INDUCED ERYTHROCYTE SUICIDAL DEATH AND ITS EFFECTS ON THE PARASITEMIA AND SURVIVAL OF Plasmodium berghei INFECTED MICE: ROLE OF AMPHOTERICIN B AND GUM ARABIC

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Dedication

To

my wife Hanadi,

my son Mohammed,

my daughters Maryam and Mayada
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Abbreviations

AIDS Acquired immune deficiency syndrome
AIF apoptosis-inducing factor
AMA1 apical membrane antigen 1
AO acridine orange
Apo3 L Apoptosis 3 ligand
ATP Adenosine triphosphate
Bel-2 B-cell lymphoma 2
[Ca^{2+}]_i (free) cytosolic ionic calcium concentration
CAD caspase-activated Dnase
CD 95, Apo-1 cluster of differentiation 95, Fas receptor
CFSF carboxyfluorescein diacetate, succinimidyl ester
CFTR cystic fibrosis transmembrane conductance regulator
COX cyclooxygenase
CT combination therapy
Df dilution factor
DIDS 4,4’- diisothiocyantostilbene – 2,2’ – disulfonic acid
DISC death-inducing signalling complex
DMSO dimethylsulfoxid
DR death receptors
EDTA ethylenediaminetetraacetic acid, [Ethylenedinitrilo]tetraacetic acid
EIA enzyme immunoassay
ELISA enzyme-linked immunosorbent assay
FACS fluorescence assisted cell sorting
FADD Fas-associated death domain
Fas-L fatty acid synthase ligand
Fas-R fatty acid synthase receptors
FITC fluorescein isothiocyanate
FP IX ferri/ferroprotoporphyrin IX, free heme
G6PDH glucose-6-phosphate dehydrogenase
GA gum Arabic
GAPDH glycerinaldehyde-3-phosphat-dehydrogenase
**Gardos channel**  calcium activated potassium channel

**GSH**  Gluthathione

**GSSG**  oxidized gluthathione

**Hb**  Hemoglobin

**HEPES**  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

**HIV**  human immunodeficiency virus

**IAP**  inhibitors of apoptosis proteins

**ICAD**  Inhibitor of Caspase Activated DNase

**ICAM**  intracellular adhesion molecule

**IFA**  immunofluorescence assay

**IFN**  interferon

**IL**  Interleukin

**L-NAME**  L-NG-Nitroarginine methyl ester (hydrochloride)

**NO**  Nitric Oxide

**LSCM**  Laser scanning confocal microscopy

**MACS**  magnetic assisted cell sorting

**MAEBL**  membrane antigen erythrocyte binding protein, paralogue of both AMA1 and DBL-EBP

**MOM**  mitochondrial outer membrane

**MPT**  Mitochondrial permeability transition

**MSP**  merozoite surface protein

**NBS**  Nile blue sulphate

**NF-κB**  nuclear factor kappaB

**NHE**  sodium/hydrogen exchanger

**NMDG⁺**  N-methyl-D-glucamine

**NPP**  New Permeability Pathway

**NPPB**  5-Nitro-2- (3-phenylpropylamino) benzoic acid

**NR**  Neutral red

**NSC**  nonselective cation

**NuMA**  nuclear mitotic apparatus

**P. (pf, Pf)**  *Plasmodium (Plasmodium falciparum)*

**PAF**  platelet activation factor

**PARP**  poly [(Adenosine-5'-triphosphate)-Ribose] polymerase

**PBS**  phosphate buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PCD</td>
<td>programmed cell death</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PE</td>
<td>phosphatidylethanolamine</td>
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<tr>
<td>PfEMP</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
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<tr>
<td>PK</td>
<td>protein kinase</td>
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<td>PL</td>
<td>phospholipase</td>
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<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>RDTs</td>
<td>Rapid Diagnostic Tests</td>
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<tr>
<td>RIP</td>
<td>receptor-interacting protein</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
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<tr>
<td>Smac/DIABLO</td>
<td>Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI</td>
</tr>
<tr>
<td>TH</td>
<td>T- helper cells</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFReceptor-associated death domain</td>
</tr>
<tr>
<td>TRAP-PfSSP2</td>
<td>thrombospondin-related anonymous protein, <em>P. falciparum</em> sporozoite surface protein type 2</td>
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<tr>
<td>TRP</td>
<td>transient receptor potential</td>
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<tr>
<td>TSP</td>
<td>thrombospondin</td>
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Abstract:

Background: Malaria is one of the most devastating diseases with lethal outcome of more than one million humans per year. This study intended for a novel drug discovery in order to prevent the *Plasmodium* related resistance focusing on identifying and targeting host factors essential for pathogen entry, survival and proliferation. The innovative methods involve induction of suicidal death in infected erythrocytes (eryptosis) and recognition by the spleen macrophages to get rid of the pathogen and prevent the further course of the disease. Eryptosis 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 eryptotic cells.

Objectives: The study has been performed to explore whether the administration of amphotericin B or gum Arabic may modify the course of malaria and survival of *Plasmodium berghei* -infected mice.

Materials and methods: Human erythrocytes were infected *in vitro* with *Plasmodium falciparum* (strain BinH) in the absence and presence of amphotericin B or butyrate, parasitaemia determined utilizing Syto16, cytosolic calcium estimated from fluo3 fluorescence intensity, phosphatidylserine exposure estimated from annexin V-binding and cell volume from forward scatter in fluorescence activated cell sorting analysis. Mice were infected with *Plasmodium berghei* ANKA by injecting parasitized murine erythrocytes (1 × 10⁶) intraperitoneally. Where indicated amphotericin B (1.4 mg/kg b.w.) was administered subcutaneously from the eighth day of infection for three successive days. 10% gum Arabic dissolved in tap water started from the day of infection for the other group of mice. Preparations of gum Arabic were refreshed every day during the treatment.

Results: Amphotericin B during *in vitro* infection of human erythrocytes with *P. falciparum*, increased phosphatidylserine exposure, decreased forward scatter and significantly increases cytosolic calcium activity. Amphotericin B did not significantly alter intraerythrocytic DNA/RNA amplification only at 1 µM but significantly (≥0.01 µM) decreased *in vitro* parasitemia. Erythrocytes from amphotericin B treated mice were more rapidly cleared from circulating blood than non-treated erythrocytes. Moreover, parasitemia in *P. berghei* infected mice was significantly decreased (from 50.61% to 39.36% of circulating erythrocytes 20 days after infection) and mouse survival
significantly enhanced (from 0% to 80% 27 days after infection) upon administration of amphotericin B (1.4 mg/kg b. w) subcutaneously from the eighth day of infection.

In the other in vitro part of this study butyrate which is one of the important gum Arabic fermentation products, significantly ($\geq 0.5$ mM) decreased the proliferation of *P. falciparum*. On the other hand it influences intraerythrocytic DNA/RNA amplification only at 10 mM concentration. Butyrate was significantly increased phosphatidylserine exposure, decreased forward scatter and significantly increases cytosolic calcium activity. Most importantly, gum Arabic treatment (10% in drinking water from the day of infection) resulted in a significant decrease of parasitemia (from 43.6 ± 4.39 and 58.08) and increased the survival of *P. berghei*-infected mice (70% of the gum Arabic-treated animals survived the infection for more than 26 days after infection).

