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The cell biology of microtubule inhibition in *Plasmodium falciparum*

A thesis submitted for the degree of Doctor of Philosophy

by

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October 2007

DECLARATION

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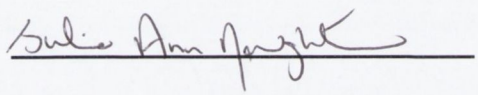

Julie Ann Naughton
October 2007

SUMMARY

Malaria is among the most prevalent human infections worldwide and both the lack of a viable vaccine and the spread of resistance to commonly used drugs have limited the options for control of the parasite, especially *Plasmodium falciparum*, the cause of the most severe form of the disease.

The cell division cycle of the blood-borne stages of *P. falciparum*, also known as schizogony, is crucial to malarial disease but is poorly characterised and has features that seem to contradict the accepted model of cell-cycle regulation in eukaryotes. Various techniques for (apparent) cell-cycle synchronisation have been used to shed light on the mechanisms involved in cell division and its control in other eukaryotes. There is no technique for cell-cycle synchronisation (as opposed to selection of parasites of a limited age-range) in *Plasmodium*. An attempt was made to do this by applying a number of inhibitors of cell cycle progression, namely inhibitors of DNA synthesis, the mitotic spindle, or cell-cycle control elements (such as cyclin-dependent kinases) to cultures of the parasite. Surprisingly, most of these compounds did not cause a block at a specific phase. Three compounds, Hoechst 33342, roscovitine and L-mimosine, did block development at the trophozoite–schizont transition (S or G2 phase). The block caused by the latter two inhibitors was reversible, suggesting that they might be used as synchronising agents. However, a consideration of the perturbing effects of inhibitors and problems with 'batch' synchronisation techniques in general lead us to believe that any results obtained using roscovitine- or L-mimosine-treated parasites may not be reflective of the normal cell cycle.

The second objective of this project was a more focused look at a single cell-cycle event in blood-stage parasites, mitosis, and the microtubules essential to this process. Microtubules are filamentous heteropolymers found in almost all eukaryotic cells. In malarial parasites, they play important roles in motility, structural integrity and cell division. Certain microtubule inhibitors such as taxol and vinblastine disrupt parasite development at very low concentrations, but most of these compounds are also toxic to mammalian cells. The dinitroaniline family of antimitotic herbicides are an exception, in that they disrupt the microtubule structures of plant cells but are relatively non-toxic to human cells. Certain members of this inhibitor class are also inhibitory to various protozoal parasites including *Plasmodium*. In this project it was demonstrated that the dinitroanilines trifluralin and oryzalin and the phosphorothioamidate herbicide

amiprophosmethyl, which has the same selectivity and herbicidal mechanism as dinitroanilines, inhibited progression of erythrocytic *P. falciparum* through schizogony. When compared with other classes of antimitotic agents, the herbicides also appeared to have a wider stage-specificity profile and parasites at all three main stages of development were to some extent irreversibly affected by short exposures. The limited potency of trifluralin, the best studied member of the dinitroaniline herbicides, was also shown to be in some part due to retention in the membranes of the parasite and its host erythrocyte, apparently limiting its access to its microtubular target. Parasitised erythrocytes accumulated trifluralin to a concentration ~300 times that of the extracellular medium whereas for vinblastine the internal concentration was ~50-100 times that of the external medium. Accumulation of trifluralin was however not saturable and did not specifically co-localise with its microtubule target. This was in contrast to the more potent vinblastine, for which accumulation was saturable and appeared to co-localise with β-tubulin. While neither agent appeared to be actively effluxed from the cells ~65% of vinblastine accumulated was retained following repeated washes compared with ~15% retention of trifluralin.

Finally, the potential of *Plasmodium* asexual stages to undergo a form of programmed cell death was suggested by the ability of taxol and vinblastine (but not trifluralin) to induce caspase-like activity in the parasite. Caspase activity is one of the first markers for programmed cell death and this finding suggests a novel pathway to exploit in the constant search for novel, effective and selective antimalarial agents.

For Mam and Dad

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PUBLICATIONS ORIGINATING FROM THIS STUDY

Fennell, B.J., Naughton, J.A., Dempsey, E. and Bell, A. 2006. Cellular and molecular actions of dinitroaniline and phosphorothioamidate herbicides on *Plasmodium falciparum*: tubulin as a specific antimalarial target. *Mol Biochem Parasitol*. **145:** 226-38.

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Abbreviations

A	.....absorbance
APAD	.....3-acetyl pyridine adenine dinucleotide
APM	.....amiprophosmethyl
APS	.....ammonium persulfate
Bp	.....base pair(s)
BSA	.....bovine serum albumin
CAR	.....cellular accumulation ratio
CDK	.....cyclin-dependent kinase
cDNA	.....complementary deoxyribonucleic acid
CHO	.....Chinese hamster ovary
CKI	.....cyclin-dependent kinase inhibitor
DDT	.....dichlorodiphenyltrichloroethane
DEVD	.....aspartic acid - glutamic acid - valine - .....aspartic acid
dH_2O	.....deionised water
DMSO	.....dimethylsulphoxide
Dpm	.....disintegrations per minute
DTNB	.....5, 5'-dithio-bis(2-nitrobenzoic acid)
DTT	.....dithiothreitol
DV	.....digestive vacuole
E-site	.....GTP-binding site on β-tubulin
EDTA	.....ethylenediaminetetraacetic acid
EGTA	.....etheleneglycolbis(aminoethyl)-Tetra acetic acid
EIF-5a	.....eukaryotic initiation factor-5a
G1	.....Gap phase 1
G2	.....Gap phase 2
HEPES	.....4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
IC_{50}	.....median inhibitory concentration
Kbp.....	.....kilobase pair

kDa	.....kilo Daltons
LDH	.....lactate dehydrogenase
M phase	.....mitosis (and cytokinesis) phase
MBP	.....maltose binding protein
MCS	.....multiple cloning site
Min	.....minute(s)
MMV	.....Medines for Malaria Venture
MSP	.....merozoite surface protein
MTOC	.....microtubule organising centre
N-site	.....GTP-binding site on α-tubulin
NBT	.....nitroblue tetrazolium
PAGE	.....polyacrylamide gel electrophoresis
PBS	.....phosphate buffered saline
PCD.....	.....programmed cell death
PCR	.....polymerase chain reaction
PES	.....phenazine ethosulphate
PfEMP1	.....*Plasmodium falciparum* erythrocyte membrane protein 1
Pfcrk.....	.....*Plasmodium falciparum* CDK-related kinase
Pfmrk	.....*Plasmodium falciparum* MO15-related kinase
PfPK5	.....*Plasmodium falciparum* protein kinase 5
PIGPA	.....50 mM sodium pyruvate, 50 mM inosine,100 mM glucose, 500 mM disodium hydrogen phosphate, 5 mM adenine, 0.72% (w/v) sodium chloride
pLDH	.....parasite lactate dehydrogenase
PMSF	.....phenylmethylsulphonyl fluoride
Ac-DEVD- *p*NA	.....Acetyl-DEVD labelled *p*-nitroaniline
PV	.....parasitophorous vacuole
PVDF	.....polyvinylidene difluoride
R	.....restriction point

RIMA	.....ring membrane antigen
RESA	.....ring-infected erythrocyte surface antigen
rpm	.....revolutions per minute
RT-PCR	.....reverse transcriptase-polymerase chain reaction
S phase	.....DNA synthesis phase
SDS	.....sodium dodecyl sulphate
SDS-PAGE	.....sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	.....standard error of the mean
SSC	.....saline sodium citrate
TAE	.....Tris-acetate-EDTA
TCA	.....trichloroacetic acid
TE	.....Tris-EDTA
TEMED	.....N, N, N', N'-tetramethyl-ethylenediamine
Tris	.....tris(hydroxymethyl)aminomethane
TVM	.....tubovesicular membrane
UV	.....ultraviolet
WHO	.....World Health Organisation
Z-VAD-fmk	.....benzyloxycarbonyl-valine-alanine-aspartic acid (OMe) fluoromethylketone

Chapter 1

General Introduction

1.1. MALARIA

1.1.1. Malaria: an introduction

Malaria is among the most prevalent human infections worldwide and is thought to kill more people than any other communicable disease apart from tuberculosis and HIV infection (Snow *et al.*, 2005).

Once endemic in much of the temperate world as well as the tropics and sub-tropics, the discovery of the insecticide dichlorodiphenyltrichloroethane (DDT) and the subsequent World Health Organisation (WHO) malaria eradication programme of the 1950's successfully eradicated malaria from Europe, most of North America and Russia by significantly reducing numbers of the mosquito vector. The emergence of DDT resistant mosquitoes and data on the public health risk of DDT spraying led to a decrease in its use (Roberts *et al.*, 1997). Endemic malaria is now confined to regions that experience more tropical and subtropical climates such as parts of Asia and the Pacific, Central and South America and Africa (Breman *et al.*, 2004) (Fig 1.1). In such endemic regions, malaria is a significant cause of morbidity and mortality and imposes enormous social and economic burdens. Of the 2.5 billion people at risk, more than 500 million become severely ill with malaria every year and more than 1 million die from its effects (WHO, 2007). Sub-Saharan Africa, where an estimated 90% of global malaria occurs, bears the greatest burden of the disease. In Africa a child has on average between 1.6 and 5.4 episodes of malaria fever each year, and an estimated one in every five (20%) childhood deaths is attributed to the effects of the disease (WHO, 2007). In recent years, the burden of this disease and death has increased significantly in malaria-endemic countries, and transmission has spread to new areas, in particular to central Asia (Moorthy *et al.*, 2004). The major causes of this resurgence are global climate and environmental change, the development of resistance to affordable antimalarial drugs and insecticides, war and civil disturbance, increasing human migration, and deterioration of health services and national control programmes. With 41% of the world's population now at risk of malaria and with worldwide cases expected to exceed 2 billion by 2010 (Breman *et al.*, 2004), efforts towards controlling this debilitating disease are urgently required.

Malaria is caused by protozoa of the genus *Plasmodium*, which contains nearly 120 different species, five of which are known to cause malaria in humans or other primates, namely *P. falciparum, P. vivax, P. malariae, P. knowlesi and P. ovale* (Bruce-

Chwatt, 1985; Singh *et al.*, 2004; Cox-Singh *et al.*, 2008). The developmental cycle of these five species occurs in two host organisms: humans or other primates and mosquitoes. There are approximately 60 different species of mosquito belonging to the genus *Anopheles* that can transmit the parasites to humans (News, 2002). *Anopheles gambiae*, native to Africa, is one of the most efficient malaria vectors in the world. Although mosquitoes are found in most countries, *Anopheles* predominates mostly in the tropics at low elevations, thereby largely restricting transmission of malaria to these regions.

1.1.2. Developmental cycle of *P. falciparum*

The developmental cycle of all species of human malaria is essentially the same (Fig. 1.2). *P. falciparum* is the most significant of the four *Plasmodium* species that infect man, causing most malaria-related deaths, and is the subject of this thesis. The developmental cycle of *P. falciparum* is one of the most complex of any human infection and involves numerous cellular differentiations and nuclear divisions.

The cycle commences when an infected female anopheline mosquito bites a human host inadvertently injecting 5 - 20 sporozoites into the tissue or bloodstream of the individual (Rosenberg *et al.*, 1990). Within ~ 30 minutes the sporozoites migrate through the bloodstream to the liver, where they invade hepatocytes and develop to become schizonts, a process known as hepatic or exoerythrocytic schizogony. Each sporozoite divides asexually to produce 20,000 - 30,000 merozoites which are released into the bloodstream upon rupture of the hepatocytes.

The intra-erythrocytic stages of the *P. falciparum* life cycle begin with the invasion of host erythrocytes by the extracellular merozoite form of the parasite. Invasion is dependent on the merozoites' ability to adhere to fresh erythrocytes and subsequently enter and seal themselves inside. The merozoite is very small (~ 1.6 μm long and ~1.0 μm wide) (Bannister *et al.*, 2000), but it contains all the tools required to escape from the remnants of its host cell and to establish itself in a fresh erythrocyte. Adhesion of the merozoite is thought to be mediated by a number of surface proteins (MSPs) (Cowman and Crabb, 2002) which allow the merozoite to adhere to the erythrocyte surface via its bristly coat. A tight junction is then formed when the merozoite aligns its apical end with the erythrocyte surface (Bannister *et al.*, 2003). Following the formation of this junction, discharges from three sets of secretory vesicles (rhoptries, micronemes and dense granules) trigger the formation of the parasitophorous

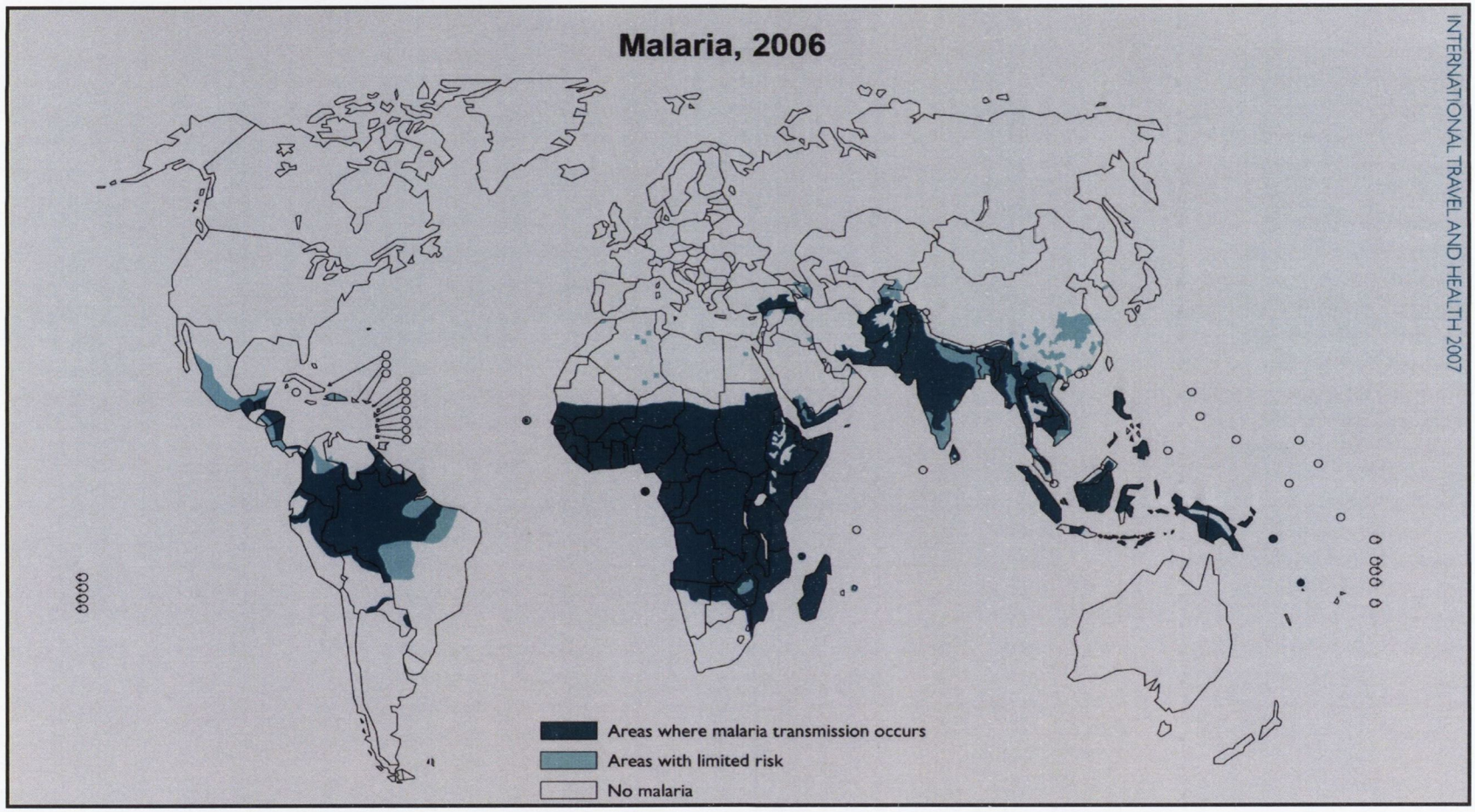


Figure 1.1. Worldwide incidence of malaria in 2006 (obtained from WHO travel guidelines at http://www.who.int/malaria/malariaendemiccountries.html).

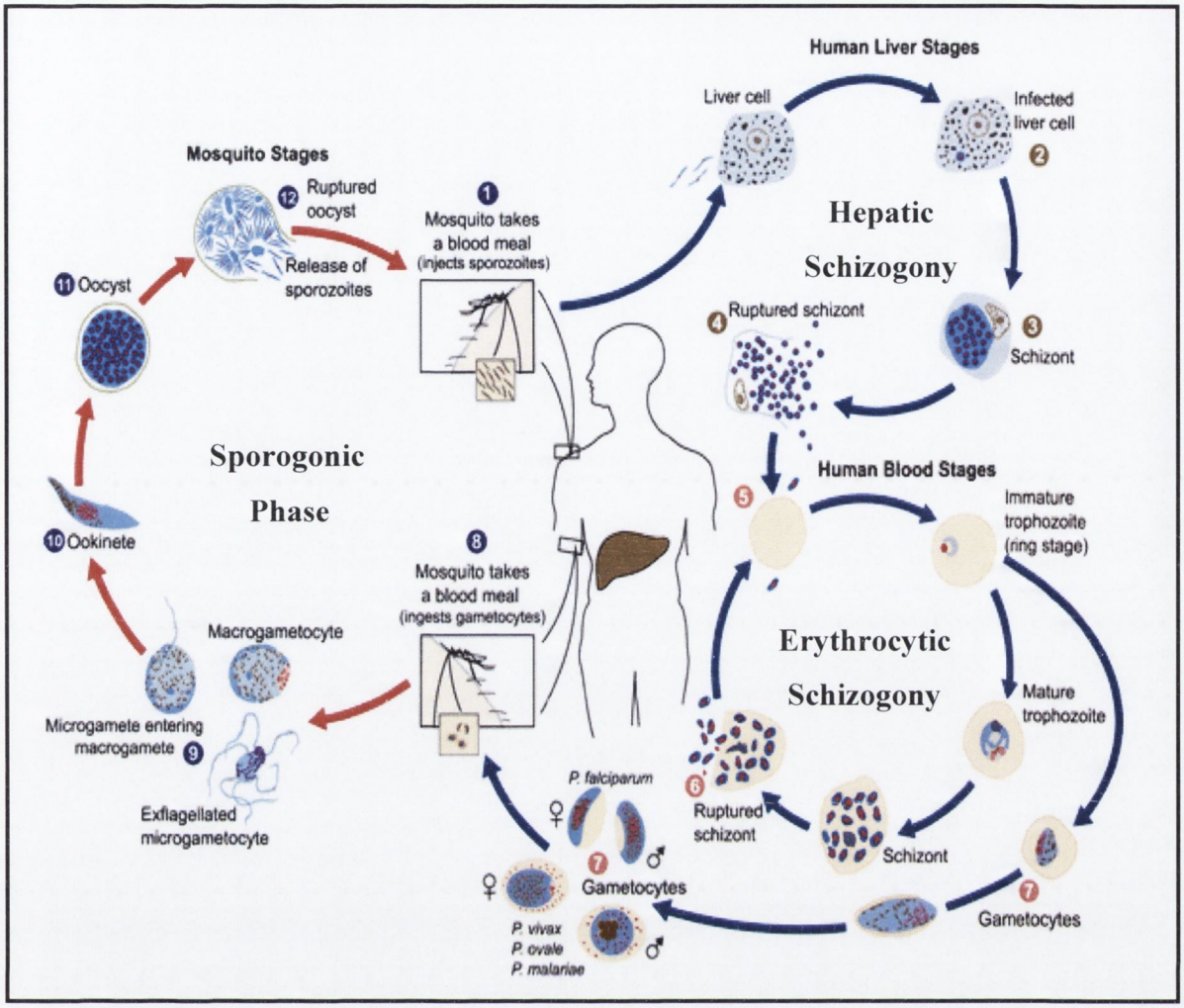


Figure 1.2. Developmental cycle of *Plasmodium falciparum*. Obtained from http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm. See text for explanation.

vacuole (PV) (Bannister *et al.*, 2000). An actin-myosin motor then aids the merozoite to glide into the cavity (Pinder *et al.*, 2000) and the bristly coat is removed. Once enclosed in the PV, the merozoite secretes a number of molecules including ring membrane antigen (RIMA) (Trager *et al.*, 1992) and ring-infected erythrocyte surface antigen (RESA) (Culvenor *et al.*, 1991) which are associated with the enlargement of the PV. Merozoites can then develop along one of two pathways.

The asexual intra-erythrocytic cycle of *P. falciparum* occurs over a 44-48-hour time period and is the phase of the parasite's development cycle responsible for the clinical manifestations of malaria in humans. The invasion related structures of the merozoite are destroyed and the merozoite matures into a disc-like structure called the ring stage.

Active feeding begins at this stage, which covers the first 14-16 hours of intra-erythrocytic growth, with portions of the host cell cytoplasm being absorbed through a dense ring on the parasite surface called the cytosome; haemoglobin degradation in the digestive vacuole (DV) is also initiated at this stage. As the ring grows, there is a corresponding increase in the area of the surrounding parasitophorous vacuole and tubovesicular membranes (TVM), narrow finger-like projections, are extended into the periphery of the erythrocyte (Haldar *et al.*, 2001). The functions of this organelle are as yet unknown, but it is believed to help uptake of nutrients from the host cell. A number of ring-specific molecules are synthesised and exported into the erythrocyte to facilitate modification of the erythrocyte membrane (Das *et al.*, 1994). Some of these modifications accelerate uptake and efflux of nutrients and waste products and/or allow the infected erythrocytes to adhere to other parasitized erythrocytes (auto-agglutination) or to uninfected erythrocytes (rosetting).

The ring-stage parasites grow and develop into the more metabolically-active trophozoite stage, accounting for approximately the next 20 hours of intra-erythrocytic development. Unlike ring-infected erythrocytes, those infected with trophozoite-stage parasites adhere to the endothelial cells of blood vessels allowing them to escape detection and removal in the spleen (Miller *et al.*, 2002). Binding to the endothelial cell is mediated by a number of molecules secreted by the parasite at this stage, such as erythrocyte membrane protein 1 (PfEMP1) (Baruch *et al.*, 1996). Additional exported molecules induce membrane and cytoskeletal components of the erythrocyte to rearrange into small knobs that increase the selective adhesiveness of the parasitised host cell membrane. Antigenic variation of PfEMP1 together with the variety of

proteins used for erythrocyte adherence allows the parasite to outwit the vast array of protective components of the host immune system.

The trophozoite continues haemoglobin proteolysis and increases in size until the parasite initiates DNA synthesis and begins erythrocytic schizogony. This involves a series of nuclear divisions to produce anywhere between 8 and 32 nuclei (Leete and Rubin, 1996) per schizont accompanied by numerous cytosolic changes in preparation for merozoite formation, such as division and segregation of organelles. New merozoites are formed following the partitioning of the nuclei, cytoplasm and organelles and the parasite is now termed a segmenter. Finally, the merozoites are released following lysis of the erythrocytic and the PV membranes.

The alternative developmental route taken by some merozoites leads to the initiation of the sexual pathway by differentiating into macro- (female) or micro- (male) gametocytes. Ingestion of gametocyte containing erythrocytes by another anopheline mosquito results in the re-initiation of the insect stage of parasite development beginning in the midgut of the insect with the release of the gametocytes from the erythrocyte.

The male gametocyte undergoes mitosis to produce eight flagellated microgametes each of which can fertilise a female macrogamete resulting in the formation of a zygote. This in turn differentiates into a motile ookinete that penetrates the mosquito gut wall and develops into an oocyst, which in turn matures and produces thousands of sporozoites by asexual multiplication (Sherman, 1979). Sporozoites are released into the mosquito hemolymph upon rupture of the oocyst, from where they migrate to the salivary glands of the mosquito. When the mosquito feeds on its next bloodmeal, the sporozoites are injected into the human host and so begins a new cycle of infection (Bruce-Chwatt, 1985; Wang *et al.*, 2005).

1.1.3. Malaria: prophylaxis and treatment

The growing global problem of malaria is largely due to the emergence of parasite resistance to our limited armoury of antimalarial drugs (Marsh, 1998; White, 2004) combined with the spread and increasing populations of the mosquito vector. Currently the most extensively used antimalarials are quinolines, antifolate drugs, artemisinins, atoquavone and antibiotics such as tetracyclines. The effectiveness of each of these agents is limited, either by emerging resistance, prohibitive cost or adverse side-effects. Fortunately, recent advances in our understanding of the mechanisms of

action of and resistance to traditional drugs, the emergence of the artemisinins as a novel antimalarial class and the recently completed genome projects should aid a rapid increase in the number of available (and affordable) antimalarials. The establishment of organizations such as the Medicines for Malaria Venture (MMV, http://www.mmv.org/) and the Bill and Melinda Gates Foundation (http://www.gatesfoundation.org/) have been instrumental in utilising information from the biological advances in the development of novel agents to treat malaria.

Chloroquine and other quinoline antimalarials such as amodiaquine and quinine have been the standards of malaria chemotherapy for much of the past 40 years. The limited host toxicity, excellent clinical efficacy, ease of use and simple, cost-effective synthesis has resulted in the successful and widespread application of these drugs as antimalarials. The development and spread of parasite resistance has significantly reduced their impact, although use in combination with agents that reverse parasite resistance may prolong their use, and continuing efforts to synthesis new and effective derivatives continues (Biagini *et al.*, 2003).

Artemisinins are among the newer agents in the fight against malaria. They have broad spectrum activity against all erythrocytic stages of the parasite (Skinner *et al.*, 1996) in addition to reducing gametocyte transmission (Chen *et al.*, 1994). Their proposed mode of action was thought to be via free radicals released upon activation of their endoperoxide moiety by ferrous haem or exogenous free iron.

As it has been noted that these compounds localise in membranes outside the DV where haem is present and that they have high toxicity to young ring forms which contain little or no haemozoin, the mechanism of action remains controversial (reviewed in Cunha-Rodrigues *et al.*, 2006). Derivatives of the original plant-derived artemisinin such as artemether, arteether and artesunate are widely used as part of combination therapies. Issues with potential neurotoxicity have led to the increased research into derivatives of artemisinin such as the water soluble artelinate and into synthetic endoperoxides, which have longer half-lives and reduced neurotoxicity (Haynes, 2001; Robert *et al.*, 2002; Bachi *et al.*, 2003; Biagini *et al.*, 2003).

A novel target receiving increased attention is the apicoplast (plastid) of *P. falciparum*, which contains a 35-kilobase circular genome that encodes elements of a prokaryotic transcription and translation system believed to be the target of certain antibiotics. Although agents such as the tetracyclines that are thought to target this system inhibit *P. falciparum*, their actions are slow. Recent advances including the

completion of the *P. falciparum* genome sequence (Gardner *et al.*, 2002) have facilitated the discovery of several key metabolic pathways in the plastid, the disruption of which results in a more rapid cell death (Ralph *et al.*, 2004). The non-mevalonate pathway that leads to synthesis of isoprenoids (chemical precursors used for the formation of numerous cellular molecules) is selectively inhibited by the actions of fosmidomycin (Jomaa *et al.*, 1999). The Type II fatty acid biosynthesis pathway, similar to that found in plants and bacteria, can be disrupted using thiolactomycin resulting in parasite growth inhibition (Ridley, 2002). As humans generate fatty acids by the Type I biosynthetic pathway this inhibition is selective. In view of the selective antimalarial activity of such agents, it would appear that the prokaryotic-like biosynthetic pathways present in the apicoplast present exciting targets for the development of novel antimalarials.

The microtubular systems of malaria parasite may also hold promise as a novel antimalarial target, due to the vital role microtubules play in cell division, motility and structural integrity of malarial parasites (Usanga *et al.*, 1986; Bell, 1998; Kappes and Rohrbach, 2007). This will be discussed further in subsequent sections.

The availability of the complete genome sequence of *P. falciparum* coupled with transcriptomic, proteomic and comparative genomic technologies should facilitate the identification of many more novel chemotherapeutic targets. For example, transcriptomic technologies have already been used to determine how each of the more than 5,400 parasite genes are switched on and off in the intra-erythrocytic stage of the parasite development cycle and to assign functions to the proteins involved (Le Roch *et al.*, 2003). With such tools to hand to enhance our understanding of the cellular and molecular biology of the malarial parasites, the future for identification, characterisation and validation of novel antimalarial drug targets appears a lot brighter.

1.2. THE CELL CYCLE

1.2.1. The eukaryotic cell cycle

1.2.1.1. General introduction

The concept of an organised cell cycle was first put forward in 1951 by Howard and Pelc (Howard and Pelc, 1951). Using autoradiography and ^{32}P labelling of *Vicia faba* seedlings, they determined that DNA synthesis occurred during a distinct period during interphase and that two intervals or gaps existed, a long one between mitosis and

DNA synthesis (G_1) and a shorter one between DNA synthesis and mitosis (G_2). Four phases of the eukaryotic cell-cycle were thus defined, G_1 phase, characterised by cell growth and protein synthesis, S phase, characterised by DNA synthesis, G_2 phase, where the cell ensures DNA synthesis has successfully completed prior to mitosis, and finally mitosis or M phase (Fig. 1.3) (Howard and Pelc, 1951). The concept of growth fraction was introduced by Mendelsohn (Mendelsohn, 1962), who stated that as an alternative to being in one of the cell-cycle stages already described, cells can also be either terminally differentiated or they can exit G_1 to enter a quiescent stage of the cycle called G_0, where they remain metabolically active but no longer proliferate unless called on to do so by appropriate extracellular signals. Human nerve cells are an example of terminally differentiated cells, while G_0 stage cells include skin fibroblasts, as well as the cells of many internal organs, such as the liver, kidney, and lung.

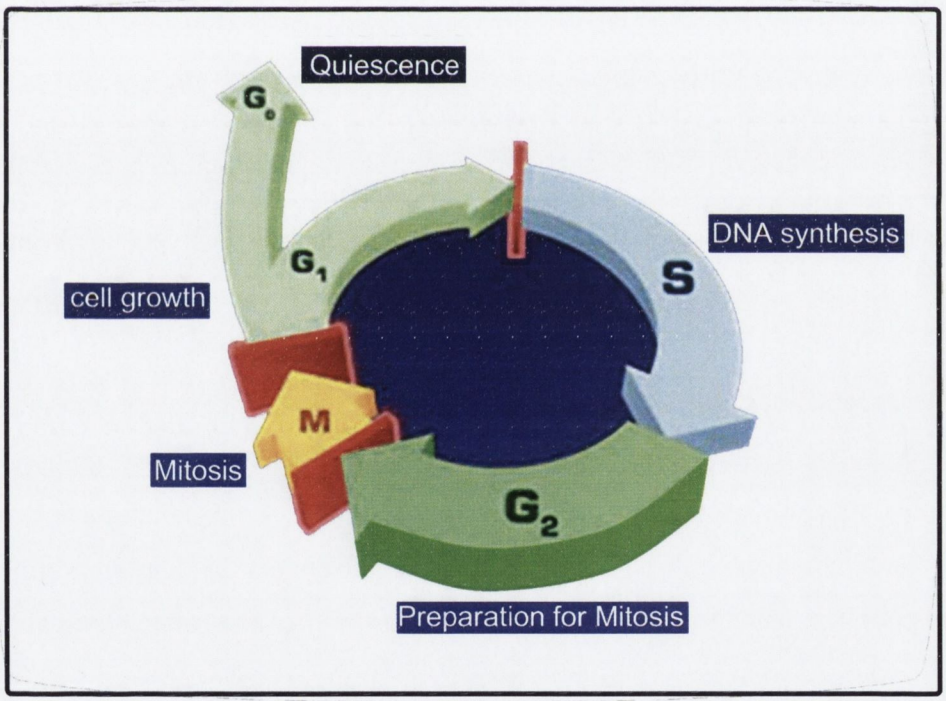


Figure 1.3. General model of the eukaryotic cell cycle (edited from http://www.learner.org). Red bars represent known cell-cycle checkpoints.

Decades of investigations since the cell cycle was originally described have shown variations in the standard cell cycle model. The most common variations occur in the duration of each cell cycle phase and in some cases the lack of one or both of the gap phases. A typical rapidly proliferating human cell has a total cycle time of approximately 24 hours. In these cells the G_1 phase might last about 12 hours, S phase

about 6 to 8 hours, G_2 about 3 to 6 hours, and M about 30 minutes (Shackelford *et al.*, 1999). Budding yeasts (*Saccharomyces cerevisiae*) have a much shorter cell-cycle, in some cases lasting only about 90 minutes, with each of the four cycle stages present but of far shorter duration than those of the typical rapidly proliferating human cell. Early embryonic cells have an even shorter cell cycle (30 minutes or less). In this case, there is no G_1 or G_2 phase and cell growth does not take place. Instead, the cell cycle consists of very short S phases alternating with M phases (reviewed in Puri, 1999; Golias *et al.*, 2004).

1.2.1.2. Control of the cell cycle

The cell cycle is a tightly controlled process with multiple regulatory mechanisms permitting or restraining its progress (Gali-Muhtasib and Bakkar, 2002). The development of checkpoints at stages along the cell-cycle helps to regulate its progression. Their presence ensures the successful completion of critical events in the cycle such as the faithful replication of the DNA complement and segregation of the chromosomes. Failure of this checkpoint regulation has been associated with events such as neoplastic transformation (Malumbres and Barbacid, 2001). The cell cycle is sequential and unidirectional, and regulated by specific molecular events. Studies carried out on S phase cells fused to G_1 or G_2 phase cells showed that DNA synthesis began prematurely in the G_1 stage cells while the G_2 stage cells did not re-replicate their DNA (Rao and Johnson, 1970).

Control of the eukaryotic cell cycle is a highly complex process involving multiple processes and molecules, but two key classes of proteins have proven to be instrumental in that control. Since their initial discovery in yeasts, cyclin-dependent kinases (CDKs) have proven to be universal regulators of the cell cycle in all eukaryotes studied to date. These are highly conserved protein serine/threonine kinases, only displaying activity when complexed with cyclins, the second vital component in regulation, to form catalytic heterodimers (Nigg, 1995; Spellman *et al.*, 1998). When activated by bound cyclin, CDKs activate or inactivate target proteins by phosphorylation to orchestrate coordinated entry into the next phase of the cell cycle (Sanchez and Dynlacht, 2005). The downstream proteins targeted are determined by the specific cyclin-CDK combinations and while CDKs are constitutively expressed it is the cyclins that are synthesised at specific stages of the cell cycle in response to various molecular signals. The genes encoding both cyclins and CDKs are quite well conserved

across eukaryotes but in general more complex organisms have more elaborate cell cycle control systems that incorporate more individual components.

Despite differences in complexity in different organisms, eukaryotic cell-cycle control tends to follow a general model (Fig. 1.4.).

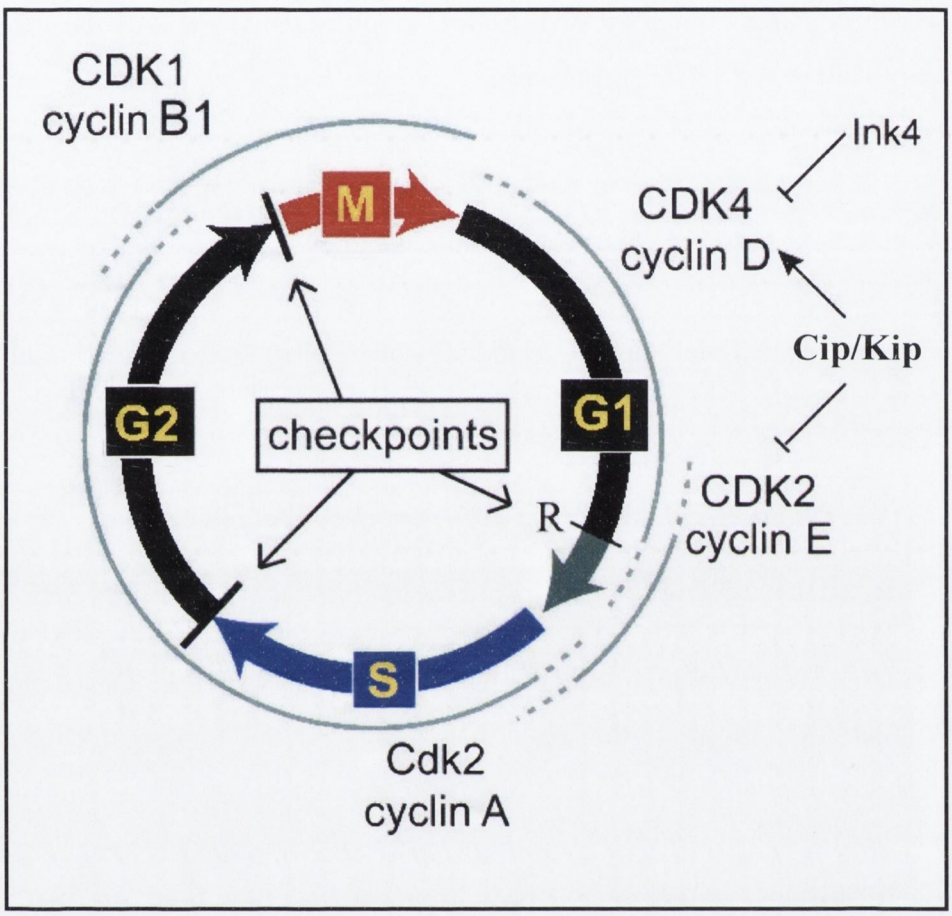


Fig. 1.4. Highly schematic diagram showing aspects of cell cycle regulation in human cells. Checkpoints exist at several points across the cell-cycle, including the restriction point (R) and those at the end of S phase and G2 phase. CDKs are constitutively expressed while cyclins are cell-cycle stage specific. CDK/cyclin complexes phosphorylate target proteins resulting in activation of processes involved in the subsequent cell-cycle stage. Cyclin-dependent kinase inhibitors (CKIs) such as Cip and Kip proteins inactivate the CDK/cyclin complex preventing their activity thus halting cell-cycle progression.

In response to extracellular signals (e.g. growth factors), G_1 cyclin-CDK complexes (e.g. cyclin D/CDK4 in humans) form and promote transcription factor expression which in turn promote the expression of proteins of the DNA replication machinery and S phase cyclins (e.g. cyclins A and E in humans). G_1 cyclin-CDK

complexes also target S phase inhibitors for ubiquitin dependent proteolytic degradation. Newly activated S phase cyclin-CDK complexes (e.g. cyclin A or E and CDK2) phosphorylate pre-assembled pre-replication complexes present on DNA replication origins thus activating them and preventing the formation of new ones. This helps ensure that the entire genome is replicated only once. Mitosis-associated cyclin-CDK complex (CDK1 together with cyclin B1 in humans) governs the G2/M transition by stimulating proteins involved in mitotic spindle assembly and chromosome condensation.

In addition to the CDKs and cyclins are a family of CDK inhibitors (CKI) which bind to and inactivate cyclin-CDK heterodimers. There are numerous CKIs including the Cip/Kip family which halt the cell-cycle in G_1 phase in response to several different factors including DNA damage or the presence of growth inhibitors (reviewed in Nigg, 1995; Spellman *et al.*, 1998; Sanchez and Dynlacht, 2005).

1.2.1.3. Endoreduplication and asynchronous nuclear division

Endoreduplication, or endoreplication, is the process whereby a single cell undergoes multiple rounds of DNA synthesis without cell division (cytokinesis) resulting in polyploid (or endopolyploid) cells. Nuclei in these cells may undergo mitosis resulting in multinucleate cells (e.g. syncytial slime moulds such as *Physarum*, early insect embryos and some mammalian hepatocytes). Other cell types can undergo multiple rounds of DNA synthesis without any chromosome separation (termed polyteny): the best known example of this is found in cells of the *Drosophila* salivary gland, where chromosomes may have up to 2048 copies of the haploid genome (Edgar and Orr-Weaver, 2001). Control of endoreduplication appears to be a simplified version of the normal regulatory processes involved in the standard model of eukaryotic cell-cycle progression (see section 1.2.2). Components that are not required may not be expressed, for instance certain cells that bypass mitosis no longer express the mitotic initiating component Cdk1 or its activators cyclin A, cyclin B and Cdc25C. Structural components such as centrosomes may also be lost (Mahowald *et al.*, 1979; Edgar and Orr-Weaver, 2001).

