2009

Functional characterization of cytochrome b5 reductase and its electron acceptor cytochrome b5 in Plasmodium falciparum

Lucio Malvisi
University of South Florida

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Functional Characterization of Cytochrome b$_5$ Reductase and its Electron Acceptor

Cytochrome b$_5$ in *Plasmodium falciparum*

by

Lucio Malvisi

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
Department of Global Health
College of Public Health
University of South Florida

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Date of Approval:
July 27, 2009

Keywords: malaria, protein expression, drug resistance, artemisinin-based combination therapy, detoxification of xenobiotics

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DEDICATION

To my family and in particular to my father Martino and my mother Natalina who have supported me throughout all these years spent in the United States, far from my home, Italy, where my heart is. I want to thank them for teaching me the important values in life that have helped me to succeed in every-day life, to fight in tough times and to come out of difficult situations with strength and pride. I also want to thank them for the beautiful and unforgettable years I spent at home which made me the person I am today.
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### TABLE OF CONTENTS

**LIST OF TABLES** ............................................................................................................ iii

**LIST OF FIGURES** .......................................................................................................... iv

**LIST OF SYMBOLS AND ABBREVIATIONS** ............................................................... v

**ABSTRACT** ....................................................................................................................... vi

**INTRODUCTION** ..............................................................................................................1

Malaria .............................................................................................................................1

History .............................................................................................................................1

Funding and Financial Contributions ...........................................................................3

Prevention, Control, and Cure .......................................................................................6

Etiology .............................................................................................................................13

Vector and Transmission ...............................................................................................15

Life Cycle .......................................................................................................................20

Public Health Impact .....................................................................................................21

Clinical Features ...........................................................................................................25

Pathophysiology ...........................................................................................................27

Diagnosis ........................................................................................................................30

Treatment .......................................................................................................................37

Treatment of Uncomplicated *P. falciparum* Malaria .................................................40

Treatment of Malaria Caused by *P. vivax*, *P. ovale*, and *P. malariae* .............43

Malaria and *Plasmodium falciparum* .........................................................................45

Epidemiology ..................................................................................................................45

Epidemiological Patterns and Transmission ...............................................................46

Geographical Distribution ............................................................................................49

Drug Resistance ............................................................................................................50

Cytochrome b₅ Reductase (*Cb₅r*) and Cytochrome b₅ (*Cb₅*) ..................................57

Cytochrome b₅ Reductase .............................................................................................57

Cytochrome b₅ ................................................................................................................59

*Cb₅r* and *Cb₅* Function ............................................................................................61

*Cb₅r* and *Cb₅* Relationship and Interaction .............................................................62

Role in Drug Metabolism and Involvement in the Detoxification of Xenobiotics through Cytochrome P450 ..........................................................66

Phylogenetic Analysis of *P. falciparum Cb₅r* and *Cb₅* ...........................................68

Structural Comparison of *P. falciparum Cb₅r* with Mammalian *Cb₅r* and Other Species ..........................................................70

**OBJECTIVES** .................................................................................................................85

**MATERIALS AND METHODS** ....................................................................................88

Experimental Design .....................................................................................................88
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Estimated contributions to malaria control activities by development agencies</td>
<td>4</td>
</tr>
<tr>
<td>Table 2</td>
<td>Funding by region (US $)</td>
<td>5</td>
</tr>
<tr>
<td>Table 3</td>
<td>Reported malaria deaths, 1990-2007</td>
<td>9</td>
</tr>
<tr>
<td>Table 4</td>
<td>Causes of death in children under five years of age, estimates for 2000-2003</td>
<td>24</td>
</tr>
<tr>
<td>Table 5</td>
<td>Effects of some commonly used antimalarials on the infectivity of <em>P. falciparum</em> to the mosquito</td>
<td>40</td>
</tr>
<tr>
<td>Table 6</td>
<td>Levels of endemicity of malaria and associated statistics and characteristics</td>
<td>47</td>
</tr>
<tr>
<td>Table 7</td>
<td>Distribution of drug-resistant <em>Plasmodium falciparum</em> malaria</td>
<td>51</td>
</tr>
<tr>
<td>Table 8</td>
<td><em>P. falciparum</em> proteins with a proven role in resistance to clinical antimalarial drugs</td>
<td>56</td>
</tr>
<tr>
<td>Table 9</td>
<td>Kinetic properties of cb5r mutants using wild-type cb5</td>
<td>65</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1  Estimated contributions to malaria control activities by development agencies (US$) ................................................................. 4

Figure 2  Trends in malaria cases (inpatients and outpatients) and deaths (inpatients) in relation to intervention, 2001 – 2006 (NMCP data) ................................................................. 7

Figure 3  Steps from malaria control to elimination ................................................. 10

Figure 4a Proportion of children sleeping under insecticide-treated bed nets in selected countries, around 2000 and 2006 (Percentage) ....................... 12

Figure 4b Number of doses of artemisinin-based combination therapies procured worldwide, 2003-2006 (Millions)................................................................. 12

Figure 5 Global distribution of dominant or potentially important malaria Vectors ..................................................................... 16

Figure 6 Life cycle of malaria .......................................................................................... 20

Figure 7 Geographical distribution of malaria ................................................................... 49

Figure 8 Malaria transmission areas and reported drug resistance .................................... 52

Figure 9 Structure of rat cb_{5r} ...................................................................... 58

Figure 10 Cytochrome b_{5} diagram ................................................................................ 60

Figure 11 Ribbon Diagram of the Docking Model of Pig Cb_{5r} and Cb_{5} ........................ 64

Figure 12 Electron transport pathways to cytochrome P450 in the endoplasmic reticulum ...................................................................... 67

Figure 13 Phylogram of cytochrome b_{5} reductases .................................................. 69

Figure 13a Phylogram of cytochrome b_{5} .................................................................. 70

Figure 14a Amino acid sequence alignment with the first two binding motifs by ClustalW analysis and view ....................................................... 71

Figure 14b Continued amino acid sequence alignment containing the first NADH-binding motif by ClustalW analysis and Jalview ............................ 72

Figure 14c Continued amino acid sequence alignment with the second NADH-binding motif by ClustalW analysis and Jalview ............................ 73

Figure 15 The ribbon model of rat and candida cb_{5r} ................................................. 74
Figure 16  Cb₅r phylogram according to the first half of the protein containing the FAD-binding domain and the FAD/FMN selectivity domain .................................................................79

Figure 17  Cb₅r phylogram according to the first half of the protein containing the first and second NADH-binding domain ........................................84

Figure 18  Protein purification by chitin agarose chromatography using pTWIN vector ..................................................................................93

Figure 19  DNA gel of cb₅ gene coding insert by agarose gel electrophoresis ........94

Figure 20  Optimization of P. falciparum cb₅r protein expression: various IPTG concentrations .........................................................................98

Figure 21  P. falciparum cb₅r purification gel: Chitin Agarose and Ni-NTA Chromatography .................................................................................99

Figure 22  Optimization of P. falciparum cb₅r expression gel: addition of triton and SDS detergents .................................................................99

Figure 23  Optimization of P. falciparum cb₅r protein expression: growth at different conditions (temperature and incubation period)...............100

Figure 24  Optimization of P. falciparum cb₅r expression: 37°C LB vs TB ........................................................................................................100

Figure 25  Optimization of P. falciparum cb₅r: 16°C and EtOH .....................101

Figure 26a  P. falciparum cb₅r protein expression screening: clones 1 through 6 .................................................................................101

Figure 26b  P. falciparum cb₅r protein expression screening: clones 7 through 12 ................................................................................102

Figure 27  Cb₅ SDS-polyacrylamide gel electrophoresis ..................................103

Figure 28  Cb₅ gel after chitin agarose chromatography ..................................104

Figure 29  Cb₅ gel after complete purification by Ni-NTA column chromatography .................................................................105
# LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbols and Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFATM</td>
<td>Global Fund to fight AIDS, Tuberculosis, and Malaria</td>
</tr>
<tr>
<td>PMI</td>
<td>President’s Malaria Initiative</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin-based Combination Therapy</td>
</tr>
<tr>
<td>INT</td>
<td>Insecticide-treated Bed Net</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>NMCP</td>
<td>National Malaria Control Programs</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent Preventive Treatment</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RBL</td>
<td>Reticulocyte Binding-Like</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy Binding-Like</td>
</tr>
<tr>
<td>RDT</td>
<td>Rapid Diagnostic Test</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CRT</td>
<td>Chloroquine Resistance Transporter</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate Reductase</td>
</tr>
<tr>
<td>DHPAS</td>
<td>Dihydropteroate Synthetase</td>
</tr>
<tr>
<td>SERCA</td>
<td>Ca^{2+} Sarco/Endoplasmic Eeticulum Calcium-dependent ATPase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
</tr>
<tr>
<td>CBD</td>
<td>Chitin Binding Domain</td>
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</tbody>
</table>

vi
<table>
<thead>
<tr>
<th>TB</th>
<th>Terrific Broth</th>
</tr>
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<tbody>
<tr>
<td>Cb₅</td>
<td>Cytochrome b₅</td>
</tr>
<tr>
<td>Cb₅r</td>
<td>Cytochrome b₅ Reductase</td>
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Functional Characterization of Cytochrome b$_5$ Reductase and its Electron Acceptor

Cytochrome b$_5$ in *Plasmodium falciparum*

Lucio Malvisi

ABSTRACT

Malaria is a disease of major public health importance, killing approximately one million people and causing about 250 million cases of fever annually. It mostly affects children under the age of five and pregnant women in many developing countries, making it a prominent issue in international health and maternal and child health. The most aggressive form of malaria is caused by the parasite *Plasmodium falciparum* which is responsible for 80% of infections and 90% of deaths from malaria, and is most prevalent in sub-Saharan Africa.

Public Health interventions include the implementation of prevention programs, health education, and chemotherapy. The latter has experienced multiple problems in the past years whereby resistance of the parasite to the available drugs has emerged, rendering the majority of them ineffective. Furthermore, the high cost of those drugs represents a major obstacle to their dispensation in areas of the world where the affected people are often the less fortunate. The enzyme Cytochrome b$_5$ Reductase (cb$_5$r) and its electron acceptor Cytochrome b$_5$ (cb$_5$) play a role in fatty acid elongation, cholesterol biosynthesis, and cytochrome P450-mediated detoxification of xenobiotics. Therefore, these proteins are suitable as potential novel drug targets for malaria. These two proteins have been thoroughly studied in mammals but have to be characterized in microorganisms such as fungi and parasites, including *Plasmodium falciparum*. It is
important to note that plant cb₅r has been identified as a novel herbicidal target.

Considering the close phylogenetic relationship between plant cb₅r and *Plasmodium falciparum* cb₅r, we conclude that these plant inhibitors may also serve as promising candidates for a new class of antimalarial drugs against the parasite.

In this project, we want to obtain the biochemical and enzymatic characterization of cb₅r and cb₅ in order to establish whether these two proteins represent potential novel drug targets in *Plasmodium falciparum* malaria.

This initial work may lead to the development of novel drugs which will consequently affect the field of public health with respect to drug delivery, drug resistance, and drug chemotherapy.
INTRODUCTION

Malaria

Malaria is an infectious disease caused by a parasite called *Plasmodium*, which is transmitted via the bites of infected mosquitoes. Symptoms of malaria include fever, headache, and vomiting, and usually appear between 10 and 15 days after the mosquito bite. If not treated, malaria can quickly become life-threatening by disrupting the blood supply to vital organs (WHO, 2009). Each year approximately 500 million cases and over one million deaths occur worldwide (mainly among children under five years of age), the majority being reported in Africa south of the Sahara. These numbers make malaria one of the most important neglected diseases and a major field of action for public health organizations and authorities. A key fact is that there are incredible margins of improvement in terms of reduction of number of cases and deaths and this could be achieved by applying simple preventive measures. However, the malaria burden faces tons of issues that have impeded the regular application of those measures. Among these, the lack of funding for the development of a vaccine and of new drugs, the insurgence of drug resistance, the lack of education and the cultural obstacles of the affected populations have all played an unfavorable role in the fight against malaria. All these factors are deeply related as we will discuss later.

History

The word malaria comes from the Italian *mala aria*, meaning bad air, because it was associated with swamps and marshland. The disease was probably first described by
the Chinese in 2700 BC and then again by the Greeks in the 4th century BC where it was partially responsible for the decline of their empire. But it was again in China where a plant, named Qinghao plant, was first discovered; this plant showed very promising anti-fever properties in people affected and it represented the only available cure for malaria for centuries. The active ingredient of Qinghao was isolated by Chinese scientists in 1971. Known as artemisinin, it is today a very potent and effective antimalarial drug, especially in combination with other medicines. Another drug that has served as one of the few known remedies to treat malaria was quinine, which was derived from the bark of a plant discovered by the Spanish conquistadores in Peru. Along with artemisinin, quinine is still one of the most effective drugs available today.

The discovery of the parasite that causes malaria was made in 1880 by Charles Louis Alphonse Laveran, a French surgeon who noticed the presence of the parasites in the blood of a patient and for which he was awarded the Nobel Prize. Then, an Italian doctor, Camillo Golgi, found that there were different types of parasites that caused malaria, one that exhibited tertian periodicity (fever every three days) and one that showed a quartan periodicity (fever every four days). He also observed that the insurgence of fever corresponded to the rupture and release of merozoites (the liver stage of the parasite) into the blood. Other scientists named the various species of the parasite until the American scientist William Welch in 1897 named the malignant tertian malaria parasite, *Plasmodium falciparum* (CDC, 2004). Ever since, the prevalence of malaria has been steadily increasing despite the efforts of various public health agencies acting at local, national, and international level, to control and prevent the spread of the disease.
Over the past twenty years the annual cases of malaria have constantly raised and it is predicted that this trend will not slow down in the next years. The goal of reaching eradication that was proposed in the past years has appeared to be little realistic, therefore the current target is to achieve control of the disease.

**Funding and Financial Contributions**

Thankfully, funding for malaria that comes from international organizations, federal agencies, global funds, and the governments of wealthy nations have substantially increased in the past few years (Table 1 & Fig. 1) (Roll Back Malaria, 2005). The “Global Fund to fight AIDS, Tuberculosis, and Malaria” (GFATM), the European Union, the President’s Malaria Initiative (PMI), the “World’s Bank Booster Program for Malaria Control in Africa”, and the new French-led UNITAID are some of the main contributors in the fight against malaria (European Alliance against Malaria, 2007). Moreover, the intervention through personal funding by generous people has also occurred. These people have decided to offer their wealth in the fight against neglected diseases, as in the case of Bill Gates who founded, together with his wife, “The Bill and Melinda Gates Foundation”. Its goal is to help create a more equal world, without the burden of those infectious diseases that affect the most disadvantageous areas of the world and the most vulnerable people (Bill and Melinda Gates Foundation, 2009).
Table 1: Estimated contributions to malaria control activities by development agencies. (Roll Back Malaria, 2005)

<table>
<thead>
<tr>
<th>Fiscal year</th>
<th>Total Contributions (US$)</th>
<th>% of total</th>
<th>No. of Respondents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>19,129,701</td>
<td>1%</td>
<td>4</td>
</tr>
<tr>
<td>2000</td>
<td>42,287,888</td>
<td>2%</td>
<td>8</td>
</tr>
<tr>
<td>2001</td>
<td>386,285,841</td>
<td>21%</td>
<td>8</td>
</tr>
<tr>
<td>2002</td>
<td>418,551,580</td>
<td>22%</td>
<td>8</td>
</tr>
<tr>
<td>2003</td>
<td>400,505,904</td>
<td>22%</td>
<td>8</td>
</tr>
<tr>
<td>2004 (projected)</td>
<td>599,416,847</td>
<td>32%</td>
<td>7</td>
</tr>
<tr>
<td><strong>Total Overall</strong></td>
<td><strong>1,875,267,762</strong></td>
<td><strong>100%</strong></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Estimated contributions to malaria control activities by development agencies (US$). (Roll Back Malaria, 2005)

Also in the Roll Back Malaria report, we see similar trends in the allocation of money that is made available for research in malaria. Analyzing the situation of the past few years, we notice that funds have been more and more abundant every year and in every aspect of the fight against malaria. Contributions from development agencies have targeted not only the governments of the countries where malaria is present, but also reached out to NGOs, research and development in malaria control, and other organizations such as Roll Back Malaria, the Malaria Consortium, and the Special Program on Research and Training in Tropical Diseases. Another important aspect is that funds also increased in terms of availability in different geographic regions; all affected regions on earth saw their resources reach very promising levels. In particular sub-
Saharan Africa, the area of the world where the majority of cases and deaths occur and where most of the fatalities are caused by the most aggressive of the malaria parasites, namely *Plasmodium falciparum*, experienced an exponential development in the funds donated by agencies (Table 2) (Roll Back Malaria, 2005). This really has a positive impact on the lives of millions of people that live in endemic areas; it is also a sign that the strategy and the administration of the budget is well organized because funding is going where it is most needed and dispersion of money, a common issue in the delivery of funds to the proper recipients, is widely avoided.

<table>
<thead>
<tr>
<th>Recipient Region</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Americas</td>
<td>305,400</td>
<td>2%</td>
<td>1,661,200</td>
<td>5%</td>
<td>3,680,900</td>
<td>8%</td>
<td>4,533,200</td>
</tr>
<tr>
<td>Europe/Central Asia</td>
<td>0</td>
<td>0%</td>
<td>15,000</td>
<td>1%</td>
<td>0</td>
<td>0%</td>
<td>964,000</td>
</tr>
<tr>
<td>South Asia</td>
<td>186,100</td>
<td>1%</td>
<td>716,000</td>
<td>2%</td>
<td>2,387,038</td>
<td>5%</td>
<td>2,254,041</td>
</tr>
<tr>
<td>South East Asia</td>
<td>7,160,400</td>
<td>42%</td>
<td>7,998,380</td>
<td>42%</td>
<td>5,003,220</td>
<td>8%</td>
<td>5,213,556</td>
</tr>
<tr>
<td>Sub-Saharan Africa</td>
<td>9,355,076</td>
<td>55%</td>
<td>21,746,251</td>
<td>68%</td>
<td>47,553,368</td>
<td>80%</td>
<td>53,068,100</td>
</tr>
<tr>
<td>Total</td>
<td>17,065,016</td>
<td>32,178,351</td>
<td>39,076,344</td>
<td>66,083,930</td>
<td>123,432,671</td>
<td>241,789,281</td>
<td>383,970,246</td>
</tr>
</tbody>
</table>

Table 2: Funding by region (US $). (Roll Back Malaria, 2005)

When looking at the data in Table 2 or many other tables of data collected by international organizations, we realize that the huge difference in funding between 1999 and 2004 (the most striking numbers are observed in the sub-Saharan African region) is certainly not reflected by the crude figures of number of new cases and deaths. In 2005, the World Health Assembly determined to ‘ensure a reduction in the burden of malaria by at least 50% by 2010 and by 75% by 2015’. This resolution has been interpreted to mean a reduction in morbidity as well as mortality.
We are facing a worldwide burden that’s causing the death of millions of people every day and it is mainly affecting the most vulnerable part of the population, namely children and pregnant women. Current public health measures of prevention and control have served of little help in spite of their excellent, at least theoretically, applicability. Public health authorities are acting on any possible form of prevention and control, attacking the problem with aggressive actions, however each procedure often encounters difficulties. These can be due to drug resistance in the development of a new drug or drug target, mosquitoes that have become resistant to insecticides, or the implementation of new programs that go against cultural practices to name a few.

