CHARACTERIZATION OF THE *PLASMODIUM FALCIPARUM* REPLICATION LICENSING FACTOR, PfMCM6, A SUBSTRATE OF TWO *PLASMODIUM FALCIPARUM* CYCLIN-DEPENDENT KINASES, PfPK6 AND PfMRK

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Characterization of the *Plasmodium falciparum* replication licensing factor, PfmcM6, a substrate of two *Plasmodium falciparum* cyclin-dependent kinases, Pfpk6 and Pfmrk

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A thesis submitted in partial fulfillment for the degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology

2012
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

To

My wife Lydia, my son Daniel and my Daughter Sharon
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# TABLE OF CONTENTS

DEDICATION .................................................................................................................. iii

ACKNOWLEDGEMENTS ............................................................................................... iv

TABLE OF CONTENTS ................................................................................................. vii

LIST OF TABLES ........................................................................................................ viii

LIST OF FIGURES ...................................................................................................... xiii

LIST OF ABBREVIATIONS .......................................................................................... xvi

ABSTRACT .................................................................................................................... xvii

CHAPTER ONE .............................................................................................................. 1

1.0 Introduction .......................................................................................................... 1

1.1 Background information ........................................................................................ 1

1.2 Problem Statement ............................................................................................... 2

1.3 Justification .......................................................................................................... 3

1.4 Research Questions .............................................................................................. 3

1.5 Hypothesis .......................................................................................................... 4

1.6 Objectives .......................................................................................................... 4

1.6.1 General objective ........................................................................................... 4

1.6.2 Specific Objectives ....................................................................................... 4
CHAPTER TWO .............................................................................................................................. 5

2.0 Literature review .................................................................................................................. 5

2.1 Complexities of Plasmodium cell cycle.............................................................................. 5

2.2 DNA Replication in Eukaryotes as model for the replication in Plasmodia................. 8

2.3 DNA Helicases and their Role in DNA replication......................................................... 12

2.4 Minichromosome Maintenance (MCM) Complex and its Role in DNA replication......... 13

2.5 Discovery and characterization of Plasmodial MCM (PfMCM) Complex ................. 16

2.6 Cyclin-Dependent Kinases and their role in cell cycle regulation ......................... 18

2.7 Plasmodial-Cyclin Dependent Kinases (CDKs).............................................................. 21

2.8 Eukaryotic MAT-1 Proteins and their role in DNA replication............................... 25

2.9 The role of Plasmodial MAT-1 Protein in DNA replication................................. 28

CHAPTER THREE ...................................................................................................................... 30

3.0 Materials and Methods.................................................................................................. 30

3.1 Identification of the putative replication licensing factor........................................... 30

3.2 Expression and purification of PfPK6............................................................................. 30

3.3 Cloning of PfMCM6...................................................................................................... 31

3.4 Expression and purification of PfMCM6.................................................................... 32

3.5 Acquisition of PfMAT-1, PfCTD and PfMRK............................................................ 33
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6</td>
<td>Kinase Phosphorylation assays</td>
<td>33</td>
</tr>
<tr>
<td>3.6.1</td>
<td>Principles of the Kinase- Kinase-Glo® Luminnescent Kinase assay</td>
<td>33</td>
</tr>
<tr>
<td>3.6.2</td>
<td>Kinase-Glo® reagent preparation</td>
<td>34</td>
</tr>
<tr>
<td>3.6.3</td>
<td>Determining optimal ATP concentration</td>
<td>34</td>
</tr>
<tr>
<td>3.6.4</td>
<td>Determining optimal substrate concentration</td>
<td>35</td>
</tr>
<tr>
<td>3.6.5</td>
<td>Determining optimal Kinase concentration</td>
<td>35</td>
</tr>
<tr>
<td>3.7</td>
<td>Radioactive kinase assays</td>
<td>35</td>
</tr>
<tr>
<td>3.8</td>
<td>Kinase assays using Kinase-Glo®</td>
<td>36</td>
</tr>
<tr>
<td>3.9</td>
<td>Kinase inhibition assays</td>
<td>36</td>
</tr>
<tr>
<td>3.10</td>
<td>Preparation of antibodies against PfMCM6 from synthetic epitopes</td>
<td>37</td>
</tr>
<tr>
<td>3.11</td>
<td>P. falciparum cultures for parasite antigen preparation</td>
<td>38</td>
</tr>
<tr>
<td>3.12</td>
<td>P. falciparum protein preparation from culture lysate</td>
<td>38</td>
</tr>
<tr>
<td>3.13</td>
<td>Immunoprecipitation of PfMCM6 from P.falciparum culture lysate</td>
<td>39</td>
</tr>
<tr>
<td>3.14</td>
<td>SDS PAGE</td>
<td>40</td>
</tr>
<tr>
<td>3.15</td>
<td>Western Blot analysis</td>
<td>40</td>
</tr>
<tr>
<td>3.16</td>
<td>Bioinformatic analysis of PfMCM6</td>
<td>42</td>
</tr>
<tr>
<td>3.17</td>
<td>Flavonoid and Chloroquine susceptibility testing</td>
<td>42</td>
</tr>
<tr>
<td>3.17.1</td>
<td>RPMI basic media preparation</td>
<td>42</td>
</tr>
<tr>
<td>3.17.2</td>
<td>RPMI complete media preparation</td>
<td>43</td>
</tr>
</tbody>
</table>
3.17.3 Flavonoid/Chloroquine sensitivity tests ............................................................. 43

CHAPTER FOUR ............................................................................................................. 45

4.0 Results .................................................................................................................. 45

4.1 PfMCM6 identified ............................................................................................... 45

4.2 Expression and purification of PfMCM6 .............................................................. 47

4.3 Expression and purification of PfPK6 and acquisition of PfMRK ....................... 47

4.4 PfMCM-6 as a substrate of PfMRK and PfPK6 ................................................... 48

4.5 Optimization of the Kinase Glo Assay ............................................................... 50

4.6 The phosphorylation of PfMCM6 by PfMRK ..................................................... 50

4.7 The effect of PfMAT 1 on the kinase activity of PfMRK in the presence
PfMCM6/PfCTD ......................................................................................................... 52

4.8 Bioinformatic analysis of motifs present in the PfMCM6 protein sequence ..... 55

4.9 Anti-PfMCM6 antibodies .................................................................................... 58

4.10 Western-Blot Analyses ...................................................................................... 60

4.11 Inhibition of PfMRK activity using PfMCM-6 as Substrate .............................. 63

4.12 Inhibition of PfPK6 with PfMCM-6 as Substrate .............................................. 64

4.13 Testing of Flavonoids as inhibitors of PfPK6 and PfMRK using PfMCM6 as
substrate .................................................................................................................. 64

CHAPTER FIVE ............................................................................................................. 69
5.0 Discussion ........................................................................................................ 69

5.1 Introduction .................................................................................................... 69

5.2 PfMCM6 and the DNA replication licensing system .................................... 70

5.3 PfMCM6 and the MCM complexes ............................................................... 74

5.4 The Motifs and Modules ................................................................................ 75

5.5 Interaction of PfMCM6 with MAPK ............................................................ 82

5.6 PfMAT1 and PfMCM6 ................................................................................ 84

5.7 Inhibitory action of flavonoids against PfMRK and PfPK6 with PfMCM6 as substrate ............................................................................................................... 85

CHAPTER SIX ........................................................................................................ 89

6.0 CONCLUSION AND RECOMMENDATIONS ............................................ 89

6.1 Conclusion ..................................................................................................... 89

6.2 Recommendations ....................................................................................... 89

REFERENCES ...................................................................................................... 91

APPENDICES ....................................................................................................... 117
LIST OF TABLES

Table 1: Activities of characterized Flavonoids against PfPK6 and PfMRK: ............ 67

Table 2: Activities of uncharacterized Flavonoids against PfPK6. ............................ 67
LIST OF FIGURES

Figure 1: The *P. falciparum* life cycle ................................................................. 6

Figure 2: Sequence of events leading to initiation of DNA replication ............ 11

Figure 3: The pQE-30 Vector (Qiagen) Map ......................................................... 31

Figure 4: The pGEX Vector Map (GE Healthcare Inc.) ........................................ 32

Figure 5: The Ultra-Glo™ Recombinant Luciferase reaction .............................. 34

Figure 6: Phylogenetic relationship of the PfMCMs and PfRLF. ....................... 46

Figure 7: Alignment of aa sequences of the putative PfRLF and PfMCM6. ....... 46

Figure 8: SDS PAGE Electrophoresis of the PfMCM6 Elute .............................. 47

Figure 9: SDS PAGE Electrophoresis of the Elute containing PfPK6. .............. 48

Figure 10: PfMCM6 Phosphorylation by PfMRK using the radioactive Assay. .... 49

Figure 11: The PfCTD/PfMRK control experiment ............................................. 50

Figure 12: PfMRK Kinase Assay with PfMCM6 as substrate ............................ 51

Figure 13: PfMAT1 effect on PfMRK phosphorylation with PfMCM6 as substrate. 53

Figure 14: Effect of PfMAT1 on PfMRK phosphorylation with PfCTD as substrate. 54

Figure 15: PfPK6 Kinase Assay with PfMCM6 as substrate .............................. 55

Figure 16: Eukaryote Linear Motif search tool result ........................................ 58

Figure 17: HPLC report on the GEEEEDDDDDEEEPS epitope ............................ 60
Figure 18: Proteomic Analysis of the PfMCM6 Epitope................................. 61

Figure 19: Elisa titration Curve of Rabbit 22 (A) and Rabbit 52 (B) anti- Serum. .... 62

Figure 20: Western blot analysis of PfMCM-6. .................................................. 63

Figure 21: PfMRK-PfMCM6 Inhibition Assay..................................................... 64

Figure 22: Inhibition of PfPK6 phosphorylation activity by Abbysinone IV. ........... 68
LIST OF APPENDICES

APPENDIX I: PfMCM 2-7 PROTEIN SEQUENCES................................. 117

APPENDIX II: PfMCM6 phosphorylation and PfPK6 and PfMRK kinase inhibition data .................................................................................................................. 123

APPENDIX III: Epitope sequences generated via several algorithms .................. 128

APPENDIX IV: Chemical Structures of some of the tested Flavonoids ............ 139

APPENDIX V: Isolated Flavonoids tested against PfPK6-PfMCM6. ............... 140

APPENDIX VI: ELM data for PfMCM6 ..................................................................... 141

APPENDIX VII: The KEMRI IRB/ERC Research Approval Letter .................... 142
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CAK</td>
<td>CDK activating Kinase complex</td>
</tr>
<tr>
<td>Cdc7-Dbf4</td>
<td>Cdc-7 dumb bell former four</td>
</tr>
<tr>
<td>CDKs</td>
<td>Cyclin Dependent Protein Kinases</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>Cdt-1</td>
<td>Cdc-dependent transcript 1</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CTD</td>
<td>C-Terminal Domain</td>
</tr>
<tr>
<td>DDK</td>
<td>Dumb Bell Kinase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyriyribonucleic Acid</td>
</tr>
<tr>
<td>ELM</td>
<td>Eukaryote Linear Motif</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra cellular-signal Regulated Kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen- Activated Protein Kinase</td>
</tr>
<tr>
<td>MCM</td>
<td>Mini-chromosome Maintenance</td>
</tr>
<tr>
<td>Mg2+</td>
<td>Magnesium ions</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleoside Triphosphate</td>
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<tr>
<td>ORC</td>
<td>Origin Recognition Complex</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<td>PfMAT1</td>
<td><em>Plasmodium falciparum</em> Ménage à trois</td>
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<tr>
<td>PfMRK</td>
<td><em>Plasmodium falciparum</em> MO15-related Kinase</td>
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<tr>
<td>Pol-α</td>
<td>polymerase-α</td>
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<tr>
<td>post-RC</td>
<td>post-replicative complex</td>
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<td><em>Plasmodium falciparum</em> Protein Kinase-5</td>
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<td>PfPK6</td>
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<tr>
<td>pre-RC</td>
<td>pre-replication complex</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein-A</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SSBP</td>
<td>single stranded DNA binding protein</td>
</tr>
<tr>
<td>SSDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>TFIIH</td>
<td>Transcription Factor IIH</td>
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ABSTRACT

Malaria remains a big challenge the world-over especially in Sub-sahara Africa. Since there is no malaria vaccine currently, chemotherapy is the only curative intervention option available. At present, there are few known drug targets in the parasite. Worse still, most of these targets have mutated leading to widespread resistance to current drugs. There is therefore an urgent need for discovery and development of new antimalarial chemotherapeutic interventions and targets. An “Achilles’ heel” in the parasite biology that could be exploited for chemotherapeutic intervention is the parasite cell cycle mechanisms. Thus, recent malaria drug discovery efforts have focused on targeting parasite-derived cyclin-dependent kinase proteins as potential new drug targets. The aim of this study was therefore to establish the functional relationships between two of these kinases, PfMRK and PfPK6, with a putative P. falciparum replication licensing factor (PfRLF) with a view of establishing their potential as drug targets.

A non-radiactive kinase assay was used to assess phosphorylation capacities of PfMRK and PfPK6 on PfRLF. Bioinformatic tools were also used to characterize PfRLF. Kinase inhibition assays using locally sourced natural products as inhibitors to PfMRK and PfPK6 were carried out to unravel the drug
target potential of these kinases. Bioinformatic analyses revealed that the putative PfRLF is actually the *P. falciparum* Minichromosome Maintenance 6 (PfMCM6) protein involved in replication. The kinase assays established that both PfPK6 and PfMRK phosphorylate PfMCM6 *in vitro* and that PfMAT1 enhances PfMRK activity on PfMCM6. Enhancement of PfMRK activity by PfMAT1 confirms previous observations that PfMRK is the plasmodial CDK7 equivalent. The Kinase inhibition assays showed that the Abyssinone class of flavonoids actively inhibits the activity of PfMRK and PfPK6 on PfMCM6.

This study has confirmed the potential of PfMRK and PfPK6 as drug targets for malaria treatment. Flavonoids, especially prenylated abyssinones are lead compounds for antimalarials targeting these two kinases. Modification and further characterization of these lead compounds may lead to therapeutic agents with higher efficacy and specificity.
CHAPTER ONE

1.0 Introduction

1.1 Background information

*P. falciparum* is the causative agent of the most lethal form of human malaria that affects between 300 and 500 million people resulting in about 2 million deaths every year (Breman, 2001). The malaria scourge is chiefly an African problem with 90% of all the reported mortalities occurring in Sub-Sahara Africa (Trape et al., 2002). Furthermore children under the age of five years are the most affected. There has been steady and progressive drug resistance development in *P. falciparum*, thus complicating efforts to combat malaria (Pradines et al., 2006, Trape et al., 1998, Rukaria et al., 1992, al-Yaman et al., 1996, Trape, 2001, Winstanley, 2002, Wongsrichanalai et al., 2002, Zucker et al., 2003, Parola et al., 2007). This calls for the need to develop novel antimalarial chemotherapeutic interventions (Ridley, 2002). Identification of enzymes that may serve as potential antimalarial drug targets is central to the drug discovery efforts currently being undertaken (Woodard et al., 2007, Lu et al., 2005, Waters and Geyer, 2003, Gelb, 2007, Jana and Paliwal, 2007); Genomic databases present a wealth of information for the identification of these new targets (Gardner et al., 2002, Kissinger et al., 2002). This is achieved through discovery of unique parasite proteins and/or enzymes that are functionally different from those of the host. Already plenty of helpful information has been gleaned from these databases leading to identification of possible drug targets (Cooper and Carucci, 2004)). The databases were developed through international efforts
that finally paid off with the publication of the complete *P. falciparum* genome sequence (Gardner et al., 2002).

1.2 Problem Statement

*P. falciparum* malaria is the most lethal form of this human disease. It has a widespread distribution affecting up to 500 million people and result in approximately 2 million deaths every year (Breman, 2001). There is resistance to varying degrees to all currently available drugs that target *P. falciparum*. This is a precarious position because malaria is known to be deadly if not promptly treated. To counter this threat, it is imperative that new drug targets in the parasite are identified with a view of developing new and more potent therapeutics against this menacing parasite. Publication of the *P. falciparum* genome has lead to the identification of potential drug targets within the malaria causing parasite. *P. falciparum* CDKs have been identified as one class of proteins that can be exploited for chemotherapy. These considerations stem from studies on mammalian CDKs which are targets in anticancer chemotherapy discovery programmes. Following some degree of success, *P. falciparum* CDKs are now increasingly being researched as possible drug targets. These include PfMRK, *PfPK5* and *PfPK6*. Whereas Mammalian CDKs have largely been characterized, Plasmodial CDK mechanisms are not yet fully studied. However, using a bacterial Two-Hybrid system, a *P. falciparum*-derived putative replication licensing factor (PfRLF/PfMCM6) has been identified as a specific protein that interacts with PfMRK and PfPK6 (Jirage et al., 2010, Eyase, 2006). This protein displays a 240 amino acid domain predicted to belong to Minichromosome Maintenance protein family. The *P. falciparum* MCM proteins have been identified and
partially characterized (Patterson et al., 2006). Since CDKs and MCM proteins are involved in regulation of replication mechanisms, they ought to be studied as potential drug targets.

1.3 Justification

Malaria affects approximately half of the global population, living in 109 countries (WHO, 2008). The malaria problem is particularly dire in Sub-Saharan Africa where 90% of the burden rests. This situation is worsened by the fact that the malaria parasite, *P. falciparum* has developed resistance to most available chemotherapies. It is therefore imperative that continued research be carried out to identify new chemotherapeutic options and potential drug targets within the genome of the malaria parasite. Availability of the *P.falciparum* genome to the malaria research community has led to important discoveries concerning differences between the parasite and its human host. Consequently these differences may be exploited for chemotherapeutic targets. The *P.falciparum* cell cycle is one such target that may be exploited as enzymes in the parasite cell show marked differences with the mamalian counterpart. It is therefore important to study the various components of the *P.falciparum* cell cycle with a view to establishing whether they may be plausible chemotherapeutic targets.

1.4 Research Questions

i) Is the *P. falciparum* replication licensing factor (RLF/PfMCM6) a substrate for PfMRK and PfPK6?

ii) What is the effect of *PfMAT1* on PfMRK in relation to the *PfRLF*/PfMCM6?
iii) Are PfMRK and PfPK6 really potential drug targets for treatment of malaria?

1.5 Hypothesis

i) *PfRLF*/PfMCM6 is not a substrate of PfPK6 and PfMRK.

ii) *PfMAT* has no effect on *PfRLF*/PfMCM6 phosphorylation by PfMRK.

iii) PfPK6, PfMRK and *PfRLF*/PfMCM6 are not drug targets.

