Antiretroviral Drugs Inhibit Malaria

Signal Peptide Peptidase

A Thesis Submitted by

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ABSTRACT

Malaria is listed on the CDC website[1] as one of the more deadly diseases in the world today. According to the World Health Organization, the global disease burden for malaria in 2015 was at about 214 million new cases with the African region accounting for 88 percent of these cases[2]. Malaria is caused by a parasitic protozoan known as Plasmodium that grows and reproduces within the blood of its host organism after an initial multiplication stage in the liver. It is transmitted via a carrier organism, also known as a vector. The female Anopheles mosquito is responsible primarily for propagating the malaria parasite during pregnancy as she craves human blood. The malaria transmitting Anopheles mosquito is prevalent in regions prone to malaria. Upon completion of parasite differentiation within the mosquito gut, the parasite is carried in the mosquito saliva and transferred to its human host when a mosquito bite occurs. Once within the host, the parasite undergoes a series of developmental phases that involve two stages; the liver stage and the blood stage, the liver stage occurring first. In the liver stage multiplication occurs followed by maturation and growth, asexual reproduction and subsequent release of the parasite into the blood. The hibernation process occurs where parasites lie dormant within the hepatocytes for extended periods during the liver stage. Parasite hibernation could last up to a year. In the blood stage, red blood cells (RBCs) are invaded by parasites that grow, mature and reproduce asexually resulting in the continuation of the malaria life cycle. Differentiation and sexual reproduction also occur in this stage. While various species of the malaria parasite have been identified for multiple hosts, four species are common for infection in humans: *P. falciparum, P. vivax,*

P. ovale, and P. malariae. The most lethal form being *P. falciparum.* Zoonotic infection by monkey *P. knowlesi,* is also common in certain parts of Asia and Africa.

Considering the emergence of drug resistance and given the high morbidity and mortality caused by the most lethal malaria species *P. falciparum,* there is an urgent need to develop new drugs and vaccines against malaria.

Signal Peptide Peptidase (SPP) is a membrane-embedded enzyme that functions to cleave transmembrane proteins, primarily in the endoplasmic reticulum (ER) of cells, releasing them to participate in multiple signaling and regulatory activities. In *P. falciparum,* SPP plays a significant role in cleaving transmembrane substrates as a clearing mechanism to reduce ER stress within the parasite. This process is a key step in the survival and further development of the parasite within its host. Failure to cleave transmembrane proteins by *P. falciparum* SPP could result in parasite death due to ER stress. This key feature of *P. falciparum* Signal Peptide Peptidase (P*f*SPP) has made it an appealing target for pharmacological intervention.

A number of antiretroviral drugs function as aspartic protease inhibitors. These drugs are known to inhibit the development of the Human Immunodeficiency Virus (HIV) since the virus relies on aspartic proteases for survival[3]. In the present study, we tested the effects of aspartic protease inhibitors on malaria parasite infected erythrocytes. Our results show that aspartic protease inhibitors inhibit P*f*SPP resulting in the death of intraerythrocytic malaria parasites. Therefore aspartic protease inhibitors have been

identified as effective antimalarial drugs with the potential to be used in combination therapy against malaria.

DEDICATION

This work is dedicated to Mr. Walter Okure my hero who gave me a one-way ticket to The United States of America forever changing the course of my life for good, and for teaching me the priceless skill of fervent prayer a gift that keeps on giving.

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I would like to thank Dr. David Greenblatt for giving me the opportunity to earn this degree at Tufts University, Professor Margery Beinfeld a source of great knowledge and support, Dr. Baleja for advice, Dr. Alexei Degterev and his laboratory for help and input. I would like to thank Professor Athar Chishti, my project mentor, and his laboratory for being a receptacle of incessant learning and growth. I would also like to thank Dr. Jennifer Nwankwo for words of encouragement, and Dr. Gbolarumi Adebayo my long time career advisor and confidant for consistent and unrelenting support.

A great deal of appreciation goes to Professor S. J. Okure my tenacious dad for teaching me the importance of acquiring a good education, my mom Mrs. Margaret S Okure for teaching me the value of being a virtuous woman, and Dr. Maxwell Okure my genius brother for igniting within me a passion for science. Mrs. Victoria Udoh my sister for advice and life lessons, and Mrs. Marianne Olaniran my sister for friendship and understanding.

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LIST OF ABBREVIATIONS

Ataz	Atazanavir
Ctrl	Control
DI	Deionized Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ER	Endoplasmic Reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
Fos	Fosamprenavir
HEK293-T	Human Embryonic Kidney Cells 293T
HIV	Human Immunodeficiency Virus
HSP101	Heat Shock Protein 101
i-CLiPs	Intermembrane Cleaving Proteases
iRBC	Infected Red Blood Cells
LL2-Ketone	2,2-(2-oxo-1, 3-propanediyl) bis [(phenyl methoxy) carbonyl] L-leucinamide ketone
Lop	Lopinavir
Mut	Mutant
NaCl	Sodium Chloride
P. *falciparum*	Plasmodium *falciparum*
P*f*SPP	Plasmodium *falciparum* Signal Peptide Peptidase
Pyri	Pyrimethamine

Quin	Quinine Sulfate
RBC	Red Blood Cells
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute (medium)
RT	Room Temperature
SPP	Signal Peptide Peptidase
SPPL2A	Signal Peptide Peptidase Like-Protein 2A
SPPL2B	Signal Peptide Peptidase Like-Protein 2B
SPPL3	Signal Peptide Peptidase Like-Protein 3

Chapter 1

INTRODUCTION

1.1 Malaria Infection, Parasite Life Cycle, and Epidemiology

Malaria is caused by a bite from a mosquito infected with the Plasmodium protozoan. Approximately 3.3 billion people in several countries across the world, specifically in Sub-Saharan Africa, South and Central America, Southern Asia and parts of the Middle East are prone to malaria burden and mortality. This in part is because mosquitoes breed in stagnant tepid water and as a result thrive in warm and swampy climates. Malaria is transmitted by the female Anopheles mosquito and transmission occurs when a pregnant mosquito infected with the plasmodium protozoan parasite bites a human being during a blood meal. The vector carries the parasite in its saliva as sporozoites and transfers them to its host. Human infection occurs both in the liver and blood stages. In the liver stage, the sporozoites undergo repeated cycles of asexual reproduction increasing exponentially. The mature form of the parasite, known as a schizont, develops and differentiates into merozoites within the hepatocytes. Merozoites are released into the blood stream where they consequently invade RBCs initiating the blood stage of infection[4].

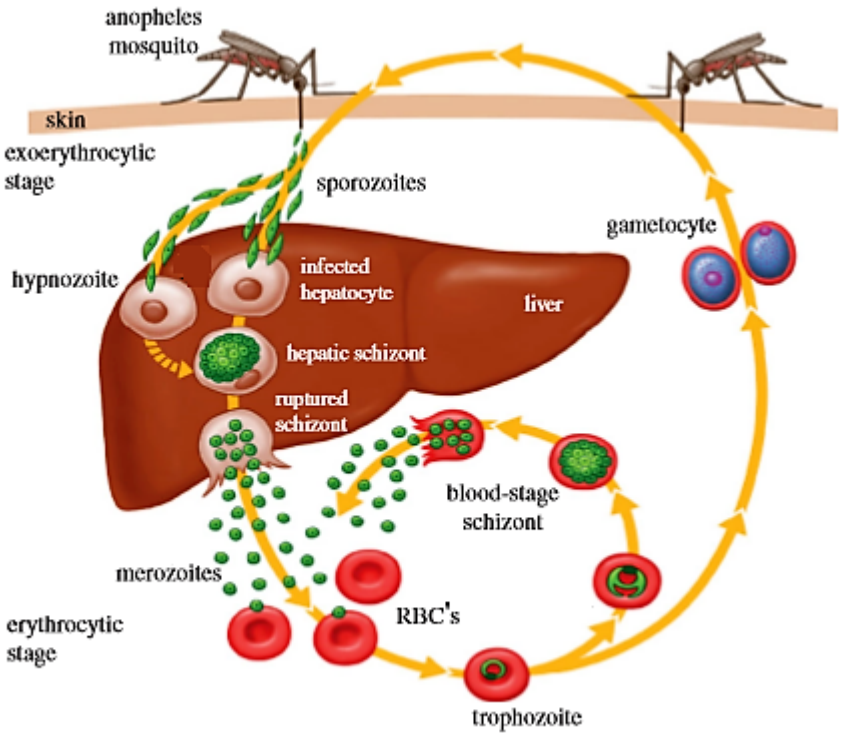

FIGURE 1.1 Malaria Life Cycle within its human host. (Adapted from Hill Adrian VS. (2011) vaccines against malaria)

After invasion of RBCs, the parasite develops into a ring stage, and then matures to trophozoites and schizonts stages, undergoing asexual differentiation into merozoites. Upon rupture of the infected RBCs, the merozoites are released into the blood stream to continue the invasion-growth and reinvasion life cycle within the RBCs. Some parasites from the ring stage will further differentiate into gametocytes with the ability to reproduce sexually within the vector. The sexual reproduction stage of the cycle occurs in the gut of an infected mosquito. When a non-carrier female mosquito bites an infected human, it ingests some of the gametocytes present in human circulation. Gametocytes migrate to the gut of the mosquito where the sexual reproduction occurs and zygote

sporozoites are formed in the vector. The mosquito will later transmit these sporozoites into the human host during a subsequent blood meal repeating the cycle.

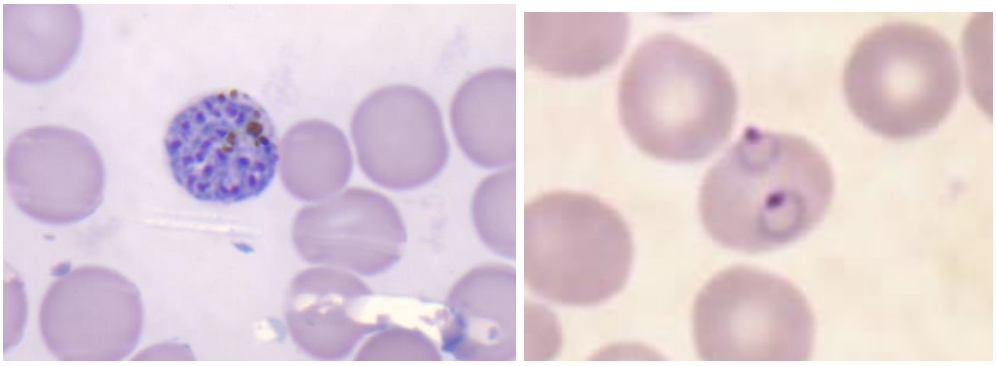

FIGURE 1.2 Malaria parasite development. Parasite cycle stages within an infected human erythrocyte showing the schizont (Left) and ring stages (Right). (Adapted from Athar Chishti's Malaria Lecture PowerPoint for Translational Pharmacology at Tufts University 2016).