Synchronous or parasynchronous DNA synthesis (and mitosis if it occurs) is a common feature of nearly all these cell types (Nygaard *et al.*, 1960; Clutterbuck, 1970; Hoepfner *et al.*, 2002). However, there have been reports of asynchronous nuclear division in a few cell types, mainly as a result to DNA damage to a proportion of the

nuclei present (Hinchcliffe *et al.*, 1999; Demeter *et al.*, 2000) or to unattached chromosomes (Rieder *et al.*, 1997). Asynchronous nuclear division independent of damage appears rare, although it has been suggested it occurs in the multinucleated filamentous fungus *Ashbya gossypii* (Gladfelter *et al.*, 2006). How this is regulated and why it occurs is still not understood.

1.2.2. The cell cycle and *Plasmodium* schizogony

1.2.2.1. General introduction

How schizogony of *P. falciparum* correlates with the standard model of eukaryotic cell-cycle progression and control is unclear. Associating known cell-cycle phases with stages in the parasite's intraerythrocytic development has proven difficult.

In the "ring" and early trophozoite stages of the parasite life cycle (fig. 1.2), the cells are highly active in protein and RNA synthesis, haemoglobin metabolism and host cell membrane restructuring. It has been suggested that this corresponds to G_1 phase (fig. 1.3). The merozoite (fig. 1.2) is thought to be metabolically inactive so may correspond to a G_0-like-phase. The first measurable cell cycle event in cultured *P. falciparum* is the initiation of DNA replication. This has been measured by incorporation of radiolabelled nucleotide precursors into alkali-insoluble macromolecules. Owing to the range of ages found in cultured parasites and the difficulty in reducing that age range without drastically reducing the parasitaemia (and therefore the rate of incorporation), it is difficult to determine the time after invasion of the start of this first S-phase: values around 30 hours have been reported (Inselburg and Banyal, 1984). After this time, DNA synthesis in the parasite population appears to be more or less continuous until around 38-40 hours post-invasion: this presumably represents a mixture of S-phase and non-S-phase parasites, as the nuclear bodies contain no more than diploid DNA amounts, at least in *P. berghei* (Janse *et al.*, 1986). The onset of the first M-phase is marked by the first nuclear division, i.e. the beginning of schizogony, which occurs around 36 h post-invasion (Leete and Rubin, 1996). Four or five divisions, presumably interspersed by periods of further DNA replication, are completed in a common cytosol before the nuclei and other organelles are partitioned into nascent merozoites. The latter events (known as segmentation) occur rapidly in the last few hours of the cycle and their exact timings are not known. Whether there is a short G_2 phase between successive genome replications is unknown (Jacobberger *et al.*, 1992; Leete and Rubin, 1996; Arnot and Gull, 1998; Doerig *et al.*, 2000).

However, schizogony does differ from the cell cycles of some of the better-characterised eukaryotes. Some atypical features include the absence of chromosome condensation and the maintenance of the nuclear membrane during mitosis (Heath and Rethoret, 1980; Sinden, 1991). Perhaps the most significant difference is that instead of a logarithmic division of nuclei, as would be expected for multiple nuclei in a single cytoplasm, i.e. 1n –2n –4n –8n –16n etc., parasite nuclear division appears to occur in an asynchronous manner, with any number between 1 and 32 nuclei counted in a single parasite (Leete and Rubin, 1996). There are two possible explanations for this: the first is that nuclei take the same length of time to divide, but some are prevented from dividing due to, for example, DNA damage. The second is that nuclear division is truly asynchronous, with different division times for different nuclei (Fig. 1.5).

Immunofluorescence studies using anti-tubulin antibodies have shown the presence in one cell of microtubular structures normally found only at different cell-cycle stages and would imply that the mechanism for controlling the cell division cycle in other eukaryotic cells does not apply to this parasite, or at least differs in some significant way.

As the cell-cycle is essential for parasite growth it is obvious that inhibiting this multiplication would prevent the disease, and molecules forming part of the cell-cycle apparatus or contributing to its regulation are proposed to be promising drug targets (Hammarton *et al.*, 2003). The possibility of novel targets is further increased by the evidence that cell-cycle regulation in these cells is atypical for a eukaryote.

1.2.2.2. *Plasmodium* cell cycle control

The presence of checkpoints in *Plasmodium* is unconfirmed. Indeed, studies with antimicrotubule agents such as taxol have shown that at least one checkpoint present in eukaryotic cells, ensuring that DNA synthesis does not recommence until after successful completion of mitosis, is absent in this parasite. In addition to this, studies on schizont-stage parasites irradiated with γ-radiation, which resulted in DNA damage, did not prevent subsequent DNA synthesis (reviewed in Doerig *et al.*, 2000). Both microtubule and DNA damage in higher eukaryotes results in a halt of cell-cycle progression at the relevant checkpoint, either to allow the damage to be repaired or to initiate programmed cell death pathways.

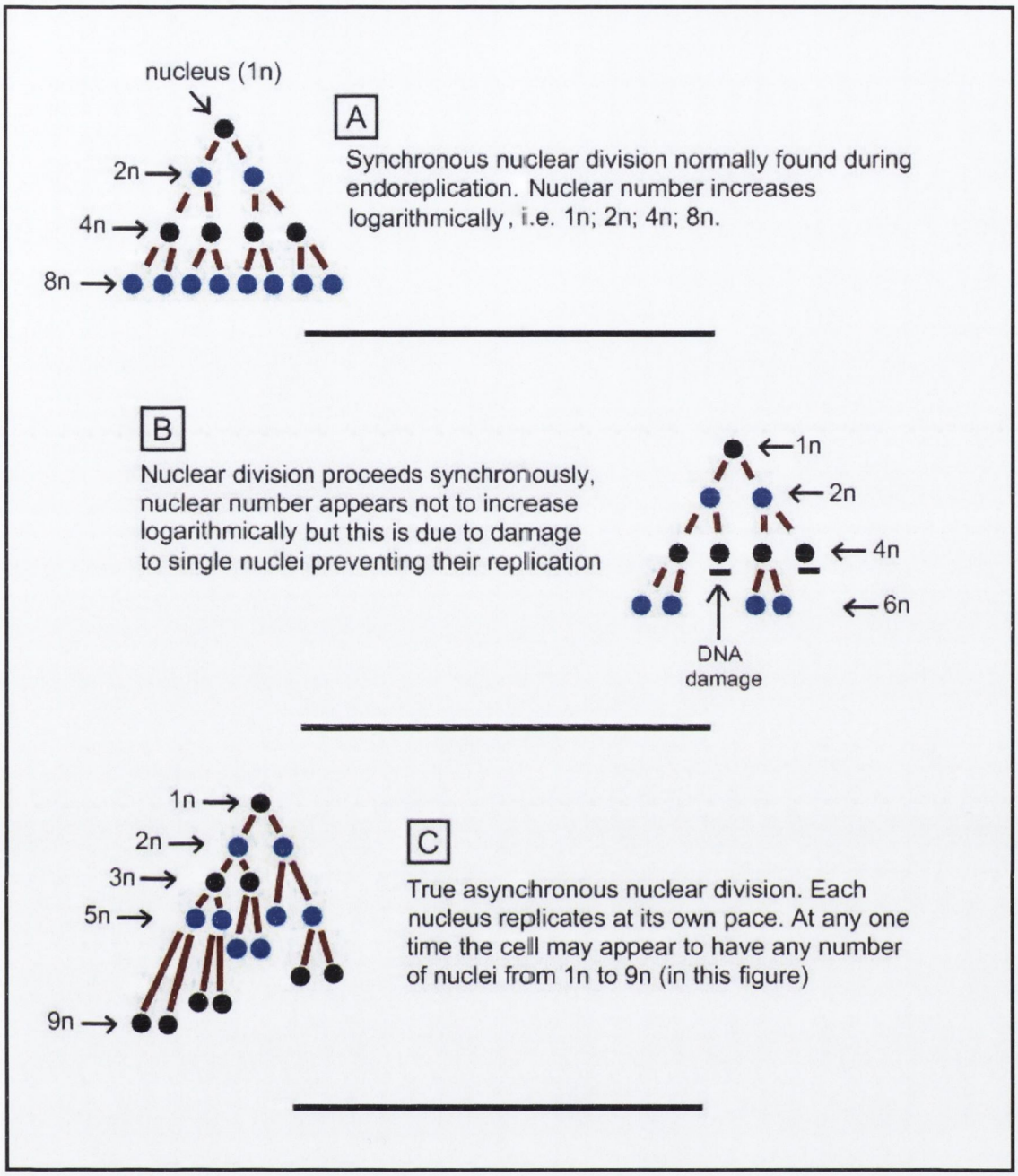


Figure 1.5. Highly schematic representation of multiple rounds of nuclear division in a single cytoplasm. (A) shows normal synchronous nuclear division (termed endoreplication or endoreduplication). (B) shows cells whose nuclear number does not increase logarithmically. In these cells nuclear division of each nucleus takes the same length of time but some nuclei do not replicate, in this case due to DNA damage. (C) shows true asynchronous division with each nucleus in the cell dividing independently of the others.

Despite these differences a number of homologues of known eukaryotic cell-cycle regulatory proteins have been found in the *Plasmodium* genome, and some of these have been expressed and characterised (Table 1.1).

	UniProt ID	Name	Size	Related mammalian protein	% Similarity	Ref.
Cyclin-dependent kinases						
	Q25826	Pfcrk1	85,993	Cdc-2	30	Doerig *et al.*, 1995
	Q07785	PfPK5	32,996	CDK2	63	Ross Mc Donald *et al.*, 1994
	*AAC61592**	PfPK6	35,000	CDK2	40	Bracchi-Richard *et al.*, 2000
	Q8IJQ1	PfPK7	37,988	CDK7	42	Dorin *et al.*, 2004
	P90584	Pfmrk	37,989	CDK7/MO15	42	Li *et al.*, 1996
Cyclins						
	Q9NG01	Pfcyc-1	39,261	cyclin H	22	LeRoch *et al.*, 2000
	Q8T358	Pfcyc-2	206,756	cyclin Y	30	Merckx *et al.*, 2003
	Q8T357	Pfcyc-3	26,846	cyclin Y	30	Merckx *et al.*, 2003
	Q8T356	Pfcyc-4	31,144	cyclin L1	37	Merckx *et al.*, 2003

*GenBank ID; no UniProt ID

Table 1.1. Cyclins and cyclin-dependent kinases of *Plasmodium falciparum*.

The first of these to be identified was *P. falciparum* protein kinase 5 (PfPK5), a CDK-like kinase with structural similarity to human CDK1 and CDK5 (Ross-Macdonald *et al.*, 1994; Graeser *et al.*, 1996). PfPK5 does however exhibit activities unusual in a CDK: monomeric PfPK5 exhibits low levels of histone H1-like kinase activity, it also undergoes autophosphorylation in the presence of cyclin (Doerig *et al.*, 2002). The kinase activity is also increased by a number of different cyclin molecules (Le Roch *et al.*, 2000; Holton *et al.*, 2003). Additional CDK-like proteins described in *Plasmodium* include *P. falciparum* CDK-related kinase 1, 3 and 4 (Pfcrk-1, Pfcrk-3 and Pfcrk-4), *P. falciparum* MO15-related kinase (Pfmrk) and *P. falciparum* protein kinase 6 (PfPK6). PfPK6 and Pfmrk have both been expressed recombinantly and shown to have CDK-like activity and interactions, while the other proteins were identified by sequence similarity and have yet to be produced in recombinant form (Doerig *et al.*, 2002; Doerig, 2004).

The identification of several *Plasmodium* CDK-like proteins resulted in a search for their cyclin partners. The lack of sequence similarity among these proteins made the genomic search difficult, but to date four have been found. The first to be identified, *P. falciparum* cyclin 1 (Pfcyc-1), has shown the ability to interact with and enhance the

activity of PfPK5 and also activates Pfmrk (Le Roch *et al.*, 2000). To date only RNA expression studies have been carried out on Pfcyc-2, 3 and 4 (Merckx *et al.*, 2003). These have shown that Pfcyc-2 and Pfcyc-3 are detected at both the gametocyte and asexual erythrocytic stages of the parasite. Pfcyc-1 was also detected in these stages but it was much more abundant in the gametocyte stage. Pfcyc-4 mRNA appeared confined to the asexual erythrocytic stages of development.

While the discovery of homologues of cell-cycle regulatory proteins has been an advance, knowledge of the cell-cycle in *Plasmodium* remains scant. In order to decipher what cell cycle stages are present in the parasite, how long they are and also to answer the riddle of the apparent asynchrony of nuclear division in the schizonts, the initial objective of this project was to develop a method for synchronising cultures of *P. falciparum* to a particular cell cycle stage, so that subsequent events could be observed.

1.2.3. Studying the cell cycle in *Plasmodium*

The difficulties in following the progression of a single cell has meant that the study of the cell-cycle has been dominated by methods of obtaining cultures of cells all synchronised to a single cell-cycle stage. The preferred of these techniques are non-inhibitor based methods including mitotic shake off and centrifugal elutriation as these are assumed to yield cells with features closest to normal cells. These techniques are based on certain physical characteristics of the cells. During mitosis in a mammalian cell growing in a monolayer the cells begin to round off, loosening their contact both with the culture dish and the surrounding cells. This allows them to be suspended easily in the medium and siphoned off without disturbing other cells not at this stage. The result of this is a population of cells all in M-phase. Centrifugal elutriation is based on separation of cells in a population based on size and density. These methods are not applicable in the case of *P. falciparum* due to the intracellular nature of the parasite stages of interest. Double thymidine block is another non-inhibitor based method commonly used in synchronisation of mammalian cells but as *Plasmodium* sp. synthesise their own pyrimidines this method cannot be applied to these parasites either.

Use of inhibitors of specific cell-cycle processes or molecules is a widely accepted and commonly used technique. This approach is based on the assumption that a series of checkpoints exists in the cell, which control whether it progresses to the next stage of the cycle or not. Inhibiting the completion of processes involved in each stage is thought to prevent cells from progressing past these checkpoints, thus arresting them in

a single stage of the cell cycle. Agents that inhibit DNA synthesis (e.g. aphidicolin and L-mimosine), mitosis (e.g. colchicines or nocodazole) or molecules involved in cell cycle control such as cyclin dependent kinases (e.g. flavopiridol), have been used on these eukaryotic cells, apparently to bring about synchronisation of populations to particular cell cycle stages (Gewirtz, 1993; Merrill, 1998; Davis *et al.*, 2001).

Once a treatment has been applied to a population, the success of the method must be assessed. Various cell-cycle events can be used to determine the success of a block and of these DNA content is one of the easiest to assay. Flow cytometry is a technique developed originally as a method of counting and sizing particles, but in the last 25 years it has been developed into a tool which is able to quantify multiple physical and chemical properties of cells or cellular fragments as they flow past a laser in suspension. By using a DNA-specific fluorescent probe it is possible to quantify the amount of DNA contained in a single cell. This technique is routinely used in cell cycle studies of other eukaryotic cells, as a means of determining the cell cycle stage(s) of a population (Hang and Fox, 2004; Zhang and Siede, 2004). Although not completely conclusive, measurement of DNA content is an excellent initial indicator of the success or otherwise of a synchronisation technique.

Once successfully synchronised to the same cell-cycle phase, populations of cells can be used to study protein and gene expression profiles. In addition, as the inhibitory effects of many of the cell cycle inhibitors can be reversed, the emerging cells can be observed to assess the duration of cell-cycle phases and whether one or both of the gap phases are present. Immunofluorescence microscopy and other techniques can be used to determine the concentrations, locations and activities of different cell-cycle regulatory elements at different times.

The term "synchronised" has been applied to cultures of erythrocytic *P. falciparum* but these techniques actually *select* a particular age-range of the asexual developmental cycle (using density gradients, temperature shifts or differential osmotic lysis of parasitised erythrocytes). Multiple applications of these techniques can produce a population covering a much narrower age range than that normally found in cultured *P. falciparum*. The smallest range reported following use of these methods was 3–5 h (Hoppe *et al.*, 1991; Rojas and Wasserman, 1993). For the purposes of investigating the regulation of processes involved in cell-cycle progression and the events occurring at each cell-cycle phase, this age range is too large. The hypothesis taken as a starting point in part of this project (Chapter 3) was that 'batch' synchronisation of parasite

cultures using inhibitors of specific cell-cycle processes might allow a population of parasites truly synchronised to a specific cell-cycle phase to be isolated and studied.

1.3. CELL DEATH

1.3.1. General introduction to cell death: Necrosis and programmed cell death

There are many different mechanisms of cell death, defined by morphological and biochemical characteristics. Initially all cell death was thought to be a passive process, however the existence of a 'suicide' type of cell death was first proposed in 1971 by Farber and Roberts in response to morphological characteristics noted on treatment of mammalian cells with cytotoxic anticancer drugs (Farber and Roberts, 1971). The most recognised term for active or programmed cell death (PCD), apoptosis, was coined in 1972 by Kerr, Wyllie and Currie (Kerr *et al.*, 1972). They proposed the classification of cell death be split into two broad categories based on morphological characteristics, with necrosis being confined to passive cell-death by collapse of internal homeostasis, and the new term, apoptosis, applied to a more organised self-destruction of the cell (Kerr *et al.*, 1972). Since then, as PCD is better understood, morphological and biochemical evidence indicate mechanisms of active, organised cell suicide other than the classical apoptotic process exist (Vaux and Strasser, 1996; Yuan, 1997; Madeo *et al.*, 2002; Newmeyer and Ferguson-Miller, 2003).

Necrosis is characterised by increase in cell size, little initial chromatin changes and an increasing disruption and disorganisation of cytoplasmic organelles. The mitochondria begin accumulating lipid-rich particles and as a result start to swell, and the inner mitochondrial membrane begins pulling away from the outer membrane. At the later stages of necrosis, the cyto-architecture of the cell is lost and proteases, nucleases and the contents of lysosomes are released. Finally the outer membrane ruptures; in metazoa this initiates an inflammatory response usually with the release of inflammatory cytokines and subsequently recruitment of macrophages and monocytes to the site (Vaux and Strasser, 1996; Yuan, 1997; Elmore, 2007).

Programmed cell death is a more controlled process, with a distinct number of sequential morphological changes. Like cell death in general, programmed cell death is divided into distinct types. Currently, three distinct types of PCD have been discriminated based on morphological characteristics. Type I: apoptotic cell death, characterised by prominent chromatin condensation and DNA fragmentation; type II:

autophagic cell death, where the formation of internal autophagic vacuoles is prominent and type III: a caspase-independent programmed cell death. PCD can be initiated in cells by multiple external and internal triggers such as cellular stress, chemotherapeutic agents such as taxol and disruption of normal cell-cycle progression and is controlled by a vast and complex network of effectors and regulators (Vaux and Strasser, 1996; Yuan, 1997; Elmore, 2007).

Determination of whether the cell is undergoing PCD is achieved by the detection of a number of different specific events. Chromatin condensation (which can be detected by acridine orange staining), DNA fragmentation (seen using terminal-deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL)), phosphatidyl-serine translocation to the outer leaflet of the membrane and activation of caspases are the major indicators of PCD in cells (Elmore, 2007).

1.3.2. Programmed cell death and protozoal parasites

PCD was originally thought to be restricted to multi-cellular organisms as a means of removing damaged cells preventing cancerous growth or damage to the organism and in the processes of embryonic development and maturation of the immune system. The possibility that PCD occurs in unicellular organisms was originally dismissed due to the lack of any obvious reason for this altruistic cellular behaviour. However it has since been suggested that PCD may be a very effective means of promoting and maintaining population clonality (Welburn *et al.*, 1997; Lee *et al.*, 2002; Madeo *et al.*, 2002), as well as perhaps preventing host death by reducing parasite numbers (Zangger *et al.*, 2002). Very little is known about mechanisms of PCD in unicellular organisms but evidence for PCD in these cells is increasing (reviewed in Debrabant *et al.*, 2003).

1.3.3. Programmed cell death in *Plasmodium*

Studies on PCD in *Plasmodium* erythrocytic stages are limited. Original reports that chloroquine treatment resulted in a PCD-like cell death were based on DNA fragmentation detected following treatment (Picot *et al.*, 1997) but this has since been disputed (Nyakeriga *et al.*, 2006). While evidence for PCD in the erythrocytic stages of the parasite are disputed, the most compelling evidence for the potential of *Plasmodium* to undergo a form of PCD has come from studies in the mosquito stages of *P. berghei* (Al-Olayan *et al.*, 2002). Morphological characteristics typical of PCD were exhibited

by both zygotes and ookinetes and many of the typical PCD markers were detected. DNA fragmentation in nuclei was detected by TUNEL analysis, acridine orange staining detected chromatin condensation and annexin labelling detected translocation of phosphatidyl-serine to the outer leaflet of the ookinete membrane. Membrane blebbing was also detected by microscopy. Finally caspase activity was also detected and so, as the cells exhibited multiple indicators of PCD at the same time it appears incontrovertible that it occurs in these cells. While the machinery involved in this process is unknown and although PCD has yet to be confirmed in the erythrocytic stages of *Plasmodium* it is likely that there is potential for these stages to undergo a form of PCD.

1.4. TUBULIN AND MICROTUBULES

1.4.1. Tubulin and microtubules: an introduction

Microtubules are filamentous cytoskeletal heteropolymers found in almost all eukaryotic organisms and are involved in a wide variety of cellular functions including transport, motility, maintenance of shape and polarity as well as in mitotic and meiotic cell division. Their principal components are two protein subunits termed α- and β-tubulin combined with guanine nucleotides to form a core cylinder (Mandelkow and Mandelkow, 1995; Nogales, 2000). The molecular properties of microtubules reflect the diverse functions they carry out, and the most significant of these properties is the ability to polymerise (assemble) and depolymerise (disassemble) reversibly based on the binding and hydrolysis of guanosine triphosphate (GTP) by tubulin subunits (Dumontet, 2000). This is regulated by numerous cellular factors including microtubule-associated proteins (MAPs) and by post-translational modifications of the tubulin subunits.

1.4.2. Tubulin and microtubule structure and dynamics

1.4.2.1. Tubulin structure

The primary heterodimeric subunit of a microtubule is comprised of α- and β-tubulin monomers, closely-related proteins of approximately equal molecular mass, about 50,000 Da each, and sequence lengths of around 450 amino acids. Studies of tubulin sequences have shown that the α- and β-tubulin monomers share approximately 36 - 42% identity with each other at the amino acid level (Ludueña *et al.*, 1992).

Sequence similarity between tubulins of different species varies, but generally exceeds 60% identity (Oakley, 2000). Tubulins from protozoa and plants/algae share approximately 88 - 93% amino acid sequence identity (Burns *et al.*, 1994), while for plants and animals amino acid identity is closer to 79-87% (Morejohn *et al.*, 1991). Despite this high level of sequence conservation, numerous pharmacological and electrophoretic differences have been noted across eukaryotic tubulins (Bell, 1998; Morejohn *et al.*, 1991; Ochola *et al.*, 2002; Werbovetz, 2002, Fennell *et al.*, 2008) making it possible selectively to target parasite tubulin with inhibitors (Lacey, 1988). The structure of the bovine tubulin dimer was solved by Nogales *et al.*, (1998) and later refined (Lowe *et al.*, 2001). The conserved nature of the tubulin sequence has led to this structure being used to model the tubulin dimer of numerous other species (Blume *et al.*, 2003; Morrissette *et al.*, 2004).

1.4.2.2. Microtubule structure and dynamics

The first step in microtubule formation is the assembly of tubulin dimers in combination with GTP into protofilaments, which assemble side to side to form the cylindrical microtubule (Fig. 1.6). Studies both *in vitro* and in cells have shown that between 9 and 18 protofilaments can combine to form the "B" lattice structure (Oakley, 2000; Li *et al.*, 2002). Microtubule length is variable and can reach several μm, while the diameter remains close to 25 nm. The principal lateral contacts are made between homologous subunits (i.e. α - α and β - β), but contact among the heterologous subunits (i.e. α - β and β - α), while rarer, may also occur along a seam of contact. The function of this seam remains to be elucidated but is believed to be involved in interactions with MAPs and/or in microtubule stability (Nogales, 2000).

The dynamics of microtubule formation appears to be due to protofilament orientation which results in one end being kinetically more active than the other. This more kinetically active end (plus end) is crowned by a molecule of GTP whereas the less active end (minus end) terminates with the α-subunit exposing its catalytic domain (Inclán *et al.*, 2001) (Fig. 1.6). It is the catalytic domain within the α-subunit that contacts the E-site of the exposed GTP nucleotide, which subsequently becomes hydrolysed to GDP. The β-tubulin subunit of the newly added tubulin dimer then exposes its GTP nucleotide allowing the addition of another tubulin dimer. This cap of β-tubulin containing GTP is sufficient to stabilise the microtubule structure despite being as little as one subunit deep. Addition of new subunits to the minus end results

from contact between the catalytic region of the last α-tubulin subunit and the E-site nucleotide of the new subunit.

Microtubules are thus highly dynamic polymers, able to switch stochastically between growing and shrinking phases both intracellularly and *in vitro* (Heald *et al.*, 2002). Microtubules have been found to exhibit two types of dynamic behaviour, namely treadmilling and dynamic instability (Mandelkow *et al.*, 1995) allowing for any necessary rearrangements of cytoskeletal structure required, as for example in nuclear division. Treadmilling occurs without a significant change in microtubule length: dimers are lost from the minus end and added to the plus end of the microtubule, resulting in a GTP hydrolysis-driven net flux of dimers through the polymer. This phenomenon is widely believed to be critical in the polar movement of chromosomes during anaphase (Dumontet, 2000). Dynamic instability involves a population of microtubules exhibiting dramatically different behaviour: some of the microtubules are growing slowly while others are shrinking or disassembling rapidly. This sudden change from growth to shrinkage is known as a "catastrophe", while the cessation of shrinkage and the re-growth of the microtubule is termed a "rescue" (Amos, 2004).

Control of microtubule dynamics is thought to be mediated by multiple factors; local environmental conditions such as low temperature (generally less than 10°C), fluctuating pH, low GTP levels and high calcium levels can increase the rate of microtubule disassembly and under certain conditions can cause catastrophic depolymerisation of the entire microtubule in a non-polar fashion (Lacey, 1988) (Fig. 1.7). Intracellular levels of free tubulin dimers can have dramatic effects on microtubule behaviour and MAPs have been identified which can destabilise microtubular structures by a variety of different mechanisms (Heald and Nogales, 2002; Amos, 2004).

By docking the high-resolution structures of the tubulin dimer into a lower resolution map of an intact microtubule a model of the atomic structure of the microtubule has emerged (Nogales, 2000; Amos, 2000; Li *et al.*, 2002).

Within cells microtubules generally grow out from a microtubule organising centre (MTOC), commonly termed a centrosome in animal cells. The microtubule plus end is exposed in the cytoplasm enabling it to switch between growth and shrinkage while the minus end is usually embedded into the MTOC (Oakley, 2000). A third member of the tubulin family, γ-tubulin, forms a key component of the MTOC (Oakley *et al.*, 1989) and has also been located in the mitotic spindle (Lajoie-Mazenc *et al.*, 1994) (Fig. 1.7). This protein shares a high sequence similarity to α- and β- tubulin.

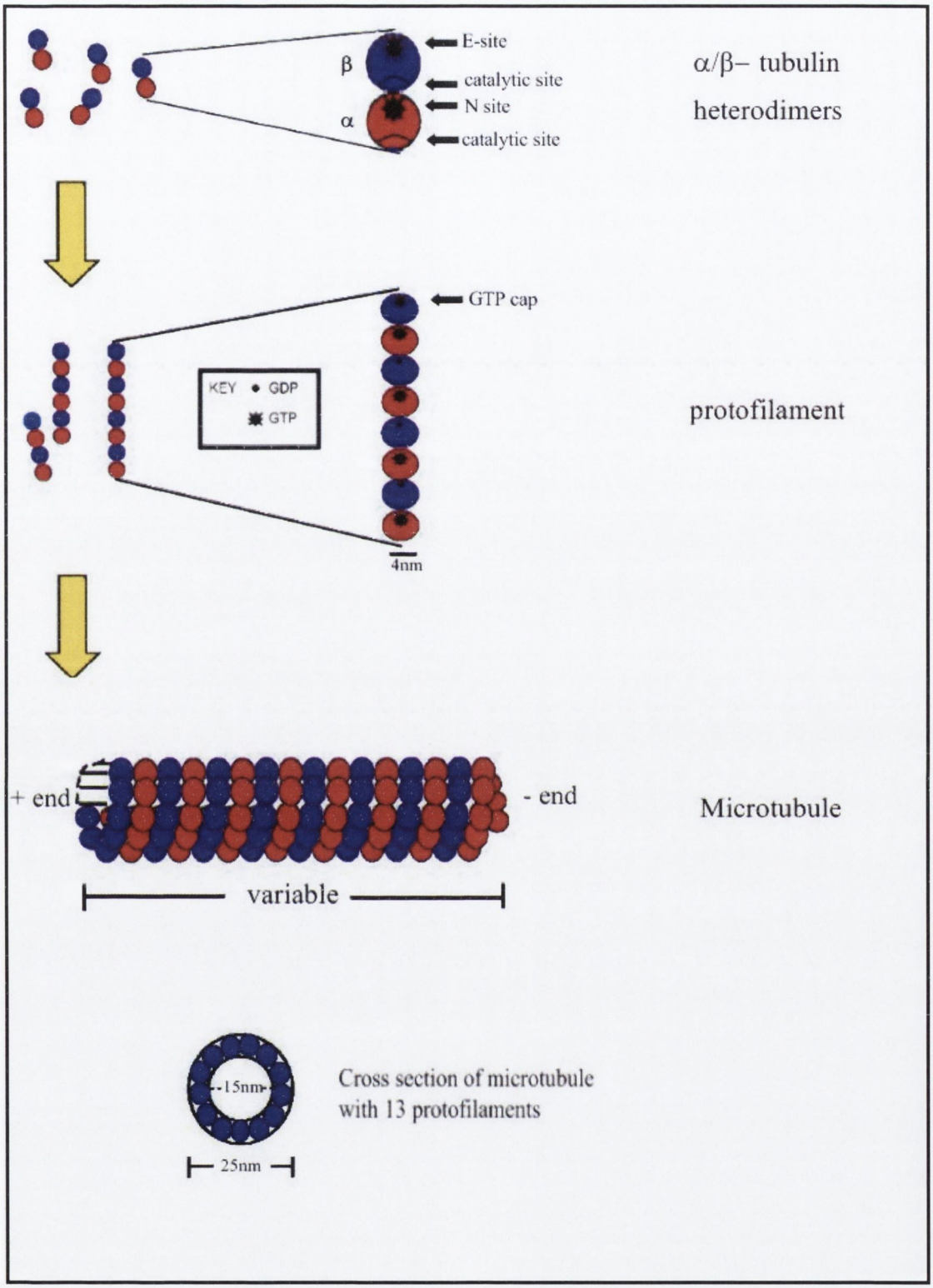


Figure 1.6. Schematic diagram of microtubule formation and structure. Free α- and β-tubulin heterodimers (containing one molecule of GTP in each of the N- and E- sites) associate linearly to form short protofilaments, accompanied by the hydrolysis of β-tubulin associated-GTP to GDP, while the α-subunit- and the β-tubulin cap-associated GTP remains unhydrolysed. Protofilaments associate laterally to form a cylindrical polar microtubule with an outer diameter of 25 nm and variable length up to several μm. Lateral contacts in the microtubule are between the subunits α-α and β-β, but there may be a line of discontinuity where α and β subunits meet, producing a seam that runs the length of the microtubule (not observed in above schematic).

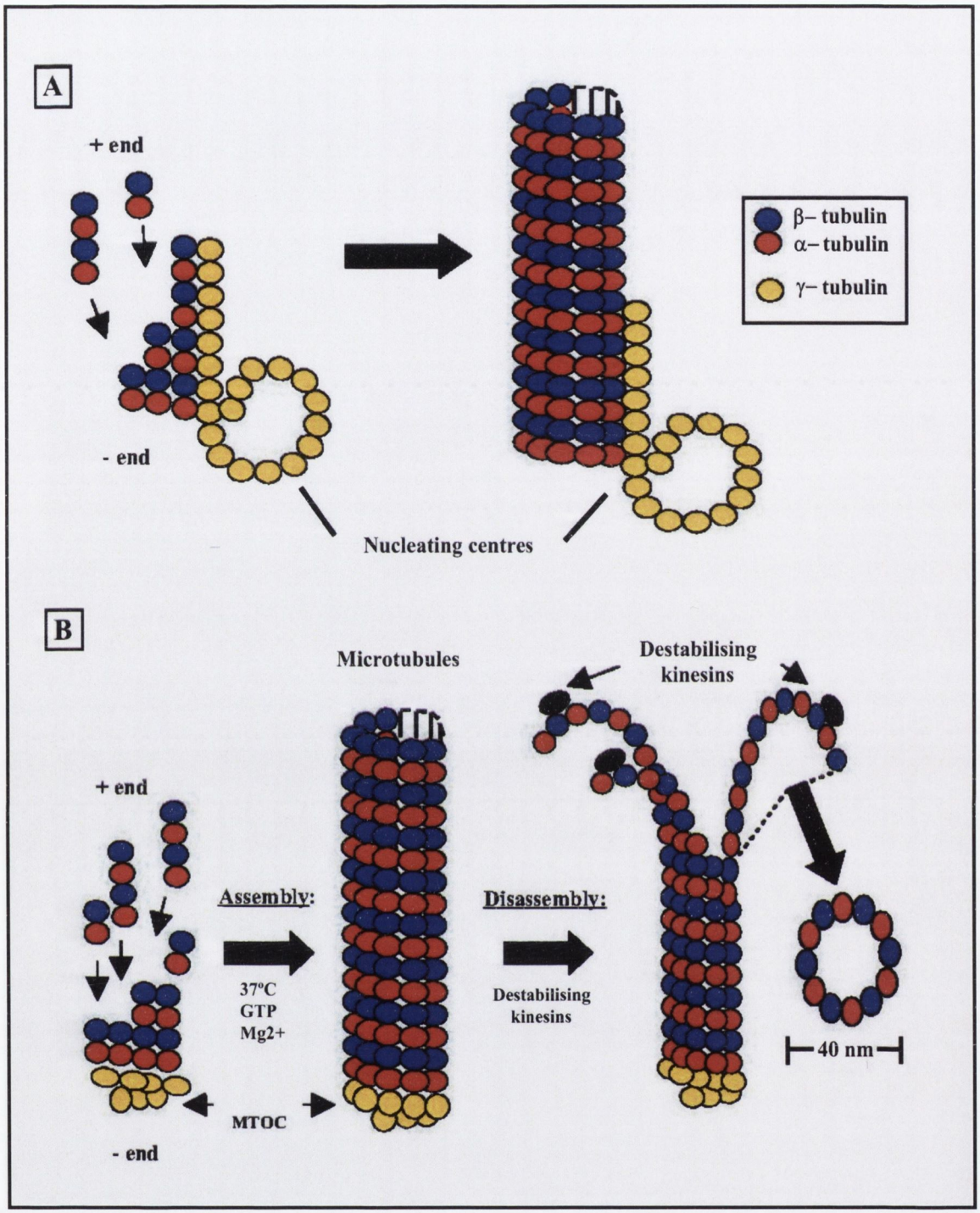


Figure 1.7. Two proposed models for microtubule nucleation, assembly and depolymerisation (modified from Nogales, 2000). **A.** A spiral-like protofilament of γ-tubulin acts to stabilise the α/β-tubulin microtubule lattice by lateral interactions, thus facilitating microtubule assembly. **B.** The first helical turn of the microtubule is comprised of γ-tubulin (shown) and various other proteins (not shown for reasons of simplicity), and the microtubule begins to form from this base. Following microtubule formation an example of microtubule disassembly is shown induced by the actions of depolymerising kinesins. Note the peeling or spiralling of protofilaments from the microtubule lattice as well as their ability to form ring-like structures up to 30 - 40 nm in diameter. MTOC = microtubule organising centre.

1.4.3. Cellular organisation of microtubules: general overview

Microtubular-based structures present in different eukaryotic cells, or at different times or places in the same cell, are of many diverse kinds, each unique in architecture and mode of function (Fig. 1.8). Mitotic microtubular spindles are essential for cell division (mitosis and cytokinesis) in nearly all eukaryotic cells. Intense networks of microtubules are built up between the MTOCs forming the mitotic spindle which, during metaphase, binds to the kinetochores (points of attachment of microtubule to chromosome) and orients the duplicated chromosomes in the central plane of the mitotic cell. At the same time, astral microtubules bypass the kinetochores and terminate elsewhere in the body of the spindle, often reaching the opposite pole (Fig. 1.8 A). The exact function of these in mitosis is not certain but they are believed to help to define the plane of cytokinesis in cells that form a cleavage furrow (McIntosh, 1994). In anaphase, microtubules facilitate chromosome separation and move them to opposite ends of the cell. Finally, in teleophase, microtubules play an important role in cytokinesis and in the formation of the cell plane, which separates the two new cells (Sakai, 1994).

Another important functional class of microtubules, together with a number of accessory proteins, are found to constitute the axoneme of cilia and flagella, which contribute to cell motility. Microtubules of the axoneme are organised into the characteristic "9 + 2" arrangement (Fig. 1.8 C) which consists of nine peripheral doublet microtubules (termed A- and B-tubules) and a central pair of singlet microtubules. Movement of cilia and flagella is believed to result from the sliding movements between the doublet microtubules. The force for such movement is supplied by the dynein motor proteins, which are attached to the A-tubules of each doublet. The central, radial spokes are believed to convert this dynein-driven microtubule sliding action into the characteristic bending motions often observed with flagella (Smith *et al.,* 1994). Flagella and cilia can be found on a wide range of cells such as human epithelial cells (cilia only) and are particularly predominant on protists such as *Paramecium* and *Chlamydomonas*.

A third functional class of microtubules can be found ramifying throughout the cytosol of many eukaryotic cells. They play a crucial role in maintaining the position of cellular organelles as well as serving as tracks along which organelles (e.g. ribosomes) and vesicles can be transported (Fig. 1.8 B). Motor proteins from the dynein and

kinesin families, which use MgATP as a source of fuel, are used to shuttle the membranous cargo along the microtubule track, which can be minus-end or plus-end directed (Bloom, 1992). For example, microtubule-based motors are believed to be involved in the organisation of the endoplasmic reticulum membranes that are often dispersed throughout the cytosol. Moreover, newly synthesised material in the endoplasmic reticulum and the Golgi system is thought to be delivered to various cellular locations by vesicle trafficking along the microtubule tracks (Ludueña *et al.*, 1995).

Finally, the submembrane microtubules comprise another major functional class of microtubules in various eukaryotic cells (Fig. 1.8) and make a key contribution to the maintenance of cell shape and integrity. However, it should be noted that numerous other microtubule-containing structures can be found in the eukaryotic cell and that this brief overview just serves to highlight some of the diverse forms that can be found in even the simplest of cells.

1.4.4. Microtubules of *Plasmodium*

1.4.4.1. Tubulin genes and proteins

To-date two α-tubulin (αI- (Holloway *et al.*, 1989) and αII- (Holloway *et al.*, 1990)), one β-tubulin (Delves *et al.*, 1989, Sen *et al.*, 1990, Wesseling *et al.*, 1989) and one γ-tubulin (Maessen *et al.*, 1993) genes from *P. falciparum* have been cloned and characterised, and αI- and β-tubulin have been expressed as maltose binding protein (MBP)-fusions (Fennell *et al.*, 2006). The two *P. falciparum* α-tubulins share ~ 94% identity at the amino acid level and both have ~ 40% identity to β-tubulin. They are also very similar to tubulins from most other organisms, with the major amino acid differences found in the last 15 residues of the C-terminal tails. For example, *P. falciparum* β-tubulin shares ~ 88 % identity with human β-tubulin, while αI- and αII-tubulins are slightly less similar to human α-tubulin, sharing ~ 83% and ~ 82%, identity, respectively (Table 1.2).

