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The Glyoxalase System, Inhibition of Thioredoxin Reductase and Use of Methylene Blue as Drug Development Strategies against the Malarial Parasite *Plasmodium falciparum*

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Dedication

To my family

Confirmation

This thesis is the original work of Akoachere Monique Bate. Other sources of information have been properly quoted. The work has not been used to obtain any other university degrees.

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Original Publications

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Abbreviations

- $A_{....nm}$ Absorption at ... nm
- ACTs Artemisinin-based combination therapies
- ad To give a concentration of; to give a volume of
- AGEs Advanced glycation endproducts
- APS Ammonium persulphate
- BlueCQ Methylene blue + chloroquine drug combination
- BSA Bovine serum albumin
- cpm Counts per minute
- CQ Chloroquine
- Da Dalton
- DDT Dichloro-diphenyl-trichloroethane
- DMSO Dimethylsulfoxide
- DNA Deoxyribonucleic acid
- dNTP Deoxyribonucleotide triphosphate
- DTE Dithioerythritol
- DTNB Dithionitrobenzene
- EDTA Ethylenediaminetetraacetic acid
- FAD Flavin adenine dinucleotide
- FIC Fractional inhibitory concentration
- G6PD Glucose-6-phosphate dehydrogenase
- GILP Glyoxalase I-like protein
- Glo Glyoxalase
- GR Glutathione reductase
- GSH/GSSG Glutathione (reduced /oxidized)
- GST Glutathione-S-transferase
- HCPC-GSH S-(*N*-hydroxy-*N*-chlorophenylcarbamoyl)glutathione
- HIV/AIDS Human immunodeficiency virus / Acquired immune deficiency syndrome
- HRP Histidine rich protein
- HTA Hemithioacetal
- IC Inhibitory concentration
- IPTG Isopropylthiogalactopyranoside

- ITN Insecticide treated nets
- LB Luria-Bertani
- LC/MS/MS Liquid chromatography / Mass spectrometry / Mass spectrometry
- LDH Lactate dehydrogenase
- MALDI-TOF Matrix-assisted laser desorption ionization – Time of flight
- MB Methylene blue
- MOPS 4-Morpholinopropane sulfonic buffer
- MSP Merozoite surface protein
- NADH/NAD^+ Reduced /oxidized nicotinamide adenine dinucleotide
- NADPH/$NADP^+$ Reduced /oxidized nicotinamide adenine dinucleotide phosphate
- NPRBC Non-parasitized red blood cells
- NTPs Nucleotide triphosphates
- PCR Polymerase chain reaction
- PEG Polyethylene glycol
- *Pf* *Plasmodium falciparum*
- PMSF Phenylmethylsulfonylfluoride
- PPP Pentose phosphate pathway
- PRBC Parasitized red blood cells
- RESA Ring-infected erythrocyte surface antigen
- RNA Ribonucleic acid
- rpm Rounds per minute
- SDLGSH S-D-lactoylglutathione
- SDS Sodium dodecyl sulphate
- SDS-PAGE Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
- TEMED N,N,N',N'-Tetramethylethylenediamine
- Trx Thioredoxin
- TrxR Thioredoxin reductase
- U Unit of enzyme activity (µmol/min)
- WHO World Health Organisation

Summary

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* and is responsible for about half a billion diseases cases and 2-3 million deaths each year. Much of the parasite's success to establish persistent infections is attributed to evasion of the human immune defense system through antigenic variation and increasing development of resistance to all currently available antimalarial drugs except the artemisinins. The difference in structure and mode of action of the artemisinins underlines the fact that new antimalarial drugs – with differential modes of action – are an urgent priority in order to circumvent plasmodial resistance mechanisms in the absence of effective vaccines or vector control measures.

By means of rational drug design and re-evaluation of an ancient antimalarial drug, three new drug development strategies against the deadliest malarial parasite, *Plasmodium falciparum,* were developed within the frame of this thesis in order to design possible new mechanism drugs and prevent resistance development to artemisinin.

First, a complete functional glutathione-dependent glyoxalase (Glo) detoxification system – comprising a cytosolic GloI (cGloI), a GloI-like protein (GILP) and two GloIIs (cytosolic GloII named cGloII, and tGloII preceded by a targeting sequence) – was characterized in direct comparison with the isofunctional human host enzymes. Kinetic and structural similarities of enzymes of both systems were described; however, striking differences – especially for the GloIs – were also detected which could be exploited for drug development. Various S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones tested as *P. falciparum* Glo inhibitors were found to be active in the lower nanomolar range and could be used as lead structures in the development of more selective inhibitors of the *P. falciparum* glyoxalase system (Akoachere *et al.*, 2005).

Secondly, the characterization of the mode of inhibition of three promising inhibitors of the previously-validated drug target *P. falciparum* thioredoxin reductase (PfTrxR) is reported in this thesis. The enzyme is a homodimeric flavoenzyme which reduces thioredoxin (Trx) via a C-terminally located CysXXXXCys pair. In this respect PfTrxR differs significantly from its human counterpart which bears a Cys-Sec redox pair at the same position. PfTrxR is essentially involved in antioxidant defence and redox regulation of the parasite and has been validated as a drug target. The inhibitors, 4-nitro-2,1,3-benzothiadiazole (IC_{50} on PfTrxR = 2 μM), 6,7-nitroquinoxaline (IC_{50} on PfTrxR = 2 μM), and bis-(2,4-

dinitrophenyl)sulfide (IC_{50} on PfTrxR = 0.5 µM), showed uncompetitive inhibition with respect to both substrates, NADPH and thioredoxin. All three inhibitors were active in the lower micromolar range on the chloroquine resistant *P. falciparum* strain K1. 4-Nitro-2,1,3-benzothiadiazole was antagonistic with known antimalarials suggesting that the inhibitor uses similar routes of uptake and/or acts on related targets or biochemical pathways (Andricopulo *et al.*, 2005; Andricopulo *et al.*, submitted).

Lastly and most importantly, the renaissance of interest in the ancient antimalarial drug methylene blue (MB) led to the identification of a potential artemisinin-based combination therapy (ACT). A strong synergistic action of MB and artemisinin might be capable of fighting resistant *P. falciparum* parasites in the field. MB is active against all blood stages of both chloroquine (CQ)-sensitive and CQ-resistant *P. falciparum* strains with IC_{50} values in the lower nanomolar range. Ring stages showed the highest susceptibility. As demonstrated by high performance liquid chromatography / tandem mass spectrometry on different cell culture compartments, MB accumulates in malarial parasites. In drug combination assays, MB was found to be antagonistic with CQ and other quinoline antimalarials like piperaquine and amodiaquine; with mefloquine and quinine MB showed additive effects. In contrast, synergistic effects of MB with artemisinin, artesunate, and artemether were observed for all tested parasite strains. Artemisinin/MB concentration combination ratios of 3:1 were found to be advantageous demonstrating that the combination of artemisinin with a smaller amount of MB can be recommended for reaching maximal therapeutic effects. *In vitro* data reported here indicate that combinations of MB with artemisinin (derivatives) might be a promising option for treating drug resistant malaria. Resistance development under this drug combination is unlikely to occur (Akoachere *et al.*, in press).

Taken together, the results support the feasibility of the rational development of new potential antimalarial drugs. In combination with existing and other promising new malarial-control measures, new antimalarial drugs could greatly contribute to reducing the intolerable global burden of this disease.

Zusammenfassung

Malaria ist eine parasitäre Infektionskrankheit, die von Protozoen der Gattung *Plasmodium* hervorgerufen wird. Pro Jahr gibt es über 500 Millionen Krankheitsfälle/Neuinfektionen mit 2-3 Millionen Todesfällen. Ein wichtiger Punkt in der Pathogenese der Malaria ist die Ausbildung persistierender Infektionen. Antigenetische Variation ermöglicht es dem Parasiten, das menschliche Immunsystem zu umgehen. Weiterhin sind Plasmodien in der Lage, auf die eingesetzten Malariamittel mit rascher Resistenzentwicklung zu reagieren. Deshalb kommen Neu- und Weiterentwicklung von Medikamenten in der Bekämpfung der Malaria neben Impfstoffentwicklung und Insektiziden Massnahmen gegen den Vektor eine zentrale Rolle zu. Eine Ausnahme in der zunehmenden Resistenzproblematik bildet Artemisinin, welches eine andere chemische Zusammensetzung und einen anderen Wirkmechanismus als andere gegenwärtige Malariamittel aufweist.

In Rahmen dieser Doktorarbeit wurden drei neue Arzneimittelentwicklungs-Strategien gegen den gefährlichsten human Malariaereger, *P. falciparum*, verfolgt. Dies erfolgte anhand von rationaler Medikamententwicklung bzw. durch eine Neubewertung ehemaliger Malariamittel mit dem Ziel, mögliche neue Wirkmechanismen aufzuzeigen und Resistenzentwicklung bei Artemisinin zu verhindern.

Der erste dieser verschiedenen Angriffspunkte war die Charakterisierung neuer Arzneimittelzielmoleküle im *Plasmodium*-Stoffwechsel. Dies umfasst die Charakterisierung eines Glutathion-abhängigen Glyoxalase (Glo) Systems im Vergleich zum isofunktionellen humanen System. Dieses System hat eine zentrale Rolle im Entgiftungsstoffwechsel der Parasiten und besteht aus einer cytosolischen GloI (cGloI), einem Glo-I ähnlichen Protein (GILP), zwei GloII (cytosolische GloII (cGloII) sowie tGloII mit einer vorangestellten Targeting-Sequenz). Hier werden kinetische und strukturelle Ähnlichkeiten im humanen und Plasmodien-System beschrieben und im Sinne einer Überprüfung als möglicher Arzneimittel-Angriffsort Verschiedenheiten aufgezeigt (vor allem bei GloI). Verschiedene S-(*N*-Hydroxy-*N*-Arylcarbamoyl)Glutathion Verbindungen wurden als Inhibitoren der Glyoxalasen an *P. falciparum* getestet, sie waren im niederen nanomolaren Bereich aktiv. Somit können diese Verbindungen als Leitsubstanzen für die Entwicklung selektiver Inhibitoren des *P. falciparum*-Glyoxalase Systems dienen (Akoachere *et al.*, 2005).

Ein zweiter zentraler Punkt dieser Doktorarbeit ist die Charakterisierung des Wirkmechanismus von drei vielversprechenden Inhibitoren der bereits als Arzneimittel-Zielmolekül validierten *P. falciparum* Thioredoxinreduktase (PfTrxR). Es handelt sich um ein homodimeres Flavoenzym, welches Thioredoxin mit Hilfe eines C-terminalen CysXXXXCys-Motives reduziert. Hierbei unterscheidet es sich vom humanen Enzym, welches an der gleichen Position ein Cys-Sec Redoxpaar beinhaltet. PfTrxR ist essentiell involviert in antioxidative Abwehrmechanismen und Redoxhomöostase im Plasmodienstoffwechsel. Die Inhibitoren 4-Nitro-2,1,3-Benzothiadiazol (IC_{50} für PfTrxR = 2 µM), 6,7-Nitroquinoxalin (IC_{50} = 2 µM) und Bis-2,4-Dinitrophenyl)sulfid (IC_{50} = 0,5 µM) zeigen eine unkompetitive Hemmung für die beiden Substrate NADPH und Thioredoxin. Alle drei Inhibitoren sind aktiv im niederen mikromolaren Bereich bei dem choroquinresistenten *P. falciparum* Stamm K1. 4-Nitro-2,1,3-Benzothiadiazol zeigt einen antagonistischen Wirkmechanismus mit anderen bekannten Malariamitteln; dies bedeutet, dass dieser Hemmstoff entweder einen ähnlichen Aufnahmemechanismus besitzt und/oder an verschiedenen Molekülen bzw. biochemischen Stoffwechselwegen angreift (Andricopulo *et al.,* 2005; Andricopulo *et al.,* submitted).

Im Sinne einer Neubewertung früherer Arzneimittel gegen Malaria fokussiert meine Doktorarbeit auf Methylenblau (MB) in Bezüg auf eine mögliche Artemisinin-gestützte Kombinationstherapie (ACT: Artemisinin-based combination therapy). Diese Kombination aus zwei Antimalariamitteln ist ein möglicher Weg, Resistenzenentwicklungen bei *Plasmodium* zu vermeiden. Methylenblau ist aktiv gegen alle Blutstadien von *Plasmodium* sowohl an chloroquinresistenten Stämmen mit IC_{50}-Werten im niedermolaren Bereich. Hierbei zeigen Ringstadien die höchste Empfindlichkeit. Darüberhinaus akkumuliert MB in verschiedenen Zellkompartimenten, dies konnte mit Hilfe von Hochdurchsatz-Flüssigkeits-Chromatographie bzw. Tandem-Massen-Spektrometrie gezeigt werden. In Arzneimittel-Kombinations-Assays konnte nachgewiesen werden, dass MB antagonistisch zu Chloroquin und anderen Quinolinen wie Piperaquin und Amodiaquin wirkt, während es mit Mefloquin und Quinine einen additiven Effekt zeigt. Im Gegenteil dazu besitzt MB einen synergistischen Effekt mit Artemisinin, Artesunat und Artemether in allen getesteten Plasmodienstämmen. Ein Konzentrationsverhältnis von 3:1 zwischen Artemisinin und MB hat sich als vorteilhaft erwiesen. Dies verdeutlicht, dass geringe Mengen von MB empfohlen werden können, um maximalen therapeutischen Effekt zu erzielen. Diese hier berichteten *in vitro*-Daten unterstützen die Thesen, dass die Kombination aus Artemisinin (bzw. Artemisininderivaten) und MB eine vielversprechende Möglichkeit in der

Behandlung therapieresistenter Malariafälle bieten kann. Weiterhin ist eine Resistenzentwicklung gegen die Arzneimittelkombination unwahrscheinlich (Akoachere *et al.*, 2005).

Zusammenfassend kann man sagen, dass diese Resultate die Eignung der rationalen Arzneimittelentwicklung für neue Antimalariamittel unterstreichen. In Kombination mit existierenden Arzneimitteln und zusammen mit anderen Kontrollmechanismen können neue Antimalariamittel dazu beitragen, die intolerierbare, weltweite Bedrohung durch Malaria zu verringern.

1 Introduction and Rationale

1.1 The disease

Malaria is a protozoan disease caused in humans by four species of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) and is transmitted by the bite of an infected female mosquito of the genus *Anopheles*. Malaria is today one of the most important parasitic diseases of man as it is responsible for approximately a million deaths and about 500 million incidences of disease each year. The different strains of *Plasmodium* are responsible for different patterns of the characteristic fevers typical of the disease: *P. falciparum* (malaria tropica), *P. vivax* (malaria tertiana), *P. malariae* (malaria tertiana), and *P. ovale* (malaria quartana). *Plasmodium vivax* and *P. falciparum* infections are the most common. *P. falciparum* is responsible for the most deadly type of malaria infection with children under the age of 5 being most of its victims. *P. vivax* and *P. ovale* form resting stages in the liver (hypnozoites) which once reactivated can cause a clinical relapse of the disease many months after the initial event. Malaria in animals and birds is caused by other *Plasmodium spp*. However, *P. knowlesii* which normally causes the infection in monkeys has also been responsible for cases of the disease in humans (Singh *et al.*, 2004).

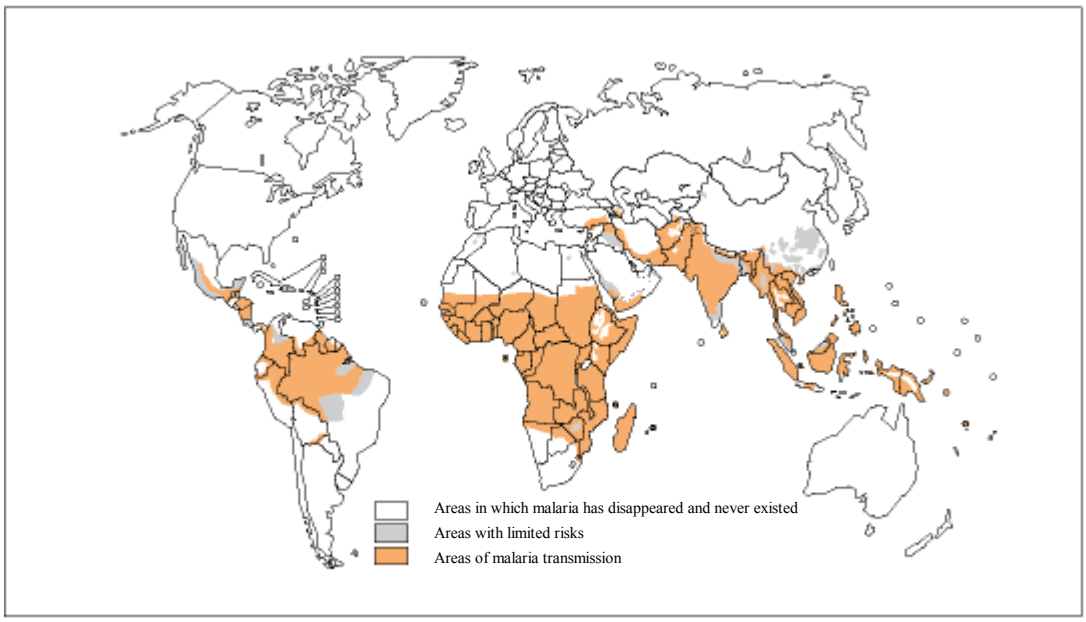


Figure 1: **Geographic distribution of malaria around the world.**
Malaria is endemic to tropical and subtropical regions (http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/96vol22/dr2220ed.html)

The disease once had a worldwide distribution but it was successfully eliminated from many countries with temperate climates during the mid 20th century. Today malaria is found throughout the tropical and sub-tropical regions of the world including parts of Africa, Asia, Central and South America, Oceania, and certain Caribbean islands (Figure 1). Almost 90% of all malaria cases occur in Africa necessitating stringent control measures within the continent (Snow *et al.*, 2005).

Malaria is characterized by extreme exhaustion associated with paroxysms of high fever, sweating, shaking chills, and anemia (Miller *et al.*, 2002). Several risk groups are found among those living in endemic areas who are subject to repeated *P. falciparum* infections: infants and young children suffer particularly from life-threatening anaemia, older children from an induced coma (Marsh and Snow, 1999), and primagravida women from severe disease related to placental sequestration (Ricke *et al.*, 2000). Malaria naive travellers, either crossing international borders or travelling from malaria-free to malaria-endemic areas in their own countries, constitute another risk group, and are susceptible to severe disease after acquiring their first infection. There is a number of severe complications that are specific to *P. falciparum* infections. In the nonimmune patient, many of the severe complications of *P. falciparum* – such as cerebral malaria, anemia, hypoglycaemia, renal failure, and noncardiac pulmonary oedema – occur in combination or as isolated complications (Miller *et al.*, 1994).

1.1.1 The *Anopheles* vector

Only female mosquitoes of the genus *Anopheles* transmit human malaria. The genus includes roughly 400 species of *Anopheles* mosquitoes worldwide, of which 60 species are malaria vectors, and some 30 species are of major importance (Bruce-Chwatt, 1985). *Anopheles gambiae*, the principal vector in sub-saharan Africa, is a particular effective malaria vector because of its preference for feeding on humans and its long life compared with some other anopheline species. The four developmental stages of an anopheline mosquito are egg, larva, pupa, and adult (or imago). The adult male feeds on nectar, while the female adult feeds primarily upon blood. Females of most *Anopheles spp.* prefer warm-blooded animals, predominantly mammals. Some species prefer humans, and are termed anthropophagic or anthropophilic. Others prefer animals such as cattle, and are termed zoophagic or zoophilic. The time of anopheline feeding is mainly between dusk and dawn.

1.1.2 Life cycle of the parasite

The life cycle of the parasite is complex involving vertebrate and invertebrate hosts as well as different locations within each of these hosts. In humans, the malaria parasite enters the host when an infected *Anopheles* mosquito takes a blood meal. The first stage of malaria infection in man leads sporozoites of *Plasmodium* to the liver via blood circulation where the sporozoites invade the hepatocytes and begin an asexual phase of reproduction (Figure 2). This stage lasts on average between 5.5 days (*P. falciparum*) and 15 days (*P. malariae*) before the hepatic schizonts rupture to release merozoites into the blood stream. In *P. vivax* and *P. ovale* infections a proportion of the intrahepatic parasites do not develop, but instead rest inert as hypnozoites, to awaken weeks or months later, and cause the relapses which characterize infections with these two species. The hepatic phase – sometimes called the pre-erythrocytic phase – is asymptomatic as only a few liver cells are infected. However, considerable asexual reproduction takes place within the hepatocyte and many thousands of merozoites are released from each infected hepatocyte.

Merozoites liberated into the bloodstream invade the erythrocytes by a vigorous wriggling or boring motion inside a vacuole composed of the invaginated erythrocyte membrane. Once inside the erythrocyte, the parasite lies within the erythrocyte cytosol enveloped by its own plasma membrane, and a surrounding parasitophorous vacuolar membrane. Within the erythrocytes, the merozoites develop from small ring forms to the trophozoites – feeding stages – after about 24 hours of invasion. Approximately 36 hours after merozoite invasion (or 54 hours in *P. malariae*) repeated nuclear division takes place to form the schizont or the segmenter form. This then ruptures and the released merozoites (on average 16 per schizont) reinvade other erythrocytes and start a new cycle. The erythrocytic life cycle is approximately 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*.

After a series of asexual cycles in *P. falciparum*, a subpopulation of parasites develops into sexual forms (male and female gametocytes) which are long lived and motile. Upon ingestion of these gametocytes in another blood meal by the mosquito, the gametocytes become activated to gametes. Male and female gametes fuse in the insect's gut to form a zygote. The zygote in turn develops into the ookinete, which crosses the wall of the gut and forms a sporozoite filled oocyst. When the oocyst bursts, the sporozoites migrate to the salivary glands to await inoculation into the next human host upon feeding. These mosquitoes normally breed in areas where there is stagnant water such as swamps and

during the rainy seasons of African countries but the female mosquito requires a blood meal for the maturation of her eggs.

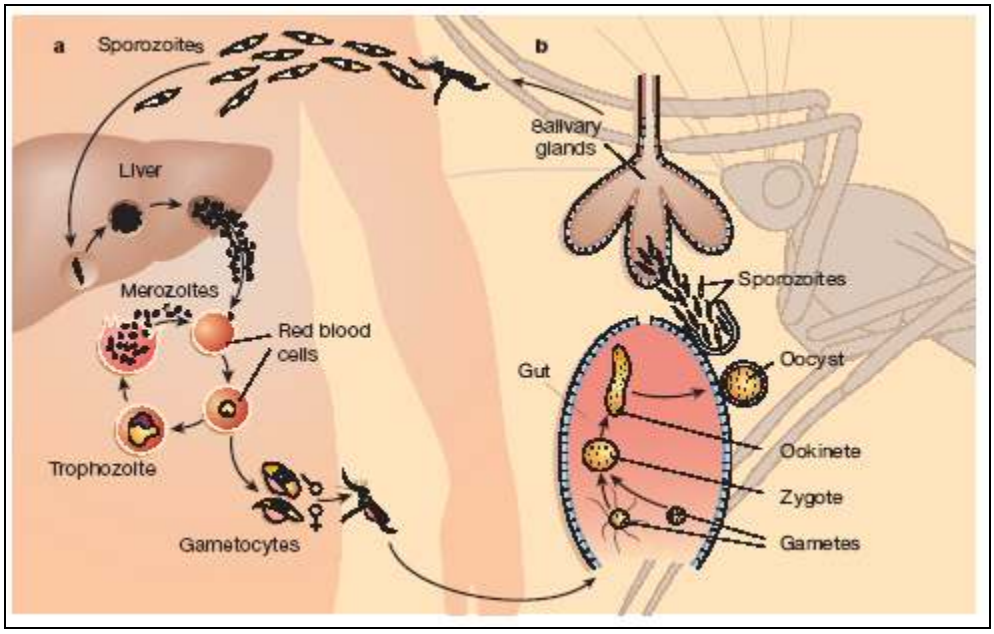


Figure 2: **Life cycle of the parasite *Plasmodium falciparum*.**
Malaria parasites engage in highly specific and varied interactions with cell types of both the mammalian host and the mosquito vector (adapted after Wirth, 2003).

1.1.3 Pathophysiology of the disease

The pathophysiology of malaria results from the destruction of erythrocytes, the liberation of parasite and erythrocyte material into the circulation, and the host reaction to these events. *P. falciparum* malaria infected erythrocytes also sequester in the microcirculation of vital organs, interfering with microcirculatory flow and host tissue metabolism. Malaria parasites do induce the release of cytokines, which are responsible for many of the symptoms and signs of the infection, particularly fever and malaise (Hunt and Grau, 2003). Sequestration of *P. falciparum* parasites in the brain is thought to be central to the pathophysiology of cerebral malaria.

1.1.4 Diagnosis of malaria

Malaria is diagnosed principally by microscopic examination of the blood for the identification of the parasites. Thick and thin blood films are made which are later on stained in appropriate staining dyes like Giemsa or Acridine orange. The thick film is approximately 30 times more sensitive than the thin film, although sensitivity and specificity depend to a great extent on the experience of the microscopist, the quality of the slides, stains and microscope, and the time spent examining the slide. Artefacts are

common and often confusing. Speciation of malaria at the trophozoite stage is easier on the thin film, although gametocytes and schizonts are more likely to be seen on the thick film. The thin film is more accurate for parasite counting. A morphological demonstration of *P. falciparum* malaria parasites is shown in Figure 3 below.

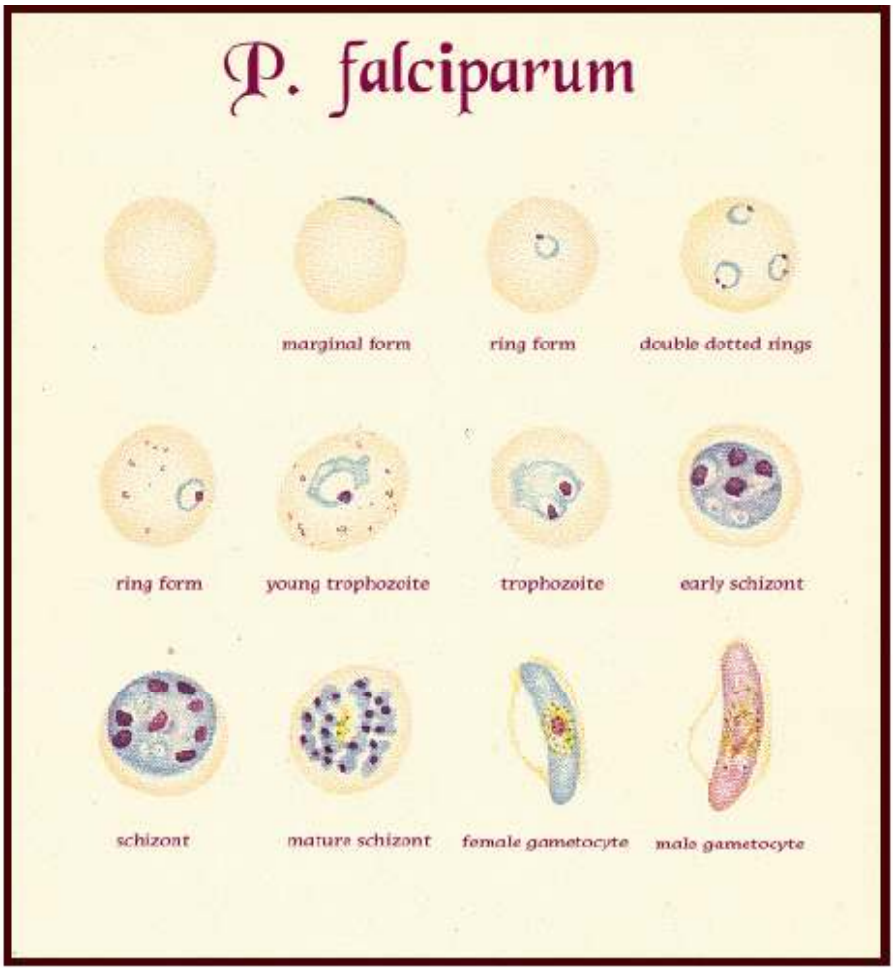


Figure 3: Blood stages of *P. falciparum* (Malaria Manual, 2003).

Although microscopic diagnosis of malaria is the gold standard, it is unreliable outside specialized centers. Moreso, the time until results from microscopic examinations become available can cause detrimental delays particularly in rapidly fatal *P. falciparum* infections due to a consequent delay in the provision of appropriate therapy (Kain *et al.*, 1998; Nüesch *et al.*, 2000). Several rapid malaria tests have been developed and they may be a useful diagnostic adjunct to microscopy in centers without major expertise in tropical medicine. Polymerase chain reaction (PCR) is a highly sensitive alternative to microscopy, but the infrastructure and expertise required preclude its routine use in many health care settings (Chiodini, 1998). By using immunochromatographic methods, these tests detect

parasite antigens in lysed blood from a fingerprick blood sample and can be performed in approximately 15 minutes without special equipment (Chiodini, 1998). Two-band tests target histidine-rich protein-2 (HRP-2) and detect *P. falciparum* only, while the more recent 3-band tests also detect other malaria parasites (*P. vivax, P. malariae, and P. ovale*) by using HRP-2 and aldolase combined or parasite lactate dehydrogenase (LDH) as targets (Marx *et al.*, 2005). Efforts are needed to optimise sensitivity for the detection of *P. vivax* and improve test performance for *P. ovale* and *P. malariae*.

1.1.5 Control

1.1.5.1 Control through prevention of transmission

Mosquito control has been at the centre of past efforts to eradicate malaria, mainly through the use of the insecticide DDT (dichloro-diphenyl-trichloroethane) for indoor residual house-spraying. Facing the ban imposed on DDT, the programme became unsustainable despite the notable successes that were achieved. Other insecticides in use include carbamates and pyrethroids, against some of which mosquito resistance has been shown to develop. Trials of insecticide treated nets (ITNs) have consistently shown reductions in overall child mortality and in episodes of clinical malaria (Lengeler, 2004). In emergency situations, insecticide-treated tarpaulins have proved very effective and materials that have long-acting insecticidal action are being developed. Repellants could provide useful protection against malaria, especially in places where vector mosquitoes bite early in the evening (Rowland *et al.*, 2004) whereby the use of ITNs has only little protection to offer against malaria infection as the mosquito biting precedes local bedtime. The use of mixtures or mosaics of insecticides on nets has been a successful approach to prevent insecticide resistance.

Environmental management (including drainage of breeding sites), improvement in house design, use of larvivorous fish, and zooprophylaxis have proved effective in some specific epidemiological situations but must be based on detailed behavioural knowledge of the main local vectors.

The completion of the sequencing of the *Anopheles gambiae* genome has provided unprecedented opportunities for scientists to study vector-pathogen interactions that are central to malaria pathogenesis and treatment. Through integration of advanced and high throughput gene expression technologies, the processes of identifying new antigens and new targets for insecticide development could be accelerated and hence morbidity and mortality caused by malaria could be reduced.

