PHARMACOLOGICAL EFFECT OF MEK INHIBITORS ON *PLASMODIUM FALCIPARUM*

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ABSTRACT

Malaria continues to kill up to 3 million people each year, and parasite resistance to drugs is a pressing problem. Protein kinases are now prime targets for chemotherapy in a variety of diseases such as cancer and neurodegenerative diseases, and have been proposed as potential targets for antimalarial intervention. Our group is engaged in research aiming at characterising *Plasmodium* protein kinases at the biochemical and functional levels, with emphasis on homologues of enzymes that are known regulators of proliferation and differentiation in eukaryotic cells.

The Mitogen Activated Protein Kinases (MAPKs) are quasi-ubiquitous in eukaryotes, where they play crucial roles in the regulation of cell proliferation and differentiation in response to extra-or intracellular stimuli. The MAPKs typically function in 3-component modules comprising the MAPK and upstream MAPKKs (MEKs) and MAPKKKs (MEKKs). Surprisingly, experimental and *in silico* analyses have demonstrated *P. falciparum* does not possess any MEK homologues, even though its kinome comprises two members of the MAP kinase family. Prior to the finding that the parasite does not possess MEKs, it had been shown in our laboratory that U0126, a MEK inhibitor, had parasitocidal activity *in vitro*. To confirm and extend these data, a panel of structurally distinct MEK inhibitors, most of which are not ATP competitors (and hence are likely to be more selective than more classical PK inhibitors) was tested on cultured *P. falciparum*. It is striking that most of the MEK inhibitors kill *P. falciparum* with EC_{50}s that compare very well with those against mammalian cells. The absence of MEK-encoding genes in the *Plasmodium* genome suggests that the target may be a host erythrocyte MEK. Consistent with this hypothesis, we detected an increase of MEK phosphorylation in infected red blood cells (RBCs) compared to uninfected RBCs, using a panel of antibodies directed against the activated, phosphorylated form of MEK1. In the presence of MEK inhibitors, the signal decreased in infected red blood cells. Furthermore, we show that U0126 does not act during invasion or within the first 24 hours of the asexual cycle, but during the trophozoite stage. Taken together, our data strongly suggest that infection with *P. falciparum* modulates the MAP kinase pathway in its host erythrocyte, and that the parasite relies on this pathway for its own survival.

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LIST OF ABREVIATIONS

ACTs: artemisinin-based combination therapies

AP-1: activating protein 1

APS: ammonium persulfate

ATF-2: activating transcription factor 2

ATP: adenosine triphosphate

BSA: bovine serum albumin

CO_2: carbon dioxide

CK2: casein kinase 2

cDNA: complementary DNA

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CPM: counts per minute

^{O}C: degree centigrade

DABCO: 1,4-diazabicyclo[2.2.2]octane

dH_2O: distilled water

DMSO: dimethyl sulphoxide

DNA: deoxyribonucleic acid

EC_{50}: half maximal effective concentration

EDTA: ethylene diaminetetracetic acid

EGTA: ethylene glyceroltetracetic acid

e.g.: exempli gratia

ERK: extracellular signal-regulated kinase

FEC_{50}: fraction of EC_{50}

Fig: figure

g: gram

GSK3: glycogen synthase kinase-3

^{3}H: tritium

h: hour

Hb: hemoglobin

HCl: hydrogen chloride

HRP: horseradish peroxidase

hrs: hours

IFA: immunofluorescence assay

IgG: immunoglobulin G

ikB: inhibitor of kappa B

iKK: ikB kinase

IRBCs: infected RBCs

ITN: insecticide-treated net

JNK: C-Jun N-terminal kinase

kDa: kilodalton

KO: knock out

l: litre

μCi: microcurie

μg: microgram

μM: micromolar

mA: milliampere

MACS: magnetic cell sorting

MAP kinase: mitogen-activated protein kinase

MEK: MAPK/ERK kinase

ml: millilitre

min: minute

mM: millimolar

NaCl: sodium chloride

NaF: sodium fluoride

NF-kB: nuclear factor kappa B

PT: parasitaemia

%: percentage

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffer solution

PDGF: platelet-derived growth factor

PI3K: phosphoinositide 3-kinase

P. falciparum: Plasmodium falciparum

P. berghei: Plasmodium berghei

PK: protein kinase

PKA: protein kinase A

PKC: protein kinase C

PMSF: phenylmethylsulfonylfluoride

RBC: red blood cell

SAPK: stress activated protein kinase

SDS: sodium dodecyl sulfate

SEM: standard error of the mean

spp: species

T. annulata: Theileria annulata

T. parva: Theileria parva

TBS: tris buffer solution

TBS/T: TBS/Tween

TEMED: N'-tetramethylenediamine

TGF: transforming growth factor

T. cruzi: Trypanosoma cruzi

TyrK: tyrosine kinase

URBCs: uninfected RBCs

V: volts

v/v: volume per volume

w/v: weight per volume

WB: western blot

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis and performed all of the work presented.

1 INTRODUCTION

1.1 Introduction

Malaria is a major public health problem in the tropical and sub-tropical regions of the world (Fig 1), large areas of Central and South America, Hispaniola (Haiti and the Dominican Republic), Africa, the Indian subcontinent, Southeast Asia, the Middle East, and Oceania being malaria-risk areas. This disease represents a severe burden to socio-economic development of the affected countries. It is a common disease with two billion people potentially at risk and 300 to 500 million people infected each year, leading to 1-3 million deaths, mostly among young children in sub-Saharan Africa (http://www.rbm.who.int/wmr2005/).

Malaria is caused by a protozoan parasite from the phylum Apicomplexa and genus *Plasmodium*. There are four species responsible for human malaria [1]: *P. falciparum*, *vivax*, *malariae*, and *ovale*. One of these, *P. falciparum*, predominant in Africa, is responsible for the vast majority of the malaria-related deaths.

1.1.1 *P. falciparum life cycle*

Malaria parasites have a life cycle that is split between a vertebrate host and an insect vector (Fig 2).

During a blood meal, a malaria-infected female *Anopheles spp* mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. After this initial replication in the liver (exo-erythrocytic schizogony) the merozoites invade red blood cells, where they undergo asexual multiplication (erythrocytic schizogony). During erythrocyte invasion the parasite establishes a parasitophorous vacuole membrane, inside which it resides. It then matures from the initial ring stage to the trophozoite stage and finally develops into a schizont, the rupture of which leads to the release of up to 32 merozoites. Erythrocytic schizogony is the stage of the life cycle that is responsible for malarial pathogenesis. Instead of undergoing asexual multiplication, and under the control of stimuli that are not understood, some parasites withdraw from proliferation and differentiate into sexual erythrocytic stages.

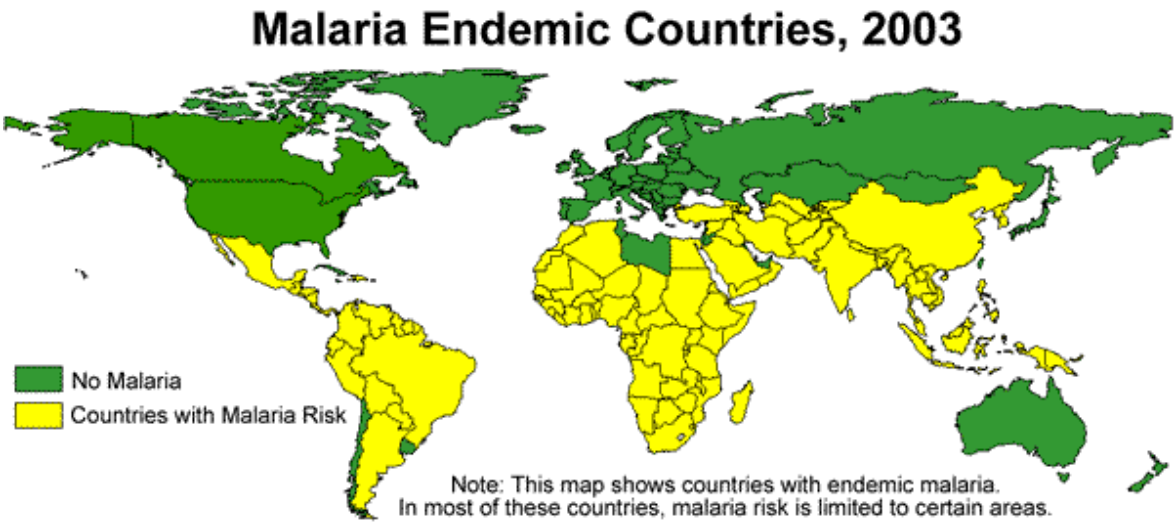


Figure 1: Geographical distribution of malaria
Malaria is widespread through the world (South America, Africa, Asia), mostly localized in tropical and subtropical regions (in yellow) (http://dpd.cdc.gov/DPDx/HTML/Malaria.htm).

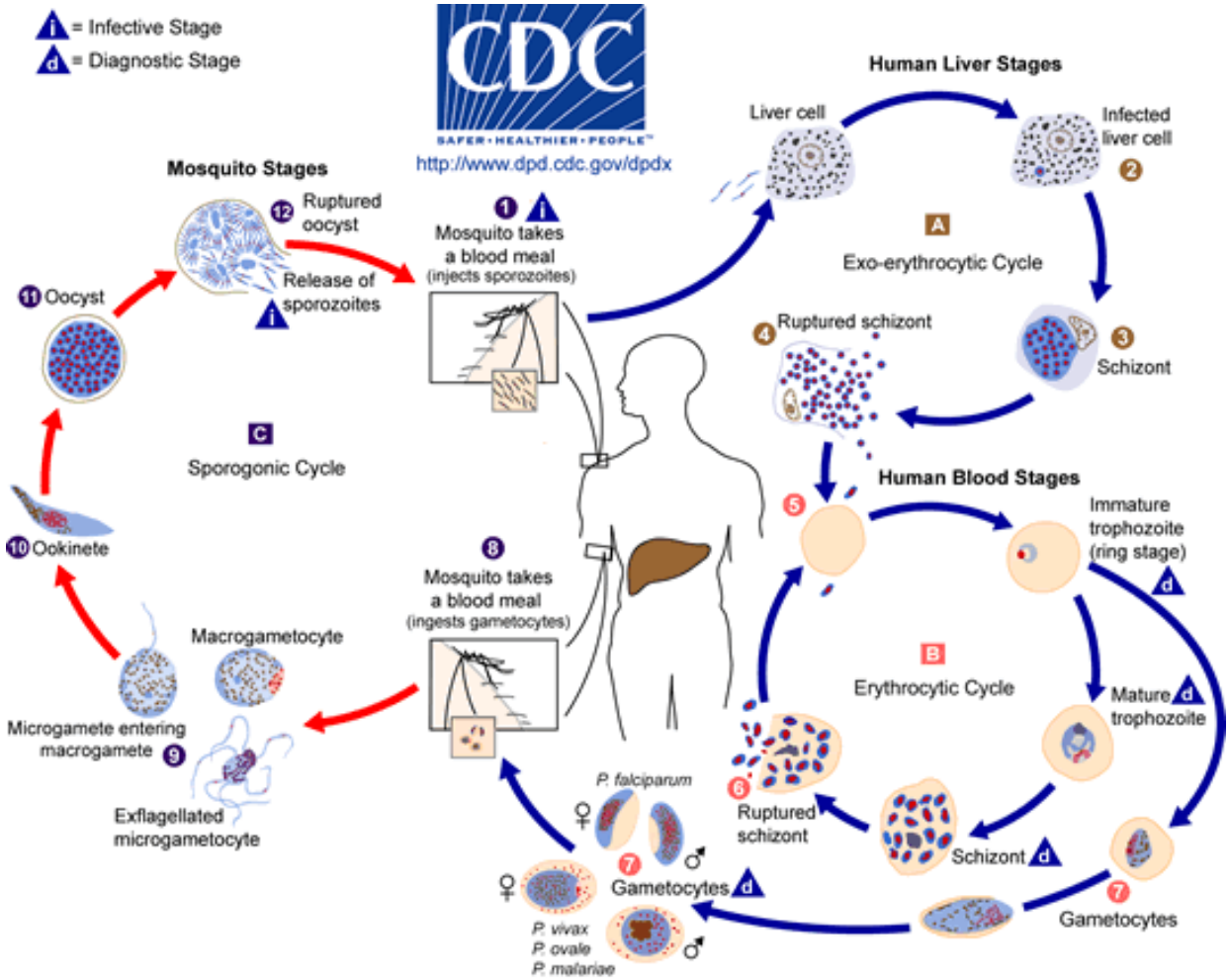


Figure 2: Life cycle of Plasmodium falciparum
(*http://dpd.cdc.gov/DPDx/HTML/Malaria.htm*)

During a blood meal a female *Anopheles spp* mosquito can ingest male (microgametocytes) and female (macrogametocytes) gametocytes, which initiate the multiplication of parasites in the mosquito (sporogonic cycle). While in the midgut of the mosquito the gametocytes develop into gametes. The male microgametocytes undergo a process called exflagellation, whereby eight flagellated gametes are formed from every microgametocyte. Fertilisation of the female macrogametocyte then leads to the generation of diploid zygotes. The zygotes in turn develop into motile and elongated ookinetes in which meiotic reduction occurs. The ookinetes cross the mosquito midgut epithelium and become attached to the outer surface of the midgut, where they develop into oocysts. The oocysts, in which intense asexual multiplication occurs, grow. Their rupture releases sporozoites, which make their way to the salivary glands of the mosquito. Inoculation of the sporozoites into a new human host reinitiates the life cycle of the parasite.

1.1.2 *Malaria chemotherapy*

Malaria incidence has been dramatically reduced in some parts of Africa by increasing deployment of anti-mosquito measures [2]. Although a new strategy using Insecticide-Treated Net (ITN) might greatly reduce malaria risks, there is still a possibility that vector mosquitoes bite outside of peak sleeping hours and undermine efficacy of this key malaria prevention measure [3]. Furthermore, attempts to develop an effective malaria vaccine have so far failed, because of variability of *P. falciparum* surface proteins [4]. Nevertheless, recent studies have shown promising phase II results of a trial to test the efficacy of RTS,S/AS02A vaccine [5]. This vaccine specifically targets the pre-erythrocytic stage of *P. falciparum* and confers protection against infection by *P falciparum* sporozoites. However, until such a vaccine reaches the market, chemotherapy remains a major element in the fight against malaria. Treatments differ according to the infecting species, the geographic area where the infection occurred, and the severity of the disease. In terms of prophylaxis for travellers to malaria-risk areas in South America, Africa, the Indian subcontinent, Asia, and the South Pacific, it is recommended to take one of the following drugs: mefloquine (Lariam®), doxycycline, or Malarone™.

Mefloquine has been found to produce swelling of the *Plasmodium falciparum* food vacuoles. It is thought act by forming toxic complexes with free heme that damage membranes and interact with other plasmodial components. Doxycycline is a member of the tetracycline antibiotics group. It has been shown that tetracyclines specifically block expression of the *P. falciparum* apicoplast genome [6]. Malarone, a combination of two drugs (atovaquone and proguanil) acts at the liver and blood stages. Atovaquone acts by inhibiting the mitochondrial electron transport through the cytochrome *c* reductase complex [7]. To solve resistance problems due to the selection of point mutation in the cytochrome *c* gene, atovaquone was combined with proguanil. Proguanil acts by inhibiting dihydrofolate reductase, an enzyme involved in the sysnthesis of DNA precursors.

Chloroquine (CQ, Aralen®) and Hydroxychloroquine sulfate (Plaquenil®) are also greatly used. Chloroquine (a 4-aminoquinoline drug) enters in the Red Blood Cell (RBC) by simple diffusion and prevents formation hemozoin, an insoluble form of polymerised heme molecules, leading to heme build up [8]. Chloroquine binds to heme, forming a complex. This complex is highly toxic to the cell and disrupts membrane function, resulting in cell lysis and ultimately parasite cell autodigestion. The mechanism of action of hydroxychloroquine sulfate is unknown but may be based on its ability to bind to and alter the properties of DNA. It has been found that it could be taken up into the acidic food vacuole of the parasite, thus interfering with vesicle functions and possibly inhibiting phospholipid metabolism [9]. *P. falciparum* is widely genetically resistant to chloroquine (WHO, 2001) and resistance to chloroquine in *P. vivax* has increased in South Asia. The best antimalarial drug for treating chloroquine-resistant malaria parasite remains quinine (or mefloquine and intravenous quinidine) but its hypoglycemic effect may be problematic [10]. However, quinine resistance is also increasing, especially in Southeast Asia, particularly in the borders areas of Thailand [11]. Chloroquine has largely been replaced by sulfadoxine pyrimethamine (SP) for the treatment of *P. falciparum* malaria, but there is evidence that malaria parasites bearing high-level pyrimethamine resistance originally arrived in Africa from Southeast Asia [12].

Despite drug resistance, chemotherapy is currently the only efficient strategy to fight against the parasite. Recently, new antimalarials have been developed, such as artemisinin-based combination therapies (ACTs) [13-15]. ACTs combine a derivative of the natural product artemisinin, an extremely potent and fast-acting antimalarial endoperoxide, with a longer-lasting partner drug that continues to reduce the parasite biomass after the short-lived artemisinin has dropped below therapeutic levels [2]. Artemisinin derivatives act rapidly against asexual blood stage parasites to alleviate symptoms and have additional beneficial effect of killing gametocytes and therefore decreasing parasite transmission. Most countries in the world have now switched to an official policy of using an ACT as the first-line treatment [2].

The discovery of novel drugs having the advantages and efficacy that once characterized chloroquine is a high priority for malarial research. This is why novel approaches to antimalarial drug development are reviewed in publications [16-18]. A better knowledge of the parasite genome allows to identify new targets. This is particularly the case for protein kinases (PKs), which represent potential drug targets. In view of their drug ability and proven potential as targets in a variety of diseases (see below), protein kinases represent an attractive class of possible targets for antimalarial intervention [19]. This forms the basis of our interest in protein kinases, especially those that regulate cell proliferation, such as MAP kinases.

1.2 MAP kinase pathways

Proper regulation of genes in all forms of cellular life is dependent on intracellular regulatory circuits or signal transduction pathways. Among eukaryotic cells, phosphorylation of intracellular factors followed by specific gene transcription is an important output of such signal transduction pathways, and the major elements of many such pathways are conserved in species as different as humans, fungi, and plants. Across the eukaryotic kingdom, these highly conserved regulatory circuits maintain the balanced gene transcription necessary for correct cell growth, differentiation, and death [20].

1.2.1 *MAP kinase pathways in higher eukaryotes*

1.2.1.1 Regulation of MAP kinases

The enzymes of the Mitogen-Activated Protein Kinase (MAPK) family are critical components of a central switchboard that coordinates incoming signals generated by a variety of extracellular and intracellular mediators [21-23]. In mammalian cells, the MAPK family is an evolutionarily conserved class of proline-directed serine/threonine kinases [24], which include ERK1/2, p38 Hog, JNK/SAPK, ERK3, ERK5 and ERK7/8. Specific phosphorylation and activation of enzymes in the MAPK module, which comprises MAPKKKs, MAPKKs MAPKs and scaffolding proteins, transmits the signal down the cascade, resulting in the phosphorylation of many proteins with substantial regulatory functions throughout the cell, including other protein kinases, transcription factors, cytoskeletal proteins and other enzymes (Fig 3).