1.6 Objectives

1.6.1 General objective

To characterize the putative *P. falciparum* replication licensing factor (*PfRLF*/PfMCM6), as a substrate of two *P. falciparum* CDKs, PfPK6 and PfMRK.

1.6.2 Specific Objectives

i. To determine the identity of *PfRLF*/PfMCM6 followed by expression and purification of PfPK6 and *PfRLF*/PfMCM6 in a bacterial system.

ii. To determine whether PfPK6 and PfMRK phosphorylated PfRLF in vitro and presence of PfRLF in-vivo.

iii. To determine the effect of PfMAT1 on the phosphorylation of *PfRLF*/PfMCM6 and presence of phosphorylation motifs in PfMCM6.

iv. To establish a natural product antimalarial CDK inhibitor screening model against PfPK6 and PfMRK with *PfRLF*/PfMCM6 as substrate.
CHAPTER TWO

2.0 Literature review

2.1 Complexities of Plasmodium cell cycle

Plasmodium undergoes a life cycle (Figure 1) that is composed of a succession of distinct developmental stages which are not fully understood (Arnot and Gull, 1998). *P. falciparum* goes through a complex obligate developmental cycle in the anopheline vector in preparation for transmission to the human host (Baton and Ranford-Cartwright, 2005). The mosquito vector, injects cell cycle arrested sporozoites into the blood stream of the human host. The sporozoites then travel to the liver, where exo-erythrocytic schizogony takes place in the hepatocytes. The schizonts are thereafter released into the blood circulation, invading the red blood cells, where asexual multiplication occurs. The erythrocytic schizogony is what causes clinical pathogenesis of the disease. Upon erythrocytic invasion, some of the merozoites, arrest their cell cycle and differentiate into gametocytes. The molecular mechanisms of this action are not understood. Thus it follows that intracellular multiplication of Plasmodium does not comply with the canonical eukaryotic model.

During the cell cycle in eukaryotic systems it is presumed that M phase-CDK activity is inhibited while S phase-CDK activity oscillates to allow DNA-replication (Read et al., 1993) This may not hold true for *P. falciparum*, since during asexual multiplication, asynchronous nuclear division occurs during DNA replication (Arnot and Gull, 1998,
Doerig et al., 2000). Therefore a clear correspondence between cellular events occurring during Plasmodium schizogony and the G1, S, G2 and M phases of the ‘‘traditional’’ cell cycle has not been established in *P. falciparum*.

**Figure 1** *The P. falciparum life cycle* (Adapted from the CDC malaria fact sheet http://www.cdc.gov/malaria/about/biology/index.html). A; Exo-erythrocytic Cycle occurs in the human liver, B; Erythrocytic Cycle occurs in the red blood cell, C; Sprogonic cycle occurs in the mosquito midgut.
The invading merozoite is presumed to be in G1 and that DNA synthesis is initiated in the trophozoite approximately 18 hours post-invasion (Klemba and Goldberg, 2002). The molecular mechanisms involved in erythrocytic schizogony are highly complicated (Doerig et al., 2002). Thus whereas in most eukaryotes DNA replication is followed by cytokinesis, leading to two identical cells, each with a single nucleus, there is no cytokinesis in *P. falciparum*. This results in the single syncytium containing 8-32 nuclei. The mechanisms behind these stages are essential for the proper development of the parasite and may play a role in the induction of antimalarial drug resistance. However, clear understanding of the signals involved in triggering cell growth, proliferation and the molecular events controlling sexual differentiation, such as gametogenesis, and fertilization remains largely unachieved.

The mosquito vector ingests the gametocytes which then mature into gametes within its midgut. Signals that control this transformation are equally not well understood. This is followed by fertilization, the only diploid stage in the life cycle of *Plasmodium*. The zygote develops into an ookinete then crosses the peritrophic membrane and the midgut epithelium and immobilizes at the epithelium basal lamina. At the epithelial basal lamina the ookinete develops into an oocyst. This is followed by asexual multiplication resulting into thousands of sporozoites. These sporozoites are then cell-cycle arrested and accumulates in the mosquito’s salivary glands awaiting inoculation into a vertebrate host (www.malaria.org). Proteins synthesized in the salivary glands of the vector have been suggested to be important in the parasite’s life cycle (Choumet et al., 2007). Studies show that there is an over-representation of proteins involved in signaling as well as
proteins involved with immune response during sporogony (Choumet et al., 2007). Levels of certain salivary gland proteins are elevated in the presence of the parasite (Choumet et al., 2007). Approximately 43% of all the *P. falciparum* expressed genes are cell cycle regulated (Le Roch et al., 2000). This underscores the need for more studies to understand the effects on the cell cycle of the various genes expressed at all cell cycle stages in *Plasmodium*.

### 2.2 DNA Replication in Eukaryotes as model for the replication in *Plasmodium*

DNA replication in *Plasmodium* has not been thoroughly elucidated. Being a eukaryote, it would be expected that the sequence of events in this process are the same or at least similar to those that occur in other eukaryotic cells. However current studies show that there are fundamental differences between the canonical eukaryotic cell and that of *Plasmodium*, giving hope for chemotherapeutic exploitation. In this section the DNA replication in other eukaryotes is described to underscore these differences between *Plasmodium* and other eukaryotes.

The eukaryotic genome is made up of multiple chromosomes, whose replication is under strict control such that only one genome equivalent of DNA is duplicated per cell cycle. Cell cycle events are regulated by signal molecules that act upon the nuclei to synchronize DNA replication appropriately. These events ensure that DNA replication does not occur under unfavorable conditions. In the canonical eukaryotic system this replication originates at multiple sites within the genome, during the entire S phase. Thus during successive cell cycles, identical genetic material must be passed on from the
mother cell to the two daughter cells with exact precision. This process is accomplished by duplicating the cell’s genomic DNA during the S-phase of the cell cycle and passing on the two copies equally to the daughter cells during the M phase. Therefore DNA replication takes place during a restricted period of the cell cycle and through a process of replication licensing chromosomes are replicated only once per cell cycle. Over-replication or under-replication of genetic material would result in undesirable genetic alterations leading to cell death or malignant cell growth. Thus under normal circumstances no DNA segment is replicated twice in a single cell cycle (Nurse, 1994, Heichman and Roberts, 1994).

Discovery of cyclin dependent kinases (CDKs) as the major regulators of the events that drive the cell cycle, as well as other proteins involved in DNA replication such as the origin recognition complex (ORC), cell division cycle (Cdc) 6/18, Cdc-dependent transcript 1 (Cdt1) and the Minichromosome maintenance (MCM) complex has led to major insights into the control of DNA replication (Morgan, 1997). Thus the initiation of DNA replication occurs through the association of a set of pre-replicative complexes (including the MCM family) with chromatin during G1, followed by activation of S-phase CDKs, which triggers DNA replication and blocks the assembly of new pre-replicative complexes (Diffley, 1996). Specifically the transition from G1 into S phase is regulated by Cyclin dependent kinases (Pagano et al., 1992).

As shown in figure 2 the initiation of chromosomal DNA replication in eukaryotes can be divided into two major events. The first event takes place at the G1 phase, during which the chromosomes are properly groomed or primed in preparation for DNA replication,
when a pre-initiation complex is formed at the origin of replication by the ORC proteins (Diffley, 1996, Dutta and Bell, 1997). The second event occurs during the G1/S transition when CDKs promote the conversion of the pre-initiation complex into an active replication form through several proteins such as CDC6, MCM proteins, CDC45, Replication protein-A (RPA), and DNA polymerase- α (Pol α) (Diffley, 1996, Dutta and Bell, 1997). The CDKs appear also to exert a negative effect on DNA replication by catalyzing a series of phosphorylation events that render these replication factors unable to re-enter the pre-initiation state, thus preventing re-replication of the genome (Frouin et al., 2002). Therefore a physical link between CDK/Cyclin complexes and essential replication factors at the origin of replication has been proposed (Fotedar and Roberts, 1991, Jaumot et al., 1994, Jallepalli et al., 1997, Tye, 1999). Studies using different model systems suggest that this highly regulated process, which involves at least 20 protein factors, is largely conserved from yeast to humans (Tye, 1999).

Replication origins take two states during a cell cycle; the first one in G1 phase, before DNA replication begins, when a multi-protein complex called the pre-replication complex (pre-RC) assembles on the origins. The second one takes place from the initiation of S-phase to the end of M-phase, when a complex with fewer components called the post-replicative complex (post-RC) persists on the origin DNA. At the end of the M-phase, low CDK activity allows assembly of the pre-RC a state competent for replication. An increase in CDK activity at the G1/S transition triggers inactivation and at the same time converts the origin to the post-RC state. The high CDK activity after then prevents the reformation of pre-RCs until the end of mitosis. The CDK cycle thus
Separates the two states at replication origins and couples the initiation of S-phase to the completion of M-phase (Wuarin and Nurse, 1996, Diffley, 1996, Stillman, 1996). When chromatin becomes competent for replication, it is referred to as being licensed. The licensed state appears to be established when pre-replicative complexes (pre-RCs) are formed on replication origins. Initiator proteins essential for pre-RC formation include Cdc6/18, Cdt-1 and MCM proteins (Tye, 1999).

**Figure 2 Sequence of events leading to initiation of DNA replication.** (i) The pre-Replicative Complex is assembled during the G1 phase (ii) Cdc7-Dbf4 kinase interacts with the MCM complex (iii) Cdc45 physically interacts with the MCM complex (iv)
Origin Recognition Complex rebinds the vacated replication origin to form the post-Replicative Complex as the Replicative Complex transforms into elongation complex (adapted from Annual Reviews in Biochemistry: Tye, 1999).

2.3 DNA Helicases and their Role in DNA replication

Genetic information is locked in the duplex DNA of the genome. To access this information the duplex DNA has to be transiently unwound. For this purpose, a diverse class of enzymes, the DNA helicases, are available. They catalyze the unwinding of duplex DNA and thus play a critical role in many cellular processes including DNA replication, repair, recombination, and transcription (Tuteja, 2003, Matson, 1991, Lohman, 1992). They are also thought to be motor proteins translocating along DNA using nucleoside triphosphate hydrolysis as the source of energy (West, 1996). Multiple DNA helicases have been isolated from different sources and accordingly named as prokaryotic, eukaryotic, bacteriophage, and viral helicases (Tuteja, 2003, Matson, 1991, Lohman, 1992, Tuteja and Tuteja, 1996). More than one helicase is present in each system because of a variety of different needs for the duplex DNA unwind in different metabolism. All DNA helicases bind Nucleoside Triphosphate (NTP) and exhibit nucleic acid independent intrinsic NTPase activity necessary for duplex unwinding.

The first malaria parasite helicase protein has recently been purified. It contains ATP/Mg$^{2+}$-dependent DNA unwinding and ssDNA-dependent ATPase activities (Tuteja et al., 2002, Tuteja, 2003) and prefers to unwind DNA containing replicative-like structures (Tuteja, 2003).
2.4 Minichromosome Maintenance (MCM) Complex and its Role in DNA replication

MCM proteins were so named because they were isolated as products of genes essential for minichromosome maintenance in *S. cerevisae* (Maine et al., 1984, Moir et al., 1982, Takahashi et al., 1994). The MCM proteins were initially identified for their role in plasmid replication or cell cycle progression. Six of the MCM proteins (MCM2-7) form a complex that is required for the initiation and elongation of chromosomal DNA (Forsburg, 2004, Tye, 1999, Labib et al., 2000). The six MCM proteins are similar to each other, especially in a 240 amino acid core domain containing an ATP binding motif. As members of the AAA+ super family of proteins, all MCM containing proteins possess an ATPase motif within this core including walker A and B motifs which are highly conserved in DNA helicases (Koonin, 1993).

The MCMs are conserved from yeast to mammals and interact with each other to form a hexameric complex with a molecular weight of about 600 kDa (Fujita et al., 1997, Kubota et al., 1997). Based on physical interaction studies *in vivo*, the complex formed is composed of stoichiometric amounts of each protein ((Forsburg, 2004). Biochemical studies now show that the MCM complex works as a replicative helicase. In these studies a purified trimeric complex consisting of MCM-4, -6 and -7 probably in a hexameric structure composed of two trimers, in the presence of forked DNA structures and single stranded DNA binding protein (SSBP) was shown to posses ATPase and processive DNA helicase activity (Bochman and Schwacha, 2009, Ishimi, 1997, Lee and Hurwitz, 2001, You et al., 2002, Kaplan et al., 2003). A mutation of the conserved lysine
residue in the walker A motif in both MCM 6 and MCM 7 subunits of the MCM 2-7 complex results in the inhibition of the ATPase activity of the complex. This ATPase deficient MCM complex is able to support pre-RC assembly but is not able to support DNA replication. This shows that ATP binding and/or hydrolysis by MCMs is dispensable for the early initiation steps but crucial for DNA unwinding during DNA replication (Ying and Gautier, 2005). Nevertheless MCMs have roles in both pre-RC formation and post initiation of DNA replication.

The six proteins (MCM 2-7) interact with each other to form multiple complexes such as MCM 2,4,6,7, MCM 2, 3, 5 and MCM 4, 6, 7 (Prokhorova and Blow, 2000). However, the predominant form is the heterohexameric complex containing all six MCMs which is relatively stable and productively binds to chromatin (Prokhorova and Blow, 2000, Fujita et al., 1997). Electron microscopy indicates that this complex has a globular structure and in vivo studies suggest that all six MCMs are recruited to replication origins during the G1 phase (Labib et al., 2000, Tye, 1999). During the G1 phase human MCM proteins first assemble at or adjacent to bound origin recognition complex along with cell division cycle 6 (Cdc6) and Cdc dependent transcript 1 (Cdt-1) proteins and move to other sites during genome replication (Schaarschmidt et al., 2002). These proteins together form a pre-replication complex at the origin of DNA replication at the beginning of the S phase. The ORC associates with replication origins as the cells complete mitosis, Cdc6/18 and Cdt-1(for yeast) are loaded on to chromatin, and they in turn load the MCM complex on to chromatin (Coleman et al., 1996, Tanaka et al., 1997, Aparicio et al., 1997, Maiorano et al., 2000). Licensing is considered complete when the MCM complex is loaded onto
chromatin. The multi-complex thus assembled corresponds to the pre-replicative complex (pre-RC). This complex is activated at the G1/S transition, and DNA replication is initiated. After assembly, the complex is activated by cyclin dependent-kinases (CDKs) and the Cdc-7 dumb bell former four (Cdc7-Dbf4, DDK) complex in the S phase to promote the initiation of DNA replication. The two protein kinases (CDK and DDK) trigger a chain reaction that results in the phosphorylation of the MCM complex and finally in the initiation of DNA synthesis (Lei et al., 1997, Labib et al., 2000, Schaarshmidt et al., 2002). Recruitment of DDK to the replication origin occurs during the S phase; and this decision is controlled locally at individual origins. Phosphorylation of MCM 2 by DDK results in a conformational change in the MCM complex. Recruitment of Cdc45 depends on the phosphorylation of the MCM complex and the activity of CDK.

The MCM component of the complex provides the DNA unwinding function to start the DNA replication which finally requires the concerted action of many enzymes/factors such as replication protein A (RPA), Polymerase α, Polymerase δ, Replication Factor C, Proliferating Cell Nuclear Antigen (PCNA), Flap Endonuclease 1, Endonuclease DNA 2 and DNA Ligase 1 (Tuteja, 2003, Schaarshmidt et al., 2002). The unwinding of the Origin DNA (the locus in the genome where DNA replication is initiated) is detected only at a later step when the MCM complex is phosphorylated by DDK at the G1 to S phase transition (Lei et al., 1997). These studies suggest an anchoring mechanism other than direct contact with DNA for the MCM complex immediately after its recruitment to replication origins (Lei et al., 1997).
Chromatin Immunoprecipitation (CHIP) experiments in *S. cerevisiae* indicates that the MCM complex moves on chromatin as replication proceeds (Aparicio et al., 1997). The disassembly of the pre-RC leaves only ORC bound to chromatin, which corresponds to the post-RC state and inhibits additional rounds of replication until the cells have passed through the mitosis and the pre-RC is re-established (Perkins and Diffley, 1998). The loading of the MCM complex may induce a change in chromatin structure thereby reducing the binding of the ORC and CDC 6/18 to chromatin. This destabilization of ORC and CDC6/18 could be important for blocking re-licensing (Nishitani and Lygerou, 2004).

Two protein kinases, CDK and DDK, are required to activate the licensed origins for initiation. CDK associated with G1/S cyclins such as CDK-2-cyclin E in higher eukaryotes have a key role in the initiation of DNA replication. Activation by protein kinases is believed to result in changes to the Pre-RC that lead to the binding of CDC-45 to the MCM complex, followed by the unwinding of replication origins subsequently, DNA replicating proteins such as RPA, DNA polymerase α and ε are recruited to initiation sites (Tye, 1999).

### 2.5 Discovery and characterization of Plasmodial MCM (PfMCM) Complex

Using BLASTP algorithm to search the Plasmodb database, six *P. falciparum* MCM subunits have been identified (Patterson et al., 2006). All the identified Plasmodium MCM subunits are large polypeptides containing the MCM signature sequence (IVT) D (DE) (FL) (DNST) KM (Patterson et al., 2006). The PfMCM subunits contain the
canonical 240 amino acid conserved region in the middle. The presence of the six subunits suggests that heterohexameric MCM complexes form in the malaria parasite just like other eukaryotes. PfMCMs 2, 4, 6, and 7 have a zinc finger motif which is a characteristic of all eukaryotic MCM proteins (Patterson et al., 2006). It is possible that the zinc finger plays a role in the binding of the PfMCMs to chromatin as the zinc finger domains are known to be involved in protein-DNA interactions (Poplawski et al., 2001). *S. cerevisiae* Zinc finger MCM mutants are non-viable, pointing to the importance of these domains in the function of the MCMs (Yan et al., 1991). The PfMCMs also contain another conserved motif characteristic of eukaryotic MCMs which is the Walker A type ATP-binding motif.