1.1.5.2 Control through therapy

Transmission of the disease malaria warrants a repertoire of infectious gametocytes which develop from merozoites in the erythrocytes. Antimalarial drugs which are mostly directed to the erythrocytic stages of the parasite control the levels of transmission of the parasite by reducing and/or preventing the development of gametocytes from these erythrocytic stages. All antimalarial drugs have their liabilities as well as their benefits and could be classified into different groups based on their site of antimalarial action. Of particular interest are the lysosomal food vacuole (the site of extensive haemoglobin degradation), the apicoplast (a plastid organelle thought to originate from a green algal symbiont), an acrystate mitochondrion with a limited electron transport system and of course, the cytosol (Figure 4).

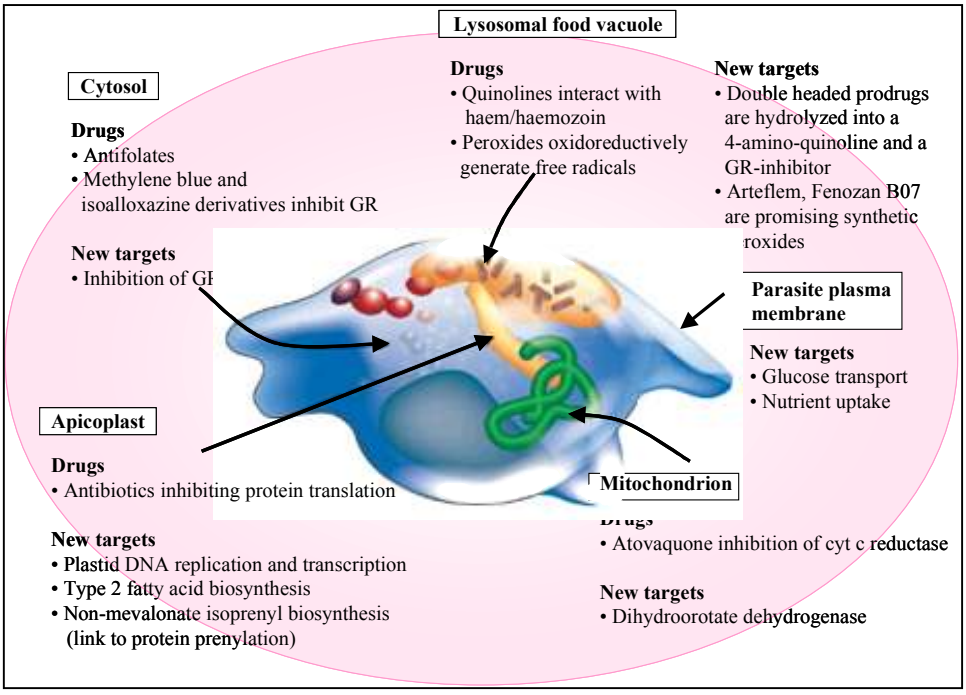


Figure 4: **Diagram of *P. falciparum* trophozoite residing in an erythrocyte.** The main organelles that are associated with drug targets are highlighted, drawing attention to both sites of current antimalarial drug action and new targets that are under investigation (Ridley, 2002)

Three broad groups of antimalarial drugs (structures shown in Figure 5) include the aryl aminoalcohols or quinoline-related compounds (quinine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, primaquine, and tafenoquine), the antifolates (pyrimethamine, sulfadoxine, proguanil, chlorproguanil, and trimethoprim) and the artemisinin compounds (artemisinin, dihydroartemisinin, artesunate, arteether, and artemether).

Artemisinins which are today the most effective antimalarial drugs are the oldest which have been in use. Artemisinins are the active ingredient in extracts of the plant qinghaosu (*Artemisia annua*) which have been used in traditional medical practice in China for over two millennia. The rediscovery of the artemisinins in the early 1970s constituted the best development in the treatment of multi-drug resistant malaria (Hien and White, 1993; Haynes, 2001). These drugs are structurally unrelated to existing antimalarial drugs. They are rapidly effective, safe, with no known clinical resistance and with the broadest time window of antimalarial effect (from ring forms to early schizonts and even gametocytes). The artemisinins act faster than any other antimalarial drug, with an approximate parasite- and fever-clearance time of 32 hours, in constrast to 2-3 days needed with conventional antimalarial drugs to resolve symptoms (Price, 2000). The essential pharmacophore is structurally and mechanistically unique: an endoperoxide bridge that undergoes iron-catalysed activation to form toxic free radicals. Recent studies indicate that artemisinins inhibit ATPase and alter intracellular calcium stores (Eckstein-Ludwig *et al.*, 2003). One liability of the artemisinins is the recrudescence of fully sensitive parasites upon monotherapy due to their short half life. The current recommended use of artemisinins is in combination therapy (WHO, 2001), where they effect a rapid and massive decrease in parasite burden and their gametocidal activity may lessen transmission of resistant parasites to the mosquito. Artemisinin and its derivatives (artemether and artesunate) are being used in combination with other antimalarial drugs for treatment of uncomplicated malaria, and as parenteral or rectal formulations for severe disease.

Quinoline antimalarials and related alcohols owe their origins to quinine – an active ingredient of *Cinchona* bark – and as such share obvious structural anology. They constitute the largest class of antimalarials with chloroquine being the pre-eminent agent in the class. Chloroquine and other 4-substituted quinolones kill malaria parasites by interfering with the detoxification of haem by effectively blocking the sequestration of toxic heme into hemozoin within the food vacuole (Sullivan *et al.*, 1996). Chloroquine accumulates in parasitized red cells, particularly in the acidic digestive food vacuole, to reach levels hundreds of times those in plasma, and the accumulation is reduced substantially in chloroquine-resistant cells (Macomber *et al.*, 1966). Chloroquine resistance has been correlated with mutations in a transporter found on the food vacuole membrane (chloroquine resistance transporter, CRT). Another food vacuole transporter gene, multi-drug resistance gene 1 (MDR1), has been implicated to play an ancillary role in resistance.

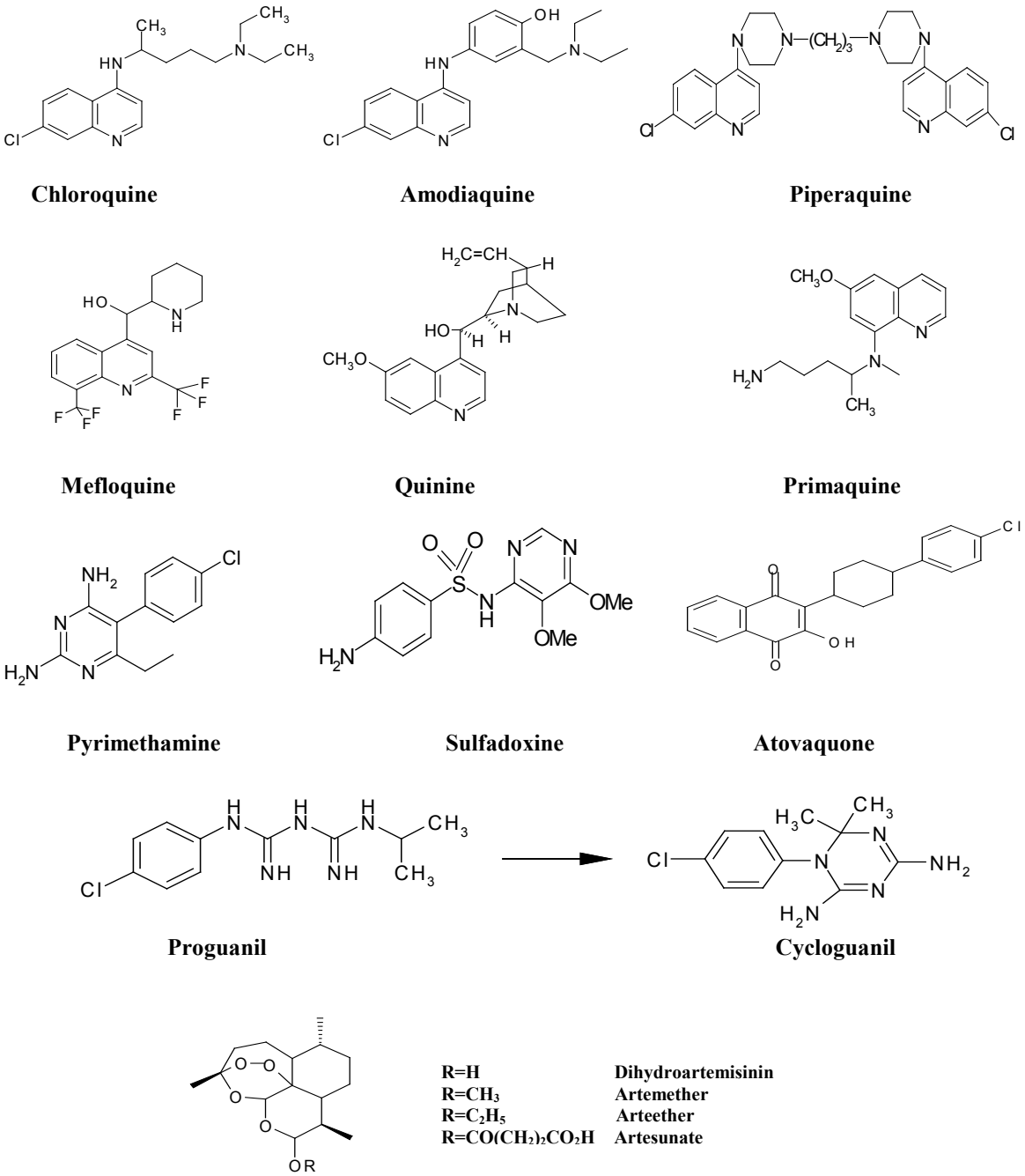


Figure 5: **Structures of selected antimalarial drugs.**
4-amino quinolines (chloroquine and amodiaquine), quinoline alcohols (mefloquine and quinine), bisquinoline (piperaquine) and 8-amino quinolines (primaquine) interfere with heme polymerization. Pyrimethamine, sulfadoxine, and cycloguanil are substrate analogs that interfere with folate metabolism. Proguanil is converted in humans to cycloguanil by cytochrome oxidase. Newer antimalarials with novel structures and mechanisms include atovaquone and the artemisinins.

Relatively small changes in the quinoline structure can enhance compound uptake into the food vacuole of chloroquine-resistant parasites (Ridley and Hudson, 1998). Resistance to quinine, mefloquine and halofantrine is probable because of mechanisms similar to those observed for chloroquine. Unlike chloroquine, however, these compounds do not currently

face high levels of resistance, and they thus remain, depending on the region, completely or partly active. The major disadvantage of the 8-amino quinolines like primaquine is the induction of haemolytic anemia in subjects with glucose 6-phosphate dehydrogenase (G6PD) deficiency as a potentially life-threatening adverse effect. This is likely related to their ability to generate ROI and the decreased reducing potential of G6PD-deficient erythrocytes.

Another important group of antimalarials constitute the antifolates which exploit the fact that folate biosynthesis in malaria parasites is distinctly different from that in other systems. Tetrahydrofolate is an essential cofactor in the methyl transfer reactions that generate monomers for protein and nucleic acid synthesis. Two kinds of antifolates exist; the first type consisting of drugs like pyrimethamine and proguanil inhibit the enzyme dihydrofolate reductase while the second type of antifolates consisting of sulfonamides like sulfadoxine and dapsone inhibit the dihydropteroate synthetase. The application of antifolates in antimalarial chemotherapy was greatly enhanced by the discovery of an extraordinary degree of synergism in drug combinations involving both classes of antifolates which is suspiciously linked to multiple blockades in a dihydrofolate metabolic pathway (Wang *et al.*, 2004). The molecular basis for resistance to antifolates, such as sulfadoxine-pyrimethamine has been well characterized. *P. falciparum* resistance to sulfadoxine-pyrimethamine is primarily conferred by successive single-point mutations in parasite genes that encode the target enzymes dihydrofolate reductase and dihydropteroate synthetase.

Atovaquone, a hydroxynaphthoquinone derivative, is an analog of ubiquinone, a parasite mitochondrial electron-carrier which is the cofactor of the dihydroorotate dehydrogenase. Atovaquone acts by inhibiting parasite mitochondrial electron-transport (Robert *et al.*, 2001). The best application of atovaquone for treatment is in a synergistic combination of atovaquone with proguanil (an antifolate) as Malarone® whereby the combination is well tolerated and more effective than CQ alone, CQ-sulfadoxine/pyrimethamine, or mefloquine, against acute uncomplicated multidrug resistant *P. falciparum*. It is also effective in regions where proguanil alone is ineffective due to resistance. The combination however, is expensive and not easily affordable in most African countries.

Antibiotics like tetracycline, doxycycline, and clindamycin targeting prokaryotic translation have antimalarial action through the inhibition of prokaryote-like protein synthesis in the apicoplast. The action of these antibiotics is slow, so they are being used increasingly in combination with other antibiotics to augment their activity (WHO, 2001).

Factors involved in the decision on the best treatment for malaria include the parasite species, the severity of disease, the patient's age and immune status, the parasite's susceptibility to the drugs (*i.e.* drug resistance), and the cost and availability of drugs. Drug resistance, and in particular chloroquine resistance, is a major public health problem in the control of malaria. Major factors involved in the development of drug resistance are shown below (Table 1). Cross-resistance between antimalarials is a phenomenon linked to the common or shared aspects of their modes of action and probably of their resistance mechanisms. A correlation analysis is the first indication of a common mode of action and perhaps a common mechanism of resistance (Le Bras and Durand, 2003).

Table 1: Factors contributing to development and spread of drug resistance

Factor	Comments
Self-treatment	Individuals may only take the drug until symptoms clear or will take lower doses to save money.
Poor compliance	Individuals may not complete the full course of treatment because of drug side effects.
Mass administration	The widespread use of a drug in an area of intense transmission increases drug pressure by exposing a larger parasite population to the drug.
Long drug half-life	Drugs that are slowly eliminated will lead to a longer exposure of the parasite to subtherapeutic drug concentrations.
Transmission intensity	High levels of transmission may allow re-infection while drugs are at sub-therapeutic levels.

Adapted after www.tulane.edu/~wiser/protozoology/notes/malaria.html#treat

1.1.5.3 Control through vaccines

Vaccines have been shown to be one of medicine's most cost-effective interventions as they induce an immune response in the infected host resulting in a marked decrease of the incidence of the disease which progressively leads to the eradication of the disease. The discovery and implementation of a safe and effective vaccine against malaria is a major priority in the control of the disease (Tongren *et al.*, 2004). However, an effective vaccine for malaria still remains elusive. The acquisition of natural protective immunity against malaria by adults living in malaria endemic zones provides hope that a vaccine against malaria could be developed. The ability to tolerate high parasitaemias without apparent adverse effects relates to the development of "antitoxic" immunity (Playfair *et al.*, 1990). The host adapts to repeated infection by producing less cytokines for a given quantum of

parasites (Riley *et al.*, 1988). Unfortunately, the immune response that correlates with protection in these life long residents of endemic areas has not been identified, so development of a vaccine that mimics this immunity remains difficult.

It may be possible to intervene at any or a combination of the stages in the parasite's life cycle to produce an effective vaccine (Figure 6). Malaria vaccines could be directed against the sporozoite stage of the malaria parasite to prevent infection, or against the stages in human blood – the asexual stage to prevent clinical disease, or the sexual stage to prevent transmission.

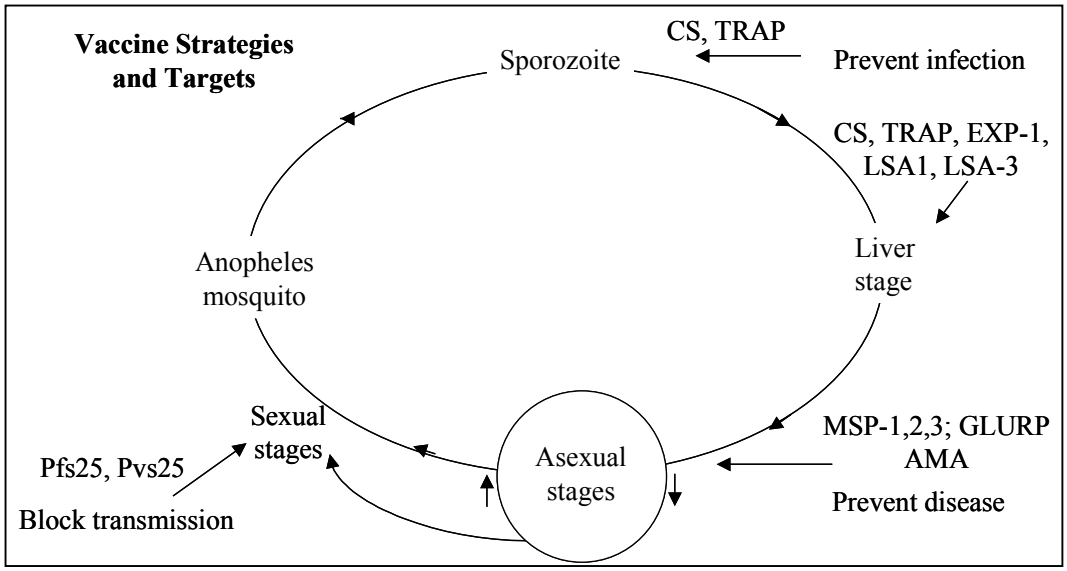


Figure 6: **Malaria life cycle and vaccine targets.**
CS = circumsporozoite; TRAP = thrombospondin-related adhesive protein; EXP-1 = exported antigen 1; LSA1 = liver stage antigen 1; MSP-1 = merozoite stage protein 1; GLURP = glutamate-rich protein; AMA-1 = apical membrane antigen 1; Pf = *Plasmodium falciparum*; Pv = *P. vivax* (adapted after Ballou *et al.*, 2004).

Much interest has been directed to pre-erythrocytic stage vaccines which are designed to prevent invasion of hepatocytes by sporozoites or to destroy infected hepatocytes (Kester *et al.*, 2001). Blood stage vaccines to eliminate or reduce the number of blood stage parasites have mainly focused on antigens contributing to erythrocyte invasion (WHO, 2004). Transmission-blocking vaccines are designed to prevent mosquitoes that feed on vaccinated individuals from becoming infected, reducing transmission and providing indirect protection to the entire population (Kaslow, 2002). However, four types of malaria vaccines namely synthetic peptide SPf66 and merozoite surface protein / ring-infected erythrocyte surface antigen MSP/RESA vaccines (against the asexual stages of the

Plasmodium parasite) and CS-NANP and RTS,S vaccines (against the sporozoite stages), have been tested in randomized controlled trials in endemic areas and have demonstrated varying levels of protective immunity.

The development of malaria vaccines faces the following challenges.

- The need to cope with diverse antigenic types
- The importance of stimulating a life long response that is boosted by natural infection.

Recent developments, including better methods for antigen production, improved adjuvants and novel delivery systems, provide optimism that sustained and appropriate long-lived immunity can be achieved (Brown and Reeder, 2002).

1.2 Rationale of the study

The difficulties that have been encountered in malarial vaccine development projects have indicated that the complete eradication of the disease malaria has to be the result of a synergistic combination of various malarial control measures as no single one can be 100% effective. Researchers, pharmaceutical companies, government policy makers, international bodies like WHO, local communities of endemic areas and the international community all have to combine their efforts in the eradication process. In this light, we – as malaria researchers searching for new drug alternatives against the parasite – developed three new projects in search of new alternative drugs as our own contribution in the fight against malaria. These projects involve the exploitation of the chemotherapeutic value of the glyoxalase detoxification system, selective inhibition of *P. falciparum* thioredoxin reductase (PfTrxR) which has been previously-validated as a drug target by gene-knockout experiments, and characterization of the *in vitro* antimalarial properties of methylene blue. An introduction to each of the above-mentioned projects as well as other related aspects is given below.

1.2.1 The glyoxalase system

The glyoxalase (Glo) system, a glutathione (GSH)-dependent detoxification system involved in the conversion of toxic 2-oxoaldehydes to the corresponding non-toxic 2-hydroxyacids, is composed of two enzymes: glyoxalase I (GloI, EC 4.4.1.5) and glyoxalase II (GloII, EC 3.1.2.6). GloI converts the non-enzymatically produced hemithioacetal from

a 2-oxoaldehyde and GSH to a thiolester of GSH which can then be readily hydrolysed by GloII producing a 2-hydroxyacid and regenerating GSH (Figure 7; Martins *et al.*, 2001).

GloI is a member of the vicinal oxygen chelate metalloenzyme family (Armstrong, 2000), and contains a paired βαββββ motif providing a metal coordination environment. The reaction types catalyzed include isomerization (glyoxalase I containing Zn^{2+} and/or Ni^{2+}), epimerization (methylmalonyl-CoA epimerase with Co^{2+}), oxidative cleavage of C-C bonds (estradiol dioxygenase, $Fe^{2+}$and/or Mn^{2+}), and nucleophilic substitutions (fosfomycin resistance proteins, Mn^{2+}, Mg^{2+}).

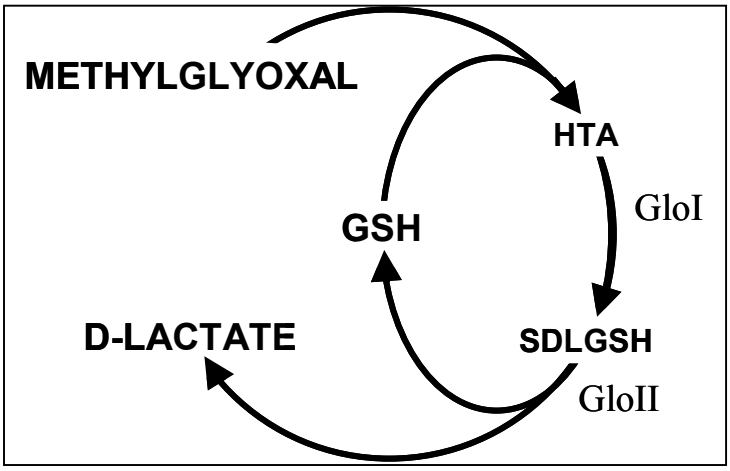


Figure 7: **Reactions of the glyoxalase system.**
GSH – glutathione, HTA – hemithioacetal, SDLGSH – S-D-lactoylglutathione (adapted from Martins *et al.*, 2001).

As revealed by sequence similarities and the X-ray crystal structure of human GloII, GloII is a member of the relatively newly defined metallohydrolase family containing the β-lactamase fold consisting of a four-layered ß sandwich (Cameron *et al.*, 1999b; Daiyasu *et al.*, 2001). This superfamily also includes class B β-lactamase, arylsulfatase, mRNA 3'-processing protein, a DNA cross-link repair enzyme, an alkylphosphonate uptake-related protein, CMP-*N*-acetylneuraminate hydroxylase, and insecticide hydrolases.

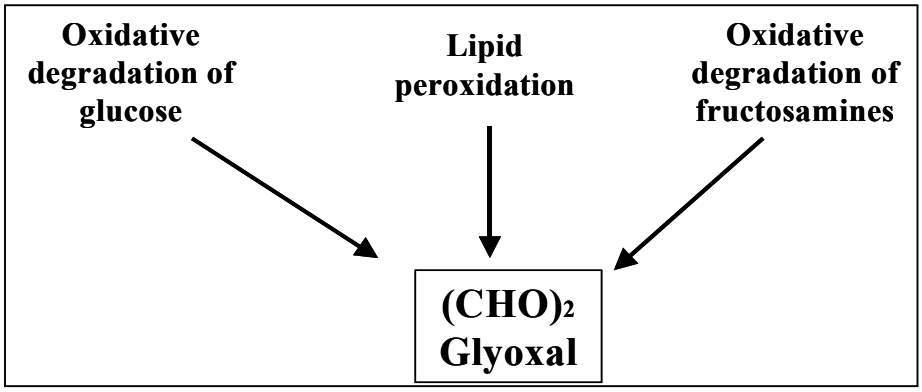
Physiological substrates (2-oxoaldehydes) of the glyoxalase system are glyoxal formed in lipid peroxidation and glycation reactions, methylglyoxal arising from triose phosphates and thus glycolysis, ketone body metabolism and threonine catabolism, as well as 4,5-dioxovalerate generated from 5-aminolevulinate and α-ketoglutarate (Figure 8). Previous studies have proven the electrophilic binding of 2-oxoaldehydes to guanyl residues of DNA and RNA as well as to lysyl, arginyl and cysteinyl residues of proteins (Lo *et al.*, 1994; Vaca *et al.*, 1994). This leads to mutagenesis, apoptosis, protein cross-linking and

degradation, as well as to the synthesis and production of pro-inflammatory cytokines (Thornalley, 1996; Thornalley, 1998). 2-oxoaldehydes do mediate the formation of advanced glycation end products (AGEs) which can lead to pathophysiological complications in diabetes and are known to be cytostatic at low concentrations and cytotoxic at high concentrations (Thornalley, 1996; Thornalley, 1998).

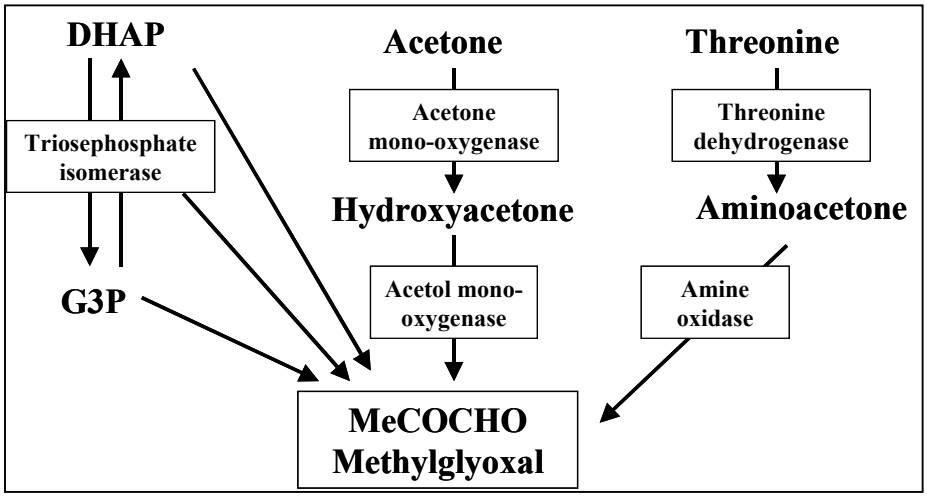
The glyoxalase system has been characterized in various organisms including man and other mammals (Marmstål *et al.*, 1979; Biswas *et al.*, 2002; Mearini *et al.*, 2002), fish (Antognelli *et al.*, 2003), higher plants (Crowder *et al.*, 1997), yeast (Frickel *et al.*, 2001), bacteria (Clugston and Honek, 2000), trypanosomes (Irsch and Krauth-Siegel, 2004), and nematodes (Sommer *et al.*, 2001). The cytosol is the main cellular location of the Glo system; however some isoenzymes have been reported to contain signal targeting sequences for mitochondria and even plastids (Cordell *et al.*, 2004). The ubiquitous distribution of the glyoxalase system, the presence of several isoenzymes in a single organism, and the high sequence similarity of Glo from different sources indicate the importance of the detoxification of 2-oxoaldehydes - produced from physiological reactions - in biological systems (Thornalley, 1993).

Blood stages of the malarial parasite *P. falciparum* are mainly responsible for the clinical manifestations of the disease. After sequencing the *P. falciparum* genome, it is still unclear whether citric acid cycle and oxidative phosphorylation are significantly involved in energy production of the blood stages (Gardner *et al.*, 2002). In any case, the parasite exhibits high glycolytic activity to cover energy demands resulting from its high proliferation rate (Jacobasch *et al.*, 1990). This activity is indicated by an appr. 75 fold increase in glucose consumption in *P. falciparum* infected erythrocytes in comparison with normal erythrocytes (Sherman, 1979) and exposes the parasite to higher fluxes of methylglyoxal produced by the spontaneous decomposition of triose phosphates (glyceraldehyde-3-phosphate and dihydroacetone phosphate) during the triose phosphate isomerase reaction of glycolysis (Thornalley, 1996).

A. Formation of glyoxal



B. Formation of methylglyoxal



C. Formation of 4,5-dioxovalerate

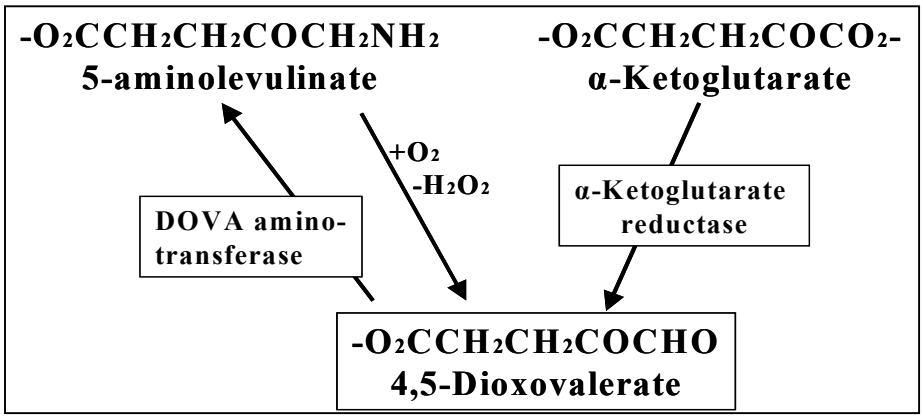


Figure 8: **Physiological formation of 2-oxoaldehydes.**
A). Glyoxal is formed by lipid peroxidation and by the slow oxidative degradation of glucose and glycated proteins. B). Methylglyoxal is formed by the non-enzymatic and enzymatic fragmentation of glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), acetone metabolism, and threonine catabolism. C). DOVA is formed by the oxidative degradation of 5-aminolevulinate and also by the reduction of α-ketoglutarate (adapted after Thornalley, 1998).

The anti-malarial activity of Glo inhibitors was demonstrated by the growth inhibition caused by S-p-bromobenzylglutathione diethyl ester with IC_{50} values in the lower micromolar range (Thornalley *et al.*, 1994). Selective inhibition of the Glo system leading to 2-oxoaldehyde stress within the parasite therefore provides a promising chemotherapeutic approach to the treatment of malaria. New chemotherapeutic approaches are urgently needed to fight against the increasing drug resistance of the parasite, to reduce the 2 million malaria deaths per year and the increased geographical spread of the disease (Olliaro, 2001; Greenwood and Mutabwinga, 2002).