**Conclusions:** In conclusion amphotericin B and gum Arabic stimulate the erythrocytic machinery responsible for the eryptosis following infection with *Plasmodium*. The acceleration of eryptosis precedes the full intraerythrocytic maturation of the pathogen, thus prevents 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 indicated that the stimulation of eryptosis in infected erythrocytes is not only a host dependent defense mechanism that can be used as a novel approach to prevent the chances of resistance in *plasmodia*. 
خلايفي: الملاحي هي واحدة من أكثر الأمراض المدممة وتسبب في وفاة أكثر من مليون إنسان سنويا. هذه الدراسة المخصصة للاكتشاف أدبي جديد من أجل مع مسببات المرض القاومة للأدوية ذات الصلة. هذا النتاج يمكن أن يكون مناسبا لتحديد واستهداف عوامل أساسية في جسم الفأر لتتعلق بدخول مسببات المرض وبدأت، وانتشرها. هذه الأساليب المبتكرة تطوري على تحريض الوفاة الانتقائية للكيرات الدم الحمراء المصابة. تلعب تعرف الخلايا البالغة في الطحال عليها من أجل التخلص من مسببات المرض ومنع المزيد من مسار المرض. وتميز الوفاة الانتقائية للكيرات الدم الحمراء بانكماش الخلايا وارتفاع نزيف الوفاة الكيسية لعشوائية النزيف الخلايا مع عرض جزيئات الفوسفاتيدسوزين على سطح الخلايا مما يؤدي إلى سهولة التعرف عليها بواسطة الخلايا البالغة. وتبدو هذه الخلايا الدموية المصابة وتكسيرها.

الأهداف: تهدف هذه الدراسة لاستكشاف ما إذا كان إعطاء دواء الأمونتريبينج ب أو الصمغ العربي قد يعدل مسار الملاحي، ونقاء المتصورة البرجية في الفنار المصابة.

المواد والأساليب: تم إعداد الكيرات الدم الحمراء البشرية في المختبر بالمتصورة المجنحة (المتصورة المجنحة) في غيل ووجود الأمونتريبينج ب أو الزبادي، واستخدام النباتات في البدء باستخدام سايو 16. تم تكثيف الهيماتوكنز في البداية بحد الإنتاج الضوئي القلبي، أما عرض الفوسفاتيدسوزين فقد تم تقديره عن طريق إرتباط بروتين الأنسين الخاص وحجم الخلية عن طريق البخور الأمامي في فاز التحليلي للخلايا المتنوعة بالإضاءة الضوئية. تم إعداد الفنار بالتصورة البرجية أولاً عن طريق حقن التجوف البريتوتي بخلايا حمراء مصابة (1*10^6). في الفنار المخصصة للدراسة الأمونتريبينج تم حقنه (1.4 ملغ / كجم من وزن الجسم) تحت الجلد من الفنار الثامن من العدد لمدة ثلاثة أيام متتالية. وفي المجموعة الأخرى من الفنار المصابة تم تقديم 10% من الصمغ العربي المذاب في مياه الحرفية اعتبارا من اليوم الأول من التحويل بعد تجريد مستحضر الصمغ العربي كل يوم خلال فترة العلاج.

النتائج: في الكيرات الدم الحمراء البشرية في المختبر المصنعة بالتصورة المجنحة أدى الأمونتريبينج ب إلى زيادة في عرض الفوسفاتيدسوزين على سطح الخلايا وتقاس في التبخر الأمامي وزيادة معتمة في نشاط الكاسبيوم داخلي السيتيتازم. لم يؤدي الأمونتريبينج ب إلى تغيير معتمد في نسبة الأحماض النووية ورنا في داخل كيرات الدم الحمراء إلا أنه أدى إلى تقلص معتمد في إنتاج الطفيل داخل الكيرات الحمراء.

الكيالات الحمراء من الفنار المعالجة بالأمونتريبينج ب تمت إزالتها من الدورة الدموية بسرعة أكبر مقارنة بالكيالات الحمراء من الفنار التي لم يتم معالجتها بالأمونتريبينج ب. و إضافة إلى ذلك انخفض عدد الكيرات المصابة بالمتصورة برجية في الفنار المصابة بشكل ملحوظ (من 50.61 إلى 39.36% من الكيرات الحمراء المنتشرة 20 يوم بعد الإصابة) كما زادت نسبة نقاء الفنار على قيد الحياة بدرجة كبيرة (من 0% إلى 80% 27 يوما بعد الإصابة) في حالة معالجتها بالأمونتريبينج ب تحت الجلد من الفنار الثامن من العدد.
في جانب آخر من الدراسة المختبرية أدت الزبيدات التي هي واحدة من أهم منتجات تخمير الصمغ العربي (كـ 0.5 ملي) إلى إنخفاض ملحوظ في تكاثر المتصورة المنجلية. من ناحية أخرى فإنها أثرت على الحمض النووي داخل الكريات المصابة وذلك بتصحيم الحمض النووي الرببي / فقط في تركيز 10 مليمول. كما أدت الزبيدات إلى زيادة كبيرة في عرض الفوسفاتديلسيرين وانخفاض التنوع إلى الأمام والى زيادة كبيرة في نشاط الكالسيوم في السيتوبلازم. الأهم من ذلك، أدت المعالجة بالصمغ العربي (10 % في مياه الشرب اعتباراً من اليوم للعدوى) في تناقص كبير في عدد الخلايا المصابة (من 43.6 ± 4.39 و58.08 وزيادة في نسبة بقاء الفئران المصابة بالمتصورة بزيادة على قيد الحياة (من 0 إلى 100 % 21 يوماً بعد الإصابة).

الخلاصة: تخلص هذه الدراسة إلى أن الامفوتيسيين ب والصمغ العربي يقومان بتحفيز الآليات المسؤولة عن إنتحار كريات الدم الحمراء بعد العدو مع المنجلية. و هذا الازدهار في إنتحار الخلايا الحمراء يسبق نضج مسببات العدوى، و يمنع بالتالي المسار القاتل من هذا المرض وتعزز بقاء المضيف أثناء الملازما. يدافع هذا الاكتشاف عن أن تنشيط إنتحار الكريات الحمراء المصابة هي آلية يعتمدها المضيف لمكافحة العدوى. النتائج التجريبية بجانب أنها تبرر إنتحار الكريات الحمراء كآلية للحد من إنشار المرض فإنها أيضا تبرز نهجاً جديداً للحيلولة دون فرص للمقاومة في المتصورات.
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1. Introduction

1. 1. Malaria

Malaria is one of the most prevalent parasitic infections in the world and certainly the most detrimental. Each year, over one million people die from the disease, with the vast majority of the deaths in children under five years old in Sub-Saharan Africa (1). In addition to the overwhelming death toll, over 213 million malarial “attacks” lead to more than 800 million days of illness in Africa annually (2, 3) The spread of drug-resistant malaria parasites threatens to compound the problem even more. The situation is so dire that economists have determined that malaria is a definitive cause of poverty in many afflicted regions (4). Furthermore, in malaria-endemic regions, the effect of the disease is also manifested by its lasting influence on human genetics, resulting in the preservation of potentially harmful variants of human genes (i.e., sickle cell trait), largely because of their advantage in heterozygotes protected from severe, complicated, and fatal malaria (1).