The expression of PfMCMs 2, 6, and 7 shows that they are all present during the intraerythrocytic life cycle (Patterson et al., 2006). Both MCM 2 and MCM 7 peak in the schizont/segmented schizont stages, PfMCM 7 is down regulated in the ring and early trophozoite stages whereas PfMCM 2 peaks in late Trophozite stage as well (Patterson et al., 2006). This is in agreement with microarray data from plasmodb database ([www.Plasmodb.org](http://www.Plasmodb.org)) which shows that the level of transcripts of PfMCM2, 6, and 7 are lowest in the ring stage (12 hrs) and peaks in schizonts (36 hrs). This expression profile is coincident with DNA replication in Plasmodium. It has been suggested that PfMCMs 2, 6 and 7 are found in stoichiometric amounts during the ring and schizont stages (Patterson et al., 2006). PfMCM formation in the schizont and ring stages suggest that in these two stages the parasite cell is in G1 phase and that there is formation of the Pre-RC. During the Trophozoite stage interaction between the subunits is not observed. This
suggests that the parasite could be in the S phase hence the disruption of the pre-RC complex. It has been observed that only PfMCM 6 subunit makes direct contact with chromatin but the other units are associated through protein-protein interaction. Evidently PfMCM proteins form a multiprotein complex whose expression and association with the chromatin coincides with the preparation for DNA replication. It is not known what proteins the parasite uses to load the MCMs onto chromatin as no homologues of Cdc6 and Cdt-11 have been identified in the _P. falciparum_.

The _Plasmodium_ genome shows that the pre-RC complex is minimal as compared to that of higher eukaryotes: only three ORC subunits are recognizable, PfORC1, PfORC2, and PfORC5 (Mehra et al., 2005, Gupta et al., 2008). This is in contrast to the six subunits usually found in other eukaryotes. ORC1 shows high similarity with Cdc6. However it is not clear if PfORC1 may function as a component of the ORC or Cdc6, or might act in both capacities. The identity of PfORC5 to ORC5 from other organisms is very low. Thus PfORC2 is the only one left as the likely protein that binds to Plasmodium replication origins (Patterson et al., 2006). Another interesting difference is that no DBF4-CDC7 (DDK) has been identified in the _Plasmodium_ genome. Consequently it has been suggested that CDKs or other kinases may play a central role in phosphorylating of PfMCMs in place of DDK (Patterson et al., 2006).

### 2.6Cyclin-Dependent Kinases and their role in cell cycle regulation

Protein Kinases and phosphatases play a key role in regulating the eukaryotic cell division (Norbury and Nurse, 1992, Hunter and Pines, 1994, Hunter, 1995). One family
of protein kinases, the Cyclin Dependent Kinases (CDKs) are central in the control of the eukaryotic cell cycle. They are designated individually by number as CDK1 to CDK8 (Morgan, 1995). The first identified CDK, CDC2 (CDK1), is the best understood. In yeast, two control points of the cell cycle, the transition through START, the point at which progression to DNA synthesis begins, and the initiation of mitosis, are regulated by CDK1, the product of the CDC2 gene in *Schizosaccharomyces pombe* and the CDC28 gene in *Saccharomyces cerevisiae* (Reed et al., 1985, Simanis and Nurse, 1986). The CDKs require their cognate partners, the cyclins to be fully activated. Cyclins are proteins that were first identified in marine invertebrates. They were observed to accumulate and degrade in a cyclical manner, thus oscillating with the cell cycle progression (Rosenthal et al., 1980). Association of CDKs and cyclins leads to specific CDKs phosphorylating their cognate substrates.

Activated CDKs are regulators that control the activities of several other proteins to precisely initiate particular cell cycle events. CDKs and cyclins exist in a number of combinations with each combination being responsible for a given cell cycle phase. CDK activation is very important in ensuring that certain critical steps are completed before others begin, and a series of checkpoint controls operates to safeguard the fidelity of the system. CDK-cyclin binding is specific such that not all cyclins bind to all CDKs. Binding of the cyclin causes profound changes in the structure of the kinase allowing access of the ATP and protein substrates to the catalytic cleft. Cyclin binding of CDKs, most of the time may results in partial phosphorylation of the specific substrate or no phosphorylation at all. In order for the CDK to be fully activated, phosphorylation of a
threonine residue in the T loop by another component called a CDK activating Kinase (CAK) is required in order to further modify the structure of the catalytic subunit. CAK itself is a CDK complex composed of a catalytic sub unit, CDK7/MO15 and a regulatory sub unit, cyclin H (Fisher et al., 1995, Fesquet et al., 1993, Morgan, 1997). It is suggested that when the cyclin binds, the T loop moves away from the active site giving room for ATP to bind (Jeffrey et al., 1995). Phosphorylation of the threonine residue within the T-loop gives the CDK its most active conformation. The conformation brought about by cyclin binding orients the γ-phosphate of ATP, facilitating the phosphotransfer reaction. After the phosphotransfer the CDK then phosphorylates proteins involved in cell cycle regulation. To make sure that cellular events occur in a controlled manner, the cyclin subunit is degraded through ubiquitin targeted degradation, thus inactivating the CDK. This is complemented by the binding of inhibitory proteins as well (Willems et al., 1996, Sherr and Roberts, 1995, Hunter and Pines, 1994). This sequential activation and de-activation ensures that the cell cycle progresses in a timely manner so that critical events are completed before the cell commits to another round of division. Some CDKs function in cellular processes which are not directly linked with cell cycle control (Hellmich et al., 1992).

Previous studies have implicated CDKs as regulators of the process of DNA replication (Nasheuer et al., 1991, Dutta and Stillman, 1992, McVey et al., 1993, Zeng et al., 1994, Prosperi et al., 1994). It has been shown that apart from modulating the activity of DNA replication enzymes, CDKs regulate the association of replication complexes to chromatin during the cell cycle. For instance it has been suggested that a stable
association of cyclin A to replication complexes during S phase acts to recruit CDK2, which then would regulate the dynamic association of the replication proteins with the chromatin (Frouin et al., 2002). It has been predicted that CDKs function both to activate initiation complexes and to inhibit further initiation complex formation (Kelly and Brown, 2000). Consequently in synchronized HeLa cells it has been observed that CDK1/cyclin B1 is associated with replication complexes in G2/M at the same time CDK2 activity ceases. This might indicate an inter-phase regulatory mechanism, which stops replication complexes from re-binding to chromatin at the G2/M phase (Frouin et al., 2002). CDKs have also been associated with the replication licensing factor Cdt-1, considered an essential component of the pre-initiation complex required for loading the MCM proteins onto chromatin. (Bell, 2002, Blow and Hodgson, 2002, Diffley and Labib, 2002, Diffley, 2001, Nishitani et al., 2000, Wohlschlegel et al., 2000, Rialland et al., 2002, Liu and Bagchi, 2004).

2.7 Plasmodial-Cyclin Dependent Kinases (CDKs)

A number of plasmodia-derived CDKs have been identified and characterized. These include *P. falciparum* Protein Kinase-5 (*PfPK-5*) (Graeser et al., 1996), *P. falciparum* Protein Kinase-6 (Bracchi-Ricard et al., 2000) and *P. falciparum* MO15 Related Kinase (Li et al., 1996). The three CDKs have been expressed and purified as active recombinant protein kinases and are being used in drug screening projects (Waters and Geyer, 2003, Geyer et al., 2005).
PfPK5 which was the first *P. falciparum* CDK to be identified is 59% identical to the human CDK-1 (Ross-Macdonald et al., 1994). Studies have shown that PfPK5 is regulated by phosphorylation of a conserved threonine residue located within the T-loop of the kinase (Graeser et al., 1996). The T-loop regulatory phosphorylation is conserved in many CDKs and is required for the correct catalytic conformation of the enzyme (Morgan, 1995, Morgan, 1996). Furthermore PfPK5 contains the PSTTIRE motif closely related to the PSTAIRE motif that is known for cyclin binding in other CDKs. Previous studies suggest that PfPK5 regulates the S-phase of the parasitic cell cycle (Graeser et al., 1996). Studies have shown that PfPK5 can be activated by human cyclin H which is the activating partner of CDK7, it can also be activated by P25 (Le Roch et al., 2000). In higher eukaryotes the two activate different catalytic units. Thus PfPK5 may be activated by cyclins but with much less specificity as compared with the higher eukaryotes. It therefore follows that PfPK5 is regulated differently as compared to its homologues in higher eukaryotes.

PfPK6 has sequence similarity to both CDKs and Mitogen- Activated Protein Kinases (MAPKs). The identity of PfPK6 with that of human CDK 2 and human p38 MAPK is 38% and 33% respectively (Bracchi-Ricard et al., 2000). PfPK6 has DIKPEN and GTLWYRAPE motifs in its subdomains VI and VIII. These are in agreement with the consensus sequence (DLKPEN and GT/SXXY/FXAPE) for serine/threonine kinases (Hanks and Hunter, 1995). A number of critical domains known to be important for the binding of CDK-activating proteins are also conserved in PfPK6 for example KALDKK, RAPE, DPNYR (Bracchi-Ricard et al., 2000). PfPK6 contains a motif very similar to the
CDC2/CDC28 family consensus sequence EV/IVTLWY in subdomain VIII (TVVTLWY in PfPK6). It lacks the traditional PSTAIRE sequence which is responsible for binding regulatory cyclin subunits (Hanks and Hunter, 1995, Bracchi-Ricard et al., 2000, Meyerson et al., 1992). This sequence is replaced by another; SKCILRE in PfPK6. PfPK6 has significant sequence identity with MAPks. Nevertheless PfPK6 is smaller in size when compared to the other MAPks. The TXY motif between kinase subdomains VII and VIII that is involved in the activation by Mitogen Activated Protein Kinases/Extra cellular-signal Regulated Kinases (MAPks/ERK) through the phosphorylation of both threonine and tyrosine is changed to TPT (Bracchi-Ricard et al., 2000). A phylogenetic tree constructed from different CDks and MAPks indicated that PfPK6 branches off between the CDks and MAPks (Bracchi-Ricard et al., 2000). However PfPK6 seems to be more related to CDks than MAPks (Bracchi-Ricard et al., 2000).

*P. falciparum* MO15 Related Kinase (PfMRK) gene is located on chromosome 10 of the genome and has no introns (Li et al., 1996). Homologous genes for MO15 or CDK7 have been isolated from a number of species, such as Xenopus (Shuttleworth et al., 1990), human (Wu et al., 1994, Darbon et al., 1994, Levedakou et al., 1994, Tassan et al., 1994), Mouse (Stepanova et al., 1994), and fission and budding yeast (Simon et al., 1986, Damagnez et al., 1995, Buck et al., 1995). In these species the product of this gene (CDK7) forms part of the CDK activating Kinase (CAK) and therefore it is largely expected that PfMRK would serve the same function in *P. falciparum* as CDK7 in other species.
CDK activity is tightly controlled by a number of highly conserved biochemical mechanisms. The cyclin subunit is a primary CDK activity regulator. The cyclin binds to one side of the catalytic cleft where it interacts with the N-terminal and the C-terminal lobes of the CDK forming a large continuous protein-protein interface (De Bondt et al., 1993, Jeffrey et al., 1995). The PSTAIRE motif is central to this interface (Li et al., 1996). In PfMRK there is a small insert consisting of five amino acids just before the PSTAIRE motif. In the human CDK2, the residues of Glu42, Gly43, Val44, Ile49, Ile52, Lys56 and Glu57 are involved in cyclin A binding (Jeffrey et al., 1995). However PfMRK, similar to mammalian MO15 contains only Gly43, Ile52, Lys56 and Glu57. Since PfMRK is the suggested equivalent of CAK in the P. falciparum genome these differences may be important in the event that PfMRK is used as a drug target. In addition to cyclin binding, complete CDK activation depends on phosphorylation of Thr160 or its equivalent in the T-loop. The kinase responsible for the phosphorylation is the CDK Activating Kinase, CAK (Solomon, 1994). Thus it is expected that PfMRK would phosphorylate P. falciparum CDKs at Thr182 (an equivalent of Thr 160) as well. Studies have previously shown that MO15/CDK7 can activate CDK1, CDK2, CDK3, CDK4 and CDK6 \textit{in-vivo} by phosphorylating this threonine residue (Fesquet et al., 1993, Solomon, 1994, Poon et al., 1993, Fisher et al., 1995, Labbe et al., 1994, Matsuoka et al., 1994, Aprelikova et al., 1995, Desai et al., 1995). In the human CDK2, Thr160 lies in the T-loop that blocks the cleft between the N- and C- termini (De Bondt et al., 1993, Jeffrey et al., 1995). Studies show that in PfMRK there is a 13 amino acid hydrophilic insert following the fifth residue in the equivalent T-loop position (Li et al., 1996). The
influence of this insert on the phosphorylation of thr182, an equivalent of thr160 in CDK2, by the Plasmodial CAK is not understood (Li et al., 1996).

Cyclin-dependent kinase inhibitors are key regulators of CDKs; they bind and inactivate CDK/Cyclin complexes. The GDSEID motif at the beginning of the α5 helix and the (S/T) FPXW motif in the L14 segment of CDKs are implicated in the CKIs binding (Li et al., 1996). PfMRK has the GDSEID motif but lacks the (S/T) FPXW motif; this suggests that the parasite PfMRK inhibitor if present would likely be different from that of the human CKI.

MO15 and cyclin H are components of the transcription factor TFIIH and phosphorylate the C-terminal repeat Domain (CTD) of the largest subunit of RNA polymerase II in yeast (Cismowski et al., 1995, Buck et al., 1995, Damagnez et al., 1995) and mammals (Feaver et al., 1994, Serizawa et al., 1995). Thus CAK may be involved in other functions such as the control of transcription initiation and nucleotide excision repair (Roy et al., 1994, Shiekhattar et al., 1995).

2.8 Eukaryotic MAT-1 Proteins and their role in DNA replication

MAT1 (Ménage à trios 1) in association with other two sub-units namely CDK7 and cyclin H have been identified as forming the cyclin-dependent kinase activating kinase (CAK) (Tassan et al., 1995, Yee et al., 1995, Devault et al., 1995). The structure of MAT1 includes an N-terminal Ring-finger motif (Tassan et al., 1995, Devault et al., 1995, Fisher et al., 1995), a central coiled coil region (Fisher et al., 1995, Lupas et al., 1991), and a C-terminal cyclin like box (Yee et al., 1996).
MAT1 functions as an assembly factor to promote the stability and activation of the CDK-7-Cyclin H complex (Tassan et al., 1995, Devault et al., 1995, Fisher et al., 1995). Each of its structural domains may function to mediate distinct protein-protein interactions. The ring-finger motif of MAT1 is not involved in the complex formation (Tassan et al., 1995, Yee et al., 1995) indicating another potential function in interaction with other cellular proteins.

CDKs and Cyclin H are able to interact in the absence of MAT1. However this interaction is dependent upon the phosphorylation of a conserved site Thr 170 in the T-loop of human CDK 7 (Fisher et al., 1995). In the presence of MAT1 stabilization and activation of the CDK-7 cyclin-H complex is independent of the phosphorylation state of Thr 170 (Devault et al., 1995, Fisher et al., 1995). This shows that MAT1 may function as an activating assembly factor which is able to bypass the requirement for T-loop phosphorylation in the enzymatic activation of CAK. In addition to its function as an assembly factor, MAT1 also serves as a targeting subunit of CAK to determine its substrate specificity (Yankulov and Bentley, 1997).

Two major classes of putative CAK substrates have been identified. The first one is the CDK subfamily and and the second one being the components of the transcriptional machinery. These components include the Carboxy-terminal domain of the large subunit of RNA polymerase II, Transcription Factor IIE, Transcription Factor IIF (Yankulov and Bentley, 1997), the TATA binding protein (Rossignol et al., 1997) and the Octamer transcription factor-1 (Inamoto et al., 1997). Most of these proteins are phosphorylated in a MAT1 dependent manner (Inamoto et al., 1997, Ko et al., 1997). MAT1 functions both
as a positive regulating subunit and a targeting subunit that ultimately determines the substrate specificity of the multifunctional CAK complex. Studies have most recently indicated that MAT1 interacts with MCM7 a member of a family of DNA licensing factors known to be involved in the regulation of DNA replication (Wang et al., 2000). This may point to other potential additional functions of the CDK-7-cyclin H -MAT1 kinase complex. It has been demonstrated that several proteins including MCM7, co-purify with pol II along with general transcription factors such as Transcription Factor IIH (TFIIH) indicating MCM7 associates either directly or indirectly with TFIIH. TFIIH is a multi-subunit complex made up of at least nine polypeptides and all the three subunits of CAK, including CDK7, Cyclin H and MAT1 (Serizawa et al., 1995, Shiekhattar et al., 1995). It has been shown that MAT1 functions as an assembly factor (Tassan et al., 1995, Yee et al., 1995, Devault et al., 1995) and as a targeting factor (Yankulov and Bentley, 1997, Rossignol et al., 1997, Ko et al., 1997).

The CAK has been proposed to be involved in cell cycle control; transcriptional regulation and DNA repair (Kaldis, 1999). As the primary targeting factor of CAK, MAT1 modulates CAK activities and functions via its physical and functional interactions with other target proteins such as transcriptional factor Octamert1 (Inamoto et al., 1997) in which case, MAT1 interacts with the POU domain of Oct1, which effectively targets CAK to Oct1 as well as the catalytic subunit of the S-phase CDKs. MCM 7 is a member of the MCM protein family (MCM2-7), which performs a critical role in DNA synthesis to ensure DNA is replicated precisely once per cell cycle (Chong et al., 1996, Chong and Blow, 1996). The initiation of DNA replication occurs through
the association of a set of pre-replicative complexes (including the MCM family) with chromatin during G1, followed by activation of S-phase CDKs which trigger DNA replication and block the assembly of new pre-replicative complexes (Diffley, 1996). It has been suggested that the loading of MCM proteins onto chromatin may be regulated by CDC6 Protein (CDC6P) and by CDKs. CAK targets various CDKs as its putative substrates (Kaldis, 1999) and is therefore suggested to regulate MCM-7 function indirectly through the activation of another associated CDK (Wang et al., 2000). It has been suggested that MAT1 and CAK are directly involved in the regulatory biology of DNA replication through their physical interaction with MCM-7.

2.9 The role of Plasmodial MAT-1 Protein in DNA replication

Binding of MAT1 to the CDK7-cyclin H complex influences substrate specificity and phosphorylation of the CDK7/cyclinH/MAT1 complex stimulates CTD kinase activity 20-fold (Larochelle et al., 2001). PfMRK shows the greatest sequence identity to human CDK7 which is regulated in a slightly different manner than most CDKs. CDK7 activation is dependent on cyclin H association and phosphorylation of the T-loop (Shuttleworth, 1995). Studies show that PfMRK is most active in a trimeric complex consisting of PfMRK/CYC1/PfMAT 1(Chen et al., 2006). This common mode of activation may highlight the similarity between human CDK7 and PfMRK. The mechanisms of CDK stimulation by MAT1 have not been structurally determined. It has been suggested that PfMAT1 binding to PfMRK triggers conformational changes that increase kinase activity. PfMAT1 may also stabilize the PfMRK/CYC1 interaction.
resulting in an active kinase complex (Larochelle et al., 2001, Fisher et al., 1995, Devault et al., 1995, Tassan et al., 1995).
CHAPTER THREE

3.0 Materials and Methods

3.1 Identification of the putative replication licensing factor

To identify the putative replication licensing factor, full-length genes of the six PfMCM proteins were phylogenetically compared. Thus a phylogenetic tree was constructed from amino acid codes of the PfMCMs (appendix I) using the Bayesian method of tree inference by Mr Bayes software. Additionally translation of the *P. falciparum* putative licensing factor DNA ORF code into amino acid code was carried out using Genedoc software followed by a pair-wise sequence alignment using the default parameters of the Muscle3.5 software.