The malaria parasite *P. falciparum* is able to invade all stages of erythrocytes[5], and completes a 48 hour life cycle within RBCs, starting with invasion, maturation, replication, lysis and reinfection. Malaria symptoms observed in patients originate from the life cycle of the malaria parasite in RBCs. Clinical symptoms of *P. falciparum* infection are caused by chronic inflammation presented as repeated waves of fever and chills every 48 hours. Moreover, *P. falciparum* species remodels the host membrane by inserting multiple malaria proteins thus changing the mechanical properties of the parasitized erythrocytes[6]. One unique feature of *P. falciparum* is its ability to insert an adhesion receptor termed *Plasmodium falciparum* erythrocyte membrane protein 1

(P*f*EMP1) into infected red blood cells (iRBCs) at the trophozoite-schizont stage[6]. This remodeling enables the parasitized erythrocytes to bind to the vascular endothelium avoiding the spleen while growing in a low oxygen microenvironment. The cytoadherence of infected red blood cells results in an accumulation of cells that causes obstructions within the vasculature ensuing pathology and leading to possible organ failure. A brain occurrence of microvascular blockage could result in cerebral malaria a potentially fatal condition. Cerebral malaria is more common in pregnant women and infants[7].

1.2 **Function of Signal Peptide Peptidase**

Signal Peptide Peptidase (SPP) is found in vertebrates, plants and eukaryotes. One of its known functions is to cleave structural transmembrane proteins freeing them to act as signaling molecules for further activation. Signaling molecules are peptide ligands attached to transmembrane proteins targeted towards activation of specific secretory downstream activities when cleaved. The transmembrane cleavage is involved also in regulatory clearance of membrane proteins. This activity reduces stress and prevents subsequent apoptosis. SPP cleaves remnant signal peptides in the endoplasmic reticulum (ER), golgi apparatus, lysosome and plasma membrane for multiple signaling processes. Mammalian SPP belongs to a family of intermembrane cleaving proteases that includes other protease homologues for example the SPP-like proteases and presenilins[8].

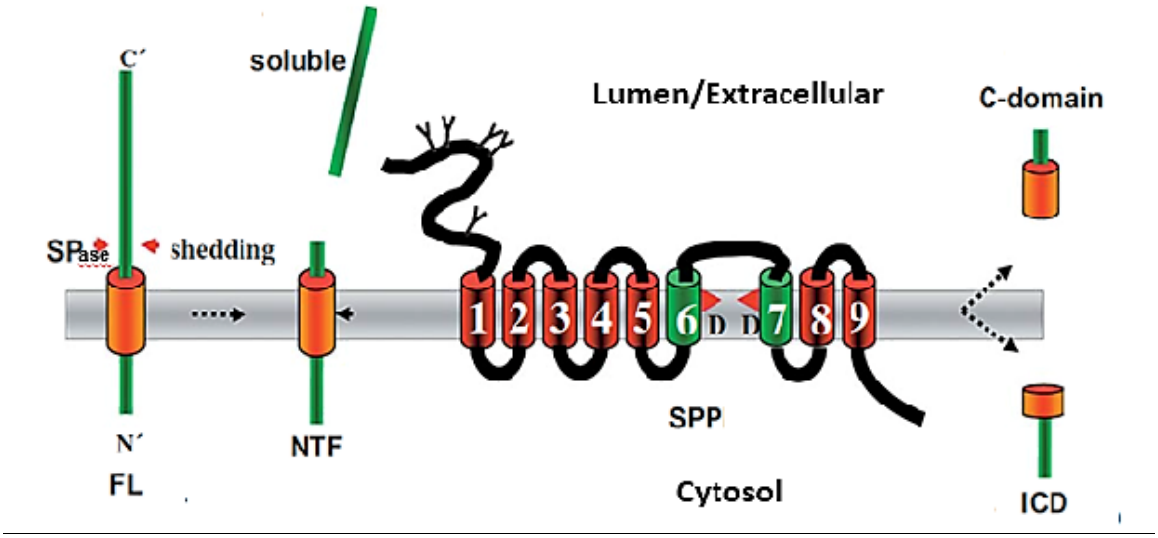

FIGURE 1.3 SPP Cleavage Mechanism. SPP cleavage mechanism is conserved across intramembrane cleaving proteases[8]. (Adapted from Fluhrer, R., and Haass, C. (2007). Neurodegenerative Diseases 4, 112-116)

Humans express five different SPP types localized across the vesicular trafficking network[9]. They are known as intermembrane cleaving proteases (i-CLiPs). Other known i-CLiP peptides include Presenilin, BACE1, ADAM 10, gamma secretase, SPP like protease 2, SPP like protease 3, and ADAM 17[8]. These are structurally different from SPP. SPP cleaves a variety of substrates such as prolactin, MHC class I, calreticulin, pro-calcitonin, TNFα, Bri2 and FasL. Cleaved signal peptides play post-targeting signaling functions. SPP is also associated with cleaving misfolded transmembrane proteins freeing them to be targeted for degradation which triggers other downstream homeostatic and stress reducing responses initiated via the unfolded protein response mechanism[10]. SPP regulates protein N-glycosylation (SPPL3) via the degradation of Golgi

glycosyltransfereases and regulates B-cell inflammatory responses (SPPL2A and 2B). It is also essential for development across several species[5].

The Plasmodium protozoan contains a single gene of SPP[11]. The Plasmodium *falciparum* SPP (P*f*SPP) gene is highly conserved across several species of malaria like the *P. vivax*, *P. knowlesi*, *P. berghei,* and *P. malariae*. It is also conserved across several strains of *P. falciparum*; 3D7, 7G8, Dd2, HB3, K1 and FCR3. It is noteworthy that the malaria parasite genome encodes multiple proteases, however, no single protease is essential for survival of the parasite in its human host. In contrast, the genetic inactivation of a single SPP gene in *P. falciparum* results in lethality underscoring the critical importance of this protease for the parasite life cycle[12].

1.3 HIV Aspartic Protease Inhibitors as Antimalarials

P. falciparum Signal Peptide Peptidase has been established as a critical molecule in the blood stage of malaria parasitemia[11]. Studies indicate that P*f*SPP cleaves membrane bound substrates in the ER during protein processing clearing the ER and enhancing continuation of the parasite life cycle[11].

Suppression of P*f*SPP activity in the ER could mean halting the ER clearing process leading to accumulation and ER stress mediated cell death (Note: mature RBCs do not have any organelles. The ER stress originates from the parasite induced pathways.) It would also prevent residue fragments cleaved by P*f*SPP from release into the cytosol and

extracellular lumen acting as signaling molecules to further propagate the life cycle. Only one SPP gene is expressed by *P. falciparum*, which makes this enzyme a very attractive drug target.

HIV aspartate protease inhibitors have been proven to exhibit antimalarial characteristics in vitro[13]. This may be accomplished through the inhibition of *P. falciparum* signal peptide peptidase, a key stress regulating peptide located in the ER membranes of cells an essential element for the survival of the parasite. There are HIV inhibitors on the market that have been tested in vitro and in vivo in human red blood cells, and in *Drosophila* and zebrafish for their antimalarial benefits. Our goal was to attempt at repurposing these inhibitors to treat malaria infection by inhibiting P*f*SPP in infected RBCs.

Chapter 2

MATERIALS AND METHODS

2.1 Luminescence Assay

HEK293T cells (50,000) were seeded in 100 µl of Dulbecco's Modified Eagle Medium (DMEM) media solution supplemented with FBS, Glutamine and Penicillin/Streptomycin and incubated at 37 ºC overnight. Cells were transfected the following day with a total of 200 ng DNA, 0.5 µl Biotool Transfection Reagent (# B35102), in a total volume of 10 µl. Malaria HSP101, a substrate of P*f*SPP[11], ATF6-Luciferase and Renilla luciferase that are not expressed by HEK293T cells, and thus 4 plasmids were co-transfected. At approximately 24 hours after transfection, Dual Glo Luciferase reagent (Promega Corporation) was added and incubated at room temperature (RT) for 10 minutes or more. Multiple readings were taken at varying time points of 10 and 20 minutes each first with Dual Glo and next with Stop and Glo (Renilla) reagent to normalize the signal. Firefly luciferase signal relates to P*f*SPP activity, while Renilla Luciferase signal relates to the transfection efficiency in each well. Firefly and Renilla activities were measured using a Veritas microplate luminometer. Luminescence intensity values were obtained and evaluated using the mean, standard deviation and student's t-test. To show that HIV inhibitors could specifically inhibit P*f*SPP cleavage of Malaria Heat Shock Protein 101 (HSP101) a known substrate of SPP, a similar procedure was performed. After transfection, well were incubated with inhibitors for 2 hours in a 37 ºC incubator at 5 % CO_2. HIV protease (1 µM) inhibitors Lopinavir and Atazanavir (Kindly provided by Professor William Bachovchin, Tufts University School of Medicine) were dissolved in

DMSO (Gold Biotechnology Inc.) and added to each clear bottom well. The plate was incubated overnight at 37 ºC and 5 % CO_2. The Luciferase and Renilla Glo assay reagents were added at varying time points of 10 minutes and 20 minutes. The concentration change over time indicating cleavage of substrate triggering release of the signal molecule fused to luciferase was measured in the luminometer.

2.2 **Flow Cytometry**

Infected red blood cells were incubated overnight at 37 ºC in the presence of 0.5 µl inhibitors Lopinavir or Atazanavir containing 2 µl of infected blood and 97.5 µl of complete malaria media under malaria gas mixture (5 % Oxygen, 5 % CO_2, and 90 % Nitrogen). HIV protease inhibitors (from Prof. William Bachovchin) at 50 µM, 10 µM, 2 µM, 0.4 µM and 0.08 µM were used. Both Lopinavir and Atazanavir at 1:1 mixtures were used at 10 µM and 50 µM. Similar concentrations were used with the antimalarials, Quinine Sulfate and Pyrimethamine, during later experiments. The most efficient concentrations for Lopinavir, Atazanavir and LL-2 ketone were 10 µM, 250 µM and 5 µM, respectively. The following day, a blood smear of the sample was made to measure parasitemia. Blood sample (20 µl) from the parasite culture was washed with PBS (Lonza). Infected RBCs were stained with a 2 µM solution of cell permeable Hoechst dye (# 40046) (Biotium) in RPMI at a 1 : 8000 ratio which binds to DNA and is detected within the FITC and Indo 1 blue spectra spanning between 10^0 and 10^4 absorbance ranges in the flow cytometer. Red blood cell counts ranged between zero and ten thousand depending upon the sample size, sample type, and other parameters affecting each

measurement. 2,2-(2-oxo-1,3-propanediyl) bis [(phenyl methoxy) carbonyl] L-leucinamide ketone, also known as LL-2 ketone[9] was used as a positive control with its established activity of effectively inhibiting SPP demonstrating that inhibition of SPP results in the reduction of parasitemia and infection. Uninhibited infected red blood cells and uninfected red blood cells were used as negative controls. Initial experiments were conducted using uninfected and stained RBCs to establish correct signal windows by flow cytometry.