This family has been implicated in a number of biological events including cell proliferation, differentiation and metabolism. Activation of MAPK family members involves the dual-phosphorylation of a conserved threonine and tyrosine (**T**X**Y**) motif located in the activation loop, just before the conserved kinase sub-domain VIII "APE" region [25, 26] (Fig 4). This event is mediated by the upstream dual specificity MAPK kinases (MAPKKs, also known as MAP/ERK Kinase or MEKs). MEK isoforms are dual specificity kinases in that they are able to phosphorylate tyrosine as well as serine and threonine residues [27, 28]. MEK isoforms include: MEK-1, -2, -3, -4, -5, -6, and –7. Activation of MEK (MEK1/2) involves the dual-phosphorylation of the conserved serines (**S**MAN**S**) motif by a MAP/ERK Kinase Kinase (MEKK) such as Raf1 [29] (Fig 4). Furthermore, it has been shown that phosphorylation of a serine localised in position 297 by a PAK1 (p21-activated kinase) stimulates the Raf1-dependent phosphorylation of the two serines localised in the activation loop [30]. Activation of the pathway occurs in response to elements lying upstream of the MEKK, in response to intra or extracellular signals.

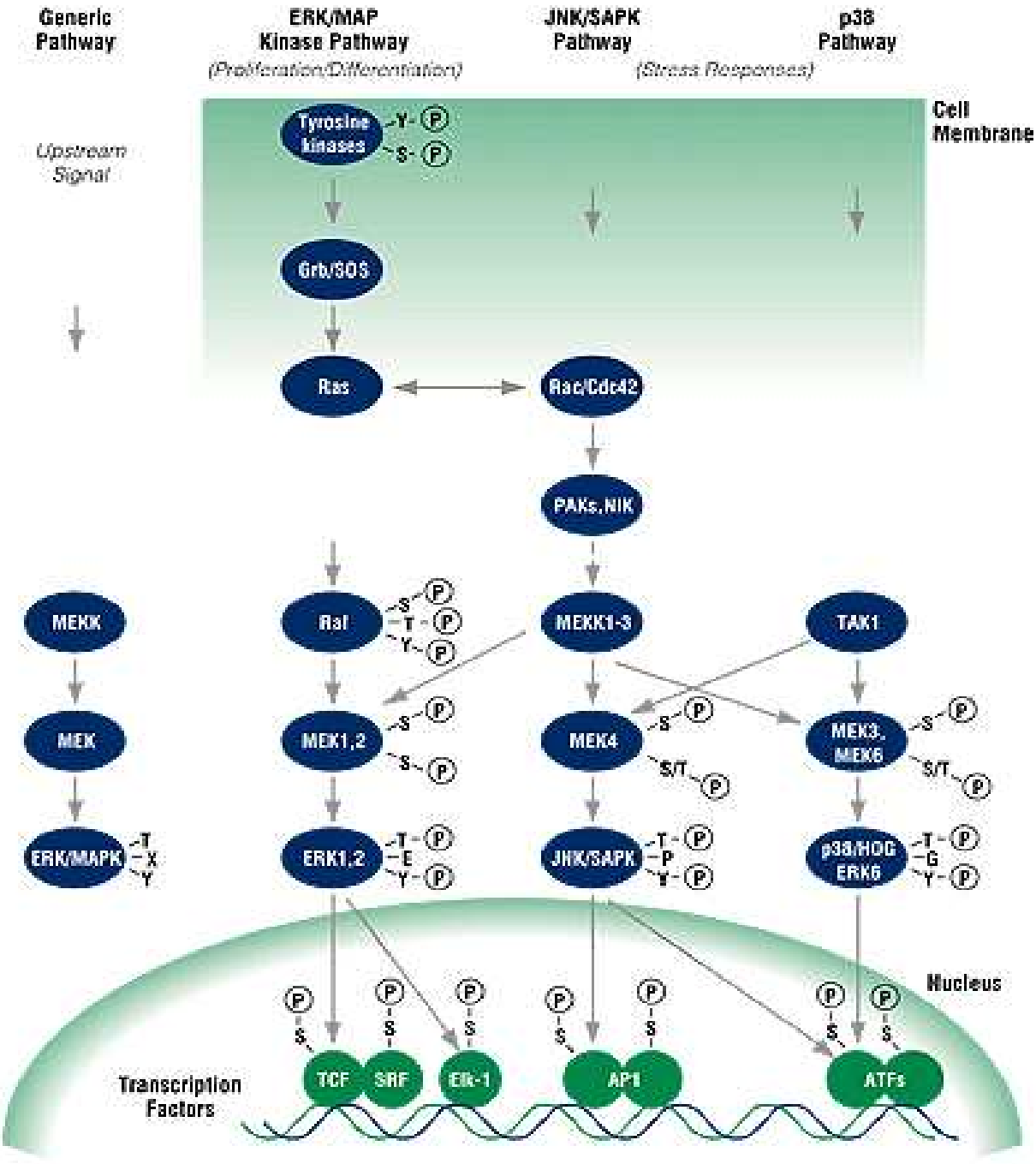


Figure 3: Parallel MAP kinases cascades involve specific MAP kinase enzyme modules Each of the MAPK/ERK, JNK and p38 cascades consists of a three-enzyme module that includes MEKK, MEK and an ERK or MAPK super family member. A variety of extracellular signals trigger initial events upon association with their respective cell surface receptors and this signal is then transmitted to the interior of the cell where it activates the appropriate cascades. The shaded area indicates those signalling molecules that become associated with the intracellular surface of the plasma membrane upon activation. (http://www.promega.com/pnotes/59/5644f/5644f_core.pdf)

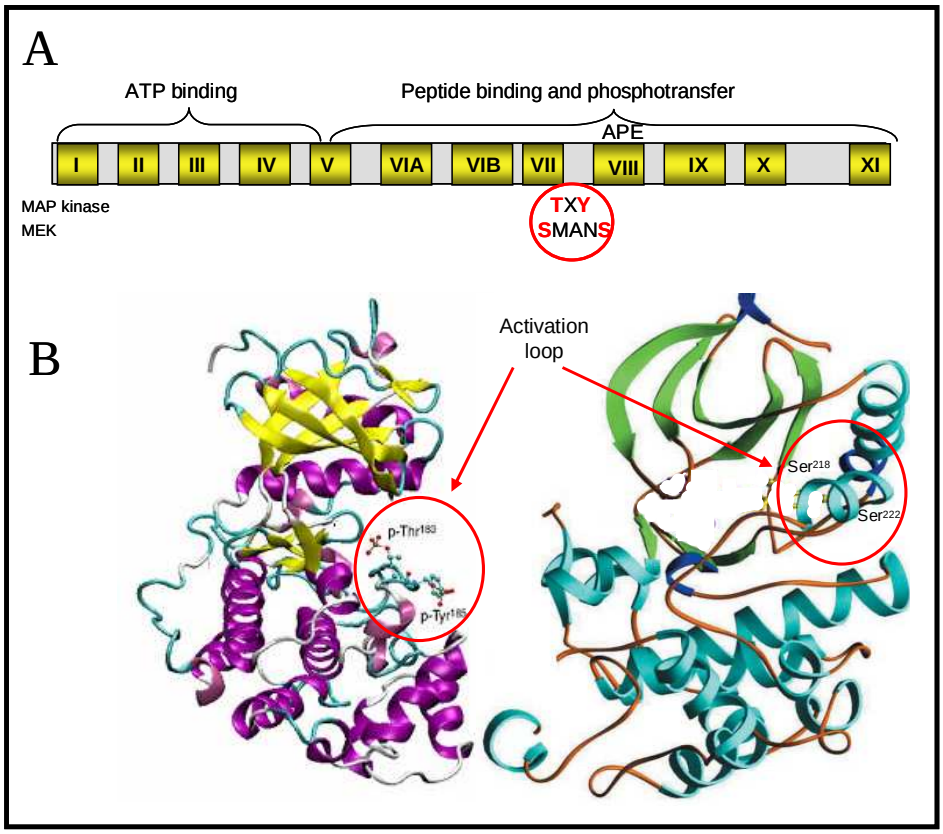


Figure 4: Structure of kinases

A: Primary structure: XI sub-domains (in yellow) of the catalytic domain based on highly conserved residues. The activation of the kinase is due to a dual phosphorylation of residues represented in red. B: Left: Crystal structure of active human ERK2 with ribbon colored according to their secondary structure, β-sheets in yellow, α-helices in purple an loops in cyan (www.nature.com/onc/journal/v26/n22/fig_tab/1210415f1.html). Right: Crystal structure of inactive MEK1 with ribbons colored according to their secondary structure, β-sheets in green, α-helices in cyan and loops in brown (www.nature.com/nsmb/journal/v11/n12/full/nsmb859.html).

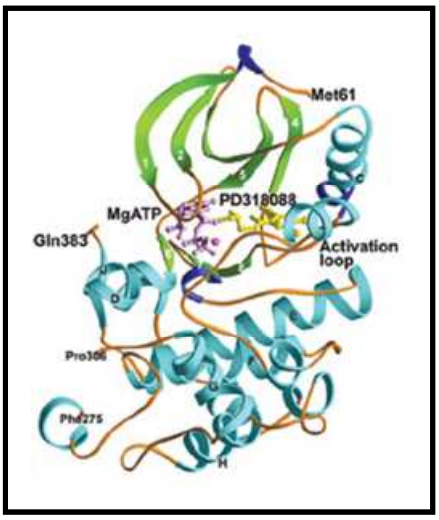


Figure 5: Three-dimensional representations of the ternary complex of PD318088 and MgATP bound to human MEK1

MEK1 protein kinase structure with the N-terminal lobe on top, the C-terminal lobe at the bottom and the kinase active site occupied by MgATP and inhibitor located in the hinge region. The α-helical regions of the protein are cyan, the β-sheet regions are green, the ATP cofactor is pink, the magnesium atom is magenta and PD318088 is gold. PD318088 is an analog of PD184352.
(http://www.nature.com.gate2.inist.fr/nsmb/journal/v11/n12/full/nsmb859.html)

The ERK signalling module was the first MAP kinase cascade to be characterised, being a vital mediator of a number of cellular fates including growth, proliferation, and survival. There are two mammalian ERK isoforms that are ubiquitously expressed, ERK1 and ERK2, and these are often referred to as p42/p44 MAP kinases [31, 32]. In this cascade, MEK1 and MEK2 function as upstream MAPKKs and the Raf proteins as MAPKKK. Duration of ERK1/2 activation depends on regulated removal of one or both phosphates by specific tyrosine or serine/threonine phosphatases (MAPK phosphatases), which radically decreases ERK activity [33]. Furthermore, the specificity of these phosphatases is dependent on their intracellular localisation. ERK1/2 as well as other MAP kinases target not only transcription factors but also membrane proteins and cytoplasmic proteins. Gene knockout experiments have illustrated theimportance of the ERK1/2 pathway [28]. Disruption of any of the three Raf proteins known to activate ERK1/2 is invariably fatal in mice [28]. Also, when MEK1 was genetically targeted, embryonic death was observed with signs of tissue necrosis [28].

Precisely how ERK1/2 affects cellular physiology *in vivo* is poorly understood. Most often an important role is attributed to ERK1/2 dependent regulation of the activating protein 1 (AP-1) family of transcription factors. Members of this family that are phosphorylated by ERK1/2 include c-Jun, c-Fos, and activating transcription factor 2 (ATF-2) but the *in vivo* relevance of this phosphorylation is not yet clear [34].

1.2.1.2MEK inhibitors

Specific and potent inhibitors of MEK1/2 have been developed [35]. By screening compound libraries on constitutively active MEK1 recombinant protein, the first MEK inhibitors, PD98059 and U0126, were identified [36, 37]. These first-generation MEK inhibitors have been utilized extensively as tools to elucidate the role of the ERK pathway in variety of biological processes. A second-generation MEK inhibitor, with enhanced bioavailibility, PD184352, has been synthesized [38]. The majority of protein kinase inhibitors developed so far are competitive with ATP and are believed to interact within the ATP-binding

site of their target protein kinase. In contrast, PD98059, U0126 and PD184352 differ in this respect since they are allosteric inhibitors that do not compete with ATP [39] (Fig 5). Such a peculiar characteristic may confer a high level of specificity of PD98059, U0126 and PD184352 towards MEK1/2; none of these compounds significantly inhibits the activity of a large panel (at least 24) of protein kinases, which includes ERK1, JNK1, and p38 kinases in an *in vitro* assay [40]. The precise action mechanism of PD98059, U0126 and PD184352 is unknown. However, studies have shown that it seems very likely that PD98059, U0126 and PD184352 act similarly as allosteric inhibitors: they bind outside the ATP and ERK1/2 binding sites on MEK1/2 and the modification of the three dimensional structure of MEK1/2 renders it not phosphorylatable by upstream kinases [40]. Therefore, these inhibitors do not inhibit MEK itself, but its phosphorylation/activation. Recently another study showed that PD184352 and U0126 are extremely potent and selective inhibitors of MEK1/2 [41]. A panel of 70-80 protein kinases was expressed in different cell lines and kinase assay were performed in presence of inhibitors (65 compounds) to determine the efficiency of every compound on each protein [41].

SL327 (or MEK inhibitor 1/2), which is a water-soluble structural homolog of U0126 [37, 42], and other ATP competitor inhibitors of MEK (e.g. MEK Inhibitor I, a cell permeable pyridine-containing vinylogous cyanamide compound and MEK Inhibitor II, also called 2-Chloro-3-(N-succinimidyl)-1,4-naphthoquinone)) [37, 43] have been developed and are commercially available. These inhibitors display significant affinity only towards ATP-bound MEK. However, as described above, those ATP competitor inhibitors also affect other protein kinases such as PKA, PKC and Raf1 at higher concentrations [43] and are therefore less specific than the U0126 or PD124352.

1.2.2 *MAP kinases of* P. falciparum

Work in the Doerig laboratory is focussed on signalling pathways that regulate cell proliferation and differentiation in *P. falciparum*. In this context, two plasmodial members of the MAPK family have been characterised: Pfmap-1 (PF14_0294) [44] and Pfmap-2 (PF11_0147) [45].

- Pfmap-1 has a conserved MAPK catalytic domain and a characteristic phosphorylation/activation site, **TDY**, which is used as an activation site by all MAPKs characterized so far. It is not related to classical MAPKs of the ERK1/2, p38 or JNK subfamilies, but to ERK7/8, a novel class of MAPK-related enzymes [46, 47]. Pfmap-1 is expressed in both asexual stages and gametocytes, but its function in parasite development has not yet been determined [45]. However, recent reverse genetics studies show that this gene is not essential for the parasite and no phenotype has been discovered so far, either on the asexual growth, gametocytogenesis or on oocyst and sporozoite formation [48].

- Pfmap-2 is a very atypical MAPK that displays a divergent putative activation site (Thr-Ser-His instead of the Thr-X-Tyr that is conserved in all other MAPKs). Phylogenetic analysis places Pfmap-2 at the very base of the MAPK cluster [49], rendering orthology assignment to any known MAPK impossible. Pfmap-2 is expressed predominantly in gametocytes [45] but the protein can also be detected by HA tagging in asexual stages [48]. Inactivating the *P. falciparum* pfmap-2 genomic locus was possible only if an episome expressing the protein was present in the transfected parasite, demonstrating that the gene is essential for asexual parasite survival [48]. In contrast, the *pbmap-2* gene is not essential in *P. berghei*. When the *P. berghei* homologue, Pbmap-2, is knocked out, the exflagellation of male gametocytes is almost entirely abolished [50-52].

Surprisingly, no typical member of the MAPKK was found in the *P. falciparum* kinome [49, 53]. Despite the absence of plasmodial genes clustering within the MAPKK family, two sequences were found with around 30% identity to human MEK1: PfPK7 (PFB0605w) and PfPK8 (PFB0150c) (Hanot, Dorin & Doerig, unpublished).

- PfPK7 displays 34% identity to vertebrate MAPKK3/6. MEK-related sequences are located in the C-terminal lobe, whereas the N-terminal lobe is more closely related to fungal cAMP-dependent protein kinases (PKA). PfPK7 is expressed in several developmental stages of the parasite, both in the mosquito vector and in the human host. It is not strictly essential for erythrocytic asexual growth, although both asexual and sexual stages are impaired when this gene is knocked out [54]: PfPK7 KO asexual parasites grow slower than the wild type

and there was no oocysts formation in the mosquito stage, and both phenotypes were rescued by complementation with an episome expressing the PfPK7 cDNA. Recombinant PfPK7 displayed kinase activity towards a variety of commercial substrates, but was unable to phosphorylate the two *P. falciparum* MAPK homologues *in vitro*, making it unlikely to function as a MEK functional homologue. Furthermore, it was insensitive to PKA and MEK inhibitors [29].

- PfPK8 possesses a MAPKK-like protein sequence showing 28% identity in the catalytic domain with both Ste20 family kinases, which act upstream of the 3-component MAPK module [55], and MEKs. PfPK8 contains a large N-terminal extension, which includes repetitive sequences. This gene product has a potential phosphorylation site motif "**S**DQ**S**" with two serine residues in close proximity within the activation loop, which is similar to the "**S**MAN**S**" signature of MEK1/2, where the C-terminal serine is phosphorylated by the upstream MEKK.

However, PfPK8 (like PfPK7, see above) is active *in vitro* against various non-physiological substrates, but do not phosphorylate plasmodial or human MAPKs and is therefore unlikely to represent MEK functional homologues.

Another protein, Pfnek-1 (PFL1370w), possesses an activation site reminiscent of that found in MEK1/2.

- Pfnek-1 displays maximal homology to the never-in-mitosis/Aspergillus (NIMA)/NIMA-like kinase (Nek) family of protein kinases, whose members are involved in eukaryotic cell division processes. Pfnek-1 possesses a large C-terminal extension in addition to the catalytic domain, which is very similar to many enzymes of the NIMA/Nek family. Surprisingly, the FXXT motif usually found in NIMA/Nek protein kinases is substituted in Pfnek-1 by a SMAHS motif, which is reminiscent of a MAP/ERK kinase (MEK1/2) activation site (SMANS). Recombinant Pfnek-1 is able to specifically phosphorylate Pfmap-2 *in vitro*, and that co incubation of Pfnek-1 and Pfmap-2 results in a synergistic increase in exogenous substrate labelling [56]. Therefore, Pfnek-1 may play a role in pfmap-2 regulation *in vivo*, although this has not been demonstrated.

Taken together, experimental and *in silico* analyses demonstrate that *P. falciparum* does not possess any typical MEK homologues, and reveal the absence of typical 3-component MAPK modules in malaria parasites [29]. Phylogenetic analysis of the *P. falciparum* kinome [49] independently confirmed that no plasmodial sequence clusters within the STE family, which comprises MEKs.

