Eryptosis as protection factor against malaria

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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aus
Warangal, India

Tübingen
2010
Tag der mündlichen Prüfung: 28. 10. 2010
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance between groups</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’ triphosphate</td>
</tr>
<tr>
<td>B.W.</td>
<td>Body weight</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CFSE</td>
<td>5,6-carboxylfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CSP</td>
<td>Circum sporozoite protein</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl) amiloride</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FL-1</td>
<td>Florescence channel 1</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gardos channel</td>
<td>Calcium activated potassium channel</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-2-hydroxyethyl-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
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<tr>
<td>---------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar (mmol/L)</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NO₃</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NPP</td>
<td>New Permeability Pathways</td>
</tr>
<tr>
<td>NSC</td>
<td>Nonselective Cation Channel</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RTC</td>
<td>Reticulocytes</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
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1. SUMMARY

Over the past centuries malaria, a dreadful pathogen born blood related disease has been the major cause of mortality among mankind. As time passed the pathogens coevolved with their hosts, simultaneously manipulating the hosts’ defence mechanisms by intruding and mimicking various host related metabolic and signaling pathways. In the course of their parasitic effect they modified themselves to proliferate and destruct the host to death. The present strategy for controlling and curing infectious diseases has targeted various metabolic or enzymatic systems within the parasite. The most severe drawback of this method of controlling the diseases has led to the development of parasitic resistance and consequent relapse of once-contained infectious diseases amidst the host. I intended for a novel drug discovery paradigm in order to prevent the pathogen related resistance focusing on identifying and targeting host factors essential for pathogen entry, survival and proliferation. The innovative methods involve stimulation of the infected erythrocytes and recognition by the spleen macrophages to get rid of the pathogen and prevent the further course of the disease.

In case of malaria the pathogen, *Plasmodium*, enters erythrocytes and thus escapes recognition by the immune system. The pathogen induces oxidative stress to the host erythrocyte, which triggers cryptosis, the suicidal death of erythrocytes. Cryptosis is characterized by cell shrinkage, membrane blebbing and cell membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are identified by macrophages which engulf and degrade the cryptotic cells. To the extent that infected erythrocytes undergo cryptosis prior to exit of *Plasmodia* and subsequent infection of other erythrocytes, the premature cryptosis may protect against malaria. Accordingly, any therapeutical intervention accelerating suicidal death of infected erythrocytes has the potential to foster elimination of infected erythrocytes, delay the development of parasitemia and favorably influence the course of malaria. Cryptosis is stimulated by a wide variety of triggers including osmotic shock, oxidative stress, energy depletion and a wide variety of xenobiotics. Diseases associated with accelerated cryptosis include sepsis, haemolytic uremic syndrome, malaria, sickle-cell anemia, beta-thalassemia, glucose-6-phosphate dehydrogenase (G6PD)-deficiency, phosphate depletion, iron deficiency and Wilson’s disease. Among the known stimulators of cryptosis, paclitaxel, chlorpromazine, cyclosporine, curcumin, azathioprine, amiodarone, anandamide, PGE$_2$ and lead have indeed been shown to favourably influence the course of malaria. Moreover, sickle-cell trait, beta-
thalassemia trait, glucose-6-phosphate dehydrogenase (G6PD)-deficiency and iron deficiency confer some protection against a severe course of malaria. The therapeutic agents were chosen on the basis of their proved eryptotic activity, which allowed rapid identification of new and existing licensed drugs for host mediated antimalarial therapy. Importantly, counteracting *Plasmodia* by inducing eryptosis is not expected to generate resistance of the pathogen, as the proteins involved in suicidal death of the host cell are not encoded by the pathogen and thus cannot be modified by mutations of its genes.

The present studies have been conducted to investigate whether the subcutaneous administration of azathioprine or aurothiomalate may modify the course of malaria and survival of *Plasmodium berghei*-infected mice and further to examine if intraperitoneal administration of amiodarone influence the course of malaria.

*In vitro* infection of human erythrocytes with *Plasmodium falciparum* increased annexin V binding and initially decreased forward scatter, effects significantly augmented by azathioprine. At higher concentrations azathioprine significantly decreased intraerythrocytic DNA/RNA content *in vitro* parasitemia at (1 µM) concentration. Administration of azathioprine significantly decreased the parasitemia of circulating erythrocytes and increased the survival of *Plasmodium berghei* infected mice (from 0% to 77% 22 days after infection).

Exposure to aurothiomalate significantly decreased the *in vitro* parasitemia of *P. falciparum*-infected human erythrocytes without influencing the intraerythrocytic DNA/RNA content. Administration of sodium aurothiomalate *in vivo* (daily 10 mg/kg b.w. s.c. from the 8th day of infection) enhanced the percentage of phosphatidylserine exposing infected and noninfected erythrocytes in blood. All non-treated mice died within 30 days of infection. Aurothiomalate-treatment delayed the lethal course of malaria leading to survival of more than 50% of the mice 30 days after infection.

The *in vitro* infection of human erythrocytes with *P. falciparum* (strain BinH) increased annexin V-binding, an effect significantly augmented by amiodarone (10 µM). Amiodarone further significantly decreased intraerythrocytic DNA/RNA content (≥ 5 µM) and *in vitro* parasitemia (≥ 1µM). Following infection of mice with *Plasmodium berghei* ANKA by intraperitoneal injection of parasitized murine erythrocytes (1x10^6) amiodarone (intraperitoneal 50 mg/kg b.w) significantly decreased the parasitemia and increased the survival of *P. berghei* infected mice (from 0% to 70% 26 days after infection). Moreover, treatment with amiodarone significantly increased the percentage of PS-exposing infected erythrocytes.
In conclusion azathioprine, aurothiomalate and amiodarone stimulate the erythrocytic machinery responsible for the eryptosis following infection with *Plasmodium*. The exhilaration of eryptosis precedes the full intraerythrocytic maturation of the pathogen, thus preventing the further lethal course of the disease and fosters host survival during malaria. The revelations defend that the stimulation of eryptosis in infected erythrocytes is a host dependent mechanism to combat against infection. The experimental results not only justify that the stimulation of eryptosis in infected erythrocytes is not only a host dependent defence mechanism but also a novel approach to prevent the chances of resistance in plasmodia. This method of identifying new host mediated antimalarial agents may be combined with the regular antimalarial agents with host directed drug therapy and increase the efficacy in eliminating the invaded and invading pathogen, thus control the resurgence of once contained disease.
2 ZUSAMMENFASSUNG

Im Verlauf der letzten Jahrhunderte war Malaria, ein schreckliches Pathogen aus der Reihe der blutsverwandten Krankheiten die Hauptsache für die Sterblichkeit der Menschheit. Mit der Zeit entwickelten sich die Pathogen gemeinsam mit ihren Wirten weiter und manipulierten dabei die Verteidigungsmechanismen des Wirts, indem sie in verschiedene wirtsspezifische Metabolismen und Signalwege eindrangen und sie imitierten. Im Verlauf ihrer parasitären Auswirkung modifizierten sie sich selbst, um zu proliferieren und den Wirt zu töten. Die gegenwärtige Strategie, um infektiöse Krankheiten zu kontrollieren und zu heilen, hat auf verschiedene metabolische oder enzymatische Systeme innerhalb des Parasiten abgezielt. Die schwerwiegendste Einschränkung von dieser Art, die Krankheiten zu kontrollieren, hat zu der Entwicklung von parasitärer Resistenz geführt und konsequentem Rückfall der once-contained infektiösen Krankheiten im Wirts. Wir zielten auf ein Entwicklungsmodell für eine neuartige Substanz ab, um die Resistenz des Pathogens zu verhindern, indem wir uns darauf konzentrierten, die Wirtsfaktoren, die für den Befall, das Überleben und die Proliferation des Pathogens essentiell sind, zu identifizieren und auf sie abzuzielen. Die innovativen Methoden beinhalten Stimulation der infizierten Erythrozyten und Erkennung der Makrophagen der Milz, um das Pathogen zu entfernen und den weiteren Krankheitsverlauf zu verhindern.


Die therapeutischen Mittel wurden auf der Basis ihrer überprüften eryptotischen Aktivität ausgewählt, was eine schnelle Identifikation neuer und existierender zugelassener Arzneimittel für eine wirtsvermittelte Antimalaria-Therapie ermöglicht. Es ist wichtig, dass nicht erwartet wird, dass beim Bekämpfen von *Plasmodia* über eine Induktion der Eryptose eine Resistenz des Pathogens entwickelt wird, weil die Proteine, die beim suizidalen Tod der Wirtszelle beteiligt sind, nicht durch das Pathogen kodiert werden und damit durch Mutationen seiner Gene nicht verändert werden können.

Die bisherigen Studien wurden durchgeführt, um zu untersuchen, ob die subkutane Verabreichung von Azathiopron oder Aurothiomalat den Verlauf von Malaria und das Überleben von Mäusen, die mit *Plasmodium berghei* infiziert wurden, modifizieren kann und weiter, um zu untersuchen, ob intraperitoneale Verabreichung von Amiodaron den Verlauf von Malaria beeinflussen kann.


Die *in vitro*-Infektion humaner Erythrozyten mit *P. falciparum* (Linie BinH) erhöhte die Annexin V-Bindung, ein Effekt der durch Amiodaron (10 µM) signifikant verstärkt wurde. Amiodaron verminderte außerdem signifikant den intraerythrozytären DNA/RNA-Gehalt (≥ 5 µM) und Parasitenwachstum *in vitro* (≥ 1µM). Nachdem Mäuse über eine intraperitoneale Injektion von parasitierten murinen Erythrozyten (1x10⁶) mit *Plasmodium berghei* ANKA infiziert worden waren, verminderte Amiodaron (intraperitoneal 50 mg/kg KG) signifikant die Infektionsrate und erhöhte das Überleben von Mäusen, die mit *P. berghei* infiziert wurden (von 0% bis zu 70% 26 Tage nach der Infektion). Darüber hinaus erhöhte die Behandlung mit Amiodaron signifikant die Prozentzahl von Phosphatidylserin-exprimierenden infizierten Erythrozyten.

3. INTRODUCTION

Malaria and its incidence

Malaria, a disease caused by parasites of the genus Plasmodium, places a huge burden on human life. Individuals in all continents are potentially at risk, but the greatest suffering falls to the people in tropical countries. The degree of endemicity varies between countries and even between different areas in the same country. In the regions of very high endemicity, the greatest suffering is borne by children less than 5 years of age, whereas in areas of low endemicity, the disease affects all age groups (Miller et al., 1994). Malaria is one of the most life threatening diseases in the world, causing 1.5-2.7 million deaths annually. Of the four species of human malaria parasites, Plasmodium falciparum causes the most severe of symptoms and the greatest number of deaths (Eda et al., 2002b). Predictions of global climate change have stimulated forecasts that the vector-borne diseases will spread into the regions that are at present too cool for their persistence (Rogers et al., 2000). The increase in global warming has a possibility of increase in the worldwide incidence of malaria.

![Figure 1 Map of world-wide malaria incidence](image-url)

Source: WHO
**Historical perspective**

Since ancient times, humankind has had to struggle against the persistent onslaught of pathogenic microorganisms. Nowadays, malaria is still the most important infectious pathogenic disease worldwide.

The early descriptions of malaria and its clinical symptoms were put forward by Hippocrates in about 400 BC. In the first quarter of the fifteenth century the bark of cinchona tree was employed in Native American medicine for a febrile disease which has similar symptoms like malaria. The modern drug for malaria, quinine, was isolated from the same tree bark. In the nineteenth century Alphonse Laveran won the Nobel prize for his discovery that malaria is caused by a protozoan in the blood(Sundberg, 2007). Earlier Lancisi suspected that mosquito may be the vector for malaria, but it wasn’t proved scientifically until Sir Ronald Ross identified plasmodium in the gut of mosquito for which he also won Nobel prize(Yoeli, 1973).