2.3 **Malaria Culture**

To start a new culture, parasites frozen in liquid nitrogen were gently thawed in varying concentrations of salt solution. Solutions of 1.6 % NaCl in deionized (DI) water, 12 % NaCl in DI water and 0.9 % NaCl plus 0.2 % dextrose in DI water were prepared. Frozen parasites were thawed in a 37 ºC water bath. Sterile techniques were employed throughout the entire procedure to eliminate any chances of contamination. Thawed parasites in 1 ml volume were transferred to a 50 ml tube and 200 µl of 12 % NaCl was slowly added while swirling the tube continuously. Parasites were allowed to settle at room temperature for 5 minutes. 10 ml of 1.6 % NaCl was carefully added to parasites drop-by-drop while swirling continuously. It was essential for parasitized erythrocytes retrieved from cold liquid nitrogen stocks to be methodically introduced to varying concentrations of salt solution as sudden salt exposure could result in dehydrated red blood cells absorbing enormous quantities of water from their isotonic environment via osmosis and rupturing. Sample was centrifuged for 5 minutes at 2000 revolutions per minute (rpm) and supernatant was discarded. The tube was swirled and tapped gently to

re-suspend pellet in the remaining solution. Pellets were handled gently to prevent rupturing of the infected RBCs. 10 mls of 0.9 % NaCl plus 0.2 % dextrose was carefully added drop-by-drop while simultaneously swirling. Sample was centrifuged at 2000 rpm for 5 minutes at room temperature (RT) and supernatant was discarded. Pellet was gently re-suspended in 5 mls of complete malaria media. Complete malaria media was made up of 0.5 % Albumax (Invitrogen), 25 mM HEPES (Invitrogen), 200 µM hypoxanthine, supplemented with 10 mg/ml gentamycin solution (Sigma Aldrich) in 1 X Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) containing L-glutamine. For weekly maintenance of parasite culture, fresh RBCs at 2 % hematocrit were added. Parasite culture was transferred daily from the 50 ml tube to a 25 ml tissue culture flask with a vented cap and flask was placed in a special portable chamber filled with malaria gas mixture as mentioned before. The chamber was placed in an incubator at 37 ºC. To maintain the culture through subsequent days, the culture was removed using a 5 ml pipette and placed in a 15 ml tube, which was centrifuged at 2000 rpm for 5 minutes at room temperature. The supernatant was aspirated and the culture was washed in 5 ml RPMI and centrifuged a second time. The supernatant was aspirated and the culture resuspended in 5 ml complete malaria media and carefully transferred to a fresh tissue culture flask. The flask placed in a chamber filled with fresh malaria gas mixture air and returned to the incubator.

2.4 **Blood Smears**

Malaria culture was placed in a 15 ml falcon tube at room temperature and centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated and tube gently tapped resuspending the pellet to maintain consistency. Pellet (2 µl) was placed on a glass slide to make the blood smear. Another identical slide was held over the slide with the pellet and the top edge of the slide was gently slid over the pellet carefully drawing it down the slide. The pellet now a thin film layer was spread out on the slide making it easy to observe under a microscope. The slide was fixed in methanol for 30 seconds to permeabilize the membrane for staining, and placed in a dryer for approximately 30 seconds. The slides were then stained in 20 % Geimsa, for 30 seconds and washed in DI water until the purple geimsa color could no longer be seen. The slide was placed in a dryer and allowed to completely dry. A drop of oil was placed directly on top of the smear for better visibility under a microscope. Parasitemia was measured with a Nikon microscope using a 60X objective. Blood smears were prepared daily to monitor parasite culture.

2.5 **Preparation of RBCs**

Fresh RBCs were either purchased or obtained from a healthy volunteer. RBCs were washed 3 times in RPMI to remove buffy coat and stored at 50 % hematocrit (in RPMI) in the fridge until needed. Washed fresh RBCs had a shelf life of 3-4 weeks under these conditions.

2.6 **Synchronization of Parasites**

Malaria parasites were synchronized to generate a particular stage of parasite development. This would enable us obtain stage specific information and determine what developmental stage was the most responsive to treatment. The parasite culture was placed in a 15 ml tube and centrifuged at 2000 rpm for 5 minutes at room temperature. The supernatant was aspirated and 5 mls of 5 % sorbitol was added. Sorbitol is a sugar alcohol that ruptures trophozoite and schizont stage infected RBCs but not the ring-stage infected RBCs. The sample was incubated under rotation at 37 ºC for about 15 minutes, and centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and 5 mls of RPMI was added. Pellets was carefully resuspended to avoid from damage to the RBCs. This process was repeated two additional times for 3 washes. After the third wash, a blood smear was made to measure parasitemia. The parasite infected RBCs were resuspended in 5 mls of complete malaria media and transferred to a fresh 25 ml tissue culture flask. The flask was placed in a chamber with malaria gas mixture and incubated overnight at 37 ºC.

2.7 **HEK293T Cell Culture**

To set up the HEK293T cell culture, Dulbecco's Modified Eagle Medium (DMEM) media solution was pre-warmed and supplemented with 10 % fetal bovine serum (FBS), Glutamine and Penicillin/Streptomycin, in a 37 ºC water bath for 5 minutes. Complete media was prepared using 5 ml of 10 % FBS, 0.5 ml of a 100 X stock of Penicillin/Streptomycin to make a 1 X stock, and volume was adjusted to 50 ml with DMEM stock media.

Cells were treated with 0.05% Trypsin plus EDTA to adequately cover the entire surface of the plate. Addition of Trypsin caused cells to dissociate becoming round. Cells were incubated at 37 ºC while occasionally pipetting to mix if clumps persisted. Once cells were loose and round, a four fifth plate volume of complete media was added to inhibit Trypsin. The culture was carefully transferred to a 15 ml tube, placed in a centrifuge and centrifuged at 2000 rpm for 5 minutes at room temperature. The supernatant was aspirated and 1 ml of complete media was added to resuspend the pellet forming a homogenous mixture. A one tenth dilution of cells in complete media was prepared and 10 µl from the mixture was placed on a hemocytometer to count the cells. Cells in only one of the four grids were counted. Total number of cells were calculated using the dilution factor with the following equation:

EQUATION FOR NUMBER OF CELLS PER MILILITERS

$$(\#\ \textbf{of Counted cells} \times 4 \times \textbf{Dilution Factor}) \times 10^4$$

The value of the number of cells per ml was used to determine the seeding density, volume of dish to use for the culture, and how much growth medium to add to the culture for normal growth of HEK cells as shown in the chart below.

2.8 **Transfecting HEK293T Cells**

HEK293T cells were used for the transfection assay. The following plasmids were used for the transfection; ATF6 the firefly expression vector, RL-SV40 the Renilla expression vector.

	Surface Area (cm^2)	**Seeding Density**	**Cells at Confluency**	**Versene (ml of 0.05% EDTA**	**Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA**	**Growth Medium (ml)**
Dishes						
35 mm	9	0.3×10^6	1.2×10^6	1	1	2
60 mm	21	0.8×10^6	3.2×10^6	3	2	3
100 mm	55	2.2×10^6	8.8×10^6	5	3	10
150 mm	152	5.0×10^6	20.0×10^6	10	8	20
Culture Plates						
6-well	9	0.3×10^6	1.2×10^6	2	2	3 - 5
12-well	4	0.1×10^6	0.4×10^6	1	1	1 - 2
24-well	2	0.05×10^6	0.2×10^6	0.5	0.5	0.5 - 1.0

Table 2.1 Cell Seeding Chart

HSP101 the SPP substrate with an ATF transcription factor attached, mSPP signal peptide peptidase and mSPP Mut. the signal peptide peptidase double mutant. All plasmids were sequenced and confirmed before transfection. Our cell culture was prepared a day before with the right confluency of 8.8×10^6 (See Table 1. Above). On the day of transfection, the culture media was replaced with fresh complete media. 387 ng/µl of ATF6, 590 ng/µl of RL-SV40, 492 ng/µl of HSP101, 213 ng/µl of mSPP, 95.3 ng/µl

of mSPP Mutant, and 726 ng/µl of the GFP tag were added to 1.2 µl of Opti-MEM in an Eppendorf tube. Biotool reagent (1.75 µl) was added to 11.25 µl of Opti-MEM in a separate tube. Both tubes were mixed and incubated at room temperature for 30 minutes. The HEK293T cells were transferred to a 36 well clear bottom plate, and plasmids were added to the HEK293T cells. The cells were placed in a 37 ºC incubator overnight. Dual Glo luciferase reagent was added the following day to measure luminescence as described before.

Chapter 3

RESULTS

3.1 HSP101 a Major Substrate for PfSPP

Our first goal was to confirm that *P. falciparum* Signal Peptide Peptidase transfected in HEK239-T cells could cleave the co-transfected Heat shock protein substrate HSP101[11]. This objective was achieved by comparing the activity of P*f*SPP with a P*f*SPP double mutant where the critical active site aspartic acid residues were mutated to alanine resulting in the loss of enzyme activity[11]. Malaria Heat Shock protein 101 (HSP101) served as the substrate. The firefly luciferase assay was used to accomplish this task with DMSO as the negative control. ATF6 transcription factor attached to HSP101 and its cleavage by P*f*SPP regulated transcription and translation of luciferase. Luciferase cleaves its chemical substrate thus emitting luminescence as a measure of cleavage activity due to an interaction between the SPP enzyme and HSP101 substrate.

