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THE CHARACTERIZATION OF THE PHOSPHATIDYL-INOSITOL -3-KINASE IN *PLASMODIUM FALCIPARUM* AND THE EFFECT OF SELECTIVE INHIBITORS OF THIS ENZYME ON THE PARASITE

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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2004

Declaration

I, Nokuhle Mtombeni, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or other university.

N. Mtombeni

N Mtombeni

4/05/2004

Publications and Presentations

Publications:

Inhibition of *Plasmodium falciparum* signal transduction pathway-alternative drug targets. Harmse L, Mtombeni N, Cummings B, van Zyl R, Havlik I & Coetzer TL. *Molecular Pharmacology* (in preparation).

Congress:

Cell cycle and signal transduction kinases in *P. falciparum*. Harmse LH, Havlik I, van Zyl C, Mtombeni N, Coetzer TL & Doerig CD. A section of this research has been presented at the British Society of Parasitology Conference, Manchester, UK, April 2003.

ABSTRACT

Malaria is the most prevalent parasitic disease in the world and the emergence of drug resistant strains of *Plasmodium falciparum* has made the search for new antimalarial drugs important. Protein kinases play an important role in cellular function and the phosphatidylinositol 3-kinase (PI3K) signal transduction pathway is implicated in diverse cellular processes such as glucose transport, cell survival and proliferation. A homology based approach identified an open reading frame (ORF) coding for the catalytic region of part of the 6.4 Kb ORF of PFE0765w gene sequence found at plasmoDB. The ORF consisted of 1 758 base pairs which coded for a 586 amino acid protein with a molecular weight of 68.5 KDa. The PfPI3K ORF was amplified from *P.falciparum* DNA, subcloned into an expression vector and the sequence verified. Analysis of the expressed protein obtained by Western blotting and probing with anti-His monoclonal antibody showed a protein of 68.5 KDa as well as some smaller products.

The effects of the PI3K inhibitors, wortmannin and LY294002 and the indirect activators of the PI3K pathway, forskolin and 1,9-dideoxy-forskolin on the *in vitro* growth of the chloroquine sensitive (3D7) strain of *P. falciparum* were determined using the [^{3}H]-hypoxanthine incorporation assay. The compounds inhibited parasite growth with IC_{50} values within the micromolar range in contrast to chloroquine and quinine which inhibited growth in the nanomolar range. LY294002 was the most active of the two PI3K inhibitors tested. The adenylate cyclase activator, forskolin was the least active. The inert analogue of forskolin, 1,9-dideoxyforskolin, inhibited the parasite growth with an IC_{50} value similar to that of LY294002. The information obtained in this study may lead to further studies in validating PI3 kinase as a potential drug target in *P. falciparum* and elucidating its function in the parasite.

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ABBREVIATIONS

A260/A280	Absorbance measured at 260 and 280 Wavelengths
AGC	Protein kinase A, G & C protein kinase group
APS	Ammonium persulphate
ATP	Adenosine triphosphates
BAD	Bcl-2 antagonist of cell death
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Btk	Bruton's tyrosine kinases
C	Molar concentration of oligonucleotide
C2	Calcium dependent/independent binding Domains
°C	Degree Celcius
cAMP	Cyclic adenosine monophosphates
Cas 9	Caspase 9
CDK	Cyclin dependent kinases
CD	Catalytic domain
cGMP	Cyclic guanosine monophosphates
CK1 family	Casein kinase 1 family
CMGC	Cyclin dependent mitogen activated glycogen synthase kinase 3 cyclin dependent kinase-like kinase group
cpm	Counts per minute
CREB	Cyclic adenosine monophosphate response element-binding protein
3D7	Chloroquine-sensitive *P. falciparum* strain

DAG	Diacylglycerol
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytosine-5'-triphosphate
ddNTPs	Dideoxynucleotide-5-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-5-triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine-5'-triphosphate
EBI	European Bioinformatics Institute
EDTA	Ethylene diamino tetracetic acid
ERK	Extracellular regulated kinase
FKHLR	Forkhead transcription factor
g	Grams
GENBANK	Genomic data bank
GSK3	Glycogen synthase kinase 3
ΔH	Sum of nearest neighbour enthalpy changes
HD	Helical domain
HEPES	4-(2-hydroxyethyl)-1-piperazinethane sulphonic acid
HRP	Horseradish peroxidase
IC_{50}	Inhibitory concentration required to decrease parasite growth by 50 %
Iκκ	IκB kinases
IP_3	Inositol-1,4,5-triphosphate

IPTG	Isopropyl-β-d-thiogalactopyranose
KDa	Kilodalton
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
M	Molar
mA	Milliamps
MAPK/ MEK	Mitogen activated protein kinase kinase
mCi/μCi	Milli/micro-Curie
mg/ml	Milligram per millilitre
Milli-Q® water	Distilled and deionised water
mM/ml/mg	Millimolar/millilitre/milligram
mPARA	Mean parasite control
mRBC	Mean red blood cell control
mRNA	Messenger ribonucleic acid
mTOR	Target of rapamycin protein kinase
μg/ml	Microgram per millilitre
μg/μl	Micrograms per microlitre
μl/μm	Microlitre per micrometre
μM/μg	Micromolar/micrograms
nM/ng	Nanomolar/nanograms
NCBI	National Center for Biotechnology Information
NIMA/NEK	Never in mitosis/Aspergillus
nM	Nanomolar
nm	Nanometre
ORF	Open reading frame

p110α, β, δ, γ	Phosphatidylinositol 3-kinase class 1 human homologues
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK	Phosphoinositide dependent protein kinase
PfCDPK1,2,3	*Plasmodium falciparum* calcium-dependent protein kinase 1, 2 & 3
Pfcrk-1	*Plasmodium falciparum* cdc2-related protein kinase
Pfcyc-1	*Plasmodium falciparum* cyclin 1
PfMAP/Pfmap-1	*Plasmodium falciparum* mitogen activated protein kinase
Pfmrk	*Plasmodium falciparum* cyclin dependent kinase activating kinase
Pfnek-1	*Plasmodium falciparum* never in mitosis protein kinases 1
PfPI3K	*Plasmodium falciparum* phosphatidylinositol 3-kinase
PfPK	*Plasmodium falciparum* protein kinase
PfPKG	*Plasmodium falciparum* cyclic GMP-dependent protein kinase
PI-3,4,5-P_3	Phosphoinositides
PI3K	Phosphatidylinositol 3-kinase
PI3K-C2α, β	Phosphatidyliositol 3-kinase calcium dependent α, β kinases
PIPES	1,4-Piperazinediethanesulfonic acid
PIP_2	Phosphatidylinositol-4,5-biphosphate
p70S6K	Ribosomal protein kinase
PKB	Protein kinase B

PKC	Protein kinase C
PlasmoDB	Plasmodium Genome Database
pRBC	Parasitised red blood cells
PVDF	Polyvinylidene fluoride
R	Molar gas constant
RBC	Red blood cells
RBD	Ras binding domain
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute
ΔS	Sum of the nearest neighbour entropy changes
SDS	Sodium dodecyl sulphate
STE kinases	Sterile 7,11,20 kinases
TAE buffer	Tris ethylene diamino tetracetic acid glacial acetic buffer
TBE buffer	Tris base boric sodium ethylene diamino tetracetic buffer
TBST buffer	Tris hydrochloride sodium chloride Tween buffer
TE buffer	Tris hydrochloride ethylene diamino tetracetic buffer
TEMED	N,N,N',x N'-tetramethyl-ethlenediamine
Tm	Melting temperature
V	Volts
v/v	Volume per volume
Vps34	*Saccharomyces cerevisiae* gene
W	Watts
w/v	Weight per volume

1.0 INTRODUCTION

Malaria remains a major problem in developing countries, where 0.7-2.7 million people die from malaria each year, with over 75 % of these being children and pregnant women (Weatheral *et al*, 2002; Maitland *et al*, 2003). This is likely to increase due to a breakdown in healthcare systems, an increase in drug resistance by the parasite to current therapeutic agents and the development of resistance to insecticides by the mosquito vector (Hougard *et al*, 2002). The protozoan parasite *Plasmodium falciparum* is the main causative agent of disease and death from malaria. This protozoa parasite causes multiple complications such as cerebral malaria, hypoglycaemia, metabolic acidosis and respiratory distress. Cerebral malaria is particularly dangerous with up to 30 % of infected patients dying (Adams *et al*, 2002). The prevalence of malaria caused by *P. falciparum* is of major concern to Africa due to its effect on productivity. The methods currently available to control malaria have proven to be inadequate. Furthermore *P. falciparum* resistance to chloroquine and the antifolates (pyrimethamine and proguanil) is now widespread (Ginsburg *et al*, 1999; Maitland *et al*, 2003). A more recent report of artemisinin and mefloquine resistance, now poses an urgent challenge for the development of affordable and more potent drugs (Ramachandran, 2002). Moreover the immediate prospects of a useful vaccine remain uncertain (Richie & Saul, 2002).

1.1 The *Plasmodium falciparum* life cycle

There are four species of *Plasmodium* responsible for human malaria, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* (Miller *et al*, 2002). *P. falciparum* causes the most severe form of malaria in humans, namely cerebral malaria which is a life threatening disease.

The protozoan parasites in the genus *Plasmodium* have a complex life cycle (Figure 1.1).

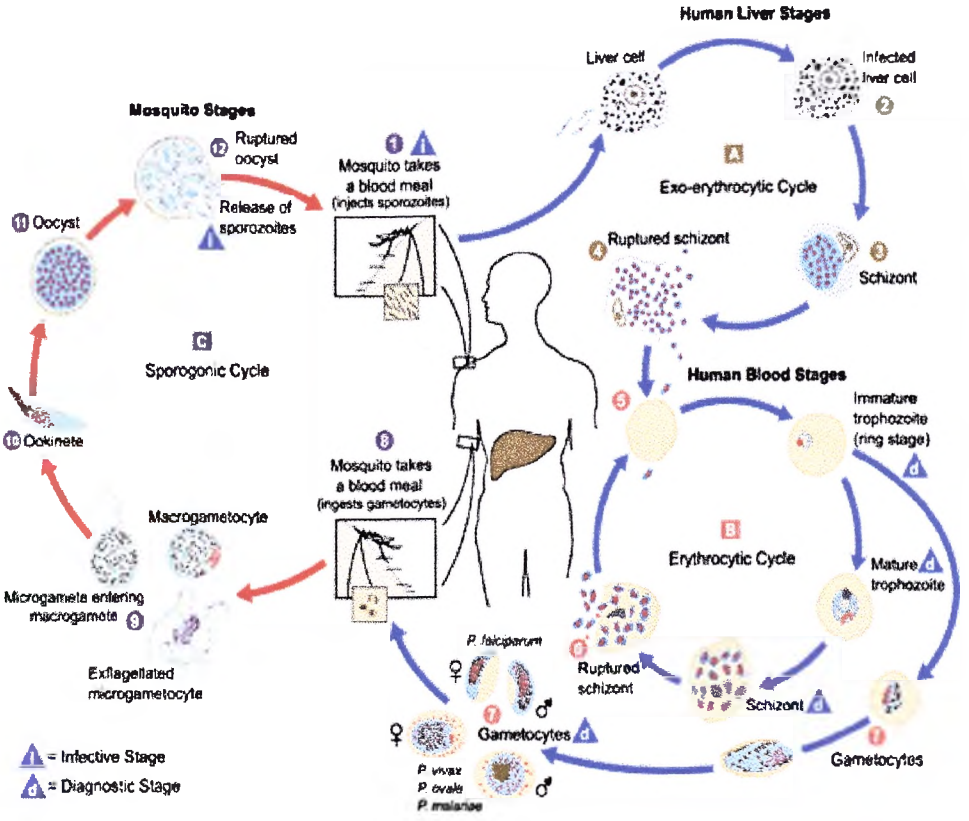


Figure 1.1: The life cycle of *Plasmodium*. An infected female *Anopheles* mosquito injects sporozoites into the human host when taking a blood meal (1). The sporozoites then infect the hepatocytes (2) and mature into schizonts (3). The schizonts then rupture and release merozoites (4) which infect erythrocytes (5) constituting the erythrocytic cycle (B). Some immature trophozoites may differentiate into gametocytes (7) which are taken up by a female *Anopheles* mosquito during a blood meal (8). The male and female gametes fuse to form zygotes (9) within the mosquito's stomach. The zygotes mature into ookinetes (10) which develop into oocysts within the mosquito's midgut wall (11). Mature oocysts release sporozoites which remain in the salivary glands until inoculation into another host (12) (http://www.dpd.cdc.gov).

The pathogenicity of this parasite is thought to be as a result from its rapid rate of asexual reproduction in the host and its ability to sequester in small blood vessels (Cowman *et al*, 2000). The asexual phase of the life cycle takes place in the vertebrate host whereas the sexual phase occurs in the female *Anopheles* mosquito (Lobo & Kumar, 1998). Infection in the human host begins with the inoculation of sporozoites from an infected female *Anopheles* mosquito during a blood meal. Upon entry, the sporozoites are transported to the liver where they invade the hepatocytes, multiply and mature into schizonts which release merozoites upon rupture (Weatheral *et al*, 2002). In the case of *P. falciparum* and *P. malariae*, the merozoites invade red blood cells initiating the intra-erythrocytic cycle (Figure 1.1 B). In *P. ovale* and *P. vivax* some sporozoites remain dormant in the hepatocytes, while the remainder develop into schizonts and enter the erythrocytic cycle. The erythrocytic cycle starts with the invasion of merozoites which form rings that mature to form trophozoites (Leete & Rubin, 1996). The parasite then develops into schizonts which rupture to release 8-32 merozoites that infect new erythrocytes (Figure 1.1, 6) (Weatheral *et al*, 2002). The clinical symptoms that ensue upon rupture include headache, myalgia, chills, fever and sweating associated with the fever(Wongsrichanalai, 2001; Weatheral *et al*, 2002). Some asexual parasites can differentiate into gametocytes which are critical for transmission of the parasites to new human hosts through the female *Anopheles* mosquito (Phillips, 2001; Weatheral *et al*, 2002). In *P. falciparum* the process of asexual development takes up to 48 hours and is repeated in the human host if not contained by antimalarial drugs or by the host's immune system.

The transition of the parasite from one stage of its complex life cycle to the next is influenced by many factors which result in changes in gene expression and cell cycle progression. The control of these various activities remains poorly understood, although they are

thought to involve signal transduction cascades in the parasite (Doerig, 1997). An understanding of the molecular mechanisms controlling parasite growth and differentiation may lead to the development of new therapeutic agents.

Signal transduction is a process whereby external chemical or physical signals facilitate a series of intracellular metabolic responses. In multicellular eukaryotes extracellular signal molecules such as epidermal growth factor, platelet derived growth factor and insulin bind to receptors localised on or in the cell membrane (Snaar-Jagalska & Schenk, 1999). Binding of a ligand (signal molecule) to its receptor can result in the activation of a transducing protein followed by the modulation of intracellular enzymatic activity. Activation of intracellular proteins may lead to the activation or inhibition of 'effector proteins'. The transducing proteins are usually protein kinases that are activated upon phosphorylation by other protein kinases and can lead to altered gene expression. This further leads to the stimulation of cell survival and differentiation (Snaar-Jagalska & Schenk, 1999). Elements of several signal transduction pathways have been identified in *P. falciparum* (Doerig, 1997). Evidence exists for the presence of the cyclic adenosine mono-phosphate dependent pathway (cAMP), the cyclic guanide mono- phosphate dependent pathway (cGMP), the mitogen activated protein kinase pathway (MAPK), the phosphatidylinositol cycle and calcium signalling (Doerig, 1997).

The availability of a complete annotated malaria genome has led to the identification of many *P. falciparum* protein kinases. The information of the genome can be exploited through molecular and biochemical studies to define the roles of specific protein kinases. Furthermore, protein kinases can be investigated as potential drug targets leading to the identification of lead compounds for drug design.

1.2 Protein kinases

Protein kinases are enzymes that can transfer phosphate groups from ATP to an amino acid residue of a protein. Protein kinases are found in signal transduction pathways where they regulate the activity of many effector molecules (Hanks, 2003). Phosphorylation is an important mechanism of regulation of protein activity. Phosphorylation can either activate or inhibit the activity of an enzyme and is therefore central to cellular signal transduction. The process can be readily reversed by protein phosphatases, making the control of phosphorylation states of protein kinases flexible (Snaar-Jagalska & Schenk, 1999). Protein kinases are essential for the regulation of diverse cellular processes including metabolism, stress responses, transcription, translation, DNA replication and cell-cycle control. In the multicellular eukaryotes they are also involved in more evolved functions such as neuronal signalling, apoptosis and cell-cell communication (Hanks, 2003).

In contrast to their involvement in normal cellular function, protein kinases have also been implicated in several diseases such as cancer and malaria (Doerig *et al*, 2002). Their potential as oncogenes has resulted in protein kinases being implicated in several human cancers, and have thus been investigated as potential drug targets. Several protein kinase inhibitors are currently in clinical development and some protein kinase inhibitors such as imatinib mesylate and sirolimus are already in clinical use (Bongiorno *et al*, 2003).

1.2.1 Protein kinase groups

Protein kinases were originally subdivided into five major groups according to similarities in their catalytic domains, modes of regulation and substrate specificities. The five groups consisted of

the AGC group; the CMGC group; the protein tyrosine group; the calcium calmodulin kinase group and the 'other kinases group'. The AGC group is composed of the protein kinase A, protein kinase G, protein kinase C, the G-protein coupled receptor family and the ribosomal S6 protein kinase family. The AGC group is regulated by cyclic nucleotides and activated upon the release of secondary messengers. The CMGC group is composed of the cyclin dependent kinases, mitogen activated protein kinase, glycogen synthase kinase 3 and cyclin dependent kinase-like kinases and located down stream of most signal pathways (Snaar-Jagalska & Schenk, 1999). The protein tyrosine group includes kinases that phosphorylate tyrosine residues. The calcium calmodulin kinase group is regulated by calcium calmodulin dependent protein kinases (reviewed by Hanks & Hunter, 1995). The 'other kinases' group, is made up of protein kinases that have not been easily classified according to modes of regulation, substrate specificities and similarities in the catalytic domain (Hanks & Hunter, 1995).

Following the completion of several genome projects, three additional major protein kinase groups have been identified; the sterile 7, 11, 20 (STE) kinases, casein kinase 1 (CK1) and the tyrosine-kinase like groups. The STE group is composed of three classes of protein kinases (STE7/MAP2K, STE11/MAP3K and STE20MAP4K) that are related to MAPKs and occur sequentially upstream of the MAPKs (Morrison *et al*, 2000). The STE group has been extensively identified in yeast sterile mutants. The CK1 group is composed of the casein kinase 1 family and play major roles in DNA repair and cell division. The CK1 group has been identified in the worm. The tyrosine-kinase like group is phylogenetically similar to the tyrosine kinases, however it includes the serine/threonine kinase receptors (Hanks, 2003).

1.2.2 Features of the conserved protein kinase catalytic domains

The serine/threonine and tyrosine kinases have a similar mode of action and have many common features, although they differ in substrate specificity. The catalytic domain consists of 250-300 amino acids which are further divided into twelve subdomains indicated by roman numerals (Figure 1.2). For the members of the serine/threonine and tyrosine superfamilies the smaller subdomains contain characteristic patterns of conserved amino acid residues and are rarely interrupted by large amino acid insertions. The catalytic kinase domain forms a two-lobed structure. The smaller N-terminal lobe includes the subdomains I-IV and is responsible for anchoring ATP. The larger C-terminal lobe whose function is to bind the substrate and initiate phosphotransfer, includes the subdomains VI-X. The subdomain V functions as a link between the N-terminal lobe and the C-terminal lobe of the catalytic region (Hanks & Hunter, 1995). The flexibility between the two lobes is due to an induced fit mechanism whereby the transition from an inactive form to an active form proceeds by a defined mechanism (Johnson *et al*, 1998).

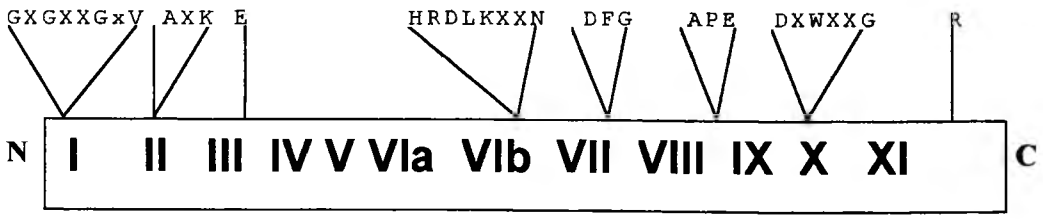


Figure 1.2: An illustration of a typical serine/threonine protein kinase catalytic domain. The protein kinase catalytic domain subdivided into twelve conserved subdomains is shown by Roman numerals. The consensus sequences in the subdomains are also shown (Snaar-Jagalska & Schenk, 1999)

1.3 Signal transduction pathways in *Plasmodium falciparum*

1.3.1 cAMP-dependent pathway

cAMP is an important secondary messenger in all eukaryotes. Its effects are exerted through the activation of cAMP-dependent protein kinases such as protein kinase A. In *P. falciparum* homologues of the cAMP-dependent pathway that include the membrane bound adenylyl cyclase and protein kinase A genes have been identified (Read & Mikkelsen, 1991). cAMP has been implicated in *P. falciparum* sexual differentiation and the gene encoding the *P. falciparum* protein kinase homologue has been isolated and identified using the Plasmodium Genome Database (PlasmoDB) (http://www.plasmodb.org).

1.3.2 Phosphatidylinositol cycle

Binding of signal molecules to cell surface receptors such as G-proteins, result in the activation of phospholipase C which causes the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2). The hydrolysis of PIP_2 results in the production of two second messengers namely diacylgylcerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 facilitates the transmission of the signal by stimulating the release of calcium ions stored in the endoplasmic reticulum. DAG transmits the signal by activating protein kinase C which phosphorylates and activates other protein kinases such as phosphatidylinositol 3-kinase (PI3K) and glycogen synthase kinase 3. The presence of the phosphatidylinositol cycle has been previously reported (Martin *et al*, 1994). The sequential accumulation of secondary messengers IP_3 and DAG at the exflagellation stage of *P. falciparum* gametocytes strongly infers that the phosphatidylinositol cycle is involved in the process (Martin, 1994). A gene PfNSM that encodes a phospholipase C homologue in

P. falciparum, has been identified in the PlasmoDB (http://www.plasmodb.org). The gene is expressed during the stage progression from trophozoite to schizont (Hanada *et al*, 2002). The presence of phosphatidylinositol-4,5-biphosphate and membrane bound phosphatidylinositol synthase in *P. knowlesi* infected erythrocytes suggests the presence of a complete phosphatidylinositol cycle (Elabbadi *et al*, 1994).

1.3.3 cGMP dependent pathway

The membrane bound protein, guanyl cyclase is activated upon binding of signal molecules resulting in the production of cGMP. The cGMP then exerts its effects by stimulating cGMP-dependent protein kinases which lead to activation of other protein kinases and changes in metabolic reactions. Exflagellation in both *P. falciparum* and *P. berghei* is stimulated by cGMP and guanylyl cyclase activators such as nitroprusside, whereas adenylyl cyclase activators have no effect on the process. This suggests that elements of the cGMP-dependent pathway are involved in gametogenesis (Kawamoto *et al*, 1990). The cyclic GMP dependent protein kinase has been identified in *P. faciparum* and found to be expressed in the ring stages of the *P. falciparum* life cycle (Deng & Baker, 2002).

1.3.4 Calcium signalling

Calcium released from the endoplasmic reticulum in eukaryotes is a secondary messenger in signal transduction cascades. In plants calcium stimulated protein kinase activities occur through calcium-dependent protein kinases. In *P. falciparum* calcium signalling has been implicated through the presence of calcium-dependent protein kinases in the asexual parasites (Read & Mikkelsen, 1990). Furthermore investigations into the role of calcium signalling in *P. falciparum* and *P. berghei* have shown that calcium antagonists such as TMB-8 and W-7 inhibited exflagellation in both species

(Kawamoto *et al*, 1990). A gene encoding a calmodulin homologue in *P. falciparum* was identified in the asexual parasites, where calmodulin was previously shown to be an intracellular receptor for calcium (Robson & Jennings, 1991; Leese, 1995). Three genes encoding predicted calcium-dependent protein kinases have been identified in *P. falciparum* and they include, PfCDPK1 (Zhao *et al*, 1993), PfCDPK2 (Farber *et al*, 1997) and PfCDPK3 (Li *et al*, 2000).

1.3.5 Mitogen activated protein kinase pathway

The mitogen activated protein kinases (MAPK) mediate responses in many signal pathways such as growth arrest, cell movement, cell proliferation and cell differentiation in eukaryotes. MAPK can be activated by molecules such as G-protein coupled receptors, receptor tyrosine kinases, activated ras, phorbol esters and protein kinase C (Cobb & Goldsmith, 1995). In *P. falciparum*, a MAPK homologue, namely *P. falciparum* mitogen activated protein kinase 1 (*Pf*map 1) has been identified and characterised in two separate laboratories and referred to as either *Pf*map-1 or *Pf*MAP (Doerig *et al*, 1996; Lin *et al*, 1996). It is suggested that the homologue is linked to sexual development due to the high levels of expression of the gene in gametocytes (Lin *et al*, 1996). Another hypothesis suggests that *Pf*map-1 has a role in the developmental phase leading to gameto-cytogenesis as the decision to differentiate seems to be made during asexual division (Doerig, 1997). A second MAPK homologue *Pf*map-2 has also been identified (Dorin *et al*, 1999; Doerig *et al*, 2002).

1.3.6 Other kinases identified in *Plasmodium falciparum*

In eukaryotic cells, the cell-cycle progression is controlled by cyclin dependent kinases (CDK) which constitute an intricate signalling system. *P. falciparum* cdc2-related protein kinase (Pfcrk-1) with homology to the p58 gene family which are down regulators of cell division, has been identified in *P. falciparum*. Pfcrk-1 may have a

role in sexual differentiation as suggested by the high level of expression of mRNA in gametocytes (Doerig *et al*, 1995). *P. falciparum* CDK activating kinase termed Pfmrk with 62 % sequence similarity and 46 % identity to human CDK activating kinases has also been identified in *P. falciparum*. Pfmrk may be involved in sexual stage development as it is predominantly expressed in gametocytes (Li *et al*, 2000). CDK-related kinases have been identified and characterised in *P. falciparum*, they include *P. falciparum* protein kinase 5 (PfPK5) a homologue of cyclin dependent kinase 1 (CDK1) (Ross-Mcdonald *et al*, 1994). Furthermore a protein Pfcyc-1 with an open reading frame (ORF) encoding a protein with homology to cyclin H from other organisms has been cloned and activates PfPK5 *in vitro* (LeRoch *et al*, 2000). *P. falciparum* protein kinase 6 (PfPK6) has sequence similarity to CDKs and MAPKs and exhibits 57.3% and 49.6 % similarity of the catalytic domain to human CDKs and human MAPKs respectively (Bracchi-Ricard *et al*, 2000). A *P. falciparum* kinase with homology to the NIMA/NEK family of protein kinases (Pfnek-1) has also been identified and cloned (Dorin *et al*, 2001). Sequence data from PlasmoDB predicts that *P. falciparum* has genes coding for seventy protein kinases (http://www.plasmodb.org).

1.4 The phosphatidylinositol 3-kinase pathway

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases that initiate signalling cascades by generating three membrane phosphoinositides, PI3-P,PI-3,4-P_2 and PI-3,4,5-P_3. The PI3Ks can also convert cellular phosphoinositides that are generally present near the cell membrane namely; PI3-P, 4-P_2, 5-P_2 to PI-3,4,5-P_3. The PI3Ks participate in a number of cellular processes such as cell growth and transformation, differentiation, motility, insulin action, cell survival, proliferation and carbohydrate metabolism. PI3Ks have

been found in all eukaryotes, including yeast and were also identified in *Drosophila* (reviewed by Vanhaesebroeck *et al*, 2001).
The PI3Ks phosphorylate inositol lipids on the position three of the inositol headgroup (Figure 1.3).

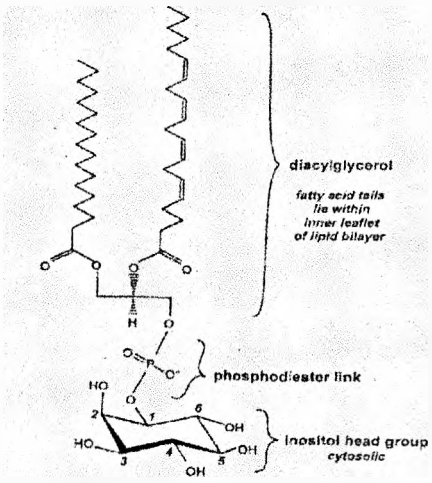


Figure 1.3: The structure of inositol lipids. The inositol lipid consists of a diacylglycerol linked by a phosphodiester bond to a inositol head group. The inositol head group is exposed to the cell cytoplasm, while the diacylglycerol is inserted in the inner layer of the membrane bilayer (Stein, 2001).