1.3 PKs as drug targets for cancer

As mentioned above, reversible phosphorylation of many proteins plays a central role in most cellular processes. In eukaryotic cells about 30% of the proteins carry phosphate groups. Many diseases, such as cancer and neurodegenerative diseases, have as an origin a deregulation of protein phosphorylation [57], and therefore protein kinases are now considered as promising drug targets. Indeed, the first kinase inhibitor (imatinib mesilate or ST1571, which is a tyrosine like kinase inhibitor) to be developed as a drug (Gleevec), has recently been made available on the market [58, 59], and a CDK inhibitor is now in clinical trial for anti-cancer evaluation [60].

The *P. falciparum* kinome comprises approximately 80 protein kinases [49]. Several of these protein kinases have been characterised, which led the observation that the properties of these enzymes diverge significantly from those of their mammalian homologues. Specific inhibition of malarial kinases might, therefore, lead to the development of novel antimalarials.

1.4 Host cell protein kinases as antimalarial drug targets

There are two reasons for considering host cell proteins as targets in the context of chemotherapy against parasitic diseases:

(i) The first one is purely scientific: evidence is accumulating that host cells are far from passive partners in the interaction with the parasite. The host cell mediates processes, which are essential to parasite survival. Targeting host components has the considerable advantage that parasite drug resistance cannot result from alterations of the drug target [19].

(ii) The second one is economical: the major problem with new antimalarials is the reluctance of the pharmacological companies to invest in expensive research if the marketing prospects are poor. One solution to resolve this situation would be to identify a drug already developed for other purposes, which would have anti-parasitic effects. Additionally, many drug companies have large libraries of compounds that been shown to be unfit for their originally intended purpose, but may still be attractive as anti-malarials.

However the question of the toxicity of the compounds on the patient remains. Would a drug that acts on the host cell kinase be safe for the patient? As mentioned before, protein kinase inhibitors are actually used as drug targets for cancer therapy. In the context of anti-malarial drugs these compounds would be required for a short time and therefore toxicity would be a lesser issue than in context (such as cancer) where chemotherapy must be used for an extended period of time.

1.4.1 *Selected instances of parasite requiring host protein kinase activity:*

Some host cell protein kinases or signalling pathways are required for survival of intracellular parasitic protists (Table 1).

1.4.1.1Theileria

Theileria spp. are apicomplexan parasites that infect cattle and bear considerable socioeconomic impact on affected countries. The parasite is transmitted by ticks and establishes an infection in cells of the immune system (*T. parva* infects T and B cells, whereas *T. annulata* infects B cells and monocytes/macrophages). Infection of the host cell results in its transformation, and the disease resembles a lymphoma in many respects (reviewed in [61]). A notable difference is that transformation is reversible and dependent on live parasites: if the parasite is killed by treatment with an appropriate drug, the host cell stops proliferating and dies.

Species	Host cell PK/pathway	Host cell type	References
Apicomplexa			
Plasmodium falciparum	PKC	Erythrocyte	[62]
Toxoplasma gondii	p38 activation	Macrophages	[63]
	NF-κB	Fibroblasts	[64]
Theileria spp	NF-κB	Lymphocytes	Reviewed in [61]
	JNK		
	Src		
	PI3K		
Cryptosporidium parvum	PI3K	Epithelial cells	[65]
	Src		[66]
Trypanosomatids			
Trypanosom cruzi	TGFβR	Epithelial cells	[67]
	PI3K		[68]
	Ca^{2+}/PKC		[69]
	ERK1		[70]
Leishmania donovani	NF-κB	Macrophages	[71]
	ERK1/2		Reviewed in [72]
	JAK2/SSTAT1		
	Ca^{2+}/PKC		

Table 1: Examples of parasites needing the host cell pathways (adapted from reference 15)

To stimulate proliferation, one of the host cell pathways, the JNK pathway (which is also a MAPK pathway), is activated in infected cells and this leads to the upregulation of the transcription factor AP1 [73]. Another transcription factor that is activated by *Theileria* infection is c-Myc, in part through the stimulation of the JAK2/STAT3 signaling pathway; elevated c-Myc levels have been shown to contribute to the prevention of apoptosis of the host cell [74]. Additionally, the NF-κb pathway is also controlled by the parasite. Normally, a "signalosome", constituted by intra-cellular stimuli or activated membrane-bounds receptors, activates the NF-κB pathway [61]. The IκB inhibitor, which sequesters the NF-κB transcription factor in the cytoplasm, forms a complex with the IKKs (kinases, which when activated by upstream effectors, phosphorylate IκB). Once the IKKs are activated, the IκB inhibitor is phosphorylated and releases the active NF-κB. This then stimulates the expression of many genes functioning in the control of cell proliferation and survival [61]. Constitutive NF-κB activation is achieved by the parasite, not by stimulation of the upstream signaling pathways leading to the IKK kinases, but instead by the parasite recruiting large amounts of IκB signalosome complexes at its surface. Hence, it appears that a large proportion of the host cell signaling pathways is diverted by the parasite to trigger proliferation [61]. Some of the relevant pathways are considered as targets for anti-cancer therapy.

Interestingly, *Theileria* is not the only intracellular parasite exploiting the NF-κB pathway of its host. The apicomplexan *Toxoplasma gondii* and the phylogenetically distant *Leishmania donovani* (a trypanosomatid), have also been reported to manipulate the NF-κB pathway of their host cell [71, 75].

1.4.1.2 Trypanosoma cruzi

In the trypanosomatid family, *Trypanosoma cruzi,* modulates several host pathways for its own development [68]. *T. cruzi* is an obligate intracellular parasite, causing a chronic debilitating illness, Chagas' disease, in millions of people in Latin America. The flagellate protozoan is transmissible to humans and other mammals mostly by hematophagous assassin bugs of the subfamily

Triatominae (Family *Reduviidae*) [68]. *T. cruzi* infects most cell types, including fibroblasts, epithelial cells, endothelial cells, myocytes and macrophages.

T. cruzi invasion may require activation of specific signaling pathways critical for parasite entry into host cells. This hypothesis is consistent with current concepts of cellular invasion by virus and bacteria, which can activate surface membrane signaling molecules in their interplay with mammalian host cells [76, 77]. It has been reported that while *T. cruzi* attaches to epithelial cells lacking signalling transforming growth factor β (TGFβ) receptor, the adherent parasites cannot penetrate and replicate inside the mutant cells, as they do in parental cells. It is still not exactly known how this pathway would be activated by the parasite but it has been hypothesised that the TGFβ pathway may be activated by a factor secreted by infective trypomastigotes [67]. Other studies have shown *T. cruzi* requires other host cells pathway for its own development, such as PI3K (Table 1).

1.4.2 *Malaria and host cell signalling pathways: liver stages*

A number of studies demonstrated that *P. berghei*, which is widely used as a model system to study the liver stage of *P. falciparum*, also interferes with host signalling pathways [78-80]. The entry of *P. berghei* sporozoites into hepatocytes is relatively well characterized (little is known about parasite-host interactions during later developmental stages of the intracellular parasite) and it has been established that the parasite induced expansion of the host cell is an important stress factor for infected cell. Cell stress is known to trigger programmed cell death. In this context apoptotic markers were examined in infected hepatocytes and it has been shown that *P. berghei* inhibits host cell apoptosis in the liver stage [78]. The parasite interferes with the apoptotic machinery of the host cell but it is not known how it does so. . However, a recent study [79] revealed that CS outcompetes NFκB nuclear import, thus downregulating the expression of many hepatocyte genes controlled by NFκB.

1.4.3 *Malaria and host cell signalling pathways: blood stages*

Even though the mammalian red blood cell is inactive in terms of gene expression, it is "not an inert bag of hemoglobin (Hb)" and evidence is accumulating for complex signalling networks involving protein phosphorylation that regulate, for example, cytoskeleton functions and transporter activity [81]. It has been shown that there is a variety of active signalling protein kinases present in the erythrocyte, such as the TyrKs, Syk and Lyn [82], PKC [83], PKA catalytic [84] and regulatory [85] subunits, Casein kinase 2 (CK2) [86], ERK1/2 (see below) and other signaling molecules, such as cGMP binding proteins [85].

It has been known for many years that infection of the red blood cell by malaria parasites requires protein phosphorylation within the erythrocyte. Early studies demonstrated that invasion is blocked by staurosporine [87], which may be mediated by protein kinases of either the host cell or the parasite (or both). Parasite entry is dependent on intra-erythrocytic levels of ATP, which is indicative of the involvement of host protein phosphorylation. Furthermore it is inseparable from a cAMP-independent kinase activity near the surface of the erythrocyte, with spectrin (a component of the sub-membrane cytoskeleton) as the probable substrate [88]. Moreover, inside the infected erythrocyte, several host proteins, such as band 4.1 [89], show increased phosphorylation after infection. Infection by *P. falciparum* appears to modulate the activity of erythrocytic PKC: enzymatic activity is decreased upon infection, although the physiological significance of this observation has not yet been established [62]. More recently, a crucial role for erythrocyte heterotrimeric G proteins and β2-adregenic receptor in the early stages of infection by the malaria parasite was uncovered [90], illustrating the complexity of the signalling events within the erythrocyte that the parasite must rely on in order to establish infection. The observation in the same study that β-antagonists and heterotrimeric G-protein inhibitory peptides affected infection of mice by *P. berghei* provides a proof of principle that interfering with host cell signalling is a promising avenue in antimalarial chemotherapy.

Little is known of the requirements for host cell kinase activity once the infection is established. Evidence is accumulating that the parasite exports

protein kinases to the cytoplasm and/or the membrane of the erythrocyte; examples include at least some FIKKs (FIKKS are a novel specific Apicomplexa protein kinase family, called on the basis of a conserved motif in sub-domain II) [91], a casein kinase 1 activity that is associated with the erythrocyte membrane [89], a GSK3 homologue [92], a histidine-rich protein (PfHRP1) that is associated with erythrocytic band 3 [93] and an ''orphan'' kinase that localizes in knobs, structures that mediate cytoadherence of *P. falciparum*-infected cells to endothelial cells [94]. It is likely that the presence of parasite protein kinases in the host cell cytoplasm allows the parasite to subvert host signalling pathways. An intriguing example may be the activation of an erythrocyte anion channel by *P. falciparum* infection. The channel is activated either by infection or by exogenous PKA, indicating that the parasite-dependent activation is probably mediated by phosphorylation [95]. Interestingly, infection apparently results in the degradation of the host cell PKA [96], and it is conceivable that channel activation is effected through a protein kinase exported by the parasite. Very recently, it has been shown that overexpression of the regulatory subunit of PKA (PfPKA-R) in parasite results in down-regulation of anion conductance [97].

1.4.4 *A role for host MAPK pathways in erythrocyte infection?*

In the course of investigations in our laboratory aimed at characterizing parasite MAPK pathways, it was demonstrated that U0126, an inhibitor that has high specificity against MEK1/2, was able to prevent parasite growth in a hypoxanthine incorporation assay, with an EC_{50} (2-3μM) that compared favourably with the EC_{50} of this molecule in other cellular systems (1-10μM, C.M. Doerig and C. Doerig, unpublished). However, as detailed in the sections above, it was eventually demonstrated that the parasite does not possess 3-component MAPK pathways.

It could be that the effect of U0126 on parasite growth is due to the inhibition of some parasite enzyme, but this is unlikely, because (i) U0126 is an allosteric inhibitor that does not target the ATP-binding site but the interaction between the MEK and the upstream MEKK, which presumably reduces the problem of lack of selectivity, and (ii) the parasite kinases with maximal homology to the MEKs, PfPK7, and Pfnek-1, are not affected by U0126 (see above). Another possibility

is that the target of the inhibitor is the host cell MAPK pathway. Indeed, there is evidence that ERK1/2 pathways are present in erythrocytes, where they regulate the activity of a sodium/proton exchanger [98].

1.5 Aims and objectives

The general objective of this project is to test the hypothesis, suggested by the observation that U0126 has parasitocidal activity that the parasite relies on host erythrocyte MAPK pathways for its own survival. In particular, we aim to establish whether or not *P. falciparum* requires host MEK activity by (i) determining whether inhibition of host erythrocyte MEKs using a variety of inhibitors (in addition to U0126) affects parasite growth and (ii) analysing the effect of infection on the activity of host MEK.

The specific aims to achieve this are:

1. To test a panel of MEK inhibitors and determine their effect on *P. falciparum* growth.

2. To determine at which stages the MEK inhibitors act.

3. To determine if there is an effect of infection on the host MAPK pathway.

2 MATERIALS AND METHODS

2.1 *P. falciparum culture*

2.1.1 *Culture of erythrocytic stages of P. falciparum*

P. falciparum (clone 3D7) was grown in human erythrocytes as described previously [99]. In brief, the parasites were grown at a 5% haematocrit in complete RPMI 1640 medium (for 5l: 79.45g RPMI 1640 powder (Invitrogen), 0.25g hypoxanthine (Sigma), 10g sodium bicarbonate – $NaHCO_3$, 0.25mg gentamycine sulphate (VWR International), 0.5% w/v of Albumax II-lipid rich bovine serum albumin (Invitrogen), pH 7.2, filter-sterilized and stored a 4°C) either in 25cm^2 (5ml stocks) or 75cm^2 (25ml preparative cultures) ventilated flasks. The flasks were kept in a 37°C incubator with a 5% CO_2 atmosphere. To remove serum and leukocytes, the blood (obtained in donation pouches from the Blood Transfusion Service, Gartnavel Hospital, Glasgow) was washed three times (centrifugation at 2000g, 5min) in RPMI 1640 before use. The medium was changed daily and the parasitaemia (PT) was controlled every day by examining Giemsa-stained blood smears (Sigma). When the PT reached 8-10%, the culture was harvested or diluted (usually to 0.5%).

2.1.2 *Synchronisation of cultures*

2.1.2.1 Synchronisation by sorbitol treatment

This synchronisation technique relies on the fact that only rings survive incubation with sorbitol. A young stage parasite culture (8% PT rich in rings or young trophozoites) was spun down, and the cells were resuspended in 10 volumes of 5% sorbitol. After incubation at room temperature for 10min, the cells were washed twice into RPMI 1640 medium and resuspended in complete medium.

2.1.2.2 Synchronisation by Percoll

This technique separates the different stages of *P. falciparum* using a density gradient. Four large flasks of asynchronous culture with a high PT (<5%) was spun down at 600g for 5min at room temperature. The supernatant was

removed and the pellet was washed twice in simple RPMI. Then, the pellet was resuspended at a 20% haematocrit with complete medium. 7ml of 60% Percoll (30ml Percoll + 17ml RPMI + 3ml 10X PBS) were added to 15ml centrifuge tubes. Slowly, 2ml of parasites were layered onto the Percoll and the tubes were spun at 700g for 10min without using brake. After spinning, the schizonts and late trophozoites formed a distinct reddish/brown band near the top of the Percoll. The rings, early trophozoites and uninfected RBC formed the pellet. Using a Pastette the schizont layer was carefully removed into a 50ml tube and washed twice in simple RPMI. The pellet was resuspended in complete medium (e.g. 5ml) and blood 50% (e.g. 500µL). A smear was done to check the PT and dilute down if necessary.

2.1.3 *Infected RBCs purification by MACS Column*

This technique was used to purify the Infected Red Blood Cells (IRBC) from the Uninfected. The magnetic cell sorting (MACS) technique uses the hemozoin (rich in iron) contained in the parasite. The hemozoin corresponds to the heme polymerization, generated from haemoglobin degradation by the digestive vacuole of *P. falciparum* [100]. A MACS® Column placed in the magnetic field of a MACS Separator creates a high gradient magnetic field strong enough to retain cells rich in iron (especially the IRBCs at late trophozoites and schizonts stages). First of all, the column was loaded with 10ml of buffer (1X Phosphate Buffered Saline (PBS, pH 7.2 from GIBCO), 2mM of EthyleneDiamineTetraceticAcid (EDTA, Sigma) and 0.5% of Bovine Serum Albumin (BSA, Sigma)) and left for incubation at room temperature at least 5min. The excess of loaded buffer was eluted and the column was rinsed with 50-60ml of buffer. During this time most of the media from four preparative cultures (25ml flasks) was removed and the cells were centrifuged 5min at 2000g at room temperature. Cultures already synchronised once using sorbitol can be used and in this case 4-5% of PT at late stages is the best. After centrifugation, the supernatant was removed and cells were resuspended to 30ml with buffer. This *P. falciparum* culture was flushed through the MACS® Column, with a very slow flow through to allow the IRBCs to bind the column. Then the column was washed with 50-60ml of buffer, and the IRBCs were eluted out of the magnetic field using 30ml of buffer. The eluted material was centrifuged at 2000g at room temperature for 5min and the

supernatant removed. The cells were then lysed to perform molecular experiments.

2.1.4 *Proteins extraction*

After MACS column purification, uninfected and infected RBCs were counted on a hematimetre and the same number of cells was taken. These cells were sonicated in lysis buffer (20mM Tris pH 7.5, 2mM EthyleneGlycerolTetraceticAcid (EGTA), 5mM EDTA, 0.5% Triton X100) with phosphatase inhibitors (30mM sodium fluoride (NaF), 40mM βglycerophosphate, 20mM sodium pyrophosphate and 1mM sodium orthovanadate) and protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 3mM benzamidine, 5μM pepstatin A and 10μM leupeptin). Lysates were cleared by centrifugation at 10,000g for 15min at 4°C. The supernatant was either used directly to perform immunoprecipitation (IP) or diluted in Laemmli 2X (20% glycerol, 0.1% bromophenol blue, 4% of Sodium Dodecyl sulfate (SDS), 0.1M Tris-Hydrochloric acid (HCl) pH 6.8, 5% β-mercaptoethanol), boiled for 3min and stored at -20°C.

2.1.5 *Western blots performed by Kinexus*

Samples prepared as described above (2.1.4) were sent to Kinexus (Vancouver, Canada). Kinexus performed western blots using phospho-specific antibodies (KCSS 7.0) for a panel of human proteins and phospho-protein levels were quantified by luminescence, which was expressed as counts per minute (CPM).

2.1.6 *Cryopreservation of parasites in liquid nitrogen*

5ml of a 5% PT culture (containing a high proportion of rings stages) were centrifuged at 2000g for 5min, and the supernatant was removed. One packed cell volume of deep-freeze solution (28% glycerol, 3% sorbitol, 0.65% Sodium Chloride (NaCl)) was added drop-wise to the pellet. Cells were then resuspended and placed into cryotubes for immediate freezing in liquid nitrogen. In order to establish a new culture from a frozen stock, the cryotube was thawed and the content transferred to a sterile 15ml tube and the volume measured. For each ml of RBCs solution, 0.2 ml of sterile solution A (12% NaCl in distilled

water) was added drop-wise, stirred constantly, and left for 3min. Secondly, 10ml of sterile solution B (1.6% NaCl in distilled water) was added to the tube drop-wise. After centrifugation for 5min at 2000g, the RBCs were resuspended in 10ml of sterile solution C (0.2% dextrose, 0.9% NaCl in distilled water) added drop-wise. Finally, after another centrifugation, the supernatant was removed and discarded. Cells were resuspended in 5ml of culture medium with fresh blood to obtain a haematocrit of 5%. The culture was then maintained as described above.

