

**Real time imaging of the glutathione redox potential
and import of host peroxiredoxin 2 in the malarial
parasite *Plasmodium falciparum***



**A thesis submitted to the Faculty of Biology and Chemistry
(FB 08) in fulfilment of the requirements of the
Doctor of Science Degree of Justus Liebig University
Giessen, Germany**

By

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Kampala, Uganda

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Berichte aus der Biochemie

Denis Kasozi Matovu

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and import of host peroxiredoxin 2 in the malarial
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Declaration

I declare that this thesis is my original work and that it has not been previously presented in this or any other university for any degree. I have complied with the principles of good scientific practice as laid down in the “Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis”.

Giessen, 8th May 2012

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Denis Kasozi Matovu

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Dedication

To my family

The thesis was presented for examination on the 22nd March 2012 to the faculty of Biology and Chemistry of the Justus Liebig University Giessen, Germany and the thesis defense was on the 3rd May 2012. The thesis was supervised by Prof. Dr. med. Katja Becker and Prof. Dr. rer. nat. Richard Göttlich.

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List of Publications

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Abstracts from conferences and scientific meetings

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5. **Kasozi DM**, Zocher K, Coulibaly B, Adler H, Dandekar T, Fritz-Wolf K, Becker K Schirmer H (2009). Methylene blue as a synthetic analogue of pyocyanin. Effects on gametocytes and blood schizonts of *P. falciparum* "Health Research in Africa". Second Scientific Days of Centre de Recherche en Santé de Nouna. Centre Badeya, Nouna, Burkina Faso, 08th -10th of December 2009.

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List of Abbreviations

• ACTs	Artemisinin-based combination therapies
• AMA-1	Apical membrane antigen 1
• AQ	Amodiaquine
• ART	Artemisinin
• ATM	Artemether
• ATP	Adenosine triphosphate
• ATS	Artesunate
• APS	Ammonium persulfate
• BFA	Brefeldin A
• BSA	Bovine serum albumin
• BSO	Buthionine sulfoximine
• CDNB	1-Chloro-2, 4-dinitrobenzene
• cpm	Counts per minute
• CQ	Chloroquine
• CQS	Chloroquine sensitive
• CQR	Chloroquine resistant
• Clat	Clathrin box motif
• CTD	Cytochalasin
• CS	Circum-sporozoite
• Da	Dalton
• DHFR	Dihydrofolate reductase
• DHPS	Dihydropteroate synthase
• Diamide	Diazenedicarboxamide
• dNTP	Deoxynucleotide triphosphate
• DDT	Dichloro-diphenyl-trichloroethane
• DMSO	Dimethyl sulfoxide
• DNA	Deoxyribonucleic acid
• DNase	Deoxyribonuclease
• DTT	1, 4-Dithiothreitol
• DYN	Dynasore
• E_{GSH}	Glutathione redox potential
• EDTA	Ethylenediaminetetraacetic acid
• EXP-1	Exported antigen 1
• FP IX	Ferriprotoporphyrin IX
• γ -GCS	γ -Glutamylcysteine synthetase
• GR	Glutathione reductase
• GLURP	Glutamate-rich protein
• Grx1	Glutaredoxin-1
• GSH/GSSG	Glutathione (reduced /oxidized)
• HF	Halofantrine

• H ₂ O ₂	Hydrogen peroxide
• hPrx2	human Peroxiredoxin 2
• IC	Inhibitory concentration
• IFA	Immunofluorescence assay
• ITNs	Insecticide treated nets
• IPTG	Isopropyl- β -Dthiogalactopyranoside
• JAS	Jasplankinolide
• kDa	Kilodalton
• LB	Luria-Bertani medium
• LSA1	Liver stage antigen 1
• MACS	Magnetic activated cell sorting
• MB	Methylene blue
• MSP-1	Merozoite stage protein 1
• μ g	Microgram
• μ l	Microliter
• μ M	Micromolar
• mg	Milligram
• ml	Milliliter
• mM	Millimolar
• MNA	Menadione
• MNS	Monensin sodium salt
• MQ	Mefloquine
• NEM	<i>N</i> -Ethylmaleimide
• NPRBC	Non-parasitized red blood cells
• Ni-NTA	Nickel nitrilotriacetic acid
• PCR	Polymerase chain reaction
• PBS	Phosphate buffered saline
• PfCRT	<i>P. falciparum</i> chloroquine resistance transporter
• PfMDR1	<i>P. falciparum</i> multi-drug resistance transporter 1
• PfMRP1	<i>P. falciparum</i> multi-drug resistance-associated protein
• PMSF	Phenylmethylsulfonyl fluoride
• PQT	Paraquat
• PRBC	Parasitized red blood cells
• Prx	Peroxiredoxin
• PVDF	Polyvinyl difluoride
• PYO	Pyocyanin
• OD	Optical density
• roGFP	Redox sensitive green fluorescent protein
• RBC	Red blood cells
• ROS	Reactive oxygen species
• RNS	Reactive nitrogen species
• rpm	Rounds per minute