1.4.1 Classes of phosphatidylinositol 3-kinases

PI3Ks are divided into three main classes: I, II and III. They are grouped on the basis of sequence homology, *in vitro* substrate preference, the method of activation and regulation (Figure 1.4). Four homologues of mammalian PI3Ks have been defined to be in class Ia and b. PI3Ks in class II (PI3K-C2) have an additional C2 domain distinguishing them from the other two classes. Class III (Vps34) has no ras-binding domain (Figure 1.4).

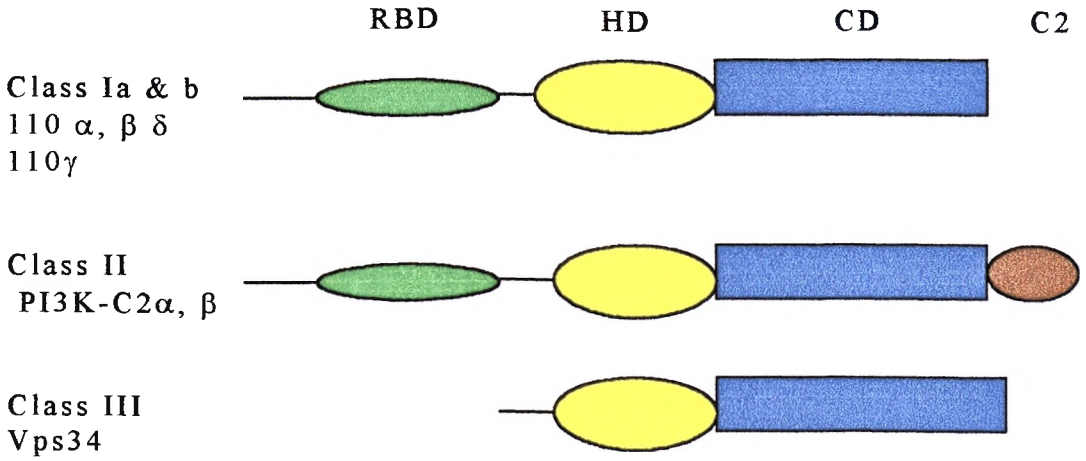


Figure 1.4: Structural features of the catalytic domain of the phosphatidylinositol 3-kinase family. The protein domains are as follows: ras-binding site (RBD), helical domain (HD), the catalytic domain (CD) and the C2 domain (C2), (reviewed by Fruman *et al*, 1998).

1.4.1.1 Class I phosphatidylinositol 3-kinase

Class I PI3Ks are divided into two subclasses namely Ia and Ib according to their means of activation. The class Ia PI3Ks include the mammalian PI3Ks, p110α, p110β and p110δ. The mammalian homologues were shown to share 42-58 % amino acid sequence identity. Class I PI3Ks have also been identified in *Drosophila melanogaster*, *Caenohabitis elegans* and *Dictotelium discoideum* and were shown to have three PI3K catalytic subunits (Figure 1.4) (reviewed in Vanhaesebroeck *et al*, 2001). The class 1a PI3Ks consists of a regulatory and catalytic unit. The catalytic subunit is 110-120 KDa in size whilst the regulatory subunit is normally 85 KDa. The class I PI3Ks mainly produce PI-3,4,5-P_3 *in vivo*.

The catalytic subunit has a ras binding domain, a helical domain and a C-terminal catalytic domain (Figure 1.4) (Fruman *et al*, 1998). Most of the membranous tyrosine kinases can activate class Ia PI3K

through binding of the Src-homology 3 domain found in the regulatory subunit. Thus receptors such as the insulin and platelet derived growth factor receptors strongly activate class Ia PI3Ks. The class Ia PI3Ks may also be regulated by interaction with the small G protein ras which interacts directly with the catalytic subunit.

The mechanism of action of the class Ia PI3K upon activation by external stimuli is the phosphorylation of a number of downstream effectors such as protein kinase B and protein kinase C, leading to changes in cell functions (Stein, 2001). A protein with 36 % identity to p110α which belongs to the class Ib, was also cloned and termed p110γ. Class Ib PI3Ks have a catalytic subunit 110 KDa in size and a regulatory subunit of 101 KDa. Class Ib kinases are mainly activated through G-protein signalling (Fry, 2001).

1.4.1.2 Class II phosphatidylinositol 3-kinase

Class II PI3Ks are large protein kinases that are 170-210 KDa in size. The catalytic subunit contains a helical domain, a catalytic domain 45-50 % similar to class I PI3Ks and a ras binding domain (Figure 1.4). All the class II enzymes have extensions to their amino acid sequence at both their N- and C-termini in comparison with class I enzymes. Class II PI3Ks have also been designated C2-containing phosphoinositide kinases due to possessing a C-terminal region with homology to C2 domains. The C2 domains are protein subunits that facilitate calcium dependent or calcium independent phospholipid binding and have been found to be involved in calcium dependent binding of protein vesicles (Stein, 2001). Class II PI3Ks include PI3Ks from *Drosophila*, mice and *C. elegans*. The mammalian class II isoforms are PI3K-C2α, β and γ which are encoded by three separate genes (reviewed in Vanhaesebroeck *et al*, 2001; Fruman *et al*, 1998). Activation of class II PI3Ks by molecular interactions through

external stimuli is still unclear, although class II PI3Ks appear to be activated by tyrosine kinase receptors (Stein, 2001).

1.4.1.3 Class III phosphatidylinositol 3-kinase

The third class of the family of PI3Ks is structurally related to the product of the *Saccharomyces cerevisiae* gene, Vps34 and the human homologue, Vps34p (Waterfield *et al*, 1997). The 94 KDa yeast protein Vps34, is associated with another protein Vps15 which is a serine/threonine kinase. Vps15 recruits Vps34 to the cell membrane leading to the activation of Vps34. The activated Vps34 is then involved in protein transport through the vacuole (Stein, 2001). PI3Ks of the third class have also been identified in *Drosophila* and *Dityostelium* (Vanhaesebrooeck *et al*, 2001). A human DNA encoding 30 % sequence identity to the Vps15 gene was cloned and also found to recruit Vps34 (Fruman *et al*, 1998). Class III PI3Ks mainly generate phosphoinositides *in vivo* (Stein, 2001).

1.4.2 Other related lipid and protein kinases

Other enzymes with catalytic domains that closely resemble PI3Ks include the phosphoinositide 4-kinase and the phosphoinositide phosphate kinases (Norris *et al*, 1995). The phosphoinositide 4-kinases are important in regulating phosphoinositide levels but have a minor direct role in signal transduction (Stein, 2001). The DNA-dependent protein kinases belong to the PI3K family and have a related kinase domain with a carboxyl terminal region but have no other regions of homology. The DNA-dependent protein kinases are larger than the class I PI3Ks with sizes of 200-400 KDa and appear to act as a protein kinase only. The DNA-dependent protein kinases are involved in the activation of DNA repair and the linking of this to cell cycle control. Other protein kinases related to PI3Ks include the target of rapamycin (mTOR). The mTOR is involved in the control of protein synthesis through activation of protein kinases such as p70S6K (Fruman *et al*, 1998).

1.4.3 The phosphatidylinositol 3-kinase/protein kinase B pathway

The PI3K/protein kinase B pathway has been extensively studied upon discovery that PI3Ks activation prevents cell death and PI3Ks also have a role in cell growth regulation (Fruman *et al*, 1998; Vivanco & Sawyers, 2002). The class Ia PI3Ks are mostly involved in the PI3K/protein kinase B pathway and their activation leads to cell survival or apoptosis (Figure 1.5) (Stein, 2001).

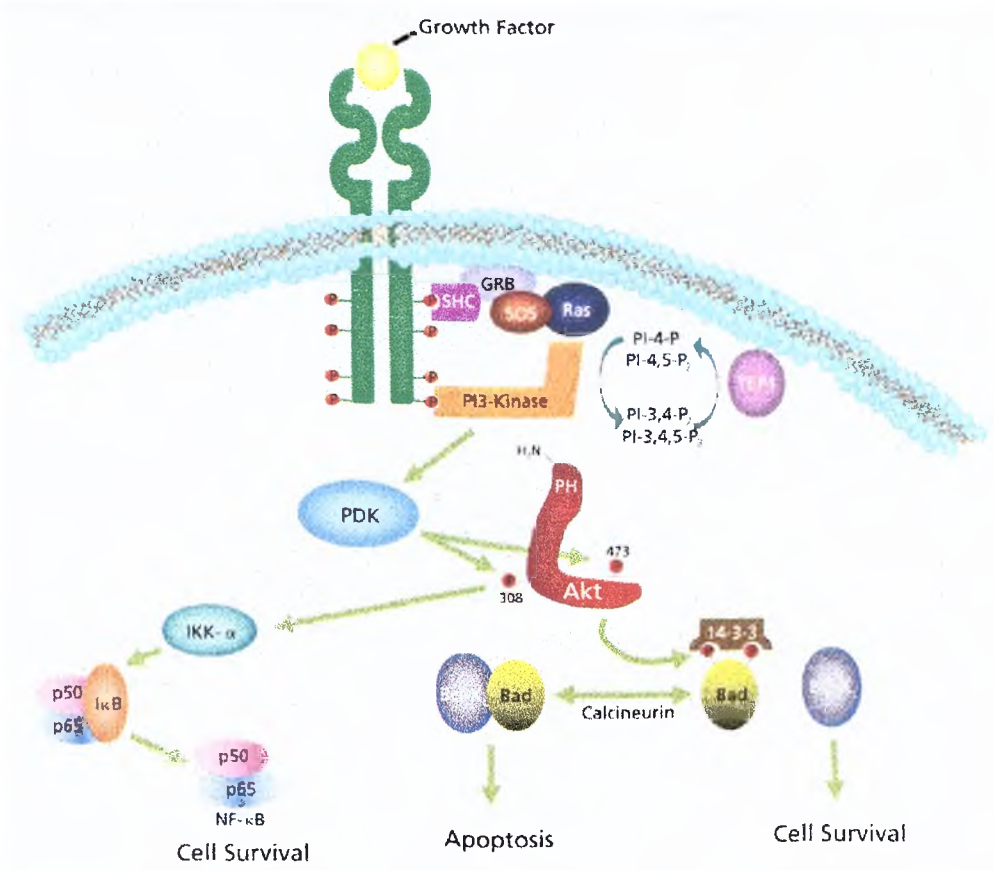


Figure 1.5: The phosphatidylinositol 3-kinase/protein kinase B pathway (Toker, 2000).

Ligands such as insulin, insulin-like growth factor, platelet derived growth factor, epidermal growth factor and basic fibroblast growth factor bind the receptor tyrosine kinase (Figure 1.5). This leads to

autophosphorylation of the receptor tyrosine kinase resulting in the production of phosphorylated tyrosine residues. The phosphorylated residues facilitate the recruitment and activation of secondary kinases such as PI3K (Figure 1.5). The phosphorylated residues recruit kinases that contain the Src-homology 2 domains. The activation of PI3K results in the production of phosphoinositides such as PI-3,4,5-P_3 at the cell membrane. These second messengers mediate signal pathways by membrane recruitment and activation of downstream effector proteins such as the phosphoinositide dependent protein kinase (PDK) (Ching *et al*, 2001). The phosphoinositides bind PDK at its pleckstrin homology domain and activate it. Upon activation PDK is able to phosphorylate other downstream effectors such as protein kinase B, which in turn leaves the plasma membrane to phosphorylate intracellular substrates such as glycogen synthase kinase 3 (Andjelkovic *et al*, 1999).

Activated protein kinase B can be translocated to the nucleus resulting in phosphorylation of transcription factors such as cAMP-response element–binding protein (CREB), forkhead transcription factors (FKHLR) and lead to the regulation of gene transcription (Coffer *et al*, 1998). PDK may also phosphorylate protein kinase C in the presence of phosphoinositides leading to the activation of MAPK, which leads to protein synthesis (Chantry *et al*, 1997).

1.4.4 Effects of the phosphatidylinositol 3-kinase/protein kinase B pathway

The diverse functions of PI3Ks contribute to mitogenesis, cellular growth, cell survival, glucose metabolism and cell motility (shown in Figure 1.6) (Stein, 2001). PI3K has a contributory role to cell survival signalling through its effector protein kinase B. The pro-apoptotic bcl-2 antagonist of cell ceath (BAD) is phosphorylated and inactivated by protein kinase B leading to cell protection from apoptosis (Vivanco & Sawyers, 2002).

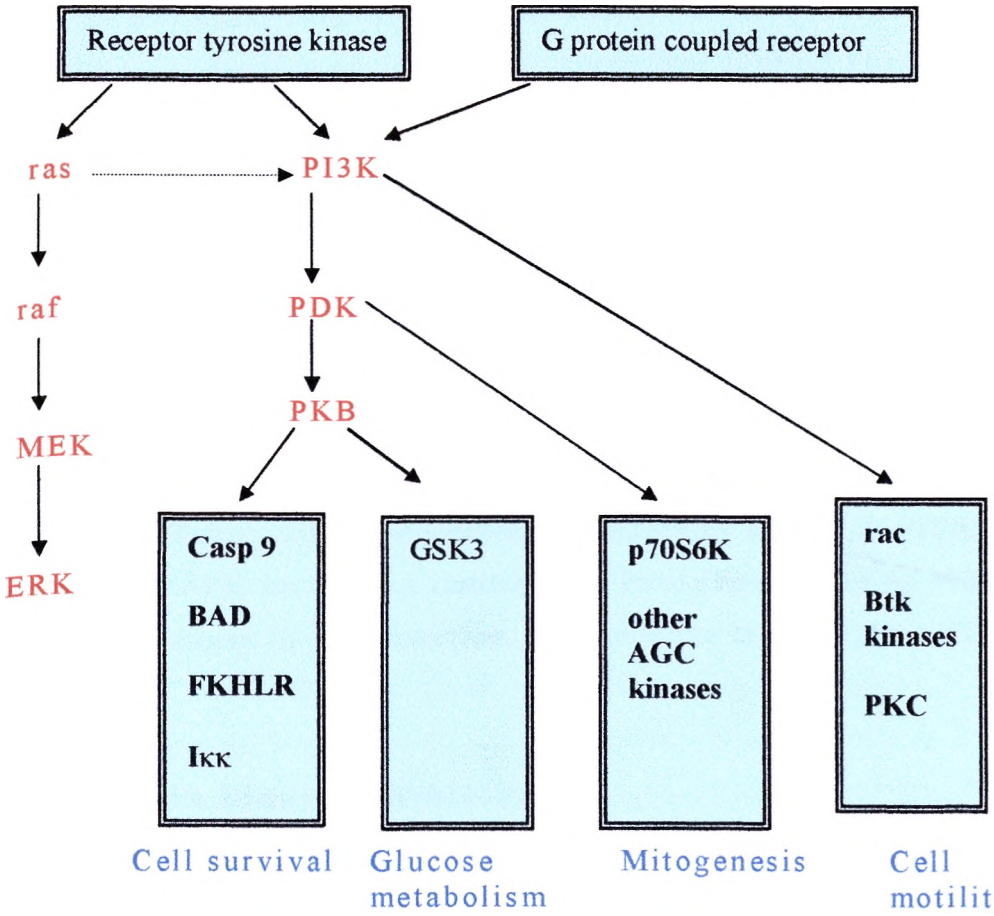


Figure 1.6: Biological effects of the phosphatidylinositol 3-kinase pathway. Three possible means by which PI3K is activated are shown and the downstream effectors of PI3K have been identified. The major functional pathways for transmission of PI3K signals lead to cell survival, glucose metabolism, mitogenesis and cell motility. The abbreviations used are as follows; protein kinase B (PKB), mitogen activated protein kinase kinase (MEK), extracellular regulated kinase (ERK), caspase 9 (Cas 9), bcl-2 antagonist of cell death (BAD), forkhead transcription factor (FKHLR), IκB kinase (Ikκ), glycogen synthase kinase 3 (GSK3), Bruton's tyrosine kinases (Btk), protein kinase C (PKC) (adapted from Stein, 2001).

Protein kinase B has also been shown to inhibit pro-apoptotic cysteine protease, caspase-9 and forkhead transcription factors (Figure 1.6). Studies on fibroblasts show that protein kinase B inhibits proteases that specifically cleave the RNA polymerase, thus promoting cell survival (Woodgett *et al*, 1996). PI3K activation of the downstream effector protein kinase C through PDK leads to the activation of ribosomal S6 kinase (p70S6K). Phosphorylation of 40S ribosomal protein S6 by p70S6K leads to the translation of several mRNAs. These mRNAs encode proteins necessary for the assembly of translational machinery and include ribosomes and elongation factors. This has led to the conclusion that p70S6K regulates protein synthesis (Toker, 2000). Potential mechanisms by which PI3K activates MAPK have been implicated. Phosphorylation of MAPK leads to changes in cell function such as gene transcription (Toker, 2000).

1.5 Protein kinase inhibitors

The importance of PI3K activity in many organisms has been investigated and proposed through the use of specific protein kinase inhibitors such as wortmannin and LY294002 (Stein, 2001).

1.5.1 Wortmannin

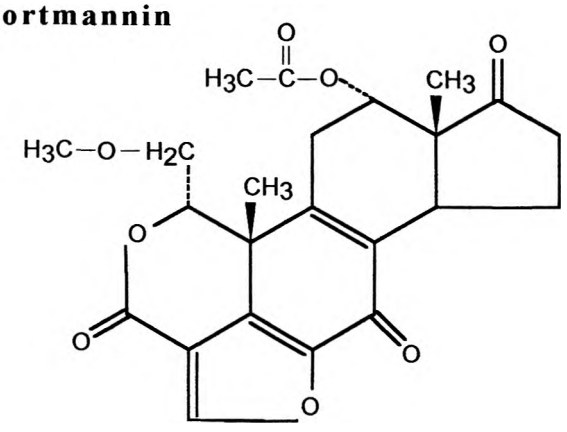


Figure 1.7: Chemical structure of wortmannin (Powis *et al*, 1994).

Wortmannin also referred to as SL-2052 is a steroid metabolite, first isolated from the fungal species *Penicillum fumiculosum* (Figure 1.7). It was later found to be produced by other fungi such as *Myrothecium roridum* and *Fusarium oxysporum* (Abbas & Mirocha, 1988). Wortmannin has been an invaluable tool in elucidating the role of PI3K in signal transduction pathways in mammalian cells as well as in many protozoa such as *Entamoeba invadens* and *Trypanosoma cruzi*. In *E. invadens* PI3K is implicated in various cellular responses which include encystation. Wortmannin was shown to inhibit encystation and protozoal growth of *E. invadens* in a dose dependent manner (0.5 μM-10 μM) (Makioka *et al*, 2001). Parasite invasion was also inhibited in a dose dependent manner when non-phagocytic and macrophage cells were treated with wortmannin. PI3K was shown to be activated upon invasion by *T. cruzi* in the host cells (Wilkowsky *et al*, 2001). Kinetic studies show that wortmannin, a non-competitive and irreversible inhibitor of PI3K kills cells at a much higher concentration (2-58 μM) than the isolated enzyme (4.2 nM) (Powis *et al*, 1994). Wortmannin has also been shown to inhibit PI3K enzymes isolated from calf thymus cells with an IC_{50} value of 2nM (Vlahos et al, 1994).

1.5.2 LY294002(2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran -4-one)

LY294002 has been extensively applied to rabbit smooth muscle cells to investigate the biological functions of PI3K activation. Studies have shown that LY294002 acts as a competive inhibitor of ATP at the catalytic site of PI3K and was found to inhibit PI3K in rabbit cell lines with IC_{50} values of 10-20 μM (Stein, 2001; Vlahos *et al*, 1994). LY294002 has also been shown to decrease the growth of ovarian carcinoma and ascite formation in a mouse model of ovarian cancer (Jaffe *et al*, 2000). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-

one (LY294002) is a synthetic morpholine derivative of the broad spectrum kinase inhibitor quercetin (Figure 1.8). Quercetin is a naturally occurring flavonoid in algae and vegetables that can inhibit a broad range of protein kinases, whilst LY294002 was developed as a selective PI3K inhibitor (Vlahos *et al*, 1994).

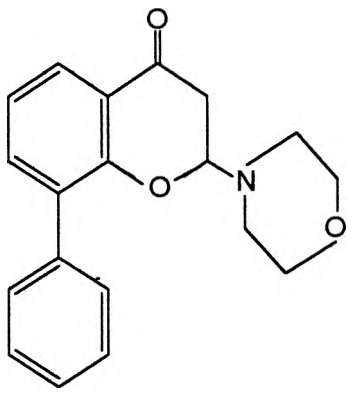
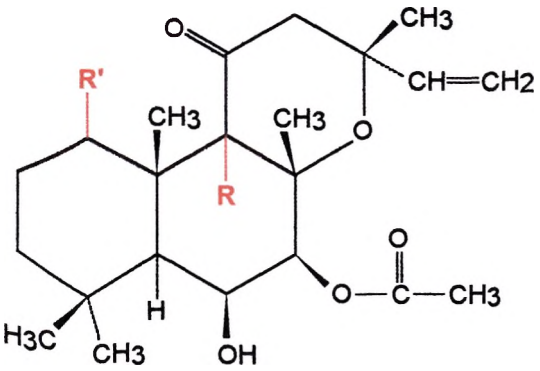


Figure 1.8: Chemical structure of LY294002 (Vlahos *et al*, 1994).

1.6 Indirect modulators of the phosphatidylinositol 3-kinase/protein kinase B pathway

1.6.1 Forskolin and 1,9-dideoxyforskolin

Forskolin (7-beta-acetoxy-8, 13-epoxy-1-alpha) also referred to as colforsin or coleonol is a lipid soluble diterpenoid isolated from the plant *Coleus forskohlii* (Figure 1.9) (Seamons & Daly, 1986). Many of its known biological effects are due to its activation of adenylate cyclase which results in an increase in intracellular cAMP concentrations in the cell and an activation of protein kinase B which is usually activated by PI3K (Seamons & Daly, 1986). Forskolin is prepared from roots of the *C. forskohlii*. Forskolin was shown to cause an increase in intracellular cAMP resulting in an activation of protein kinase B in a PI3K independent manner (Coffer *et al*, 1998).



	R	R′
Forskolin	OH	OH
1,9-Dideoxyforskolin	H	H

Figure 1.9: Chemical structure of forskolin and 1,9-dideoxyforskolin (Seamons & Daly, 1986).

cAMP is involved in development and differentiation in a number of lower eukaryotic species including *P. falciparum* (Read & Mikkelsen, 1991). Forskolin has also been shown to inhibit erythrocytic hexose transport and alter voltage dependent potassium channels in a clonal pheochromocytoma cell line (Wadler & Huang Yang, 1991). Forskolin was also shown to reverse acquired resistance in murine sarcoma cell line. Acquired resistance was shown to be as a result of the expulsion of drugs by the presence of the plasma membrane associated P-glycoprotein (Wadler & Huang Yang, 1991)

In mammalian cells P-glycoproteins are energy-dependent membrane proteins that transport ions and sugars, however may also act as drug transporters that actively pump a wide variety of drugs out of the cells such as doxorubicin (Crandall *et al*, 2000). Studies have shown that forskolin inhibits MAPKs in rat adipose cells resulting in the inhibition of glucose transport leading to decreased growth of cells (Joost *et al*, 1988).

Other pharmacological *in vivo* actions with therapeutic significance include firstly, the ability of a low concentration (2 nM) of topically applied forskolin to lower intra-occular pressure and reverse glaucoma within systemic cardiovascular effects. Secondly, forskolin is capable of producing smooth muscle relaxation in lung tissue, thus this bronchodilatory effect suggests it might be suitable as an anti-asthmatic agent. Thirdly, forskolin produces reversible positive inotropic and chronotropic effects in *in vivo* experiments, as well as being a potent antihypertensive agent proposed to be as a result of a decrease in peripheral resistance caused by the relaxation of the arteriolar smooth muscle. However therapeutic potential is limited due to its short half life (Seamons & Daly, 1986).

1,9-Dideoxyforskolin (7b-Acetoxy-6b-hydroxy-8,13-epoxy-labd-14-en-11-one) is an inert analogue of forskolin with respect to adenylate cyclase activation and produced by *Coleus forskohlii*. The minor chemical differences between these two compounds (Figure 1.9) results in 1,9-dideoxyforskolin being unable to stimulate adenylate cyclase and is useful as a negative control for forskolin (Seamons & Daly, 1986). As for forskolin previous studies have shown that 1,9-dideoxyforskolin inhibits the activated anion channels and chloride channel independently of their interactions with the P-glycoproteins (Chesnoy-Marchais, 2003). Binding of 1,9-dideoxyforskolin to the P-glycoprotein leads also to a reversal in acquired resistance to doxorubicin (Wadler & Huang Yang, 1991).

1.7 Chloroquine and quinine

Chloroquine is mainly effective against the intraerythrocytic stage of *P. falciparum*. However due to the onset of resistance there has been an urgent need to obtain other antimalarials that are effective against the intraerythrocytic stages of the malaria parasite. In this study

chloroquine and quinine are used as standard antimalarials for comparison against selected compounds tested.
The antimalarial activity of any drug including chloroquine relies on their accumulation within the parasitised erythrocytes. Chloroquine is a hydrophilic weak base which accumulates in the parasite's acidic food vacuole along a pH gradient. Heme also referred to as ferriprotoporphyrin IX (FPIX) accumulates as a result of haemoglobin digestion in the food vacuole. The drug interfers with the conversion of free heme to haemozoin. Polymerisation of FPIX into haemozoin is the mechanism by which the parasite is protected from the toxic properties of FPIX. The free FPIX can lyse membranes leading to the generation of reactive oxygen intermediates that can inhibit other metabolic processes in the parasite (Ginsburg et al, 1999). Chloroquine inhibits the polymerisation of FPIX into haemozoin by forming a complex with FPIX. An accumulation of FPIX-chloroquine haemolytic complex will eventually kill the parasite (Ginsburg et al, 1999). Quinine's antimalarial action is believed to be similar to the antimalarial action of chloroquine.

1.8 Gene identification using homology based methods

The DNA and amino acid sequence databases continue to grow in size thus providing useful information in the analysis of newly sequenced genes and proteins. The available databases include the PlasmoDB the official database of the malaria parasite genome project (http://www.plasmoDB.org), Entrez and the GENBANK. The *P. falciparum* genome project provides the finished sequence for *P. falciparum* (3D7 strain). The homology based approach involves the use of a set of algorithms such as the BLAST (Basic Local Alignment Search Tools) programme, introduced in 1990. The BLAST programme compares a query sequence to all the sequences in a specified database. Comparisons are made in a pairwise manner and

given a score reflecting the degree of similarity between the query and the sequence being compared. The higher the score, the greater the degree of similarity. The alignments made may either be global or local. Global alignments include characters from each sequence, while a local alignment includes only the most similar local regions. Distinguishing between real and artifactual matches is done using an estimate of probability, that the match might occur by chance. The probability value is known as the p value, where the most highly significant values will be close to zero. The expectation value may be used where the lower the expectation value the more significant the score (Altschul *et al*, 1997).

1.9 Aims of this study

PI3K is an important enzyme for intracellular signalling and is implicated in cell growth, proliferation and differentiation. A study to verify the sequence presence and function of this enzyme in *P. falciparum* will contribute towards the understanding of signal transduction pathways in *P. falciparum*. Therefore the specific aims of this study are;

1. To clone and verify the sequence coding for the catalytic region of the PI3K in *P. falciparum*.
2. To investigate the effects of specific PI3K protein kinase inhibitors such as wortmannin and LY294002 on the *in vitro* growth of *P. falciparum*.
3. To investigate the effects of indirect stimulation of the phosphatidylinositol 3-kinase pathway by forskolin and its inactive congener in *P. falciparum*.

2.0 METHODS

2.1 *In vitro* culturing of *Plasmodium falciparum*

2.1.1 Preparation of complete culture medium

Incomplete culture media was prepared by mixing 10.4 g RPMI 1640, 5.9 g N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer, 4.0 g glucose, 44 mg hypoxanthine and 50 µg/ml gentamicin to one litre. The media was sterilised by filtration through a 0.22 µm membrane and stored at 4°C until required. Complete culture media was prepared by adding 10 % (v/v) heat inactivated human plasma and 4.2 ml of 5 % (w/v) $NaHCO_3$ to incomplete culture media. All the buffers, media and solutions were prepared with Milli-Q® water.

2.1.2 Preparation of plasma

Three bags of human plasma from the South African National Blood Services were pooled into a one litre Schott bottle and inactivated at 56°C for 2 hours. The inactivated plasma was centrifuged at 1 750×g in 50 ml tubes at 25°C for 10 mins. The supernatant was decanted and aliquoted into sterile 50 ml tubes and stored at -20°C.

2.1.3 Preparation of erythrocytes

Whole blood with anticoagulant acid citrate dextrose (ACD) was obtained from the South African National Blood Services and stored at 4°C for a maximum period of three weeks. For the daily culture maintenance, erythrocytes were washed three times with phosphate buffered saline (PBS) buffer (pH 7.2) (8 g NaCl, 0.3 g KCl, 0.73 g $Na_2HPO_4.2H_20$ and 0.2 g KH_2PO_4 to one litre) by centrifuging at 400×g for 5 mins at 25°C. The supernatant was removed along with the thin layer of white blood cells. An equal volume of culture media was added to the washed erythrocytes and stored at 4°C for two weeks.