**The pathogen and vector of malaria**

The phylum Apicomplexans are responsible for a wide range of serious diseases of humans, livestock, wild animals and invertebrates and there are an estimated 5000 species of parasites in the group. Human apicomplexan diseases include malaria and toxoplasmosis; babesiosis, theileriosis and coccidiosis are common problems in livestock(Lim et al., 2010). The pathogen of malaria is from the Protozoan family Plasmodiidae that contains only one genus: *Plasmodium*, and two subgenera: *Plasmodium* and *Laverania*. Only four *Plasmodium* species are responsible for malarial infection in man; *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. *P. vivax*, *P. ovale* and *P. malariae* belong to the *Plasmodium* subgenus. *P. falciparum* on the other hand belongs to the subgenus *Laverania*(Esposito et al., 1991) which is the only species that may lead to severe, often fatal, complications of the disease. In addition to these four human malaria parasites, man is occasionally exposed to infection with simian malaria parasites such as *P. cynomolgi* and *P. knowlesi* (Kissinger et al., 1998). The vector largely responsible for distribution of malaria is the female *Anopheles* mosquitoes. Anopheles female mosquitoes are able to transmit *Plasmodium* to humans, and, among the more than 450 Anopheles species known about 60 are considered to be the actual vectors in the wild (Cohuet et al., 2010). *A. gambiae* is a highly efficient, highly adapted vector that feeds nearly exclusively on humans and has a long lifespan, greater than 30 days. As a
consequence, *A. gambiae* mosquitoes feeding on a single infected individual are able to transmit malaria to hundreds of others (Pierce et al., 2009). Parasite transition from the human host to the mosquito vector is mediated by gametocytes, sexual stages that are formed in human erythrocytes, which therefore play a crucial part in the spread of the tropical disease (Kuehn et al., 2010).

**Life cycle of the malaria parasite**

The life cycle of malaria parasite is highly complicated, it is comprised of two stages (Figure 2), a sexual phase with multiplication in the female of the *Anopheles* species of mosquito also called as mosquito cycle or vector cycle, and an asexual phase with multiplication in a vertebrate host, which in turn has two cycles, one within and one outside the erythrocyte called endo and exo erythrocytic cycle respectively.

**Mosquito cycle (Sexual cycle):**
Female *Anopheles* mosquito is the predominant vector that can either infect mammals or itself be infected during a blood meal. Of the two hosts, humans are the intermediate host and the *Anopheles* mosquito is the definitive host, as in it the sexual phase of the cycle (Fujioka et al., 2002). Each species of *Plasmodium* infecting humans exhibits different morphological stages (i.e. sporozoites, merozoites, etc.) that elicit unique host immune responses, and each of these is a potential target for an antimalarial vaccine (Targett et al., 2008). Parasites taken up by the mosquito during a blood meal as gametocytes transform into the male exflagellated microgametocytes or female macrogametocytes and are released from the erythrocytes in the midgut of the insect where fertilization occurs, forming the ‘zygote’ within 18h of the blood meal. The zygote within the midgut then elongates becoming a motile ookinete which enters the midgut epithelium and comes to rest beneath the basal lamina where it forms an oocyst 24-72h after the blood meal. The oocyst then matures in 7-15 days after the blood meal giving rise to up to 10,000 sporozoites. Finally, the sporozoites move through pores in the oocyst membrane into the haemocoelic fluid to accumulate in the acinal cells of the salivary gland (Bannister et al., 2000). The vacuolar membrane then undergoes lysis to leave the sporozoites unprotected inside the salivary gland. Here they mature into infective sporozoites that are again inoculated into the vertebrate host during the next blood meal.

**Hepatic cycle:**
Sporozoites injected incidentally during the mosquito bite move within minutes to invade hepatocytes if they are not first cleared by the spleen. They are usually not injected directly
into a blood vessel but, as the mosquito searches, it releases vasodilators with the saliva to increase its chances of finding one, thus depositing any sporozoites present into the epithelium (Kappe et al., 2003). In the liver, the exo- or pre-erythrocytic stage is played out over a period of less than a week of asexual divisions in hepatic parenchymal cells (Miller et al., 1998). The exact duration depends on the size of the fully grown schizont and the number of merozoites it contains, and this depends on the species of the malaria parasite involved. *P. falciparum* takes a minimum of five and a half days for merogony to be completed. Here, even in their intracellular form, they are liable to attack, this time from cytotoxic cells that target the infected hepatocytes (Good et al., 1999). *P. vivax*, *P. ovale*, and the simian malaria *P. cynomolgi* may develop hibernating forms, termed hypnozoites.

**Erythrocytic cycle:**
Surviving, they will break their containment to release thousands of merozoites through the liver sinusoids and into the blood-stream from each single infected hepatocyte. At this point, there is a way of microscopically differentiating amongst species, as *P. falciparum* will produce about 30,000 mononucleated merozoites per cell, whereas *P. vivax* produces about a third of these, *P. ovale* makes a few more than *P. vivax* and *P. malariae* produces only some 2000. These free merozoites make a perilous journey, avoiding host immune defences, in search of a red blood cell to invade. The merozoite is now committed to invasion. On entering, the parasitophorous vacuole is formed around it and the two junctions continue to move as a circumferential band towards the posterior of the merozoite where they will fuse thus leaving it wholly within the vacuole. Anywhere between 36 and 48 hours from the onset of invasion the merozoite will multiply by schizogony (Mitchell et al., 1988). Inside the red cell development continues to the trophozoite stage and early on they look like rings under a light microscope with Giemsa staining. This part of malaria life cycle is thus called the ‘ring’ stage. Infected erythrocytes become distorted and irregular during the mature trophozoite stage. Protein synthesis increases dramatically as nuclear division begins. Each nucleus will form a new parasite that, upon rupture of the erythrocyte will liberate invasive merozoites into the blood stream. Some parasites will not undergo schizogony but differentiate into sexual stage, extra-cellular, male or female gametocytes (micro- or macro-gametes). The gametocytes are then ingested by the mosquito when it takes its fateful blood meal. Though the mature gametocytes are present in the blood of infected vertebrate hosts, mating only occurs in the midgut of female Anopheline mosquitoes. Gametogenesis takes about 20 minutes, beginning immediately after the parasitized RBC reaches the gut. The parasite dissolves the two erythrocyte membranes surrounding it and, in the case of the male gamete, undergoes the
nuclear reorganization necessary for the formation of several flagellar, free swimming
gametes (Sinden et al., 1996). Within the erythrocyte the parasite ingests hemoglobin for its
amino acid needs, and uses the cell’s glucose and other nutrients which are digested inside
numerous phagosomes. Its diet becomes its main enemy as toxic heme is released into the cell
as a by-product of hemoglobin digestion. This is, however, polymerized into hemozoin, the
insoluble malarial pigment that is harmless to the parasite. The parasitemia increases
exponentially as mature schizont-bloated erythrocytes rupture in synchrony releasing 10-30
merozoites each, along with the hemozoin and other by-products of the parasite’s metabolism.
It is at this point that the high fever characteristic of malaria can be observed. In order to
attack the malaria parasite we must target at those stages in the life cycle where it is the most
susceptible to immune attack, as when it is extracellular rather than intracellular. The
extracellular stages, sporozoites, merozoites and gametes express molecules with functional
roles in the biology of the parasite where immune effector mechanisms may be most effective,
and it is here that one can look for potential vaccine candidates (Good, 1995).
Apoptosis is defined as programmed cell death activated by an internally controlled suicide program. It is a subtly orchestrated disassembly of cellular components designed to eliminate unwanted cells during embryogenesis in various physiological processes (Lancellotti et al., 2009). Apoptosis is currently one of the hottest areas of modern biology. It describes the orchestrated collapse of a cell, staging membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by spleen macrophages. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs...
as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury et al., 2001). Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus. Irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a $p53$-dependent pathway. Some hormones, such as corticosteroids, may lead to apoptotic death in some cells (e.g., thymocytes) although other cells are unaffected or even stimulated. The alternative to apoptotic cell death is necrosis, which is considered to be a toxic process where the cell is a passive victim and follows an energy-independent mode of death. But since necrosis refers to the degradative processes that occur after cell death, it is considered by some to be an inappropriate term to describe a mechanism of cell death (Elmore, 2007). Apoptosis allows the elimination of the cells without the release of intracellular materials into the extra cellular space and hence it usually does not cause inflammation (Gulbins et al., 2000b), whereas necrosis leads to cell disintegration and the induction of unspecific and/or specific immune response. The two main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each requires specific triggering signals to begin an energy-dependent cascade of molecular events. Each pathway activates its own initiator caspase (8, 9, and 10) which in turn will activate the executioner caspase-3. However, granzyme A works in a caspase-independent fashion. The execution pathway results in characteristic cytomorphological features including cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies and finally phagocytosis of the apoptotic bodies by adjacent parenchymal cells, neoplastic cells or macrophages. The role of apoptosis in normal physiology is as significant as that of its counterpart, mitosis. It demonstrates a complementary but opposite role to mitosis and cell proliferation in the regulation of various cell populations. It is estimated that to maintain homeostasis in the adult human body, around 10 billion cells are made each day just to balance those dying by apoptosis (Renehan et al., 2001). And that number can increase significantly when there is increased apoptosis during normal development and aging or during disease.

**Suicidal erythrocide death or eryptosis**

Usually, erythrocytes within 100-120 days undergo senescence, which eventually results in the clearance of the aged erythrocytes (Arese et al., 2005; Bosman et al., 2005; Kiefer et al., 2000). Senescence comprises binding of hemichromes to band 3, clustering of band 3, and deposition...
of complement C3 fragments and anti band 3 immunoglobulins (Lutz, 2004). Prior to senescence-dependent removal, erythrocytes may, similar to nucleated cells, undergo a suicidal death program which accomplishes the disposal of abundant, defective or potentially harmful cells (Green et al., 1998b; Gulbins et al., 2000a). Suicidal death of nucleated cells or apoptosis (Green et al., 1998a; Gulbins et al., 2000c) is paralleled by cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing and breakdown of phosphatidylserine asymmetry of the plasma membrane (Bortner et al., 1999; Bortner et al., 2002; Bortner et al., 2004; Javadov et al., 2007; Maeno et al., 2000; Okada et al., 2001; Yu et al., 2001). Stimulators of apoptosis include activation of death receptors such as CD95 (Daniel et al., 2001; Grassme et al., 2000; Lang et al., 1999) or TNFα (Rieger et al., 2007), cell injury due to oxidative stress (Han et al., 2004; Varela et al., 2007), cytostatic drugs (Bachmeier et al., 2007), radiation (Rosette et al., 1996), osmotic shock (Shimizu et al., 2006) alkaline stress (Chen et al., 2007), Na⁺/H⁺ exchanger inhibitors (Konstantinidis et al., 2006) or bile salts (Becker et al., 2007). Apoptotic cells are identified by macrophages recognizing (Fadok et al., 2000), engulfing and subsequently degrading (Boas et al., 1998e) phosphatidylserine-exposing cells.

Mature erythrocytes have lost their nuclei and mitochondria, important organelles in apoptosis. Nevertheless, erythrocytes may undergo suicidal death characterized by cell shrinkage, membrane blebbing and phosphatidylserine exposure, all features typical of apoptotic nucleated cells (Berg et al., 2001b; Bratosin et al., 2001c; Daugas et al., 2001a).

A major stimulator of eryptosis is an increase in cytosolic Ca²⁺ activity (Berg et al., 2001a; Bratosin et al., 2001b; Daugas et al., 2001b), which leads to cell membrane vesiculation (Allan et al., 1977), stimulates cell membrane scrambling (Akel et al., 2006b; Nicolay et al., 2006; Niemoeller et al., 2006b) and activates the cysteine endopeptidase calpain, an enzyme degrading the cytoskeleton and thus causing cell membrane blebbing (Pant et al., 1983). Ca²⁺ may enter through nonselective cation channels (Kaestner et al., 2002; Kaestner et al., 2004; Lang et al., 2005e; Bernhardt et al., 2007; Ivanova et al., 2008). The molecular identity of those channels is not completely understood but may include TRPC6 (Foller et al., 2008d). The cation channels are activated by osmotic shock (Huber et al., 2001b; Lang et al., 2003b), oxidative stress (Lang et al., 2003c; Duranton et al., 2002b) and Cl⁻ removal (Duranton et al., 2002c; Huber et al., 2001c; Lang et al., 2005d).