- SIN-1 3-Morpholinosydnonimine hydrochloride
- SIS Sorting and internalization signal
- SDS Sodium dodecyl sulphate
- SDS-PAGE Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
- SNP Sodium nitroprusside
- SP Sulphadoxine-pyrimethamine
- TBE Tris-borate EDTA
- TCTP Translationally controlled tumour protein
- TE Tris-EDTA
- TEMED N,N,N',N'-Tetramethylethylenediamine
- Trx Thioredoxin
- TrxR Thioredoxin reductase
- TSS Tyrosine-based sorting signal
- U Unit of enzyme activity ($\mu\text{mol}/\text{min}$)
- WHO World Health Organisation

Summary

Malaria caused by the most lethal *Plasmodium* species, *P. falciparum*, remains a major global health problem to almost half the world's population. With the lack of a vaccine and the emergence of both drug and insecticide resistance, the identification of novel drug targets and the development of rationally effective combination therapies are urgently required. To contribute to the efficient control or the elimination of malaria, three approaches to support novel drug discovery strategies were explored in this thesis.

First, the role of the glutathione redox potential (E_{GSH}) in the mechanism of drug action and resistance in malaria parasites was systematically studied. The E_{GSH} in *P. falciparum* influences drug action and resistance by detoxification of reactive oxygen and nitrogen species. However, real-time determination of the compartmentalization of E_{GSH} has been limited so far because conventional approaches disrupt sub-cellular integrity. Using a E_{GSH} biosensor, comprising human glutaredoxin-1 linked to a redox sensitive green fluorescent protein (hGrx1-roGFP2), the basal cytosolic E_{GSH} as well as the antimalarial drug-induced changes in E_{GSH} were determined in drug-sensitive (3D7) and resistant (Dd2) strains of *P. falciparum*. By confocal microscopy, the ability of hGrx1-roGFP2 to rapidly react to changes in E_{GSH} due to oxidative and nitrosative stress was demonstrated. Importantly, the cytosolic basal E_{GSH} of 3D7 and Dd2 strains was found to be -314.2 ± 3.1 mV and -313.9 ± 3.4 mV, respectively, which is suggestive of a highly reducing cytosol. Among the tested antimalarial drugs, only methylene blue (MB) rapidly, on scale of minutes, oxidized glutathione (GSH). In contrast, quinoline and artemisinin based drugs required 24 h to significantly change the E_{GSH} thus suggesting downstream effects on GSH metabolism. Notably, following 24 h incubation at 4-fold IC_{50} , artemisinin derivatives exerted, by far, the strongest impact on E_{GSH} . In accordance with the higher levels of reduced GSH in Dd2 than 3D7 parasites, the effects on E_{GSH} were more pronounced in the 3D7 than in the Dd2 strain which indicates a role of GSH in drug action and resistance. In conclusion, for the first time, the applicability of a highly specific E_{GSH} biosensor for spatiotemporal measurement of the intracellular E_{GSH} , in real time, in *P. falciparum* was established, illustrating its feasibility for the use in other parasites and pathogens (Kasozi *et al.*, 2012 submitted).

Secondly, the mechanism of uptake of host human peroxiredoxin 2 (hPrx2) into *P. falciparum* and its inhibition were investigated in order to identify new drug targets. During its erythrocytic cycle, *P. falciparum* imports several host proteins to play crucial roles in specific processes of parasite biochemistry, physiology, and antioxidant defense. However, the molecular mechanism of the uptake of host erythrocytic proteins remains elusive. By bioinformatic analysis of host proteins (~ 30) significantly abundant in parasite protein lysates that exhibited specific abundance profiles across the intraerythrocytic cycle, 4 endocytic vesicle associated motifs were identified including: the sorting and internalization signal (SIS), the tyrosine-based sorting signal (TSS), the clathrin box motif (Clat) and the WXXXY|F motif. Surprisingly, TSS was found 3-5 times and in contrast SIS and Clat occurred on average 1-2 times in nearly all proteins with the exception of superoxide dismutase. Additionally, the WXXXY|F motif was identified in the β -subunit of hemoglobin, biliverdin reductase B, and carbonic anhydrase I. Notably, hPrx2 had all three endocytic