Another very striking number that is closely associated to those just mentioned is the coverage of the population with curative and preventive measures such as Artemisinin-based Combination Therapy (ACT) and Insecticide-treated Bed Nets (ITN), respectively. Considering 2005 as the baseline year for the evaluation of the results obtained with the implementation of the new strategies and directives set by the WHO, such as control programs, epidemiological surveillance, health policies and health system structure, the target is to reach a coverage of over 80% of the population by 2010. It is a very complicated scenario and the data obtained from the surveillance reports of many countries are contradictory, often due to unreliable data collection. Between 2001 and 2006, many countries in Africa experienced a growth in cases and deaths even though this may be due to improved surveillance or more complete records for those years. On the other hand, a few other African countries such as Eritrea, Madagascar, and Rwanda, achieved high coverage of interventions and saw cases and deaths diminished (Fig. 2).
The scenario in terms of curative and preventive measures in Africa is very variegated and difficult to interpret. But thanks to an improved epidemiological surveillance and more thorough records, more reliable data will be available in the future that will enable us to draw more accurate conclusions.

![Figure 2: Trends in malaria cases (inpatients and outpatients) and deaths (inpatients) in relation to intervention, 2001 – 2006 (NMCP data). (WHO, 2008)](image)

These are two clear examples of how the start of the implementation of malaria control programs, such as the distribution of insecticide-treated nets (ITN), indoor residual spraying (IRS), long-lasting insecticidal nets (LLIN), and artemisinin-based combination therapy (ACT) coincide with a stunning decrease in the number of cases and deaths.

Outside Africa, the picture is similar. In a few countries of Central and South America, the reduction in malaria has coincided with improved policies on malaria control; these included efficient surveillance and early case detection to prevent and contain epidemics, integrated vector management, prompt diagnosis and treatment, and health system strengthening. In the WHO Eastern Mediterranean Region, the countries that have shown the greatest reductions in malaria are those where National Malaria Control Programs (NMCPs) have strong political and financial support from the government, and which operate within health systems that are well developed at the central and peripheral level. As we can see, the available data do not allow us to draw definite conclusions about the efficacy of the whole range of activities being
implemented and developed by the partnership between various international organizations and programs.

An important aspect is that the process of reduction of the incidence of malaria in successful countries was guaranteed by a more advanced political and economic status. On the other hand, the absence of a firm and little corrupted head of state, the devastations caused by never-ending civil and military conflicts, the simultaneous presence of other deadly diseases, a history of centuries of subordination to colonial empire, and the scarcity of natural resources have impeded a decent administration of the malaria control interventions in certain countries such as Burkina Faso, Nigeria, Senegal, and Ghana. Here the predispositions to a well-rounded execution of preventive and control measures are absent, therefore the logical outcome was an increase in the cases and deaths from malaria over the last few years (Table 3).
From the table we notice the increase in deaths in the countries just mentioned: Burkina Faso, Nigeria, Senegal, and Ghana where certain conditions such as lack of leadership, corruption, war, and extreme poverty prevented the malaria control programs from being successful.

In an effort to a comprehensive fight against malaria, WHO has also identified four programmatic phases on the way to achieving and maintaining elimination: control, pre-elimination, elimination, and prevention of reintroduction (Fig. 3) (WHO, 2008).
Figure 3: Steps from malaria control to elimination. (WHO, 2008)

According to this scheme, countries make the transition from control to the pre-elimination phase when less than 5% of all suspected malaria cases have a laboratory confirmation of malaria. The elimination phase begins when there is less than 1 malaria case per 1000 people at risk per year. Elimination is achieved when the “prevention of reintroduction”, without local transmission by mosquitoes, has been successful for three or more consecutive years (WHO, 2008).

Analysis of Goal 6 of the Millennium Development Goals: Combat HIV/AIDS, malaria and other diseases. Interestingly, Goal 6 of the Millennium Development Goals, which foresees the eradication of malaria by 2015, is falling short of global targets. Here, the two main means to reduce the incidence of malaria are considered 1) prevention through a higher distribution and use of insecticide-treated mosquito nets and 2) treatment through the use of antimalarial medicines. In particular the United Nations are now tending to shift their focus and interest on the artemisinin-based combination therapy, especially in sub-Saharan Africa where resistance to traditional drugs has developed. But “There has been less progress in treating malaria than in preventing it” UN officials say in their last report in August 2008. In fact, according to data gathered from 2000 to 2006, the use of insecticide-treated mosquito nets has increased (Fig. 4a) (even though not with
the impressive figures the organization was expecting), while the proportion of children with fever who have received antimalarial medicines in 2006 has declined in comparison with the 2000 figures. Even more stunningly, treatment with ACT is, in general, still at extremely low levels, even though it has shown hopeful signs of growth since 2005 (Fig. 4b). Funding for the more expensive ACT is recent but has increased markedly since 2005; moreover, the implementation of programs that try to promote the use of ACT as standard treatment for malaria is just a recent shift, therefore the governments and the ministries of health of the affected nations are slowly but effectively mandating the introduction of this fairly new line of treatment. In general, evidence suggests that both the increased distribution of insecticide-treated nets and household spraying, and the use of antimalarial medicines are giving promising results in the reduction of cases and deaths from malaria around the world, even though the idea of eradication by 2015 seems to be rather utopist (United Nations, 2008).
Figure 4a: Proportion of children sleeping under insecticide-treated bed nets in selected countries, around 2000 and 2006 (Percentage). (United Nations, 2008)

Figure 4b: Number of doses of artemisinin-based combination therapies procured worldwide, 2003-2006 (Millions). (United Nations, 2008)
In 2007 the European Alliance against Malaria said that there is an estimated global requirement of about US$ 3.2 billion a year to make sure malaria is eradicated by 2015. However, at present less than US$ 1 billion per year is available for malaria resources (European Alliance against Malaria, 2007). All the elements needed for a successful eradication, such as funding, research, leadership, experience, awareness, and dedication are present but need to be used wisely. The results are promising.

**Etiology**

Malaria is caused by obligate intracellular protozoan parasites of the genus *Plasmodium*. They are members of the class Sporozoa, therefore alternating asexual and sexual reproductive cycles are observed.

There are currently over 200 species in the genus, of which at least ten infect humans but only five are known to cause malaria in humans:

- ***Plasmodium falciparum***: found in tropical regions and not established in colder climates; causes the most severe and fatal disease.

- ***Plasmodium vivax***: most common and widely distributed (subtropical and temperate) malarial parasite; relapsing malaria.

- ***Plasmodium malariae***: limited to subtropical areas. This species is less common than *P. falciparum* or *P. vivax*.

- ***Plasmodium ovale***: the least common malarial species, usually found in Africa; relapsing malaria.

- ***Plasmodium knowlesi***: causes malaria in macaques but can also infect humans.
Plasmodium falciparum has the highest rate of mortality. It is also responsible for 80% of malarial infections and 90% of deaths. Episodes of infection and death from P. falciparum occur mainly in sub-Saharan Africa.

P. vivax is less virulent than P. falciparum and usually not fatal. Sometimes death is observed due to splenomegaly, a pathologically enlarged spleen. Its distribution comprises Asia, Latin America, and some parts of Africa.

P. malariae causes a ‘benign malaria’ and it is not nearly as dangerous as the infections caused by P. falciparum and P. vivax. It is found worldwide from sub-Saharan Africa to Southeast Asia, the islands of the Western Pacific, and the Amazon Basin of South America. It is also the least studied of all five malaria-causing parasites due to its low prevalence and milder clinical manifestations.

P. ovale is very limited in its distribution; it is endemic mainly in West Africa, the Philippines, Eastern Indonesia, and Papua New Guinea. It is considered less virulent than P. falciparum and P. vivax.

P. knowlesi is a primate malaria parasite commonly found in Southeast Asia. As mentioned, it primarily causes malaria in macaques but it can also infect humans, especially those who work in the rain forest. With the increasing amount of deforestation in Southeast Asia, the macaques are now coming in close contact with humans, therefore even the people that live in semi-urban areas are now found to be infected with P. knowlesi. The first case of a natural infection with P. knowlesi was reported in 1965 in an American man who visited the Malaysian rainforest. P. knowlesi accounts for up to 70% of cases of malaria in Southeast Asia. This parasite is not prevalent in West Africa. This may be due to the fact that West African populations lack the Duffy antigen, a protein on
the surface of red blood cells (RBC) that the parasite uses to start the invasion (National Institute of Health, 2000).

**Vector and Transmission**

Malaria is transmitted to humans through the bite of infected female anopheles mosquitoes. *Anopheles* is the only recognized genus of mosquitoes that is able to transmit the parasite *Plasmodium* with the consequent development of malaria in the host. Of the 460 recognized species of anopheles, approximately 60 have been identified as vectors for malaria. *Anopheles gambiae* is probably the best known vector because of its ability to transmit the deadly *Plasmodium falciparum*, and it is also the most abundant vector in Africa together with *A. funestus*. The vector in North America is *Anopheles freeborni*. In India, approximately 45 mosquitoes have been identified to be vectors or at least potential vectors for malaria; among these, the most prominent are *A. fluviatilis* and *A. minimus* which are found in the foot-hill regions, *A. stephensi* and *A. sundaicus* which are numerous in the coastal regions and *A. culicifacies* and *A. philippinensis* which are characteristic of the plains (Fig. 5) (Malaria Site, 2007).
Understanding the biology and behavior of *Anopheles* mosquitoes can help understand how malaria is transmitted and can aid in designing appropriate control strategies. One very important aspect in terms of the biology and behavior of anopheles mosquitoes is that different species have different capacities to successfully carry and transmit the parasite to humans. Some have immune mechanisms that encapsulate and kill the parasites after they have invaded the mosquito stomach wall (Tahar et al, 2002). This ability of the mosquito immune system to identify and attack the parasites renders...
the mosquito refractory to the parasite and does not facilitate the transmission of the
disease, a fundamental characteristic in terms of public health control measures. In fact,
there have been studies where this immune response mechanism has been analyzed with
the hope of producing genetically modified mosquitoes that are refractory to malaria and
that will replace the wild ones, thereby limiting the transmission of malaria. In particular,
scientists have found that the C-type lectin CEL-III from *Cucumaria echinata*, a sea
cucumber found in the Bay of Bengal, impaired the development of the parasite
*Plasmodium* when produced in genetically modified *A. stephensi* (Yoshida et al., 2007).
This and further studies are setting up the base for the creation of transgenic mosquitoes
with the goal of replacing them with the wild mosquitoes; this would be considered a
huge relief in the efforts that are being made in the control and prevention of malaria and
it is a sort of revolution in how the methods used to fight malaria are viewed. This
would be considered a victory over malaria by molecular biology rather than public
health (CDC, 2008).

Other behavioral characteristics that influence the transmission of malaria and that
have implications on the design of control programs are 1) the sources of blood and its
relation to the time required for the development of the parasite in the mosquito, 2) the
patterns of feeding, and 3) insecticide resistance. These elements are explained as
follow:

1) It is known that some mosquitoes are anthropophilic and feed on humans while others
are zoophilic and feed on animals. The two major species found in Africa, *A. gambiae*
and *A. funestus*, are strongly anthropophilic. The next necessary process that has to take
place in order for the transmission cycle to continue, is the development of the parasite
within the mosquito. This process is known as extrinsic incubation period and it ranges from 10 to 21 days, depending on the parasite species and the temperature at which it develops. If a mosquito does not live long enough to allow for parasite development, the transmission cannot occur. Since it is not feasible to measure the life span of mosquitoes in nature, researchers estimated that the rate of survivorship in *A. gambiae* in Tanzania ranged between 0.77 and 0.84, meaning that at the end of one day between 77% and 84% will have survived. Assuming that this survivorship is constant throughout the adult life of a mosquito, less than 10% of female *A. gambiae* would survive longer than a 14-day extrinsic incubation period (CDC, 2008).

2) Anopheles mosquitoes are crepuscular (active at dawn or dusk) or nocturnal (active at night). They enter the house between 5 p.m. and 9.30 p.m. and again in the early hours of the morning. They start biting by late evening and the peak of biting activity is at midnight and early hours of the morning. Anopheles mosquitoes can be usually differentiated into endophagic/endophilic (mosquitoes which prefer to feed indoors and rest indoors after feeding) and exophagic/exophilic (those which prefer to feed outdoors and rest outdoors after feeding); the biting pattern of the first group can be easily prevented by indoor spraying of insecticides while the activity of the second category is controlled through the destruction of the breeding sites (Malaria Site, 2007).

3) Insecticides are used in the two most efficacious methods of control recommended by the WHO and implemented by the ministries of health and local authorities of malaria-endemic nations: indoor residual spraying and insecticide-treated nets. Unfortunately, mosquitoes throughout the planet have shown various levels of resistance to all
insecticides available. According to the CDC, there are over 125 mosquito species with documented resistance to one or more insecticides (CDC, 2008).

Since resistance is a fundamental aspect in control program planning, resistance management has become an integral part of resistance surveillance. As a consequence, program planning can also be more proficient with an efficient surveillance. If for example, a certain vector is no longer considered susceptible to DDT based on field surveillance and subsequent verification with molecular tools, then an alternative insecticide may be used in that particular area. Moreover, if the vector shows some resistance to a particular insecticide, it does not mean that control activities are compromised. Resistance to a particular insecticide must be high enough not to have any effect on preventing transmission. A good example that delineates this case scenario was Western Kenya, where resistance to pyrethroid applied to bed nets appeared soon after their introduction and was calculated to be approximately 10% of the vector population. After a couple of years, the levels of resistance were still the same, therefore no changes in control policies were necessary (Brogdon & McAllister, 1998).

The current available insecticides are DDT, pyrethroids (deltamethrin and permethrin), bendiocarb, and malathion. Mosquitoes have shown some degree of resistance to all of these. The most widely used in recent years have been pyrethroids because of their low toxicity and relative safety for humans, but resistance, particularly in the application to ITNs, has become a growing problem throughout Africa (Kerah-Hinzoumbè et al., 2008). Furthermore, cases of cross-resistance to DDT and pyrethroids have been documented. This occurred because these two chemicals have a similar mode of action and act on the same target, namely the sodium channels of the nerve sheath. In
In order to deal with an increasing level of resistance to our available insecticides, it is imperative that we establish a well-organized system of early detection and constant field surveillance for resistance and that we make progress in those molecular genetics studies that will lead us to a deeper understanding of how resistance arises and maintains in populations (Brogdon & McAllister, 1998).

**Life Cycle**

![Figure 6: Life Cycle of Malaria. (CDC, 2006)](image)

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 4. (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 A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 6. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. 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 8. The parasites’ multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10 which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 13 into a new human host perpetuates the malaria life cycle (CDC, 2006).

Public Health Impact

Malaria is one of the most severe public health problems worldwide and a leading cause of death and illness in many developing countries. According to the last ‘World Malaria Report 2008’ there were an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5
years. 109 countries were endemic for malaria in 2008, of which 45 were within the WHO African Region (WHO, 2008). Other significant figures:

Malaria Cases:
- 91% or 230 million cases were due to *Plasmodium falciparum*
- 86% of all cases occurred in the African Region, followed by South-East Asia (9%) and the Eastern Mediterranean Region (3%)
- The percentage of cases due to *P. falciparum* exceed 75% in most African countries but only in a few countries outside Africa
- In Africa, 19 of the most populous countries accounted for 90% of estimated cases in 2006

Malaria Deaths:
- There were an estimated 881,000 deaths worldwide in 2006, of which 90% in the African Region and 4% in each of the South-East Asia and Eastern Mediterranean Region
- An estimated 85% of deaths occur in children under 5 years, but the proportion is much higher in the African (88%) and Eastern Mediterranean regions (76%) than in other regions (16-40%)
- Eighteen countries accounted for 90% of deaths in the African Region, and seven countries had 90% of deaths outside the African Region, dominated by Sudan and India

So which are the most affected groups of the population? For the most part children under the age of five who have not yet developed immunity to malaria, pregnant women, whose immunity is decreased by pregnancy, especially during the first and second
pregnancies, and travelers or migrants coming from areas with little or no malaria transmission who lack immunity.

A central factor that contributes to the expansion of the burden of malaria is the infection during pregnancy. Malaria can have adverse effects on both the mother and the fetus. Health consequences include maternal anemia, fetal loss, premature delivery, intrauterine growth retardation, and delivery of low birth-weight infants (<2500 g or <5.5 pounds). The health condition that an infection causes differ according to the type of malaria transmission area. There are two types of transmission areas: stable (high) or unstable (low).

- In high transmission areas, women have gained a level of immunity to malaria that wanes somewhat during pregnancy. Malaria infection is more likely to result in severe maternal anemia and delivery of low birth-weight infants.
- In low transmission areas, women generally have developed no immunity to malaria. Malaria infection is more likely to result in severe malaria disease, maternal anemia, premature delivery, or fetal loss.

In sub-Saharan Africa, the region of the world with the highest incidence of malaria, infections are estimated to cause 400,000 cases of severe maternal anemia and 75,000-200,000 infant deaths annually. Maternal anemia contributes significantly to maternal mortality and causes an estimated 10,000 deaths per year. Low birth weight is the greatest risk factor for neonatal mortality and a major contributor to infant mortality. Although many factors contribute to low birth weight, malaria is a major factor and one
of the few, along with poor nutrition, anemia, and other infections, that is amenable to intervention once a woman becomes pregnant (CDC, 2004; Steketee et al., 2001).

In an effort to combat this problem, the World Health Organization currently recommends a three-pronged approach to prevent these adverse effects in areas of Africa with high levels of transmission of *Plasmodium falciparum* malaria:

1. Intermittent preventive treatment (IPT) with antimalarial drugs
2. Insecticide-treated bed nets (ITN)
3. Febrile malaria case management

One very important point that’s worth to be mentioned here is that the available health information for much of sub-Saharan Africa is of very poor quality. The annual estimates provide a useful tool that greatly aids in the types, number, and extensiveness of malaria control initiatives but there has been skepticism about their origin and validity. Therefore, all present approximations are derived from evidence-based approaches that provide greater credibility to malaria control projects (Snow et al., 1999). Often times estimates of the number of malaria deaths are made by: (1) multiplying the estimated number of *P. falciparum* malaria cases by a fixed case-fatality rate for each country; or (2) from an empirical relationship between measures of malaria transmission risk and malaria-specific mortality rates (WHO, 2008).