1.4.4.2.Functional classes of *Plasmodium* microtubules

While the microtubular systems of *Plasmodium* have not been as well characterised as their mammalian counterparts, at least three functional classes of microtubules have been identified in *Plasmodium*; the mitotic spindle microtubules, the subpellicular microtubules and the axonemal microtubules (reviewed in Bell, 1998). A

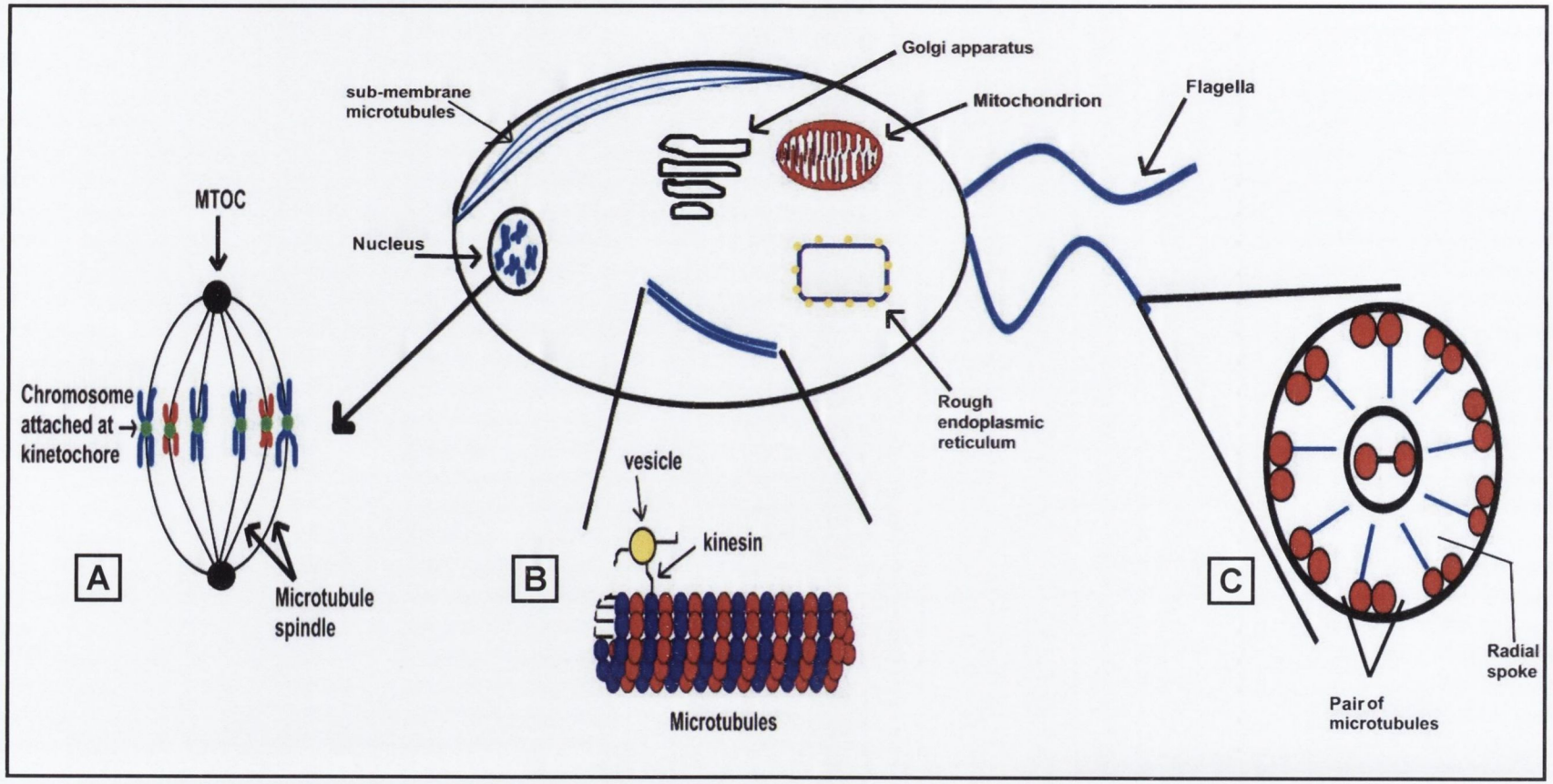


Figure 1.8. Highly schematic diagram showing microtubule-containing structures in a simple eukaryotic cell. Submembrane microtubules confer stability of shape on the cell. **A.** Shows spindle microtubules bound to chromosomes at the kinetochores and extending from the spindle pole body or centriole. **B.** Intracellular vesicle being transported via kinesin-mediated transport along the cytoplasmic microtubular cytoskeleton. **C.** Cross-section through a flagellum showing arrangement of microtubules. The nine peripheral doublet microtubules are shown with the radial spokes attached; not shown are the dynein arms that project to the next adjacent doublet. Adapted from Fennell, B.J. PhD Thesis.

cytosolic microtubular cytoskeleton may also be present as kinesin and dynein, both microtubule-associated motor proteins, have been identified and shown to be present at various stages on the asexual stages of the parasite (Fowler *et al.*, 2001).

UniProt ID	Name	Size (Da)	Related mammalian protein	% Similarity	Ref.
Q6ZLZ9	αI-tubulin	50,297	Human α-tubulin	83	Holloway *et al.*, 1981
Q8IFP3	αII-tubulin	49,691	Human α-tubulin	82	Holloway et al., 1990
Q7KQL5	β-tubulin	49,751	Human β-tubulin	88	Delves *et al.*, 1989; Sen *et al.*, 1990; Hall *et al.*, 2002
Q81210	δ-tubulin (putative)	86,520	Human δ-tubulin	39	Hall *et al.*, 2002
P34787	γ-tubulin	51,538	Human γ-1-tubulin	64	Maessen *et al.*, 1993

Table 1.2. Tubulin Proteins of *Plasmodium falciparum*

The mitotic microtubular spindles play a crucial role in eukaryotic cell division and cytokinesis and have been identified in erythrocytic (Read *et al.*, 1993) and exoerythrocytic schizonts (Suhrbier *et al.*, 1993), male gametocytes, zygotes and oocysts (Sinden *et al.*, 1978; Sinden and Strong, 1978). The MTOC is usually in the form of a centriolar plaque embedded in a pore of the nuclear membrane and from this the hemi-spindle microtubules nucleate (Read *et al.*, 1993). The centriolar plaque duplicates and the associated hemispindle splits into two daughter hemispindles. Apart from disruption at the centriolar plaque, the nuclear membrane remains intact during mitosis allowing migration of the newly duplicated centriolar plaques towards opposite poles of the nucleus. Interestingly, the centriolar plaques do not have to be aligned directly opposite each other for the successful movement of chromosomes along the spindle microtubules in *P. falciparum* (Read *et al.*, 1993). There complete mitotic spindle is formed on the combination of the daughter hemispindles. In the case of sporogony in *P. berghei*, there are three sets of microtubules, namely those connecting each kinetochore to either spindle pole body, those extending from pole to pole of the spindle (astral microtubules) and those that radiate from either pole to a location somewhere beyond the equatorial plane of the spindle (reviewed in Morrissette and Sibley, 2002). Mitotic spindles at different stages of division can be observed in a single parasite cytoplasm (Vickerman and Cox, 1967; Canning and Sinden, 1973; Read *et al.*,

1993). Such asynchrony of nuclear division in a single cytoplasm is in contrast to most models of mitosis in eukaryotic cells and the reasoning for its occurrence has yet to be determined (see section 1.2.3 for a discussion of the *Plasmodium* cell cycle). Following nuclear division in erythrocytic schizonts, the mitotic microtubular apparatus is reorganised into structures believed to assist in the separation of merozoites and their organelles (Read *et al.*, 1993; Morrissette and Sibley, 2002). Post-mitotic microtubule-associated structures have also been observed following schizogony and sporogony in *P. berghei* and after gametogenesis in *P. yoelii* (Sinden *et al.*, 1976).

A second functional class of microtubules, the subpellicular microtubules, has been identified in most stages of the parasites development cycle, including erythrocytic segmenters, extracellular merozoites, both micro- and macro- gametocytes as well as in the sporozoites and ookinete (Bell, 1998). They confer shape, integrity and apical polarity to the parasite cell, and, in general, extend longitudinally from the cell apex (see Fig. 1.8 for schematic representation) that tethers the pellicular membranes (a series of flat vesicles found below the plasma membrane that comprise the so-called internal membrane complex). Their number and length can vary greatly among *Plasmodium* species and stages. For example, the merozoite of *P. falciparum* contains only a narrow band of 2 – 3 subpellicular microtubules, (designated f-MAST [falciparum merozoite assemblage of subpellicular tubules] (Fowler *et al.*, 1998)) while the *Plasmodium* ookinete contains a more extensive array of ~ 60 (Aikawa, 1967; Aikawa, 1971). Despite the reduced number of *P. falciparum* merozoite subpellicular microtubules, they appear to play a crucial role in invasion as microtubule inhibitors previously shown to depolymerise the f-MAST (Fowler *et al.*, 1998) prevented merozoite invasion of uninfected erythrocytes (Bejon *et al.*, 1997; Fowler *et al.*, 1998). This inhibition may be as a result of a failure in microneme trafficking which is thought to be an essential part of invasion (Bannister *et al.*, 2003).

Parasite motility is facilitated by a third class of microtubules together with a number of accessory proteins which constitute the axoneme of the male gamete flagellum (Fig. 1.8) (reviewed in Tilney *et al.*, 1996).

1.4.5. Microtubule inhibitors

Microtubules have emerged as one of the most important targets for anti-cancer agents (Islam and Iskander, 2004; Nagle *et al.*, 2006), of fungicides and herbicides (Morejohn and Fosket, 1991) and of anthelminthics (Katiyar *et al.*, 1994). Investigations into the

ability of these agents to inhibit a number of protozoal parasites have shown that microtubule inhibitors successfully inhibit the causative parasites of some of the most common and severe human protozoal parasite infections, including *Plasmodium* (Usanga *et al.*, 1986; Dieckmann-Schuppert and Franklin, 1989; Dieckmann-Schuppert and Franklin, 1990; Bell *et al.*, 1993; Pouvelle *et al.*, 1994; Schrevel *et al.*, 1994; Skinner-Adams *et al.*, 1997; Bell, 1998; Fennell *et al.*, 2003; Navarrete-Vazquez *et al.*, 2003; Fennell *et al.*, 2006; Navarrete-Vazquez *et al.*, 2006), *Leishmania* (Chan *et al.*, 1993; Katiyar *et al.*, 1994; Callahan *et al.*, 1996; Werbovetz *et al.*, 1999; Bhattacharya *et al.*, 2002; Fennell *et al.*, 2003; Werbovetz *et al.*, 2003; Jayanarayan and Dey, 2005; George *et al.*, 2006) and *Trypanosomes* (Filho *et al.*, 1978; Baum *et al.*, 1981; Kaminsky and Zweygarth, 1989; Chan *et al.*, 1993; Grellier *et al.*, 1995; Turrens *et al.*, 1996; Bogitsh *et al.*, 1999; Lubega *et al.*, 2002; Werbovetz *et al.*, 2003) (see Tables 1.3 and 1.4).

Several classes of microtubule inhibitor exist, some of which have shown selective antiprotozoal activity while others, mainly those developed from anti-cancer agents, are highly toxic to mammalian cells (for a review see Kappes and Rohrbach, 2007).

1.4.5.1. Antimitotic herbicides

Although few selective inhibitors of protozoal parasite microtubules in general and *Plasmodium* microtubules in particular have been found among anticancer and related agents, several synthetic herbicides that bind to plant tubulins (Morejohn and Fosket, 1991; Hugdahl and Morejohn, 1993) have shown antiprotozoal and antimalarial activity but little toxicity for mammalian cells (Chan and Fong, 1990; Stokkermans *et al.*, 1996; Werbovetz *et al.*, 2003; Fennell *et al.*, 2006). These herbicides fall into three classes, the dinitroanilines, phosphoric amides, and N-phenyl carbamates. Studies in plants have shown that treatment with the dinitroanilines oryzalin and trifluralin and the phosphorothioamidate amiprophos-methyl resulted in damage to areas with rapidly dividing cells and depolymerisation of microtubular structures, indicating microtubules as their target (Hoffman and Vaughn, 1994). Additional plant studies using purified tubulin highlighted the potential selectivity of these agents: low micromolar concentrations of oryzalin failed to inhibit taxol-induced bovine brain tubulin polymerisation, while similar concentrations applied to rose tubulin inhibited the rapid phase of taxol-induced tubulin assembly (Hugdahl and Morejohn, 1993).

As shown in tables 1.3 and 1.4, various tubulin-binding herbicides have activty against a number of protozoal parasites and as in plants, cell-based studies have indicated in some cases that the target is tubulin. Oryzalin and several of its analogs have been shown to inhibit *in vitro* polymerisation of purified *Leishmania* tubuin (Werbovetz *et al.*, 2003; Bhattacharya *et al.*, 2004; George *et al.*, 2006). [^{14}C]trifluralin has been shown to bind both to *P. falciparum* αI- and β-tubulin expressed as MBP-fusion proteins but not to bovine α/β-tubulin dimers (Fennell *et al.*, 2006), and to purified *Leishmania* tubulin (Chan and Fong, 1990).

The effect on *Plasmodium* of exposure to these agents was not yet fully characterised before the work in this thesis was undertaken. Previous reports have shown modest antimalarial activity in culture for trifluralin (IC_{50} range, <1 – 1.3 μM), oryzalin (IC_{50} = 4.3 μM), pendimethalin (IC_{50} range, 0.67 – 10 μM), chloralin (IC_{50} = 16 μM) and the phosphorothioamidate amiprophosmethyl (IC_{50} = ~3.5 μM) (Nath and Schneider, 1992; Fowler *et al.*, 1998; Dow *et al.*, 2002; Fennell *et al.*, 2006). This modest antimalarial activity when compared with some of the agents described below may be as a result of lower-affinity tubulin binding and/or poorer uptake of these compounds into cells. Micromolar and nanomolar concentrations of trifluralin can inhibit erythrocytic development and exflagellation of the male gametocytes respectively (Nath and Schneider, 1992). Autoradiographs of *P. falciparum* gametocytes showed that radiolabelled trifluralin was closely associated with microtubules (Kaidoh *et al.*, 1995). In the same study, trifluralin treatment of *P. falciparum* gametocytes resulted in fragmentation of microtubules, increased microtubule diameter and dissolution of the subpellicular complex (Kaidoh *et al.*, 1995). Trifluralin and pendimethalin exposure of *P. falciparum* merozoites resulted in the disassembly of the narrow bands of subpellicular microtubules (f-MAST) (Fowler *et al.*, 1998). However, the very high drug concentrations used (0.1 – 1 mM) cast some doubt over the relevance of these observations to the primary mechanism of inhibition of asexual parasites. A more likely explanation of the antimalarial action of the dinitroanilines is the failure of parasites to maintain their characteristic mitotic architecture in the presence of such agents, thus resulting in inhibition of nuclear and cell division. This was suggested by the results of Fennell *et al.*, (2006) where exposure of the asexual stages of *P. falciparum* to 10 μM of trifluralin or oryzalin results loss of normal microtubular structures and their replacement by speckles or dots of discrete tubulin labelling throughout the parasite. The effects of these agents on mitotic division

Table 1.3. Microtubule inhibitors active on protozoal parasites in culture

Parasite	Colchicine & related compounds	Benzimidazoles	"*Vinca*"-site binders	Taxol & related compounds	Dinitroanilines and phosphothioamidates	References
Plasmodium spp.	++	+/++	++/+++	++/+++	++	Bell, *et al* 1993, 1998; Dieckmann-Schuppert and Franklin, 1989, 1990; Fennell, *et al* 2003, 2006; Navarrete-Vazquez *et al*, 2006; Pouvelle *et al*, 1994; Schrevel *et al*, 1994; Skinner-Adams *et al*, 1997; Usanga *et al*, 1986
Toxoplasma gondii	++/+++	-	ND	+++	++/+++	Aguirre-Cruz, *et al* 1996; Estes *et al*, 1998; Morrissette *et al*, 2002a, 2002b; Stokkermans *et al*, 1996
Cryptosporidium parvum	-	-	+++	ND	+ to +++	Armson *et al*, 1999b; Mead *et al*, 2003; Wiest *et al*, 1993
Eimeria spp.	-	-	-	ND	ND	Russell *et al*, 1981
African trypanosomes	++	+++	ND	+++	- to +++	Chan *et al*, 1993a, 1993b; Kaminsky and Zweygarth, 1989; Lubega *et al*, 2002; Turrens *et al*, 1996; Werbovetz, *et al* 2003
Trypanosoma cruzi	-/+	-/++	ND	++/+++	- to +++	Baum *et al*, 1981; Bogitsh *et al*, 1999; Filho, *et al* , 1978; Grellier *et al*, 1995; Turrens *et al*, 1996
Leishmania spp.	-/+	-/+	- to +++	- to ++	- to +++	Armson *et al* 1999b; Bhattacharya, *et al* 2002; Callahan *et al*, 1996; Chan *et al*, 1993a; Fennell, *et al* 2003; George *et al*, 2006; Jayanarayan and Dey, 2005; Katiyar *et al* , 1994; Werbovetz *et al*, 1999, 2003
Giardia spp.	++	++/+++	+++	++	-	Dawson *et al*, 2007; Katiyar *et al*, 1994; Menon *et al*, 2002; Morgan *et al*, 1993; Navarrete-Vazquez *et al*, 2003, 2006; Valdez *et al*, 2002
Trichomonas vaginalis	-	++/+++	ND	++	ND	Juliano *et al*, 1985; Katiyar *et al*, 1994; Menon *et al* 2002 ; Navarrete-Vazquez *et al*, 2003, 2006
Theileria annulata	-	ND	ND	++	ND	Fritsch *et al*, 1988
Entamoeba histolytica	-	++/+++	ND	++	++	Dastidar *et al*, 2007; Makioka *et al*, 1999, 2002; Navarrete-Vazquez *et al*, 2003, 2006
Encephalitozoon spp.	ND	- to +++	ND	ND	ND	Katiyar *et al*, 1997; Ridoux, *et al* 1998
Naegleria spp	ND	ND	ND	ND	+++	Levy *et al*, 1998

* Ineffective ND, Not determined

** Effects demonstrated but IC_{50} not measured - Ineffective at ≥100 times IC_{50} of reference agent (drug in current use)

\+ Effective at 10-100 times IC_{50} of reference agent + Effective at 2-10 times IC_{50} of reference agent

+++ Effective at ≤ IC_{50} of reference agent

Reference agents and their IC 50 were as follows: *Plasmodium* : chloroquine, < 100 nM; *Toxoplasma* : pyrimethamine, 20 – 900 nM; *Cryptosporidium* : nitazoxanide, IC 90 32 µM (no IC 50); African trypanosomes: pentamidine, 1.7 µM; *T. cruzi* : benznidazole; 5 – 10 µM; *Leishmania* : pentamidine, 15 µM; *Giardia* : albendazole, 0.1 – 1 µM; *Trichomonas* : metronidazole, 1.89 – 18.2 µM; *Theileria* : parvaquone, 18 nM; *Entamoeba* : nitazoxanide, 7.6 – 8.8 µM; *Encephalitozoon* : albendazole, 16.6 nM; *Naegleria* : amphotericin B, 1.6 µM; *Acanthamoeba* : chlorohexidine, 1.5625 – 3.125 µM.

Table 1.4. Efficacy of microtubule inhibitors in animal models of protozoal infections

Parasite	Colchicine & related compounds	Benzimidazoles	Taxol & related compounds	Dinitroanilines and Phosphothioamidates	References
Plasmodium spp.	N.D.	-/+	-/+	-	Dow *et al*, 2002; Morrissette *et al*, 2002a, 2002b; Pouvelle *et al*, 1994; Seitz *et al*, 1998; Sinou *et al*, 1996
Toxoplasma gondii	-	ND	ND	ND	Aguirre-Cruz *et al*, 1996; Estes *et al*, 1998 ; Morrissette *et al*, 2002
Cryptosporidium parvum	ND	-	ND	++	Farthing *et al*, 2006; Fayer *et al*, 1995;
Trypanosoma cruzi	ND	N.D	ND	++	Zaidenberg *et al*, 2006
Leishmania spp.	ND	-	ND	++	Travi *et al*, 1998; Chan *et al*, 1993a, 1993b
Giardia spp.	ND	+++	ND	ND	Escobedo *et al*, 2003a, 2003b ; Fayer *et al*, 1995; Hall *et al*, 1993; Pengsaa *et al*, 1999; Yereli *et al*, 2004; Karabay *et al*, 2004
Encephalitozoon spp.	ND	++/+++	ND	ND	Dore *et al*, 1995; Farthing *et al*, 2006; Molina *et al* , 1998
Enterocytozoon bieneusi	ND	++	ND	ND	Farthing *et al*, 2006; Gross, *et al* 2003

\- inactive or reversible parasite clearing
\+ ≤10% cure rate
++ 10-90% cure rate
+++ 90-100% cure rate

ND, Not determined.

and other aspects of developmental progression in asexual, erythrocytic parasites are explored in this thesis.

The site at which these agents bind has yet to be characterised. However, studies on both *Toxoplasma gondii* and the weed *Eleusine indica* indicate that low-level resistance to the dinitroanilines is often associated with point mutations in α-tubulin (Anthony and Hussey, 1999; Morrissette *et al.*, 2004). These observations have been combined with computational tubulin modelling to propose dinitroaniline-binding sites on plant (Blume *et al.*, 1998) and apicomplexan (Morrissette *et al.*, 2004) α-tubulins, in two separate locations. Neither of these sites has been experimentally confirmed and the modelling studies suffer from certain drawbacks. First, resistance resulting from these mutations may not affect the dinitroaniline binding site itself but could merely stabilize the microtubules, diminishing the effect of the herbicides. Second, the models were based on the known three-dimensional structure of the bovine tubulin heterodimer, to which the herbicides do not bind with high affinity: therefore, the regions alleged to contain dinitroaniline-binding sites are also those about which there is least structural certainty. A third issue is raised by *Chlamydomonas reinhardtii*, where most cases of dinitroaniline resistance are linked to mutations which map to loci other than tubulin genes (James and Lefebvre, 1989; James *et al.*, 1989; Lux and Dutcher, 1991; James and Lefebvre, 1992).

1.4.5.2. "*Vinca*" domain agents

The "*Vinca* domain" refers to a region on the tubulin subunits where a number of structurally highly dissimilar compounds are proposed to bind. These agents all show varying degrees of competitive inhibition of each other's binding to tubulin and because of such differences, it has been proposed that their binding sites do not overlap completely (Downing and Nogales, 1999).

The vinca alkaloids, originally isolated from the periwinkle *Catharanthus roseus*, were the first class of compounds to bind this site giving it its name. Vinblastine, vincristine and vinorelbine are used in the treatment of numerous cancers including lymphomas, leukaemia and some solid tumours (Verrills and Kavallaris, 2005). Vinblastine is perhaps the most extensively studied of these agents and is known to bind rapidly, reversibly and with high affinity to a single site at the interface between two tubulin heterodimers (Gigant *et al.*, 2005) while weakly inhibiting GTP hydrolysis. Vinblastine is effective at both substoichiometric concentrations, where it binds to free

protofilament ends interfering with microtubule dynamics without resulting in depolymerisation (Dumontet, 2000) and higher concentrations where it binds stoichiometrically to tubulin subunits preventing their assembly and promoting microtubule dissassembly (Downing, 2000). Dolastatin 10, originally isolated from *Dolabella auricularia* (sea hare), and several synthetic derivatives have shown potent antimicrotubule activity affecting tubulin-dependent GTP hydrolysis and microtubule assembly by binding near the vinblastine binding site.

Agents binding to the "*Vinca*" domain have shown potent antimalarial activity with IC_{50} values ranging from high pM (for dolastatin 10 and certain auristatins (Fennell *et al.*, 2003)) to between 0.028 to 1.7 μM for vinblastine (Bell, 1998) against the asexual stages of the parasite (Table 1.3). Both vinblastine and dolastatin were shown to exhibit maximum toxicity when applied at the trophozoite stage (Usanga *et al.*, 1986; Fennell *et al.*, 2003) and both were shown to disrupt cellular microtubular structures by mass microtubule depolymerisation (Bell, 1998; Fennell *et al.*, 2003). Vinblastine treatment of *P. nigeriensis* caused a loss of axonemal and spindle microtubular structures resulting in an inhibition of male gametogenesis (Sinden *et al.*, 1985). While these agents do inhibit *Plasmodium,* and so are useful as tools for studying microtubule dynamics, there potency against the mammalian host limits their potential for clinical antimalarial use.

1.4.5.3. Taxol-site agents

Taxol (paclitaxel), a complex poly-oxygenated diterpene, is a natural product isolated from the bark of the western yew *Taxus brevifolia.* It has shown potent inhibitory activity against a wide range of higher and lower eukaryotes and, along with taxotere (docetaxel), a more soluble cogener, has become an effective tool in the treatment of several types of cancer (Saville *et al.*, 1995). Taxol supresses microtubule dynamics either by promoting assembly or preventing depolymerisation of microtubular structures and results in cell death by apoptosis at lower concentrations and necrosis at higher ones (Yeung *et al.*, 1999). It interacts with polymerised microtubules, binding to a hydrophobic pocket in the intermediate domain of β-tubulin situated on the luminal side of the microtubule wall (Nogales *et al.*, 1998; Ganesh *et al.*, 2004). Competitive inhibition assays have indicated a number of antimicrotubule agents including epothilones and eleuthesides have a similar binding site and mechanism of action to taxol, and these have also shown potential as anti-cancer agents (Bergstralh and Ting,

2006). Few, if any agents known to bind at or near the taxol site have been tested against protozoal parasites, perhaps due to the toxicity of these to against host cells.

Of the taxol-site agents, only the effects of taxol and taxotere have been studied to any great extent, and both have proved effective against a wide range of protozoal parasites (table 1.3) with activities comparable if not superior to agents currently in use. Cellular studies indicated that the protozoal target of these agents appeared to be tubulin (reviewed in Werbovetz 2002) and studies with purified tubulin from *Leishmania* confirmed an interaction (Kapoor *et al.*, 1999).

Against asexual erythrocytic stages of *P. falciparum*, taxol has an IC_{50} of between 0.071 and 1 μM while taxotere, a semisynthetic taxol derivative, is more potent with an IC_{50} range of between 3.1 and 5 nM, although both agents required 72 h to exhibit these levels of potency (Pouvelle *et al.*, 1994; Schrevel *et al.*, 1994). Studies in rodent models of *Plasmodium* have shown effective parasite clearing by taxol and taxotere, albeit at levels close to host toxicity (Pouvelle *et al.*, 1994; Sinou *et al.*, 1996). As with the "*Vinca*" domain binding agents, few additional studies with taxol and related agents have been carried out *in vivo* due to the toxicity these compounds exhibit against host cells (table 1.4).

1.4.5.4. Colchicine-site agents

Colchicine, a tricyclic acetylated alkaloid is a natural product produced by members of the lily family including *Colchicum autumnale* (autumn crocus). It is highly active against mammalian cells (in the sub-micromolar range) and, as with other microtubule inhibitors, blocks mitosis by disrupting normal microtubular structures (Morejohn and Fosket, 1991). It binds to a single high-affinity site on unpolymerised β-tubulin monomer (Ravelli *et al.*, 2004). As with vinblastine, colchicines disrupts microtubules both at substoichiometric and stoichiometric concentrations (Downing, 2000). A number of antimitotic *Colchicum* alkaloids have been found which also display high levels of activity against mammalian tubulin. Therapeutic use of these agents for treatment of cancer and other ailments is limited as the concentrations required for activity are close to the limits for toxicity (Nagle *et al.*, 2006; Kappes and Rohrbach, 2007). The benzimidazoles which bind at or close to the same site are more successful as therapeutic treatments for helminth infections and are currently among the most commonly used agents for the treatment of such infections (Katiyar *et al.*, 1994).

Activity of colchicines and related agents against *Plasmodium* is markedly less than the toxicity exhibited against mammalian cells. Against erythrocytic stages of *P. falciparum* colchicine has an IC_{50} range of between 10 and 13 μM and colcemid is even less potent with an IC_{50} range of between 10 and 110 μM (Kappes and Rohrbach, 2007). The benzimidazoles also exhibit only modest activity against *Plasmodium* (Dieckmann-Schuppert and Franklin, 1989). This difference in potency between mammalian and *Plasmodium* tubulin (and indeed plant tubulin) indicates that the site to which these agents bind may not be as conserved in the tubulin of *Plasmodium* and plants and so as a potential antimalarial target site, this holds little promise.

1.5. PROJECT OBJECTIVES

The cell cycle of any eukaryotic cell is essential for its growth and propagation. This is true for the disease causing stages of *P. falciparum*, where erythrocytic schizogony results in the formation of new merozoites. The potential for novel antimalarial targets in this process may be hindered by our lack of understanding of the processes involved. Microtubules are known to play a vital role in the cell cycle and the potent antimalarial activity of certain classes of microtubule inhibitor demonstrates the potential of these structures as antimalarial targets. The selective nature of two classes of antimitotic herbicide has shown that these may represent the best chance of developing novel antimalarial agents targeting these structures.

The work documented in this thesis was undertaken to investigate means of studying the cell-cycle as a whole and the potential of microtubule inhibiting herbicides as a possible basis for developing novel antimalarial chemotherapy. This project had three main aims:

1) to determine if batch synchronisation methods could result in populations of parasites synchronised to a single cell-cycle stage;
2) to characterise the cellular effects of several dinitroaniline and phosphorothioamidate herbicides on the erythrocytic stages of *P. falciparum*;
3) to determine if the potencies of two antimitotic agents, one dinitroaniline and one vinca alkaloid against *P. falciparum* were related to the ability of the parasites to accumulate each agent.

Chapter 2

Materials and Methods

2.1. CHEMICALS, REAGENTS AND INHIBITORS

Chemicals and reagents used in this project were purchased from Sigma-Aldrich (Dublin, Ireland), unless otherwise stated. All general chemicals were of analytical grade. Water used was filter-sterile, double deionised (ddH_2O), and was dispensed from a Milli-Q Synthesis A10 (Millipore, Billerica, Massachusetts, USA). Trifluralin was a gift from Dow Elanco, UK. Glycoconjugated trifluralin derivatives (Mead *et al.*, 2003) were the gift of Dr. Jan Mead, Atlanta VA Medical Center, Decatur and Department of Pediatrics, Emory University, Atlanta, Georgia, USA. CN trifluralin derivatives and liposomal trifluralin were the gift of Dr. Eugénia Cruz, INETI, Estrada Paço Lumiar, 22, 1649-038, Lisboa, Portugal. Pancratistatin prodrug (Pettit *et al.*, 1995) was a gift from Dr. George Petit (Cancer Research Institute, Arizona State University, Tempe, Arizona, USA) (Torres-Labandeira *et al.*, 1991). All reagents used during electrophoresis were of electrophoresis grade, unless otherwise stated. All chemicals used for cell culture were of cell culture grade.

2.2. CELL CULTURE, PREPARATION AND ANALYSIS

2.2.1. Routine culture of *P. falciparum*

P. falciparum strain 3D7 (obtained from M. Grainger, National Institute of Medical Research, London, U.K.) adapted to grow in Albumax (Gibco, Auckland, New Zealand), was cultivated in continuous culture in human O^+ erythrocytes (obtained from the National Blood Centre, Dublin, as needed) at a haematocrit of 2.5% or 5% (v/v), according to the method of Trager and Jensen (Trager and Jensen, 1976). Whole blood was washed weekly using a Sorvall RT6000D benchtop centrifuge (Du Pont Ltd., Stevenage, Herts, UK). Prior to washing, erythrocytes were incubated in PIGPA solution (50 mM sodium pyruvate, 50 mM inosine, 100 mM glucose, 500 mM disodium hydrogen phosphate, 5 mM adenine, 0.72% (w/v) sodium chloride) for 1 hour at 37°C by combining 10 parts whole blood with 1 part PIGPA solution. Erythrocytes were then washed by centrifugation at 2000 rpm (650 x *g*) at 4°C for 10 min, removing the buffy coat, washing twice in sterile, cold phosphate-buffered saline (PBS) (OXOID Ltd., Basingstoke, Hampshire, UK), and once in complete culture medium by centrifuging as before and removing the buffy coat each time. Washed erythrocytes were resuspended to 50% haematocrit in complete culture medium (see below) and stored at 4°C. To

check for the presence of yeast or bacterial contamination, a sample of washed erythrocytes was added to complete medium and incubated at 37°C for 24 hours after which Giemsa-stained smears were prepared. Smears were made on twin frosted microscope slides (76 x 26 mm) (Western Laboratory Service, Aldershot, Hampshire, UK) which were stained with a 1:10 dilution of Giemsa stain (Fluka Chemie AG, Buchs, Switzerland) in Giemsa buffer (1% (w/v) $Na_2HPO_4.2H_2O$, 1% (w/v) KH_2PO_4). Parasites were cultured routinely in complete medium, pH 7.0, which consisted of RPMI 1640 medium, supplemented with 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.002% (v/v) gentamicin, 0.18% (w/v) sodium bicarbonate, 50 µg/ml hypoxanthine and either 5% (w/v) Albumax® or 10% human serum (heat inactivated at 56°C for 30 min). Parasites were maintained in petri dishes (Sarstedt Ltd., Driminagh, Co. Wexford) at 37°C in a candle jar. Parasitemia was observed by microscopic assessment of Giemsa-stained smears. Routine cultures were diluted with fresh erythrocytes twice a week or as needed, and culture medium was replaced depending on parasitaemia.

2.2.2. Age-selection of parasites

Under normal conditions, routine cultures of erythrocytic *P. falciparum* typically consist of an asynchronous population of parasites i.e. rings, trophozoites, schizonts, segmenters and a few free merozoites. To reduce the age-range of parasites in the population, two-step sorbitol treatment (Lambros and Vanderberg, 1979) was employed. Cultures to be treated were centrifuged at 2000 rpm (650 x *g*) for 10 min at room temperature using a Sorvall RT6000D benchtop centrifuge, the supernatants removed and the resulting pellets resuspended in warm filter-sterilised 5% (w/v) D (-) sorbitol (Merck, Darmstadt, Germany) and left at room-temperature for 5 min to accumulate in host erythrocytes resulting in the osmotic lysis of those containing mid/late trophozoite-, schizont- and segmenter-stage parasites. The suspension was centrifuged as before, the resulting supernatant removed, and the pellet resuspended in warm wash medium (RPMI 1640 medium supplemented with 25 mM HEPES, 0.18% (v/v) sodium bicarbonate, and 50 µg/ml hypoxanthine). The suspension was centrifuged again as before and the resulting pellet resuspended in complete culture medium to 2.5% haematocrit and cultured as normal. A single round of sorbitol treatment is estimated to reduce the age of the culture from 0 - 46 h (the cycle in this line lasts ~46 h) to ~ 0 - 20 h post-invasion. Cultures with a narrower age-range were

obtained by repeating sorbitol treatment a second time. The treatment itself takes ~1.5 hours and the gap between treatments is calculated as the time between the end of the first sorbitol treatment to the start time of the second. To achieve populations ~18-28 h old (trophozoite cultures used in section 2.4.5), double sorbitol treatment was carried out 38 hours apart, followed by parasite maturation and multiplication for an additional 64 hours. For cultures used in assessing cell-cycle inhibitors (described in section 2.3) and in microtubule inhibitor stage-specific susceptibility assays as described in section 2.4, a narrow age range was achieved by repeated sorbitol treatments 34 hours apart, followed by parasite maturation and multiplication for an additional 46, 49, 52, and 58 hours (for rings ~ 0 - 6, 3 - 9, 6 - 12, and 12 - 18 h post-invasion), 64, 65, 70, and 76 hours (for trophozoites ~ 18 - 24, 19 - 25, 24 - 30, and 30 - 36 h post-invasion) and finally 80, 82 and 88 hours (for schizonts/segmenters ~ 34 - 40, 36 - 42 and 42 - 48 h post-invasion). The extra 46 h incubation time, in addition to that needed for the cells to achieve the required age, was to increase the parasitaemia and allow recovery of the parasites from the sorbitol treatment to ensure those used in subsequent experiments are healthy at the outset.

2.2.3. Isolation of infected erythrocytes by Percoll®-alanine gradients

Percoll®-alanine density gradients can be used to separate infected from uninfected erythrocytes (Kutner *et al.*, 1985; Kanaani and Ginsburg, 1989). Cultures of 15-30% parasitaemia were centrifuged at 2000 rpm (650 x *g*) for 10 min at room temperature using a Sorvall RT6000D benchtop centrifuge, the supernatants removed and the resulting pellets washed in warm wash medium (RPMI 1640 medium supplemented with 25 mM HEPES, 0.18% (v/v) sodium bicarbonate, 50 µg/ml hypoxanthine). Pellets were suspended in warm PBS supplemented with 3% (w/v) alanine to a final haematocrit of 50%. A stock solution of 90% (v/v) Percoll® (Amersham Biosciences, Uppsala, Sweden), 3% (w/v) alanine and 0.595% (w/v) HEPES in RPMI 1640 was used to make a series of solutions containing 80%, 70% and 60% Percoll® by diluting the stock in a solution of PBS containing 3% (w/v) alanine. Gradients were made by careful layering of each solution beginning with the highest concentration of Percoll® upon which the re-suspended parasite culture was gently layered. Final gradients were made with a ratio of 3:3:2:2:1 of Percoll® 90% : 80% : 70% : 60% : parasite culture. Gradients were centrifuged (without braking) at 10000 rpm (18000 x *g*) for 20 min at 30°C using a Beckman L8-M ultracentrifuge (Beckman

Instruments Inc., Harbour Boulevard, CA, USA) in a Beckman Sw40Ti rotor. Layers of interest were removed (uninfected erythrocytes were layered near the bottom of the tube while infected erythrocytes were contained in the higher layers), resuspended in warm wash medium and centrifuged at 2000 rpm (650 x *g*) for 10 min at room temperature. The pellets were washed twice more in warm wash medium before being resuspended in complete medium as required.

2.2.4. Isolation of infected erythrocytes by magnetic purification of parasitised erythrocytes

Cultures containing a high parasitaemia of late trophozoite- and schizont- stage parasites were obtained using a VarioMACS® Separator (Miltenyi Biotec, Surrey, UK). The suspension of parasitised erythrocytes were applied to a CS column. The high haem content of late-stage *P. falciparum* parasites causes them to remain in the column while it is in the magnetic field, which allows uninfected erythrocytes or those infected with ring-stage parasites to flow through. On removing the column from the magnetic field, those erythrocytes containing trophozoite- or schizont- stage parasites can easily be removed by passing wash medium through the column. Cultures of 15-30% parasitaemia, 2.5% haematocrit were centrifuged at 2000 rpm (650 x *g*) for 10 min at room temperature using a Sorvall RT6000D benchtop centrifuge, the supernatants removed and the resulting pellets washed in warm wash medium, re-centrifuged and re-suspended in warm wash medium to a haematocrit of 30-50%. The CS column (Miltenyi Biotec) of the magnet purification system was placed in the magnet, the outlet needle (0.9 mm x 40 mm 20G) and adaptor (Miltenyi Biotec) were attached, and the column was washed three times with warm wash medium ensuring that the column did not run dry at any point. The cultures to be separated were then added to the top of the column and allowed to pass through. Once all the cells had been added the column, and the loading chamber was almost empty, warm wash medium was passed through the column until the effluent was clear to the eye. The column was then removed from the magnet apparatus and washed again with warm wash medium to removed attached cells. These were centrifuged at 2000 rpm (650 x *g*) for 10 min at room temperature, washed once in warm wash medium and then resuspended in complete growth medium to the required haematocrit. A sample of the pellet was also taken to assess the parasitaemia by Giemsa staining.

2.2.5. Harvesting parasites from infected erythrocytes

Parasites were released from erythrocytes by the saponin treatment method as outlined by Zuckerman (Zuckerman *et al.*, 1967). Cultures of between 5 and 20% parasitaemia, 2.5% haematocrit, were washed twice in ice-cold PBS and sedimented by centrifugation at 2000 rpm (650 x *g*) at 4°C for 10 min using a Sorvall RT6000D benchtop centrifuge. Pellets were resuspended in 0.05% (w/v) saponin in ice-cold salt sodium citrate (SSC) (150 mM NaCl, 15 mM sodium citrate, pH 7.0) buffer and kept on ice for 20 min with vigorous shaking every 5 min for ~30 s to rupture erythrocyte membranes thus releasing the parasites. Liberated parasites were sedimented by centrifugation for 15 min at 3000 rpm (975 x *g*) at 4°C and washed twice with ice-cold SSC to remove remaining lysed erythrocytes. Parasite pellets were resuspended in PBS containing 9.5% (v/v) glycerol, 1 μg/ml pepstatin A, 21 μg/ml leupeptin and 2 mM phenylmethylsulphonyl fluoride (PMSF) or PBS containing 9.5% (v/v) glycerol alone. Parasite preparations were snap frozen in liquid nitrogen and if not used immediately for preparation of cell extracts and stored at -70°C in a freezer (Revco Ltd., Asheville, NC, USA).

2.2.6. Preparation of crude parasite extracts

Harvested parasites (see section 2.2.5) were thawed on ice to minimise protease activity and lysed by three rounds of freeze-thaw lysis followed by centrifugation for 5 min at 4°C at 14,000 rpm (18,000 x *g*), using a Sigma 1-15 microfuge, to remove insoluble debris. The resulting supernatant was clarified twice more by this process. Pellets were resuspended in PBS, pooled together and defined as the "insoluble fraction". The final supernatant was defined as the "whole cell extract" and its protein concentration was determined by the Bradford method (see section 2.6.1).

