huang 2000). This reaction is shown in figure 4.

Two additional modifications are required to obtain a finished product. First, the three tailing amino acids of the prenylated protein are removed by an endopeptidase.
Figure 4. Structures along the FTase reaction path. 0, Structure of unliganded FTase6 (PDB number 1FT1; red, α-subunit; blue, β-subunit). 1–4, Observed ligand conformations in FTase crystal structures. 1, Farnesyl diphosphate substrate (FPP, blue) bound to FTase
The second and final additional step involves the addition of a methyl group to the prenylated cysteine’s carboxyl group. In this step, an S-adenosyl-L-methionone acts as a methyl donor and the reaction is mediated by a methyltransferase (Zhang, 1996). The entire protein prenylation mechanism is illustrated in figure 5.

Geranylgeranyltransferase II, also known as Rab GGTase, modifies proteins that do not contain the CAAX sequence. Instead, GGTase modifies proteins with -CC, -CXC, -CCX or -CCXX (in single letter amino acid and where X is any amino acid) c-terminal motif (Farnsworth, 1994). Three polypeptides are required for prenylation. Two of these peptides form an $\alpha\beta$ heterodimer, similar other prenyltransferases, and a 95kDa protein known as Rab escort protein (Rep1). The 50 kDa $\alpha$ subunit and the 38 kDa $\beta$ subunit compose GGTase II (Seabra, 1992). The $\alpha\beta$ heterodimer shares about 30% sequence similarity with their FTase and GGTase counterparts and is responsible for the enzymatic function of GGTase II. True to its name, the role of Rep 1 is to binding the protein substrates in a way that enzymatic action by the $\alpha\beta$ heterodimer is still permitted (Andres, 1993). Thus, the amino acid sequence on the protein substrate that mediates binding to Rep1 plays a crucial role in prenylation. This explains why GGTase II can neither farnesylate nor be inhibited by short peptides. The $\alpha$ subunit of GGTase II contains two unique domains, an IG-like domain and an LRR domain, not found in its analogs that are responsible for binding of the Rep 1/protein substrate complex to the $\alpha\beta$ heterodimer (Zhang, 2000). Rep will remain bound to the substrate protein throughout the prenylation process. After prenylation, Rep delivers the modified protein to its target membrane. Similar to FTase I, GGTase II requires zinc for proper activity (Zhang 200). The mechanism of geranygeranylation is shown in figure 5.
Figure 5. Overview of protein prenylation. Modification of C-terminal Cys residues (designated "C") for both the CaaX motif and Rab proteins are depicted. In the case of CaaX motif proteins, famesylation is the modification shown. The minus sign (-) on particular forms designates those containing a free carboxyl group on the Cys residue and "C-Me" indicates that the carboxyl group has been methylated. Text gives further details. [Originally from Casey, 1996; p. 243]
1.9 Inhibitors of Protein Prenylation

As previously mentioned, inhibitors of mammalian protein farnesyltransferase have been developed due to their ability to arrest the growth of transformed cells and cause shrinkage of tumor in experimental animals. The design of PFT inhibitors has followed three main approaches: (i) pseudopeptide or peptidomimetic inhibitors which act competitively with the protein to be prenylated and noncompetitively with the isoprenoid FPP, (ii) FPP analogue inhibitors which, conversely, act competitively with the isoprenoid FPP and noncompetitively with the substrate protein and (iii) bisubstrate inhibitors which mimic the transition state of the two substrate complex. Pseudopeptide inhibitors are molecules that closely resemble the CAAX motif. In these molecules, some the peptide bonds have been reduced to their methylamino forms as a means to improve membrane permeability. Complications with such inhibitors include the relative abundance of hydrolizable bonds, low absorption, rapid metabolism and low oral bioavailability. Peptidomimetic inhibitors take this approach further by replacing the peptidic features of the tetrapeptide structure with simple, stable hydrophobic subunits such as a 4-aminobenzoic acid ring and benzodiazepine (Qian, 1997). The development of FPP analogue inhibitors encountered stifling problems such as the critical role FPP plays in cholesterol synthesis. Thus, nonspecific binding of FPP analogues causes several side effects. Bisubstrate analogues contain the hydrophobic part of the farnesyl group and a mimic of the protein recognition site. The potency and specificity reported for these initial compounds, against mammalian FTase, is very similar to that observed with the smaller CAAX mimetics (Graham,
1995; Gibbs, 1994). Although further modifications of bisubstrate analogs may improve potency beyond that achievable with FPP or CAAX mimetics alone, this gain in potency may be offset by the larger size of these molecules, which may compromise their pharmacological properties in vivo. Figure 6 shows the structures of various inhibitors.

This vast knowledge and abundance of farnesyltransferase inhibitors has stimulated researchers to determine the effect of these compounds on various organisms. It has been previously shown that inhibition of plasmodial farnesyltransferase can be achieved in vitro (Chakrabarti, 1998). Furthermore, the growth of *P.falciparum* in erythrocytic culture system is arrested efficiently by peptidomimetics. Inhibition of farnesyltransferase activity as well as growth arrest by FTase inhibitors has been shown, in lower eukaryotes such as *S. cervisiaecerae* and trypanosomes.

**1.10 Prenylated Proteins**

It has been estimated that about 0.5% of all mammalian proteins are prenylated (Epstein, 1991). Protein prenylation is essential for the proper localization and function of proteins central to signaling, nuclear architecture, and cell proliferation, such as small G proteins (Takai, 1992), heterotrimeric G protein γ-subunits (Yamane, 1990), nuclear lamins (Farnesworth, 1989), protein kinases (Inglese, 1992) and members of the Ras G protein superfamily, including Rab, Rho and Rac, that play crucial roles in protein trafficking and cytoskeletal organization (Desnoyers, 1996; Takai, 1993). Other proteins such as lamins, DNAJ, Ykt6 SNARE, PRL class of tyrosine
Figure 6. Structural comparison of Ftase inhibitors between FTase CAAX substrate, Farnesyl Pyrophosphate, Farnesylated CAAX residue and inhibitors. Adapted from Gibbs 1994, p.176.
phosphatases, CENP-E and CENP-F have been found to be prenylated in mammalian systems. Scientist have yet to indentify the entire percentage of prenylated proteins in mammals, which would tally to some 150 to 200 proteins. Protein prenylation also plays a critical role in diverse cellular processes in many organisms other than mammalian cells including yeasts and plants (Zhang, 1996; Yang, 2000; Pei, 1998).