<table>
<thead>
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<th>Rank</th>
<th>Cause</th>
<th>Numbers (thousands per year)</th>
<th>% of all deaths</th>
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<td>Neonatal causes</td>
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</tr>
<tr>
<td>2</td>
<td>Acute respiratory infections</td>
<td>2,027</td>
<td>19</td>
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<tr>
<td>3</td>
<td>Diarrheal diseases</td>
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<tr>
<td>4</td>
<td>Malaria</td>
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<td>8</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>Measles</td>
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</tr>
<tr>
<td>6</td>
<td>HIV/AIDS</td>
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<tr>
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<td>Injuries</td>
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<tr>
<td>Other causes</td>
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<tr>
<td>Total</td>
<td>10,596</td>
<td>100.0</td>
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</tr>
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</table>

**Table 4: Leading Causes of Death in Children Under Five Years of Age, Estimates for 2000-2003.** (WHO, 2005)

**Clinical Features**

Infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death (CDC, 2006). In general, malaria presents itself as a febrile illness characterized by fever and related symptoms (Malaria Site, 2006). The incubation period can range from 7 to 30 days, the shortest time being typically associated with *P. falciparum* and the longest interval with *P. malariae*. In the case where antimalarial drugs are being taken for prophylaxis and in the event of an infection with *P. vivax* or *P. ovale*, the incubation period can even reach months or years because these two parasites can produce dormant liver stages and reactivate after months or even years after the first manifestation, a physiological process known as relapse.

Malaria can be categorized as uncomplicated and severe (complicated). In the uncomplicated malaria a typical attack lasts about 6-10 hours. It consists of:

- a cold stage (sensation of cold, shivering)
- a hot stage (fever, headaches, vomiting; seizures in young children)
- a sweating stage (sweats, return to normal temperature, tiredness).
Attacks occur every second day with tertian parasites (*P. falciparum*, *P. vivax*, and *P. ovale*), every third day with the quartan parasite (*P. malariae*), whereas a quotidian fever (every day) is observed in *P. knowlesi*. Additional clinical manifestations of *P. falciparum* include mild jaundice, enlargement of the liver, and increased respiratory rate. In countries where cases of malaria are infrequent, the typical symptoms may be attributed to influenza, a cold, or other common infections, especially if malaria is not suspected. Conversely, in countries where malaria is frequent, residents often recognize the symptoms as malaria and treat themselves without seeking diagnostic confirmation ("presumptive treatment").

Severe malaria occurs when *P. falciparum* infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The manifestations of severe malaria include:

- Cerebral malaria, abnormal behavior, impairment of consciousness, seizures, coma, or other neurologic abnormalities
- Severe anemia due to hemolysis (destruction of the red blood cells)
- Hemoglobinuria (hemoglobin in the urine) due to hemolysis
- Pulmonary edema (fluid buildup in the lungs) or acute respiratory distress syndrome (ARDS), which may occur even after the parasite counts have decreased in response to treatment
- Abnormalities in blood coagulation and thrombocytopenia (decrease in blood platelets)
- Cardiovascular collapse and shock
Severe malaria occurs most often in persons who have no immunity to malaria or whose immunity has decreased. These include all residents of areas with low or no malaria transmission, and young children and pregnant women in areas with high transmission (CDC, 2006).

**Pathophysiology**

An important and fairly new concept in describing malaria is that of a multisystem disorder even when the clinical manifestations appear to involve a single organ, such as the brain or the liver. For instance, metabolic acidosis that is often observed in severe cases of malaria, such as cerebral malaria or malarial anemia, may lead to a syndrome of respiratory distress (Taylor et al., 1993). In most cases, this is predominantly (but not exclusively) a lactic acidosis (English et al., 1996). To emphasize the complexity, variety, and complementary nature of clinical and molecular events of malaria, it was determined that lactic acidosis in children has multiple causes: from increased production by parasites, through direct stimulation by cytokines, to decreased clearance by the liver. However, most significantly by far is likely to be the combined effects of several factors in reducing oxygen delivery to tissues (English et al., 1997).

A second characteristic is that there is no simple one-to-one correlation between clinical syndromes of severe disease and pathogenic processes. As a result, severe anemia may arise from multiple poorly understood processes, including acute hemolysis of uninfected RBCs and dyserythropoiesis, as well as through the interaction of malaria infection with other parasite infections and with nutritional deficiencies (Newton et al., 1997).
But how does the infection begin at the physiological level? Mosquitoes inject parasites (sporozoites) into the subcutaneous tissue and less frequently directly into the bloodstream; from there, sporozoites travel to the liver. Recent evidence indicates that sporozoites pass through several hepatocytes before invasion is followed by parasite development (Mota et al., 2001). The co-receptor on sporozoites for invasion involves, in part, the thrombospondin domains on the circumsporozoite protein and on thrombospondin-related adhesive protein. These domains bind specifically to heparin sulfate proteoglycans on hepatocytes in the region in apposition to sinusoidal endothelium and Kupffer cells (Frevert et al., 1993). Within the hepatocyte, each sporozoite develops into tens of thousands of merozoites, each able to invade a RBC upon release from the liver. Disease begins only once the asexual parasite multiplies within RBCs. This is the only gateway to disease. *P. falciparum* and *P. vivax* within RBCs develop over 48 hours, producing around 20 merozoites in a mature parasite, each able to invade other RBCs. A small proportion of asexual parasites convert to gametocytes that are critical for the transmission of the infection to other hosts through female anopheline mosquitoes. This does not cause any disease.

In this context, the strategy of *P. vivax* differs from that of *P. falciparum*. *P. vivax* develops into gametocytes soon after release of merozoites from the liver while *P. falciparum* gametocytes develop much later. Early treatment of clinical malaria by anti-bloodstage chemotherapy for *P. falciparum* also kills the developing gametocytes; *P. vivax* transmits before the symptomatic stage of the disease (Weatherall et al., 2002).

All the typical clinical symptomology and severe disease pathology associated with malaria is caused by the asexual erythrocytic or blood stage parasites (CDC, 2004).
The sequence of invasion into the red blood cells is probably similar for all *Plasmodium* species. The merozoite first attaches to red cells. In *P. falciparum*, Erythrocyte Binding Antigen 175 and Merozoite Surface Protein 1, 2 with sialoglycoproteins have been identified as the ligands while in *P. vivax*, the Duffy antigen on RBC is the site of binding. After the attachment to the red cell, the merozoite re-orientates itself so that apposition of apical end occurs. This is followed by localized invagination and interiorization of the merozoite. The entire process is completed in 30 seconds. The growth and multiplication cycle within the RBCs (*Erythrocytic schizogony*) takes about 48 hours for one cycle (72 hours in the case of *P. malariae*). Each merozoite divides into 8-32 (average 10) fresh merozoites. The merozoites grow in stages into rings called trophozoites (trophos means nourish) and divide in a Schizont - meaning split - to release more merozoites (mero means separate). At the end of this cycle, the mature schizonts rupture the RBCs and release the new merozoites into the blood, which in turn infect more RBCs. The merozoite ingests hemoglobin from RBCs to form a food vacuole where it is degraded and heme is released. The toxic heme is in turn detoxified by heme polymerase and sequestrated as hemozoin. All the clinical features of malaria are caused by these events in the blood. Eventually, the growing parasite progressively consumes and degrades intracellular proteins, principally hemoglobin, resulting in the formation of the 'malarial pigment' and hemolysis of the infected red cell. This also alters the transport properties of the red cell membrane, and the red cell becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins, which could directly induce the release of cytokines such as TNF and interleukin-
1 from macrophages, resulting in chills and high grade fever. This occurs once in 48 hours, corresponding to the erythrocytic cycle (Weatherall et al., 2002).

Both *P. falciparum* and *P. vivax* can cause severe anemia, but only *P. falciparum* causes the multiple complications of cerebral malaria, hypoglycemia, metabolic acidosis, and respiratory distress. Certain differences in the biology of the two parasites partially explain the differences in patterns of disease. First, *P. falciparum* can invade a large percentage of the RBCs, whereas *P. vivax* is limited to reticulocytes. A second difference between the two parasites is that there is a surprising redundancy of invasion pathways in *P. falciparum*. *P. vivax* invades only Duffy blood group-positive RBCs and is largely limited to reticulocytes. In West Africa, where the Duffy blood group is missing on RBCs, *P. vivax* essentially disappeared. The limitations in invasion of *P. vivax* have led to the discovery of two families of parasite receptors: 1) the parasite molecule that binds to the Duffy blood group system and Duffy binding-like (DBL) homologous proteins of *P. falciparum* and *P. knowlesi* (Kappe et al., 1998) and 2) the parasite reticulocyte-binding proteins of *P. vivax* (Galinski et al., 1992) and reticulocyte binding-like (RBL) homologous proteins of *P. falciparum* (Rayner et al., 2001). The various members of the DBL and RBL families may recognize different RBC receptors than the Duffy blood group or the receptor on reticulocytes.

**Diagnosis**

The diagnosis of malaria involves identification of malaria parasite or its antigens/products in the blood of the patient. Although this seems simple, the efficacy of the diagnosis is subject to many factors. The different forms of the four malaria species,
the different stages of erythrocytic schizogony, the endemicity of different species, the population movements, the interrelation between the levels of transmission, immunity, parasitemia, and symptoms, the problems of recurrent malaria, drug resistance, persisting viable or non-viable parasitemia, and sequestration of the parasites in the deeper tissues all affect proper diagnosis. Finally the use of chemoprophylaxis or even presumptive treatment on the basis of clinical diagnosis can all have a bearing on the identification and interpretation of malaria parasitemia on a diagnostic test (Malaria Site, 2006).

Prompt and accurate diagnosis is the key to effective disease management, which is one of the main interventions of the Global Malaria Control Strategy (WHO, 1993). It is thus of concern that poor diagnosis continues to hinder effective malaria control. This is due to a combination of factors, including non-specific clinical presentation of the disease, high prevalence of asymptomatic infection in some areas, lack of resources and insufficient access to trained health care providers and health facilities, and widespread practice of self-treatment for clinically suspected malaria.

The first and most practical method for diagnosing malaria is through clinical examination. Clinical diagnosis is the most widely used approach in rural areas and at the periphery of the health care system where laboratory support to clinical diagnosis does not exist (WHO/USAID, 2000). The problem with clinical diagnosis is that the symptoms of malaria are very non-specific, especially those of mild and moderate malaria. A diagnosis of malaria based on clinical grounds alone is therefore unreliable. As a result, when malaria is suspected, the clinical examination should be followed by a microscopic diagnosis, which represents the established method for laboratory confirmation of malaria. In most settings, the procedure consists of collecting a finger-
prick blood sample, preparing a thick blood smear (in some settings a thin smear is also prepared), staining the smear (most frequently with Giemsa), and examining the smear through a microscope (preferably with a 100X oil-immersion objective) for the presence of malaria parasites (Makler et al., 1998).

Microscopy offers many advantages.

- It is sensitive. When used by skilled and careful technicians, microscopy can detect densities as low as 5 – 10 parasites per µl of blood (WHO, 1990). Under general field conditions, however, the detection capabilities of a typical microscopist might be more realistically placed at 100 parasites per µl of blood (WHO, 1998).

- It is informative. When parasites are found, they can be characterized in terms of their species (P. falciparum, P. vivax, P. ovale, and/or P. malariae) and of the circulating stage (e.g. trophozoites, schizonts, gametocytes). Occasionally, expert microscopists can detect morphological alterations induced by recent drug treatment. In addition, the parasite densities can be quantified (from ratio of parasites per number of leukocytes or erythrocytes). Such quantifications are needed to demonstrate hyperparasitemia (which may be associated with severe malaria) or to assess parasitological response to chemotherapy.

- It is relatively inexpensive. Cost estimates for endemic countries range from about US$ 0.12 to US$ 0.40 per slide examined (Palmer, 1999). Such figures, however, do not reflect the true cost to the health system or to the patient, which may be substantially higher. In addition, the cost per test will increase if
utilization is low, or if microscopy in the health facility is used only for malaria diagnosis.

- It is a general diagnostic technique that can be shared with other disease control programs, such as those against tuberculosis or sexually transmitted diseases.

- It can provide a permanent record (the smears) of the diagnostic findings and be subject to quality control.

Microscopy suffers from three main disadvantages.

- It is labor-intensive and time-consuming, normally requiring at least 60 minutes from specimen collection to result.

- It is exacting and depends absolutely on good techniques, reagents, microscopes and, most importantly, well trained and well supervised technicians.

Unfortunately these conditions are often not met, particularly at the more peripheral levels of the health care system.

- There are often long delays in providing the microscopy results to the clinician, so that decisions on treatment are often taken without the benefit of the results.

There are also non-microscopic tests that can be grouped in a category named ‘Rapid Diagnostic Tests’ (RDT). These tests involve identification of the parasitic antigen or the anti-plasmodial antibodies, or the parasitic metabolic products. Some of the most common RTDs are: OptiMal Assay, Polymerase Chain Reaction (PCR), the immune-chromatographic test, the detection of antibodies by Radio-immuno assay, and immunofluorescence or enzyme immune assay.
The advantages of RDTs over microscopy are:

- RDTs are simpler to perform and to interpret. They do not require electricity, special equipment or training in microscopy. Peripheral health workers (and other health providers as well as community volunteers) can be taught the procedure in a matter of hours, with good retention of skills over a one-year period.
- RDTs are relatively robust and test performance and interpretation vary relatively little among individual users. Moreover, most kits can be shipped and stored under ambient conditions.
- Since RDTs detect circulating antigens, they may detect *P. falciparum* infection even when the parasites are sequestered in the deep vascular compartment and thus undetectable by microscopic examination of a peripheral blood smear. In women with placental malaria (as demonstrated by placental smears), RDTs have detected circulating HRP-II even though the blood smears were negative due to sequestration of *P. falciparum* in the placenta (Leke et al., 1999).

Disadvantages include:

- Commercially available RDTs targeting HRP-II can detect only *P. falciparum*. Such kits will detect only a portion of cases in areas where other *Plasmodium* species are co-endemic. They are not suitable for diagnosing cases of imported malaria from areas where *P. falciparum* is not necessarily the most prevalent species.
- RDTs that target HRP-II of *P. falciparum* can give positive results for up to two weeks following chemotherapy and parasite clearance as confirmed by
microscopy. The reason for this antigen persistence needs to be clarified. Pending such clarification, RDTs targeting HRP-II might yield confusing results in relation to the assessment of treatment failure or drug resistance.

- The current RDTs are more expensive than microscopy, with costs per test varying from US$ 0.60 to US$ 2.50 and possibly more, depending on the marketing area.

- RDTs are not quantitative. They thus fail to provide information of possible prognostic importance and are not suitable for detailed investigations on the therapeutic efficacy of antimalarial drugs.

- Kits that detect both *P. falciparum* and non-falciparum species cannot differentiate between *P. vivax*, *P. ovale* and *P. malariae*, nor can they distinguish pure *P. falciparum* infections from mixed infections that include *P. falciparum*.

- RDTs that detect antigens produced by gametocytes can give positive results in infections where only gametocytes are present. Gametocytes are not pathogenic, and gametocytes of *P. falciparum* can persist following chemotherapy without implying drug resistance. Such positive RDT results can lead to erroneous interpretations (false positives) and unnecessary treatment of people not suffering from malaria.

Now a differentiation has to be made between the diagnostic tools of choice in areas of high and low malaria transmission. In high malaria endemicity areas, if a case is suspected of severe malaria, laboratory confirmation can guide initial therapy. In facilities at the central and district levels, microscopy should be the confirmatory diagnostic test of choice. In peripheral locations where microscopy is not available,
RDTs might prove particularly useful since they can be performed by health workers with limited training and skills. Compared to blood smears, RDTs provide more timely results for disease management. In low malaria endemicity areas, clinical diagnosis should be rapidly followed by microscopy and RDTs if necessary. In the extreme case of remote communities or highly mobile populations, where microscopic diagnosis is not available and where patients do not have adequate access to health care facilities, treatment is frequently based on clinical diagnosis alone. Here, the use of RDTs by local health workers or community volunteers has proved valuable.

Another important factor to consider is the development of drug resistance. In fact, when the diagnosis of malaria is based merely on a symptomatic evaluation, many problems can emerge. First of all, since malaria has very unspecific symptoms, a diagnosis can never be accurate, and using malarial medicines when the agent causing the disease is not a parasite in the genus *Plasmodium*, greatly enhances the development of resistance. Secondly, resistance to available drugs in many areas of the world is an emerging problem, it is species specific and it has a geographical distribution; therefore prescribing a certain medicine ignoring what *Plasmodium* species is causing the infection, can strengthen their resistance and expand the problem. A series of wrong diagnosis has certainly played a role in the development of drug resistance worldwide, especially to the two traditional medicines that have represented first- or second-line treatment for many decades: chloroquine and sulfadoxine–pyrimethamine. This has lead to the need of developing new antimalarial drugs; today public health authorities, in order to avoid an increase in the resistance to the traditional medicines, recommend the use of a fairly new group of drugs to which all *Plasmodium* species have shown susceptibility:
artemisinin and its derivatives. The downside of artemisinin is its relatively high cost and lower safety.

**Treatment**

The development of resistance to the available drugs in the last few decades has posed a serious challenge in our goal of achieving malaria eradication and has evolved our way of facing and managing the disease. It is undoubtedly a complicated problem. Until a few decades ago, chloroquine was a very effective medicine and no signs of resistance had yet appeared. Then, the indiscriminate use of chloroquine posed a very heavy selective pressure on the parasite and this process slowly lead to the insurgence of resistant strains that survived the action of the drug. As time went by, resistant strains were effectively reproducing and allowed for a successful transmission; this created a worldwide resistance of *P. falciparum* to most of the available medicines, such as amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine-pyrimethamine. As we know, *P. falciparum* is the most prevalent and dangerous malarial parasite, hence, in this discussion, we will mainly focus on the treatment of this form of malign malaria.

There is a series of factors that allowed the parasite to develop resistance. Some of these factors are: the extremely high prevalence of the disease throughout the world, the administration of drugs when malaria was only suspected through clinical symptoms (and the patient was malaria negative), the poor adherence to the recommended therapy, the prescription of ineffective drugs against a specific species of parasite, and the use of monotherapy (single drug against the parasite) as opposed to combination therapy, in
which two different drugs have two distinct modes of action on the parasite (Hastings & Mackinnon, 1998).

Resistance to antimalarials has been documented for \textit{P. falciparum}, \textit{P. vivax}, and, recently, \textit{P. malariae}. In \textit{P. falciparum}, resistance has been observed to almost all currently used antimalarials (amodiaquine, chloroquine, mefloquine, quinine and sulfadoxine–pyrimethamine) except for artemisinin and its derivatives. The geographical distribution and rates of diffusion have varied considerably. \textit{P. vivax} has rapidly developed resistance to sulfadoxine–pyrimethamine in many areas. Chloroquine resistance is confined largely to Indonesia, East Timor, Papua New Guinea and other parts of Oceania. There are also documented reports from Peru. \textit{P. vivax} remains sensitive to chloroquine in South-East Asia, the Indian subcontinent, the Korean peninsula, the Middle East, North-East Africa, and most of South and Central America (WHO, 2006).