Ca²⁺, in addition, stimulates Ca²⁺-sensitive K⁺ channels (Bookchin et al., 1987; Brugnara et al., 1993; Franco et al., 1996) with subsequent efflux of K⁺, hyperpolarization of the cell
membrane and Cl⁻ exit (Lang et al., 2003g). The cellular loss of KCl with osmotically obliged water causes cell shrinkage (Lang et al., 2003h).

A second major stimulator of cell membrane scrambling is ceramide (Lang et al., 2004d). Eryptosis is further stimulated by energy depletion (Klarl et al., 2006), oxidative stress (Barvitenko et al., 2005; Bracci et al., 2002; Lang et al., 2002) or impaired antioxidative defence (Bilmen et al., 2001b; Damonte et al., 1992b; Mavelli et al., 1984). Oxidative stress involves activation of the Ca²⁺-permeable cation channels (Duranton et al., 2002a). Moreover, oxidative stress activates erythrocyte Cl⁻ channels (Huber et al., 2002a; Tanneur et al., 2006b) which contribute to cryptotic cell shrinkage (Myssina et al., 2004). Oxidative stress further activates caspases (Bratosin et al., 2001a; Mandal et al., 2003; Matarrese et al., 2005).

Suicidal erythrocyte death may be triggered by a wide variety of stimulators (Lang et al., 2008b) including ligation of specific surface antigens, such as glycophorin-C (Head et al., 2005a), the thrombospondin-1 receptor CD47 (Head et al., 2005b) and the death receptor CD95/Fas (Mandal et al., 2005). Eryptosis may further be stimulated by ceramide (acylsphingosine) (Lang et al., 2004c), prostaglandin E₂ (Lang et al., 2005c), platelet activating factor (Lang et al., 2005f), anti A IgG antibodies (Attanasio et al., 2007), hemolysin from Vibrio parahaemolyticus (Lang et al., 2004e), listeriolysin (Foller et al., 2007b), paclitaxel (Lang et al., 2006c), amantadine (Foller et al., 2008b), azathioprine (Geiger et al., 2008c), amiodarone (Nicolay et al., 2007b), retinoic acid (Niemoller et al., 2008b), Ciglitazone (Niemoller et al., 2008a), chlorpromazine (Akel et al., 2006a), peptidoglycan (Foller et al., 2009a), cyclosporine (Niemoller et al., 2006a), methylglyoxal (Nicolay, Schneider, Niemoeller, Artunc, Portero-Otin, Haik, Jr., Thornalley, Schleicher, Wieder, and Lang, 2006), amyloid peptides (Nicolay et al., 2007c), anandamide (Bentzen et al., 2007b), Bay-Y5884 (Shumilina et al., 2006), curcumin (Bentzen et al., 2007a), arsenic (Mahmud et al., 2009a), methylidopa (Mahmud et al., 2008), valinomycin (Schneider et al., 2007), aluminium (Niemoller et al., 2006c), mercury (Eisele et al., 2006), lead (Kempe et al., 2005), gold (Sopjani et al., 2008f), selenium (Sopjani et al., 2008c), vanadium (Foller et al., 2008e), cadmium (Sopjani et al., 2008a), tin (Nguyen et al., 2009), and copper (Lang et al., 2007b).

Eryptosis participates in the pathophysiology of several clinical conditions, including sepsis (Kempe et al., 2007b), hemolytic uremic syndrome (Lang et al., 2006b), renal insufficiency (Myssina et al., 2003a), malaria infection (Brand et al., 2003), sickle-cell anemia (Wood et al., 1996a; Browning et al., 2007), beta-thalassemia (Lang, Roll, Myssina, Schittenhelm, Scheel-Walter, Kanz, Fritz, Lang, Huber, and Wieder, 2002), glucose-6-phosphate dehydrogenase (G6PD)-deficiency (Lang, Roll, Myssina, Schittenhelm, Scheel-Walter, Kanz, Fritz, Lang,
Huber, and Wieder, 2002), phosphate depletion (Birka et al., 2004), and Wilsons disease (Lang et al., 2007a). Enhanced eryptosis in sepsis (Kempe et al., 2007a) and hemolytic uremic syndrome (Lang et al., 2006a) is secondary to the capability of serum from respective patients to trigger eryptosis.

The Ca\textsuperscript{2+}-permeable cation channels and thus eryptosis are inhibited by erythropoietin (Myssina et al., 2003b), which similarly inhibits apoptosis of erythrocytic progenitor cells (Jelkmann, 1992; Polenakovic et al., 1996a). The inhibitory effect of erythropoietin on eryptosis increases the life span of circulating cells (Polenakovic et al., 1996b). Interestingly, however, in vitro eryptosis of erythropoetin-overexpressing animals is enhanced (Foller et al., 2007a), an effect possibly accounting for enhanced death of erythrocytes upon decreasing erythropoietin plasma levels (Rice et al., 2005). Dopamine, isoproterenol and epinephrine inhibit eryptosis by decreasing the activity of the Ca\textsuperscript{2+}-permeable cation channels (Lang et al., 2005b). The cation channels (Huber et al., 2001a; Lang et al., 2003d) and thus eryptosis (Lang et al., 2003f) are further inhibited by amiloride (Lang et al., 2003a) and ethylisopropylamiloride (EIPA) (Lang et al., 2003e). Additional inhibitors of eryptosis include flufenamic acid (Kasinathan et al., 2007), adenosine (Niemoeller et al., 2007), zidovudine (Kucherenko et al., 2008) and caffeine (Floride et al., 2008). The most powerful known inhibitor of eryptosis is nitric oxide (Nicolay et al., 2008b), which could be released from erythrocytes (Crawford et al., 2006; Dejam et al., 2005a; Grubina et al., 2007), whereby oxygenated hemoglobin binds and desoxygenated hemoglobin releases NO (Angelo et al., 2006; Dejam et al., 2005b; Diesen et al., 2007; Power et al., 2007; Reynolds et al., 2007; Yang et al., 1996). Nitric oxide is partially effective through activation of cGMP-dependent protein kinases (Das et al., 2006; Li et al., 1999; Nicolay et al., 2008b). Accordingly, deficiency in cGMP-dependent protein kinase type I (cGKI) leads to excessive eryptosis, severe anemia and splenomegaly (Foller et al., 2008a).

Eryptosis in malaria

As indicated above, Plasmodium falciparum depends on the activation of ion channels in the erythrocyte cell membrane, as they allow the uptake of nutrients, Na\textsuperscript{+} and Ca\textsuperscript{2+} and the disposal of waste products (Kirk, 2001b). Plasmodium falciparum activates the ion channels by inducing oxidative stress (Duranton et al., 2003; Huber et al., 2002b; Tanneur et al., 2006a). The opening of the Ca\textsuperscript{2+}-permeable cation channels is followed by Ca\textsuperscript{2+} entry and stimulation of eryptosis (Bilmen et al., 2001a; Damonte et al., 1992a; Mavelli, Ciriolo, Rossi, Meloni, Forteleoni, De Flora, Benatti, Morelli, and Rotilio, 1984). The clearance by macrophages limits the life span of the infected eryptotic cells (Lang et al., 2004b). Thus, the pathogen faces a
dilemma. On the one hand, it requires the channels for the uptake of Na\(^+\), on the other hand, the associated Ca\(^{2+}\) uptake eventually triggers eryptosis of the parasitized erythrocyte (Brand, Sandu, Duranton, Tanneur, Lang, Huber, and Lang, 2003) and the opening of those channels thus limits the period of safe dwelling in the host cells.

The intraerythrocytic pathogen deals with this dilemma by delaying the execution of eryptosis. It sequesters Ca\(^{2+}\) and thus keeps the intra-erythrocytic free Ca\(^{2+}\) concentration together with the erythrocyte Ca\(^{2+}\) pump low (Huber et al., 2005) In addition it prevents premature hemolysis of its host erythrocyte by decreasing the colloidal osmotic pressure of the erythrocyte cytosol. This is accomplished by excess haemoglobin digestion and by export of the haemoglobin-derived amino acids through the NPP (Lew et al., 2003). Eventually, Plasmodium infection results in breakdown of the phospholipids asymmetry across the erythrocyte membrane and exposure of phosphatidylserine (Brand, Sandu, Duranton, Tanneur, Lang, Huber, and Lang, 2003;Eda et al., 2002a;Joshi et al., 1987;Joshi et al., 1988;Maguire et al., 1991;Schwartz et al., 1987).

Phosphatidylserine exposure, in turn, has been demonstrated for trophozoite-infected erythrocytes to stimulate their phagocytotic clearance (Ayi et al., 2002b;Turrini et al., 1992a) On the other hand, phosphatidylserine exposure reportedly facilitates tissue sequestration of trophozoite-infected cells and, thus, contributes to the partial immune evasion of the late stages (Eda and Sherman, 2002a;Sherman et al., 2003). In addition to triggering of phosphatidylserine exposure, the intraerythrocytic pathogen ages its host cell. i.e., it induces a dramatic, time-compressed enhancement of normal erythrocyte senescence which contributes to the clearance of infected cells (Sherman et al., 2004;Turrini et al., 1992b). In particular, this occurs in infected erythrocytes with sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency which fosters the clearance of these erythrocytes already in the ring stage of infection and which underlies the partial resistance to malaria of the carriers of these erythrocytes (Arese, Turrini, and Schwarzer, 2005;Ayi et al., 2002a;Ayi et al., 2004c;Cappadoro et al., 1998b).

Erythrocytes with hemoglobinopathies or G6PD-deficiency are highly prone to enter eryptosis (Ayi et al., 2004b;Cappadoro et al., 1998a;de Jong et al., 2001b;Kean et al., 2002b;Kuypers et al., 1998b;Lang, Roll, Myssina, Schittenhelm, Scheel-Walter, Kanz, Fritz, Lang, Huber, and Wieder, 2002) and pharmacological induction of phosphatidylserine exposure of ring stage-infected erythrocytes reportedly accelerates their clearance (Ayi et al., 2002c). Thus, maneuvers accelerating eryptosis may result in premature clearance of the intraerythrocytic parasite. As a matter of fact, iron deficiency (Koka et al., 2007a), lead (Koka et al., 2007b), chlorpromazine (Koka et al., 2008a) and inhibition of NO synthase by L-NAME (Koka et al.,
2008c) decrease parasitemia and partially enhance the survival of *Plasmodium berghei*-infected mice eventually by accelerating erythrocyte death. Thus, several conditions are known, which are associated with enhanced susceptibility to eryptosis and at the same time with a milder course of malaria. Among those, sickle cell trait is an example of a condition which is not associated with the problem of developing resistance of the pathogen since the physiology of the host cell is not at the genetic disposition of the pathogen.

