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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from

Kampala, Uganda

March 2012

Berichte aus der Biochemie

Denis Kasozi Matovu

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

D 26 (Diss. Universität Giessen)

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes

Shaker Verlag
Aachen 2012

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.d-nb.de.

Zugl.: Giessen, Univ., Diss., 2012

Printed in Germany.

ISBN 978-3-8440-1072-5
ISSN 1434-5536

Shaker Verlag GmbH • P.O. BOX 101818 • D-52018 Aachen
Phone: 0049/2407/9596-0 • Telefax: 0049/2407/9596-9
Internet: www.shaker.de • e-mail: info@shaker.de

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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Acknowledgements

I wish to sincerely thank Prof. Katja Becker and Prof. Richard Göttlich for supervising my PhD project. I am greatly indebted to Prof. Katja Becker for not only hosting me in her research group but also sharing with me her exceptional experience in the redox metabolism of the malaria parasite *Plasmodium falciparum*. I wish to express my sincere gratitude to Prof. Andreas J. Meyer (Bonn University) for the support with the real time imaging of the glutathione redox potential using the redox sensitive green fluorescent protein as part of my PhD project.

Special thanks go to the entire working group of Prof. Katja Becker. To Stefan Rahlfs, I say thank you for your support throughout the PhD project especially with the cloning of the ten human peroxiredoxin 2 (hPrx2) mutants. To Elisabeth Fischer, thanks for the support with the cell culture experiments. Thanks a lot to Marina Fischer, for the help with expressing hPrx2 mutants. Many thanks go to Timothy Bostick for critically reading my PhD thesis. To the other co-workers of the group, R Iozef, H Prieto, B Ulrike, B Hecker, M Stumpf, D Heinke, I really do appreciate the help received from you. To the other PhD colleagues in the lab, E Jortzik, J Ma, Z Tao, L Wang, J Preuss, J Pretzel, F Mohring, J Bathke and K Zocher, special thanks for all the ideas I have gained from you during our numerous discussions.

Notably, I wish to acknowledge the "Deutscher Akademischer Austauschdienst" (DAAD) for the generous financial support right from the German language course at Speak + Write in Marburg and throughout the PhD program at Giessen University.

I would like to thank in a special way the entire Matovu family for their encouragement throughout the PhD project. I am indebted to our late mother Suzan and all our mothers for the continuous support and encouragement in our careers. Many thanks go to my brothers David, Bosco and sisters Rose, Harriet and Tina. To all our sons (Ronnie, Paul, Nathan, Amos Jude, Kato and Wassawa) and daughter (Suzan), I dedicate to you this thesis to work hard for greater horizons. Special thanks go to the entire family of Eng. J. Kabanga for your inspiration throughout our studies. Last but not least, my profound gratitude goes to all my friends some of whom I met in Marburg and Giessen for the encouragement, support and joy you gave me in all aspects of life.

List of Publications

1. **Kasozi DM**, Rahlfs S, Meyer A, Becker K (2012). Real time imaging of intracellular glutathione redox potential changes in *Plasmodium falciparum. In preparation*

2. **Kasozi DM**, Gromer S, Adler H, Zocher K, Rahlfs S, Wittlin S, Fritz-Wolf K, Schirmer RH, Becker K (2011). The bacterial redox signaller pyocyanin as an antiplasmodial agent: Comparisons with its thioanalog methylene blue. Redox Rep 4:154-65.

3. **Kasozi DM**, Rahlfs S, Becker K (2011). Current aspects of endoperoxides in antiparasitic chemotherapy. In: Drug discovery against apicomplexan parasites. pp. 413 - 430. (Becker K, vol. ed., Selzer P, series ed.) Wiley-Blackwell, Weinheim.

4. Meissner P, Adler H, **Kasozi DM**, Fritz-Wolf K and Schirmer RH (2011). The reducing milieu of parasitized cells as a target of antimalarial agents. Methylene blue as an ethical drug. In: Drug discovery against apicomplexan parasites. pp. 115-136. (Becker K, vol. ed., Selzer P, series ed.) Wiley-Blackwell, Weinheim. ……..
5. Baingana RK, **Kasozi DM**, Garrett D (2008). Application of retinol-binding protein enzyme immunoassay to dried blood spots to assess vitamin A deficiency in a population-based survey: the UDHS 2006. Food Nutr Bull 29:297-305.