2.1.4 Preparation of a *P. falciparum* culture from cryopreserved stock

A cryopreserved stock of the 3D7 strain of *P. falciparum* was thawed in a preheated waterbath at 37°C (section 2.1.8). The culture was then aseptically transferred to 15 ml calibrated tubes (Elkay, U.S.A). For every millilitre of thawed suspension 0.1 ml of 12 % (w/v) NaCl was slowly added and mixed gently. The suspension was left to stand for 5 mins at room temperature. Nine volumes of 1.6 % (w/v) NaCl was slowly added to the suspension which was then centrifuged for 5 mins at 400×g. The pellet was resuspended in 9 volumes of a 0.9 % (w/v) NaCl and 0.2 % (w/v) glucose solution. After a further centrifugation step at 400×g for 5 mins, the pellet was resuspended in complete media containing 20 % (v/v) heat inactivated human plasma to obtain a 5 % haematocrit. The suspension was transferred to a flask and gassed with a mixture of 3 % O_2, 92 % N_2 and 5 % CO_2. The flask was then incubated at 37°C (Freese *et al*, 1988).

2.1.5 Culture maintenance

The 3D7 *P. falciparum* strain is a cloned chloroquine-sensitive strain obtained from C. Doerig, Inserm U511, Paris, France, which was cultured *in vitro* according to the method described by Jensen & Trager (1977) and modified by Freese *et al* (1988). The *P. falciparum* 3D7 culture of 25 ml was maintained in 175 cm^2 culture flasks at a 5 % haematocrit. The culture media was aspirated and replaced every 24 hours. The parasitaemia was maintained between 5-10 % and determined daily by preparing Giemsa stained thin blood smears. When the parasite percentage was >8 %, 80 % of the culture was transferred to a 50 ml centrifuge tube (Nunc, Denmark) for harvesting by centrifugation and saponin lysis (section 2.1.9). The harvested parasites were replaced with freshly washed erythrocytes (section 2.1.3). The suspension was then resuspended in 25 ml complete culture media and gassed for one minute with a 5 % CO_2,

3 % O_2 and 92 % N_2 gas mixture and incubated at 37°C.

2.1.6 Determination of percentage parasitaemia

Giemsa stain (Merck, Germany) was diluted five-fold in phosphate buffer (pH 7.2) which was prepared by dissolving 3.5 g KH_2PO_4 and 14.5 g $Na_2HPO_4.12H_2O$ to one litre. The buffer was autoclaved and stored at 4°C. Thin blood smears were prepared from cultured parasites and air-dried before being fixed with 100 % methanol. The blood smears were again air-dried and stained in 20 % (v/v) Giemsa stain (Merck, Germany) for 20 mins. The slides were rinsed with water and dried. The slides were examined microscopically to determine the percentage parasitaemia and parasite morphology at a 1000X magnification under oil immersion (Jensen and Trager, 1976). The percentage parasitaemia was determined as follows:

$$\% \text{ Parasitaemia} = \frac{\text{Infected RBC}}{\text{Infected + Uninfected RBC}} \times \frac{100}{1}$$

Where RBC = Red blood cells

2.1.7 Synchronisation of the culture

P. falciparum 3D7 cultures were synchronised using the procedure described by Lambros & Vanderberg (1979). Cultures in the early ring stage were centrifuged at 400×g for 5 mins at room temperature in 50 ml centrifuge tubes. The supernatant was discarded and the pellet was resuspended in 20 ml of sterile 5 % (w/v) D-sorbitol and incubated for 20 mins at room temperature. The culture was then centrifuged for 5 mins at 400×g at 25°C. The supernatant was aspirated, the pellet resuspended in complete culture media, transferred back to a culture flask, gassed and returned to the incubator. To maintain a synchronous culture synchronisation was repeated every 48 hours when the parasites were in the early ring stage.

2.1.8 Cryopreservation

P. falciparum 3D7 cultures were cryopreserved according to the method described by Freese *et al* (1988). The freezing solution was prepared by dissolving 7.2 g NaCl, 1.48 g $Na_2HPO_4.2H_2O$ and 0.44 g KH_2PO_4 in 100 ml of sterile water. Of the freezing solution 7.2 ml was mixed with 28 ml glycerol and filter sterilised. A culture in the ring stage was centrifuged at 400×g for 5 mins at 25°C. The supernatant was aspirated and the pellet resuspended in a 1:1 ratio with the freezing solution. The suspension was aliquoted into 1 ml cryotubes (Nunc, Denmark). The tubes were left to stand for 10 mins at room temperature and were then placed in liquid nitrogen.

2.1.9 Harvesting of parasites

The 3D7 *P. falciparum* culture was maintained as described in section 2.1.5. Cultures with a 10 % parasitaemia of predominantly trophozoites were used for harvesting. The parasite culture was centrifuged in a 50 ml centrifuge tube (Nunc, Denmark) at 400×g for 10 mins. The supernatant was aspirated and the pellet resuspended in 30 ml of 0.015 % (w/v) saponin to lyse the erythrocytes and release the *P. falciparum* parasites. The suspension was centrifuged at 10 000×g for 10 mins, the pellet resuspend in 5 ml PBS buffer and centrifuged again at 10 000×g for 10 mins. The supernatant was aspirated, the pellet resuspended in PBS buffer (1 ml) and transferred to a 1.5 ml microfuge tubes (Eppendorf, Germany) and centrifuged at 13 000×g for 5 min at 4°C. The supernatant was aspirated and the microfuge tube containing the parasite pellet frozen in liquid nitrogen and then stored at -70°C.

2.2 Molecular methods

2.2.1 Data mining of the *Plasmodium falciparum* genomic database

A homology based approach was used to identify an ORF coding for a kinase with high homology to PI3K in the malaria genomic database. The method is based on the fact that proteins with similar functions are known to have conserved regions at their catalytic sites. The malaria sequence database at NCBI (http://www.ncbi.nlm.nih.gov/malaria/plasmodiumbl.html) was screened using the BLAST programme, tblastn, which compares a specific protein/amino acid query sequence obtained from the Entrez protein database against a dynamically translated nucleotide database. The contigs containing nucleotide sequences with statistically significant similarity (low E values) were then subjected to an ORF search using the open reading frame finder at the NCBI website (http://www.ncbi.nlm.nlm.nih.gov/gorf.html). The ORF obtained was compared to the GENBANK using the BLAST programme, blastp, which compares an amino acid query sequence against all known protein sequences in the GENBANK.

2.2.2 DNA isolation

The frozen isolated parasites were resuspended in 50 µl autoclaved water and 950 µl lysis buffer (0.1 M EDTA, 0.5 % sodium dodecyl sulphate (SDS), 10 mM Tris pH 8). EDTA is a chelating agent which chelates Mg^{2+} and Ca^{2+} ions required as cofactors for DNase activity. SDS is a detergent which disrupts the cell membranes (Brown, 1990). An equal volume of phenol (pH 8) was added to denature and remove proteins from the DNA solution. Phenol has to be buffered to an alkaline pH to prevent the DNA from being deposited at the interface of the organic and aqueous phases (Sambrook *et al*, 1989). The suspension was then mixed to create an emulsion. The suspension was centrifuged at room temperature at 10 000×g for 15 mins and the

phenol extraction was repeated at least once to clear the upper phase. The upper phase was removed to a fresh tube and an equal volume of chloroform was added to remove phenol and protein contaminants and centrifuged as before.

The upper phase was then removed to a fresh tube and genomic DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of ice-cold isopropanol followed by mixing. The mixture was immediately centrifuged at 10 000×g for 20 mins at 4°C. The pellet was washed with 70 % (v/v) ice-cold ethanol and centrifuged again at 10 000×g for 5 mins at 4°C. The pellet obtained was resuspended in 500 µl of 1×TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.5) and digested with 50 µl of RNase A with a final concentration of 50 µg/ml. The solution was then incubated at 37°C for 30 mins. The DNase-free RNase was prepared by dissolving the lyophilised RNase in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl to a final concentration of 10 mg/ml. The solution was then boiled for 15 mins to inactivate the DNase and slowly cooled to room temperature before aliquots were stored at -20°C (Sambrook *et al.* 1989).

This was followed by a re-extraction of DNA as before with phenol (pH 8) and chloroform to remove the RNase. The upper aqueous phase was recovered to a fresh tube. The high molecular weight DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100 % ethanol and left at -70°C overnight. The DNA pellet was collected by centrifugation at 10 000×g at 4°C for 20 mins and washed with ice-cold 70 % (v/v) ethanol. The pellet was air dried in a sterile flow cabinet and resuspended in 50 µl of autoclaved water. To determine the purity and concentration of the DNA preparation, a UV spectrophotometer was used to scan a 10-fold diluted DNA sample between 200 and

300 nm. An absorbance peak observed at 270 nm indicated the presence of phenol contamination. The absorbance measured at 260 nm was used to calculate the DNA concentration where an A260 reading of 1 is equivalent to 50 µg/ml of DNA (Sambrook *et al*, 1989). The concentration of DNA was calculated as follows:

$$\text{DNA concentration } (\mu g/\mu l) = \frac{A260 \times 50\ \mu g/ml \times \text{dilution factor}}{1000}$$

The ratio obtained between the absorbance reading at 260 nm and 280 nm (A260/A280) is an estimation of purity of the DNA preparation. This indicates an absence of residual phenol, salts and protein. Pure preparations of DNA have A260/A280 values of ≥1.8. Contamination with protein and phenol decreases the A260/A280 ratio (Sambrook *et al*, 1989).

2.2.3 The polymerase chain reaction

The polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. The ORF from the 3D7 *P. falciparum* culture was amplified by PCR using the PCR Master Kit® and Expand High Fidelity PCR System® (Roche Molecular Biochemicals, Germany). All reagents were thawed and thoroughly mixed before the experiment and the reactions were prepared on ice in 0.2 ml PCR tubes (Molecular Bioproducts, U.S.A).

2.2.3.1 Primer design

Specific primers were designed from the ORF obtained for the sense and antisense strands. Nucleotides corresponding to the *Bam HI* restriction site were added to the 5'-end of the primer for the sense strand. Nucleotides corresponding to the *Hind III* restriction site were added to the 5'-end of the primer for the antisense strand to facilitate directional cloning into the expression vector.

A few nucleotides were added to the primers between the 5'-ends and restriction sited to serve as a support for the restriction enzyme during digestion. Their omission would prevent the enzyme from cleaving the DNA (Innis *et al*, 1988). The primers were manually analysed for complimentarity between the 3'-ends to prevent primer-dimer artifacts. The primers were synthesised by Integrated DNA Technologies (IDT), Inc, U.S.A. IDT uses the Allawi and SantaLucia set of nearest neighbour thermodynamic parameters for the equation to determine the melting temperature (T_m) of the oligonucleotides (http://www.idtna.com).

$$T_m = \frac{\Delta H}{\Delta S + R \ln (C)} - 273.15 + 12.0 \log[Na^+]$$

where, T_m = Melting temperature

ΔH = Sum of the nearest neighbour enthalpy changes

ΔS = Sum of the nearest neighbour entropy changes

R = Molar gas constant (1.987 cal. K^{-1} $mole^{-1}$)

C = Molar concentration of oligonucleotide

2.2.3.2 The PCR Master Enzyme reaction

The PCR Master Enzyme reaction was used to optimise the PCR reaction by determining the optimum annealing temperature, primer and DNA concentration. The PCR Master Kit® (Roche Molecular Biochemicals, Germany) contained all the reagents for the PCR in a premixed double concentrated Master mix. The PCR reactions were performed in a Perkin Elmer System 2400 thermal cycler (Perkin Elmer, U.S.A.) The PCR reaction with a final volume of 25 µl,

consisted of 1 μg of template DNA, primers to a final concentration of 0.5 μM and nuclease-free water to dilute the PCR Master by two-fold. The final concentrations of the PCR Master mix components were 1.5 mM $MgCl_2$, 0.2 mM of each of dATP, dGTP, dCTP and dTTP, 10 mM Tris-HCl, 50 mM KCl, pH 8.3 and 1.25 U *Taq* DNA polymerase in 0.005 % (v/v) Brij 35.
The programme used was as follows:

Step	Temperature (°C)	Time (mins)	Number of cycles
Denaturation	94	2	1
Denature	94	0.30	35
Anneal	54 - 64	1	
Elongation	72	1	
Final extension	72	7	1

The generation of template-independent artefacts like primer-dimers and non-specific amplification can occur at room temperature once all the reagents are mixed. This can be avoided by means of a modified hot-start method (Kellog *et al*, 1994). The tubes were kept on ice until the thermal cycler had reached a temperature of 90°C. This was also done for the Expand High Fidelity PCR reactions.

2.2.3.3 The Expand High Fidelity PCR System®

The Expand High Fidelity PCR System® contains a thermostable *Taq DNA polymerase* and *Pwo DNA polymerase* which has a 3'-5' exonuclease proof reading activity which minimises nucleotide misincorporations. Thus the Expand High Fidelity PCR System® results in a 3-fold increased fidelity of DNA synthesis.

Two separate master mixes were prepared to prevent the enzyme mix interacting with primers or template DNA without dNTPs. The interaction between the enzyme mix and dNTPs may lead to degradation of primer and template DNA through the

3'-5'exonuclease activity of the *Pwo* proof reading polymerase. The reaction mixtures were prepared on ice in PCR tubes and were carried out in a final volume of 50 µl.

Master mix 1 consisted of dNTPs, template DNA, primers and sterile water to a final volume of 25 µl. The final concentrations of the reagents used in the master mix were: 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer, 1 µg template DNA and 1× Expand HF® buffer (composition is proprietary). The reagents were thoroughly mixed and kept on ice.

Master mix 2 consisted of 1× Expand HF® buffer with a final concentration of 1.5 mM $MgCl_2$, 2.6 U enzyme mix and sterile water to a final volume of 25 µl. The reagents of the master mix 2 were thoroughly mixed and also kept on ice. The two master mixes were combined and mixed well just before transferring the PCR tubes to the thermal cycler. The PCR tubes were kept on ice until thermal cycler had reached 94°C. The final concentration of the reagents used were: 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 µM of each primer, 1 µg template DNA, 1x Expand HF® buffer with a final concentration of 1.5 mM $MgCl_2$ (pH 8.3) and 2.6 U of enzyme mixture.
The programme used for the Expand High Fidelity PCR System® was as follows:

Step	Temperature (°C)	Time (mins)	Number of cycles
Denaturation	94	2	1
Denature Anneal Elongation	94 63 72	0.30 0.30 1	35
Final Extension	72	7	1

The extension temperature for both *Taq polymerase* and *Pwo polymerase* enzyme mix in the Expand High Fidelity PCR System® is dependent on the length of amplification product. The optimum extension temperature for amplification of DNA sequences up to 3 kilobases is 72°C, therefore 72°C was used as the optimum extension temperature for the reactions.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the analysis of the PCR products and products obtained from the restriction digests. A 1 % (w/v) agarose gel was prepared in 1× TAE buffer (0.02 M Tris, 0.04 ml of 0.5 M EDTA, 0.571 ml glacial acetic acid to 500 ml water) and 1 µg/ml ethidium bromide was then added to the gel. Prior to loading onto gel, gel-loading buffer (10 % v/v) was added to the products being analysed. The gel loading buffer comprised of 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in 1× TAE buffer.

The gel was submerged in 1× TAE buffer and electrophoresis was carried out at 30 V for 3 hours. A 100 base pair molecular weight marker (Roche Molecular Biochemicals, Germany) was used to estimate the sizes of the PCR and digestion products. The bands were viewed using a White/Ultraviolet light transilluminator UVP®.

2.2.5 DNA sequencing

DNA was sequenced using the T7 Sequenase version 2.0™ DNA Sequencing Kit (Amersham Life Science, Inc, USA) which uses the dideoxy-chain termination method developed by Sanger *et al* (1977).

The method involves the *in vitro* synthesis of a DNA strand by DNA polymerase using a single-stranded DNA template. The sequencing kit has a sequenase 2.0 DNA polymerase for DNA sequencing with

properties that include a reduction in nucleotide misincorporations (Innis *et al*, 1988). Synthesis of a DNA strand is initiated at the site where a primer anneals to the template. The synthesis is terminated by the incorporation of a dideoxy analogue that will not support continued DNA elongation such as 2', 3'-dideoxynucleoside 5'-triphosphates (ddNTPs). The ddNTPs lack the 3'-OH group necessary for DNA chain elongation. The nucleic acid sequence of PCR products and plasmids containing inserts were verified by sequencing.

2.2.5.1 Preparation of sequencing gel

An 8 % acrylamide gel of 0.4 mm thickness was cast in a Sequi-Gen® GT Nucleic Acid Electrophoresis Cell (Bio-Rad, USA). Prior to assembling the electrophoresis cell, the glass plates were washed thoroughly and wiped with 100 % ethanol before the plates were assembled with 0.4 mm spacers. One of the plates was siliconised using silanising reagent, Repel-silane ES® (Amersham Pharmacia Biotech, UK) to facilitate separation of plates and the removal of the sequencing gel after electrophoresis.

The 8 % acrylamide gel solution was prepared by dissolving 7.6 g acrylamide and 0.4 g bis-acrylamide in 40 ml autoclaved water. To this mixture 42 g urea and 10 ml of 10× TBE buffer (108 g Tris base, 55 g boric acid, 9.3 g $Na_2EDTA.2H_2O$) was added. After the urea had dissolved the solution was adjusted to 100 ml with autoclaved water and degassed for 10 mins under vacuum. Polymerisation was initiated by adding one ml of 10 % ammonium persulphate (APS) and 25 µl N,N,N'x N'-tetramethyl-ethlenediamine (TEMED) just before the gel was cast. The gel was left to polymerise overnight at room temperature.

Before use the gel surface was rinsed with 1× TBE buffer and the gel assembly transferred to the electrophoresis apparatus where the sharkstooth comb was inserted to form wells. The buffer chambers were filled with 1× TBE and the gel was pre-run at 80 W to reach a temperature of 50°C and then the samples were loaded.

2.2.5.2 Annealing of primers

The PCR products were purified as described in the High Pure PCR Product Purification Kit® (Roche Molecular Biochemicals, Germany). The annealing mixture consisted of 7 µl (2 µg) PCR DNA product, 2 µl reaction buffer and 0.5 µM primer. The annealing mixture was denatured by heating it at 99°C for 2-3 mins and cooled by placing the vial immediately on ice for 5 mins. The tube was briefly centrifuged and chilled on ice until required.

2.2.5.3 Labelling reaction

To the ice-cold annealed DNA primer mixture the following reagents were added while the tubes were kept on ice:

1. 2 µl of 5× reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl)
2. 1 µl of 0.1 M dithiothreitol (DTT)
3. 2 µl of 1:5 diluted labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP)
4. 0.5 µl of ^{32}P-dATP (3000 Ci/mmol, 10 µCi/µl)
5. 2 µl of sequenase enzyme diluted 1:7 with enzyme dilution buffer (10 mM Tris-HCl (pH 7.5), 5 mM DTT)

The reaction mixture was mixed and incubated at room temperature for 5 mins to allow the DNA to incorporate the labelled isotope.

2.2.5.4 Termination reaction

Four tubes were labelled G, A, T, C and 2.5 µl of the dideoxy termination mixture (80 µM dGTP, 80 µM dATP, 80 µM dTTP, 80 µM dCTP and 8 µM of the appropriate dideoxy nucleotide) was added for each sequencing reaction and the tubes were preheated for 1 min at 37°C. After 5 min, 3.5 µl of the labelling mixture was transferred to each of the preheated termination tubes labelled G, A, T or C, mixed and incubated at 37°C for 5 mins.

The reactions were terminated by adding 4 µl of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol FF) and placed on ice. The reactions were briefly centrifuged in a microfuge at 13000×g to collect the reaction mixture at the bottom of the tube. The reaction mixtures were denatured at 80°C for 5 mins in a heating block immediately before loading 3 µl of the reaction mixture onto the gel in the order G, A, T, C. Three loadings of 3 µl each were obtained from each termination reaction and were separated by approximately 2 hours, with the run time for the first loading being 6 hours. The gel was electrophoresed at a constant power (80 W) using a PowerPac 3000 Power Supply (Bio-Rad, USA).

The glass plates were separated following electrophoresis and the gel was transferred to an old X-ray film and covered with cling-wrap. This was placed in an autoradiograph cassette and exposed to a CURIX medical X-ray film (35 cm × 43 cm, AGFA) overnight at -20°C. The film was developed with the kind permission of the X-ray unit staff within the Johannesburg General Hospital.

2.2.5.5 Subclone DNA sequencing

Plasmid DNA (section 2.2.7) was alkaline denatured by adding 4 µl of 2 M NaOH, 2 mM EDTA to 2 µg of DNA and incubated for 5 mins at room temperature. The reaction was neutralised by adding 20 µl of a 3 M sodium acetate (pH 5.0) solution. The plasmid DNA was precipitated with 150 µl ice-cold absolute ethanol at -70°C for 1 hour, centrifuged at 13 000×g in a microfuge at room temperature for 5 mins and washed with 400 µl 70 % ethanol. The pellet was air dried in a sterile flow hood overnight and resuspended in 7 µl water.

The plasmid DNA was sequenced using the sequencing protocol (section 2.2.7) except for the annealing mixture which consisted of: 7 µl DNA, 2 µl 5x reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 0.5 µM primer. The annealing mixture was heated to 65°C for 2 mins and slowly cooled to < 35°C over a 30 min period. The mixture was placed on ice and used for sequencing within 60 mins as described in section 2.2.6.

2.2.6 Subcloning of PCR products

PCR products were obtained using the specific primers for the PI3K gene. The PCR products were then inserted into the pQE-80L Histidine Tag Expression vector (Qiagen, Germany).

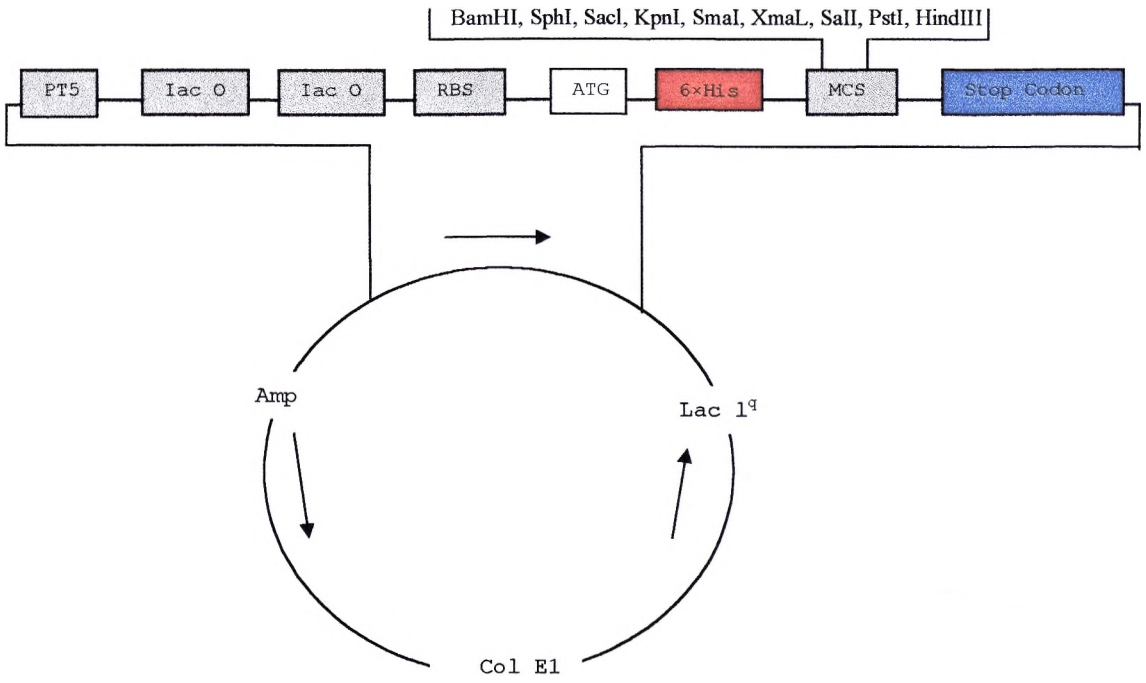


Figure 2.1: **A map of the pQE-80L His-tag expression vector**. The abbreviations used are as follows; T5 promoter (PT5), lac operator (lacO), ribosome-binding site (RBS), start codon (ATG), 6×His tag sequence (6×His), multiple cloning site with restriction sites indicated (MCS), stop codons in all three reading frames (Stop Codons), Col E1 origin of replication (Col E1), ampicillin resistance gene (Amp), lacI^q repressor gene (lacI^q).

2.2.6.1 Preparation of competent cells

Escherichia coli DH5α glycerol stock cells were streaked on a Luria-Bertani agar plate and grown overnight at 37°C. A single colony from the streaked plate was inoculated in 5 ml Luria-Bertani medium (10 g tryptone peptone, 5 g yeast extract, 5 g NaCl, 1 g D-glucose) in 15 ml culture tubes (Elkay, U.S.A). The cultures were incubated overnight at 37°C with shaking. Following the overnight incubation, the cultures were mixed and 1 ml of the culture transferred to 100 ml Luria-Bertani media pre–heated to 37°C. The 100 ml culture was

incubated at 37°C and shaken at 250 rpm until the absorbance value of the culture at 600 nm was 0.4. The *E. coli* cells were allowed to cool to 0°C in ice water. The bacteria was then split between two 50 ml polypropylene tubes and collected by centrifugation at 3 000×g for 20 mins at 4°C. The supernatant was aspirated and the bacterial cells were resuspended in 10 ml transformation buffer (60 mM $CaCl_2$, 10 mM Pipes (pH 7), 15 % glycerol) and left on ice for 20–30 mins.

The cells were again collected by centrifugation at 3 000×g for 20 mins at 4°C and the supernatant aspirated. The cell pellet was resuspended in 3 ml transformation buffer until a homogenous suspension was obtained. The competent bacterial cells were aliquoted (200 µl) into 1.5 ml sterile Eppendorf tubes, frozen in liquid nitrogen and stored at –70°C.

2.2.6.2 Restriction digests

2.2.6.2.1 Preparation of vector and PCR for ligation

The PCR product was purified using the High Pure PCR Product Purification Kit® (Roche Biochemicals, Germany). In the presence of a chaotropic salt, nucleic acids bind specifically to pre-treated glass fibres. Nucleic acids can thus be separated and purified from impurities such as salts, unincorporated nucleotides, primers and polymerases present in the PCR reaction mixture (Vogelstein & Gillespie, 1979). Nucleic acids were eluted in 100 µl of a 1× TE buffer and stored at -20°C.

The purified PCR product and pQE-80L vector were digested overnight at 37°C with the restriction enzymes *Hind III* (Roche Biochemical, Germany) and *Bam HI* (Promega, USA) in reaction buffer B (10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl and 1 mM mercaptoethanol, pH 8). Both enzymes are 100 % effective in this

buffer. The restriction-digest reaction of the PCR products contained, 2 U *Bam HI*, 2 U *Hind III*, 2 µl of 10x reaction buffer B, 10 µl of PCR product and sufficient sterile water to make up the volume to 20 µl. To digest 2 µg pQE-80L vector, 4 U *Hind III*, 4 U *Bam HI*, 4 µl of 10x reaction buffer B and sterile water to a volume of 20 µl were added. The reactions were incubated at 37°C overnight. The enzymes and fragment cut from the pQE-80L vector were removed using the High Pure PCR Purification kit. This was followed by dephosphorylation of the vector with calf intestinal phosphorylase and the vector was again cleaned using the High Pure Purification kit to remove the enzyme. The vector and PCR products were then stored at -20°C until required. The restriction digests (5 µl aliquots) were separated on a 1 % (w/v) agarose gel (section 2.2.4) along with a 100 base pair molecular weight marker to obtain an accurate indication of the molecular weight.

2.2.6.3 Ligation reaction

The known concentrations of each band in the 100 base pair mass ladder were used to estimate the concentrations of the PCR products and expression vectors. This was done by comparing the intensity of bands of PCR products and vectors on the agarose gel with the intensity of the bands of the 100 base pair mass ladder. The Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Germany) was used for the ligation reactions. The ligation reaction was catalysed using the T4 DNA ligase (Roche Molecular Biochemicals, Germany) in a 20 µl reaction. The ligation reactions were carried out at an insert:vector molar ratio of 3:1. The quantity of insert used was determined as follows:

$$\text{Insert (ng)} = \frac{\text{vector (ng)} \times \text{size of insert (kb)}}{\text{size of vector (kb)}} \times \text{ratio}\left[\frac{\text{insert}}{\text{vector}}\right]$$

The restricted vector was also dephosphorylated to prevent recircularisation. To the known quantity of insert, the restricted vector was diluted in DNA buffer to a volume of 10 µl and 1x T4 DNA ligation buffer (30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP was added to a final volume of 20 µl. The solution was mixed thoroughly before adding 2 U of the enzyme T4 ligase. A negative control consisting of a restricted vector and nuclease-free water was added. The ligation reactions were incubated at room temperature for 30 mins.