**Synopsis of apoptosis, eryptosis and malaria**

Erythrocyte shrinkage and phosphatidylserine exposure (“eryptosis”) mimic features of apoptosis in nucleated cells which however, involves several mechanisms lacking in erythrocytes. Several conditions trigger premature eryptosis thus favouring the development of anemia. On the other hand, eryptosis may be a mechanism of defective erythrocytes to escape hemolysis. Beyond their significance for erythrocyte survival and death the mechanisms involved in “eryptosis” may similarly contribute to apoptosis of nucleated cells (Lang et al., 2005a). “Apoptosis” of erythrocytes is triggered by redox-sensitive, Ca2+-permeable, non-selective cation channels, activation of the Gardos K+ channel and cell shrinkage. The malaria parasite *P. falciparum* most probably utilizes these Ca2+-permeable non-selective cation channels to adapt the ionic composition of the host cytosol to its needs. Buffering of the cytosolic free [Ca2+] by the parasite probably prevents Gardos K+ channel activation and substantial host cell shrinkage during early parasite development. Activity of the cation channels, however, may be paralleled by entry of the host cell into “apoptosis” during the late stages of parasite development, resulting in breakdown of the membrane phospholipid asymmetry and exposure of phosphatidylserine at the outer membrane leaflet. Host cell “apoptosis” may contribute to tissue sequestration of the late stage-infected erythrocyte. It may also foster phagocytotic clearance of the infected cells by macrophages. Accelerated “apoptotic” clearance of the infected erythrocyte might contribute to the partial malaria resistance observed in sickle cell anaemia, thalassaemia and glucose-6-phosphate dehydrogenase deficiency, all conditions associated with increased susceptibility of erythrocytes to enter “apoptosis”.
A. Non-infected erythrocytes

The consecutive activation of Ca²⁺-permeable cation channels, Gardos K⁺ channels, sphingomyelinase and scramblase in dying erythrocytes results in cell shrinkage and phosphatidylserine exposure at the outer membrane, both of which foster phagocytotic clearance of the cell.

B. Plasmodium falciparum-infected erythrocytes

Hypothetical model of mechanisms underlying cytoadherence and “apoptosis” of parasitized erythrocytes *P. falciparum* infection (left) activates a Ca²⁺-permeable cation channel and organic osmolyte permeable anion channels by oxidation (middle). Infection also activates sphingomyelinase leading to formation of ceramide that in turn facilitates Ca²⁺-dependent scramblase activation. Exposure of phosphatidylserine (phospholipids in red) at the outer membrane leaflet stimulates cytoadherence to endothelial cells and/or triggers phagocytotic clearance of infected erythrocytes by macrophages (right).

Figure 3 Physiological changes in infected and non infected erythrocytes (Lang et al., 2004a)
4. OBJECTIVE OF THE STUDY

Malaria has always been a major killer of populations throughout the tropics for thousands of years. Despite important advances in our understanding of the disease, it continues to be one of the greatest causes of serious illness and death in the world. Over 75% of 2–3 million deaths occurring to African children, and about 500 million new cases reported annually, is a challenge to drug therapy and discovery (Na-Bangchang et al., 2007) (Ringwald, 2007). Global efforts to treat malaria have focused largely on provision of effective antimalarial treatment, mainly through public health services. The private sector (although a key source of antimalarial treatments in most countries) has been mostly ignored in the effort to find solutions to the issues of availability, and affordability of effective drugs. The conventional modes to control over malaria relied on the provision of proven antimalarial drugs, on environmental sanitation and on the application of insecticides. But the increase of drug resistant strains made the traditional mode of control impossible to combat malaria, hence the approach of identifying the new chemical entities with high potential in curing have been increased. Many organisations started funding for research where there is search going on for potential drug molecules which act against malaria.

To start with a new lead molecule and end it as a newly identified chemical entity which can fight against malaria may need a lot of money, man power and years of time which is similar for all the therapeutic agents used for other ailments. The initiation of identifying may be a small leap but also a giant step for the mankind. The major problem nowadays which we come across in discovery of new drugs is that they may not be so fruitful at the end since they may be dropped at any stage of the phase oriented trials, hence it’s better to check the alternate drugs which hamper the growth and destroy the pathogen. This is only possible if we can understand the various metabolic pathways of the pathogen and host-pathogen interactions.

The emergence and spread of drug resistant strains of Plasmodium falciparum, the most dangerous of the four human malaria parasites, has emphasized concerns about the use and efficacy of other available antimalarial drugs. An additional concern, however, are recent reports on other Plasmodium species about wide spread and increasing resistance to other antimalarial drugs.

Increase of Plasmodium resistance towards antimalarial agents has raised an alarm in the world health authorities. Multiple general approaches to the identification of new antimalarials are being pursued at this time. Due to the high demand for new drugs or
alternative methods in treatment, researchers are checking the chance of interfering with host mediated pathways than altering the parasite’s pathways.

The future of antimalarial chemotherapy is particularly alarming in view of the spread of malaria parasite cross-resistance to drugs that are not even structurally related. Various approaches can be envisaged to prevent or reduce the pace at which resistance develops. One clear need is to develop new drugs with host mediated mechanism, which may prevent the resistance of the pathogen. It is essential that the older approaches continue and that they are supplemented by the rational development of new agents directed against newly defined host targets. The better approach is to use the host oriented treatment and further prevention of the resistance of the pathogen may be appropriate. The mechanism of eryptosis seems to be a good tool to fight against malaria by triggering the host erythrocytes containing the pathogen to expose the phosphatidylserine, be early recognised by the spleen macrophages, and get cleared from the circulation. Moreover new combination therapies may include one of these trail components either in clinical development or recently approved therapies. This mechanism will indeed prevent further course of the disease.

Organism homeostasis requires a delicate balance between formation of new cells, by cell proliferation and their elimination by apoptosis. Apoptosis eliminates abundant and potentially harmful cells (Green et al., 1998c). The maintenance of an adequate number of cells requires the replacement of apoptotic cells or formation of additional cells by cell proliferation. Similar to apoptosis of nucleated cells, suicidal erythrocyte death or eryptosis is characterized by cell shrinkage, membrane blebbing and membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are identified by macrophages which engulf and degrade the eryptotic cells. To the extent that infected erythrocytes undergo eryptosis prior to exit of Plasmodium pathogens and subsequent infection of other erythrocytes, the premature eryptosis may protect against malaria. Accordingly, any therapeutical intervention accelerating suicidal death of infected erythrocytes has the potential to foster elimination of infected erythrocytes, delay the development of parasitemia and favorably influence the course of malaria. Hence, our present study was designed to clarify the following questions

1. Is eryptosis a helpful functional mechanism to fight against malaria?
2. Does the exposure of phosphatidylserine play a significant role in the early clearance of infected erythrocyte and prevent the further time course of the disease?
3. Do the agents that stimulate eryptosis also aid in the in vitro and in vivo treatments of malaria?

4. Do eryptotic agents like azathioprine, sodium aurothiomalate and amiodarone modulate eryptosis of infected cells and influence the course of malaria in *P. berghei* infected mice?
5. MATERIALS AND METHODS

Plasmodium culture and its maintenance

The following methods were used in our study where ever applicable.

Preparation of human and murine erythrocytes

Human erythrocytes were drawn from healthy volunteers. They were either used without purification or after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). Experiments with non-purified or Ficoll-separated erythrocytes yielded the same results. After washing, the buffy coat and upper 10-20% of the red blood cells were discarded and the remaining pellet was used for experiments. The RBCs were then resuspended at 10% hematocrit and stored at 4°C until use (2-5 days). Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO$_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl$_2$. Erythrocytes were washed three times in Ringer solution, stored in SAG mannitol (Haemonetics, Munich, Germany) at 4°C, and washed twice in Ringer solution before use. For incubation, the final hematocrit was adjusted to 0.3 %. Mouse erythrocytes were drawn from the animals by retroorbital venopuncture. Experiments on mouse erythrocytes were performed at 37°C in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM Glucose, and 1 mM CaCl$_2$, pH 7.4.

Thawing of parasites for transfection

The frozen parasites are kept in cryotubes and stored in liquid nitrogen. The cryotubes containing parasitized RBCs (>20% parasitemia) were thawed quickly at 37 °C in a water bath. The contents were transferred into a 15 ml Falcon Tube and centrifuged (1800rpm at RT for 5 min). The supernatant was discarded. Sterile filtered 3.5 % NaCl-solution was added corresponding to the left over pellet in the falcon tube at the rate of 1-2 drops per second under gently shaking conditions, eventually mixed by a pipette. After centrifugation the supernatant was discarded. Original RPMI 1640 medium double the volume of the pellet in the falcon tube was added and mixed gently. The above step was repeated until the supernatant becomes clear. The remaining cell pellet was resuspended in RPMI complete medium, and then transferred into a culture flask. Fresh RBCs were added (amount depending
on volume of the left pellet in the falcon tube). The culture flask was filled with 90% N₂/ 5% O₂/ 5% CO₂.

**Maintenance of in vitro culture**

For infection of human erythrocytes, the human pathogen *P. falciparum* strain BinH [Binh et al., 1997] was grown in vitro [Huber et al, 2002] in human erythrocytes. Cultures were maintained continuously by routine passage in fresh and stored human erythrocytes. Parasites were cultured as described earlier [Jensen and Trager 1978; Trager and Jensen 1976] at a hematocrit of 2-5% and a parasitemia of 2-10% in RPMI 1640 medium supplemented with 0.5% Albumax II (Gibco, Karlsruhe, Germany), 0.13 µM hypoxanthine, 2 mM L-glutamine (Gibco, Karlsruhe, Germany), 25 mM HEPES/NaOH pH 7.4 (Sigma-Aldrich, Schnelldorf, Germany), 20 µg/ml gentamycin (Gibco, Karlsruhe, Germany) in an atmosphere of 90 % N₂, 5 % CO₂, 5 % O₂. The parasitemia of a maintained culture flask was measured with the help of flowcytometer, then the culture suspension is split into new sterile culture flasks containing fresh erythrocytes in RPMI complete medium.

The practical protocol below is followed to maintain

**Calculations:**

\[
\text{Percentage parasitemia of old culture flask} \times \frac{\text{Vol of old media RBC}}{\text{Vol of new media with RBC}} = N \text{ (dilution factor)}
\]

Then,

\[
\frac{500\mu l}{N} = \text{amount of infected erythrocytes to be added (OB)}
\]

\[
500\mu l - OB = FB \text{ (fresh erythrocytes)}
\]

**Amount of old culture suspension to be added from the old culture flask…**

\[
\frac{OB}{500\mu l} \times M \text{ (old culture suspension)}
\]

**Giemsa staining for determination of parasitemia**

A drop of the parasite culture was transferred by a transfer pipette on an object slide. With the help of a second object slide a thin blood smear was obtained. This smear was immediately dried by the air-flow of a hair-drier to avoid the development of echinocytes (crenated RBCs) or that parasites will leave RBCs before being dried. The blood smear was fixed in methanol for 1 minute and air-dried. The object slide with the blood smears were flooded with freshly
Methods

prepared Giemsa staining solution for 25 – 30 minutes, rinsed under tap water to float off the stain and to prevent deposition of the precipitate on the film. The slides were vertically air-dried, and then examined using the 100x objective of Leica CME microscope with immersion oil.

Isoosmotic sorbitol synchronization of P.falciparum infected human erythrocytes

The *P. falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [BinH et al. 1997]. Briefly, the infected RBCs (>5% parasitemia) were spun down at 1200rpm at RT for 5 min and resuspended in isoosmotic sorbitol solution (in mM: 290 sorbitol, 5 glucose, 5 HEPES/NaOH, pH 7.4) for 20 min at 21°C in continuous shaking. Then the cells were washed twice in malaria culture medium and subcultured for further experiments.

In vitro proliferation assay

For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 µl aliquots, 0.5-1% hematocrit, 0.5 –2% parasitemia) and grown for 48 h in serum-free Albumax II (0.5%)-supplemented RPMI medium as described earlier (Tanneur et al., 2005). The erythrocytes were grown in the presence or absence of (0.001 µM- 100 µM) azathioprine or aurothiomalate or amiodarone. The parasitemia was assessed at time 0 and after 48 h of culture by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany). Parasitemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescence dye Syto16 (20 nM final concentration, Molecular Probes, Göttingen, Germany). Briefly, RBCs were incubated with Syto16, diluted in PBS or annexin binding buffer at 37°C. The staining procedure was performed for around 30 – 40 min for infected human RBCs. Syto16 green fluorescent nucleic acid stain bound to DNA has a maximum excitation/absorption wavelength of 488 nm, which corresponds to the argon line of the single-laser of the FACS Calibur used, and a maximum emission wavelength of 518 nm. Bound to RNA the absorption maximum is at 494 nm, the emission maximum at 525 nm. This green emission is detected in the Fluorescence 1 (FL-1) channel with a detector for an emission wavelength of 530 ± 15nm. FACS analysis proved a more sensitive technique for determining parasitemia than either Giemsa or Field’s rapid staining.