vesicle associated motifs namely SIS (¹⁵⁴VDEALRL¹⁵⁹), TSS (³⁷YVVL⁴⁰, ¹¹⁵YGVL^{118,126}YRGL¹²⁹), and Clat (¹²⁹LFIID¹³³). To validate the role of endocytic vesicle associated motifs in uptake of proteins to different compartments/organelles, several mutants of hPrx2 were generated. By deletion mutagenesis, SIS and Clat mutants of hPrx2 were constructed. By site directed mutagenesis, active site mutants as well as N- and C-terminal deletion mutants of hPrx2 were generated. Next all hPrx2 mutants were heterologously over-expressed in *E. coli* and added to cultures of *P. falciparum*. Notably, these endocytic vesicle associated motifs may have differential effects on the uptake of hPrx2. Furthermore, by Western blot analysis of parasite lysates after 24 h incubation at a concentration of 4 x IC₅₀, actin inhibitors [cytochalasin D (CTD) and jasplakinolide (JAS)], alkalizing agents [monensin (MNS) and ammonium chloride (NH₄Cl)], brefeldin A (BFA), paraquat (PQT), and the ATP depleting agent sodium azide (NaN₃), were found to differentially inhibit the uptake of hPrx2 into *P. falciparum*. The IC₅₀ values of CTD, JAS, MNS, NH₄Cl, NaN₃, BFA, and PQT against the 3D7 strain were determined to be 13.1 nM, 73.5 nM, 1.01 nM, 2050 nM, 209 nM, 1310 nM and 45 μM, respectively. After 24 h of incubation MQ, ART, CTD, JAS, DYN, PQT MNS, CQ and NaN₃ inhibited uptake and digestion of host hPrx2. By contrast, NH₄Cl increased uptake of hPrx2 while BFA had no effect on inhibition of hPrx2 uptake. Together the evaluation of endocytic vesicle associated motifs may lead to the development of novel drugs that inhibit uptake of proteins into *P. falciparum*.

Thirdly, the *in vitro* gametocytocidal activity of MB was evaluated. To eliminate malaria, clinical studies and mathematical models predict that artemisinin combination therapies (ACT) must incorporate a drug with gametocytocidal activity to block the transmission of *P. falciparum*. Until now, ACTs have efficacy against young but induce no or only moderate inhibition of mature gametocytes. Notably, the IC₅₀ (95% confidence interval) of MB against young (stage II-III) and mature (stage IV-V) gametocytes was found to be 33.8 (32.1-35.7) nM and 59.5 (37.3-94.8) nM, respectively, indicating that MB has significant activity against all stages of gametocyte development. To eliminate malaria, incorporation of MB into currently used ACTs would reduce transmission of *P. falciparum* (Kasozzi *et al.*, 2011). In addition, a simple, rapid SYBR green-1 fluorescence-based gametocytocidal assay was developed which should speed up the characterization of transmission blocking drugs or drug candidates.

ZUSAMMENFASSUNG

Malaria stellt eine Gefahr für fast die Hälfte der Weltbevölkerung dar. Die schwerste Form der Malaria, *Malaria tropica*, wird durch *Plasmodium falciparum* verursacht. Da Malariaparasiten zunehmend Resistenzen gegen verfügbare Medikamente entwickeln und bisher keine effektive Vakzine zur Verfügung steht, werden neue Medikamente dringend benötigt. Im Rahmen dieser Arbeit wurden drei Fragestellungen untersucht, die für die Entwicklung von neuen Therapieansätzen von grundlegender Bedeutung sind.

Der erste Schwerpunkt dieser Arbeit beschäftigt sich mit der systematischen Untersuchung der Rolle des Redoxpotentials von Glutathion (E_{GSH}) für die Wirkung von und der Resistenz gegenüber Antimalariamedikamenten. In *P. falciparum* beeinflusst das Redoxpotential von Glutathion die Effektivität von Medikamenten sowie Resistenzen durch die Detoxifikation von reaktiven Sauerstoff- und Stickstoffspezies. Bisher war es nicht möglich, E_{GSH} in verschiedenen Kompartimenten des Malariaerregers in Echtzeit zu untersuchen, da konventionelle Experimente mit einer Zerstörung der subzellulären Integrität einhergehen. Durch den Einsatz eines E_{GSH} -Biosensors bestehend aus humanem Glutaredoxin 1 und einem redox-sensitiven *green fluorescent protein* (hGrx1-roGFP2) konnten wir das basale zytosolische E_{GSH} untersuchen. Außerdem haben wir die Änderungen des E_{GSH} nach der Behandlung von sensitiven (3D7) und resistenten (Dd2) Malariaparasiten mit Antimalariamedikamenten analysiert. Mittels konfokaler Lasermikroskopie konnten wir zeigen, dass hGrx1-roGFP2 Änderungen des E_{GSH} als Antwort auf oxidativen und nitrosativen Stress schnell detektiert. Das basale E_{GSH} im Cytosol der *Plasmodium*-Stämme 3D7 und Dd2 beträgt -314.2 ± 3.1 mV und -313.9 ± 3.4 mV, was auf stark reduzierende Bedingungen im Cytosol hinweist. Von den getesteten Antimalariamedikamenten induzierte nur Methylenblau innerhalb weniger Minuten eine Oxidation von Glutathion. Quinolin und Artemisinin führten nach 24 Stunden zu einer Änderung des E_{GSH} , was auf *Downstream*-Effekte auf den Glutathionmetabolismus zurückzuführen sein könnte. Interessanterweise zeigten sich die stärksten Effekte auf das E_{GSH} nach einer 24-stündigen Inkubation mit Artemisinin. In Übereinstimmung mit höheren Konzentrationen an reduziertem Glutathion in dem *P. falciparum* Stamm Dd2 im Vergleich zu 3D7 waren auch die Auswirkungen der Medikamente auf das E_{GSH} im Stamm 3D7 stärker ausgeprägt als im Stamm Dd2, was auf eine Rolle von Glutathion für die Wirkung der Medikamente hinweist. Im Rahmen dieser Arbeit konnten wir zum ersten Mal die Anwendbarkeit eines hochspezifischen E_{GSH} -Biosensors zur Messung des intrazellulären Redoxpotentials in *P. falciparum* aufzeigen (Kasazi *et al.*, 2012, eingereicht).