2.2.6.4 Transformation

The transformation reactions were carried out according to the method described by Sambrook *et al*, (1989). The competent cells (section 2.2.6.1) were thawed on ice and 50 µl cells were added to each reaction (5 µl), including the negative control containing linearised plasmid DNA. Transformation reactions were also performed with the positive control (uncut plasmid DNA) to check transformation efficiency. The suspensions were mixed gently and incubated on ice for 30 mins. The transformation reactions in 1.5 ml Eppendorf tubes were then subjected to heat shock at 42°C for 90 seconds to facilitate DNA uptake by the competent bacteria. The tubes were returned to ice before the addition of pre-warmed Luria-Bertani media (450 µl) without antibiotics and incubated with shaking for one hour. The transformation mixtures were plated on Luria-Bertani ampicillin selection plates by spreading 150 µl and 300 µl of the bacteria over the surface of respective plates with a sterile glass rod. The plates were incubated at 37°C overnight. Following incubation the number of colonies were counted and screened for inserts by plasmid isolation and restriction digestion or PCR.

2.2.7 Screening of colonies by plasmid isolation

The alkaline lysis method (Sambrook *et al*, 1989) was used to isolate plasmids. Single bacterial colonies from the tranformation plates were inoculated into 5 ml Luria-Bertani media containing 100 µg/ml carbenicillin in 15 ml culture tubes. The culture was incubated overnight at 37°C with shaking at 200 rpm. Glycerol stocks were prepared by mixing 500 µl of the suspended culture with an equal volume of 60 % (v/v) glycerol in a 1.5 ml microfuge tube and stored at -70°C. The remaining culture was transferred to a centrifuge tube and centrifuged at 4 000×g for 15 mins at 4°C. The supernatant was aspirated and the pellet resuspended in 300 µl lysis buffer (50 mM glucose, 25 mM Tris-Cl (pH 8), 10 mM EDTA (pH 8)) followed by incubation for 10 mins at room temperature. To this suspension 600 µl of a freshly prepared solution of 0.2 M NaOH and 1 % (w/v) SDS was added, mixed by gentle inversion and incubated on ice for 10 mins. After incubation, 450 µl of ice-cold 5 M potassium acetate solution (pH 4.8) was added, mixed by gentle inversion and incubated for 10 mins on ice. The suspension was centrifuged at 10 000×g for 10 mins, the supernatant transferred to a fresh tube and RNase added to a final concentration of 20 µg/ml. The solution was then incubated at 37°C for 1 hour. The solution was then extracted with an equal volume of phenol (pH 8) and vortexed for one minute before centrifugation at 10 000×g for 10 mins at room temperature.

The upper phase was transferred to a fresh tube and an equal volume of chloroform was added. The suspension was vortexed for one minute and centrifuged at 10 000×g for 5 mins. The upper aqueous phase was transferred to a fresh tube and precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of ice-cold isopropanol. The pellet was collected by centrifugation at 10 000×g for 20 mins at 4°C and washed with 1 ml of prechilled 70 % ethanol. The pellet was air dried and resuspended in 50 µl of sterile

water. The concentration of the plasmid DNA was determined using a UV spectrophotometer. The isolated plasmids were also subjected to restriction analysis to verify the presence of inserts or sequencing.

2.2.8 Protein Expression

2.2.8.1 Time scale analysis of protein expression

Luria-Bertani media (5 ml) containing 50 µg/ml carbenicillin was inoculated with a single colony and incubated overnight at 37°C with shaking. Carbenicillin was used in place of ampicillin as it is more stable. From this culture 500 µl was inoculated in 20 ml pre-warmed 2xYT media (17 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl to one litre) containing 50 µg/ml carbenicillin and incubated at 37°C with vigorous shaking until the absorbance at 600 nm reached 0.6. Expression was induced by adding isopropyl-β-d thiogalactopyranose (IPTG) (Invitrogen, USA) to a final concentration of 1 mM. Immediately before induction with IPTG, 1.5 ml culture sample was centrifuged at 10 000×g and resuspended in 2× SDS-PAGE sample buffer (0.09 M Tris-HCL (pH 6.8) 20 % glycerol, 2 % SDS, 0.02 % bromophenol blue and 0.1 M DTT) and taken for analysis on SDS-PAGE. Samples were taken just prior to adding IPTG and then at hourly intervals for 4 hours. The samples were immediately centrifuged, the supernatant discarded and the pellets were resuspended in 2× SDS-PAGE sample buffer to lyse the cells and frozen at -20°C.

2.2.8.2 Growth and induction of *E. coli*

A colony was picked from an agar plate and was grown (section 2.2.8) in 2xYT media containing 50 µg/µl carbenicillin. From this culture 1 ml was inoculated in 50 ml prewarmed 2xYT media containing 50 µg/µl carbenicillin. The culture was incubated at 37°C with vigorous shaking until the absorbance at 600 nm reached 0.5.

Expression was then induced by adding IPTG to a final concentration of 1 mM and the culture further incubated at 37°C for 4 hours. The bacteria were collected by centrifugation at 3 000×g at 4°C for 20 mins. The supernatant was discarded and the pellet frozen at -70°C until ready for use.

2.2.8.3 Protein extraction and purification

The frozen pellets were thawed on ice and 5 ml lysis buffer containing a protease inhibitor cocktail was added. The protease inhibitor cocktail was composed of 0.02 mg/ml pancreas extract, 0.002 mg/ml chymotrypsin, 0.0005 mg/ml thermolysin, 0.02 mg/ml trypsin, 0.33 mg/ml papain and phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 100 µg/ml. The samples were then sonicated at 6× 10 seconds at 40 % power using a sonicator with a microtip probe (Heat systems-Ultrasonics, Inc, NY, U.S.A). The lysate was centrifuged at 10 000×g at 4°C for 20 mins. The supernatant was transferred to a new tube and 100 µl 50 % nickel-NTA (nickel-nitrilotriacetic acid) resins which were pre-equilibrated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8 to one litre) were added. The His tag sequence binds to the nickel-NTA resins, after the unbound proteins are washed away the target protein is recovered by elution with imidazole. The system enables proteins to be purified under non-denaturing conditions. The solution was incubated with gentle shaking on ice for 60 mins to allow the nickel-NTA resins to bind protein containing the histidine tag and then centrifuged as before. The supernatant was removed and the resins washed three times in 5 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8 to one litre). Sample of resins (20 µl) was added to sample buffer and boiled for 5 mins. The samples were then run on a 12 % SDS-PAGE (section 2.2.8.4). The samples were further eluted with the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8 to one litre).

To 70 µl of sample, 2×SDS-PAGE sample buffer was added and the samples separated on a 12 % SDS-PAGE.

2.2.8.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

2.2.8.4.1 Preparation and running of SDS-PAGE gels

In order to separate the proteins, a denaturing discontinuous SDS-PAGE system was used (Laemmli *et al*, 1970). The 12 % resolving gel mixture for a 0.7 mm thick mini gel was prepared as in Table 2.1. Prior to casting the gel 50 µl of 10 % APS and 5 µl of TEMED were added to the gel mixture as prepared in Table 2.1. The gel mixture was poured into the assembled mini gel plates leaving sufficient space for the stacking gel.

Table 2.1: 12 % Resolving gel mixture

Water	2.72 ml
30 % acrylamide/0.8 % bis-acrylamide solution	3.2 ml
Resolving gel buffer stock (1.5 M Tris-HCl, pH 8.8)	2 ml
10 % SDS	0.08 ml
Total volume	8.0 ml

The gel was overlayed with water and left overnight to polymerise. After polymerisation the overlay was removed and the surface rinsed with 1× resolving gel buffer to remove unpolymerised acrylamide. The gel was then overlayed with 1 ml of 1× resolving gel buffer to overcome diffusion effects. The stacking gel mixture was prepared as in Table 2.2.

Table 2.2: Stacking gel mixture

Sterile water	3.05 ml
30 % acrylamide/0.8 % bis-acrylamide solution	0.665 ml
Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)	1.25 ml
10 % SDS	0.050 ml
APS	0.025 ml
TEMED	0.0025 ml
Total volume	4.99 ml

The stacking gel mixture as prepared in Table 2.2 was poured onto the polymerised separating (resolving) gel and the comb inserted immediately. After the stacking gel had polymerised the comb was removed and the gel surface was rinsed with 1x stacking gel buffer. The glass plates were placed in the electrophoresis chamber (Bio-Rad) which was filled with 1× electrophoresis buffer (0.1 M Tris base, 0.38 M glycine, 0.1 % SDS), prior to loading 20 µl of the prepared samples. The gel was then electrophoresed at a constant voltage of 200 volts for 30 mins until the Multi-Tag protein marker (mixture of purified protein tagged with glutathione-S-tansferase, influenza hemagglutinin protein, human c-myc gene and poly-histidine-tagged proteins) reached the bottom of gel. Following electrophoresis the gel sandwich was removed from the electrophoresis. The glass plates were separated to release the gel and the gel was immediately equilibrated in Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol to one litre) in preparation for protein transfer (Towbin *et al*, 1979).

2.2.8.5 Western blot transfer

Western blotting is a method based on enzyme-linked immunodetection of antigen specific antibodies. Detection occurs when the antigens specifically interact with a primary anti-histidine

antibody which then interacts with an anti-IgG secondary antibody conjugated with horseradish peroxidase, a colour development enzyme (Mathews & van Holde, 1990). The Trans-blot® SD semi dry electrophoretic transfer assembly (Bio-rad) was used for protein transfer from an SDS-PAGE gel to a polyvinylidene fluoride (PVDF) membrane.

Following electrophoresis the gel was equilibrated in 100 ml Towbin transfer buffer for 30 mins, to remove electrophoresis buffer salts and detergents. The Towbin transfer buffer was used to prevent an increase in conductivity and amount of heat generated during the transfer. This was also to optimise protein binding to the (PVDF) membrane during transfer. The hydrophobic PVDF membranes were cut to the dimensions of the gel and briefly soaked in methanol to wet them and then in Towbin transfer buffer for 30 mins. The membrane was handled with forceps to prevent contamination with proteins throughout the transfer.

Two thick filter papers were cut to the dimensions of the gel and soaked in transfer buffer. The pre-soaked filter paper was placed onto the platinum anode of the Trans-blot® SD semi dry electrophoretic transfer assembly. The equilibrated PVDF membrane was placed on top of the filter paper avoiding air bubbles. The equilibrated SDS-gels were placed on top of the membranes and covered with pre-soaked filter papers. The cathode was then placed onto the stack, along with the safety cover and the protein transfer occurred at a constant current of 175 mA for 30 mins.

2.2.8.6 Horseradish peroxidase detection

The membrane was subsequently washed and briefly rinsed in TBST buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.005 % Tween). The PVDF membrane was incubated in blocking solution (5 % bovine serum albumin prepared in TBST) for 30 mins and then soaked in

TBST with 1 % (w/v) bovine serum albumin containing the primary anti-His antibody (Biotechnology, Inc, Santa Cruz,USA) with a final concentration of 0.2 mg/ml for one hour. The membrane was washed with TBST eight times for 5 min periods to remove the unbound antibody. The membrane was transferred to the TBST buffer containing the secondary antimouse antibody with conjugated horseradish peroxidase conjugate (Biotechnology, Inc, Santa Cruz, USA) with a final concentration of 0.1 mg/ml and incubated for 60 mins. The membrane was then washed eight times in TBST buffer for 5 mins each time and then submerged in horseradish colour development solution (18 mg 4-chloro-1-naphthol, 6 ml methanol, 60 µl 30 % hydrogen peroxide and 24 ml 1× Tris-saline) for 30 mins. The membranes were blotted, air dried and observed for band development.

2.2.8.7 Chemiluminescent detection using the Lumi-light western blotting substrate

The Lumi-Light Western Blotting Substrate Kit (Roche Diagnostics, Mannheim, Germany) was used for the chemiluminescent detection. The membranes were soaked in 8 ml of lumi-light substrate solution for 30 mins. The membranes were then removed from the lumi-light substrate solution and placed protein side up on a transparency film. The membranes were blotted to remove excess liquid and covered with a light transparency film. The membranes were then placed in an X-ray cassette and exposed onto an X-ray film for periods of 3 mins, 30 mins and 2 hours respectively.

2.3 Tritiated hypoxanthine incorporation assay

Hypoxanthine, a nucleotide precursor is required for DNA synthesis. The parasite is not capable of synthesising hypoxanthine and thus relies on the human host for supply. Hypoxanthine is taken up from

the human host through the nucleoside/nucleobase transporter and incoorporated into the parasite`s nucleic acid (Parker *et al*, 2000). The uptake of radiolabelled tritiated hypoxanthine was used as an indicator of the sensitivity of the parasites to potential antimalarial agents. The malaria parasites were subcultured in microtitre plates along with various drug concentrations for a single (48 hours) and double cycle (72 hours) of DNA synthesis (Desjardins *et al*, 1979).

2.3.1 Preparation of drugs

Wortmannin, forskolin, 1,9-dideoxyforskolin (Sigma, USA) were dissolved in dimethyl sulphoxide (DMSO) to 10 mM stock solutions. Concentrations within the range of 6.5 mM to 500 nM were prepared from the stock solution. LY294002 (Promega, USA) was dissolved in DMSO to prepare a 5 mM stock solution. The inhibitor solutions were diluted ten-fold in the wells of the microtitre plate, thus the final concentrations of inhibitors were in the range of 650 µM to 50 nM. Chloroquine and quinine were both dissolved in sterile water to stock concentrations of 1 mM and 0.1 mM, respectively. The drugs were aliquoted and stored at -70°C. For each experiment dilutions were freshly prepared in incomplete media that was deficient of hypoxanthine.

2.3.2 Preparation of the intra-erythrocytic *P. falciparum* culture

The percentage parasitaemia of the synchronised *P. falciparum* culture was determined as in section 2.1.6. The culture was then washed three times with PBS, to remove the complete culture media and waste products which may interfere with experimental results. The culture was adjusted to a 0.5 % parasitaemia and a 1 % haematocrit.

2.3.3 Preparation of microtitre plates

Flat bottomed microtitre plates with 96 wells, arranged in an eight row by 12 column matrix were used in the experiments.

Of the prepared drug dilutions, 25 µl was added to triplicate or quadruplicate wells and 25 µl of hypoxanthine free media was added to the control wells (row H). Two hundred microlitres of a suspension of uninfected red blood cells (1 % haematocrit) were added to the four control wells, constituting the red blood cell controls. Two hundred microlitres of the adjusted *P. falciparum* suspension was added to the remaining eight wells which constituted the parasite drug free control wells. The plate was then transferred to a sterile glass desiccator with candles and 20 ml of sterile water added. Candles were lit to provide an anaerobic atmosphere suited for optimal parasite growth. When the flame was extinguished the glass desiccator was incubated at 37°C for 24 hours (Desjardin *et al*, 1979).

2.3.4 Preparation of radioactive isotope and labelling of parasites

Ethanol was evaporated from the 3[H]-hypoxanthine isotope (17.9 Ci/mmol, 5 mCi) (Amersham Pharmacia Biotechnology, UK) and hypoxanthine-free culture medium was added to prepare a 0.03 µCi/ml solution prior to adding 25 µl isotope to each well. The plates were incubated for a further 24 hours at 37°C in a sterile candle jar after which the parasite DNA was precipitated (section 2.3.5) for the single cycle experiment. An identical set of plates were further incubated for another 24 hour period to determine the parasite growth after a double cycle of DNA synthesis.

2.3.5 Precipitation of parasite DNA

After the respective incubation periods, a semi-automated cell harvester (Titertek® Cell Harvester, Flow Laboratory, Norway) was

used to precipitate the parasite DNA from each plate onto glass fibre filter paper. Water lysed the parasites and the parasite DNA was deposited onto glass fibre filter papers (Wallac, Finland), which were left to air dry and then transferred to sample bags filled with 10 ml Betaplate® scintillantion fluid (Wallac, Finland). The sample bags were transferred to the Wallac® Betaplate® scintillation counter (Wallac 1205) and counted for 1 min/well.

2.3.6 Data evaluation

The calculated percentage values were used to generate a sigmoidal dose response curve using the non-regression data analysis curve fitting programme, Enzfitter® (Biosoft, UK) and Graph Pad Prism® (Graph Pad Software, Inc, USA). The drug concentration which inhibited 50 % parasite growth (IC_{50} value) was determined from the sigmoid dose response curve.

The following equation was used to determine the percentage parasite growth:

$$\% \text{ Parasite growth} = \frac{\text{drug (cpm) - mRBC control (cpm)}}{\text{mPARA control (cpm) – mRBC control}} \times \frac{100}{1}$$

where :mRBC = mean red blood cell control

mPARA = mean parasite control

cpm = counts per minute

2.3.7 Statistical analysis

The IC_{50} values of the drugs were expressed as means ± standard deviation and the statistical analysis comparison between the drugs

was done using the Student's t-test, where a p value less than 0.01 was considered significant.

2.3.8 Confirmation of hypoxanthine assay data

Effects of the selected compounds on the single and double cycle of parasite growth were investigated by means of Giemsa stained thin blood slides (section 2.1.6). Differential parasite counts and parasite appearances were determined for select high concentrations (0.005 μM-500 μM) and compared to an untreated parasite control.

3.0 RESULTS

3.1 Identification of phosphatidylinositol 3-kinase in *P. falciparum*

The tblastn program at the NCBI was used to compare the amino acid sequence of PI3K from other organisms to the dynamically translated nucleotide sequences present in the *P. falciparum* genome database. A *P. falciparum* sequence which had significant alignment to PI3K in *Saccharomyces cerevisae*, *Drosophila melanogaster* and *Arabidopsis thaliana* was selected. This contig containing the sequence was submitted to an open reading frame search at the NCBI website (http://www.ncbi.nim.nih.gov/gorf/orfig.cgi). The blastp program was used to align the various ORFs against protein sequence data of the GENBANK. Figure 3.1 below is a complete ORF as depicted in PlasmoDB and the sequence used in this study also found at NCBI is shown in black and is located at the C-terminus of the PF0765w gene sequence.

```
   1 atgaaaattagatatgacaagtgctccagtacaaaagacctaaa
     M   K   I   R   Y   D   K   C   S   S   T   K   D   L   N
  46 tacttttttcatttaaaattaggtttttttgtttgttataaaaat
     Y   F   F   H   L   K   L   G   F   F   V   C   Y   K   N
  91 cacaatgataaatattcctttaaaaataaaatcttacaaaaaaat
     H   N   D   K   Y   S   F   K   N   K   I   L   Q   K   N
 136 gacactatattatttttaagaaaaaaaagaaattcatgtatctc
     D   T   I   L   F   F   K   K   K   K   K   F   M   Y   L
 181 agaaaaaaaaaaaaaaaaaaaaaaaaaaaaatactcattcagatt
     R   K   K   K   K   K   K   K   K   K   I   L   I   Q   I
 226 atacaagaatataataaatataatgaatattttaaatataattca
     I   Q   E   Y   N   K   Y   N   E   Y   F   K   Y   N   S
 271 aatttagaaggtaatcaaggattcaataaaaaaccagaaaaaaat
     N   L   E   G   N   Q   G   F   N   K   K   P   E   K   N
 316 aaaaacacaaaaggaaatgtatatactgatcatacaaatcaaaat
     K   N   T   K   G   N   V   Y   T   D   H   T   N   Q   N
 361 gcaaaatcaaaaatatataattatgatatgaatgatgattcttat
     A   K   S   K   I   Y   N   Y   D   M   N   D   D   S   Y
 406 tctaattatgtgaacaacaataatgttttagaatatcatctttt
     S   N   Y   V   N   N   N   N   V   F   R   I   S   S   F
```

```
451  ttgatattaaataatgaatttttttggatatccccttcaatttgtt
       L   I   L   N   N   E   F   F   G   Y   P   L   Q   F   V
496  tgtgaaacagaagggagaagtaggaaccatgaacattatccggac
       C   E   T   E   G   R   S   R   N   H   E   H   Y   P   D
541  gtacatggggataatattaagtataacaaatgtgatgataataag
       V   H   G   D   N   I   K   Y   N   K   C   D   D   N   K
586  tataacaaatgtgatgataataagtatgataaatgtgatgataat
       Y   N   K   C   D   D   N   K   Y   D   K   C   D   D   N
631  aagtataacaaatgtgatgataataagtatgatacatgtgatgat
       K   Y   N   K   C   D   D   N   K   Y   D   T   C   D   D
676  aataagtatgatacatgtgatgataataagtatgatacatgtgat
       N   K   Y   D   T   C   D   D   N   K   Y   D   T   C   D
721  gataataagtataacaaatatgatgatgataagtatgatacatgt
       D   N   K   Y   N   K   Y   D   D   D   K   Y   D   T   C
766  gatgataataagtataacaaatatgatgatgataagtataacaaa
       D   D   N   K   Y   N   K   Y   D   D   D   K   Y   N   K
811  tatgatgatgataagtataacaaatatgatgatgataagtatgaa
       Y   D   D   D   K   Y   N   K   Y   D   D   D   K   Y   E
856  aaaagtagaaaaaagaaaaaacttaataatttatataaaaccata
       K   S   R   K   K   K   K   L   N   N   L   Y   K   T   I
901  ttaacaaaaaaaaaaagaaaaaaaatgaactctaatttatgtgta
       L   T   K   K   K   R   K   K   M   N   S   N   L   C   V
946  ataaataaaatatacaagtatcctataaaatattgtgaattaaat
       I   N   K   I   Y   K   Y   P   I   K   Y   C   E   L   N
991  tccaaagcgtttgtatttttattattaaaaatgtaggtgttcat
       S   K   A   F   V   F   F   I   I   K   N   V   G   V   H
1036 aaaataacatattattcatataataaattatttttcgaaagatggt
       K   I   T   Y   Y   S   Y   N   K   L   F   S   K   D   G
1081 gttttaaatcaaggaattcaaatatgcaaactttatcatgtgaac
       V   L   N   Q   G   I   Q   I   C   K   L   Y   H   V   N
1126 aaaaataaaaaaattaaacaaattatttttgaagccttgaaaaat
       K   N   K   K   I   K   Q   I   I   F   E   A   L   K   N
1171 aaaataacctttttcatatgataacaatccaaacaatatcaagaaa
       K   I   T   F   S   Y   D   N   N   P   N   N   I   K   K
1216 aaaatatacaaatttcttaaaaaaaattgtgcttatcatgatctc
       K   I   Y   K   F   L   K   K   N   C   A   Y   H   D   L
1261 ataaaattgttttatttcaagggtcacaaacaaagggaaaaatgt
       I   K   L   F   Y   F   K   G   H   K   Q   R   E   K   C
1306 aacaaaaaattaaatatggaaaaaacctttggggtgcacaaatcc
       N   K   K   L   N   M   E   K   T   F   G   V   H   K   S
1351 agtcgttacaattataagacatataaaaaagaaaaagaaaattgat
       S   R   Y   N   Y   K   T   Y   K   K   K   K   K   I   D
1396 atgtgtaaaaattattgtgatgatatacttgatacatataatagt
       M   C   K   N   Y   C   D   D   I   L   D   T   Y   N   S
1441 aaatattacaagggtgaattatccgggcagcataaacatatcaag
       K   Y   Y   K   G   E   L   S   G   Q   H   K   H   I   K
1486 atgacggggaacaaaaagaagagcatcatataaaatatacacat
       M   T   G   E   Q   K   E   E   H   H   I   K   Y   T   H
1531 ttgaattttaatcatgggaaagatgaaacattttataaagaatta
       L   N   F   N   H   G   K   D   E   T   F   Y   K   E   L
1576 tataaatgtaattatattgaaaaatatatatcgtctgtaaattat
       Y   K   C   N   Y   I   E   K   Y   I   S   S   V   N   Y
1621 tttttattggagagaagacgaatgtttaacaaatataaacaacaa
       F   L   L   E   R   R   R   M   F   N   K   Y   K   Q   Q
1666 gagttgtgtgtgaacaaaaatgaggagaataataaaaataaaaat
```

```
      E  L  C  V  N  K  N  E  E  N  N  K  N  K  N
1711  gatgatgataataaaaatgatgatgataacaaaaatgatgatgat
      D  D  D  N  K  N  D  D  D  N  K  N  D  D  D
1756  aataataaaaatgatgatgataataaaaatgatgatgatgataat
      N  N  K  N  D  D  D  N  K  N  D  D  D  D  N
1801  aaaaatgatgatgataataaaaatgatgatgatgataataaaaat
      K  N  D  D  D  N  K  N  D  D  D  D  N  K  N
1846  gatgatgatgataataataaaaataatattcaatgtgataaccat
      D  D  D  D  N  N  K  N  N  I  Q  C  D  N  H
1891  tcagataatatttatatgtgtggtacctatggtaatatggaaaac
      S  D  N  I  Y  M  C  G  T  Y  G  N  M  E  N
1936  tataatgttcctcatagtacgaataatacaaatctccaatccata
      Y  N  V  P  H  S  T  N  N  T  N  L  Q  S  I
1981  aagaaaagaattattaacatgaacatattagacaatataaggtgt
      K  K  R  I  I  N  M  N  I  L  D  N  I  R  C
2026  aataaaacttacaaatacattgataaaaataaattcaaatgtttt
      N  K  T  Y  K  Y  I  D  K  N  K  F  K  C  F
2071  acatattattcatgtaaaaattataatgtatgcaaaaaaattata
      T  Y  Y  S  C  K  N  Y  N  V  C  K  K  I  I
2116  gaaaaatataaactttataaatttttaaaaaaaaaaaaaattgaa
      E  K  Y  K  L  Y  K  F  L  K  K  K  K  I  E
2161  ggatacatgattttgaatttccttaattttaataaagaattaata
      G  Y  M  I  L  N  F  L  N  F  N  K  E  L  I
2206  tattataatgaacataaaaaagatatgtctacattacatgataat
      Y  Y  N  E  H  K  K  D  M  S  T  L  H  D  N
2251  ttatttgatgtaatttccaataatcaaaatgaaaatgtgaaatat
      L  F  D  V  I  S  N  N  Q  N  E  N  V  K  Y
2296  aatcatatttgtaataataataagtatgattggtttttttaattcc
      N  H  I  C  N  N  N  K  Y  D  W  F  F  N  S
2341  tttgattatgtaggtaatctagaggaatccatcacatgtttcaat
      F  D  Y  V  G  N  L  E  E  S  I  T  C  F  N
2386  aatcacaaaaaaaaaagaaaatatgaaaaatataaaaaaatataaaa
      N  H  K  K  K  E  N  M  K  N  I  K  N  I  K
2431  aaaaaaaaaaaaaaaaatttgttttacaatgaacaacataatata
      K  K  K  K  K  N  L  F  Y  N  E  Q  H  N  I
2476  aaaaataataaaaatgattaccattttgataagtatccttcttct
      K  N  N  K  N  D  Y  H  F  D  K  Y  P  S  S
2521  ttatatagtcatttaacaaataaaaaaatggtaaacaatacagag
      L  Y  S  H  L  T  N  K  K  M  V  N  N  T  E
2566  gtaaataatataaaagatgaaaattcccttcagatgtatattatt
      V  N  N  I  K  D  E  N  S  L  Q  M  Y  I  I
2611  aataaagatgttacaaaaaataaggatggtaacttactattaaat
      N  K  D  V  T  K  N  K  D  G  N  L  L  L  N
2656  tcatattataattctaaattgggtaaatcaataaatacatgttct
      S  Y  Y  N  S  K  L  G  K  S  I  N  T  C  S
2701  aaggaaatatataaagaggaacataaaaatgtttatatatataat
      K  E  I  Y  K  E  E  H  K  N  V  Y  I  Y  N
2746  aaaaaaattacaaaaatgaatataaaaatgaagacggaacaaaaa
      K  K  I  T  K  M  N  I  K  M  K  T  E  Q  K
2791  tatatctgcgtcgattcgaaaaggaatacaagaacatataatagt
      Y  I  C  V  D  S  K  R  N  T  R  T  Y  N  S
2836  aaaaacatacgaacatataatagtaaaaacatacgaacatataat
      K  N  I  R  T  Y  N  S  K  N  I  R  T  Y  N
2881  agtaaaaacatacgaacgtataataggaaaaacatacgaacgtat
      S  K  N  I  R  T  Y  N  R  K  N  I  R  T  Y
```

```
2926 aataggaaaaacatacgaacgtataataggaaaaacatacgaacg
     N  R  K  N  I  R  T  Y  N  R  K  N  I  R  T
2971 tataataggaaaaacataagatgtaataacagaaaaaaattccat
     Y  N  R  K  N  I  R  C  N  N  R  K  K  F  H
3016 ctaaatagaaataaaaaaaaaaatggttgtgtaaaaaaatacaaa
     L  N  R  N  K  K  K  N  G  C  V  K  K  Y  K
3061 ttatatgatgaaaggaataccttggtatataagaacaaaataggg
     L  Y  D  E  R  N  T  L  V  Y  K  N  K  I  G
3106 agtaaccatttttttttgaaggaagaaattggaaaaagtacaaaa
     S  N  H  F  F  L  K  E  E  I  G  K  S  T  K
3151 aagttgaacgatatatttgaacatataagtaactatacaaatcgt
     K  L  N  D  I  F  E  H  I  S  N  Y  T  N  R
3196 attagtaagaatataaatataacaaataaaaatagatatgatgat
     I  S  K  N  I  N  I  T  N  K  N  R  Y  D  D
3241 tatccttttgatttttttgtcaaaagataaaattgaatatatctct
     Y  P  F  D  F  L  S  K  D  K  I  E  Y  I  S
3286 atgttatcaccaactataaacgagattaaaacattaaatactatt
     M  L  S  P  T  I  N  E  I  K  T  L  N  T  I
3331 cttaccattccacttattaaaatgaatgagtatgaaaaaaattgt
     L  T  I  P  L  I  K  M  N  E  Y  E  K  N  C
3376 atatggagattcagatttcaattattaaatagaaaagagaccttta
     I  W  R  F  R  F  Q  L  L  N  R  K  E  T  L
3421 ggaaaattcctaaaatctattaattggaataataaagaagaagaa
     G  K  F  L  K  S  I  N  W  N  N  K  E  E  E
3466 gaagaagccataatttttattaaataagtgggcaaaaccaggtata
     E  E  A  I  I  L  L  N  K  W  A  K  P  G  I
3511 gagaattgtatagaattattttattcacatctacatcattatgtt
     E  N  C  I  E  L  F  Y  S  H  L  H  H  Y  V
3556 attaaaaaatatattatagatattataaagaatagtaaaaaagaa
     I  K  K  Y  I  I  D  I  I  K  N  S  K  K  E
3601 gaaatcaaattatatctatttcaattggtccaaagtttaagaact
     E  I  K  L  Y  L  F  Q  L  V  Q  S  L  R  T
3646 tttaattatcaacatatagataatctatttattaatactttaata
     F  N  Y  Q  H  I  D  N  L  F  I  N  T  L  I
3691 caaaaatgtattaaatcaaaaaaactatccatatattttattgg
     Q  K  C  I  K  S  K  K  L  S  I  Y  F  Y  W
3736 tttttattaagtgaagcgaaagataaaattaaaggcaaattgtat
     F  L  L  S  E  A  K  D  K  I  K  G  K  L  Y
3781 ttacatatacataaattatttattaataaattaatgacttctaat
     L  H  I  H  K  L  F  I  N  K  L  M  T  S  N
3826 attagaaaaaataaaattatcctagatattttaaaaaatcaaaac
     I  R  K  N  K  I  I  L  D  I  L  K  N  Q  N
3871 cgtttccgtaatcaattattatatctaacaaaaattgccaagaat
     R  F  R  N  Q  L  L  Y  L  T  K  I  A  K  N
3916 aaaacagacagaatacaaaataaaaccagaaaattaagaaacttt
     K  T  D  R  I  Q  N  K  T  R  K  L  R  N  F
3961 ctattttattatagaactaattatggatatataaatatcaaagac
     L  F  Y  Y  R  T  N  Y  G  Y  I  N  I  K  D
4006 tttataaaaaataatatttttatatctgatcataatgtctatgat
     F  I  K  N  N  I  F  I  S  D  H  N  V  Y  D
4051 tttttggatatatgtaaaatgaaaagggaaaattcgctggatact
     F  L  D  I  C  K  M  K  R  E  N  S  L  D  T
4096 cctatgaggggtgataacattggtcaacctagttacctgggaatg
     P  M  R  G  D  N  I  G  Q  P  S  Y  L  G  M
4141 gtccctggtatgggtaaatccactgacgatagtaaaaatgtttac
```

```
      V  P  G  M  G  K  S  T  D  D  S  K  N  V  Y
4186  ggtgatgataataaaaatgtttatggtgatgataataaaaatgtt
      G  D  D  N  K  N  V  Y  G  D  D  N  K  N  V
4231  tatggtgacgatagtaaaaatatttattgtgacgataataaaaat
      Y  G  D  D  S  K  N  I  Y  C  D  D  N  K  N
4276  gtttatggtgatgataataaaaatatttatggtgacgatagtaaa
      V  Y  G  D  D  N  K  N  I  Y  G  D  D  S  K
4321  aatatttatggtgacgataataaaaatatttttagtgatgataat
      N  I  Y  G  D  D  N  K  N  I  F  S  D  D  N
4366  aaaaacctttatagtgacaataataataaaacacataagatat
      K  N  L  Y  S  D  N  N  N  N  K  H  I  R  Y
4411  aataagtacgtaaaaaatatatcctatgaacactttaatgaatat
      N  K  Y  V  K  N  I  S  Y  E  H  F  N  E  Y
4456  ccttacgataataaaaagagtagaaatatatatacatgtaataaa
      P  Y  D  N  K  K  S  R  N  I  Y  T  C  N  K
4501  gatatatgtaattctatttattatttagataacgaacttacaata
      D  I  C  N  S  I  Y  Y  L  D  N  E  L  T  I
4546  aattatgatataaaagatgatttatatttttttcaatataaaaga
      N  Y  D  I  K  D  D  L  Y  F  F  Q  Y  K  R
4591  agttcagatgaaaaattattaaatacagatttaagtaatgattct
      S  S  D  E  K  L  L  N  T  D  L  S  N  D  S
4636  aatgatatgattcattatattgatgattctaaaaatgttaaaatt
      N  D  M  I  H  Y  I  D  D  S  K  N  V  K  I
4681  gaacgaaatagagataactctttcttttctaatttttacaatttt
      E  R  N  R  D  N  S  F  F  S  N  F  L  Q  F
4726  aatgataatttagatttttttttaaatgctacttatagtgatgaa
      N  D  N  L  D  F  F  L  N  A  T  Y  S  D  E
4771  gataataattatgaaatattagatgactctataaattttgttcag
      D  N  N  Y  E  I  L  D  D  S  I  N  F  V  Q
4816  aaacaaaaaattaaaaaaataaaaacacctttaatttacctata
      K  Q  K  I  K  K  I  K  T  P  L  I  L  P  I
4861  gatcctaatattgaattattatctttcttacctgaacaatcatat
      D  P  N  I  E  L  L  S  F  L  P  E  Q  S  Y
4906  gtattacgttcttctttatatcctatagttattgcttgtttagtt
      V  L  R  S  S  L  Y  P  I  V  I  A  C  L  V
4951  cgaaaaaaaattaaattatataatgaaaattataataatttaatt
      R  K  K  I  K  L  Y  N  E  N  Y  N  N  L  I
4996  attaataatcatacctttttataaaaatgaccaaaataaggataat
      I  N  N  H  T  F  Y  K  N  D  Q  N  K  D  N
5041  atcataaacaatttgtcatatgataagtcatatcattcatattat
      I  I  N  N  L  S  Y  D  K  S  Y  H  S  Y  Y
5086  aactcgcaatttattaaaactttacaaaattctttttgaaagtaca
      N  S  Q  F  I  K  T  L  Q  N  S  F  E  S  T
5131  acaagtcttaattatcattataatttttttgaaatgttcaaataat
      T  S  L  N  Y  H  Y  N  F  L  K  C  S  N  N
5176  aatatttttttataaaaacaaaaaaattgaaagaattaaaccaaac
      N  I  F  Y  K  N  K  K  I  E  R  I  K  P  N
5221  acatcaattcaaaaagcctttccgtccaatgaaaatatattaaat
      T  S  I  Q  K  A  F  P  S  N  E  N  I  L  N
5266  agaaatcaacatgtgtattatagtaataaccaaattgttcataat
      R  N  Q  H  V  Y  Y  S  N  N  Q  I  V  H  N
5311  attaaaaaaatgaacaagcataaaagggatgattacatgattaat
      I  K  K  M  N  K  H  K  R  D  D  Y  M  I  N
5356  gaaaaggtccttccatgtgttagtaattcgtgtttgggcgataaa
      E  K  V  L  P  C  V  S  N  S  C  L  G  D  K
```

```
5401 ctgatgccgtcgcatgataaaatgaggtcgtcgcatgataaaatg
     L  M  P  S  H  D  K  M  R  S  S  H  D  K  M
5446 atgccgtcacatgataaaatgatgccatcgcatgataaacttatg
     M  P  S  H  D  K  M  M  P  S  H  D  K  L  M
5491 tcgccgcattatactcttatgtcgtcgcatgataaacctgtggca
     S  P  H  Y  T  L  M  S  S  H  D  K  P  V  A
5536 ccatcaggcgtttcctcgttaggagagaaaaagtcaaaagatgaa
     P  S  G  V  S  S  L  G  E  K  K  S  K  D  E
5581 aaaaagaatagaaaaaaatataacgaaatctaccagctgtcgata
     K  K  N  R  K  K  Y  N  E  I  Y  Q  L  S  I
5626 aaaaagtatatctataaagcaggtgatgatttaagacaagatcat
     K  K  Y  I  Y  K  A  G  D  D  L  R  Q  D  H
5671 ttagttatacaaataatttatgttatggataatatatggaaaaga
     L  V  I  Q  I  I  Y  V  M  D  N  I  W  K  R
5716 tatggtttggatttaaaaatgacactatatagagtattagcgtta
     Y  G  L  D  L  K  M  T  L  Y  R  V  L  A  L
5761 tcgacagatgatggttttattgaatttgtggattatgcagaatct
     S  T  D  D  G  F  I  E  F  V  D  Y  A  E  S
5806 atatcttcaataaaaaaaaattataaaggagaaataagacaatat
     I  S  S  I  K  K  N  Y  K  G  E  I  R  Q  Y
5851 tttatagataatagtacatgttcaagttccccttttaggatttgat
     F  I  D  N  S  T  C  S  S  S  P  L  G  F  D
5896 acagaaatattacaaaactttatatcaagctgtgcaggatatagt
     T  E  I  L  Q  N  F  I  S  S  C  A  G  Y  S
5941 gttattacctatatattaggtataggagatagacatctagataat
     V  I  T  Y  I  L  G  I  G  D  R  H  L  D  N
5986 ttaatggtaacaaaagatggacgcttttttcatatcgactttggt
     L  M  V  T  K  D  G  R  F  F  H  I  D  F  G
6031 tatatatttggagaggatccaaaaccattttctccacctatgaaa
     Y  I  F  G  E  D  P  K  P  F  S  P  P  M  K
6076 ttatgtaaagaaatgattgaagctatgggaggagctcatagtata
     L  C  K  E  M  I  E  A  M  G  G  A  H  S  I
6121 ggttatgaacaattcttaaaaaaatgttgtctagcatataaatat
     G  Y  E  Q  F  L  K  K  C  C  L  A  Y  K  Y
6166 ttaagataccattctcaattaattattagcttgttagatgcaatg
     L  R  Y  H  S  Q  L  I  I  S  L  L  D  A  M
6211 tgtgatgcaggattaaaggatatgaaaatgagtccagagttatgt
     C  D  A  G  L  K  D  M  K  M  S  P  E  L  C
6256 gtcttaaaagtacaagaaaaatttagacttgatttaaatgatgaa
     V  L  K  V  Q  E  K  F  R  L  D  L  N  D  E
6301 gcagcagaaatttatttcttagtgttatcaatgcatccgttaaa
     A  A  E  I  Y  F  L  S  V  I  N  A  S  V  K
6346 acgttatttcctgttgttgtggataaattgcatgagtgggcctta
     T  L  F  P  V  V  V  D  K  L  H  E  W  A  L
6391 aattggaaatga 6402
     N  W  K  *
```