The goal of the proposed research is to characterize and understand protein prenylation in *P. falciparum*. This will enable us to establish inhibitors of protein preylation as potential antimalarials. Specifically, following aims will be pursued:

1. Analyze protein prenylation in *P. falciparum*

2. Test the effect of FTase inhibitors

3. Characterize prenylated proteins in *Plasmodium*.

We will attempt to elucidate the types of proteins prenylated, the temporal localization of protein prenylation, and the effects of FTase inhibitors on protein prenylation, cell cycle and subcellular localization of prenylated proteins.
2. MATERIALS AND METHODS

2.1 Culture

P. falciparum strain 3D7, was maintained in a water-jacketed incubator at 37°C with an atmosphere of 5% CO2 and 95% air. Parasite culture is stored in 10mm petri dishes, containing 10mL culture per dish. The parasites' development and proliferation was monitored daily by light microscopy of Leukostat (Fisher) stained thin smears. The nutrient media of infected RBC culture plates was exchanged daily with fresh RP10S media: RPMI 1640 enriched with 11 mM glucose, 100 uM hypoxanthine, 0.2% (w/v) sodium bicarbonate, 25 mM HEPES pH 7.2, and 10% human A (Rh+) blood serum. Culture plates were split according to estimated parasitemia within the next 24 hours. RBC from type A (Rh+) blood was acquired from the Central Florida Blood Bank. The blood is washed, and diluted to a hematocrit of 5%, in a serum-free medium (Trager, 1976).

2.2 Synchronization

Synchronization was achieved through sorbitol treatment. Sorbitol lyses parasites in trophozoite and schizont stages by osmotic pressure; leaving the ring infected RBC’s intact. Culture plates were pooled in a 50 mL sterile conical centrifuge tube and spun at 200 × g for 5
minutes. The pellet was resuspended, in a volume five-fold greater than its own, in pre-warmed 5% sorbitol solution and incubated in a 37°C water bath for 15 minutes. After incubation, the solution was centrifuged at $200 \times g$ for 7 minutes. The pellet was washed twice with RPMI 1640. Original media volume was added to resuspend the RBC pellet and solution was returned to petri dishes.

### 2.3 Collection of Parasites

Culture plates were allowed to grow to a parasitemia of 10-20% for harvesting. Pooled parasites in a 50 mL conical centrifuge tube were treated with 0.1% (w/v) saponin and incubated for 5 minutes at room temperature. After incubation the solution was centrifuged at 4°C with a speed of $1800 \times g$ for 7 minutes. The pellet was washed twice in PBS, pH 7.2. The yield was approximately $4-5 \times 10^7$ parasites mL$^{-1}$ of culture. All pellets were stored at -80°C until further use.

### 2.4 Hypoxanthine Incorporation Assay

Freshly synchronized parasitic culture was pooled in a 50 mL conical centrifuge tube and spun at 2000 μg for five minutes at 4°C. The pellet is washed twice with five volumes and re-suspended with hypoxanthine free medium. If necessary, culture was split to a 3% - 5%
parasitemia. [3H] Labeled hypoxanthine was added to a concentration of 1 mCi per ml. Inhibitors were dissolved in DMSO to a concentration of 25mM. This stock solution was subsequently diluted immediately prior to assay. Costar's Cell Culture Cluster 96 well plates were used. In each well, 200µL of radiolabeled culture and 1µL of diluted drug or DMSO (as control) was added. Culture was returned to incubator for 24 hours, allowing hypoxanthine incorporation. A Tomtec's Mach II M Harvester 96 was used to transfer samples form 96 well plate to glass fiber membranes. Corresponding sections of the membrane were cut, placed in five ml scintillation vials and immersed in three ml, Fisher Scintiverse, Scintillation Fluid. The radioactive count of each vial was detected by a Beckman LS500TD. The entire assay was performed in triplicate and the average of the results was plotted using Prism 2.0 software. The effectiveness of each inhibitor is reported as an IC50 value, the concentration of inhibitor that reduces the count (i.e. the incorporation of hypoxanthine into nucleic acids) by 50%.

2.5 Radiolabeling of P.falciparum Prenylated Proteins

Synchronized parasite cultures were labeled with [3H]farnesol or [3H]geranylgeraniol, (American Radiolabeled Chemicals) at 6, 20, 32 hr. post synchronization. These time points reflect ring, trophozoite and schizont stages, respectively. Time points were incubated with radiolabeled prenyl group for 12 hours. Radiolabeled parasite proteins were isolated, resolved by SDS-PAGE, and detected by fluorography (details in section)
2.6 Inhibition of in vivo Radiolabeling of P. Falciparum Prenylated Proteins with Peptidomimetics

Costar's Cell Culture Cluster 24 well plates were used. Inhibitors FTI-277 and GGTI-298 were purchased from Calbiochem. To each well, 1 mL of asynchronous P. falciparum culture and either 10 µCi of [3H]farnesol or [3H]geranylgeraniol was added. For inhibition studies, 5µL of inhibitor (dissolved in DMSO) were added. Samples were returned to the incubator for 24 hours, allowing the prenyl groups to enter the cell and become attached to proteins. The contents of each well were transferred to a 1.5mL minicentrifuge tube and spun at 1800 × g for 7 minutes. The pellet was washed twice with PBS. The labeled parasites were released from RBC by treatment with 0.1% (w/v) saponin for 5 min followed by two washes with phosphate-buffered saline, pH 7.2 (Gibco) and centrifugation at 1800 × g for 7 min. The parasite pellet was washed twice with PBS and lysed by addition of 24 µl of M-Per lysis reagent (Pierce). The lysed parasite extracts were mixed with 12µl 4X loading buffer and run on a 12% SDS-PAGE gel. The gel will ultimately be subjected to fluorography (as described below).

2.7 Effect of Farnesyltransferase Inhibitors on P. falciparum Protein synthesis

Synchronous culture with a parasitemia around 6% was pooled in a 50 mL conical centrifuge tube and spun at 2000 × g for five minutes at 4°C. The RBC pellet was washed twice
with RPMI deficient in methionine and cysteine. Washed RBC pellet was resuspended in methionine and cysteine-deficient RP10S media enriched with Pro-mix L-[35S] in vitro cell labelling mix (Amersham Biosciences). Radiolabeled malarial culture was transferred, in 1 ml aliquots, to a 24 well plate (costar). Each well received either 5µl DMSO (control) or inhibitor. Samples were returned to the incubator for 24 hours to allow labeled amino acids to be used in protein synthesis. Cell extracts were prepared (as described in previous paragraph), resolved on SDS-PAGE, and subjected to autoradiography (as described below)

### 2.8 Effect of FTI-277 on *P. falciparum* Maturation

Freshly synchronized (6 h post-synchronization) parasitic culture, with approximately 6% parasitemia was transferred, in 1ml aliquots, into 24 well plates (costar). Wells were treated with: 5 l of DMSO (control), 5µM FTI-277 or 10µM FTI-277. Samples were returned to incubator and timepoints at 12, 24 and 36 hours past inhibitor treatment were collected and used to produce stained thin smears. Images of stained thin smears were captured with a Zeiss Axioplan2 microscope equipped with a Hamamatsu color chilled 3CCD camera.
2.9 Indirect immunofluorescence microscopy