Intraerythrocytic DNA amplification

To estimate the DNA/RNA amplification in a further series of experiments, the culture was ring stage-synchronized and re-synchronized after 6 h of culture (to narrow the developmental
parasite stage), aliquoted (200 µl aliquots, 2 % hematocrit and 10 % parasitemia) and cultured for further 16 h. The erythrocytes were cultured in the presence or absence of (0.001 µM- 100 µM) azathioprine or aurothiomalate or amiodarone. Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

**Animals**

Animal experiments were performed according to the German animal protection law and approved by the local authorities (registration number PY 3/09). Experiments were performed in healthy SV129/J wild type mice (aged 4 months, both male and female). The animals had free access to standard chow (C1310, Altromin, Lage, Germany) and drinking water. Murine erythrocytes were drawn from the animals by incision of the tail vein.

Mice were infected with *P. berghei* ANKA-parasitized murine erythrocytes (1x10⁶) injected intraperitoneally (Bienvenu et al., 2008; Cabrales et al., 2010; Lacerda-Queiroz et al., 2010; Lackner et al., 2008; Lang et al., 2009; Muniz-Junqueira et al., 2005; Steeg et al., 2009; White et al., 2010). The control mice had free access to tap water while the treated groups received azathioprine (5 mg/kg b.w. s.c), aurothiomalate (10 mg/kg b.w.s.c) and amiodarone (50 mg/kg b.w. i.p.) from the 8th day of infection. In addition, from the eighth day after infection blood was collected daily from the mice by incision of the tail (Brand et al., 2008). The parasitemia was assessed daily by flow cytometry and by counting of Giemsa-stained blood smears. Parasitemia was defined as the percentage of erythrocytes stained with the DNA/RNA-specific fluorescence dye Syto16 or by identification of Giemsa-stained infected erythrocytes using light microscopy.

**Determination of PS-exposure**

FACS analysis was performed as described (Foller et al., 2007b, 2006]. Suspensions of non infected erythrocytes were stained with annexin-fluos (Roche, Mannheim, Germany), suspensions of *P. falciparum* infected erythrocytes were stained with annexin V-568 (Roche, Germany) and/or with the DNA dye Syto16 (Molecular Probes) to depict phosphatidylserine exposing and infected erythrocytes, respectively. For annexin binding, erythrocytes were washed, resuspended in annexin-binding buffer (140 mM NaCl, 10 mM HEPES, 5 mM Glucose, 5 mM CaCl2, pH 7.4), stained with annexin V-568 (dilution 1:50) or annexin V-fluos (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin binding buffer. Syto16 (final concentration of 20 nM) was either directly added to the diluted
erythrocyte suspension (or incubated for 30 min at 37°C) or co-incubated in the annexin binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson) using FL-1 for Syto16 or annexin V-fluos fluorescence intensity and with FL-2 for annexin V-568 fluorescence intensity.

**Determination of intracellular Ca\(^{2+}\) influx in the erythrocytes**

Intracellular Ca\(^{2+}\) measurements were performed as described previously (Foller et al., 2008c). Erythrocytes were loaded with fluo-3 AM (Calbiochem, Bad Soden, Germany) by addition of fluo-3 AM stock solution (2 mM diluted in DMSO) to 1 ml of erythrocyte suspension (0.16% hematocrit in Ringer solution; 4 \(\mu\)M fluo-3 AM final concentration). The cells were incubated at 37°C for 15 min under protection from light. An additional 2-\(\mu\)l aliquot of fluo-3 AM was added, and then the mixture was incubated for 25 min. Fluo-3-loaded erythrocytes were centrifuged at 1,000 \(g\) for 5 min at 22°C and then washed twice with Ringer solution containing 0.5% bovine serum albumin (Sigma) and once with Ringer solution and incubated. For flow cytometry, fluo-3-loaded erythrocytes were resuspended in 1 ml of Ringer solution (0.16% hematocrit) containing 5mM calciumchloride and incubated at 37°C for 30 min. As a positive control, erythrocytes were stimulated with 1 \(\mu\)M Ca\(^{2+}\) ionophore ionomycin (Sigma) for 3 min prior to analysis to increase intracellular Ca\(^{2+}\) activity. For negative control, cells were incubated for 30 min at 37°C with vehicle alone. Subsequently, Ca\(^{2+}\)-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

**Blood count**

Erythrocyte density, packed cell volume, mean erythrocyte volume, white blood cell counts, and hemoglobin content were determined using an electronic haematology particle counter (type MDM 905) from Medical Diagnostics Marx (Butzbach, Germany), equipped with a photometric unit for hemoglobin determinations.

**FACS analysis of PS exposure and forward scatter**

FACS analysis was performed as described (Nicolay, Gatz et al. 2007). After incubation, cells were washed in Ringer solution containing 5 mM CaCl\(_2\). Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V fluorescence
intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

**Freezing of parasites**

From time to time samples with high parasitemia, grown to ≥ 20% parasitemia, predominantly containing ring stages, were deep frozen in liquid nitrogen for stock purposes. The cell pellet obtained after centrifugation (1800/4 min at RT) was mixed with the same volume of freezing solution, sterile transferred into a 2.0 ml CryoTube Vial, and directly deep frozen in liquid nitrogen.

**Data analysis and statistics**

Data are provided as means ± standard error of mean (SEM), n represents the number of independent experiments. Statistical analysis was made by paired or unpaired $t$ test or by ANOVA using Dunnett’s or Tukey’s test as post hoc test, where appropriate. $P \leq 0.05$ was considered statistically significant.
6. RESULTS

Azathioprine favourably influences the course of malaria

Azathioprine, a widely used immunosuppressive drug (Anstey et al., 1998b; Casetta et al., 2007; Hollander et al., 1998b; Wise et al., 2007a), has recently been shown to similarly trigger eryptosis (Geiger et al., 2008a). The present study thus explored whether azathioprine accelerates eryptosis of *P. falciparum*-infected erythrocytes and thus influences parasitemia and survival during malaria. Azathioprine (6-mercaptopurine) has previously been shown to inhibit a purine phosphoribosyltransferase of the parasite and thus to interfere with *in vitro* growth of the parasite (Queen et al., 1989; Queen et al., 1990). An effect on the survival of infected erythrocytes or *in vivo* efficacy, to our knowledge, has never been reported.

![Figure 4](image-url)

**Figure 4** Effects of azathioprine on intraerythrocytic amplification and *in vitro* parasitemia

A. *In vitro* parasitemia with *P. falciparum* in human erythrocytes as a function of azathioprine concentration (arithmetic means ± SEM, n=8). * indicates significant difference (p ≤ 0.05) from absence of azathioprine.

B. Intraerythrocytic DNA amplification as a function of the azathioprine concentration (arithmetic means ± SEM, n = 6).

To explore whether infection of erythrocytes triggers eryptosis, phosphatidylserine-exposing erythrocytes were identified by determination of annexin V-binding in FACS analysis. Prior to infection, the percentage of annexin V-binding erythrocytes was low (1.25 ± 0.20%, n = 6; data not shown). Infection within 24 hours led to a marked increase in annexin V-binding of both, infected erythrocytes and noninfected bystander cells (Fig. 5). The percentage of annexin V-binding was more than twice as high in infected than in noninfected erythrocytes (Fig. 5), a difference statistically significant both, in the absence and presence of azathioprine.
The phosphatidylserine exposure of infected erythrocytes was significantly augmented by azathioprine (Fig. 5), an effect observed at 1 µM azathioprine.

![Diagram of PS-exposing cells](image)

**Figure 5 Effects of azathioprine on phosphatidylserine exposure of infected and noninfected erythrocytes**

Arithmetic means ± SEM (n=12) of annexin V-binding of infected (closed bars) and noninfected (open bars) erythrocytes following infection of human erythrocytes with *P. falciparum* at 0 µM (left bars) and 1 µM azathioprine. *** indicates significant difference (p≤0.001; paired ANOVA) from noninfected erythrocytes, ## indicates significant difference (p≤0.01; paired ANOVA) from absence of azathioprine.

Depending on the stage of the parasite development, infection of erythrocytes decreased (early stages; Fig. 6A) or increased (late stages; Fig. 6B) erythrocyte forward scatter, indicating that early stages initially decreased the host cell volume. Subsequently, during later parasite development, the volume-expanding trophozoites increased the host cell volume. Azathioprine at concentrations of 5 and 10 µM decreased the forward scatter of late stage infected erythrocytes, which was probably due to azathioprine-induced inhibition of intraerythrocytic parasite development (see Fig. 4B). In the early stage of infection, however, a statistically significant shrinking effect of azathioprine on infected cells was evident at lower concentrations of azathioprine (≥ 0.1 µM). In summary, these experiments indicate that low concentrations of azathioprine augment eryptosis of the host erythrocyte.
Results

Figure 6  Effects of azathioprine on forward scatter of infected and noninfected erythrocytes

A. Normalized forward scatter (n=12) of the early stage-infected erythrocytes (closed symbols) and noninfected (open symbols) erythrocytes as a function of the azathioprine concentration. * indicates significant difference (p≤0.05; ANOVA) from absence of azathioprine. ### indicate significant difference (p≤0.05, p≤0.01; ANOVA) from noninfected erythrocytes. Noninfected erythrocytes and erythrocytes infected with early and late parasite stages were defined by background, intermediate and high staining of the cells with the DNA/RNA-specific fluorescence dye syto16.

B. Normalized forward scatter (n=12) of late stage-infected erythrocytes (closed symbols) and noninfected (open symbols) erythrocytes as a function of the azathioprine concentration. * indicates significant difference (p≤0.05; ANOVA) from absence of azathioprine.

In a last series of experiments, mice were infected with *P. berghei* to determine the *in vivo* efficacy of azathioprine treatment. The administration of azathioprine (daily injections of 5 mg/kg b.w. azathioprine subcutaneously) was initiated 8 days after infection. At this time, parasitemia was less than 5% (Fig. 7B). The percentage of infected erythrocytes gradually increased in both, treated and untreated mice. The percentage of parasitized erythrocytes was lower in azathioprine-treated animals than in animals without azathioprine treatment, an effect reaching statistical significance between day 17 and day 20 of infection (Fig. 7A, B). Accordingly, azathioprine treatment at least transiently decreased parasitemia (Fig. 7A, right panels and Fig. 7B). Azathioprine treatment further affected the survival of *P. berghei*-infected mice. As illustrated in Fig. 7C, all untreated animals died within 22 days after the infection. In contrast, 77% of the azathioprine-treated animals survived the infection for more than 22 days.
Results

<table>
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<th>Cell counts</th>
<th>parasitemia (%)</th>
<th>survival (%)</th>
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Survival is stimulated by aurothiomalate, a gold-containing drug effective against rheumatoid arthritis (Sopjani et al., 2008e). Gold complexes have indeed been shown to counteract malaria (Blackie et al., 2003; Navarro, 2009; Navarro et al., 1997d; Navarro et al., 2004a; Sanchez-Delgado et al., 1996d; Sannella et al., 2008b; Wasi et al., 1987). They are considered to be effective through inhibition of heme aggregation, haemozoin formation and/or parasitic thioredoxin reductase as well as interaction with the DNA of the parasite.
The present study explored, whether sodium aurothiomalate augments the death of *Plasmodium falciparum*-infected human erythrocytes and/or *Plasmodium berghei* infected mouse erythrocytes and whether this effect correlates with a favourable influence on parasitemia and host survival during murine malaria.