Recently, a glyoxalase I from *P. falciparum* (Iozef *et al.*, 2003) was characterized. This cGloI belongs to a group of large glyoxalases I earlier characterized in yeast (Frickel *et al.*, 2001), later found in plants (Clugston *et al.*, 1998), *Plasmodium yoelii*, and in insects such as *Anopheles gambiae* and *Drosophila melanogaster* (Iozef *et al.*, 2003). Genes of these large glyoxalases have almost twice the size of the smaller glyoxalases of which human GloI is a good example. Amino acid sequences of the N- and C-terminal halves of their monomeric gene products are homologous to each other and to the two subunits of homodimeric small glyoxalases I (Ridderström and Mannervik, 1996a). These observations led to the gene duplication hypothesis on the evolution of these large glyoxalases, which was further supported by the fact that the recombinantly produced C-terminal half of cGloI showed glyoxalase I activity (Iozef *et al.*, 2003). In the homodimeric human Glo I, the active site is located at the dimer interface where side chains from both subunits interact with GSH and the metal ion which is zinc in most Glo I proteins and nickel in *E. coli*.

The genes of a second putative GloI (GILP) as well as of two GloII (tGloII and cGloII) in *P. falciparum* have been identified. This was followed by the comparative structural and functional analysis of the four putative glyoxalases with their isofunctional human counterparts within the frame of this thesis. First inhibitor studies serve as a basis for further elucidating the functions of the glyoxalase system in *P. falciparum* and for estimating its potential as target for the development of antimalarial drugs.

1.2.1.1 Non-glyoxalase metabolism of methylglyoxal

The ubiquitous glyoxalase system appears to act mainly against methylglyoxal toxicity. However, other enzymes have also been implicated in the detoxification of methylglyoxal and other 2-oxoaldehydes.

Aldehyde reductase or aldose reductase (EC 1.1.1.21) can catalyze the conversion of methylglyoxal to hydroxyacetone and lactaldehyde, using NADPH as cofactor. The product distribution is GSH-dependent. The non-enzymatic pre-equilibrium reaction between methylglyoxal and GSH to form the hemithioacetal in equilibrium with methylglyoxal (free and hydrated) provides two substrates for aldose reductase. Unhydrated methylglyoxal is converted to hydroxyacetone through reduction of the aldehyde functional group while the hemithioacetal is converted into lactaldehyde through reduction of the ketone functional group followed by dissociation of the resulting hemithioacetal (Vander Jagt and Hunsaker, 2003). The human placental enzyme has been well characterized. The K_M and k_{cat} value for methylglyoxal are 8 µM and 2.36 sec^{-1}, respectively (Vander Jagt *et al.*, 1972). Lactaldehyde and hydroxyacetone could still be further reduced by aldose reductase to L- and D-propane-1,2-diol. A putative aldose reductase gene has been identified on the *P. falciparum* genome. The gene has been cloned into plasmid expression vectors but overexpression of the gene in *E. coli* and characterization of the gene product remain elusive (AG Becker-Brandenburg).

Methylglyoxal reductase also catalyzes the conversion of methylglyoxal to L-lactaldehyde. Such activity has been found in mammalian liver homogenates of rat and goat as well as from yeast *Saccharomyces cerevisiae* and mould *Aspergillus niger* (Thornalley, 1993). The mammalian enzyme (89 kDa) requires NADH as cofactor and has broad substrate specificity for 2-oxoaldehydes and other aldehydes. The yeast enzyme (43 kDa) requires NADPH as cofactor, is specific for 2-oxoaldehydes and may be induced by exposure to methylglyoxal (Murata *et al.*, 1986).

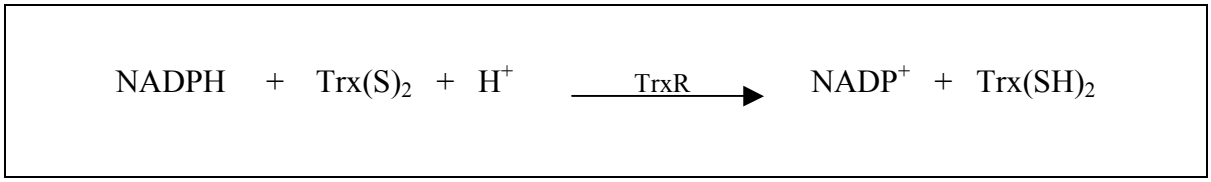
Other methylglyoxal metabolising enzymes include betaine aldehyde dehydrogenase which is able to catalyze the NAD^+-dependent oxidation of methylglyoxal to pyruvate, 2-oxoaldehyde dehydrogenase catalyzing the $NADP^+$-dependent oxidation to pyruvate, and pyruvate dehydrogenase which converts methylglyoxal to acetyl-CoA and formate (Izaguirre *et al.*, 1998). Methylglyoxal is however a poor substrate for these enzymes. Methylglyoxal has been shown to be a slow competitive inhibitor for the oxidation of pyruvate as reported K_i values are in the lower millimolar range (Thornalley, 1993).

Thus the various methylglyoxal metabolising pathways involve NADPH dependent reduction, glutathione dependent disproportionation and both NAD-dependent and NADP-dependent oxidations.

1.2.2 The thioredoxin reductase system

Plasmodium falciparum can not efficiently develop in glucose-6-phosphate dehydrogenase deficient erythrocytes and is sensitive to reactive oxygen species (ROS) resulting from the host immune system as well as from hemoglobin breakdown (Becker *et al.*, 2004). The small amount of free heme escaping the various detoxification pathways, causes oxidative damage to host proteins and membranes, inhibits parasite enzymes and finally lyses erythrocytes. Two systems interact to protect malarial parasites against ROS: the glutathione system, comprising glutathione, glutathione reductase (GR), glutathione *S*-transferase, and different glutaredoxin-like proteins and the thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR), and thioredoxin dependent peroxidases (Becker *et al.*, 2003; Becker *et al.*, 2004, Rahlfs *et al.*, 2002). Both systems are NADPH dependent.

The redox-active small protein Trx is involved in ribonucleotide reduction and thus DNA synthesis as well as in the redox control of many cellular processes, including transcriptional control, protein folding, and enzyme regulation (Holmgren *et al.*, 1998; Arnér and Holmgren, 2000). Trx is reduced by thioredoxin reductase (TrxR), a homodimeric FAD-dependent protein as indicated in the reaction below.



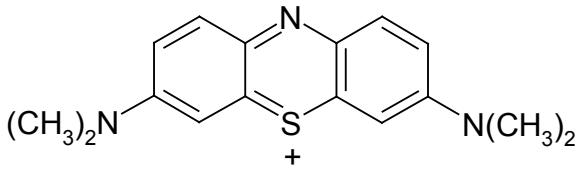
TrxR is found in nature in two forms. The low M_r form, with a subunit of 35 kDa, is present in prokaryotes and lower eukaryotes including yeast, and has one FAD and one redox active disulfide-dithiol in each monomer. The high M_r form, with a subunit of ca. 55 kDa, contains one FAD and two redox active disulfide-dithiols per monomer and is found in higher eukaryotes including man and *P. falciparum* (Williams *et al.*, 2000; Novoselov and Gladyshev, 2003). The second redox center in large TrxRs is located on a flexible C-terminal arm of the protein. It picks up reducing equivalents from the N-terminal redox center of the other subunit and transfers them to Trx and other substrates. This second redox center is represented by a Cys-Sec pair in hTrxR. The accessibility and reactivity of this redox pair is presently exploited as target for chemotherapeutic agents – an approach that is supported by the fact that TrxR is found at ten-fold higher levels in many cancer cell lines than in normal cells (Kahlos et al., 2001; see Gromer *et al.*, 2004, for review). In

PfTrxR the C-terminal redox pair is represented by a CysXXXXCys sequence. This difference in an essential structural and functional motif of host cell and parasite enzyme represents a most promising starting point for the development of specific PfTrxR inhibitors (Wang *et al.*, 1999; Becker *et al.*, 2000). The inhibition of PfTrxR is most likely to result in enhanced oxidative stress, to ineffective DNA synthesis and cell division, and to disturbed redox regulatory processes in the parasites. As demonstrated by knock out studies on *P. falciparum*, PfTrxR is essential for the parasite thus representing a validated drug target (Krnajski *et al.*, 2002).

The approach of our collaborative effort to develop a new generation of antimalarial drugs has been two-pronged. The first approach was based on a high throughput screen of 350,000 compounds in the Pfizer library seeking compounds that discriminated between human and PfTrxR. The screen yielded 15 compounds 13 of which were Mannich bases, 4 saturated and 9 unsaturated. A series of unsaturated mannich bases that act as mechanism based inhibitors (Davioud-Charvet *et al.*, 2003) were synthesized. In the second approach, the subject of the present study, a series of inhibitors that act in the low micro- to nanomolar range and show high specificity for the *P. falciparum* enzyme have been developed. As described here, the inhibitors were evaluated on isolated *P. falciparum* and human TrxR as well as on *P. falciparum* in cell cultures.

1.2.3 Methylene blue in antimalarial therapy

Use of methylene blue (MB - a phenothiazonium salt, structure see scheme 1) in antimalarial therapy dates back to the 1890s when it became the first chemically synthesized drug to be employed in the treatment of malaria (Guttman and Ehrlich, 1891).



Scheme 1: Structure of methylene blue

Subsequently, chemical modification of chromophore and side chains of MB led to other antimalarials such as the 8-aminoquinolines and – via sontoquine – the 4-aminoquinolines (Figure 9).

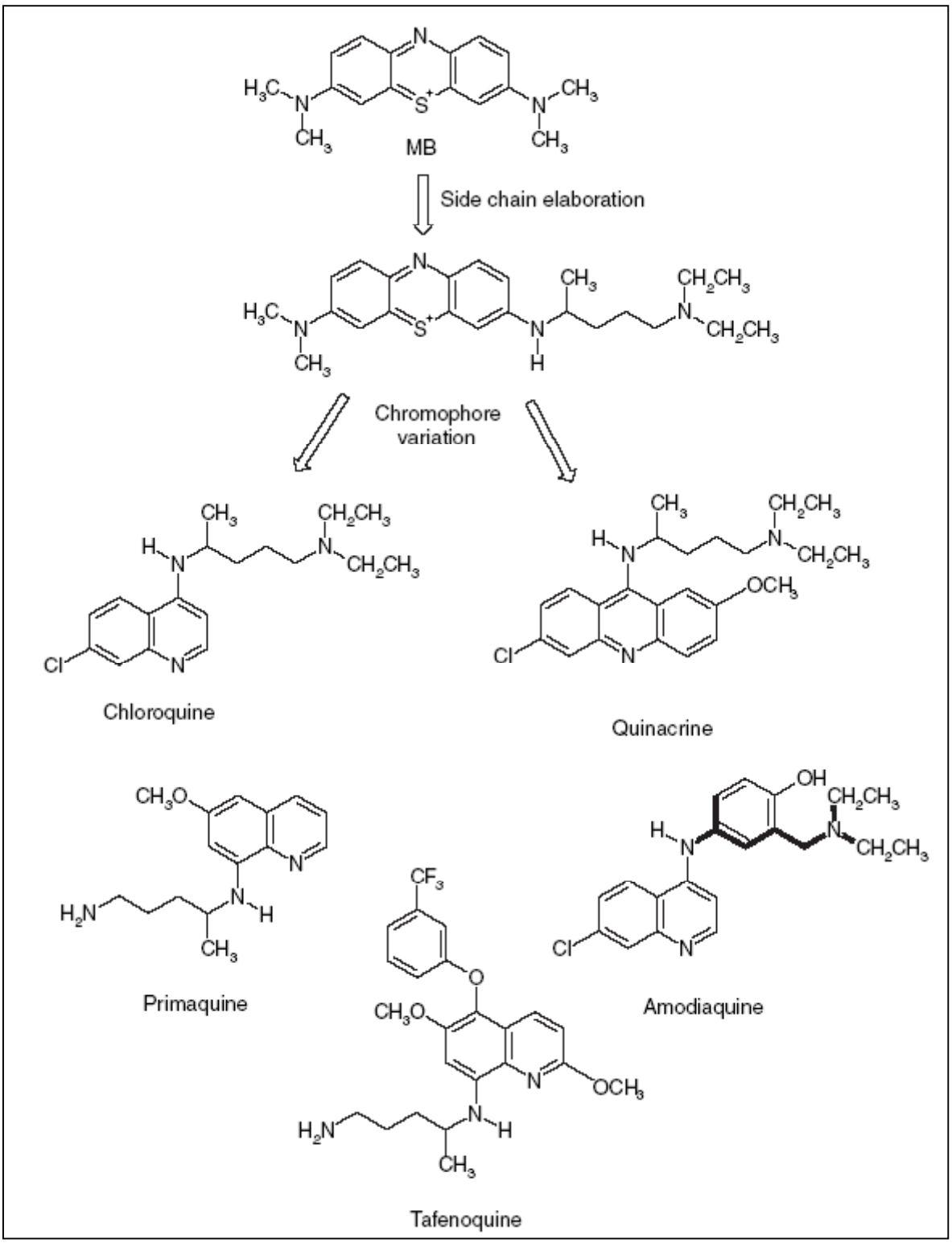


Figure 9: **Chemical development of antimalarial drugs from methylene blue.** The dialkylaminoalkylamino side chain equivalent to amodiaquine is shown in bold (Wainwright and Amaral, 2005).

Currently, the major indication for MB is the treatment of inherited and acquired methaemoglobinaemias due to its ability to reduce methaemoglobin (Mansouri and Lurie, 1993; Coleman and Coleman, 1996). In addition, it is used as a neuroprotective agent in ifosfamide-based chemotherapy regimes. Since doses of the drug applied in methaemoglobinopathies have also been shown to have antimalarial effects and since MB was identified as a specific inhibitor of *P. falciparum* glutathione reductase, MB was reconsidered as useful antimalarial drug (Farber *et al.*, 1998; Schirmer *et al.*, 2003, Mandi *et al.*, 2005). Further advantages of MB include its intrinsic inhibition of haem polymerization within the food vacuole (Atamna *et al.*, 1996), its prevention of methaemoglobinaemia – a serious complication of malaria anaemia (Anstey *et al.*, 1996) – and its relatively low price. Furthermore, MB shows high selectivity indices with respect to the viability of the human monocytic leukemia-derived cell line J-111 (Vennerstrom *et al.*, 1995; Atamna *et al.*, 1996) indicating that its cytotoxicity for mammalian cells is low. Considerable side effects of MB have been reported (Goluboff and Wheaton, 1961; Peters, 1970) but they are probably restricted to persons with certain forms of inherited G6PD-deficiency.

Malaria control has been greatly hampered by the continuous increase of parasite resistance to available anti-malarial drugs, chloroquine (CQ) – a highly effective and affordable drug – being the most prominent example (Le Bras and Durand, 2003; Arav-Boger and Shapiro, 2005). Several drug combinations were formulated to prevent or delay resistance development (Ohrt *et al.*, 2002; Yeung *et al.*, 2004; Kremsner and Krishna, 2004) and the use of CQ sensitizers in combination with CQ has been given high priority (Valecha *et al.*, 1992). CQ-resistant parasites were shown to possess significantly increased concentrations of reduced glutathione (GSH) when compared with sensitive parasites (Dubois *et al.*, 1995; Meierjohann *et al.*, 2002; Ginsburg and Golenser, 2005). Thus, the reduction of glutathione disulfide by the flavoenzyme glutathione reductase (GR) as well as the *de novo* synthesis of GSH seem to be more efficient in resistant parasites. In *Plasmodium*, GSH is likely to be involved in buffering the reducing milieu, in antioxidant defense, redox signalling, DNA-synthesis, haem degradation, and in detoxification reactions catalysed by glutathione *S*-transferase (Ginsburg, 2003; Becker *et al.*, 2003 & 2004; Krauth-Siegel *et al.*, 2005). Furthermore, the combination of CQ derivatives with the selective PfGR inhibitor 6-[2-(3-methyl)-naphthoquinolyl]hexanoic acid as double drug conjugate inhibited the growth of CQ-resistant *P. falciparum* both *in vitro* and *in vivo* (Davioud-Charvet *et al.*, 2001). These and other observations suggest that the combination

of CQ with MB – as PfGR inhibitor and thus CQ sensitizer – might be useful. This approach has recently been tested in clinical trials in the Nouna District of Burkina Faso (Coulibaly *et al.*, 2005; Mandi *et al.*, 2005). In this study, cases of MB toxicity or intolerance were not reported, however, a clear advantage over a CQ monotherapy could not be observed, either (Meissner *et al.*, 2005). Several reasons were proposed to be responsible for this clinical failure. They include (i) the possibility that the dosage of the highly water soluble MB was not high enough (Meissner *et al.*, 2005), (ii) the possibility that MB and CQ do act antagonistically, and (iii) the possible formation of stable blue pigments in blood cells by MB leading to false positive counts of parasitaemia.

In this work, the effects of MB on *P. falciparum* in cell culture including growth inhibitory effects on different parasite strains, stage specificity, uptake, and staining effects were systematically studied. In addition, the combination of MB with other clinically used antimalarials was tested in order to search for potential clinical drug combination therapies.

1.2.4 Other aspects of the PhD project

1.2.4.1 Glutathionylation of thioredoxin of *P. falciparum*

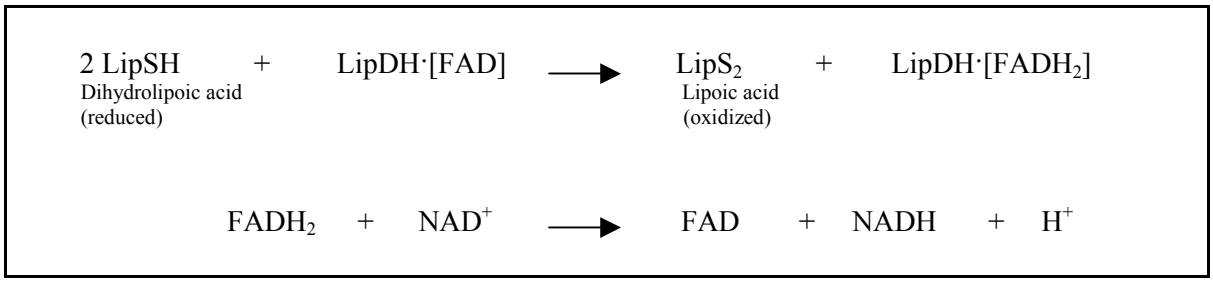
Protein glutathionylation describes the formation of a disulfide bond between a cysteine residue in a protein and the cysteine in the tripeptide GSH (γ-glutamylcysteinylglycine). The modification can be induced in cells by oxidative stress as a protective mechanism to prevent the disruption of amino acid residues by reactive oxygen species (ROS). Such modification in *P. falciparum* is most likely to occur as the parasite shows high susceptibility to oxidative stress. Glutathionylation can occur by a direct oxidation of a protein and GSH, by thiol disulfide exchange between a protein and oxidized glutathione (GSSG), and also with the glutathione disulfide *S*-oxide (GS(O)SG) intermediate produced by decomposition of S-Nitrosoglutathione (GSNO, Li *et al.*, 2001). Glutathionylation of proteins is reversible, as these proteins can be reduced by glutaredoxins (Holmgren, 1989), and the process serves to regulate protein functions depending on the GSH/GSSG ratio in the environment.

Thioredoxin is a small (~ 12 kDa) ubiquitous redox protein with biological activities ranging from electron donor for ribonucleotide reductase, regulatory protein for NF-kB and ASK, to an extracellular cytokine. This study of the glutathionylation of *P. falciparum* thioredoxin (PfTrx) was triggered by the discovery of glutathionylation of certain cysteine residues on human Trx (Casagrande *et al.*, 2002) under conditions of oxidative stress. PfTrx contains two cysteines at its conserved redox active site CGPC at positions 41 and

44, with an additional cysteine at position 54 of the recombinantly-synthesized histidine-tagged protein. This study tries to investigate the susceptibility of PfTrx to the glutathionylation under conditions of oxidative stress.

1.2.4.2 Lipoamide dehydrogenases of *P. falciparum*

Lipoamide dehydrogenases (LipDH) belong to the homodimeric FAD-dependent cysteine-oxidoreductase enzyme family which is mostly involved in the maintenance of redox balance and is considered as chemotherapeutic target for *P. falciparum* (Becker *et al.*, 2000). Members of this family catalyse the transfer of electrons between pyridine nucleotides and disulphides. LipDH constitutes part of the following enzyme complexes; pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain 2-oxo-acid dehydrogenase, as well as the glycine cleavage system in plants (Williams, 1992). The first two enzyme complexes are very important in the energy metabolism of the organisms in which they occur. The pyruvate dehydrogenase complex links glycolysis to the citric acid cycle and consists of pyruvate dehydrogenase (E1), lipoamide transacetylase (E2) and LipDH (E3). In these complexes, LipDH catalyzes the re-oxidation of protein-bound dihydrolipoyl residues transfering reducing equivalents to NAD^+ via FAD (Scheme 2).



Scheme 2: The lipoamide dehydrogenase reaction

Glucose consumption in *P. falciparum* infected erythrocytes increases ~100 fold in comparison to normal erythrocytes. This renders the enzymes involved in glucose metabolism within the parasite attractive drug targets. Inhibition of *P. falciparum* LipDH (PfLipDH) should have inhibition effects on the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. With the identification of two putative PfLipDH genes on chromosome 8 and 12, we set out to functionally characterize their recombinant gene products as well as investigate their potential as drug targets for *P. falciparum*. The inhibitory/stimulatory effect of MB on PfLipDH was also investigated.

1.3 Objectives of the study

With the overall goal to contribute to the fight against drug resistant malaria through the rational design of new drugs and prevention of resistance development to artemisinin, the thesis aimed at exploring the chemotherapeutic value of the glyoxalase detoxification system, selective inhibition of *P. falciparum* thioredoxin reductase (PfTrxR), and the antimalarial properties of methylene blue. In order to do this, the following objectives with respect to each project were drawn.

1.3.1 The glyoxalase system

- Cloning, overexpression and purification of the recombinant Glos of *P. falciparum*.
- Biochemical and kinetic characterization of Glos of *P. falciparum*.
- Analysis of the effects as well as the mode of action of Glo inhibitors on Glos of *P. falciparum* in a search for selectivity.
- Analysis of the effects of Glo inhibitors on cultures of *P. falciparum*.
- Development of X-ray-diffractable crystals – and as such structures – of Glo enzymes which could facilitate the design of selective inhibitors of Glos of *P. falciparum*.
- Development of model structures of Glo enzymes of *P. falciparum* based on X-ray structures of human Glos.

1.3.2 The thioredoxin reductase of *P. falciparum*

- Study of the mechanism of inhibition of some selective inhibitors of PfTrxR.
- Analysis of the effects of these inhibitors on cultures of *P. falciparum*.
- Analysis of the effects of drug combination of these inhibitors with CQ, MB and artemisinin.

1.3.3 Methylene blue in antimalarial chemotherapy

- Study of the susceptibility of *P. falciparum* parasites to MB.
- Determination of the *P. falciparum* growth stage-specificity of MB.
- Analysis of the parasitic uptake of MB.
- Analysis of the drug combination effects of MB with clinically-used antimalarials.

2 Materials and methods

2.1 Materials

Most of the reagents used were obtained at their highest purity from Roth, Merck or Sigma.

2.1.1 Chemicals

Acrylamide solution	BioRad, München
Ammonium persulphate	Merck, Darmstadt
Ampicillin	Sigma, Steinheim
Bacto-Agar	Roth, Karlsruhe
Bovine serum albumin	Roth, Karlsruhe
Chloramphenicol	Roth, Karlsruhe
Ethidium bromide	Sigma, Steinheim
Ethylenediaminetetraacetate (EDTA)	Roth, Karlsruhe
Gentamycin	Gibco, Karlsruhe
Glucose	Merck, Darmstadt
Glycerin	Roth, Karlsruhe
Hypoxanthine	Sigma, Steinheim
Isopropylthiogalactoside (IPTG)	Roth, Karlsruhe
Kanamycin	Roth, Karlsruhe
Lipid rich bovine serum albumin (Albumax)	Gibco, Karlsruhe
Mercaptoethanol	Sigma, Steinheim
Milk powder	Biorad, München
Pepstatin	Sigma, Steinheim
Phenylmethylsulfonylfluoride (PMSF)	Sigma, Steinheim
RNase I	Roth, Karlsruhe
Saponin	Roth, Karlsruhe
Sodium dodecyl sulphate (SDS)	Merck, Darmstadt
Sorbitol	Roth, Karlsruhe
Taq polymerase reaction buffer (10x)	Fermentas, St. Leon-Rot
Tris	Roth, Karlsruhe
Trypton	Roth, Karlsruhe
Tween-20	Merck, Darmstadt
Yeast Extract	Roth, Karlsruhe

2.1.2 Malaria drugs

Mefloquine	Roche, Mannheim
Artemisinin	Aldrich, Steinheim
Chloroquine	Sigma, Steinheim
Primaquine	Aldrich Chem Co, Milwaukee; USA
Pyrimethamine	Sigma, Steinheim
Quinine	Acrös Organics, Geel; Belgium
Amodiaquine	Sigma, Steinheim
Methylene Blue	Roth, Karlsruhe

2.1.3 Enzymes

DNase I	Roche, Mannheim
Pfu polymerase	Promega, Mannheim

Restriction Enzymes	MBI Fermentas, St. Leon-Rot
Taq polymerase	MBI Fermentas, St. Leon-Rot

2.1.4 Antibodies

Mouse anti-glutathione antibodies	Virogen, USA
Rabbi anti-mouse immunoglobulins	DAKO A/S, Denmark
Mouse anti-histidine tag IgGs	Qiagen, Hilden

2.1.5 Kits

Bradford kit	Biorad, München
Crytallization kit (crystal screen 1&2)	Hampton Research, Laguna Niguel, U.S.A
Plasmid extraction kit	Qiagen, Hilden
QIAquick gel extraction kit	Qiagen, Hilden
QIAquick PCR-purification kit	Qiagen, Hilden
Ready-To-Go ligase	Amersham, Freiburg
Western lightning chemiluminescence reagent	Perkin Elmer, Boston, U.S.A.

2.1.6 Instruments

Accurate scale	Scaltec Instruments, Göttingen
Beckmann spectrophotometer DU® 650	Beckman, München
Biophotometer	Eppendorf, Hamburg
Bosch PE626 normal scale	Gebr. Bosch, Jungingen
Gel set up (agarose gel)	Peqlab
Hitachi spectrophotometer U-2001	Hitachi Ltd, Tokyo
Incubator shaker	Thermo Life Sciences, Egelsbach
LKB multiphor II nova blot	Pharmacia Biotech, Erlangen
Megafuge 1.0 R	Heraus Instruments, Hanau
Microscope axiostar	Zeiss, Jena
Mini-Spin table centrifuge	Eppendorf, Hamburg
Optima™ TLX ultracentrifuge	Beckman, München
PCR –normal and –gradient cyclers	Eppendorf, Hamburg
pH-Meter (Φ 350 pH/Temp/mV Meter)	Beckman, München
Quarz cuvettes	Hellma, Müllheim
Salvant, SpeedVac, vacuum centrifuge	Thermo Life Sciences, Egelsbach
Sonicator	Bandelin Electronics, Berlin
Sorvall centrifuge RC5C	Du Pont Company, Wilmington

2.1.7 Biological materials

2.1.7.1 cDNA libraries and erythrocytes

***P. falciparum* cDNA libraries:** cDNA banks from blood stages of the *P. falciparum* 3D7 strain were kindly provided by Prof. David Kaslow (National Institute of Health, Bethesda, USA) and were used as templates for the amplification of all *P. falciparum* glyoxalase genes.

Human lung cDNA library was kindly provided by Dr. Lutz Schomburg (Charité, Berlin, Germany) and used for the amplification of the human glyoxalase I gene.

Clones of the human glyoxalase II gene in the pKK-D vector were kindly provided by Prof. Bengt Mannervik (Biomedical Center, Uppsala, Sweden)

Human erythrocytes were obtained from the Red Cross, Frankfurt.

2.1.7.2 Plasmids

Plasmid	Antibiotic Resistance, other importance	Source
pQE 30	Ampicillin	Qiagen, Hilden
pKK-D	Ampicillin	Gift from Professor Mannervik (Uppsala, Sweden)
pREP	Kanamycin, lac repressor gene	Qiagen, Hilden
pRIG	Chloramphenicol, tRNAs for R, I and G	Gift from Professor Wim Hol (Baca & Hol, 2000)
pRARE	Chloramphenicol	Novagen, Darmstadt

2.1.7.3 *Escherichia coli* cells

E. coli strain	Genotype	Reference / Manufacturer
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsd-r17, supE44, relA1, lac, [F' pro AB, $lacl^qZ\Delta M15$, Tn10, (Tet^r)]	(Bullock *et al.*, 1987) Stratagene, LaJolla, USA
M15 [pREP4]	nal^S, Str^S, rif^S, Km^R, lac^-, ara^-, gal^-, mtl^-, F^-, $recA^+$, uvr^+	(Villarejo and Zabin, 1974) Qiagen, Hilden
BL21	F^- ompT $hsdS_B(r_B^-m_B^-)$ gal dcm	(Wood, 1966) Stratagene, LaJolla, USA
JRG	LipDH deficient	AG Prof. Krauth-Siegel, Heidelberg

2.1.7.4 Strains of *P. falciparum*

Strain	Origin	Source
K1 (Chloroquine-resistant)	South-East Asia	AG Prof. Lanzer, Heidelberg
Dd2 (Chloroquine-resistant)	Indochina	AG Prof. Lanzer, Heidelberg
HB3 (Chloroquine-sensitive)	Honduras	AG Prof. Lanzer, Heidelberg
3D7 (Chloroquine-sensitive)	The Netherlands	AG Prof. Lanzer, Heidelberg

2.1.8 Buffers and solutions

2.1.8.1 Solutions for DNA electrophoresis

10 x TBE:

1 M	Tris
1 M	Boric acid
20 mM	EDTA

pH 8.0 with acetic acid

DNA Sample buffer

0.1%	Bromophenol blue
1 mM	Tris
60%	Saccharose

pH 8.3 with HCl

2.1.8.2 Solutions for protein electrophoresis

Sample buffer

62.5 mM	Tris-HCL
25%	Glycerin
2%	SDS
0.01%	Bromophenol blue
5%	Mercaptoethanol

Running buffer

1.5 M	Tris-HCl	pH 8.8

Stacking buffer

0.5 M	Tris	pH 6.8

Electrophoresis buffer

25 mM	Tris
192 mM	Glycin
0.1%	SDS

Coomassie staining solution

0.5%	Coomassie brillant blue R250
10%	Acetic acid
30%	2-Propanol

Coomassie destaining solution

10%	Acetic acid
40%	Methanol

2.1.8.3 Solutions for western blotting

Anode buffer I

300 mM	Tris

Anode buffer II

25 mM	Tris

Cathode buffer

40 mM	6–Aminohexanic acid

TBS buffer

10 mM	Tris
155 mM	NaCl
pH 8.0 with HCl	

TBST buffer

0.05%	Tween 20
ad 1000 ml with TBS Buffer	

Blocking buffer 5% milk powder in TBS Buffer

Ponceau staining solution

1%	Ponceau S
1%	Acetic acid

Ponceau destaining solution

1%	Acetic acid

2.1.8.4 Extraction of parasites from infected red blood cells

Saponin lysis Buffer

	0,02 % (w/v)	Saponin
in:	7 mM	K_2HPO_4
	1 mM	NaH_2PO_4
	11 mM	$NaHCO_3$
	58 mM	KCl
	56 mM	NaCl
	1 mM	$MgCl_2$
	14 mM	Glucose,

pH 7.4 with HCl

2.1.9 Growth medium

LB medium	10 g	Tryptone
	5 g	Yeast extract
	10 g	NaCl
	per litre of H_2O	
ZY medium	20 g	Tryptone
	15 g	Yeast extract
	5 g	NaCl
	per litre of H_2O	
2 x YT medium	16 g	Tryptone
	10 g	Yeast extract
	5 g	NaCl
	per litre of H_2O	

3 Methods

3.1 Gene identification

Complete open reading frames of two putative GloI genes on chromosome 6 and chromosome 11, two putative GloII genes on chromosome 4 and chromosome 12, and two PfLipDH genes on chromosome 12 (PfLipDH1) and chromosome 8 (PfLipDH2) were identified by online screening of the *P. falciparum* genome project (www.ncbi.nlm.nih.gov/Malaria/plasmodium-blcus.html) using other well known glyoxalases and LipDH as search strategies.