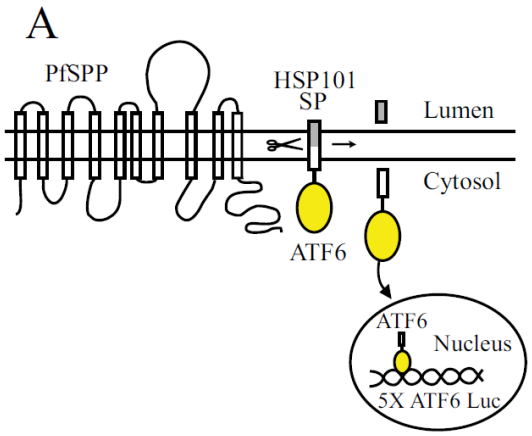

FIGURE 3.1 Luciferase Activation of SPP Substrate HSP101. Transcription factor ATF6 dissociates from the intermembrane protein complex once cleaved by enzyme. The free

ATF6 interacts with luciferase to produce a detectable emission[11] (Adapted from M. Baldwin et al, BBRC, 2014).

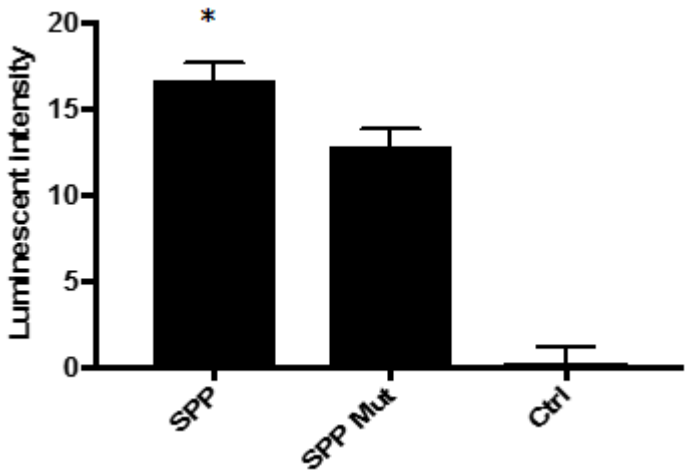

FIGURE 3.2 HSP101 a Substrate for P*f*SPP. P*f*SPP (10 μM) showing the strongest signal indicative of significant * 0.003 substrate cleavage using two degrees of freedom and standard error. The mutant shows less signal while control has almost no signal. Keep in mind that there might be other types of SPP present in the cells hence residual mutant signal.

The transfected P*f*SPP showed greater luminescence intensity than its double mutant and the control. Addition of a double mutant to the experiment was necessary to demonstrate the double mutant's inability to cleave the substrate given the fact that it had no enzyme activity, whereas cells expressing endogenous SPP can still cleave substrates. Luminescence signal was verified originating from the cleavage of SPP substrate due to the fact that cleaved fragments of transmembrane protein HSP101 with an attached

transcription factor produced a luminescence signal on a luminometer. The release of the transcription factor coupled with the luciferase vector generated detectable luminescence. Necessary controls were included to ensure equal expression of both P*f*SPP and P*f*SPP's double mutant in the transfected cells.

3.2 **HIV Protease Inhibitors Inhibit SPP**

Once the activity of *P. falciparum* signal peptide peptidase as an intramembrane cleaving protease of HSP101 was confirmed, the next step was to evaluate the ability of HIV aspartic protease inhibitors to inhibit P*f*SPP activity. This was accomplished using three known HIV protease inhibitors; Lopinavir, Fosamprenavir, and Atazanavir. A global inhibitor of signal peptide peptidases (LL2-ketone) was used as a positive control, and DMSO was used as negative control. The P*f*SPP double mutant served as a negative control to verify specific enzyme activity on its substrate HSP101 by releasing the signaling molecules for detectable luminescence. After the specificity of P*f*SPP activity was confirmed using the P*f*SPP double mutant, it was considered not necessary to include a P*f*SPP double mutant in all subsequent experiments with HEK293T cells. All future experiments performed in human blood were carried out without the inclusion of P*f*SPP double mutant.

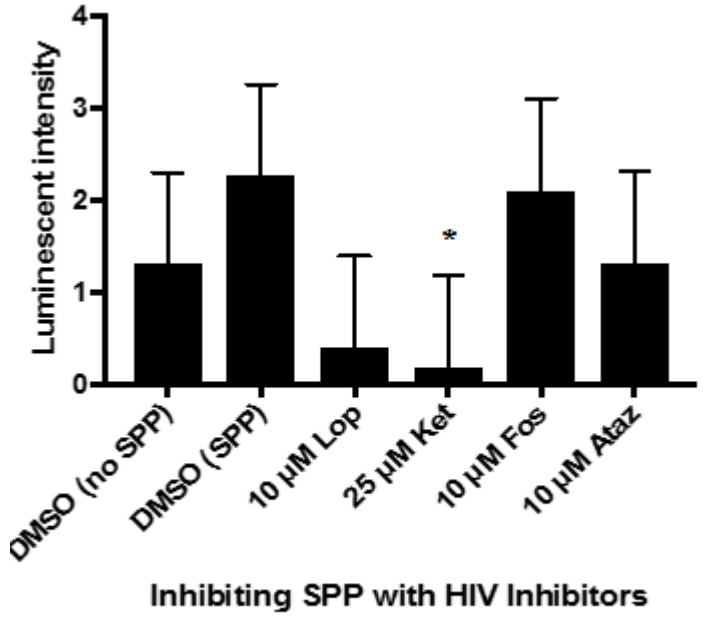

FIGURE 3.3 Inhibition of SPP activity by Antiretroviral Drugs. Graphical representation of the relative intensity of 3 HIV Protease Inhibitors (10 μM) tested with both positive and negative controls (LL-2 ketone and DMSO). The p value *0.04 with one degree of freedom and standard error. Lopinavir shows the most significant inhibition (lowest signal intensity after LL-2 ketone) while Fosamprenavir shows relatively little inhibition. HEK293T cells were used for transfection.

Lopinavir showed the highest level of inhibition for P*f*SPP second only to the positive control LL2-Ketone while Fosamprenavir showed minimal inhibition. Atazanavir showed inhibition at the basal level equivalent to no SPP treatment, indicating that Atazanavir has the capacity only to inhibit malaria SPP but no other SPPs present within the cell. The specificity could potentially minimize possible side effects that patients may encounter

due to the inhibition of human SPP alongside malaria SPP. This finding suggest that Atazanavir may function as an inhibitor of choice for antimalarial activity. Section 4.1 will provide a more detailed explanation regarding the use of this inhibitor as an antimalarial agent.

3.3 **Dose Curve and IC_{50} for HIV Inhibitors**

The next series of experiments evaluated transfected HEK293T cells to identify an optimal inhibitory concentration for the protease inhibitors that would later be used to inhibit malaria parasite growth in red blood cells. Titration curves were generated for Lopinavir and Fosamprenavir with LL-2 ketone as a positive control. Concentrations for Lopinavir and Fosamprenavir were as follows: 0.08 μM, 0.4 μM, 2 μM, 10 μM, 50 μM and 250 μM. Concentrations for LL-2 ketone were as follows: 0.20 μM, 1 μM, 5 μM and 25 μM. All concentrations were used in duplicate to obtain values. A plot of luminosity intensity versus inhibitor concentration was generated.

Lopinavir showed the characteristic sigmoidal curve with an IC_{50} value at 10 μM. This places Lopinavir at an optimal inhibitory concentration of 10 μM.

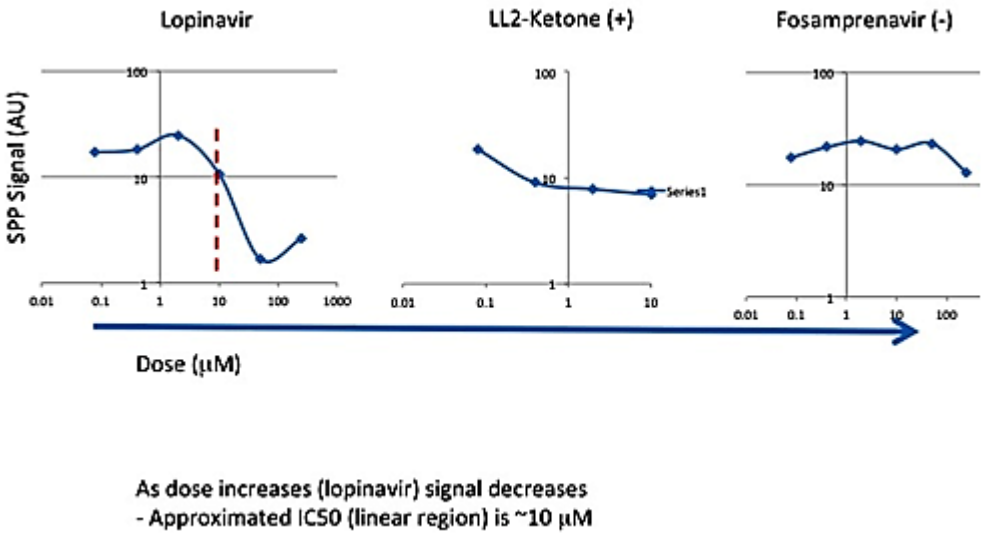

FIGURE 3.4 Dose curves for Protease Inhibitors. Dose Curves for Lopinavir, Fosamprenavir and LL2-ketone. All concentrations were used in duplicate.

The curve for LL-2 ketone showed minimal inhibitory variation from start to finish, inhibition rate initially increasing with time but then tapering off to an almost constant rate of inhibition which was expected for an inhibitor as potent as LL-2 ketone. The Fosamprenavir curve also showed a random profile giving relatively little information in terms of its optimal inhibitory concentration, which was expected for a weak inhibitor like Fosamprenavir.

Once the optimal inhibitory concentration for Lopinavir activity was determined, a similar experiment was conducted with malaria parasites in infected red blood cells to determine an optimal inhibitory concentration for Lopinavir in blood. Lopinavir was used as an inhibitor of SPP in the malaria parasite at varying concentrations of 0.4 μM, 2 μM, 10 μM and 50 μM. The experiment was performed using a universal inhibitor of SPP,

LL-2 ketone at varying concentration of 0.02 µM, 0.1 µM and 0.5 µM as a positive control. The optimal parasite inhibitory concentration by LL-2 ketone was determined to be at 10 µM (Figure 3.5)

3.4 **Inhibiting Malaria SPP in Human Blood**

The next step was to determine whether HIV protease inhibitors would be effective for inhibition of malaria parasite development in human red blood cells. *P. falciparum* strain 3D7 was used for these sets of experiments.