1. 1. 1. History

The malaria-like intermittent fevers and other symptoms such as enlarged spleen, periods of high fever, headaches, shaking chills, and weakness were already known more than 3000 years ago in Chinese, Assyrian, Indian, and Egyptian manuscripts. Hippocrates (460-370 B.C.) in Greece is credited with having accurately described the clinical symptoms in his medical writings (5). Malaria was widespread in Europe during the middle ages, but malaria tropica was endemic only in the warmer South Europe. The name is derived from the Italian word *mala aria* (Latin: *malus* = bad, *aeris* = air) because the disease often appeared near swamps with their typical odor (6). It was thought that “miasma”, which literally means “bad air”, caused the disease (7). The “miasma” theory was proved wrong in the 19th century. In 1880 Charles Louis and Alphonse Laveran identified the parasite in its blood stage in the act of exflagellation under the microscope (8). In 1897 Sir Ronald Ross, whose teacher was Patrick Manson, who discovered the mosquito transmission of filariasis, demonstrated the transmission of malaria via mosquitoes (9). However, it was only in 1948 that Short and Garnham described the liver stage of primate and human malaria *Plasmodium vivax* (*P. vivax*) (5). Finally, in 1966 the liver stage of *P. falciparum* was discovered (10).
1.1.2. Epidemiology of malaria

The "many epidemiologies" of malaria have been characterized by degrees of endemicity (11). Malaria is described as endemic when there is a constant incidence of cases over a period of many successive years. At the other extreme malaria transmission may be epidemic when there is a periodic or occasional increase in the incidence of cases. A more general classification into stable and unstable malaria has been introduced. Stable malaria refers to high transmission without any marked fluctuations over years, although seasonal fluctuations may exist. Unstable malaria describes transmission that varies from year to year with frequent epidemics. The former situation is characterized by high degrees of collective immunity whereas the latter is not. These terms describe extremes of a wide range of situations (12).

Malaria is transmitted primarily by the bite of infected female *Anopheline* mosquitoes. The source of infection can be either a sick person or an otherwise asymptomatic carrier of the parasite. Malaria can also be transmitted congenitally or by inoculation of infected blood. Anophelines feed at night and their breeding sites are primarily in rural areas. The greatest risk of malaria infection is therefore from dusk to dawn in rural areas. In many malaria-endemic areas, there is little or no risk in urban areas. However, urban transmission is common in some parts of the world, especially Africa (13, 14).

The incidence of severe clinical manifestations varies seasonally within endemic areas and it is not possible at present to predict which asymptomatic individual will develop severe disease (14). The epidemiological profile and clinical pattern of severe malaria in Africa has been shown to vary according to the intensity of exposure in persons living in rural areas with different levels of transmission (15). The degree of endemicity varies between countries and even between different areas in the same country. In Sub-Saharan Africa cerebral malaria and severe malarial anaemia are the leading causes of mortality (16).

The impact of global climate change poses an obvious threat to human health. The insect-vectors of *Plasmodium* spp. thrive in warm climates of tropical regions. Global warming leading to increased temperature in temperate areas, could provide a habitat suitable for the increased distribution of Anopheline vectors. Whether the potential increase in vector populations will lead to a concomitant increase in malaria transmission is not clear (17).
Malaria is unstable in the semi-arid savanna of central and northern Sudan and the great majority of infective bites take place immediately prior to the seasonal peak in number of malaria cases in September and October (18). Malaria morbidity and mortality, with special reference to central Sudan, affects all age groups (19). In Sudan it has been found that, despite variation between districts, urbanization tends to lead to reduced human malaria transmission (20).

1.1.3. Clinical manifestations

The most characteristic symptom of malaria is fever. Other common symptoms include chills, headache, myalgia, nausea, and vomiting. Diarrhea, abdominal pain, and cough are occasionally seen. As the disease progresses, some patients may develop the classic malaria paroxysm with bouts of illness alternating with symptom free periods. The malaria paroxysm comprises three successive stages. The first is a 15 to 60 minute cold stage characterized by shivering and a feeling of cold. Next, the 2 to 6 hour hot stage, in which there is fever, sometimes reaching 41°C, flushed, dry skin, and often headache, nausea, and vomiting. Finally, there is the 2 to 4 hour sweating stage during which the fever drops rapidly and the patient sweats. In all types of malaria the periodic febrile response is caused by rupture of mature schizonts. In *P. vivax* and *P. ovale* malaria, a brood of schizonts matures every 48 hr, so the periodicity of fever is tertian ("tertian malaria"), whereas in *P. malariae* disease, fever occurs every 72 hours ("quartan malaria").

The fever in *P. falciparum* malaria may occur every 48 hr, but is usually irregular, showing no distinct periodicity. These classic fever patterns are usually not seen early in the course of malaria, and therefore the absence of periodic, synchronized fevers does not rule out a diagnosis of malaria (21).

A variety of laboratory abnormalities may be seen in a case of uncomplicated malaria. These include normochromic, normocytic anemia, thrombocytopenia, leukocytosis or leukopenia, hypoglycemia, hyponatremia, elevated liver and renal function tests, proteinuria. Patients with complicated malaria may occasionally show evidence of massive intravascular hemolysis with hemoglobinemia and hemoglobinuria.

If the diagnosis of malaria is missed or delayed, especially with *P. falciparum* infection, potentially fatal complicated malaria may develop. The most frequent and serious complications of malaria are cerebral malaria and severe anemia. Other complications
include: hyperparasitemia (more than 3 to 5% percent of the erythrocytes parasitized); severe hypoglycemia; lactic acidosis; shock; pulmonary, cardiac, hepatic, or renal dysfunction; seizures; spontaneous bleeding; or massive diarrhea or vomiting. These manifestations are usually associated with poor prognosis (21).

1.1.4. The parasites
There is approximately 400 species of Anopheles throughout the world; about 60 are malaria vectors under natural conditions, 30 of which are of major importance. Malaria is transmitted by the bite of an infected female Anopheles mosquito. Malaria parasites are eukaryotic single-celled microorganisms that belong to the genus Plasmodium. More than 100 species of Plasmodium can infect various animal species such as reptiles, birds and various mammals, but only four species of parasite can infect humans under natural conditions: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. These four species differ in their morphology, antigenicity, pathogenicity, drug sensitivity and geographical distribution. *P. falciparum* is species responsible for the severe complications of malaria and is the principal cause of malaria deaths in young children in Africa (22, 23). The widely worldwide distributed *P. vivax* is rarely fatal, while *P. malariae* has a relatively low frequency. On the other hand, the least common *P. ovale* is restricted to West Africa. Although both *P. falciparum* and *P. malariae* can cause anaemia, mild anaemia is more common in *P. vivax* infections whereas severe anemia is usually caused by *P. falciparum*. *P. ovale* and *P. vivax* have dormant liver stages named hypnozoites that may remain in this organ for weeks to many years before the onset of a new round of pre-erythrocytic schizogony, resulting in relapses of malaria infection. In some cases *P. malariae* can produce long-lasting blood-stage infections, which, if left untreated, can persist asymptotically in the human host for periods extending into several decades (24).

1.1.5. Life cycle of malaria parasites
The life cycle of malaria parasites is extremely complex and requires specialized protein expression for survival in both the invertebrate and vertebrate hosts. These proteins are required for the invasion of a variety of cell types and for the evasion of host immune responses. Once injected into the human host, *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony, whereas *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or lead to delayed schizogony as they pass through the
hypnozoite stage. The life cycle of the malaria parasite can be divided into several stages as shown in figure 1.1 below, starting with sporozoite entry into the bloodstream (21).