3.2 Expression and purification of PfPK6

PfPK6 had been previously cloned (Bracchi-Ricard et al., 2000). Overnight cultures of the PfPK6 cloned in PQE-30 (Figure3) were diluted 1:50 in LB broth supplemented with 100μg/ml ampicilin and grown at 37°C until an OD$_{600}$ of 0.6 was reached. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1mM to induce expression and the cell cultures left to grow overnight at 20°C. The cells were harvested by centrifugation and resuspended in sonication buffer (50mM NaH$_2$PO$_4$ pH 8.0, 300mM NaCl, 5% glycerol, 1% CHAPS, 10mM 2-β-Mecarptoethanol, 10mM Imidazol and 1 mM 1x Halt$^{\text{TM}}$ protease phosphatase inhibitor cocktail) (Thermo Scientific Asheville, NC, USA). To lyse the cells 1mg/ml of lysozyme was added followed by three rounds of
sonication. Cell debris were removed by centrifugation and PfPK6 was purified from the supernatant using the GSTrap™ column (GE Healthcare Life Sciences, Uppsala, Sweden).

Figure 3: The pQE-30 Vector (Qiagen) Map; the map contains the following features, PT5: T5 promoter; lac O: lac operator; RBS: Ribosome-binding site; ATG: Start codon; 6xHis: 6xHis tag sequence; FXa Recognition Site: factor Xa protease recognition site; MCs: Multiple Cloning Site; Stop codons in all the 3 reading frames; ColE1: Col E1 origin of replication; Ampicillin resistance gene.

3.3 Cloning of PfMCM6

PfMCM6 was amplified from *P. falciparum* strain 3D7 cDNA by polymerase chain reaction using the following primers. Forward primer: 5’-GGG AAT TCT CAG CTA TAT TTA ATG AAA GTG AAT TAT C-3’ Reverse primer: 5’-GGG AAT TCT TAA AAA TTG TCA ATT TCT TCT TG-3’ (Applied Biosystems, USA). This was followed by digesting with EcoRI and cloning into PGEX-5X-3 (Figure 4; GE Healthcare Life Sciences, Uppsala, Sweden) (Jirage et al., 2010). The cloning step incorporated a GST tag at the 5’ end of the PfMCM6 gene.
Figure 4: The pGEX Vector Map (GE Healthcare Inc.). The Map contains a protease cleavage site; The Glutathione S-Transferase (GST) sequence; the multiple cloning site; the ampicilin resistance gene; the pBR322 origin of replication and The Lac 1° Operator.

3.4 Expression and purification of PfMCM6

Clone pQE30-MCM6 was transformed into E. coli host strain XL10 GOLD (stratagene). Overnight cultures of pQE30-MCM6 were diluted 1:50 in LB broth supplemented with 100μg/ml ampicilin and grown at 30°C until an OD₆₀₀ of 0.7 was reached. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1mM to induce expression and the cell cultures left to grow overnight at 20°C. The cells were harvested by centrifugation and resuspended in sonication buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 5% glycerol, 1% CHAPS, 10mM 2-β-Mecarptoethanol, 10mM imidazole 1x Halt™ protease and phosphatase inhibitor cocktail). To lyse the cells 1mg/ml of lysozyme was added followed by three rounds of sonication. Cell debri were removed by centrifugation and PfMCM6 was purified from the supernatant using glutathione 4B sepharose column (Qiagen). The lysate was passed over the column and bound material washed with sonication buffer. PfMCM6 was eluted with sonication buffer containing 15mM
Glutathione. Eluted PfMCM6 was dialysed into kinase dialysis buffer (25mM Tris-HCL pH8.5; 20mM NaCl, 1mM EDTA, 0.25mM 1, 4-dithiothreitol, and 5% glycerol) to remove salts and other impurities.

3.5 Acquisition of PfMAT-1, PfCTD and PfMRK

PfMRK, PfCTD and PfMAT-1 had previously been cloned and expressed at the Walter Reed Army Institute of Research. These were therefore acquired from the Laboratory of Dr. Norman Waters and used for downstream experiments.

3.6 Kinase Phosphorylation assays

3.6.1 Principles of the Kinase- Kinase-Glo® Luminnescent Kinase assay

The assay used in this study was the Kinase-Glo®Luminnescent Kinase assay (Promega). This assay is a high-throughput screening (HTS) method of measuring kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction. The assay was performed in single wells of a 96-well plate by adding a volume of kinase Glo® reagent equal to volume of solution in the well of a completed kinase reaction and measuring luminescence. The luminescent signal is correlated with the amount of ATP present and inversely correlated with the amount of kinase activity. The kinase Glo® assay does not require any radioactively labeled components as the assay is dependent on the properties of a thermostable luciferase (Figure 5).
Figure 5: The Ultra-Glo™ Recombinant Luciferase reaction. Mono-oxygenation of luciferin is catalyzed by Ultra-Glo™ luciferase in the presence of Mg2+, ATP and molecular oxygen and produces one photon of light per turnover (Kinase glo manual Promega Inc.).

3.6.2 Kinase-Glo® reagent preparation

The Kinase-Glo® buffer was thawed and equilibrated to room temperature prior to use. The kinase-glo substrate was also equilibrated to room temperature. The buffer and substrate were thereafter mixed to form the Kinase-Glo® reagent. The reagent thus reconstituted was dispensed into single use aliquots and stored at -20°C.

3.6.3 Determining optimal ATP concentration

Two-fold serial dilutions of ATP were made across the plate using as much kinase (PfMRK) as was practical and excess kinase substrate (PfMCM6). As a control the same ATP titration was made without kinase or kinase substrate present in the well. The kinase was then allowed up to two hours to consume as much ATP as possible. After the two hours an equal volume of Kinase-Glo® reagent was added. The plate was then mixed and incubated at room temperature for 10 minutes to stabilize the luminescent...
signal. The signal was recorded on a genios® plate reader (Tecan Group Ltd. Switzerland).

3.6.4 Determining optimal substrate concentration

Two-fold serial dilutions of kinase substrate (PfMCM6) were made across the plate using as much kinase (PfMRK) as possible with the optimal amount of ATP (determined above). As a control, the same titration was done without the kinase. An equal volume of Kinase-Glo® reagent was added to each well. The luminescence was recorded on a genios® plate reader.

3.6.5 Determining optimal Kinase concentration

Two-fold serial dilutions of kinase were made across the plate using the optimal amount of ATP and kinase substrate (as determined above). The kinase reaction was left to incubate at room temperature for two hours. An equal volume (50µl) of Kinase-Glo® reagent was added to the kinase reaction. The reaction was then mixed and left to incubate at room temperature for 10 minutes to stabilize the luminescence signal. The luminescence was recorded on a genios® plate reader.

3.7 Radioactive kinase assays

Before the main kinase assay experiments were done using the new Kinase-Glo® non-radioactive method, a test run of the purified PfMCM6 as a substrate of PfMRK was done with the existing radio-active method. The purpose of this was to ensure that using PfMCM6 as a substrate was feasible. Briefly, purified PfMRK was assayed in a 15µl
reaction containing kinase buffer (50mM Tris-Hcl, 10mM MgCl2 and 1mM DTT) 3µl of PfMCM6, 3µl of Pfcyc, 3 µl of PfMRK, 5µci (³²P) ATP 3000Ci mmol⁻¹ (Amersham). As a control, ATP alone was incubated in reaction buffer. Reaction mixtures were incubated at 30°C for 15 minutes.

3.8 Kinase assays using Kinase-Glo®

Using Kinase-Glo® reagent kinase activity assays were carried out to determine whether PfMRK and PfPK6 phosphorylate PfMCM6. Additionally PfCTD, a known substrate of PfMRK was tested as a control. Briefly purified PfMRK was assayed in a 50µl reaction containing 38 µl kinase buffer (50mM Tris-Hcl, 10mM MgCl2 and 1mM DTT) 3µl of PfMCM6/PfCTD, 3µl of Pfcyc, 3 µl of PfMRK, 3 µl; (12.5µM ATP) (Amersham). As a control, first ATP alone was incubated in reaction buffer. Secondly, PfMCM6 and ATP were incubated in reaction buffer. Finally, PfMRK and ATP were incubated in reaction buffer. Reaction mixtures were incubated at 30°C for 15 minutes. For kinase activity assay using PfPK6, the experiment was done similar to that of PfMRK above only that Pfcyc was not used as a cyclin is not required for PfPK6 activity. In order to test the effect of PfMAT 1on PfMRK, the PfMAT1 protein was added to the full PfMRK-PfMCM6 kinase activity assay and level of activity measured on a genios® plate reader.

3.9 Kinase inhibition assays

For kinase inhibition tests, purified PfMRK was assayed in a 37.5µl reaction containing 25.5 µl kinase buffer (50mM Tris-Hcl, 10mM MgCl2 and 1mM DTT) 3µl of PfMCM6/PfCTD, 3µl of Pfcyc, 3 µl of PfMRK, 3 µl; (12.5µM ATP) (Amersham) in solid
black, flat bottom 96 well plates. Kinase inhibitors were screened at reducing concentrations by adding 12.5 µl of compound (listed in table 1 and 2) from the drug plate in which serial dilutions had been made with concentrations ranging from 50000ng/mL to 48.8ng/mL. The compounds had been dissolved in DMSO and diluted in kinase reaction buffer. The plate contents were mixed on a shaker and incubated at room temperature for two hours. Subsequently 50 µl of the Kinase-Glo® reagent was added and the plate mixed and left to incubate for 10 minutes at room temperature. The luminescence was recorded on a genios® plate reader. Kinase inhibition assays with against PfPK6 were carried out in a similar fashion to PfMRK above, using PfMCM6 as substrate. Pfcyc was omitted as it is not required for PfPK6 activity. The reaction buffer in this case was therefore adjusted to 28.5 µl to bring total reaction volume to 50 µl. The 50% inhibition (IC$_{50}$) concentration was determined.

3.10 Preparation of antibodies against PfMCM6 from synthetic epitopes

A number of algorithms were used to determine the most appropriate synthetic epitope that would give the desired antibody against PfMCM-6 protein. The algorithms used included Parker, Chouf_turn, Kyte82, Zimmerman, Wolfenden 79 and Levitt. The peptide epitope thus obtained was subjected to HPLC analysis to determine its purity. The peptide was also run through proteomic analysis (Electrospray Ionisation) to determine the correct molecular mass of the epitope. The epitope thus obtained was injected into two rabbits designated as rabbit number 22 and rabbit number 54. The immunization and antibody raising protocol (Immuno Precise Antibodies Ltd Canada) involved 78 days where day 0 was pre-immune bleeding followed by first boost on day
This was then followed by a second boost on day 47 before a test bleed was done on day 57 which was followed by the third and final boost on day 66. The Terminal bleed was done on 78 and the antiserum recovered. The antiserum was then freeze dried and kept at -20 for future use.

3.11 *P. falciparum* cultures for parasite antigen preparation.

Media for the growth of cultures for antigen preparation were prepared as explained in section 3.17 below. Clone 3D7 *P. falciparum* cultures were subsequently grown at 6% hematocrit and maintained until majority were early trophozoites (rings) at between 5% and 10% parasitemia. The parasite culture was then centrifuged to pellet. The volume of the packed cells was estimated and for every 100µl of the pellet 2mLs of 5% sorbitol was added. This was then incubated at room temperature for 10 minutes with shaking 3 times. This was followed by centrifugation at 3000rpm and the supernatant aspirated. The pellet was washed two times in RPMI 1640 and diluted to 5% hematocrit using malaria culture media. Finally the parasites were counted and subcultured. This procedure was then repeated after one cycle of subculture.

3.12 *P. falciparum* protein preparation from culture lysate

Trophozoite/shizont infected red blood cells were lysed by incubating with 0.1% saponin for 10 minutes at 37°C to make the red blood cell membrane permeable. The lysate was then centrifuged at 5,000 rpm and the supernatant containing the red blood cell membrane fragments discarded. The pellet containing intact parasites was washed twice with 0.1% saponin solution and once with PBS. The parasites were later lysed in 10
volumes of cold distilled water and then centrifuged at 15,000 rpm on a cooled table top centrifuge (Eppendorf) for 30 minutes. The lysate was resuspended in PBS and subjected to five freeze-thaw cycles in dry ice/ethanol bath. The protein acquired was stored at -70°C.

3.13 Immunoprecipitation of PfMCM6 from *P. falciparum* culture lysate

Protein-A magnetic beads suspension (Pureproteome™) was gently mixed so that all the beads were uniformly resuspended. 50µl of the resuspended beads were aliquoted into a 1.5 mL microcentrifuge tube. The tube was then placed in the Magna Grlp magnetic Rack (Pureproteome™), the beads were allowed to adhere to the side of the tube, and the storage buffer was removed using a pipette. The beads were then washed by adding 500µL of PBS containing 0.1% Tween 20 surfactant and vortexing vigorously for 10 seconds. The magnetic beads were thereafter returned to the magnetic rack and allowed to adhere to the side of the tube. The buffer was removed with a pipette and washed beads were resuspended in 100µL of PBS containing 0.1% Tween 20 Surfactant, anti-PfMCM6 antibodies were added to the resuspended beads. The mixture was incubated at room temperature with continuous mixing. The tube was then placed in the magnetic rack, the beads allowed to adhere to the side of the tube and the buffer removed using a pipette. The beads were washed 3 times with 500µL of PBS containing 0.1% Tween 20 surfactant. Following this the tube was removed from the rack and the sample added. The sample and the immobilized capture antibody were incubated at 4°C with continuous shaking overnight.
In the morning the tube containing the mixture was placed in the magnetic rack, the beads were allowed to adhere to the side of the tube and the sample removed with a pipette. The beads were then washed three times with 500 µL PBS containing 0.1% Tween 20 surfactant. After the last wash, the tube was removed from the magnetic rack and 60 µL of 0.2 M Glycine (pH2.5) was added and mixed to resuspend the beads. The suspension was then incubated at room temperature for 2 minutes. The tube was later put in the magnetic rack and the beads were allowed to adhere to the side. Following this the supernatant which was the immunoprecipitate was transferred to a new tube using a pipette and neutralized by adding 5 µL of 1 M Tris (pH 8.5).

3.14 SDS PAGE

All samples were run as follows; 6 µL of sample was added to 3.75 µL of 4X LDS sample buffer (Invitrogen), 1.5 µL of reducing agent and 3.75 µL of distilled water making a total volume of 15 µL. The mixture was then vortexed and heated at 70°C for 10 minutes. After the incubation 10 µL were loaded into the pre-cast SDS gel (Invitrogen). The gel was then run in 1x NuPAGE SDS running buffer (Invitrogen) at a constant voltage of 200 Volts for 35 minutes.

3.15 Western Blot analysis

For western blot analysis transfer buffer was prepared by adding 25mLS of 10x transfer buffer (Invitrogen) to 50 mL of methanol, 425 mL of distilled water and 1 mL of antioxidant (Invitrogen). Two sponges were wetted and in water to expel any air bubbles, nitrocellulose paper was wetted as well. The two sponges were placed on the blot
module one after the other. Following this, the gel was disassembled from the SDS PAGE apparatus and using a wetted filter paper the gel was picked and placed over the two wetted sponges with the filter paper directly over the sponges on blot module aligning well with the cathode. The wetted nitrocellulose paper was placed on top of the gel a wetted filter paper was placed on top of the gel before two wetted sponges were placed on top. The whole assembled apparatus was pressed gently to expel any air bubbles. The blot module was closed, put into the electrophoresis tank and run at 30V for 1 hour.

The blot was then removed from the transfer apparatus and nonspecific sites blocked with Casein for 1 hour at room temperature with shaking. Thereafter the blot was removed from the Casein and the Anti-PfMCM-6 antibody added at a dilution of 1:800. The blot was then incubated overnight at 4°C with shaking. After this the membrane was washed by resuspending in wash buffer (PBS containing Tween 20 at 0.05%) for six times 5 minutes each. Following this, the blot was incubated in HRP Goat anti-Rabbit antibody at a dilution of 1:20,000. The blot was then washed in wash buffer (PBS containing Tween 20 at 0.05%) six times 5 minutes each with agitation to remove unbound secondary anti-body. 6mL of peroxide and 6mL of the luminal enhancer solution (Thermo Scientific) were mixed to give the substrate solution. The blot was then incubated in the substrate solution for five minutes at room temperature. After the incubation the blot was removed from the substrate solution and placed in a plastic membrane protector. Absorbent tissue was used to remove excess liquid and to remove all bubbles from between the blot and the surface of the membrane protector. The
protected membrane was then placed in a film cassette with the protein side facing up. All lights were turned off except the red safe light appropriate for film exposure. A piece of film was carefully placed and exposed for 60 seconds. This was followed by immersing the film into developer solution followed by fixative and finally washed in water and dried.

3.16 Bioinformatic analysis of PfMCM6

Motifs and domains in PfMCM6 that are relevant to its phosphorylation by PfMRK and PfPK6 were characterized. The protein sequence of PfMCM6 was searched for these motifs and domains by using the Eukaryote Linear Motif search tool (ELM), computational biology free software that searches for functional sites in proteins. The PfMCM6 sequence was thus posted at the ELM database (www.elm.eu.org) to identify unique features in the protein that predispose it to phosphorylation.

3.17 Flavonoid and Chloroquine susceptibility testing

3.17.1 RPMI basic media preparation

RPMI 1640 basic media was prepared for flavonoid and Chloroquine susceptibility testing. It consisted of RPMI 1640 powder 10.4 grams; (Invitrogen, Inc., Carlsbad, California, USA) combined with 2 grams of glucose (Sigma Inc., St Louis, Missouri, USA;) and 5.95 grams HEPES (Sigma Inc. St Louis, Missouri , USA) dissolved to homogeneity in 1 liter of de-ionized water and sterilized with a 0.2µM filter.
3.17.2 RPMI complete media preparation

Complete RPMI 1640 media, used for all parasite culture drug dilutions and drug testing, consisted of RPMI 1640 basic media with 10% (vol/vol) human ABO pooled plasma, 3.2% (vol/vol) sodium bicarbonate (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and 4µg/ml hypoxanthine (Sigma Inc.) added. Complete RPMI 1640 media was stored at 4°C and used within 2 weeks.