2.1.7 *Hypoxanthine incorporation assay*

The EC_{50} values of the MEK inhibitors were determined by the [3H] hypoxanthine incorporation assay [100]. Parasites were exposed to the inhibitors for 48 hours. Briefly, asynchronous parasites were aliquoted in 96-wells plate at a 0.5% parasitaemia and 5% haematocrit in the presence of inhibitor (0.04–100μM). Labeled [3H]-hypoxanthine (0.1μCi/well) was added after 24hrs and the cells were harvested on a filter mat with a cell harvester after 48hrs of exposure. Scintillation liquid was added on the filter mat and radioactivity counted using a β-scintillation counter (in CPM). The CPM were then transformed in percentage of activity (100% corresponding to the higher counts) and blotted against the log [drug concentration]. All assays were carried out using untreated parasites with DMSO as controls. The inhibitors were dissolved in DMSO at stock concentrations of 1 or 10mM. Assays were run twice in triplicates for each inhibitor.

To measure the hypoxanthine incorporation along the life cycle, tightly synchronized parasites, using a Percoll gradient, were plated at 3-4% parasitaemia (early rings) and 5% haematocrit in the presence of inhibitor at EC_{100} and labeled [3H]-hypoxanthine. The reaction was arrested by freezing down at –20°C the wells at every 8 hours. Then the plate was harvested as below and CPM blotted against time (in hours).

2.1.8 *Isobolograms*

Two drugs that produce overtly similar effects will sometimes produce exaggerated or diminished effects when used concurrently [101]. A quantitative assessment is necessary to distinguish these cases from simply additive action.

This distinction is based on the classic pharmacologic definition of additivity that, briefly stated, means that each constituent contributes to the effect in accord with its own potency. Accordingly, the relative potency of the agents, allows a calculation using dose pairs to determine the equivalent of either agent or the effect by using the equivalent in the dose-response relation of the reference compound. The calculation is aided by a popular graph (isobologram) that provides a visual assessment of the interaction. Isobolograms between MEK and Src inhibitors (U0126 and PP2 respectively) were performed and analyzed using the technique as described [102]. Inhibitors dilutions were prepared for the drug interaction experiment on the basis of estimated EC_{50}s. The concentrations in these final solutions were adjusted to a range between approximately 10^{-2} and 10^{2} times the EC_{50}s of the respective inhibitors, using geometric progressions with a factor of 4. These were eight different concentrations for each inhibitor. EC_{50} of inhibitor A (e.g. U0126) was determined against the intra-erythrocytic growth of *P. falciparum* at various concentrations, in the presence of inhibitor B (e.g. PP2) and reciprocally. Results were expressed in fraction of EC_{50}: FEC_{50}=1 for inhibitor A in the absence of inhibitor B. The assessment of drug interaction is based on calculation of the sum of the fractional inhibitory concentrations (ΣFECs) at the given IC by the formula (EC_x of inhibitor A in the mixture/EC_x of inhibitor A alone)+(EC_x of inhibitor B/EC_x of inhibitor B alone). ΣFECs<1 denote synergism, ΣFECs≤1 and <2 denote additive interaction, ΣFECs≥2 and >4 denote slight antagonism, and ΣFECs≥4 denote marked antagonism.

2.1.9 *Growth assays in culture*

Tightly synchronized parasites by Percoll (late stages of the life cycle) were kept in culture until another cycle was initiated. When the majority of the culture was at the ring stage, the parasitaemia of the culture was estimated by counting and diluted to 2% of rings. 200µl of the diluted culture was pipetted into 96 well plates and the inhibitor (U0126, PD184352 or 1.5% DMSO for the control) added at an EC_{100} at T0. Smears were then carried out at different times of the life cycle: T0, T12, T30, T40 and T48 for each well and the parasitaemia was estimated for each stage and at each time by counting 10 fields.

2.2 Molecular methods

2.2.1 *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed using a 5% stacking gel (5% acrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate (APS) and 0.001% of N'-tetramethylenediamine (TEMED)) and a 12% resolving gel (containing 12% of acrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS and 0.001% of TEMED). Gels were run under standard conditions (80V, 15min; 180V, 1h30).

2.2.2 *Coomassie staining*

SDS-PAGE were stained in fresh Coomassie stain (for 500ml: 0.5g of Coomassie Brilliant Blue G-250, 25ml of acetic acid, 225ml of ethanol and 250ml dH_2O) for 15min at room temperature with gentle agitation. Then gels were destained in Destain solution (for 1l: 100ml of acetic acid, 450ml of methanol and 450ml of dH_2O) for 1h with gentle agitation and dried.

2.2.3 *Immunoprecipitation experiments*

The total cell lysates were prepared as described in 2.1.4 and precleared as follows. 0.25µg of mouse IgG and 20µl (25% v/v) agarose conjugate (Protein A-Agarose) were added to 1ml of whole cell lysate and the mix incubated at 4°C for 30min. The beads were then pelleted by centrifugation at 1,000g for 30sec at 4°C. To 1ml of the above cell lysate, 10µl of primary antibody was added and incubated for 1h at 4°C. 20µl of Protein A-agarose was then added and the tubes incubated at 4°C on a rocker platform for another hour. The pellet was collected by centrifugation at 1,000g for 30sec at 4°C; and the supernatant carefully aspirated and discarded. The pellet was then washed 4 times with lysis buffer with proteases and phosphatases inhibitors. After the final wash, the supernatant was aspirated carefully and the pellet resuspended in 20µl of Laemmli 4X. An aliquot of samples was kept after each step and samples were boiled for 3min, centrifuged at 1,000g for 3min, and the whole volume of

supernatant loaded onto a 12% polyacrylamide gel for Western blotting (cf 2.2.4).

2.2.4 *Western blotting*

Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (23V, 150mA for 45min, Biorad Trans-Blot® SD semi-dry transfer cell). Transfer buffer contained 14.4g/l glycine, 20% methanol and 25mM Tris-HCl pH 8.3. The nitrocellulose membrane was blocked one hour at room temperature in 1X Tris-buffered saline (TBS) containing 0.1% Tween-20 with 5% w/v non-fat dry milk. The nitrocellulose membrane was then washed (3x, 10min) with wash buffer 1X TBS, 0.5% Tween-20 (TBS/T) and then exposed to the primary antibody (1:1000 dilution in blocking buffer) overnight at 4°C. The membrane was washed again (3x, 10min) with TBS/T and incubated for 1hour at room temperature with 1:2000 anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit or anti-mouse). The membrane was finally washed three times and signal developed using the chemiluminescence system (ECL, Perkin-Elmer).

2.2.5 *ImmunoFluorescence Assay (IFA)*

RBCs were fixed using the method described by Tonkin *et al* [103]. Briefly, cells were settled onto a chamber previously coated with 0.1% poly-L-lysine for 30min. Then the cells were washed once in PBS and fixed with 4% EM grade paraformaldehyde and 0.0075% EM grade glutaraldehyde in PBS for 30min. Slides of fixed cells were washed once in PBS and then permeabilized with 0.1% Triton X-100/PBS for 10min. Slides were washed again in PBS, treated with ~0.1mg/ml of sodium borohydride ($NaBH_4$)/PBS for 10min and blocked in 3% BSA/PBS for one hour. Primary antibody (diluted 1:50 for anti phospho-MEK1/2) was then added and allowed to bind for a minimum of 1hour in 3% BSA/PBS. Slides were then washed three times in PBS for 10min each to remove excess primary antibody. AlexaFluor goat anti-rabbit 594 secondary antibody (Molecular Probes) was added at 1:1000 dilution (in 3% BSA/PBS) and allowed to bind for an hour. Slides were washed three times in PBS and mounted in 50% glycerol with 2.5% of 1,4-diazabicyclo [2,2,2] octane (DABCO, Sigma) and DAPI 1μg/ml.

3 RESULTS

3.1 Effect of MEK inhibitors on parasite growth

3.1.1 *Treatment with MEK inhibitors affects parasite growth*

Preliminary results obtained in the laboratory using the hypoxanthine incorporation assay [100] showed an effect of the MEK inhibitor, U0126, on *P. falciparum* growth, with an EC_{50} at 2-3μM. To confirm this result, this experiment was repeated and extended to include a panel of additional commercially available MEK inhibitors (Calbiochem, UK) (Table 2). All MEK inhibitors tested showed an effect on *P. falciparum* growth. The EC_{50} value for each MEK inhibitor compares very well with EC_{50} values of the same inhibitors in various cellular tests in mammalian cells [37, 40, 42, 104, 105]. Two molecules, U0126 and PD184352, show a strong effect on *P. falciparum*, with EC_{50} values at 3μM and 6-7μM respectively, whereas the lowest EC_{50} they display in mammalian systems are 1-10μM and >10μM, respectively (Fig 6). These inhibitors were used to carry out all subsequent experiments.

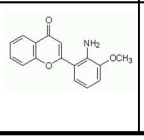
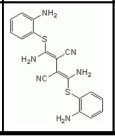
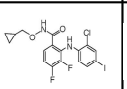
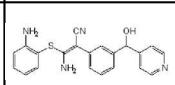
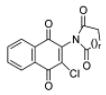
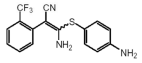
	PD98059	U0126	PD184352	MEK Inhibitor I	MEK Inhibitor II	SL327
EC_{50} (μM) in *P. falciparum*	30	3.3±0.7	6.75±0.75	26.5±3.5	42.5±7.5	24.5±3.5
EC_{50} (μM) in mammalian cells	10-50 [40]	1-10 [40]	<10 [105]	N/A	N/A	10-50 [42]
Structure						

Table 2: EC_{50}s of different MEK inhibitors tested by hypoxanthine incorporation assays

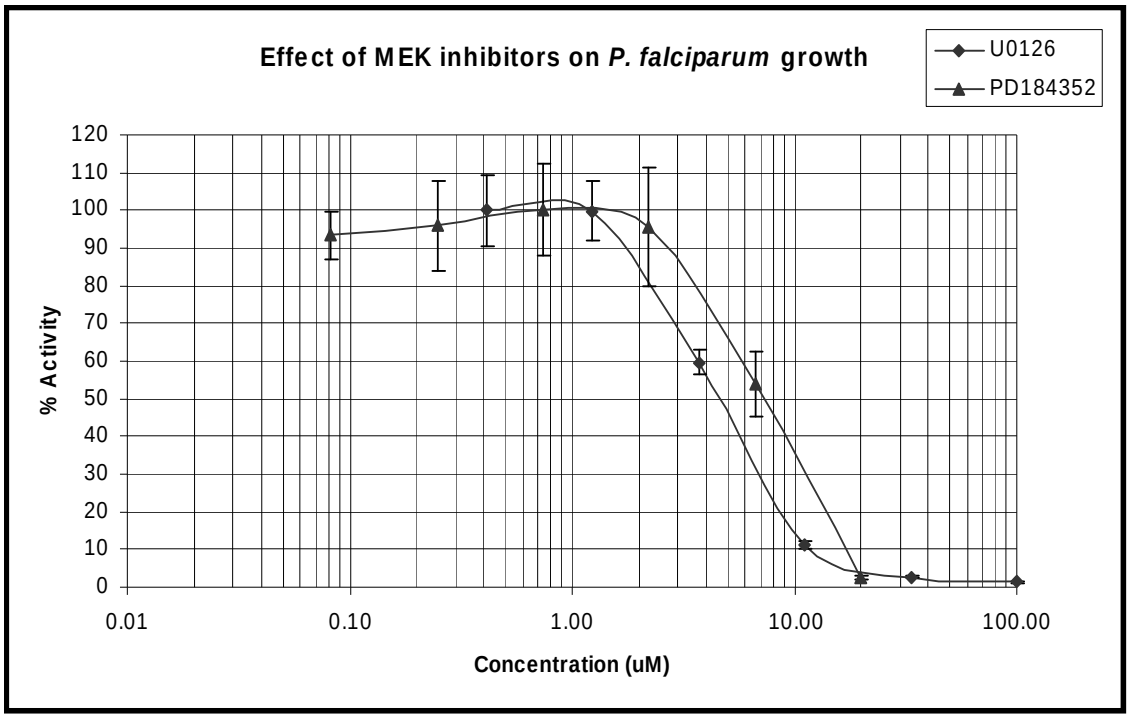


Figure 6: Effect of U0126 and PD184352 on *P. falciparum* growth

***P. falciparum* culture at a 0.5% parasitaemia were plated in presence of inhibitors and tritiated hypoxanthine (0.1μCi/well) and incubated at 37°C for 48 hours. The range of concentration is 0.41 to 100μM for U0126 and 0.08 to 20μM for PD184352. The quantity of PD184352 available was very low so the hypoxanthine incorporation experiment was performed with a lower concentration (20μM instead of 100μM). The percentage of activity was determined by the hypoxanthine incorporated relative to the uninhibited controls. Assays were run twice in triplicate and error bars correspond to SEM.**

3.1.2 *Determination of stages affected by MEK inhibitors*

3.1.2.1 Effect of MEK inhibitors on parasitaemia

Having secured evidence that MEK inhibitors affect parasite proliferation, we then investigated at which stage(s) of the asexual cycle these inhibitors act. Preliminary experiments were performed using an EC_{90} dose. However, by using this concentration, some parasites were able to initiate a new life cycle. Because the results were very difficult to analyse and interpret, the experiment was repeated using an EC_{100} dose (20μM for both inhibitors). Parasites were synchronised at the late-trophozoites/schizonts stage using the Percoll method (cf 2.1.2.2) and put back in culture. When the majority of the parasites were at the ring stage, the inhibitors (U0126 or PD184352, 20μM, i.e. close to EC100) were added (T0) and smears were prepared to measure parasitaemia at different times (hours post treatment): T0, T12 (ring stage), T30 (trophozoites), T40 (schizont) and T48 (new ring stage).

There were no significant differences in the number of early ring stage parasites between the control and the treated cultures (T12). The parasites appeared viable and no morphological changes were observed (Fig 7).

In contrast, at T30 there was a significant difference between the numbers of late rings/early trophozoites in the treated cultures, compared to the control cultures. In the presence of PD184352 it was very difficult to differentiate the late rings from the early trophozoites, suggesting the parasites treated with this inhibitor were delayed in entering the trophozoite stage. There was a significant difference in the number of trophozoites, as well as morphological changes, between the inhibitor-treated and the control cells. The morphological aspect of cells treated by PD184352 appeared to be blocked at the late rings-early trophozoite stages. Normal mature trophozoites fill the erythrocyte, however, the cells treated with U0126 do not show this morphology, are much smaller and displayed a very condensed cellular content (Fig 7A).

At T40, there was a significant difference in the number of schizonts between the treated and untreated cells. No cells treated with U0126 or PD184352

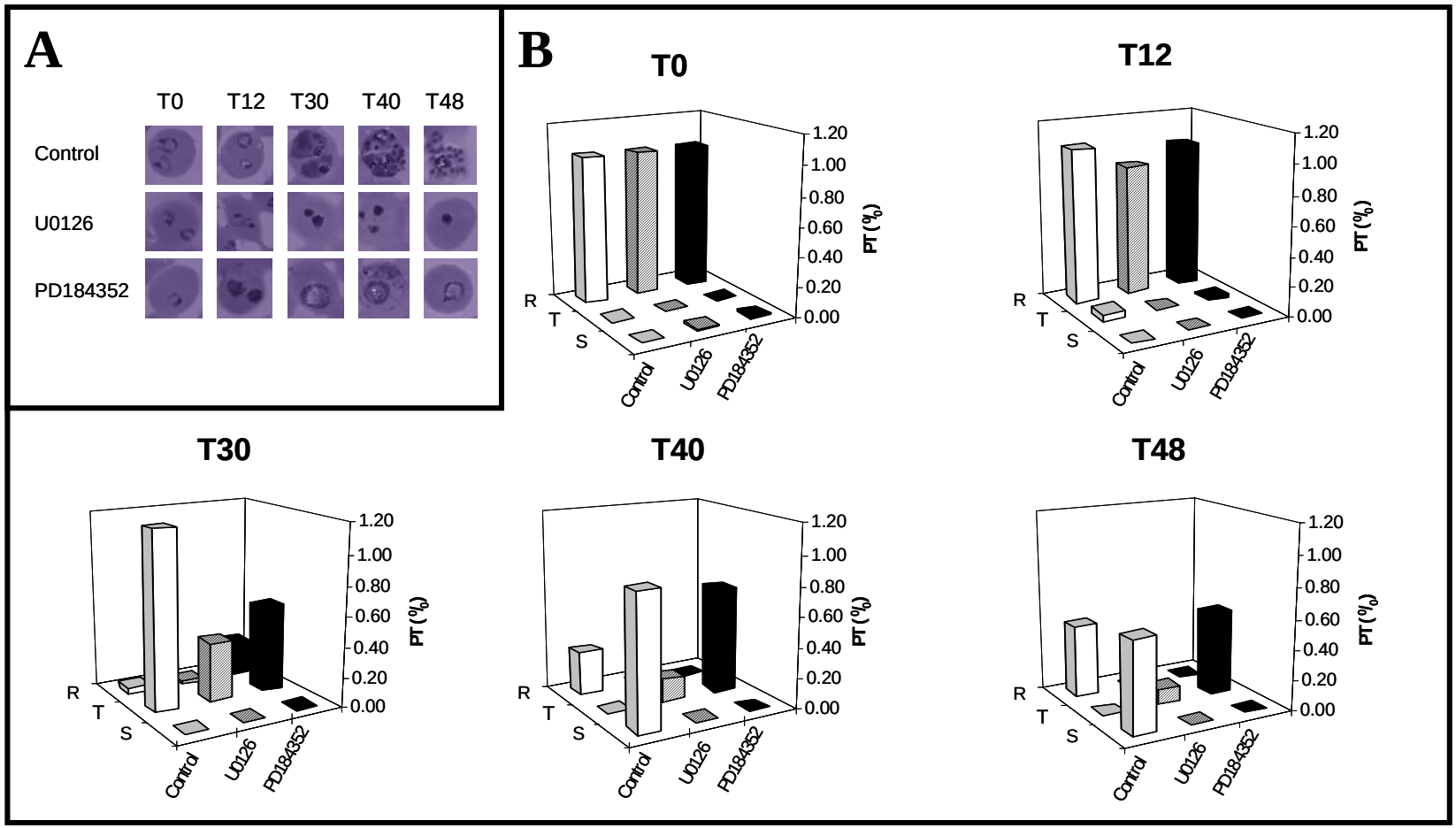


Figure 7: Effect of MEK inhibitors (U0126 and PD184352) on *P. falciparum* along the life cycle

A: Giemsa stained parasites at different time of the life cycle treated or not by MEK inhibitors. B: Parasitaemia (PT) at different time for each stage in presence of 20μM of MEK inhibitors (U0126 and PD184352). The number of cells has been estimated by counting 10 fields. The experiment was done twice in triplicate.

together with schizonts, which was to be expected because it is difficult to perfectly synchronise *P. falciparum* cultures and the schizont stage is a very short stage of the plasmodial erythrocyte cycle. Cells treated with U0126 were all seen to be at the trophozoite stage at T40, and the majority of them seemed blocked at this stage, the parasites appearing very condensed as observed at T30. There was a lot of debris in the culture (probably dead trophozoites) and the parasitaemia was very low. With PD184352, the parasitaemia was not as low as with U0126 but the parasites also seemed developmentally blocked, albeit at an earlier stage (late rings/early trophozoites). There were no dead parasites observed, as opposed to treated effect of U0126 treatment.