**Fig. 1.1. Life cycle of the malaria parasite *P. falciparum* (24).**

1. 1. 5. 1. **Tissue schizogony (pre-erythrocytic schizogony)**

Infected sporozoites from the salivary gland of the *Anopheles* mosquito are injected into the human host along with anticoagulant-containing saliva to ensure an even-flowing blood meal. It was thought that sporozoites move rapidly away from the site of injection, but a recent study using the rodent parasite (25) species *Plasmodium yoelii* as a model system indicates that, at least in this case, the majority of infective sporozoites remain at the injection site for hours, with only slow release into the circulation (26). Once in the human bloodstream, *P. falciparum* sporozoites reach the liver and penetrate the liver cells (hepatocytes) where they remain for 9–16 days and undergo asexual replication known as exo-erythrocytic schizogony. The mechanism of targeting and invading the hepatocytes is not yet well understood, but studies have shown that sporozoite migration through several hepatocytes in the mammalian host is essential for completion of the life cycle. Each sporozoite gives rise to tens of thousands of merozoites inside the hepatocytes, upon
release these merozoites invades red cells to undergo successive waves of schizogeny releasing more merozoites to reinvade new red blood cells (RBCs). The time taken to complete the liver stage of the parasite life cycle, called the prepatent period, varies, depending on the infecting species; (8–25 days for *P. falciparum*, 8–27 days for *P. vivax*, 9–17 days for *P. ovale* and 15–30 days for *P. malariae*) (27).

1. 1. 5. 2. Erythrocytic schizogony

Merozoites enter erythrocytes by a complex invasion process, which can be divided into four phases: (a) initial recognition and reversible attachment of the merozoite to the erythrocyte membrane; (b) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of substances from the rhoptry and microneme organelles, leading to formation of the parasitophorous vacuole; (c) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat; and finally (d) resealing of the parasitophorous vacuole and erythrocyte membranes after completion of merozoite invasion. Asexual division starts inside the erythrocyte and the parasites develop through different stages therein. The early trophozoite is often referred to as the 'ring stage', because of its characteristic morphology. Trophozoite enlargement is accompanied by highly active metabolism, which includes glycolysis of large amounts of imported glucose, the ingestion of host cytoplasm and the proteolysis of hemoglobin into constituent amino acids. Malaria parasites cannot degrade the by-product heme which is potentially toxic to the parasite. Therefore, during hemoglobin degradation, most of the liberated heme is polymerized into hemozoin (malaria pigment), a crystalline substance that is stored within the food vacuoles (28).

The end of this trophic stage is marked by multiple rounds of nuclear division without cytokinesis resulting in the formation of schizonts. Each mature schizont contains around 20 merozoites which are released upon lysis of the RBC to reinvade further uninfected RBCs. This release coincides with the sharp increases in body temperature during the progression of the disease. This repetitive intraerythrocytic cycle of invasion–multiplication–release–reinvasion continues, taking about 48 h in *P. falciparum*, *P. ovale* and *P. vivax* infections and 72 h in *P. malariae* infection. It usually occurs quite synchronously and the merozoites are released at approximately the same time of the day. The contents of the infected RBC that are released upon its lysis stimulate the production
of tumour necrosis factor (TNF) and other cytokines, which are responsible for the characteristic clinical manifestations of the disease (24).

A small proportion of the merozoites in the RBCs eventually differentiate to produce micro- and macrogametocytes (male and female, respectively), which have no further activity within the human host. These gametocytes are essential for transmitting the infection to new hosts after undergoing an sporogonic cycle in female *Anopheles* mosquitoes. Normally, variable numbers of cycles of asexual erythrocytic schizogony occur before any gametocytes are produced. In *P. falciparum*, erythrocytic schizogony takes 48 h and gametocytogenesis takes 10–12 days. Gametocytes appear on the fifth day of primary attack in *P. vivax* and *P. ovale* infections, and thereafter become more numerous; they appear at anything from 5 to 23 days after a primary attack by *P. malariae* (24).

1. 1. 5. 3. Sexual phase in the mosquito (Sporogony)

A mosquito taking a blood meal on an infected individual may ingest these gametocytes into its midgut, where macrogametocytes form macrogametes and exflagellation of microgametocytes produces microgametes. These gametes fuse, undergo fertilization and form a zygote. This transforms into an ookinete, which penetrates the wall of a cell in the midgut and develops into an oocyst. In a recent study (29), it has been shown that gamete surface antigen *Pfs230* mediates human RBC binding to exflagellating male parasites to form clusters termed exflagellation centers, from which individual motile microgametes are released. This protein thus plays an important role in subsequent oocyst development, which is a critical step in malaria transmission. Sporogony within the oocyst produces many sporozoites and when the oocyst ruptures, they migrate to the salivary glands for onward transmission into another vertebrate host. This infective “sporozoite” form of the parasite reaches the salivary glands after 10–18 days and thereafter the mosquito becomes infective for 1–2 months (30).

1. 1. 6. Malaria immunity

There are different types of protective immunity against malaria, one type is the clinical immunity which reduces the intensity of clinical symptoms and the risk of death from malaria, the other type is the antiparasitic immunity which directly reduces the number of parasites in the infected individual (31). In the case of *P. falciparum* malaria, it is possible
that a certain degree of immunity to some aspects of severe disease may be attained after only one or two infections (32). However, effective antiparasitic immunity is only attained after several frequent infections (33).

1.1.6.1. Innate immunity
Some individuals are either naturally resistant to malaria infection or less likely to develop a severe form of the disease. Innate resistance to infection is generally only a partial resistance, and may be linked to the fact that malaria parasites find it harder to invade certain types of human erythrocytes. Also, it could be due to the fact that certain host erythrocytes have a reduced ability to sustain the growth of parasites (33).

1.1.6.2. Acquired immunity
In endemic areas, most adults have a degree of immunity that controls parasite replication but not sterile immunity which eliminates parasite from blood. Acquired immunity to malaria develops after repeated exposure to the parasite (34).

1.1.6.3. Strain specific immunity
The long time required to develop protective immunity against malaria may be explained by poor immunogenicity of the parasite and/or that immune responses are strain specific. A large number of infections would then be required to encounter the whole local repertoire of different antigens. Early studies of induced malaria indicated that malaria immunity is strain specific (35, 36).

1.1.7. Malaria diagnosis
The standard method for detection of *Plasmodium* in human blood is by microscopical examination of Romanovsky-stained thick and thin blood films. This technique can, when used in optimal conditions by a competent microscopist, detect a parasitaemia as low as 0.001% (10-40 parasites per µl of blood), but it is a time-consuming technique for the detection of scanty parasites and often difficult to use accurately to identify mixed infections. Several alternative diagnostic methods have been developed in order to reduce the time spent examining slides or to enable fairly competent personnel to achieve equally reliable results. These include:

Antigen detection: Various test kits are available to detect antigens derived from malaria parasites. Such immunochromatographic tests most often use a dipstick or cassette format,
and provide results in 2-15 minutes. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available. Malaria RDTs are currently used in some clinical settings and programs. However, before malaria RDTs can be widely adopted, several issues remain to be addressed, including improving their accuracy; lowering their cost; and ensuring their adequate performance under adverse field conditions (37).

Molecular diagnosis: Parasite nucleic acids are detected using polymerase chain reaction (PCR). This technique is more accurate than microscopy. However, it is expensive, and requires a specialized laboratory even though technical advances will likely result in field-operated PCR machines (37).

Serology: Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology does not detect current infection but rather measures past experience (37).

1.1.8. Malaria chemotherapy
Chemotherapy is important in alleviating the suffering and reducing the mortality caused by the disease. The action of antimalarial drugs ties in their effect on the metabolic process or the vital structure of the parasite which lead to the disruption of its activity and then to parasite death. However the efficacy of the drug depends also on the species and strain of the parasite and its sensitivity towards a given drug, as well as the state of host immunity (38).

Patients successfully treated with antimalarial drugs may thus be healthy but may remain infective for up to two months until the *P. falciparum* gametocytes die off naturally, or until another drug such as primaquine is given to eliminate the mature gametocytes (39).