Freshly synchronized (6 h post-synchronization) parasitic culture, with approximately 6% parasitemia was transferred, in 1ml aliquots, into 24 well plates (costar). Wells were treated with: 5 of DMSO (control), 5µM or 10µM of FTI-277 and GGTI-298. Samples were returned to incubator and timepoints at 12, 24 and 36 hours past inhibitor treatment were allowed to adhere to poly-L-lysine-coated coverslips at 37 °C for 30 min. The slides were washed four times with phosphate-buffered saline, fixed in 4% paraformaldehyde (10 min at room temperature) and once again washed four times with phosphate-buffered saline. The cells were permeabilized and blocked by incubating slides in blocking solution (phosphate-buffered saline, 0.05% saponin, 5% bovine serum albumin) for 20 min at room temperature. The primary antibody, an affinity purified rabbit anti-farnesyl polyclonal antibody (Calbiochem), was diluted into a ration of 1:50 in blocking solution. The secondary antibody, an affinity purified Cy2-labeled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories), was diluted into a ration of 1:200 in blocking solution. Samples were incubated for 90 minutes in with each antibody solution followed by three washes with phosphate-buffered saline. Nuclear staining was done with 1 µM 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The coverslips were mounted with gel/mount (Biomed) on slides. The slides were viewed on a DeltaVision restoration microscope system (Applied Precision) equipped with a Nikon TE200 microscope and a Photometrix cooled CCD camera. DeltaVision software (SoftWoRx) was used for image deconvolusion.
2.10 Preparation of ammonium sulfate fraction

All steps were performed at or under 4°C. Parasite pellet from 2 liters of culture (200 asynchronous plates at 10-20 % parasitemia) was resuspended in 20 mL of buffer A [50 mM Tris HC1, pH 7.5, containing one tablet of EDTA free protease inhibitor (Roche) 10mL-1]. The cell suspension was then sonicated with a microtip, using six 15-second cycles, and the resulting lysate was centrifuged at 100,000 × g with a 50.1 Ti rotor for 2 h (Beckman). The cleared lysate (S-100) was subjected to 0-50% ammonium sulfate [(NH4)2SO4] precipitation. It is important to grind the ammonium sulfate with a mortar and pestle and add it slowly to the cleared lysate in order to ensure complete dissolution and avoid temporary pH spikes. Once all the ammonium sulfate has been completely added, the solution is left on ice for 15 minutes. The solution was then centrifuged at 12,000 × g for 20 minutes. The pellet was resuspended in 2 mL of buffer B (50 mM Tris-HCL, pH 7.5, 1mM DTT, 20 µM ZnCl2, 20 mM NaCl), injected into a Slide-A-Lyzer 10,000 MWCO (Pierce) cassette and dialyzed against the same buffer for 4 hr. The dialysis buffer is exchanged at least once during this process. The dialysed sample was then transferred into 1.5 mL cryogenic vials and stored at -80°C until further use.
2.11 Determination of Protein Concentration

All protein concentrations were determined using the Bradford Assay (Bradford, 1976; Sambrook, 1989). A mixture of 200 µl of Coomassie blue (Bio-rad protein assay concentrated dye reagents), 1,2,4,6, or 8 µg of a 1 µg/ul bovine serum albumin (Sigma) stock solution and ddH2O to bring the volume up to 1 mL was used to make a standard curve. Protein absorbance was read by UV spectroscopy (Beckman DU 640B Spectrophotometer) at 595 nm. Mixture of unknown samples was made in the same fashion as the standards with the exception that 2µL of Unknown sample was used instead of BSA standard. The concentrations of protein samples were determined by interpolation.

2.12 Mono-Q Chromatography of P. falciparum protein farnesytransferase activity

Mono-Q chromatography was performed by modification of previously described methods (Vogt, 1995). After dialysis of the reconstituted proteins from ammonium sulfate precipitation and determination concentration, 10ug of sample were loaded onto a Mono-Q (anion exchange) HR 5/5 column (Pharmacia Biotech) that had been equilibrated with buffer B (i.e. dialysis buffer). The column was then washed with 10 mL of Buffer B and the elution method consisted of a 15 mL linear gradient from 0.02 to 0.27 M NaCl, a 10mL step maintaining NaCl at 0.27 M and a 10 mL linear gradient from .27 to 1M NaCl. The FTase was expected to
elute at a salt concentration of 270mM (Vogt, 1995). The buffer flow was maintained constant at the rate of 1 mL/min and 1 mL fractions were collected. An aliquot of 20 μL from every other fraction was used to determine FTase activity (described next). Fractions with FTase activity were pooled and concentrated using a Centricon filter with a 10kDa cut-off. Samples were spun at 3000 g until a minimal volume was reached. The concentration and activity of the partially purified FTase were determined. Samples were transferred, in 250 μL aliquots, into 1.5 mL cryogenic vials and stored at -80°C until further use.

2.13 Assay for Farnesyltransferase Activity

The scintillation proximity assay (SPA) was performed as directed by manufacturer’s recommendations (Amersham Biosciences). Protein samples incubated in assay buffer pH7.5 (50mM HEPES, 30mM MgCl2, 20mM KCl, 5mM DTT, 0.01% Triton X-100) with the laminin-B peptide and [3H] farnesylpyrophosphate for 1 hr at 37°C. The reaction is stopped by the addition of a mixture of STOP solution and reconstituted SPA bead. This mixture was left at room temperature for 30 min, followed by their count on with a Beckman LS500TD scintillation counter. The mechanism behind this assays goes as follows. If farnesyltransferase is present and uninhibited, human laminin-B carboxy-terminus sequence peptide (biotin-YRASNRSCAIM) is [3H] farnesylated at the cysteine residue. The resultant [3H]farnesyl-(CYS)-biotin laminin-B is captured by a streptavidin-linked SPA bead. The scintillation fluid in this reaction is contained within the fluoromicrospherical SPA beads, therefore photons will only
be emitted if the scintillation bead comes, and remains, in close proximity to a radioactive compound, namely $[^3\text{H}]$farnesyl attached to the laminin peptide. The high-affinity bond formed by biotin and streptavidin mediates this proximity. If farnesyltransferase is absent or it has been inhibited, the $[^3\text{H}]$farnesyl will not be attached to the laminin peptide. Therefore, no radioactive material will remain constantly in close proximity to the scintillation fluid to sustain the emission of photons. Thus, little or no counts will be observed. This mechanism is depicted in figure 7.