3.2 Polymerase chain reaction (PCR)

The PCR is an *in vitro* procedure for synthesizing enzymatically, specific sequences of DNA (Mullis and Faloona, 1987). Two oligonucleotide primers that flank the DNA region of interest and hybridise to opposite strands of the DNA are utilised in this technique. A series of repetitive cycles involving template denaturation, primer annealing and the extension of the annealed primers using the thermostable Taq DNA polymerase accomplishes the DNA amplification. At the completion of the reaction, amplification products are analyzed by size fractionation using agarose gel electrophoresis and then purified with a QIAquick PCR purification kit.

Conditions under which a PCR is carried out varies depending mainly on the specific DNA sequence to be amplified and the oligonucleotide primers which are to be used for the amplification. Detailed descriptions of the conditions under which the Glo and LipDH genes investigated in this study were amplified are shown in Tables 2 and 4, respectively.

For the *P. falciparum* glyoxalase and LipDH genes, N-terminal and C-terminal primers were designed from the sequence of the 5' and 3' ends of the genes, respectively. A *P. falciparum* gametocyte cDNA library from the strain 3D7 was used as template for the PCR amplification.

Primers used for the amplification of the human GloI gene were designed from the gene sequence obtained from the GenBankTM/EMBL Data Bank (Acc. No. NP_006699). Primers were designed such that the 5' and 3' ends of the genes to be amplified would be flanked by additional DNA containing restriction sites necessary for subsequent cloning of the genes into plasmid vectors. A human lung cDNA library was kindly provided by Dr. Lutz Schomburg (Charité, Berlin) and used as a template. Amplification of the human

GloII gene was unnecessary as a clone of the gene present in pKK-D vector was provided by Professor Mannervik (Uppsala, Sweden).

3.3 Cloning and overexpression of genes

All the enzymes used in this study were synthesized by plasmid-directed expression in *E. coli*. Plasmids carrying resistance genes to particular antibiotics were used as vectors so that treatment with antibiotics could be used as an indicator of plasmid/vector maintenance in *E. coli* cultures. Appropriate restriction enzymes were used to cut both the flanking ends of the amplified genes (to make them sticky) and the plasmid vector. Amplified genes cut with particular restriction enzymes could then be introduced into the multiple cloning site of a plasmid vector which had been cut with the same restriction enzymes. The sticky ends of both the gene and the vector then anneal to each other by complementary base pairing since they were generated by the same restriction enzyme. The gene was then ligated into the vector by the use of a ligation kit which exploits the action of DNA ligase. All products were verified by DNA sequencing. The generated recombinant plasmid was then used to transform the appropriate competent *E. coli* by heat shock treatment. The transformed *E. coli* were then grown in the most suitable growth medium and IPTG induction of the expression of the recombinant genes was carried out at optimized levels during the growth of the *E. coli*.

All glyoxalase genes were cloned into the expression vector pQE30 and overexpressed in *E. coli* cells. The human GloI gene was also cloned into pQE30 and overexpressed using *E. coli* BL21(DE3) plus S cells. The gene encoding cytosolic human GloII in the vector pKK-D (Ridderström *et al.*, 1996) was overexpressed in *E. coli* JM109. Table 2 delineates gene cloning and overexpression procedures of the various human and *P. falciparum* glyoxalase genes that were used in this study.

Genes of PfLipDH1 and PfLipDH2 were either cloned into the pQE30 or pRSET(A) expression vector and the overexpression was optimized in several different *E. coli* strains. The pRIG was employed in all of the overexpressions in order to adapt the codon usage of the *E. coli* expression to that of *P. falciparum*.

PfTrxR and PfTrx were prepared as described in Kanzok *et al.* (2000).

3.4 Site-directed mutagenesis

The Tyr185Phe mutant of cGloII was generated by site directed mutagenesis using standard PCR with *Pfu* DNA polymerase using the following primers: OPfG4Ys 5' GCG GAC ATG AGT TTA CCC TTA ATA ATT TAA GG 3' and OPfG4Yas 5' CCT TAA ATT ATT AAG GGT AAA CTC ATG TCC GC 3'. The expression clone of cGloII was used as a template. The resulting PCR product was sequenced to make sure that no unwanted mutations had been introduced.

3.5 Purification of glyoxalase enzymes

After recombinant production, the *E. coli* cells were harvested and resuspended in their various lysis buffers containing lysozyme and DNase and supplemented with PMSF, cystatin and pepstatin as protease inhibitors. The cells were broken by ultrasonic treatment, extracts centrifuged at 17000 rpm for 30 minutes and all proteins of interest (glyoxalases) were purified to homogeneity from the supernatants using *S*-hexylglutathione and Ni-NTA affinity matrices. *S*-hexylglutathione sepharose columns are widely used in the purification of glutathione-related enzymes such as glyoxalases, GSTs, and thiol transferases. Table 3 indicates the purification procedure used for particular proteins as well as the yield obtained per litre of overexpression medium. Purity and concentration of purified proteins were determined by Coomassie blue staining after SDS–PAGE and the Bradford assay (Bio-Rad) or A_{280} method.

Table 2: Cloning and overexpression of human and *P. falciparum* glyoxalase genes. Restriction endonuclease sites added to the primer sequences are underlined

Gene / Accession numbers	**Primer Sequence** 1. Sense 2. Antisense	**Restriction site**	**PCR**	**Expression vector**	***E. coli* strain**	**Overexpression**
cGloI AF486284	Iozef *et al.*, 2003	Iozef *et al.*, 2003	Iozef *et al.*, 2003	pQE30	M15	Overnight at 30°C after induction with 0.25 mM IPTG at OD_{600} = 0.3 using LB medium containing 8 mM $MgSO_4$, 1 mM $ZnSO_4$ and 10 mM Tris, pH 7.5
GILP NP_703709	1. CGCGGGATCCAAACTTTTTGTAGTCGTTATATTTGTTC 2. GCGCAAGCTTTTATTTGTCTTTTAAGTAAACATTATATCCG	*Bam*HI *Hin*dIII	94°C, 30´´ 65°C, 45´´ 72°C, 60´´ 25 cycles	pQE30	M15 BL21	Overnight at 24 – 30°C after induction with 0.25 mM IPTG at OD_{600} = 0.7 – 0.8 using LB medium containing 8 mM $MgSO_4$ and 10 mM Tris, pH 7.5
tGloII AF486285	1. CGCGGGATCCGCACAAGAAATATCAAATTTAG 2. GCGCAAGCTTTTATGAGGCTTTAAAATTATCC	*Bam*HI *Hin*dIII	94°C, 30´´ 50°C, 45´´ 72°C, 90´´ 35 cycles	pQE30	M15	6 hours at 37°C after induction with 1 mM IPTG at OD_{600} = 0.7 using LB medium containing 8 mM $MgSO_4$ and 10 mM Tris, pH 7.5
cGloII AY494055	1. CGCGGGATCCAAGCCATGCGCACAAGTACTTGTAGTGC 2. CGCGGAGCTCTTAAAAATTATTTTTTAATTGTCTTAATTTAT	*Bam*HI *Sac*I	94°C, 30´´ 60°C, 30´´ 72°C, 90´´ 35 cycles	pQE30	XL-I Blue	4 hours at 37°C after induction with 1 mM IPTG at OD_{600} = 0.5 using LB medium
human GloI NP_006699	1. CGCGGGATCCAAGCCATGCGCACAAGTACTTGTAGTGC 2. CGCGAAGCTTCTACATTAAGGTTGCCATTTTG	*Bam*HI *Hin*dIII	94°C, 30´´ 64°C, 30´´ 72°C, 45´´ 35 cycles	pQE30	BL21 (DE3)	Overnight at 30°C after induction with 0.25 mM IPTG at OD_{600} = 0.3 using ZY medium containing 1 mM $ZnSO_4$
human GloII CAA62483	Ridderström *et al.*, 1996	Ridderström *et al.*, 1996	Ridderström *et al.*, 1996	pKKD	JM109	Overnight at 37°C after induction with 0.25 mM IPTG at OD_{600} = 0.3 using ZY medium

Table 3: Purification of *P. falciparum* and human glyoxalases

Enzyme	First Purification	Second Purification	Yield/l culture
cGloI	*S*-hexylglutathione column in 10 mM Tris-HCl, pH 7.8; elution with 5 mM *S*-hexylglutathione	Ni-NTA column; elution with 100-200 mM imidazol	3 mg
GILP	Ni-NTA column; elution with 50-75 mM imidazol	Not necessary	5 mg
tGloII	*S*-hexylglutathione column in 10 mM Tris, pH 7.8; elution with 3 M NaCl	Ni-NTA column; elution with 50 mM imidazol	0.2 mg
cGloII	Ni-NTA column; elution with 30-200 mM imidazol	Not necessary	5 mg
human GloI	*S*-hexylglutathione column in 10 mM Tris-HCl, pH 7.8; elution with 5 mM S-hexylglutathione	Ni-NTA column; elution with 30-50 mM imidazol	0.5 mg
human GloII	*S*-hexylglutathione column in 10 mM MOPS, pH 7.2; elution with 3 M NaCl	Not necessary	2 mg

Washing steps on Ni-NTA columns were carried out with 50 mM sodium phosphate buffer, pH 8.0 containing 300 mM NaCl. Imidazol concentrations used during elution were diluted in the same buffer. Washing and elution steps of all GloIs on *S*-hexylglutathione columns contained 200 mM NaCl.

Table 4: Optimization of the expression conditions for PfLipDH1 and PfLipDH2

Gene	Expression Vector and *E. coli* strain	Protein yield after purification
PfLipDH1 **Forward primer** CGCGGAGCTCAACAGCGT TATTTTTAGAGCAC **Reverse primer** GCGCAAGCTTTTACATGT GTATAGGTTTATCAAAAG **PCR** 94°C, 30´´ 57°C, 30´´ 72°C, 90´´ 35 cycles	pQE PfLipDH1 in M15 cells plus pRIG	PfLipDH1 obtained with about 30% purity
	pQE PfLipDH1 in XL1 Blue / Nova Blue cells plus pRIG	Large amounts of PfLipDH1 obtained in inclusion bodies
	pQE PfLipDH1 in JRG 1342 cells plus pRIG	No PfLipDH1 obtained
	pQE PfLipDH1 in BL21 cells plus pRIG	No PfLipDH1 obtained
	pRSETPfLipDH1 in M15 cells plus pRIG	No PfLipDH1 obtained
PfLipDH2 PCR conditions (AG Becker-Brandenburg)	pQE PfLipDH2 in M15 cells plus pRIG	No PfLipDH2 obtained
	pQE PfLipDH2 in XL1 Blue cells plus pRIG	No PfLipDH2 obtained

In all the above-stated overexpression systems, the following parameters were varied; time allowed for overexpression (from 4 hours to overnight overexpressions), overexpression temperature (from 25°C to 37°C), and amount of IPTG used for induction of gene expression (from 0.2 mM to 1 mM).

3.6 SDS-PAGE

The discontinuous buffer system for running SDS-PAGE was performed according to Laemmli (1970). This method separates denatured and reduced proteins according to their size and gives an indication of the purity of elution fractions after purification of proteins.

The proteins are initially mixed in a volume ratio of 1:2 with sample buffer and heated at 95°C for 5 minutes to denature the proteins. The proteins are then loaded into a pre-cast two-part polymerized acrylamide gel. The first part consists of a stacking gel (Tris-Glycine-Buffer pH 6.8, 4% acrylamide/bisacrylamide, 1% SDS) into which the proteins are loaded. The second part consists of a separation gel (Tris-Glycine-Buffer pH 8.8, 10-15% acrylamide/bisacrylamide, 1% SDS) on which the proteins are separated. Polymerization of the acrylamide in both the stacking and the separation gels is catalyzed by TEMED and initiated by APS. The amount of acrylamide/bisacrylamide on the gels influences the pore size and elasticity of the gel. The gels are run in an electrophoresis tank containing electrophoresis buffer at a voltage of 200V.

After the separation process, the gel was either stained with Coomassie and further destained in Coomassie destaining solution or used directly for western blotting.

3.7 Western blotting

The semi-dry western blot method was performed according to Towbin *et al.* (1979). This method involves the transfer of proteins from gels into nitrocellulose membranes. It enables the immunological identification of proteins on nitrocellulose membranes by specific antibodies to the proteins *e.g.* the identification of a 6–histidine tag at the N-termini of proteins by anti-histidine antibodies.

Filter papers, gels and nitrocellulose membranes previously soaked in different appropriate buffers are aligned in between the graphite plates of the anode and cathode. The arrangement of the whole assembly is as follows: graphite cathode followed by 5 filter papers and the polyacrylamide gel soaked in cathode buffer, followed by the membrane and 2 filter papers soaked in anode buffer 2, followed by 3 filter papers soaked in anode buffer 1 and finally, the graphite anode. The transfer process is carried out at 0.8 mA/cm^2 of gel for 55 minutes.

To view the efficiency of the transfer process, the nitrocellulose membrane is stained with Ponceau staining solution (for 30 seconds) and immediately de-stained with 1% acetic acid until the protein bands become invisible. Non-protein-bound sites on the membrane are

blocked by incubation of the membrane in TBS-buffer containing 5% milk powder overnight at 4°C or for 1 hour with gentle shaking at room temperature. Then the membrane is incubated in TBS-buffer containing a dilution of the primary antibody (e.g. mouse anti-histidine IgGs) overnight or for 1 hour with gentle shaking at room temperature. The primary antibody is washed off by two-times incubation in TBST for 10 minutes followed by another wash in TBS for 10 minutes. The membrane is then incubated in a dilution of the secondary antibody (e.g. rabbit anti-mouse immunoglobulins) solution in TBS buffer. The secondary antibody should be able to recognize the primary antibody. After another washing step, immunostaining of the membrane is carried out with an enhanced chemiluminescence (ECL)-Kit in a dark room. The membrane is incubated with an equal mixture of detection solution 1 & 2, after which it is wrapped in a plastic foil and exposed to an X-ray film for periods of about 30 seconds to 10 minutes. The exposure time is dependent on the strength of the signals obtained.

3.8 Concentration of proteins

To increase the protein concentrations of pure protein solutions, the proteins were forced through centriprep and microcon columns (YM 3, 10, or 30 – Amicon, Beverly, USA) which contain membranes with pore sizes that do not allow flowthrough of the protein of interest. The process is carried out by centrifugation at 3700 rpm and 4°C until a desired volume or a minimal volume of 600 µl and 270 µl for the centriprep and microcon columns, respectively, is attained. The amount of protein in the concentrate is then measured.

3.9 Determination of protein concentrations

Two methods were employed.

3.9.1 A_{280} method

This method depends on the absorption of light by aromatic amino acid side chains of proteins such as tyrosin and tryptophan at 280 nm. The specific molar extinction coefficient ε is obtained through online tools (Justbio, ProtCalc; www.justbio.com) and by taking into account the absorption of specific prosthetic groups (e.g. FAD).

The protein concentration is calculated with the following formula.

$$[\text{Pr otein}]\ \text{in mM} = \frac{A_{280}}{\varepsilon\ (\text{cm}^{-1}\text{mM}^{-1}) \cdot \text{d (cm)}}$$

whereby ε= molar extinction coefficient at λ of 280 nm, d= distance moved by the light in a cuvette = 1 cm

3.9.2 Bradford method

The Bradford method is based on the detection of a blue colouration that results after mixing a protein sample with Coomassie brilliant blue R-250. The blue colouration is proportional to the amount of protein in the sample. By determining the optical density (OD) at a wavelength of 595 nm and by extrapolation of this OD_{595} on a standard linear graph, the concentration of the protein can be determined. The standard linear graph is obtained by plotting known concentrations of BSA against their corresponding OD_{595}s after treatment with the Coomassie reagent. The process is carried out according to the manufacturer's (BIORAD) instructions.

3.10 Enzymatic assays

Enzyme activity is determined by measuring either the rate of disappearance of substrate or the rate of appearance of a product.

GloI activity was determined by the rate of formation of the thiol ester S-D-lactoylglutathione (from the methylglyoxal(MGO)-glutathione(GSH) hemithioacetal) at 240 nm, the extinction coefficient ε being 3.37 mM^{-1} cm^{-1}(Ridderström and Mannervik, 1996b). The GloI standard assay mixture was defined as follows: 100 mM potassium phosphate (with 100 mM KCl for cGloI), pH 7.0, 0.01-0.5 mM of the hemithioacetal (MGO-GSH) were incubated for 5 minutes and the reaction was started by adding GloI. The total assay volume was 1 ml. For a desired concentration of MGO-GSH, the required concentrations of MGO and glutathione (GSH) were calculated from the equations below. Excess free GSH in the assay was 0.1 mM and the dissociation coefficient for MGO-GSH was 3 mM.

One unit of GloI catalyzes the formation of 1 µmol of S-D-lactoylglutathione per minute. Specific activity and k_{cat} values were calculated according to the Michaelis-Menten equation and derived from Lineweaver-Burk plots, respectively. Kinetic measurements were carried

out at 30°C to compare values obtained with those previously reported for human GloI (Ridderström and Mannervik, 1996b).

$$K_{d(MGO-GSH)} = \frac{[MGO]\cdot[GSH]}{[MGO-GSH]} = 3\,mM$$

$$[MGO_{total}]\,in\ assay = ([MGO]+[MGO-GSH])\,mM$$

$$[GSH_{total}]\,in\ assay = (0.1+[MGO-GSH])\,mM$$

For measuring GloII activity, the decrease in absorbance resulting from S-D-lactoylglutathione ($\varepsilon_{240\ nm}$ = 3.1 $mM^{-1}cm^{-1}$) hydrolysis was measured at 25°C in 100 mM MOPS buffer, pH 7.2 in a total volume of 1 ml. S-D-lactoylglutathione concentrations varied from 0.05 mM to 0.5 mM; higher concentrations of S-D-lactoylglutathione were limited by its high absorbance at 240. The reaction was started by addition of GloII. One unit of GloII catalyses the hydrolysis of 1 µmol of S-D-lactoylglutathione per minute. K_m and k_{cat} values of human GloII for S-D-lactoylglutathione had previously been reported (Ridderström *et al.*, 1996) using the GloII DTNB [5,5'-dithiobis(2-nitrobenzoate)] assay. The release of 5-thio-2-nitrobenzoate from the reduction of DTNB by GSH (product of the GloII reaction) is monitored spectrophotometrically at 412 nm ($\varepsilon_{412\ nm}$ = 13.6 $mM^{-1}\ cm^{-1}$). Both cGloII and human GloII were tested in the DTNB assay with S-D-lactoylglutathione concentrations ranging from 20 to 1800 µM at 37°C.

TrxR was assayed in a total volume of 500 µl in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, with 100 µM NADPH and appr. 10 mU/ml TrxR. The reaction at 25 °C was monitored by the decrease in absorbance at 340 nm and was started without preincubation (< 30 sec.) with 20 µM PfTrx.

The activity of LipDH was normally assayed in 47 mM potassium phosphate buffer, pH 6.9 containing 200 mM KCl and 1 mM EDTA by monitoring at 340 nm the decrease in NADH concentration (ε being 6.22 $mM^{-1}\ cm^{-1}$) upon transfer of reducing equivalents to lipoamide. However, due to the photoreactive effects of MB with EDTA, assays in which the stimulatory/inhibitory effects of MB on PfLipDH1 were tested, were carried out simply in 50 mM potassium phosphate buffer pH 7.4. All assays were carried out at 25°C in the presence of either 100 µM NADH (for normal assays) or 200 µM NADH (for MB-related assays)

Activity of all the enzymes was determined according to the following equation.

$$\text{Volume activity V in U/ml} = \frac{\Delta A/\text{min} \cdot V_o}{\varepsilon(\text{cm}^{-1}\text{mM}^{-1}) \cdot v_i}$$

whereby $\Delta A/\text{min}$ = change in absorbance per minute, V_o = total volume of assay, ε = extinction coefficient and V_i = volume of enzyme solution in assay

3.11 Inhibition studies

The S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones were tested as inhibitors of *P. falciparum* GloI and GloII. GloI inhibition was studied at 25°C in the assay system described above. GloII inhibition by S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones was studied in the standard GloII assay. Three inhibitor derivatives were tested which differ in the nature of their aryl group, namely S-(*N*-hydroxy-*N*-phenylcarbamoyl)glutathione (HPC-GSH), S-(*N*-hydroxy-*N*-chlorophenylcarbamoyl)glutathione (HCPC-GSH) and S-(*N*-hydroxy-*N*-bromophenylcarbamoyl)glutathione (HBPC-GSH) (Figure 10; Murthy *et al.*, 1994). *P. falciparum* Glos were directly compared with their human counterparts. S-p-bromobenzylglutathione, a GloI inhibitor whose diethyl ester prodrug was previously reported to inhibit the growth of *P. falciparum* in culture with IC_{50} values approximating 5 µM (Thornalley *et al.*, 1994), was also tested as cGloI inhibitor. The DTNB assay described above was employed for testing weak glyoxalase II inhibitors (namely; *S*-(p-azidophenacyl)glutathione, *S*-propylglutathione and *S*-hexylglutathione) which showed high absorbance at 240 nm.

Eleven novel TrxR inhibitors were obtained from Professor Charles Williams (Ann Arbor, USA) within the frame of a scientific collaboration. All 11 inhibitors were initially assessed in direct comparison on *P. falciparum* and human TrxR by Christine Nickel and based on a selective inhibition of PfTrxR criteria – as determined by IC_{50}-values under standardized conditions – three were chosen for further characterization of their mode of inhibition and influence on *P. falciparum* cultures. These three inhibitors were 4-nitro-2,1,3-benzothiadiazole (compound **1**), 6,7-nitroquinoxaline (compound **2**) and bis-(2,4-dinitrophenyl) sulfide (compound **3**). For characterization of the inhibition type, varying amounts of inhibitor and substrates (PfTrx and NADPH) were employed.

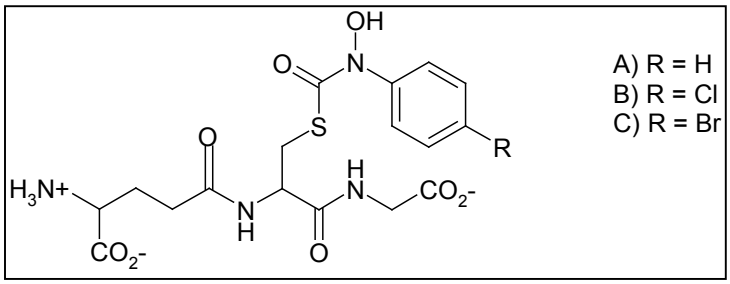


Figure 10: **Structure of the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones**
A). S-(*N*-hydroxy-*N*-phenylcarbamoyl)glutathione (HPC-GSH), B). S-(*N*-hydroxy-*N*-chlorophenylcarbamoyl)glutathione (HCPC-GSH) and C). S-(*N*-hydroxy-*N*-bromophenyl carbamoyl)glutathione (HBPC-GSH)

3.12 Glutathionylation assays

In order to induce glutathionylation, fully reduced (to ensure availability of free cysteine-SH groups) PfTrx was incubated at a concentration of 10 µM with 5 mM GSSG at room temperature for 2 hours or at 4°C for a couple of days. After the incubation period, control samples without GSSG as well as GSSG-treated samples were further treated with 2 mM iodoacetamide in order to block residual cysteine-SH groups. Identification of glutathione on PfTrx was carried out either by western-blotting using anti-glutathione antibodies or by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) after tryptic digestion of the PfTrx samples by Sasa Koncarevic.

3.13 Metal ion analysis

Zinc, iron and nickel contents of all *P. falciparum* glyoxalases were determined by atom absorption spectroscopy (Dr. V. Muntean, Seelig Analytical Laboratories, Karlsruhe, Germany). The protein samples were exhaustively dialysed against a 4 mM potassium phosphate buffer, pH 7.0, which also served as a blank in these experiments.

3.14 Structure prediction of glyoxalase enzymes

Models of cGloI, tGloII, and cGloII are based on the crystal structures of homodimeric human glyoxalase I in complex with HIPC-GSH (Cameron *et al.*, 1999a) and human glyoxalase II (Cameron *et al.*, 1999b), respectively (Protein Data Bank accession numbers 1qin and 1qh5, respectively). Alignments were optimized manually in the Swiss-PDB Viewer (spdbv). Computations of the models were carried out at the Swiss-Model server

(Guez and Peitsch, 1997; Schwede *et al.*, 2003), and force field energies of the models were calculated with the GROMOS96 implementation of spdbv.

3.15 Protein crystallization

3.15.1 Crystallization screening

The goal of the crystallization experiments was to produce crystals of the glyoxalase enzymes that are suitable for X-ray diffraction analysis. This diffraction pattern can then be analyzed to discern the protein's three-dimensional structure. As such, highly concentrated protein samples (minimum of 8 mg/ml) with purity of at least 95% are required. Based on the yields obtained after protein purification, crystallization trials were carried out for cGloI, GILP and cGloII. tGloII was very unstable and only small amounts could be obtained after purification, so crystallization trials could not be carried out. PfLipDH1 and PfLipDH2 could likewise not be set for crystallization due to the small quantities obtained in overexpressions.

In trials to develop protein crystals of the glyoxalase enzymes, the hanging drop method was used. The hanging drop method relies on the vaporisation of water or a volatile agent between a micro-drop of mother liquor and much larger and highly-concentrated reservoir solution, thereby causing a slow precipitation of the protein within the drop. Ideally, individual protein molecules align themselves in a repeating series of "unit cells" by adopting a consistent orientation. In the hanging drop method, drops are prepared on siliconized microscope glass cover slips by mixing 2 µl of protein solution with the same volume of precipitant solution. The cover slip is placed over a small well containing 800 µl of the precipitating solution (Figure 11). By use of a crystallization kit (Crystal screen I and II, Hampton Research), up to 100 different solutions with variations in pH, ionic strength, metallic content, and buffer systems were tested initially on each of the glyoxalases. The proteins were previously dissolved in 50 mM sodium phosphate buffer pH 8.0, containing 300 mM NaCl. The proteins were kept under different temperatures (4°C, 15°C, 24°C or 30°C) after being set as shown below for at least one week to allow for the development of the crystals.

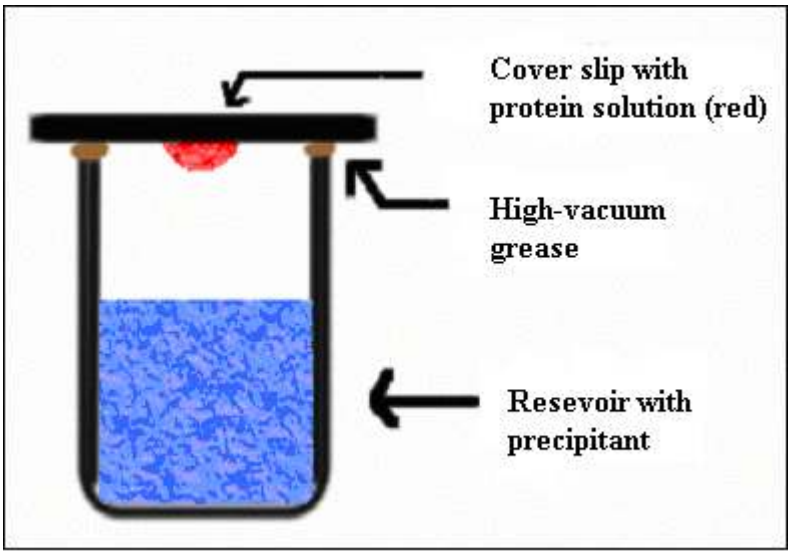


Figure 11: **Diagram of hanging drop method.**
Reservoir solution (blue) usually contains buffer and precipitant. Protein solution (red) contains the same compounds, but in lower concentrations.

3.15.2 Crystallization optimization

Optimal conditions for crystallization of a novel protein are difficult to predict. Every macromolecule is unique in its physical and chemical properties and its three-dimensional structure with distinctive surface characteristics. The variables influencing crystal growth are too many to allow an exhaustive search.

Optimization for the development of crystals generally involved the addition of additives (e.g. β-octylgalactoside, maltoside) in the protein drop of the coverslide. In order to prevent the development of salt crystals, the glyoxalase proteins were dissolved in 50 mM sodium phosphate buffer containing 100 mM NaCl instead of 300 mM NaCl. By use of the buffer system and conditions (25-30% w/v PEG 2000 monomethyl ether in 50 mM MES pH 5.8 containing 100 mM NaCl; 15°C) under which crystals were obtained for the human GloI (Cameron *et al.*, 1999a), cGloI and GILP were optimized for crystal development. Several variations of this buffer system included bidirectional changes in the pH, concentration of PEG 2000 monomethyl ether and MES, and temperature.

3.16 Cell culture experiments

Intraerythrocytic stages of *P. falciparum* strains were cultured according to Trager and Jensen (1976) with slight modifications. Typically, parasites were propagated in leukocyte-free erythrocytes (A^+) at 3.3 % haematocrit at 37°C under reduced oxygen (typically 3% O_2, 3% CO_2, 95% N_2) in tissue culture (RPMI 1640 – Gibco) medium containing 4% human serum (A^+) and 0.2% Albumax (lipid-rich bovine serum albumin). Albumax appears to reduce both the rate at which erythrocytes deteriorate *in vitro* and pH drift when cultures are exposed to ambient air. The media were further supplemented with 9 mM (0.16%) glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, and 22 µg/ml gentamycin. CQ sensitive (3D7 and HB3) and resistant (K1 and Dd2) strains of *P. falciparum* (Su *et al.*, 1997) were grown in continuous culture according to the needs of each experiment. Characteristics of the *P. falciparum* strains employed in this study are shown in Table 5. Day to day monitoring of parasite growth was carried out by observation of parasitaemia on Giemsa-stained thin-blood films. Depending on the strain, parasites typically propagate 3-8 fold every 48 hours, thus care was taken to avoid parasite cultures attaining too high parasitaemia for adequate growth (3-10%). Percentage parasitaemia used in experiments also varied according to the experiment.