3.17.3 Flavonoid/Chloroquine sensitivity tests

The SYBR green I-based in vitro drug susceptibility assay technique described in details earlier (Bacon et al., 2007, Johnson et al., 2007, Smilkstein et al., 2004) et al., 2007; Bacon et al., 2007) was used with modifications. Briefly *P. falciparum* reference clone W2 classified as CQ “resistant” and clone D6, classified as CQ “sensitive” (Desjardins et al., 1979) were grown in continuous culture with 90% N₂, 5% CO₂ and 5% O₂ using RPMI-1640 (GIBCO-BRL) supplemented with 40.5 mM TES, 27.6 mM Sodium Bicarbonate, 11 mM Glucose and 10% heat inactivated human serum at 37°C, which were donated by the Walter Reed Army Institute of Research (WRAIR). Both D6 and W2 clones were culture-adapted at 6% hematocrit, to reach 3 to 8% parasitemia, for 7 to 30 days, then adjusted to 2% hematocrit and 1% parasitemia, and cultured in the presence of various flavonoid dilutions in complete RPMI 1640. As a control chloroquine was tested against both W2 and D6 as well.

To prepare Chloroquine/flavonoids, Chloroquine was dissolved in 70% ethanol at 1mg/ml while flavonoids were dissolved in 100% dimethyl sulfoxide at 5mg/ml and diluted in complete RPMI 1640 media to 1000ng/ml for CQ and 50000ng/ml for the flavonoids as starting concentration. They were further subjected to twofold serial
dilution across ten wells using the biomek 2000 (Beckman Coulter, Inc Fullerton, CA USA). The structures and names/codes of flavonoids used in this study are listed in appendix IV and V.

For the susceptibility assay, the ten varying twofold serial dilutions (10 concentrations and the first row was control wells) of the drug/flavonoid (12.5 µl/well) were dispensed into 96-well microculture plates (Nunc Inc., Roskilde, Denmark) by a semiautomated microdilution technique using Beckman 2000 laboratory workstation. A total of 100 µl of cell medium mixture at 2% hematocrit and parasites at 1% (for reference isolates W2 & D6) was added to each well, and the plates were incubated for 72 h in a gas mixture (5% CO2, 5% O2, and 90% N2) at 37°C. The plates were subsequently freeze-thawed for hemolysis. For further haemolysis, 100µl of lysis buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100] containing SYBR green I (1x final concentration) were added directly to the plates and gently mixed by using the Beckman Coulter Biomek 2000 automated laboratory workstation (Beckman Coulter, Inc., Fullerton, CA).

The plates were incubated for 5 to 15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per well relative fluorescence units (RFU) of SYBR green I dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC) with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and with the gain set at 60.
CHAPTER FOUR

4.0 Results

4.1 PfMCM6 identified

A multiple protein sequences alignment of the recently annotated six PfMCMs (Patterson et al., 2006)(Dr Chakrabati un-published data, Appendix I), followed by phylogenetic analysis of the six PfMCMs and the *P. falciparum* putative replication licensing factor showed that the PfMCM-6 and the putative replication licensing factor clustered on the same branch with 100% posterior probability value and equal branch length (Figure 6). A pairwise alignment of the amino acid codes of PfMCM-6 and the putative replication licensing factor showed that the two genes are identical amino acid for amino acid (Figure 7).
Figure 6: Phylogenetic relationship of the PfMCMs and the putative PfRLF. The percentages represent posterior probabilities of the branching amongst the different PfMCM proteins.

Figure 7: Alignment of amino acid sequences of the putative PfRLF and PfMCM-6. The alignment shows amino acid for amino acid comparison of the two sequences.
4.2 Expression and purification of PfMCM6

For further analysis PfMCM6 was expressed as a GST tagged fusion protein at high levels and remained soluble during expression (Figure 8). Purification and dialysis resulted in fairly pure protein fractions observed at about 135 kDa (Figure 8) after removal of Glutathione as well as salts. The observed size was the expected size taking into account the GST tag at 25kDa and protein at 110 kDa.

![Figure 8: SDS PAGE Electrophoresis of the PfMCM6 Elute. MW=Molecular weight marker; lanes 1, 2, & 3 represents Successive elutions of the 110kDa PfMCM-6 protein with a 25kDa GST tag.]

4.3 Expression and purification of PfPK6 and acquisition of PfMRK

The HIS tagged PfPK6 protein was observed at approximately 37 kDa (Figure 9) which is the expected molecular size taking into account the HIS tag at 1 kDa. The protein which was expressed in measures of 2mL from an FPLC (Armersham™) was observed
most clearly during the 4\textsuperscript{th}, 5\textsuperscript{th} and 6\textsuperscript{th} elutes out of a total of 12 elutes. The other Kinase used in this study is PfMRK. This was a kind gift from Dr. Norman C. Waters.

![Figure 9: SDS PAGE Electrophoresis of the Elute containing PfPK6. MW= Molecular Weight Marker, lane 1, 2, 3, 4, 5, & 6 = successive elutions containing the PfPK6 protein.](image)

**4.4 PfMCM-6 as a substrate of PfMRK and PfPK6**

The feasibility of PfMCM-6 as a substrate of PfMRK was confirmed using the gold standard $^{32}$P radioactive protein kinase assay. From this experiment there were two observations. (i) PfMCM6 auto-phosphorylates and (ii) in presence of PfMRK there is increased incorporation of radioactivity (Figure 10).
Figure 10: PfMCM6 Phosphorylation by PfMRK using the radioactive Assay. First bar on the left represents Background reading alone in reaction buffer, followed by the bar representing PfMCM6 alone in reaction buffer and lastly the bar representing PfMCM6 and PfMRK with CYC1 in reaction buffer.
4.5 Optimization of the Kinase Glo Assay

The optimization of the Kinase glo assay was carried out using PfCTD as the substrate for PfMRK and CYC1 as the cyclin. There was an immediate uptake of ATP by the kinase reaction leaving enough ATP to produce only 4,000 RFUs compared to the expected 18,000 RFUs (Figure 11). The difference (Δ) in the relative fluorescent units of 14,000 units corresponded to the ATP used in the kinase reaction to phosphorylate CTD.

![Figure 11: The PfCTD/PfMRK control experiment.](image)

PfCTD was used as substrate to determine feasibility of the kinase glo Assay.

4.6 The phosphorylation of PfMCM6 by PfMRK

To characterize the nature of the interaction between PfMCM6 and PfMRK, phosphorylation experiments were carried out. The control reaction (Consisting of ATP only in reaction buffer) against which all other reactions were compared yielded the
highest measure of Relative Light Units (RLUs, Figure 12). The high RLU value was due to presence of maximum unincorporated ATP in the reaction media.

The reaction incorporating ATP, reaction buffer, kinase Glo reagent and PfMCM6 resulted in a slight drop in RLU (2000 RLU, Figure 12). PfMRK auto-phosphorylated in the presence of ATP and the ΔRFU value for this auto-phosphorylation was 18,000 RFU (Figure 12). The reaction consisting of PfMRK, ATP, PfMCM6 and PCYC-1 in the kinase reaction buffer, resulted in ATP being depleted from the reaction mixture at a much higher level giving ΔRLU of 8,000. The Δ value between the PfMRK auto-phosphorylation reaction and the PfMRK with PfMCM 6 reaction was approximately 12,000 RLUs (Figure 12).

Figure 12: PfMRK Kinase Assay with PfMCM6 as substrate. PfMRK Autophosphorylates in the presence of ATP & and actively Phosphorylates PfMCM6.
4.7 The effect of PfMAT 1 on the kinase activity of PfMRK in the presence PfMCM6/PfCTD

This study sought to establish whether PfMAT1 would augment phosphorylation of PfMCM-6 by PfMRK in the presence of Cyclin 1. Previously, study of the enhancement of phosphorylation of PfCTD by PfMAT 1 used a radio-active assay (Chen et al., 2006). The same experiment was repeated in this study using the Kinase Glo non-radioactive kinase assay. Similarly, the effect of PfMAT 1 on the phosphorylation of PfMCM6 by PfMRK was determined using the non-radioactive Kinase-Glo® assay. The control reaction containing ATP in reaction buffer showed the highest value of 48,000 RLU (Fig 13). The reaction containing ATP and PfMCM6 in kinase buffer showed basal uptake of ATP of 39,000 RLU (Figure 13). Similarly the reaction containing ATP, PfMCM6 and PfCYC1 in Kinase buffer showed 39,000 RLU (Figure 13). The kinases activity assay involving PfMRK, PfMCM6 and PfCYC1 in presence of the Kinase-Glo® reagent showed value of 22,000 RLU. The result indicated that PfMRK phosphorylates PfMCM6 (Figure 13). The Δ value between the control reaction and this reaction was approximately 20,000 RLUs (Figure 13). Finally, in order to test and quantify the effect of PfMAT1 on the phosphorylation of PfMCM6 by PfMRK, PfMAT 1 was included in the reaction. This resulted in the least measure of RLU at approximately 13,000 units (Figure 13). The ΔRFU value between the Kinase activity reaction and the PfMAT 1 reaction was approximately 10,000 RLUs (Figure 13).

For comparison, the assays were carried using PfCTD as the substrate instead of PfMCM6. The control experiment with only ATP in the kinase buffer yielded 44,000
RLU whereas when PfCTD was included in the assay, 40,000 RLU were seen indicating ΔRLU of 4,000 units (Figure 14). When PfCYC1 was included in the assay, there was no change in RLUs emitted (Figure 14). Upon addition of the PfMRK to this assay, a dramatic reduction of 18,000 RLUs was observed (Figure 14). This confirmed that PfCTD is a substrate for PfMRK as has been reported before (Chen et al., 2006). Addition of PfMAT 1 further reduced the RLUs by 7,000 units (Figure 14). This confirmed that PfMAT 1 enhances phosphorylation of PfCTD (Chen et al., 2006). This control experiment thus proved the feasibility of the Kinase-Glo® non-radio-active assay in conducting these experiments.

![Bar chart](image.png)

**Figure 13: PfMAT1 effect on PfMRK phosphorylation with PfMCM6 as substrate.**

PfMAT1 enhanced PfMRK activity when added to the complete kinase activity assay.
Figure 14: Effect of PfMAT1 on PfMRK phosphorylation with PfCTD as substrate.

PfMAT1 enhanced PfMRK activity when added to the complete kinase activity assay.

To investigate the interaction between PfMCM6 and PfPK6, kinase phosphorylation assays were carried out. When ATP was incubated in the presence of the kinase buffer and the kinase reagent the assay showed 38,000 RLUs (Figure 15). Addition of PfMCM-6 resulted in basal autophosphorylation (Figure 15) as previously observed (Figs. 13 & 14). The ability for PfPK6 to autophosphorylate was tested by addition of ATP to the kinase in the appropriate buffer. As expected there was a dramatic decrease in the amount of free ATP in this reaction by 20,000 RLU compared to the control (Figure 15). This was not surprising because Malarial CDKs especially PfPK6 have been shown to autophosphorylate (Doerig et al., 2002, Bracchi-Ricard et al., 2000, Doerig et al., 2008). When PfMCM6 was added to the assay containing ATP, PfPK6 and the kinase reagent in the presence the appropriate buffer, a further drop of 6,000 RLUs was observed (Figure 15). This indicated that a smaller amount of ATP remained in the reaction mixture after addition of the substrate due to incorporation of the phosphate groups from ATP to
PfMCM6 substrate by PfPK6. However, a relatively small ΔRLU value was observed compared to the PfMRK-PfMCM6 assay (Figure 17).

![Graph](image)

**Figure 15: PfPK6 Kinase Assay with PfMCM6 as substrate.** PfPK6 Autophosphorylates in the presence of ATP & and actively Phosphorylates PfMCM6.

4.8 Bioinformatic analysis of motifs present in the PfMCM6 protein sequence

To better understand the molecular basis for the phosphorylation of PfMCM6 by both PfMRK and PfPK6, a bioinformatic analysis of the PfMCM6 protein was carried out by searching against the Eukaryote Linear Motif (ELM) resource server (www.elm.eu.org). The search returned a number of motifs and domains that may point to the nature of molecular mechanisms involved in phosphorylation of PfMCM6 by PfMRK and PfPK6. The main domain observed is the canonical MCM domain that is common to all MCM proteins. This domain is found between amino acid positions 206 and 751 (Figure 16, appendix VI). The other motif observed within the PfMCM6 sequence is the cyclin recognition site designated as LIG_CYCLIN_1. This motif is found at the C-terminus of the protein covering residues 133-137 (KVLIV) and 169-173 (RILDI) (Figure 16, Appendix VI).
Also observed were motifs designated as LIG_MAPK_1 and LIG_MAPK_2. The first site for the LIG_MAPK_1 covers amino acid positions 164 to 170. The amino acid sequence in this region is KRHFLRI. The second site for LIG_MAPK_1 motif covers amino acids at positions 189 to 198; the amino acid sequence in this region is KTKEVGKICL. A related motif assigned the tag LIG_MAPK_2 was observed and covers amino acid 103-106. The amino acid sequence of this motif is FDFP.

Another functional site observed in PfMCM6 is the phosphoinositide-3-OH-kinase phosphorylation site designated as MOD_PIKK_1. The motif is found at several sites at both the C and N-termini. The two C-terminal sites are found at amino acid residue 35-41 with the sequence DENSQMS and residues 154-160 with the sequence DIGSQLY. Likewise, there are two such sites at the N-terminus of the protein. They occur at amino acids 822-828 whose sequence is ESITQDQ and at amino acids 908-914 with the sequence NYQTQEK.

Several Src Homology (SH2) domains were also observed. These include the LIG_SH2_PTP2 domain covering amino acid residue 117-120 with the sequence YVLL, the LIG_SH2_SRC domain covering amino acid residue 801-804 with the sequence YQYI, the LIG_SH2_STAT3 domain covering residue 909-912 with the sequence YQTQ and the LIG_SH2_STAT_5 domain. This last domain had 4 repeats, two on the N-terminus and 2 on the C-terminus covering amino acid residues 64-67 with the sequence YVQD, 162-165 with the sequence YIKR, 803-806 with the sequence YISA, and 833-836 with the sequenceYLQV. Other motifs present on the C-terminus of the PfMCM6 protein are the classical nuclear localization signals. These include
TRG_NLS_MonoCore_2 motif covering residues 28-33 with the amino acid sequence QKKKRR, the TRG_NLS_MonoExtC_4 motif covering residues 29-34 with amino acid sequence KKKRRI. The MOD_SUMO motif which is recognized for modification by SUMO-1 was identified covering amino acid residues 838-841 with the amino acid sequence AKAE. The LIG_14-3-3_3 motif was also observed and covers residues 839-844 with the protein sequence KAESSE. In addition, the LIG_PDZ_3 was observed on the N terminus covering amino acid 924-929 represented by the sequence EEIDNF.

The other motifs identified by ELM for PfMCM6 are the forkhead associated domains, LIG_FHA_1 and LIG_FHA_2. The LIG_FHA_1 covers amino acid residues 188-194 with the sequence IKTKEVG on the C-terminus while LIG_FHA_2 covers two sites at residues 78-84 with the sequence LKTFSEK on the C-terminus and amino acid residues 869-875 with the sequence SETNEDD towards the N terminus. The protein also has the LIG_BRCT_BRCA1_1 motif which has a central role in cell-cycle check point and DNA repair functions. This motif occurs at two sites in PfMCM6, the first at the extreme N-terminus covering residues 1-5 with the sequence MSAIF and the second site at residues 99-103 with the sequence DSLNF. PfMCM6 protein also has the APCC-binding destruction motif designated as LIG_APCC_Dbox_1. This motif is found at residues 164-172 whose sequence is KRHFLRILD.
Figure 16: Eukaryote Linear Motif search tool result (generated at www.elm.eu.org). The protein sequence of PfMCM6 was searched against the elm resource to identify the motifs present. The numeral next to each feature represents the number of each motif feature.

4.9 Anti-PfMCM6 antibodies

Several short sequences were identified in the PfMCM6 that had the potential of producing synthetic epitopes. From these epitopes the ones that were considered the most immunogenic were used to generate antibodies against PfPK6. Notably the peptide sequence, GEEEEDDDDEEEPS which appears in the region 49-63 of the PfMCM6 sequence was selected by all the search algorithms as the most appropriate for generating antibodies against PfMCM6. One reason why it was selected was due to its potential to
be highly charged and therefore mostly likely exposed to the solvent and therefore a good epitope. Other peptides that were identified to produce potential epitope include EKLNSSKDKNNFDEQ found between the amino acid 394-408 region, AVHRDPDQGDTVLEA in region covered from amino acids 520-535, and VNKDQETENTEQQNK in the region between amino acids 777-791 (Appendix III).

The GEEEEDDDDEEEEPS peptide was synthesized by Immunoprecise Antibodies Ltd as a white lyophilized powder with a solubility of 1.0mg/ml in water at a pH of 8.5. HPLC analysis of the GEEEEDDDDEEEEPS peptide gave one major peak at 10.936 milli-absorbance unit (mAU) with an associated purity of 96.479% (Figure 17).

To confirm the amino acid sequence in the synthesized peptide, the peptide was analyzed using Electrospray Ionisation proteomic tool. The molecular mass analysis of the peptide showed a major peak at 1023.36 with the sequence Cys-Gly-Glu-Glu-Glu-Glu-Glu-Asp-Asp-Asp-Asp-Asp-Glu-Glu-Glu (Figure 18). The molecular mass of this product was determined to be 2,044.72 Da in agreement with the computed value for the peptide at 2,027.82 Da. The reconstituted peptide was inoculated into two rabbits to immunize and raise antibodies. At the end of the immunization period serum was collected from terminal bleed of rabbit, pooled and aliquoted into 3 tubes of 22.5ml each. This serum was lyophilized. Serum collected from the second rabbit was aliquoted into 4 tubes each containing 22.5 ml and then lyophilized. Titration analysis of the serum from both rabbits against the cognate peptide showed that the antibodies could be used at a dilution of between 1/400-1/800 (Figure 19).
4.10 Western-Blot Analyses

The western blot analyses on *P.falciparum* parasite protein lysate using the generated anti-PfMCM6 antibodies identified a protein of 110kDa (Figure 20).