As it can easily be extrapolated, treatment of malaria today is a very complex topic. First, a number of general properties of the current antimalarial drugs will be delineated; this will be followed by an in-depth discussion of the specific medicines against different parasites and their different types of combination, settings, and interventions. Generally speaking, antimalarials can help bring about a reduction in malaria transmission by their effect on parasite infectivity. This can be a direct effect on the gametocytes, the infective stages found in human infections (gametocytocidal effect) or, when the drug is taken up in the blood meal of the mosquito, an effect on the parasite’s development in the insect (sporonticidal effect). Chloroquine (CQ) acts against young gametocytes but has no suppressive effects on mature infective forms (Bruce-
Chwatt, 1981). Chloroquine has even been shown to be capable of enhancing the infectivity of gametocytes to the mosquito (Hogh et al., 1998). In contrast, sulfadoxine-pyrimethamine (SP) increases gametocyte carriage but, provided there is no resistance, reduces the infectivity of gametocytes to mosquitoes (Hogh et al., 1998; Robert et al., 2000; von Seidlein et al., 2001). Artemisinins are the most potent gametocytocidal drugs among those currently being used to treat an asexual blood infection (von Seidlein et al., 1998; Targett et al., 2001; Drakeley et al., 2004). They destroy immature gametocytes, preventing new infective gametocytes from entering the circulation, but their effects on mature gametocytes are less, hence they will not affect the infectivity of those already present in the circulation at the time a patient presents for treatment (Pukrittayakamee et al., 2004). Primaquine, an 8-aminoquinoline antimalarial that has been widely used as a hypnozoiticidal drug, is the only antimalarial medicine that had been deployed in the treatment of *P. falciparum* infections due to its effects on infectivity. It acts on mature infective gametocytes in the circulation and accelerates gametocyte clearance (Pukrittayakamee et al., 2004), as opposed to artemisinins, which mainly inhibit gametocyte development. Amodiaquine is an antimalarial with schizonticidal activity. It is effective against the erythrocytic stages of all four species of *Plasmodium falciparum*. It is as effective as chloroquine against chloroquine-sensitive strains of *Plasmodium falciparum* and it is also effective against some chloroquine-resistant strains. Amodiaquine accumulates in the lysosomes and brings about loss of function. The parasite is unable to digest hemoglobin on which it depends for its energy (‘Crusade Against Malaria’, 2008). Lastly, mefloquine is indicated for the treatment of mild to moderate acute malaria caused by mefloquine-susceptible strains of *P. falciparum* (both
chloroquine-susceptible and resistant strains) or by *Plasmodium vivax*. There are insufficient clinical data to document the effect of mefloquine in malaria caused by *P. ovale* or *P. malariae*. Patients with acute *P. vivax* malaria, treated with mefloquine, are at high risk of relapse because mefloquine does not eliminate exoerythrocytic (hepatic phase) parasites (RxList, 2008).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect of treatment</th>
<th>Gametocytocidal</th>
<th>Sporonticidal</th>
<th>Overall effect on suppressing infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viability</td>
<td>Viability</td>
<td>Infectivity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>of young</td>
<td>of mature</td>
<td>of gametocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sequestered</td>
<td>circulating</td>
<td>gametocytes</td>
<td>mosquitoes</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Reduces</td>
<td>No effect (4)</td>
<td>Enhances (5)</td>
<td>+</td>
</tr>
<tr>
<td>Sulfadoxine-pyrimethamine</td>
<td>No effect</td>
<td>Increases (5-7)</td>
<td>Suppresses (5-7)</td>
<td>+/-</td>
</tr>
<tr>
<td>Artemisinin derivatives</td>
<td>Reduces greatly (8-11)</td>
<td>Little effect (11)</td>
<td>Unknown</td>
<td>+++</td>
</tr>
<tr>
<td>Primaquine</td>
<td>Unknown</td>
<td>Reduces greatly (11)</td>
<td>Unknown</td>
<td>+++</td>
</tr>
<tr>
<td>Quinine (4)</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>None</td>
</tr>
</tbody>
</table>

*Table 5: Effects of Some Commonly Used Antimalarials on the Infectivity of *P. falciparum* to the Mosquito. (WHO, 2006)*

*Treatment of Uncomplicated *P. falciparum* Malaria*

Today the World Health Organization recommends combination therapy for the treatment of falciparum malaria. Monotherapy is strongly discouraged as either resistance is already present or it may lead to the process of resistance development. The parasites that survive have genes that make them resistant to a certain drug and these
same genes can then be transmitted to the mosquito when it bites and, consequently, to humans (Hallett et al., 2004).

Antimalarial combination therapy is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite. The concept is based on the potential of two or more simultaneously administered schizontocidal drugs with independent modes of action to improve therapeutic efficacy and also to delay the development of resistance to the individual components of the combination (WHO, 2006).

The recommended treatment for the cure of falciparum malaria is the Artemisinin-based Combination Therapy (ACT). There are four derivatives of artemisinin that induce a rapid clearance of parasitemia and a resolution of the symptoms. They reduce the parasite number by a factor of approximately 10000 in each asexual cycle, which is more than other current antimalarials (100- to 1000-fold per cycle). Artemisinin and its derivatives are also rapidly eliminated. When given in combination with rapidly eliminated compounds (tetracyclines, clindamycin), a 7-day course of treatment with an artemisinin compound is required; but when given in combination with slowly eliminated antimalarials, shorter courses of treatment (3 days) are effective. A trial that compared the effects of a single drug such as amodiaquine, mefloquine, or sulfadoxine-pyrimethamine with that of one of those same drugs with the addition of artesunate (an artemisinin derivative), found that there was a clear benefit in the use of the combination treatment. It also resulted in fewer parasitological failures at day 28 which is the current recommended duration of follow-up. This is when the treatment is considered to be successful, even though recrudescence may occur and another cycle
needs to be begun with the same firs-line treatment or, if the circumstances suggest that
sensitivity is not sufficient, then the second-line treatment may be used. Another
characteristic that make artemisinin compounds ideal for the treatment of malaria is that
they are active against all four species of malaria parasites and are generally well
tolerated. As previously mentioned, these drugs also have the advantage, from a public
health perspective, of reducing gametocyte carriage and thus the transmissibility of
malaria. This contributes to malaria control in areas of low endemicity (WHO, 2006).

As mentioned, the concept of ACT is based on the use of an artemisinin
derivative and the choice of another drug. Whereas no significant differences in the
efficacy of the artemisinin product in terms of absorption and bioavailability have been
demonstrated, it is the properties of the partner medicine that determines the effectiveness
and choice of combination. Various combination therapies have been evaluated in
multiple clinical trials where an artemisinin compound was given in conjunction with
another drug such as amodiaquine, chloroquine, lumefantrine, mefloquine, piperaquine,
and doxycycline. The combinations that proved to be the most advantageous were (in
alphabetical order):

- artemether-lumefantrine
- artesunate + amodiaquine
- artesunate + mefloquine
- artesunate + sulfadoxine-pyrimethamine

Although for many countries, artemether-lumefantrine and artesunate +
mefloquine may give the highest cure rates, there may be problems of affordability and
availability of these products. Also, there is currently insufficient safety and tolerability
data on artemisinin + mefloquine at the recommended dose of 25mg/kg in African children to support its recommendation there. Trials with mefloquine monotherapy (25mg/kg) have raised concerns of tolerability in African children. Countries may therefore opt to use artemisinin + amodiaquine and artemisinin + sulfadoxine–pyrimethamine, although they may have lower cure rates because of resistance (WHO, 2006).

Finally, a last note on a crucial aspect of malaria treatment: treatment management. In order to optimize the benefit of deploying ACTs, and to have an impact on malaria, it will be necessary to deploy them as widely as possible; this means that they will have to reach the most peripheral health centers and communities. The dissemination of clear national treatment guidelines, use of appropriate information, education, communication materials, monitoring of the deployment process, access and coverage, and the provision of adequately packaged antimalarials are needed to optimize the benefits of providing these new effective treatments (WHO, 2006).

Treatment of Malaria Caused by *P. vivax*, *P. ovale*, and *P. malariae*

*P. vivax* has been characterized thoroughly in terms of pharmaceutical sensitivity and resistance due to its high prevalence in endemic areas of the Middle East, Oceania, and Central and South America. In Africa, it is rare except in the Horn and it is almost absent in West Africa. In most areas where *P. vivax* is prevalent, malaria transmission rates are low, and the affected populations therefore achieve little immunity to this parasite. Consequently, people of all ages are at risk.
The other two human malaria parasite species, *P. malariae* and *P. ovale*, are generally less prevalent but are distributed worldwide, especially in the tropical areas of Africa.

Of these three parasites, only *P. vivax* and *P. ovale* form hypnozoites, the liver stage of the parasite that can result in relapses of infection. This affects the development and schooling of children and debilitates adults, thereby impairing human and economic development in affected populations. Therefore, when treating these two forms of malaria, the goal is to cure both the blood stage and the liver stage infections, thereby preventing both relapses and recrudescence. This is known as radical cure.

Recent data on the *in vivo* susceptibility of *P. ovale* and *P. malariae* are very few. Both are regarded as very sensitive to chloroquine, although there is a single recent report of chloroquine resistance in *P. malariae*. They are also thought to be susceptible to amodiaquine, mefloquine, and the artemisinin products. On the other hand, *P. vivax* has received much more attention and therefore we possess very extensive studies that assess the susceptibility to drugs of this parasite. It is still generally very sensitive to chloroquine, although resistance has emerged in some areas such as Oceania, Indonesia, and Peru. Resistance to pyrimethamine has increased rapidly in some areas, and sulfadoxine-pyrimethamine is consequently ineffective. In general, *P. vivax* is sensitive to all the other antimalarial drugs; it is more sensitive than *P. falciparum* to the artemisinin derivatives, and slightly less sensitive to mefloquine (although mefloquine is still effective). The only drugs with significant activity against the hypnozoites are the 8-aminoquinolines.
As far as *P. ovale* is concerned, the recommended treatment for the relapsing malaria is the same as that given for radical cure in *P. vivax*, i.e. with chloroquine and primaquine. Instead, *P. malariae* should be treated with the standard regimen of chloroquine as for vivax malaria, but it does not require radical cure with primaquine as no hypnozoites are formed in infection with this species.

**Malaria and Plasmodium falciparum**

**Epidemiology**

First of all, it is important to consider that there are three main factors that determine the occurrence of malaria. These factors also correspond to the three components of the malaria cycle:

1. In order for the parasite to complete the "invertebrate host" half of their life cycle, *Anopheles* mosquitoes must be present and must be in contact with humans.

2. In order for the parasites to complete the "vertebrate host" half of their life cycle, humans must be present and must be in contact with *Anopheles* mosquitoes.

3. Malaria parasites must be present (CDC, 2004).

Climate can also influence all three components of the life cycle. It is thus a key determinant in the geographic distribution and the seasonality of malaria. An essential resource for the successful reproduction and development of the mosquito eggs is the presence of water. The required time for the eggs to develop into larvae, pupae, and adults, is approximately 9-12 days in tropical areas. In case rain is scarce and water dries...
up too quickly, the egg is not able to complete its evolution; on the other hand, when rains are too excessive, eggs are can be damaged and flushed away.

Once adult mosquitoes have emerged, the ambient temperature, humidity, and rains will determine their chances of survival. Once an adult mosquito takes a bite on an infected human, the parasite needs to go through a whole new growth cycle inside the mosquito, known as the extrinsic cycle. This cycle takes about 9 to 21 days to complete at 25°C, meaning that the mosquito needs to survive long enough to allow the extrinsic cycle to complete. Warmer climates shorten the extrinsic cycle, thus increasing the chances of transmission. Conversely, below a certain temperature (15°C for *Plasmodium vivax*, 20°C for *P. falciparum*), the extrinsic cycle cannot be completed and malaria cannot be transmitted (Craig et al., 1999).

**Epidemiological Patterns, Transmission, and Distribution**

There is a fundamental concept that describes and influences the epidemiology and distribution of malaria: transmission. Transmission is described in terms of intensity, stability, and seasonal patterns. The intensity of transmission can be measured as the average number of infectious bites received during a given period of time by an individual living in the area. Stability regards the situation over a period of years. In areas of stable transmission, the pattern of transmission remains roughly unchanged from year to year, whereas areas with unstable malaria are characterized by considerable variation in the intensity of transmission between years. In areas of stable malaria transmission, the transmission intensity is roughly reflected in the spleen rates and the point prevalence of parasitemia in children (Gilles, 1993).
According to the levels of spleen rate and parasitemia, endemicity is comprised of four categories: holoendemic, hyperendemic, mesoendemic, and hypoendemic. In holoendemic area, transmission occurs all year long. In hyperendemic areas, transmission is intense but with periods of no transmission during the dry season. In mesoendemic regions, there is a regular seasonal transmission. Finally, in hypoendemic areas, transmission is very intermittent (Shiff, 2006). Table 6 summarizes the characteristics of the four different levels of endemicity.

<table>
<thead>
<tr>
<th></th>
<th>Spleen rate* in &lt; 5 year old</th>
<th>Parasitemia &lt; 5 year old</th>
<th>Transmission</th>
<th>Clinical Manifestations</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Holoendemic</strong></td>
<td>&gt; 75%</td>
<td>60 – 70 %</td>
<td>Stable**</td>
<td>Anemia severe in early life</td>
<td>High in 1st and 2nd year of life</td>
</tr>
<tr>
<td><strong>Hyperendemic</strong></td>
<td>50 – 70 %</td>
<td>50 – 70 %</td>
<td>Seasonal but intense. Stable</td>
<td>Cerebral malaria in older children</td>
<td></td>
</tr>
<tr>
<td><strong>Mesoendemic</strong></td>
<td>20 – 50 %</td>
<td>&lt; 20%</td>
<td>Seasonal. Unstable***</td>
<td>Cerebral malaria common</td>
<td></td>
</tr>
<tr>
<td><strong>Hypoendemic</strong></td>
<td>0 – 10 %</td>
<td>0 – 10 %</td>
<td>Periodic/Unusual rainfall. Unstable</td>
<td>Severe outbreaks in children/adults</td>
<td>High in all groups and ages</td>
</tr>
</tbody>
</table>

Table 6: Levels of Endemicity of Malaria and Associated Statistics and Characteristics. (Shiff, 2006)

* Spleen Rate: number of palpable enlarged spleens per 100 individuals of similar ages.

** Stable malaria occurs in areas of high prevalence, cases are frequently asymptomatic, fever occurrence is infrequent, anemia is high at younger ages (particularly under 2 years of age), and mortality is greatest under 2 years of age (Shiff, 2006).

*** Unstable malaria is based on immunity achieved through personal exposure to *Plasmodium* antigens. Since exposure in areas of low endemicity is usually low, the immune system is naive to the antigens and is not able to effectively produce a strong
immune response; a broad range of disease situations is possible, including cerebral malaria in children 4 – 6 years old and adult malaria. Since the infection is highly symptomatic, a rapid diagnosis is critical and prompt effective treatment is vital to avoid fatalities (Shiff, 2006).

Now why do children over five years of age and adults living in areas of stable malaria transmission are immune to malaria? It has been proven that individuals develop immunity to the malarial parasites through repeated exposure all year round and over the years. In this type of settings, children over the age of five and adults are progressively more immune to malaria because they have been exposed to the parasite for a prolonged and constant period of time. Those living in an area of low transmission setting should progress to clinical disease and severe manifestations and the age is a less determining factor since no one is constantly exposed to the parasite over the course of the his life (Reyburn et al., 2005). In fact, recent studies in Tanzania and Kenya have highlighted the fact that the epidemiology and clinical manifestations of severe malaria vary with the intensity of transmission (Snow et al., 1994). Theoretically, individuals living in an area of low transmission should suffer from complicated disease but this is not always the case. A study conducted in Daraweesh, Sudan, an area of low and unstable transmission where the season of heavy rain is followed by the dry season, found that local dwellers did acquire the ability to control some infection. However, the protection is far from solid and malaria occurs in all age-groups, although the risk of getting malaria decreases with age (Theander, 1998).
Geographical Distribution

Figure 7: Geographical Distribution of Malaria. (WHO 2006)

The distribution of malaria depends mainly on climatic factors such as temperature, humidity, and rainfalls (CDC, 2004). As mentioned early, in tropical and subtropical areas two factors are needed for the transmission of malaria to be successful: 

I) *Anopheles* mosquitoes can survive and multiply and II) Malaria parasites can complete their growth cycle in the mosquitoes (extrinsic incubation period).

There are also some geographical features that prevent the transmission from occurring:

- high altitudes
- cooler seasons in some areas
- deserts (excluding the oases)
- some islands in the Pacific Ocean which have no local *Anopheles* species capable of transmitting malaria
The highest transmission is found in Africa south of the Sahara, where the
closeness to the equator makes the climate warmer; in these conditions, transmission is
more intense, malaria is transmitted year-round, and *P. falciparum* predominates.

In cooler regions, transmission will be less intense and more seasonal. There, *P.
vivax* might be more prevalent because it is more tolerant of lower temperatures.

**Drug Resistance**

The World Health Organization defines antimalarial drug resistance as the ability
of a parasite strain to survive and/or multiply despite the administration and absorption of
a drug given in doses equal to or higher than those usually recommended but within
tolerance of the subject (WHO, 2001). This definition was later modified to specify that
the drug in question must gain access to the parasite or the infected red blood cell for the
duration of the time necessary for its normal action (Bruce-Chwatt et al., 1986). The first
scientific reviews in the literature that discussed the emerging problem of drug resistance
appeared in the mid ‘80s and reported that *Plasmodium falciparum* in Central and West
Africa was becoming resistant not only to chloroquine, but also to all existing alternative
treatments except quinine (Ambroise-Thomas & Rossignol, 1986). Today, drug
resistance varies largely depending on the geographic region, the drug being used, and
the different species of the *Plasmodium* parasite. In *P. falciparum*, resistance has been
observed to almost all currently used antimalarials (amodiaquine, chloroquine,
mefloquine, quinine and sulfadoxine–pyrimethamine) except for artemisinin and its
derivatives. The geographical distributions and rates of diffusion have varied
considerably. *P. vivax* has developed resistance rapidly to sulfadoxine–pyrimethamine in
many areas. Chloroquine resistance is confined largely to Indonesia, East Timor, Papua New Guinea and other parts of Oceania though there are also documented reports from Peru. *P. vivax* remains sensitive to chloroquine in South-East Asia, the Indian subcontinent, the Korean peninsula, the Middle East, north-east Africa, and most of South and Central America (Table 7 & Fig. 8) (WHO, 2006).