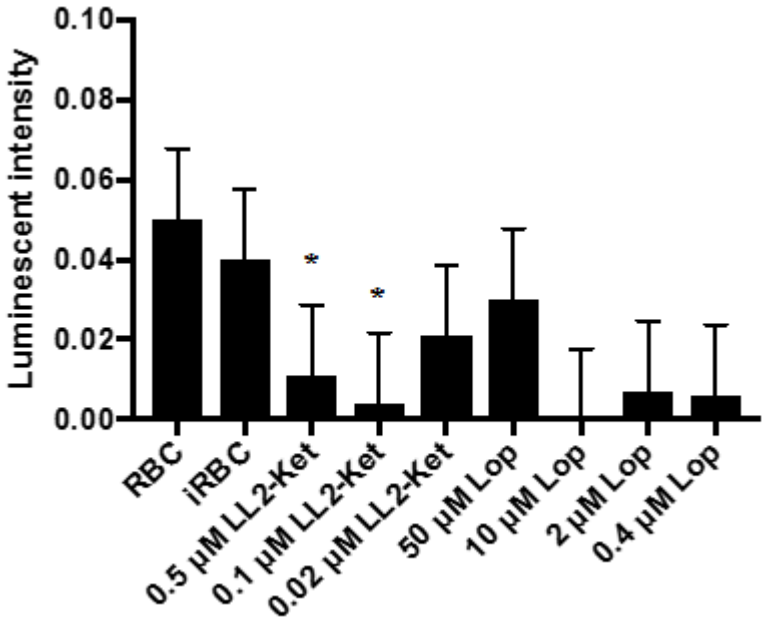

FIGURE 3.5 Lopinavir Dose Response in Blood. Lopinavir and LL-2 ketone inhibition of malaria parasites at varying doses. The percent parasitemia was relatively low, however, inhibition was significant with a p value of *0.02 at two degrees of freedom and standard

error. The data indicates that Lopinavir at 10 μM functions as the most effective inhibitor.

We were interested to test the effects of these inhibitors on both ring and schizont developmental stages of parasitemia. Therefore it was necessary to obtain a synchronous culture of parasites at specific stages to determine the more vulnerable developmental stages of parasites by these inhibitors. The percent parasitemia was determined from blood smears. It was found to be essential to have significant infection before attempting inhibition with protease inhibitors in order to definitively establish the efficiency of these protease inhibitors.

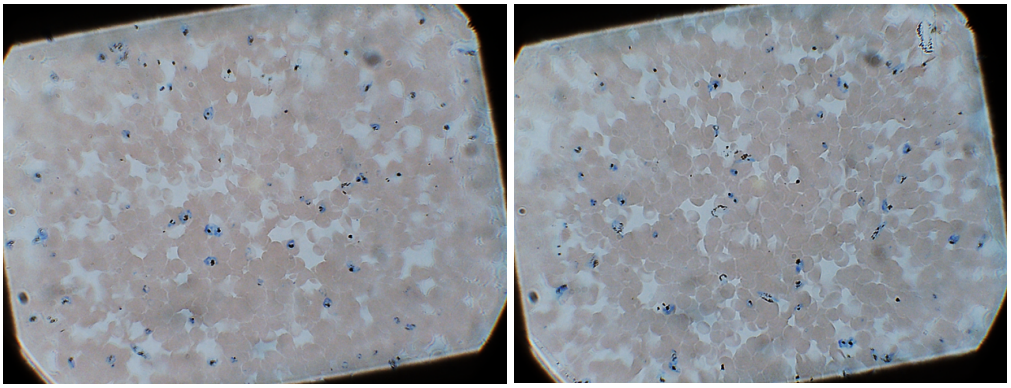

FIGURE 3.6 Malaria Culture Slides. Cultured red blood cells on a slide showing malaria parasite growth (blue areas).

First, we evaluated infected RBCs by flow cytometry; which can differentiate different stages of infected cells via their sizes and DNA content. Infected red blood cells stained with Hoechst dye generated the fluorescence signal due to the presence of malaria DNA

as mature red blood cells do not have DNA and therefore could not produce any signal within the fluorescence window. All fluorescence intensity was therefore attributed to the presence of malaria parasites at different stages of development.

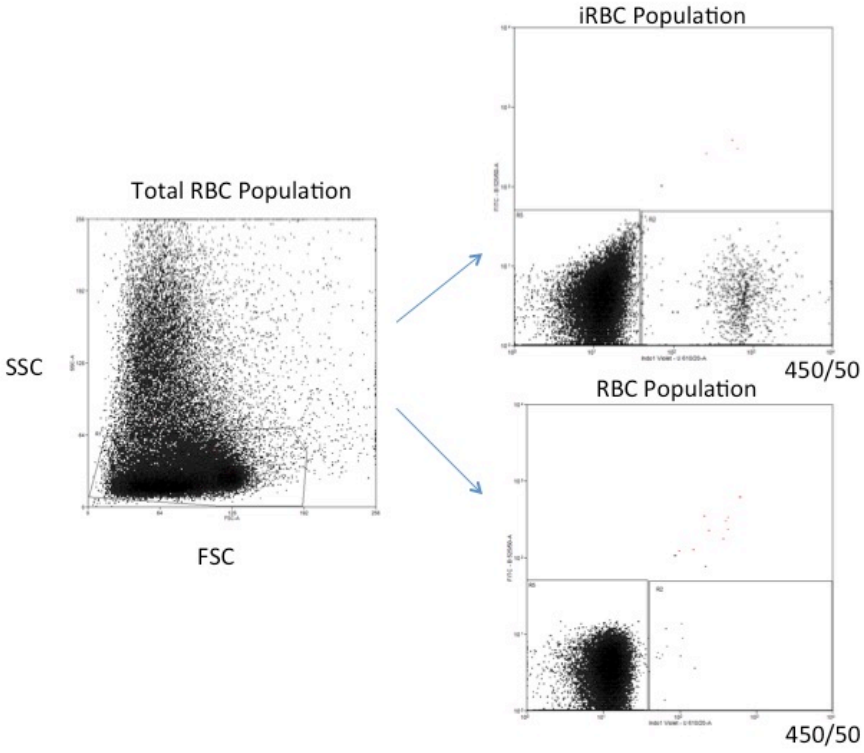

FIGURE 3.7 Flow Cytometry of Infected RBCs. Flow Cytometry showing the ratio of infected red blood cells to total red blood cell population.

The fluorescence signal was used to measure parasitemia in the presence and absence of HIV protease inhibitors. A reduction in percent parasitemia after application of the HIV protease inhibitors would be an indication that HIV protease inhibitors were inhibiting malaria parasite growth under these conditions.

Lopinavir Flow Cytometry Data

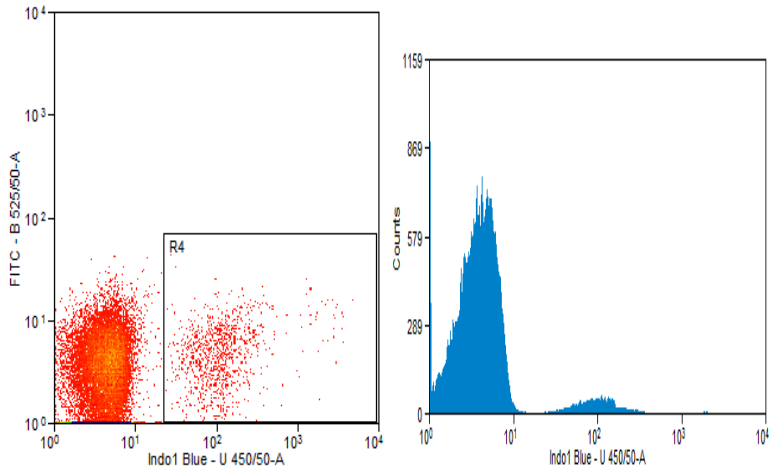

FIGURE 3.8 Flow Cytometry after Lopinavir Treatment. Flow Cytometry data showing the ratio of uninfected red blood cells and infected red blood cells treated with Lopinavir at a concentration of 10 μM. The R4 window denotes infected RBCs, Indo blue, the spectrum for quantification of infected RBC emissions measured in the absorbance ranges shown. Average parasitemia after Lopinavir inhibition was about 3%. (See Figure 3.10 Histogram).

Atazanavir Flow Cytometry Data

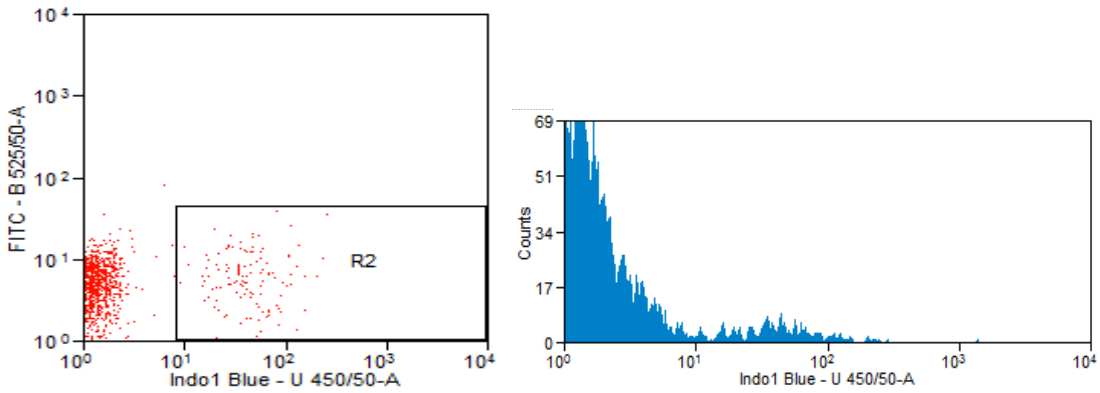

FIGURE 3.9 Flow Cytometry after Atazanavir Treatment. Flow Cytometry data showing inhibition of malaria parasites growth in red blood cells treated with 0.4 μM Atazanavir. Inhibition was negligible due to a low dose of Atazanavir used for the experiment. The R2 window denotes the infected RBCs, Indo blue the spectrum for quantification of infected RBC emissions measured in the absorbance range shown (See Figure 3.11 Histogram). Atazanavir inhibits optimally at 250 μM.

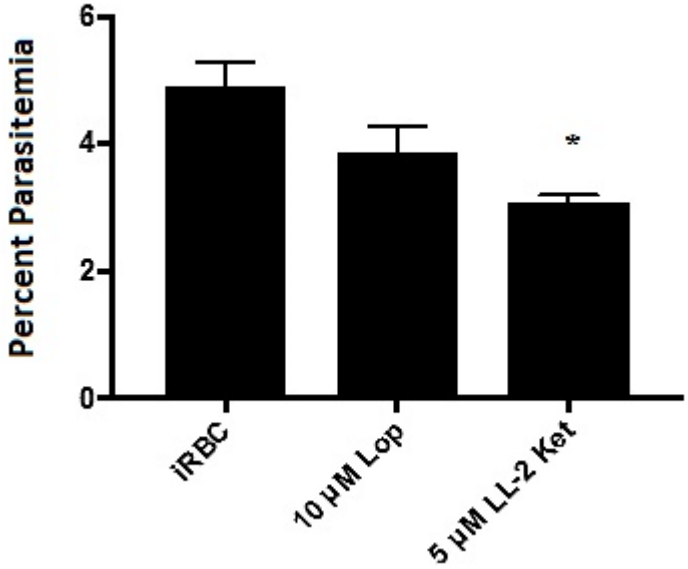

FIGURE 3.10 Lopinavir Inhibition in Blood. Flow Cytometry data showing the HIV protease inhibitor Lopinavir inhibiting malaria parasites at 10 μM, LL2-ketone (positive control) inhibiting significantly at 5 μM, p value *0.03 using two degrees of freedom and standard error. Infected red blood cells without inhibitors were used to show no inhibition (See Figure 3.8).