**Figure 17:** HPLC report on the GEEEDDDDEEEPS epitope. The major peak represents purity of the synthesized epitope.
Figure 18: Proteomic Analysis of the PfMCM6 Epitope. Mass spectrometry analysis (Electrospray ionisation) shows the sequence and molecular weight of the epitope.
Figure 19: Elisa titration Curve of Rabbit 22 (A) and Rabbit 52 (B) anti-Serum. The titration was done using the Rabbit anti-GEEEEDDDDEEEPS serum, both the pre-bleed and terminal bleed serum against the GEEEEDDDDEEEPS free peptide.
Figure 20: Western blot analysis of PfMCM-6. A=Parasite Lysate probed with Rabbit anti-PMCM6 antibodies and B= purified recombinant protein probed with Anti-GST antibodies. Part A Lane 1 = Invitrogen NU-Page protein maker Lane 2 = *P. falciparum* protein lysate, Part B, Lane 1&2= purified GST tagged recombinant protein, lane 3=PfMRK, lane 4=PfPK6.

4.11 Inhibition of PfMRK activity using PfMCM-6 as Substrate

Having established PfMCM-6 is a substrate of PfMRK; in this section the applicability of PfMCM6 as a substrate of PfMRK for use in antimalarial drug discovery studies was investigated. Previously, compound WRAIR 692 had been established by the Walter Reed Army institute of research, Department of Experimental therapeutics, as an inhibitor of PfMRK with PfCTD as substrate using the radioactive kinase assay. Therefore compound WRAIR 692 was used to test inhibition of PfMRK with PfMCM-6 as the alternate substrate while employing the non-radioactive Kinase-Glo® assay. The
PfMRK inhibition assay generated a good sigmoidal dose response curve (Figure 21). This Curve gave an IC₅₀ value of 76.78µM.

![Graph showing sigmoidal dose response curve](image)

**Figure 21: PfMRK-PfMCM6 Inhibition Assay.** Sigmoid curve generated by Dose response inhibition reaction of PfMRK in the presence of CDK inhibitor (compound WRAIR 692).

### 4.12 Inhibition of PfPK6 with PfMCM-6 as Substrate

Similar to the previous section the WRAIR inhibitor 692 was tested for its ability to inhibit kinase activity of PfPK6 with PfMCM-6 as substrate. The inhibitor gave an IC₅₀ of 29 mM.

### 4.13 Testing of Flavonoids as inhibitors of PfPK6 and PfMRK using PfMCM6 as substrate

This study sought to establish the ability of recently characterized flavonoids to inhibit phosphorylation of PfMCM6 by PfPK6 and PfMRK. A serial dilution of each of the
following prenylated flavanones was done and subsequently tested for inhibition: Abyssinone IV, Abyssinone V, and Abbysinine III, (Appendix, (Yenesew et al., 2004)).

The initial Abyssinone IV assay generated a good sigmoidal dose response curve with an IC$_{50}$ value of 31.17µM, while a repeat assay with the same compound gave, an IC$_{50}$ of 37.49 µM (Fig 2). To determine if Abyssinone IV could also inhibit PfMRK, the compound was tested against PfMRK in the presence of PfMCM6 as substrate. The IC$_{50}$ of this inhibition reaction was 15.99 µM. When the PfMCM6-PfPK6 system was tested against a similar molecule Abyssinone V which has an OH group on the ‘A’ ring (Appendix IV), a higher IC$_{50}$ of 117.5 µM was observed (Table 1). When Abyssinone V was assayed against PfMRK in the presence of PfMCM6, IC$_{50}$ of 5.64µM was obtained. The inhibitory profile of a third related flavanone called Abyssinine III (appendix IV) was tested using the PfPK6-PfMCM6 system. This flavonone gave an IC$_{50}$ value of 86.4 µM (Table 1). When tested against PfMRK in the presence of PfMCM6 as the substrate, Abyssinine III gave an IC$_{50}$ of 1.18 µM (Table 1). The last flavonoid tested for inhibitory activity against PfPK6 and PfMRK using PfMCM6 as the substrate was Abyssinone V-4-O-methylether (Appendix IV). In these assays, Abyssinone V-4-O-methylether gave an IC$_{50}$ of 113.5 µM for PfPK6 (Table 1) and 0.26 µM for PfMRK (Table1).

To broaden the number of compounds that could potentially be used as inhibitors of the Plasmodial PfPK6, other compounds were tested against PfPK6 in the presence of PfMCM6 substrate. The compounds included a prenylated flavonoid called 5’-prenyl Pratensein and Flavogaulin (Appendix IV). The 5’-prenyl Pratensein assay yielded an IC$_{50}$ of 112.3 µM (Table 1). Previous work had shown that 5’-prenyl Pratensein gives an
IC\textsubscript{50} value of \(\sim5.3\) μM when tested against \textit{P. falciparum} clone D6 and an IC\textsubscript{50} value of \(\sim8.7\) μM against \textit{P. falciparum} clone W2 (Yenesew et al., 2004). The Flavogaulin inhibition assay in the PfPK6-PfMCM6 system gave an IC\textsubscript{50} value of 126.59 μM (Table 1). When tested against \textit{P. falciparum} clones D6 and W2, the IC\textsubscript{50} values were 2.2 μM and 2.03 μM respectively.

To further test the PfPK6-PfMCM6 system in identifying potential kinase inhibitors, several newly purified uncharacterized Flavonoids with only the skeletal structures available (Appendix V) were tested. The IC\textsubscript{50} values of these compounds against PfPK6 and clones D6 and W2 are shown in table 2. These IC\textsubscript{50} are reported as ng/ml since complete structures of these natural products are not yet available. From these data compound BA4Awas as active against PfPK6 as it was against \textit{P.falciparum} clones.
<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-PfPK6 μM</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-PfMRK μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abyssinone IV</td>
<td>31.17</td>
<td>15.99</td>
</tr>
<tr>
<td>Abyssinone V</td>
<td>122.6</td>
<td>5.89</td>
</tr>
<tr>
<td>Abyssinine III</td>
<td>86.4</td>
<td>1.18</td>
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<tr>
<td>Abyssinone-V-4-O-methylether.</td>
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<td>5.64</td>
</tr>
<tr>
<td>Flavogaulin</td>
<td>126.59</td>
<td>-</td>
</tr>
<tr>
<td>5’-prenyl-Pratensein</td>
<td>112.3</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1: Activities of characterized Flavonoids against PfPK6 and PfMRK:**

Chemical Structures shown in Appendix IV.

<table>
<thead>
<tr>
<th>Flavonoid Code</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-PfPK6 μg/mL</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-D6 μg/mL</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;-W2 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA4T</td>
<td>28.83</td>
<td>40.60</td>
<td>13.14</td>
</tr>
<tr>
<td>BA7D</td>
<td>16.50</td>
<td>39.45</td>
<td>12.31</td>
</tr>
<tr>
<td>BA4A</td>
<td>7.95</td>
<td>8.41</td>
<td>-</td>
</tr>
<tr>
<td>BA4N</td>
<td>21.09</td>
<td>3.97</td>
<td>5.10</td>
</tr>
<tr>
<td>BA5G</td>
<td>62.77</td>
<td>4.13</td>
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<tr>
<td>BA7P</td>
<td>206.75</td>
<td>10.88</td>
<td>7.68</td>
</tr>
<tr>
<td>BA5J</td>
<td>30.545</td>
<td>19.118</td>
<td>19.44</td>
</tr>
<tr>
<td>BA7M</td>
<td>4.555</td>
<td>37.750</td>
<td>13.241</td>
</tr>
</tbody>
</table>

**Table 2: Activities of uncharacterized Flavonoids against PfPK6.** Backbone structures have been shown in Appendix V.
Figure 22: Inhibition of PfPK6 phosphorylation activity by Abbysinone IV. Curves A and B represent replicate inhibitory profiles over a range of compound concentrations.
CHAPTER FIVE

5.0 Discussion

5.1 Introduction

This study has characterized two P. falciparum CDKs; PfPK6 and PfMRK in relation to a DNA replication licensing factor. Additionally their potential as drug targets for the treatment of malaria has been evaluated. Mechanistically, PfPK6 or PfMRK-specific drugs may inhibit the CDK itself or may target the CDK activity through a proxy CDK-interacting partner. Thus, a major component of the present work focused on characterization of a plasmodial putative replication licensing factor that has previously been demonstrated as a specific interacting partner of PfPK6 and PfMRK (Eyase, 2006). Results obtained in the present study have shown that the putative replication licensing factor identified by Eyase (in 2006) and PfMCM-6 are similar.

CDKs are highly regulated through processes such as phosphorylation, ubiquitin targeted protein degradation, and association with stimulatory and inhibitory proteins (Morgan, 1995, Morgan, 1996). Due to their central role in growth regulation, CDKs are currently under investigation as drug targets for disease treatment especially cancer (Lapenna and Giordano, 2009). CDKs may not serve as drug targets in cancer alone but in parasites as well. Thus, since their role is central to the survival of the cell, parasite CDKs have potential of serving as drug targets for treatment of their cognate infectious agents. If distinct differences exist in the protein substrates and functional activity of host and
parasite CDKs. In this regard the role of PfMCM6 as a substrate of the two Plasmodial CDKs is discussed.

5.2 PfMCM6 and the DNA replication licensing system

In eukaryotes the initiation of DNA replication is a complex process involving multiple regulated steps. MCM proteins are key components to this process. In a study by Le-roch and his colleagues, six replication licensing factors have been observed in the *P. falciparum* genome (Le Roch et al., 2003). These six factors correspond to the six *P. falciparum* MCM proteins which include PfMCM2, PfMCM3, PfMCM4, PfMCM5 PfMCM6 and PfMCM7 (Patterson et al., 2006). The six MCM proteins are present in other organismal systems and constitute the replication licensing system (Mahbubani et al., 1997).

Since PfMCM6 was shown to interact with PfMRK and PfPK6, I sought to further characterize this interaction by first obtaining recombinant proteins. Protein expression and purification of a GST-fused PfMCM6 using an *E. coli* expression system resulted in a soluble protein whose molecular weight was approximately 130 kDa by both SDS PAGE and Western blot analysis. This molecular weight was in the expected cumulative weight range having derived 110kDa from the PfMCM6 and 25kDa for the GST tag. The PfPK6 and PfMRK proteins were also expressed in *E. coli* as HIS-taged fusions with six histidine amino acid residues fused at the N-termini. These proteins were also found to be soluble and expressed at high levels in the bacterial system. Most of the time, expressions of foreign proteins in *E. coli* at high levels as was the case in this study,
results in the formation of inclusion bodies. These inclusion bodies are composed of insoluble aggregates of the expressed protein which are functionally inactive due to lack of correct folding. Such inclusion bodies may be recovered from bacterial lysates by centrifugation and washed with Triton X-100 and EDTA to remove much of bacterial protein as possible from the aggregated foreign protein.

To renature the proteins in the inclusion bodies requires that the inclusion bodies are dissolved in denaturing agents to release the protein followed by refolding by gradual removal of the denaturing reagents through dilution or dialysis. It was fortunate in this study that the PfMCM6-GST, HIS-PfPK6 and HIS-PfMRK fusions did not form inclusion bodied but were expressed as a soluble protein. The solubility of these protein fusion may be explained by considering the isoelectric point (pI) of the proteins. The theoretical pI and molecular weight (Mw) of PfMCM6 calculated by the “Compute pI-Mw” tool at Expasy were pH 5.14 and 105674.08Da respectively. Furthermore, those of PfPK6 and PfMRK were pH 5.87 / 35843.69Da and pH 7.49 / 37989.21Da respectively. These results showed that PfMCM6, PfPK6 and PfMRK would have minimum solubility in water or salt solutions at pH 5.14, 5.87 and 7.49 respectively. Since the pH in the bacterial cell is close to 7 and the sonication buffer had a pH of 7.5 for PfMCM6 and PfPK6 and 8.5 for PfMRK, all the proteins maintained a net negative charge maintaining solubility during the expression and purification processes.

Using the radio-isotope (32P) method of Kinase activity assay as well as the non radioactive Kinase-Glo® assay, it was demonstrated that PfMCM-6 protein utilizes ATP in the absence of any other protein. In an attempt to explain this utilization of ATP
in the absence of an exogenous factor(s), there are three scenarios to choose from. (i) PfMCM6 may act as a kinase and utilize ATP to auto-phosphorylate itself. If this were the case PfMCM6 would take up ATP from the reaction media until a certain saturation point when all the acceptor residues in the protein have been phosphorylated. The amount of ATP remaining in solution after this would depend on the initial concentration of PfMCM-6 protein in the reaction. The higher the PfMCM-6 concentration in the medium, the less the ATP amount would remain after the incubation due to incorporation into the PfMCM6. (ii) Acting as an ATPase, PfMCM6 may catalyze the decomposition of ATP into ADP and a free phosphate ion. The energy released in such ATPase driven reactions is usually harnessed to drive other chemical reactions that would not otherwise occur. Since MCM proteins have helicase activity (Ishimi, 1997, Ishimi et al., 2000), this may be the energy used in the helicase reaction. Acting as a helicase in the presence substrate (DNA) would completely deplete ATP from the reaction as long as the substrate is not limiting in the reaction media. The fact that in the reaction the substrate (DNA) was absent, the initial uptake of ATP would not be expected to proceed to depletion. (iii) Acting as ATPase, PfMCM6 may use energy from the hydrolysis of ATP to perform some work such as refolding itself. If this was the case, then only a fraction of the added ATP would be utilized and most of the ATP would remain in the medium. Indeed studies have shown that MCM proteins have ATPase activity (Ishimi, 1997, Koonin, 1993) and structurally the MCM proteins including the P. falciparum MCMs are part of the large AAA ATPase family that forms large ATP-dependent complexes that are involved in a myriad of cellular functions. Scenario (i) is unlikely because there is no experimental
evidence to support the notion that PfMCM6 autophosphorylates. Scenario (ii) is unplausible due to the lack of the DNA substrate within the reaction medium. Therefore scenario (iii) is the most probable explanation for the observed basal removal of ATP from the reaction mixture. ATP is essential in the assembly of AAA ATPases into hexameric complexes. The PfMCMs which are members of the AAA ATPase family are therefore naturally amenable to activity in the presence of ATP.

A search of the full-length PfMCM-6 protein sequence against the Eukaryote Linear Motif (ELM) database indicated presence of the canonical MCM domain which includes the MCM box. The MCM box is present between residues 206 and 751 in PfMCM-6 (Figure 14). The MCM box consists of three motifs viz: two ATPase consensus (designated walker A and walker B) motifs and the short SRFD motif. Walker A motif includes the P-loop of the active site of ATPase which has the traditional lysine residue found in every ATP-binding protein (Bochman and Schwacha, 2009). Walker A consensus motif may have an alanine or serine residue which together with other conserved residues creates the MCM-specific consensus GDPxx(S/A)KS. In comparison walker B is a bulky and hydrophobic element which is believed to be involved in the hydrolysis of ATP (Forsburg, 2004). The walker B motif contains in part the sequence IDEFDKM. The short SRFD sequence is situated approximately 70 residues downstream of the walker B motif and constitutes the arginine finger. The presence of the MCM domain containing highly conserved walker A and walker B motifs, suggest that PfMCM-6 protein has inherent ATPase activity as observed in this work.
5.3 PfMCM6 and the MCM complexes

Of the six PfMCM proteins that have been reported in *P. falciparum*, only PfMCM6 has been demonstrated to interact with *P. falciparum* CDKs (Jirage et al., 2010). This study has refined the nature of this interaction by showing that PfMCM6 is a substrate for PfPK6 and PfMRK. What is the role of PfMCM6 in the plasmodial MCM complex? The mammalian MCM complex has subunits which associate with different affinities. Among these, there is a tightly associated core of MCM4, MCM6 and MCM7. This core complex in the human has been associated with helicase activity *in vitro* hence the supposition that MCM proteins are the eukaryotic replicative helicase (Ishimi et al., 2000). Similarly, it would appear that PfMCM-6 belongs to the respective core complex of PfMCM4, PfMCM6 and PfMCM7 thus forming part of the *P. falciparum* replicative helicase (Patterson et al., 2006). According to Ishimi and others both the MCM 4 and MCM 6 play a major role in the mammalian helicase activity initiated by the MCM 4, 6, 7 complex (Ishimi et al., 2000). It is suggested that an ATP-binding site in MCM4 takes part in the helicase activity by binding single stranded DNA while an MCM6 site plays a role through high affinity binding with ATP (Ishimi et al., 2000).

Since PfMCM6 is hologous to the mammalian counterparts, it is therefore suggestive that PfMCM6 plays its role PfMCM complex by binding with ATP. Furthermore, findings in this study support the suggested role of ATP in the affinity binding since PfMRK and PfPK6 are observed to phosphorylate PfMCM6. Whereas the PfMCM4 role may not be clear compared to its mammalian counterpart the role played by PfMCM6 in the PfMCM triad appears similar to that played by the mammalian counterpart. This conclusion is
supported by results showing that of all the *P. falciparum* MCMs, PfMCM-6 is the only member that is tightly associated with chromatin (Patterson et al., 2006). This also explains why of the six PfMCM proteins only PfMCM6 was found to specifically interact with and was phosphorylated by both PfMRK and PfPK (Jirage et al., 2010). It has been observed that PfMCMs bind to chromatin only during the G1/S and get dislodged as the S phase proceeds (Patterson et al., 2006).

Observations of motifs within Plasmodium CDKs and PfMCMs suggest that these proteins are involved in DNA replication. In fact, phosphorylation of the mammalian triad of MCM4, 6, 7 inactivates the helicase activity of this complex (which functions at the initiation of DNA replication) during the S phase (Ishimi et al., 2000). This technically ensures one round of replication per cell cycle. Whether the same happens in the case of the PfMCM4, 6, 7 complex, is currently not clear.

### 5.4 The Motifs and Modules

Using the ELM resource this study has demonstrated the presence of interesting motifs in the PfMCM6 protein at both the C and N termini that are consistent with DNA replication. For instance ELM prediction of the presence of LIG_CYCLIN_1 motifs in the N-terminal end of the PfMCM-6 sequence (Figure 14.) was largely expected. This cyclin recognition site that is sometimes referred to as Cy or RxL motif ([http://elm.eu.org](http://elm.eu.org)) is said to be present in many cyclin/CDK interacting proteins (Takeda et al., 2001, Lowe et al., 2002). Presence of this motif serves to hype the level of phosphorylation at (ST)Px(KR) sites. This motif would bind in a similar manner to that
of the p21kip cyclin inhibitor (Russo et al., 1996). Therefore presence of this LIG_CYCLIN_1 motif within Pf MCM6 is indicative of the protein’s potential interaction with CDK(s) that require a cyclin for complete activity. Notwithstanding, this study has shown that PfMCM6 is phosphorylated by PfPK6 in the absence of cyclin. However this study has shown that PfMRK requires a cyclin in order to phosphorylate PfMCM6. Thus these data show that PfMCM6 is phosphorylated by disparate kinases. Thus PfMCM6 is unique among proteins since it may be phosphorylated in the presence or absence of a cyclin.