2.2.7. Flow cytometric analysis of parasitized erythrocytes

Parasite DNA content was determined by measuring propidium iodide staining as previously described (Pattanapanyasat *et al.*, 1997) with some modifications. The culture medium was aspirated off to leave the cultures in each well at ~50-75% haematocrit. The cells were resuspended in the remaining medium and a 50-μl sample of each culture to be analysed and of uninfected erythrocytes (at 50% haematocrit) was taken, placed in an eppendorf tube and centrifuged at 2000 rpm (650 x *g*) at room temperature for 2 min using a Sorvall RT6000D benchtop centrifuge. The pellets were

then washed once in warm PBS, re-centrifuged as before and the pellets resuspended 1% (w/v) paraformaldehyde in PBS. Cells were kept at 4°C for at least 4 h to allow complete fixation. Following the fixation step, the cells were centrifuged for 5 min at room temperature at 14,000 rpm (18,000 x *g*) using a Sigma 1-15 microfuge and the resulting pellets were washed twice with ice-cold PBS. The pellets were then resuspended in 100 mM glycine and incubated at room temperature for 30 min with a buffer change after 15 minutes. Cells were then centrifuged for 2 min at room temperature at 14,000 rpm (18,000 x *g*) using a Sigma 1-15 microfuge and the resulting pellets were resuspended in room-temperature PBS containing 0.25% (v/v) Triton X-100 and incubated for 2 min at 20°C. After a further wash in PBS, cells were resuspended in PBS containing propidium iodide (10 µg/ml) and RNAse (50 µg/ml) and incubated for 2 h in the dark at 37°C. The stained cells were analysed using a FACScan flow cytometer (Becton-Dickinson, CA, USA) or an EPICS XL flow cytometer (Beckman Coulter, High Wycombe, UK) and the resulting histograms analysed using FACStation 1.0 'CellQuest' Macintosh System Software 7.1.0 (Becton-Dickinson, CA, USA) or EPICS XL flow cytometry software (Beckman Coulter, High Wycombe, UK).

2.2.8. Culture of chinese hamster ovary cells

Chinese hamster ovary (CHO) cells (ATCC, Middlesex, UK) were maintained in continuous culture according to supplier's instructions, in Dulbecco modified Eagle medium-F12 (DMEM-F12) (Gibco, Auckland, New Zealand) supplemented with 10% heat-inactivated foetal calf serum (Gibco) and 50 µg/ml penicillin-streptomycin (Pen-Strep) (Gibco) in standard cell culture flasks (Sarstedt Ltd., Drimnagh, Co. Wexford) at 37°C and 5.8% CO_2 using a Forma Scientific CO_2 water-saturated incubator (Biosciences, Dublin, Ireland). Culture medium was replaced twice a week and cells re-seeded once a week. To re-seed cells, the cell layer was briefly rinsed with 0.25% (w/v) trypsin-0.53 mM EDTA (Gibco) solution to remove all traces of the serum which inhibits trypsin action. Cells were then treated in the flask with the trypsin-EDTA solution and observed under a light microscope until the cells had rounded off and dispersed. The action of trypsin was stopped by adding complete growth medium, and cells were removed from the flask, centrifuged at 1500 rpm (487.5 x *g*) using a Sorvall RT6000D benchtop centrifuge for 2 min, and the resulting pellet resuspended in complete growth medium. Appropriate amounts of the cell suspension

were added to new culture vessels and cells were reincubated at 37°C as before. The count of cells in suspension was calculated using a standard haemcytometer.

2.2.9. Assessment of effects of dinitroaniline and phosphorothioamidate herbicides on CHO cells using the MTT assay

The effects of trifluralin, oryzalin and APM on CHO cell growth were determined using the colorometric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of Mosmann (1983). The assay is based on the reduction of yellow MTT to purple formazan in the mitochondria of living cells. A solubilization solution (dimethyl sulfoxide (DMSO) BDH Laboratory Supplies, Poole, UK) is added to dissolve the insoluble purple formazan product into a coloured solution which is then quantified by measuring at 570 nm by a spectrophotometer. The assay was carried out by adding 100 µl of a suspension of CHO cells ($1x10^6$ cells/ml) in complete culture medium to each well in a 96-well flat-bottom culture plate (Sarstedt Ltd., Drimnagh, Co. Wexford) and incubated overnight to allow the cells to form a monolayer. The medium was subsequently removed and replaced by 90 µl of complete culture medium containing titrations of inhibitor. DMSO was diluted in culture medium and added to cultures at a similar percentage (0.5% (v/v) DMSO) as that used for inhibitor studies as a control for the solvents in which the inhibitor stock solutions were prepared. Cultures were then incubated for 72 h at 37°C following which 10 µl of MTT reagent were added to each well, and the culture dishes re-incubated at 37°C for a further 4 hours. At this point 100 µl of DMSO were added, the plates left in the dark at RT for a further 2 hours and then the absorbance measured at 570 nm using a Titertek Multiskan® Plus spectrophotometer (Eflab, Helsinki, Finland). CHO cells cultured in the absence of inhibitors were used as positive controls. All inhibition assays were repeated three times in duplicate. Dose-response curves for each drug were constructed from the absorbance readings, and the median inhibitory concentrations (IC_{50}) were determined graphically.

2.3. ASSESSMENT OF THE EFFECTS OF CELL-CYCLE INHIBITORS ON *P. FALCIPARUM*

2.3.1. Assessment of anti-malarial effect of cell-cycle inhibitors using the parasite lactate dehydrogenase (pLDH) assay

The effects of cell-cycle inhibitors on *P. falciparum* growth in culture were determined using the spectrophotometeric parasite lactate dehydrogenase (pLDH) assay of Makler *et al.* (Makler *et al.*, 1993). The assay is based on measurement of the biochemical reaction in which L-lactate is converted to pyruvate in the presence of parasite LDH and 3-acetyl pyridine adenine dinucleotide (APAD). In a standard assay, asynchronous parasite cultures of 0.8% parasitemia, 2% haematocrit were grown in 96-well flat-bottom culture plates (Sarstedt Ltd., Drimnagh, Co. Wexford) in complete culture medium containing titrations of inhibitor. DMSO or NH_4OH alone were diluted in culture medium and added to cultures at a similar percentage (v/v) as that used for inhibitor studies as a control for the solvents in which the inhibitor stock solutions were prepared. For the determination of pLDH activity, 10-µl samples of parasitised erythrocytes were removed following 48 and 72 h incubation, and added to 50 µl of Malstat® (Flow Inc., Portland, OR, USA), which provides APAD and L-lactate substrate. pLDH is released from the parasites into the extracellular environment resulting in the production of pyruvate from L-lactate and the reduction of APAD. Ten µl of nitro blue tetrazolium (NBT) : phenazine ethosulfate (PES) (ratio 1 : 1 of NBT (2 mg/ml) and PES (0.1 mg/ml)), were added to each sample and the presence of reduced APAD resulted in the formation of a blue formazan product that was detected using a Titertek Multiskan® Plus spectrophotometer at 650 nm. The resulting absorbance is proportional to pLDH activity, which in turn correlates with parasite growth (Makler *et al.*, 1993). Background levels of LDH from human erythrocytes were determined using uninfected erythrocytes as negative controls. Parasites cultured in the absence of inhibitors were used as positive controls for the measurement of uninhibited pLDH activity. All inhibition assays were repeated three times in duplicate. Dose-response curves for each drug were constructed from the absorbance readings, and the median inhibitory concentrations (IC_{50}) were determined graphically.

2.3.2. Effects of inhibitors on parasite progression through schizogony

Inhibitors used in this assay are outlined in Table 2.1. Inhibition of parasite progression through schizogony was determined using cultures ~12–18 h post-invasion (obtained by double sorbitol treatment as outlined in section 2.2.2) grown in 24-well microculture plates at 3.0% parasitaemia and 2.5% haematocrit. Cells were exposed to between 2 and 10 times the IC_{50} value of each agent (Table 2.1) as well as the inclusion of an untreated control and a control for the solvent in which the inhibitor stock

Table 2.1 Inhibitors used in cell-cycle experiments

Agent	Concentrations used	Target process/molecule(s)	72 h IC_{50} value (μM) (for asynchronous cultures)	Reference
Roscovitine	50-100μM	Cell cycle control/cyclin dependent kinase(s)	25	Woodard, 2003
L-mimosine	390μM	DNA synthesis/ EIF-1A hydroxylation	76	McCaffrey, 1995; This study
Hoechst 33342	200-300nM	DNA synthesis/AT-rich regions of DNA	0.021	Chen, 1993; This study
Olomucine	100 μM	Cell cycle control/cyclin dependent kinase(s)	62	Woodard, 2003
Nocodazole	100 μM	Mitosis and cytokinesis/microtubules	64	Bell, 1998
Pancratistatin prodrug	132- 440nM	Microtubules	0.044	Bell, personal communication; Pettit, 1995
Aphidicolin	5-20μM	DNA synthesis/DNA polymerase α	1	Inselburg, 1984; Ikegami, 1978
Phyllanthoside	360-1200nM	Protein synthesis	0.120	Bell, personal communication
Vinblastine	750-2500nM	Mitosis and cytokinesis/microtubules	0.250	Bell, 1998

solutions were prepared containing DMSO at the maximum percentage (0.5% v/v) as that present in the inhibitors. Following 18, 24 and 30 hours' incubation at 37°C in a candle jar, samples were taken and the parasitaemia and stage spread of each were observed by microscopic assessment of Giemsa-stained smears.

2.3.3. Effects of inhibitors on DNA synthesis

The effects of the cell-cycle inhibitors on DNA synthesis were analysed by flow cytometry as described in section 2.2.7. As with the previous section, the inhibitors used in this assay are outlined in Table 2.1. Inhibition of DNA synthesis was determined using cultures as described in section 2.3.2. Following 18, 24 and 30 hours incubation at 37°C in a candle jar samples were taken and processed for flow cytometric analysis as outlined in section 2.2.7.

2.3.4. Investigating the reversibility of the block on schizogony

Inhibitors that were found to block the cell cycle at a specific stage were then tested for reversibility. The lowest concentrations of Hoechst 33342, L-mimosine and roscovitine (0.2 μM, 390 μM and 50 μM respectively) that successfully blocked schizogony as judged by observation of Giemsa stained smears and flow cytometric analysis of DNA synthesis were used for this assay. Cultures with age ranges of ~12-18 h post invasion were grown in 24-well microculture plates at 3.0% parasitemia and 2.5% haematocrit. Cells were then exposed each of the three agents to be tested at the concentration stated above. Again an untreated control and a control for the DMSO solvent in which the inhibitor stock solutions were prepared were also included. Following 18 h incubation at 37°C in a candle jar, a sample was taken for microscopic analysis of Giemsa-stained smears. The cultures were resuspended and centrifuged at 2000 rpm (650 x *g*) at room temperature, the resulting pellets were washed three times in warm wash medium and then re-incubated in complete growth medium for a further 30 h, at which time a sample was again taken for microscopic analysis of Giemsa-stained smears. The cultures was then diluted 1:3 into fresh erythrocytes and incubated for a further 48 h as before, at which time samples were again taken for microscopic analysis of Giemsa-stained smears.

2.4. ASSESSMENT OF THE ANTIMALARIAL EFFECTS OF ANTIMITOTIC HERBICIDES

2.4.1. Effects of inhibitors on cultures of different initial ages

The relationship between the initial age of a culture and susceptibility to antimitotic herbicides was determined using the pLDH method as outlined in section 2.3.1. Eight distinct parasite cultures with age ranges of ~ 6 h covering the whole parasite erythrocytic cycle (0 - 6, 6 - 12, 12 - 18, 18 - 24, 24 - 30, 30 - 36, 36 - 42, 42 – 46/0-2 hours post-invasion) were obtained by double sorbitol treatment as described in section 2.2.2. These were exposed to serial two-fold dilutions of trifluralin, oryzalin and amiprophos-methyl (APM) from 64 μM to 0.125 μM in growth medium at 0.8% parasitemia and 2.0% haematocrit. Taxol (ICN Biomedicals Inc., Aurora, Ohio, USA) (8000 nM - 15.62 nM) was included as a positive control as the stage-specific susceptibility profile for this agent has already been characterised (Fennell *et al.*, 2003). After 48 h and 72 h incubation, samples of parasitised erythrocytes were taken and the pLDH activity was determined. Dose response curves were constructed and IC_{25}, IC_{50} and IC_{75} were determined for the eight initial parasite ages tested. All experiments contained duplicates and were repeated three times.

2.4.2. Effects of antimitotic herbicides on parasite morphology

Parasite cultures of trophozoite stage (20 - 26 h post-invasion) were established in 24-well microculture plates at 3.0% parasitaemia and 5.0% haematocrit. Cultures were treated with 20 μM of each inhibitor (trifluralin, oryzalin or APM). Untreated cultures established in the corresponding concentration of the solvent DMSO (0.2% v/v) were also included. Plates were incubated at 37°C and after incubation for 6, 12, 24, 30, 42 and 48 h, samples of parasitised erythrocytes were removed and used to make duplicate Giemsa-stained smears (see section 2.2.1). Smears were examined by light microscopy at 1000 X magnification using a Nikon SE microscope (The Micron Optical Co. Ltd, Co. Wexford) for obvious morphological or developmental abnormalities of parasites, and for calculating parasitaemias of inhibitor-treated and control parasites. Photographs were taken with a Nikon Coolpix 950 digital camera using full auto-exposure mode and Best Shot Selection attached to a Nikon Eclipse E400 microscope (The Micron Optical Co. Ltd.).

2.4.3. Effects of antimitotic herbicides on DNA synthesis

The effects of the antimitotic herbicides on DNA synthesis were analysed by flow cytometry as described in section 2.2.7. Parasite cultures of trophozoite stage (20 - 26 h post-invasion) were established in 24-well microculture plates at 3.0% parasitemia and 5.0% haematocrit. Cultures were treated with 20 μM of each inhibitor (trifluralin, oryzalin or APM). Untreated cultures established in the corresponding concentration of the solvent DMSO (0.2% v/v) were also included. Plates were incubated at 37°C in a candle jar and after incubation for 6, 12, 24, 30, 42 and 48 h, samples of parasitised erythrocytes were removed and processed for flow cytometric analysis as outlined in section 2.2.7.

2.4.4. Activity of inhibitors on different developmental stages

For determination of susceptibility of different stages of *P. falciparum* to antimitotic herbicides, parasites were synchronised and grown to 3 - 9, 19 - 25 and 34 - 40 h post-invasion (see section 2.2.2) and 1-ml samples of the cultures at 2.5% haematocrit and 2% parasitemia were exposed to inhibitors at ~ 7 x IC_{50} (see section 2.4.4), or corresponding concentrations of 0.2% (v/v) DMSO, for 6 h. Cultures were then washed three times in 10 ml of warm wash medium and recultured for 48 h in inhibitor-free medium, with a change of medium after 24 h. After incubation, samples of parasitised erythrocytes were removed and used to make Giemsa-stained smears as described in section 2.2.1. Slides were prepared in duplicate and the parasitaemia of at least 1000 erythrocytes was counted on each slide.

2.4.5. Assessment of effect of antimitotic inhibitors on caspase-3 activation

Caspase-3 (DEVDase; cleaves at the peptide sequence aspartic acid - glutamic acid - valine - aspartic acid) protease activity is an early regulatory event in programmed cell death. Activity of this caspase (or a homologue) has been shown to be induced in *P. falciparum* mosquito-stage parasites as consequence of stress (section 1.3). Activity of this protease was detected by the colorimetric CaspACE® Assay System (Promega, supplied by Medical Supply Co., Dublin, Ireland).

2.4.5.1. *P. falciparum*: inhibitor exposure, harvesting and isolation of cell extract

Parasite cultures (aged 18-28 h post-invasion), were grown in 6-well culture dishes at 2% haematocrit and 4% parasitaemia, and exposed to 50 nM taxol, 20 μM trifluralin or 1 μM vinblastine. Untreated cultures and treated cultures, to which 50 μM

of the potent, irreversible and cell-permeable pan-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-valine-alanine-aspartic acid (OMe) fluoromethylketone) were also added prior to exposure to the inhibitor, were included as negative controls. Following 16 h incubation at 37°C in a candle jar, parasites were isolated by saponin lysis (see section 2.2.5), pellets were washed once in ice-cold PBS and resuspended in cell lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml pepstatin A, 10 μg/ml leupeptin). Cell extract was prepared by three rounds of freeze-thaw lysis followed by 15 min incubation on ice at which point samples were centrifuged for 30 min at 4°C at 14,000 rpm (18,000 x *g*) using a Sigma 1-15 microfuge. The supernatant was removed, its protein concentration obtained using the assay of Bradford (see section 2.3.1.1) and this fraction used in the caspase detection assay.

2.4.5.2. CHO cells: inhibitor exposure, harvest and isolation of cell extract

CHO cells were grown as described in section 2.2.8, and the cell density adjusted to 10^6 cells/ml. Cells were incubated overnight to allow a monolayer to be established and then the cells were exposed to 1 μM taxol or no inhibitor and reincubated at 37°C for 6 h in a humidified, 5% CO_2 atmosphere. Cells were dispersed by trypsin-EDTA treatment (see section 2.2.8) and centrifuged at 4°C at 1500 rpm (487.5 x *g*) using a Sorvall RT6000D benchtop centrifuge for 5 min. The cell pellet was then washed once with ice-cold PBS, re-centrifuged as before and resuspended in ice-cold cell lysis buffer. Cell extract was prepared by three rounds of freeze-thaw lysis followed by 15 min incubation on ice at which point samples were centrifuged for 30 min at 4°C at 14,000 rpm (18,000 x *g*) using a Sigma 1-15 microfuge. The supernatant was removed, the protein concentration obtained using the assay of Bradford (see section 2.3.1.1) and used in the caspase detection assay.

2.4.5.3. Detection of Caspase-3 activity

The assay was performed in a total volume of 100μl in flat bottomed 96-well dishes as instructed by the manufacturers. Cell extracts were adjusted to contain 1 mg/ml total protein using caspase assay buffer (312.5 mM HEPES (pH 7.5), 31.25% (w/v) sucrose, 0.3125% (w/v) CHAPS (3-[(3-cholamido-propyl)-dimethylammonio]-1-propane-sulphonate)) and 50 μg total protein were used in the assay. Replicate wells containing blank (no cell extract), negative control 1 (extract from untreated parasites),

negative control 2 (extract from untreated CHO cells), extract from inhibitor-treated parasites, extract from taxol-treated CHO cells and extract from inhibitor- and Z-VAD-FMK-treated parasites were prepared. To each well 32 µl caspase assay buffer, 2 µl DMSO, 10 µl 100 mM DTT and dH_2O were added to give a final volume in each well of 98 µl. Two µl of the 10 mM stock of the colorimetric substrate, *p*-nitroaniline (pNA)-labelled Ac-DEVD (Ac-DEVD-pNA) were then added to all wells and the plates covered and incubated at 22°C overnight. pNA is released from the substrate upon cleavage by caspase-3 (DEVDase). Free pNA produces a yellow colour that is monitored by a spectrophotometer at 405 nm. The amount of yellow colour produced upon cleavage is proportional to the amount of DEVDase activity in the sample.

2.5. ASSESSMENT OF CELLULAR ACCUMULATION OF TRIFLURALIN AND VINBLASTINE

Parasite cultures used in these experiments were of high parasitaemia obtained by Percoll®-alanine density gradient separation (section 2.2.3) or by magnetic separation (section 2.2.4). The cell accumulation ratio (CAR) is the ratio of cell associated compound to the amount of compound remaining in a volume of supernatant equal to the calculated volume of the cells. The number of cells present in each sample was determined using a standard haemcytometer and cell volume was calculated using the estimated volume of a parasite infected erythrocyte as 75×10^{-15} (Saliba *et al.*, 1998).

2.5.1. Kinetics and extent of accumulation

Measurements of cellular uptake of [^{14}C] trifluralin (Sigma Aldrich, Dublin, Ireland; specific activity 16.8 mCi/mM) and [^{3}H] vinblastine (Amersham Biosciences, Dublin, Ireland; specific activity 9.8 Ci/mM) were carried out using cultures of intact uninfected erythrocytes of final haematocrit 1-2%, or intact trophozoite (~24-30 h post-invasion)- or schizont (~34-40 h post-invasion)-infected erythrocytes of >85% parasitaemia and 1-2% haematocrit. Cells were suspended in complete growth medium containing 3 µM [^{14}C] trifluralin or 25 nM [^{3}H] vinblastine and incubated at 37°C. Duplicate 200 µl samples were taken at graded time intervals and the reaction terminated upon centrifugation of the cells (14,000 x *g* for 2 min) through silicon oil (Dow Corning, Coventry, UK). Ten-µl samples of both the extracellular medium (which remains on top of the silicon oil) and the silicon oil were placed in separate scintillation

vials. To quantify cell-associated compound, the eppendorf tube containing the pellet was inverted and the base which contained the pellet alone clipped off, placed in a scintillation vial and the cell pellet lysed by addition of 100 µl hot dH_2O. To each scintillation vial, 3 ml of EcoLite® scintillation cocktail (ICN Biomedicals Inc., Aurora, Ohio, USA) were added and radioactivity was counted in a liquid scintillation spectrometer (Packard Tri-Carb model 2100TR, Perkin-Elmer, FOSS Ireland, Dublin, Ireland). The absolute amounts of radioactivity (dpm) were determined using either a [^{14}C] or [^{3}H] internal standard.

2.5.2. Kinetics of efflux and reversibility of accumulation

To determine the proportion of compound reversibly accumulated, cultures of intact uninfected erythrocytes of final haematocrit 1-2%, or intact schizont (~34-40 h post-invasion)-infected erythrocytes of >85% parasitaemia and 1-2% haematocrit were suspended in warm complete medium containing either 3 µM [^{14}C] trifluralin or 25 nM [^{3}H] vinblastine. Following 2h incubation at 37°C, duplicate 200 µl samples were removed for determination of total cell-associated compound by centrifuging through silicon oil and processing for scintillation counting as above and the remaining suspension separated into 200-µl aliquots. To measure reversible accumulation each aliquot was washed by centrifugation (14,000 x *g*, 2 min) and resuspended in 200 µl of warm RPMI 1640 supplemented with 50 µg/ml sodium bicarbonate, with two of the aliquots removed at each point for determination of cell-associated compound. Cell-associated radioactivity was determined by centrifuging through silicon oil and scintillation counting as described in section 2.5.1.

Rates of compound release were measured using cultures of intact uninfected erythrocytes of final haematocrit 1-2%, or intact schizont (~34-40 h post-invasion)-infected erythrocytes of >85% parasitaemia and 1-2% haematocrit resuspended in 3 µM [^{14}C] trifluralin or 25 nM [^{3}H] vinblastine. Following 2 h incubation at 37°C, duplicate 200-µl samples were removed for determination of total cell-associated compound at time zero. Following centrifugation of the remaining culture (14,000 x *g*) and resuspension in warm growth medium, the resulting cell suspensions were incubated at 37°C. At graded intervals 200-µl samples were removed, the cells were separated from the extracellular medium by centrifuging through silicon oil and the cell-associated radioactivity and that in the extracellular medium were determined by scintillation counting as described in section 2.5.2.1.

2.5.3. Saturability of accumulation

Intact uninfected erythrocytes or schizont-infected erythrocytes (parasites aged 34-40 h post-invasion) (>90% parasitaemia) were suspended in complete medium containing either 0.1 μM [^{14}C] trifluralin and increasing concentrations of unlabeled trifluralin or 5 nM [^{3}H] vinblastine and increasing concentrations of unlabeled vinblastine to a final haematocrit of 1-2%. Cells were incubated at 37°C for 2h at which time duplicate 200-μl samples were taken from each. Cellular accumulation of the radiolabeled compound was measured as before.

2.5.4. Sub-cellular locations of cell-associated microtubule inhibitors

To determine the approximate subcellular distribution of each compound, erythrocytes were suspended in warm complete medium with either 3 μM [^{14}C] trifluralin or 25 nM [^{3}H] vinblastine. Following 2h incubation at 37°C parasites were released from infected erythrocytes using saponin lysis (see section 2.2.5. for details) and resuspended in ice-cold PBS. Crude parasite extracts were then prepared immediately by three cycles of freeze-thaw lysis followed by centrifugation at 14,000 x *g* for 1 h at 4°C. The supernatant (soluble fraction) was placed in a clean eppendorf tube and the pellet (particulate or insoluble fraction) was resuspended in warm PBS. Samples from both fractions were taken, placed in scintillation vials with 3ml of EcoLite® scintillation fluid and the radioactivity determined by scintillation counting. Protein concentrations of all fractions was obtained using the Bradford assay (see section 2.6.1). Distribution of tubulin was determined by SDS-PAGE analysis of 10-μl samples containing followed by western blotting using anti-β-tubulin antibodies (affinity purified rabbit antiserum to a synthetic *P. falciparum* β-tubulin peptide) (described in sections 2.6.2 and 2.6.3).

2.5.5. The effect of increased parasitaemia on the IC_{50} for trifluralin

The IC_{50} of trifluralin against cultures of asynchronous parasites of 2% haematocrit and starting parasitaemia of 0.2 or 1.5% and were obtained using the pLDH method as described in section 2.3.1.

2.6. PROTEIN QUANTITATION AND ANALYSIS

2.6.1. Protein quantitation using the assay of Bradford

Protein concentrations were determined using the colourimetric assay of Bradford (Bradford, 1976), which is based on measuring the binding of Coomassie Brilliant Blue G250 to the protein of interest. A bovine serum albumin (BSA) standard curve was used to determine protein amounts in experimental samples. The assay involved a series of BSA solutions of 15.0, 12.5, 10.0, 7.5, 5.0, 2.5 and 1.0 μg/ml in a final volume of 100 μl of PBS diluted from a filter-sterile stock solution of 0.5 mg/ml BSA. Experimental samples used for protein quantification were also diluted in PBS to a final volume of 100 μl. One ml of Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 0.25% (v/v) ethanol and 10% (v/v) orthophosphoric acid) was added to experimental samples and BSA standards, with vortexing to ensure sufficient mixing. Samples and standards were incubated at room temperature for 20 min and absorbances determined at 595 nm in a Shimadzu UV-1601PC spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

2.6.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoretically separated by SDS-PAGE according to the method of Laemmli (Laemmli, 1970) using 10% (w/v) acrylamide mini-gels (9cm x 9 cm) to ensure maximum resolution of the proteins of interest. Samples were solubilised in an equal volume of 2x reducing SDS sample loading buffer (0.125 M Tris-HCl, pH 6.8, 2.3% (v/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.009% (w/v) bromophenol blue as a tracking dye), heated to 90 - 100°C for 10 min, cooled to room temperature, then centrifuged at 10,000 rpm (9,000 x g) for 5 s before being loaded on the gel. A mixture of proteins of known molecular weight (New England Biolabs, Hertfordshire, UK) was loaded on each gel as reference standards (Tables 2.2 and 2.3). The protein samples (10 - 20 μl) were electrophoresed at 100 V through the stacking gel, then 147 V through the separating gel, using an EC105 power supply pack (E-C Apparatus Corp., Holbrook, New York, USA) until the sample tracking dye reached the bottom.

2.6.3. Visualisation of protein samples on polyacrylamide gels

SDS-PAGE gels were stained with filtered Coomassie blue reagent: 0.15% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) methanol (BDH), 10% (v/v) glacial acetic acid (BDH), overnight, followed by destaining with 20% (v/v) methanol, 7.5% (v/v)

Table 2.2. Standard Broad-Range molecular weight protein markers

Protein	Source	MW (Da)
Myosin	rabbit muscle	212,000
[a]MBP-β-galactosidase	*Escherichia coli*	158,194
β-Galactosidase	*E. coli*	116,351
Phosphorylase *b*	rabbit muscle	97,184
Albumin	Bovine serum	66,409
Glutamic dehydrogenase	bovine liver	55,561
[a]MBP2	*E. coli*	42,710
Lactate dehydrogenase M	porcine muscle	36,487
Triosephosphate isomerase	rabbit muscle	26,625
Trypsin inhibitor	soybean	20,040
Lysozyme	chicken egg white	14,313
Aprotinin	bovine lung	6,517
Insulin A, B chain	bovine pancreas	2,340

[a]MBP: Maltose-binding protein

Table 2.3. Prestained Broad Range molecular weight protein markers

Protein	Source	MW (Da)
[a]MBP-β-galactosidase	*E. coli*	175,000
[a]MBP-paramyosin	*E. coli*	83,000
Glutamic dehydrogenase	bovine liver	62,000
Aldolase	rabbit muscle	47,500
Triosephosphate isomerase	rabbit muscle	32,500
β-Lactoglobulin A	bovine milk	25,000
Lysozyme	chicken egg white	16,500
Aprotinin	bovine lung	6,500

[a]MBP: Maltose-binding protein

glacial acetic acid until the background was clear. Gels were photographed with the Alphaimager 2200 (Alpha Innotech Corp.) and dried onto cellophane (Bio-Rad Laboratories, Hercules, CA, USA).

2.6.4. Western immunoblotting

Following electrophoresis (section 2.6.2), unstained polyacrylamide gels were soaked in transfer buffer (1.44% (w/v) glycine, 0.30% (w/v) Tris-HCl, pH 8.3, 20% (v/v) methanol) and sandwiched with polyvinylidiene difluoride (PVDF) membrane (Roche Diagnostics GmbH, Mannheim, Germany) previously treated in 100% (v/v) methanol for 5 s. The gel-membrane sandwich was then inserted into a blotting tank with ~ 1 l transfer buffer and transferred overnight at 100 V, 4°C with an ice block present to keep the tank from over-heating. After transfer, the blot was blocked in 5% (w/v) skimmed milk in Towbin's buffer (10 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl) overnight with shaking. Primary antibodies (affinity-purified rabbit antiserum to a synthetic *P. falciparum* β-tubulin peptide) used were diluted to a final concentration of 1 : 4000 in 3% (w/v) skimmed milk in Towbin's buffer, and applied to the blocked membrane for 2 h. The blot was washed 5 - 10 times for 25 min in Towbin's buffer with 0.05% (v/v) Tween 20. These steps were repeated for the secondary antibody, which in all cases was goat anti-rabbit immunoglobulin–peroxidase (GARIg-PO) diluted to a final working concentration of 1 : 2000. Bands were detected using a chemiluminescence system (Roche) according to the manufacturer's instructions in a darkroom with use of safety lights. The blot was exposed to X-OMAT UV film (Kodak, Dublin) for the desired length of time prior to passage through a Kodak X-OMAT 1000 automatic developer which involved automatic carriage of X-ray film through Industrex developer (Kodak) for 30 s, washing in dH_2O for 30 s and passage through Industrex fixative (Kodak) for 30 s. The film was air-dried and photographed using an Alphaimager 2200 (Alpha Innotech Corp.).

Chapter 3

Investigations into Cell Cycle Synchronisation of *P. falciparum*

3.1. INTRODUCTION

Eukaryotic cell-cycle progression has been extensively studied (Baserga, 1999; Puri, 1999; Golias *et al.*, 2004) and the methods used to study it well documented (Johnson *et al.*, 1993; O'Connor and Jackman, 1995). Attempts have been made to link development of *P. falciparum* through the erythrocytic stages to the accepted model of eukaryotic cell-cycle progression (Leete and Rubin, 1996; Arnot and Gull, 1998; Doerig *et al.*, 2000; Doerig, 2004). Despite the discovery of apparent *P. falciparum* homologues of known eukaryotic cell-cycle regulatory proteins (Doerig, 2004), aspects of schizogony, including the apparent asynchronous division of multiple nuclei in a single parasite (Rao and Johnson, 1970; Read *et al.*, 1993), have thus far indicated that the parasite does not follow the consensus model of eukaryotic cell-cycle progression (see section 1.2.2).

While a variety of tools exists to allow the study of the cell cycle, non-inhibitor based methods used in mammalian cells, yeasts and other organisms, including mitotic shake off (Urakawa *et al.*, 2004) and centrifugal elutriation, are not applicable in the case of *P. falciparum* due to the intracellular nature of the stages of interest. Double-thymidine block is another non-inhibitor based method that is commonly used (O'Connor and Jackman, 1995) but as *Plasmodium* sp. synthesise their own pyrimidines this method cannot be applied to these parasites either. Synchronising cells in a population by using inhibitors of a specific target or event necessary for progression to the next cell-cycle phase has been widely used in mammalian cells among others for many years, therefore this approach was adopted. The method is based on the assumption that a series of checkpoints exists in the cell, which control whether it progresses to the next stage of the cycle or not. For example, exit from S phase (when DNA synthesis occurs) can be prevented by DNA damage (Gewirtz, 1993; Bishay *et al.*, 2000).

The aim of the work described in this chapter was to assess whether this technique could result in a reversible block of cell-cycle progression resulting in populations of intra-erythrocytic *P. falciparum* parasites synchronised to specific cell-cycle stages. Isolation of such parasites would allow the study of cell-cycle-stage specific gene and protein expression, as well as characterisation of how the microscopically-defined stages of development in the erythrocyte relate to the cell cycle. Aphidicolin was used in a previous report of an attempt to develop a technique in *P.*

falciparum but while recovery from the treatment was reversible it was felt by Graeser *et al.* that the time taken for recovery was too long (Graeser *et al.*, 1996).

A number of agents (Table 2.1), which have been used previously against mammalian cells or have known cell-cycle targets were selected for this purpose, including aphidicolin as this was the only agent used for this purpose in *P. falciparum*. Several of these were first assessed for antimalarial activity and the active compounds were added to those previously known to inhibit *P. falciparum*. The method used to determine inhibitor susceptibility was an assay based on the activity of the parasite LDH (pLDH) enzyme (Makler *et al.*, 1993). The activity of this malarial parasite enzyme correlates directly with parasite growth, and measuring this yields an indication of the metabolic state of the parasite at the time of sampling.

In order to determine the success of an agent in halting the progression through schizogony, parasite development was examined using two methods. The first was the use of microscopic analysis of Giemsa-stained smears to analyse the effect of exposure to each compound on (i) parasite morphology, (ii) nuclear numbers, (iii) the distribution of parasite stages present and (iv) parasitaemia. The second method was the use of flow cytometry to analyse the effects of treatment on DNA synthesis in the parasite populations. The addition of a DNA-binding, fluorescent dye allowed for the comparison of cellular DNA contents.

The success of a method for cell-cycle synchronisation would depend on the ability to develop a reversible block, so once candidate compounds were obtained which successfully halted cell-cycle progression at a particular stage, investigations were carried out to see if the block could be removed and the parasites would regrow synchronously. Following removal of the compound and re-incubation of the parasites, similar methods to those outlined above were used to determine if the resulting parasite populations were indeed progressing synchronously through subsequent cell-cycle stages.

3.2. RESULTS

3.2.1. Growth inhibition of *P. falciparum* by cell cycle inhibitors

3.2.1.1. Hoechst 33342

Hoechst 33342 is a DNA-binding agent whose potency against *P. falciparum* was unknown. The potency of this agent on asynchronous cultures of *P. falciparum* was

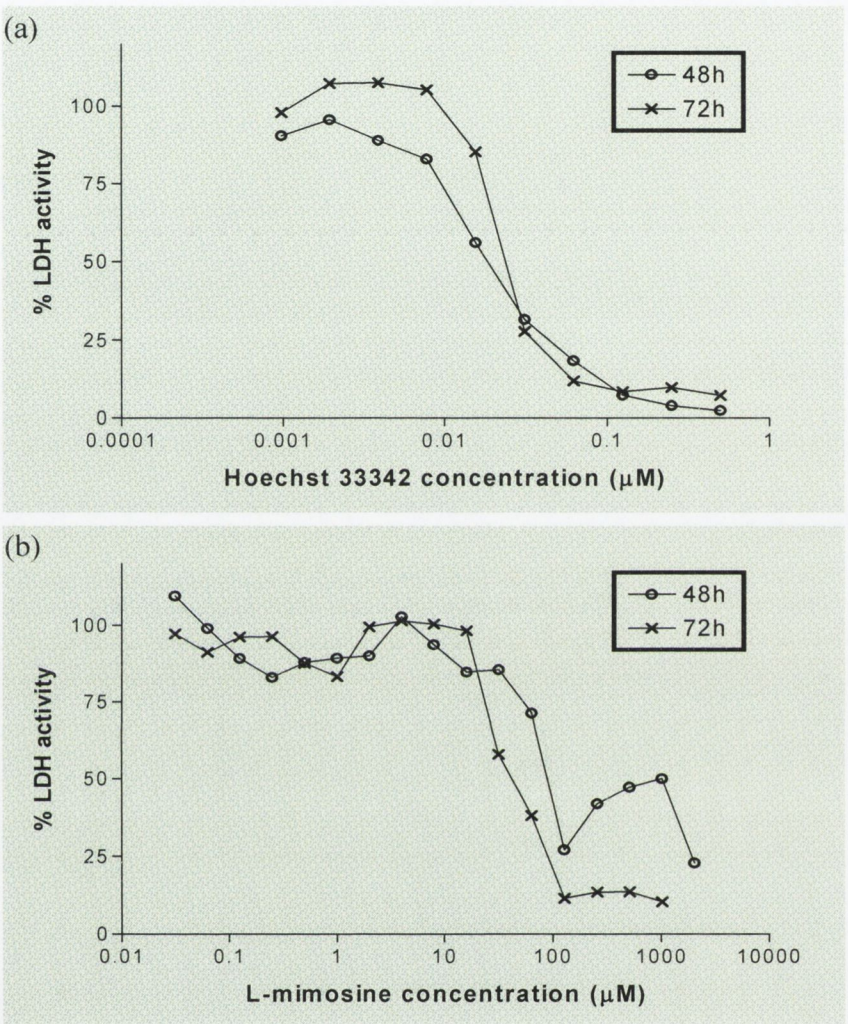


Figure 3.1. Susceptibilities of asynchronous cultures to Hoechst 33342 (a), and L-mimosine (b), as measured by the pLDH method. Arithmetic mean values of four to five determinations after 48 h (circles) and 72 h (x) are shown; the initial parasitemias were 0.8%.

elucidated using the pLDH method as outlined in section 2.3.1. Hoechst 33342 was highly active against these cultures, with 48h and 72h IC_{50}s of 0.017μM and 0.021μM respectively (Fig. 3.1).

3.2.1.2. L-mimosine

L-mimosine is a plant amino acid which inhibits specific protein hydroxylases involved in posttranslational hydroxylation of eukaryotic initiation factor-5A (eIF-5A) (McCaffrey *et al.*, 1995). It has been used successfully to inhibit cell-cycle progression in mammalian cells (Merrill, 1998). Its potential to inhibit *P. falciparum* has never been investigated. To fill this gap the effect on asynchronous cultures of *P. falciparum* was elucidated using the pLDH method as outlined in section 2.3.1. The compound was inhibitory but only at relatively high concentrations, with 48h and 72h IC_{50}s being 84μM and 76μM respectively (Fig. 3.1).

3.2.2. Optimisation of flow cytometric analysis

Previously published techniques for flow-cytometric analysis of malaria parasites have shown that accurate parasitaemias can be ascertained using propidium iodide (Pattanapanyasat *et al.*, 1997) and Hoechst 33258 (Janse and Van Vianen, 1994) as nuclear stains. However, I have found that the techniques, as they were reported, did not allow consistently accurate calculations of parasitaemia when compared to Giemsa-stained smears. The distinction between ring- and schizont-infected cultures was also unclear.

Glutaraldehyde fixation of cultures results in the presence of free aldehyde groups on the surface of the fixed cells, which interact with the dye, and a blurring of the distinction between infected and uninfected erythrocytes and between parasites of different DNA content. This effect was reduced by using formaldehyde as a cellular fixative, as this is monovalent and as such leaves fewer free aldehydes, and by incubation of the fixed cultures with glycine, which binds the free aldehyde quenching their effect. An additional problem is the tendency of more than one parasite to invade a single erythrocyte. In order to combat this, I found that by incubating the cultures on a platform shaker, multiple infections were reduced from 10-20% to less than 0.1% of infected erythrocytes. This confirmed previous reports (Jacobberger *et al.*, 1983; Bianco *et al.*, 1986; Hare and Bahler, 1986; Janse *et al.*, 1987; Dertinger *et al.*, 2000; Contreras *et al.*, 2004) allowed a distinction to be made between single parasites with multiple

nuclei and single erythrocytes containing multiple parasites. Following each modification to the technique, the parasitaemias as measured by flow cytometry and the counting of Giemsa-stained smears were compared. The parasitaemias of cultures grown using the platform shaker to reduce multiply-infected erythrocytes, fixed in formaldehyde and incubated with glycine, as measured by flow cytometry and the counting of Giemsa stained smears, matched to within <1%. This technique also allowed populations of rings (i.e. parasites containing a single nucleus) to be distinguished from asynchronous cultures) i.e. containing one or more nucleus: fig. 3.2).