Figure 3.1: ORF of PfPI3K. The complete 6.4 Kb ORF of PI3K as annotated in PlasmoDB. The ORF identified in April 2001 and which is the subject of this study is shown in black. The sequences used for the design of the primers are underlined and coloured blue.

3.1.1 The blastp results of the open reading frame

The blastp program was used to compare the ORF of the C-terminus used in this study against the Genbank. The descriptions and E values of the top ten sequences producing significant alignment obtained are shown below;

	Score	E value
sp|P54676|P3K4 PHOSPHATIDYLINOSITOL 3-KINASE VPS34-like	266	4e-70
gb|AAB71971.1| (AC002292) Phosphatidylinositol 3-kinase (Arabidopsis thaliana)	263	3e-69
sp|P42339|PI3K PHOSPHATIDYLINOSITOL 3-KINASE (PI3-KINASE)	263	3e-69
ref|NP_075247.1| phosphatidylinositol 3-kinase (Rattus norvegicus)	259	3e-68
pir||T08420 1-phosphatidylinositol 3-kinase	259	3e-68
ref|XP_008788.3| phosphoinositide-3-kinase, class 3 (Homo sapiens)	259	3e-68
sp|P42347|P3K1_SOYBN PHOSPHATIDYLINOSITOL 3-KINASE (root nodule)	259	3e-68
sp|P42348|P3K2_SOYBN PHOSPHATIDYLINOSITOL 3-KINASE (root nodule)	258	8e-68
pir||S57219 phosphatidylinositol 3-kinase - human	257	2e-67
ref|NP_002638.1| phosphoinositide-3-kinase, class 3; Vps34	257	2e-67

The top alignment of *P. falciparum* ORF with the most significant alignment (low E values) in the blastp sequence of April 2001 is shown below. The query sequence refers to the *P. falciparum* ORF sequence, whilst the subject refers to the Genbank. The positive sign (+) between the query and subject sequence indicates specific amino acid residues that belong to the same class and have a similar 3-D structure.

sp|P54676|P3K4_DICDI PHOSPHATIDYLINOSITOL 3-KINASE VPS34-LIKE (PI3-KINASE)

pir||A59003 phosphoinositide 3-kinase - slime mold (Dictyostelium discoideum)

gb|AAA85726.1| (U23480) phosphatidylinositol 3-kinase [Dictyostelium discoideum]

Length = 816, Score = 266 bits (680), Expect = 4e-70
Identities = 126/255 (49%), Positives = 175/255 (68%)

```
Query: 332 IYKAGDDLRQDHLVIQIIYVMDNIWKRYGLDLKMTLYRVLALSTDDGFIEFVDYAESISS 391
           I+K  DDLRQD L+IQ+I +MD + K+  LDLK+T Y+VLA + +DG +E V+ +E+++S
Sbjct: 561 IFKTNDDLRQDQLIIQLISLMDRLLKKENLDLKLTPYKVLATAEEDGIVEMVNPSEAMAS 620

Query: 392 IKKNYKGEIRQYFIDNSTCSSSPLGFDTEILQNFISSCAGYSVITYILGIGDRHLDNLMV 451
           +   Y G+I ++F  ++  + SP G   E++  F+ SCAGY VITYILGIGDRHLDNL++
Sbjct: 621 VLSKYDGDILKFFKTHNPDADSPYGIAPEVMDTFVKSCAGYCVITYILGIGDRHLDNLLL 680

Query: 452 TKDGRFFHIDFGYIFGEDPKPFSPPMKLCKEMIEAMGGAHSIGYEQFLKKCCLAYKYLRY 511
           T +G+ FHIDFGYI G+DPK   PPMKLCKEM+  MGG +S  YE+F + CC AY  LR
Sbjct: 681 TPNGKLFHIDFGYILGKDPKILPPPMKLCKEMVLGMGGENSKHYEKFKQLCCEAYNILRK 740

Query: 512 HSQLIISLLDAMCDAGLKDMKMSPELCVLKVQEKFRLDLNDEAAEIYFLSVINASVKTLF 571
            S LI++L   M DA +  +    E  +LKVQEK +L+L D+ A    L ++N SV  LF
Sbjct: 741 SSHLILNLFALMVDASIPSISDDKEKSILKVQEKLQLELTDQEASNSLLQLLNDSVTALF 800

Query: 572 PVVVDKLHEWALNWK 586
           PV+++  H+W   WK
Sbjct: 801 PVIIEMAHKWLQYWK 815
```

```
 Score = 33.9 bits (76), Expect = 3.9
 Identities = 15/59 (25%), Positives = 35/59 (58%), Gaps = 14/59 (23%)

Query: 53  SINFVQKQKIKKIKT--------------PLILPIDPNIELLSFLPEQSYVLRSSLYPI 97
           ++N  +++KI+K++               P+ LP++P+IE++  +PE+S + +S+  P+
Sbjct: 488 AMNITREKKIEKLRIMLAEGEYKDLSDFQPIRLPVNPDIEIIGIVPEKSNIYKSAKSPL 546
```

The amino terminus of the PfPI3K does not show significant similarity to other PI3K in other organisms.

3.2 Primer design for PI3K amplification by PCR

Primers specific for PfPI3K were designed using the sequence information obtained in the ORF (section 3.1). To facilitate directional cloning into an expression vector additional nucleotides for the *Bam HI* restriction site and the *Hind III* restriction site were added to the sense

and antisense primers respectively. The primer sequences designed were as follows: *Bam HI* restriction site denoted in blue underlined print and *Hind III* restriction site by red underlined print.

Primer for the sense strand:

T_m= 59.1°C

Amino acid sequence: I H Y I D D S

Bam HI

Primer sequence: 5'CAT CAC GGA TCC ATT CAT TAT ATT GAT GAT TCT 3'

Primer for the antisense strand:

T_m= 60.5°C

Amino acid sequence: E W A L N W K

Hind III

Coding sequence: 5'GAG TGG GCC TTA AAT TGG AAA AAG CTT AAT TAG3'

Hind III

Complementary: 3'CTC ACC CGG ATT TTA ACC TTT TTC GAA TTA ATC 5'

Sequence

Hind III

Primer sequence: 5'CTA ATT AAG CTT TTT CCA ATT TTA GGC CCA CTC 3'

Primer positions in pQE-80L:

5'-PROMOTER REGION-*Xhol*-OPERATOR 1-TAACAATTAT AATAGATTCA

Sense primer

5'-CG GATAACAATT TCACACAG-3'

ATTGTGAGCG GATAACAATT TCACACAGAA TTCATT-RBS-MET-ARG-GLY-SER-6×HIS TAG-*Bam Hl-Sphl-Sacl-Kpnl-Smal-Sall-Pstl-Hindlll*-AATTAGCTGA GCTTGGACTC CTGTTGATAG ATCCAGTAAT GACCTCAGAA C-3'

3'-GGTCATT ACTGGAGTCT TG-5'

antisense primer

3.2.1 Optimisation of PCR using the PCR Master Enzyme System®

The PCR reaction was optimised by modifying experimental variables that influence specificity and yield of the desired product. Therefore annealing temperature, primer concentrations and DNA concentrations were investigated to achieve optimum conditions for the PCR reactions. The DNA used for the PCR reactions was isolated from the 3D7 strain of *P. falciparum*.

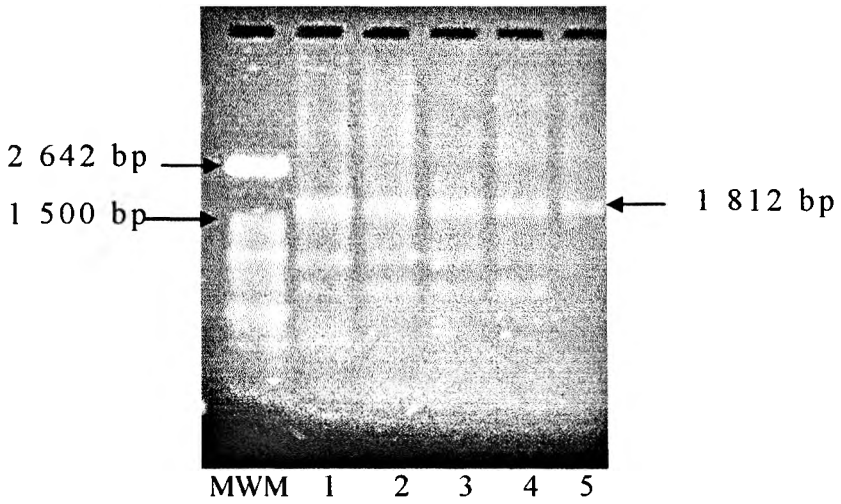


Figure 3.2: PCR of PI3K at different annealing temperatures. The PCR products obtained using primers designed for PfPI3K over a range of annealing temperatures are represented as follows:
1 = 58°C, 2 = 61°C, 3 = 62°C, 4 = 63°C, 5 = 64°C.

The prominent band obtained at the different annealing temperature (58-62°C) was 1 812 bp, which was more than the expected base pair length (1 758 bp) since the restriction sites and additional nucleotides will be included. Double bands at 1 812 bp were noted at the lower annealing temperatures (58-62°C). Minor bands of 900 bp and 400 bp

were also observed in the reactions. The most specific annealing temperature was shown to be 64°C however the PCR product decreased significantly as 64°C is higher than the Tm for the primers.

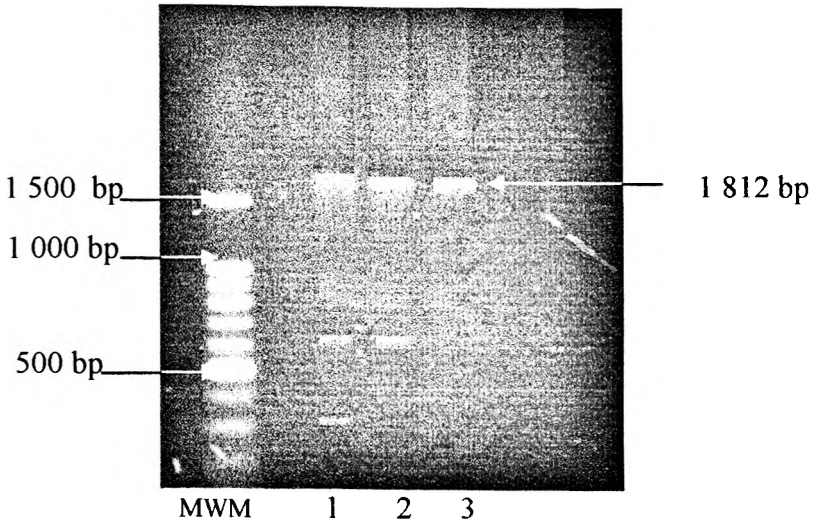


Figure 3.3: Optimisation of primer and DNA concentration. PCR products were obtained when varying the primer and DNA concentration with an annealing temperature of 64°C and using the PCR Master Kit®. The lanes were represented as follows:

1 = 0.5 µg/25 µl DNA concentration/ 0.5 µM primer concentration,

2 = 0.5 µg/25 µl DNA concentration/ 0.1 µM primer concentration,

3 = 1 µg/25 µl DNA concentration/ 0.1 µM primer concentration.

Lower primer (0.1 µM) and high DNA (1µg) concentrations produced a more prominent band at 1 812 bp (3). Minor bands were also produced (600 bp, 300 bp) when a primer concentration of 0.5 µM was used.

3.2.2 PCR using the Expand High Fidelity Enzyme System®

The Expand High Fidelity PCR system® composed of a thermostable *Taq DNA polymerase* and a proof reading polymerase, *Pwo DNA polymerase* was used after optimising the conditions for template and primers. The enzyme system is designed to give PCR products with high yields and specificity. Figure 3.4 displays the PCR products obtained from the two enzymes used.

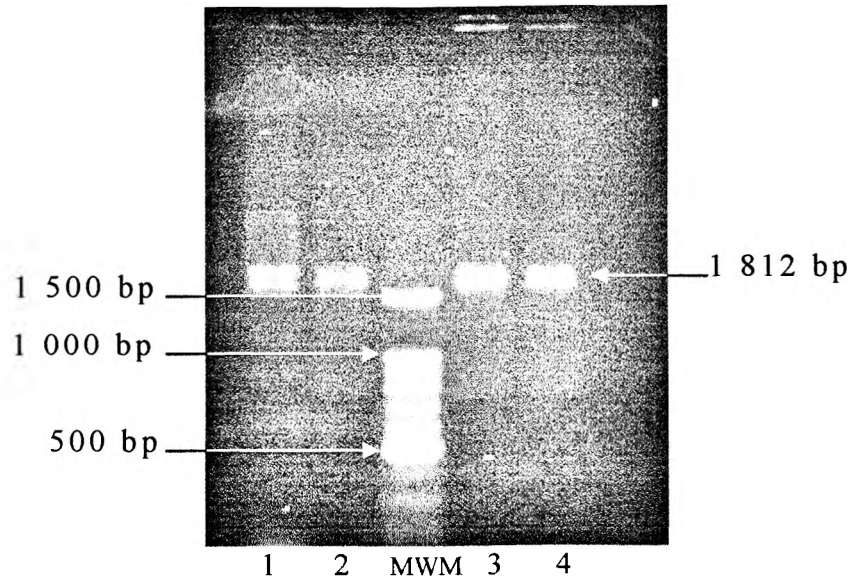


Figure 3.4: Comparison of PCR products obtained using the PCR Master Enzyme system® and the Expand High Fidelity Enzyme system®. The lanes were represented as follows:
1 & 2 = PCR Master product
3 & 4 = Expand High fidelity system product

The PCR Master Enzyme System® produced a distinct double band at the expected length of 1 812 bp (lanes 1 and 2), while the PCR reactions using the Expand High Fidelity Enzyme system® produced a

single prominent band at the expected length of 1 812 bp lanes 3 and 4). Double bands were observed when using the PCR Master kit® due to the lack of fidelity of the enzyme system.

3.2.3 Sequencing of the PfPI3K PCR product

The PfPI3K PCR product obtained using Expand High Fidelity Enzyme system® was sequenced from both ends of the ORF to verify the nucleotide sequence (Figure 3.5).

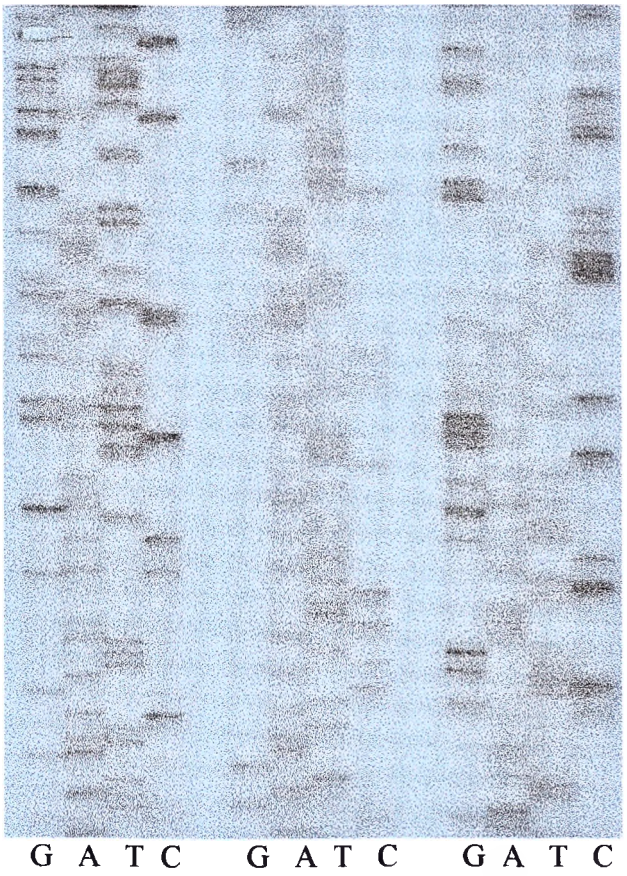


Figure 3.5: DNA sequence of PCR product. Autoradiogram of sequenced PCR products are shown. The primers used were designed from the sense and antisense strands of the ORF of contig P42336. The reactions were loaded in the order of G, A, T, C.

The nucleotide sequence obtained was used in the blastn search of the PlasmoDB (http//:www.plasmodb.org) to determine the identity of the sequence. The sequence obtained of PfPI3K using primers for the sense strand:

```
5'TTACAATTTA ATCATAATTT AGATTTTTTT TTAAATGCTA CTTATAGTGA TGAAGATAAT
  AATTATGAAA TATTAGATGA CTCTATAAAT TTTGTTCAGA AACAAAAAAT TAAAAAAATA
  AAAACACCTT TAATTTTACT ATAGATCCTA ATATTGAATT ATTAATCTTT CTTACCTGAA
  GAATCATATC TA3'
```

The pairwise alignment which had the most significant similarity (lowest E value) of the sequence obtained using the primer for the sense strand is shown below:

```
>gi/97739977   P. falciparum 3D7  GENOMIC   pfal_chr5
         Length = 1,343,552

  Plus Strand HSPs:

 Score = 960 (150.1 bits), Expect = 6.0e-38, P = 6.0e-38
 Identities = 190/192 (98%), Positives = 190/192 (98%),
Strand = Plus / Plus

Query:     1 TTACAATTTAATCATAATTTAGATTTTTTTTTAAATGCTACTTATAGTGATGAAGATAAT 60
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633695 TTACAATTTAATGATAATTTAGATTTTTTTTTAAATGCTACTTATAGTGATGAAGATAAT 633754

Query:    61 AATTATGAAATATTAGATGACTCTATAAATTTTGTTCAGAAACAAAAAATTAAAAAAATA 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633755 AATTATGAAATATTAGATGACTCTATAAATTTTGTTCAGAAACAAAAAATTAAAAAAATA 633814

Query:   121 AAAACACCTTTAATTTTACCTATAGATCCTAATATTGAATTATTATCTTTCTTACCTGAA 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633815 AAAACACCTTTAATTTTACCTATAGATCCTAATATTGAATTATTATCTTTCTTACCTGAA 633874

Query:   181 GAATCATATCTA 192
              |||||||| ||
Sbjct: 633875 CAATCATATGTA 633886
```

The sense sequence obtained for the PfPI3K with primer for the antisense strand:

```
5'TACCATTCTC AATTAATTAT TACCTTGTTA GATGCAATGT GTGATGCAGG ATTAAAGGAT
  ATGAAAATGA GTCCAGACTT ATGTGTCTTA AAAGTACAAG AAAAATTTAG ACTTGATTTA
  AATCATGAAG CAGCAGAA 3'
```

The pairwise alignment with the most significant similarity (lowest E value) using the primer for the antisense strand with contig P42336 is shown below:

```
>gi|23504570|emb|AL929352.1|PFA929352 plasmodium falciparum strain 3D7,
chromosome 5, segment 2/4
          Length = 313050
Score =  258 bits (130), Expect = 2e-66
 Identities = 136/138 (98%)
 Strand = Plus / P

Query: 1      taccattctcaattaattattaccttgttagatgcaatgtgtgatgcaggattaaaggat 60
               |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 288150 taccattctcaattaattattagcttgttagatgcaatgtgtgatgcaggattaaaggat 288209


Query: 61     atgaaaatgagtccagacttatgtgtcttaaaagtacaagaaaaatttagacttgattta 120
               |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288210 atgaaaatgagtccagagttatgtgtcttaaaagtacaagaaaaatttagacttgattta 288269

Query: 121    aatgatgaagcagcagaa 138
               ||||||||||||||||||
Sbjct: 288270 aatgatgaagcagcagaa 288287
```

The blastn results of the manual sequencing data confirmed the sequence to be part of the ORF identified in contig P42336. The sequence is part of the C-terminal end of the PfPI3K sequence. The sequence data obtained is not 100 % identical to the PlasmoDB sequence, this may result in the misincorporation of amino acids in protein expression resulting in a different amino acid being expressed.