A first series of experiments explored the influence of aurothiomalate on the *in vitro* growth of *Plasmodium falciparum* in human erythrocytes. To this end, *P. falciparum* infected erythrocytes were cultured in human erythrocytes and synchronized to the ring stage by sorbitol treatment. Within 48 hours the percentage of infected erythrocytes increased from 6.0% to 21.0% in the absence and to 9.8% in the presence of 100 µM aurothiomalate (Fig. 8A). Accordingly, aurothiomalate blunted the increase in the percentage of parasitized erythrocytes, an effect reaching statistical significance at ≥ 10 µM aurothiomalate (Fig. 8A). The half maximal inhibition (IC50) was achieved by 68 µM aurothiomalate. In contrast, at the concentrations tested, the presence of aurothiomalate did not influence the intraerythrocytic DNA amplification of the parasite (Fig. 8AB).
Results

Figure 8: Effects of sodium aurothiomalate on intraerythrocytic amplification and in vitro parasitemia

A. In vitro parasitemia with *P. falciparum* (left panel) in human erythrocytes as a function of the aurothiomalate concentration (arithmetic means ± SEM, n = 16). *, ***, indicate significant difference (p<0.05, p<0.001) from the absence of aurothiomalate. Intraerythrocytic DNA amplification (right panel) as a function of the aurothiomalate concentration (arithmetic means ± SEM, n = 12).

B. Intraerythrocytic DNA amplification (right panel) as in B for different time periods (arithmetic means ± SEM, n = 8).

In order to determine the effect of infection and of aurothiomalate on eryptosis, phosphatidylserine-exposing erythrocytes were identified by measurement of annexin V-binding in FACS analysis. Within 24 hours, the infection with *P. falciparum* markedly increased the annexin V-binding of infected and noninfected erythrocytes (Fig. 9). The percentage of annexin V-binding was, however, significantly higher in infected than in noninfected erythrocytes (Fig. 9). The phosphatidylserine exposure of infected erythrocytes was significantly increased in the presence of aurothiomalate (Fig. 9), an effect reaching statistical significance at 50 µM aurothiomalate.

![Figure 9 Effects of aurothiomalate on phosphatidylserine exposure of infected and noninfected human erythrocytes](image)

Arithmetic means ± SEM (n = 6) of the percentage of annexin V-binding infected (open bars) and non-infected (closed bars) erythrocytes following infection of human erythrocytes with *P. falciparum* in the presence of 0 – 100 µM aurothiomalate. **, ### indicate significant difference (p<0.01, p<0.001) from non-infected erythrocytes, ***, **** indicate significant difference (p<0.01, p<0.001) from the absence of aurothiomalate.

In a next series, mice were infected with *P. berghei* with or without sodium aurothiomalate treatment. Sodium aurothiomalate was administered daily from the 8th day of infection. Similar to the in vitro infection of human erythrocytes with *P. falciparum*, the infection of
mice with *P. berghei* was followed by a marked increase in the percentage of phosphatidylserine-exposing erythrocytes. The phosphatidylserine exposure of infected erythrocytes was significantly more pronounced following treatment with sodium aurothiomalate than the phosphatidylserine exposure of noninfected erythrocytes (Fig. 10).

The parasitemia was still low on the 8th day of infection (Fig. 11B). The percentage of infected erythrocytes gradually increased with or without sodium aurothiomalate treatment. However, the percentage of parasitized erythrocytes was significantly lower in sodium aurothiomalate-treated animals than in animals without sodium aurothiomalate treatment (Fig. 11A, B). Since the FACS-dependent determination of parasitemia utilizes a DNA/RNA-specific dye, reticulocytes may also be counted as parasitized erythrocytes. Therefore, a second series of experiments was performed to compare the values for parasitemia determined by FACS analysis to those obtained from Giemsa staining. As shown in Fig. 11D, E, parasitemia was lower in the aurothiomalate-treated group of mice, irrespective of the methods applied. The treatment with sodium aurothiomalate further resulted in enhanced survival of *P. berghei*-infected mice. As shown in Fig. 11C, all untreated animals died within 30 days after the infection. In contrast, 57% of the sodium aurothiomalate-treated animals were still alive 30 days after infection. All treated mice died, however, by day 44 after infection.
Figure 11: Effect of sodium aurothiomalate treatment on the parasitemia and survival of *Plasmodium berghei*-infected mice

A: Original histograms of parasitemia-dependent Syto16 fluorescence in untreated animals (upper panels) and in animals treated daily from day 8 daily with 10 mg/kg b.w. sodium aurothiomalate s.c. (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*.

B: Arithmetic means ± SEM of the parasitemia in mice without treatment (open circles, n = 8 mice) or with daily 10 mg/kg b.w. s.c. of sodium aurothiomalate (closed circles, n = 6 mice) as a function of days after infection with *P. berghei*. Significant difference (* p<0.05, ** p<0.01, *** p<0.001; t-test) from the untreated animals on days 12 - 20. The results presented are one of three independent series.

C: Survival of mice without treatment (open circles) or with daily 10 mg/kg b.w. sodium aurothiomalate s.c. (closed circles) as a function of days after infection with *Plasmodium berghei*.

D-E: Arithmetic means ± SEM of the parasitemia in mice without treatment (open circles, n = 4 mice) or with daily 10 mg/kg b.w. s.c. of sodium aurothiomalate (closed circles, n = 4 mice) as a function of days after infection with *P. berghei*. The parasitemia was determined daily either by staining with Syto16 and subsequent FACS analysis as in B (D) or by daily Giemsa staining of blood smears and light microscopy-dependent analysis (E).

To investigate whether aurothiomalate treatment influences inflammation, the plasma levels of the inflammatory mediator TNFα, were determined on the 16th day of infection. As a result,
the TNFα concentration in non-treated mice was 53.4 ± 29.7 pg/ml whereas the TNFα was below the detection limit in mice treated with sodium aurothiomalate (both n= 4).

As shown earlier, TNF-α may exert an antiparasitic effect in animal models (Clark et al., 1987; Neifer et al., 1989; Taverne et al., 1987), and high TNF production is associated with more rapid clinical and parasitologic recovery in humans (Kremsner et al., 1995). Even though aurothiomalate does not seem to affect induction of TNF-alpha in phagocytic cell cultures (Bondeson, 1997), the present observations clearly demonstrate an effect of the drug on TNF production in vivo.

Malaria is paralleled by loss of erythrocytes leading to anemia. As shown in Fig. 12, the hematocrit of aurothiomalate-treated mice was significantly reduced. The effect could have been due to enhanced eryptosis or hemolysis. In noninfected erythrocytes aurothiomalate has previously been shown to trigger eryptosis rather than hemolysis (Sopjani et al., 2008h).

![Figure 12: Effect of sodium aurothiomalate treatment on the hematocrit of Plasmodium berghei-infected mice](image)

Arithmetic means ± SEM of packed cell volume (hematocrit) in mice without treatment (open circles, n = 8 mice) or with daily 10 mg/kg b.w. s.c. of sodium aurothiomalate (closed circles, n = 8 mice) as a function of days after infection with *P. berghei*. * indicates significant difference (p<0.05; t-test)

The present study demonstrates that aurothiomalate had only mild effects on the parasite burden and moderately delayed the lethal course of malaria following infection of mice with *P. berghei*. Similar to what has been observed earlier (Huber et al., 2004c), the infection of mice with *P. berghei* was followed by an invariably lethal course of malaria without aurothiomalate treatment. More than 50% of the sodium aurothiomalate-treated animals survived the infection for 30 days, even though they all died by day 44.
The effect of sodium aurothiomalate treatment may in part be due to a toxic effect on the pathogen, which compromises the intraerythrocyte growth of the parasite. As a matter of fact, gold-containing drugs have previously been shown to be toxic for *Plasmodia* (Blackie, Beagley, Chibale, Clarkson, Moss, and Smith, 2003; Cohen et al., 1965a; Egan, 2003; Egan, 2008c; Martinez et al., 2008b; Navarro, 2009; Navarro et al., 1997c; Navarro et al., 2004b; Sanchez-Delgado et al., 1996c; Sannella et al., 2008c; Slater et al., 1992a; Wasi, Singh, Gajanana, and Raichowdary, 1987; Ziegler et al., 2001c). Drugs could specifically enter infected erythrocytes, as the pathogen dramatically enhances the permeability of the erythrocyte membrane (Kirk, 2001a).

Alternatively, sodium aurothiomalate may exert a protective effect by accelerating the death of infected erythrocytes. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages (Boas et al., 1998d; Yamanaka et al., 2005c) and are thus rapidly cleared from circulating blood (Kempe et al., 2006d). As eryptosis mainly affects infected erythrocytes, accelerated eryptosis should decrease the parasitemia and thus favourably influence the course of the disease (Foller et al., 2009b) and Wilson’s disease (Wood et al., 1996b; Andrews et al., 1999a; Closse et al., 1999b; Gallagher et al., 2003b; Pandolfi et al., 2007a). Accordingly, eryptosis has been suggested to foster vascular derangements of metabolic syndrome (Zappulla, 2008a).

The discrepancy between the moderate influence of aurothiomalate on parasitemia and the effect on survival of the infected host is suggestive for an additional effect of the drug on mouse survival. Possibly it is in part the anti-inflammatory effect of the drug, which accounts for at least part of the effect on host survival and the stimulation of eryptosis. As a matter of fact, aurothiomalate treatment virtually abolished the increase in TNF-α plasma concentration following infection.

**Protective effect of amiodarone in malaria**

Amiodarone, an ion channel blocker effective in the treatment of cardiac arrhythmias (Nishimura et al., 1989; Yamashita et al., 2006), has previously been shown to trigger eryptosis (Nicolay et al., 2007a). The present study explored, whether amiodarone would augment the phosphatidylerserine exposure of *Plasmodium*-infected erythrocytes and thus favorably influence parasitemia and host survival during malaria.

In a first series of experiments, the influence of amiodarone on the *in vitro* growth of the parasite was analysed. To this end, *P. falciparum*-infected erythrocytes were cultured in human erythrocytes and synchronised to ring stage by sorbitol treatment. Within 48 hours the
percentage of infected erythrocytes increased from the initial value of 2.4 ± 0.6% to some 12.3 ± 2.6%, a result of intraerythrocytic amplification with subsequent evasion from the host cell, and invasion into new erythrocytes (Fig. 1A). In the presence of amiodarone the increase in the percentage of parasitized erythrocytes was decreased, an effect reaching statistical significance at ≥ 1 µM amiodarone concentration (Fig. 13A). The presence of amiodarone further decreased the intraerythrocytic DNA amplification of the parasite, an effect reaching statistical significance at ≥ 5 µM amiodarone concentration (Fig. 13B). Thus, at concentrations ≥ 5 µM, amiodarone exerts direct inhibitory effects on parasite amplification, evasion and/or reinvasion.

**Figure 13:** Effects of amiodarone on intraerythrocytic amplification and *in vitro* parasitemia

A. *In vitro* parasitemia with *P. falciparum* in human erythrocytes as a function of the amiodarone concentration (arithmetic means ± SEM, n = 16 (4 cultures studied in quadruplicates). *, ** indicate significant difference (p<0.05, p<0.001) from the absence of amiodarone.

B. Intraerythrocytic DNA amplification as a function of the amiodarone concentration (arithmetic means ± SEM, n = 3 cultures studied in quadruplicates).

To elucidate the effect of infection and of amiodarone on eryptosis, the percentage of phosphatidylserine-exposing erythrocytes was determined by measurement of annexin V-binding in FACS analysis. The percentage of annexin V-binding erythrocytes was low prior to
the infection (2.7 ± 0.4 %, n = 6). Within 24 hours infection was followed by a marked increase in the percentage of annexin V-binding erythrocytes (Fig. 14). The percentage of annexin V-binding was significantly higher in infected than in noninfected erythrocytes both in the absence and presence of amiodarone (Fig. 14). The phosphatidylserine exposure of infected erythrocytes was significantly augmented by amiodarone (Fig. 14), an effect reaching statistical significance at 10 µM amiodarone.