Table 5: Characteristics of *Plasmodium falciparum* strains employed (Fidock *et al.*, 2004; Su *et al.*, 1997; Akoachere *et al.*, in press).

Name	Clone	Drug resistance	Multiplication rate	Chloroquine IC_{50} value in nM
3D7	Yes (from NF54)	-	4-5	8.6 ± 0.4
HB3	Yes	PYR	6	16.8 ± 0.5
Dd2	Yes (from WR'82)	CQ, QN, PYR, SDX	5-6	90.2 ± 10.6
K1	No	CQ, PYR	4-5	155 ± 11.4

CQ, chloroquine; QN, quinine; PYR, pyrimethamine; SDX, sulfadoxine.

3.16.1 Synchronization

Synchronisation of parasites in culture to ring stages was carried out by treatment with 5% (w/v) sorbitol (Lambros and Vanderberg, 1979) which warrants a predominantly ring phase culture of about 10% parasitaemia. Five millilitres of 5% sterile sorbitol preheated to

37°C was added to 0.5 ml parasitized erythrocytic pellet from culture and left for 5 minutes at 37°C. The sorbitol mixture was then centrifuged at 1900 rpm for 3 minutes at room temperature to spin down the erythrocytes. The sorbitol was then off-aspirated and the pellet rapidly resuspended in complete medium for a quick wash after which the pellet was returned to culture. If the culture was not sufficiently synchronised, the procedure was repeated after 48 hours when the culture was in the ring form again.

3.16.2 Studies on MB uptake

Young *P. falciparum* trophozoites were incubated for 12 h with 13 nM, 26 nM, or 39 nM MB. Non-parasitized red blood cells (NPRBC) were treated identically. After 12 h, cell cultures were centrifuged at 750 × *g* for 3 minutes at 25°C. The supernatants representing the cell culture medium were immediately frozen. In parasitized red blood cells (PRBC), erythrocytic and parasitophorous vacuolar membranes were lysed by treatment with 0.02% w/v saponin for 10 minutes at 37°C. After another centrifugation step the supernatants were stored at -80°C. The parasites in the pellet were washed three times in saponin buffer and disrupted by three times freezing and thawing in liquid nitrogen followed by sonication on ice. After centrifugation (31000 × *g*, 30 min, 4°C) parasite lysate was obtained in the supernatant and parasite debris in the pellet. NPRBC were washed twice in phosphate buffered saline (PBS) before being disrupted by freezing and thawing. MB was determined in various compartments by high performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) (Rengelshausen *et al.*, 2004). In brief, *in vitro* samples (up to 100 µl of incubation media, cell suspensions, or parasite lysates) were processed by adding 200 µl of precipitation reagent (acetonitrile), vortex-mixing for 15 s, and centrifugation for 10 min at 2500 × *g*. The clear supernatants were transferred into autosampler vials for LC/MS/MS analysis.

3.16.3 Determination of enzymatic activity from parasitic extracts

In order to test the effects of Glo inhibitors on Glo activity in the parasites, *P. falciparum* cultures with 15% parasitaemia and 3.3% haematocrit were treated for 8 hours with 10 µM (2 x IC_{50}) S-p-bromobenzylglutathione cyclopentyldiester. Then the parasites were isolated by suspending the erythrocytes in a 20-fold volume of saponin buffer containing 7 mM K_2HPO_4, 1 mM NaH_2PO_4, 11 mM $NaHCO_3$, 58 mM KCl, 56 mM NaCl, 1 mM $MgCl_2$, 14 mM glucose, and 0.02% saponin (pH 7.5 at 25°C) for 10 minutes at 37°C. Following centrifugation (2700 rpm, 3 min, 25°C) the pellets were washed three times at 25°C with a

20-fold volume of buffer. The parasites were disrupted by four times freezing in liquid nitrogen and thawing, followed by sonication for 2 x 10 seconds. After a cleaning spin (50000 rpm, 60 min, 4°C), protein content of the parasite extracts was determined using the BioRad (München, Germany) protein dye assay with bovine serum albumin serving as a standard (Bradford, 1976). GloI and GloII activities were determined in the same extracts.

3.16.4 Drug susceptibility tests

3.16.4.1 WHO microtest

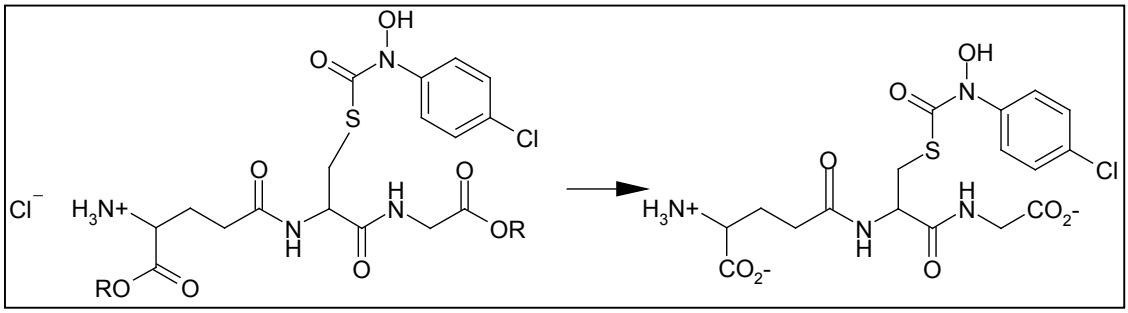
The microtest was developed by Rieckman *et al.* (1978) and adopted by WHO for epidemiologic monitoring of drug-resistant malaria, requires a parasite count under the microscope to measure the capacity of antimalarial drugs to inhibit parasite maturation to the schizont stage. The assay was modified such that the ability of the drug to inhibit growth of the parasites after a complete cycle of 48 hours (rings to rings) was measured.

In a 24 well culture plate containing parasite culture at 1% parasitaemia and 3.3% haematocrit in each well, the drug of interest is applied in duplicates at various concentrations. Control wells with no drug or no drug plus necessary vehicule (DMSO or 100% ethanol) are also reserved on the same culture plate. The parasites are exposed to the drug for a considerable amount of time depending on the experiment after which the medium is replaced with fresh medium and allowed to grow for another 24 hours to allow completion of a full erythrocytic cycle of the parasites. Thin-filmed smears on glass slides are then prepared from the culture material of each well. The smears are then methanol fixed and then stained in freshly prepared Giemsa solution. The respective percentage parasitaemia is counted under a light microscope and the difference in percentage parasitaemia as compared to that of untreated slides, is considered proportional to the effect of growth inhibition of the parasites by the drug. The counted parasitaemia is plotted against the corresponding drug concentration on a logarithmic dose response curve and the IC_{50} value (drug concentration that inhibits 50% of the parasite growth in comparison with drug-free controls) of the drug is extrapolated from the curve.

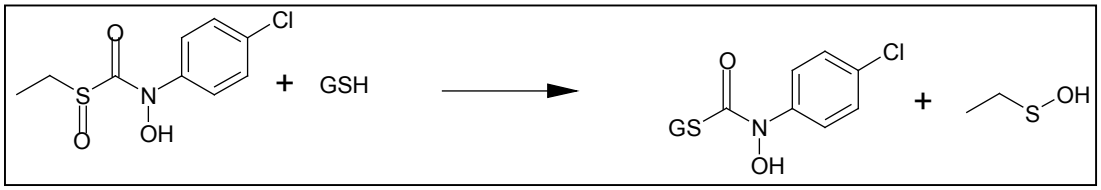
3.16.4.1.1 Effects of the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones – Glo inhibitors - in *P. falciparum* cultures

Intraerythrocytic stages of the chloroquine resistant *P. falciparum* strains K1 and Dd2 were applied in the above described WHO microtest as follows. Sorbitol-synchronised parasites

in the ring stage were used for testing the effects of two different pro-drug forms of HCPC-GSH, namely the HCPC-GSH diethylester and the S-(*N*-hydroxy-*N*-chlorophenyl carbamoyl)sulfoxide on *P. falciparum* in culture. It is worth noting that potent inhibitors of glyoxalases are usually GSH derivatives which as a result of the diacid nature of GSH are not membrane-permeable; therefore pro-drug derivatives are being used. The dialkylester pro-drug of the inhibitor depends on the presence of unspecific intracellular esterases to generate the active drugs within the cell (Lo and Thornalley, 1992; Kavarana *et al.*, 1999) as indicated below.



The alkylsulfoxide depends on an acyl interchange with GSH within the cell (Hamilton *et al.*, 1999).



Synchronised parasites (1.5% parasitaemia, 3.3% haematocrit, 500 µl total volume) were exposed for 24 hours to 0.1 µM -100 µM prodrug. The parasites were then incubated in inhibitor-free medium for another 24 hours to complete the *P. falciparum* life cycle of 48 hours. Growth inhibition was quantified by counting parasitized red blood cells using Giemsa-stained slides of thin blood smears.

3.16.4.1.2 Testing of PfTrxR inhibitors compounds 1, 2 and 3, in cultures

The strain used was the chloroquine-resistant strain K1, which was synchronized at the ring stage. Parasitemia was adjusted to 1-1.5 % and hematocrit to 3.3 %. For each inhibitor, 12 wells with 495 µl and 2 with 500 µl of this culture were prepared. The

inhibitors were dissolved in DMSO *ad* 10 mM. This stock was stepwise diluted *ad* 10 µM, 1µM, 0.1 µM and 0.01 µM with DMSO. All inhibitor solutions were sterile filtered.
Of each solution 5 µl was added to an incubation well reaching 500 µl end volume, 1% DMSO and final inhibitor concentrations of 100, 10, 1, 0.1 and 0.01 µM, respectively. Every approach was prepared twice for each inhibitor. Also carried out twice were controls without any addition and with 1% DMSO, respectively.
Cultures were monitored after 24 and 48 h. Parasitemia was determined by counting Giemsa-stained parasites under the microscope.

3.16.4.1.3 Stage specificity of MB action

IC_{50} values for MB were determined on different synchronized blood stage forms (rings, trophozoites, and schizonts) of the *P. falciparum* strain K1. For this purpose, aliquots of a parasite culture synchronized to the ring stage (t = 0) were drawn every 3 to 6 h throughout the 48 h cycle and exposed to various MB concentrations. Considering the multiplication of the parasites towards the end of the cycle, the parasites to be treated at 39, 42, and 48 h, were diluted with A^+ erythrocytes by factors of 1:2, 1:4 and 1:7, respectively. Parasites were exposed to the drug for 6 h followed by a change of the medium and undisturbed growth for 42 h to complete a full cycle. Parasite growth and parasitaemia were monitored by assessing Giemsa-stained blood smears under the microscope.

3.16.4.2 Semi-automated microdilution method

Isotopic drug sensitivity assays by means of the semi-automated microdilution technique (Desjardins *et al.*, 1979) were employed to investigate the susceptibility of the malaria parasites to clinically-used antimalarials as well as TrxR and Glo inhibitors of particular interest to us which had shown anti-enzymatic effects in the nanomolar range. The method depends on the incorporation of radioactive ^{3}H-hypoxanthine – which is taken up by the parasite as a precursor of purine deoxynucleotides for DNA synthesis – and was performed according to the modifications of Fivelman *et al.* (2004).
In 96 well microtitre plates (Nunc^R), a two-fold serial dilution of the starting concentration of each drug to be tested (B1-B12) was carried out so that each well contained 100 µl of half the drug concentration of the above-preceding well (Figure 12). 100 µl of a 2 times stock of previously diluted parasites at a parasitaemia of 0.5% and a haematocrit 2.5% were added onto each well so that the parasites were finally at a parasitaemia of 0.5% (> 70% rings) and 1.25% haematocrit. These dilutions were carried out in hypoxanthine-free medium. Positive control wells contained parasitized red blood cells (PRBC) while the

negative control wells contained non-parasitized red blood cells (NPRBC). The starting concentration of each drug was chosen such that the IC_{50} value (drug concentration that produces 50% reduction in the uptake of ^{3}H-hypoxanthine) falls within the middle of the plate. The plate was then incubated as normal culture and after 48 hours, 50 µl (final concentration of 0.5 µCi/well) of [3H]-hypoxanthine was added per well and the plate was further incubated. After a further 24 hours, the plates were placed at -80°C for at least 1 hour to freeze the cells. Plates were then thawed, each well was harvested on a glass fibre filter (Perkin-Elmer, Rodgau-Jügesheim, Germany), dried, and radioactivity in counts per minute (cpm) from each well measured in a β-counter was considered to be proportional to the respective growth of *P. falciparum* in the well. For the experiments, *P. falciparum* strains with different CQ sensitivity (3D7, HB3, Dd2, and K1) were employed (Su *et al.*, 1997). Percentage reductions were used to plot percentage inhibition of growth as a function of drug concentrations. IC_{50} values were determined by linear regression analyses on the linear segments of the curves (IC_{90} values could also be determined by curve-fitting and could provide a useful measure of variation between experiments).

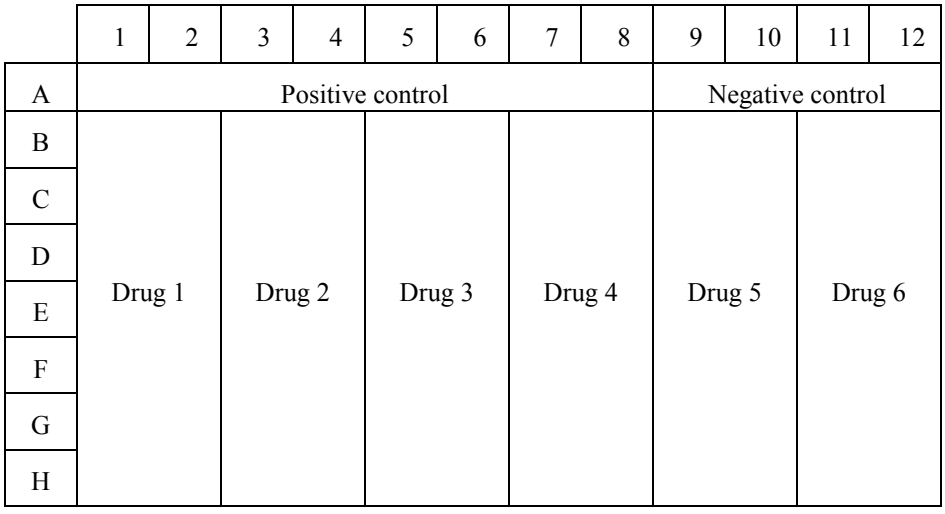


Figure 12: **Semi-automated microdilution method: Arrangement of drugs on a 96 well plate.**
A1-A8: positive control, red cells and parasites
A9-A12: negative control, red cells only (no parasites)
B1-H12: Serial drug dilutions for 6 compounds in duplicate. Arrow indicates direction of decreasing drug concentration

Drugs that were tested for parasitic susceptibility by this method included CQ, MB, artemisinin, artesunate, artemether, piperaquine, amodiaquine, quinine, mefloquine, primaquine, pyrimethamine, and the PfTrxR inhibitor, compound **1**. Assays were typically

repeated on two or three separate occasions. Within each experiment standard deviations were typically less than 10% of the mean. Differences in parasite stages could lead to up to two-fold shifts in the IC_{50} values between experiments; however, these differences rarely affected the overall relationships between the parasite strains with respect to their differences in drug response.

3.16.4.3 Drug combination assays

In order to assess the effects of combined drugs, isobologram analysis was performed to assess whether two drugs were additive, synergistic or antagonistic. This was mostly applied to test the effects of MB in combination with other clinically used antimalarials. The two drugs to be tested in combination were applied alone and in fixed concentration ratios of 1:1, 1:3, and 3:1 as described by Fivelman *et al.* (2004). A CQ control is applied in the position of the sixth drug as the IC_{50} values of CQ had already been standardized in the laboratory for all the strains of *P. falciparum*. This had the advantage that day to day variations in the IC values could be easily monitored. IC_{50} and IC_{90} values were calculated; the fractional inhibitory concentrations (FIC) of the respective drugs were determined (Fivelman *et al.*, 2004) based on the following definitions below (Ohrt *et al.*, 2002).

$$FIC_{50} = FIC_{50}A + FIC_{50}B \qquad FIC_{50}A = \frac{IC_{50}(A+B)}{IC_{50}A} \qquad FIC_{50}B = \frac{IC_{50}(B+A)}{IC_{50}B}$$

For these studies *P. falciparum* strains with different CQ sensitivity (3D7, HB3, Dd2, and K1) were employed (Su *et al.*, 1997).

Drug combination effects of the PfTrxR inhibitor compound **1** with CQ, MB and artemisinin were assessed using this method. Most importantly were the combinations involving MB and some clinically used antimalarials like CQ, artemisinin, artesunate, artemether, piperaquine, amodiaquine, quinine, mefloquine, primaquine and pyrimethamine. The above combinations were carried out on the CQ-resistant *P. falciparum* strain K1 in order to determine which drug combinations could be clinically useful in the treatment of resistant falciparum malaria. Drug combination assays of MB with the artemisinins were extended to other strains of *P. falciparum* with varying CQ sensitivies (Dd2, HB3 and 3D7).

3.16.5 Statistics

IC_{50} values were determined for each drug alone and for drugs in fixed concentration ratios by fitting a logistic dose-response equation to the concentration-response curves. IC_{50} and IC_{90} values were used to calculate 50% and 90% fractional inhibitory concentrations, respectively (FIC_{50} and FIC_{90}). Mean IC and FIC values obtained for the various drugs and corresponding parasite strains were used for statistical analyses (Ohrt *et al.*, 2002; Fivelman *et al.*, 2004). The unpaired Student's *t*-test was used to compare differences between IC_{50} values of MB obtained for CQ-sensitive and CQ-resistant strains. Pearson correlation was used to assess the correlation between IC values of MB and CQ as well as between MB and the artemisinins. Least square means (LSMeans) was used to compare differences in FICs obtained in the combination of MB with the artemisinins with respect to the three different artemisinins and the different combination ratios. Statistical Analysis Software (SAS) was used for statistical computations. For all statistical tests, the significance level (*P*) was set at 0.05.

4 Results

4.1 The glyoxalase system

4.1.1 Recombinant production of *P. falciparum* glyoxalases

Screening the *Plasmodium falciparum* genome sequencing database with different known GloI and GloII genes resulted in the identification of a second putative GloI gene from chromosome 6 and two putative GloII genes from chromosome 4 and chromosome 12, respectively. Designed perfect match primers were employed in PCR reactions (Table 2) using *P. falciparum* cDNA as template and the resulting products of expected size were cloned into the expression vector pQE30 and sequenced. The sequences were in full agreement with the respective sequences found in the genomic database.

The basic characterization of the cGloI gene product (see Figure 13 for sequence information referring to the 3-D model) had been reported previously (Iozef *et al.*, 2003). The GILP gene from chromosome 6 consists of 924 bp and its gene product of 307 amino acids contains an N-terminal apicoplast targeting sequence with high probability (>98%, PATS 1.2.1N, Zuegge *et al.*, 2001). High sequence identities were obtained with the respective proteins from other *Plasmodium* species (e.g. 67% identity with *P. yoelii*) but only low identities were found with proteins from other species, the highest value being 25% identity with *Arabidopsis thaliana.* The gene product (without His-tag, with targeting sequence) had a calculated molecular mass of 35.8 kDa, an isoelectric point of pH 8.59 and an $\varepsilon_{280\ nm}$ of 33.6 $mM^{-1}cm^{-1}$.

The tGloII and cGloII genes from chromosome 12 and 4 consist of 840 bp and 792 bp, respectively. Alignments of the amino acid sequences of *P. falciparum* glyoxalases II with those from other species showed identities of up to 32% (tGloII with *Gallus gallus* and *Cicer arietinum*) and 43% (cGloII with man and *Danio rerio*) (Figure 14). tGloII contains an N-terminal putative targeting sequence which was deleted in the cloning process to improve solubility and stability of the protein. As deduced from its DNA sequence, tGloII is composed of 322 amino acids; the N-terminally truncated active form of the protein has 267 amino acids with a molecular mass of 31.8 kDa and an isoelectric point of pH 7.25. cGloII is composed of 263 amino acids with a molecular weight of 30.5 kDa and an isoelectric point of pH 7.64. Extinction coefficients ε at 280 nM were 27.12 $mM^{-1}cm^{-1}$ and 17 $mM^{-1}cm^{-1}$ for tGloII and cGloII, respectively.

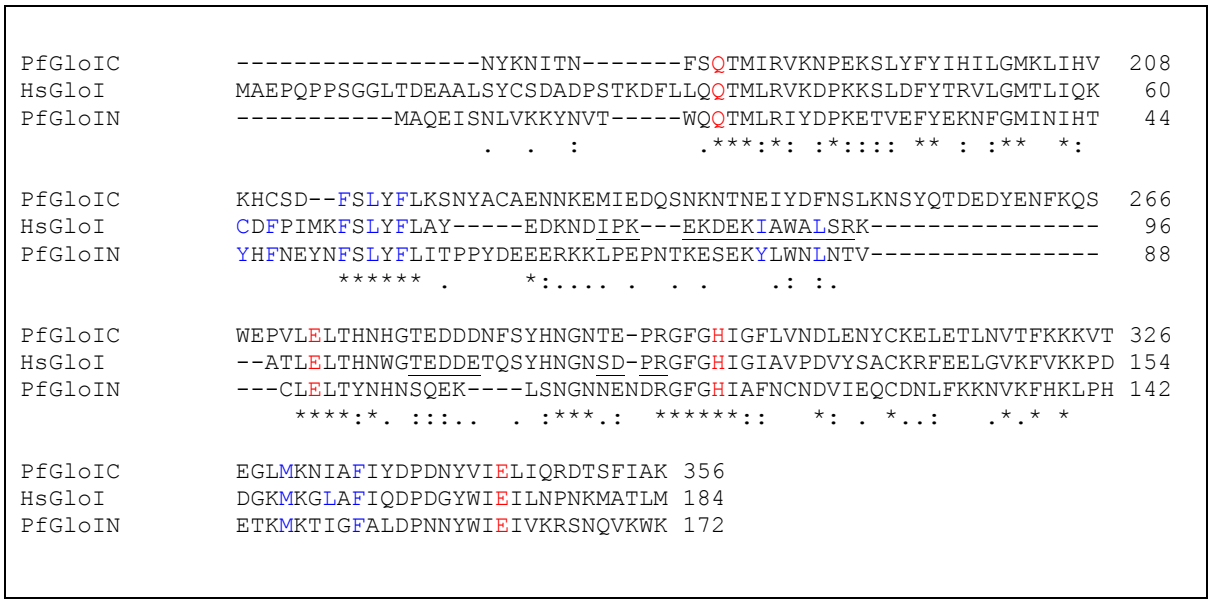


Figure 13: **Alignment of N- and C-terminal halves of *P. falciparum* glyoxalase I with human glyoxalase I.**
Residues involved in the formation of the active Zn^{2+} coordination site are indicated by red letters, while those involved in the formation of the hydrophobic binding pocket are shown in blue. Putative metal-defining inserts (Sukdeo *et al.*, 2004) are underlined.

Freshly transformed *E. coli* cells were employed for overexpression of the *P. falciparum* Glo genes (Table 2) and, as shown in Table 3, the hexahistidyl-tagged recombinant proteins were purified over Ni-NTA columns. cGloI and tGloII were pre-purified on an *S*-hexylglutathione agarose column. Yields and experimental details are shown in Table 3.

After overexpression in *E. coli* M15 cells, the gene product of GILP was insoluble. Three shorter variants of the GILP gene were then cloned and overexpressed in *E. coli* BL21 cells in order to remove the N-terminal apicoplast signal sequence from the gene products. Gene products of these shorter variants designated Δ20GILP, Δ23GILP and Δ43GILP lacked the first 20, 23 and 43 amino acids of the complete GILP sequence, respectively. These variants were soluble and could be purified to homogeneity on Ni-NTA columns with yields of about 5 mg per litre cell culture.

The theoretical molecular weights of the glyoxalase enzymes corresponded to those seen in SDS-PAGE (Figure 15).

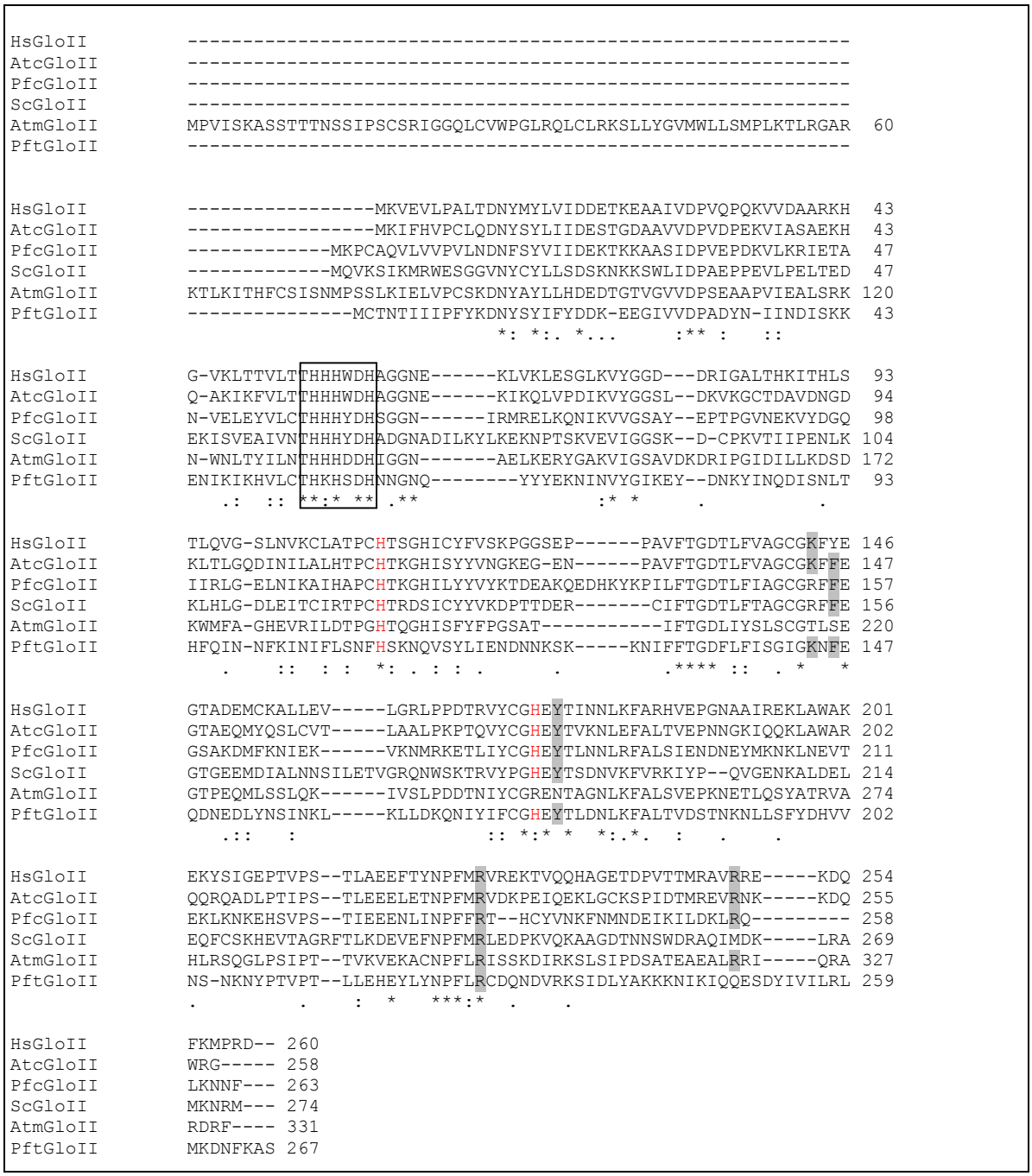


Figure 14: **Alignment of glyoxalases II.**
Residues involved in binding of the substrate S-lactoyl-glutathione as shown for the human enzyme (Cameron *et al.*, 1999b; Ridderström *et al.*, 2000) are shadowed; the active site motif THxHxDH is boxed; two other conserved histidines required for zinc binding at the active site are indicated by red letters. GenBank accession numbers are given in parentheses: HsGloII, human glyoxalase II (CAA62483); AtcGloII, cytoplasmic *Arabidopsis thaliana* glyoxalase II (NM_111922); PfcGloII, *Plasmodium falciparum* cytoplasmic glyoxalase II (AY494055, Akoachere *et al.*, 2005); ScGloII, S*accharomyces cerevisiae* (CAA71335); AtmGloII, mitochondrial *Arabidopsis thaliana* (NP_565999); PftGloII, *Plasmodium falciparum* targeted glyoxalase II (AF486285, Akoachere *et al.*, 2005); the sequence of an N-terminally truncated form (55 aa) is given in this alignment. This short form of the protein was approximately 3.5-fold more active than the full-length protein.

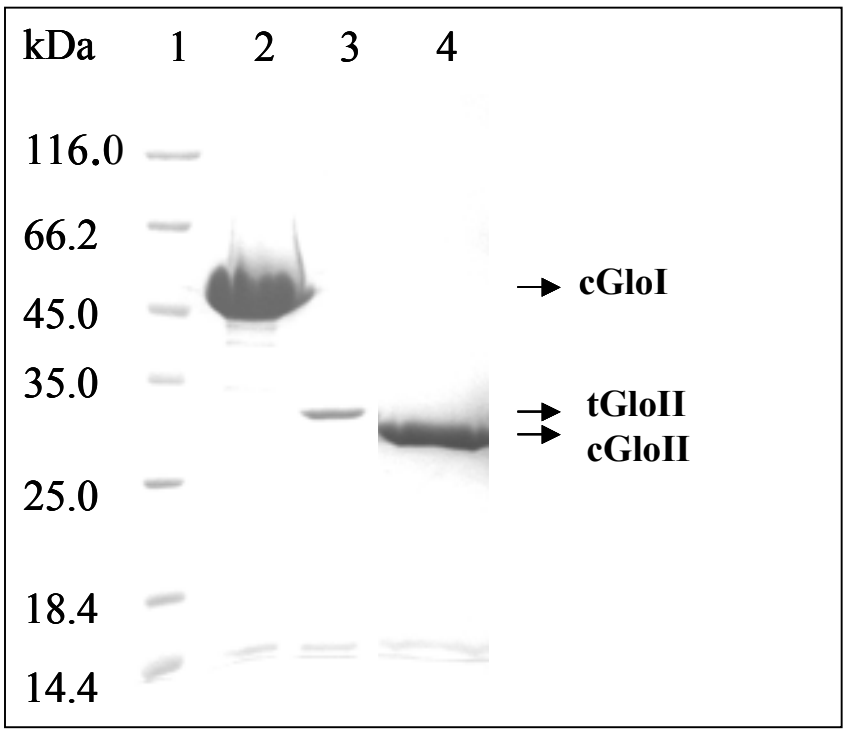


Figure 15: **SDS-PAGE of active recombinantly produced *P. falciparum* glyoxalases.**
Lane 1 – molecular weight markers, lane 2 – cGloI, lane 3 – tGloII and lane 4 – cGloII.