Protease inhibitor Lopinavir (Prof. William Bachovchin at Tufts University School of Medicine) was added to malaria parasite infected RBCs to determine inhibition of parasite growth. Flow cytometry was used to assess the parasitemia, which is graphically represented in Figure 3.10. A reduction in parasitemia was observed with both the positive control (LL-2 ketone) and Lopinavir. A similar experiment conducted with Atazanavir (Figure 3.11) did not produce any conclusive results due to the fact that Atazanavir does not function as an SPP inhibitor at the tested concentration. Atazanavir however operates more effectively at a higher inhibitory concentration of 250 μM. This was not known at the time the experiment was conducted hence the use of low concentrations of Atazanavir to inhibit SPP. Higher concentrations of Atazanavir may be required to further investigate the role of this inhibitor in malaria infection under these conditions.

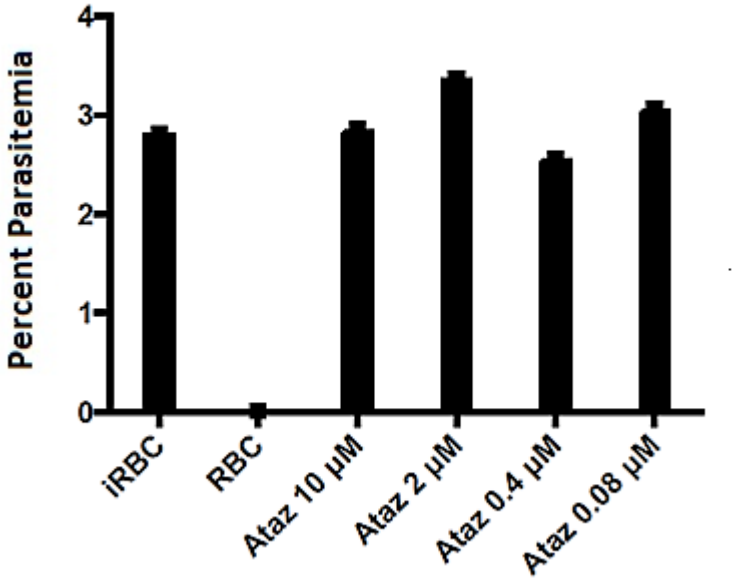

FIGURE 3.11 Atazanavir inhibition in Blood. Flow Cytometry data showing that HIV protease inhibitor Atazanavir does not inhibit malaria parasite infected RBCs at varying concentrations. Inhibition was negligible due to low dose Atazanavir used.

3.5 **Optimal Inhibitory Concentration with Atazanavir**

Atazanavir a protease inhibitor with inhibitory activity specific to *P. falciparum* SPP (Figure. 3.3) was added to parasite infected RBCs to determine optimum inhibition efficiency. The results show that a higher dose of Atazanavir can achieve a similar effect as the effect observed with a low dose of Lopinavir. Lopinavir's optimal inhibitory concentration was determined to be at 10 µM (Figure 3.5) whereas Atazanavir had an optimal inhibitory concentration at about 250 µM (Figure 3.12). Therefore, Lopinavir was used in combination with Atazanavir to determine whether there would be an

increase in efficiency of inhibition by simultaneously suppressing parasite development through the employment of a mixture of two inhibitors. An equal concentration of Lopinavir and Atazanavir at 50 µM proved to be of higher efficiency than their combination at the low concentration of 10 µM. Atazanavir at 250 µM was found to be the most optimal inhibitory concentration for the inhibition of parasite growth under these conditions (See Figure 3.12).

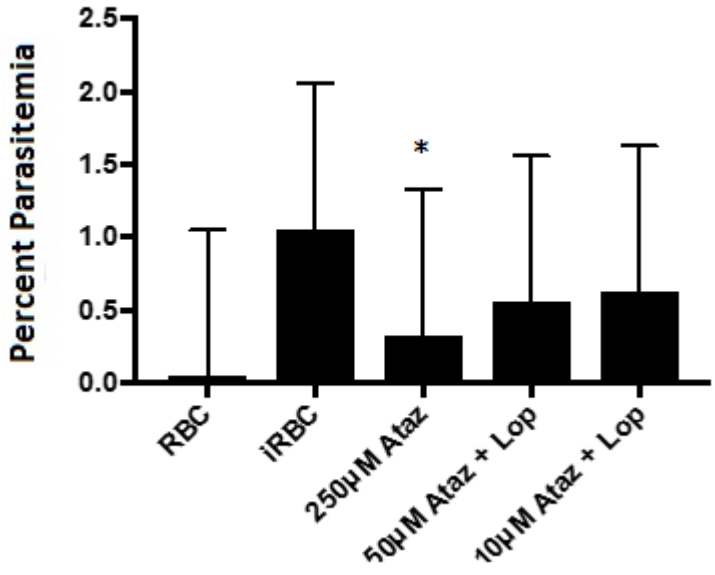

FIGURE 3.12 Effect of Atazanavir & Lopinavir on Malaria Parasites. Atazanavir at high concentrations was used alone and in combination with Lopinavir in infected red blood cells to determine inhibition efficiency. Significant Inhibition p value *0.01 and one degree of freedom occurred with Atazanavir at 250 μM. This concentration was determined to be the optimal inhibitory dose for Atazanavir. Standard error was used.

3.6 **Minimal Resistance Combination Therapy with Quinine Sulfate**

Development of drug resistance by malaria parasites is a reason of major concern. One contributing factor to the rapid development of resistance to malaria drugs could be attributed to the fact that antimalarial medications are handed out needlessly even without adequate diagnosis of the disease. The frequent and pointless use of drugs triggers the onset of parasite tolerance to drugs. Malaria parasites in an attempt to evade the lethal effects of antimalarial drugs develop multiple mechanisms to circumvent the effects of these drugs ultimately resulting in the development of drug resistance.

Quinine Sulfate a long-standing antimalarial agent[7] was combined with Atazanavir and used at equal ratios of 50 μM, and 250 μM concentrations. A separate 1:1 mixture with Lopinavir at 10 μM, Lopinavir only at 10 μM, Quinine Sulfate only at 10 μM, Atazanavir only at 50 μM, Quinine Sulfate only at 50 μM and Atazanavir only at 250 μM were used to inhibit malaria parasite growth in red blood cells. These experiments were conducted to further assess the composition of multiple potent antimalarials as an effective therapy to delay the onset of parasite induced drug resistance. Our results indicated that Atazanavir and Quinine mixture at 250 μM was the most efficient inhibitor combination for antimalarial activity under these conditions (Figure 3.13).

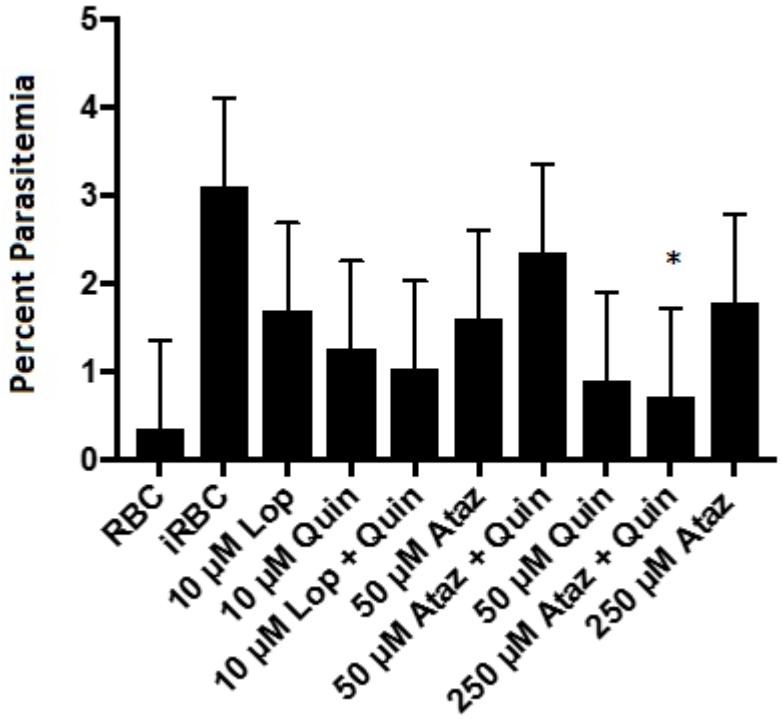

FIGURE 3.13 Protease Inhibitor Cocktail with Quinine Sulfate. Inhibition of malaria parasites with Quinine Sulfate, Lopinavir and Atazanavir using their individual dose and cocktail mixtures. Atazanavir and Quinine mix at 250 μM showed the most significant inhibition (p value *0.001) with one degree of freedom and standard error. Atazanavir and Quinine at 50 μM showed the least inhibition. Lopinavir and Quinine at 10 μM showed relatively more inhibition together than when used individually.

3.7 **Minimal Resistance Combination Therapy with Pyrimethamine**

To minimize drug resistance, a combination therapy consisting of the antimalarial agent Pyronaridine Tetraphosphate, also known as Pyrimethamine, was tested at varying

concentrations with Lopinavir and Atazanavir. The concentrations used to inhibit malaria parasite growth in RBCs were as follows; 10 μM Lopinavir, 10 μM Pyrimethamine, 50 μM Atazanavir, 50 μM Pyrimethamine, 250 μM Atazanavir, 250 μM Pyrimethamine, a 1:1 mixture of 10 μM Pyrimethamine and Lopinavir, a 1:1 mixture of 50 μM Pyrimethamine and Atazanavir, and a 1:1 mixture of 250 μM Atazanavir and Pyrimethamine. A combination of Atazanavir with Pyrimethamine at 250 μM concentration showed the most efficient inhibition of parasite growth in human RBCs.