At T48 a majority of late schizonts with some early rings were observed in the control culture. However, treated cultures produced neither schizonts nor rings. The number of trophozoites decreased from T40 for both inhibitors, which suggests that the majority of treated parasites had died. The cells treated by PD184352 died slowly than those treated by U0126.

3.1.2.2 Effect of MEK inhibitors on hypoxanthine incorporation

Having established that the MEK inhibitors prevent the maturation of trophozoites into schizonts, we next wanted to determine whether the block occurred prior to or after S-phase. This was investigated by a hypoxanthine incorporation assay. MEK inhibitors and tritiated hypoxanthine were added to synchronised parasites at the ring stage. Every 8 hours an aliquot of the culture was harvested. After 56 hours, the quantity of hypoxanthine incorporated by the infected erythrocyte was estimated as described in 2.1.7. It appeared there were no differences between the control and the treated cells during the first 24 hours (Fig 8). This part of the life cycle corresponds to the ring stage and correlates very well with the parasitaemia measurements described above. However, at T24, there was significantly less hypoxanthine incorporated in the treated cells than in the control. This difference became more pronounced during the rest of the life cycle. This indicates that MEK inhibitors interfere with parasite development before completion of genome replication, although residual DNA synthesis still occurs in the presence of the compounds (this is not the case with other inhibitors we tested - see below).

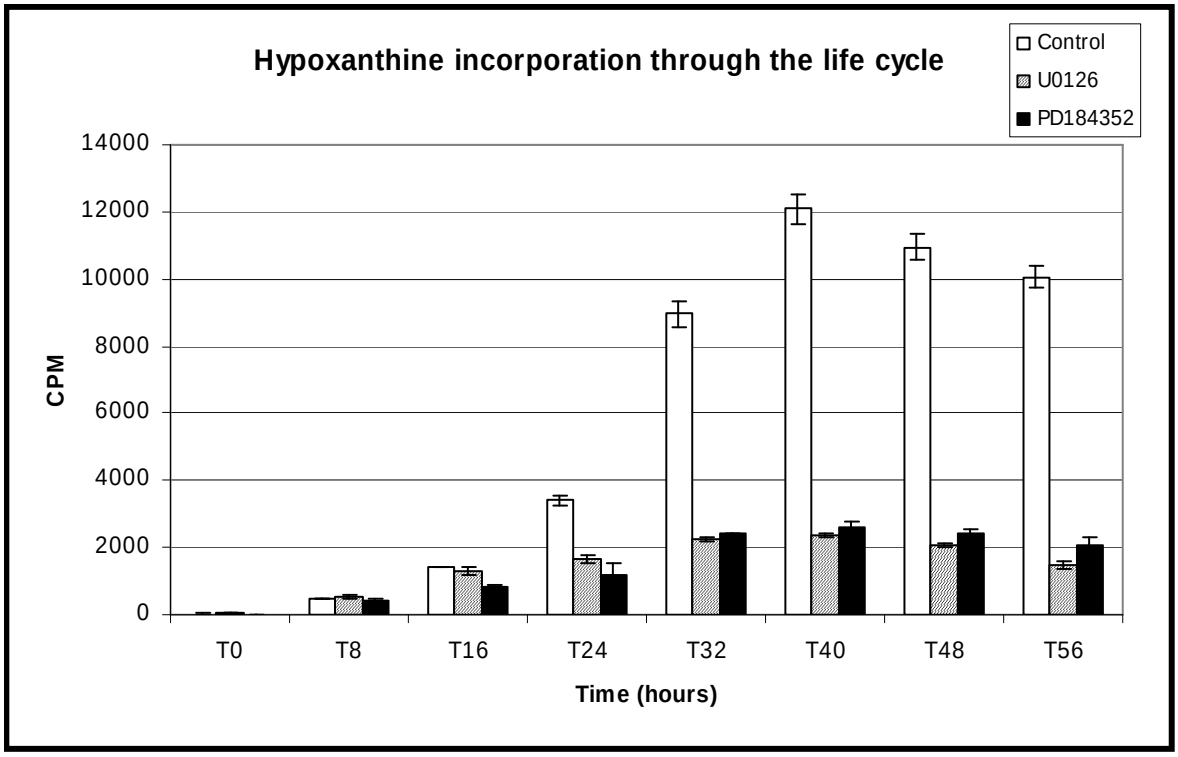


Figure 8: Hypoxanthine incorporation during *P. falciparum* life cycle in presence of MEK inhibitors (U0126 and PD184352).

MEK inhibitors (20µM), DMSO control, and tritiated hypoxanthine (0.1µCi/well) were added at T0. The cells were harvested every 8 hours and hypoxanthine incorporation was determined by scintillation. The experiment was performed in triplicate and error bars correspond to the SEM.

3.2 Effect of infection on host proteins phosphorylation

3.2.1 *Effect of infection on host protein phosphorylation*

Having demonstrated that MEK inhibitors do affect *P. falciparum* growth, we wanted to investigate whether there was an effect of infection on phosphorylation of host erythrocyte MEKs. Extracts of infected or uninfected erythrocytes, prepared following the description in 2.1.5, were normalized using the same number of cells. They were then sent to a company, KINEXUS, which performed Western blots using a panel of 70 antibodies directed specifically against the phosphorylated form of a variety of signalling proteins. Interestingly, this analysis revealed human phospho-proteins in the infected erythrocytes that were not phosphorylated in the uninfected cells (Table 3).

The proteins giving a signal only for IRBCs were: CDK1/2 (a cyclin-dependent kinase), Dok2 (an effector of tyrosine kinase), eIF2α (a translation initiation factor), MEK1 (but only for the site S297, see below), PTEN (a phosphatase) and PRAS40 (proline-rich Akt/PKB substrate 40 kDa, a tensin homologue), and Src (a membrane bound tyrosine kinase). Some of these proteins are undoubtedly of human origin, but other signals, such as CDK1/2 and eIF2α, may be of parasitic origin. Conversely, some antibodies reacted more strongly with the uninfected erythrocyte extract than with infected cell extract: MEK1 (T291 and T385, which are not the activation site), MEK2 (T394, which again is not the activation site, was targeted) and RSK1/2, a ribosomal S6 kinase 1/2. Of particular interest to us was the observation that an antibody against the phosphorylated (S297) form of MEK1 gave a much higher signal in infected red blood cells (IRBCs) than in uninfected erythrocytes (URBCs) (Fig 9A). The phosphorylated signal was quantified by luminescence and expressed in CPM (Fig 9B). This suggested that host cell MEK1 is activated upon *P. falciparum* infection. However, this antibody does not recognise the serines (217/221) of the activation loop but another serine located at position 297. Phosphorylation of serine 297 by PAK1 was shown to "prime" the enzyme for activation [26]. Focussed on the initial themetic of this project, we decided to investigate further this observation and carry on the work on the MEK1 protein by looking at the effect of *P. falciparum* infection on MEK1 phosphorylation using different approaches.

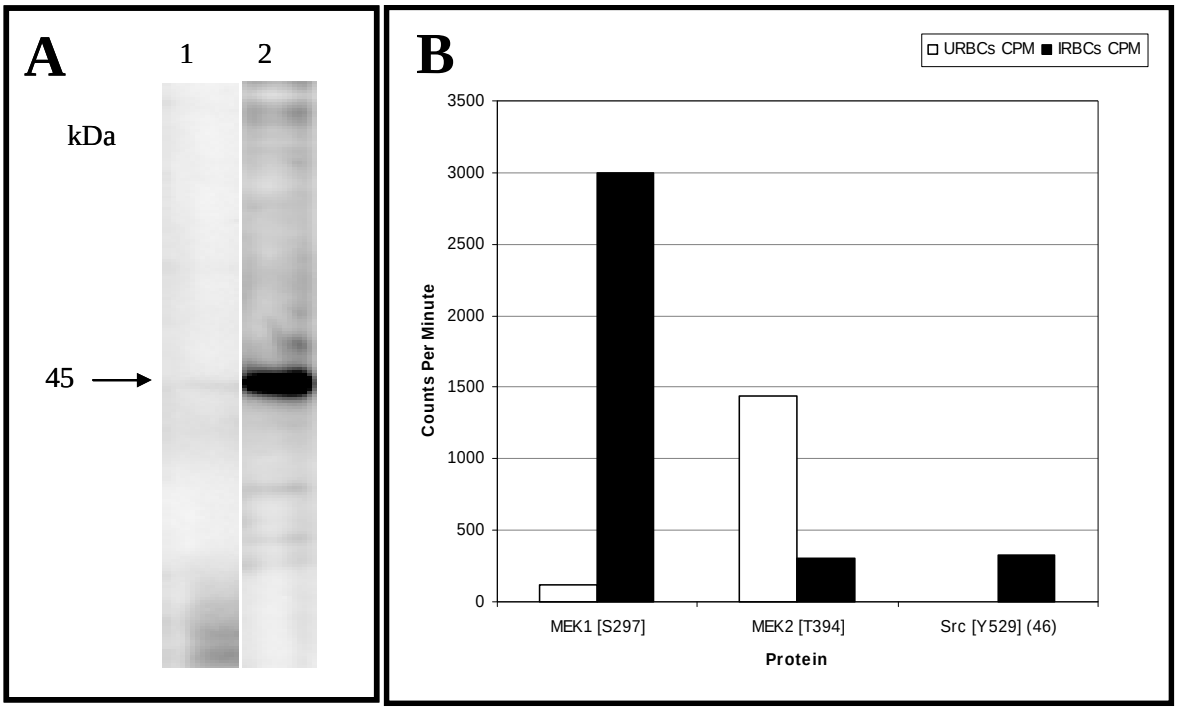


Figure 9: Effect of infection on host proteins phosphorylation
A: Western-blot on Uninfected RBCs (lane1) versus Infected RBCs (lane 2) blotted with anti phospho-MEK1. B: Histogram of protein phosphorylation for MEK1, MEK2 and Src. The quantity of phosphorylated protein was determined by luminescence.

URBCs	IRBCs
-	CDK1/2 [T14+Y15]
-	Dok2 [Y142]
-	eIF2a [S51]
-	Erk1 [T202+Y204]
MEK1 [S297] MEK1 [T291] MEK1 [T385]	MEK1 [S297] - -
MEK2 [T394]	MEK2 [T394]
-	PTEN [S380+T382+S385]
-	PRAS40 [T246]
RSK1/2 [S221/S227]	-
-	Src [Y529]

Table 3: Phosphorylated proteins identified by Kinexus

3.2.2 *Effect of infection on MEK phosphorylation*

3.2.2.1 Phosphorylation of the activation site (S217/S221):

We first wanted to see if the activation site of the MEK1/2 protein was phosphorylated using different approaches.

3.2.2.1.1 Western blot analyses:

A western blot was performed on URBCs versus IRBCs with an antibody recognising the phosphorylated serines 217/221 of MEK1/2 (Fig 10). The expected size is around 45kDa. No band appeared for the URBCs extract when there were a lot of parasitic proteins recognized by the antibody for the IRBCs extract. However, there was a major band at 45kDa, which appeared at the same size than the band in the positive control lane. This suggests the major band recognized in IRBCs extract correspond to the MEK1/2 protein and the phosphorylated signal is increased in IRBCs compared to the URBCs.

3.2.2.1.2 Immunofluorescence analyses:

Immunofluorescence assay (IFA) was used to confirm results obtained by Western blotting. To enhance our confidence that any signal observed can be assigned to phosphorylated MEK1/2, parallel IFAs were performed using two antibodies against the activated form of MEK1/2 from two different companies (Santa Cruz inc, CA, USA and Cell Signaling, MA, USA). Similar results were obtained with both antibodies (Fig 11). There was no fluorescence observed when the cells were incubated only with secondary antibody (negative control, not shown). The basal level of MEK phosphorylation was very low in URBCs. In contrast, and IRBCs yielded a robust signal, especially at the late stages. As a signal for the phosphorylated protein was observed, the next step was to check the non-phosphorylated MEK expression in both cells types (uninfected and infected). So IFA was carried out with our anti-MEK1 monoclonal antibody. Unfortunately, no signal was observed, and information form the supplier indicated that this antibody is not suitable to perform IFA.

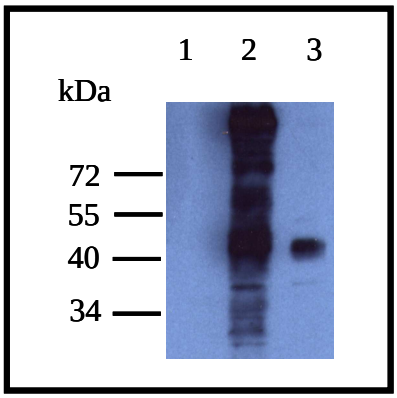


Figure 10: Effect of infection on phosphorylation of MEK1/2 activation site (Ser 217/221) Uninfected RBCs (lane1) versus Infected RBCs (lane 2) blotted with anti phospho-MEK1/2 (Santa Cruz inc, CA, USA) and positive control (Cell Signaling, MA, USA): Hela cells treated with TPA (tetradecanoyl phorbol acetate, 200nM for 15min) (lane3). TPA treatment activates the MAP kinase pathway in Hela cells [106].

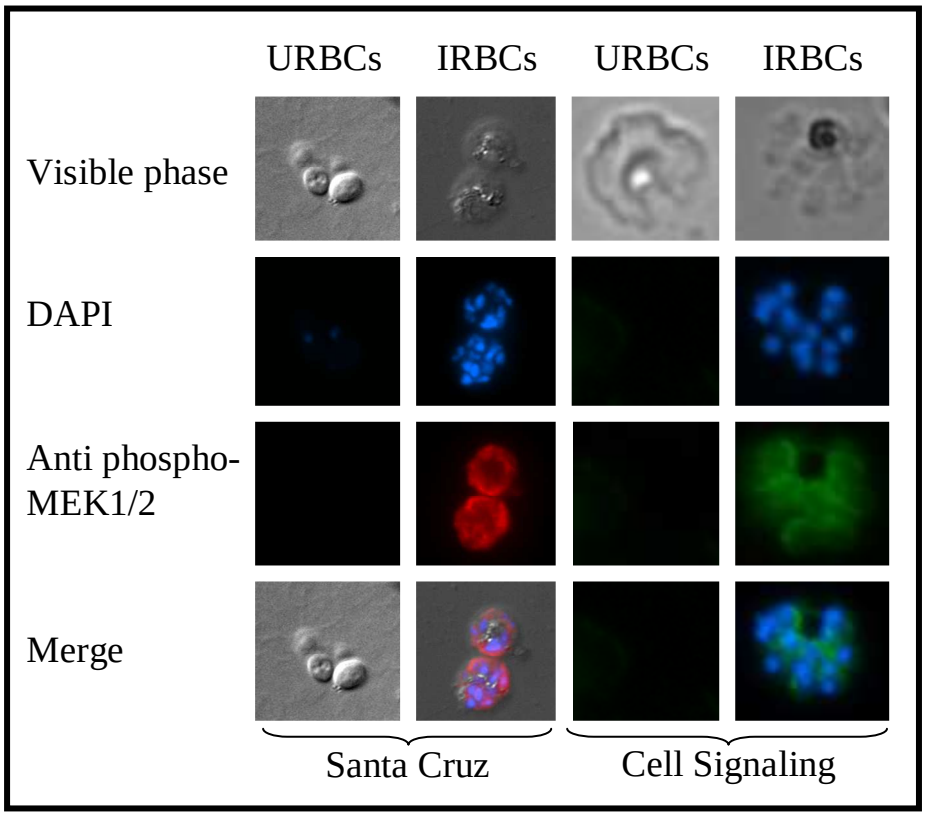


Figure 11: ImmunoFluorescence Assay
Smears from asynchronous *P. falciparum* cultures were fixed using paraformaldehyde and glutaraldehyde and incubated with anti-phospho MEK antibodies (Ser 217/221) (supplied from Santa Cruz inc, CA, USA and Cell Signaling, MA, USA).

3.2.2.2 Phosphorylation of S297:

To validate and extend the interesting observation that the Kinexus antibody used to detect MEK1 phosphorylation gave a dramatically increased signal in infected (versus uninfected) erythrocytes, we purchased the same antibody and performed additional experiments.

We wanted to reproduce the Kinexus Western blot results (Fig 12A). A protein at approximately 45kDa, which is the expected size for MEK, was detected in positive controls (mouse 3T3 cells treated or not with PDGF) and there was also a band at the same size for the URBCs and IRBCs. The signal was stronger in the IRBC than in the URBC cell extract, confirming the results received from Kinexus. The identity of the band was verified by probing the same membrane with a highly specific anti-MEK1 monoclonal antibody recognising all forms (phosphorylated or not) of the enzyme (Fig 12B). As with the phospho-antibody, a band was recognized at 45kDa for the positive controls and for the URBCs and IRBCs. Interestingly, this revealed that the total amount of MEK1 is higher in the extract from uninfected cells than in the extract from infected cells, despite a much stronger signal in the latter when the anti-phospho-MEK1 antibody was used (Fig 12A). This suggested that the differences in signal were due to an increase in phosphorylation.

However the best experiment to prove the protein recognized by the phospho-antibody is the human MEK was to immunoprecipitate the MEK (non-phosphorylated) from total extracts in URBCs and IRBCs and to perform a Western blot with the anti-phospho-MEK1 antibody (Fig 13A). No signal was seen with beads incubated with cell extract in the absence of antibody. For extracts (URBCs and IRBCs) incubated with the antibody recognizing non phosphorylated MEK1, a band was seen at about 55kDa. This band corresponded to the mouse IgG (heavy chain). Additionally, there was a signal at 45kDa for the URBCs only. The membrane was blotted with the MEK1 antibody (Fig 13B) and the same band was recognized. There was a band at 45kDa for the IRBCs too, but this protein was not phosphorylated. The inability to immunoprecipitate the MEK protein and particularly the phosphorylated form was investigated further.

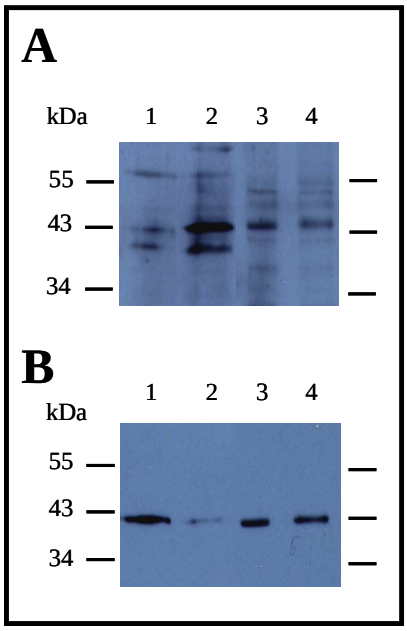


Figure 12: Effect of infection on phosphorylation of MEK S297
A: Uninfected RBCs (lane1) versus Infected RBCs (lane 2) blotted with anti phospho-MEK1 (BioSource International, USA) and positive controls (Invitrogen, CA, USA): m3T3 cells non-treated with PDGF (lane3) and m3T3 cells treated with PDGF (lane 4). B: Uninfected RBCs (lane1) versus Infected RBCs (lane 2) blotted with anti-MEK1 (BioSource International, USA) and positive controls m3T3 cells non-treated with PDGF (lane3) and m3T3 cells treated with PDGF (lane 4).