4.1.2 Kinetic characterization

The kinetic characterization of cGloI has previously been reported by Iozef *et al.,* 2003. As shown in the present study, addition of $ZnSO_4$ during the overexpression of cGloI resulted in increased catalytic activity and a decreased K_m value. In addition, the former measurements were carried out under conditions of excess MGO and the assumption that GSH would be readily converted to the MGO-GSH hemithioacetal (Vander Jagt *et al.*, 1972; Iozef *et al.*, 2003). In the present study, the substrate concentrations in each assay were calculated on the basis of the dissociation constant of the MGO-GSH adducts (Ridderström and Mannervik, 1996b). Differences in protein quality and the assay system chosen thus explain the kinetic values obtained here. In direct comparison with human GloI, cGloI showed higher k_{cat} and lower K_m values (Table 6).

Table 6: Kinetic properties of *P. falciparum* and human glyoxalases I

Glyoxalase I	K_m	k_{cat}	**Specific activity**	k_{cat}/K_m
cGloI	28 ± 3 µM	6637 min^{-1}	150 U/mg	4.0 x 10^6 $M^{-1}s^{-1}$
human GloI	90 ± 10 µM	2375 min^{-1}	110 U/mg	0.44 x 10^6 $M^{-1}s^{-1}$

All values were obtained at 30°C. The *P. falciparum* glyoxalase I-like protein (GILP) was not active under the conditions tested (see Results).

Different N-terminally truncated variants of GILP were successfully overexpressed in *E. coli* and purified. However, none of these variants showed GloI activity under the various conditions tested. Buffers tested were: i) 100 mM potassium phosphate, 0-300 mM KCl, pH 7.0, 6.6 or 6.0. ii) 100 mM Hepes, 1 mM EDTA, pH 7.4. iii) 50 mM Tris, pH 7.4. iv) 100 mM MOPS, 1 mM EDTA, pH 6.0 or 6.7. Addition of several metals ($ZnCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, $FeCl_2$, $FeCl_3$) in concentrations up to 100 µM or addition of 0.5 mg/ml BSA to stabilize GILP did not lead to detectable activity, either. The tested GloI substrates were the methylglyoxal-GSH and glyoxal-GSH hemithioacetal adducts from methylglyoxal and glyoxal, respectively.

Both *P. falciparum* glyoxalases II were capable of hydrolysing S-D-lactoylglutathione into D-lactate and GSH with a pH optimum of pH 7.6-7.8 for tGloII and 7.2-7.8 for cGloII. Their kinetic properties along with those of human GloII obtained in our laboratory are shown in Table 7.

Table 7: Kinetic properties of *P. falciparum* and human glyoxalases II

Glyoxalase II	K_m	k_{cat}	**Specific activity**	k_{cat}/K_m
tGloII	225 ± 25 µM	3860 min^{-1}	117 U/mg	0.29 x 10^6 $M^{-1}s^{-1}$
cGloII	100 ± 10 µM	7175 min^{-1}	226 U/mg	1.19 x 10^6 $M^{-1}s^{-1}$
human GloII	248 ± 25 µM	2782 min^{-1}	96 U/mg	0.19 x 10^6 $M^{-1}s^{-1}$

All values were obtained at 25°C. K_m values obtained at 37°C were 190 µM for cGloII, 208 µM for human GloII and 463 µM for the cGloIITyr185Phe mutant.

4.1.3 Inhibition studies

The inhibitory effects of S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathione derivatives on both *P. falciparum* and human glyoxalases are shown in Table 8. These derivatives, being hydrophobic transition state analogs of S-D-lactoylglutathione, showed K_i values on cGloI in the nanomolar range (see Figure 16A for HBPC-GSH) and on tGloII and cGloII in the micromolar range. When performing assays with these compounds as substrates in the absence of S-D-lactoylglutathione, no activity of cGloII could be observed at concentrations of up to 1 mM.

Table 8: IC_{50} and K_i values of S-(N-aryl-N-hydroxycarbamoyl)glutathiones on human and *P. falciparum* glyoxalases.

S-(*N*-hydroxy-*N*-arylcarbamoyl) glutathione derivative	**IC_{50} / K_i in µM**				
	cGloI	**human GloI**	**tGloII**	**cGloII**	**human GloII**
HPC-GSH	10 / ND	2.5 / ND	185 / ND	6 / 6	10 / 8
HCPC-GSH	0.11 / 0.08	0.09 / 0.05	30 / 50	2 / 0.7	1 / 0.7
HBPC-GSH	0.06 / 0.06	0.06 / 0.03	20 / 30	1.6 / 0.5	0.85 / 0.7-1

ND – Not determined; Values represent means of three independent determinations.

S-p-bromobenzylglutathione competitively inhibited cGloI with a K_i value of 20 µM (Figure 16B). Human GloI was inhibited much stronger, the K_i being 0.17 µM.

When tGloII was tested in inhibition assays with *S*-propylglutathione, *S*-hexylglutathione and *S*-(p-azidophenacyl)-glutathione, K_i values of 1.9 mM, 1.5 mM and 1.3 mM, respectively, were obtained. cGloII was even less sensitive to the compounds (data not shown). *S*-(p-azidophenacyl)-glutathione slightly activated the enzyme at concentrations of 0.2 mM to 1 mM.

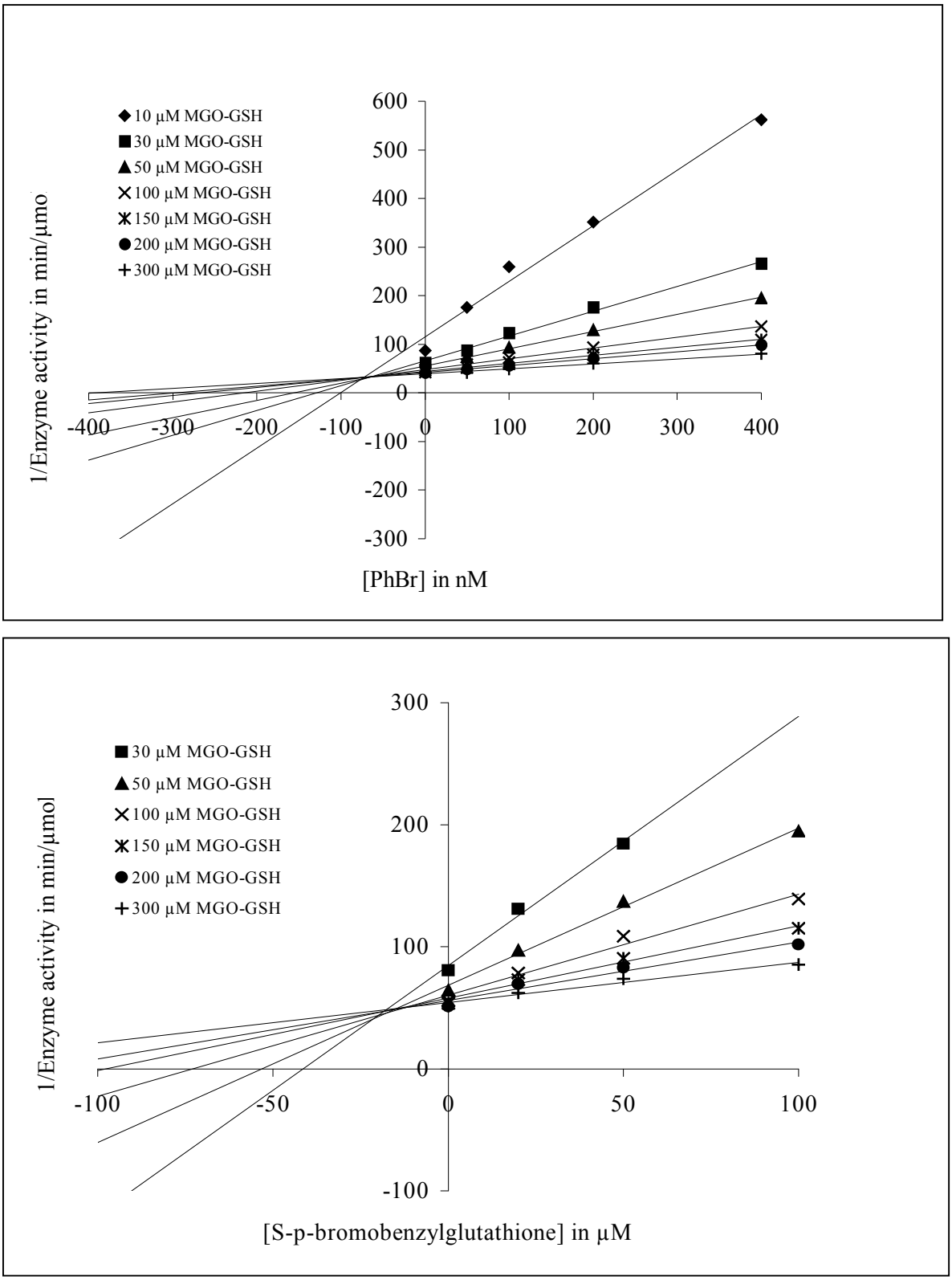


Figure 16: **Dixon plots comparing competitive inhibition of (A) HBPC-GSH and (B) S-*p*-bromobenzylglutathione on *P. falciparum* glyoxalase I using the methylglyoxal-GSH adduct as substrate.**

4.1.4 Metal ion analysis

The catalytic mechanism of the GloI enzymes requires an octahedrally-coordinated divalent metal ion which coordinates the two oxygen atoms of the glyoxal moiety of the substrate, polarising them and facilitating the rearrangement, via an enediolate intermediate, to D-lactate (Himo and Siegbahn, 2001). GloII enzymes like other members of the metallo-beta-lactamase superfamily of proteins possess a characteristic dinuclear active site (Wenzel et al., 2004).

A metal content of up to 1.2 zinc and 0.1 nickel ions was obtained for cGloI (Iozef *et al.*, 2003). According to sequence alignments, particular putative inserts responsible for defining GloI metal specificity to Zn^{2+} were found to be present in cGloI (Figure 13; Sukdeo *et al.*, 2004). However, metal coordinating residues were not entirely conserved in GILP and accordingly the metal content measured was negligible. Furthermore, the gene fusion demonstrated for cGloI (Iozef *et al.*, 2003) does not apply for GILP.

tGloII showed a Zn^{2+} ion content of 1.7 ± 0.3 with insignificant amounts of other metals tested. cGloII had a rather low metal content of 0.05 and 0.26 for Zn^{2+} and Fe^{2+}, respectively. This indicated that the enzyme was not completely saturated with metals at its active site. GloII activity was therefore tested in buffers enriched with Zn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Ca^{2+} and Cu^{2+} ions, respectively. Only Zn^{2+} activated the enzyme by a factor of ~ 2.

As previously shown, cGloI is a zinc dependent enzyme (Iozef *et al.*, 2003) which was further supported by the fact that overexpression in the presence of Zn^{2+} enhanced catalytic activity. GILP, however, did not bind significant amounts of metal ions and has no known central metal coordinating motifs, either. tGloII was shown to be clearly Zn^{2+} dependent whereas cGloII had a rather low metal content of 0.05 and 0.26 for Zn^{2+} and Fe^{2+}, respectively, and could be slightly activated by zinc. A zinc dependency has been reported for other GloII, e.g. for GloII from *Arabidopsis thaliana* by Crowder *et al.* (1997). Also other metals were shown to bind to GloII, namely iron and manganese, especially if the enzymes were produced in media enriched with these metals (Wenzel *et al.*, 2004). The Zn/metal binding motif T-H-X-H-X-D-H as well as the two other histidines required for zinc-binding are conserved in both *P. falciparum* GloIIs (Figures 14 and 19).

4.1.5 Cell culture experiments

IC_{50} values of 30 µM and 10 µM were obtained for HCPC-GSH diethyl ester and HCPC-sulfoxide, respectively. Haemolysis could be observed when higher concentrations of both pro-drugs were employed.

In extracts from isolated trophozoites of the *P. falciparum* strain Dd2 the specific activities of glyoxalase I and glyoxalase II were 0.2 U/mg and 1.0 U/mg, respectively. Treatment with 10 µM S-p-bromobenzylglutathione cyclopentyl diester, a potent GloI inhibitor, in a parallel culture (see methods) led to 0.2 U/mg GloI and 0.7 U/mg GloII, respectively. The lack of induction of the glyoxalase system by the S-p-bromobenzylglutathione cyclopentyl diester might be explained by the fact that the *P. falciparum* enzymes, in contrast to human GloI, are not significantly affected by the inhibitor.

4.1.6 Crystallization experiments

Several attempts to develop glyoxalase crystals did not yield any real protein crystal from the three glyoxalase (cGloI, GILP and cGloII) enzymes that were used. On exploitation of the conditions that were used to obtain the human GloI crystals (PEG 2000 monomethyl ether as precipitant – Cameron *et al.*, 1999a), microcrystals were received for cGloI. Further optimization did not lead to any improvement of the quality of the microcrystals. Crystallographic studies of the recombinant glyoxalase enzymes should have helped in the design and development of glyoxalase inhibitors that could have applications as anti-malarial drugs. This is because the X-ray structure that would have resulted from the crystals could have served in facilitating the design of specific *P. falciparum* glyoxalase inhibitors. The fact that the X-ray structures of both human GloI and GloII have been completely solved and published (Cameron *et al.*, 1997; 1999a and 1999b) supports the development of more specific and highly selective inhibitors of *P. falciparum* glyoxalases as the inhibitors would have been designed after a close structural comparison of human and *P. falciparum* target sites of the inhibitors.

However, optimization of the crystallization screens of the glyoxalases continued with further crystallization trials of cGloI and GILP at the High Throughput Screening Laboratory of the Hauptmann Woodward Medical Research Institute (USA). With the help of crystallization robotics at this institute, about 1400 crystallization settings with varying precipitants, pH, buffer systems, and ionic strength, were simultaneously tested for the development of crystals. The results of these experiments indicated several promising conditions under which small-sized crystals (see examples in Figure 17) of cGloI developed. These conditions can now be tested further for the development of X-ray diffractable crystal sizes.

Moreover, on the basis of the structure of GILP, it would be possible to decipher if the enzyme is actually a glyoxalase and also by exploiting its plasmodial specificity to validate it as a target for rational drug design against *P. falciparum*.

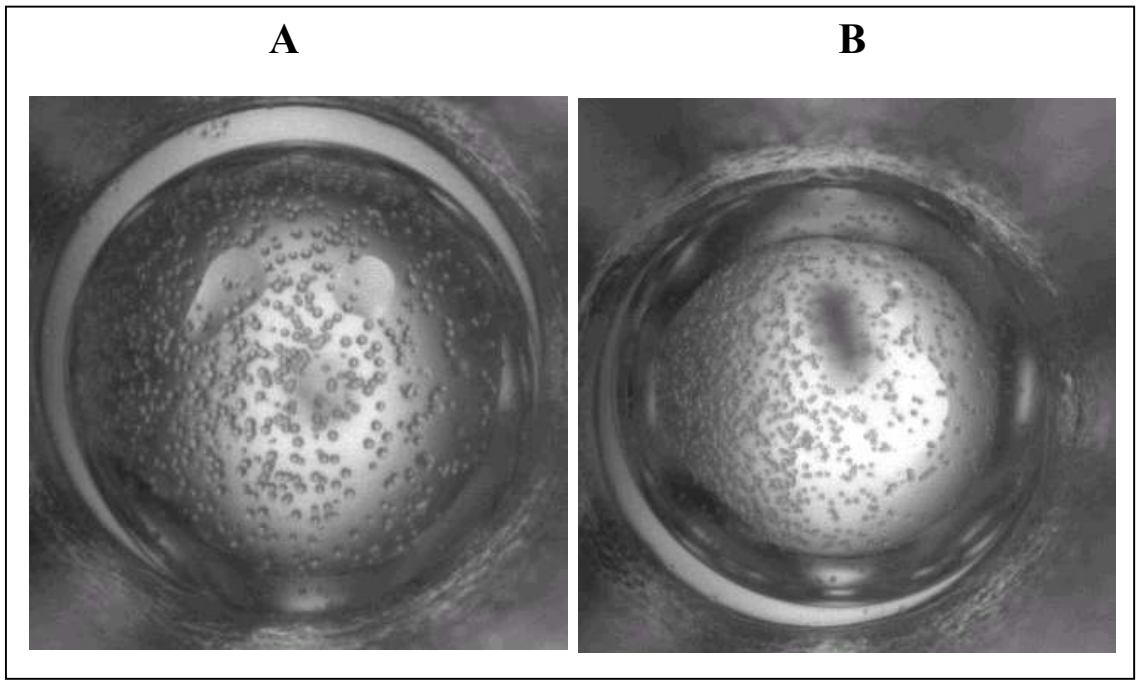


Figure 17: **Crystals of cGloI**
Crystals were developed in the following precipitating solutions. A. 0.09 M HEPES-Na, pH 7.5 containing 0.18 M manganese chloride, 27% isopropanol and 10% anhydrous glycerol. B. 0.1 M Hepes, pH 7.0 containing 2.48 M magnesium chloride.

4.1.7 *Plasmodium falciparum* glyoxalase structure predictions

Models of cGloI, tGloII and cGloII were generated based on the crystal structures of the human glyoxalases I and II, respectively. Monomeric cGloI comprises two potential active sites which are presumably very similar to the active sites of the human homodimer (Figures 13 and 18A). Gln18, Glu91, His299, and Glu345 coordinate the first zinc ion, and His115, Glu161, Gln182, and Glu272 bind a zinc ion at the second active site. Like in yeast GloI both active sites presumably act independently of each other (Frickel *et al.*, 2001). Most of the residues involved in the formation of a hydrophobic binding pocket at the active site of human GloI (Kalsi *et al.*, 2000) are also conserved in cGloI (Figures 13 and 18B). Conserved residues of the hydrophobic binding pocket in cGloI are Phe47, Phe52, Leu54, Phe56, Leu85, Phe335 and Met330. Cys60, Ile88 and Leu160' (of the other subunit) in human GloI are replaced by Tyr45, Tyr81 and Ile333 in cGloI, respectively. Moreover, greater binding affinity (reflected by lower K_i) to cGloI with increase in

hydrophobicity of the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones was observed. This is in support of the postulated hypothesis of Kalsi *et al.* (2000) that occupancy of this pocket maximizes polar interactions between the enzyme and the bound enediol analogues. Despite the sequence similarities, a complete model of cGloI could not be generated due to a distance of 2.5 nm between Met138 and Ser25' of human GloI which are aligned to Lys172 and Tyr174 of cGloI. In addition, the residues corresponding to Met1-Pro24 of human GloI are missing in cGloI. Thus, the real structure of monomeric cGloI is likely to differ significantly from the human dimeric enzyme. Such significant differences could be exploited in the synthesis of specific inhibitors of cGloI. It was not possible to generate a model of GILP based on human GloI due to limited sequence similarity.

Structures and calculated force field energies (-8.8 MJ/mol) of tGloII and cGloII are very similar to the used template (Figure 19). The residues coordinating two zinc ions at the active site are conserved and the resulting metal binding site is also very similar for the three glyoxalases II (Figures 14 and 20). However, several residues contributing to the glutathione-binding site differ between tGloII, cGloII and human GloII (Figures 14 and 21). Arg249, Lys143 and Lys252 are thought to interact with the carboxylate group of the glycine of glutathione. In tGloII, two of these basic residues are replaced by Gln249 and Asp252. Tyr145 of human GloII is replaced by Phe as it is also the case for many other glyoxalases II (Cameron *et al.*, 1999b), whereas Tyr175 and Lys143 are conserved or replaced by Arg, respectively.

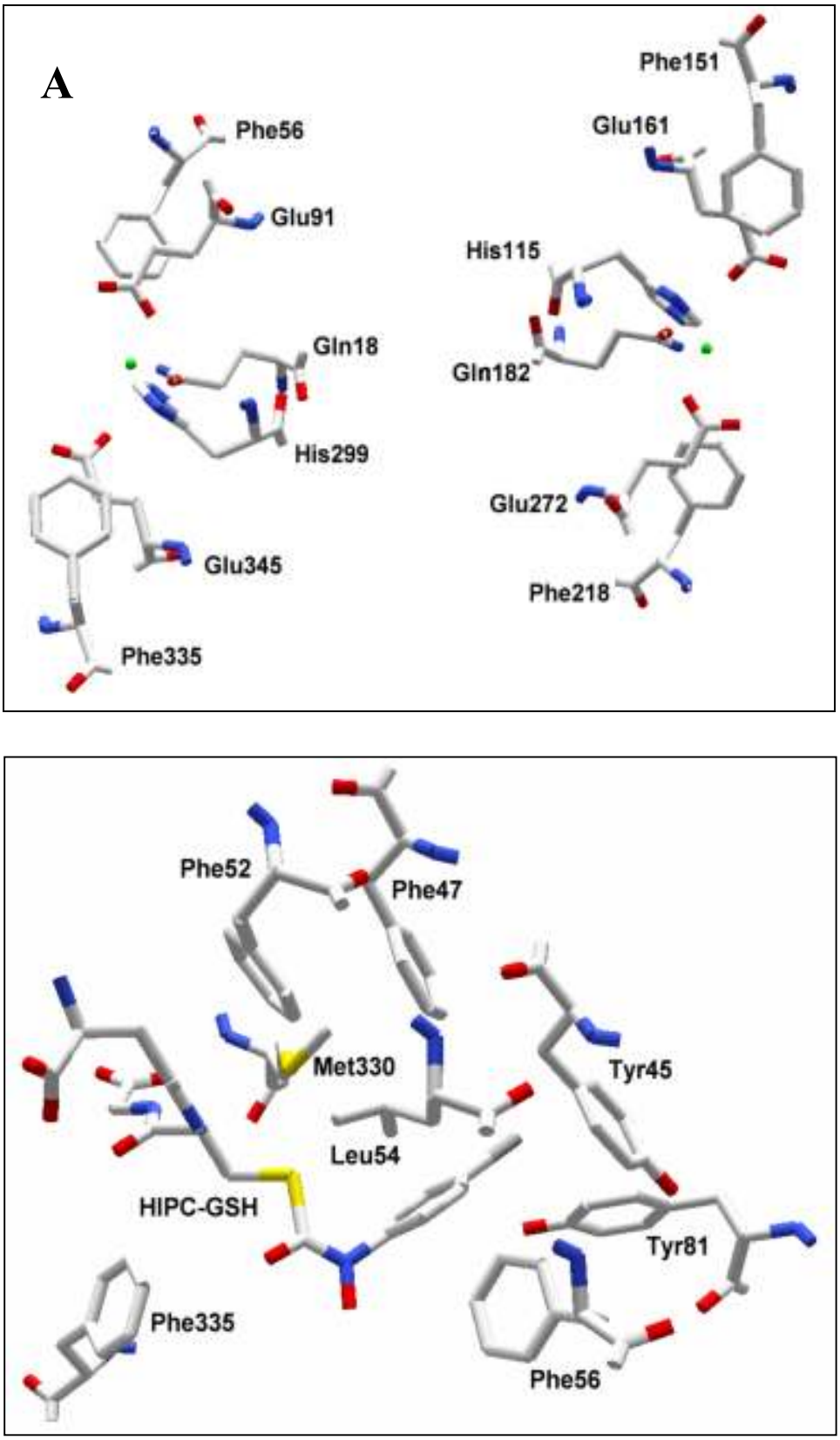


Figure 18: **Model of the active sites and hydrophobic binding pocket of GloI based on the crystal structure of human GloI (Cameron *et al.*, 1999a).**
(A) The putative active sites of monomeric GloI with each containing a zinc ion (given as sphere). (B) Model of the putative hydrophobic binding pocket of monomeric cGloI.

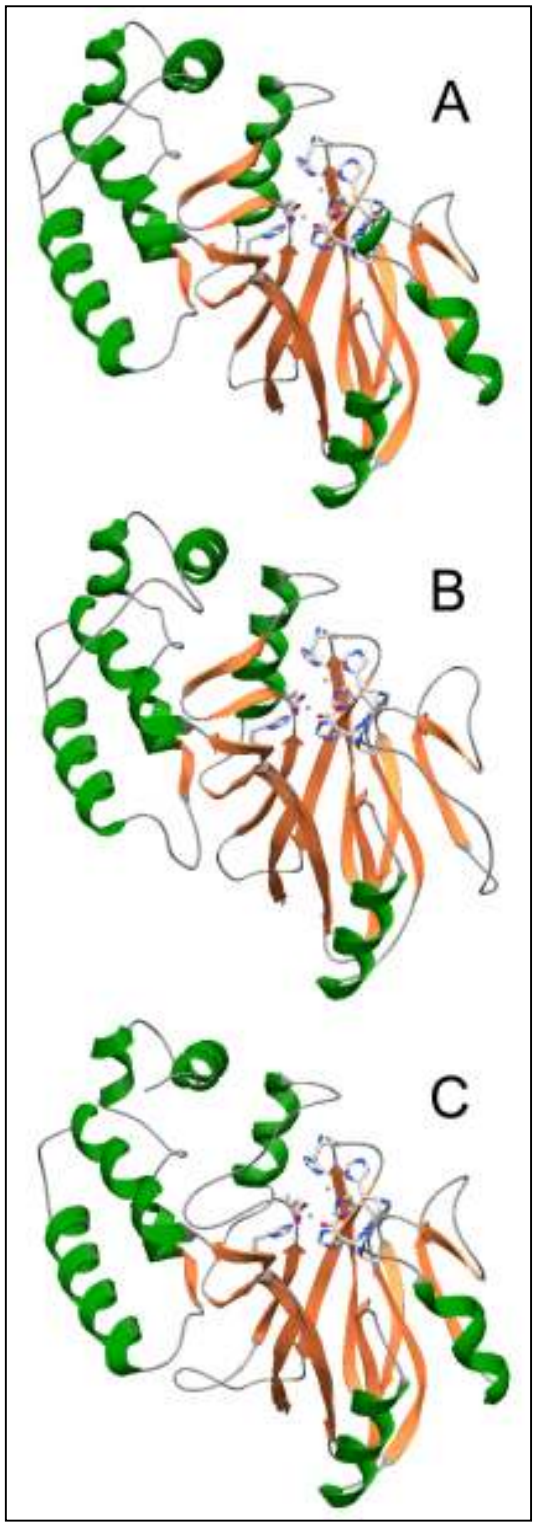


Figure 19: **Model of tGloII and cGloII based on the crystal structure of human GloII (Cameron *et al.*, 1999b).**
Residues coordinating two zinc ions are highlighted. (A) Structure of human GloII showing an N-terminal domain, containing predominantly β-strands similar to metallo-b-lactamases, and a smaller second domain comprising predominantly a-helices; (B) model of tGloII; and (C) model of cGloII.

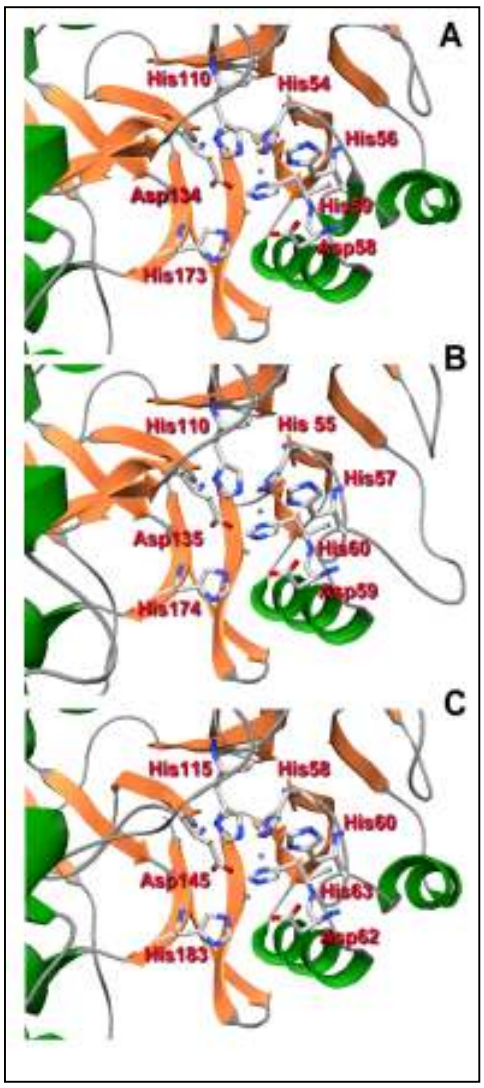


Figure 20: Model of the metal binding site of tGloII and cGloII based on the crystal structure of human GloII (Cameron *et al.*, 1999b).
Residues coordinating two zinc ions are highlighted. (A) Structure of human GloII; (B) model of tGloII; and (C) model of cGloII.

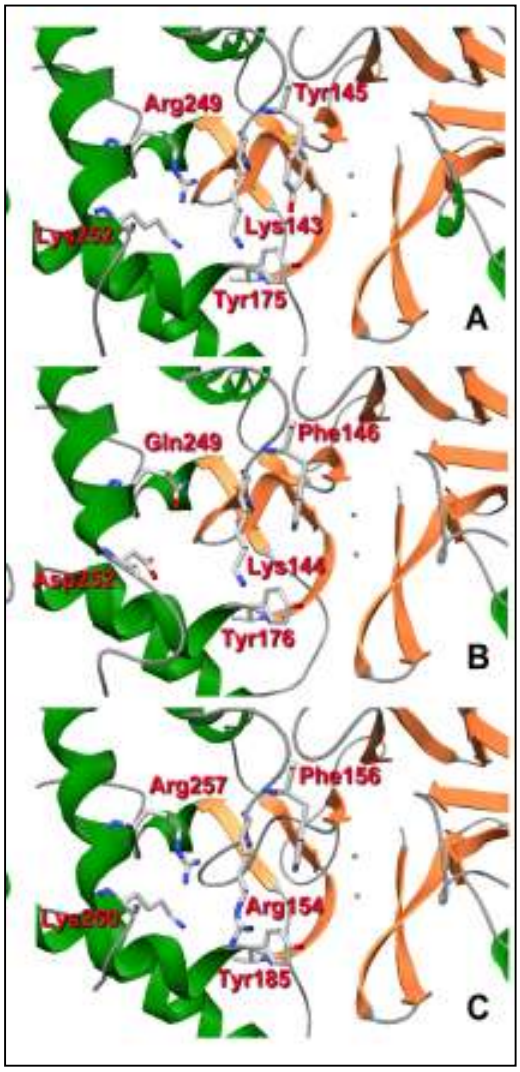


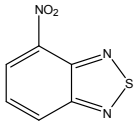
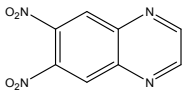
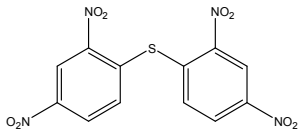
Figure 21: Model of the glutathione-binding site of tGloII and cGloII based on the crystal structure of human GloII (Cameron *et al.*, 1999b).
Residues involved in glutathione-binding are highlighted. (A) Structure of human GloII; (B) model of tGloII; and (C) model of cGloII.