Pyrimethamine's mechanism of action is distinct from the action mechanism of more common quinoline antimalarials that target hemoglobin degradation within the parasite. Pyrimethamine inhibits malaria parasite growth by inhibiting dihydrofolate reductase a key enzyme in the malaria parasite folic acid biosynthetic pathway. This inhibitory activity is selective to malarial folic acid synthesis and not the mammalian enzyme because the drug functions at concentrations much lower than those required to produce comparable inhibition of the mammalian enzyme. Inhibition of the folic acid synthesis enzymes is manifested late in the life cycle of malarial parasites by failure of nuclear division at the time of schizont formation in erythrocytes and liver[7].

Pyrimethamine the antimalarial used for this experiment was chosen due to its unique line of action which would bring an alternative inhibitory perspective to the combination drug therapy. Moreover, Pyrimethamine's exhibition of fewer side effects than its quinoline counterparts, one major side effect of quinolines being incessant itching, made

it a desirable drug of choice[14].

(https://pubchem.ncbi.nlm.nih.gov/compound/pyrimethamine)

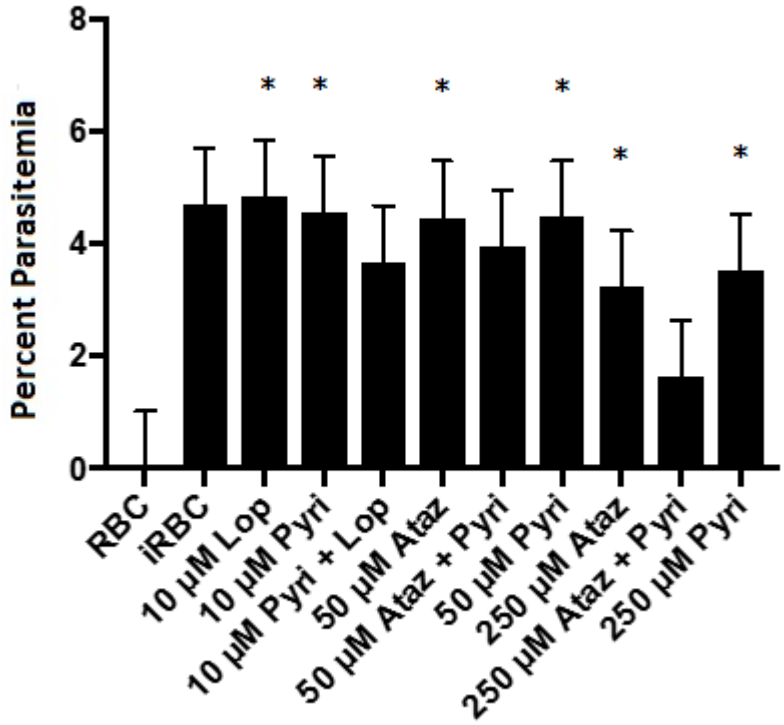

FIGURE 3.14 Effect of Pyrimethamine on Malaria Parasite Growth. Inhibition of malaria parasites with Pyrimethamine, Lopinavir, Atazanavir, using their individual doses and cocktail mixtures. Atazanavir and Pyrimethamine mixture at 250 μM showed the greatest inhibition. While Lopinavir at 10 μM showed the least inhibition. Inhibition achieved via the single drug therapy was significantly (p value *0.03) different from that achieved via the combination therapy, with 5 degrees of freedom and standard error.

Chapter 4

DISCUSSION

4.1 Minimal Side Effects with Atazanavir

P. falciparum signal peptide peptidase activity was inhibited by several HIV inhibitors, the most potent being Lopinavir with values close to those for the positive control LL2-ketone, a universally accepted inhibitor of SPP. In contrast, Atazanavir showed inhibition at levels similar to no malaria SPP. These data suggest that Atazanavir inhibits only P*f*SPP and does not inhibit endogenous human SPP of which there are currently five known types. This conclusion was drawn from the experiment with Atazanavir shown in Figure 3.3. depicting a signal intensity for Atazanavir inhibition of SPP at the same level as that displayed in the basal cells. Luminescence intensity was verification of SPP cleavage of its HSP101 substrate. Reduced luminescence emission in the assay used in this study was a further demonstration of inhibition of SPP activity by protease inhibitors. Both LL2-ketone and Lopinavir exhibited significant inhibition of SPP as detected by reduced luminescence (Figure 3.3). Atazanavir on the other hand inhibited SPP as indicated by a stronger luminescence intensity of Atazanavir in comparison to Lopinavir and LL-2 ketone which both had weaker luminescence intensities. Keeping in mind that luminescence intensity corresponds to inhibitory capabilities, a strong intensity signal would mean decreased inhibition and vice versa, and this result thereby depicted moderate inhibition by Atazanavir. The luminescent intensity of Atazanavir coincided with the luminescent signal of the basal cells with no malaria SPP, suggesting that Atazanavir might be sensitive to only P*f*SPP and not other SPPs. Thus, our data suggests that Atazanavir may inhibit only malaria SPP with a residual signal from other intrinsic

non-malaria SPPs. Therefore, Atazanavir could possibly inhibit SPP without deleterious side effects observed from other anti-retroviral therapies, as it selectively works on malaria SPP. Additional studies will be required to confirm this hypothesis

4.2 **High Efficiency Quinine Cocktail**

A 1:1 cocktail mixture of Quinine Sulfate and Lopinavir at 10 μM showed greater inhibitory activity than Quinine Sulfate alone at 10 μM or Lopinavir alone at 10 μM. This observation indicates that Quinine Sulfate and Lopinavir work together synergistically to produce a more favorable inhibitory outcome and that combining both treatments may also decrease drug resistance arising from single treatment modalities. Further work is needed to confirm this hypothesis.

4.3 **Pyrimethamine Cocktail a Work in Progress**

The Atazanavir, Lopinavir and Pyrimethamine combinatorial treatment data (Figure 3.14) appear to be inconsistent with our theory which places HIV aspartic protease inhibitors combined with antimalarials as synergistically effective drugs in malaria parasite growth inhibition. The signal intensity of parasites treated with Lopinavir at 10 μM measured on the graph as percent parasitemia (Figure 3.14) appeared from the data to be higher than the signal intensity for infected red blood cells that were given no protease inhibitors. This result is quite misleading given the fact that previous experiments have shown Lopinavir to be a highly potent inhibitor of SPP specifically at the concentration

of 10 μM. Obtaining results that show Lopinavir at 10 μM with no inhibition could possibly be attributed to random experimental error. It is to be noted that Pyrimethamine does not dissolve well with DMSO and therefore could contribute to overestimation of its effective inhibitory dose under these conditions. Pyrimethamine in general is selectively soluble in both polar and non-polar solvents which explains its poor solubility in DMSO. Follow up work is required to confirm the data generated from these experiments[14]. (https://pubchem.ncbi.nlm.nih.gov/compound/pyrimethamine).

4.4 **Combination Therapy and Drug Resistance**

Studies indicate that it could take as little as two months for the malaria parasites to develop effective resistance through genetic recombination, gene expression or alternative coopting to certain drugs[15]. Use of multiple agents to minimize the likelihood of subsequent development of drug resistance is a common applied strategy[16]. This strategy works by presenting more than one recognizable ligand at the binding site of a target receptor preventing the receptors from easily recognizing a ligand and developing ways to circumvent the effect of the ligand. For example, the presence of two or more ligand structures with different binding conformations creates competition at a target site thereby challenging the parasites process to develop resistance. If the parasite develops resistance to one structure and or mechanism it might take a while to come up with another mechanism in the therapeutic mixture of multiple drugs.

4.5 **Summary**

We used standard conditions practiced in the malaria research field to test the effect of pharmacological inhibitors in human red blood cells. Protocols used throughout my thesis work involved well characterized temperatures, humidity and air concentrations identical to those found within the red blood cell of the host carrying the parasite.

Our results indicate that Signal Peptide Peptidase, an enzyme present in both malaria and human cells, could be specifically inhibited by HIV aspartic protease inhibitors particularly Lopinavir and Atazanavir as an antimalarial therapy by specifically targeting the malaria SPP.

Our hypothesis that P*f*SPP inhibition would lead to cell death has been exploited experimentally as a means to induce cell death within the cells of the plasmodium protozoan, thwarting further development of the parasitic life cycle and stopping the propagation of the disease. Antiretroviral aspartic protease inhibitors demonstrated marked inhibition of *P. falciparum* signal peptide peptidase. The inhibitory activity of these antiretroviral HIV protease inhibitors will as expected produce antimalarial outcomes.

Prior experiments conducted with ten HIV aspartic protease inhibitors[5] on the market revealed some inhibitors were more potent than others at inhibiting malaria parasite growth. These were Lopinavir, Atazanavir, Fosamprenavir, and Nelfinavir. Our experiments placed Lopinavir as a highly potent low dose inhibitor able to optimally

inhibit the growth of malaria parasites at the concentration of 10 μM. Lopinavir's inhibition was indiscriminate, it inhibited both human and malaria SPP. Atazanavir another HIV protease inhibitor, was able to inhibit SPP at much higher doses. The dosage for optimal Atazanavir inhibition was at 250 μM. Atazanavir emerges as an inhibitor of choice due to its relative specificity to *P. falciparum* SPP. Our data indicate that P*f*SPP inhibition by Atazanavir closely correlates with basal level of enzyme activity in cells with no malaria SPP. Thus, these observations suggest that other intrinsic human SPPs were apparently not inhibited by Atazanavir. This observation could mean relatively low side effects in humans making Atazanavir a more attractive choice for use in research and future drug development. This hypothesis has not yet been directly tested, and still remains a supposition however further work on each specific type of human SPP could shed more light and produce more convincing results.

Upon characterization of the effective dose of HIV protease inhibitors as antimalarials, we sought to combine these inhibitors with known and widely used antimalarial remedies creating a cocktail that would not only inhibit malaria growth but also could withstand the subsequent emergence of malaria parasite drug resistance. Quinine, a highly efficient and well-known antimalarial in use since the early 17th century[7], was selected as the antimalarial to be used in conjunction with Lopinavir and Atazanavir. The cocktail was determined through our experiments to be highly effective as these compounds worked synergistically together to produce favorable inhibitory outcomes. Quinine sulfate and other quinoline antimalarials have significant side effects one major side effect[7] being debilitating itching of the extremities. Pyrimethamine did not share the common side

effects associated with other quinoline antimalarial medications like Quinine and Chloroquine specifically the unremitting itching of body extremities. This attribute as well as Pyrimethamine's unique action mechanism made pyrimethamine an attractive choice for combined treatment therapy.