3.2.3. Effects of inhibitors on parasite maturation

The purpose of this experiment was to assess the potential of each agent to stop progression of the parasite through schizogony, thus producing a synchronous population. Each agent targets a molecule or process essential for normal cell-cycle progression, or in the case of roscovitine and olomucine, inhibits proteins homologous to those involved in regulation of the eukaryotic cell-cycle. The fundamental basis for the success of this technique is the assumption of the existence of checkpoints at each stage of the cell-cycle, which control its progression.

The effect of each agent on the normal stage progression and parasitaemia of the parasites in culture was assessed using Giemsa stained smears (Fig 3.3). A narrow age range of parasites was selected and these were allowed to progress to the late ring/early trophozoite stage at which time various agents believed or expected to block cell-cycle progression were added (see section 2. for details). Agents used and their respective targets are outlined in table 2.1.

Of those agents tested only three resulted in a successful block of schizogony: Hoechst 33342, L-mimosine and roscovitine. This can be seen in Fig 3.3: no schizonts appeared in cultures treated with these agents. Pancratistatin prodrug inhibited stage progression, but it prevented ring-stage parasites developing further and so appeared to affect a target not necessarily associated with the cell cycle.

Aphidicolin and phyllanthoside appeared to prevent the majority of parasites entering schizogony, but they did not succeed in inhibiting all parasites present (Fig. 3.3).

Vinblastine and nocodazole treatment resulted in significantly reduced numbers of schizonts, but neither prevented them from appearing altogether (Fig. 3.3). The method of determining what stages of parasites were present depended on both cell size

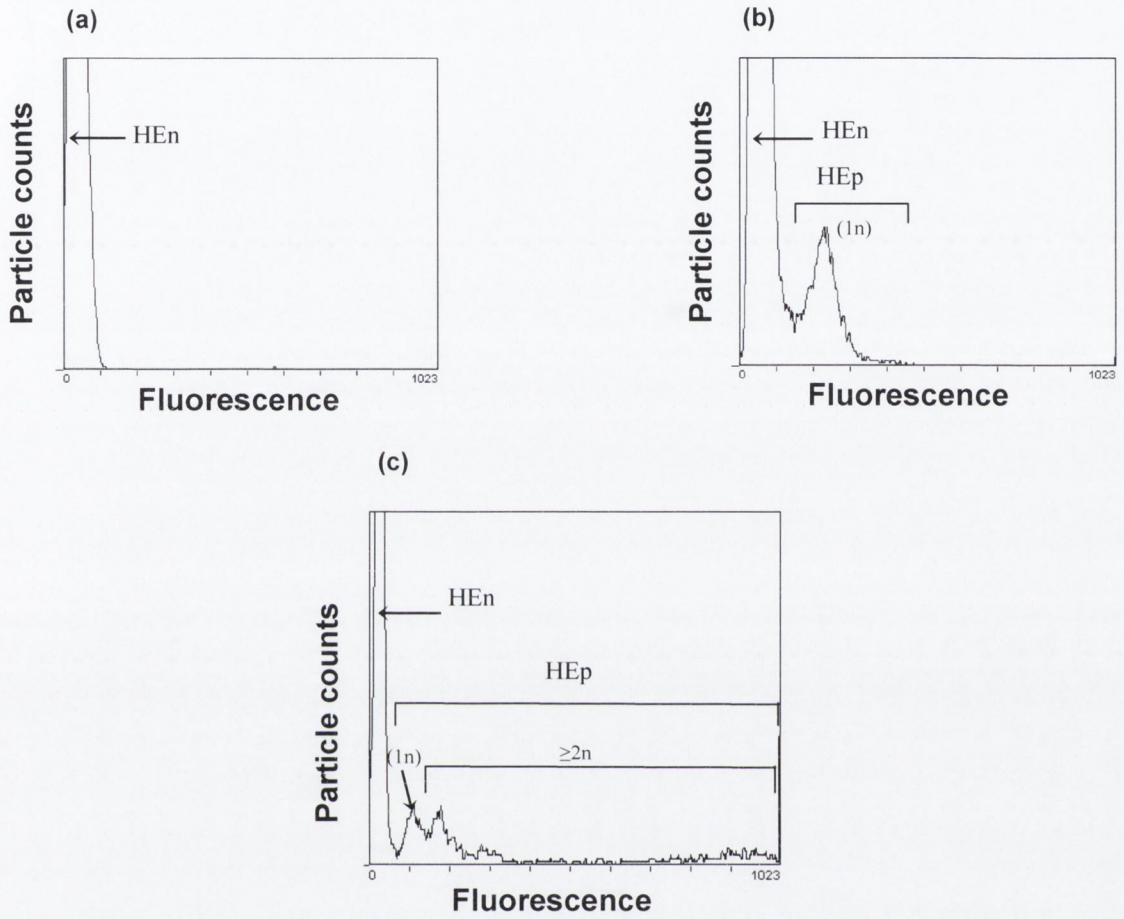
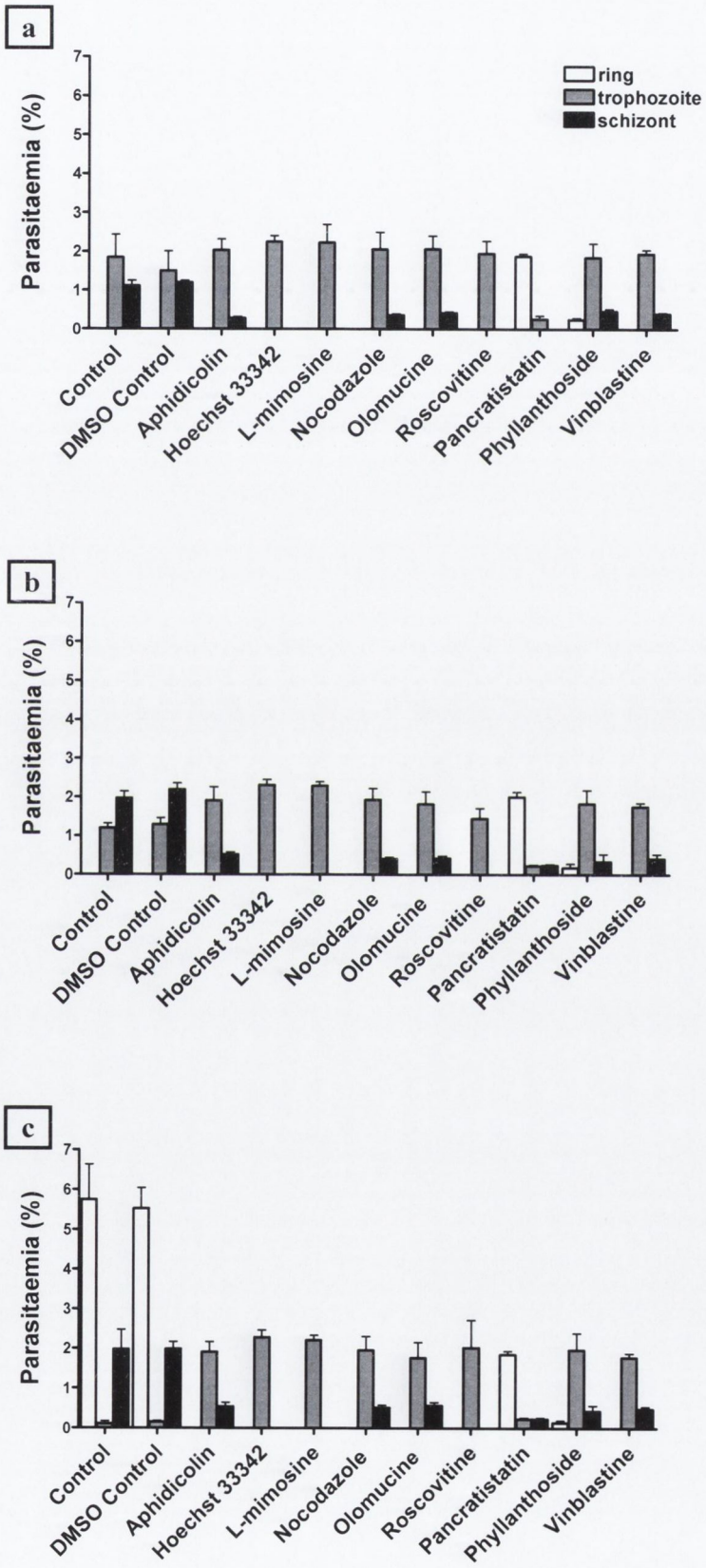


Fig. 3.2. Flow-cytometric analysis of uninfected erythrocytes (a), ring infected-erythrocytes (1n) (b) and asynchronous parasite-infected erythrocytes (1n-32n) (c). Uninfected and infected erythrocytes were fixed in formaldehyde, stained with propidium iodide and analysed using an EPICS flow cytometer (see section 2.2.7). HEn and HEp represent uninfected and infected erythrocytes, respectively.

Fig. 3.3. Effects of inhibitors on progression into schizogony. Cells were selected to an age range of 12-18 h post-invasion and incubated with the inhibitors. Samples were taken 18 h (a); 24 h (b) and 32 h (b) post-treatment for microscopic analysis of Giemsa-stained smears. Shown are results from cultures exposed to 5 μM aphidicolin; 200 nM Hoechst 33342; 390 μM L-mimosine; 100 μM nocodazole; 100 μM olomucine; 50 μM roscovitine; 132 nM pancratistatin prodrug; 360 nM phyllanthoside and 750 nM vinblastine. Results were qualitatively similar for all concentrations of each agent mentioned in Table 2.1. Shown are the mean + SD for triplicate experiments.


a
b
c
Parasitaemia (%)
ring
trophozoite
schizont
Control
DMSO Control
Aphidicolin
Hoechst 33342
L-mimosine
Nocodazole
Olomucine
Roscovitine
Pancratistatin
Phyllanthoside
Vinblastine

and number of nuclei; schizonts in cultures treated with these microtubule inhibitors appeared to have 2-3 nuclei. Both these agents disrupt microtubular structures so it is possible that staining of DNA attached to these disrupted microtubules gave the appearance of multiple nuclei.

None of the agents tested appeared to arrest the cultures exclusively in the schizont stage. Both the untreated control and parasites treated with DMSO progressed through the intra-erythrocytic cycle as expected, with trophozoites developing into schizonts which subsequently released new merozoites which in turn invaded erythrocytes, resulting in the appearance of new rings and the corresponding increase in parasitaemia.

3.2.4. Inhibitor effects on DNA synthesis

Information in addition to that obtained from the Giemsa-stained smears was needed to confirm that a cell-cycle block had indeed been achieved. The inhibition of DNA synthesis was thus assessed by staining the parasite DNA with propidium iodide and analysing these cells using flow cytometry.

As with section 3.2.3, a narrow age-range of parasites in culture was allowed to progress to late ring/early trophozoite stage at which time they were exposed to one of a number of cell-cycle inhibitors. Following 18, 24 and 32 h exposure the DNA contents of cells in each culture were analysed by flow cytometry.

The control and DMSO treated cultures (Fig. 3.4) showed that at all time points selected, cultures contained parasites with 1n and ≥2n amounts of DNA. This concurs with examination of Giemsa-stained smears which showed the presence of rings or trophozoites (containing 1n) and schizonts (≥2n). The inclusion of the DMSO-treated control ensured that this solvent at the highest concentration used had no effect on DNA synthesis.

Aphidicolin did not completely inhibit the appearance of parasites with ≥2n amounts of DNA (Fig 3.4), which concurs with the presence of a reduced number of schizonts in cultures treated with all concentrations of this agent (Fig. 3.3) seen in the Giemsa-stained smears.

Following 18 and 24 h exposure to Hoechst 33342, the peak corresponding to DNA content appears to migrate from a position corresponding to ~1n-2n to that of a 2n amount of DNA (Fig. 3.4). This would concur with previous reports that Hoechst 33342 can be used to synchronise mammalian cells to the G2 phase. This peak does however

disappear following 32 h exposure. Parasites appeared distorted under the microscope and this may result in the loss of DNA through a porous or broken membrane due to exposure to the compound. Alternatively, preparation for flow cytometric analysis may result in the membranes of already fragile treated parasites being further disrupted resulting in DNA loss. As during analysis of data through the flow cytometer, particles below a certain size threshold were discounted to prevent the inclusion of dust and debris and free DNA would be too small to register.

Results for both L-mimosine and roscovitine (Fig. 3.4) were qualitatively similar to those for Hoechst 33342, including the loss of detection of parasitized erythrocytes following 32 h exposure to either agent.

Pancratistatin prodrug inhibited parasites at the ring and trophozoite stages (Fig. 3.3) following 18 h treatment and as expected the flow cytometry results showed a fluorescence peak corresponding to parasites with a DNA content of 1n (Fig. 3.4). Following 24 and 32 h exposure Giemsa-stained smears indicated the presence of a small number of schizonts (Fig. 3.3) and a level of DNA synthesis can be seen in the flow cytometry data, with parasites containing ≥2n evident (Fig. 3.4).

Vinblastine exposure reduces the appearance of schizonts in culture when compared with the control (Fig. 3.3), and as discussed in section 3.2.1 it may be possible that the appearance of schizonts is merely caused by disrupted microtubules and their associated DNA. Parasites containing ≥2n amounts of DNA can be clearly seen in the Fig. 3.4. While it neither confirms nor negates the hypothesis mentioned above, it does indicate that a successful cell-cycle block is not achieved following treatment with this drug. As with Hoechst 33342, following 32 h exposure there is an absence of a fluorescence signal corresponding to parasitized erythrocytes. As stated above, this could be attributed to substantial membrane perturbation allowing release of DNA due to cell damage either resulting from exposure to the compound or to the preparation of cells for flow cytometric analysis.

3.2.5. Reversibility of cell cycle inhibitors

The purpose of the work described in this section was to see if a technique of analysing cells emerging from a inhibitor-induced block could be developed for *P. falciparum*. The first step of showing that Hoechst 33342, L-mimosine and roscovitine all successfully blocked both nuclear division and resulted in cells accumulating with equal amounts of DNA (presumably prior to the first S-phase in the

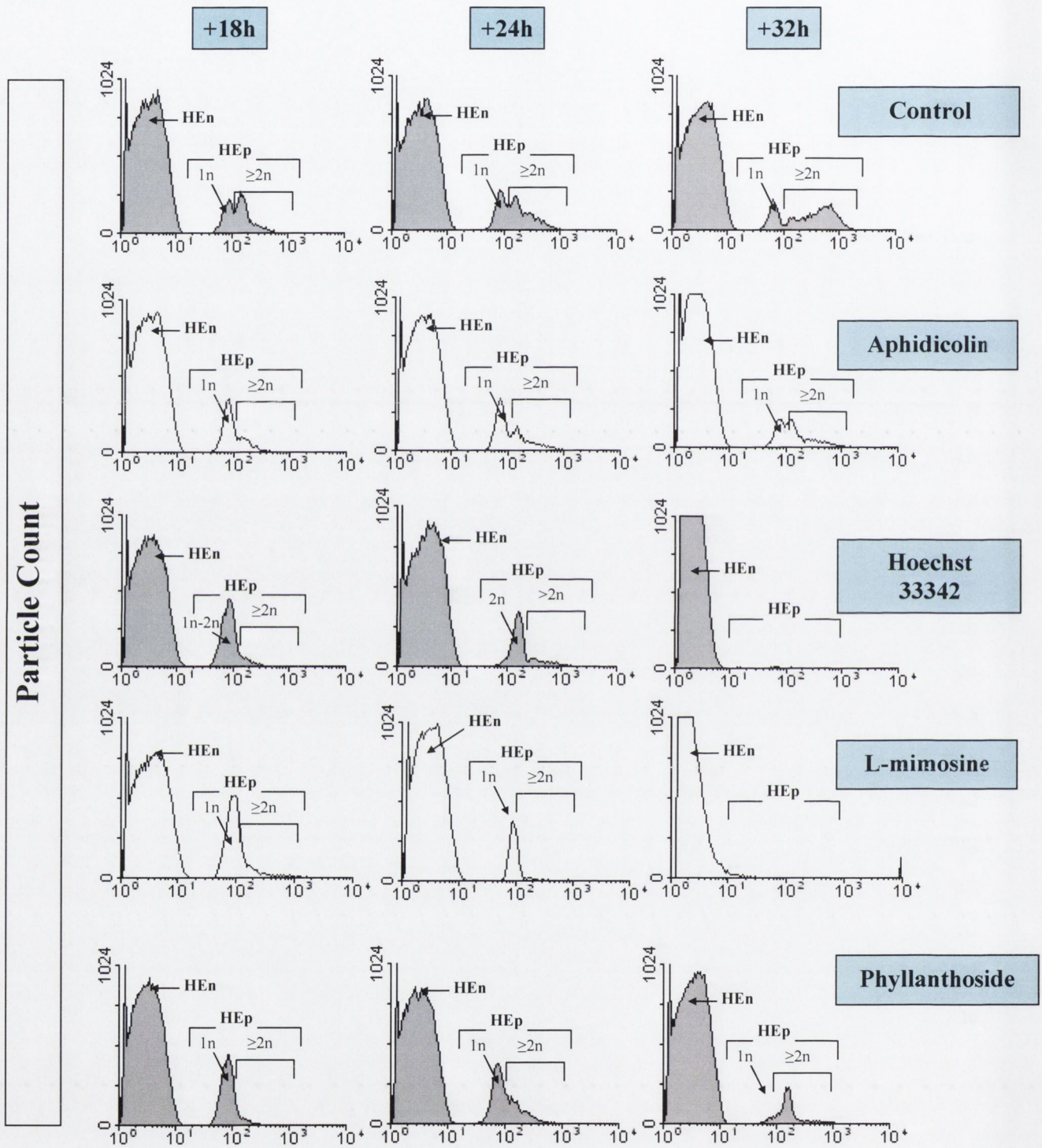
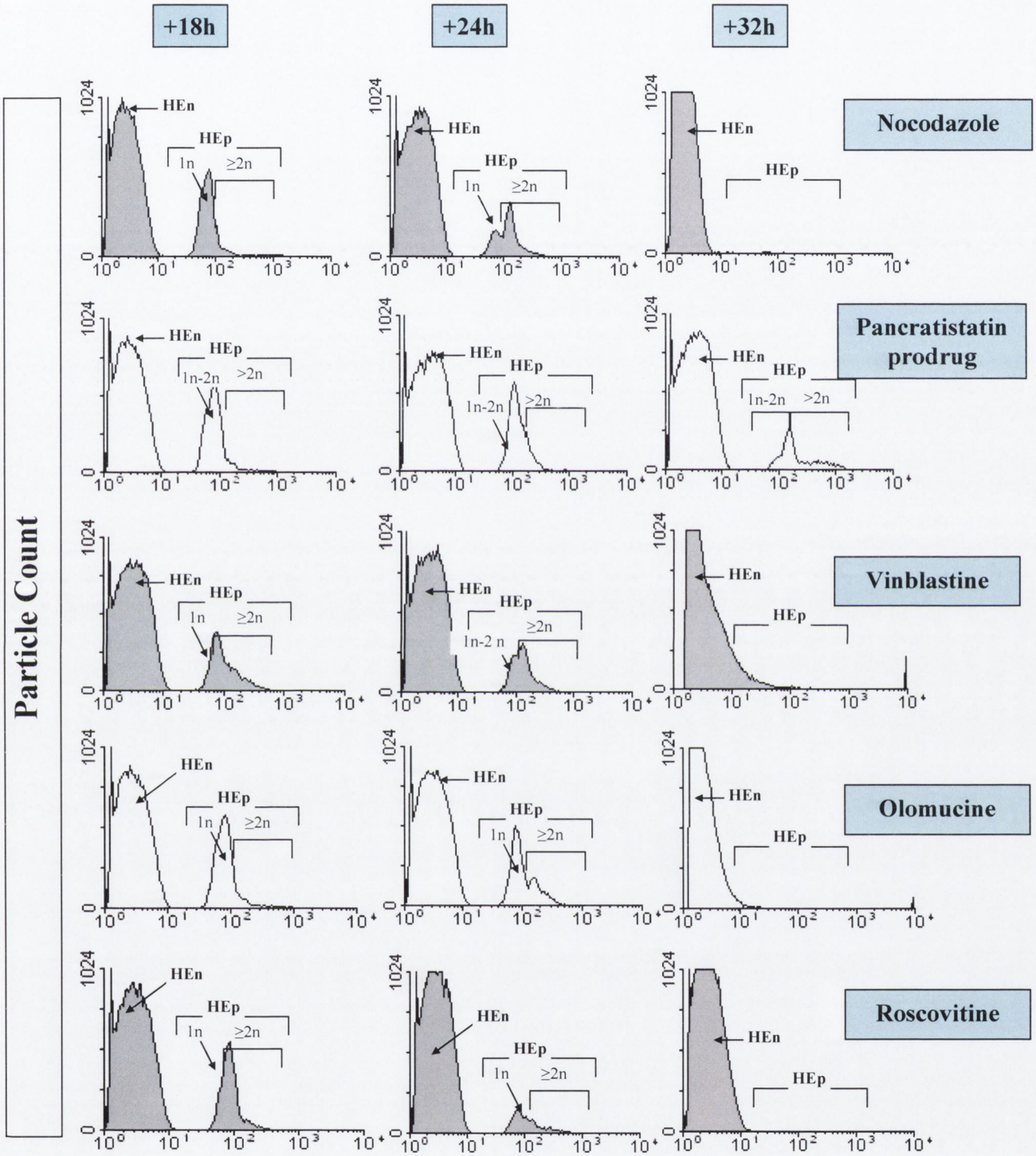


Fig. 3.4. Flow-cytometric assay of the cell-cycle inhibitor-treated parasites. Cultures of parasites 12-18 h post-invasion were exposed to no inhibitor (Control), 10 μM aphidicolin, 200 nM Hoechst 33342, 390 μM L-mimosine, 1.2 μM phyllanthoside, 100 μM nocodazole, 440 nM pancratistatin prodrug, 1μM vinblastine, 100 μM olomucine or 50 μM roscovitine.



Samples were taken at intervals as in Fig. 3.3, fixed in 1% formaldehyde, stained with propidium iodide and fluorescence levels measured (x-axis). Shown are the results from an untreated culture and the corresponding cultures exposed to each agent for 18 h, 24 h or 32 h. DMSO-treated cultures exhibited similar traces to the untreated control. HEn and HEp represent uninfected and infected erythrocytes, respectively.

case of L-mimosine and roscovitine, and in the first S-phase, or G_2 phase if it exists when treated with Hoechst 33342: figs. 3.3 and 3.4) had been achieved (see section 3.2.3 and 3.2.4). Next, the ability to remove the block applied by these agents was assessed. Following 18 h exposure to the inhibitor, samples were taken for microscopic analysis (Fig. 3.5 (a)), and the cells were washed extensively with culture medium to remove the inhibitor (or at least that proportion of the inhibitor that was not tightly bound to the cells) and then re-incubated at 37°C for 30 h. For both roscovitine- and L-mimosine-treated cultures, slowing down of development was apparent, with parasitaemias lower than the control and schizonts with multiple nuclei being numerous (Fig. 3.5 (b). Following a further 48 h incubation (Fig. 3.5 (c)), the roscovitine- and L-mimosine-treated cultures had increased parasitaemias approaching that of the control culture, including new rings and trophozoites. This confirmed that the block was removed and that cytokinesis and re-invasion of new merozoites occurred. Somewhat disappointingly, the cultures did not remain completely synchronous following the removal of the block, progression through schizogony and reinvasion, although this might be improved by multiple staggered treatments. This was not attempted for the reasons discussed in section 3.3. In the case of Hoechst 33342 (Fig. 3.5 (b)), neither schizonts nor new rings were apparent after the inhibitor was removed, indicating that the block was irreversible. In agreement with this conclusion, the Hoechst 33342-treated cultures did not recover after subsequent incubation (Fig. 3.5 (c)).

3.3. DISCUSSION

The limited knowledge available about the processes involved in the progression of the malaria parasite *P. falciparum* through intraerythrocytic schizogony is in part due to the difficulty in obtaining truly cell-cycle-synchronised populations of parasites. Schizogony is a process essential for parasite viability and understanding the complex processes involved may yield potential novel chemotherapeutic targets. The cell cycles of mammalian cells, yeasts and other organisms have been extensively studied but non-inhibitor based methods used for synchronising the cycle in these eukaryotic cell types cannot be applied to *P. falciparum* for a variety of reasons including the intracellular location of the parasite stages in question. Use of inhibitor-based and other 'batch' synchronisation methods for the study of eukaryotic cell-cycle phases have been widely reported (Harper, 2005; Harper and Brooks, 2005). For this reason development of an

inhibitor-based method similar to that applied in other eukaryotic cell types was attempted. Data presented in this chapter and elsewhere (Naughton and Bell, 2007) represent the results of attempts to synchronise the cell-cycle of *P. falciparum* using inhibitors.

In order to determine if a successful cell-cycle block had been achieved a technique by which the quantity of DNA contained in each parasite cell could be compared was also required. For this reason a technique previously used merely to detect *Plasmodium*-infected erythrocytes was modified to make this possible. Flow cytometry is able to distinguish between parasitised and non-parasitised erythrocytes based on the level of fluorescence observed following staining with the DNA-binding dye propidium iodide, but accurate distinction between the developmental stages of the parasite was difficult. This problem was overcome by growing the cultures using the platform shaker to reduce multiply-infected erythrocytes, fixing the cells in formaldehyde and incubating with glycine. The parasitaemias as measured by flow cytometry of cultures treated in this way and the counting of Giemsa stained smears matched to within <1%. This technique also allowed populations of rings (i.e. parasites containing a single nucleus) to be distinguished from asynchronous cultures (i.e. containing one or more nuclei: fig. 3.2). Once an accurate and consistent method for distinguishing between cultures of parasites containing only 1n and those containing parasites with multiple nuclei was achieved an assessment of the potential of each cell-cycle inhibitor reversibly to block schizogony could be carried out.

'Batch' synchronisation techniques are based on the ability of an inhibitor to prevent cell-cycle progression past a certain point. Once sufficient time has been allowed for all cells in the population to reach this point, the effects of the inhibitor are reversed and the cells allowed to proceed through the remaining cell-cycle phases uninhibited.

By this definition, both roscovitine and L-mimosine appear to achieve this in *P. falciparum*, by reversibly inhibiting progression into schizogony. All other agents examined either failed to produce a successful block of schizogony, as seen with aphidicolin, pancratistatin prodrug, phyllanthoside and the microtubule inhibiting agents nocodazole and vinblastine, or, as was the case with Hoechst 33342, inhibition of schizogony was irreversible.

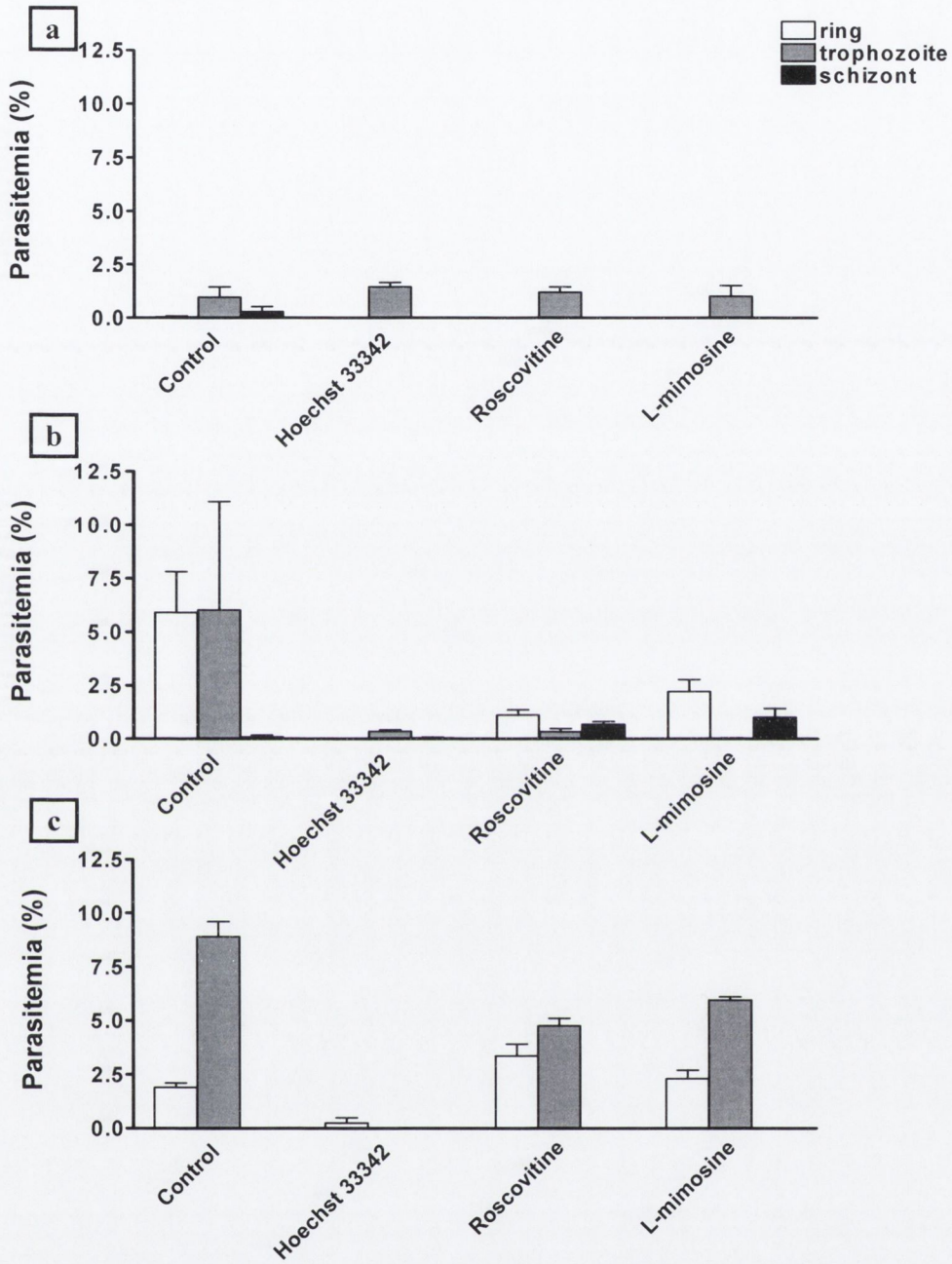


Fig. 3.5: **Reversibility of inhibition by Hoechst 33342, roscovitine and L-mimosine.** Cells were selected to an age range of 12-18 h post-invasion and incubated with the inhibitors. A sample was taken 18 h post-treatment for microscopic analysis, when the control parasites were aged ~30-36 h post-invasion (a). The cultures were washed and re-incubated in culture medium for a further 30 h, at which time a sample was again taken for microscopic analysis (b). The cultures was then diluted 1:3 into fresh erythrocytes and incubated for a further 48 h, at which time samples were again taken for microscopic analysis (c). Results represent the mean + SD for triplicate experiments.

As previously stated, the success of batch synchronisation of cultures depends on the presence of checkpoints at the end of each cell-cycle phase preventing the cell from progressing past a certain point until it is ready to do so. Results shown in this chapter indicate that at least one of these checkpoints is absent. Treatment with the microtubule inhibitors showed that DNA synthesis occurs despite the lack of completion of mitosis. A previously published study using Taxotere (Sinou *et al.*, 1998) also showed this continuation of DNA synthesis despite disruption of the normal microtubular structures. Based on both these finds it is unlikely that a conventional checkpoint exists at the end of G_1 phase.

Additional problems arise when the targets of both agents used to achieve a reversible block on schizogony are examined. Roscovitine is a known inhibitor of mammalian CDK molecules (reviewed in Meijer and Raymond, 2003) and has been shown to interact with a recombinant form of a putative *P. falciparum* cyclin-dependent kinase (Harmse *et al.*, 2001). The known targets in mammalian cells include several CDKs including CDK2 and CDK9 and recent reports suggest it may also inhibit a non-protein kinase target pyridoxal kinase, the enzyme responsible for phosphorylation and activation of vitamin B_6 (Bach *et al.*, 2005). These multiple mammalian targets in addition to the lack of an extensive examination of the targets of this molecule in the malaria parasite indicate the likelihood of this agent having multiple cellular targets in the parasite. In addition to this, the cell-cycle target of roscovitine is related to cell-cycle control which in this parasite may not follow the accepted model, as the lack of at least one of the conventional cell-cycle checkpoints indicates. Perhaps more promising is L-mimosine, the molecular target of which is the hydroxylation and thus activation of eukaryotic initiation factor 5A (McCaffrey *et al.*, 1995). Despite this, the effects of L-mimosine on *Plasmodium* have yet to be fully characterised and the potential for interactions with alternative cellular targets cannot be ruled out, especially at the high concentrations of the compound required.

The doubt about cellular targets in *Plasmodium* in conjunction with the lack of knowledge of the control of the cell cycle makes it impossible to assume that all the parasites in a single population are in the same phase when treated with these inhibitors. Moreover, the results in this chapter indicate that at the concentrations of roscovitine and L-mimosine required to achieve the (apparent) block, there was a degree of irreversible damage to the parasites.

In recent years (and largely while the work described in this chapter was under way) the validity of using inhibitor-based methods (and other 'batch' methods such as nutrient starvation, double thymidine block and treatment with hormones such as *Saccharomyces cerevisiae* alpha-factor) for synchronising the cell cycle of populations of eukaryotic cells has been questioned (Shedden and Cooper, 2002; Cooper *et al.*, 2006). The objection stems from a belief that while a population of cells can be obtained which exhibit a common property, e.g. all cells have a similar DNA content, such an alignment does not mean that the cells are arrested in the same cell-cycle stage. An additional problem is that the inhibitors may disrupt the treated cells to the extent that they are no longer truly representative of normal cycling cells. Cooper (Shedden and Cooper, 2002; Cooper *et al.*, 2006) is one of the main critics of batch-synchronisation and has called into question the results of certain pioneering and influential studies of cell-cycle-dependent gene expression in human or yeast cells based on batch-synchronised cultures. This viewpoint may still be a minority one and hotly contested (Cooper, 2004; Spellman and Sherlock, 2004a; Spellman and Sherlock, 2004b; Cooper *et al.*, 2006), but there is an additional problem to be considered in the case of *P. falciparum*. As previously stated, the success of batch synchronisation of cultures depends on the presence of checkpoints at the end of each cell-cycle phase preventing the cell from progressing past a certain point until the cell is ready to do so, and at least one of these checkpoints appears to be absent.

In summary, the results of this chapter demonstrate the effect of numerous cell-cycle inhibitors on *P. falciparum*. Certain inhibitors previously reported or assumed to be specific for particular cell-cycle phases or to 'synchronise' erythrocytic *P. falciparum* cells did not block development at specific points as expected. Three agents were found to block development at the trophozoite–schizont boundary. L-mimosine and roscovitine appeared to block DNA synthesis from initiating (G1- or early S-phase), while Hoechst 33342 treatment resulted in cells accumulating with a 2n quantity of DNA (late S or G2-phase). The effects of two of these (roscovitine and L-mimosine) were (at least partially) reversible, and these two agents might in principle have potential as *P. falciparum* cell-cycle synchronising agents. However, on the basis of the properties of the recovering roscovitine- and L-mimosine-treated parasites described here and the arguments that such methods "cannot and do not" synchronise other cell types (Cooper *et al.*, 2006), contrary to the original aim of this work, any results

obtained using inhibitor-treated parasites are unlikely to be reflective of the normal cell cycle. Study of the *Plasmodium* cell cycle will continue to be difficult; live-cell microscopy using fluorescently-tagged molecules would seem to offer the best hope of understanding this important and unusual process.

Chapter 4

Antimalarial Activities and Mechanisms of Action of Dinitroaniline and Phosphorothioamidate herbicides

4.1. INTRODUCTION

Microtubule inhibitors have previously been identified as potential chemotherapeutic agents for the treatment of *P. falciparum* malaria (reviewed in (Bell, 1998; Kappes and Rohrbach, 2007). The ability of numerous agents to interact with microtubules and thus disrupt the cell cycle has led to their successful use as fungicides (Hollomon *et al.*, 1998), anticancer drugs (Altmann, 2001; Kavallins *et al.*, 2001) and anthelmintic agents (Lacey, 1988).

A number of microtubule inhibitors have demonstrated the ability to interfere with the tubulin/microtubule system of the parasite and thus affect the development of both sexual and asexual forms of *P. falciparum* (Usanga *et al.*, 1986; Dieckmann-Schuppert and Franklin, 1989; Dieckmann-Schuppert and Franklin, 1990; Pouvelle *et al.*, 1994; Schrevel *et al.*, 1994; Kaidoh *et al.*, 1995; Bejon *et al.*, 1997; Skinner-Adams *et al.*, 1997; Bell, 1998; Fowler *et al.*, 1998; Sinou *et al.*, 1998; Taraschi *et al.*, 1998; Dow *et al.*, 2002; Fennell *et al.*, 2003; Navarrete-Vazquez *et al.*, 2003; Fennell *et al.*, 2006; Navarrete-Vazquez *et al.*, 2006). While these inhibitors do provide useful tools for elucidating the roles of microtubules throughout the various developmental stages of *P. falciparum*, their use as potential anti-malarial drugs is limited by the high toxicity exhibited by these agents against mammalian cells and the high level of amino acid sequence conservation shown between tubulin proteins of humans and *P. falciparum* (Bell, 1998). Nonetheless, it may be possible to discover or develop microtubule inhibitors which have high parasite toxicity but little effect on the host. This is supported by reports that members of the "*Vinca*" alkaloid and taxoid groups may exhibit higher potency against cultured parasites than host cells (Bell, 1998). In addition to this, benzimidazole anthelmintics are used successfully due to their selective toxicity, which results from a differential binding to host and parasite tubulin (Lacey, 1988). The most promising indication of this potential as far as malaria is concerned is found among certain antimitotic herbicides, which have been shown to inhibit plant microtubules. Some of these, namely certain dinitroaniline herbicides such as trifluralin and oryzalin and the phosphorothioamidate herbicide amiprophosmethyl (APM; Fig. 4.1) have shown moderate activity against *P. falciparum* in culture while having very low toxicity to mammalian cells (Chan and Fong, 1990; Morejohn and Fosket, 1991; Hugdahl and Morejohn, 1993; Stokkermans *et al.*, 1996; Bell, 1998; Werbovetz *et al.*, 2003; Fennell *et al.*, 2006).

As these compounds may form the basis for the development of novel anti-malarials, it is important to understand how they interact with and inhibit *P. falciparum*. The purpose of the work reported in this chapter was to characterise the cellular effects of trifluralin, oryzalin and APM on intra-erythrocytic *P. falciparum*. This characterisation involved: (i) investigation of the relative effects of these compounds on differently-aged parasites; (ii) analysis of the effects of inhibitors on parasite maturation, morphology and DNA synthesis, (iii) inhibitor reversibility studies and (iv) investigating the possibility of a controlled, programmed cell death (PCD) response to treatment by these and other microtubule inhibitors.

The occurence of PCD in unicellular organisms has received increasing attention over the last decade (see sections 1.3.2 and 1.3.3). Recent publications by Hurd *et al.* (2006) on *P. falciparum* mosquito stages in addition to those on other protozoal parasites (Debrabant *et al.*, 2003; Jayanarayan and Dey, 2005), have indicated that a form of PCD may play a role in the cell death of these organisms. In the asexual parasites, original reports of a PCD-like cell-death in response to chloroquine treatment (Picot *et al.*, 1997; Meslin *et al.*, 2007) have been disputed (Nyakeriga *et al.*, 2006). The marker(s) for detecting PCD used in these studies were DNA fragmentation and mitochondrial membrane disruption (Picot *et al.*, 1997; Nyakeriga *et al.*, 2006; Meslin *et al.*, 2007) and PCD has been confirmed in mammalian cells in the absence of these markers (Cohen *et al.*, 1992; Fournel *et al.*, 1995; Zamai *et al.*, 1996; Boix *et al.*, 1997; Yuste *et al.*, 2001; Newmeyer and Ferguson-Miller, 2003) so these studies do not represent a comprehensive investigation of the process in asexual *Plasmodium* stages. Microtubule inhibitors such as taxol (Fig. 4.1) are known to induce apoptosis in mammalian cells Mollinedo and Gajate, 2003) as well as a form of PCD in *Leishmania* (Jayanarayan and Dey, 2005). To see if there was any indication of a PCD type response to treatment with taxol, trifluralin or vinblastine in *P. falciparum* erythrocytic stages, an assay to detect DEVDase (caspase-3) activity, which is one of the markers of PCD, was carried out. Evidence of caspase-3 like activity induced in mosquito stages has been previously noted (Al-Olayan *et al.*, 2002; Hurd *et al.*, 2006).