1.1.8.1.4, 8-aminoquinoline drugs
Such as chloroquine, amodiaquine, and primaquine. Chloroquine is a 4-aminoquinoline that has marked and rapid schizonticidal activity against all infections of *P. malariae* and *P. ovale* and against chloroquine-sensitive infections of *P. falciparum* and *P. vivax*. It is also gametocytocidal against *P. vivax*, *P. malariae* and *P. ovale* as well as immature gametocytes of *P. falciparum*. It is not active against hepatic stages, and should therefore be used with primaquine to effect radical cure of *P. vivax* and *P. ovale*. It was the most important antimalarial for more than 40 years because of its activity against the four
plasmodia that infect human (39). In addition, chloroquine is known to be safe for children and pregnant women (38, 40). However, the efficacy of chloroquine has been severely compromised by the spread of chloroquine-resistant strains of *P. falciparum*, which are now prevalent in Southeast Asia, South and Central America, and sub-Saharan Africa (41) where drug-resistance led to increased morbidity and mortality of malaria and forced the use of alternative drugs (42).

1. 1. 8. 2. Antifolate drugs

The only useful combinations of antifolate drugs for the treatment of malaria are synergistic mixtures that act against the parasite-specific enzymes, dihydropteroate synthetase and dihydrofolate reductase. Available combinations include the sulfa and pyrimethamine combinations sulfadoxine-pyrimethamine and sulfalene-pyrimethamine, the former being more widely available (43).

Antifolate drugs such as pyrimethamine and proguanil are slow blood schizonticides but they have the advantage that they are effective in smaller doses, safe and free from side effects. The ability of sulfa (sulfonamides and sulfones) to show potentiating effect with proguanil or pyrimethamine (44) suggested that combination of these two groups of drugs would not only be more effective antimalarial agent, but would also delay, if not avoid, the development of resistance. The most commonly used combination is that of sulfonamide, sulfadoxine, and pyrimethamine (fansidar), which has been particularly effective in areas such as Africa where chloroquine resistance is now widespread (45). However, resistance to the pyrimethamine sulfadoxine combination became widespread and the successive failure of these two drugs in Africa was described in 1998 as malaria disaster (45).

1. 1. 8. 3. Quinine

It is normally effective against *P. falciparum* infections that are resistant to chloroquine and sulfa drug-pyrimethamine combinations. Decreasing sensitivity to quinine has been detected in areas of South-East Asia where it has been extensively used for malaria therapy. This has occurred particularly when therapy was given in an unsupervised and ambulatory setting with regimens longer than 3 days. In these settings, patient adherence to therapy is low, leading to incomplete treatment; this may have led to the selection of resistant parasites (46).
1.1.8.4. Artemisinin and its derivatives
Artemisinin is poorly soluble in oils or water but the parent compound has yielded dihydroartemisinin, the oil-soluble derivatives artemether and arteether, and the more water-soluble derivatives sodium artesunate and artetinic acid. These derivatives have more potent blood schizonticidal activity than the parent compound and are the most rapidly effective antimalarial drugs known. They are used for the treatment of severe and uncomplicated malaria (47). They are not hypnozoiticidal but gametocytocidal activity has been observed. These compounds are not recommended for use in the treatment of malaria due to *P. vivax, P. malariae* or *P. ovale* since other effective antimalarial drugs are available for this purpose (39).

1.1.8.5. Combination therapy to combat the spread of drug resistance
Due to the emergence and rapid spread of resistance to almost all available antimalarial drugs, much of interest has been focused upon the use of drugs in combination. The underlying science behind the therapeutic effect of the combinations that include an artemisinin derivative is that the artemisinin rapidly kills most of the parasites and then those that remain are killed by the companion drug (39).

Combination therapy (CT) is defined as "the simultaneous use of two or more antimalarial drugs with different biochemical targets in the parasite or in tissue hosting the parasite, which are synergistic, additive or complementary in their effect". Using this definition, two or more drugs which have the same biochemical target in the parasite such as sulfadoxine, and pyrimethamine, chlorproguanil-dapsone, atovaquone-proguanil are not considered CT. Similarly, an antimalarial which is co-administered with a non-antimalarial drug which enhances its action is also not classified as CT. Suffice to say that CT therefore can be either fixed combinations, where all components are coformulated in a single tablet/capsule or free combinations where the components are in separate tablets/capsules but are coadministered (39).

In sub-Saharan Africa, the first two combinations considered in a larger scale were pyrimethamine/sulfadoxine plus chloroquine and pyrimethamine/sulfadoxine plus amodiaquine (39). Recently interest has been directed towards the use of drugs in combination with artesunate in purpose of delaying the emergence of resistance (48).
antifolate product proguanil has been formulated together with a new type of inhibitor, atovaquone, to yield malar-one, recently licensed for clinical use (49).

1. 2. Apoptosis

Apoptosis is the process of programmed cell death (PCD), that may occur normally during development and aging as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defence mechanism such as in immune reactions or when cells are damaged by disease or noxious agents. 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. Apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called “caspases” and a complex cascade of events that link the initiating stimuli to the final demise of the cell (50).

1. 2. 1. Morphology of apoptosis

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (51). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (52). With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. On histological examination with hematoxylin and eosin stain, the apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. Early during the chromatin condensation phase, the electron-dense nuclear material characteristically aggregates peripherally under the nuclear membrane although there can also be uniformly dense nuclei. Blebbing which is an extensive irregular bulging of the plasma membrane detached from the cytoskeleton occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding.” Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained and all of this is enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by
macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called “tingible body macrophages” and are frequently found within the reactive germinal centers of lymphoid follicles or occasionally within the thymic cortex. The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated neither with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines (53, 54).

1.2.2. Classification of cell death: apoptosis and necrosis

Mechanisms of cell death varies with cell type, developmental stage, metabolism and the kind of death signal. Apoptosis is a major form of programmed cell death, although there are still other types of programmed cell death, especially in pathological situations (autophagic death, cytoplasmic death, parapoptosis etc.). These modes of cell death are, however, much less studied (55, 56).

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 to be an inappropriate term to describe a mechanism of cell death. Oncosis is therefore used to describe a process that leads to necrosis with karyolysis and cell swelling whereas apoptosis describes the process that involves cell shrinkage, pyknosis, and karyorrhexis. Necrosis is an uncontrolled and passive process that usually affects large fields of cells whereas apoptosis is controlled and energy-dependent and can affect individual or clusters of cells. Necrotic cell injury is mediated by two main mechanisms; interference with the energy supply of the cell and direct damage to cell membranes (57).

Although the mechanisms and morphologies of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the “apoptosis-necrosis continuum” (58). For example, two factors that convert an ongoing apoptotic process into a necrotic process include a decrease in the availability of caspases and intracellular ATP (58, 59). It is not always easy to distinguish apoptosis from necrosis.
by conventional histological examinations. Both processes can occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of ATP depletion and the availability of caspases (58).

Some of the major morphological changes that occur with necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane (52, 57, 60). This loss of cell membrane integrity results in the release of the cytoplasmic contents into the surrounding tissue, sending chemotactic signals with eventual recruitment of inflammatory cells. Because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue and are quickly phagocytosed by macrophages or adjacent normal cells, there is essentially no inflammatory reaction (53, 54). It is also important to note that pyknosis and karyorrhexis are not exclusive to apoptosis and can be a part of the spectrum of cytomorphological changes that occurs with necrosis (58).