The combination of pyrimethamine and two of the HIV aspartic protease inhibitors Lopinavir and Atazanavir produced results somewhat inconsistent with results from prior experiments involving the other antimalarial Quinine Sulfate. We had expectations that the HIV protease inhibitors would produce parasite inhibition alone and that in combination with pyrimethamine, there would be a more decisive increase in malaria parasite inhibition. Lopinavir at 10 μM showed no inhibition which was inconsistent with prior experiments that have shown Lopinavir to inhibit optimally at this concentration. Pyrimethamine at 10 μM showed some inhibition, the 1:1 mixture of Lopinavir and pyrimethamine at 10 μM showed even more inhibition which was expected. Pyrimethamine and Atazanavir at two different concentrations of both 50 and 250 μM showed some inhibition apart and more inhibition when together in a 1:1 mix (Figure 3.14). These results were also consistent with our projection and hypothesis. The pyrimethamine experiment was an overall success excluding the part where Lopinavir at 10 μM failed to adequately inhibit malaria parasites as expected.

The relative lack of success for the results from our experiment with pyrimethamine could be attributed to a flaw in the method or protocol for dissolution of the powder in solvent (DMSO in our case).

One however consistent theme through the course of our cocktail experiments was that malaria parasites were better inhibited by cocktail combination compounds than via single compound therapy. Thus, indicating that the combination of these compounds into one antimalarial cocktail mix could not only delay the subsequent development of malaria parasite drug resistance, but equally proved to be a more potent killer of the malaria parasite.

4.6 **Future Directions**

The overall success of the experiment creates a need for future steps to be taken towards further investigation of the SPP enzyme. The genes that express SPP should be studied across various strains of malaria as well as in humans and mice in order to identify any existing conserved sites on the DNA. This could mean finding a common ancestor or identification of the essentiality of varying nucleotides in the SPP gene sequence. In silico examination of protein sequence is particularly important when targeting the conserved enzymatic area of P*f*SPP but not of mammalian homologs. It involves comparative studying of genes that express SPP with archived database sequences, identifying regions of local similarity and deciphering evolutionary relationships leading to identification of possible members of gene families[17]et cetera. Once identified, the conserved nucleotides found in *P. falciparum* SPP could be mutated by either deletions or base pair substitutions using any of the promising new gene editing techniques like CRISPR/cas9 to target specific sequences on the gene coding for SPP and modifying them thus editing the gene to produce desirable outcomes permanently preventing the

enzyme from performing homeostatic regulatory activity within the parasite. This could cripple the protein synthesis process for *P. falciparum* SPP shutting down enzyme production which would result in the parasitized erythrocytes undergoing senescence, shrinking and displaying signal molecules on their surface membrane facilitating macrophage mediated degradation or splenic clearance. Inhibition of the protein synthesizing process of these enzymes could equally release the parasitized cells into a downward spiral of apoptotic destruction. The P*f*SPP sequence could also be identified and aligned with the rodent SPP sequence for a rodent malaria model closest to P*f*SPP. The closest rodent malaria sequence could then be injected into mice and the mice could be treated with the antimalarial cocktail to observe for any possible inhibition in vivo. (See last paragraph for more detailed information on in silico examination). This study could be closely followed by a dose dependence evaluation of the antimalarial combination cocktail in vivo in mice and even possibly in a primate such as a monkey.

Another interesting area of future research would be to investigate and identify other possible substrates for signal peptide peptidase elucidating their primary roles to determine if they possess any probable antimalarial indication.

One potential future direction would be to investigate other members in the transmembrane cleaving protease family, or other proteases in general to identify substrates that hold promising potential to kill malaria parasites. A particular group of enzymes of interest are Plasmepsins. These enzymes have proteolytic capabilities across varying cell types within the organism and are specific to *P. falciparum*. They possess

high hemoglobin degrading ability and are responsible for some of the symptoms demonstrated by malaria patients. Plasmepsin V shares a close resemblance to signal peptide peptidase, localized in the endoplasmic reticulum it was initially thought to function identically[18]. However further investigation placed Plasmepsin V's primary sphere of operation with the degradation of hemoglobin[19] away from direct involvement with the malaria parasitic life cycle. Plasmepsin V has also been reported to cleave *P. falciparum* export elements (PEXEL), a transmembrane protein that facilitates export of parasite structural proteins from the parasitophorous vacuole into the host RBCs [18]. This Plasmepsin V characteristic should be further investigated for possible antimalarial implications although it does not play a part in the key role of survival and development in the case of the parasite. Plasmepsin V could also be a possible therapeutic target for sickle cell anemia[19]as its line of action is directly related to hemoglobin and its degradation an underlying contributor to the sickle cell disease. Sickle cell disease is associated with the rapid deterioration of red blood cell count as a result of cells with distorted conformation being destroyed by the spleen. The sickle shape is caused by malformed hemoglobin structures due to mutation hence the name of the disease.

One last step would be to infect a rodent with one of these strains of malaria that exist in rodents; *P. berghei*, *P. yoelii*, *P. chabaudi* or *P. vinckei*. Each strain would be treated with one of our cocktails to observe for inhibition of malaria parasite growth in vivo[13]. This would shed more light on the workings of malaria in vivo as these mice share many common genetic features with humans. Valuable information could be obtained from the execution of a study of this nature in regards to the safety and efficacy of new therapies

specifically at which operating dose desirable results could be achieved[18]. Moreover, a search for an SPP target sequence on a local gene alignment search tool[17] has determined both *P. berghei* and *P. chabaudi* to display conserved sequences on their genes of high significance similarity at about 88 percent to the *P. falciparum* SPP sequence. A primary structure analysis of P*f*SPP revealed conservation of aspartate-containing catalytic site motifs YD spanning amino acids 221-22, and LGLGD spanning amino acids 259 – 263, that are required for the activity of Presenilin-type intramembrane aspartyl proteases.

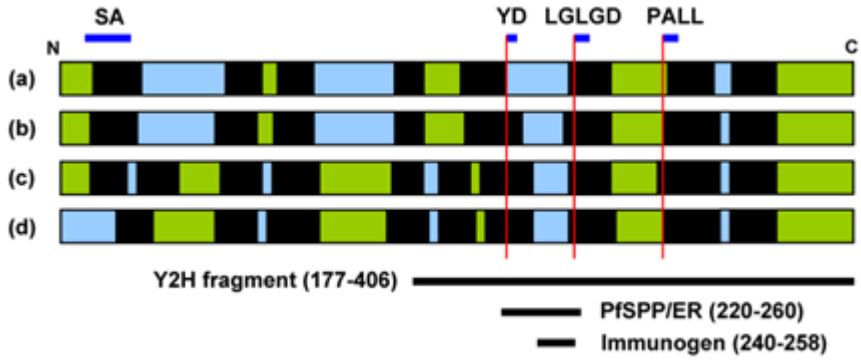

FIGURE 4.1 In silico Examination of *P. falciparum* SPP alignment with other rodent malaria strains namely *P. berghei* and *P. chabaudi* showing alignment along the aspartate-containing catalytic site motifs YD and LGLGD. (Adapted from Li et al 2008)

The C-terminal PAL motif [29] spanning amino acids 335 – 337 essential for a proper conformation of the active site is also present in P*f*SPP.

SPP - Berghei

MNLLKLIGKNKKMKNHDKRNSAIYYSCYAIIVLTIILSRFIVIPLILQMTLYTFITIYIG
SHESIRQLEADDKTQKTDHITTYDAIMFPIIGSAALLTLYFAYKFLDPYYVNLLLTVYLT
MAGVFSLQSVFSTVLEPFFPKIFKKDEFVKTINAPKFISKDPIVFNTNKGEIMSLIVCFI
IGARWIFYKDFVTHNILAISFCFQALSLVILSNFVIGFILLSGLFVYDIFWVFGNDVMVT
VAKSFEAPVKLLFPVSLDPLHYSMLGLGDIIIPGILISLCLRFDYYLHRNKIHKGNVKKM
FNDISIHESFKKYYFYTITVFYQAGLILTYCMLFYFEHAQPALLYLVPACILAIVGCALF
KKEFKIMIKYQEITDKSSNADDGKKKTLEKEETLKSQESIMSVTKRKINAK

SPP - Chabaudi

MNLLKLIGKNKKMKNPDKRNSIIYYSCYAIIVLIIILSRFIVIPLVAQMSLYTLITIYIG
SHESIRQLEADDKSQKTDHITTYDAIMFPIIGSAALLTLYFAYKFLDPYYVNMLLTVYLT
MAGVFSLQGVFSTLLEPFFPKLFKKDEYVKTINAPKFISKDPIVFNTNKGEILSLIVCFI
IGARWLFYKDFITHNILAISFCFQALSLVILSNFVIGFILLSGLFVYDIFWVFGNDVMVT
VAKSFEAPVKLLFPVSLDPLHYSMLGLGDIIIPGILMALCLRFDYYLHRNKIHKGNTKKM
FNDISIHEPFKKYYFYTITAFYQAGLLLTYFMLFYFQHAQPALLYLVPACIIAIVGCALC
KKEFKIMIKYQEITDKGSNTDEGKKKILEKEETLKSQESIMSVAKKRVNAK

SPP - Falciparum

MNLLKLIGKNKKMKNENMGNSIFYYSCYVIIVLTIILSKFVVIPLMAQMFLYTFITIYIG
SHDSLKQLEIDDKTKKSDNITAYDAMMFPVIGSAALLTLYFAYKFLDPFYVNLLLTLYLT
LAGVFSLQGVFTTILEPVFPNFFKKDEYVKTFKLPNFIYKEPIVFNTNKGEIVCLILSFA
IGLRWIFYKDFITHNVLAVSFCFQAISLVILSNFLIGFLLLSGLFVYDIFWVFGNDVMVT
VAKSFEAPVKLLFPVSSDPVHYSMLGLGDIIIPGILMSLCLRFDYYLFKNNIHKGNLKKM
FNDISIHESFKKYYFYTIIIFYELGLVVTYCMLFYFEHPQPALLYLVPACILAILACSIC
KREFKLMIKYQEITDKSNTVDDASKNKKKDKEEIPKIQETPVSNAKKRITNK

FIGURE 4.2 SPP Alignment across Three Plasmodium Strains in mice and humans. Data acquired from the Basic Local Alignment Search Tool (BLAST) database depicting an 88 percent alignment.

These genes could be further researched extensively to obtain clarity in their functionality and structure in terms of alignment and identity which could provide valuable information for creating a malaria drug remedy (See Figures 4.1 and 4.2).

An epidemiological study could be evaluated in Africa involving patients infected with both HIV and malaria, taking drugs concurrently for both diseases. A study of this nature would reveal whether or not patients with HIV taking malaria drugs have a better

response to antimalarials or vice versa. This would shed more light to the workings and relations between these two groups of pharmacological agents.

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