2.14 Cloning PfYKT6.2

PfYKT6.2 was amplified by RT-PCR using mRNA isolated from asynchronous parasites. The amplified cDNA was cloned into the T7 polymerase-driven bacterial expression vector pET30 vector using Ligation Independent Cloning according to manufacturers instructions (Novagen). Primers were designed using Primer Select (Lasergene) [forward: GACGACGACAAGATGAATTTATTAGCAA and reverse: GAGGAGAAGCCCGGTTCACATAATGCTG] and used to amplify the 627bp open reading frame. The PCR product was purified using QIAquick PCR purification kit according manufactures instructions (Qiagen). The DNA was resuspended in ddH$_2$O and quantified by Spectrometry (Beckman Coulter DU 640). T4 polymerase treatment and transformation of competent cell were performed according to manufacturer’s instructions. After plating 150µl of transformed cells on kanamycin plates (50µg/ml), colonies were allowed to grow overnight. A few colonies were selected and grown over night for mini-prep using Qiagen’s spin mini-prep kit.
Figure 7. Scintillation Proximation Assay. Technique used to assay farnesyltransferase activity as described in the text.
according to protocol. Positive clones were identified by digestion with NdeI and XhoI restriction endonucleases (promega) in a 20 µl reaction followed by sequencing (as described below). Plasmids with correct insert sequence were electroporated into BL21 DE3 electrocompetent cells using a Bio RAD Gene Pulser II, incubated in 300µl S.O.C. medium and plated on kanamycin plates. Glycerol stocks were also made.

2.15 Expression of Recombinant Protein

Bacterial cultures (BL21-DE3) were grown in 5ml LB broth over night at 37°C with shaking at 220 rpm. Overnight culture was added to 500ml LB and grown at 37°C with shaking until the optical density (at 600nm) of 0.6 was reached. At this point culture was induced by the addition of IPTG to 0.5 µM. Induction continued for eight hours at 25°C with shaking. Culture was subsequently spun at 4000 × g for twenty minutes at 4°C. Pellets were then washed in Extraction/Wash Buffer (50mM sodium phosphate, 300mM NaCl, 10% glycerol, pH 7.0) and spun at 4000 × g for twenty minutes. Pellets were stored at -80°C.

2.16 Purification of PfYKT6

Purification of recombinant proteins was performed using TALON™ Super-flow Resin (Clontech Laboratories, Inc.). Induced frozen cell pellets were thawed and re-suspendedby
vortexing in Extraction/Wash Buffer (50mM sodium phosphate, 300mM NaCl, 10% glycerol, pH 7.0) containing one tablet of EDTA free protease inhibitor/10ml (Roche). Cell lysis was achieved by passing samples through a French press (LSM-Aminco) and by sonication with a microtip for 4–15 second cycles. Cell extracts were then centrifuged at 12,000 \( \times \) g for 20 minutes at 4°C and the pellet is discarded. The supernatant was subsequently mixed with TALON™ Superflow Resin, which had been previously equilibrated with Extraction/Wash buffer, in a 50ml centrifuge tube on a platform shaker for 20 minutes at 4°C. The loaded resin is then centrifuged at 700 \( \times \) g and the supernatant is removed. The resin in washed twice with 10 bed volumes of Extraction/Wash (either Extraction/wash or Denaturing, depending on the sample) containing 20M and 50mM imidazole. After clearing the sample, the resin is transferred into a 2ml column (Cell Thru- Clontech Laboratories, Inc.) and washed with 5 bed volumes of Extraction/Wash containing 50 mM imidazole. Purified protein was then eluted by adding 10 bed volumes of Extraction/Wash containing 150 mM imidazole. Ten 1 ml fractions were collected and run on a SDS-PAGE. Fractions with greatest amount of purified protein are concentrated using a Centricon filter with a 10kDa cut-off.

2.17 DNA Sequencing

Cloned fragments were sequenced using a dye terminator kit (Beckman Coulter CEQ DTCS Quick Start). Fifty femto moles of template were used in each reaction. If the DNA to be sequenced was located in a plasmid, then the DNA was diluted in five \( \mu \)l ddH2O and heated to
96°C for one minute. This mixture was then allowed to cool to room temperature. One µl of a 5 µM primer and 4 µl of DTCS was added. After the PCR reaction, (96°C twenty sec., 50°C twenty sec., 60°C four min.) peptide sequences were precipitated by adding 2 µl of a 50:50 mix of NaOAc and EDTA. One half-microliter glycogen was added to act as a carrier. Next 60 µl of 95% EtOH at -20°C as added. This was then spun for fifteen minutes at 14,000 × g at 4°C. The precipitate was washed twice with ice cold 75% EtOH and spun for two minutes at 14,000 × g at 4°C. The pellet was then spun to dryness in a speedvac and re-suspended in 20 µl sample loading solution (Beckman Coulter). The purified sequencing product was analyzed by a Beckman Coulter CEQ 2000 XL running CEQ 2000 software. Contigs were aligned and analyzed with Megalign and Seqman.

2.18 In vitro Inhibition of Ftase Activity.

In vitro inhibition assay was performed using 2µg partially purified Ftase, 20 g recombinantly expressed and purified PfYKT6, 0.25µCi [3H] farnesylpyrophosphate (American Radiolabeled Chemicals) and buffer (50 mM Tris, 3mM MgCl, 1mM DTT, 50 MZnCl2) in a final volume of 25 µl. Inhibitor (FTI-276), where applicable, was added in a volume of 1µl and the volume of buffer was adjusted accordingly so that the final volume remained the same. Samples were mixed, incubated for one hour in 37°C water bath, resolved on a 12% SDS-
PAGE. The gel was fixed, dried and subjected to fluorography for 21 days (details in next section).

2.19 SDS PAGE, Gel Fixing and Protein Transfer

Proteins samples were denatured in 4X sample buffer (250mM Tris pH 6.8, 8% SDS, 40% glycerol, 20% p-mercaptoethanol) and boiled for 5 minutes before separating on a SDS poly-acrylamide gel. Protein samples were resolved on SDS-polyacrylamide gels according to the method of Laemmli, 1970. If the gel contains tritiated chemicals, it must be fixed with a 60:25:10 solution of Water:Ethanol:Acetic acid and with Amplify (Amersham Biosciences) for thirty minutes on a platform shaker. Gels are subsequently dried and exposed to x-ray film using an intensifying screen (Kodak BioMax MS film and BioMax TranScreen LE screen.) for 7 to 28 days at -80°C. Gels containing 35S need not be treated. They are dried and exposed to film (Kodak) for 45 min at -80°C.
3. RESULTS