Most protein phosphorylation by CDKs principally occurs in the nucleus. However, there is evidence showing that some of this phosphorylation may occur in the cytoplasm (Jirage et al., 2010). Regarding phosphorylation of PfMCM6 by PfPK6 cytoplasmic and nuclear phosphorylation may apply as PfPK6 has characteristics that are consistent with both a CDK and MAPK (Bracchi-Ricard et al., 2000). Since PfMCM6 is phosphorylated by PfMRK as well, it is not apparent whether both Kinases phosphorylate PfMCM6 in the same Subcellular compartment and whether the two phosphorylation events have temporal differences in their expression profiles. Additionally, this study has also shown that PfMCM6 has several nuclear export signal motifs, TRG_NES_CRM1_1, TRG_NLS_Monocore_2, TRG_NLS_MonoExtC_3, and TRG_NLS_MonoExtN_4. Presence of these motifs in PfMCM6 indicates involvement of nuclear export/import mechanisms in its physiology. These further augments the notion that phosphorylation of PfMCM6 may occur either in the cytoplasm or inside the nucleus. Interestingly, it has been observed that all MCM proteins show cell cycle-dependent subnuclear localization.
Thus, MCM proteins are found within the nucleus only during late mitosis and G1 (Mahbubani et al., 1997, Yan et al., 1993, Dalton and Whitbread, 1995). Whether the observed motifs have any bearing as to where the phosphorylation of PfMCM6 takes place, or whether they are just a consequence of PfMCM6 being a nuclear protein remains to be determined.

The presence of the LIG_APC/C_Dbox_1 binding motif within the PfMCM6 protein sequence further indicates how this protein operates. The Anaphase-promoting ubiquitin ligase complex APC/C selectively targets numerous cell cycle-regulatory proteins for ubiquitin-mediated proteosome-dependent degradation (Castro et al., 2006). Proteins containing LIG_APC/C_Dbox_1 motif utilize this motif as a signal for ubiquitin-dependent degradation. The counter part protein in this process is the ubiquitin specific protein 7 (USP7) which acts as a de-ubiquitination enzyme and belongs in the family of thiol and metallo-proteases that are responsible for processing ubiquitin and ubiquitin-like proteins (Amerik and Hochstrasser, 2004). The presence the USP7 binding motif would suggest the presence of a complete ubiquitination/de-ubiquitination system involved in the control of PfMCM6 during replication. Degradation via ubiquitin pathway is a regulator in initiaiation of DNA replication. In mammals, CDC6 protein which is required together with MCMs for initiation of DNA replication contains the same degradation motifs and is consequently ubiquinated by the APC/C sytem (Petersen et al., 2000). Whereas the Dbox motif was originally observed in cyclin B and therefore its presence largely expected in cyclins, it has been shown that other proteins may possess this motif (Arlot-Bonnemains et al., 2001). Therefore does the presence of
LIG\_APC/C\_Dbox\_1 within the PfMCM6 protein sequence mean that the PfMCM protein undergoes proteolysis using the ubiquitin pathway during the cell cycle as well? Petersen and others have suggested that the APC-CDH1 dependent proteolysis of CDC6 in early G1 and in quiescent cells is evidence that the proteolysis process is part of a mechanism that ensures the timely licensing of replication origins during G1 (Petersen et al., 2000). Studies using eukaryotic systems have shown that CDC6 and CDC18 both of which are members of the pre-replication complex (of which MCM proteins are part) are phosphorylated by CDKs leading to ubiquitin mediated proteolysis (Piatti et al., 1996, Drury et al., 1997, Jallepalli et al., 1997). This study has actively demonstrated the phosphorylation of PfMCM6, a replication licensing factor, by PfMRK and PfPK6. Thus phosphorylation of PfMCM6 by PfMRK and PfPK6 may wholly or in part target the PfMCM6 for ubiquitin mediated proteolysis. In support of this, a recent study has shown that PfMCM proteins oscillate during the various developmental stages of *P. falciparum* akin to the cyclins (Patterson et al., 2006). Therefore the presence of both the LIG\_APCC\_Dbox\_1 binding motif and the USP7 binding motif suggests a complete ubiquitination/de-ubiquitination system involved in the control of actions of PfMCM6 within the plasmodial cell cycle.

The MOD-SUMO motif peptide, AKAE was located between amino acids 838 and 841 of the PfMCM6 sequence. Sumoylation affects hundreds of proteins and regulates many cellular processes including gene expression, signal transduction, cell division (mitotic processes in mammalian cells), DNA repair and ubiquitin-dependent proteolysis (Zhang et al., 2008). Therefore the presence of this important motif in PfMCM6 may suggest
involvement in any of the mentioned processes especially cell division and ubiquitin-dependent proteolysis.

The LIG_WW_4 domain was also observed in PfMCM6. The LIG_WW_4 motifs are small modular domains of 38-40 residues long that are also involved in mediating protein-protein interaction through binding of short proline rich regions within proteins (Kato et al., 2002). The LIG_WW_4 motif recognition is serine-phosphorylation dependent. WW domain-containing proteins are involved in many cellular processes such as ubiquitin mediated protein degradation and mitotic regulation (Sudol et al., 2001). The presence of LIG_WW_4 domain in PfMCM6 suggests that PfMCM6 is regulated by the ubiquitin system. To illustrate the importance of WW containing proteins in the cell cycling in general, the human pin 1 protein that regulates mitotic arrest, binds to its cognate partners through the WW domain (Lu, 2000). In addition human pin 1 protein binds to the human RNA Pol II C-terminal domain (CTD) through the WW ligands (Sudol et al., 2001). The formation of complexes between CTD and other proteins containing the WW motif is mediated by phosphorylation of the CTD (Sudol et al., 2001). It is a well established fact that *Plasmodium falciparum* RNA Pol II C-terminal domain (PfCTD) is a specific substrate of the Plasmodium CDK PfMRK (Chen et al., 2006). This study has established PfMCM6 as a specific substrate of PfMRK as well. These observations strongly suggest that LIG_WW_4 motifs which depend on phosphorylation for activity have a role in the interactions involving PfMRK and its substrates including PfCTD and PfMCM6 (by inference).
A related motif identified in PfMCM6 at the ELM resource is the 14-3-3 ligand. 14-3-3 proteins interact with specific phosphoserine or phosphothreonine containing motifs, these proteins are involved in among other things cell cycle control and signal transduction (Tzivion and Avruch, 2002). 14-3-3 proteins’ chief role is to act as adaptor molecules to mediate protein-protein interaction. Since PfMRK and PfPK6 phosphororylate PfMCM6 there must be interaction of each kinase and PfMCM6. It is therefore easy to visualize PfMCM6 interacting with the kinases through this 14-3-3 scaffold motif.

The class three binding motif, LIG_PDZ_3, was also found within the carboxy terminus of PfMCM6. PDZ domains frequently occur in multiple copies or in association with other protein binding motifs in multi-domain scaffold proteins (Sheng and Sala, 2001). These modules found on the C- Termini of the proteins are important in mediating interactions for the assembly of large multi-protein complexes at specific subcellular locations (Stricker et al., 1997, Hung and Sheng, 2002). The presence of this important module within C-Terminal of the PfMCM6 sequence (Figure 16) confirms that PfMCM6 forms part of a multi-protein complex resulting in the Pre-RC (Tye, 1999).

The fork-head associated motif (FHA) was also found in PfMCM6. FHA domains comprise ~65-100 amino acids and form a β-sandwich fold consisting of a 3-stranded and a 4-stranded anti-parallel β sheet. While the N- and C terminal ends of the domain are located on adjacent β strands, the opposite side is bound by loops that act to coordinate phosphopeptide binding. FHA domains are found primarily in eukaryotic nuclear proteins that play a role in the DNA-damage response. The FHA domain mediates
phospho-peptide interactions with proteins phosphorylated by serine/threonine kinases. Most FHA domains recognize phospho-threonine with specificity provided by residues C-terminal to the phospho-threonine residue, particularly the +3 position.

FHA-domain containing proteins have roles in cell cycle checkpoint control, DNA repair, signal transduction, transcriptional regulation, and pre-mRNA splicing (Durocher et al., 1999, Durocher and Jackson, 2002, Durocher et al., 2000a, Durocher et al., 2000b). FHAs bind to Phosphothreonine motifs; it has been observed that all high affinity interactions use phosphothreonine which may be an essential requirement for biological ligands (Yuan et al., 2001). The role of FHA interaction domain in PfMCM6 may involve PfMCM proteins during S phase DNA damage, since recent studies indicate that MCM proteins sense and respond to S-phase DNA damage (Bailis and Forsburg, 2004).

Phosphoinositide-3-OH-kinase related kinases (PIKK) phosphorylation site was also found in PfMCM6. PIKKs are atypical protein kinases exclusive to eukaryotes. The PIKK proteins are large proteins with Ser/Thr kinase activity serving important role in DNA repair and DNA damage checkpoints (Bailis and Forsburg, 2004). The presence of the PIKK recognition motif together with the FHA domain within PfMCM6 show that this protein is involved in DNA repair/remodeling.

LIG_BRCT, an important protein motif was also observed in PfMCM6. BRCT domains are protein modules mainly found in Eukaryota. BRCT domains are present in proteins that are associated with DNA damage response. They recognize and bind specific phosphorylated serine (pS) sequences. This phosho-protein mediated interaction of the
BRCT domain has a central role in cell-cycle check point and DNA repair functions. Studies have shown that yeast Yph1p, an essential protein containing a BRCT domain, interacts with yeast origin recognition complex, ORC (Du and Stillman, 2002). This interaction in yeast may illustrate what is happening in Plasmodia where PFMCNM6 interact with ORC to form the Pre-replication complex (Figure 2).

The presence of a set of Src Homology 2 (SH2) ligands within PFMCNM6 furthermore suggests that this protein requires phosphorylation for its activity. The Src Homology domains are small modular domains that are involved in different signaling pathways. They recognize phosphorylated tyrosine residues, propagating the signal downstream therefore facilitating protein-protein interaction (Yaffe, 2002). The various SH2 domains observed (Figure 14) are an indicator to the requirement of Kinases in actions involving PFMCNM6 (Songyang et al., 1993). Another phosphorylation related motif identified in PFMCNM6 is the MOD_GSK3_1 representing a GSK phosphorylation site. GSK3 comprises two highly related proteins (GSK3-alpha and GSK3-beta). Its phosphorylation of several substrates is inhibitory in nature suggesting that GSK3 does phosphorylate PFMCNM6 thereby inhibiting its activity.

5.5 Interaction of PFMCNM6 with MAPK

Two motifs found within the PFMCNM6 protein (LIG_MAPK_1 and LIG_MAPK_2) are Mitogen Activated Protein Kinases (MAPK) docking sites. The presence of these two motifs within the sequence of PFMCNM6 is not surprising since one of the proteins that PFMCNM6 interacted with is PFPK6. PFPK6 has been observed to exhibit both the
characteristics of a MAPK as well as those of a CDK. PfPK6 has a 286-residue catalytic domain similar to that found in eukaryotic protein kinases. The identity of PfPK6 ORF to that of the human was shown to be 38% similar to CDC-2 and 33% similar to human p38MAPK (Bracchi-Ricard et al., 2000). This shows that PfPK6 is related to both MAPKs and CDKs. MAPKs are involved in signaling pathways characterized by a cascade of multiple kinases involved in the regulation of several extracellular stimuli and therefore control the cellular environment.

The MAPKs are serine threonine kinases that will usually phosphorylate substrates at the S/TP motif. However, S/TP sequence is found in most proteins and therefore additional factors are thought to be involved in directing individual kinases towards the correct substrate (Grewal et al., 2006, Bardwell, 2006, Bardwell and Shah, 2006). Substrate specificity is therefore ensured through interaction at docking motifs which are short amino acid stretches found on MAPK-interacting proteins. The most common docking motif consists of a group of two or more positively charged amino acids preceding a spacer of two-to-six residues from a hydrophobic–x-hydrophobic sequence where the hydrophobic residues are long chain aliphatics (Bardwell et al., 2001). The other type of motif is usually a short peptide containing the sequence FxFP which is normally downstream of the phosphorylation site (Galanis et al., 2001). Substrates can contain the classical docking motif or the FxFP or often may contain both (Sharrocks et al., 2000).

PfMCM6 is prominently expressed during the erythrocytic cycle peaking during the late schizont stage with a reduced expression only at the early trophozoite stage and peaking up again at the late trophozoite stage (Patterson et al., 2006). Likewise PfPK6 is mostly
expressed during the trophozoite stage (Bracchi-Ricard et al., 2000). Clearly, the expression of both proteins peak at the trophozoite and schizont stages. Furthermore, PfPK6 localizes both within the nucleus and the cytosol (Bracchi-Ricard et al., 2000) as does PfMCM6 during the erythrocytic cycle (Patterson et al., 2006). Presence of the nuclear export signal on the C-terminus of the PfMCM6 sequence is deliberate since this would bring it in proximity with its cognate kinase, the PfPK6. The co-expression of both PfMCM6 and PfPK6 during the same stages of parasite development and the colocalization of the two proteins in both the nucleus and the cytoplasm suggest that they are both available for the kinase to phosphorylate the substrate and confirm the previous two-hybrid interaction results (Eyase, 2006).

The expression of mammalian MCM proteins is controlled by MAPKs (Bruemmer et al., 2003). Similarly, the presence of MOD_ProDKin_1 site in PfMCM6, further suggests that, PfPK6 which has CDK and MAPK characteristics phosphorylates PfMCM6. MOD_ProDKin_1 site is phosphorylated by proline directed kinases, for both MAPKs and CDKs. From just these results, it is not clear whether or not PfPK6 acting as a MAPK is involved in signal transduction pathways leading to expression of PfMCM. More physiological studies are required to ascertain this.

5.6 PfMAT1 and PfMCM6

Previous studies have shown that the PfMAT1 protein directs PfMRK to its substrates. Furthermore, PfMAT1 stimulates phosphorylation of PfCTD substrate by PfMRK (Chen et al., 2006). In this study, it has been demonstrated that PfMAT1 stimulates
phosphorylation of PfMCM6 by PfMRK. This result concurs with studies indicating that MAT1 protein is a substrate targeting factor, directing certain proteins to their interacting partners (Devault et al., 1995). It has previously been observed that Mammalian MCM7 physically interacts with MAT1 both in vitro and in vivo through its C terminus (Wang et al., 2000). Whether PfMAT1 interacts with Plasmodial MCM proteins, and specifically PfMCM6, is beyond the scope of this thesis. However what is demonstrated here is that indeed PfMCM6 is a specific substrate of PfMRK.

5.7 Inhibitory action of flavonoids against PfMRK and PfPK6 with PfMCM6 as substrate

Flavonoids are ubiquitous phenolic compounds derived from plants and they consist of two aromatic rings linked by a three-carbon bridge. In plants, they are suggested to function as growth regulators and antimicrobial agents (Havsteen, 2002). Flavonoids have long been tested as possible anti-cancer agents and more recently as possible antimalarials (Lehane and Saliba, 2008, Manthey and Guthrie, 2002). The targets of these compounds are presumed to be cyclin dependent protein kinases which control the eukaryotic cell cycle (Kim et al., 2008). This study sought to characterize the potential use of PfPK6 and PfMRK with PfMCM6 as substrate to identify natural products that inhibit the two plasmodial kinases.

The activities of flavonoids in the class of abyssinones were characterized. When abyssinone IV was added to the phosphorylation reaction of PfMCM6 by PfMRK, the IC$_{50}$ was 15.99 µM. This was in contrast to an IC$_{50}$ of 31.17 µM when the kinase in this
reaction was changed to PfPK6 (Figure yyy). These results show that the inhibitory effect of abyssinone IV on PfMRK is twice compared to PfPK6 (Table 1). Thus, abyssinone IV is a good lead as a drug targeting inhibition of PfMRK. Incidentally this was the most active of all the flavonoids against PfPK6. The published IC$_{50}$ values of abyssinone IV against the chloroquine sensitive P. falciparum clone D6 and the chloroquine resistant clone W2 are 5.4 ± 1.5 µM and 5.9 ± 1.8 µM respectively (Yenesew et al., 2004). Clearly, there is a 3-5fold difference in the IC$_{50}$s obtained in this work compared to those of Yenesew et al. The differences in the two IC$_{50}$ values could be explained by the difference in the design of the two experiments. Whereas the data from Yenesew et al., was obtained using an in vivo assay system in live parasites, the data in the present work was derived from an in vitro assay system using purified recombinant enzyme. It is understandable then that the IC$_{50}$ results from these two systems are not the same. The difference of IC$_{50}$ values in the two systems may be explained by the fact that the in vitro system had only one kinase enzyme present at a time (either PfMRK or PfPK6) whereas the in vivo system many kinases including both PfMRK and PfPK6 were present in the assay. The inhibitory activity of abyssinone IV in the in vivo system may be multifocal involving a number of kinases present in the parasite whereas in the in vitro assay system could test only a single targeted kinase at a time. In fact, these conclusions are confirmed by the the results here showing that abyssinone IV inhibits both PfPK6 and PfMRK albeit to different levels.

As a pointer to which of the two CDKs may be more sensitive to flavonoids, the remaining flavonones tested showed much higher inhibitory activities in the ranges of
several-fold against PfMRK as compared to PfPK6. Abyssininine III showed the highest activity against PfMRK and second highest activity against PfPK6 (table 1). The published value for D6 and W2 remained largely the same as with Abyssinone IV above, at 5.8 ± 1.1 and 5.2 ± 1.5 respectively. This suggests that overall targets of the two flavonoids in the parasite may be the same. Abyssininone V and AbyssinoneV-4’-O-Methylether showed identical profiles for Pfpmrk. Similarly the difference between the two Abyssinones as regarding PfPK6 showed minimal difference. However there is a big difference between the D6 and W2 published values for these two Abbyssinones with Abyssinone-V-4-O-methylether giving the highest IC\(_{50}\) at 11.3± 2.1 μM for D6 and 11.1± 2.4 μM for W2 while those for Abyssininone V are 4.9± 0.8 and 6.1± 1.3 for D6 and W2 respectively. Notably the structural differences between the two Abbysinones lies in the replacement of an OH group in between two prenyl groups on the B ring of Abyssinone V with a methyl-ether group to create Abyssinone-V-4-O-methylether (Appendix). These data underscores the cardinal role that may be played by the OH group in parasite chemotherapy. The data also suggest that there may be other targets of the Abbysinone V and by extension the Flavonoids, other than the two CDKs.