3.3 Cloning of PfPI3K products into an expression vector

The PfPI3K PCR products were cloned into the pQE-80L expression vector in a directional manner. The colony counts obtained are shown below in Table 3.1.

The high counts obtained for the plasmid positive control indicated that the transformation of the competent DH5α cells with the

pQE-80L vector was successful. The plasmid negative control had no colonies indicating that the restriction digested vector did not recircularise (Table 3.1).

Table 3.1: The colony counts obtained from the transformation of competent DH5α *Escherichia coli* cells.

PCR product insert (samples)	Number of colonies
A	202
B	567
C	1200
Plasmid negative control	0
Plasmid positive control	1586

Three different High Fidelity PCR reactions were cloned to check for mutations and misincorporation of nucleotides. Plasmids were isolated from the transformed clones and screened by restriction digestion and PCR to verify the presence of insert. Plasmids from eight clones from each PCR reaction were subjected to restriction digest with *Bam HI* and *Hind III*. The products were separated on a 1 % agarose gel and the inserts were shown to be of the correct size of 1 812 bp (Figure 3.6).

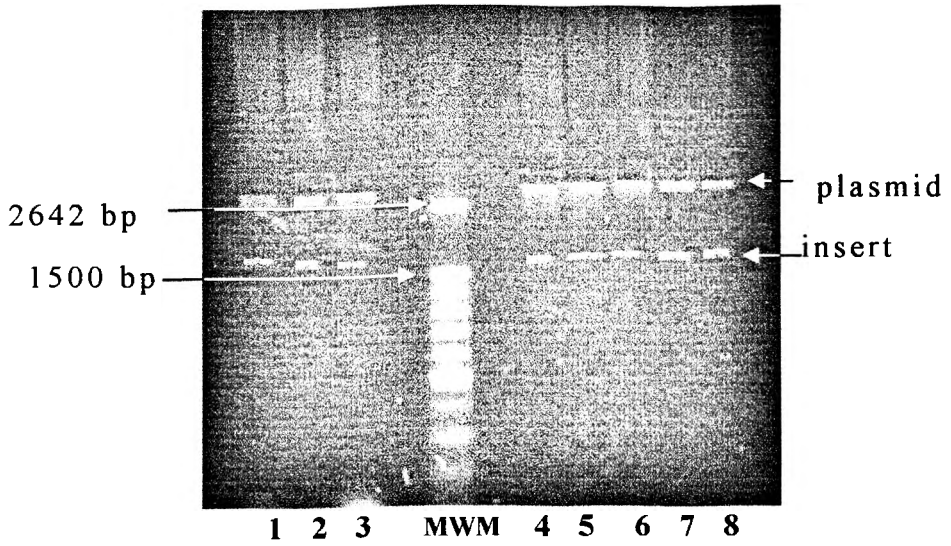


Figure 3.6: Restriction analysis of plasmids obtained from transformed bacteria. The products of the restriction digest on the plasmids containing the PI3K insert are shown.
1 & 2 = Clones from PCR A;
3, 4 & 5 = Clones from PCR B;
6, 7 & 8 = Clones from PCR C.

The plasmids isolated from clones which were obtained from three different PCR reactions A, B and C had inserts of the correct size of 1 812 bp. The colonies were further screened by PCR to determine the transformation efficiency of the three different PCR product inserts (A-C). High transformation efficiency was observed for all three PCR reactions, where all the plasmids screened had insert as shown in Figure 3.7. Negative control PCR reactions were also performed and did not show the 1 812 bp band (data not shown).

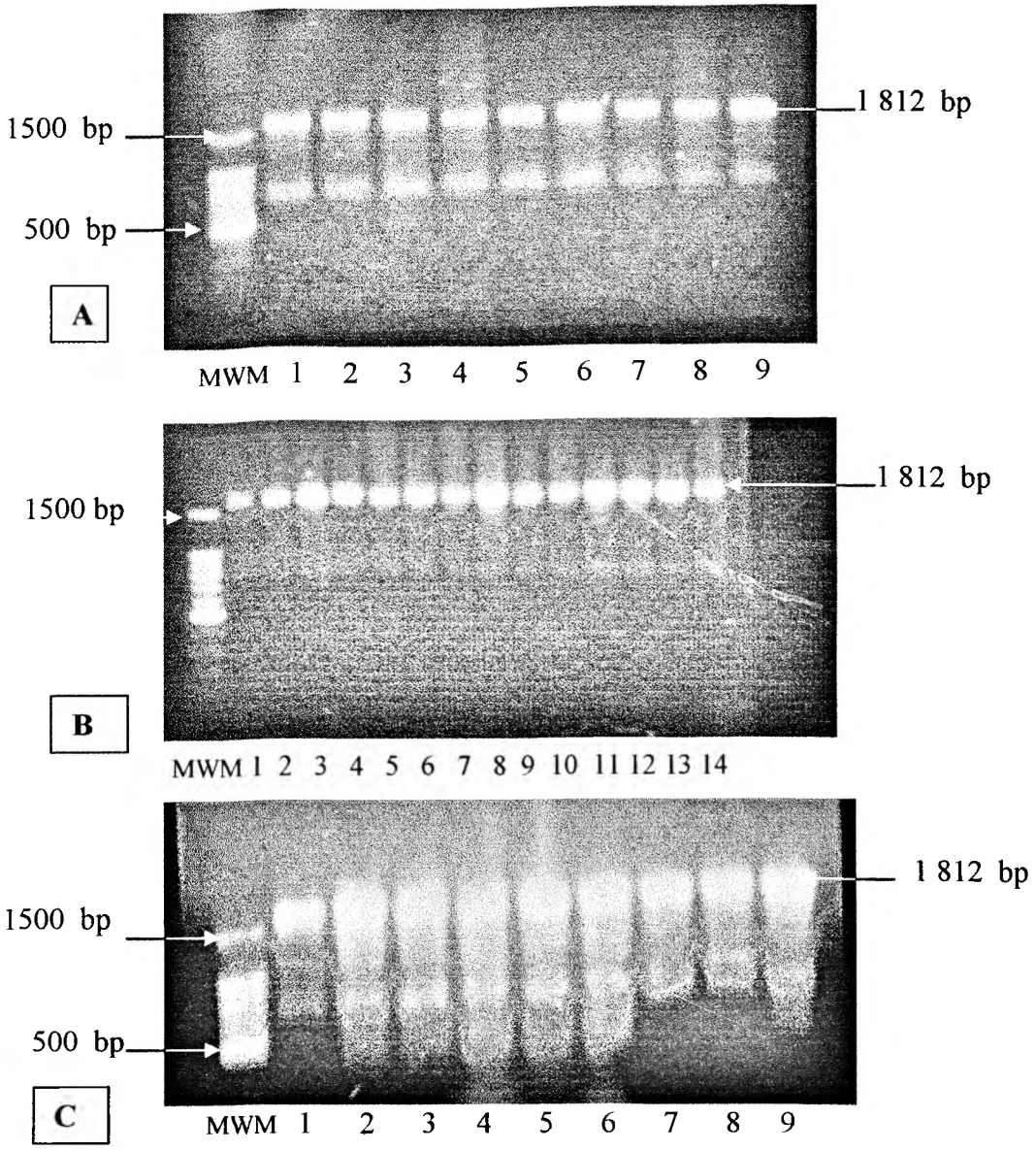


Figure 3.7: Agarose gel electrophoresis of PI3K clones screened by PCR. The products of PCR screens on the transformed clones of the three PCR reactions, A (lanes 1-9), B (lanes 1-9) and C (lanes 1-9) containing PfPI3K inserts are shown.

All the plasmids contained an insert with a correct size of 1 812 bp. Screening by PCR produced additional bands (800 bp) but plasmid isolated and digested did not form this band.

3.3.1 Sequencing of the PfPI3K inserts

The PfPI3K inserts in some of the isolated plasmids were sequenced at both ends of the inserts for verification. To verify the sequences, the obtained sequences were subjected to a blastn search at the malaria genome database. The sequence products with the most significant alignment are shown.

The sequence of PfPI3K inserts obtained with the primer for the sense strand by manual sequencing:

```
5'AAAATTGAAC GAAATAGAGA TAACTCTTTC TTGTCTAATT TTTACAATTT AATGATAAT
  TAGATTTTTT TTTAAATCCT ACTTATAGTG ATGAAGATAA TAATTATGAA ATATTAGATG
  ACTCTATAAA TTTTGTTGAG AAACAAAAAA TTAAAAAATA AAAA3'
```

The pairwise alignment with the most significant similarity (lowest E value) obtained using the sense strand with the contig P42336 is shown below:

```
>97739977 P. falciparum 3D7 GENOMIC pfal_chr5
Length = 1,343,552 Plus Strand HSPs: Score = 764 (120.7
bits), Expect = 4.3e-29, P = 4.3e-29 Identities = 163/165
(98%), Positives = 163/165 (98%), Strand = Plus / Plus


Query: 1      AAAATTGAACGAAATAGAGATAACTCTTTCTTGTCTAATTTTTTACAATTTAATGATAAT 59
              ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 633653 AAAATTGAACGAAATAGAGATAACTCTTTCTTTTCTAATTTTTTACAATTTAATGATAAT 633712

Query: 60     TTAGATTTTTTTTTAAATCCTACTTATAGTGATGAAGATAATAATTATGAAATATTAGAT 118
              |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
       633713 TTAGATTTTTTTTTAAATGCTACTTATAGTGATGAAGATAATAATTATGAAATATTAGAT 633772

Query: 119    GACTCTATAAATTTTGTTGAGAAACAAAAAATTAAAAAATTAAAAA 164
              |||||||||||||||||| |||||||||||||||||| ||||||
Sbjct: 633773 GACTCTATAAATTTTGTTCAGAAACAAAAAATTAAAAAAATAAAAA 633818
```

The sense sequence of the PfPI3K insert obtained with primer for the antisense strand:

```
5'GCATATAAAT ATTTAACATA CGATTCTCAA TTAATTATTA CCTTGTTAGA TGCAATGTGA
  TCCAGGATTA AAGGATATGA AAATGAGTCC AGAGTTTTGT CTTAAAAGTA CAAGAAAAAT
  TTACACTT 3'
```

The pairwise alignments of the most significant similarity obtained using primer for the antisense strand with contig P42336 is shown below:

```
>gi|23504570|emb|AL929352.1|PFA929352 Plasmodium falciparum strain 3D7,
chromosome 5, segment 2/4
          Length = 313050

 Score =  186 bits (94), Expect = 5e-45

Identities = 129/132 (96%), Gaps = 1/132 (0%)
 Strand = Plus / Plus


Query: 1      gcatataaatatttaagataccattctcaattaattattagcttgttagatgcaatgtgt 58
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 288132 gcatataaatatttaagataccattctcaattaattattagcttgttagatgcaatgtgt 288191


Query: 59     gatccaggattaaaggatatgaaaatgagtccagagttttgtgtcttaaaagtacaagaa 116
               ||| |||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 288192 gatgcaggattaaaggatatgaaaatgagtccagagttatgtgtcttaaaagtacaagaa 288251


Query: 117    aaatttacactt 128
               ||||||| ||||
Sbjct: 288252 aaatttagactt 288263
```

The sequence was 96 % identical to that of the PlasmoDB. Discrepancies were due to misincorporation of nucleotides during amplification in PCR.

The insert was also sequenced by automated sequencing. The sequence produced from the automated sequencer using vector primer from the sense strand extended further into the ORF.

```
5'TTCTTGTCTA ATTTTTTACA ATTTAATGAT AATTTAGATT TTTTTTTAAA TCCTACTTAT
  AGTGATGAAG ATAATAATTA TGAAATATTA GATGACTCTA TAAATTTTGT TCAGAAACAA
  AAAATTAAAA AAATTAAAAC ACCTTTAATT TTACCTATAG ATCCTAATAT TGAATTATTA
  TCTTTCTTAC CTGAACAATC ATATGTATTA CGTTCTTCTT TATATCCTAT ACTTATTGCT
  TGTTTAGTTC GAAAAAAAAT TAAATTATAT AATGAAAATT ATAATAATTT AATTATTAAT
  AATCATACCT TTTATAAAAA TGACCAAAAT AAGGATAATA TCATAAACAA TTTGTCATAT
  GATAAGTCAT ATCATTCATA TTATAACTCG CAATTTATTA AAACTTTACA AAATTCTTTT
  GTAAGTACAA CAAGTCTTAA TTATCATTAT AATTTTTTGA AATGTTCAAA TAATAATATT
  TTTTATAAAA ACAAAAAAAT TGAAAGAATT AAACCAAACA CATCAATTCA AAAAGCCTTT
  CCGTCCAATG AAAATATATT AAATAGAAAT CAACATGTGT ATTATAGTAA TAACCAAATT
```

GTTCATAATA TTAAAAAAAT GAACAAGCAT AAAAGGGATG ATTACATGAT TAATGAAAAG
GTCCTTCCAT GTGTTAGTAA TTCGTGTTTG GGCGATAAAC TGATCCCGTC GCATGATAAA
ATGAGGTCGT CGCATGATTA AATGATGCCG TCACATGATA AAATCATGCC ATCGCATG3'

The pairwise alignment with the most significant similarity is shown below:

>97739977 P. falciparum 3D7 GENOMIC pfal_chr5

Length = 1,343,552

Plus Strand HSPs: Score = 3818 (578.9 bits), Expect = 4.9e-167,

P = 4.9e-167, Identities = 770/778 (98%), Positives = 770/778 (98%), Strand

= Plus / Plus

```
Query:      1 TTCTTGTCTAATTTTTTACAATTTAATGATAATTTAGATTTTTTTTTTAAATCCTACTTAT 60
              ||||| ||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 633680 TTCTTTTCTAATTTTTTACAATTTAATGATAATTTAGATTTTTTTTTTAAATGCTACTTAT 633739

Query:     61 AGTGATGAAGATAATAATTATGAAATATTAGATGACTCTATAAATTTTGTTCAGAAACAA 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633740 AGTGATGAAGATAATAATTATGAAATATTAGATGACTCTATAAATTTTGTTCAGAAACAA 633799

Query:    121 AAAATTAAAAAAAATTAAAACACCTTTAATTTTACCTATAGATCCTAATATTGAATTATTA 180
              |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633800 AAAATTAAAAAAATAAAAACACCTTTAATTTTACCTATAGATCCTAATATTGAATTATTA 633859

Query:    181 TCTTTCTTACCTGAACAATCATATGTATTACGTTCTTCTTTATATCCTATACTTATTGCT 240
              |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct: 633860 TCTTTCTTACCTGAACAATCATATGTATTACGTTCTTCTTTATATCCTATAGTTATTGCT 633919

Query:    241 TGTTTAGTTCGAAAAAAAATTAAATTATATAATGAAAATTATAATAATTTAATTATTAAT 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633920 TGTTTAGTTCGAAAAAAAATTAAATTATATAATGAAAATTATAATAATTTAATTATTAAT 633979

Query:    301 AATCATACCTTTTATAAAAATGACCAAAATAAGGATAATATCATAAACAATTTGTCATAT 360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 633980 AATCATACCTTTTATAAAAATGACCAAAATAAGGATAATATCATAAACAATTTGTCATAT 634039

Query:    361 GATAAGTCATATCATTCATATTATAACTCGCAATTTATTAAAACTTTACAAAATTCTTTT 420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 634040 GATAAGTCATATCATTCATATTATAACTCGCAATTTATTAAAACTTTACAAAATTCTTTT 634099

Query:    421 GTAAGTACAACAAGTCTTAATTATCATTATAATTTTTTGAAATGTTCAAATAATAATATT 480
              | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 634100 GAAAGTACAACAAGTCTTAATTATCATTATAATTTTTTGAAATGTTCAAATAATAATATT 634159

Query:    481 TTTTATAAAAACAAAAAAAATTGAAAGAATTAAACCAAACACATCAATTCAAAAAGCCTTT 540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 634160 TTTTATAAAAACAAAAAAAATTGAAAGAATTAAACCAAACACATCAATTCAAAAAGCCTTT 634219

Query:    541 CCGTCCAATGAAAATATATTAAATAGAAATCAACATGTGTATTATAGTAATAACCAAATT 600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 634220 CCGTCCAATGAAAATATATTAAATAGAAATCAACATGTGTATTATAGTAATAACCAAATT 634279
```

```
Query:    601 GTTCATAATATTAAAAAAATGAACAAGCATAAAAGGGATGATTACATGATTAATGAAAAG 660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 634280 GTTCATAATATTAAAAAAATGAACAAGCATAAAAGGGATGATTACATGATTAATGAAAAG 634339

Query:    661 GTCCTTCCATGTGTTAGTAATTCGTGTTTGGGCGATAAACTGATCCCGTCGCATGATAAA 720
              |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct: 634340 GTCCTTCCATGTGTTAGTAATTCGTGTTTGGGCGATAAACTGATGCCGTCGCATGATAAA 634399

Query:    721 ATGAGGTCGTCGCATGATTAAATGATGCCGTCACATGATAAAATCATGCCATCGCATG 778
              |||||||||||||||||| |||||||||||||||||||||||| ||||||||||||
Sbjct: 634400 ATGAGGTCGTCGCATGATAAAATGATGCCGTCACATGATAAAATGATGCCATCGCATG 634457
```

The sequences obtained of part of the PfPI3K insert by sequencing and those of the ORF identified in contig P42336 showed 96-98 % identity. However additional errors due to polymorphism may have been present in the overall sequence and identity may be lower. The part of the insert sequenced which had previously been cloned in a pQE-80L vector which has an N-terminal His tag, was thus verified to be part of the ORF for PfPI3K.

The sequence produced using an automated sequencer using the pQE-80L vector antisense primer is as follows:

```
5'TTTCCAATTT AAGGCCCACT CATGCAATTA TCCACAACAA CAGGAAATAA CGTTTTAACG
  GATGCATTGA TAACACTAAG AAAATAAATT TCTGCTGCTT CATCATTTAA ATCAAGTCTA
  AATTTTTCTT GTACTTTTAA GACACATAAC TCTGGACTCA TTTTCATATC CTTTAATCCT
  GCATCACACA TTGCATCTAA CAAGCTAATA ATTAATTGAG AATGGTATCT TAAATATTTA
  TATGCTAGAC AACATTTTTT TAAGAATTGT TCATAACCTA TACTATGAGC TCCTCCCATA
  GCTTCAATCA TTTCTTTACA TAATTTCATA GGTGGAGAAA ATGGTTTTGG ATCC3'
```

The pairwise alignment with the most similar homology is shown below:

```
>97739977 P. falciparum 3D7 GENOMIC pfal_chr5 Length = 1,343,552

Minus Strand HSPs: Score = 1760 (270.1 bits), Expect = 4.4e-74,
P = 4.4e-74 Identities = 354/355 (99%), Positives = 354/355 (99%),
Strand = Minus / Plus

Query: 354    GGATCCAAAACCATTTTCTCCACCTATGAAATTATGTAAAGAAATGATTGAAGCTATGGG 295
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 635023 GGATCCAAAACCATTTTCTCCACCTATGAAATTATGTAAAGAAATGATTGAAGCTATGGG 635082

Query: 294    AGGAGCTCATAGTATAGGTTATGAACAATTCTTAAAAAAAATGTTGTCTAGCATATAAATA 235
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 635083 AGGAGCTCATAGTATAGGTTATGAACAATTCTTAAAAAAAATGTTGTCTAGCATATAAATA 635142
```

```
Query: 234    TTTAAGATACCATTCTCAATTAATTATTAGCTTGTTAGATGCAATGTGTGATGCAGGATT 175
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 635143 TTTAAGATACCATTCTCAATTAATTATTAGCTTGTTAGATGCAATGTGTGATGCAGGATT 635202

Query: 174    AAAGGATATGAAAATGAGTCCAGAGTTATGTGTCTTAAAAGTACAAGAAAAATTTAGACT 115
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 635203 AAAGGATATGAAAATGAGTCCAGAGTTATGTGTCTTAAAAGTACAAGAAAAATTTAGACT 635262

Query: 114    TGATTTAAATGATGAAGCAGCAGAAATTTATTTTCTTAGTGTTATCAATGCATCCGTTAA 55
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 635263 TGATTTAAATGATGAAGCAGCAGAAATTTATTTTCTTAGTGTTATCAATGCATCCGTTAA 635322

Query: 54     AACGTTATTTCCTGTTGTTGTGGATAATTTGCATGAGTGGGCCTTAAATTGGAAA 1
              |||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 635323 AACGTTATTTCCTGTTGTTGTGGATAAATTGCATGAGTGGGCCTTAAATTGGAAA 635377
```

The sequences obtained of the C-terminal part of the PfPI3K insert using an automated sequencer showed 98 %-99 % identity to the complete PFE0765w sequence gene found in the PlasmoDB (http//www:plasmodb.org). Furthermore the sequences obtained by automated sequence had less discrepancies compared to the manual sequences. However one consistent error noted in both the manual and automated sequences may be due to polymorphism, while the other errors in the manual sequence may be due to experimental error. The N-terminus extremity and 726 bp fragment middle of the ORF have not been verified due to time constraints.

Inspection of the automated sequence data in relation to the amino acid sequence of the PFE076w ORF shows discrepancies indicated in blue and red in the pairwise alignment. The discrepancies shown in blue may have minimal effect on the overall amino acid sequence in comparison to the ORF encoding the PFE076w gene. The discrepancies shown in red indicate that the changes in the nucleotides may lead to changes in the overall protein configuration.

Tryptophan replaced lysine 22 on the automated sequence obtained using sense vector primers. Tryptophan is an amino acid with nonpolar side chains whilst lysine has charged polar side chains. Histidine 74 a basic amino acid also replaced aspartic acid which is acidic. Asparagine 578 which is an amino acid with uncharged polar side chains replaced lysine which has polar side chains on the automated sequence obtained using antisense vector primers.

These changes in amino acids are likely to cause changes in the secondary and tertiary structure of the protein and will influence the function of the protein. The N-terminus part of the sequence encoding the ORF of PFE0765w was not verified therefore the complete inspection of the sequence coding PFE0765w is not shown below. The C-terminal part of the ORF encoding PFE0765w sequence is shown below.

The amino acids that were changed due to the discrepancies shown in the nucleotide sequence relative to the PF0765w ORF gene sequence are shown below in blue. The amino acids that may cause adverse changes to the structure of the protein are shown in red below.

```
  1 MIHYIDDSKNVKIERNRDNS 20  FFSNFLQFNDNLDFFLNATY 40
 41 SDEDNNYEILDDSINFVQKQ 60  KIKKIKTPLILPIDPNIELL 80
 81 SFLPEQSYVLRSSLYPIVIA 100 CLVRKKIKLYNENYNNLIIN 120
121 NHTFYKNDQNKDNIINNLSY 140 DKSYHSYYNSQFIKTLQNSF 160
161 ESTTSLNYHYNFLKCSNNNI 180 FYKNKKIERIKPNTSIQKAF 200
201 PSNENILNRNQHVYYSNNQI 220 VHNIKKMNKHKRDDYMINEK 240
241 VLPCVSNSCLGDKLMPSHDK 260 MRSSHDKMMPSHDKMMPSHD 280
281 KLMSPHYTLMSSHDKPVAPS 300 GVSSLGEKKSKDEKKNRKKY 320
321 NEIYQLSIKKYIYKAGDDLR 340 QDHLVIQIIYVMDNIWKRYG 360
361 LDLKMTLYRVLALSTDDGFI 380 EFVDYAESISSIKKNYKGEI 400
```

401 RQYFIDNSTCSSSPLGFDTE 420 ILQNFISSCAGYSVITYILG 440

441 IGDRHLDNLMVTKDGRFFHI 460 DFGYIFGEDPKPFSPPMKLC 480

481 KEMIEAMGGAHSIGYEQFLK 500 KCCLAYKYLRYHSQLIISLL 520

521 DAMCDAGLKDMKMSPELCVL 540 KVQEKFRLDLNDEAAEIYFL 560

561 SVINASVKTLFPVVVDKLHE 580 WALNWK 586

3.4 Protein expression of PfPI3K

Clones from three separate PCR reactions were screened for expression by induction of cultures with IPTG. The protein expressed was isolated by nickel-affinity chromatography from IPTG-induced PfPI3K clones in the pQE-80L vector with an N-terminal His tag and probed using the anti-His monoclonal antibody. The His-tagged proteins were identified with a horseradish peroxidase conjugate secondary antibody and lumi-light western blotting substrate.

3.4.1 Expression of cloned ORF

A pilot expression of the clones produced no significant bands and a larger scale expression study produced bands of the expected size (68 KDa) (Figure 3.8 & 3.9). The western blot analysis showed the presence of expressed protein with a size of 68 KDa. The presence of other minor bands may be due to degradation products of proteins (Figure 3.8a). Negative and positive controls from IPTG induced cells with an expression vector without the insert and expression vector containing the insert were also included to evaluate the specificity of the detected bands (data not shown).

Coomassie gels of protein extracts before (data not shown) and after purification were also analysed (Figure 3.8b). The numerous additional bands may be degradation products of protein and truncated translation products (Figure 3.8b).

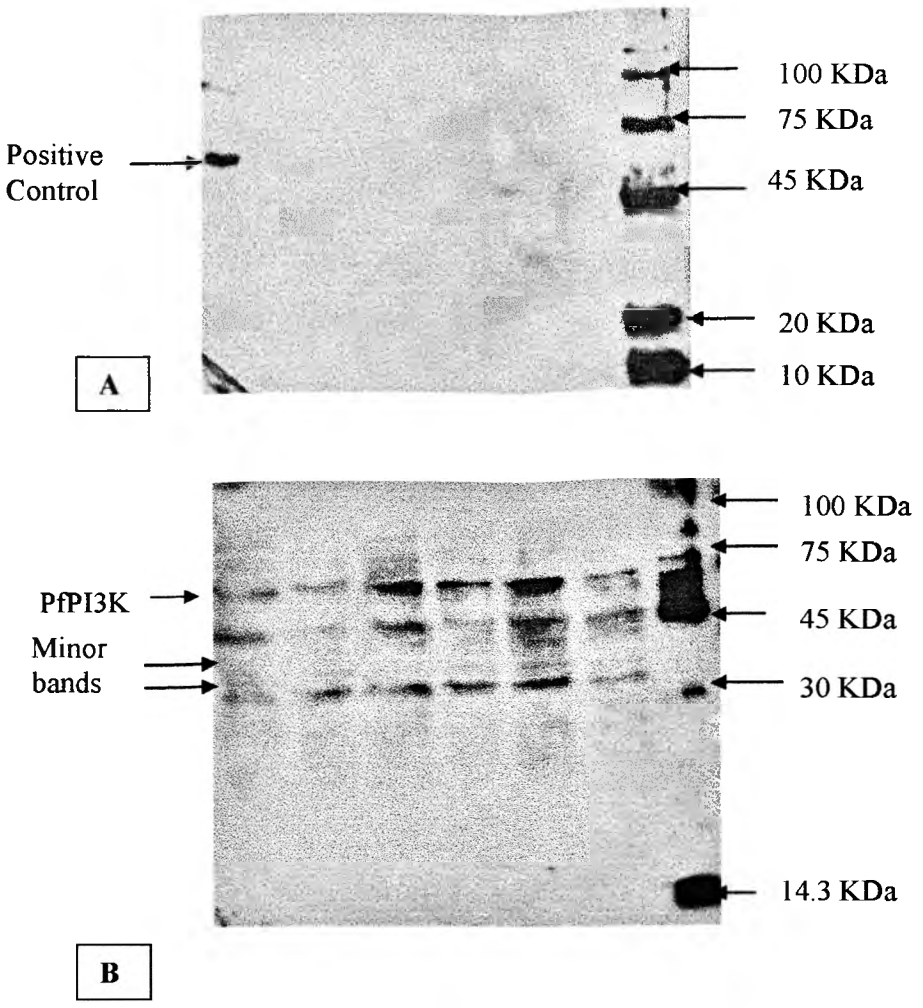


Figure 3.8a: Western blot analysis of PfPI3K.