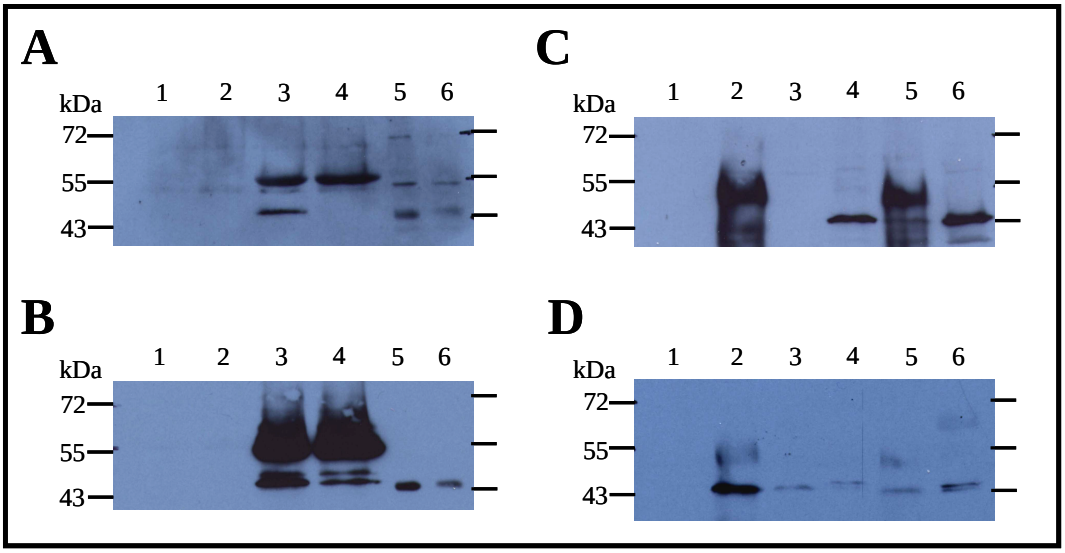


Figure 13: Immunoprecipitation of human MEK in IRBCs and URBCs followed by Western blot using an anti-phospho MEK1 antibody (Ser 297)
A+B: Human MEK1 was immunoprecipitated using anti-MEK1 antibody (BioSource International, USA) from URBCs and IRBCs. Lane1: URBCs beads only, lane 2: IRBCs beads only, lane 3: URBCs after immunoprecipitation, lane 4: IRBCs after immunoprecipitation, lane 5: positive control (m3T3 cells-PDGF), lane 6: positive control (m3T3 cells+PDGF). (A): Western blot was performed using anti phospho-MEK1 antibody (BioSource International, USA) and (B): Western blot was performed using anti MEK1 antibody (BioSource International, USA). C+D: Protein samples were kept after each step of the IP. Lane 1: URBCs total extract before IP, lane 2: URBCs after pre-clear (using a mouse IgG antibody, SIGMA), lane 3: URBCs supernatant after IP, lane 4: IRBCs total extract before IP, lane 5: IRBCs after pre-clear (using a mouse IgG antibody, SIGMA), lane 6: IRBCs supernatant after IP. (C): Western blot was performed using anti phospho-MEK1 (Ser 297) antibody (BioSource International, USA) and (D): Western blot was performed using anti MEK1 antibody (BioSource International, USA). The pre-clear step reduces non-specific binding to the Protein A agarose beads and to the antibody used to perform the IP.

Total extracts (before immunoprecipitation), extracts incubated with mouse IgG and supernatant after incubation with anti-MEK1 antibody were blotted with phospho anti-MEK1 (Ser 297) antibody (Fig 13C). A band at 45kDa was observed for the total extract from IRBCs only, confirming the results obtained previously. A huge background was observed for the extracts after pre-clear as well for URBCs than for IRBCs but no particular bands were observed at 45kDa. Nevertheless, from the supernatant kept after incubation with anti-MEK1 antibody, a band at 45kDa was recognized by the phospho-antibody in IRBCs only. The membrane was blotted again with anti-MEK1 antibody (Fig 13D) and the same band was recognized. Taken together, these data suggest that the phosphorylated form of MEK cannot be immunoprecipitated from IRBCs (see Discussion, p65).

IFA were performed using this phospho antibody, but unfortunately no fluorescence was observed, suggesting the antibody is not suitable to perform IFAs experiment.

3.2.3 *Inhibitors effect on phosphorylation of MEK activation site*

We next wanted to check if the IFA signal obtained in IRBCs using the anti-phospho–MEK1/2 antibody (Ser 217/221) would be decreased, as expected, in the presence of MEK inhibitors. Synchronised cultures were maintained until parasites initiated a new erythrocytic cycle and then MEK inhibitors (U0126 and PD184352 at 20μM) were added (T0). IFA was performed on cells at T0 and T36 (we showed earlier [Fig 6] that at T36 the MEK inhibitors have detectable effects on parasite development) (Fig 14). At T0, there were still some late schizonts and fluorescence was observed for 96% of RBCs infected with schizonts for the control (DMSO), 97% for IRBCs (schizont stage) treated with U0126 and 97% for IRBCs (schizonts) treated with PD184352 (Table 4). The early ring stage present on the same slides showed fluorescence in the majority of the IRBCs (Table 4). This observation was consistent with previous IFA data, demonstrating that there was a higher signal for the IRBCs at the late stages of the life cycle. It thus appears there were no differences at T0, i.e at the time the inhibitors were added to the cultures. At T36, the majority of the control culture was at the trophozoite stage, with some schizonts already visible. Again fluorescence was visible in IRBCs at the late stages, either late trophozoites or schizonts. However, in the presence of MEK inhibitors, the number of fluorescent IRBCs decreased considerably relative to controls. No schizonts were observed in cells treated with U0126 or PD184352, confirming the results described in 3.1.2 and showing that treatment blocks schizont development. Of the trophozoite-infected cells treated with U0126 only 34% were fluorescent and in these cells the fluorescence was dramatically reduced (Fig 14). In cells treated with PD184352, the number of cells presenting fluorescence is even lower. The experiment was also performed using URBCs; the number of cells for which there was fluorescence in URBCs was very low (Table 4).

Taken together, this set of data shows that interfering pharmacologically with MEK activation results, as expected, in a significant decrease in the signal obtained with the phospho-specific MEK1/2 antibody (see discussion).

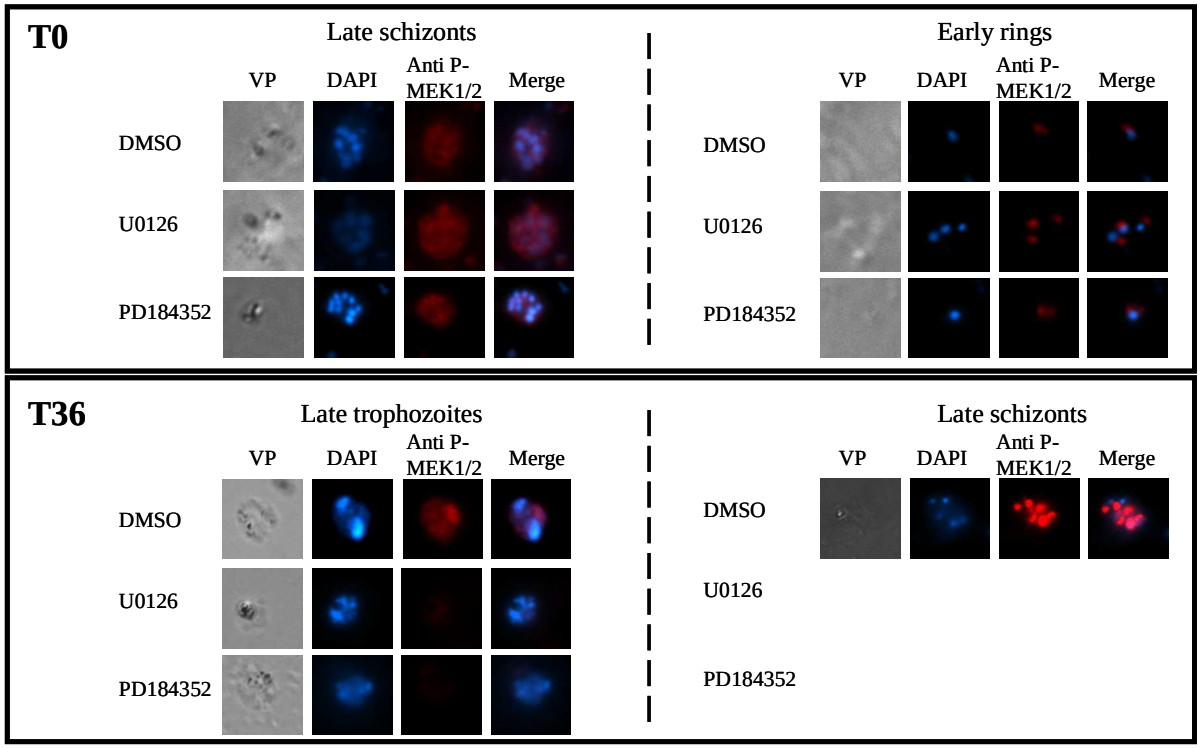


Figure 14: Effect of inhibitors on phosphorylation of MEK activation site
ImmunoFluorescence Assay: smears were made at T0 and T36 from synchronous *P. falciparum* cultures treated with MEK inhibitors and incubated with anti-phospho MEK1/2 (Ser217/221) antibodies (Santa Cruz inc, CA, USA), (VP: Visible Phase). The number of cells presenting fluorescence was counted on 100-cell samples (table 4, below). Since treatment prevents schizont development, there were no schizonts to be observed in the treated cultures at T36 bottom right.

	T0						T36					
	DMSO		U0126		PD184352		DMSO		U0126		PD184352	
URBCs	1%		1%		0%		0%		0%		0%	
	Schizonts	Early rings	Schizonts	Early rings	Schizonts	Early rings	Trophozoites	Schizonts	Trophozoites	Schizonts	Trophozoites	Schizonts
IRBCs	96%	95%	97%	90%	97%	85%	89%	64%	34%	-	34%	-

Table 4: Percentage of cells presenting fluorescence in presence of MEK inhibitors

3.3 Effect of a Src inhibitor on parasite proliferation

It is well established that Src, a membrane-bound tyrosine kinase, can stimulate the MAP kinase pathway via different proteins such as Grb2, Sos, Ras and Raf and thereby act as an upstream element of MAP kinase pathways. We were therefore very interested by the observation from the Kinexus experiment that Src phosphorylation is higher in infected than in uninfected erythrocytes (Table 3 and Fig 8).

We wanted to investigate whether a Src inhibitor would have an effect on *P. falciparum* growth, and, if this were the case, whether the block in parasite development would occur at the same stage as the block caused by treatment with MEK inhibitors. A similar approach (^{3}H hypoxanthine incorporation test) was taken with the Src inhibitor (PP2) as has been with the MEK inhibitors described earlier (see Fig 6). The EC_{50} value of PP2 is 2μM (Fig 15), which is comparable with the EC_{50} value of this inhibitor in various mammalian cell systems (0.6-18μM, [107, 108]).

The Src inhibitor appeared to have an effect earlier in the life cycle than the MEK inhibitors (Fig 16). At T12, the number of rings was lower than in the control and MEK inhibitor-treated cultures. At T30, the majority of the cells were still at the ring stage. The parasite life cycle progression hence seemed to be blocked at the ring stage, and it was very difficult to differentiate the late rings from the early trophozoites. Furthermore, the morphology of the treated cells with the Src inhibitor was completely different than that of cells treated with MEK inhibitors. The parasites treated with Src inhibitor appeared to be vacuolated. The parasitaemia continued to decrease and the parasites never progressed beyond the early trophozoite stage.

There was a significant difference in hypoxanthine incorporation between the cells treated by Src inhibitor on one hand, and the control cultures or those treated with the MEK inhibitors on the other hand. Incorporation was extremely low and the treated parasites synthesised little or no DNA (Fig 17).

An isobologram was performed between MEK and Src inhibitors to determine whether or not there was any synergy between these two inhibitors. This experiment required large quantities of inhibitors and so was performed between U0126 and PP2 only (the supply of PD184352 was from non-commercial source and very limited). Hypoxanthine incorporation assays were carried out as described in 2.1.8. The EC_{50} for U0126 was 3μM and 2.1μM for PP2, so the range chosen for U0126 was (225-0.014μM) and for PP2 (200-0.0122 μM). The EC_{50} values were determined for each range and an isobologram was plotted (Fig 18). No synergy was detected (see discussion).

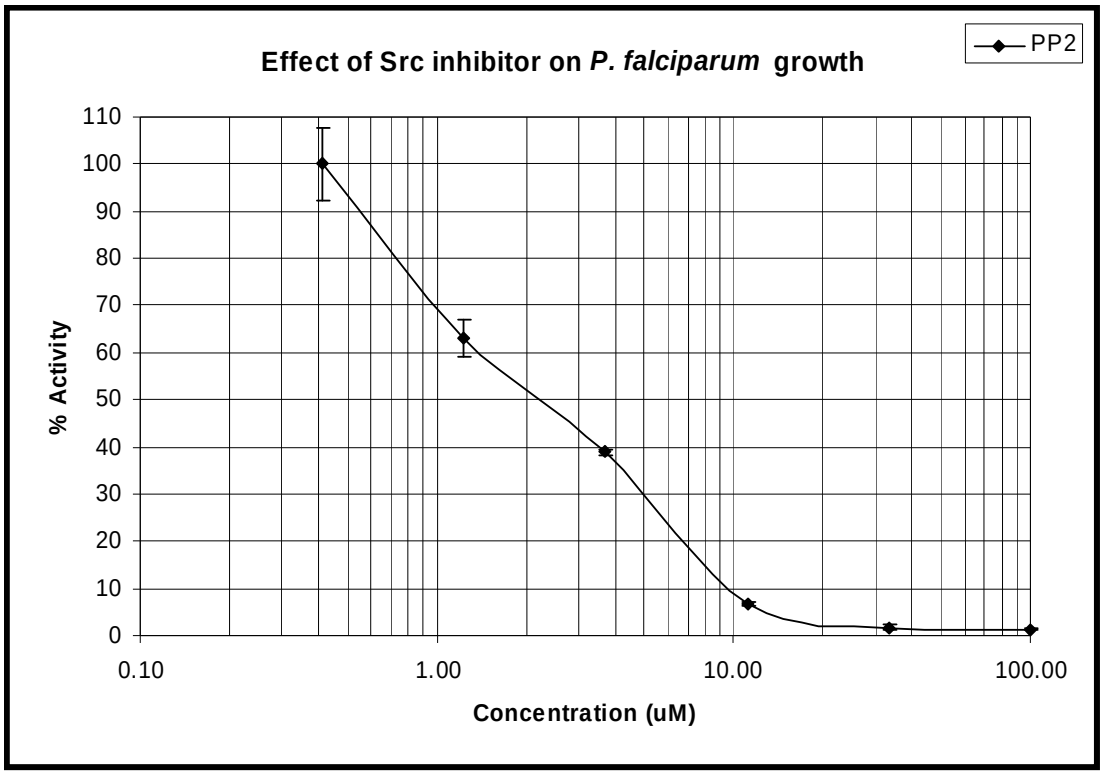


Figure 15: Effect of PP2 on *P. falciparum* growth

***P. falciparum* culture at 0.5% of parasitaemia were plated in presence of inhibitor and tritiated hypoxanthine (0.1μCi/well) and incubated at 37°C for 48 hours. The range of concentration is 0.412 to 100μM for PP2. The percentage of activity was determined by the hypoxanthine incorporated relative to the uninhibited controls. Assays were run twice in triplicates and error bars correspond to SEM.**

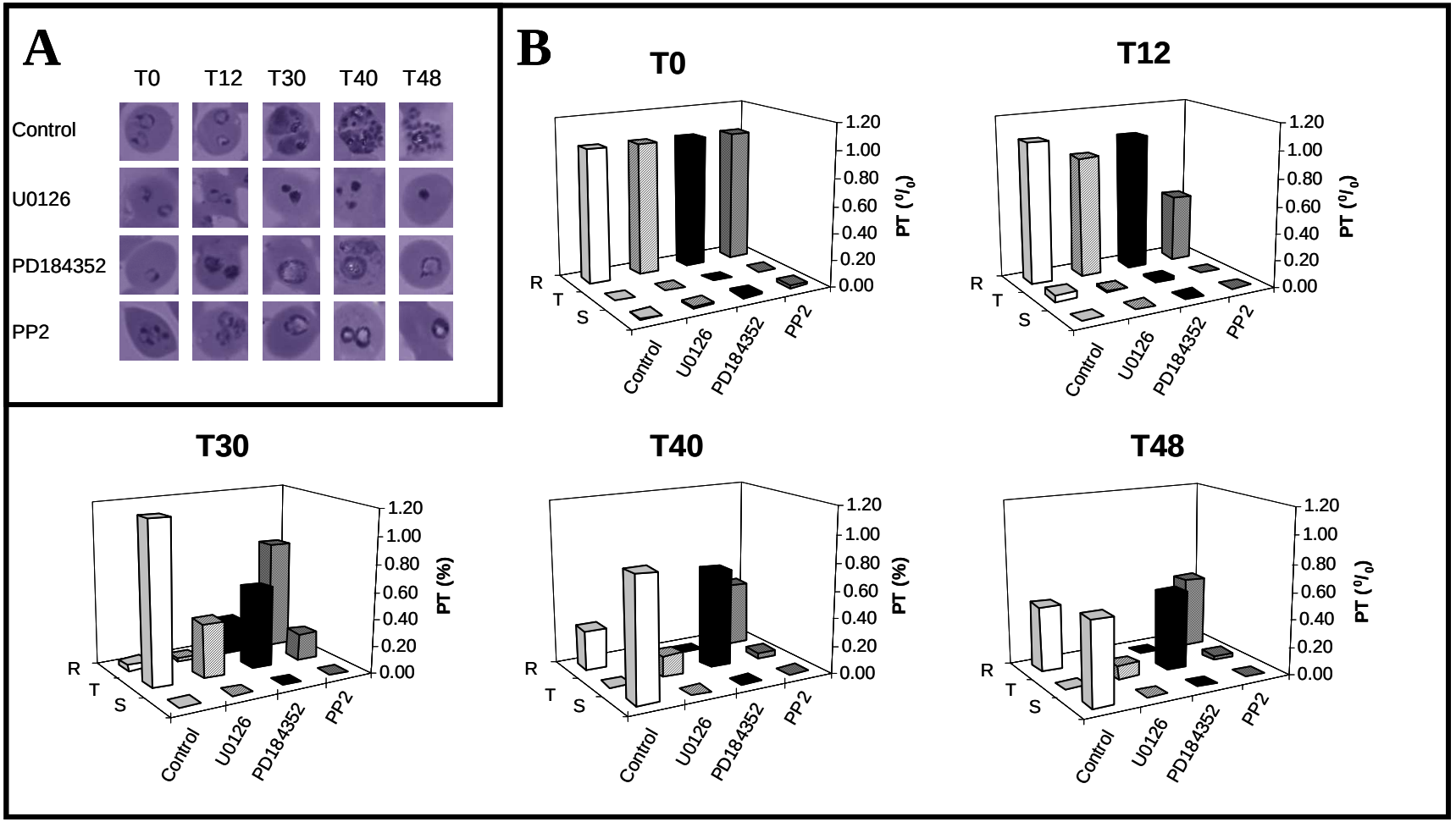


Figure 16: Effect of Src inhibitor on *P. falciparum* along the life cycle compared to MEK inhibitors

A: Giemsa stained parasites at different time of the life cycle treated or not by MEK or Src inhibitors. B: Parasitaemia at different time for each stage in presence of 20µM of MEK inhibitors (U0126 and PD184352) and of Src inhibitor (PP2). The number of cells has been estimated by counting 10 fields. The experiment was done twice in triplicate.