<table>
<thead>
<tr>
<th>Region</th>
<th>Resistance reported</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central America (Mexico, Belize, Guatemala, Honduras, El Salvador, Nicaragua, Costa Rica, NW Panama)</td>
<td>N N N</td>
<td>North-west of Panama Canal only</td>
</tr>
<tr>
<td>Caribbean (Haiti and Dominican Republic only)</td>
<td>N N N</td>
<td></td>
</tr>
<tr>
<td>South America (SE Panama, Columbia, Venezuela, Ecuador, Peru, Brazil, Bolivia)</td>
<td>Y Y Y QN</td>
<td>Resistance to MQ and QN, although reported, is considered to occur infrequently</td>
</tr>
<tr>
<td>Western Africa</td>
<td>Y Y Y</td>
<td>Incidence of resistance to CQ variable, but very common in most areas</td>
</tr>
<tr>
<td>Eastern Africa</td>
<td>Y Y N</td>
<td>Incidence of resistance to SP highly variable, with some reports of focally high incidence, but generally uncommon</td>
</tr>
<tr>
<td>Southern Africa</td>
<td>Y Y N</td>
<td>Resistance to SP, although reported, is considered to be generally uncommon</td>
</tr>
<tr>
<td>Indian Subcontinent</td>
<td>Y N N</td>
<td></td>
</tr>
<tr>
<td>South-East Asia and Oceania</td>
<td>Y Y Y HAL, QN</td>
<td>Border areas of Thailand, Cambodia, and Myanmar highest risk for multiple-drug-resistant infections; in other areas, incidence of resistance to SP and MQ highly variable and absent in many areas</td>
</tr>
<tr>
<td>East Asia (China)</td>
<td>Y Y ?</td>
<td>Resistance greatest problem in southern China</td>
</tr>
</tbody>
</table>

Table 7: Distribution of Drug-Resistant *P. falciparum* Malaria. (WHO, 2001)
A number of factors that are thought to contribute to the development and intensification of drug resistance have been identified. These are: incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor or erratic absorption, and misdiagnosis (WHO, 2001).

In terms of the molecular processes that lead to the formation of resistance, there are a number of mechanisms that characterize each category of drugs.

- Chloroquine has the capacity of capture harmful heme moieties which are produced by the parasites as a by-product of hemoglobin digestion, its major source of amino acids. This process occurs in digestive vacuoles in which chloroquine can exert its function if the parasite is chloroquine sensitive, otherwise, if the parasites are chloroquine resistant, this drug will not be able to
accumulate at sufficient levels that will allow it to play its role of eliminating heme moieties. The different behavior is due to mutations in the *pfcrt* (*Plasmodium falciparum* chloroquine resistance transporter) gene; resistant strains of the parasite have several nucleotide substitutions due to the great variety of chloroquine resistant parasites, but one amino acid is found constantly in resistant strains, K76T (Hyde, 2007). These mutations are thought to play a role in determining the characteristics of CRT (chloroquine resistance transporter) with respect to the drugs; scientists suggest that the K76T amino acid alters the activity of the CRT in a way that chloroquine exits the vacuole more rapidly (Johnson et al., 2004). One possible explanation for this modification is that when K76 is present, the expulsion of drug outside the vacuole through CRT is limited due to the amino acid positive charge; when the strain is resistant and K76T comes into action, the movement of chloroquine towards the outside of the vacuole is excessive and accelerated (Hyde, 2007).

- Another category of drugs that is worth mentioning are the antifolates. The principal drugs of interest within this class are pyrimethamine and sulfadoxine. Pyrimethamine targets the dihydrofolate reductase (DHFR) activity of the DHFR-thymidylate synthetase protein, while sulfadoxine inhibits the dihydropteroate synthetase (DHPS) activity of the DHPS-hydroxymethylpterin pyrophosphokinase. DHFR enzyme is present in both the host and the parasite and its role pertains the constant supply of fully reduced forms of folate conenzymes that help, for instance, in the provision of nucleotides for DNA synthesis and the metabolism of certain amino acids. DHPS is only found in the
parasite and it is also used in the biosynthesis of essential folate enzymes. These
drugs bind to these two enzymes several hundred times more strongly in the
parasite than in the human orthologue, therefore effectively exerting its action on
the parasite and not affecting the functions of the enzyme in humans in the case of
DHFR. The genetic basis of resistance to the antifolate drugs is similar to that
underlying chloroquine resistance, in that a small number of point mutations in
these two target genes appear to be responsible for the major part of resistance
(Hyde, 2007).

Finally artemisinin is thought to function, upon activation by Fe$^{2+}$, by inhibiting
the Ca$^{2+}$ sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA)
PfATPase6, a transporter found on membranous structures within the parasite
cytoplasm (Hyde, 2007). Resistance to artemisinin products has not quite
emerged yet, primarily due to its use in combination therapy, even though in vitro
resistance has been reported in isolates from French Guiana, Cambodia, and
Senegal. In a study conducted by Jambou and colleagues, the coding sequences of
PfATPase6 isolates from these three countries were established and substantial
polymorphism was observed, especially in the Cambodian strains. More
specifically, the polymorphism of S769N, found in many of the isolates, was
associated with an increased mean IC$_{50}$ (measure of the effectiveness of a
compound in inhibiting biological or biochemical function) for artemether
(Jambou et al., 2005). In fact, for instance, the S769N mutation differs from the
engineered L263E replacement, which eliminates artemisinin inhibition of the
PfATPase6 enzymatic activity (Uhlemann et al., 2005). In certain species,
residue 769 is essential to the structural transitions needed for the progress of the ATPase cycle and calcium binding and release. By analogy with the inhibition of the rabbit SERCA by thapsigargin, a structural analogue of artemisinins, it is inferred that the S769N mutation prevents artemisinin derivatives from interfering with these conformational changes (Jambou et al., 2005).
Table 8: *P. falciparum* Proteins with a Proven Role in Resistance to Clinical Antimalarial Drugs. (Jambou et al., 2007)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Location</th>
<th>Principal drugs affected&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT</td>
<td>Transporter</td>
<td>Membrane of food vacuole</td>
<td>Chloroquine, Mefloquine, halofantrine, lumefantrine, artemisinins, quinine</td>
<td>Major determinant Minor determinant</td>
</tr>
<tr>
<td>Pgh1 (P-glycoprotein homologue 1) or MDR1 (multidrug resistance 1)</td>
<td>Transporter</td>
<td>Membrane of food vacuole</td>
<td>Mefloquine, halofantrine, lumefantrine, quinine (possibly) Chloroquine, artemisinins</td>
<td>Major determinant Minor determinant</td>
</tr>
<tr>
<td>DHPS</td>
<td>Folate pathway enzyme</td>
<td>Cytoplasm (principally)</td>
<td>Sulfadoxine, clapsone</td>
<td>DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates</td>
</tr>
<tr>
<td>DHFR</td>
<td>Folate pathway enzyme</td>
<td>Cytoplasm (principally)</td>
<td>Pyrimethamine, proguanil, chlorproguanil</td>
<td>DHPS and DHFR targeted simultaneously in synergistic combinations of antifolates</td>
</tr>
<tr>
<td>Cytochrome b</td>
<td>Subunit of complex III (cytochrome bc&lt;sub&gt;1&lt;/sub&gt; complex) electron transport chain</td>
<td>Mitochondrion</td>
<td>Atovaquone</td>
<td></td>
</tr>
<tr>
<td>ATP6 (sarco/endoplasmic reticulum calcium-dependent ATPase [SERCA] orthologue)</td>
<td>Membrane-bound Ca&lt;sup&gt;2+&lt;/sup&gt;-transporting ATPase</td>
<td>Membranous structures within cytoplasm</td>
<td>Artemisinins</td>
<td>Likely major determinant</td>
</tr>
</tbody>
</table>
Cytochrome b<sub>5</sub> Reductase (cb<sub>5</sub>r) and Cytochrome b<sub>5</sub> (cb<sub>5</sub>)

*Cytochrome b<sub>5</sub> Reductase*

Cytochrome b<sub>5</sub> reductase (cb<sub>5</sub>r) is a member of the FNR superfamily of flavoprotein oxidoreductases and contains a single flavin adenine dinucleotide (FAD) group (Shirabe et al., 1997). The function of this enzyme is to catalyze the transfer of two electrons from NADH to two molecules of cytochrome b<sub>5</sub> in one catalytic cycle (Strittmatter, 1965).

Cb<sub>5</sub>r exists in two forms, a membrane-bound form that is comprised of both a hydrophobic membrane-anchoring region (M<sub>r</sub> ~ 3 kDa, residues 1-25) and a hydrophilic, catalytic portion (M<sub>r</sub> ~ 30 kDa, residues 26-300), and a soluble form comprising only the soluble reductase. The soluble form of cb<sub>5</sub>r exists in circulating erythrocytes and participates in methemoglobin reduction, while the membrane-bound form is embedded in the membranes of the somatic cells such as those of the endoplasmic reticulum, mitochondria, and plasma membrane (Bewley et al., 2001).

For the purpose of this project, the membrane-bound form of cb<sub>5</sub>r will have greater importance and will represent the predominant topic. As mentioned, membrane-bound cb<sub>5</sub>r consists of a hydrophobic N-terminal membrane-anchoring domain, and a larger hydrophilic, soluble catalytic domain. It contains four conserved motifs: two NADH binding motifs, a flavin-binding motif, and a FMN/FAD selectivity motif (Marohnic et al., 2005).

A well known cb<sub>5</sub>r protein structure is represented by the rat cb<sub>5</sub>r. The structure has a typical two-domain arrangement, with an amino-terminal binding domain (residues 33-147) and a carboxyl-terminal NADH-binding domain (residues 171-300). These two
domains are linked by a 25-amino acid, three-stranded antiparallel \( \beta \)-sheet (residues 148-170) that is thought to serve as a hinge to orient the NADH and FAD-binding domains, so that an efficient transfer of electrons from NADH to flavin can occur. There is also an FAD molecule that interacts with both domains through hydrogen bonds (Fig. 9b). The FAD-binding domain is a six-stranded, antiparallel \( \beta \)-barrel with a \( \sim 30^\circ \) twist of each strand. The barrel is capped by the only \( \alpha \)-helix in this domain. A long loop (residues 110-125), located between strand 5 and the \( \alpha \)-helix, forms a lid that contributes to the majority of interactions of the FAD-binding domain with the adenine dinucleotide moiety of the FAD. The NADH-binding domain contains a typical Rossman fold (three \( \alpha/\beta/\alpha \) layers that pack into a five-stranded parallel \( \beta \)-sheet in the order 3, 2, 1, 4, 5) (Fig. 9a) (Bewley et al., 2001); these folds are able to bind only one nucleotide at a time, thus in this case there are two paired Rossman folds that bind to the incoming NADH molecule (Rao & Rossman, 1973).

Figure 9: Structure of Rat Cb_{5r}. (Bewley et al., 2001)
(a) Stereo ribbon diagram of rat cb_{5r}. The FAD-binding domain is colored red, the NADH-binding domain is blue, the three-stranded linker domain is green, and the FAD molecule is shown in a ball and stick.
representation using standard atom colors. (b) Interactions of the FAD and visible region of the NAD.
Water-mediated hydrogen bonds are shown as cyan dotted lines.

**Cytochrome b$_5$**

Cytochrome b$_5$ (cb$_5$) is an electron transfer protein with a redox potential of 20 mV which is capable of accepting and transferring a single electron (Velick & Strittmatter, 1956). It is a small protein of about 17 kDa found throughout the phyla, from yeasts to insects, seed plants and animals. In all species and tissues (except erythrocytes), it is anchored in the membranes by a hydrophobic, carboxyl-terminal end of the peptide chain. In erythrocytes, a truncated, soluble form of the protein is found (Schenkman & Jansson, 1999). The protein has a heme prosthetic group that lacks a free coordination position and consequently, it serves as a physiological electron transfer component facilitating a number of reactions, including fatty acid desaturation, fatty acid elongation, and cytochrome P450 (P450) monooxygenation (which in turn is involved in the catabolism of xenobiotics) (Schenkman & Jansson, 1999; Keyes et al., 1979; Conney et al., 1957).

Structurally, cb$_5$ is a small, cylindrical, acidic membrane protein consisting of 6 helices and 5 $\beta$-strands. The protein is folded into two domains, the larger of which is the cytosolic heme-containing, amino-terminal, hydrophilic region. The smaller domain is the hydrophobic, membrane-binding carboxyl portion of 14–18 residues, connected to the globular domain by a proline-containing hinge region of ~7 amino acids, and followed by 7 polar amino acids at the very end of the polypeptide chain (Fig. 10) (Mathews, 1985). Proteases can clip before or after the proline-containing hinge region, releasing the soluble, catalytic heme-containing domain. In mammals, 23 of the 134 amino acids are
either glutamate or aspartate and are completely conserved. Most of them are located in the hydrophilic region of the protein. The heme is located in a hydrophobic pocket, and the heme iron is coordinated with two completely conserved histidine side chains, H68 and H44. This binding prevents direct interaction of cb₅ with molecular oxygen (Schenkman & Jansson, 2003).

Another important characteristic of cb₅ is its site of biosynthesis and subsequent association with the membrane of the organelles in which they are embedded. It has been shown that cb₅ and its reductase cb₅r are synthesized in free polysomes and therefore, their association with the membrane must be established post-translationally, as opposed to the case of another reductase, P450, which plays a variety of roles in conjunction with cb₅ and cb₅r. P450 is synthesized in bound polysomes and cotranslationally inserted into the endoplasmic reticulum membranes (Okada et al., 1981).

Figure 10: Cytochrome b₅ Diagram. (Ramamoorthy, 2009)
This figure shows the hydrophobic portion imbedded in the phospholipid bilayer of the membrane and the larger hydrophilic segment that lays in the cytosol.
**Cb₅r and Cb₅ Function**

Cb₅r and cb₅ work in an interrelated fashion and depend on each other for the exchange and flow of electrons. More specifically, cb₅r catalyzes the transfer of two electrons from NADH to two molecules of cb₅ in one catalytic cycle (Shirabe et al., 1997). When these two proteins are found in the soluble form in erythrocytes, the electrons donated to cb₅ are used to reduce the ferric heme iron of hemoglobin to the ferrous state, resulting in the formation of methemoglobin (Hultquist & Passon, 1971). The membrane-bound forms on the other hand, serve a different function, participating in the elongation and desaturation of fatty acids, cholesterol biosynthesis, and some of cytochrome P450 mediated drug metabolism (Schenkman & Jansson, 1999; Keyes et al., 1979; Conney et al., 1957). Physiological electron donor of cb₅ is NADH-cytochrome b₅ reductase and acceptors are methemoglobin, fatty acid desaturase, and cytochrome P450 (Shirabe et al., 1997).

In fatty acid desaturation, cb₅ participates in the synthesis of unsaturated fatty acids by donating electrons, which it receives from NADH-cb₅r, to desaturases which are microsomal, non-heme, iron-containing proteins. The desaturases use the electrons from cb₅ to generate an electron-deficient, activated oxygen species which removes electrons from the saturated hydrocarbon. Unsaturated fatty acids possess a structural role in cellular membranes where they greatly contribute to the fluidity of the cellular membranes. They are also precursors of polyunsaturated fatty acids which are important mediators and regulators of various cell functions (Vergeres & Waskell, 1995).
In cholesterol biosynthesis, cb₅ has been shown to provide the reducing equivalents to the 19-step microsomal conversion of lanosterol to cholesterol; more precisely, it supplies electrons in the reaction in which 4-methyl sterol oxidase (a desaturase) oxidizes the C-30 methyl group of lanosterol (the cholesterol precursor) to a steroid acid which can be consequently decarboxylated (Fukushima et al., 1981).

The role and mechanism of action of cb₅r and cb₅ in drug metabolism is still subject of much research; many studies link the involvement of cb₅r and cb₅ in cytochrome P450-mediated reactions, which include drug metabolism (Porter, 2002). The interests are concentrated on the possibility of an NADH-cb₅ driven, P450 reductase independent pathway of drug metabolism (Yamazaky et al., 1996). In particular, the cb₅r/cb₅ action is thought to be involved in the donation of the second of the two required electrons for the reduction of P450 in the catalytic cycle (Pompon & Coon, 1984). The complete process and set of reactions will be explained in the next section.

**Cb₅r and Cb₅ Relationship and Interaction**

The link between cb₅r and cb₅ comes from the knowledge that there exist a transfer of electrons and a consequent electrostatic interaction between the reductase and the hemoprotein (Shirabe et al., 1997). There are propionate groups of the heme in cb₅, which are thought to be an electron pathway to heme iron from cb₅r, that protrude from the molecular surface. All charged residues around this heme-protruding site are negative (Mathews et al., 1971). These negatively charged residues are very numerous and are able to form several hydrogen bond pairs with positively charged residues on the flavin protruding site of cb₅r. The actual interaction occurs between some carboxyl
residues of cb$_5$ have been proven to be involved in the charge-pairing with the lysil residues of cb$_5$r (Strittmatter et al., 1992). In the same study, it was found that lysil residues around the flavin-protruding site of cb$_5$r are implicated in the formation of charge pairs with carboxylate residues of cb$_5$. The specific links are found between Lys13 and Glu48 (or Glu 56), Lys 97 and Glu43 (or Glu44), and Lys134 and the heme propionate; in all three pairs, the former residue is of cb$_5$r and the latter is of cb$_5$.

In figure 11, a docking model of cb$_5$r and cb$_5$, the positively charged residues around the flavin of cb$_5$r are involved in hydrogen bonding with cb$_5$. Here, Lys 97 and Lys134 of cb$_5$r and Glu44 and propionate of cb$_5$ form two hydrogen-bonded pairs. However, Lys13 and Glu48 or Glu56 are separated. All three hydrogen-bonded pairs (Lys13/Glu48, Lys 97/Glu43, and Lys134/propionate) cannot be formed simultaneously in a single docking form like this one because the three residues of each of the two molecules are arranged counterclockwise when viewed from the exterior (Nishida & Miki, 1996).
Another consideration to be made is that \( \text{Cb}_5 \) also interacts with the NADH-binding domain of \( \text{Cb}_5 \) through the formation of four salt bridges. There is evidence through a study on transient kinetics, that the binding of NADH modulates the formation of the \( \text{Cb}_5\text{r/Cb}_5 \) complex. It is also thought that these two NADH and FAD domains are tightly packed under NADH-binding state. Hydrogen-bonding of \( \text{Cb}_5 \) to both domains of \( \text{Cb}_5\text{r} \) is reasonable in the view of the modulation of \( \text{Cb}_5\text{r/Cb}_5 \) complex formation by the NADH binding (Meyer et al., 1995).

In order to prove that the ionic interactions and specific chemical bonding between these two proteins are exact, \( \text{Cb}_5\text{r} \) and \( \text{Cb}_5 \) mutants were constructed by means of
bacterial expression and site-directed mutagenesis (Strittmatter et al., 1992). In this study, the amino acids that are believed to interact are lysine residues K41, K125, K162, and K164 in cb$_5$r and glutamate residues E47, E48, E52, E60 and aspartate residue D64 in cb$_5$. The activity of cb$_5$r mutants was measured with potassium ferricyanide as an electron acceptor; it was found that the activity in the mutant was almost the same as that of the cb$_5$r wild type. This suggests that electron transfer from NADH to cb$_5$r remains intact in all mutants. Also the specific activity of all cb$_5$r mutants was reported to be considerably less efficient than that of the wild type, and the $K_m$ values were significantly elevated when the wild type cb$_5$ was used as the electron acceptor (Table 9).