Im zweiten Schwerpunkt dieser Arbeit wurde der Aufnahmemechanismus sowie dessen Hemmung von humanem Peroxiredoxin 2 (hPrx2) in *P. falciparum* untersucht. Während der intraerythrozytären Phase importiert *P. falciparum* verschiedene Wirtsproteine, die spezifische Funktionen für die Biochemie, Physiologie, und die antioxidative Abwehr des Parasiten haben. Allerdings ist bisher nicht bekannt, wie diese humanen Proteine durch den Malariaparasiten aus dem Erythrozyten aufgenommen werden. Durch bioinformatische Analysen der importierten Wirtsproteine wurden vier Motive identifiziert, die mit endozytischen Vesikeln assoziiert sind: das Sortier- und Internalisierungssignal (SIS), das Tyrosinbasierte Sortierungssignal (TSS), das Clathrinbox-Motiv (Clat) und das WXXXV/F-

Motiv. Interessanterweise enthält hPrx2 drei der mit endozytischen Vesikeln assoziierten Motive: SIS (¹⁵⁴VDEALRL¹⁵⁹), TSS (³⁷YVVL⁴⁰, ¹¹⁵YGVL¹¹⁸, ¹²⁶YRGL¹²⁹) und Clat (¹²⁹LFIID¹³³). Um die Funktion dieser Motive für die Aufnahme von Proteinen in verschiedenen Kompartimente zu untersuchen, wurden hPrx2 Mutanten ohne SIS bzw. Clat-Motiv, sowie Mutanten mit verändertem aktiven Zentrum oder N- und C-terminalen Deletionen erstellt, in *E.coli* heterolog überexprimiert, und zu den *P. falciparum* Kulturen dazugegeben. So konnte die Funktion der Motive auf die Aufnahme von hPrx2 in die Parasiten untersucht werden. Außerdem wurde gezeigt, dass Inhibitoren von Actin (Cytochalasin D, Jasplakinolide), alkylierende Verbindungen (Monensin, Ammoniumchlorid), Brefeldin A, Paraquat, und Natriumazid die Aufnahme von hPrx2 in *P. falciparum* hemmen. Die Untersuchung der mit endozytischen Vesikeln assoziierten Motive ist für die Entwicklung von Medikamenten, welche die Aufnahme von Wirtsproteinen durch *Plasmodium* hemmen, von großem Interesse.

Der dritte Schwerpunkt dieser Arbeit beschäftigt sich mit der Wirkung von Methylenblau auf Gametozyten. Klinische Studien und mathematische Modelle schlagen vor, dass Artemisinin in Kombination mit einem auf Gametozyten wirkenden Medikament die Transmission von *P. falciparum* blockieren könnte. Methylenblau hemmt junge (Stadium II-III) und reife (Stadium IV-V) Gametozyten mit einem IC₅₀ von 33.8 nM und 59.5 nM. Methylenblau zeigt daher eine signifikante Aktivität gegen alle Entwicklungsstadien der Gametozyten. Die Verwendung von Artemisinin in Kombination mit Methylenblau könnte daher die Transmission von *P. falciparum* effektiv reduzieren (Kasozzi *et al.*, 2011). Außerdem wurde ein schneller SYBR green-1 Fluoreszenz-basierter Assay entwickelt, der die Untersuchung von Wirkstoffen zur Hemmung der Transmission vereinfacht.