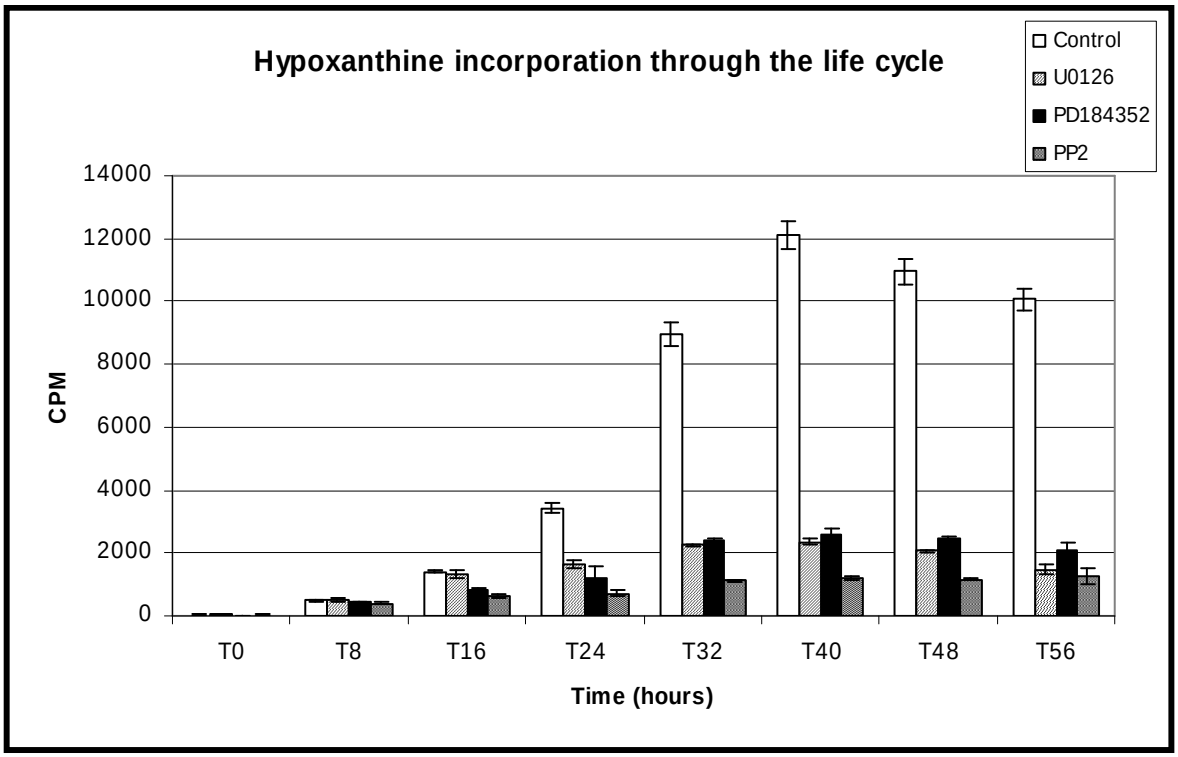


Figure 17: Hypoxanthine incorporation along *P. falciparum* life cycle in presence of MEK inhibitors (U0126 and PD184352) and Src inhibitor (PP2).

MEK inhibitors (20μM), Src inhibitor (μM), DMSO control, and tritiated hypoxanthine (0.1μCi/well) were added at T0. The cells were harvested every 8 hours and hypoxanthine incorporation was determined by scintillation. The experiment was performed in triplicate and error bars correspond to the SEM.

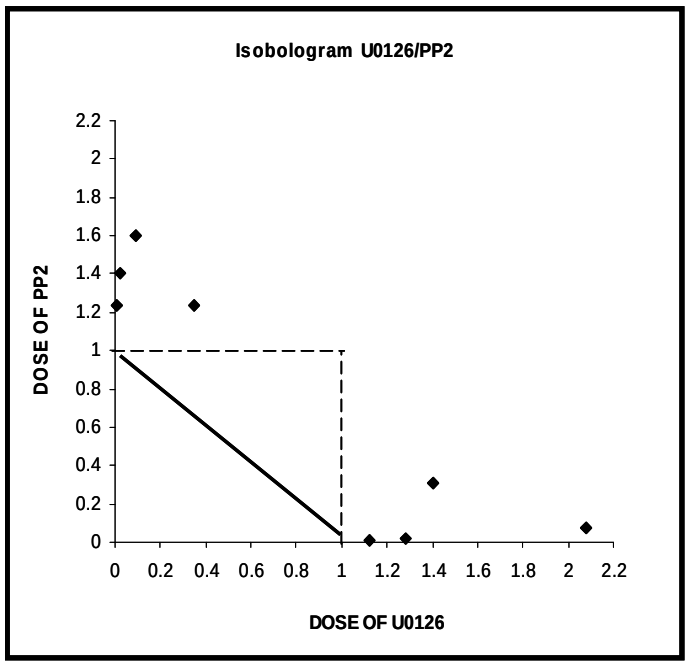


Figure 18: Isobologram of the interaction between MEK inhibitor (U0126) and Src inhibitor (PP2) against *P. falciparum* at the IC50

Results were expressed as fraction of EC_{50}: FEC_{50}=1 for MEK inhibitor, U0126, in the absence of Src inhibitor, PP2. The assessment of drug interaction is based on calculation of the sum of the fractional inhibitory concentrations (ΣFECs) at the given IC by the formula (EC_x of U0126 in the mixture/EC_x of U0126 alone)+(EC_x of PP2/EC_x of PP2 alone). ΣFECs<1 denote synergism, ΣFECs≤1 and <2 denote additive interaction, ΣFECs≥2 and >4 denote slight antagonism, and ΣFECs≥4 denote marked antagonism. Data points below the lines connecting the IC_{50} denote no synergism (ΣFIC>1).

4 DISCUSSION

4.1 Effect of MEK inhibitors on parasite growth

Malaria is one of the world's most important infectious diseases and effective drugs are vital to the lives of millions of people. Unfortunately, resistance to existing therapies is an increasing problem so new strategies are urgently needed to help control this pathogen. Our work suggests that the host cell signalling pathways might represent attractive novel targets.

As detailed in the introduction, *P. falciparum* does not possess any MEK homologues. However, we found that MEK inhibitors were able to kill *P. falciparum*, suggesting the parasite might rely on the host MAP kinase pathway for its own survival. All the MEK inhibitors we tested do inhibit *P. falciparum* growth with EC_{50} values that compare very well with those in mammalian cellular assays. It appears that the inhibitors do differ in efficiency to inhibit MEK activation, which is expected due to their different modes of action.

(i) Studies have shown that U0126, PD98059 and PD184352 prevent MEK1 phosphorylation [40, 109] and not by targeting the ATP pocket. Even though these three inhibitors prevent parasite growth in a range, which is similar to those found in mammalian cells, PD98059 seems to have a lower efficiency than the other two. Indeed, for U0126 and PD184352, the EC_{50} is lower than 10μM, when the EC_{50} for PD98059 is around 30μM. This is not surprising because it has been already hypothesised that U0126 and PD98059 are both inhibiting MEK phosphorylation but with a different mode of action. There is at least one study [110] in which a cellular process was inhibited by U0126 but not by PD98059, supporting that both inhibitors may have distinct modes of action in some contexts. Furthermore, it has been proved PD98059 is actually a compound that stops one protein kinase (Raf) from activating another (MEK1) [40]. U0126 and PD184352 also exert their effect on cells by suppressing the activation of MEK1, and not by blocking its activity [40]. Nevertheless, it is not known where those inhibitors bind on the enzyme.

(ii) SL327, is a water-soluble structural homologue of the specific MEK1/2 inhibitor U0126 [37, 42]. Its EC_{50} on parasite growth is around 25μM. Although this inhibitor is a structural homologue of U0126, its EC_{50} is different than the

one found for U0126. Slight structural modifications can affect different parameters and so the efficiency of the inhibitor on the protein. However this EC_{50} is comparable to the range found for mammalian cells for the same inhibitor.

(iii) The mechanism of action of the two other MEK inhibitors (MEK Inhibitor I and MEK inhibitor II) is not known and they are not commonly used. No data was found in the literature about the efficacy of these inhibitors in cellular systems.

All the MEK inhibitors we tested have an effect on *P. falciparum*. The facts we observe (i) an inhibition of *P. falciparum* growth and (ii), no MEK homologues are present in *P falciparum*, strongly suggest that the host MEK might be involved.

To determine at what stage(s) of the asexual life cycle the MEK inhibitors act, the inhibitors (U0126 or PD184352) were added to tightly synchronized parasites at the ring stage. The parasitaemia was then estimated along the life cycle and DNA synthesis was monitored. Both techniques showed there were no differences from control cultures during the ring stage. In contrast, at 24 hours a significantly lower number of trophozoites were observed for the cells treated with MEK inhibitors and, furthermore, the trophozoite shape was abnormal. The morphology of the cells was slightly different depending on the inhibitor the cells were treated with. These results were confirmed by the hypoxanthine incorporation assay. From T24 until the end of the life cycle, the cells treated by the inhibitors compared to the control incorporated a significantly lower quantity of hypoxanthine. This suggests the inhibitors were acting at the trophozoite stage. We were surprised to observe that parasites in the treated cultures display different morphologies depending on which inhibitor was used. As both inhibitors prevent MEK phosphorylation, a similar effect on the parasite shape was expected. Similar to PD98059 exerting different effects from those of other MEK inhibitors in some systems (see above), it thus appears that U0126 and PD184352 may have different mechanisms of action. Finally, the treated parasites were not able to form schizonts. An experiment was performed to observe the effect of MEK and Src inhibitors on the initiation of a new cycle, by adding the inhibitors at the late stages of the life cycle. This experiment was performed once, that is why the results have not been incorporated in the

results section. In this single experiment, there were no effects on the initiation of a new cycle, neither with MEK inhibitors nor Src inhibitors. This is in line with the absence of effect of U0126 on invasion rate (H. Taylor, personal communication).

4.2 Effect of infection on phosphorylation of host proteins

As described in the results section, human proteins have been found to have a higher signal for IRBCs only: CDK1/2, Dok2, eIF2a, MEK1, PTEN, PRAS40 and Src. All these proteins are in pathways involved in cell cycle regulation and are therefore playing a key role for cancer disease. Indeed, for the majority of them, inhibitors have been found to have an effect on the proliferation and are therefore potential drug targets against cancer. However, some of these proteins are implicated in processes absent in erythrocytes and we suspect those proteins to be parasitic and not human (e.g. CDK1/2 and eIF2a). The other proteins have not been studied and we focussed the research on MEK1 and Src.

We had a particular interest for the MEK1 protein. On the panel of proteins screened by Kinexus, there was no antibody against the MEK1 activation site (Ser217/221). However, an antibody gave a higher signal for the phosphorylated form of the MEK1 at the Serine located at 297. The phosphorylation of this site is necessary for efficient activation of MEK1 [111]. S297 is phosphorylated by PAK-1 and this phosphorylation is robustly stimulated during cellular adhesion to extracellular matrix proteins [111]. It has been shown that the rapid and efficient activation of MEK and phosphorylation on S297 induced by cell adhesion to fibronectin is influenced by FAK and Src signaling [111]. Recently a study provided evidence that PAK phosphorylation of MEK1 S297 stimulates MEK1 autophosphorylation on the activation loop [30]. The observation of an increase of phosphorylation for the MEK S297 and Src Y529 residues suggests there could be some synergy between those two kinases and they could even be implicated in a same pathway (see 4.4).

4.3 Effect of infection on host MEK phosphorylation

Having shown that MEK inhibitors act on parasite growth at the trophozoite stage, it was interesting to look at the effect of *P. falciparum* infection on the MAP kinase pathway.

It appeared there was an increase of MEK phosphorylation in the infected versus uninfected erythrocytes, as showed by the results from Kinexus. The same result was found using two different techniques: western blot and IFA on synchronised culture at the late stages. This strongly suggests the increased signal corresponds to the MEK protein. However, when the total form of the MEK protein was immunoprecipitated using a highly specific monoclonal antibody and blotted with an anti-phospho-MEK antibody, the increased signal previously observed in the infected red blood cells extracts disappeared. Two possible explanations come to mind: (i) Either the protein recognized is not the human MEK, but a parasite protein with a similar molecular weight. (ii) Or the protein is the human MEK but once phosphorylated it becomes refractory to immunoprecipitation with the MEK1 antibody. Concerning the first explanation, it would be really unlikely that a parasite protein would be inhibited by a panel of structurally different MEK inhibitors and would have exactly the same molecular weight than the host protein. From PlasmoDB alignment, only one parasitic protein was found to possess a similar activation site to MEK1: PfNek-1. Indeed, as detailed in the introduction, this protein has a SMAHS activation motif, closely related to the SMANS motif found in MEK1. However, PfNek-1 does not co-migrate with MEK1 and has a molecular weight around 110kDa whereas the molecular weight of MEK1 is close to 43kDa. Secondly, it is possible the phosphorylated form of the protein interacts with other proteins in such a way that the site recognized by the antibody (against the non-phosphorylated form) becomes not accessible.

To link the results found *in vivo* in the presence of MEK inhibitors with the MEK phosphorylation, MEK inhibitors were incubated with infected erythrocytes and IFAs performed to determine whether the phosphorylated MEK signal was affected. An important decrease in the IFA signal was detected in erythrocytes

treated with MEK inhibitors. In URBCs no decrease was observed as no signal was detected in absence of inhibitors (Fig 11).

4.4 Effect of Src inhibitor on MEK phosphorylation

The Kinexus results showed the MEK protein was not the only protein to be up-phosphorylated in infected red blood cells; Src protein was up-phosphorylated too. The effect of a Src inhibitor was tested on *P. falciparum* culture to investigate whether MEK and Src inhibitors act at the same development stage. Furthermore, to determine whether Src inhibitors synergise with MEK inhibitors, an isobologram was performed.

Concerning *P. falciparum* growth, there was a significant difference between the control and the cells treated by Src inhibitor only 16 hours after the drug was added, while an effect from MEK inhibitors appeared only 24 hours after the drugs were added. The quantity of hypoxanthine incorporated did not increase along the life cycle. It appears that the Src inhibitor acts at an earlier stage than the MEK inhibitors, and that the effects on parasite morphology are different depending on the inhibitors used. These two experiments (parasitaemia of life cycle stages and hypoxanthine incorporation) gave different information but correlated very well, confirming the Src and MEK inhibitors do not act at the same stage, with the Src inhibitor acting earlier than the MEK inhibitors.

Concerning the isobologram, all the points were out of the square, suggesting there were no synergy and even a slight antagonism. Unfortunately we were not in a position to repeat this experiment because of limited available amounts of the inhibitors.

5 PERSPECTIVES AND GENERAL CONCLUSION

By testing a panel of structurally different MEK inhibitors, we observed a very strong inhibition of parasite growth. Most classical kinase inhibitors are ATP competitors. In contrast, the MEK inhibitors used in this study are allosteric inhibitors, interacting with the protein outside the ATP-binding cleft. This confers a high level of selectivity to these molecules and supports the hypothesis that erythrocyte MEKs are the targets. The results from Kinexus and our own Western blot concur to show MEK phosphorylation is stimulated by infection. Overall, results from the pharmacological experiment using a panel of MEK (and Src) inhibitors and from the immunological approaches (WB and IFAs) are consistent with a role for the host cell signalling pathway in erythrocyte infection by *P. falciparum*. It will be interesting to investigate further and characterise the pathway that leads to MEK activation, and the downstream effectors that are required for parasite survival. Preliminary data suggest the human ERK is not phosphorylated by infection-activated MEK (data not shown). Perhaps the parasite is using the host MEK to phosphorylate proteins of parasite origin. The Kinexus analysis revealed that Src is activated too upon *P. falciparum* infection, and we showed that treatment with a Src inhibitor is lethal to the parasite; however, we cannot exclude at this stage that the parasitocidal effects of the Src inhibitor are mediated through other (host cell or parasite) targets.

Our discovery that MEK inhibitors prevent parasite development has important implications in the context of antimalarial drug discovery, because it suggests that host cell pathways represent potential targets. Indeed, by targeting a host protein to kill *P. falciparum*, the resistance problem, which emerged years ago with chloroquine and other treatments, would probably be slower to appear, because the most direct mechanism to develop resistance, i.e. selection of parasites expressing mutated target unable to bind to the drug, would not be available. Furthermore, the cost to develop a new drug is huge and as detailed in the introduction, inhibitors targeting human kinases (including those in the MAPK pathways) are already in the drug development pipe-line for cancer, diabetes or neurodegenerative disorders. It would be of tremendous economic interest to piggy-back on those molecules, for which significant investment has already been done. Finally, this study can be repeated for every protein found to have a higher signal for the IRBCs only by Kinexus.