<table>
<thead>
<tr>
<th>$b_5$R</th>
<th>Cytochrome $b_5$ (wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}^a$</td>
</tr>
<tr>
<td>wild</td>
<td>(s$^{-1}$)</td>
</tr>
<tr>
<td>K41A</td>
<td>864 ± 95</td>
</tr>
<tr>
<td>K125A</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>K162A</td>
<td>472 ± 67</td>
</tr>
<tr>
<td>K163A</td>
<td>75 ± 4.5</td>
</tr>
<tr>
<td>K162A/K163A</td>
<td>256 ± 18</td>
</tr>
</tbody>
</table>

Table 9: Kinetic Properties of Cb$_5$r Mutants Using Human Erythrocyte Wild-Type Cb$_5$. (Shirabe et al., 1998)

This result may be explained by the fact that the residues from cb$_5$ that are involved in the interaction with cb$_5$r are negatively charged and normally are neutralized by the positive charge of the cb$_5$r residues. The difference in this case is that the cb$_5$r mutants have lost their positive charge. From these results it can be concluded that the four lysine residues K41, K125, K162, and K163 may participate in the electrostatic interaction with cb$_5$ (Shirabe et al., 1998). Based on the same study, cross-linking studies
of the binding of residues E47, E48, E52, E60, and D64 (group A residues) of cb₅ with cb₅r were undertaken. Cb₅ mutants of the glutamate residues were generated. One or all of these residues were changed to Ala. The obtained $K_m$ and $K_{cat}$ values were not significantly different than those of the wild type, indicating that none of these residues participates in the interaction with cb₅r. The role in binding to cb₅r by other acidic residues located close to the heme group of cb₅ was examined. The substitution of E41, E42, D57, and E63 (group B) with Ala caused a five-fold increase in the $K_m$ with no notable change in the $K_{cat}$. This indicates that some of the group B residues participate in the interaction of cb₅ with cb₅r (Shirabe et al., 1998).

**Role in Drug Metabolism and Involvement in the Detoxification of Xenobiotics through Cytochrome P450**

In the past few decades, hundreds of experiments have been performed to study the effect and the mechanism of action of the NADH, NADPH, cb₅r, cb₅, P450 reductase, and P450 sequence of reactions on the substrate metabolism of P450 (Vergeres & Waskell, 1995). Cytochrome P450 is a mixed function oxidase which catalyzes the oxidation of numerous endogenous and exogenous compounds (Siegel et al., 2007). The activation of P450 for oxidation of its substrates and activation of molecular oxygen, requires two electrons which are provided by NADPH or, possibly, cb₅ (Ortiz de Montellano, 1986). The first electron is transferred to P450 by NADPH P450 reductase while the second electron can either be transferred by NADPH P450 reductase or, as speculated, cb₅ (Vergeres & Waskell, 1995). Cb₅ cannot transfer the first electron to P450 because the potential of cb₅ is ~ 20 mV, while that of ferric P450 (that needs to be reduced to ferrous P450) is ~ -330 mV. Therefore the reduction of P450 by
NADH/cb₅r/cb₅ chain of electron flow would be too slow to support the necessities of P450-dependent metabolism of various substances, including drugs (Fig. 12) (Porter, 2002).

**Figure 12: Electron Transport Pathways to Cytochrome P450 in the Endoplasmic Reticulum. (Porter, 2002)**

After being reduced by NADPH and/or cb₅, P450 reacts with oxygen to form an active oxygen complex, which in turn hydroxylates the various substrates (Gillette, 1971). The reaction is as follows:

- \( \text{NADPH} + \text{A(P450)} \rightarrow \text{AH}_2 + \text{NADP} \)

- \( \text{AH}_2 + \text{O}_2 \rightarrow \text{Active oxygen complex} \)

- \( \text{Active oxygen complex} + \text{drug} \rightarrow \text{Hydroxylated drug} + \text{A} + \text{H}_2\text{O} \)

As mentioned, when the source of reducing equivalents is NADH, the reduction of P450 is very slow, about 10% of the rate with NADPH, even in the case of the input of the second electron (Shenkman & Jansson, 1999). Early studies on P450 indicated that the rate-limiting step in the monooxygenase reaction was the input of a second electron. It was also suggested that the role of cb₅ was to speed up this electron transfer by providing the second electron more rapidly from NADH than could be provided from NADPH (Hildebrandt, 1971).
A hypothesis has been presented to explain this stimulatory effect of cb\textsubscript{5}. Cb\textsubscript{5} forms a two-electron acceptor heterodimeric complex with P450 that causes cb\textsubscript{5} to stimulate the substrate oxidation by P450. This would also allow for a single interaction, rather than two, with the reductase to provide the two electrons needed for the activation of molecular oxygen. Cb\textsubscript{5} was suggested to act by oxidizing ferrous P450, thereby allowing the input of a second electron to the oxidized P450, and then returning the electron to the oxyferrous P450 intermediate. This function of cb\textsubscript{5} was suggested to be due to the need for only single interaction with NADPH-P450 reductase by the heterodimeric complex rather than two separate interactions with P450 alone (Shenkman & Jansson, 1999).

**Phylogenetic Analysis of P. falciparum Cb\textsubscript{5r} and Cb\textsubscript{5}**

Cb\textsubscript{5r} and cb\textsubscript{5} have a high pharmacological significance due to their role in drug metabolism and detoxification of xenobiotics. With the increasing levels of drug resistance to essentially all of our available antimalarial medicines, there is an urgent need to find new parasitic drug targets. A new important aspect that has not yet been mentioned in this review, is the identification of plant cb\textsubscript{5r} as a novel herbicidal target (Reindl, 2005), thus making this class of agents as potential candidates for new antiparasitic drugs against *P. falciparum* malaria. The phylogenetic relationship between plant and *P. falciparum* cb\textsubscript{5r} and cb\textsubscript{5} is a useful tool to evaluate the evolutionary distance. Analyzing the phylogenetic tree can give us precious insights into the possible relatedness of these proteins which belong to these two different kingdoms (plantae and protista, respectively).
*Plasmodium falciparum* cb₅r formed a distinct clade with other protozoan, having the closest homology to plants, and all protozoan isoforms were distinct from their mammalian orthologues (Fig. 13). Considering that plant cb₅r is an herbicidal target, it can be speculated that *P. falciparum* cb₅r may also represent a potential novel drug target. An additional consideration that can be extrapolated from the phylogenetic tree is that protozoan cb₅r is more closely related to plant cb₅r than mammalian cb₅r. This is a further advantage in terms of novel drug target and drug efficacy: the closeness in the evolutionary course of protozoan cb₅r to plant cb₅r may lead to promising treatment outcomes. This is further supported by the fact that the host cb₅r (human) will not be affected by the action of the drug since human cb₅r is phylogenetically too far away from its protozoan orthologue.

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**Figure 13: Phylogram of Cytochrome b₅ Reductases.**

Amino acid sequences of cb₅r were aligned using ClustalW.
*P. falciparum* cb\(_5\) also formed a distinct clade separate from their mammalian orthologues (Fig. 13a). The oldest cb\(_5\) orthologues are found in prokaryotes, including purple bacteria and Gram-positive species.

**Figure 13a: Phylogram of Cytochrome b\(_5\).**
Various cb\(_5\) amino acid sequences were aligned using ClustalW.

**Structural Comparison of *P. falciparum* \(Cb_5r\) with Mammalian \(Cb_5r\) and Other Species**

Several cb\(_5\)rs from a variety of species were aligned using ClustalW and Jalview to compare the sequences of the four conserved binding motifs (Fig. 14a, 14b, 14c). In particular, *Plasmodium falciparum* and mammalian \(Cb_5r\) were analyzed. In the flavin-binding domain, at the second amino acid position, *P. falciparum* has a serine (S) while human \(Cb_5r\) has a proline (P); also, in the same domain, at the sixth position, there is a
valine (V) in *P. falciparum* cb₅r and an isoleucine (I) in the human code. Lastly a tyrosine (Y) in *P. falciparum* and a serine (S) in human are found at the seventh position.

There are a total of three substitutions in amino acids in the flavin-binding domain sequence (which consists of seven amino acids) between *P. falciparum* and human cb₅r. *P. falciparum* cb₅r only shares two differences with the other mammalian species presented here: Pro becomes Ser and Ser becomes Tyr in mammalian and *P. falciparum*, respectively. Compared to many plant and parasite species, *P. falciparum* cb₅r shares many differences, especially in the first two and last two amino acids. *P. falciparum* cb₅r is the also the only protein with a Tyrosine at the seventh position in the flavin-binding domain. The third, fourth, and fifth amino acid in the domain are conserved throughout the species (Fig. 14a).

### Figure 14a: Amino Acid Sequence Alignment with the First Two Binding Motifs by ClustalW Analysis and Jalview.

Comparison of mammalian with *P. falciparum* cb₅r shows three amino acid substitutions in the FAD-binding motif: Pro → Ser, Ile → Val, and Ser → Tyr. The 9-amino acid gap preceding the FAD/FMN selectivity motif that is present in many fungal and plant cb₅r s, is reduced to a 2-amino acid gap in *P.
The first NADH-binding domain has the signature sequence GxGxxP. The sequences in mammals are very conserved, all being GTGITP, while *P. falciparum* has GTGMTP, with only one substitution from Isoleucine (I) in mammals to Methionine (M) in the parasite. The presence of Met at the fourth position is unique to *P. falciparum* (Fig. 14b).

**Figure 14b:** Continued Amino Acid Sequence Alignment Containing the First NADH-binding Motif by ClustalW Analysis and Jalview.

The NADH-binding motif is very conserved and has the signature sequence GxGxxP. In all mammals the sequence is GTGITP, while the *P. falciparum* one is GTGMTP. Here the only substitution is Ile that becomes Met in *P. falciparum*. The presence of Met at the fourth position is unique to *P. falciparum*. There is a 16-amino acid gap preceding the NADH-binding motif in fungi and plants that is reduced to a 5-amino acid gap in *P. falciparum*. This gap is also seen as a potential novel drug target.

There is a 9-amino acid gap preceding the FAD/FMN selectivity motif in many fungal and plant species which corresponds to a “lid” that covers the FAD prosthetic group.
group in all mammalian and Trypanosoma species (T. brucei and T. cruzi), and in Leishmania infantum as well. This variation in the protein structure may decrease the stability of the molecule and could be considered as a target in the design of drugs. This gap in P. falciparum cb₅r is made up of only two amino acids so its properties should be investigated (Fig. 14a & 15).

Figure 14c: Continued Amino Acid Sequence Alignment with the Second NADH-binding Motif by ClustalW Analysis and Jalview.

This second NADH-binding domain is extremely conserved throughout the organisms. Mammalian and P. falciparum cb₅r possess exactly the same sequence in the NADH-binding domain. All possible sequences are also presented in bold below the Jalview alignment.

There is another larger 16-amino acid gap in the sequence preceding the NADH-binding motif in all fungal and plant species. This gap is absent in all mammalian
species. This region connects the NADH- and FAD-binding motifs and it is represented by two $\beta$-sheets which are missing in those species that lack this 16-amino acid sequence. This also decreases the stability and catalytic efficiency of the protein and may constitute a potential target in the development of novel drugs. There is only a 5-amino acid sequence gap in the structure of *P. falciparum* cb$_{5r}$ (Fig. 14b & 15).

![Figure 15: The Ribbon Model of Rat and Candida Cb$_{5r}$](image)

This model compares a mammalian (rat) cb$_{5r}$ to a fungal (Candida) cb$_{5r}$. In the absence of a ribbon model of *P. falciparum* cb$_{5r}$, it shows the lack of the “lid” (9-amino-acid gap) over the FAD prosthetic group in Candida (only partial in *P. falciparum*) and the lack of two $\beta$-sheets (16-amino acid gap) in the region that connects the NADH- and FAD-binding motifs in Candida (only partial in *P. falciparum*).

The amino acid sequences of the species that were aligned and analyzed by ClustalW and Jalview were also evaluated by separating them into their catalytic domains. From this separation, phylograms were created that reflected the evolution of the individual separated domains. The first amino acid sequence contains the first two domains (NADH-binding domain and the FAD/FMN selectivity domain) and the second amino acid sequence includes the third and fourth domains i.e. the first and second NADH-binding domain.
Cytochrome b₅ Reductases Amino Acid Sequence Containing the FAD-binding Domain and the FAD/FMN Selectivity Domain (Underlined are the Two Domains)

>Human - CytB5Red AAP88823.1 [Homo sapiens]
MGAQLSTLGHMLFVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDREIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPISSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESMQIGDTIEFRGPGSLLVYQYQGKFAIRPDKKSNP
IIR

>Chimp - CytB5Red XP_001171082.1 [Pan troglodytes]
MGAQLSTLGHVVLFPVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDREIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPVSSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESMQIGDTIEFRGPGSLLVYQYQGKFAIRPDKKSNP
VIR

>Rat - CytB5Red P20070 [Rattus norvegicus]
MGAQLSTLSRVVLPVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDKEIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPVSSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESQMNNGDTIEFRGPGSLLVYQYQGKFAIRADKKSNP
VR

>Dog - CytB5Red ABA12483.1 [Canis familiaris]
MGAQLSTLGHVVLFPVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDKEIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPVSSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESQMNNGDTIEFRGPGSLLVYQYQGKFAIRPDKKSNP
PIIK

>Chicken - CytB5Red XP_416445.1 [Gallus gallus]
MGAQLSTLGHVVLFPVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDKEIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPVSSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESQMNNGDTIEFRGPGSLLVYQYQGKFAIRPDKKSNP
PIIK

>Xenopus - CytB5Red AAH45265.1 [Xenopus laevis]
MGAQLSTLGHVVLFPVWFLYSLMLKLFVRSTPATLLESPIDKYPLRLIDREIISHD
TRRRFPALPSQHILGLPVGQHIYLSARIDGNLVVRPYTPVSSDSDKGFVDVLVIKV
YFKDTHPKFPAGGKMSQYLESQMNNGDTIEFRGPGSLLVYQYQGKFAIRPDKKSPPVP
K
>Zebrafish - CytB5Red AAQ97765.1 [Danio rerio]
MSYAMSTTVAVTGVVVLSTAGLGYYFRKRKILITLIDPSEKYKLRLVDKEI
ISHDTRRFRFALPSPEHVLGLPGKHVYLSARIDGNLIVRPYTPVSSDDDKGFVDDL
VVKIYFRDVHPKFPAGGKMSQYLESLRIGDVIDFRGPGGLLEYKAGRLDIAK
KAPAETK

>Drosophila - CytB5Red AAF50004.1 [Drosophila melanogaster]
MTEFDFVPLAVGVVAVLAGALIVHYLLNKKSTKPRREPNTRARLRLTVDPNDKY
LLPLIEKENLSDHTRRFGLPSKHIVGLGVQGHIHILATIDNELIRPYTPISSDE
DVGYVDLVVKVVFKDSHPKFPAGGKMTQHLEQLDGLKDIFSGPSGRLQYLGN
GTFSIKLRKDPPKHAV

>Caenorhabditis elegans - CytB5Red AAB66010.1 [Caenorhabditis elegans]
MVENNTLAITGGVVLISSVSLFYLRLQRAEKKSKRTEEDSVKKYLLPLIEKEFIS
HINTKFRFGLPSKDHILPGHVYLSANIKGLKLSRYSVPSVCDLDDLGVYVDL
MIKVKYFKNTHFRPDGGKMSQHLESLKIGDTVSPGRPIISIIYKGSLFTVRMDK
KAEKPKNKR

>Saccharomyces cerevisiae - CBR1 [ER] (Z28365) 322aa ScCBR1
MYKSYSYIRKNERESKKEVVKLVQICQLAQLQETQSKSKMAIDAQKLVVVVIVVV
VPQLFKFIQPKTPVLDPOQSFPLVEKTLTHNTSMYKGLPHADDVLGLPI
GHIVIKANINGKDIRTSPSYTPTSLDGDTRGNFELLVKSYNGKVSKIMIGELKIGDSI
QIKGPRGNYHYERN

>Saccharomyces cerevisiae - MCR1 [Mito] -(Z26877) 302aa ScMCR1
MFSRLSRSHKAPIALGTVIAAAATAFYFANRNQHHSVNFESNKVFKGDKWID
LPISKIEEESHDTRRIFFKPLTEDSEMLVLASKALAFKVPKGSNVRPYTPVSD
LSQKGFQLVVKHYEGGKMTSHLFGLKPDNTVFSFKPIMKKWQPN

>Candida albicans - CBR1 [ER] –CaCBR1 chr4 (294aa) EAK92238 [Candida albicans SC5314]
MSETTTTVPIETVSEPNNPFIIVFATVATIISAFIGYLLQQFSKHKHPVLKPDQFKPL
IEKIRVSHNSIYRFGLPKSTDRGLPQGQHISIAGTDGKVEVRSYTPSTDDLQG
FDLLIKTYENGNISVRHAGKNVGEHEIEIRPGKFGFTYTPN

>Candida albicans - MCR1 [Mito] –CaMCR1 chr6(301aa) XM_705739 [Candida albicans SC5314]
MLTHHLSKATPKFLVPFAGATALSIGLQYSRTSNYIANGTETFDSNEWVD
LKLKSDILTHNTHKLVLKLDENDVSSGLITASCCLLTFKVPKGNVIRPYTPVSD
VNQSEQEFIVKDYDGKMSHISFDALKEGETLSFKPVIKVWKWEPN

>Candida glabrata –CytB5Red XM_448811 [Candida glabrata CBS138]
MDGIKILATFSVLVFYKLFTYSKKGVSQKEAVKALLKTEFELVEKEQLTH
NTAKYKFKLADESHVGLPGQHIVKTIIGKPVSRSYTPTSLDEECVGFELLV
KSYPEGNISKHIGDMKIKEGINKISGPRGIFYEVN

76
>Cryptococcus_neoformans_CBR1_[ER]  –CnCBR1(AAW46852, 294 aa, C. neoformans JEC 21 strain, chr 13)
MFTIEVLAQKLAPHASFLGGLVVAAILGLFIIFQEEKDRKVLDPVEWRSFKLVDKD
HLSSHNTALYRFALPRASDSLGLPIQPQHISVAEEINGKVVRSYTPPTTLDKKGHF
DLVVKTYEKGNSISRYLSSLTTGQIEIKVKGPGKGFYTPN

>Cryptococcus_neoformans_MCR1_[Mito]  –CnMRC1(XM_572314, 352 aa, C. neoformans JEC 21 strain, chr 8)
MAAARFLSSARIARPVISRTAAQYRGYSSGAAPGGLVVLAVGATGVGAY
AYLQYNPSVKKEVEAKGAEAEKAAAERTAAGISAFVKDTWPFTLEKVGKY
HTNTIYHFSFGEEGKDGSEGGEVAVVLRSPEPQIKDEKDPIIPYTPISPPD
QKGSIEFMIKSYSGKFTFLSMLSPQGQLFKIQFQYPQPN

>Mortierella -CytB5Red (AB020034) [Mortierella alpine]
MTLSNPAIAAASGVILAGAYLIDPSALPFVAAGVAATVARVLFKKTAVKTPMD
PKEYRKFKLVDKVHCSPNTAMYKFALPHEDDDLNLPIQPQHISIMANINGKDISRS
YTPSSSDVGVFVLCIKSYSGQNGKFSELSIGDSINARGPKGQSFYTPN

>Physarum_[slime_mold] -CytB5Red (AB259870) [Physarum polycephalum]
MTVTQLLTSLSFEAKLGIVIAAAAVVVISKFAFGSSSKREPALNPEYKKFMLRE
KQINHHTLFRFNLHHHDVEVGVPQGQHSMVKATDGIEYRPYTPVSSDDEKG
YFDLIIKVEKQMSQYIDHLPNDLQVRPGPKQFYKPN