Other two characterized flavonoids that were tested against the PfPK6-PfMCM6 system were Flavogaulin (Yenesew et al., unpublished data) and 5’ prenyl-pratensein. Interestingly these compounds had similar PfPK6 inhibitory profile as compared to Abyssininone V and Abyssinone-V-4-O-methylether. This is an indicator that mechanisms of inhibition may be the same. It is also worthwhile noting that the Flavogaulin result indicates a high sensitivity against both D6 and W2. In fact at ~2.2 μM and 2.0 μM for
D6 and W2 Flavogaulin has the highest sensitivity observed against the parasite in this study. It should however be noted that Flavogaulin IC\textsubscript{50} against PfPK6 at 126.59 \(\mu\text{M}\) was the highest reported in this study. Taken together, these results suggest that flavougalin may have a different target in the parasite other than PfPK6.

The differences in IC\textsubscript{50} values of the various flavonoids on PfMRK and PfPK6 may indicate functional differences between PfPK6 and PfMRK. In fact, PfPK6 phosphorylation activity may be constitutive whereas the activity of PfMRK is not (Doerig et al., 2000, Doerig et al., 2002). The fact that all tested flavonoids here were manifold active against PfMRK compared to PfPK6 suggests PfMRK is more responsive to flavonoid inhibition than PfPK6. It is interesting to note that PfPK6 contains a comparatively large insertion (15 amino acid residue) in its L loop which is absent in all other CDKs (Doerig et al., 2002). This insertion is supposedly involved in its constitutive activity (Doerig et al., 2002). Furthermore, structural models of PfPK6 indicate that this kinase is more compatible with the active or partially active structure of CDK2 as compared to that of inactive CDK2 (Doerig et al., 2002). In contrast, PfMRK requires presence of a cyclin for maximal phosphorylation. Overall, the inhibitory assays in this study suggest that PfPK6 poseses constitutive phosphorylation activity against its substrates. However, PfMRK is more sensitive towards flavonoid inhibitors compared to PfPK6. Therefore PfMRK may be considered a better target for development of Flavonoid derived antimalarials. On the other hand one may speculate that PfPK6-PfMCM6 would make an excellent screening system since it may only pick the most potent of Flavonoids.
CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has established that PfMCM6, a plasmodial replication licensing factor, is a specific substrate of two *P. falciparum* CDKs PfPK6 and PfMRK. Furthermore, the phosphorylation of PfMCM6 by PfMRK is enhanced by PfMAT1, a substrate targeting factor. Key motifs that indicate phosphorylation potential are present in the PfMCM6 protein sequence. Thus the presence of both cyclin and MAPK interactions motifs within the PfMCM6 sequence indicate a functional association between PfMCM6 and both CDKs and MAPKs. Presence of several NLS in the primary structure of PfMCM6 shows that this protein has a tropism for the nucleus where interaction with CDKs occurs. PfMRK was found to be more responsive to kinase inhibitors as compared to PfPK6. Finally this work has demonstrated that the cheaper non-radioactive Kinase-Glo® method of testing for kinase activity is as good as the radio-active method.

6.2 Recommendations

More studies will be required to ascertain the suggested compartmental co-localization of PfMRK-PfMCM6 and PfPK6-PfMCM6 to further delineate the physiological relevance of the PfMRK and PfPK6 phosphorylation of PfMCM6. More-over further testing of kinase inhibitors is required to establish the best experimental conditions for the PfPK6-PfMCM6 kinase inhibition model. The lead compounds (flavonoids) identified in this study ought to be further characterized/modified and studied to identify compound that
will have higher efficacy against plasmodium. The non-radioactive Kinase-Glo® method of testing kinase activity should be employed in place of radio active methods as it is safer and cheaper.
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APPENDICES

APPENDIX 1: PfMCM 2-7 PROTEIN SEQUENCES.

>PfMCM2:

1 MEDKKKLEED LESNKYDIDE EDLLEDEGRL NEEERQAELGE GESLFEDEDEG FIGGADDEKK
61 EMQKLRNLGL DNDYDDDFI DDELDDYDNL KARRAAERHM QMQRQ#EGKY EKMKFWKITLE
121 DQLEGDDEEE DIFDKVAEKV AKRRENHLT AEETDIPDLS NLESAKICLS VPNKDVVFEDE
181 RYQQAADTCF RYFLHKFSLK DSMGLNIDSE NTDEHEEEEM NSHQYYIDK IEKMILNDKH
241 TLIVSALKHL QFHCENLVQW IEFPKEQILE VLHECLMVEA YRISPKLKYG RICKVVRDWS
301 PYSQQLRNLR CETELTLIKV TGVCIKRGVY LPKLRVMYLK CNSCTTTLSE VPIYFADGKK
361 PVLPRRCPHC QSATFSVDRI KTAYTQYQI TLQESPCSVP AGRAPRQREV VVTGDVLKD
421 KPGEEVEVLG IYKTKYDGL NIKYGFPILO TEIEANNIER KEDIQLSELT EDDIKDILKL
481 SKDPNIHERI ITSIAPAIGW HKDIKTSIAY ALFGGVQKGG DKSFSKNET NNFGVQNRDII
541 LNNFKGOGTI RGDINVLLLQ DPGGLKSQVL QVYKMTNLRT YTTGKGSAS VGLTAGVRKD
601 HTTNEWTLEG GALVLADEGI CIIDEFDKMT DKDRVSIHEA MEQQISISIK AGIVTLRAR
661 CAVIAAANPI YGRYDSPSLF KENVDSLPDI LSIPDDLTVL RDIIPNVDDEFI YLAEPVVTRH
721 QLSHPKLENT QNYQKRIENL KNVISSSSAY EPIPQDLLQK YIIYARTNCK PSLSDVPYAE
781 ISAKLSNFYS RVRQKASASG GYPLTRHLIE SIIRIIEANA KMRLSHQIYS KDDVDIAITL
841 LESYVSCQRF AVAKQLSKEF ARYRALFRGG YEVRLELLLRR TVQHMDQQRN LKNASAKDFQ
901 NDSESGTGEAE AELNPNNVF LPLHIFMKTA MQNKFSEYQV VNWMKSVFN EHYAVIKRDG
961 VEGIISSKFK V

>PfMCM3:

1 MESLSIEKNS TPFGRSEYRT FNSELNYTIM DSSLNHSSIL DNSMKIEKDS RDKRLQKLNNQ
61 NIVSEYESGR QSVVFQQQKY QQOLLEGFFLF VQINKYIHQK ITELRTEAID EYNRMQNKNI
121 PNIIIHRQLI CNINNFQTTGN EFELLKACL IKEPYALPA YQAIAIHELWK SEDSKVVDIP
>PfMCM4:

1  MGTPRRRLGQ QNNNNNSPFA LSSSNIFGSN NEIFGSNFHM TPMSSRTTN SKSFLNSMLN
61  ESYLNNQSNAGSQFIKYGHT PLAIRRKACA RADIGDVGRE AFMEDVESGR LPHFIDSNLE
121  QIKELFNQFF DEFNITNYSDE VLDFTDEDRIS ISEYILLHRD NKLVYLAYY WKMIFKETG
181  RQCNECRLNNT NYEDDDENNE NSEGIRNLEH IKSFEIDLTH IFFNKKLYK LIEEYPSDCI
241  SEIDKIISTK YNSLALVLVE GDTRSSSSDK YPLSSTKQDY CRVRFNNKHK KDTPRKLGPN
301  QIETLVCVKVG VIIRCNIIP EMTMMAFKCT SKKRIGVNYE EKCNEEYEH VIQGEVQEPV
361  TCSNCCNKNT FELWHNNCFF SSSQLKLSE VTEHLKQGET PGSISIYAYD DLIDYTKPGD
421  TVELTGILKA SPVRNPNSR CYNSVHRTYI NVIHINKENT QKMpkttEQDND TANIILKRN
481  DGTVEENFEK LNEQGNNLFT TEVIQKMEQL SKDPNIYQRL VDSIAPSIYG REDIKKLLCC
541  QLFGGSKITD KYNKORSEI HILLCGDPSA AKSQOLLHYVH KLSPRGIYTS GKGSSSVGLT
601  AFISKDSETK EYILESAGAVV LSDKGICCID EFDKMDSSAR AILHEVMEQQ TVTIAKAGIV

118
661 ATLNARTSIL ASANPINSRY DKNKAVVENI NLPPSLFSRF DLIYLVIDQA NEDEDRLAT
721 VLCKNFSYNP EEEEEDEQED QEEDEPYIT QQRARKSKG SRKNERENYY NGGDNDDDDD
781 ISNYLNDNSD AQNKRGSWAN VNISYDEYNN SSNKKTSKNY LIDSNTLALY IAYCRITCNP
841 IISLESKKII IEYIKMRCK EGTKSPTASP RQLEGVLRLS QSLAKMKLR VVSPEEANEA
901 VRLMNIATFQ SLIDPLSGRI DFDQVNLGQT SQHHKKSDDL KDIIMNALVL KNMTKDELLT
961 HCHETIMNDF QHTTSMDRKS FEEAFYDLEK SQEITRCG SLYKKK

>PfMCM5:

1 MIGIQEGRAF FNPTRNPNNR PENNRIEEIN LPSLNEVNY FQDFLSRYSS NTLKEGNLKR
61 TLFENVKNNN FTLDLKLDI YKQQTTEFN EISATPRSER KMKKPENYSA ILARALSERP
121 LTLYLPITERV CYEVCTANIL NDEDEHLNY IQINLLNTFI RPTPIRGLLA ATQERFVVVP
181 GIIVQASKPQ HKMRKTLQC RYCDHKSID VPLWRDKPQL PPYCRRSSTM KSSMGLGNA
241 DSQGLCNGVL EPYVLIPNEC TFVDIQSLKM QELPEAVPTG DMRPLQRLNV TRYLCEKMP
301 GDRVYVHGVL TSYNPNPRT RADGTNSFSL HVLGFQKYDD MSGNDLNFDV EERELTLA
361 AEHNIHEKIF KSIAPELSYGM DEVKKACACL LFGGTRKRGB EETKIRGDIN MLMLGDFSVA
421 KSQILKFNVR CAPVVSYITSG KGSSAAGLTA AVMRDSQGFV SLEGGAVALA DGGVCCIDF
481 DCMRDDDVVVA IHEAMEQQT I SISKAGITTM LNTRCSVIAA ANFSFGSYDD SQDTTYQHDF
541 KTTILSRFDI IFLLRNKQDV EKDTILCNHI VALHASKHS QEGEIPLSLK TRYIYAKRE
601 IAPLLSKEAR DLSRNAYQVT RAERYGDRRS VTKKIPITLR QLESILRLAY SFAKMLSQF
661 ATDKHVQMSI DLFSASTAEKT AKQCLIFETM SPLEQKAVQK AEDAILGRLG KGQRASRVL
721 FRELQLRGFD RSALSKALAI LIKKGELQER GDRSVRRT

>PfMCM6:

1 MSAIFNESEL SGLDAHSAFG NNETSSFQKK KRRIDENSQM SVNDLVDDGE EEEDDDDEE
61 EPSYVQDQM KAVKFKFLKT FSEKKSEEDD DDGDSIWKDS LNFDFPSNLE IAKDSHYVLL
121 LFSILQNSYS RNKVLIVVMDK HVLMNPEFTDK NRFDIGSQLY MYIKRHLIRI LDIFEKKVL
181 LAESINPIKT KEGKICLRF YNKKNPIHSL RSLRCMLGEL MIVRGQVTR TSDVPELT
241 AAFKCNECGN IINGVKQQFR YTQPSKCPSA SCSNMSDWSL VLEQSYFVDW QKIRLQEIAQ
301 ESPPGSMPRN MDVILRNDIV DSVHAGDRII VTGCLIVVPD IPTLMKPGDV PRSVARQLLK
361 KNENSLVSQG LTGIKGVGVQ DLNHKLCIYA CQIEKLNSSK KDNNFDEQTQ VDINCEEILN
421 CDDLKWLRDI AMHPNTIDIL AEClAPKIWG NLEIJKGALL MMTGQVQKIT SNCKLRGDIN
481 MCIVGDPGTA KSEILKYMES FAPRAIFTSG KGSTAAAGTA AVHRDPDQGD TVLEAGALMY
541 ADQQICCCIDE FDKMDEKDRV AIHEAMEQQT ISITKAGIQA TLNARASVLA ACNPKVGRVD
601 TLKTFAQQVN IPAPLSRFD LFYTMLDID IDKDTSIANH LVSMHCGEEA EKHLRANAGK
661 LDSVKLEIYL ELSKRVKPLL TDEAKYKLIQ YYVSRNIEY SPGAQRSMRM TVRQLESLIR
721 LSEAVAKLKF SHFVDVKHVE IACSIFKASM KKISNEKEIN LDEEFDKVNN SLMGNKVNKL
781 DQETENTEQN KKTITIKASE YQYISAIIFE IIKEYEFNNN NESITQDQLI ETYLQVYAKA
841 ESSEQVDDEWI YKLKKINRL INQDIKLLE TNEDDPKVI IRHNPYAGP IVEGVSNKNT
901 YGYNTFKNYQ TQEKAPEDDV DFQEEIDNF
>PfMCM7:

1 MGERRRDIRS YLDDSHTKYL EAVKNYNAHI DELVRFDLTF EDPAVNHTNW GKLKYKGYLQ
61 KIYNHDTEVL PIYLGDLREY FSKENNDVDY SVYNGIMINT HRYMELLYSA ADKCLSDECY
121 KRFMRGYPEE DESEKIKKRN LRRINNEDNS GYSTDESEKE AFNNLFRDMI KPIEEIRQER
181 MKEYKLPAYL RVPNFEIIILP SSRDLVRKMR IVPNADCGSLL STFCEVIRA TQLKPRIQVA
241 TYECDRCHVF AYKAVDGFF MPLFDCPGCT NVHGRGSLK FQSKLSKFVK YQEIKVQELS
301 SQLPEGDIPR SMNCIIHGES TTSIQPGMSV TLTVLVMPVT KSGYQALKKG LIAEKVFHIY
361 YVQNNKENFN EHIDNYDKIM EQVQELKNSP NLYEKLAYNI GPEIYGHDDV KKAALLQLIG
421 GCTKKKKDG GG LIRGDHIILL MGDPGVAKSQ LMKKVCLIAS RSIYTTGKGS SSVGLTAADV
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661 QLESQQERSYS DTRINYTPPR ALLAIRISQ ALARLRDSV IETADFEAII RLTEQSKASV
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781 IDKYVEVTVF TINENNTAIA FPNVYNEHHF EYDEMEQEDY E

>gi|124513486|ref|XP_001350099.1| protein kinase 6 [Plasmodium falciparum 3D7]

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>gi|1695919|gb|AAC72269.1| MO15-related protein kinase PfMRK

[Plasmodium falciparum]
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241 LGTPNENNWP EALCLPLYTEFKATKDKFTYFKIDDDCIDLTLTLKLNAHERISAE
301 AMKHRYFFND PLPCDISQLPFNDL
APPENDIX II: PfMCM6 phosphorylation and PfPK6 and PfMRK kinase inhibition data

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Table 1 Data showing Phosphorylation values for the PfMCM6-PfPK6 substrate CDK combination. Rows A and B contained ATP, Reaction Buffer and the Kinase Reagent. Rows C and D contained ATP, PfPK6, Reaction Buffer and Kinase Reagent. Rows E and F contained ATP, PfPK6, PfMCM6, Reaction Buffer, and Kinase reagent. Rows G and H contained ATP, PfMCM6, Reaction Buffer and Kinase Reagent. Column 1, 2 and 3 had 10μl ATP columns 4, 5 and 6 had 12μl ATP and Columns 7, 8 and 9 had 15μl ATP.
Table 2. Data showing Phosphorylation values (Relative Light Units, RLU) for the PfMCM6-PfPK6 substrate CDK combination. Rows A and B contained ATP, Reaction Buffer and the Kinase Reagent. Rows C and D contained ATP, PfMRK, Reaction Buffer and Kinase Reagent. Rows E and F contained ATP, PfMCM6, Reaction Buffer, and Kinase reagent, Rows G and H contained ATP, PfMCM6, PfMRK, Reaction Buffer and Kinase Reagent. Column 1,2 and 3 had 10μl ATP columns 4,5 and 6 had 12μl ATP and Columns 7,8 and 9 had 15μl ATP.
Table 4: Data showing the Dose response inhibition of PfMCM6 Phosphorylation by PfMRK. These was done in three repeats, in Rows A, B and C
Table 4 Data showing the Dose response inhibition of PfPK6 kinase activity with PfMCM6 as the substrate. This was done in two repeats, in Rows A and B for inhibitor 692 and rows C and D inhibitor 707.
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APPENDIX IV: Chemical Structures of some of the tested Flavonoids.

Abbyssinone IV

Abbyssinone V

Abbyssinone V-4-O-Methylether

Abbyssinone V

Flavogaulin

5'-Prenyl-Pratensein
APPENDIX V: Isolated Flavonoids tested against PfPK6-PfMCM6.
## APPENDIX VI: ELM data for PfMCM6

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APPENDIX VII: The KEMRI IRB/ERC Research Approval Letter
KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel. (254) (020) 2722541, 2713549, 0722-265801, 0733-400009, Fax: (254) (020) 2720320
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1

January 20, 2010

TO: FREDRICK EYASE (PRINCIPAL INVESTIGATOR) MALARIA MOLECULAR LABORATORY KEMRI/WRP, EMAIL: feyase@erp-nbo.org

THRO': DR. JUMA RASHID, THE ACTING DIRECTOR, CCR NAIROBI

RE: SSC PROTOCOL NO. 1727 (INITIAL SUBMISSION): CHARACTERIZATION OF A PLASMODIUM FALCIPARUM PUTATIVE REPLICATION LICENCING FACTOR (PFRLF) AS A SUBSTRATE OF TWO PLASMODIAL CYCLIN DEPENDENT KINASES, PFPK6 AND PFMK.

Make reference to your letter received on the January 15, 2009. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 173rd meeting of KEMRI/National Ethics Review Committee held on Tuesday 15th December 2009, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this 20th day of January 2010, for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on 19th January 2011. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by 9th December 2010.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R. C. KITHINJI, FOR: SECRETARY, KEMRI/NATIONAL ETHICS REVIEW COMMITTEE