3.1 Stage-Dependent prenylation of P. Falciparum Proteins

In an effort to determine the prenylation profile in *P. falciparum* parasite cultures, parasite cultures were incubated with \(^{3}H\)farnesol or \(^{3}H\)geranylgeraniol. Also, to follow the dynamics of *Plasmodium* protein prenylation with intraerythrocytic maturation radiolabeled prenols were added at 6, 20, 32 hr. post synchronization and the culture was incubated for an additional 12 hours. Parasites were released from erythrocytes by saponin lysis, and cell-free extracts were prepared from parasite pellets. Cell extracts were then resolved on SDS-PAGE and dried gels were subjected to fluorography. As can be seen from Figure 8, culture incubated with \(^{3}H\)farnesol resulted in appearance of prenylated proteins of 50 kDa or 22-28-kDa. Figure 8A indicates that prenylated proteins of these sizes are predominantly found during the trophozoite-schizont transition (lane 2) with a significantly smaller amounts in the schizont-ring transition (lane 3). During ring-early trophozoite transition (lane 1) very little prenylated proteins were present. Incubation with \(^{3}H\)geranylgeraniol indicated proteins only at the 22-28-kDa range. When compared to labeling cells with \(^{3}H\)farnesol, \(^{3}H\)geranylgeraniol labeling resulted in an ubiquitous distribution of prenylated proteins in all three time points (figure 8B). There is, still, a slightly greater amount of labeled proteins in the trophozoite-schizont transition. As a control to this experiment, uninfected RBCs were incubated with \(^{3}H\)farnesol or \(^{3}H\)geranylgeraniol.
Figure 8. Stage Dependent prenylation of parasites. Synchronized parasite cultures were labeled with [3H]farnesol (A) or [3H]geranygeranyol (B) for 16 h starting at ring (1), trophozoite (2), and schizont (3) stages.
and treated in the same way as the infected RBC samples. No signal was seen on autoradiograph, suggesting that protein prenylation in the RBC did not play a role in the results obtained from infected parasites (data not shown).

3.2 Two-dimensional separation of radiolabeled P. falciparum prenylated proteins

In a further attempt to enumerate the proteins prenylated during the intraerythrocytic stage of the malarial parasite, samples labeled with $[^3]H$farnesol or $[^3]H$geranylgeraniol at 6, 20, 32 hr. post synchronization were separated by two-dimensional electrophoresis (figures 9 and 10). Samples treated with $[^3]H$farnesol showed multiple species of proteins in both the 22-28-kDa and the 50kDa range. As before, the 50kDa range band is mainly seen in the trophozoite-schizont transition; interestingly, this band seems to be composed of various proteins with different isoelectric points. There are several proteins in the 22-28-kDa range. Some of these proteins seem to be exclusively labeled in either the trophozoite-schizont transition or schizont-ring transition. The two-dimensional separation of $[^3]H$geranylgeraniol-treated samples did not appear as resolved as the separation of samples treated with $[^3]H$farnesol. Still, the 22-28kDa band seems to be composed of proteins of various sizes and charges. Samples from the trophozoite-schizont transition and schizont-ring transition seem to have greater numbers of basic proteins when compared with sample from the ring-early trophozoite transition.
Figure 9. Two-dimentional separation of proteins labeled with $[^3]H$ farnesol during erythrocytic stages of differentiation. Parasites were labeled with $[^3]H$farnesol during the ring (A) trophozoite (B) and schizont (C) stages of *P.falciparum* growth. $^{14}C$-labeled molecular weight markers (myosin, 200,000; phosphorylase b, 97,400; bovine serum albumin, 68,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and lysozyme, 14,300) appear at the basic edge of the autoradiographs. The arrows represent the proteins, which are observed uniquely at that stage of differentiation.
Figure 10. Two-dimensional separation of proteins labeled with $[^3]$H geranylgeraniol during erythrocytic stages of differentiation. Parasites were labeled with $[^3]$Hfarnesol during the ring (A) trophozoite (B) and schizont (C) stages of *P.falciparum* growth. $^{14}$C-labeled molecular weight markers (myosin, 200,000; phosphorylase b, 97,400; bovine serum albumin, 68,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; and lysozyme, 14,300) appear at the basic edge of the autoradiographs. The arrows represent the proteins, which are observed uniquely.
3.3 Mode of prenylation of *P. falciparum* Proteins

Once the prenylation profile of *P. falciparum* had been established, we focused on the prenyl composition of its major bands. We wanted to investigate whether the detected bands are farnesylated or geranylgeranylated. Asynchronous culture was labeled with \[^{3}\text{H}]\text{prenols and proteins were separated by SDS-PAGE. Gels segments corresponding to prenylated proteins are treated with methyl iodide (releases the prenyl moieties as free prenols) followed by reverse phase thin layer chromatography (TLC) analysis. The results from this analysis revealed that prenols released from the 50-kDa band, are only farnesol (figure 11). In contrast, the prenols released from each of the three bands in the 22-28-kDa range is geranylgeraniol, regardless of whether \[^{3}\text{H}]\text{farnesol (figure 4) or [}^{3}\text{H]}\text{geranylgeraniol is used as the prenol precursor and of stage of maturation of the culture.}

3.4 Inhibition of *P. falciparum* growth by prenyltransferase inhibitors.

We decided next to determine the efficacy of peptidomimetic inhibitors FTI-277, GGTI-298 and GGTI-286 as antimalarials. For this, culture is grown in media supplemented with \[^{3}\text{H}]\text{hypoxanthine and incubated with inhibitor under study. Hypoxanthine is a purine precursor and cell cycle progression (i.e. DNA and RNA synthesis) requires its uptake. Peptidomimetic inhibitor concentrations were 125\text{µM, 12.5µM, 1.25µM, 125nM, 12.5nM. Each treatment was done in triplicate. Incorporation of [}^{3}\text{H]}\text{-hypoxanthine into nucleic acids following treatment...}
Figure 11  Mode of prenylation of P. falciparum proteins. Proteins in asynchronous cultures of P. falciparum were radiolabeled with \[^{1}H\text{farnesol. Gel segments corresponding to the indicated labeled prenylated proteins were cut out. The prenyl moieties were released as free prenols and separated by reverse phase TLC. The mobilities of farnesol (FOH) and geranylgeraniol (GGOH) standards are shown.}
with peptidomimetics were plotted in graphs described in figures 12 thru 14. The IC$_{50}$ values were quite similar for all three inhibitors tested - around 5µM.

3.5 **Morphological changes in inhibitor treated parasites.**

After we had established that peptidomimetic inhibitors indeed have an effect on Plasmodial replication and protein prenylation, it became imperative to study their effects on cell cycle progression. Synchronous culture at ring stage was treated with either 5µM or 10µM FTI-277 and stained thin smears were made 24 h thereafter (figure 15). Parasites treated with inhibitor contained an enlarged vacuole. Control parasites at 24hr. post treatment appeared much to be further along in the life cycle whereas inhibitor treated parasites seem incapable to mature past the early trophozoite stage.