4.2. RESULTS

4.2.1. Effect of herbicide microtubule inhibitors on *P. falciparum*, CHO cells and uninfected erythrocytes

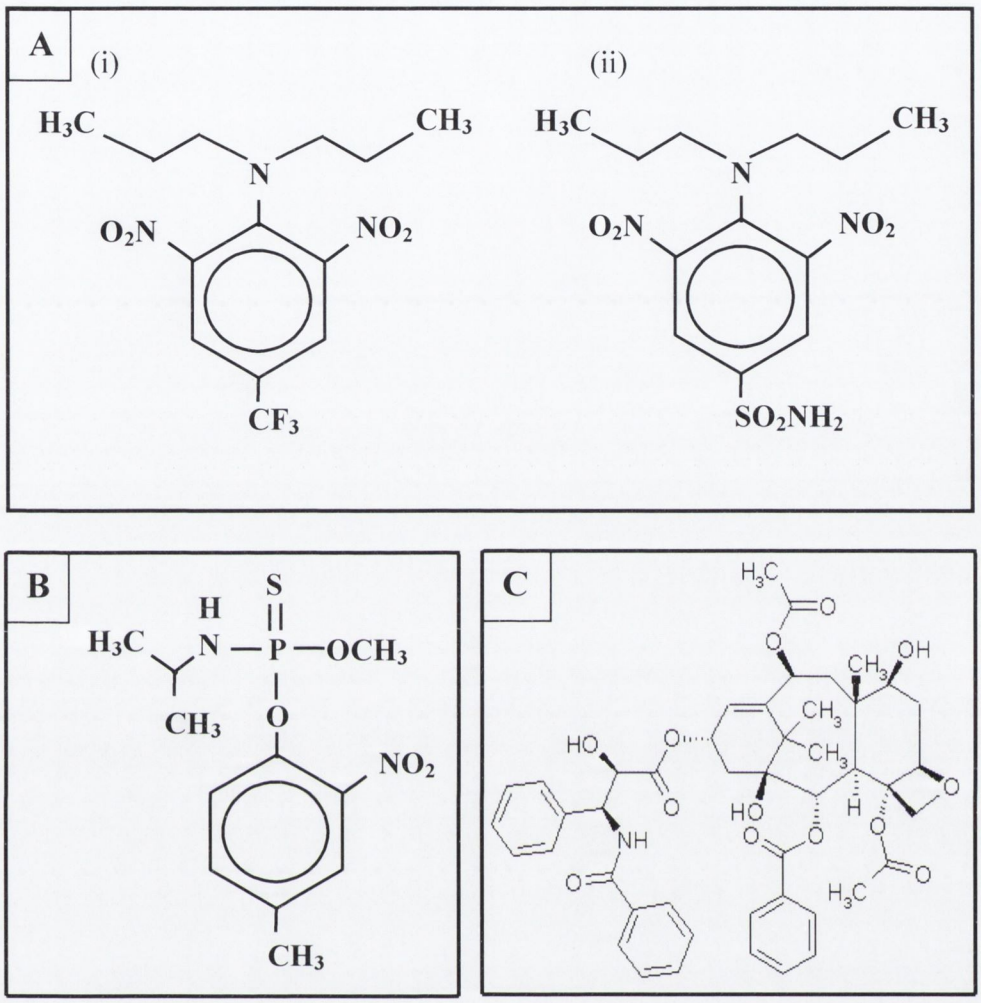


Figure 4.1. Chemical structures of (A) the dinitroaniline herbicides (i) trifluralin and (ii) oryzalin; (B) the phosphorothioamidate herbicide amiprophosmethyl (APM); and (C) taxol

Previous studies have shown that trifluralin, oryzalin and APM have quite low toxicity to mammals (Chan and Fong, 1990; Stokkermans *et al.*, 1996; Werbovetz *et al.*, 2003) but to confirm this, an assay was carried out on two lines of mammalian cells. None of the inhibitors tested displayed an inhibitory effect on the mammalian cell lines up to concentrations of 32–64 μM (table 4.1). It was not possible to test above these concentrations owing to the poor solubility of the compounds. There was a marked and reproducible difference in the activity of these compounds against *P. falciparum* and the mammalian cells, which is consistent with data previously reported for other parasites such as *Trypanosoma cruzi*, *Leishmania* and *Cryptosporidium* (Traub-Cseko *et al.*, 2001; Bhattacharya *et al.*, 2002; Mead *et al.*, 2003).

4.2.2. Effects of herbicides on parasite cultures of different initial ages

The purpose of this experiment was to determine at which point during the parasite's erythrocytic cycle the initial addition of trifluralin, oryzalin or APM caused maximal inhibition of parasite growth.

Previous work has demonstrated that parasite populations of different initial ages respond differently following 48 h exposure to the microtubule inhibitors dolastatin 10, auristatin PE, vinblastine and taxol (Fennell *et al.*, 2003). To see if a similar trend occured upon exposure to the dinitroanilines and APM, eight populations of parasites were selected, each with an age range of ~ 6 h, covering the parasite's whole intra-erythrocytic developmental cycle. Inhibitor susceptibilities upon 48 h exposure were determined for each of these populations. The data in Table 4.2 clearly indicate that application of these agents at different time points in the parasite's intra-erythrocytic cycle results in different inhibition profiles. Cultures that were ~0-6, 6-12 and 42-46/0-2 h post-invasion on initial exposure appeared least susceptible to all inhibitors at the concentrations used. Those parasites aged ~18-24, 24-30 and 30-36 h post-invasion at initial exposure were highly susceptible, while those aged ~12-18 and 36-42 h were slightly less so. Both the two dinitroanilines tested and APM displayed similar susceptibility profiles. Unlike taxol, however, the ring stages do show some measurable susceptibility to 48h exposure to trifluralin and APM, and to a lesser extent oryzalin. These differences were found in spite of the fact that all of the cultures were exposed to inhibitor for the same 48 h total. Therefore, oryzalin and trifluralin and APM appear to display their most intoxicating effect when initially applied at the trophozoite / early schizont stage of asexual parasite development, but do exhibit the ability to inhibit

parasites both at earlier and later stages of the asexual life cycle when compared with taxol.

4.2.3. Effects of inhibitors on parasite morphology and maturation

In order to investigate the effects of these inhibitor classes on parasite development through the trophozoite and schizont stages, cells aged 20-26 h post-invasion were selected and treated with 20 μM of trifluralin or APM and these parasites were subsequently examined at several time points for morphological abnormalities (Fig. 4.2 A) and changes in parasitaemia and stages present (Fig. 4.2 B). When new rings were appearing in the control cultures, both trifluralin- and APM-treated cultures lacked new rings, had much lower parasitaemias and higher proportions of damaged cells than the control (Fig. 4.2 B). The herbicide-treated cells also appeared distorted and seemed to be prevented from entering into or progressing properly through the schizont stage (Fig. 4.2 A).

4.2.4. Effect of antimitotic herbicides on DNA synthesis

To investigate the effects of these inhibitor classes on parasite DNA content, cells aged 20-26 h post-invasion were selected and treated with 20 μM of trifluralin or APM and these parasites were subsequently examined at several time points by flow cytometry. When the DNA content of the parasitised erythrocytes was measured, untreated control cultures showed progression from parasites aged 26-32 h post invasion, which showed a profile with an initial peak of low-level fluorescence representing DNA-free uninfected erythrocytes (HEn) (seen in all subsequent profiles), followed by a distinct peak representing parasitised erythrocytes with a haploid (1n) amount of DNA (Fig. 4.3A). This second peak shifted as a slightly higher fluorescence signal was detected, corresponding to DNA synthesis in parasites aged 32-38 h post-invasion, which contain between 1n and 2n amounts of DNA. Following reinvasion, this peak returned to its original position corresponding to a 1n amount of DNA, as expected for parasite populations of median ages 7 h and 13 h post-invasion.

After 6 h of exposure to trifluralin (Fig. 4.3 B) or APM (Fig. 4.3 C), where the parasites were aged ~26-32 h post-invasion, a similar fluorescence trace to that of the control was observed. Following a further 6 h of exposure (Fig. 4.3 B and C), again the trace resembled the untreated control (Fig. 4.3 A), the second peak shifted as slightly higher fluorescence signal was detected, corresponding to DNA synthesis in parasites

Table 4.1. Inhibitory effects of antimitotic herbicides on *P. falciparum* and mammalian cells.

Compound	Class	IC_{50} (72 h)		
		P. falciparum **(μM)**	**Vero monkey kidney cells (μM)**	**CHO cells (μM)**
Trifluralin	Dinitroaniline	1.9*	> 64**	> 64**
Oryzalin	Dinitroaniline	6.1*	> 32**	> 64**
Amiprophos-methyl	Phosphorothioamidate	3.5*	> 64**	> 64**

*IC_{50} determined using asynchronous parasite cultures (starting parasitaemia 0.8%; haematocrit 0.4%) by the parasite lactate dehydrogenase (pLDH) assay outlined in section 2.3.1 and represents the geometric mean of 4 determinations.

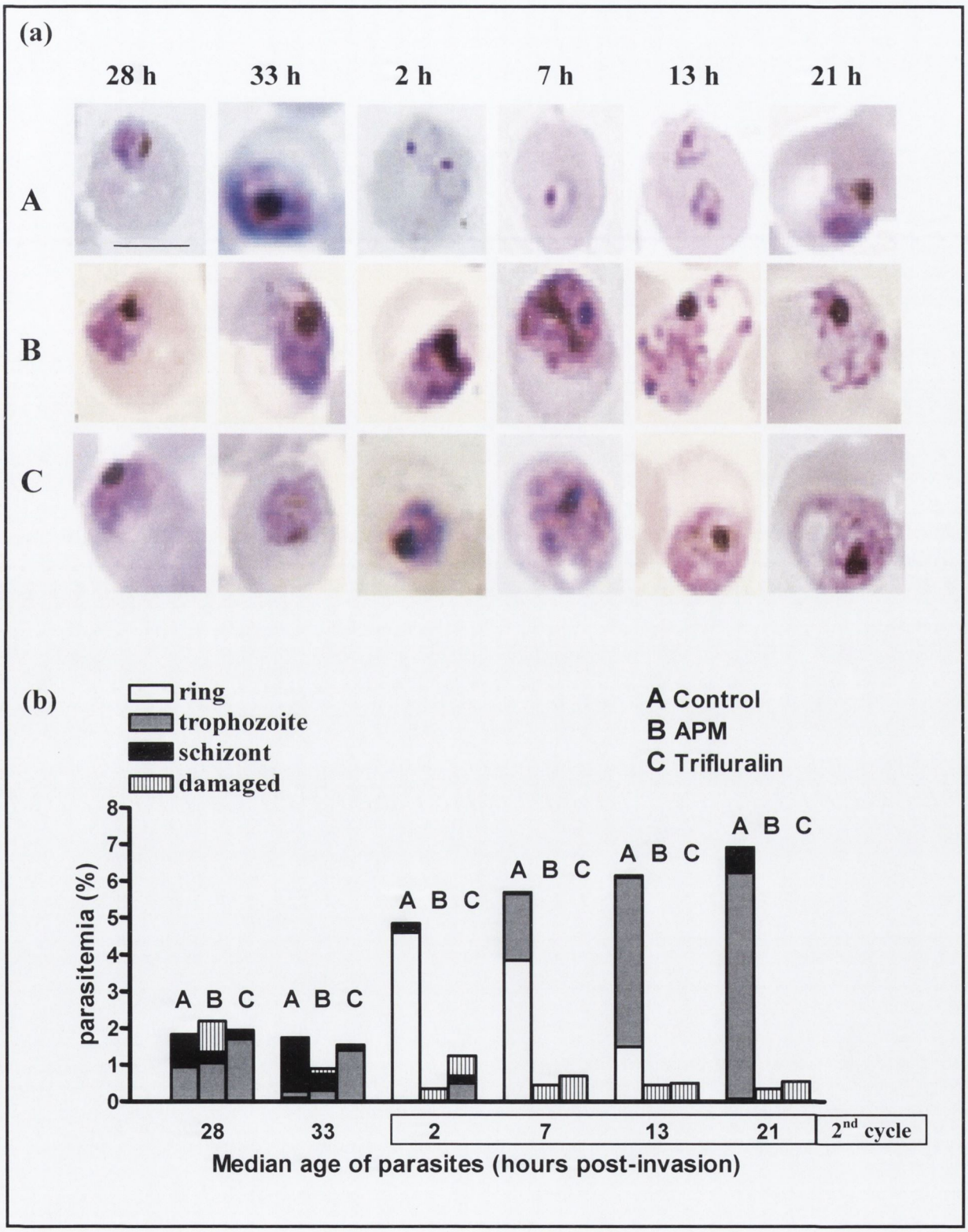
** IC_{50} value represents the geometric mean of 4 determinations and were obtained using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of Mosmann (1983) as outlined in section 2.2.9.

Table 4.2. Susceptibilities of age-selected *P. falciparum* cultures of different initial ages upon 48 h exposure to inhibitors.*

Approx. age of culture (h post-invasion)	Trifluralin			Oryzalin			APM			Taxol		
	IC_{25}(nM)	IC_{50}(nM)	IC_{75}(nM)	IC_{25}(nM)	IC_{50}(nM)	IC_{75}(nM)	IC_{25}(nM)	IC_{50}(nM)	IC_{75}(nM)	IC_{25}(nM)	IC_{50}(nM)	IC_{75}(nM)
0-6	5.9	43.9	>64	>64	>64	>64	54	>64	>64	>8	>8	>8
6-12	14.2	45.2	>64	>64	>64	>64	8	>64	>64	>8	>8	>8
12-18	15.2	13.2	>64	49.7	63	>64	4	63	>64	>8	>8	>8
18-24	1	1.8	33.2	2.5	3.5	11.2	2	3.4	4.1	0.022	0.046	0.11
24-30	1.6	2.3	11.4	3.9	5.3	13.7	2.5	4	15	0.016	0.026	0.16
30-36	1.95	4	21.3	4.2	11.2	14.3	3	7.3	16	0.018	0.041	0.175
36-42	2.6	9.6	35.6	5.3	14.9	51.6	7	12	30	0.057	0.409	>8
42-46/0-2	16.8	>64	>64	11.3	>64	>64	16	>64	>64	0.3	>8	>8

* Eight populations of *P. falciparum* parasites, each with an approximate age range of 6 hours, were isolated by sorbitol treatment (see section 2.2.2) and resuspended to a parasitaemia of 0.8% and haematocrit of 0.4%. They were subsequently exposed to titrations of each agent for 48 h at which point pLDH activity was measured. IC_{25} IC_{50} and IC_{75} values shown represent the concentration required to inhibit 25%, 50% and 75% of the parasites respectively and are the geometric mean values of at least 4 separate determinations.

Figure 4.2. Development of parasites in the presence of inhibitors. (a) Cultures of 20 - 26 h post-invasion were exposed to no inhibitor (A) or 20 μM of APM (B) or trifluralin (C), and the microscopic appearance of parasitised erythrocytes was then examined and photographed at 6 or 12 h intervals. The ages of parasites shown indicate the median age of the parasites in the culture over an ~ 6 h range and the parasites shown are representative of those examined. Scale bar is 5 μm. (b) Cultures were treated as described above, and smears were counted at 6 or 12 h intervals, as described in section 2.4.3. The ages of parasites shown on the x-axis indicate the median age of the parasites in the culture over an ~ 6 h range. The data are those of a representative experiment.


(a)
28 h
33 h
2 h
7 h
13 h
21 h
A
B
C
(b)
ring
trophozoite
schizont
damaged
A Control
B APM
C Trifluralin
parasitemia (%)
0
1
2
3
4
5
6
7
8
A B C
28
33
2
7
13
21
2nd cycle
Median age of parasites (hours post-invasion)

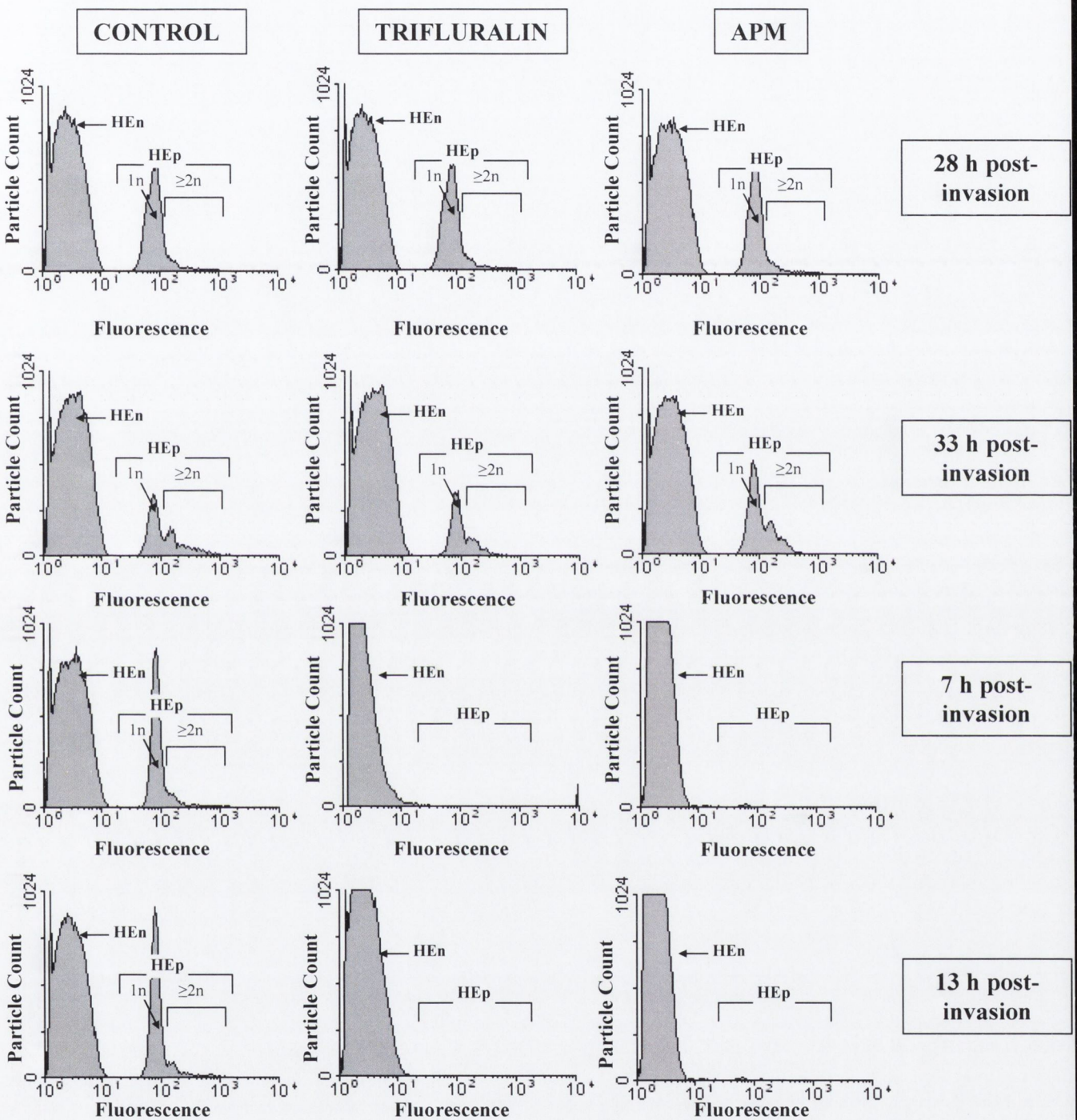


Fig. 4.3. Flow-cytometric assay of the herbicide-treated parasites. Cultures of parasites 20-26 h post-invasion were exposed to no inhibitor (Control), to 20 μM of trifluralin, or 20 μM of APM. Samples were taken at intervals similar to Fig. 4, fixed in 1% formaldehyde, stained with propidium iodide and fluorescence levels measured. HEn represents uninfected erythrocytes and infected erythrocytes (HEp) are marked by their DNA content (1n representing the amount of DNA present in a single nucleus) determined by comparison with cultures known to contain only ring stage parasites i.e. those with only a single nucleus. Shown are data from a single representative experiment, with 50000 cells being counted in each.

aged 32-28 h post-invasion. A further 18 h exposure to either agent led to the loss of the distinct peak corresponding to parasitized erythrocytes and the merging of this peak with that of uninfected erythrocytes. A final additional 6-h exposure led to the complete disappearance of any peak corresponding to parasitized erythrocytes (Fig. 4.3 B and C). The results showed that exposure to these agents did not inhibit an initial level of DNA synthesis. The disruption of the trace seen following longer exposures to all three agents could be caused by fragmentation of the DNA resulting in loss of the double-helical structure required for PI binding or loss of low-molecular weight DNA fragments from the cell, as well as the break-up of parasite cells that is apparent in Fig. 4.2 (a).

4.2.5. Stage-dependent susceptibility to and reversibility of herbicides.

Previous work using several different microtubule inhibitors has shown that different developmental stages of the parasite exhibit differing levels of susceptibility to short exposures to the inhibitors (Schrevel *et al.*, 1994; Fennell *et al.*, 2003). For example, 6-h pulses of dolastatin 10, vinblastine and taxol were all most effective when applied to schizont-stage cultures, less so when applied to trophozoites, and largely ineffective on rings (Fennell *et al.*, 2003). The stage-dependent susceptibility of *P. falciparum* to short exposures (6 h) of oryzalin, trifluralin and APM was evaluated by subjecting ring-infected (4 - 10 h post- invasion), trophozoite infected (18 - 24 h post-invasion) and schizont-infected (34 - 40 h post-invasion) erythrocytes to various concentrations of oryzalin, trifluralin and APM. At the end of the incubation period, cultures were carefully washed and recultured for 52 h in inhibitor-free medium. The results in Fig. 4.4 reveal that the susceptibility of *P. falciparum* towards the agents tested differed according to its particular erythrocytic stage of development. There was a slight reduction (~ 30%) in parasitaemia on addition of any inhibitor to ring-stage parasites (Fig. 4.4). The development of trophozoite and schizont forms pulsed for 6 h with 20 μM oryzalin, trifluralin or APM resulted in ~ 60-65% inhibition, suggesting that these drugs had the ability to penetrate parasites of both stages within the 6 h incubation period. This result suggests that the inhibitors affect the parasitised erythrocytes at all stages of development, albeit to different extents, and correlates well with the previous observation of activity of oryzalin, trifluralin or APM on parasites of different initial ages (see section 4.2.2). This result suggests that the herbicides had a somewhat wider stage-specificity than the other microtubule inhibitors mentioned above.

4.2.6. Detection of caspase-like activity in *P. falciparum* following exposure to antimitotic compounds

Previous investigations into cell death in the ookinetes of *P. berghei* detected caspase activity by labelling whole cells with a fluorescently labelled caspase inhibitor (a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor, FAM-VAD-fmk (carboxyfluorescein-Val-Ala-Asp-fluoromethylketone)) (Al-Olayan *et al.*, 2002). A previous report in *Leishmania* suggested that while exposure to taxol resulted in a PCD-like cell death in these cells, trifluralin did not, and both taxol and vinblastine have both demonstrated the ability to induce apoptosis in mammalian cells (reviewed in (Mollinedo and Gajate, 2003). For this reason it was felt that rather than restricting the investigation to the ability of trifluralin to induce caspase activity, which has yet to be demonstrated in any cell type, both taxol and vinblastine were included. To see if similar caspase-like activity was detected in *P. falciparum* cells exposed to either taxol or vinblastine, a colourometric assay to detect DEVDase activity (Fig 4.4) based on the inhibitor DEVD-fmk labelled with the colourometric compound *p*NA (*p*-nitroaniline) was used. A certain level of substrate hydrolysis releasing the *p*NA molecule is expected due either to non-specific hydrolysis by other non-caspase cellular proteases or by substrate degradation. Activity in untreated cultures was taken to represent this non-specific hydrolysis. Both taxol and to a lesser extent vinblastine appeared to induce the DEVDase activity in treated cultures (Fig. 4.5 A) when compared with untreated ones. Addition of the pan-caspase inhibitor *Z*-VAD-fmk (benzyloxycarbonyl-valine-alanine-aspartic acid (OMe) fluoromethylketone) to the cultures prior to either compound appeared to negate the ability of these agents to induce this DEVDase activity (Fig 4.4 A) or repressed DEVDase activity once activated. Exposure to trifluralin appeared not to induce any such activity (Fig 4.4 A). As expected, taxol induced DEVDase activity in CHO cells, and this activity was repressed by the addition of the caspase inhibitor *Z*-VAD-fmk (Fig 4.4 A). Substrate hydrolysis specifically resulting from exposure to each compound and so representing specific DEVDase activity was calculated by subtracting *p*NA released from the substrate in both the un-induced cultures and those exposed to the caspase inhibitor *Z*-VAD-Fmk (Fig. 4.4 B) from activity in cultures exposed to the antimitotic agents alone. DEVDase specific activity for CHO cells was seen to be the highest (as expected), followed by *P. falciparum* cells exposed to taxol then vinblastine. Trifluralin induced negligible DEVDase-specific activity.

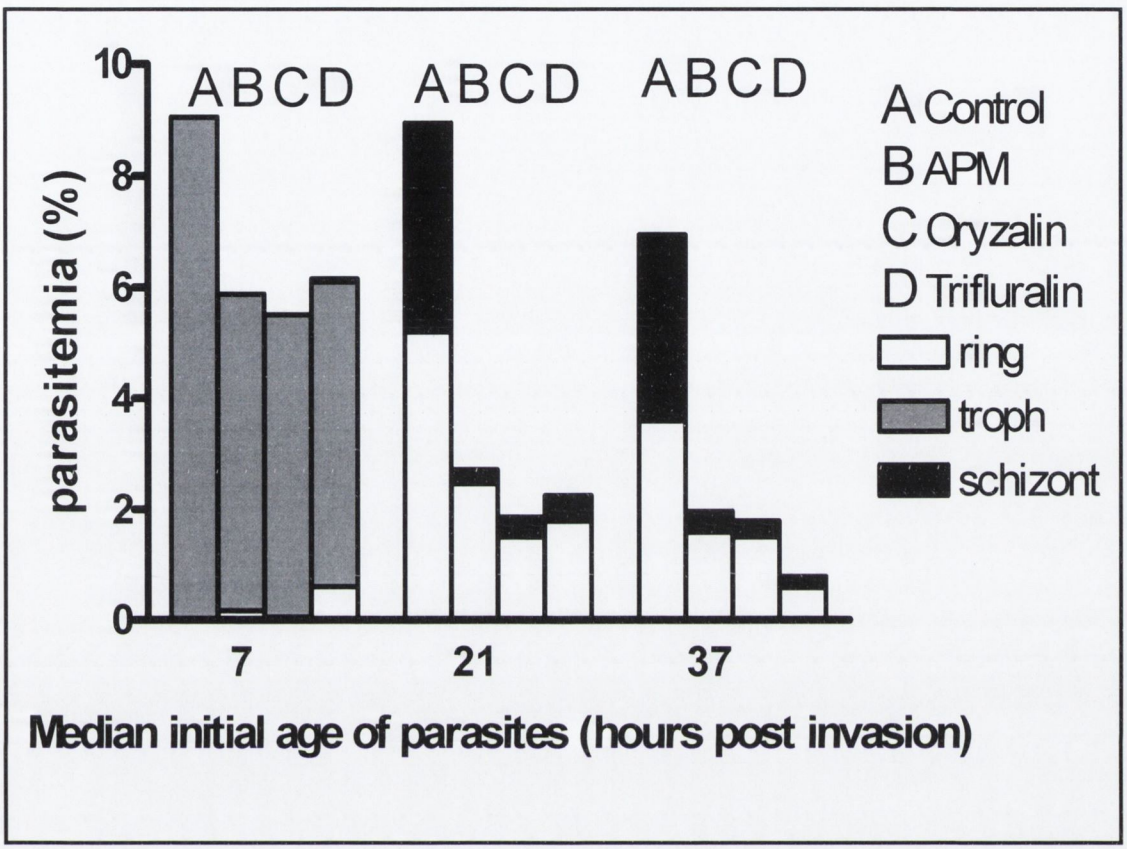


Figure 4.4. Effects of microtubule inhibitors on different developmental stages. Cultures of ring stage (4 - 10 h post-invasion), trophozoite stage (18 - 24 h) and schizont stage (34 - 40 h) were selected and each incubated for 6 h in the presence of no inhibitor or 20 μM of APM, oryzalin or trifluralin as indicated. The cells were then washed, recultured for 52 h in inhibitor free medium, and finally examined on Giemsa-stained smears; the parasitemias obtained at this time are indicated on the y-axis and the x-axis shows the average age of parasites over the 6 h range used at the start of the experiment. This experiment was repeated 3 times and the data here are from a single representative experiment. Troph=trophozoite

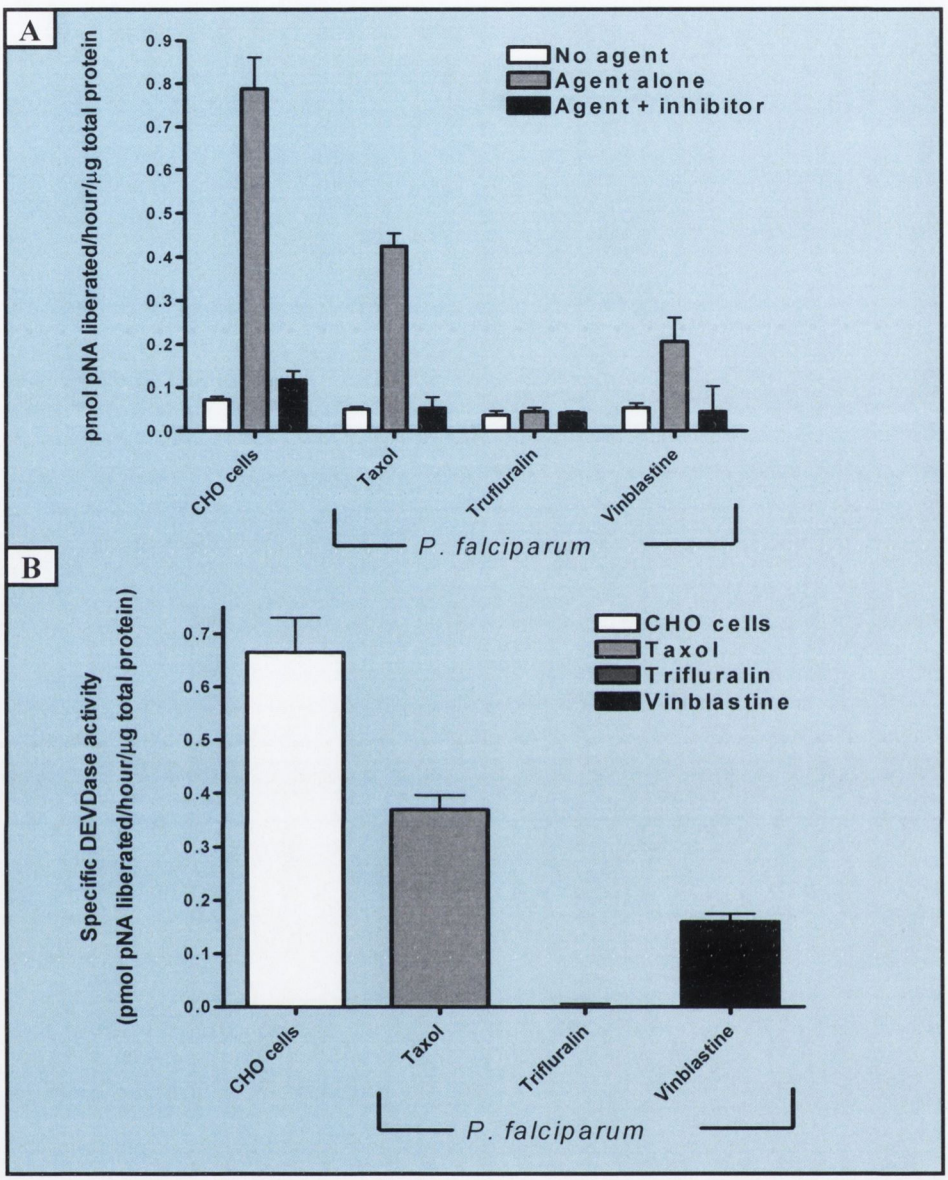


Fig. 4.5. Induction of DEVDase activity by anti-mitotic agents in CHO cells and *P. falciparum*. Cultures of CHO cells were exposed to no agent, 1 µM taxol, or both 1 µM taxol and the caspase inhibitor *Z*-VAD-fmk. *P. falciparum* cultures were exposed to no agent, 1 µM Taxol, 10 µM trifluralin, 1 µM vinblastine or a combination of the anti-mitotic inhibitor and *Z*-VAD-fmk. Following 12 h incubation, cells were harvested and crude cells extracts prepared. Hydrolysis of acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-*p*NA) resulting in the release of the *p*NA moiety was detected at 405 nm. Release of *p*NA is displayed as picomoles released per hour per µg total protein for each treatment group as calculated from a standard curve using free pNA of known concentrations (A). The specific DEVDase activity in cultures exposed to anti-mitotic inhibitor alone was calculated by subtracting *p*NA released on addition of extracts from untreated cultures and those exposed to *Z*-VAD-fmk from that found in cultures exposed to the microtubule inhibitor (B). Values shown are mean ± SEM for 3 replicate experiments.

4.3. DISCUSSION

Studies to date have indicated that potent anti-malarial activity can be observed with several classes of microtubule inhibitors such as taxoid and "*Vinca*" alkaloid groups, the dolastatins and their synthetic derivatives the auristatins (see Kappes and Rohrbach, 2007 for a review). However, the cytotoxicity of these agents for mammalian cells has lead to the search for more selective agents. Antimitotic herbicides of the dinitroaniline and phosphorothioamidate groups appear to have the requisite selective activity against *Plasmodium* (Chan and Fong, 1990; Morejohn and Fosket, 1991; Hugdahl and Morejohn, 1993; Stokkermans *et al.*, 1996; Bell, 1998; Werbovetz *et al.*, 2003; Fennell *et al.*, 2006). This selectivity was confirmed, as no activity was detected against either CHO or Vero monkey kidney cell lines for the concentrations allowed by the solubility of these agents (see section 4.2.1).

Few studies on the cellular effects of these herbicides on *P. falciparum* have been carried out, and those have been limited to dinitroanilines, most often trifluralin, and to concentrations far in excess of those required to inhibit the parasite (Bell, 1998; Fowler *et al.*, 1998). Investigations outlined in this chapter aim to better understand the effect of more relevant inhibitory concentrations of these agents on *P. falciparum*.

Fennell *et al.* (2006) published molecular data supporting the contention that the tubulin of mitotic microtubules is the primary target of the dinitroanilines and APM. Immunofluorescence microscopy showed the disruption of microtubular structures and [^{14}C]trifluralin was shown to bind to both α- and β-tubulin recombinant proteins. However, certain features of the antimalarial activity of the antimitotic herbicides were found to differ from those of the dolastatin/auristatin, "*Vinca*" alkaloid and taxoid classes of microtubule inhibitor.

The biphasic dose/inhibition curve and plateau of partial inhibition observed after 48 h exposure of asynchronous parasites to the microtubule inhibitors vinblastine, dolastatin 10 and taxol (Schrevel *et al.*, 1994; Fennell *et al.*, 2003; Fennell *et al.*, 2006) is not as pronounced for APM, oryzalin or trifluralin (Fennell *et al.*, 2006). This biphasic dose-response is thought to imply that two distinct populations of parasites were present in the cultures tested, with differing susceptibilities, but that the less susceptible population disappears upon longer exposure to inhibitors, as shown by the disappearance of the plateau (Fennell *et al.*, 2006). An additional contributory factor may be the lack of a mitotic checkpoint and so a delayed response to the damage caused

by microtubule inhibitors (Graeser *et al.*, 1996; Sinou *et al.*, 1998). It is also worth remembering that a lag period may exist as the drug infiltrates the parasite cell to its site of action and takes full effect, so although parasites may be at the stage most susceptible to inhibition, they may not be exposed to sufficient concentrations of inhibitor to impede their subsequent progression through the erythrocytic cycle. This is however unlikely to apply in the case of trifluralin (see chapter 5).

As with antimitotic agents tested previously (Fennell *et al.*, 2003) parasite asexual stages exhibited differing susceptibilities to trifluralin, oryzalin and APM depending on initial age of exposure (see section 4.2.2). While the concentrations of agents used are not strictly comparable due to the lower potency of the antimitotic herbicides when compared to agents of other classes, it is the difference in susceptibility between parasites of different ages to the same agent that is examined, rather than the absolute value of the inhibitory concentration. There was a wider susceptibility profile to treatment with the antimitotic herbicides, as parasites of initial age 12-18 h post-invasion were susceptible to these agents, while parasites at the same early stage were resistant to treatment with taxol (see table 4.2), auristatin PE, dolastatin 10 or vinblastine (Fennell *et al.*, 2003). The reduced effect of treatment on young parasites (those initially aged 0–12 h post-invasion) (Table 4.2) may be caused by arrest in schizogony and the fact that schizonts contain higher pLDH activity per parasite than rings. Only after the control parasites had entered the more metabolically active trophozoite stage, with its higher pLDH levels, would the inhibitory effect be apparent. The different susceptibilities of cultures of different initial ages appears to agree with the proposal that the biphasic dose response curve is, at least in part, due to the presence in asynchronous parasite cultures of two individual populations of parasites with differing susceptibilities. The less pronounced biphasic dose response seen upon treatment with the dinitroanilines and APM could then be a result of a wider range of parasites in the asynchronous population being susceptible to these agents when compared with taxol and other antimitotic agents.

Reasons for this broader susceptibility may be that compounds such as trifluralin and APM, which have simple aromatic chemistry in contrast to the complex chemical structures of the dolastatins and taxoids, may accumulate more rapidly within the parasite. This rapid accumulation has been suggested by previous reports on the inhibition of the *in vivo* flagellar regeneration on *Chlamydomonas* zoospores almost directly upon addition of trifluralin at concentrations >5 μM (Hess, 1979). It is also

possible that in addition to the rapid accumulation of the herbicides they bind to tubulin that is present but not yet assembled into mitotic spindles while other agents may bind only to assembled microtubular structures.

Maturation, morphology and DNA content assays were carried out on cultures with a starting age of 20-26 h post-invasion as parasites appeared most susceptible when the herbicides were applied initially at this stage of development. Upon exposure, parasites appeared to be arrested at the schizont stage of development (Fig. 4.2 (a)). These cells had an abnormal appearance however and lacked distinct nuclear bodies. This could be explained by the disruption of microtubular structures seen upon treatment with these agents (Fennell *et al.*, 2006), so even though there may be some level of DNA synthesis (Fig. 4.3), microtubule-dependent nuclear division does not occur. The lack of merozoite development could also be attributed to this lack of normal microtubular structures. Previous electron microscopic studies have demonstrated that merozoite morphogenesis and formation can be disrupted by certain microtubule inhibitors (Taraschi *et al.*, 1998). A similar inhibition of schizont maturation and merozoite formation was noted in *P. falciparum* upon treatment with dolastatin 10, vinblastine or taxol. Continuing exposure to these agents eventually results in the disruption of cellular integrity and the appearance of "broken" parasites within infected erythrocytes (fig. 4.2).

The effect on cellular DNA content and synthesis also indicated inhibition at the early schizont stage of development. As with microtubule inhibitors of other classes, the antimitotic herbicides did not inhibit initial DNA synthesis (Fig. 4.3 and section 3.2.4). Longer exposure to these agents resulted in the loss of cellular DNA content (Fig. 4.3). This disappearance of the fluorescent signal corresponding to parasitised erythrocytes may be caused by fragmentation of the DNA resulting in loss of the double-helical structure required for PI binding or loss of low-molecular weight DNA fragments from the cell, which may be due to the break-up of parasite cells that is apparent in Fig. 4.2 (a). So it appears that while in the short term antimitotic herbicides do not inhibit DNA synthesis, cellular DNA content is drastically reduced by longer exposures and occurs prior to complete disruption of parasite cellular integrity.

In a series of drug-pulse experiments (Fig. 4.4) both trophozoite and schizont infected cultures were highly susceptible to irreversible inhibition upon short term-exposure to trifluralin, oryzalin and APM. While mostly unaffected, there was also a proportion of the ring-infected cultures which appeared susceptible to the short term-

exposure to these agents. The concentration of tubulins increases with increasing parasite age (Fennell *et al.*, 2008) and tubulins undergo an active process of polymerisation and depolymerisation in schizont stages. This could explain the differences in susceptibility of each stage. Previous studies into dolastatin 10, vinblastine and taxol showed a similar result, but there was a marked increase in susceptibility of schizont-stage in comparison with trophozoite-stage parasites, and ring-stage parasites appeared unaffected by exposure to these agents, which is a profile not quite mirrored by the antimitotic herbicides. This difference corresponds to the broader stage susceptibility profile seen for the antimitotic herbicides as discussed above and may be a result of the more rapid cellular accumulation of the herbicides and/or binding to tubulin that is present but not yet assembled into mitotic spindles.