LIST OF REFERENCES

1. Nikolaev, B.P., *[Fourth specie of the malarial parasite in man (Plasmodium ovale) and its discovery in the USSR.].* Zool Zhurnal, 1951. **30**(3): p. 211-6.
2. Greenwood, B.M., et al., *Malaria: progress, perils, and prospects for eradication.* J Clin Invest., 2008. **118**(4): p. 1266-76.
3. Killeen, G.F., et al., *Quantifying behavioural interactions between humans and mosquitoes: evaluating the protective efficacy of insecticidal nets against malaria transmission in rural Tanzania.* BMC Infect Dis, 2006. **6**: p. 161.
4. Staalsoe, T., et al., *Novel Plasmodium falciparum malaria vaccines: evidence-based searching for variant surface antigens as candidates for vaccination against pregnancy-associated malaria.* Immunol Lett, 2002. **84**(2): p. 133-6.
5. Alonso, P.L., et al., *Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial.* Lancet, 2004. **364**(9443): p. 1411-20.
6. Dahl, E.L., et al., *Tetracyclines specifically target the apicoplast of the malaria parasite Plasmodium falciparum.* Antimicrob Agents Chemother, 2006. **50**(9): p. 3124-31.
7. Fry, M. and M. Pudney, *Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80).* Biochem Pharmacol, 1992. **43**(7): p. 1545-53.
8. Sullivan, D.J., Jr., I.Y. Gluzman, and D.E. Goldberg, *Plasmodium hemozoin formation mediated by histidine-rich proteins.* Science, 1996. **271**(5246): p. 219-22.
9. Titus, E.O., *Recent developments in the understanding of the pharmacokinetics and mechanism of action of chloroquine.* Ther Drug Monit, 1989. **11**(4): p. 369-79.
10. AlKadi, H.O., *Antimalarial drug toxicity: a review.* Chemotherapy, 2007. **53**(6): p. 385-91.
11. Panisko, D.M. and J.S. Keystone, *Treatment of malaria--1990.* Drugs, 1990. **39**(2): p. 160-89.
12. Roper, C., et al., *Intercontinental spread of pyrimethamine-resistant malaria.* Science, 2004. **305**(5687): p. 1124.
13. Barnes, K.I., et al., *Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa.* PLoS Med, 2005. **2**(11): p. e330.
14. Bhattarai, A., et al., *Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar.* PLoS Med, 2007. **4**(11): p. e309.
15. Nyarango, P.M., et al., *A steep decline of malaria morbidity and mortality trends in Eritrea between 2000 and 2004: the effect of combination of control methods.* Malar J, 2006. **5**: p. 33.
16. Winstanley, P.A., *Chemotherapy for falciparum malaria: the armoury, the problems and the prospects.* Parasitol Today, 2000. **16**(4): p. 146-53.
17. Ridley, R.G., *Medical need, scientific opportunity and the drive for antimalarial drugs.* Nature, 2002. **415**(6872): p. 686-93.

18. Biagini, G.A., et al., *Antimalarial chemotherapy: young guns or back to the future?* Trends Parasitol, 2003. **19**(11): p. 479-87.
19. Doerig, C., et al., *Protein kinases as targets for antimalarial intervention: Kinomics, structure-based design, transmission-blockade, and targeting host cell enzymes.* Biochim Biophys Acta, 2005. **1754**(1-2): p. 132-50.
20. Hommes, D.W., M.P. Peppelenbosch, and S.J. van Deventer, *Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets.* Gut, 2003. **52**(1): p. 144-51.
21. Robinson, M.J. and M.H. Cobb, *Mitogen-activated protein kinase pathways.* Curr Opin Cell Biol, 1997. **9**(2): p. 180-6.
22. Cobb, M.H., *MAP kinase pathways.* Prog Biophys Mol Biol, 1999. **71**(3-4): p. 479-500.
23. Ishikawa, Y. and M. Kitamura, *Dual potential of extracellular signal-regulated kinase for the control of cell survival.* Biochem Biophys Res Commun, 1999. **264**(3): p. 696-701.
24. Dunn, P.P., J.M. Bumstead, and F.M. Tomley, *Sequence, expression and localization of calmodulin-domain protein kinases in Eimeria tenella and Eimeria maxima.* Parasitology, 1996. **113 (Pt 5)**: p. 439-48.
25. Cobb, M.H. and E.J. Goldsmith, *How MAP kinases are regulated.* J Biol Chem, 1995. **270**(25): p. 14843-6.
26. Goldsmith, E.J., M.H. Cobb, and C.I. Chang, *Structure of MAPKs.* Methods Mol Biol, 2004. **250**: p. 127-44.
27. Robbins, D.J., et al., *MAP kinases ERK1 and ERK2: pleiotropic enzymes in a ubiquitous signaling network.* Adv Cancer Res, 1994. **63**: p. 93-116.
28. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. **22**(2): p. 153-83.
29. Dorin, D., et al., *PfPK7, an atypical MEK-related protein kinase, reflects the absence of classical three-component MAPK pathways in the human malaria parasite Plasmodium falciparum.* Mol Microbiol, 2005. **55**(1): p. 184-96.
30. Park, E.R., S.T. Eblen, and A.D. Catling, *MEK1 activation by PAK: a novel mechanism.* Cell Signal, 2007. **19**(7): p. 1488-96.
31. Boulton, T.G., et al., *An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control.* Science, 1990. **249**(4964): p. 64-7.
32. Boulton, T.G., et al., *ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF.* Cell, 1991. **65**(4): p. 663-75.
33. Todd, J.L., K.G. Tanner, and J.M. Denu, *Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. A novel role in down-regulating the ERK pathway.* J Biol Chem, 1999. **274**(19): p. 13271-80.
34. Gupta, S., et al., *Transcription factor ATF2 regulation by the JNK signal transduction pathway.* Science, 1995. **267**(5196): p. 389-93.
35. Kohno, M. and J. Pouyssegur, *Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs.* Prog Cell Cycle Res, 2003. **5**: p. 219-24.
36. Dudley, D.T., et al., *A synthetic inhibitor of the mitogen-activated protein kinase cascade.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7686-9.

37. Wityak, J., et al., *Beyond U0126. Dianion chemistry leading to the rapid synthesis of a series of potent MEK inhibitors.* Bioorg Med Chem Lett, 2004. **14**(6): p. 1483-6.
38. Sebolt-Leopold, J.S., et al., *Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo.* Nat Med, 1999. **5**(7): p. 810-6.
39. Ohren, J.F., et al., *Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition.* Nat Struct Mol Biol, 2004. **11**(12): p. 1192-7.
40. Davies, S.P., et al., *Specificity and mechanism of action of some commonly used protein kinase inhibitors.* Biochem J, 2000. **351**(Pt 1): p. 95-105.
41. Bain, J., et al., *The selectivity of protein kinase inhibitors: a further update*. Biochem J, 2007. **408**(3): p. 297-315.
42. Atkins, C.M., et al., *The MAPK cascade is required for mammalian associative learning.* Nat Neurosci, 1998. **1**(7): p. 602-9.
43. Bakare, O., et al., *Synthesis and MEK1 inhibitory activities of imido-substituted 2-chloro-1,4-naphthoquinones.* Bioorg Med Chem, 2003. **11**(14): p. 3165-70.
44. Doerig, C.M., et al., *A MAP kinase homologue from the human malaria parasite, Plasmodium falciparum*. Gene, 1996. **177**(1-2): p. 1-6.
45. Dorin, D., et al., *An atypical mitogen-activated protein kinase (MAPK) homologue expressed in gametocytes of the human malaria parasite Plasmodium falciparum. Identification of a MAPK signature*. J Biol Chem, 1999. **274**(42): p. 29912-20.
46. Abe, M.K., et al., *Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth.* Mol Cell Biol, 1999. **19**(2): p. 1301-12.
47. Abe, M.K., et al., *ERK8, a new member of the mitogen-activated protein kinase family.* J Biol Chem, 2002. **277**(19): p. 16733-43.
48. Dorin-Semblat, D., et al., *Functional characterization of both MAP kinases of the human malaria parasite Plasmodium falciparum by reverse genetics.* Mol Microbiol, 2007. **65**(5): p. 1170-80.
49. Ward, P., et al., *Protein kinases of the human malaria parasite Plasmodium falciparum: the kinome of a divergent eukaryote*. BMC Genomics, 2004. **5**(1): p. 79.
50. Rangarajan, R., et al., *A mitogen-activated protein kinase regulates male gametogenesis and transmission of the malaria parasite Plasmodium berghei.* EMBO Rep, 2005. **6**(5): p. 464-9.
51. Khan, S.M., et al., *Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology.* Cell, 2005. **121**(5): p. 675-87.
52. Tewari, R., et al., *An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite.* Mol Microbiol, 2005. **58**(5): p. 1253-63.
53. Anamika, N. Srinivasan, and A. Krupa, *A genomic perspective of protein kinases in Plasmodium falciparum.* Proteins, 2005. **58**(1): p. 180-9.
54. Dorin-Semblat, D., et al., *Disruption of the pfPK7 gene impairs schizogony and sporogony in the human malaria parasite Plasmodium falciparum.* Eukaryot Cell, 2007.
55. Gustin, M.C., et al., *MAP kinase pathways in the yeast Saccharomyces cerevisiae*. Microbiol Mol Biol Rev, 1998. **62**(4): p. 1264-300.

56. Dorin, D., et al., *Pfnek-1, a NIMA-related kinase from the human malaria parasite Plasmodium falciparum Biochemical properties and possible involvement in MAPK regulation.* Eur J Biochem, 2001. **268**(9): p. 2600-8.
57. Cohen, P., *The role of protein phosphorylation in human health and disease. The Sir Hans Krebs Medal Lecture.* Eur J Biochem, 2001. **268**(19): p. 5001-10.
58. Yamamoto, K., et al., *[Neoadjuvant therapy with imatinib mesilate for gastrointestinal stromal tumor of the stomach before subsequent successful surgical resection].* Gan To Kagaku Ryoho, 2006. **33**(12): p. 1875-7.
59. Artigau Nieto, E., et al., *Gastrointestinal stromal tumors: experience in 49 patients.* Clin Transl Oncol, 2006. **8**(8): p. 594-8.
60. Whittaker, S.R., et al., *The cyclin-dependent kinase inhibitor seliciclib (R-roscovitine; CYC202) decreases the expression of mitotic control genes and prevents entry into mitosis.* Cell Cycle, 2007. **6**(24): p. 3114-31.
61. Dobbelaere, D.A. and P. Kuenzi, *The strategies of the Theileria parasite: a new twist in host-pathogen interactions.* Curr Opin Immunol, 2004. **16**(4): p. 524-30.
62. Hall, B.S., et al., *Modulation of protein kinase C activity in Plasmodium falciparum-infected erythrocytes.* Blood, 1997. **89**(5): p. 1770-8.
63. Kim, L., et al., *p38 MAPK autophosphorylation drives macrophage IL-12 production during intracellular infection.* J Immunol, 2005. **174**(7): p. 4178-84.
64. Molestina, R.E., et al., *Activation of NF-kappaB by Toxoplasma gondii correlates with increased expression of antiapoptotic genes and localization of phosphorylated IkappaB to the parasitophorous vacuole membrane.* J Cell Sci, 2003. **116**(Pt 21): p. 4359-71.
65. Chen, X.M., et al., *Phosphatidylinositol 3-kinase and frabin mediate Cryptosporidium parvum cellular invasion via activation of Cdc42.* J Biol Chem, 2004. **279**(30): p. 31671-8.
66. Chen, X.M., et al., *Cryptosporidium parvum invasion of biliary epithelia requires host cell tyrosine phosphorylation of cortactin via c-Src.* Gastroenterology, 2003. **125**(1): p. 216-28.
67. Ming, M., M.E. Ewen, and M.E. Pereira, *Trypanosome invasion of mammalian cells requires activation of the TGF beta signaling pathway.* Cell, 1995. **82**(2): p. 287-96.
68. Woolsey, A.M., et al., *Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation.* J Cell Sci, 2003. **116**(Pt 17): p. 3611-22.
69. Villalta, F., et al., *Signal transduction in human macrophages by gp83 ligand of Trypanosoma cruzi: trypomastigote gp83 ligand up-regulates trypanosome entry through protein kinase C activation.* Mol Cell Biol Res Commun, 1999. **2**(1): p. 64-70.
70. Villalta, F., et al., *Signal transduction in human macrophages by gp83 ligand of Trypanosoma cruzi: trypomastigote gp83 ligand up-regulates trypanosome entry through the MAP kinase pathway.* Biochem Biophys Res Commun, 1998. **249**(1): p. 247-52.
71. Singh, V.K., et al., *Leishmania donovani activates nuclear transcription factor-kappaB in macrophages through reactive oxygen intermediates.* Biochem Biophys Res Commun, 2004. **322**(3): p. 1086-95.

72. Olivier, M., D.J. Gregory, and G. Forget, *Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view.* Clin Microbiol Rev, 2005. **18**(2): p. 293-305.
73. Chaussepied, M., et al., *Upregulation of Jun and Fos family members and permanent JNK activity lead to constitutive AP-1 activation in Theileria-transformed leukocytes.* Mol Biochem Parasitol, 1998. **94**(2): p. 215-26.
74. Dessauge, F., et al., *c-Myc activation by Theileria parasites promotes survival of infected B-lymphocytes.* Oncogene, 2005. **24**(6): p. 1075-83.
75. Molestina, R.E. and A.P. Sinai, *Detection of a novel parasite kinase activity at the Toxoplasma gondii parasitophorous vacuole membrane capable of phosphorylating host IkappaBalpha.* Cell Microbiol, 2005. **7**(3): p. 351-62.
76. Gooding, L.R., *Virus proteins that counteract host immune defenses.* Cell, 1992. **71**(1): p. 5-7.
77. Bliska, J.B., J.E. Galan, and S. Falkow, *Signal transduction in the mammalian cell during bacterial attachment and entry.* Cell, 1993. **73**(5): p. 903-20.
78. van de Sand, C., et al., *The liver stage of Plasmodium berghei inhibits host cell apoptosis.* Mol Microbiol, 2005. **58**(3): p. 731-42.
79. Singh, A.P., et al., *Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite.* Cell, 2007. **131**(3): p. 492-504.
80. Leiriao, P., et al., *HGF/MET signalling protects Plasmodium-infected host cells from apoptosis.* Cell Microbiol, 2005. **7**(4): p. 603-9.
81. Barvitenko, N.N., N.C. Adragna, and R.E. Weber, *Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance.* Cell Physiol Biochem, 2005. **15**(1-4): p. 1-18.
82. Maccaglia, A., C. Mallozzi, and M. Minetti, *Differential effects of quercetin and resveratrol on Band 3 tyrosine phosphorylation signalling of red blood cells.* Biochem Biophys Res Commun, 2003. **305**(3): p. 541-7.
83. Manno, S., Y. Takakuwa, and N. Mohandas, *Modulation of erythrocyte membrane mechanical function by protein 4.1 phosphorylation.* J Biol Chem, 2005. **280**(9): p. 7581-7.
84. Del Carlo, B., M. Pellegrini, and M. Pellegrino, *Modulation of Ca2+-activated K+ channels of human erythrocytes by endogenous protein kinase C.* Biochim Biophys Acta, 2003. **1612**(1): p. 107-16.
85. Montoliu, C., et al., *Increased protein kinase A regulatory subunit content and cGMP binding in erythrocyte membranes in liver cirrhosis.* J Hepatol, 2004. **40**(5): p. 766-73.
86. Uhle, S., et al., *Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome.* Embo J, 2003. **22**(6): p. 1302-12.
87. Ward, G.E., et al., *Staurosporine inhibits invasion of erythrocytes by malarial merozoites.* Exp Parasitol, 1994. **79**(3): p. 480-7.
88. Rangachari, K., et al., *Control of malarial invasion by phosphorylation of the host cell membrane cytoskeleton.* Nature, 1986. **324**(6095): p. 364-5.
89. Chishti, A.H., et al., *Phosphorylation of protein 4.1 in Plasmodium falciparum-infected human red blood cells.* Blood, 1994. **83**(11): p. 3339-45.
90. Harrison, T., et al., *Erythrocyte G protein-coupled receptor signaling in malarial infection.* Science, 2003. **301**(5640): p. 1734-6.

91. Nunes, M.C., et al., *A novel protein kinase family in Plasmodium falciparum is differentially transcribed and secreted to various cellular compartments of the host cell.* Mol Microbiol, 2007.
92. Droucheau, E., et al., *Plasmodium falciparum glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors.* Biochim Biophys Acta, 2004. **1697**(1-2): p. 181-96.
93. Magowan, C., et al., *Plasmodium falciparum histidine-rich protein 1 associates with the band 3 binding domain of ankyrin in the infected red cell membrane.* Biochim Biophys Acta, 2000. **1502**(3): p. 461-70.
94. Kun, J.F., et al., *A putative Plasmodium falciparum exported serine/threonine protein kinase.* Mol Biochem Parasitol, 1997. **85**(1): p. 41-51.
95. Egee, S., et al., *A stretch-activated anion channel is up-regulated by the malaria parasite Plasmodium falciparum.* J Physiol, 2002. **542**(Pt 3): p. 795-801.
96. Syin, C., et al., *The H89 cAMP-dependent protein kinase inhibitor blocks Plasmodium falciparum development in infected erythrocytes.* Eur J Biochem, 2001. **268**(18): p. 4842-9.
97. Merckx, A., et al., *Plasmodium falciparum Regulatory Subunit of cAMP-Dependent PKA and Anion Channel Conductance.* PLoS Pathog, 2008. **4**(2): p. e19.
98. Sartori, M., G. Ceolotto, and A. Semplicini, *MAPKinase and regulation of the sodium-proton exchanger in human red blood cell.* Biochim Biophys Acta, 1999. **1421**(1): p. 140-8.
99. Lin, D.T., N.D. Goldman, and C. Syin, *Stage-specific expression of a Plasmodium falciparum protein related to the eukaryotic mitogen-activated protein kinases.* Mol Biochem Parasitol, 1996. **78**(1-2): p. 67-77.
100. Desjardins, R.E., et al., *Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique.* Antimicrob Agents Chemother, 1979. **16**(6): p. 710-8.
101. Tallarida, R.J., *Drug synergism: its detection and applications.* J Pharmacol Exp Ther, 2001. **298**(3): p. 865-72.
102. Gupta, S., et al., *In vitro interactions of artemisinin with atovaquone, quinine, and mefloquine against Plasmodium falciparum.* Antimicrob Agents Chemother, 2002. **46**(5): p. 1510-5.
103. Tonkin, C.J., et al., *Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method.* Mol Biochem Parasitol, 2004. **137**(1): p. 13-21.
104. Vrana, J.A. and S. Grant, *Synergistic induction of apoptosis in human leukemia cells (U937) exposed to bryostatin 1 and the proteasome inhibitor lactacystin involves dysregulation of the PKC/MAPK cascade.* Blood, 2001. **97**(7): p. 2105-14.
105. Delaney, A.M., et al., *Identification of a novel mitogen-activated protein kinase kinase activation domain recognized by the inhibitor PD 184352.* Mol Cell Biol, 2002. **22**(21): p. 7593-602.
106. Rivedal, E. and H. Opsahl, *Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells.* Carcinogenesis, 2001. **22**(9): p. 1543-50.

107. Salazar, E.P. and E. Rozengurt, *Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells.* J Biol Chem, 1999. **274**(40): p. 28371-8.
108. Hanke, J.H., et al., *Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation.* J Biol Chem, 1996. **271**(2): p. 695-701.
109. Ahn, N.G., et al., *Pharmacologic inhibitors of MKK1 and MKK2.* Methods Enzymol, 2001. **332**: p. 417-31.
110. Newton, R., et al., *The MAP kinase inhibitors, PD098059, UO126 and SB203580, inhibit IL-1beta-dependent PGE(2) release via mechanistically distinct processes.* Br J Pharmacol, 2000. **130**(6): p. 1353-61.
111. Slack-Davis, J.K., et al., *PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation.* J Cell Biol, 2003. **162**(2): p. 281-91.