>Leishmania_infantum -CytB5Red (L.infantum clone JPCM5, MCAN/ES/98/LLM-877; 308 aa)
MVGVLVIIAIFSMAAFFAFMFTRTTTKVAMDPTMFKFKLKRETEVTHDFTIFRFAL
ENETQTGLPIQPQIHVLRADCTTAGKTETVTHSYTPISSDDEKGYVDFMIKVFYFA
GVHPSPFHGGRLSQYMYHMLGKEIMRPQPGKFIYLGNQTSRIHKPGKIVTE

>Leishmania_braziliensis -CytB5Red (XM_001563404) [MHOM/BR/75/M2904; 275aa]
MQQSEHLAASCAGKAPNTFTSDEYKPFKLISSRYESHDRFORFYAPLSADDVSFFYM
PVASCIAAKYTDADKDVARPYTIISSNSTKGHFELEVKKYPKGKMGNHLFAMQ
PGDELLIKGFPEFAKYKN

>Leishmania_major -CytB5Red (XM_001685505) [strain Friedlin; 338aa]
MAKFMFTGAATALGAAFHTYTSQRMAAAECAAKKEAFTTFKFKPFVGLGALNLE
EDVAILFRFLNLDPSDVFDLVPCSTLQAHIKEGANMVDPQMRFYTPITPNGTKGYF
DLLVKQKQRPGRFTEHLFSMDVGESLLFRAIYQLTYKKN

>Trypanosoma_brucei -CytB5Red (XP828456; 307 aa, TREU 927/4 GUTat 10.1 clone)
MMLAVIVAFAVFIFFTALAGRLLPFYRFYPPALNPVQSFKLVKTRVTHDS
FIIFRFALHASHQCLGLPTGHHRFVASKHNTGTPQVQHSPYTISSNDDKGFVDD
FLVKIYKYKSNAPFPGGLSQHDLSSIGEAEVEMGPGKGFQMNGDYTVEM
MGKGEVKQR
>Trypanosoma_cruzi -CytB5Red (XP807822; 306 aa, CL Brener strain)
MLTYVVAAVIVLTVFLLFVKGVFFSSSMGVALDASKQNFKLFVKDITVSHNSFIF
RFALHSPQRLGLPIGQLHHLHMRCTTNPDKPXMVQHAYTPVSSDDDLGHVDLFI
KVYFKNVHPNFPGRGLSQUALYDLPLGMVIEIRGPVGNFEYLGKGNYTVDGK
GLKMKM

>Plasmodium_falciparum –CytB5Red (XP_001350386; 362 aa, strain 3D7)
MKRSFRSMRSLRSIFMRILYFLTSNIIIFCVSISIFGLYFGKHELHNKNKLFNLKSHK
EYEELDTKEKSKQFPLNONGKNQSFKYIKIKLTPVFIIFSYPEDEYEYLGLGICHH
IKFNASNIEGKIKGKWNNDDEKEKNLQISRSYTPVYIDKKhVHIIRVYYPD
DEYIDGGKMSIQLKNKNNDEIDINGPGFGLLEYKGNKELLHLKSVK

>Arabidopsis -CytB5Red (Arabidopsis thaliana, NM_121783)
MDTEFLRTLDRQILLGQVAFVAVGAAAYFLTSKRRVCLDPENFKFKLKV
RHQLSHNVAKFVFLPSTSVLGLPIGQHISCRGKDGQGQDVIKPYTPTTLDSDVG
RFELVIKMYQGRMSHFFREMVDLAVGBKPGFKYQPG

>Squash -CytB5Red (Cucurbita maxima, AF274589)
MAAFRLRRLATAAPALRNYALCNQGQRSIESKFRFPIGTTITATAGFSYASTS
VHLAPNCEEGKQVALKPDQWIEFELDVARSHTMLRYSCFDGPSEWGMML
LHASLQELQLIKTNREGVKYVARYSTPIPEDEAKGFDILILKIPQMGMTQHFAKL
KPGDKEVKGPRKLYSPN

>Maize -CytB5Red (Zea mays, AF077372)
MDHFLQEQRFSYETTVAVAVAVAAVAAAGAFLLLLLRSRKPGBKLCPLDENFKFKL
VEKKQISHNVARKFALKPTPTSVLGLPIGQHISCRGQDATGEEVIKPYTPTTLDSD
LGYFELVIKMYQGRMSHFFREMKVGYLVSKPGFKFYM

>Rice -CytB5Red (Oryza sativa (japonica cultivar-group), NM_001051147)
MDLHHGESQTTTAVAVAVAVAAAGAFLLLLLRSRKPGBKLCPLDENFKFKLVEK
KQISHNVARKFALKPTPTSVLGLPIGQHISCRGQDATGEEVIKPYTPTTLDSDLGH
FELVIKMYQGRMSHFFREMKVGYLYSVKPGFKFYQ

>Chlamydomonas_[green_alga] -CytB5Red (Chlamydomonas reinhardtii(green algae), XM_001695672)
MRUSITLSSYQVAGLLLVLVLIQALVFRLFRRKTKKPKPFLDPEFQPVPVLVEKTLIT
HTVRLRFALPDQERVGLPIGQHISFKAQGEDKGKDVIRPYTPVSSDDQLGAVDVF
VIKLYPTGMQSVIAKMQVGDMLMKGKPGFRFYT

>Tung-oil_Tree -CytB5Red (Vernicia fordii, AY819697)
MDLEFLQRTLQVILVGVAVAVLAIGIGAVFLSSKKPKGBKLCPLDENFKDFKLVPNRT
QLSHNVAKFSFALKPTPTSVLGLPIGQHISCRGKDSQGEVEIKPYTPTTLDSDVGHF
ELVIKMYQGRMSHFFREMVGYLVSKPGFKFYQ
The phylogram below (Fig. 16) depicts the evolutionary distance among all the different species based on the first catalytic domain containing the FAD-binding domain and FAD/FMN selectivity domain. *P. falciparum* and other parasites belong to a clade different from the mammalian species and have the closest homology to fungal species. Also plants form a distinct clade which is very far from the mammalian one, reflecting a high evolutionary distance and a high degree of amino acid substitution between the two groups.

**Figure 16: Cb3r Phylogram According to the First Half of the Protein Containing the FAD-binding Domain and the FAD/FMN Selectivity Domain.**

*P. falciparum* along with other parasites and mammalian belong to two distinct clades. The closest proximity for *P. falciparum* is to fungal species. A high evolutionary distance between plants and mammalians is also observed in this phylogram.
Cytochrome b$_5$ Reductases Amino Acid Sequence Containing the First and Second NADH-binding domain (Underlined are the Two Domains)

>Human -CytB5Red AAP88823.1 [Homo sapiens]
TVKSGMIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Chimp -CytB5Red XP_001171082.1 [Pan troglodytes]
TVKSGMIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Rat -CytB5Red P20070 [Rattus norvegicus]
TVKSGMIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Dog -CytB5Red ABA12483.1 [Canis familiaris]
TVKSGMIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Chicken -CytB5Red XP_416445.1 [Gallus gallus]
KVKYVGMIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Xenopus -CytB5Red AAH45265.1 [Xenopus laevis]
KANHLMGIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Zebrafish -CytB5Red AAQ97765.1 [Danio rerio]
TVKSLGLIAGGTGITPMLQVIRAISKDPDHTVCHLLFANQTEKDILLRPEEELRNKHSARFKLWYTLDRAPEAWDYQGFVNEEMIRDHLPPPEEPLVLMCGPPP
MIQYACLPLNLDHVGHPERTCFVF*

>Caenorhabditis -CytB5Red AAB66010.1 [Caenorhabditis elegans]
>Saccharomyces_cerevisiae_CBR1_[ER]  (Z28365)  322aa  ScCBR1
CRSHLGMIAAGGTGIAPMYQIMKAIAMDPHDTTVKVLFGNVHEDILLKKELEA
LMVAKPSQFKIVYLYDSPREDWTTGGVGYITKDVKEHLPAATMDNVQILICGPP
AMVASVRRSTVDLGFRSRKPLSKMEDQVFVF*

>Saccharomyces_cerevisiae_MCR1_[Mito]  -(Z26877)  302aa  ScMCR1
QFKSITLLGAGTGINPLYQLAHVIVENPNKDTLKVNLLYGNKTPQDILLMKELDAL
KEKYPDIFNYTFVDDKQDDQDFDGEISFISKDFIQEHVPGPK ESTHLV GCPPP
F M N A Y S G E K K S P K D Q G E L I G I L N L G Y S K D Q V F F K* 

>Candida_albicans_CBR1_[ER]  –CaCBR1 chr4 (294aa)  EAK92238 [Candida albicans SC5314]
MVKSFGMIAGGTGIAPMYQIIITA LKNPDEKTIKHLVYANVTSEDILLKEE LDK N F A
ARHPDLKIHYYVLNEAPANWQGSVGFVPTEIIDTHLPK ASNTDNL LCGPPPMVS
AMKKAAL E LV GFKAKPV SKLGDQVFVF*

>Candida_salivarius_MCR1_[Mito]  –CaMCR1 chr6(301aa)  XM_705739 [Candida salivarius SC5314]
QFKSIALG GGTGITPQLHQLIqn P KDN NT KVN LII MG L KKE IDA I A S K
HKDQVKHYFV DKADEKKE WWGQGI G IT KELQ KELE PKGSDFKFV VCGPPGLYK
AIGPKVSPTDQGELTGA LKLGDGEKEHVFKF*

>Candida_glabrata  –CytB5Red XM_448811 [Candida glabrata CBS138]
VHKHLAMVAGGTGITPQLHQLIqn P KDN NT KVN LII MG L KKE IDA I A S K
HKDQVKHYFV DKADEKKE WWGQGI G IT KELQ KELE PKGSDFKFV VCGPPGLYK
AIGPKVSPTDQGELTGA LKLGDGEKEHVFKF*

>Cryptococcus_neoformans_CBR1_[ER]  –CnCBR1(AAW46852, 294 aa, C. neoformans JEC 21 strain, chr 13)
MAPHL VMIAGGTGITPQLHQLIqn P KDN NT KVN LII MG L KKE IDA I A S K
KSNGRF DVYVLNP PEGW GGFVPTKEMIEAMPSVGSGAN HEGKVL
MCGPPPMITAMKGHLAQI G YPAPR PVSKLGDQVFVF*

>Cryptococcus_neoformans_MCR1_[Mito]  –CnMRC1(XM_572314 , 352 aa, C. neoformans JEC 21 strain, chr 8)
SFEGKL CAGGTGITP WL N HSLI PEDKTK WTL YS NVS EADILL KEF D ALA
QKYPGRDLKYYVLDGPGWKGGETGY TAD L KKT FPK N EG NIRA F VCGPPQ
M KAVS GKD GMK QG E L A K L E G Y TSDEVFKY*

>Mortierella  -CytB5Red (AB020034) [Mortierella alpine]
MCRAIGMIAGGTGLTPMLQIIRAIKNPEDKTQVNFIFANVEEIDILKAEELLDSQKHPQFKVYYVLNNAPEGWTTGVGMVNAKLHMPAPADIKVLLCGPPPMVSAKAMITQDLGYDKVNASVLKPQDFQVF R\n
>Physarum [slime mold] -CytB5Red (AB259870) [Physarum polycephalum] MVKEMGIAGGTGITPMLQVARAIKKNPKETIKINLIFANVNNEDDLIRTELDDM AKYNSNFLTVYVLNNPAGWTTGVGMVNASMKQHFSPPSDIKVMMCGPPPM NKAMQGHLETGVLTYPEQWFI R\n
>Leishmania infantum -CytB5Red (L.infantum clone JPCM5, MCAN/ES/98/LLM-877; 308 aa) KVDAYAAAIAGGTGTPILQIIHAIKKKEDPTKVFLVYNQTERDILLRKLDEAAANDSFHWVYTDREATPEWKYDICYVCEEMFRKHLVDPMLGSVDSPQNAGIKKVMALMCIPPPPMQVAIMTPNERYGATD N\n
>Leishmania braziliensis -CytB5Red (XM_001563404) [MHOM/BR/75/M2904; 275aa] MWKHVGMIAGGTGIAPMYQVIPRLENPDKTNISLITYASQRRDILLANELIEMQKINNNFMYLTTLEPVRHWLGGIGYVNSAMVTTFMPKPEGINTKILVCGPPPMMQAISGDLFEPGKQPPQVGGILLLETGQKEDVFKY F\n
>Leishmania major -CytB5Red (XM_001685505) [strain Friedlin; 338aa] RWTDVGMCIGCGTGLCPIQLFMNAS LTEGDSLRNMLFAONEKSKILLGGLDEKAREHDKLQIFYTVDFSDPDIIANPVYKKGAPLTVDFASQPSTPQGRKYFHGFKYIDPMLQATMPRPSPSTLLLVCGPDPMVKKVGAAPMVLRAMSGLAYQPSGAVLNNADVSFGFLGTGLQYKEMV YRF\n
>Trypanosoma brucei -CytB5Red (XP828456; 307 aa, TREU 927/4 GUTat 10.1 clone) HVAGFAMVAGGTGTPPMMQIIHAILKSPDTRLWLYSNHTEDILLRDLALAEACKDPVRKVWWHTLRSAPPDWAYGRGRVNEEMRLTHLPPPQLEEGSVTVLLCGPPPLMLQDAVKPNLNGYSQDFIFTTF\n
>Trypanosoma cruzi -CytB5Red (XP807822; 306 aa, CL Brener strain) HTDAFTMVAGGTGTPPMMQLIRAIMKNDRDNTNYLNYANQTEDDRLRKEELDD CAKDPRMLKWYMLDRETSQPWQKYGTVYDEVATLRAHVLPVQRPRSDYNRVV ALMCIPPPMLQKAVKPNLEKLGATEDAEMSF\n
>Plasmodium falciparum –CytB5Red (XP_001350386; 362 aa, strain 3D7) IKKHIVMIAAGGTGTPFFRILLNNLTLKEKLEPSDPVYITFYIARYNENNEILLSIFDDYENRNFENKVRYSVIDKCLNTQMGNFENIGFINEELLKRLKVLKLYELNIEVKNKDTLILLCGPPPMQSSKISKLDQIHMNIIFV F\n
>Arabidopsis -CytB5Red (Arabidopsis thaliana, NM_121783) QFRAFGMLAGGSGITPMPQVARAILENPTDKTCKVIYANVNYDILLKKEEELG LTTNYPEQFKIFYVLNPQPPHVWDGGVGFKSMQIETHCPAQPASDIQLRGCIPPMN KAMAANLEALGYSPEMQFQF\n
82
The second half of the amino acid sequences of various cb5r containing the two NADH-binding domains presents interesting differences in terms of evolutionary distance compared to the cb5r first half (Fig. 17). P. falciparum has the greatest evolutionary distance from human. Its closest homology is to other protozoan and fungal species. Compared to the first half of the protein, this phylogram shows less ramifications and more highly grouped clades, suggesting that there has been a higher degree of conservation in the evolution of this two binding domain-containing part of the protein.
Figure 17: Cb$_5$r Phylogram According to the Second Half of the Protein Containing the First and Second NADH-binding Domain.

This phylogram assumes a very large evolutionary distance between this section of cb$_5$r in *P. falciparum* and human. The closest homology for *P. falciparum* is to protozoan and fungal species. There is also a higher degree of evolutionary conservation in this segment of the protein.
OBJECTIVES

Cytochrome b$_5$ reductase and its electron acceptor cytochrome b$_5$ have been studied extensively over the last fifty years and a great variety of aspects has been investigated, leading to a plethora of information in the literature. Their known roles in fatty acid desaturation and elongation, cholesterol biosynthesis, and cytochrome P450-mediated detoxification of xenobiotic in many different organisms, ranging from mammalians to fungi and plants, have been explored by many scientists.

In a time in which resistance to available medicines is emerging, and affecting the cure of numerous infectious agents, there is a need to discover new and promising therapeutic targets. In particular malaria treatment is experiencing an unprecedented crisis in terms of efficacy, safety, and availability of antimalarial drugs. Resistance to the most feared and aggressive form of malaria that is caused by *Plasmodium falciparum*, has developed to most of the available drugs, with the exception of Artemisinin. The structure, function, biochemical and pharmacological properties (in particular the role in the detoxification of xenobiotics), and the interaction with other cytoplasmic proteins of *P. falciparum*, such as cytochrome b$_5$ reductase and its electron acceptor cytochrome b$_5$ should be investigated.

Cb$_5$r and its electron acceptor cb$_5$ may serve as potential drug targets but yet, there are no formal studies that have addressed this possibility. An elucidation of the mechanisms and processes in which it is involved may generate important information about the possible role of these two proteins as drug targets. Interestingly, phylogenetic analysis has revealed that *P. falciparum* cb$_5$r is closely related to plant cb$_5$r, and plant cb$_5$r
has been identified as a novel herbicidal target. If we are to base our decision on weather or not cb₅r would be a fortunate target by comparing its position in the phylogenetic tree to that of plant cb₅r, we would likely be prompt to give an affirmative answer.

In this project, an analysis of the amino acid sequence, binding domains, and protein structure was undertaken. Furthermore, cloning of *P. falciparum* cytochrome b₅ reductase and cytochrome b₅, and the optimization of recombinant protein expression in *E. coli* was performed. A further objective of this study is the biochemical and kinetic characterization of *P. falciparum* cytochrome b₅ reductase in order to elucidate its enzymatic properties. Here, a comparison with mammalian cb₅r is necessary in order to identify differences in biochemical and kinetic activity. A conclusive and useful aim of this study (that has not been investigated here) would be the pharmacological characterization of *P. falciparum* cb₅r through the use of potential inhibitors of this enzyme. There are a number of inhibitors that have been found to alter the enzymatic activity of human cb₅r, but none of them has been tested as potential antiparasitic compounds against *P. falciparum* cb₅r. It is important to remember that, ideally, this drug should possess high specificity for the *P. falciparum* cb₅r but minimal toxicity to the human host.

Further research will be needed in order to obtain useful knowledge of the pharmacological properties of this ubiquitous enzyme and draw conclusions on its suitability as an opportune novel drug target.

In this project, observing the biochemical and enzymatic behavior of cb₅r and cb₅ can reveal very important information about the suitability of these two proteins as drug targets. This investigation will open the doors for future studies that will have the
advantage of using these results and apply them to further studies involving the pharmacological characterization of cb₅r and cb₅ and drug trials.
MATERIALS AND METHODS

The first step in this project to characterize a novel drug target must start with the source of all future assays: a purified protein. The process that leads to the expression of a protein consists of cloning of the Plasmodium falciparum cb5r and cb5 genes, followed by optimization of the recombinant protein expression and further purification. The membrane-bound form of the proteins will be used for this study; these are the truncated versions that lack the transmembrane domain since we are interested in characterizing the soluble form of the proteins.