4.2 The inhibition of PfTrxR

4.2.1 Kinetic analyses on isolated enzymes

All 11 compounds were tested for their ability to inhibit PfTrxR and the isofunctional host cell enzyme hTrxR in direct comparison by Christine Nickel. The respective IC_{50} values were determined under quasi-physiological conditions, i.e., in phosphate buffer, pH 7.4, in the presence of 100 µM NADPH and 20 µM of the corresponding thioredoxin. Since wild type Trx tends to form dimers in solution which disturb the determination of exact kinetic constants, the hTrx mutant C72S for the studies (Irmler *et al.*, 2002) was employed. Seven compounds inhibited PfTrxR with IC_{50} values of $\leq$ 10 µM. The most potent inhibitor was compound **3** with an IC_{50} of 0.5 µM. Except for two compounds; all inhibitors were more effective on the parasite enzyme than on hTrxR. Compounds **1** and **2** were by a factor of 25 and 70, respectively, more effective on PfTrxR.

Table 9: Structures of the most potent PfTrxR inhibitors; compounds 1-3 and corresponding IC_{50} values on *P. falciparum* TrxR and human TrxR.

No	Structure	IC_{50} PfTrxR [µM]	IC_{50} hTrxR [µM]	K_i PfTrxR varied substrate: PfTrx [µM]	K_i PfTrxR varied substrate: NADPH [µM]
1		2	50	0.65	1
2		2	140	n.d.	n.d.
3		0.5	4	0.2	0.2

n.d., not determined.

K_m values in the presence of varying inhibitor concentrations were determined on PfTrxR for both substrates, PfTrx and NADPH. For all three inhibitors, compounds **1-3**; uncompetitive inhibition was clearly observed as K_m and V_{max} values decreased with increasing inhibitor concentrations. In accordance, Cornish-Bowden plots showed intercepts of all lines (obtained at different substrate concentrations) in the upper left

quadrant. Data for the most potent PfTrxR inhibitor **3** are shown in Figure 22. K_i values obtained for this uncompetitive inhibition were in the submicromolar range and are shown in Table 9.

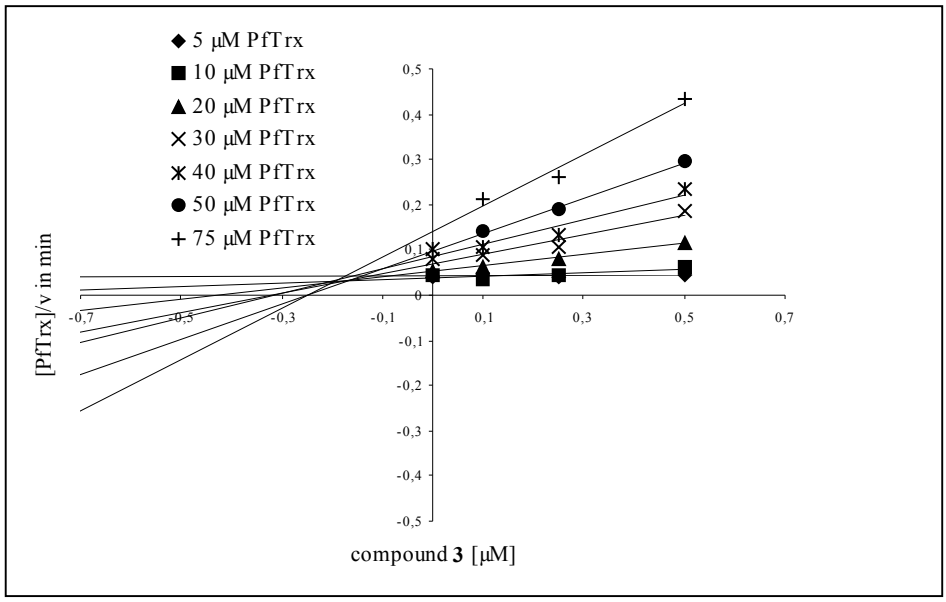


Figure 22: Cornish-Bowden plot showing that compound 3 inhibits PfTrxR uncompetitively with respect to PfTrx.

4.2.2 Effects of the inhibitors on *P. falciparum* in culture

The effects of the three most potent PfTrxR inhibitors **1-3** on the growth of malarial parasites were tested on the chloroquine resistant *P. falciparum* strain K1. IC_{50} values - indicating the drug concentration required for 50% parasite growth inhibition – were determined to be 11 µM, 15 µM, and 18 µM, respectively. Taking into account activity losses due to incomplete uptake and metabolic turnover of the inhibitor, values are in good agreement with the kinetic data. The most effective compound on cell culture, compound **1**, was also studied using the incorporation of ^{3}H-hypoxanthine as metabolic parameter to determine growth inhibition. In this assay, an IC_{50} value of 2.9 $\pm$ 0.82 µM was determined. This result is in good agreement with the fact that most drugs exhibit lower IC_{50} values when assessed with this metabolic method rather than with the determination of parasitemia. Furthermore, drug combination assays of compound **1** with the clinically used antimalarials chloroquine, methylene blue, and artemisinin were carried out. Data obtained demonstrate that the PfTrxR inhibitor acts antagonistically with all three established antimalarial drugs (Figure 23). This result might indicate that compound **1** uses similar uptake mechanisms and/or acts on similar targets/pathways as the other three drugs.

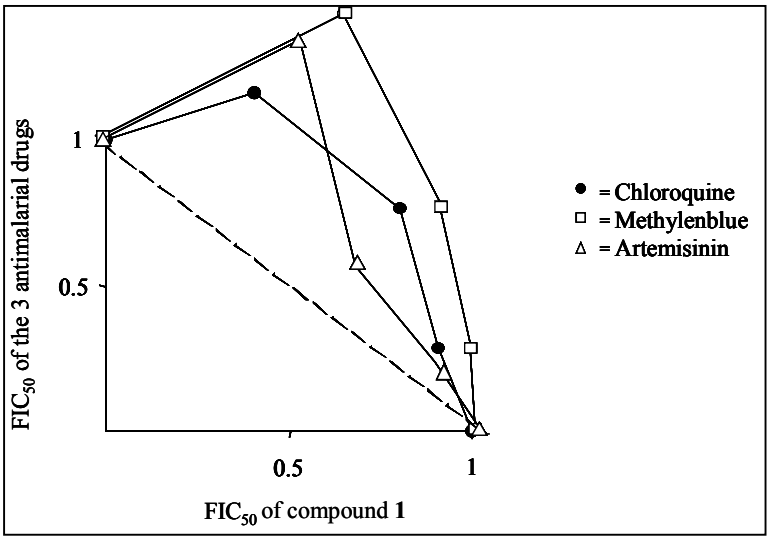


Figure 23: Convex isobologram indicating the antagonistic effects of compound 1 upon combination with chloroquine, methylene blue and artemisinin.

4.3 The *in vitro* antimalarial effects of MB

4.3.1 Stage-specificity of MB action

The susceptibility of the different *P. falciparum* intraerythrocytic stages to MB as determined by the WHO microtest is given in Figure 24. The lowest IC_{50}-values of about 11 nM were found in the very young ring stages of the parasites indicating that this developmental stage is the most susceptible one. Schizonts showed the lowest susceptibility to the drug with IC_{50}s as high as 88 nM. The fact that the IC_{50}s determined in these experiments are higher than in the drug combination assays is based on the fact that the stage-specificity data were obtained by counting parasitaemia under the microscope whereas for the drug combinations the incorporation of ^{3}H-hypoxanthine was determined. The differences in drug exposure times in both assay conditions could have played a significant role as the parasites were exposed to MB for only 6 hours during the microtest whereas in the drug combination assays they were exposed for 72 hours.

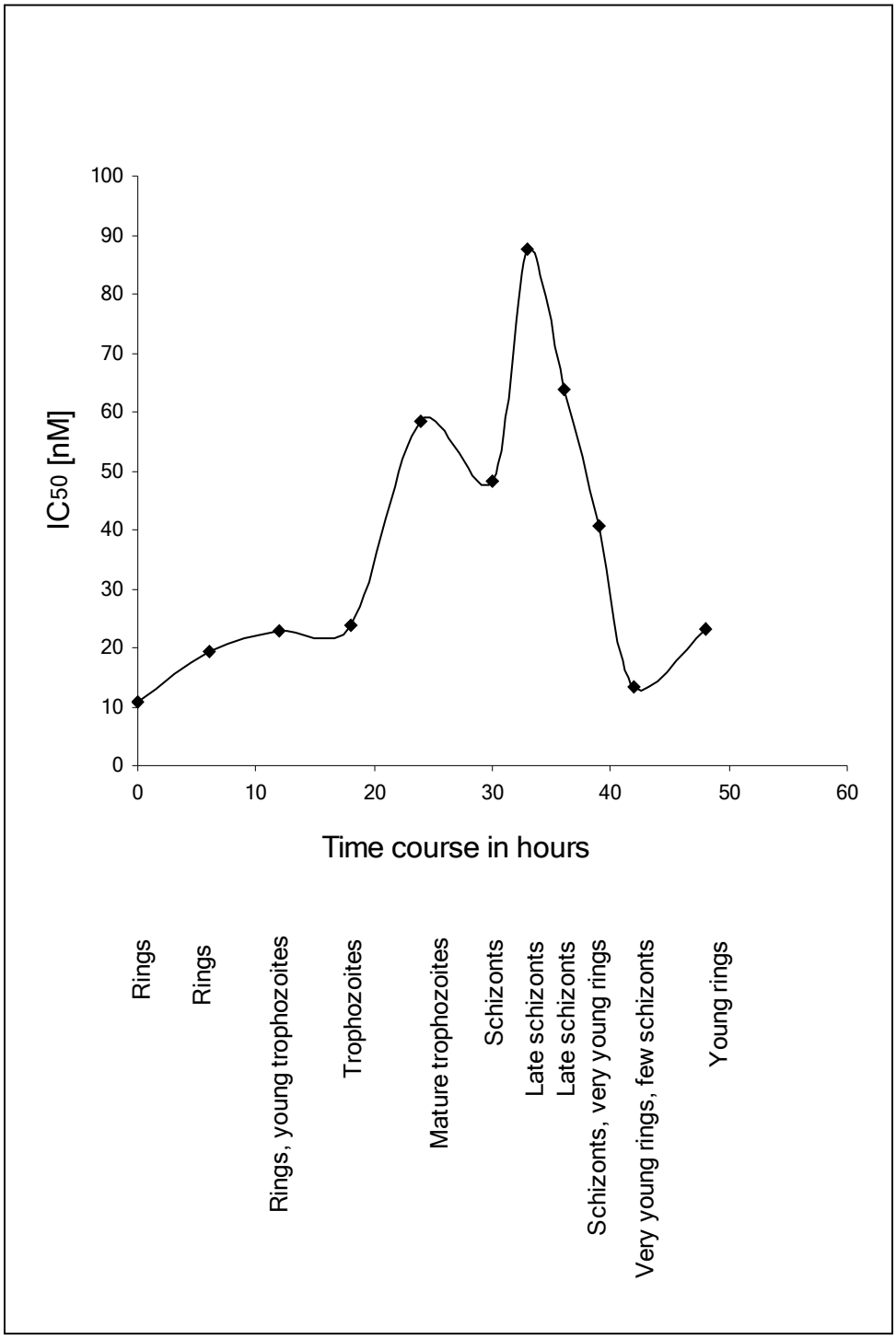


Figure 24: **Stage specificity of methylene blue action on the CQ resistant *P. falciparum* strain K1.** IC_{50}-values obtained for the various developmental stages of the 48 h life cycle are given. Data shown were derived from two parallel experiments which differed by less than 10 %.

4.3.2 Studies on MB uptake

In another experiment, the uptake of MB into various compartments of parasitised (PRBC) and non-parasitised red blood cells (NPRBC) were studied. After incubation with MB (13, 26, and 39 nM) and preparation of the different lysates, MB was determined by LC/MS/MS. The data are shown in Table 10. The total amount of MB added to the cell cultures could not be recovered quantitatively in the different fractions. This is most likely due to the various washing steps required and to the fact that MB binds strongly to surfaces of reaction tubes (Clark *et al.*, 1925).

Table 10: Clearance of the medium from MB by *P. falciparum* parasitized erythrocytes

Initial [MB] in the medium	**MB concentration [nM] after 12 hours incubation**				
	Medium without cells	**Medium of healthy erythrocytes**	**Medium of parasitized erythrocytes**	**Parasite lysate**	**Parasite membrane pellet**
13 nM MB	6.1	4.0	<2	8.2	79
26 nM MB	14.7	6.0	<2	16.3	152
39 nM MB	16.6	19.2	<2	21.7	265

4.3.3 Effects of MB upon combination with other drugs

4.3.3.1 Combination of MB with clinically-used antimalarials

In a parallel approach, the effects of several drug combinations including MB on the growth of the *P. falciparum* strain K1 were tested *in vitro*. Data are given in Table 11. The combinations of MB with mefloquine or quinine were additive whereas the combinations of MB with chloroquine (Figure 25) and all other tested quinolines as well as with pyrimethamine were antagonistic. A more pronounced antagonism was observed when the MB and chloroquine combination was performed on the chloroquine sensitive strain 3D7 (Figure 25).

Most interestingly, the combinations of MB with artemisinin and its derivatives artemether and artesunate were found to act synergistically on the CQ-resistant strain K1 (Table 11). The combinations of MB with artemisinin or artemether were advantageous over the

MB/artesunate combination. All four drugs exhibited IC_{50} values in the low nanomolar range when given alone.

4.3.3.2 Combination of MB with artemisinins

In order to investigate whether the synergistic action of MB and artemisinin derivatives differs between *P. falciparum* strains, strains with different degrees of CQ sensitivity were employed in the drug combination assays. Based on data obtained by Su *et al.* (1997) and our own experience, strains 3D7 and HB3 were chosen as CQ-sensitive strains while Dd2 was chosen in addition to K1 as CQ-resistant strain. The CQ sensitivities expressed as IC_{50}-values of these strains were 8.58 ± 0.43 nM, 16.82 ± 0.54 nM, 90.23 ± 10.55 nM, and 155.17 ± 11.38 nM, respectively. The drug combinations acted synergistically on all strains tested as the FIC_{50} and FIC_{90}-values were significantly lower than one as tested separately for all drug combinations ($P < 0.0001$). The degree of synergism was highest on the HB3 strain (mean $FIC_{50} = 0.59$, mean $FIC_{90} = 0.59$), followed by K1 (mean $FIC_{50} = 0.76$ ($P = 0.013$ describing the significance of difference when compared with HB3), mean $FIC_{90} = 0.78$ ($P = 0.0003$)), and then 3D7 (mean $FIC_{50} = 0.97$ ($P < 0.0001$), mean $FIC_{90} = 0.86$ ($P < 0.0001$)) and finally Dd2 (mean $FIC_{50} = 0.99$ ($P < 0.0001$), mean $FIC_{90} = 0.90$ ($P = <0.0001$)). When taking into account the FICs and isobolograms obtained (Table 12 and Figure 26), synergistic effects were most pronounced in the artemisinin/MB combination although artemether and artesunate were more potent than artemisinin when given as monotherapy (see IC_{50} values in Table 12). FIC-values for the three different artemisinins in combination with MB were reproducibly lowest for artemisinin (mean $FIC_{50} = 0.64$, mean $FIC_{90} = 0.62$) in comparison with artesunate (mean $FIC_{50} = 1.00$ ($P = 0.0001$); mean $FIC_{90} = 0.88$ ($P = 0.0001$)) and artemether (mean $FIC_{50} = 0.82$ ($P = 0.0008$). FIC_{50}s were reproducibly lowest at combination ratios of 3:1 (mean $FIC_{50} = 0.69$) than at a combination ratio of 1:1 (mean $FIC_{50} = 0.80$; $P = 0.049$) or 1:3 (mean $FIC_{50} = 0.98$; $P = 0.0001$). Thus, only low MB concentrations are required for maximal therapeutic effects.

Table 11: Effects of combination of MB with clinically-used antimalarials

Drugs given in combination	IC_{50} in nM	IC_{90} in nM	Mean FIC_{50} value at fixed partner:MB ratio	Type of Combination Effect
Artemether	1.8 ± 0.1	3.7 ± 0.1	0.75 at 1:1 0.65 at 1:3 0.65 at 3:1	Synergism
Artesunate	1.3 ± 0.2	3.1 ± 0.2	0.85 at 1:1 1.00 at 1:3 0.90 at 3:1	Additive effect / Synergism
Artemisinin	4.0 ± 0.8	8.0 ± 0.6	0.67 at 1:1 0.73 at 1:3 0.73 at 3:1	Synergism
CQ (4-aminoquinoline)	117 ± 27	199 ± 30	1.28 at 1:1 1.18 at 1:3 1.25 at 3:1	Antagonism
Piperaquine (Bisquinoline)	10.6 ± 2.7	20.6 ± 1.8	1.97 at 1:1 1.94 at 1:3 2.01 at 3:1	Antagonism
Amodiaquine (4–aminoquinoline)	7.8 ± 1.4	12.1 ± 0.4	1.60 at 1:1 1.20 at 1:3 1.05 at 3:1	Antagonism
Quinine (quinoline methanol)	90 ± 39	227 ± 82	0.97 at 1:1 1.05 at 1:3 0.97 at 3:1	Additive effect
Mefloquine (quinoline methanol)	7.8 ± 2.0	15.7 ± 2.5	1.04 at 1:1 1.03 at 1:3 1.09 at 3:1	Additive effect
Primaquine (8-aminoquinoline)	1820 ± 40	3860 ± 560	1.22 at 1:1 1.17 at 1:3 1.12 at 3:1	Antagonism
Pyrimethamine (antifolate)	7540 ± 810	16700 ± 880	1.02 at 1:1 1.52 at 1:3 1.41 at 3:1	Antagonism

*IC values for the partner drugs of MB (columns 2 and 3) are means of at least two independent assays conducted on different days. For MB itself IC_{50} is 6.5 ± 1.8 nM and IC_{90} is 12.4 ± 2.3 under the chosen conditions. FIC_{50} values were determined according to the fixed-ratio method of Fivelman *et al.* (2004). $FIC_{50} < 1$ indicates synergistic drug action; $FIC_{50} = 1$, additive action; $FIC_{50} > 1$, antagonistic action.
FIC_{50} A = $[IC_{50}$ (A + B)]/IC_{50} A; FIC_{50} B = $[IC_{50}$ (B + A)]/IC_{50} B; FIC_{50} = FIC_{50} A + FIC_{50} B (Ohrt *et al.*, 2002).

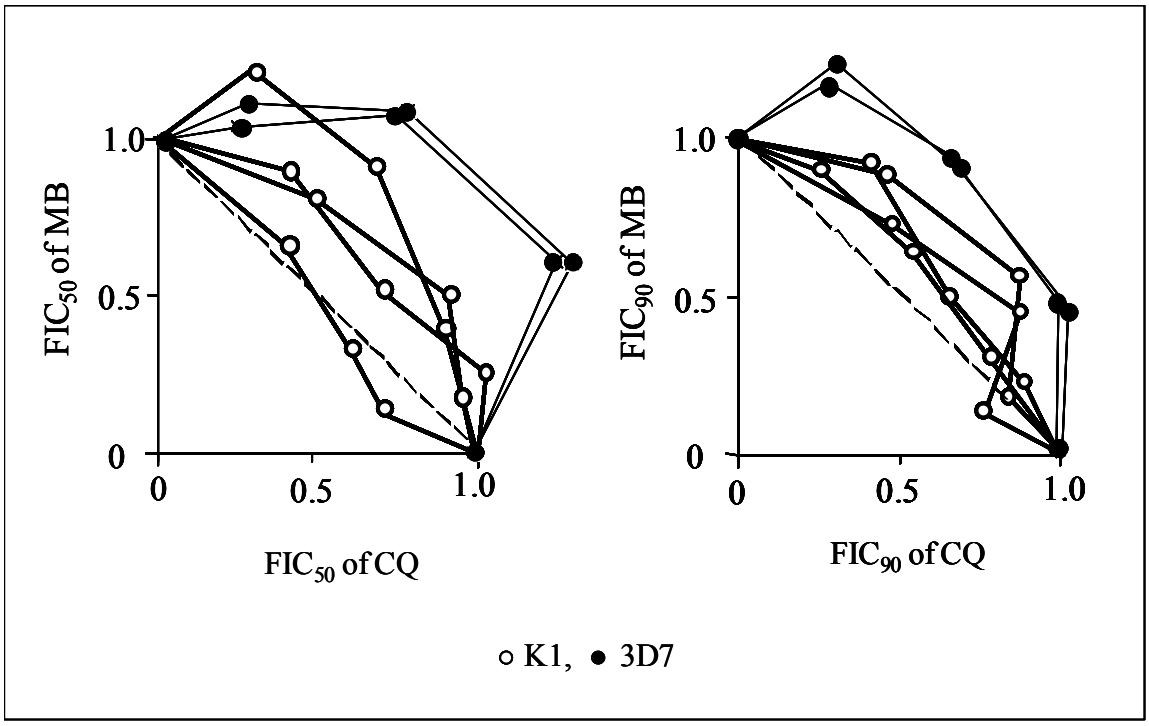


Figure 25: **FIC_{50} (left) and FIC_{90}-values (right) of MB and CQ determined at various dosage ratios (1:1, 1:3, and 3:1) and in independent experiments (indicated by the lines) on CQ-resistant (K1) and CQ-sensitive (3D7) strains of *P. falciparum*.**
The resulting antagonistic effect of the drug combination is indicated by the convex isobolograms.

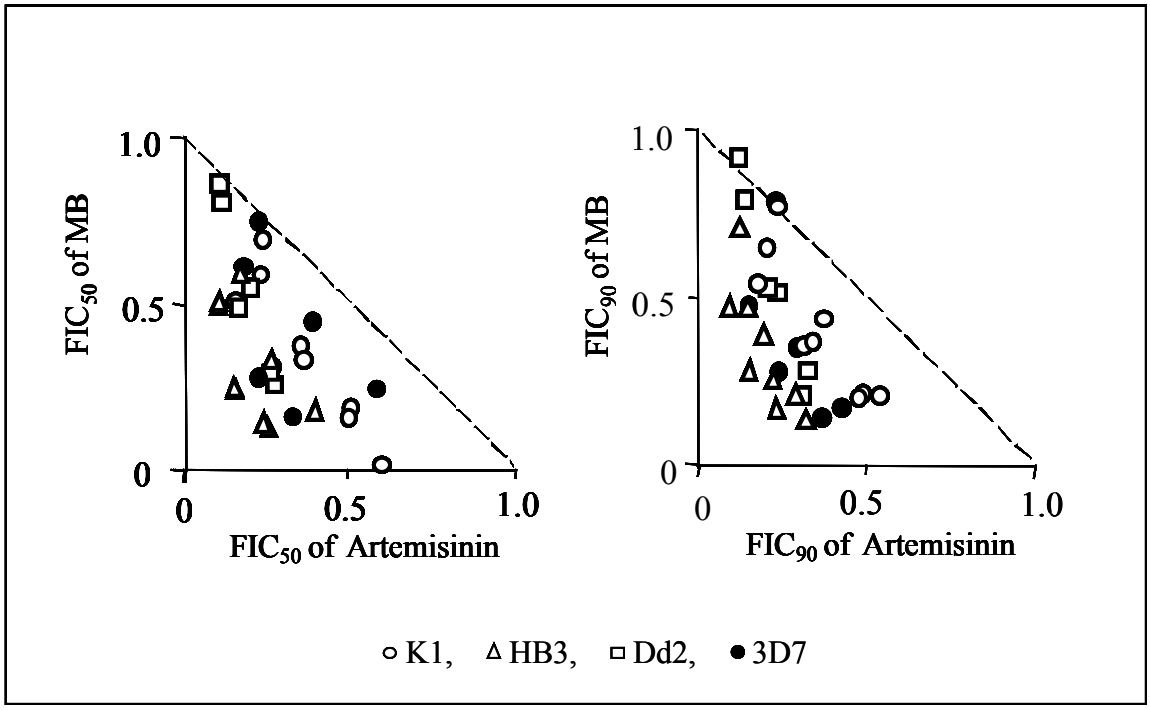


Figure 26: **FIC_{50} (left) and FIC_{90} values (right) of MB and artemisinin at various fixed dosage ratios on different *P. falciparum* strains.**
All data points were plotted to represent the complete range of interactions seen at the concentration ratios evaluated instead of presenting individual representative isobolograms. The concave isobolograms for these data points indicate a synergistic effect of the combination. A characteristic curve can be drawn for the triangles representing the strain HB3.

Table 12 : *In vitro* drug combination assays of the artemisinins, piperaquine and chloroquine with MB on *P. falciparum* strains with different CQ sensitivity.

Drug	*Plasmodium falciparum* strains tested	IC_{50} in nM		Sum (1:1 ratio)		Sum (1:3 ratio)		Sum (3:1 ratio)	
		Test Drug	MB	FIC_{50}	FIC_{90}	FIC_{50}	FIC_{90}	FIC_{50}	FIC_{90}
Artemether	K1	1.76 ± 0.07	7.94 ± 0.35	0.75 ± 0.21	0.80 ± 0.14	0.65 ± 0.21	0.90 ± 0.14	0.65 ± 0.07	0.70 ± 0.14
	Dd2	8.40 ± 1.63	5.24 ± 0.18	1.00 ± 0.14	0.90 ± 0.10	1.35 ± 0.35	1.20 ± 0.10	0.70 ± 0.14	0.70 ± 0.10
	HB3	5.10 ± 0.83	4.98 ± 2.59	0.63 ± 0.12	0.63 ± 0.12	0.80 ± 0.17	0.80 ± 0.17	0.53 ± 0.12	0.57 ± 0.12
	3D7	5.87 ± 0.45	3.26 ± 0.57	0.95 ± 0.35	0.95 ± 0.21	1.25 ± 0.21	1.10 ± 0.14	0.70 ± 0.14	0.75 ± 0.21
Artesunate	K1	1.28 ± 0.16	5.45 ± 2.05	0.85 ± 0.07	0.70 ± 0.10	1.00 ± 0.10	0.95 ± 0.07	0.90 ± 0.14	0.80 ± 0.10
	Dd2	5.22 ± 0.40	6.00 ± 0.32	1.25 ± 0.07	1.05 ± 0.07	1.30 ± 0.14	1.15 ± 0.07	1.05 ± 0.07	0.90 ± 0.14
	HB3	6.49 ± 0.81	6.10 ± 3.46	0.50 ± 0.14	0.55 ± 0.07	0.80 ± 0.14	0.90 ± 0.14	0.55 ± 0.07	0.50 ± 0.10
	3D7	4.35 ± 0.29	2.99 ± 0.34	1.30 ± 0.14	1.00 ± 0.10	1.55 ± 0.07	1.10 ± 0.10	1.00 ± 0.28	0.95 ± 0.07
Artemisinin	K1	3.98 ± 0.77	6.37 ± 1.10	0.67 ± 0.06	0.70 ± 0.10	0.73 ± 0.15	0.77 ± 0.21	0.73 ± 0.06	0.73 ± 0.06
	Dd2	20.36 ± 2.09	5.72 ± 0.06	0.65 ± 0.07	0.70 ± 0.10	0.95 ± 0.07	0.95 ± 0.07	0.50 ± 0.10	0.55 ± 0.07
	HB3	13.67 ± 2.92	3.84 ± 0.24	0.47 ± 0.12	0.43 ± 0.06	0.60 ± 0.10	0.63 ± 0.15	0.43 ± 0.06	0.37 ± 0.06
	3D7	17.29 ± 1.53	3.22 ± 0.21	0.65 ± 0.21	0.55 ± 0.07	0.90 ± 0.14	0.80 ± 0.28	0.60 ± 0.28	0.55 ± 0.07
Piperaquine	K1	8.7	5.7	2.0	1.3	1.9	1.3	2.0	1.3
	Dd2	16.6	6.2	1.5	1.2	1.4	1.2	1.4	1.1
	HB3	17.2	3.5	1.9	1.9	1.5	1.2	1.6	1.9
	3D7	17.6	5.8	2.0	2.0	1.3	1.8	1.2	1.0
Chloroquine	K1	117	6.4	1.3	1.3	1.2	1.1	1.3	1.2
	Dd2	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	HB3	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	3D7	8.8	4.0	1.9	1.6	1.4	1.5	1.9	1.5

Values are means of at least two independent assays carried out on different days. However, values from piperaquine/MB and chloroquine/MB were obtained from single experiments. When $FIC_{50} < 1$, synergistic drug action; $FIC_{50} = 1$, additive action; $FIC_{50} > 1$, antagonistic action.

4.4 The characterization of PfLipDHs

Bioinformatic analysis of the two PfLipDH genes revealed putative mitochondrial and apicoplastic target sequences for PfLipDH1 and PfLipDH2, respectively. The PfLipDH1 gene encodes a protein of 513 amino acids and a theoretical mass of 57 kDa while the PfLipDH2 gene encodes a larger protein of 666 amino acids and a molecular mass of 75.6 kDa. Both genes of PfLipDH1 and PfLipDH2 present on chromosome 12 and 8, respectively, of the *P. falciparum* genome were successfully amplified by PCR and cloned into appropriate expression vectors (pQE30 and pRSET-A). Both expression vectors allowed the overexpression of the respective genes as six-histidyl tagged proteins making possible the purification of the PfLipDHs by affinity chromatography on Ni-NTA columns. Several *E. coli* strains were used in order to optimise the overexpression process (Table 4). Yields of both PfLipDHs obtained after overexpression and purification were however very small. Active PfLipDH1 was obtained with a low purity of about 30% after overexpression in *E. coli* M15 cells. Identity of PfLipDH1 was also demonstrated by western blot analysis with anti-histidine tag antibodies. In other overexpression systems, PfLipDH1 was either obtained in inclusion bodies or was not overexpresed at all (Table 4). Attempts to purify PfLipDH1 from inclusion bodies by denaturation and subsequent renaturation in appriopriate buffer systems were not successful either. PfLipDH2 could not be overexpressed in the various overexpression systems tested.

A kinetic characterization of PfLipDH1 – which was also obtained in low amounts only – as well as the intraparasitic localization studies of both PfLipDHs was reported in McMillan *et al.* (2005).

Based on previous knowledge that MB inhibits lipoamide dehydrogenases from other sources (e.g. from trypanosomes), the inhibitory effects of MB on PfLipDH1 was tested. However, the preliminary results indicated that MB acts more as a substrate for PfLipDH1 than as an inhibitor.

4.5 Glutathionylation of PfTrx

Mass spectrometric analysis of the tryptic digests of GSSG-treated and control PfTrx revealed two distinct glutathionylated peptides whose masses corresponded to theoretically calculated glutathionylated peptides of PfTrx. Both peptides (peptide 1 and 2 – Figure 27) spanning Ile47 and Lys60, and Arg46 and Lys60, respectively indicated that the glutathionylated cysteine was Cys54. Mass spectra of peptides 1 and 2 from PfTrx treated with and without GSSG are shown below (Figure 28). Based on the relative intensities of

the glutathionylated peptides to the intensities of the non-glutathionylated peptides in the spectrum of the treated sample it could be estimated that roughly 70% of PfTrx was glutathionylated under the conditions tested.