3.6 **Immunofluorescence microscopy of inhibitor treated parasites.**

To further study the subcellular effect of prenyltransferase inhibitors in *P.falciparum*, we used a commercially available rabbit farnesyl polyclonal antibody in an indirect immunofluorescence experiment. The farnesyl-specific polyclonal antibody recognizes a farnesyl-cysteine epitope and may have the possibility cross react with the geranylgeranyl-
Figure 12. Inhibition of [³H]hypoxanthine incorporation by FTI-277. IC₅₀ concentration for FTI-277 is approximately 5µM.
Figure 13. Inhibition of $[^3]$Hypoxanthine incorporation by GGTI-298. IC$_{50}$ concentration for FTI-298 is approximately 5µM.
Figure 14. Inhibition of [{\textsuperscript{3}}H]hypoxanthine incorporation by GGTI-286. IC\textsubscript{50} concentration for GGTI-286 is approximately 5\muM
Fig. 15. Effect of FTI-277 on the intraerythrocytic maturation of *P. falciparum*. A synchronized parasite culture was treated with 5 µM FTI-277 at the late ring stage. Panel 1, control culture containing 2.5% dimethyl sulfoxide at 0 h; panel 2, control culture after 24 h; panel 3, culture containing 5 µM FTI-277 after 24 h.
cysteine epitope. Synchronous culture at ring stage was incubated with both FTI-277 and GGTI-298 in either 5µM or 10µM concentrations for 24 and 36 hours. As can be seen in figure 16 the blue color represents DAPI stained DNA and indicates the position of the nucleus and the green represents the location of prenylated proteins. The control samples show normal malarial development: at 24 hours the parasite has progressed into the mature trophozoite stage and at 36 hours the parasite is in the segmenter stage, where partitioning of the nuclear material precedes merozoite formation and erythrocyte rupture. Treatment of both FTI-277 and GGTI-298 yielded similar results for each concentration. Treatment of inhibitors at a concentration of 5µM seems to substantially decrease the amount of prenylated proteins within the parasite whereas at 10µM fluorescence-labeled prenylated proteins are almost completely disappears. The inhibition of cell cycle progression is clearly visible when one compares the slides from the control samples with that of slides treated with 5µM inhibitor, where no evidence of nuclear segmentation can be seen. The inhibitor stops the cell cycle at the trophozoite stage with an enlarged vacuole. Treatment of parasites with 10µM inhibitor completely disrupts parasite maturation, resulting in an uncharacteristically small and un-segmented nucleus.

3.7 Inhibition of invivo Radiolabeling of P. falciparum Prenylated Proteins.

Next, we tested the effect of prenyltransferase inhibitors on protein prenylation in P. falciparum. Synchronous culture at the ring stage was incubated with radiolabeled isoprenol groups in
Figure 16. Localization of prenylated proteins. Panel I, the synchronized control culture matured from the ring stage (0 h) to late trophozoites (24 h) to segmenters (36 h). The panel for 24 h shows two trophozoites in an erythrocyte. Panel II, 5 µM FTI-277 exposed cells at 24 and 36 h treatment. Panel III, 10 µM FTI-277 exposed cells at 24 and 36 h treatment; panel IV, 5 µM GGTI-298 exposed cells at 24 and 36 h treatment. Panel V, 10 µM GGTI-298 exposed cells at 24 and 36 h treatment.
presence of either FTI-277 or GGTI-298. The concentration of inhibitor was based on the results from the hypoxanthine assay. FTI-277 and GGTI-298 were used at the following final concentrations: 1µM, 5µM, 10µM and 25 µM. Figure 17 shows a labeling profile that is congruent to one described previously, where samples treated with either [3H]farnesol or [3H]geranylgeraniol have multiple bands between 22-28-kDa and samples treated with [3H]farnesol had an additional band at 50kDa. A significant loss of labeling was observed at 5µM and complete loss was observed at 25µM. Interestingly, these effects on labeling were observed regardless the inhibitor used with [3H]farnesol or [3H]geranylgeraniol. Thus both FTI-277 and GGTI-298 can inhibit overall protein prenylation in the malarial parasite equally well.

3.8 Effect of prenyltransferase inhibitors on protein synthesis in P. falciparum

Once we had established that treatment of parasitic culture with peptidomimetic inhibitors showed a significant effect on parasite maturation, we investigated whether the effect was due to specific inhibition of the protein prenyltransferase or due to non-specific inhibition causing a complete breakdown of protein synthesis. For this, we allowed parasitized RBC’s to grow in media containing [35S] methionine and [35S] cysteine. Inhibitors were added to a final concentration of 5µM and 10µM. After 24 hr. incubation, the samples were resolved on SDS-PAGE and the gel was subjected to autoradiography. Interestingly, none of the peptidomimetic
Figure 17. Inhibition of \textit{in vivo} radiolabeling of \textit{P. falciparum} prenylated proteins with peptidomimetics. Asynchronous cultures of \textit{P. falciparum} were labeled with 10 \( \mu \)Ci of [\(^3\)H]farnesol or [\(^3\)H]geranylgeraniol for 24 h in the presence or absence of different concentrations of the FTI-277 (A) or GGTI-298 (B). Labeled parasite proteins were isolated and resolved by SDS-PAGE. Radiolabeled prenylated proteins were detected by fluorography.
inhibitors used showed an effect on protein synthesis. This can be inferred by comparing the lanes that contain samples incubated with inhibitor with the control lane (figure 18).

3.9 Identification of potentially prenylated proteins in *P.falciparum*.

Identification of proteins that may be prenylated in the malarial parasite was obtained by performing an *in-silico* search of the genome for possible prenylation targets with a c-terminus sequence of CAAM (where C stands for cysteine, A stands for any aliphatic amino acid and M stands for methionine). We have chosen this as the search sequence because it was observed in our collaborator Dr. Allen’s laboratory that CAAX peptide sequences with terminal methionine residue serves as the best substrates for Plasmodium farnesyltransferases. The search yielded, among many others, a predicted polypeptide with the C-terminus sequence of CCSIM. The BLAST search of this protein showed a high homology to yeast SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) YKT6 sequence. In yeast, YKT6 has been implicated in several trafficking steps, including vesicular transport from the endoplasmic reticulum (ER) to the Golgi, intra-Golgi transport, and homotypic vacuole fusion (Zhang, 2001). YKT6 is also the only SNARE protein that is prenylated.
Figure 18. Effect of prenyltransferase inhibitors on protein synthesis in *P. Falciparum*. Parasitized RBCs were grown in media containing $[^{35}\text{S}]$ methionine and $[^{35}\text{S}]$ cysteine. Inhibitors were added to a final concentration of 5mM and 10mM. After 24 hr. incubation, samples were resolved on SDS-PAGE and the gel was subjected to autoradiography.
3.10 Cloning of *Pf*YKT6

The open reading frame for *Pf*YKT6 was amplified from *P. falciparum* cDNA using primers (forward: GACGACGACAAGATGAATTTATTAGCAA and reverse: GAGGAGAAGCCCGGTTCACATAATGCTGC) which also contained the ligation-independent cloning sequences required for inserting into Novagen's pET30 EK/LIC vector. The PCR reaction yielded a fragment of correct size (figure 19). The fragment was subsequently cloned into the pET30 EK/LIC vector, electroporated into XL1Blue competent cells, and plated onto kanamycin plates. Clones with correct insert were determined by digestion and sequencing. Positive clones were electroporated into *E. coli* BL21 DE3 host cells and an overnight culture was made in preparation for test expression.