Experimental Design

For cloning of the desired genes coding for cb5r and cb5, a biotechnology company (GENEART, Würzburg, Germany) was employed to synthesize both genes obtained from the P. falciparum genome database. Importantly, Plasmodium has an AT-rich codon bias that complicates heterologous protein expression in E. coli. Therefore, for synthesis of the genes, we designed the P. falciparum cb5r and cb5 using codon optimization for E. coli to obtain optimal protein expression. Both synthesized genes were subcloned into the vector plasmid vector called pTWIN (New England Biolabs). This vector has several features: (a) the expression of the fusion gene is under the control of the T7 promoter and is regulated by IPTG due to the presence of a lacI gene and (b) that the expression requires an E. coli host that carries the T7 RNA polymerase gene [ER2566, BL21 (DE3) and derivatives]. Each primer also contains a His6-tag, an Intein protein, and a Chitin Binding Domain (CBD) fragments for further protein purification.
When the genes have been successfully inserted into the plasmid, the transformation into bacterial cells could proceed. This was where the actual project begun. The bacteria used for this purpose was *E. coli* BL21 (DE3) RIPL, which is a special strain of bacteria optimized for heterologous protein expression.

Cells were grown in the presence of LB broth and plated on Terrific Broth (TB) agar containing 100µg/ml ampicillin. Our original genes that were ligated into plasmids contained an ampicillin-resistant gene that allowed growth of those cells that retained the plasmid. Positive clones were then sequenced to confirm that no mutations occurred during the cloning process. Followed transformation, minipreparation of bacterial plasmid DNA and digestion of minipreparation by restriction enzyme were performed. Agarose gel electrophoresis was then performed to obtain a DNA gel in which the bands corresponding to the base pairs number of plasmid and insert could be observed.

Optimization of recombinant protein expression was determined according to temperature (23 °C), time of induction (16 hours), and IPTG concentration (0.2, 0.5, 1.0 mM). Cells were sonicated and separated into pellet and supernatant. This separation also aided in identifying the presence of cb₅r and cb₅ in either the pellet or the supernatant; since the two proteins are soluble, they were expected to be found in larger amounts in the supernatant.

The purification process started with the expression in *E. coli* of the inserted plasmid; later on the recombinant protein expression was achieved by the addition of IPTG, followed by sonication. Finally, purification was obtained by Chitin Agarose Chromatography and Nickel Column Chromatography.
Methods

Vectors with the inserted clones were transformed in *E. coli* BL21 (DE3) RIPL competent cells and plated on TB agar containing 100µg/ml ampicillin. Positive clones were selected from previously streaked backup plates and sent to Moffitt Core Sequencing Laboratory for sequencing. Bacteria were grown overnight in 2ml LB broth with 75µl/ml of ampicillin; the sample was subsequently washed (minipreparation) with a series of buffers with the ultimate purpose of lysing the cells and cleaning DNA plasmids from proteins and other substances. The following buffers in the following concentrations were added:

- 300µl TENS Lysis buffer – to break the cells apart and release content
- 150µl 3M NaAc – caused proteins that were present in the released content to precipitate and separate from DNA and forms a protein precipitate (solid)
- 900µl 100% EtOH – helped precipitate DNA

Following precipitation at – 20°C for 15 minutes, pellet precipitate was washed first with 500µl 70% EtOH (cleans the plasmids from salts and buffers) followed by 500µl 100% EtOH (dries up DNA and gets rid of water left on DNA from previous wash)

DNA gel analysis was performed using Agarose Gel Electrophoresis to confirm the correct insert size in the pTWIN vector.
Optimization of Protein Expression

ΔP 138 *P. falciparum* cb₅ and cb₅r in *E. coli* BL21 (DE3) RIPL cells were grown overnight in 2mL TB-Amp<sup>75</sup>-chloramphenicol (Cm) 50µg/mL at 37°C and 250 rpm. A 1:100 dilution of overnight culture was added to a 70mL fresh TB-Amp<sup>75</sup> broth containing either 100µM riboflavin or 100µM ferric citrate; the sample was then grown for 2-2.5 hours at 37 °C at 250 rpm until an Optical Density at 600 nm (OD<sub>600</sub>) was reached. IPTG was then added to the broths at concentrations of 0.2, 0.5, and 1.0mM and grown at either 37°C for another 3 hours or overnight (16 hours) at room temperature (23°C). In order to better localize our proteins, the cells were disrupted by sonication and centrifuged to separate pellet (P) and supernatant (S/N). SDS-Polyacrylamide Gel Electrophoresis was then performed in 4-20% acrylamide gels.

Protein Purification

The cells were harvested by centrifugation at 2500 rpm for 10 minutes at 4 °C. The pellet was washed with PBS and the resuspended in lysis buffer. Cells were then sonicated and centrifuged at 10000 rpm for 5 minutes. The product is the separation of the sample into pellet and supernatant. Our proteins are soluble therefore we expect them to be found in the supernatant. The pellet was then washed with PBS to remove all traces of supernatant and spun. Pellet and supernatant are now ready to be run into a gel.

The proteins were further purified by two processes: Chitin Agarose Chromatography and Nickel (Ni-NTA) Column Chromatography. In Chitin Agarose Chromatography the protein passes through chitin resin beads which bind the target protein and the intein by the CBD. Subsequently, the target protein is cleaved by the use
of a thiol-induced cleavage buffer of the intein, and the soluble protein of interest is eluted (Fig. 18). In the purification process, a 200µl chitin bead slurry was spun at 10000 rpm for 30 seconds. The beads were washed with lysis buffer and added to the gel bed. After incubation, beads were washed again with lysis buffer for five times. An additional wash with lysis buffer containing 40mM DTT was then applied. Centrifugation led to a supernatant (elution 1), then addition of DTT buffer, incubation for 1 hour at room temperature, and subsequent centrifugation led to elution 2. DTT buffer was added again and centrifuging led to elution 3.

Before being run through a nickel column, the protein was bound to the Histidine-tag only. The His-tag gave the protein an enhanced affinity that would aid in the purification. Usually the target protein is the only protein in a mixture that would have such a strong bond to a long chain of histidine residues (usually six residues). This tag has high affinity towards nickel. When the protein is run through a resin with an abundance of nickel, the His-tag will bind the nickel and gets retained in the resin while all other proteins are will pass through. Imidazole would then be added to the resin, which will compete with his-tags for nickel binding since they have similar structure. The protein was then released from the resin in a process called elution. In the Ni-NTA column chromatography, the column was equilibrated with lysis buffer and centrifuged at 2000 rpm for 2 minutes. The lysate from chitin agarose purification was loaded into the column and allowed to sit for 30 minutes at room temperature. The sample was then centrifuged again at 2000 rpm for 2 minutes and the column was washed with lysis buffer. Finally the protein was eluted with elution buffer and spun as before, leading to a
supernatant (Ni-NTA elution 1). Elution buffer was again added and centrifuging the sample led to elution 2. A repeat of the same process led to elution 3.

Figure 18: Protein Purification by Chitin Agarose Chromatography Using pTWIN Vector.

Fusion of the intein protein to the C-terminus of *P. falciparum* cb5r target protein and release of the *P. falciparum* cb5r by thiol-induced cleavage. In this method, the target gene is cloned adjacent to an intein endoprotease (thiol inducible) linked to a chitin binding domain (CBD) that allows purification by chitin chromatography. Subsequently, thiol-induced cleavage of the intein releases the His6-cb5r or His6-cb5 protein that is further purified by standard Ni-NTA column chromatography.
RESULTS

The pTWIN vector containing both *P. falciparum* cb5r and cb5 genes, was transformed into *E.coli* BL21 (DE3) RIPL cells and confirmed by sequencing. After minipreparation and digestion of minipreparation, an Agarose Gel Electrophoresis was performed to visualize if the inserts were successfully digested by restriction enzymes (Fig 19).

![DNA Gel of Cb5 Gene Coding Insert by Agarose Gel Electrophoresis.](image)

Figure 19: DNA Gel of Cb5 Gene Coding Insert by Agarose Gel Electrophoresis.
In this DNA gel the first column is the DNA ladder, while the following columns represent four different plasmids that were picked by selecting four different bacterial colonies in TB agar. The top band with the red spot in all four pTWIN columns is the 5426 base pairs vector containing the insert. The bands with the green spot represent the 1234 base pairs insert. The cb5 is composed of 432 base pairs that are included in the 1234 base pair insert.

**Heterologous Expression of P. falciparum Cb5r Protein in E. coli**

The expression of *P. falciparum* cb5r could not be achieved during this seven-month project. Numerous different experiments were attempted in order to optimize the expression of the protein but none of these succeeded. One conclusion drawn from our studies and experiments was that *Plasmodium* cb5r may be toxic to the strain of *E. coli* used throughout our research. Specifically it may use up or deplete the cells of crucial molecules, such as NADH, that play a role in vital biochemical processes. In the same laboratory, a very similar result was observed in the expression of cb5r of the pathogenic fungus *Cryptococcus neoformans* when the same strain of *E. coli* [*E. coli* BL21 (DE3) RIPL] was used for protein expression. Interestingly, cb5 expression was very abundant while cb5r expression was not obtained. Since expression in the bacterium *E. coli* has not been successful for *Plasmodium* cb5r, one possible solution would be the use of a different organism, such as the fungus *Pichia pastoris*. Plans have been made for this alternative future approach in the laboratory.

Specifically, as for cb5 expression in *E.coli*, our first attempt of heterologous expression in *Plasmodium* cb5r was performed by (1) induction at various IPTG concentration (Fig. 20) followed by standard protein purification steps (Fig. 21). Then a series of different assays were performed using different procedures with the goal of reaching optimal expression; (2) the addition of detergents during the purification steps (Fig. 22), (3) growth at different temperatures and for different incubation periods (Fig.
23), (4) growth in different media (Fig. 24), (5) addition of alcohol to the growth medium (Fig. 25), and (6) the screening of different bacterial clones (Fig. 26 & Fig. 27). The results of these different optimization attempts are described in detail as follows:

1) Optimization of *P. falciparum* cb₅r protein expression using different concentrations of IPTG (0.2, 0.5, and 1.0 mM). Also shown are the pellet and supernatant obtained by sonication of the cells (Fig. 20). The samples were then purified by standard Chitin Agarose Chromatography and Ni-NTA Column Chromatography but no significant improvement was obtained; hence, the uninduced and induced samples looked the same (Fig. 21).

2) Optimization of cb₅r expression was performed by adding Triton X-100 and SDS detergents to the pellet and supernatant in order to solubilize the content, such as inclusion bodies, from the pellet. The protein may be found in the pellet, therefore by solubilizing the content we may obtain a better yield of cb₅r, but again, not differences could be observed (Fig. 22). More specifically, proteins expressed with bacteria sometimes end up in inclusion bodies that serve as “waste compartment” for the bacteria. These inclusion bodies are usually found as a solid form or as precipitates, therefore the addition of detergent might help solubilize these structures and release the proteins (cb₅r) into the supernatant fraction.

3) Despite our finding that for most other protein expression experiments the optimal growth conditions were overnight (16 hours) culture at room temperature (23°C), growth
was also performed at 37 °C for 2 hours to compare the outcomes. It was thought that cb5r may need a more rapid and sudden expression to prevent bacterial degradation, but no substantial difference was found, and the bands corresponding to the molecular weight of cb5r have the same intensity for the two different growth conditions (Fig. 23).

4) In the early stages of optimization of protein expression, two different growth media were used (LB broth and Terrific broth), and samples were selected at different times of incubation (30, 60, and 120 minutes). LB broth is less rich in nutrients than terrific broth, to stimulate growth, hence this medium was chosen as an alternative to the nutrient-rich Terrific broth. The rationale here is that since cb5r is toxic to *E. coli*, the protein may need a less powerful growth medium. It could also be the case that cb5r needs less time for proper folding, after which it begins degrading. Therefore three different expression times were chosen (30, 60, and 120 min). Again, the two media and the incubation time at which the samples were collect did not affect expression of *Plasmodium* cb5r (Fig. 24).

5) As a next step, two different temperatures were used (16ºC and 30ºC) with two growth media (LB and Terrific broth) and with different incubation times (30, 60, 120, and 180 min). Additionally, 2% ethanol was added to some of the growth conditions with the rationale that ethanol can substitute the lack of chaperone proteins in *E. coli* as a prokaryote, which can help facilitate protein folding in eukaryotic cells such as *Plasmodium*. Ethanol can take the role of chaperones and aid in the protein folding/unfolding during cb5r expression in the heterologous bacterial system. Nevertheless, no significant differences in expression were observed (Fig. 25).
6) In figure 26a and 26b, twelve new clones were picked and run on a gel to compare them for protein expression levels with the previously picked clone, using *Plasmodium* cb₅ as positive control for optimal protein expression (see next chapter on cb₅ expression). Again, no difference was observed between any of the twelve newly picked additional clones when comparing uninduced and induced conditions on a protein gel (Fig. 26a and 26b).

Molecular Weight of *P. falciparum* cb₅r/Intein2-CBD: 66.17 kDa

![Figure 20: Optimization of *P. falciparum* Cb₅r Protein Expression: Various IPTG Concentrations.](image)

Induction by IPTG at different concentrations (0.2, 0.5, 1.0 mM) for 16 hours at 23 °C. Also shown are the pellet and supernatant obtained after sonication of the bacteria.
Figure 21: *P. falciparum* Cb5r Purification Gel: Chitin Agarose and Ni-NTA Chromatography.

Standard protein purification with Chitin Agarose and Ni-NTA Chromatography was performed with 3 and 2 elutions, respectively. Since no significant results were observed in protein expression by IPTG induction, further purification did not show anything either.

Figure 22: Optimization of *P. falciparum* Cb5r Expression Gel: Addition of Triton X-100 and SDS Detergents.

Detergents were added to the pellet at 0.1% concentration to solubilize the content and “free” any protein trapped in inclusion bodies. The overexpressed protein may be found in the pellet, therefore by solubilizing the pellet we may extract the protein but no differences are visible between the pellet and the supernatant.
Figure 23: Optimization of *P. falciparum* Cb$_5$r Protein Expression: Growth at Different Conditions (Temperature and Incubation Period).

Optimal growth was tested at two different temperatures and incubation periods (37°C for 2 hours and 23°C for 16 hours) to see which of these two conditions positively affected expression. No significant differences were observed.

Figure 24: Optimization of *P. falciparum* Cb$_5$r Expression: LB Broth vs Terrific Broth.

The use of more or less rich media can affect the proper expression of a protein. Since cb$_5$r may be toxic to the bacterial cell, we tried to use an alternative less rich medium (LB broth) to compare it with expression in our standard medium (Terrific broth). No significant differences were observed.
Figure 25: Optimization of *P. falciparum* Cb₅r: 16°C and EtOH.

Growth was also tested at 16°C in both LB and Terrific broth. Furthermore, growth was performed with the addition of 2% ethanol to the growth medium at 30°C. Ethanol takes up the role of chaperones and is supposed to help Cb₅r fold and unfold properly since *E. coli*, being a prokaryote, lacks chaperones. In vivo, *Plasmodium* parasites, which are eukaryote organisms, use chaperones to perform such function but, since *E. coli* does not have chaperones, ethanol is thought to substitute chaperones. No significant differences were observed.

Figure 26a: *P. falciparum* Cb₅r Protein Expression: Screening of Clones 1 through 6.

Six new clones were picked and run on a gel to compare their protein expression levels with the previously induced clone, and Cb₃ as positive control that shows proper expression when compared with the uninduced sample. No significant differences were observed.
Figure 26b: *P. falciparum* Cb₅r Protein Expression: Screening of Clones 7 through 12.

Six additional clones were picked and run on a gel to compare their protein expression levels with the one of the previously picked clone, using cb₅ as positive control that shows proper expression when compared with uninduced bacteria. No significant differences were observed.

**Heterologous Expression of *P. falciparum* Cb₅ Protein in E. coli**

Optimization of protein expression for ΔLys44-cb₅r-pTWIN and ΔPro138-cb₅-pTWIN revealed that the optimal growth conditions are overnight (16 hours) culture at room temperature (23°C) at 100 rpm after being induced with 0.2 mM IPTG. After sonication, ΔPro138-cb₅-pTWIN is located in the cell lysate supernatant (Fig. 27).
Figure 27: Cb₅ SDS-Polyacrylamide Gel Electrophoresis.
The band that corresponds to cb₅ (with His-tag, intein, and CBD still attached) is labeled in red and weighs 44.78 kDa. This gel clearly shows the difference in expression between the uninduced (column 2) and the 0.2, 0.5, and 1.0mM IPTG induced cb₅ in columns 3, 4, and 5 respectively. Also included are the 0.2 and 1.0mM IPTG induced pellet (columns 6 and 8 respectively) and the 0.2 and 1.0mM IPTG induced supernatant (columns 7 and 9 respectively) obtained after sonication.

Further purification was achieved by Chitin Agarose Chromatography. This purification breaks the bond between the Intein and the His-tag so that the protein (cb₅) is left with only the six histidine residues attached (Fig. 28).
We subjected cb₅ through an additional purification through Ni-NTA Column Chromatography. Here, the protein was separated from the His-tag and it became a completely pure protein. Cb₅ final molecular weight was calculated to be 16.94 kDa (Fig. 29).
Figure 29: Cb$_5$ Gel after Complete Purification by Ni-NTA Column Chromatography.

Bands in column 9 and 10 (red stars) represent the completely purified cb$_5$. 
DISCUSSION

Our main objective of expressing a recombinant protein generated ambiguous results, one of which was that the expression of cb5 was very successful. The type of vector and the strain of bacteria used in cloning and expression of a recombinant protein gave positive outcomes with cb5 and the amount of protein yielded was extremely abundant. On the other hand, the expression of cb5r was very problematic and could not be achieved. Numerous attempts were made to achieve the expression of cb5r, but none was successful. Several approaches were attempted but none of them worked. Transformation of plasmids into E. coli and protein expression was attempted twice from the beginning; it was thought at first that maybe the clones that were chosen had not picked up our gene. The gel made after a new transformation attempt failed to show any differences between the uninduced and IPTG induced clones. In another instance, expression by IPTG induction was aided by ethanol which was thought to promote protein expression, unfortunately however, the results did not change, and the protein failed to be expressed. Additionally, the expression of cb5r was carried out at different temperatures and with different incubation periods but none of the assays produced satisfactory results. A possible solution to achieve successful purification is to use a different organism for heterologous expression, such as the fungus Pichia pastoris. Given the time constraints, further experiments could not be performed and the biochemical and kinetic characterization of cb5r and cb5 would have to be conducted at a later time.
The future production of knock-outs of cb₅r and cb₅ will help us to identify which amino acids are responsible for the binding with the molecules with which they interact. This will aid to a more complete and thorough understanding of the biochemical and pharmacological characteristics of these two proteins.

In the context of public health, the identification of new successful drug targets for falciparum malaria will have great implications, the first and most important being the possible use of alternative drugs and the consequent alleviation of the current drug resistance. As it was stated throughout the text, the emergence of resistance represents a huge obstacle in the cure of malaria, and this obviously leads to a greater number of deaths. The first goal of public health is to prevent or at least reduce the number of deaths, therefore the discovery of new drug targets represents one of many methods that leads to the reduction of the burden of malaria worldwide.

The successful expression of cb₅r will certainly be achieved in the near future. In this way, the desired biochemical, kinetic, and pharmaceutical characterization will be investigated and a possible novel drug target discovered.
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