1. 2. 3. Mechanisms of apoptosis
The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events (Figure 1. 2). To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (61). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (62).
1.2.4. Biochemical features of apoptosis

Apoptotic cells exhibit several biochemical changes such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the distinctive structural pathology. Cysteine-aspartic proteases (Caspases) are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signalling pathway and thus leads to rapid cell death (63).

Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighbouring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. Fourteen major caspases have been identified and were broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (64, 65). The other caspases that have been identified include caspase-11 which regulates apoptosis and cytokine secretion during septic shock, caspase-12 which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β, caspase-13 which is suggested to be a bovine gene and caspase-14 which is highly expressed in embryonic tissues but not in adult tissues (66-68).

Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (69). DNA breakdown by Ca²⁺-and Mg²⁺-dependent endonucleases also occurs, resulting in DNA fragments of 180 to 200 base pairs. A characteristic “DNA ladder” can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination (70).

Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell’s lipid bilayer to expression on the outer layers of the plasma membrane (71). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, other proteins are also being exposed on the cell surface during apoptotic
cell clearance. These include Annexin I and calreticulin. Calreticulin is a protein that binds to an LDL-receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (72). Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (73).

1. 2. 4. 1. Extrinsic pathway of apoptosis

The extrinsic signalling pathways that initiate apoptosis involve trans-membrane receptor-mediated interactions. These involve death-receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily (74). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain” (75). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. To date, the best-characterized ligands and corresponding death receptors include fatty acid synthase ligand/ fatty acid synthase receptors (FasL/FasR), tumor necrosis factor-α/ tumor necrosis factor receptor (TNF-α/TNFR1), apoptosis 3 ligand/death receptors 3 (Apo3L/DR3), and apoptosis 2 ligand/death receptors 4 (Apo2L/DR4) (76-78).

The sequences of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein, Fas-associated death domain (FADD) and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TNFReceptor-associated death domain (TRADD) with recruitment of FADD and receptor-interacting protein (RIP) (79). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signalling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (80). Once caspase-8 is activated, the execution phase of apoptosis is triggered (76).
Fig. 1. 2: Schematic representation of apoptotic events. 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, 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 (69).

1. 2. 4. 2. Perforin/granzyme pathway of apoptosis

T-cell are able to exert their cytotoxic effects on tumour cells and virus-infected cells via a novel pathway that involves secretion of the trans-membrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through the pore and into the target cell (79). The serine proteases granzyme A and granzyme B are the most important component within the granules (80). Granzyme B will cleave proteins at aspartate residues and will therefore activate procaspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNase) (81). Granzyme B also can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome c release (82).
Granzyme A is also important in cytotoxic T cell induced apoptosis and it activates caspase independent pathways. Once in the cell, granzyme A activates DNA nicking through DNAse NM23-H1, a tumor suppressor gene product (83). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. The nucleosome assembly protein SET normally inhibits the NM23-H1 gene. Granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1, resulting in apoptotic DNA degradation. In addition to inhibiting NM23-H1, the SET complex has important functions in chromatin structure and DNA repair. The proteins that make up this complex (SET, Ape1, pp32, and HMG2) seem to work together to protect chromatin and DNA structure. Therefore, inactivation of this complex by granzyme A is likely to contribute to apoptosis by blocking the maintenance of DNA and chromatin structure integrity (84).

1.2.4.3. Intrinsic pathway of apoptosis
The intrinsic signalling pathway of apoptosis involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals which act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals (87).

All apoptotic stimuli cause changes in the inner mitochondrial membrane and result in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial trans-membrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (87). The first group consists of cytochrome c, Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI (Smac/DIABLO), and the serine protease HtrA2/Omi (88). These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an “apoptosome” (89, 90). The second group of pro-apoptotic proteins, include the apoptosis-inducing factor (AIF), endonuclease G and caspase-activated Dnase (CAD) are released from the mitochondria.
during apoptosis, but this is a late event that occurs after the cell has been committed to death. AIF translocates to the nucleus and causes DNA fragmentation into ~50–300 kb pieces and condensation of peripheral nuclear chromatin (91).

The control and regulation of these apoptotic mitochondrial events occurs through members of the B-cell lymphoma 2 (Bcl-2) family of proteins (92). The tumour suppressor protein \( p53 \) has a critical role in regulation of the Bcl-2 family of proteins; however the exact mechanisms have not yet been completely elucidated. The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome \( c \) release from the mitochondria via alteration of mitochondrial membrane permeability (93).

1. 2. 4. 4. Execution pathway of apoptosis
The extrinsic and intrinsic pathways both end at the point of the execution phase which is the final pathway of apoptosis. It is the activation of the execution caspases that triggers this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, 6 and 7 act as effectors or “executioner” caspases, cleaving various substrates including cytokeratins, Poly (ADP-ribose) polymerase (PARP), the plasma membrane cytoskeletal protein alpha fodrin, the nuclear mitotic apparatus protein (NuMA) and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (94).

1. 2. 5. Physiologic apoptosis
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. And that number can increase significantly when there is increased apoptosis during normal development and aging or during disease (95).

Apoptosis is critically important during various developmental processes. Apoptosis is necessary to rid the body of pathogen-invaded cells and is a vital component of wound healing in that it is involved in the removal of inflammatory cells and the evolution of
granulation tissue into scar tissue (96). Additionally, apoptosis is central to remodelling in the adult, such as the follicular atresia of the postovulatory follicle and post-weaning mammary gland involution (97, 98). Furthermore, as organisms grow older, some cells begin to deteriorate at a faster rate and are eliminated via apoptosis. It is clear that apoptosis has to be tightly regulated since too little or too much cell death may lead to pathology, including developmental defects, autoimmune diseases, neurodegeneration or cancer (99, 100).

1. 2. 6. Pathologic apoptosis
Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome, acquired immune deficiency syndrome (AIDS), ischemia, and neurode-generative diseases such as Parkinson’s disease, Alzheimer’s disease, Huntington’s disease, and Amyotrophic Lateral Sclerosis. Some conditions feature insufficient apoptosis whereas others feature excessive apoptosis. Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional, with either an over-proliferation of cells and/or decreased removal of cells (95, 101).

Excessive apoptosis may also be a feature of some conditions such as autoimmune diseases, neurodegenerative diseases, and ischemia-associated injury. Acquired immune deficiency syndrome (AIDS) is an example of an autoimmune disease that results from infection with the human immunodeficiency virus (HIV) (102).

1. 2. 7. Inhibition of apoptosis
There are many pathological conditions that exhibit excessive apoptosis (neurodegenerative diseases, AIDS, ischemia, etc.) and thus may benefit from artificially inhibiting apoptosis. However, understanding of the field evolves, the identification and exploitation of new targets remains a considerable focus of attention (103). A short list of potential methods of anti-apoptotic therapy includes stimulation of the inhibitors of apoptosis proteins (IAP) family of proteins, caspase inhibition, PARP (poly [ADP-ribose] polymerase) inhibition, stimulation of the protein kinase B (PKB) pathway, and inhibition of Bcl-2 proteins. The inhibitor of apoptosis (IAP) family of proteins is perhaps the most important regulators of apoptosis due to the fact that they regulate both the intrinsic and extrinsic pathways (104).
1. 2. 8. Assays for apoptosis
Since apoptosis occurs via a complex signalling cascade that is tightly regulated at multiple points, there are many opportunities to evaluate the activity of the proteins involved. As the activators, effectors and regulators of this cascade continue to be elucidated, a large number of apoptosis assays are devised to detect and count apoptotic cells. However, many features of apoptosis and necrosis can overlap, and it is therefore crucial to employ two or more distinct assays to confirm that cell death is occurring via apoptosis. One assay may detect early (initiation) apoptotic events and a different assay may target a later (execution) event. The second assay, used to confirm apoptosis, is generally based on a different principle. Multiplexing, which is the ability to gather more than one set of data from the same sample, is another methodology for apoptosis detection that is becoming increasingly popular. There are a large variety of assays available, but each assay has advantages and disadvantages which may make it acceptable to use for one application but inappropriate for another application (105, 106). Therefore, when choosing methods of apoptosis detection in cells, tissues or organs, understanding the pros and cons of each assay is crucial. However, apoptosis assays can be classified into six major groups:

1. Cytomorphological alterations
2. DNA fragmentation
3. Detection of caspases, cleaved substrates, regulators and inhibitors
4. Membrane alterations
5. Detection of apoptosis in whole mounts
6. Mitochondrial assays.