3.11 Test expression of *Pf*YKT6

A small amount of the overnight culture was used to inoculate fresh test tubes LB media. These test tubes were incubated at 37°C until they reached an optical density (OD$_{600}$) of 0.6. At this time a small volume of the sample was set aside for later testing. IPTG was added to a final concentration of 1mM and test tubes were divided into two groups. One group was incubated at 37°C and the other was incubated at 26°C. All samples, were subsequently resolved on a SDS-PAGE gel and subjected to immunoblotting using anti-his antibodies (figure 20).
3.12 Induction and Purification of PfYKT6

PfYKT6 was purified using Talon cobalt His binding resin. To obtain sufficient material a 500 ml culture was induced with 0.5mM IPTG for eight hours at 25°C. Figure 21 shows a SDS-PAGE gel analysis of the purified and concentrated sample. Despite the presence of a few contaminating proteins, we achieved a considerable enrichment of PfYKT6, which yielded approximately 500µg with a final concentration of 0.99µg/µl. Some precipitation was detected after concentration, which may have reduced the actual yield of soluble protein in the final sample. All fusion proteins were detected by western blot analysis using antibodies against the His-tag sequence (data not shown).

3.13 In Vitro prenylation of PfYkt6.

In an effort to test whether PfYKT6 is indeed prenylated we conducted an in-vitro prenylation assay. For this, purified recombinant PfYKT6, a potentially farnesylated protein found in the plasmodial genome, was incubated with partially purified farnesyltransferase. We also tested whether or not the prenylation of PfYkt6 is inhibited by inhibitors of protein prenylation. We used 4µg of MONO-Q purified plasmodial protein farnesyltransferase, 20 µg PfYKT6 and 240 nCi and [3H] FPP. Inhibitor was used in 1nM, 5nM, and 10nM concentrations.
Figure 19. Agarose gel electrophoresis of P/YKT6 PCR product used in cloning

Figure 20. Western blot analysis of P/YKT6 test expression. Solubility of P/YKT6 is achieved with expression at 26°C. Panel A. Expression carried out at 37°C. Panel B. Expression carried out at 26°C. Lane 1. Uninduced crude lysate. Lane 2. Soluble fraction of induced sample. Lane 3. Insoluble fraction of induced sample.
Figure 21. SDS-PAGE analysis of PfYKT6 TALON purification. Lane 1. Uninduced crude lysate. Lane 2. Induced crude lysate. Lane 3. Purified and concentrated PfYKT6. Arrow indicates band corresponding to PfYKT6.
The autoradiograph (Figure 22) indicates that *in-vitro* prenylation of PfYKT6 occurs and that the inhibitor FTI-276 does exert a negative effect on this prenylation.
Figure 22. *In Vitro* inhibition of plasmodial protein farnesyltransferase. Purified PfYKT6 was incubated with partially purified farnesyltransferase in the presence and absence of peptidomimetic inhibitor FTI-276. Lane 1. Control sample containing 4µg of MONO-Q purified plasmodial protein farnesyltransferase, 20 µg PfYKT6 and 240 nCi and [3H] FPP. Lane 2. Sample containing additional 1nM FTI-276. Lane 3. Sample containing 5nM FTI-276. Lane 4. Sample containing 10 nM FTI-276.
4. DISCUSSION

Major pharmaceutical companies are intensively developing inhibitors of mammalian protein farnesyltransferase because such compounds show great promise as anti-cancer agents in experimental animal models. The vast knowledge database and diversity of putative prenyltransferase inhibitors makes researching the effects of such inhibitors in other organisms (such as *Plasmodium*) an attractive proposition. This "piggy back" approach is very important for the development of drugs to treated tropical diseases in developing countries because it will lower the price of these drugs due to the minimal amount of venture capital invested in their design. This is a crucial point since most of people affected by malaria reside in third world countries and thus cannot afford the price of new drugs.

The ultimate goal of is to find an inhibitor that would have a higher affinity for plasmodial farnesyltransferase instead of its human counterpart. Previous studies performed in our laboratory have indicated that PfPFT inhibitors affect plasmodial life-cycle (Chakrabarti, 1998). Thesepeptidomimetic inhibitors, however, showed **IC\(_{50}\)** concentrations in the micromolar range and therefore may not be attractive as a lead compound.is. An in-depth understanding of the prenylation mechanisms in Plasmodium is crucial for the identification of an optimal inhibitors. There are differences between Plasmodial and Mammalian metabolism which are of importance when considering inhibitor design. *P. falciparum* lack a *de novo* pathway for cholesterol synthesis (Mbaya, 1990; Vial, 1984) and Plasmodial isoprenoid metabolism has a closer resemblance to that of plants, utilizing the methyl erythritol phosphate biosynthetic pathway instead of the mevalonate pathway (Arigoni, 1997; Flesch, 1988). Studies on
metabolic labeling of prenylated proteins with radiolabeled prenols in mammalian cells have shown that both $[^3]{\text{H}}$farnesol and $[^3]{\text{H}}$geranylgeraniol can be used to label proteins (Crick 1994; Crick 1995). Prenols are used because the diphosphate derivatives would not be able to penetrate the cell membrane. The ability to utilize prenols in such labeling experiments depends on enzymes that convert prenols into their diphosphate derivative (Crick, 1997).

Incubation of *P. falciparum*-infected erythrocytes with either $[^3]{\text{H}}$farnesol or $[^3]{\text{H}}$geranylgeraniol yielded several radiolabeled protein bands on a SDS-PAGE. Labeling with $[^3]{\text{H}}$farnesol showed a prenylation profile that included proteins at 50-kDa and between 22-28 kDa range. Prenylation was most prevalent during trophozoite to schizont and schizont to ring stages of differentiation, whereas few prenylated proteins are observed in the ring to trophozoite stage. Labeling with $[^3]{\text{H}}$geranygeranyl showed a prenylation profile that was confined to the 22-28 kDa range. The level of geranylgeranylation does not fluctuate as much as the level of farnesylation throughout the lifecycle. Similar to farnesylation, geranylgeranylation was at its lowest level during the ring to trophozoite transition. In 1998, Chakrabarti et. al. demonstrated significant PFT and PGGT-I activity in all stages of Plasmodial intraerythrocytic development, thus suggesting that alterations in the prenylation profile is due to a decrease in the production of protein substrates for prenylation.