![Graph A](image1.png)  
![Graph B](image2.png)  
![Graph C](image3.png)  
![Graph D](image4.png)

**Figure 14: Effects of amiodarone on phosphatidylserine exposure of infected and noninfected erythrocytes**

Arithmetic means ± SEM (n = 3 cultures studied in duplicates) of the percentage of annexin V-binding infected (open bars) and non-infected (closed bars) erythrocytes following infection of human erythrocytes with *P. falciparum* in the presence of the indicated concentrations of amiodarone. *, **, and *** indicate significant difference (p<0.05, p<0.01, and p<0.001) from non-infected erythrocytes, ### indicates significant difference (p<0.001) from absence of amiodarone.

A second series of experiments explored the effect of amiodarone on *Plasmodium* infection *in vivo*. To this end, mice were infected with *P. berghei* with or without amiodarone treatment. Amiodarone (daily injections of 50 mg/kg b.w. amiodarone intraperitoneally) was administered daily from the 8th day of infection, when parasitemia was still low (Fig. 15B). In both, treated and untreated mice, the percentage of infected erythrocytes increased gradually. However, the percentage of parasitized erythrocytes was significantly lower in amiodarone-
treated animals than in animals without amiodarone treatment, an effect reaching statistical significance between day 13 and day 21 after infection (Fig. 15A, B).

The treatment with amiodarone further influenced the survival of *P. berghei*-infected mice. As shown in Fig. 15C, all untreated animals died within 26 days after the infection. In contrast, 70% of the amiodarone-treated animals survived the infection for more than 26 days.

**Figure 15: Parasitemia and survival of Plasmodium berghei infected mice**

A: Original histograms of parasitemia-dependent Syto 16 fluorescence in untreated animals (upper panels) and animals treated from day 8 until day 20 with 50 mg/kg b.w. of amiodarone i.p. (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*.

B: Arithmetic means ± SEM of parasitemia in mice without treatment (open circles, n = 10) or with 50 mg/kg b.w. of amiodarone i.p. (closed circles, n = 6) as a function of days after infection with *P. berghei*. **, *** indicate significant difference (p<0.05) from the untreated animals.

C: Survival of mice without treatment (open circles) or with 50 mg/kg b.w. of amiodarone i.p. (closed circles) as a function of days after infection with *Plasmodium berghei*.

To check whether the beneficial effect of amiodarone on the course of malaria was dependent on the stimulation of PS exposure, erythrocytes were taken from the mice on day 8 after
infection and phosphatidylserine exposure of infected and non-infected erythrocytes determined. As shown in Fig. 16, infected erythrocytes from both, amiodarone-treated and non-treated mice showed significantly more PS exposure. More importantly, treatment with amiodarone significantly increased eryptosis of infected erythrocytes while having no appreciable effect on non-infected erythrocytes.

![Graph](image)

**Figure 16: In vivo effect of amiodarone on phosphatidylserine exposure of infected and noninfected erythrocytes**

Arithmetic means ± SEM (n = 6-10) of the percentage of annexin V-binding infected (open bars) and non-infected (closed bars) erythrocytes retrieved from mice infected with *Plasmodium berghei* on day 8 after infection treated without (left bars) or with amiodarone (right bars). *** indicates significant difference (p<0.001) from non-infected erythrocytes, ### indicates significant difference (p<0.001) from control.
7. DISCUSSION

A wide variety of antimalarial drugs are available in the market to treat malaria. In the last five years, treatment of *Plasmodium* infections in endemic countries has been transformed by the use of combinations of drugs containing artemesinin derivatives. All the recent combination therapies are not so clear and target oriented since there is a high possibility of resistance of *Plasmodium* towards these agents. To counteract the resistance of *Plasmodium* various therapeutic agents are to be developed, which must act either through host-mediated or show prophylactic action.

The better way to apt is host-mediated treatment since millions of people in the tropical countries suffer with this dreadful disease and the incidence is increasing. Utilising mechanisms involved in eryptosis, especially with therapeutic agents, is quite interesting in so much as their weaknesses can equally be advantageous here. I used this principle in evading *Plasmodium* from proliferation and prevent the further course of malaria as I knew the facts and physiological changes involved in eryptotic and *Plasmodium* infected erythrocytes. Comparing the both conditions I found that the infected erythrocytes also show some similar physiological changes with that of eryptotic erythrocytes. Coming to a conclusion of my theoretical findings I combined these two physiological phenomena and make up the early apoptosis of the infected erythrocytes and thus prevent the further course of the disease.

Based on my hypothesis, upon experimentation to my surprise I found some favourable findings which might combat this dreadful disease. Owing to the frequent appearance of drug-resistant *Plasmodium* strains, the classical antimalarial drugs tend to become rapidly obsolete, generating a continuous need for new therapeutic agents, possibly endowed with innovative mechanisms of action hence eryptosis may be a better option.

**Azathioprine favourably influences the course of malaria**

Earlier findings indicated that azathioprine is widely used as an immunosuppressive drug. The side effects of azathioprine included anemia, which has been attributed to bone marrow suppression. Alternatively, anemia could result from accelerated suicidal erythrocyte death, which is characterized by exposure of phosphatidylserine (PS) at the erythrocyte surface and by cell shrinkage. They have shown exposure to azathioprine (>2 µg/mL) with increased cytosolic Ca$^{2+}$ activity and annexin V binding and decreased forward scatter for 48 hours (Geiger et al., 2008b). This is more or less equal to 7.2 µM concentration. When I used a
range of concentrations from 0.001 µM to 10 µM concentrations in the in vitro experimentation I found that 1 µM concentration and above showed decrease in the proliferation as well as the intraerythrocytic DNA amplification of Plasmodium. The effect of azathioprine in the in vitro studies may be due to direct effect on the parasite as well as on the infected erythrocyte. This might be an opportunity to move further in my work to check whether there is any eryptotic effect on co-incubated healthy erythrocytes. Performing double staining to check the eryptosis in both infected and non infected erythrocytes, my results indicated that there is not any significant effect of azathioprine on the non infected erythrocytes.

Several mechanisms may contribute to the efficacy of azathioprine. In theory, the effect of azathioprine could have been due to its immune-suppressing potency [(Anstey et al., 1998a; Casetta, Iuliano, and Filippini, 2007; Hollander et al., 1998a; Wise et al., 2007b)]. However, it is not likely that immunosuppression achieves both, a significant reduction of parasitaemia and a milder course of the disease. Moreover the dose administered is comparatively very low than its regular therapeutic dose used for immunosuppressive activity of azathioprine.

The in vitro experimentation results indicate the direct effect of azathioprine on the Plasmodium and infected erythrocytes. Its potent activity in the in vitro studies is because of absence of any metabolic machinery in the medium used for the regular growth of the plasmodium. Azathioprine could further affect parasitaemia and host survival by directly affecting the survival and replication of the pathogen or its ability to evade parasitized erythrocytes and to invade noninfected erythrocytes.

The in vivo study unravels antimalarial effect of azathioprine, i.e. the favourable influence on the course of malaria. Most importantly, azathioprine treatment significantly enhances the percentage of surviving animals after infection with P. berghei. As shown previously, without treatment, the infection of mice with P. berghei is followed by an invariably lethal course of malaria within 22 days (Huber et al., 2004b). In contrast, most of the mice treated with azathioprine survived the infection for 22 days. The reason for increase in the survival of the mice may be due to increase in the accelerated clearance of the infected erythrocytes and decreased in further invasion of new erythrocytes.

The effect of azathioprine could further be secondary to its ability to stimulate suicidal death of erythrocytes (Geiger, Föller, Herrlinger, and Lang, 2008a), an effect, which could contribute to or even account for the blunted parasitaemia and the survival of the infected mice. The drug could be effective by accelerated clearance of infected erythrocytes due to
eryptosis. Moreover, the enhanced eryptosis may promote the release of pro-inflammatory cytokines from activated macrophages (Boas et al., 1998c; Yamanaka et al., 2005b), thereby resulting in the activation of the hormonal stress response (Kempe et al., 2006c).

Other than eryptotic activity of azathioprine its effect on the parasite immunity may also be one of the un-noticed mechanisms. Along side, azathioprine acts as purine inhibitor which is necessary for the fast growing cells. This might be one of the hindrances for the further proliferation of the parasite and need to be verified to reveal the exact mechanism involved in the decrease in the course of malaria other than eryptosis as one of the major factor.

Further experiments like usage of the drug in pre-clinical evaluation studies with different standard malaria drug combinations may confirm it for clinical trials.

**Beneficial effect of aurothiomalate on murine malaria**

Eryptosis is stimulated by aurothiomalate, a gold containing drug effective against rheumatoid arthritis (Sopjani et al., 2008g). Gold complexes have indeed been shown to counteract malaria (Blackie, Beagley, Chibale, Clarkson, Moss, and Smith, 2003; Navarro, 2009; Navarro et al., 2009; Navarro et al., 1997b; Navarro et al., 2004c; Sanchez-Delgado et al., 1996b; Sannella et al., 2008d; Wasi, Singh, Gajanana, and Raichowdary, 1987). But exact mechanism involved is still in question. Premature death of *Plasmodium*-infected erythrocytes is considered to favourably influence in the clinical course of malaria since phosphatidylinerine-exposing cells are rapidly cleared from circulating blood. They are considered to be effective also through inhibition of heme aggregation, haemozoin formation and/or parasitic thioredoxin reductase as well as interaction with the DNA of the parasite (Cohen et al., 1965c; Egan, 2003; Egan, 2008a; Martinez et al., 2008c; Slater et al., 192c; Ziegler et al., 2001b).

The treatment with aurothiomalate had only mild effects on the parasite burden and moderately delayed the lethal course of malaria following infection of mice with *P. berghei*. Similar to what has been observed earlier (Huber et al., 2004a), the infection of mice with *P. berghei* was followed by an invariably lethal course of malaria without aurothiomalate treatment. More than 50% of the sodium aurothiomalate-treated animals survived the infection for 30 days, even though they all died by day 44. The other chances of the mice to survival may be the activity of aurothiomalate on the thioredoxin reductase enzyme of the *Plasmodium* (Sannella et al., 2008e) which prevented in survival and proliferation of the plasmodium.
The effect of sodium aurothiomalate treatment may in part be due to a toxic effect on the pathogen, which compromises the intraerythrocytic growth of the parasite. As a matter of fact, gold-containing drugs have previously been shown to be toxic for *Plasmodium* (Blackie, Beagley, Chibale, Clarkson, Moss, and Smith, 2003; Cohen et al., 1965d; Egan, 2003; Egan, 2008b; Martinez et al., 2008d; Navarro, 2009; Navarro et al., 1997a; Navarro et al., 2004d; Sanchez-Delgado et al., 1996a; Sannella et al., 2008f; Slater et al., 1992d; Wasi, Singh, Gajanana, and Raichowdary, 1987; Ziegler et al., 2001a). Drugs could specifically enter infected erythrocytes, as the pathogen dramatically enhances the permeability of the erythrocyte membrane (Kirk, 2001c).

Alternatively, sodium aurothiomalate may exert a protective effect by accelerating the death of infected erythrocytes. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages (Boas et al., 1998b; Yamanaka et al., 2005a) and are thus rapidly cleared from circulating blood (Kempe et al., 2006b). As eryptosis mainly affects infected erythrocytes, accelerated eryptosis should decrease the parasitemia and thus favourably influence the course of the disease (Foller, Bobbala, Koka, Huber, Gulbins, and Lang, 2009b) and Wilson’s disease (Wood et al., 1996c; Andrews et al., 1999b; Closse et al., 1999a; Gallagher et al., 2003a; Pandolfi et al., 2007b). Accordingly, eryptosis has been suggested to foster vascular derangements of metabolic syndrome (Zappulla, 2008a).

