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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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Berichte aus der Biochemie

Monique B. Akoachere

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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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2. Andricopulo, A. D., **Akoachere, M.**, Krogh, R., Nickel, C., McLeish, M. J., Davioud-Charvet, E., Kenyon, G. L., Arscott, D. L., Williams, C. H. Jr. and Becker, K. (2005). Thioredoxin reductase of the malarial parasite *Plasmodium falciparum* – Inhibitor development as a basis for novel chemotherapeutic strategies. *Flavins and Flavoproteins* in press.
3. **Akoachere, M.**, Buchholz, K., Fischer, E., Burhenne, J., Haefeli, W., Schirmer, H. and Becker, K. (2005). *In vitro* assessment of methylene blue on chloroquine sensitive and resistant *Plasmodium falciparum* strains reveals synergistic action with artemisinins. *Antimicrobial Agents and Chemotherapy* **49:** 4592-4597.
4. **Akoachere, M.**, Iozef, R., Rahlfs, S., Deponte, M., Mannervik, B., Creighton, D. J., Schirmer, H., Becker, K. (2005) Characterization of the glyoxalases of the malarial parasite *Plasmodium falciparum* and comparison with their human counterparts. *Biological Chemistry*, **386:** 41-52.
5. Cho-Ngwa, F., **Akoachere, M.** and Titanji, V. P. (2003). Sensitive and specific serodiagnosis of riverblindness using *Onchocerca ochengi* antigens. *Acta Tropica* **89:** 25-32.

Abstracts in Meetings

- **Akoachere, M.**, Buchholz, K., Fischer, E., Burhenne, J., Haefeli, W., Schirmer, H. and Becker, K. (2005). Methylene blue in antimalarial drug combinations. *In vitro* effects on *Plasmodium falciparum* strains.
  54th Annual Meeting of the American Society of Tropical Medicine and Hygiene (ASTMH), Washington D. C., USA.
- **Akoachere, M.,** Iozef, R., Rahlfs, S., Deponte, M., Schirmer, R. H. and Becker, K. (2005). Characterization of the glyoxalases of the malarial parasite *Plasmodium falciparum* and comparison with their human counterparts.
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  15th International Symposium on Flavins and Flavoproteins, Shonan Village, Japan.
- **Akoachere, M. B.,** Iozef, R., Rahlfs, S., Deponte, M., Schirmer, R. H. and Becker, K. (2005). Characterization of the glyoxalases of the malarial parasite *Plasmodium falciparum* and comparism with their human counterparts.
  First Annual BiolMalPar Conference Meeting, EMBL, Heidelberg.
- Rahlfs, S., Iozef, R., **Akoachere, M.**, Schirmer, R. H. and Becker, K. (2004). Glyoxalase I of the malarial parasite *Plasmodium falciparum*: Evidence for subunit fusion.
  Jahrestagung der Deutschen Gesellschaft für Parasitologie, Würzburg.
- **Akoachere, M.**, Iozef, R., Rahlfs, S., and Becker, K. (2003). The glyoxalase system of the malarial parasite *Plasmodium falciparum*.
  MBML (Molecular Biology and Medicine of the Lung) Retreat Program, Marburg.

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List of Schemes

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.