A = Small scale experiments of expressed PfPI3K clones in the absence of IPTG induction. A Multi-Tag protein marker is indicated on the extreme right.

B = Large scale experiments of expressed PfPI3K clones in the presence of IPTG induction.

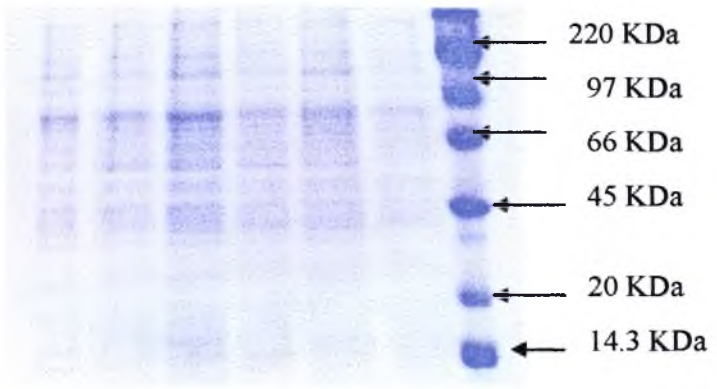


Figure 3.8b: Commassie stained gel of expressed PfPI3K clones after purification.

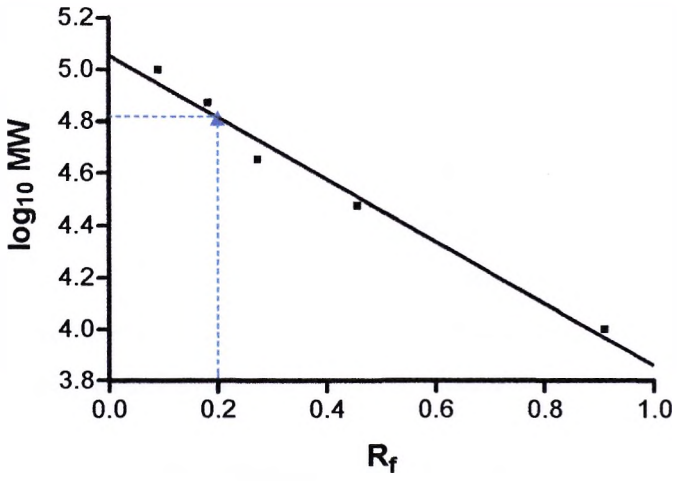


Figure 3.9: Standard curve for the determination of the molecular weights of His-tagged proteins in the western blot analysis. The symbol ▲ represents the relative distance migrated by the expressed PfPI3K protein (68 Kda = 0.2 R_f) against its molecular weight $\log_{10}$. The curve was generated by plotting the relative distance migrated by protein (R_f) against the $\log_{10}$ of its molecular weight.

3.4.2 Expression of phosphatidylinositol 3-kinase in the malaria parasite

The microarray data obtained by Le Roch, *et al* (2003) shows that the PFE0765w gene is expressed in trophozoites, schizonts and gametocytes. Mass spectrometry-based evidence at PlasmoDB also shows that the protein encoded by PFE0765w is present in sporozoites.

3.5 Sequence analysis of *P. falciparum* PI3K

The percentage identity between the compared sequences refers to the number of amino acids residues that are identical in the alignment. The pairwise alignment obtained after comparing the PfPI3K ORF (1 758 bp) against PI3K sequences in other organisms demonstrated similarity between the catalytic domains. The pairwise alignment is shown in Figure 3.10 obtained from the Clustal W alignment program at EBI (http://www.ebi.ac.uk). The pairwise blastp program was used to compare the amino acid sequence of the amino-terminus of PfPI3K with that of the pleckstrin homology domains that are present in the amino-termini of other organisms. No significant similarity was found between the sequences thus indicating that PfPI3K does not have a pleckstrin homology domain.

Table 3.2: Percentage identity of ORF in contig P42336 (PfPI3K) with PI3K in other organisms.

PI3K in various organisms	% Identity of PfPI3K to other organisms
Arabidopsis thaliana	27
Drosophila melanogaster	34
Homo sapiens	30
Saccharomyces cerevisiae	42
Rattus musculus	20
Dictostelium discoideum	22
Root nodule	31
Human	32

The overall identity of all the species is lower compared to the values for the C-terminal only. Therefore though all the species such as Homo sapiens show a 50 % similarity to the PfPI3K catalytic domain, the overall gene similarity is lower (Table 3.2).

3.5.1 Catalytic domain analysis

The catalytic domains of PI3K differ significantly from those described by Hanks and Quinn (1991). This is due to the fact that PI3Ks have been shown to phosphorylate both proteins and lipids, whilst serine/threonine kinases phosphorylate proteins (Wymann & Pirola, 1998). The PfPI3K catalytic domain contains the C-terminal which consists of the activation and catalytic loops that are important in the determination of substrate specificity (Figure 3.10). One of the highly conserved features of the nucleotide binding site is a conserved lysine residue important for ATP binding and coordination shown in Figure 3.10 (Vanhaesebroeck *et al*, 2001). The C-terminal lobe has four critical residues that are critical for ATP binding, three lysine residues and an arginine residue (Figure 3.10) (Vanhaesebroeck *et al*, 2001). The catalytic site responsible for initiating phosphorylation inferred from homology to other PI3Ks is defined by the consensus sequence GIGDRHLDN in PfPI3K. The consensus sequence GIGDRHLDN is located between amino acids 440 and 448 in PfPI3K (Figure 3.10).

```
D.melanogaster     FSGLYSEEHPSFQVRLQVFNQGRPYCLPVTSSYKAFGKRWSWNEWVTLPL 96
H. sapiens                FSGLYQETCSDLYVTCQVFAEGKPLALPVRTSYKAFSTRWNWN
A.thaliana         ------------------------------------------------
Vps34              YSSLYRDTTIDLYITCTLYSDGKQLSTSEHTSYNPFSVSNKWDETIIFPL 99
PfPI3K             --------------MIHYIDDSKNVKIERNRDNSFFSNFLQFNDNLDFFL 36

D. melanogaster    QFSDLPRSAMLVLTILDCSGAGQTTVIGGTSISMFGKDGMFRQGMYDLRV 146
H. sapiens         KYPDLPRNAQVALTIWDVYGPGKAVPVGGTTVSLFGKYGMFRQGMHD
A. thaliana        ------------------------------------------------
Vps34              KHQDLPFDTMIVITIWDIYSPMKKVPIGGTSFNIFGMNKIERKGKHKLLI 149
PfPI3K             NATYS---------------------------------------------- 41

D. melanogaster    WLGVEGDGNFPSRTPGK-GKESSKSQMQRLGKLAKKHRNGQVQKVDWLDR 195
H. sapiens         WPNVEADGSEPTKTPGRTSSTLSEDQMSRLAKLTKAHRQGHMVKVDWLDR 19
A. thaliana        ------------------------------------------------
Vps34              WQDREGDGEKETTTPGQ---IQGKDEQYRLEKLKKKYDRKLINHCQWLDK 1
PfPI3K             ------DEDNNYEILDDSINFVQKQKIKKIKTPLILPIDPNIELLSFLP- 84
```

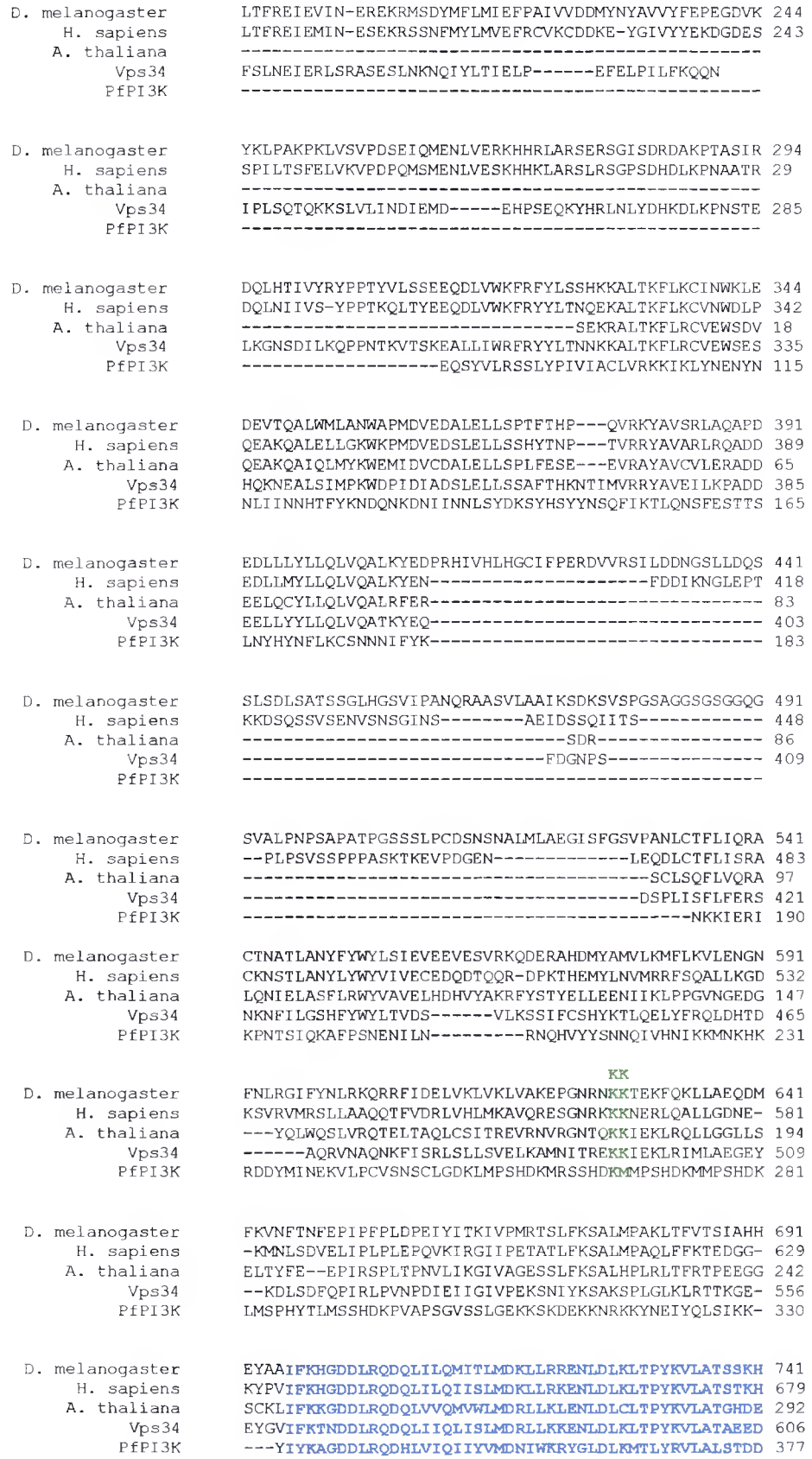

D. melanogaster LTFREIEVIN-EREKRMSDYMFLMIEFPAIVVDDMYNYAVVYFEPEGDVK 244
H. sapiens LTFREIEMIN-ESEKRSSNFMYLMVEFRCVKCDDKE-YGIVYYEKDGDES 243
A. thaliana
Vps34 FSLNEIERLSRASESLNKNQIYLTIELP------EFELPILFKQQN
PfPI3K
D. melanogaster YKLPAKPKLVSVPDSEIQMENLVERKHHRLARSERSGISDRDAKPTASIR 294
H. sapiens SPILTSFELVKVPDPQMSMENLVESKHHKLARSLRSGPSDHDLKPNAATR 29
A. thaliana
Vps34 IPLSQTQKKSLVLINDIEMD-----EHPSEQKYHRLNLYDHKDLKPNSTE 285
PfPI3K
D. melanogaster DQLHTIVYRYPPTYVLSSEEQDLVWKFRFYLSSHKKALTKFLKCINWKLE 344
H. sapiens DQLNIIVS-YPPTKQLTYEEQDLVWKFRYYLTNQEKALTKFLKCVNWDLP 342
A. thaliana ----------SEKRALTKFLRCVEWSDV 18
Vps34 LKGNSDILKQPPNTKVTSKEALLIWRFRYYLTNNKKALTKFLRCVEWSES 335
PfPI3K ----------EQSYVLRSSLYPIVIACLVRKKIKLYNENYN 115
D. melanogaster DEVTQALWMLANWAPMDVEDALELLSPTFTHP---QVRKYAVSRLAQAPD 391
H. sapiens QEAKQALELLGKWKPMDVEDSLELLSSHYTNP---TVRRYAVARLRQADD 389
A. thaliana QEAKQAIQLMYKWEMIDVCDALELLSPLFESE---EVRAYAVCVLERADD 65
Vps34 HQKNEALSIMPKWDPIDIADSLELLSSAFTHKNTIMVRRYAVEILKPADD 385
PfPI3K NLIINNHTFYKNDQNKDNIINNLSYDKSYHSYYNSQFIKTLQNSFESTTS 165
D. melanogaster EDLLLYLLQLVQALKYEDPRHIVHLHGCIFPERDVVRSILDDNGSLLDQS 441
H. sapiens EDLLMYLLQLVQALKYEN----------FDDIKNGLEPT 418
A. thaliana EELQCYLLQLVQALRFER---------- 83
Vps34 EELLYYLLQLVQATKYEQ---------- 403
PfPI3K LNYHYNFLKCSNNNIFYK---------- 183
D. melanogaster SLSDLSATSSGLHGSVIPANQRAASVLAAIKSDKSVSPGSAGGSGSGGQG 491
H. sapiens KKDSQSSVSENVSNSGINS--------AEIDSSQIITS---------- 448
A. thaliana ----------SDR---------- 86
Vps34 ----------FDGNPS---------- 409
PfPI3K
D. melanogaster SVALPNPSAPATPGSSSLPCDSNSNAIMLAEGISFGSVPANLCTFLIQRA 541
H. sapiens --PLPSVSSPPPASKTKEVPDGEN----------LEQDLCTFLISRA 483
A. thaliana ----------SCLSQFLVQRA 97
Vps34 ----------DSPLISFLFERS 421
PfPI3K ----------NKKIERI 190
D. melanogaster CTNATLANYFYWYLSIEVEEVESVRKQDERAHDMYAMVLKMFLKVLENGN 591
H. sapiens CKNSTLANYLYWYVIVECEDQDTQQR-DPKTHEMYLNVMRRFSQALLKGD 532
A. thaliana LQNIELASFLRWYVAVELHDHVYAKRFYSTYELLEENIIKLPPGVNGEDG 147
Vps34 NKNFILGSHFYWYLTVDS------VLKSSIFCSHYKTLQELYFRQLDHTD 465
PfPI3K KPNTSIQKAFPSNENILN---------RNQHVYYSNNQIVHNIKKMNKHK 231
KK
D. melanogaster FNLRGIFYNLRKQRRFIDELVKLVKLVAKEPGNRNKKTEKFQKLLAEQDM 641
H. sapiens KSVRVMRSLLAAQQTFVDRLVHLMKAVQRESGNRKKNERLQALLGDNE- 581
A. thaliana ---YQLWQSLVRQTELTAQLCSITREVRNVRGNTQKKIEKLRQLLGGLLS 194
Vps34 ------AQRVNAQNKFISRLSLLSVELKAMNITREKKIEKLRIMLAEGEY 509
PfPI3K RDDYMINEKVLPCVSNSCLGDKLMPSHDKMRSSHDKMMPSHDKMMPSHDK 281
D. melanogaster FKVNFTNFEPIPFPLDPEIYITKIVPMRTSLFKSALMPAKLTFVTSIAHH 691
H. sapiens -KMNLSDVELIPLPLEPQVKIRGIIPETATLFKSALMPAQLFFKTEDGG- 629
A. thaliana ELTYFE--EPIRSPLTPNVLIKGIVAGESSLFKSALHPLRLTFRTPEEGG 242
Vps34 --KDLSDFQPIRLPVNPDIEIIGIVPEKSNIYKSAKSPLGLKLRTTKGE- 556
PfPI3K LMSPHYTLMSSHDKPVAPSGVSSLGEKKSKDEKKNRKKYNEIYQLSIKK- 330
D. melanogaster EYAAIFKHGDDLRQDQLILQMITLMDKLLRRENLDLKLTPYKVLATSSKH 741
H. sapiens KYPVIFKHGDDLRQDQLILQIISLMDKLLRKENLDLKLTPYKVLATSTKH 679
A. thaliana SCKLIFKKGDDLRQDQLVVQMVWLMDRLLKLENLDLCLTPYKVLATGHDE 292
Vps34 EYGVIFKTNDDLRQDQLIIQLISLMDRLLKKENLDLKLTPYKVLATAEED 606
PfPI3K ---YIYKAGDDLRQDHLVIQIIYVMDNIWKRYGLDLKMTLYRVLALSTDD 377



Figure 3.10: The amino acid sequence alignment of the catalytic domain of PI3K in different organisms. The Clustal W program at EBI (http://www.ebi.ac.uk) was used to generate sequence alignments comparing amino acid sequences of PI3K in other organisms with that of PfPI3K. The catalytic core (homology region 1) shows highly conserved amino acid residues (shown in blue). The residues critical for ATP binding (lysine and arginine residues) are shaded in green. The signature residues such as the ATP binding sites are coloured in red, while the proline-rich region is coloured in pink. The abbreviations used are as follows: *D. melanogaster*: *Drosophila melanogaster* (class II); *H. sapiens*: *Homo sapiens* (classs I); *A. thaliana*: *Arabidopsis thaliana*; *Vps34*: *Saccharomyces cerevisiae* (yeast) (class III) and PfPI3K: *Plasmodium falciparum* phosphatidylinositol 3-kinase.

The following motifs were identified using a motif finder at the EBI website (http//:www.ebi.ac.uk). Figure 3.11 illustrates the different motifs found in the amino acid sequence of the ORF.

```
  1 MIHYIDDSKN VKIERNRDNS FFSNFLQFND NLDFFLNATY SDEDNNYEIL DDSINFVQKQ  60
 61 KIKKIKTPLI LPIDPNIELL SFLPEQSYVL RSSLYPIVIA CLVRKKIKLY NENYNNLIIN 120
121 NHTFYKNDQN KDNIINNLSY DKSYHSYYNS QFIKTLQNSF ESTTSLNYHY NFLKCSNNNI 180
181 FYKNKKIERI KPNTSIQKAF PSNENILNRN QHVYYSNNQI VHNIKKMNKH KRDDYMINEK 240
241 VLPCVSNSCL GDKLMPSHDK MRSSHDKMMP SHDKMMPSHD KLMSPHYTLM SSHDKPVAPS 300
301 GVSSLGEKKS KDEKKNRKKY NEIYQLSIKK YIYKAGDDLR QDHLVIQIIY VMDNIWKRYG 360
361 LDLKMTLYRV LALSTDDGFI EFVDYAESIS SIKKNYKGEI RQYFIDNSTC SSSPLGFDTE 420
421 ILQNFISSCA GYSVITYILG IGDRHLDNLM VTKDGRFFHI DFGYIFGEDP KPFSPPMKLC 480
481 KEMIEAMGGA HSIGYEQFLK KCCLAYKYLR YHSQLIISLL DAMCDAGLKD MKMSPELCVL 540
541 KVQEKFRLDL NDEAAEIYFL SVINASVKTL FPVVVDKLHE WALNWK 586
```

Figure 3.11: Amino acid motifs in PI3K. The amino acid sequence of PI3K showing the ATP binding motifs (red). A proline-rich motif (pink) and the leucine zipper-like motif (blue) are shown.

3.6 The effects of selected compounds on *P. falciparum*

3.6.1 Antimalarial effects of the phosphatidylinositol 3-kinase inhibitors and forskolin and 1,9-dideoxyforskolin

The IC_{50} values for chloroquine, quinine, wortmannin, LY294002, forskolin and 1,9-dideoxyforskolin over a single and double cycle were determined from sigmoid dose response curves as shown in Figure 3.12. Inhibition assays were repeated at least five times for each agent tested. Figure 3.12 represents the IC_{50} values obtained from five experiments. The standard deviation is for the five experiments.

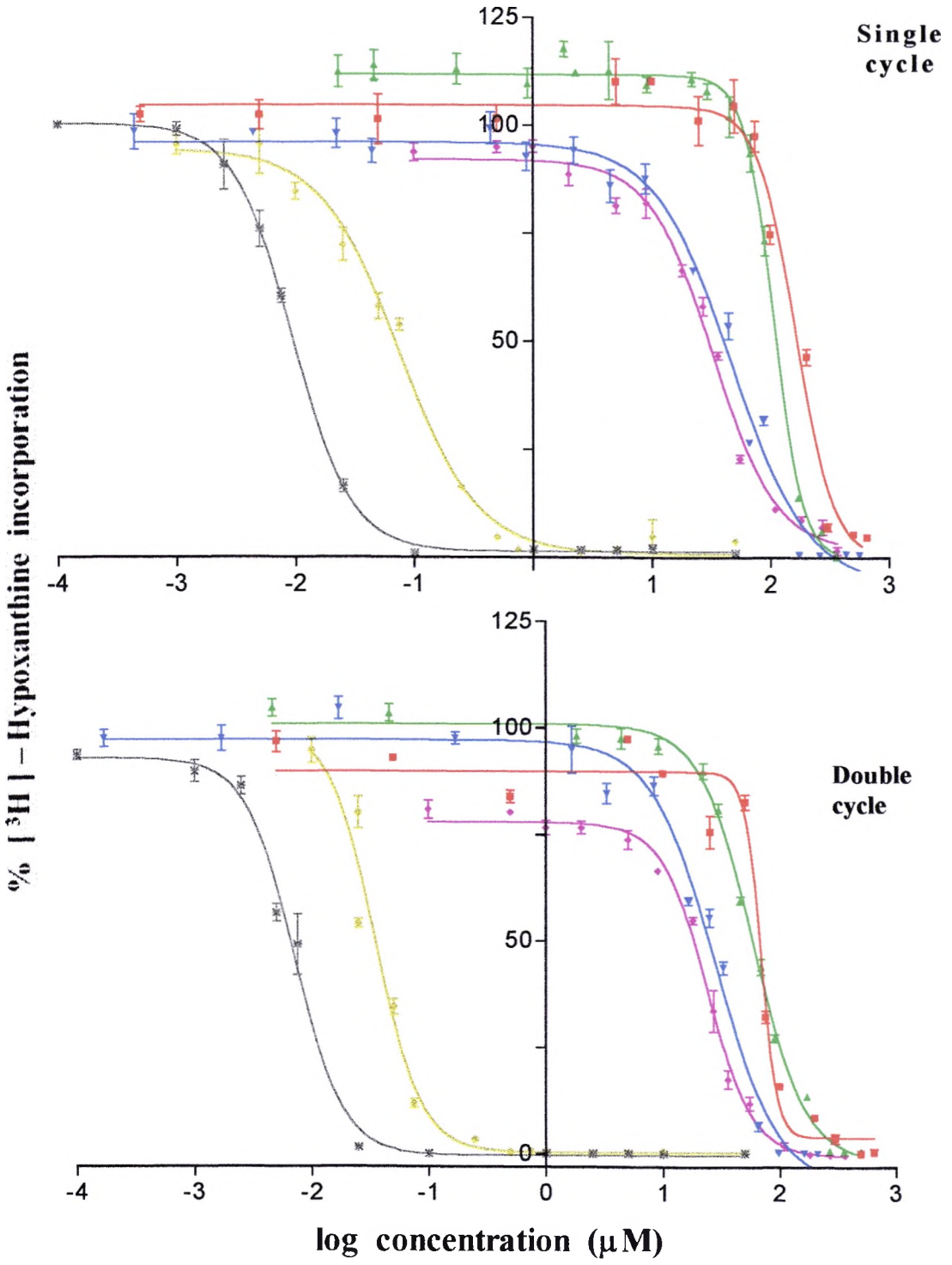


Figure 3.12: Sigmoid dose response curves of selected compounds and antimalarial agents over a single (top) and double (bottom) cycle of DNA synthesis representative of five experiments. Chloroquine (✱), quinine (●), LY294002 (◆), 1,9-dideoxyforskolin (▼), wortmannin (▲), forskolin (■), (1) standard deviation.

The IC_{50} values obtained for wortmannin over a single (110 ± 4.90 μM) cycle and double (66.12 ± 8.93 μM) cycle are higher than the documented results of wortmannin on whole cells such as human neutrophils (10 μM) (Powis *et al*, 1994). The IC_{50} values obtained for LY294002 over a single (32.14 ± 2.80 μM) cycle and double (27.45 ± 5.68 μM) cycle are comparable to the IC_{50} values obtained on cultured rabbit cell lines (20 μM) (Vlahos *et al*, 1994). The obtained IC_{50} values for forskolin over a single (169.76 ± 2.41 μM) and double (80.80 ± 7.84 μM) cycle are higher compared to the IC_{50} values obtained on smooth muscle cell lines (50-100 μM) (Seamons & Daly, 1986). Chloroquine and quinine remain a thousand-fold more potent than the protein kinase inhibitors investigated (wortmannin and LY294002. The PI3K specific inhibitors wortmannin and LY294002 had significant differences with regard to their effects on *P. falciparum* over a single ($p<0.0002$) and double ($p<0.008$) cycle. LY294002 was shown to be the most potent of the protein kinase inhibitors investigated (Figure 3.13).

The adenylate cyclase activator, forskolin and its inert analogue 1,9-dideoxyforskolin were also significantly different over a single ($p<0.0001$) and double ($p<0.0088$) cycle of DNA synthesis. 1,9-dideoxyforskolin has a more potent inhibitory effect on *P. falciparum* than forskolin (Figure 3.12 & 3.13).

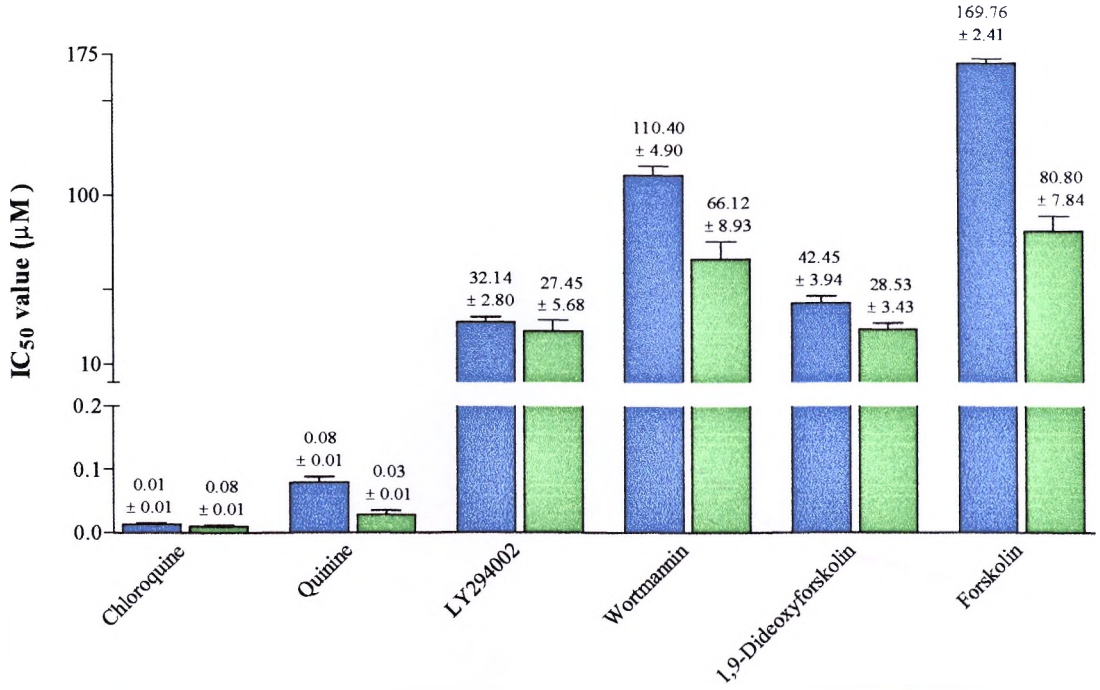


Figure 3.13: Comparison of IC_{50} values over a single (■) and double (■) cycle of DNA synthesis. The values indicate the mean ± standard deviation of at least three experiments

3.6.2 Morphological effects of the selected compounds on *P. falciparum* 3D7 strain

The morphological effects of three concentrations of these selected compounds were compared to the drug free parasites. At the high drug concentration the trophozoites appeared to be more vacuolated than the untreated control trophozoites (Figure 3.14). Whilst the parasites in the ring stage appeared smaller compared to the control parasites (not shown). The red blood cell membrane appeared to remain unaltered compared to the control red blood cells but more refined methods such as electron microscopy would need to be done to verify this observation. At the high drug concentration the trophozoites appeared to be smaller than the untreated control trophozoites (Figure 3.15). Whilst the parasites in the ring stage appeared smaller compared to the control parasites (not shown). The

percentage rings, trophozoites and schizonts decreased as concentrations increased.