The discrepancy between the moderate influence of aurothiomalate on parasitemia and the effect on survival of the infected host is suggestive for an additional effect of the drug on mouse survival. Possibly it is in part the anti-inflammatory effect of the drug, which accounts for at least part of the effect on host survival and the stimulation of eryptosis. As a matter of fact, aurothiomalate treatment virtually abolished the increase in TNFα plasma concentration following infection.

The observed antimalarial effects are probably mediated by severe oxidative stress originating from *P. falciparum* thioredoxin reductase inhibition. Which was already been demonstrated (Sannella et al., 2008a). Of course, further experimental work is absolutely necessary to prove aurothiomalate can be used in clinical trials. Hopefully both the mechanisms will lead to the potent antiplasmodial activity and also helps in preventing the resistance of *Plasmodium* towards other antimalarial agents when used in combination.
Protective effect of amiodarone in malaria

Haematological side effects of amiodarone have been reported rarely. Cases of bone marrow suppression resulting in anemia or thrombocytopenia have been reported which may be caused by decreased formation or accelerated death of erythrocytes. Suicidal erythrocyte death (eryptosis) is characterized by cell shrinkage and cell membrane scrambling leading to phosphatidylserine exposure at the cell surface. Stimulators of erythrocyte membrane scrambling include increase of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]i) following activation of Ca$^{2+}$-permeable cation channels. According to previous observations, amiodarone triggers suicidal erythrocyte death or eryptosis. Amiodarone treatment may in turn accelerate the clearance of Plasmodium-infected erythrocytes.

Administration of amiodarone showed an influence on the in vitro parasitemia and host erythrocyte survival as well as the in vivo course of malaria. When compared along with other standard malarial drugs artemensinin and chloroquine, amiodarone showed significant eryptotic effect but these trials were only performed in vitro experimentation.

Amiodarone significantly decreased the in vitro parasitemia and intraerythrocytic DNA amplification of P. falciparum within human erythrocytes, a result of intraerythrocytic amplification with subsequent evasion from the host cell, and invasion into new erythrocytes. Amiodarone may thus influence survival, proliferation and/or the ability of the parasite to exit from or enter into erythrocytes. Moreover, amiodarone augmented cell membrane scrambling with phosphatidylserine exposure of infected erythrocytes, a hallmark of suicidal erythrocyte death (Lang et al., 2008a). Further there may be many effects of amiodarone when used directly on the plasmodium and may lead to increase in the polymorphisms. Amiodarone being a potent potassium channel blocker may also exert effect on the potassium channel of the Plasmodium, which is essential for cell survival and in the regulation of the cell membrane potential and electrochemical gradient. During its lifecycle, Plasmodium falciparum parasites must rapidly adapt to dramatically variant ionic conditions within the mosquito mid-gut, the hepatocyte and erythrocyte cytosol and the human circulatory system(Waller et al., 2008).

Most importantly, treatment with amiodarone significantly enhanced the percentage of surviving animals following infection with P. berghei. As shown in earlier studies, the infection of mice with P. berghei is followed by an invariably lethal course of malaria (Koka et al., 2008b). In contrast, two thirds of the amiodarone-treated animals survived the infection for 26 days. The dose utilized (50 mg/kg day) was similar to that used previously without
evidence of toxicity (Benaim et al., 2006; Breitenstein et al., 2008; DeWitt et al., 2005; McCarthy et al., 2004; Mendez et al., 1999; Sanchez et al., 2007).

The present observations do not allow safe conclusions on the mechanisms underlying the beneficial effect of amiodarone treatment in *Plasmodium* infected mice. In theory, amiodarone could have affected parasitemia and host survival by directly affecting the survival and replication of the pathogen or by its ability to evade parasitized erythrocytes and to invade noninfected erythrocytes. A direct toxic effect on the parasite would be expected to decrease the DNA/RNA content of infected erythrocytes. Compromised evasion and subsequent invasion would decrease the number of infected erythrocytes. As a matter of fact, as indicated above, high concentrations of amiodarone decreased the number of erythrocytes parasitized *in vitro* and the DNA/RNA content of parasitized erythrocytes.

Alternatively, amiodarone may have affected parasitemia and host survival by accelerating the suicidal death of infected erythrocytes (Foller *et al.*, 2009a). As reported earlier (Nicolay, Bentzen, Ghashghaeinia, Wieder, and Lang, 2007a), amiodarone increases \([\text{Ca}^{2+}]\) and triggers annexin V-binding, but does not significantly decrease forward scatter and does not significantly influence ceramide formation. Thus, amiodarone triggers phosphatidylserine scrambling at least in part by increasing \([\text{Ca}^{2+}]\) entry into the cell. The lack of cell shrinkage despite of increases in cytosolic \([\text{Ca}^{2+}]\) points to some additional effect of amiodarone, which may account for the different thresholds for a significant amiodarone action on the different parameters. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages (Boas *et al.*, 1998a; Yamanaka *et al.*, 2005d) and are thus rapidly cleared from circulating blood (Kempe *et al.*, 2006a).

Amiodarone being an anti arrhythmic drug needs to be monitored initially in mice model. I indeed did some observations which were not so prominent signs of adverse effects of the drug. The dose selected was also very low when compared with that of the normal therapeutic dose of amiodarone used to treat arrhythmias.

In final concern I like to show attention much on the new uses of old drugs which are clinically established and patient friendly. This would be a modern approach and an advantageous choice of effective drug discovery which will have most promising effects. Indeed, much work concerning the safety profile can be avoided leading to a drastic reduction of times and costs.
8. CONCLUSION

Eryptosis is triggered by a variety of endogenous mediators and xenobiotics (Lang, Gulbins, Lerche, Huber, Kempe, and Foller, 2008a; Mahmud et al., 2009b; Niemoeller et al., 2008c; Niemoeller, Foller, Lang, Huber, and Lang, 2008b; Sopjani et al., 2008b; Sopjani et al., 2008d; Wang et al., 2008) and enhanced in a variety of clinical conditions including iron deficiency, sickle-cell anemia, beta-thalassemia, glucose-6-phosphate dehydrogenase (G6PD)-deficiency, phosphate depletion, Hemolytic Uremic Syndrome, sepsis, malaria and Wilson’s disease (Lang, Gulbins, Lerche, Huber, Kempe, and Foller, 2008a). Some of those diseases and xenobiotics have been shown to favorably influence the course of malaria, i.e. sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency (Ayi et al., 2004a; Cappadoro et al., 1998c; de Jong et al., 2001a; Kean et al., 2002a; Kuypers et al., 1998a), iron deficiency, lead, chlorpromazine and cyclosporine (Foller, Bobbala, Koka, Huber, Gulbins, and Lang, 2009a; Koka, Lang, Boini, Bobbala, Huber, and Lang, 2008b). Moreover, the enhanced eryptosis may promote the release of pro-inflammatory cytokines from activated macrophages, thereby resulting in the activation of a hormonal stress response (Zappulla, 2008b).

In conclusion azathioprine, aurothiomalate and amiodarone stimulate the erythrocytic machinery responsible for the eryptosis following infection with Plasmodium. They revealed the antiplasmodial effect both in human and mice malaria; these drugs at lower dose when compared to that of their original therapeutic dose showed stimulation of eryptosis in the infected erythrocytes and hence confirmed eryptosis might be one of the possible mechanisms involved for the early recognition and removal of the eryptosed erythrocytes by the spleen macrophages. The experimental results not only justify that the stimulation of eryptosis in infected erythrocytes is not only a host dependent defence mechanism but also a novel approach to prevent the chances of resistance in plasmodia.

In final concern my experimental results demonstrate the usefulness of the approach for the selection and design of new lead drugs active against P. falciparum with minimal chances of resistance. Hence, I like to show my attention much on the new uses of old drugs which are clinically established and patient friendly. This would be a modern approach and an advantageous choice of effective drug discovery which will have most promising effects. Indeed, much work concerning the safety profile can be avoided leading to a drastic reduction of times and costs.
9. REFERENCES

Reference List


References


References


References


10. ACKNOWLEDGEMENTS

This thesis has been acquired between September 2007 and October 2010 at the Institute of Physiology-1 at University of Tuebingen, Tuebingen, Germany.

I am indebted to many people for their long-lasting support and encouragement which was invaluable for the successful completion of this research work. In the following lines some of them are gratefully acknowledged. However, I am aware of the fact that there are many more and these words cannot express the gratitude and respect I feel for all of those.

Professor Dr. Florian Lang is gratefully acknowledged for offering me the opportunity to work in this interesting field of research, for his constant interest in the project and many constructive discussions. During my tenure, he contributed by giving me intellectual freedom in my work, supporting my attendance at various conferences, engaging me in new ideas, and demanding a high quality of work in all my endeavours.

Additionally, I would like to thank my committee members Professor Dr Giesela Drews, Professor Dr. Peter Ruth, and Professor Dr. Stephan Laufer for their interest in my work.

Initially I would be much indebted to Professor Dr. Stephan Huber, Who welcomed me into his group. His timely scientific discussions helped me a lot to be potential with enough knowledge in the area where I did my thesis.

I am thankful to my group leader Dr. Dr. Michael Föller, his support, encouragement and critiques helped me lot in compiling my thesis. Mostly his help is remarkable in my life to come up to this position.

Every result described in this thesis was accomplished with the help and support of fellow lab-mates and collaborators. I am happy to be the colleague of malaria group where I worked with Dr. Camelia Lang, Dr. Sai Sudha Koka, Ioana Alesutan, Adil Ballal, Adriana and Bala Saheb Siraskar. I will never forget their support in maintaining the friendly environment in the lab. I never felt that I am away from home or in stress of working.

I would like to express my sincere gratitude to all my senior colleagues in the lab Dr. K.M. Boini, Dr. Anand Rotte, Dr. Madhuri Bhandaru, Dr. Shumilina E., Dr. Rexhepaj R., Dr. Teresa Ackeman, Dr Omaima Nasir, Dr. Maherdad G. Dr. Goverdhan .P, Dr. Shefalee Bhavsar, Dr. Hasan Mahmud, Dr. Henning .F and Dr. Diana Michael for their help during the period of my thesis.

I deeply express my heartfelt thanks to my colleagues, Christina Liebrock, Christine Z, Cai Tang, Wenting.Y, Meerim M, and rest who always helped me in providing me the required things necessary for my practical work.

I owe my deepest gratitude to my friends Venkanna Pasham, Syed Qadri, Ganesh Pathare Khaelullah Saheb, TKB Gandhi, Prajwal, Shuchen Gu, Syeda Tasneem, Carlos Munoz, Carlos Lamfus, Anja Umbach, Lisan Pelzl, Katja Merches, Abul Fajol and Kashif Jilani who encouraged me to finish my practical as well as my thesis work in time. Their timely advises helped me in completion of thesis in time.
I like to memorise the kind help done by my lab assistants Mr. Uwe.S, Mrs. Efi Faber and Mr. Farooq feel hats off for them for their dedication and kind nature in helping us when ever we needed them.

I would have never done this if I could not thank my beloved parents Mr. Bobbala Buchireddy, Mrs. Bobbala Sumathi and my sisters Mrs. Divya Krishna Reddy and Mrs. Deepthi Sharath Reddy who have done really a better part in encouraging me and making me feel home though I am miles away.

Last but first from my heart I really thank my better half, my wife Mrs. Rajani Kandhi who really made her support in a number of ways during the final days of my thesis. I am really thankful to god who blessed me with such a girl as my wife.

Finally I like to dedicate my thesis to the mice which I used them in my research studies. Without them I would not even have done anything in completion of my research work.
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<td>School education</td>
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<td>April, 1996</td>
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Master’s thesis: In vitro and In vivo anticancer activity of Vitex trifolia Linn (verbenaceae) leaf extract towards malignant cell lines and on mouse Ehrlich Ascites carcinoma. (Supervisor: Prof Dr Akalanka Dey, Department of pharmacy, Annamalai University, Tamil Nadu, India).

2007 – 2010

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12. LIST OF PUBLICATIONS


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