1. 2. 8. 1. Cytomorphological alterations
The evaluation of hematoxylin and eosin-stained tissue sections with light microscopy does allow the visualization of apoptotic cells. Although a single apoptotic cell can be detected with this method, confirmation with other methods may be necessary. Because the morphological events of apoptosis are rapid and the fragments are quickly phagocytosed, considerable apoptosis may occur in some tissues before it is histologically apparent. Additionally, this method detects the later events of apoptosis, so cells in the early phase of apoptosis will not be detected (107).
1. 2. 8. 2. DNA fragmentation

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. The assay results in a characteristic “DNA ladder” with each band in the ladder separated in size by approximately 180 base pairs. This method is easy to perform, has a sensitivity of $1 \times 10^6$ cells (i.e., level of detection is as few as 1,000,000 cells), and is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively. On the other hand, it is not recommended in cases with low numbers of apoptotic cells. There are other disadvantages to this assay. Since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis. Additionally, DNA fragmentation can occur during preparation making it difficult to produce a nucleosome ladder and necrotic cells can also generate DNA fragments (107).

1. 2. 8. 3. Membrane alterations

Externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells allows detection via Annexin V in tissues, embryos or cultured cells. Once the apoptotic cells are bound with fluorescein isothiocyanate (FITC)-labeled Annexin V, they can be visualized with fluorescent microscopy. The advantages are sensitivity (can detect a single apoptotic cell) and the ability to confirm the activity of initiator caspases. The disadvantage is that the membranes of necrotic cells are labelled as well (108).

1. 2. 8. 4. Detection of apoptosis in whole mounts

Apoptosis can also be visualized in whole mounts of embryos or tissues using dyes such as acridine orange (AO), Nile blue sulphate (NBS), and neutral red (NR). Since these dyes are acidophilic, they are concentrated in areas of high lysosomal and phagocytotic activity. The results would need to be validated with other apoptosis assays because these dyes cannot distinguish between lysosomes degrading apoptotic debris from degradation of other debris such as microorganisms. Although all of these dyes are fast and inexpensive, they have certain disadvantages. AO is toxic and mutagenic and quenches rapidly under standard conditions whereas NBS and NR do not penetrate thick tissues and can be lost during preparation for sectioning (109).
1. 2. 8. 5. Mitochondrial assays
Mitochondrial assays and cytochrome c release allow the detection of changes in the early phase of the intrinsic pathway. Laser scanning confocal microscopy (LSCM) creates submicron thin optical slices through living cells that can be used to monitor several mitochondrial events in intact single cells over time (110). Mitochondrial permeability transition (MPT), depolarization of the inner mitochondrial membrane, Ca\(^{2+}\) fluxes, mitochondrial redox status, and reactive oxygen species can all be monitored with this methodology. The main disadvantage is that the mitochondrial parameters that this methodology monitors can also occur during necrosis. The electrochemical gradient across the mitochondrial outer membrane (MOM) collapses during apoptosis, allowing detection with a fluorescent cationic dye (69).

1. 3. Erythrocyte suicidal death (Erypyosis)
Erythrocytes are devoid of nuclei and mitochondria and thus lack crucial elements in the machinery of apoptosis. However, similar to other cell types, erythrocytes have to be eliminated when they are defective or after their physiological life span of 120 days (111) in case of human beings. Due to the lack of key organelles involved in the process of apoptosis it was considered that erythrocytes are unable to undergo apoptosis and hence have to be eliminated by mechanisms other than apoptosis. It has been observed that erythrocyte senescence is associated with cell shrinkage, plasma membrane microvesiculation, a progressive shape change from a discocyte to a spherocyte, cytoskeleton alterations associated with protein (spectrin) degradation, and loss of plasma membrane phospholipid asymmetry leading to the externalization of phosphatidylinerse in the erythrocyte membrane (111, 112). The exposure of phosphatidylinerse and further eat-me-signals at the cell surface triggers, and the decrease of cell volume facilitate, the engulfment of the dying cells by phagocytes (113). Hence, the term “eryptosis” was coined recently (114) to describe erythrocyte cell death characterised by cell shrinkage, membrane blebbing, activation of proteases, and phosphatidylinerse exposure at the outer membrane leaflet which are typical features of apoptosis in nucleated cells (115-117).

1. 3. 1. Normal erythrocyte membrane and cation transport
The non-nucleated erythrocyte is unique among human cells in that its plasma membrane accounts for all of its diverse antigenic, transport, and mechanical characteristics. The
discoid shape of the red cell evolves from the multilobulated reticulocyte during 48 hours of maturation first in the bone marrow and then in the circulation (118).

The structural organization of the human red cell membrane enables it to undergo large reversible deformations while maintaining its structural integrity during its 4-month life span in the circulation. The red cell membrane exhibits unique material behaviour. It is highly elastic (100-fold softer than a latex membrane of comparable thickness), rapidly responds to applied fluid stresses (time constants in the range of 100 milliseconds), and is stronger than steel in terms of structural resistance. While a normal red cell can deform with linear extensions of up to approximately 250%, a 3% to 4% increase in surface area results in cell lysis. Hence an important feature of induced red cell deformations, both in vitro and in vivo, is that they involve no significant change in membrane surface area. These unusual membrane material properties are the result of an evolution-driven “engineering” process resulting in a composite structure in which a plasma membrane envelope composed of cholesterol and phospholipids is anchored to a 2-dimensional elastic network of skeletal proteins through tethering sites on cytoplasmic domains of transmembrane proteins embedded in the lipid bilayer (118). Direct interaction of several skeletal proteins with the anionic phospholipids affords additional attachments of the skeletal network to the lipid bilayer (113).

The lipid bilayer is composed of equal proportions by weight of cholesterol and phospholipids (119). While cholesterol is thought to be distributed equally between the two leaflets, the four major phospholipids are asymmetrically disposed. Phosphatidylcholine and sphingomyelin are predominantly located in the outer monolayer, while most phosphatidylethanolamine and all phosphatidylserine (PS), together with the minor phosphoinositide constituents, are confined to the inner monolayer (120). Several different types of energy-dependent and energy-independent phospholipid transport proteins have been implicated in generating and maintaining phospholipid asymmetry. “Flippases” move phospholipids from the outer to the inner monolayer while “floppases” do the opposite against a concentration gradient in an energy-dependent manner. In contrast, “scramblases” move phospholipids bi-directionally down their concentration gradients in an energy-independent manner (121, 122).
The maintenance of asymmetric distribution of phospholipids, in particular exclusive localization of PS and phosphoinositides to the inner monolayer, has several functional implications. Because macrophages recognize and phagocytose red cells that expose PS at their outer surface, the confinement of this lipid in the inner monolayer is essential if the cell is to survive its frequent encounter with macrophages of the reticuloendothelial system, especially the spleen. Loss of lipid asymmetry leading to exposure of PS on the outer monolayer has been suggested to play a role in premature destruction of thalassemic and sickle red cells. Furthermore, the restriction of PS to the inner monolayer also inhibits the adhesion of normal red cells to vascular endothelial cells, thereby ensuring unimpeded transit through the microvasculature (123, 124).