It is clear that antimitotic herbicides and other microtubule inhibitors block parasite maturation and consequently inhibit re-invasion of erythrocytes, but the unanswered question of how they kill the parasites remains. The potential of antimitotic agents to induce PCD in mammalian cells (Mollinedo and Gajate, 2003) and *Leishmania* (Jayanarayan and Dey, 2005) has been documented. PCD has a number of markers which have to be present to confirm that cell death occurs by this mechanism. One of the most common and well established of these markers is the activation of caspases, a group of cysteine proteases of the clan CD that possess a catalytic dyad, His and Cys, and a conserved caspase fold essential for activity (Barrett and Rawlings, 2001) and are defined by their target specificity. Evidence of caspase-3 (DEVDase)-like activity in mosquito stages of *P. berghei* has been previously noted (Al-Olayan *et al.*, 2002; Hurd *et al.*, 2006) and linked to additional PCD markers (such as DNA fragmentation, phosphatidylserine translocation and chromatin condensation). In addition to this and subsequent to the completion of this study, a report was published detailing the presence of two *P. falciparum* genes encoding putative metacaspases. One of these, designated PfMCA1, contains both a universally conserved catalytic cysteine and histidine dyad (required for catalytic activity) and a putative caspase recruitment domain in the N-terminal amino acid sequence (Meslin *et al.*, 2007).

An assay to detect DEVDase activity in *P. falciparum* asexual stages in response to treatment with taxol, trifluralin or vinblastine was carried out. Results showed that both taxol and vinblastine induced DEVDase activity which is absent in untreated control cultures. Addition of the pan-caspase inhibitor *Z*-VAD-fmk prior to the microtubule inhibitors prevented this induction of DEVDase activity. Trifluralin

treatment did not result in a similar induction of DEVDase activity (fig. 4.5). A previous report on the response of *Leishmania* to treatment with taxol and trifluralin showed a similar result: taxol appeared to induce a PCD-type response while trifluralin treatment did not (Jayanarayan and Dey, 2005). A combination of these two studies may indicate that microtubular disruption, which occurs for all three agents tested, is not sufficient to produce the activation of caspase-like activity and thus a PCD–type response.

Despite this evidence for inducible caspase-3 activity, it cannot be stated that PCD definitely occurs upon exposure to taxol and vinblastine: additional markers of PCD must first be demonstrated (time constraints did not allow this to be done). The difference in response to treatment with trifluralin must also be explained. If, however, PCD-like cell death is confirmed in *Plasmodium* there is potential for such a pathway to be exploited for drug development and such an approach warrants further investigation.

The antimalarial effects of the antimitotic herbicides, while broadly similar to those of other microtubule inhibitors, do appear distinct. The stage susceptibility profile exhibited by these agents appeared broader than for agents previously investigated (Table 4.2) and all three stages were to some extent irreversibly inhibited by a short exposure (Fig. 4.4). The ability of taxol and vinblastine to induce DEVDase activity in treated parasites was also not mirrored upon exposure to trifluralin (Fig. 4.5). The binding site of the antimitotic herbicides has yet to be determined, but recent reports have proposed two different locations on the tubulin dimer based on molecular modeling studies (Anthony and Hussey, 1999; Mitrofanova *et al.*, 2003; Morrissette *et al.*, 2004), all of which are distinct from those previously identified for other microtubule inhibitors. The effects of antimitotic herbicides on *P. falciparum* microtubular architecture are also distinct from those of dolastatin 10/vinblastine and taxol (Fennell *et al.*, 2006).

Despite these differences the primary mode of action of the antimitotic herbicides appeared to be the inhibition of schizont maturation and merozoite production, with consequently a reduction in parasitaemia.

In summary, the studies of this chapter demonstrate the selective activity of several antimitotic herbicides, with their primary effect to inhibit progression through schizogony, and their distinct stage-susceptibility profile. In addition, the ability of taxol and vinblastine to induce caspase-3 like activity in *P. falciparum* was also demonstrated suggesting the possibility that the parasite may undergo a PCD-like response.

Chapter 5

Accumulation of Trifluralin and Vinblastine by *P. falciparum*

5.1. INTRODUCTION

The activity of antimitotic herbicides against erythrocytic stages of *P. falciparum* has been investigated in the previous chapter. The potencies of these agents, in the low μM range, are considerably less than those found for antimitotic agents such as vinblastine and taxol, that have IC_{50} values down to the nM range (Schrevel *et al.*, 1994; Sinou *et al.*, 1998; Fennell *et al.*, 2003; Fennell *et al.*, 2006). Studies in mammalian cells with a number of antimitotic anticancer agents have indicated the close correlation between cytotoxicity and efficiency of drug accumulation (Ferguson and Cass, 1985; Verdier-Pinard *et al.*, 2000).

The importance of cellular accumulation of drugs in *P. falciparum* was emphasised when extensive studies on the cellular accumulation of chloroquine and related compounds in *P. falciparum* led to the confirmation that in addition to some level of detoxification by malarial cytochrome P450 enzymes, resistance to these agents correlated with a reduction in cell associated drug (Gluzman *et al.*, 1987; Ndifor *et al.*, 1990; Ndifor *et al.*, 1993; Bray *et al.*, 2006).

The work outlined in this chapter focuses on two antimitotic agents, the '*Vinca*' alkaloid vinblastine, isolated from the Madagascar periwinkle *Catharanthus roseus* (formerly *Vinca rosea*) and trifluralin, a synthetic dinitroaniline. While both these agents cause breakdown of mitotic microtubular structures, they differ greatly in their potency against asexual cultures of *P. falciparum* (Bell, 1998; Fennell *et al.*, 2006). Vinblastine has a range of reported IC_{50} values against these cultures of 0.028 to 1.7 μM while that for trifluralin is <1 to 3 μM (Bell, 1998). Studies in mammalian cells among '*Vinca*' alkaloids have shown the close correlation between cytotoxicity and efficiency of drug accumulation as mentioned above (Ferguson and Cass, 1985). A similar correlation was noted for vinblastine and the more potent dolastatin 10, where the ability of mammalian cells to retain dolastatin 10 once accumulated exceeded the same cells ability to retain the less potent vinblastine (Verdier-Pinard *et al.*, 2000).

The work outlined in this chapter describes the first investigation into the accumulation of microtubule inhibitors into erythrocytes infected with *P. falciparum,* with the aim of determining whether the relatively modest activity of trifluralin could be accounted for by limited uptake into parasite cells. Several aspects of accumulation were investigated: (i) the extent and kinetics of accumulation into uninfected erythrocytes and those infected with trophozoite- and schizont-stage parasites, (ii)

whether each compound co-localised with its tubulin target, (iii) whether accumulation was saturable, (iv) whether accumulation was reversible and (v) if there was any evidence for efflux of either compound. The concentrations of both agents chosen for these studies were their respective IC_{50} values; for this reason there is a large absolute difference in concentrations used. The effect, if any, of non-specific accumulation of trifluralin was investigated by exploring the effect of inoculum size on parasite susceptibility to trifluralin.

Understanding of the factors limiting accumulation of trifluralin into parasites and comparison with uptake of the more potent vinblastine may aid in the design of new dinitroaniline derivatives with higher antimalarial potency. With this in mind, a number of trifluralin derivatives were assessed for antimalarial activity. The glycoconjugated derivatives analysed had been found to have moderate activity against *Cryptosporidium* (Mead *et al.*, 2003) and the N-substituted compounds had improved anti-leishmanial activity (Esteves *et al.*, 2006) over their trifluralin precursor. It was assumed by both groups that the increased potency resulted from improving the ability of trifluralin to accumulate in cells. The possibility that delivery of trifluralin could be enhanced using a liposomal formulation was also explored.

5.2. RESULTS

5.2.1. Extent and kinetics of accumulation

5.2.1.1. Accumulation in uninfected erythrocytes

In order to study accumulation of both agents by intact parasitised erythrocytes it was first necessary to identify what portion of accumulation was due to the erythrocyte itself. For this reason uninfected erythrocytes were exposed to 3μM of [^{14}C] trifluralin or 25nM [^{3}H] vinblastine and incubated at 37°C. Samples were taken periodically and processed for scintillation counting as described in section 2.5.1. These cells showed modest accumulation of both compounds: as they lack microtubules this is as expected. However the CAR were still approximately 20-40 for trifluralin and between 1 and 5 for vinblastine (Fig. 5.1 (A)). Previous studies with chloroquine have shown a CAR of 14 in uninfected erythrocytes (Fitch, 1969). There is no known erythrocytic target for any of these compounds so the ability of erythrocytes to concentrate them at all is unexplained. It is possible however that as both vinblastine and chloroquine have much higher aqueous solubilities (both defined as "freely soluble

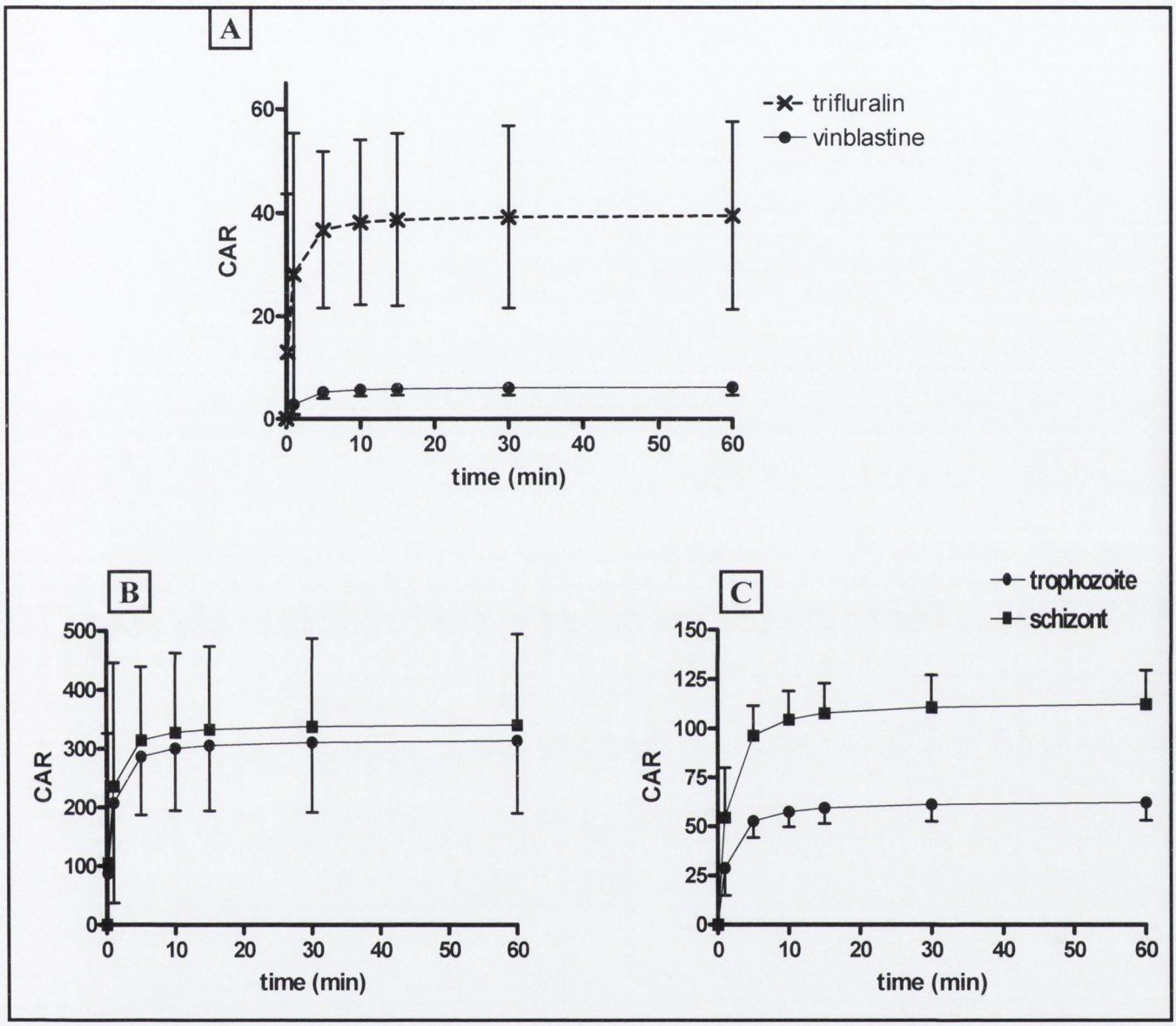


Fig. 5.1 Accumulation of [^{14}C]trifluralin and [^{3}H]vinblastine in uninfected erythrocytes (A) or those infected with trophozoite- or schizont- stage *P. falciparum* parasites ([^{14}C]trifluralin (B) and [^{3}H]vinblastine (C)). Uninfected (A) or infected erythrocytes (parasitaemia >85%; B and C) were suspended to a final haematocrit of 1-2%, exposed to either 3 μM [^{14}C]trifluralin (A and B) or 25 nM [^{3}H]vinblastine (A and C) and incubated at 37°C. Samples were taken over a 1-hour period and cell-associated accumulation of the compounds was measured by scintillation counting (see section 2.5). Accumulation is measured as the cell accumulation ratio (CAR) and results shown represent the mean ±95% CI for at least 3 replicate experiments.

in water" by the Merck Index (Budavari, 2001) than trifluralin (0.2 to 0.4 mg/l (Council, 1977)) this may indicate that more trifluralin than vinblastine (or chloroquine) is partitioned by the erythrocyte and parasite membranes.

5.2.1.2. Accumulation in infected erythrocytes

Once accumulation of the agents by uninfected erythrocytes was assessed and shown to be low, but not quite negligible it was felt that cultures of parasitised erythrocytes should be of as high a parasitaemia as possible. Magnetic purification and Percoll®-alanine gradients were both used as techniques for achieving parasite cultures of >85% parasitaemia (see sections 2.2.3 and 2.2.4). Due to the difficulties in achieving this high parasitaemia in cultures of ring-stage parasites, only cultures of trophozoite- and schizont-infected erythrocytes were used in this study. As with the uninfected erythrocytes, infected erythrocytes were exposed to 3μM [^{14}C] trifluralin or 25 nM [^{3}H] vinblastine and incubated at 37°C. Samples were taken periodically and processed for scintillation counting as described in section 2.5. The ability of parasitized erythrocytes to concentrate both compounds far exceeded that of uninfected erythrocytes, with CARs of ~300 for trifluralin and ~50 to 110 for vinblastine (fig. 5.1 (B) and (C)). This would correspond to the presence of a target or 'sink' for both agents in these cells that is absent from uninfected erythrocytes, thus allowing them to concentrate the compounds to a much greater extent.

Accumulation of both compounds in intact erythrocytes parasitized with both trophozoite- and schizont-stage parasites was shown to be rapid. Trifluralin accumulation reached an apparent maximum within 10 minutes (fig. 5.1 (B)), while vinblastine reached an apparent plateau following 15-20 minutes of exposure (fig. 5.1 (C)). The parasite stage present had no noticeable effect on the kinetics of accumulation of either compound.

5.2.2. Stage-dependence of accumulation

The presence of increased amounts of tubulin in the latter stages of asexual growth (Fennell *et al*, 2008) would indicate the likelihood of higher levels of accumulation of microtubule inhibitors in these stages. However, accumulation of trifluralin in parasitized erythrocytes was unaffected by the stage of parasite present (Fig. 5.1 (B)), with both trophozoite- and schizont-infected cultures exhibiting maximum CARs of ~300. Due to the need for a parasitaemia in excess of 85% to reduce the

impact of accumulation in uninfected erythrocytes, ring-infected erythrocytes were excluded from the current study as such high parasitaemias could not be achieved in these cultures.

Vinblastine accumulation in schizont-infected erythrocytes was ~2-fold higher than that observed for trophozoite-infected erythrocytes (CAR ~110 vs. ~55), (Fig. 5.1 (C)). The observation for vinblastine would concur with the increase in quantity of the presumed target, tubulin, in the latter stages of the intraerythrocytic cycle of the parasite (Fennell *et al.*, 2008).

5.2.3. Sub-cellular locations of [^{14}C]trifluralin and [^{3}H]vinblastine in intact schizont-infected erythrocytes

Trifluralin, unlike vinblastine, appeared not to accumulate to increased levels in schizont-infected erythrocytes, which was unexpected due to the presence of increased amounts of tubulin present in these cells. To investigate whether both compounds co-located with their presumed microtubular targets, a crude sub-cellular fractionation was carried out on isolated parasites, dividing the parasites into particulate and soluble fractions following release of the parasites from erythrocytes by saponin lysis, breakage of the cells by freeze/thaw lysis and centrifugation for 5 min at 4°C at 18,000 x *g* (see section 2.2.6). Figure 5.2(A) shows the presence of β-tubulin mainly in the soluble fraction, which includes the cytosol and presumably the nuclear lumen, with little in the membrane-containing particulate fraction. Although tubulins are the presumed molecular targets of trifluralin (Fennell *et al.*, 2006) the distribution of [^{14}C]trifluralin was quite different from that of β-tubulin. Instead of a higher level in the soluble fraction, trifluralin was distributed fairly evenly with a ratio of approximately 4.5:5.5 particulate: soluble (Fig. 5.2(B)). This is consistent with both binding to a cytosolic target, presumed to be microtubules, and accumulation in membranes, presumably due to the high lipophilicity of trifluralin. The distribution of [^{3}H] vinblastine more closely mimics that of β-tubulin with ~75% co-locating with tubulin in the soluble fraction (Fig. 5.2(B)).

5.2.4. Effects of unlabeled trifluralin or vinblastine on the accumulation of their respective radiolabeled forms

Vinblastine was accumulating in parasitised erythrocytes as would be expected for a microtubule inhibitor specifically binding to its microtubular target. Trifluralin,

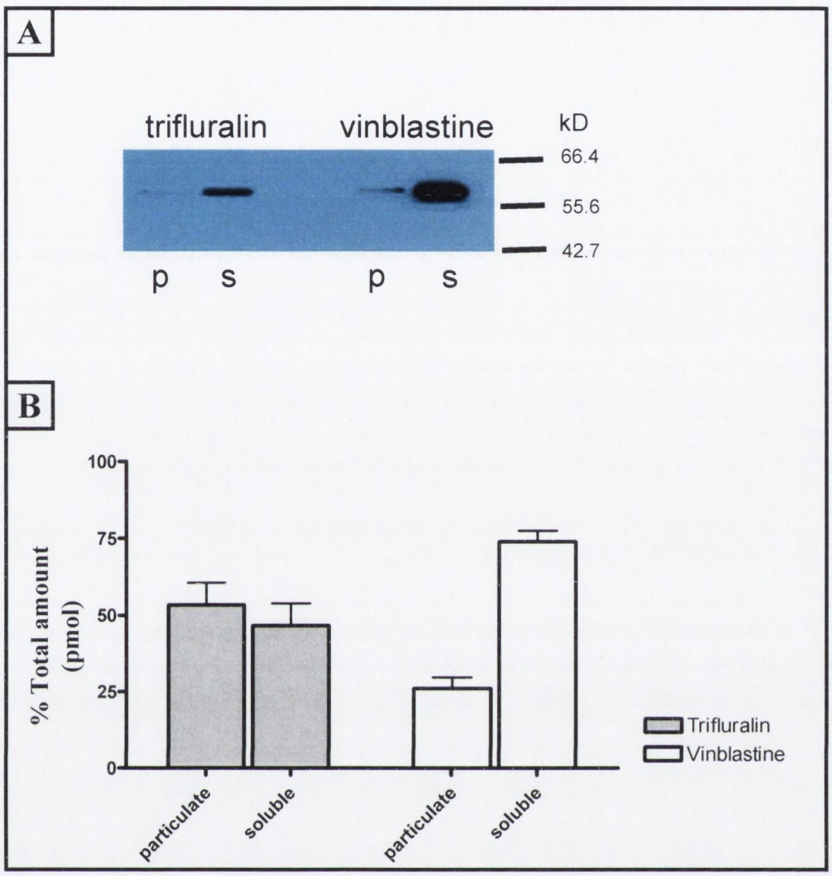


Fig. 5.2. Location of β-tubulin (A) in relation to the accumulation of [^{14}C]trifluralin and [^{3}H]vinblastine (B). Schizont-infected erythrocytes (1-2% haematocrit; >85% parasitaemia) were incubated in the presence of either 3 μM [^{14}C]trifluralin or 25 nM [^{3}H]vinblastine for 2 h, freed from the erythrocytes using saponin lysis and split into two fractions, a particulate (p) and soluble (s) fraction, by three rounds of freeze-thaw lysis (see section 2.5.4) followed by centrifugation. Protein concentrations of each sample were determined using the assay of Bradford. Samples were analysed by SDS-PAGE followed by western blotting using anti-β-tubulin antibodies (A) and by scintillation counting (B). All lanes contain 1.5 μg total protein and the running positions of molecular weight markers are shown in kDa on the left of the immunoblot. The data in fig. 5.2(B) represent the mean ± SEM for at least 3 replicate experiments and the y-axis shows the amount of agent in each fraction as a % of the sum total of agent present.

however, was displaying evidence that in addition to co-locating with its microtubular target, a significant proportion of accumulation was occurring in the particulate fraction. If this was non-specific accumulation due to, for example accumulation in membranes due to the hydrophobic nature of this agent, the presence of excess trifluralin should have little or no effect on accumulation of the radiolabelled form. The effects of increasing concentrations of unlabeled compound on the ability of schizont-infected erythrocytes to accumulate the labelled form were therefore examined. The poor solubility and low specific activity of trifluralin prevented a broader range of concentrations being used than those shown in Fig. 5.3(A). However, it was shown that the addition of 1000-fold excess of unlabeled compound had little effect on the ability of the cells to accumulate [^{14}C]trifluralin (Fig. 5.3(A)). This indicates that at these concentrations no saturation of either target or transport mechanism (passive or active) occurs. This is not replicated for vinblastine, where a significant reduction in accumulation of the radiolabelled drug was seen as the unlabeled compound concentration was increased (Fig. 5.3(B)) indicating saturation of either target or transport mechanism above ~200 nM vinblastine.

5.2.5. Reversibility of accumulation of [^{14}C]trifluralin and [^{3}H]vinblastine in intact schizont-infected erythrocytes

Previous work with the dinitroaniline oryzalin in plant cells indicated that accumulation could be reversed by repeated washing in herbicide free medium (Upadhyaya and Nooden, 1980). A similar result was noted for trifluralin accumulation in *P. falciparum*-parasitized erythrocytes with only ~15% of the accumulated compound remaining after 10 washes. The reduction was continuous with additional trifluralin lost following each subsequent wash (Fig. 5.4(A)). This would indicate that a high proportion of the compound is accumulated via a reversible low-affinity association. In contrast, ~65% of accumulated vinblastine was retained by the parasitized erythrocytes following multiple washes (Fig. 5.4(B)). The reduction in accumulated vinblastine was noted following the first and second wash with little or no significant release of vinblastine after this (Fig. 5.4(B)). So while there does appear to be some level of low-affinity association of vinblastine, most of the drug is retained by the cells through binding to a higher-affinity target.

5.2.6. Release of [^{14}C]trifluralin and [^{3}H]vinblastine from intact schizont infected erythrocytes

Mammalian tumour cell resistance to vinblastine has been attributed to the active efflux of the drug through a P-glycoprotein pump (Miyamoto *et al.*, 1996). No such release of either vinblastine (Fig. 5.5(B)) or trifluralin (Fig. 5.5(A)) was noted following 24 h incubation of parasitized erythrocytes pre-loaded with either of the radiolabelled compounds. A reduction in accumulated compound was seen immediately following the wash step prior to re-incubation but this could be attributed to reversible or low-affinity binding of the compound and not to an active efflux process.

5.2.7. Susceptibilities of asynchronous *P. falciparum* cultures of different parasitaemias to trifluralin treatment

Non-specific trifluralin accumulation would reduce the amount of compound available to inhibit its tubulin target. This explanation can be confirmed by investigating trifluralin sensitivity at two inoculum sizes, 0.2% and 1.5% parasitaemia. If this hypothesis is true, a lower IC_{50} value would be expected for cultures with a higher starting parasitaemia. Restrictions of assay sensitivity and parasite growth prevented a broader range being used, although a seven-fold increase in inoculum size should be sufficient to indicate any difference. The 48-h and 72-h IC_{50} values for cultures with an initial parasitaemia of 0.2% were ~1.3 and ~0.5 μM respectively, compared with ~3.9 and ~1.4 μM for cultures of initial parasitaemia of 1.5% (Fig. 5.6). This is consistent with the presence of an inoculum effect.

5.2.8. Growth inhibition of *P. falciparum* by trifluralin derivatives

5.2.8.1. N-substituted trifluralin derivatives

None of the N-substituted trifluralin derivatives showed improved antimalarial activity when compared to trifluralin (Fig. 5.7(B)). In fact, the IC_{50} values of these agents were 3- to 5-fold higher than trifluralin. Against *Leishmania* these compounds did appear more active (Esteves *et al.*, 2006) so the reason for this loss of potency against *P. falciparum* is unclear. Given that the added side groups are more polar and as such assumed to improve passage through and release from membranes it is possible that the replaced groups are important for binding to tubulin in *P. falciparum*.

5.2.8.2. Glycoconjugated trifluralin

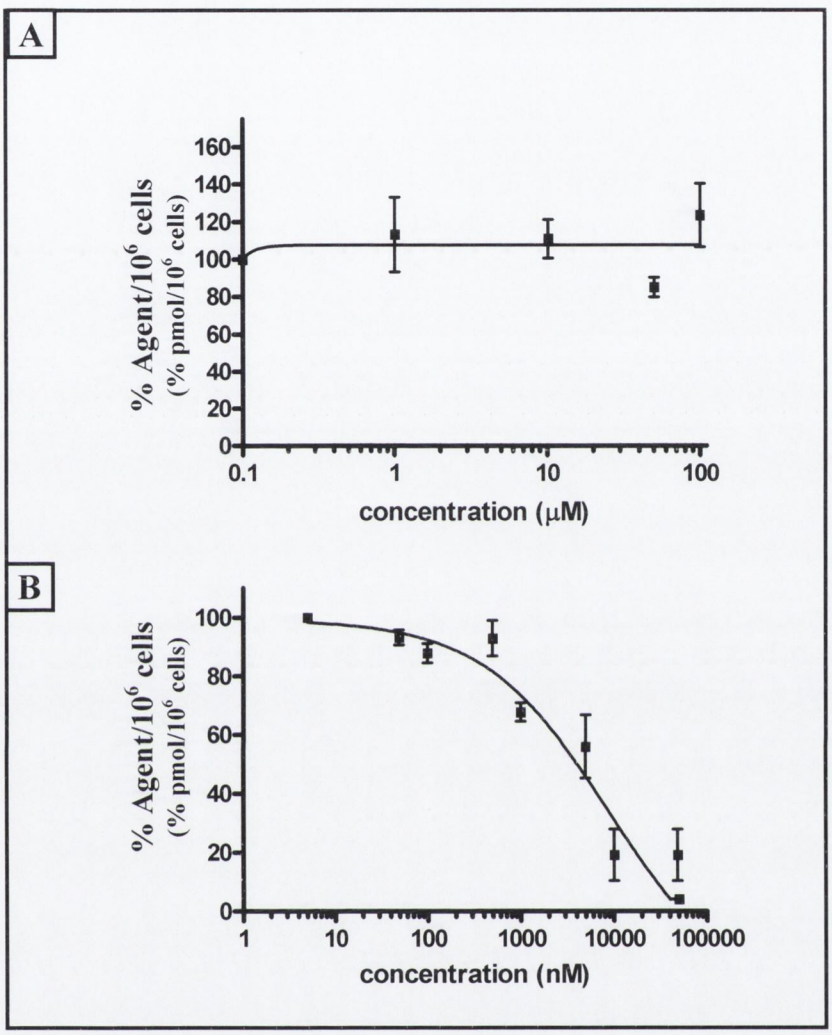


Fig. 5.3. The effects of unlabelled trifluralin (A) or vinblastine (B) on accumulation of the labelled form. Schizont-infected erythrocytes were incubated with 0.1 μM [^{14}C]trifluralin and increasing concentrations of the unlabeled trifluralin (A) or 5 nM [^{3}H]vinblastine and increasing concentrations of the unlabeled vinblastine (B) for 2 h at 37°C. Samples were taken and processed for scintillation counting. The cumulative concentration of both the labeled and unlabeled compound is plotted on the x-axis. The y-axis shows the amount of cell-associated compound present after 2h as a percentage of that found when no unlabeled compound is present. Data points shown represent the mean ± SEM for at least 3 replicate experiments and the curves represent the line of best-fit calculated to minimise the distance from all points.

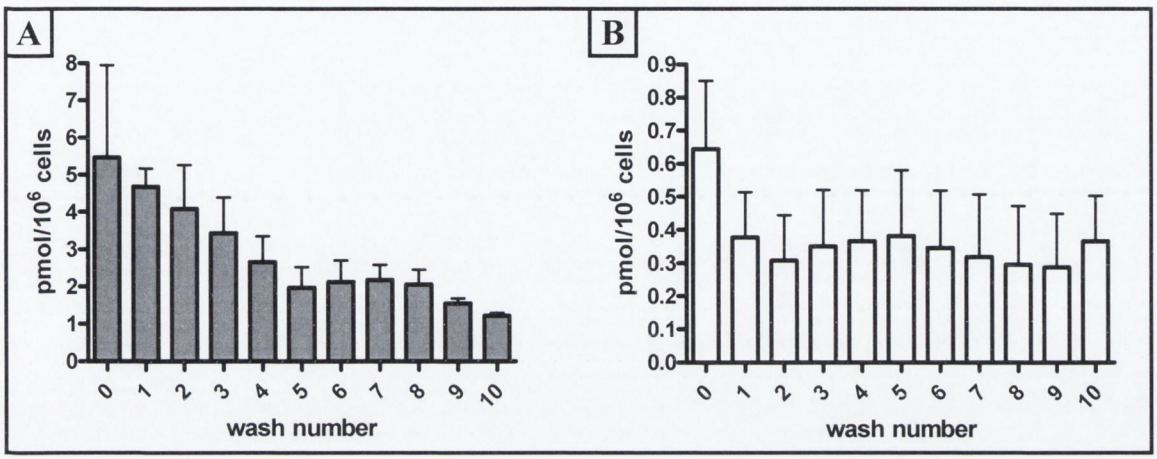


Fig. 5.4. Release of [^{14}C]trifluralin (A) and [^{3}H]vinblastine (B) accumulated in schizont-infected erythrocytes following repeated washing of the cells. Schizont-infected erythrocytes (1-2% haematocrit; >85% parasitaemia) were incubated in the presence of either 3 μM [^{14}C]trifluralin or 25 nM [^{3}H]vinblastine for 2 h, following which they were repeatedly washed in warm PBS with samples taken following each wash and cell-associated agent measured by scintillation counting (described in section 2.5.2). Results represent the mean ± SEM for 4 replicate experiments.

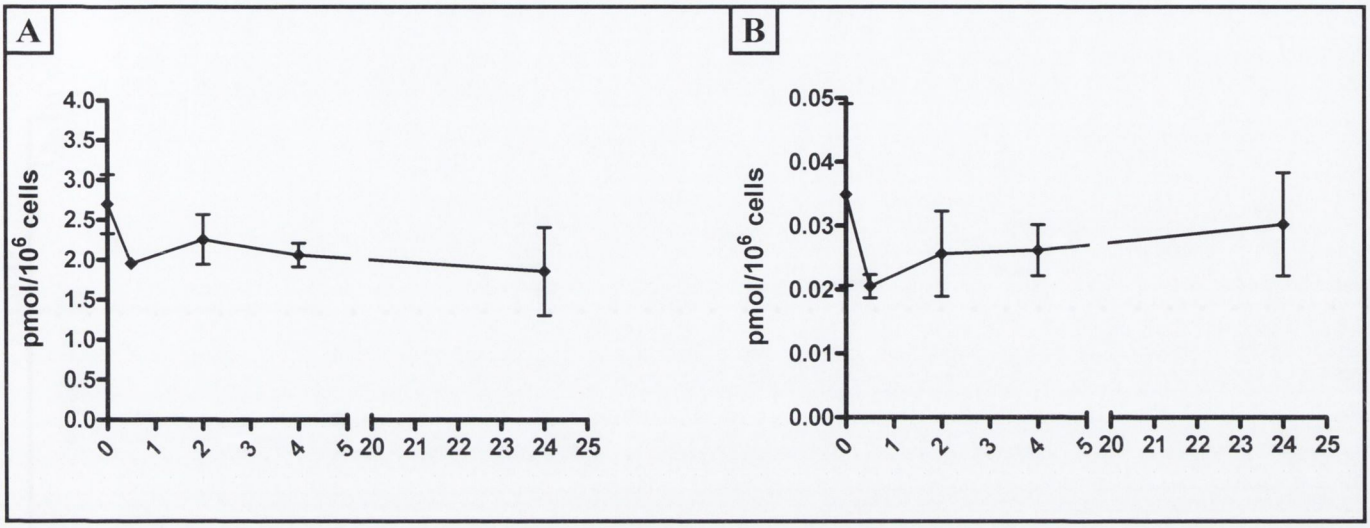


Fig. 5.5. Time course of release of [^{14}C]trifluralin (A) and [^{3}H]vinblastine (B) accumulated in schizont-infected erythrocytes. Schizont-infected erythrocytes (>85% parasitaemia; 1-2% haematocrit) were incubated with either 3 μM [^{14}C]trifluralin (A) or 25 nM [^{3}H]vinblastine (B) for 2 h at 37°C, cells were then washed in warm PBS twice and re-incubated in compound-free medium. Cell associated compound was measured by scintillation counting. Results represent the mean ± SEM for 3 replicate experiments.

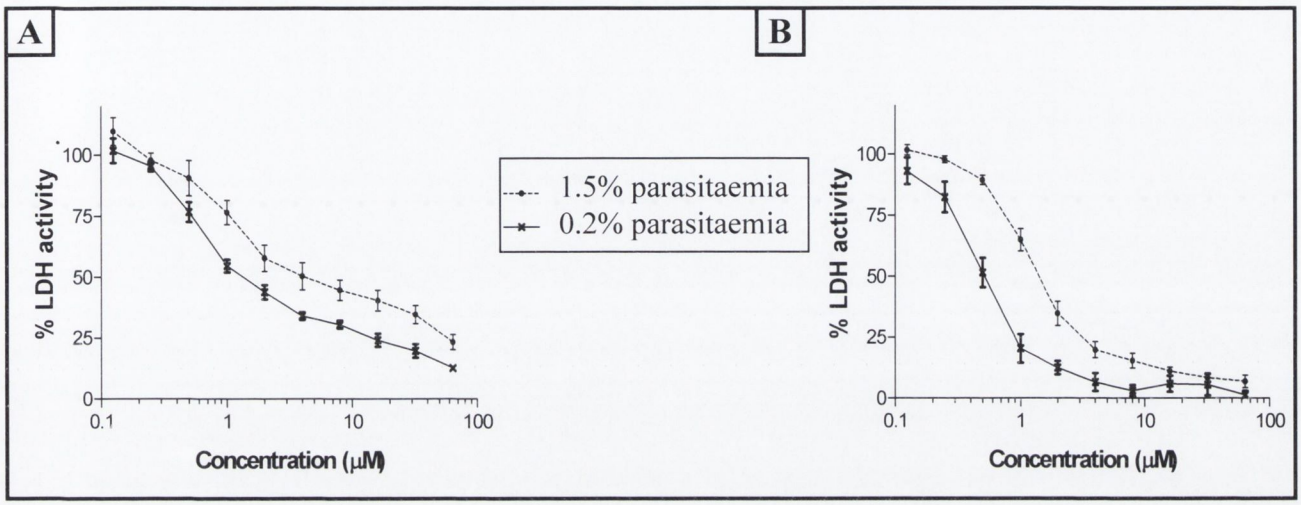


Fig. 5.6. Susceptibilities of asynchronous *P. falciparum* cultures of different parasitaemias to trifluralin treatment. Parasite cultures of initial parasitaemias of 0.2% and 1.5% and initial haematocrit 0.4% were exposed to titrations of trifluralin. Parasite susceptibility was determined using the pLDH assay (section 2.3.1) following incubation times of 48 h (A) and 72 h (B). Geometric mean values ±SEM of eight determinations are shown.

None of the glycoconjugated derivatives showed improved antimalarial activity when compared with trifluralin (Fig. 5.8(B)). In fact, the IC_{50} values of these agents were 3- to 5-fold higher than trifluralin. Reasons for this decreased potency could be linked to the sheer size of the added sugar moieties, which may hinder the passage of trifluralin through the multiple membranes. They may also prevent trifluralin binding its target, although it is unlikely as it is assumed the binding site is the same for *Plasmodium* as it is in *Cryptosporidium* and no such hindrance to binding is apparent in these parasites (Mead *et al.*, 2003).

5.2.8.3. Liposomal trifluralin

Liposomal trifluralin is intended to improve the pharmacokinetic properties of this agent and has shown to improve activity against canine leishmaniosis *in vivo* (Marques *et al.*, 2007) but was investigated here with a view to possibly achieving better delivery to the target within parasites. As expected, both liposomal and normal trifluralin showed almost identical inhibitory curves following both 48 and 72 h incubation (Fig. 5.9 (i) and (ii)).

5.3. DISCUSSION

A number of microtubule inhibitors have been shown to inhibit growth of erythrocytic malarial parasites (Bell, 1998). Some, including the anticancer agents vinblastine, dolastatin 10 and taxol, have highly potent antimalarial activity but are equally active against mammalian cells (Schrevel *et al.*, 1994; Bell, 1998; Sinou *et al.*, 1998; Fennell *et al.*, 2003). Others, especially the dinitroaniline herbicides, of which trifluralin has been most investigated, are less potent but do not affect mammalian cells (Fennell *et al.*, 2003). An obvious goal would be to design an antimalarial compound that would combine the potency of the anticancer microtubule inhibitors with the selectivity of the herbicides. One explanation for the modest potency of trifluralin may be related to aspects of cellular accumulation of this compound, so the uptake of trifluralin into erythrocytic parasites was compared with that of the more potent antimitotic agent vinblastine, for which based on previous publications (Ferguson and Cass, 1985; Verdier-Pinard *et al.*, 2000) there were expected to be no major barriers to penetration. It has been suggested that the hydrophobic nature of trifluralin may restrict its access to intracellular targets, thus increasing the dose required to effectively kill the

target organism (Benbow *et al.*, 1998). Previous attempts to synthesise derivatives with polar groups have resulted in compounds with greater activity against human parasites such as trypanosomes and *Cryptosporidium parvum* (Benbow *et al.*, 1998) but there are as yet no publications to show that this works in *Plasmodium*.

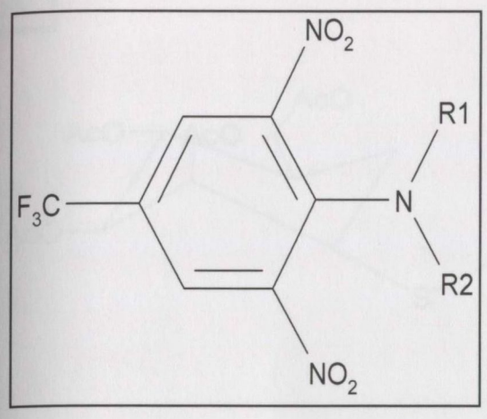
Trifluralin and vinblastine were both concentrated by parasitised erythrocytes. Trifluralin achieved a CAR 3- to 6-fold higher than vinblastine in *P. falciparum* infected erythrocytes, reaching a final cell associated concentration of approximately 5.5 pmol/10^6cells compared to 0.65 pmol/10^6 cells for vinblastine. No significant differences in the kinetics of uptake were noted, with both trifluralin and vinblastine accumulating rapidly in cells. Due to the increased quantity of microtubular target in schizont-stage parasites relative to trophozoites it was expected that there would be a corresponding increase in cell-associated compound in schizonts. This was true for vinblastine, with a 2-fold higher CAR in schizont-infected erythrocytes. It was however not the case for trifluralin, where the CAR for the two stages were similar.