Glutathionylated PfTrx could also be identified by western-blot analysis using anti-glutathione antibodies. However, results of the experiment could not be effectively reproduced on subsequent trials and are therefore not shown.

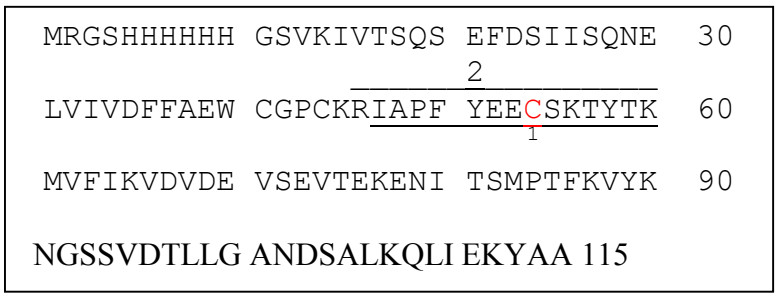


Figure 27: Amino acid sequence of recombinant hexa-histidyl-tagged PfTrx indicating the two peptide fragments spanning the glutathionylated Cys54 (red) of interest.

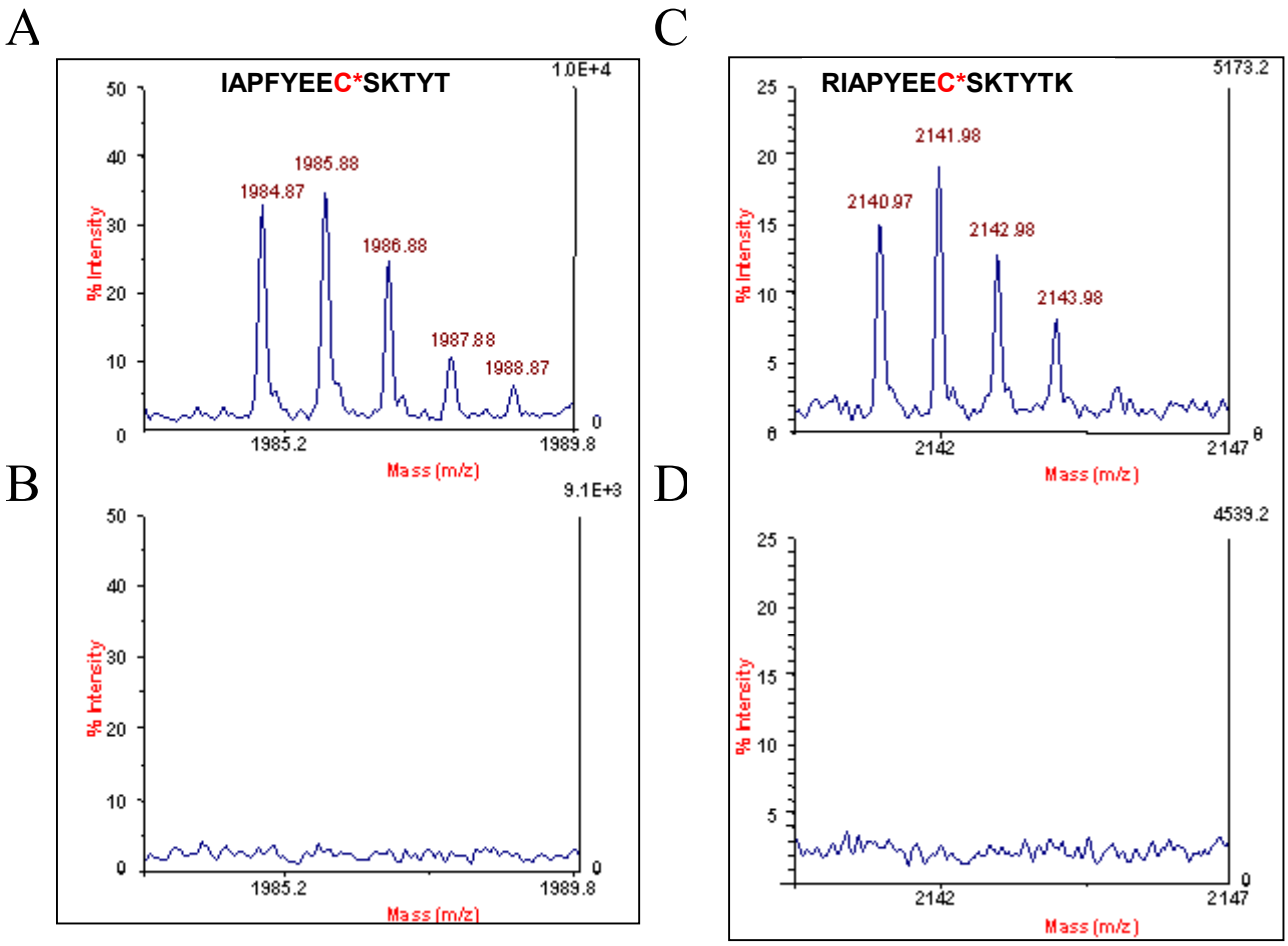


Figure 28: **MALDI-TOF mass spectra of PfTrx peptides.**
A). Peptide 1 generated from GSSG-treated PfTrx. The mass is corresponding to the glutathionylated peptide Ile47–Lys60: IAPFYEEC*SKTYTK. B). Corresponding spectrum trace of the non-treated sample corresponding to the non-glutathionylated peptide 1: IAPFYEECSKTYTK. C). Peptide 2 Arg46–Lys60: RIAPYEEC*SKTYTK generated from GSSG-treated PfTrx. D). Corresponding spectrum trace of the non-treated sample corresponding to the non-glutathionylated peptide 1: RIAPFYEECSKTYTK.

5 Discussion

Records of malaria date back to the earliest human civilizations when it was described as the distinct periodic fevers. Malaria is a serious infection with *Plasmodium* parasites, which are spread by the bite of *Anopheles* mosquitoes. For this reason, nearly all malaria control strategies target either the parasite or the mosquito. Inspite of worldwide efforts, however, an eradication of malaria is far from being achieved. There are no fewer than four species of *Plasmodium* that infect people, each with thousands of genetic variants, and about thirty-five different species of malaria-transmitting mosquitoes. It is the complex diversity of the parasites, the mosquitoes, the local ecologies, socio-economic conditions, and human responses to disease that conspire to make malaria notoriously hard to control. As a result, there is no single prescription which can successfully control malaria in all areas.

Malaria was described by Robert Desowitz as "the oldest emerging disease". The disease is not only well-known as one responsible for the death of an African child approximately every 20 seconds but also as one that has baffled the minds and brains of many profound scientists. According to Desowitz, the much heralded malaria vaccine is a goal, an illusion that has not been realized and may never be realized in combating the disease at a population level (Desowitz, 2004). This understatement came up after hundreds of millions of dollars, the energies and resources of some of the best scientific minds have been – and still are – devoted to an intensified malaria vaccine research in the past 30 years. The accuracy of malaria re-emergence – as described by Desowitz – stems from the acquisition of increasing resistance by the parasites to almost all available clinically-used antimalarial drugs and the *Anopheles* mosquitoes to insecticides. Antimalarial drug resistance is worsening, with the geographic spread of resistance widening to previously unaffected areas and a remorseless increase both in the prevalence and degree of drug resistance. Chloroquine-resistant *P. falciparum* now predominates in Southeast Asia, South America and increasingly in Africa. Sulfadoxine-pyrimethamine resistance is widespread in Asia and South America, and increasingly in Africa (Roper *et al.*, 2005). Southeast Asia is now well-known as the epicentre for multi-drug resistant *P. falciparum* malaria (Yeung *et al.*, 2004). Chloroquine and the alternative sulfadoxine-pyrimethamine are cheap, safe and previously highly effective. By mechanisms such as mutations in target genes (sulfadoxine-pyrimethamine), decreased accumulation of a drug within the parasite (including increase in efflux – quinolines) and upregulation of target genes (*P. falciparum*

multiple drug resistance (Pfmdr) gene); these drugs are increasingly being rendered useless in the treatment of malaria. Factors affecting parasite resistance can be categorized as pharmacological, host parasite and operational (Figure 29; Olliaro, 2005).

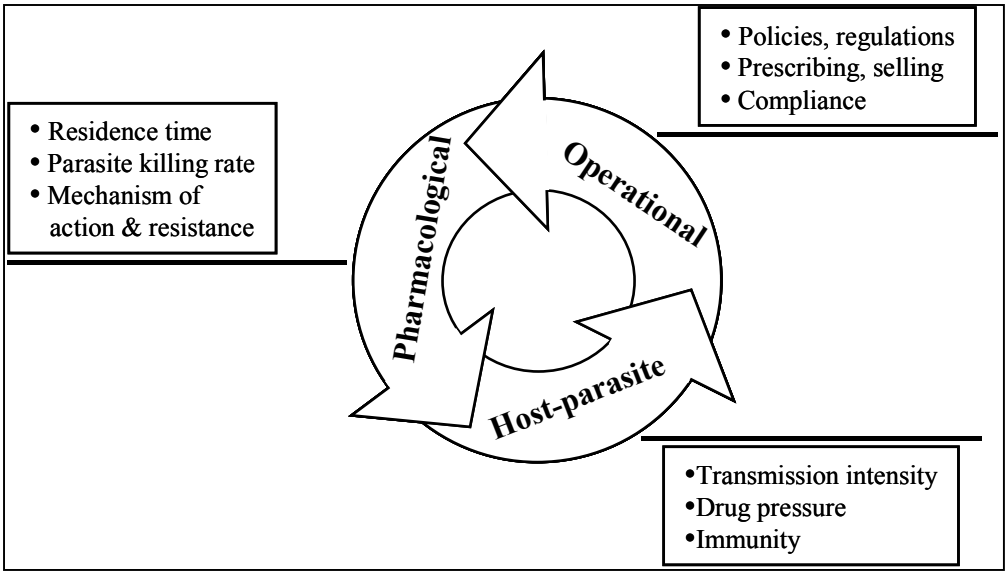


Figure 29: Factors affecting parasite resistance.
Varieties of factors contribute to, and have an impact on, the development of resistance in the malarial parasite, including pharmacological, host-parasite, and operational factors. Pharmacological factors include the drug's pharmacokinetic and pharmacodynamic characteristics, as well as its intrinsic propensity to generate resistance. Host-parasite factors include epidemiology and transmission intensity (the higher the entomological inoculation rate, the higher the chance of being infected and diseased early in life but also the sooner immunity develops and the more diverse the genetic pool; in areas of low transmission, resistance, once established tends to spread more rapidly) and operational aspects (inadequate drug treatment policies, irrational prescribing and drug use, uncontrolled drug market, counterfeit products, and noncompliance) (adapted from Olliario, 2005).

With the exception of the artemisinins, resistance to all known antimalarials has been reported. Given the inexorable spread of drug resistance, and until the development of an effective antimalarial vaccine, the search for effective, safe, and affordable drugs for falciparum malaria is one of the most pressing health priorities worldwide. New antimalarial drugs must meet the requirements of rapid efficacy, minimal toxicity and low cost.

5.1 The glyoxalase system

In an initial development project for new drugs with new mechanisms of action against the *P. falciparum* parasite, the glyoxalase system was exploited for rational drug design. Over

the last years the glyoxalase system has received increasing attention as a chemotherapeutic target for antitumour drug development. Several compounds interfering with the glyoxalase system have been shown to inhibit the growth of cancer cells *in vitro* and *in vivo* (Hamilton and Creighton, 1992; Creighton *et al.*, 2003). Blood stages of the malarial parasite *P. falciparum* are highly proliferative and of high glycolytic activity thus depending on the glyoxalase system for the detoxification of methylglyoxal. Inhibition of this system within the parasite therefore provides a promising chemotherapeutic strategy (Thornalley *et al.*, 1996).

As reported in this thesis, a complete glyoxalase system in *P. falciparum* comprising at least one GloI and two GloII isoenzymes has been characterized and direct structural and functional comparisons with the isofunctional human host enzymes have been carried out.

cGloI and cGloII seem to be localized in the cytosol and act together as the major defense line against toxic 2-oxoaldehydes. In direct comparison with the respective host enzyme, cGloI shows a three times lower K_m for methylglyoxal-GSH and a slightly higher specific activity. Also the K_m of cGloII is by a factor of at least 2 lower than for human GloII and the k_{cat} value is more than two times higher. This indicates the need for an efficient methylglyoxal detoxification in the malarial parasites. Both glyoxalases start to act in the lower micromolar range. The involvement of Tyr185 in the catalysis of cGloII was studied by site directed mutagenesis. In analogy to human GloII (Ridderström *et al.*, 2000) Tyr185 was proven to contribute to substrate binding as reflected by a 2.5 fold increase in K_m.

tGloII carries a putative apicoplast import sequence and also GILP is preceded by a targeting sequence which might direct the protein to the apicoplast and/or the mitochondrion. This fact points to the possibility that the apicoplast, a bacteria derived cell organelle of *Plasmodium*, possesses glyoxalase activity and pathways generating toxic 2-oxoaldehydes. The catalytic properties of tGloII are comparable to human GloII. For GILP, however, no typical glyoxalase activity could be determined the physiological substrate remaining unknown. This fact is of particular interest since GILP is only present and highly conserved in *Plasmodium* species. Highest sequence identities with proteins from other species were only 25% with *A. thaliana*, other plants having a monomeric large GloI, and photosynthetic bacteria, which have the dimeric form of small GloI. These observations strongly suggest GILP as a promising target for the development of specific antiparasitic drugs and a diagnostic tool for clinical and epidermiological studies. Furthermore, they point to the "green origin" of *Plasmodium*. The function of GILP will be studied in further detail.

As a basis for further inhibitor development, different S-(*N*-hydroxy-*N*-arylcarbamoyl) glutathione derivatives were tested as *P. falciparum* GloI and GloII inhibitors. The compounds acted as strong competitive inhibitors of both Glo isoenzymes – on cGloI in the nanomolar range and on tGloII and cGloII in the micromolar range (Table 5). The inhibitors had originally been synthesized as inhibitors of GloI and slow substrates of GloII based on the very low GloII activity in tumour cells compared to normal cells (Murthy *et al.*, 1994). The fact that the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones are substrates for mammalian glyoxalase II but not for *P. falciparum* glyoxalases II indicates that these compounds might selectively inhibit human glyoxalases since normal human cells – in contrast to malarial parasites - would be able to hydrolyse the inhibitor. Tight binding of the inhibitors to the enzymes arises in part from mimicking the stereoelectronic features of the enediol intermediate formed from the methylglyoxal-glutathione-hemithioacetal that acts as a substrate for GloI and also in part by the interaction of the N-aryl substituent with a hydrophobic pocket at the active site of GloI (Cameron *et al.*, 1999a). The latter reason is clearly supported by the increase in binding affinity with increase in hydrophobicity of the inhibitors which is further supported by our data. An alignment of the amino acid sequences of human GloI and cGloI showed that 80% of the residues involved in the composition of the hydrophobic binding pocket of human GloI are conserved in cGloI (Figures 13 and 18B). HBPC-GSH showed a lower K_i value for cGloII than for human GloII and could thus serve as a starting point for the development of more selective inhibitors.

Unexpectedly, S-p-bromobenzylglutathione, the diethyl ester prodrug form of which has been demonstrated to inhibit the growth of *P. falciparum in vitro* (Thornalley *et al.*, 1994), is a weak cGloI inhibitor with a K_i of 20 µM. A much stronger competitive inhibition is observed for human GloI the K_i value being in the range of 0.17 µM (Murthy *et al.*, 1994; Aronsson *et al.*, 1981; and results from this study). This strongly indicates that the antiparasitic effects of the compound are not primarily based on the inhibition of the parasite glyoxalase system but of other enzymes, or on interference with the human methylglyoxal detoxification in red cells.

With the characterization of a complete, functional, and most likely essential glyoxalase system in *Plasmodium falciparum* a novel potential target for antimalarial drug development has become accessible. First inhibitor studies revealing inhibition in the nanomolar range and the identification of a novel GloI-like enzyme which is likely to be unique for malarial parasites represent most promising steps towards the possibility of

specific interference with this pathway. Furthermore, the emergence of suitable prodrug forms of the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones which mask their diacid nature thus allowing membrane permeation are most encouraging for successful drug development.

One great advantage for exploiting the glyoxalase system of *P. falciparum* as a therapeutic target is that the host enzyme is also being targeted for the development of drugs for cancer. As a result, the cost of plasmodial antiglyoxalase drug discovery can be greatly reduced because initial work directed towards more profitable targets, have been undertaken. Most of the inhibitors used in this study were based on previous knowledge acquired from the application of the inhibitors on the glyoxalase system of cancer cells. In fact, the S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones were kindly provided to us by Professor Donald J. Creighton (Maryland, USA) who is involved in the design and development of new classes of tumoricidal agents that specifically target the glyoxalase detoxification pathway in order to induce elevated concentrations of cytotoxic methylglyoxal in tumour cells. More potent bivalent transition state analogs of S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones have shown K_i values as low as 1 nM for human glyoxalase I. All presently known glyoxalase inhibitors – bivalent and monovalent analogs of S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones, S-aryl and S-alkyl GSH derivatives, dicarbonyl GSH derivatives – coupled with the to-be-known structures of *P. falciparum* glyoxalases would serve as lead structures in the design of *P. falciparum* selective glyoxalase inhibitors. Prodrug strategies for the efficient delivery of inhibitors into plasmodial cells both *in vitro* and *in vivo* also stand to benefit.

5.2 The thioredoxin system

PfTrxR like human TrxR belongs to the high molecular weight group of TrxRs which contain an FAD moiety linked to an internal redox center and another C-terminally located redox center in each subunit of the homodimeric protein (Williams *et al.*, 2000). Electrons are shuttled from the FAD via an internal redox-active disulphide to the flexible C-terminally located redox center of the other subunit in the dimeric enzyme. The reduced C-terminal active site is then highly accessible for reducing substrates. However, hTrxR like other mammalian TrxRs contains a highly reactive selenocysteine residue at its C-terminal redox site. The fact that – in spite of the high reactivity of the selenocysteine-containing hTrxR – a selective PfTrxR inhibition could be achieved, points to the involvement of other binding moieties than the C-terminal redox center.

To study the mechanism of inhibition in more detail different approaches were made. For the most interesting compounds **1**, **2**, and **3** the inhibition of the closely related enzyme glutathione reductase were tested and K_i values and the type of inhibition with respect to the physiological substrates Trx and NADPH were determined. GR is a homodimeric FAD-dependent enzyme which is structurally and mechanistically closely related to TrxR. The most prominent difference between the two enzymes is the absence of the second, the C-terminal, redox-active site in GR (Williams *et al.*, 2000). In GR the substrate glutathione is directly reduced at the N-terminal redox center. PfGR has been well characterized and closely resembles its human counterpart. The results obtained on hGR and PfGR indicate that at inhibitor concentrations leading to 50 % PfTrxR inhibition (namely 2 μM, 2 μM, and 0.5 μM) compounds **1-3** lead to quite efficient inhibition between 18 % and 81 % for hGR and between 39 % and 69 % for PfGR. This result further supports the theory that the inhibitors do not significantly interact with the C-terminal redox center of TrxR but rather with other structural motifs or amino acid residues, possibly the intersubunit region, which are similar in PfTrxR and GRs.

All three compounds showed uncompetitive inhibition on PfTrxR with K_i values in the submicromolar range. This indicates that the inhibitors bind to one of the enzyme-substrate intermediate complexes that occur during catalysis, as previously observed for 1-chloro-2,4-dinitrobenzene with hGR (Bilzer *et al.*, 1984).

In the earlier attempt to selectively inhibit PfTrxR with mannich bases, irreversible inactivation of the PfTrxR by bisalkylation of α,β-unsaturated mannich bases of the C-terminal thiols was postulated after observation of competitive inhibition with DTNB (Davioud-Charvet *et al.*, 2003). The inhibitors in the present study act uncompetitively and to approximately equal extents in the presence of both physiological substrates of PfTrxR. All compounds tested were active on malarial parasites in the low micromolar range. Taken together, these data indicate that specificity for PfTrxR over hTrxR can be achieved by inhibitors that bind differentially to the enzyme-substrate complex. A possible binding site of these inhibitors is the interface between the two subunits of the enzyme – a hypothesis that should be verified and utilized for further inhibitor development.

5.3 Methylene blue in antimalarial chemotherapy

Methylene blue has been employed as a lead compound for antimalarial research for over a century as antimalarial quinolines like chloroquine and amodiaquine are being synthesized based on modification of the MB chromophore (Wainwright and Amaral, 2005). Use of MB in the treatment of malarial patients contributed to the birth of chemotherapy in the late 1800s. Toxicity reports after intake of MB led to its withdrawal as an antimalarial in the mid 1900s especially as other active and less toxic drugs like chloroquine and quinine became available at the time. With the increasing development of resistance by parasites to these quinolines as well as other antimalarial drugs, a renaissance of interest in the antimalarial properties of MB has been developed. Toxicity episodes after MB consumption are now highly linked to persons with certain levels of glucose-6-phosphate dehydrogenase deficiency. Several phenothiazinium derivatives of MB like azure B and new methylene blue have been developed (Atamna *et al.*, 1996). These derivatives are however more potent as antibactericidal drugs than as antimalarials (Vennerstrom *et al.*, 1995; Wainwright *et al.*, 1998). Intrinsically, MB is promising as antimalarial drug acting *in vitro* in the nanomolar range and in patients in the micromolar range (Atamna *et al.*, 1996; Mandi *et al.*, 2005).

Stage specificity experiments indicated that MB sensitivity parallels the shift in metabolic processes during development of the parasite. The developmental cycle of *P. falciparum* in erythrocytes lasts 48 hours, involving a gradual progression from early to late rings (0 – 24 h), small, pigmented to large uninuclear trophozoites (24 – 38 h) and binucleate to segmenting schizonts (38 – 48 h). As indicated by the studies on stage specificity of MB action, young ring stages of *P. falciparum* with low metabolic rates show the highest susceptibility to the drug while the trophozoites and schizonts which are involved in protein synthesis and nuclear division, respectively, show lower susceptibilities. The differences in IC_{50} values are as high as a factor of 8.

In addition, the uptake of MB into various compartments of PRBC and NPRBC was studied (Table 10). The half-life of MB in the blood after intravenous administration was estimated to be 5.25 h (Peter *et al.*, 2000). Thus in cell culture it is likely to be much longer. The MB concentrations in the medium of PRBC were repeatedly below the detection limit of 2 nM whereas the medium of NPRBC showed concentration dependent MB levels. This indicates that MB is taken up particularly by PRBC and is thus available for antiparasitic activity. This hypothesis is further supported by the fact that in the parasite lysates a higher MB concentration was determined than in the medium. In the parasite

membrane pellet the accumulation was even more pronounced (factor 9-12). Lysates of PRBC and NPRBC had MB concentrations below 4 nM. Thus, the data demonstrate selective uptake of MB by malarial parasites and indicate that concentrations required for efficient turncoat inhibition of PfGR can be reached in *Plasmodium*.

The activity of MB against *P. falciparum* glutathione reductase (PfGR, Farber *et al.*, 1998), led to its recent suggestion as an adjuvant drug for use with chloroquine (Becker *et al.*, 2003) as chloroquine resistant parasites of *P. falciparum* have been shown to possess higher amounts of reduced GSH. Moreover, double drug conjugates of CQ and the selective PfGR inhibitor 6-[2-(3-methyl)-naphthoquinolyl]hexanoic acid restricted the growth of *Plasmodium* species both *in vitro* and *in vivo* (Davioud-Charvet *et al.*, 2001). The present study on the antimalarial effects of MB was triggered by the fact that a clear advantage of the MB/CQ combination over MB monotherapy could not be observed during field trials in the Nouna district of Burkina Faso (Mandi *et al.*, 2005; Meissner *et al.*, 2005). *In vitro* combinations of MB with CQ as well as with other clinically-used antimalarials were therefore tested in a search for potential clinical drug combination therapies and also to facilitate the design of future field studies.

The combinations of MB with mefloquine or quinine were additive whereas the combinations of MB with chloroquine (Figure 25) were antagonistic. A significant positive correlation was detected between the IC_{50} values of CQ and MB ($r = 0.701$, $P = 0.0001$) indicating an increased likelihood for cross resistance. The antagonistic action of chloroquine and methylene blue were also shown for the chloroquine sensitive strain 3D7 (Figure 25). The mode of action of CQ involves the inhibition of haem polymerization into non-toxic haemozoin (Ridley *et al.*, 1997). MB has been reported to inhibit growth of *P. falciparum* through pleiotropic mechanisms (Schirmer *et al.*, 2003) involving nucleic acid intercalation, food vacuole basification, parasite redox cycle interference and haem polymerization inhibition (Atamna *et al.*, 1996) in addition to its inhibitory effects on PfGR. Thus one could speculate that a similar mode of action concerning haem-polymerization explains the antagonistic effects of the drugs. Since MB is taken up by the parasite passively, a competition for uptake mechanisms is unlikely to explain this phenomenon. The effect of MB on glutathione reduction (Ginsburg and Golenser, 2003) is probably reflected in the observation that the antagonism between MB and CQ is less pronounced for the CQ resistant strain K1 than for the sensitive strain 3D7.

However, synergistic drug combinations were observed upon combination of MB with all three artemisinins i.e. artemisinin, artesunate and artemether. However, the highest degree

of synergism was observed with the artemisinin/MB combination (Table 12 and Figure 26) at a fixed concentration ratio of 3:1.

Artemisinins are now the focus of international attention as the frontline defense against resistant falciparum malaria (Yeung *et al.*, 2004). They represent a new family of compounds, with a novel mode of action and faster antimalarial activity than any of the other drugs (Nosten and Brasseur, 2002). They have proven themselves robust over at least a decade of consistent use in Asia, in terms of effectiveness, safety and in the lack of any documented drug resistance (Haynes and Krishna, 2004). To date, there have been no reported cases of stable, clinically relevant genetic resistance to artemisinin, although tolerance can be produced through repeated *in vitro* culture of parasites in the presence of the drug (Meshnick, 2002).

The evolution of drug resistance is an inevitable consequence of genetics and natural selection when drugs are used against parasites. As effective and robust as the artemisinin are today, it is only a matter of time before genetically resistant strains emerge and spread. The key, therefore, to preserving the artemisinins is to eliminate their routine use as monotherapies, and to treat patients with uncomplicated malaria (the vast majority of cases) with artemisinin-based combination therapies (ACTs) instead (Woodrow *et al.*, 2005, Khan *et al.*, 2004). ACTs constitute a combination of an artemisinin derivative with another unrelated anti-malarial drug, ideally coformulated in a single pill so that individual drugs can not be knowingly or inadvertently used as monotherapies. Combining drugs in this way – already a practice in the treatment of tuberculosis and HIV/AIDS infection, minimizes the likelihood that a single parasite with drug resistance will propagate and spread.

According to Nosten and Brasseur (2002), an ideal drug combination should have the following characteristics.

- Components have different modes of action
- No interactions
- Short course regimens (3 days at most)
- At least one drug which clears asexual forms rapidly
- At least one drug with long half-life (> 4 days)
- Well tolerated, low toxicity
- Broad spectrum of action (including against gametocytes)
- Coformulation possible
- Inexpensive

The artemisinin/MB combination at a fixed dose ratio meets all but one of the above characteristics. In this combination, neither artemisinin nor MB has a half-life greater than 4 days. The half life of the artemisinins is usually very short with that of artemisinin being only 2-3 hours (Davis *et al.*, 2005). The half life of MB is also not much longer, only 6 hours (Peter *et al.*, 2000). However, in Africa where transmission rates of the disease are high, a partner drug for the artemisinins with a very long half life may be unsuitable as subsequent infections could occur before the partner drug is cleared from the circulation but after the artemisinins have long been cleared (Ridley, 2002). Resistance is more likely to develop against the partner drug in such a situation. In the artemisinin/MB, the parasites would be exposed to subtherapeutic concentrations for only a short time, thereby deterring the emergence of resistance to any of the partner drugs as the selection pressure for resistance would be lowered. A similar currently-in-use drug combination Lap-dap – consisting of chlorproguanil and dapsone – whereby both partner drugs also have short halflives showed a much slower parasitic resistance development in clinical studies in East Africa in comparison to the similarly acting sulfadoxine-pyrimethamine drug combination with longer halflives (Wiesner *et al.*, 2003). However, although the short halflives of both artemisinin and MB would warrant longer treatment duration, the period of treatment could greatly be reduced in comparison to artemisinin monotherapy thereby reducing the problem of compliance to treatment. Otherwise, the other aspects of an ideal drug combination can be reached by the proposed artemisinin/MB combination. Both partner drugs have different modes of action and according to the correlation analysis of their IC values, there is no form of interaction between artemisinin and MB as both drugs are highly negatively correlated. This indicates a less likelihood of cross resistance development between MB and artemisinin.

Spread of drug resistance is facilitated by the exposure of malarial parasites to sub-therapeutic levels of antimalarial drugs, that kill sensitive parasites but allows parasites with a resistance mutation to survive and reproduce. With this in mind, the following instructions for application of the Artemisinin/MB combination are recommended in order to reduce the risk of a possible emergence and spread of drug resistance to it.

- The combination should be taken at a sufficient dose and over a sufficient duration.
- Restriction of usage to patients with a definitive diagnosis of malaria.

- Restriction of usage to patients who are compliant with respect to the treatment regimen.
- Sale of the drug only in pharmacies to avoid the use of ineffective duplicate formulations

5.4 Glutathionylation of PfTrx

The Cys54 residue of PfTrx was shown to be glutathionylated on two different peptide fragments obtained upon tryptic digest of GSSG-treated PfTrx. As was the case with human Trx (Casagrande et al., 2002), a possible crosstalk between the thioredoxin and glutathione systems could be shown to exist in *P. falciparum*. Involvement of the redox active cysteines (Cys41 and Cys44) in glutathionylation could not be shown, implying that modification of the functions of PfTrx upon glutathionylation is unlikely. However, involvement of the cys54 in dimerization or polymerization of PfTrx as well as in interaction with other proteins can not be ruled out.

6 References

6.1 General references

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6.2 Websites

Factors Contributing to Development and Spread of Drug Resistance (www.tulane.edu/~wiser/protozoology/notes/malaria.html#treat)

Malaria Manual (http://www.up.ac.za/academic/biochem/afr/malaria/MANUAL.pdf)

Malaria Vaccine Initiative (http://www.malariavaccine.org)

Plasmodium genome resource (http://plasmodb.org/)

Plasmodium falciparum genome (www.ncbi.nlm.nih.gov/Malaria/plasmodium-blcus.html)

Online biological tools (www.justbio.com)