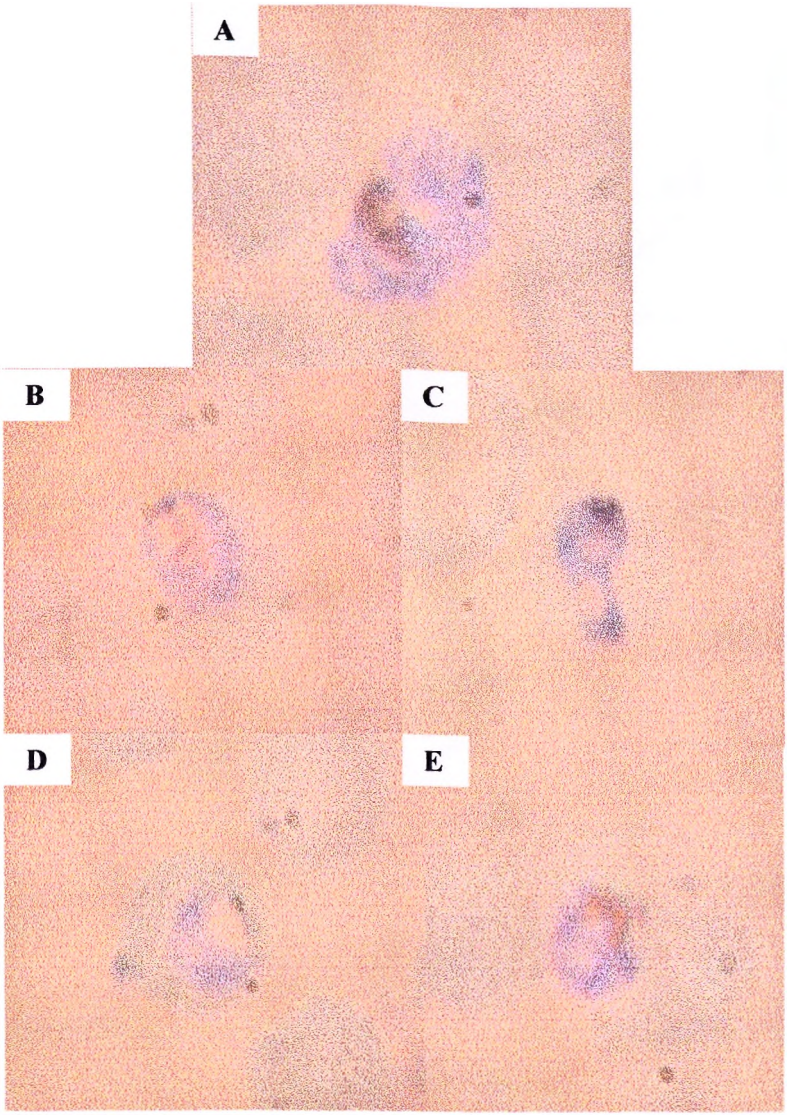


Figure 3.14: Effects of selected compounds on the trophozoite stage of *Plasmodium falciparum* compared to an untreated control (under 1000x oil immersions). A: control, B: 1,9-dideoxyforskolin, C: forskolin, D: LY294002, and E: wortmannin.

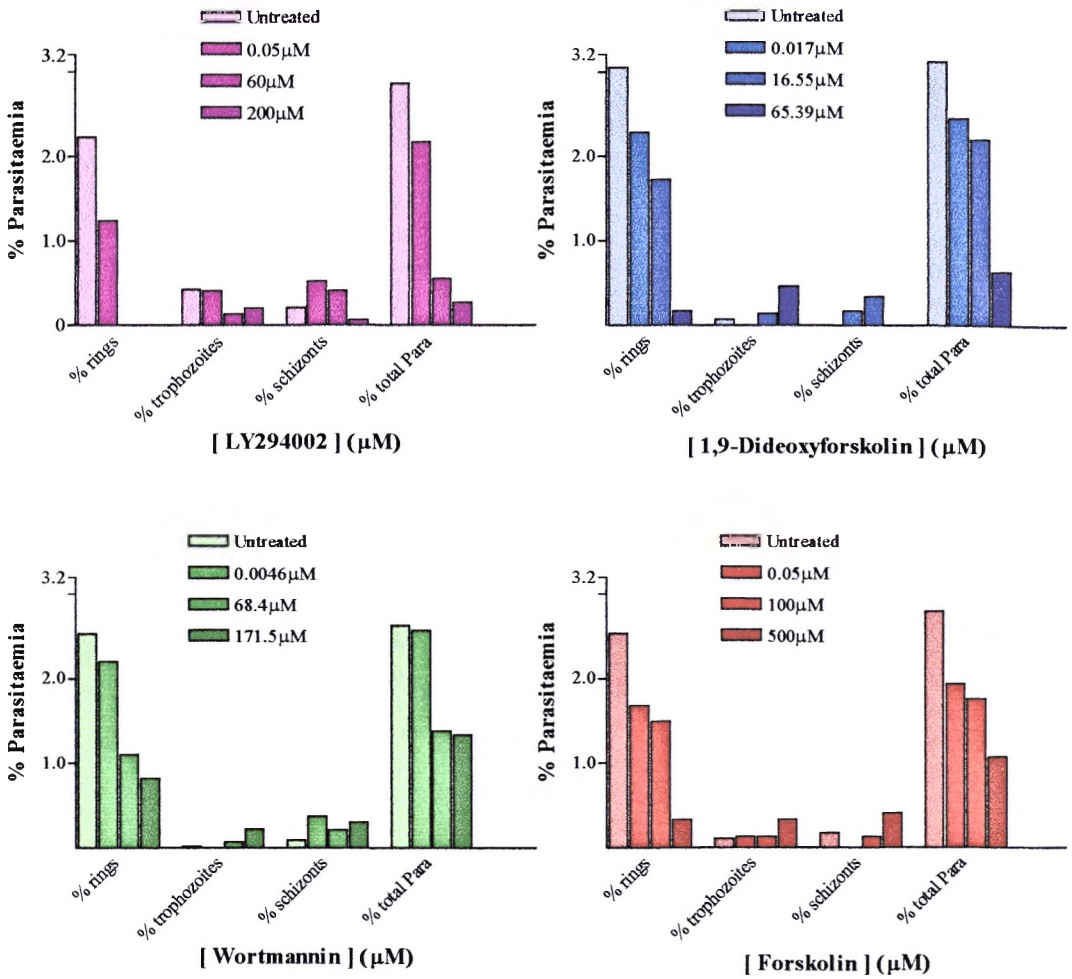


Figure 3.15: The effects of selected compounds on the growth of *P. falciparum*.

LY294002 was the more effective protein kinase inhibitor against the ring stage of *P. falciparum* compared to wortmannin, and also had a lower IC_{50} values (Figure 3.13 & 3.15). However both PI3K inhibitors do not show a significant effect against the schizont and trophozoite stages of *P. falciparum*. 1,9-dideoxyforskolin has a lower IC_{50} value compared to forskolin and showed to be more effective against the ring stages of *P. falciparum* (Figure 3.13 & 3.15).

4.0 DISCUSSION

4.1 Identification of a phosphatidylinositol 3-kinase homologue in *P. falciparum*

An open reading frame encoding a putative PI3K homologue was used to design specific primers to amplify the sense strand of the DNA coding for the ORF (Figure 3.1). The PCR Master kit® was used to optimize some of the PCR parameters such as annealing temperature, primer and DNA concentration. The optimum annealing temperature was found to be 64°C, where a single band of the expected size of 1 812 bp was obtained, with no other bands due to non-specific binding being generated. At the lower annealing temperatures (58-62°C) minor bands (900 bp & 400 bp) were observed (shown in Figure 3.3). This may have been due to non-specific binding of primers at lower annealing temperatures. The optimum primer (0.1 µM) and DNA concentration (1 µg/30 µl) generated a PCR product of the expected size of 1 812 bp at an annealing temperature of 64°C (Figure 3.3).

The 1 812 bp PCR product generated when using the Expand High Fidelity kit®, was partially sequenced from both ends of the ORF to determine the sequence (Figure 3.5). Analysis of the DNA sequence of the PCR product with the Blastn programme at PlasmoDB showed 98-99 % identity to the same part of the sequence of PfPI3K (PF0765w) located on chromosome 5. However the overall sequence may have a lower identity in comparison to the ORF of PF0765w sequence due to polymorphism. The changes in the amino acid sequence may lead to overall changes in the secondary and tertiary structure of the protein.

The PfPI3K PCR products from three PCR reactions were cloned into the pQE80L expression vector. The transformed clones were screened by restriction digestion and PCR (Figure 3.6 & 3.7). Products obtained by restriction digestion of plasmids show an insert of the expected size of 1 812 bp when separated on a 1 % agarose gel. When clones were screened by PCR a minor band of 800 bp was also seen in addition to the 1 812 bp band when the PCR Master kit® was used. This may be due to unspecific binding of primers to a region of complementarity to the sense and antisense primers within the ORF sequence. When the PfPI3K inserts of the pQE-80L plasmid were sequenced by automated sequencing using vector primers, a longer sequence was obtained and analysed to establish the identity essential for protein expression. The PfPI3K insert was sequenced with vector primers and was found to be in frame.

A small (5 ml) time scale protein expression study of the putative PfPI3K gene produced no detectable product by western blot analysis using anti-His monoclonal antibody (Figure 3.8a). A larger scale protein expression experiment of the putative PfPI3K gene produced three bands of the sizes 68.5 KDa, 45 KDa and 35 KDa (Figure 3.8b). The band of 68.5 KDa is most likely to be the expressed protein and the smaller bands (45 KDa & 35 KDa) are probably truncated translation products or proteolytic degradation products of the protein (Figure 3.8b). Further investigations are required to optimise the expression of PfPI3K and to verify the identity of the 68.5 KDa band.

4.1.1 Sequence analysis of the predicted gene in PlasmoDB containing the catalytic domain

Blast analysis of the ORF containing the catalytic domain produced significant alignment to the Vps34-like phosphatidylinositol 3-kinase (Figure 3.2). The Vps34-like PI3K is implicated in the sorting of vacuolar proteins in yeast cells and in mammalian cells

(Stack & Emr, 1994). In *P. falciparum* the PfPI3K may have a similar function as to that of the Vps34-like PI3K. Furthermore the PfPI3K may be involved in the development and functioning of the food vacuole in *P. falciparum*, thus PfPI3K may be an important potential drug target in malaria. The PfPI3K open reading frame contains the sequences DRHLDN and DFG conserved in the catalytic loop of most protein kinases (Figure 3.10). Like other PI3Ks, PfPI3K does not contain the glycine rich GxGxxG sequence motif which is present in serine/threonine kinases.

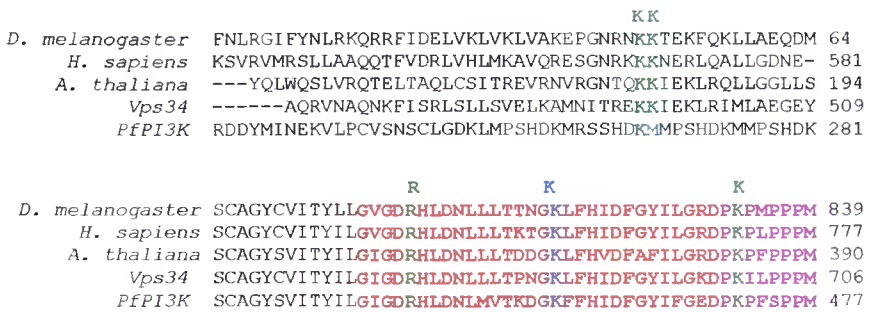


Figure 4.1: Sequence of the catalytic domain in PfPI3K. Alignment of the PfPI3K with other PI3Ks in other organisms shows the four characteristic lysine residues involved in phosphorylation of PI3Ks shown in green. PfPI3K has a methionine in place of lysine in the position coloured in turquoise. The amino acid residues characteristic of the catalytic loop are shown in red. The proline-rich region is shown in pink. The lysine residue critical for protein kinase activity is shown in blue.

4.1.2 Sequence analysis of the phosphatidylinositol 3-kinase predicted in PlasmoDB

Other regions identified in the complete PFE076w gene includes the helical domain and a catalytic core domain (Fruman *et al*, 1998). A proline-rich region and a leucine zipper-like region have also been identified.

4.1.2.1 The catalytic domain

The C-terminal of PfPI3K contains one of the highly conserved features of the catalytic domain, namely a conserved lysine residue within the ATP binding site which is characteristic of most protein kinases (Figure 4.1). The lysine residue adjacent to the GDRHLDN and DFG region is critical for protein kinase activity in all protein kinases. Obstruction of this residue by the PI3K inhibitor wortmannin, was shown to impair phosphorylation (Vanhaesebrock *et al*, 2001). The C-terminal region in the ORF of PfPI3K contains the ATP binding and activation sites which determine substrate specificity. Four residues are critical in the activation of the catalytic loop, three lysine residues and an arginine residue (Figure 4.1)

In PfPI3K the lysine residue in position 808 is replaced by a methionine residue and since lysine has charged polar side chains whilst methionine has non polar side chains this may lead changes in the structure of the protein (Figure 4.1). Thus the ATP coordination and transfer of phosphates to the lipid head groups in PI3Ks is different from the catalysis displayed by other PI3Ks (Figure 4.1). The catalytic loop is responsible for deprotonating the substrate thereby generating the nucleophile that associates with the γ-phosphate of ATP (Vanhaesebrock *et al*, 2001). The catalytic loop is defined by the consensus sequence GIGDRHLDN (Waterfield *et al*, 1997). This consensus sequence is present in PfPI3Ks.

4.1.2.2 The helical domain

The domain adjacent to the N-terminus of the catalytic domain in the complete PfPI3K gene sequence in the PlasmoDB is the helical domain (not shown) which acts as the structural spine on which other domains are anchored (Vanhaesebrock *et al*, 2001). The domain consists of five parts of antiparallel helices that are arranged in a pattern. The arrangement suggests a possibility that the helical

domain is involved in protein-protein interactions (Vanhaesebrock *et al*, 2001).

4.1.3 Additional structural motifs in PfPI3K

Two additional structural motifs were identified in the 1 758 bp ORF that are similar to those identified in other PI3Ks. The first identified region is a proline-rich region which is important in the mediation of protein-protein associations (Figure 3.10). The proline-rich region was shown to form a left-handed helical domain in PI3Ks found in *Drosophila* (Vanhaesebrock *et al*, 1997). The second motif identified in the putative PfPI3K is a region similar to the basic leucine zipper-like domain. The leucine zipper-like motif has the typical leucine/valine/isoleucine arrangement of residues (Figure 4.2). The basic leucine zipper-like motif may form helical structures that can form a coiled dimeric protein zipper complex. In transcription factors the leucine zipper motif functions in DNA binding and transcription factor dimerisation. It has been suggested that the leucine zipper may function as a binding domain for the pleckstrin homology domain in PI3K and may thus have a similar function in *P. falciparum* (Figure 4.2) (Vanhaesebrock *et al*, 2001).

```
D. melanogaster     EYAAIFKHGDDLRQDQLILQMITLMDKLLRRENLDLKLTPYKVLATSSKH 741
     H. sapiens     KYPVIFKHGDDLRQDQLILQIISLMDKLLRKENLDLKLTPYKVLATSTKH 679
   A. thaliana      SCKLIFKKGDDLRQDQLVVQMVWLMDRLLKLENLDLCLTPYKVLATGHDE 292
         Vps34      EYGVIFKTNDDLRQDQLIIQLISLMDRLLKKENLDLKLTPYKVLATAEED 606
        PfPI3K      ---YIYKAGDDLRQDHLVIQIIYVMDNIWKRYGLDLKMTLYRVLALSTDD 377
```

Figure 4.2: The leucine zipper-like motif. The leucine zipper-like motifs are indicated in blue in the above Clustal alignment.

The presence of a pleckstrin homology domain in the complete gene sequence predicted in the PlasmoDB was investigated using the motif finder at the EBI website (http://www.ebi.ac.uk) and no typical match was found. The pleckstrin homology domain is found in many signalling proteins that mediate protein-lipid interactions.

In the case of most downstream effectors of PI3K such as PDK and protein kinase B the pleckstrin homology domain is required for its recruitment to the plasma membrane.

The recruitment occurs through binding of phosphoinositides produced by the activated PI3Ks (Hill & Hemmings, 2002). A PDK homologue which provides a missing link between PI3K and its downstream effectors in the PI3K signaling pathways has not been predicted in the PlasmoDB. Further investigations can clarify the specific means of PI3K signaling in the absence of PDK and a pleckstrin homology domain or identify other proteins which may fulfill the function of PDK.

4.2 Antimalarial properties of selected compounds

The triated hypoxanthine incorporation assay was used as a direct measure of the antimalarial activity of the selected compounds, wortmannin, LY294002, forskolin and 1,9-dideoxyforskon. Wortmannin and LY294002 were less potent than chloroquine and quinine in inhibiting *P. falciparum* growth. They inhibited *P. falciparum* in the micromolar (20-200 μM) range compared to chloroquine and quinine which inhibited in the low nanomolar (9-100 nM) range (Figure 3.13). LY294002 was more effective than wortmannin over a single and double cycle of DNA synthesis.

The adenylate cyclase activator forskolin inhibited *P. falciparum* growth with a relatively high IC_{50} compared to its inert analogue 1,9-dideoxyforskolin, over a single and double cycle of DNA synthesis (Figure 3.13).

4.2.1 Antimalarial effects of wortmannin

High concentrations of wortmannin were required to inhibit whole cell activity in comparison wortmannin inhibited isolated PI3K

enzymes with IC_{50} values in the nanomolar ranges (shown in Table 4.1) (Stack & Emr, 1994; Walker *et al*, 2000). This may be due to the increased availability of the drug to the active site on isolated enzymes compared to whole cells. Wortmannin was not as effective on *P. falciparum* as compared to *S. pombe* and *S. cerevisae* yeast cells (Table 4.1)

Table 4.1: The inhibitory effects of wortmannin on different enzyme systems.

Organism	Isolated enzyme (IC_{50})	Whole cell (IC_{50})
Vps34-like *S. cerevisae* yeast cells	—	20-50 μM[1]
Human platelet cells	2-5 nM[2]	—
Human leukocytes	10-20 nM[3]	—
Schizosaccharomyces pombe	—	20 μM[3]
P. falciparum Single cycle Double cycle	— —	 110.40 ± 4.90 μM 66.12 ± 8.93 μM

1: Stack & Emer, 1994; 2:Walker *et al*, 2000; 3: Powis *et al*, 1994

Blast analysis of the ORF have suggested that the PI3K homologue in *P. falciparum* is similar to the Vps34-like PI3K found in yeast cells (Figure 3.10). The close shape complementarity between the PI3K active site and wortmannin enables wortmannin to irreversibly inhibit most isolated PI3Ks at nanomolar concentrations (10-50 nM) (Vanhaesebrock *et al*, 1997). Although the catalytic domain of PfPI3K is similar to the class III Vps34-like PI3K it differs to those found in other PI3K in class I and II. One of the lysine residues in the region for ATP binding in PfPI3K is replaced by a methionine

residue (Figure 4.1). Methionine has sulfur containing side chains and less hydrophilic compared to lysine which is a basic amino acid and strongly polar. It is possible that this may cause a change in the 3D configuration of the active site, resulting in a decrease in the inhibitory effect of wortmannin on the enzyme.

Studies have shown that most protein kinase inhibitors show discrepancies when applied to cultured cells, due to their limited selectivity. Therefore it remains unclear whether the results obtained on cultured cell lines were the consequence of low specificity in the inhibition of PI3K, or possible inhibition of other related kinases (Stein, 2001). Furthermore, in *P. falciparum* the drugs have to cross several membranes before reaching the target enzyme.

In toxicity studies carried out on rats, wortmannin was shown to be toxic at 7 mg/kg body weight, where gastric and myocardial haemorrhages were noted, along with necrosis of the spleen, lymph nodes and gastro intestinal tract associated lymphoid tissue (Gunther *et al*, 1989). A single dose of 1 mg/kg body weight resulted in Rhesus monkeys having weakness, decreased motility, incoordinated behaviour and anorexia (Wiesinger *et al*, 1974). Wortmannin (1 μM) has also been reported to increase platelet aggregation and serotonin release (Hashimoto *et al*, 1992). This could have clinical implications such as potentiating the pathology of malaria. *In vitro* exposure of wortmannin to neutrophils or macrophages results in the inhibition of respiratory bursts during phagocytosis and exocytosis (Baggolini *et al*, 1987). In malaria infected patients this may decrease the capacity of the system to clear *P. falciparum*-infected erythrocytes and merozoites. From the toxicity data and high concentrations required to achieve a therapeutic effect, wortmannin should not be used for therapeutic purposes. However, less toxic analogues may be a worthwhile future development for investigation.

4.2.2 Antimalarial effects of LY294002

The specific PI3K inhibitor LY294002 was shown to be the most effective of all the compounds investigated against the *P. falciparum* 3D7 strain (Figure 3.13). The IC_{50} obtained for *P. falciprum* is comparable to those of rabbit smooth cells and yeast cells (Table 4.2).

Table 4.2: The inhibitory effects of LY294002 on various organisms and isolated enzymes.

Organism	Isolated enzyme (IC_{50})	Whole cells (IC_{50})
Vps34-like *S. cerevisae* yeast cells	—	50 μM[1]
Human neutrophils	10 nM[2]	20 μM[2]
Rabbit smooth muscle cells	—	32 μM[2]
P. falciparum Single cycle Double cycle	— —	32.14 ± 2.8 μM 27.45 ± 5.68 μM

1: Stack & Emr, 1994; & 2:Vlahos *et al*, 1994

LY294002 is also more effective on isolated enzyme PI3Ks compared to its effects on whole cells (Table 4.2) (Stack & Emr, 1994; Vlahos *et al*, 1994). The IC_{50} values on the effects of LY294002 on the parasite are similar to other living cells (Table 4.2). Though no experimental evidence indicates whether PI3K kinase activity is present in uninfected erythrocytes the inhibition of the host cell PI3K may contribute to the effects of the inhibitor on parasite growth. LY294002 has also been extensively applied to cultured cells inorder to investigate the biological effects of PI3K activation. Studies have

shown that LY294002 acts as a competitive inhibitor of ATP at the catalytic site of PI3K (Stein, 2001). The main determinant of PI3K inhibition by LY294002 is the bulky 8-phenyl group. LY294002 was shown to display very little selectivity within the PI3K family and shown to lose selectivity for PI3K at concentrations between 50 and 100 μM *in vivo* on rats which appears to parallel the effect of LY294002 on *P. falciparum* (32.14 ± 2.80 μM) (Jaffe *et al*, 2000).

In rats, administration of LY294002 to treat ovarian cancer at a dose of 100 mg/kg resulted in skin toxicity, in the form of dry scaly skin and dermatological lesions (Jaffe *et al*, 2000). Thus LY294002 requires extensive further preclinical and clinical testing before considering its use in the management of malaria and cancer. Less toxic analogues of LY294002 may be useful to screen for antimalarial activity.

4.2.3 Antimalarial effects of forskolin and 1,9-dideoxyforskolin

Forskolin was shown to elevate cAMP in many cells. Elevated cAMP levels were shown to activate protein kinase B in a PI3K idependent manner (Woodgett *et al*, 1996). In contrast 1,9-dideoxyforskolin, the inert analogue of forskolin, has no effect on the cAMP levels and is shown to have lower IC_{50} values compared to forskolin. This suggests that the effects of 1,9-dideoxyforskolin may not be specifically on the expected targets. Forskolin had a minimal inhibitory effect on the *P. falciparum* 3D7 strain with an IC_{50} value of 169.76 ± 2.41 μM over a single cycle and 80.80 ± 7.84 μM over a double cycle of DNA synthesis. However its inert analogue 1,9-dideoxyforskolin had an IC_{50} value of 42.45 ± 3.94 μM over a single cycle and 28.53 ± 3.43 μM over a double cycle, which is similar to the inhibitory effects of LY294002 and wortmannin. Further investigation into the antimalarial effects of 1,9-dideoxyforskolin and other derivatives may be worthwhile.

However the toxicity data of 1,9-dideoxyforskolin should be taken into consideration where doses of 16 μM were shown to potentiate neurotoxicity in rat spinal neurons (Chesnoy-Marchais, 2003). Although the structural differences between 1,9-dideoxyforskolin and forskolin favour antimalarial activity it also appears to change the toxic potential. Forskolin was shown to indicate a low toxicity on whole rabbit smooth muscle cells at concentrations of 2-10 μM, however caution is to be taken in hypotensive patients as forskolin potentiates low blood pressure. Caution is also to be taken in patients suffering from peptic ulcers as forskolin stimulates gastric acid secretion (Seamons & Daly, 1986).

4.3 Comparison of the selected compounds with other therapeutic drugs used for malaria

The tested PI3K protein kinase inhibitors LY294002 and wortmannin inhibited the growth of *P. falciparum* with IC_{50} values in the micromolar range (20-200 μM). In contrast, drugs used in the prophylaxis and treatment of malaria inhibited the growth of *P. falciparum* with IC_{50} values in the nanomolar range (9-100nM) (Figure 3.13). Therefore the therapeutic drugs are still more effective against *P. falciparum* than the PI3K inhibitors LY294002, wortmannin. The inert forskolin analogue 1,9-dideoxyforskolin was also less effective compared to the classically used drugs. The high degree of homology between the human and PfPI3K catalytic domain may result in major complications in developing the PI3K specific protein kinase inhibitors for the treatment of malaria. A larger dose of the PI3K inhibitors would have to be administered to eliminate malaria infection than the dose of drug presently used in the treatment of malaria. The need to treat malaria with such high doses also results in the problem of specificity of action. This would lead to an unacceptable profile of adverse effects in patients.

Although the compounds tested were less potent than chloroquine and quinine, it may be beneficial to develop derivatives of these compounds for further evaluation.

5.0 CONCLUSION

Signal transduction pathways transduce extracellular signals into intracellular messages that control various aspects of cellular functions. Protein kinases are essential in signal transduction processes that relay the intracellular signal to effector proteins that regulate cell growth, differentiation, survival and the cell cycle. Several signal transduction pathways have been identified in *P. falciparum* and they include the cAMP-dependent pathway, cGMP-dependent pathway, phosphatidylinositol cycle, calcium dependent pathway and the MAPK pathways. The main aim of this study was to identify and clone a PfPI3K homologue in the malaria parasite, *P. falciparum*.

Several components of the PI3K pathway have been identified and characterised in *P. falciparum*. These include a downstream effector of PI3K, protein kinase B which has been identified in *P. falciparum* (Harmse, unpublished). Another protein kinase downstream of protein kinase B, glycogen synthase kinase 3 was identified in the *P. falciparum* genome database. PFE0765w a putative PI3K homologue was identified on chromosome 5 in the PlasmoDB. This study focused on cloning the catalytic region, verifying part of the PfPI3K sequence homologue found in the malaria parasite and testing the effect of selected compounds on *P. falciparum*.

A homology based approach was used to obtain an open reading frame encoding a putative PfPI3K homologue. The open reading frame was used to design specific primers. Restriction endonuclease sites were added to the 5'ends of the primers to facilitate directional cloning into an expression vector. A PCR product of the predicted size (1 812 base pairs) was obtained using the specified primers. The cloned sequence was verified by manual and automated sequencing. The PfPI3K insert was also sequenced with vector primers and was

found to be in frame. Protein expression was induced with IPTG and the protein purified by nickel-affinity chromatography. The protein was separated by SDS-PAGE, transferred to a PVDF membrane and probed with an anti-His antibody. Several bands were identified (68.5 KDa, 45 KDa and 35 KDa). The smaller bands could be truncated protein whilst the band at 68.5 KDa is of the predicted size. However, further investigations and optimisation of the expression is required. Sequence analysis indicated that the putative PfPI3K homologue obtained contained some catalytic signatures such as $DRx_3N_{12-13}DFG$ conserved in some protein kinase catalytic domains. Structural motifs typical of PI3Ks were identified in the analysis, in addition a leucine zipper-like motif and proline rich motif were identified. Most of the catalytic motifs of serine/threonine kinases were absent since PI3K is a lipid kinase.

Effects of selected compounds on the 3D7 strain was determined using the tritiated hypoxanthine incorporation assay. The effects of the inhibitors on the *P. falciparum* 3D7 strain were comparable to the *in vitro* effects on other organisms such as mammals. LY294002 and 1,9-dideoxyforskolin, an inert analogue of forskolin, were the most potent against *P. falciparum* 3D7 strain. The adenylate cyclase activator forskolin was the least effective against *P. falciparum*. Of the two PI3K inhibitors tested LY294002, was three times more potent than wortmannin.

The protein kinase inhibitor LY294002 and wortmannin had no effect on the infected and uninfected red blood cells, which remained the same after 48 hours of exposure, though more refined methods such as electron microscopy would need to be done to verify this observation. Trophozoites were observed to be smaller compared to the untreated control parasites.

Further investigations in protein expression experiments and protein kinase activity is required to confirm the function of the putative PfPI3K homologue in *P. falciparum*. Based on its function in other organisms, the PfPI3K homologue may be involved in a signal transduction pathway that results in cell survival, differentiation and apoptosis. Protein-protein interactions with *P. falciparum* lysates and factors influencing the enzyme activity of expressed proteins may contribute to attaining a specific role for PI3K in the malaria parasite. More investigation into the structure of the enzyme may be used to design a more effective and specific antimalarial drug.

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