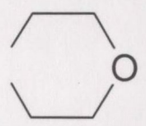
Unlike vinblastine, which accumulated to a low level in uninfected erythrocytes, trifluralin appears to be concentrated in these cells by a factor of approximately 30. When compared to other anti-malarial agents such as chloroquine, which also have no known target in erythrocytes, this concentration of trifluralin is unusually high. Based on the hydrophobic nature of the trifluralin molecule it is likely that this is a result of association with membrane lipids. This association is further indicated by the higher than expected level of association of trifluralin with the particulate fraction of the parasites. Vinblastine predominantly co-located with β-tubulin in the soluble fraction, which is consistent with tubulins being the primary target of this drug, with very little associated with the particulate fraction. Accumulation in the membrane could also mask any increase in trifluralin accumulation due to an increase in the target microtubules.

Experiments into the effect of unlabeled vinblastine on accumulation of the labelled form indicated that accumulation of vinblastine was saturable, either by saturating the target or transport mechanism. Previous studies in mammalian cells have indicated that vinblastine accumulation is mainly by passive diffusion (Ferguson and Cass, 1985), and the speed of accumulation in parasitized erythrocytes concurs with this. As this drug is known to bind the end of microtubules, which represent a finite number of target sites and accumulation by passive diffusion is unlikely to be saturable at the concentrations used in this study, this effect is likely to be due to saturation of target. Uptake of trifluralin was non-saturable, consistent with its proposed accumulation

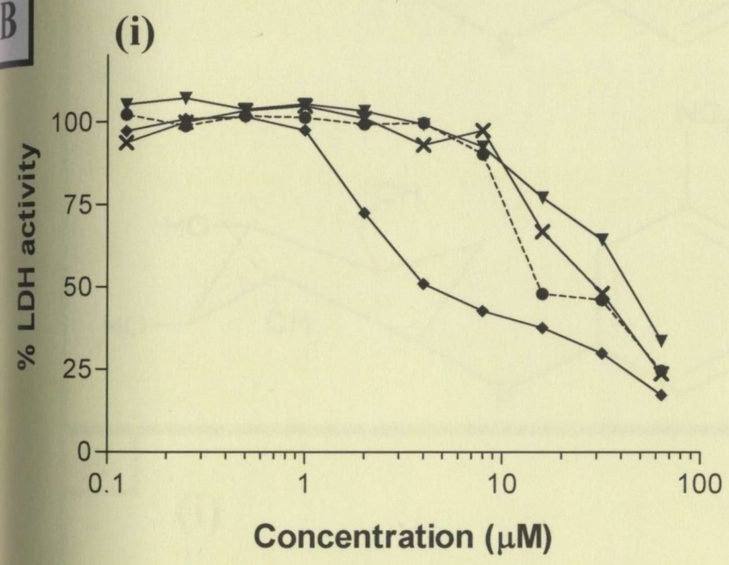
Fig. 5.7. Chemical structures of (A) and susceptibilities of asynchronous cultures to (B) trifluralin derivatives. Parasite cultures of initial parasitaemia 0.8% and haematocrit 0.4% were exposed to titrations of each compound. Parasite susceptibility was determined using the pLDH assay (section 2.3.1). Geometric mean values of three to four determinations after 48 h (i and iii) and 72 h (ii and iv) are shown. Tfl=trifluralin.

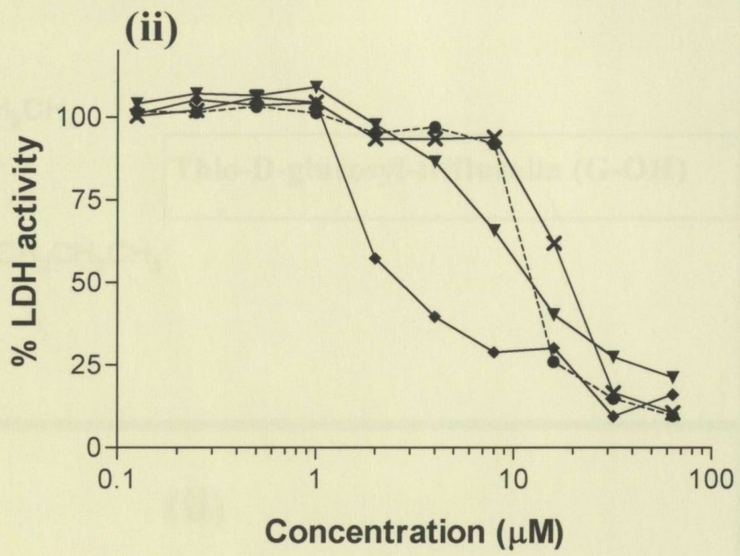
A

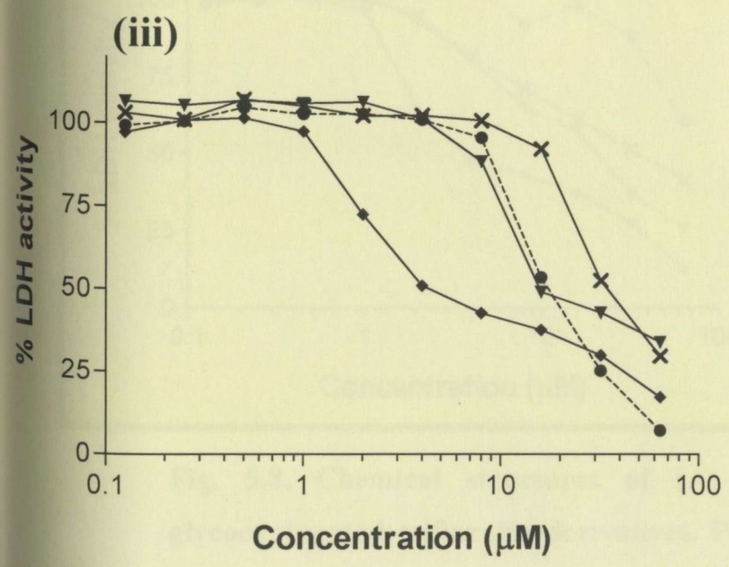


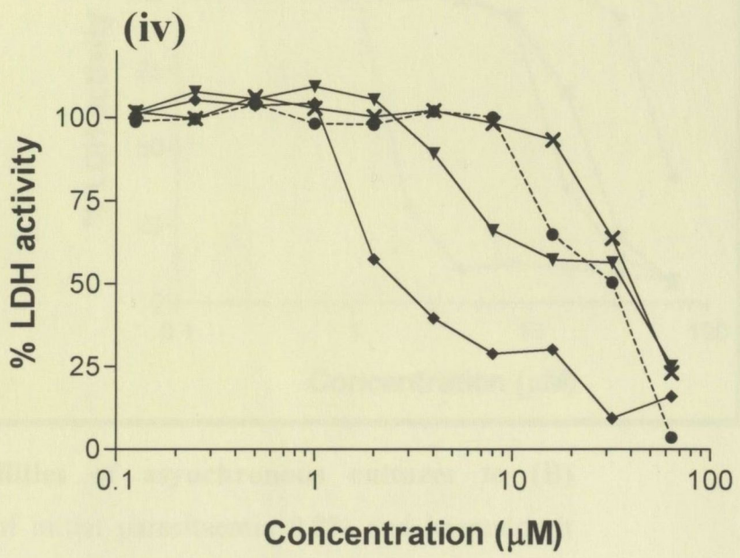
	R1		R2
Trifluralin	$CH_2CH_2CH_3$		$CH_2CH_2CH_3$
CN 1	CH_2CH_3		$(CH_2)_2OH$
CN 2	H		*i*- $CH_2CH_2CH_3$
CN 3			
CN 6	H		$-C_6H_5-OH$
CN 7	Bu		$(CH_2)_2OH$
CN 8	$CH_2CH_2CH_3$		$(CH_2)_2OH$

B









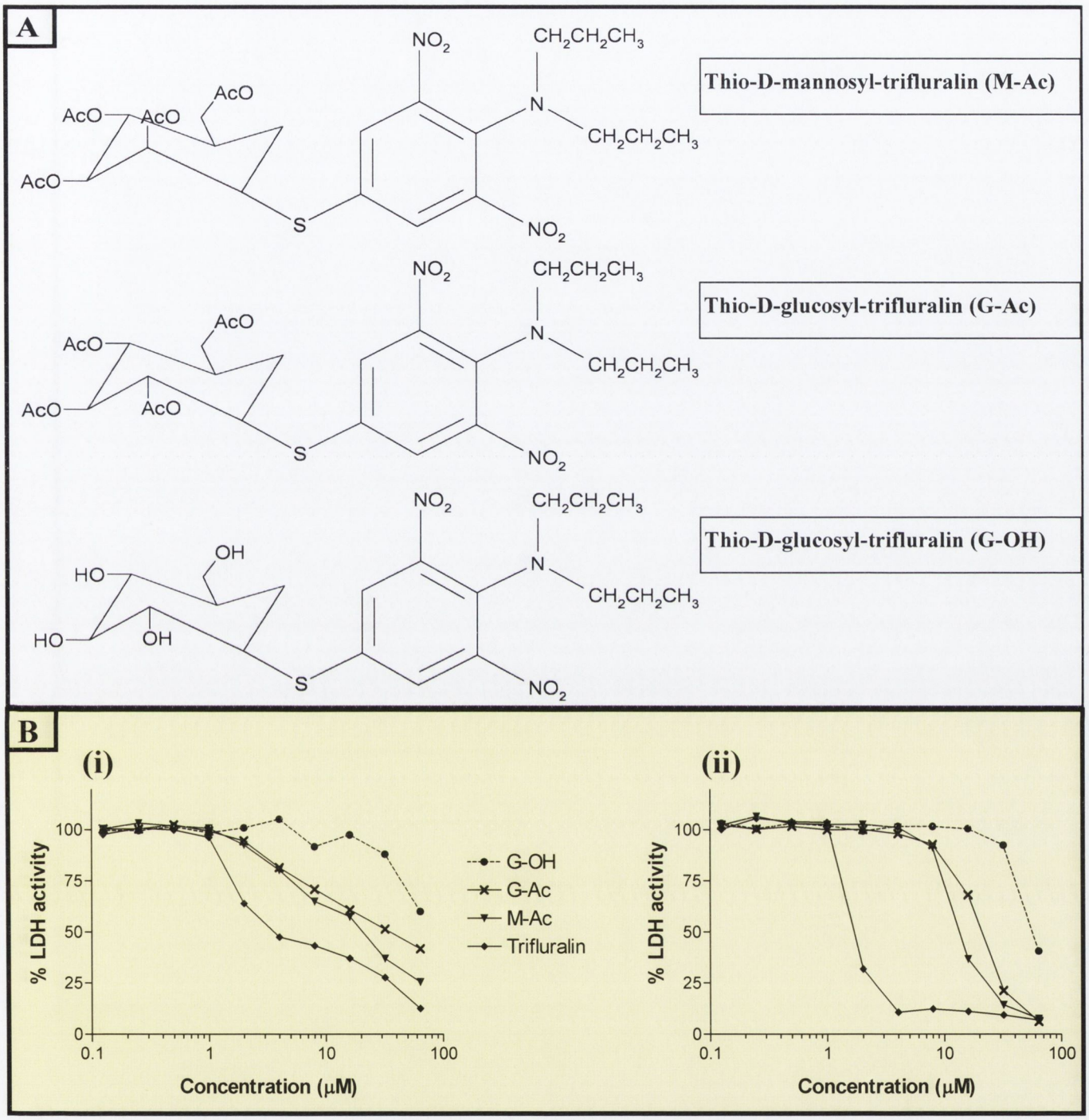


Fig. 5.8. Chemical structures of (A) and susceptibilities of asynchronous cultures to (B) glycoconjugated trifluralin derivatives. Parasite cultures of initial parasitaemia 0.8% and haematocrit 0.4% were exposed to titrations of each compound. Parasite susceptibility was determined using the pLDH assay (section 2.3.1). Geometric mean values of three to four determinations after 48 h (i) and 72 h (ii) are shown. M-Ac=Thio-D-mannosyl-trifluralin; G-Ac=Thio-D-glucosyl-trifluarlin; G-OH=Thio-D-glucosyl-trifluralin.

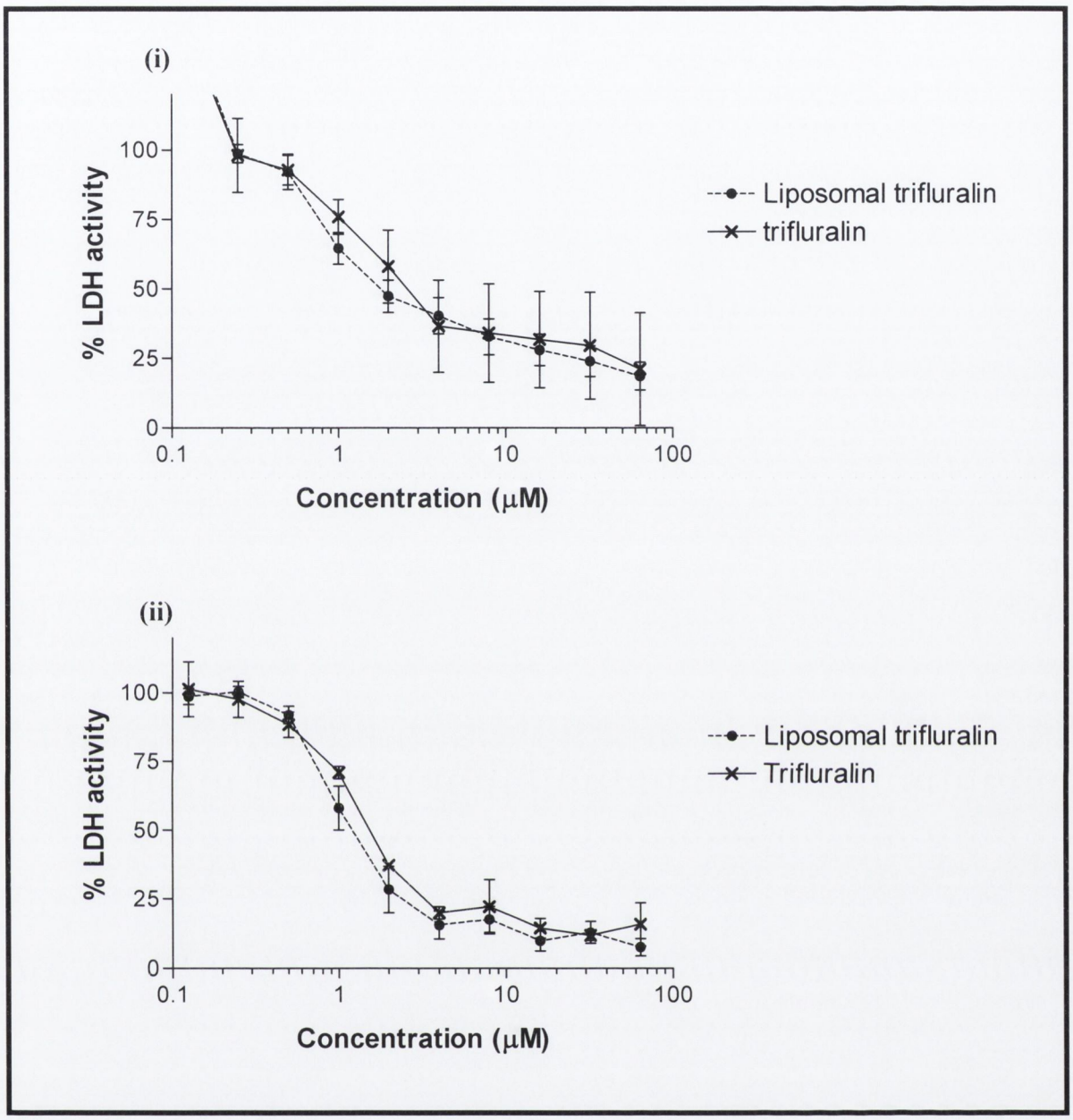


Fig. 5.9. Susceptibilities of asynchronous cultures to liposomal trifluralin. Parasite cultures of initial parasitaemia 0.8% and haematocrit 0.4% were exposed to titrations of each compound. Parasite susceptibility was determined using the pLDH assay (section 2.3.1). Geometric mean values ±SEM of three to four determinations after 48 h (i) and 72 h (ii) are shown.

predominantly in cellular membranes. In addition to this membrane accumulation of trifluralin, it is possible that, as this agent is thought to bind to unpolymerised tubulin, the abundance of targets may also contribute to the lack of saturation by unlabelled excess trifluralin. Further evidence for a low-affinity association of trifluralin with a cellular target was the ease with which accumulation was reversed by several wash steps. Almost 85% of cell associated trifluralin was removed following ten wash steps. In contrast, approximately 65% of accumulated vinblastine was retained by the parasitized erythrocytes following multiple washes. So while there does appear to be some level of low-affinity association of vinblastine, most of the drug is retained by the cells through binding to a higher-affinity target.

There was no detectable efflux of either trifluralin or vinblastine. This may have importance in the development of resistance to potential future antimalarial agents based on these compounds, as active efflux contributes to the conferring of resistance to certain agents such as quinolines in *P. falciparum* (Le Bras and Durand, 2003).

The assumption that non-specific accumulation of trifluralin is reducing the amount available to bind to its target can be corroborated by the presence of an inoculum effect. Differences in susceptibility of two otherwise identical cultures of *P. falciparum* at the same haematocrit but different parasitaemia would indicate the presence of such an effect, and this was shown to be the case (Fig. 5.6). Following both at 48 and 72 h incubation, cultures with an initial parasitaemia of 0.2% had an IC_{50} value ~3-fold lower that the corresponding cultures with initial parasitaemia of 1.5%.

In summary, results indicate that accumulation of trifluralin into parasites is not a barrier to its antimalarial potency but partitioning in both erythrocyte and parasite membranes may reduce the amount of compound available to bind the target. Several trifluralin derivatives, each designed to help reduce lipophilicity or improve transport through the membrane, were tested in parallel with trifluralin for any increase in potency. Neither the addition of mannose or glucose sugars (Fig. 5.6) nor the replacement of side-groups with less polar groups (Fig. 5.5) improved activity; in fact each modification appeared to reduce the antimalarial potency of the agents. It may be that the bulky sugar moieties added, hinder passage through the multiple membranes before reaching the parasite cytoplasm, rather than aid it. It may also be the case that the sugar moiety interferes with binding of the trifluralin to its tubulin target. While it appears unlikely that the polar side chains reduce passage of trifluralin through membranes this may be the case. The replaced side-chains of the original trifluralin

molecule may also play a role in binding of trifluralin to *Plasmodium* tubulin and their loss results in reduced potency. Liposomal trifluralin had an almost identical susceptibility profile to that of unmodified trifluralin against asynchronous cultures of *P. falciparum* (Fig. 5.7). This was not entirely unexpected as this modification is intended only to improve the pharmacokinetic properties of this agent *in vivo* (Marques *et al.*, 2007).

While the trifluralin derivatives assessed in this chapter showed no improved antimalarial potential, the indication that membrane accumulation of this compound may reduce available compound, further derivatives reducing this membrane accumulation may have the desired improved potency. In addition, it will be important to measure the binding affinity of trifluralin for tubulin and microtubules and to investigate whether derivatives with higher affinity can be designed. Due to the high potential of dinitroanilines as a group it is important to continue to characterise interactions with *Plasmodium* in the hope that a viable anti-malarial agent can be developed.

Chapter 6

General Discussion

Antimalarial chemotherapy still remains among the most important tools in the fight against malaria as current vaccine development programs have yet to produce a viable vaccine, although recent reports have been more promising (Macete *et al.*, 2007a; Macete *et al.*, 2007b). As with most drug-based antimicrobial therapies the emergence of resistance to these agents remains a constant problem (Breman *et al.*, 2004). Altering existing compounds or the use of combination therapies to avoid resistance mechanisms has prolonged the use of agents such as chloroquine, but the ever evolving nature of the parasite means that this reprieve is temporary. New targets for novel treatments for which no resistance yet exists, appear to be a more promising source of drug therapies with a longer life-span.

In the context of this thesis the cell cycle of the asexual intra-erythrocytic stages of the parasite's life-cycle and the processes therein were thought to represent a promising source of such new targets. It was felt that uncovering how the eukaryotic cell-cycle progression model applies to this parasite was an important step in understanding this essential process. The studies carried out encompassed studying the cell cycle as a whole and a more specific focus on mitosis and its inhibition by members of two classes of antimitotic herbicides.

6.1. STUDYING THE *PLASMODIUM FALCIPARUM* CELL-CYCLE

The first aim of this project encompasses the study of the cell cycle, or intraerythrocytic schizogony, as a whole. How *P. falciparum* schizogony fits in to the general model of the eukaryotic cell-cycle remains unknown due, in part, to the difficulty in studying this process in these cells. Synchronising a population of the parasites to a specific cell-cycle stage was seen as a promising method for elucidating what cell-cycle stages were present, their durations and events occurring within them. In response to this requirement, members of several classes of inhibitors targeting different process involved in cell-cycle progression were assessed for their ability reversibly to prevent *P. falciparum* schizogony, allowing accumulation of a population of parasites at a particular cell-cycle stage. An accurate method of measuring parasite DNA content by flow cytometry was also refined.

'Batch' synchronisation techniques are based on the ability of an inhibitor to prevent cell-cycle progression beyond a certain point. Once sufficient time has been

allowed for all cells in the population to reach this point, the effects of the inhibitor are reversed and the cells allowed to proceed through the remaining cell-cycle phases uninhibited. By this definition, both roscovitine and L-mimosine alone of all those agents tested appeared to achieve synchronisation in *P. falciparum*, by reversibly inhibiting progression into schizogony. Despite the perceived success of the technique, its value as a tool for investigating cell-cycle events is questionable. The first problem is quite specific to the parasite being studied here. As previously stated, the success of batch synchronisation of cultures depends on the presence of checkpoints at the end of each cell-cycle phase preventing the cell from progressing past a certain point until it is ready to do so. It has been established, both by work outlined in this thesis and elsewhere (Sinou *et al.*, 1998; Fennell *et al.*, 2006; Naughton *et al.*, 2007), that treatment with numerous different classes of microtubule inhibitors does not inhibit DNA synthesis despite successful disruption of mitosis, so it is unlikely that a conventional checkpoint exists at the end of G_1 phase. The presence or absence of the other known eukaryotic cell-cycle checkpoints in *P. falciparum* schizogony has yet to be fully investigated. The asynchronous nuclear divisions that occur in schizogony (Chen *et al.*, 1994; Leete and Rubin, 1996) in combination with the lack of at least one common cell-cycle checkpoint indicate that the control of schizogony may not conform to the accepted model of eukaryotic cell-cycle control.

Additional problems arise when the targets of both agents used to achieve a reversible block on schizogony are examined. Roscovitine targets cell-cycle control as a means for synchronising the cell cycle but may have multiple targets in *P. falciparum*. The use of CDK inhibitors to synchronise the cell-cycle is based on what is known of control of the cell cycle of other eukaryotic cell types (Baserga and Rubin, 1993; Grana and Reddy, 1995; Nigg, 1995; O'Connor and Jackman, 1995; Merrill, 1998; Shackelford *et al.*, 1999; Davis *et al.*, 2001; Golias *et al.*, 2004; Bach *et al.*, 2005; Sanchez and Dynlacht, 2005; Thomas, 2005) and as has been stated already (see section 1.2.3), this model may not apply to *P. falciparum* schizogony. Perhaps more promising is L-mimosine, which targets eukaryotic initiation factor 5A (McCaffrey *et al.*, 1995). A homologue of this protein has been identified in *P. falciparum* by sequence similarity and expressed as a recombinant protein (Molitor *et al.*, 2004). Despite this, the effects of L-mimosine on *Plasmodium* have yet to be fully characterised and the potential for

interactions with alternative cellular targets cannot be ruled out, especially at the high concentrations of the compound required.

The final problem with this method is a more general questioning of the basis for the technique itself. A general questioning of all batch synchronisation methods (including inhibitor-based methods, double thymidine block and nutrient starvation) has occured (Shedden and Cooper, 2002; Cooper *et al.*, 2006) which stems from a belief that while a population of cells can be obtained which exhibit a common property, e.g. all cells have a similar DNA content, such an alignment does not mean that all other properties of the cells are also synchronised to the same cell-cycle stage. An additional problem, and perhaps a stronger argument against the use of this technique, is that the inhibitors may disrupt the treated cells to the extent that they are no longer truly representative of normal cycling cells. This viewpoint may still be a minority one and hotly contested (Breman *et al.*, 2004; Cooper, 2004; Cooper *et al.*, 2006), but there is merit in the warning it contains. The presence of additional methods for the cell-cycle synchronisation of mammalian cells such as mitotic shake off, which do not involve the addition or removal of anything from normal cells allow comparisons to be made between cells obtained via the different techniques. This would allow the validation (or rebuttal) of inhibitor based batch synchronisation techniques. To date no such technique exists for *Plasmodium* and the intracellular nature of the stages of interest prohibit the use of techniques already in use, so the accuracy of any observations made using parasite populations obtained by inhibitor-based synchronisation must remain in doubt.

The prospect of studying the cell-cycle of the malaria parasite in this way remains a difficult one, and the use of populations synchronised to a single stage to analyse cell-cycle events seems as yet out of reach Studies using single cells by microscopy using fluorescently-tagged molecules would seem to offer the best hope of allowing the study of cell-cycle progression as a whole.

Despite this difficulty in analysing the *Plasmodium* cell-cycle as a whole, individual events contained therein still remain a promising prospect for the development of novel and much needed antimalarial drug treatments. The discovery and characterisation of homologues to proteins known to play roles in mammalian cell-cycle control may aid understanding of this process in *Plasmodium*. Microtubules, which are crucial to mitosis, have been touted as a promising potential target (reviewed in (Bell, 1998; Kappes and Rohrbach, 2007). Increasing our understanding of the interaction

between microtubule inhibitors and the parasite may aid in the development of novel approaches to the treatment of malaria.

6.2. MICROTUBULE INHIBITORS AND *P. FALCIPARUM*

Studies on the interaction between microtubule inhibitors and *P. falciparum* took two routes in this thesis. The first was a look at the potential of three microtubule inhibitors each from a different class and with distinct binding sites on tubulin, to induce programmed cell death in the parasite. The second was a focused look at members of two classes of antimitotic herbicides. The potential of members of the class to become the basis for a new family of antiprotozoal agents has received much attention in recent times but their cellular effects on *P. falciparum* had yet to be characterised. Currently the potency of these agents also means that they are unsuitable for use as clinical therapeutics. For this reason, the ability of parasitised erythrocytes to accumulate the dinitroaniline herbicide trifluralin was investigated as a possible explanation for this limited potency.

6.2.1 Initiation of programmed cell death in *P. falciparum*

While the possible use of microtubule inhibitors as antimalarial inhibitors has been investigated (reviewed in Kappes and Rohrbach, 2007), the possibility that their use may open up a new pathway to exploit as a source for novel treatments has to date not been examined. The potential of microtubule inhibitors to cause programmed cell death in mammalian cells has been well characterised (Mollinedo and Gajate, 2003), and taxol has also demonstrated the ability to cause PCD-like cell death in *Leishmania* (Jayanarayan and Dey, 2005). The occurrence of PCD in the asexual stages of *P. falciparum* is still unproven, but given that it was confirmed in the mosquito stages (Hurd *et al.*, 2006) the potential for microtubule inhibitors to cause PCD-like cell death in the intra-erythrocytic stages was seen as a possibility. Taxol, vinblastine and trifluralin are all known to disrupt the mitotic architecture of *P. falciparum* (Fennell *et al.*, 2003; Fennell *et al.*, 2006) and so these three agents were used in the study. Caspase(s) activation was chosen as the marker to detect PCD for several reasons, (i) DEVDase activity was linked to other PCD markers during studies in the mosquito stages of the parasite (Al-Olayan *et al.*, 2002), (ii) DNA fragmentation is not noted in

all types of PCD (Nagata, 2000), (iii) the number of membranes present in parasite infected erythrocytes complicates the detection of phosphatidyl-serine translocation and (iv) two putative metacaspase genes have been identified in the *P. falciparum* genome. One of these genes, designated PfMCA1, contains both a universally conserved catalytic cysteine and histidine dyad (required for catalytic activity) and a putative caspase recruitment domain in the N-terminal amino acid sequence (Meslin *et al.*, 2007). Results showed the activation of DEVDase activity in response to both taxol and vinblastine treatment, indicating that these parasites may indeed possess the ability to undergo a PCD-like cell death. The lack of DEVDase activation in response to trifluralin treatment does call into question the exact activating factor for this response. In mammalian cell types it is thought that the disruption of the cell-cycle process of mitosis results in the failure of passage beyond the M-phase checkpoint resulting in the initiation of the apoptotic pathway. However, this does not appear to be the case in these cells for two reasons: the first is that trifluralin does not elicit the same response despite its known ability to disrupt mitotic architecture and the second is the apparent lack of this particular checkpoint in *Plasmodium*. While the presence of only one PCD marker is by no means confirmation of PCD in these cells it does pose an interesting avenue for further study of these agents. If it is confirmed that asexual stages of *P. falciparum* can indeed undergo PCD-like cell death understanding the mechanisms involved may yield yet more potential antimalarial targets.

6.2.2 Antimitotic herbicides and *P. falciparum*: Current knowledge

Prior to the commencement of this work, studies had suggested that tubulin from the malarial parasite, *P. falciparum*, may present a potential drug target (Bell, 1998; Kappes and Rohrbach, 2007) and further studies showed the selective nature of antimitotic herbicides of the dinitroaniline and phosphorothioamidate groups (Chan and Fong, 1990; Stokkermans *et al.*, 1996; Bell, 1998; Werbovetz *et al.*, 2003; Fennell *et al.*, 2006). While the target of these agents in the parasite was demonstrated to be microtubules by immunofluorescent microscopy and studies using recombinant α- and β-tubulin (Fennell *et al.*, 2006), studies on the cellular effects of these herbicides on *P. falciparum* had previously been limited to dinitroanilines, most often trifluralin, and to concentrations far in excess of those required to inhibit the parasite (Bell, 1998; Fowler *et al.*, 1998). In response to this deficit, investigations were carried out with the aim to

better understand the effects of more relevant inhibitory concentrations of these agents on *P. falciparum*.

As with other antimitotic agents the antimitotic herbicides inhibited schizogony, affecting the trophozoite and early schizont stages of the asexual life-cycle while permitting initial rounds of DNA synthesis. There are differences in the cellular response to treatment with these agents.

(i) As discussed above, vinblastine and taxol appear to induce DEVDase activity in *P. falciparum* while trifluralin does not.

(ii) A second difference between the antimitotic herbicides assessed and the dolastatin/auristatin, "*Vinca*" alkaloid and taxoid classes of microtubule inhibitor is that these herbicides applied for 48 h exhibited the ability to inhibit a wider range of initial ages of the parasite, with cultures composed of younger rings and of older schizonts both affected at concentrations relatively close to that of the most susceptible (initially trophozoite) stages (Table 4.2) (Fennell *et al.*, 2003). While the concentrations of agents used are not strictly comparable due to the lower potency of the antimitotic herbicides when compared to agents of other classes, it is the difference in susceptibility between parasites of different ages to the same agent that is examined, rather than the absolute value of the inhibitory concentration. When taken in conjunction with the less pronounced biphasic dose response curve seen upon treatment with the dinitroanilines and APM (Schrevel *et al.*, 1994; Fennell *et al.*, 2003; Fennell *et al.*, 2006) it appears that a wider range of parasites in the asynchronous population are susceptible to these agents when compared with taxol and other antimitotic agents. In the development of antimalarial agents, a broader stage susceptibility profile is of benefit. Unlike in laboratory cultures, intra-erythrocytic parasite development *in vivo* tends to be quite synchronous for *P. falciparum* in that all the blood-stage parasites appear to be of similar ages (Doerig, 1997; Hotta *et al.*, 2000). This means that if you have an agent that is specifically active only at a particular stage of the parasite's 46-48 h asexual erythrocytic cycle, the time at which you can effectively administer the drug is determined by the stages present. If a compound has a wider stage specificity it broadens the range of times at which treatment can be administered.

(iii) Trifluralin and the other antimitotic herbicides showed much reduced potency against *P. falciparum* when compared with members of other microtubule inhibitor classes. Analysis of the accumulation of trifluralin and vinblastine into parasite infected erythrocytes, while not necessarily helping to explain the differences in stage

susceptibility, did shed some light on a potential explanation for differences in potency between these two agents. The K_i's for binding of trifluralin and vinblastine to tubulin of *P. falciparum* are, as yet, unknown. While trifluralin and vinblastine both accumulated at comparable rates, the intracellular trifluralin concentration reached ~300 times the extracellular amount (CAR of ~300) while the CAR for vinblastine was lower at ~100 for schizont-infected erythrocytes and ~50 for those infected with trophozoites. Such a higher concentration of trifluralin would be expected to correlate with an increased potency unless, as appears to be the case here, there was a high proportion of the agent binding to a non-specific target in the cell. Vinblastine accumulation occurs as would be expected for a microtubule inhibitor, with both saturable accumulation of the compound and co-location of vinblastine with its microtubule targets (demonstrated as co-location with β-tubulin). Trifluralin accumulation is neither saturable nor does it appear to co-locate specifically with any target as the proportions are split between particulate and soluble fractions almost evenly. Further evidence for a low-affinity association of trifluralin with a cellular target was the ease with which accumulation was reversed by several wash steps. Almost 85% of cell associated trifluralin was removed following ten wash steps. In contrast, approximately 65% of accumulated vinblastine was retained by the parasitized erythrocytes following multiple washes. So while there does appear to be some level of low-affinity association of vinblastine, most of the drug was retained by the cells apparently through binding to a higher-affinity target.

We propose that the reason for the lack of saturation of accumulation and target co-location and the high proportion of low-affinity accumulation is that the hydrophobic nature of these compounds results in their accumulation in membranes of both the erythrocyte and the parasite. This non-specific accumulation could also, at least in part, account for the reduced potency of this agent. Such non-specific "mopping up" of compound is often associated with an inoculum effect. Increased non-specific binding reduces the available compound to bind to and inhibit its primary target. A simple experiment showed that an inoculum effect was indeed seen when parasite cultures of two different parasitaemias were incubated with titrations of trifluralin. There was a 3-fold increase in IC_{50} with a 7.5-fold increase in parasitaemia.

6.2.3. Antimitotic herbicides and *P. falciparum*: Future directions

The selective antimalarial activities dinitroaniline and phosphorothioamidate herbicides and their interaction with *P. falciparum* tubulin both in culture and *in vitro* provides motivation for the further exploration of the potential of these compounds as a basis for novel antimalarial drugs. Problems still exist that need to be addressed before derivatives of antimitotic herbicides can be considered for clinical use. Firstly, the potencies of dinitroanilines and the phosphorothioamidate APM for parasite growth inhibition lie far behind those of established antimalarials such as chloroquine and mefloquine (Geary *et al.*, 1989; Kappes and Rohrbach, 2007) and herbicides effective at least in the sub-micromolar range will be required. Derivatives of both trifluralin and oryzalin with increased potency against *Leishmania* (Bhattacharya *et al.*, 2002; Werbovetz *et al.*, 2003; Bhattacharya *et al.*, 2004; Esteves *et al.*, 2006) *T. b. brucei* (Werbovetz *et al.*, 2003; Bhattacharya *et al.*, 2004) and *Cryptosporidium* (Benbow *et al.*, 1998; Armson *et al.*, 1999a; Armson *et al.*, 1999b) have been reported. Esteves *et al.* (2006) noted that with the addition of polar side-chains to trifluralin, thus increasing the hydrophilicity of the dinitroaniline, improved activity against cultured *Leishmania* was achieved. Werbovetz *et al.* used the more water soluble oryzalin as the basis for their analogs which were found to be more potent in culture against *L. donovani* and *T. brucei brucei* than the parent compound (Bhattacharya *et al.*, 2002; Werbovetz *et al.*, 2003; Bhattacharya *et al.*, 2004). Studies on the ability of these oryzalin derivatives to bind to *Leishmania* tubulin *in vitro* showed that there wasn't an exact correlation between anti-leishmanial activity and tubulin binding. This led to the hypothesis that the modifications made to oryzalin improved passage of the agent across the parasite membrane (Bhattacharya *et al.*, 2002). One agent (compound 35) showed little effect on purified mammalian tubulin while inhibiting polymerisation of *Leishmania* tubulin with an IC_{50} of 6.9 μM and also inhibited cultured *T. brucei* with an IC_{50} value of 120 nM. Based on these studies it does seem reasonable that antimitotic herbicides with heightened efficacy against cultured *P. falciparum* are a realistic target. Despite these promising derivatives, studies both in this thesis and on *Cryptosporidium* have indicated that improving water solubility of the dinitroanilines may not be sufficient to improve potency (Benbow *et al.*, 1998) and knowing the details of the antimitotic herbicide-binding site on *P. falciparum* tubulin would surely prove to be the most rational way to aid in the design of more effective ligands.

A number of putative sites, based predominantly on homology modelling of the crystal structure of the bovine αβ-tubulin heterodimer (Nogales *et al.*, 1998) have been

suggested (Blume *et al.*, 2003; Morrissette *et al.*, 2004), although these remain to be experimentally verified. An X-ray crystal structure of recombinant *P. falciparum* tubulins would provide greater insight into structural differences between *P. falciparum* and mammalian tubulins. Crystal structures of fusion proteins with such tags have been reported (Smyth *et al.*, 2003) although it is possible that the large affinity-tag, MBP, may hinder crystal growth due to conformational heterogeneity. Affinity labelling experiments using colchicine, taxol and "*Vinca*" alkaloids provided information pertaining to these binding sites on mammalian tubulin (Nogales *et al.*, 1998; Ravelli *et al.*, 2004) so a similar approach using analogues of antimitotic herbicides may aid in locating the binding site of these agents on *Plasmodium* tubulin.

Secondly, the low aqueous solubility of dinitroanilines, in addition to possibly decreasing activity *in vitro*, may contribute to the poor pharmacokinetics noted for these agents (Morejohn *et al.*, 1991). In a rodent model of malaria, the lack of *in vivo* antimalarial activity of trifluralin was thought to be the result of poor adsorption and insufficient plasma concentrations due to the low water-solubility of trifluralin (Dow *et al.*, 2002). In an attempt to overcome the low solubility and poor transport profiles of the dinitroanilines, trifluralin has been linked to a number of different sugar moieties to give glycoconjugated dinitroanilines (Mead *et al.*, 2003). The resulting analogues were found to retain moderate activity (IC_{50} 3 - 8 μM) against cultured *Cryptosporidium parvum*. Liposomal trifluralin was also developed and tested against a form of experimental canine leishmaniosis. The results, while not optimal, did show that the liposomal form of trifluralin was more effective against the disease than trifluralin alone (Marques *et al.*, 2007). While neither glycoconjugated derivatives nor liposomal trifluralin showed improved anti-malarial activity in culture, they may still have improved pharmacokinetic properties in a similar rodent model of malaria and may merit further investigation.

Thirdly, there is a possibility that the presence of aromatic nitro groups may have implications for host toxicity. The dinitroaniline trifluralin is classified as a Category C (possible human) carcinogen by the U.S. Environmental Protection Agency (EPA) (U.S. Environmental Protection Agency, 1987) and a recent study on the effects of trifluralin in rats did indicate some induction of side effects in animals at the highest dose tested (six doses of 600 mg/kg), although histopathological examination of sacrificed rats displayed no abnormalities (Dow *et al.*, 2002). Despite these indications there are many studies that fail to demonstrate that trifluralin is carcinogenic. For

example, no increase of benign or malignant neoplasms was observed during a 2-year study in which trifluralin was fed to $B6C3F_1$ mice (Francis *et al.*, 1991). In a study using A/J mice, trifluralin was in fact found to protect against the induction of lung and forestomach tumors by exposure to benzo(a)pyrene (Triano *et al.*, 1985). The presence of any prospect of the potential carcinogenicity of these agents means that extensive testing must first be carried out to assure that the risk is minimal, but the potential of these agents in the treatment of not just malaria, but a wide variety of other debilitating parasitic diseases, is too great to discard them.

The work described in this thesis clearly shows that the cell-cycle of *P. falciparum* contains characteristics not congruent with the general model of the eukaryotic cell cycle. However, individual aspects, namely the M-phase and microtubules in particular, still hold potential as a chemotherapeutic target. While a synchronisation technique was not successfully confirmed it did provide some useful insights into the differences in regulation of the cell-cycle in this organism. Interactions of a number of microtubule inhibitors with the parasite has suggested the potential existence of a novel pathway for PCD which may also be exploited as a source of novel antimalarial drug targets. The potential for the development of novel antimalarial agents with low host toxicity, suitable pharmacokinetic properties, and potent activity, are furthered by additional knowledge of the interactions between antimitotic herbicides and *P. falciparum*. The further development of these agents as lead compounds could be facilitated by uncovering the site at which they bind tubulin, allowing the synthesis of derivatives with improved affinity for the target in addition to improving the pharmacokinetic properties of the agents and targeting them to the cytoplasmic site of action, leading to the development of selective antimalarial agents of possible chemotherapeutic value.

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