Abstracts from conferences and scientific meetings

1. **Kasozi DM**, Rahlfs S, Meyer A, Becker K (2011). Real time imaging of drug induced intracellular glutathione redox potential changes in *P. falciparum.* Abstract T24, 4th GGL Conference on life sciences, Sept. 21-22, 2011.

2. **Kasozi DM**, Rahlfs S, Meyer A, Becker K (2010). Glutathione redox potential measurements using a redox sensitive green fluorescence protein in *P. falciparum.* Abstract P94, 3rd GGL Conference on life sciences, Sept. 29-30, 2010.

3. Zocher K, **Kasozi DM**, Adler H, Schirmer RH, Becker K, Fritz-Wolf K (2009). The redox-active signaler pyocyanin binds to the site of antimalarial phenothiazine drugs in glutathione reductase. Abstract T 18 - DOI: 10.3288/contoo.paper.438. Tri-national GBM Meeting on "Signal transduction and disease" in Aachen, Sept. 27-30, 2009.

4. Adler H, **Kasozi DM**, Zocher K, Coulibaly B, Gromer S, Becker K, Schirmer H (2009). Pyocyanin and its synthetic analogue methylene blue have antimalarial activity against gametocytes and asexual erythrocytic forms of *P. falciparum.* "New Trends in Infectious Disease Research" 5th Joint Ph.D. Students Meeting of the Collaborative Research Centers 544 (Heidelberg), 630 (Würzburg) and 766 (Tübingen) Heidelberg, November 19th-21st, 2009.

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 sulfhoxide
- DNA Deoxyribonucleic acid
- DNase Deoxyribonuclase
- 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_2O_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
- µg Microgram
- µl Microliter
- µM Micromolar
- mg Milligram
- ml Milliliter
- mM Millimolar
- MNA Menadione
- MNS Monensin sodium salt
- MQ Mefloquine
- NEM *N*-Ethylmaleimide
- NPRBC Non-parasitized red blood cells
- Ni-NTA Nickel nitrilotriacetic acid
- PCR Polymerase chain reaction
- PBS Phosphate buffered saline
- PfCRT *P. falciparum* chloroquine resistance transporter
- PfMDR1 *P. falciparum* multi-drug resistance transporter 1
- PfMRP1 *P. falciparum* multi-drug resistance-associated protein
- PMSF Phenylmethylsulfonylfluoride
- 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 (µmol/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 (^{154}VDEALRL^{159}), TSS (^{37}YVVL^{40}; ^{115}YGVL^{118};^{126}YRGL^{129}), and Clat (^{129} LFIID^{133}). 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 IC_{50}, actin inhibitors [cytochalasin D (CTD) and jasplakinolide (JAS)], alkalizing agents [monensin (MNS) and ammonium chloride (NH_4Cl)], brefeldin A (BFA), paraquat (PQT), and the ATP depleting agent sodium azide (NaN_3), were found to differentially inhibit the uptake of hPrx2 into *P. falciparum*. The IC_{50} values of CTD, JAS, MNS, NH_4Cl, NaN_3, 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 NaN_3 inhibited uptake and digestion of host hPrx2. By constrast, NH_4Cl 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 IC_{50} (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* (Kasozi *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 Minute eine Oxidation von Glutathion. Quinolin und Artemisinin führten nach 24 Stunden zu einer Änderungen 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 (Kasozi *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 (^{154}VDEALRL^{159}), TSS (^{37}YVVL^{40}, ^{115}YGVL^{118}, ^{126}YRGL^{129}) und Clat (^{129}LFIID^{133}). 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 IC_{50} 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 (Kasozi *et al*., 2011). Außerdem wurde ein schneller SYBR green-1 Fluoreszenz-basierter Assay entwickelt, der die Untersuchung von Wirkstoffen zur Hemmung der Transmission vereinfacht.