Studies on the mode of prenylation pursued in this thesis indicated that the 50-kDa band was predominantly labeled with $[^3]{\text{H}}$farnesol. Proteins in the 22-28 kDa range were almost exclusively geranylgeranylated, regardless of whether cells were incubated with $[^3]{\text{H}}$farnesol or $[^3]{\text{H}}$geranylgeraniol. Evidently, the radiolabeled prenols are phosphorylated to their diphosphate
derivatives and used for prenylation. It is also apparent that some of the $[^3\text{H}]$farnesyl diphosphate is elongated to $[^3\text{H}]$geranylgeranyl diphosphate to account for the appearance of $[^3\text{H}]$geranylgeranyl labeled proteins in 22-28-kDa range isolated from cells labeled exclusively with $[^3\text{H}]$farnesol.

Treatment of plasmodial cell cultures with peptidomimetic inhibitor FTI-277 caused a block in maturation of the parasite during the trophozoite stage and the appearance of an enlarged vacuole in retarded parasites. These morphological disturbances are quite novel and were not seen in earlier studies with different peptidomimetic inhibitors (Chakrabarti, 1998).

The indirect immunofluorescence data indicates the presence of prenylated proteins in all stages of *P. falciparum* intraerythrocytic lifecycle. These proteins are usually contained at distinct subcellular foci. In the segmenter stage, these foci seem to encircle chromosomal units that will eventually be organized into individual nuclei. It is to be noted that at the IC50 concentration of 5 µM FTI-277 or GGTI-298 (panels II and IV, Fig. 16), separation of chromosomes into individual nuclei is affected and the whole chromosomal structure appears to be in the form of an unsegregated mass. The prenylation activity data, which shows insignificant prenylation activity in early ring stage, can be combined with the indirect immunofluorescence data, which indicates the presence of prenylated proteins in rings, to suggest a low turnover rate of pre-existing prenylated proteins and that proteins made during the early to late trophozoite stages may be critical for the trophozoite to schizont transition and the viability of the parasite.

Previous studies have reported cell-cycle or differentiation dependent protein prenylation in synchronized HepG2 cells (Sepp-Lorenzino, 1989) and in the seminiferous epithelium of rats
at different stages of spermatogenesis (O'Farrell, 1975). The latter study led the authors to suggest that protein prenylation could constitute an obligatory step leading to the duplication of the cellular genome. Studies on FTI-277 account its effects on cell cycle such as G0/G1 or G2/M block, depending on the cell line. Similarly, studies on GGTI-298 activity report its ability to halt the cell cycle at G0/G1 (Sebti, 2000; Tamanoi, 2001). PFT inhibitor FTI-2153 has been shown to inhibit formation of bipolar spindle formation and chromosome alignment (Crespo, 2001) and SCH66336 inhibits microtubule association of farnesylated kinetochore proteins CENP-E (Ashar, 2000). At this point we believe that our results indicate that FTI-277 inhibit plasmodial lifecycle in a manner analogous to what is seeing in mammalian cells, where bipolar spindle formation is inhibited following PFT inhibitor treatment.

The resolution of malarial samples in a 2-D gel yielded a novel observation for parasites: the appearance distinctive prenylated proteins during specific stages of \textit{P. falciparum}. Proteins in the 50-kDa range were predominately found in the in trophozoite stage and appeared to be of the same molecular weight but different charge. These mobility differences are probably caused by differences in phosphorylation – a scenario described in previous publications describing multiple prenylated proteins of the same molecular weight that appeared to be phosphorylated (Dugan, 1995; Sepp-Lorenzino, 1991). Prenylated proteins of various charges and molecular weights were also observed in the 22-28-kDa range from parasites at both the trophozoite and schizont stages. Although several proteins appear to be common to the two stages, judging from their position of migration, there is differential labeling with some and at least one protein is labeled specifically at the schizont stage.
The specificity of PfPFT for the amino acid at the carboxyl terminus has similarities with mammalian PFT, where peptides ending in methionine and glutamine are substrates. However, plasmodial prenyltransferase specificity differs from its mammalian analogue in that peptides ending in serine, threonine, and cysteine are not substrates. Plasmodial Farnesyltransferase specificity has a greater similarity to specificities of other parasites’ prenyltransferases such as *T. brucei* and *Leishmania amazonesis*, which farnesylate peptides terminating in methionine and glutamine most effectively and have poor specificity for peptides terminating with other amino acids (Buchner, 2000; 2002). This data, however, only indicates part of the equations. Buckner et al. (28) have demonstrated that the specificity of the T. brucei enzyme changed when a CAAX containing peptide with different amino acid sequence was used.

Previous studies done in our lab have show that PFT and PGGT-I inhibitors are both effective at inhibiting PfPFT in vitro activity (Chakrabarti, 1998). Our study has shown that such inhibitors also have an adverse effect in labeling of P. falciparum proteins in culture with disregard to what radiolabeled prenol substrate is used. This unusual characteristic of PfPFT implies the potential for development of PFT inhibitors with specificity for the malarial parasite.

In order to gain a deeper understanding of the specificity of malarial PfPFT and characterization of its substrates, we designed an *in vitro* prenylation assay with MonoQ purified malaria extract containing PfPFT and a recombinantly expressed malarial protein that would serve as a substrate. This substrate, PfYkt6, was identified through an *in silico* search of the malarial genome. We were able to show that the substrate is indeed prenylated in vitro by the purified enzyme and that the farnesyltransferase inhibitor FTI-276 could inhibit this reaction.
Thus we have been able to establish a basic understanding based on which future research could be done on the specificity of malarial prenyltransferase. Our attempts at further characterization of PfYkt6 substrate met with many difficulties. The substrate protein was highly insoluble and repeated attempts at purifying it under denaturing conditions resulted in precipitation of product during the refolding. The yield of soluble protein was small and multiple cycles of large scale purification were required to obtain viable data (data not shown). The purification of at least two other possible PfPFT substrates, PfPTP and PfDnaJ, resulted in greater difficulties. Future attempts will focus on purification of these substrate proteins through GST-tag instead of His-tag. Substrates with variant CAAX sequence were also made using site directed mutagenesis (data not shown). Studies being currently conducted in our lab with these mutated substrates will take us one step closer to understanding malarial prenylation and its role on the malarial life cycle.
LIST OF REFERENCES