More than fifty trans-membrane proteins with variable abundance, ranging from a few hundred to a million copies per red cell, have been characterized. A large fraction of these trans-membrane proteins define the various blood group antigens. The membrane proteins exhibit diverse functional heterogeneity, serving as transport proteins. Most proteins associated with the erythrocyte membrane are involved in active and passive ion transport, i.e. processes to maintain erythrocyte homeostasis. Other erythrocyte membrane proteins may act as adhesion proteins involved in interactions of red cells with other blood cells and endothelial cells, and also as signalling receptors (123, 125).


1. 3. 1. 1. Na⁺/K⁺ pump in non-infected erythrocytes
The Na⁺/K⁺ pump mechanism of human erythrocytes is well understood. It builds up and maintains a high intracellular K⁺ ([K⁺]i) and a low intracellular Na⁺ ([Na⁺]i) concentration. The ouabain-sensitive Na⁺/K⁺-ATPase in the erythrocyte membrane pumps 2 K⁺ ions into versus 3 Na⁺ out of the cell, thereby generating opposing chemical gradients for the two ions. The pumping helps to build up the membrane potential and counterbalances the "leak" of the Na⁺ and K⁺ ions down their respective concentration gradients via various cotransporters, exchangers, and channels that adjust a steady-state cytoplasmic [Na⁺]-to-
[K$^+$] ratio of 0.12-0.16 in normal human erythrocytes (127). Intraerythrocytic Na$^+$ and K$^+$ concentrations have been determined as approximately 10 to 20 mM and 140 mM, respectively. Several cation channels that may contribute to the cation leak have been identified electrophysiologically (128).

1. 3. 1. 2. Nonselective cation channels in non-infected erythrocytes

Whole-state and nystatin-perforated patch-clamp single-channel recordings have led to the discovery of nonselective cation (NSC) channels in the human RBC membrane that are activated by strong depolarization of the membrane potential (129, 130). These voltage-gated NSC channels are stimulated through nicotinic acetylcholine and PGE$_2$ receptors. They are permeable to divalent cations such as Ca$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ (131, 132), and exhibit a hysteresis-like voltage-dependent gating (133). In addition, voltage-independent NSC channels have been discovered. Experiments with simultaneous activity of both channel types suggest their differential regulation (114).

Activation of the voltage-independent NSC channels occurs within minutes upon replacement of extracellular Cl$^-$ by gluconate (115, 116), similar when extracellular Cl$^-$ is replaced by NO$_3^-$, Br$^-$, or SCN$^-$. Furthermore, voltage-independent NSC channels are activated when the ionic strength of the bath solution is lowered by isosmotic substitution of NaCl with sorbitol, indicating that the underlying process is probably identical with that of the Na$^+$ and K$^+$ permeability increase upon incubation of human erythrocytes in low ionic strength medium (129, 132). Decrease of the extracellular Cl$^-$ concentration activates these cation channels. Remarkably, the anion channel/transporter inhibitor diisothiocyanatostilbene-2′,2-disulfonic acid (DIDS) directly inhibits K$^+$ efflux in low ionic strength medium paralleled by inhibition of Cl$^-$ exchange. Moreover, whole-cell recordings show that upon extracellular Cl$^-$ removal DIDS prevents activation of the NSC channel, while having no effect on the activated cation channels (134). Furthermore, cation channel activity strongly depends on the cytosolic (i.e., pipette) Cl$^-$ concentration (135), suggesting that a DIDS-sensitive pathway equilibrates the Cl$^-$ concentrations between the cytoplasmic and extracellular membrane face and that intracellular rather than extracellular Cl$^-$ ions modulate the cation channel activity (135). The voltage-independent channels hardly discriminate between monovalent cation channels (cation permselectivity in the rank order of Cs$^+ > K^+ > Na^+ = Li^+ >>$ NMDG$^{+}$) and have a Ca$^{2+}$-permeability similar to that of voltage-gated NSC channels (128).
1. 3. 1. 3. Ca$^{2+}$ activated K$^+$ channels (Gardos channels)

The erythrocyte uses at least four well-characterized K$^+$ transport mechanisms: the K$^+$/Cl$^-$ cotransporter, a NaK2Cl cotransporter, a Na$^+$/K$^+$ ATPase (a sodium pump) and a Ca$^{2+}$ activated K$^+$ channel (Gardos channels) (136). The gene for the Gardos channel in erythrocytes is the 4$^{th}$ member of the potassium intermediate/small conductance calcium-activated channel subfamily N (KCNN4; aliases: hsK4 (human small conductance potassium channel 4), hIK1 (human intermediate conductance potassium channel) (115, 137). Gardos channels have been characterized by single channel recordings (138). They play an important role in the regulation of cell volume and are major factors in HbS/S RBC dehydration (125).

Gardos channel activity is dependent on the free Ca$^{2+}$ concentration at the cytoplasmic membrane face. Concomitant with an increase in free [Ca$^{2+}$] from 500 nM to 60 µM its open probability increases from 0.1 to 0.9. Ca$^{2+}$ acts via binding to calmodulin constitutively associated with the Gardos channels (139). In addition to Ca$^{2+}$, channel activity is dependent on intracellular K$^+$, and inhibited by extracellular Na$^+$, K$^+$. In addition, protein kinase A (PKA) reportedly induces a dramatic enhancement of Gardos channel activity, possibly by modulating the Ca$^{2+}$ sensitivity (138).

In non-stressed and non-stimulated human erythrocytes the fractional Gardos K$^+$ channel activity is low, resulting in a very low membrane potential ($\leq$ -10 mV) (140). As a consequence, human erythrocytes relatively have high intracellular Cl$^-$ concentrations. Activation of Gardos channels by increased intraerythrocytic Ca$^{2+}$ levels ($\geq$ 150 nM) leads to hyperpolarization of the RBC membrane potential close to the K$^+$ equilibrium potential (115, 141). This imposes an outwardly directed electrochemical Cl$^-$ gradient across the RBC membrane, driving Cl$^-$ and K$^+$ into the extracellular space, followed by osmotically obliged water efflux. The Gardos channel inhibitors clotrimazole and charybdotoxin (138, 142), or Cl$^-$ channel blocker 5-Nitro-2- (3-phenylpropylamino) benzoic acid (NPPB) blunt phosphatidylserine (PS) exposure, hyperosmotic and Ca$^{2+}$-stimulated isosmotic cell shrinkage, confirming the contribution of Gardos and anion channels to KCl and water efflux during RBC shrinkage (143, 144).

Increase in free [Ca$^{2+}$], therefore favors RBC shrinkage (Gardos effect) (144) subsequently decreased RBC deformability under isosmotic conditions, measured as decreased cell size.
or filterability (141). The Gardos effect is dependent on the extracellular Ca\(^{2+}\) concentration and is sensitive to amiloride (115). During complement activation rising cytosolic calcium triggers the Gardos effect, thus limiting the colloidosmotic swelling and lysis of erythrocytes (145).

1. 3. 2. Mechanisms of erythrocyte cell death or eryptosis
Eryptosis is triggered by erythrocyte injury after several stressors, including oxidative stress. Besides caspase activation after oxidative stress, two signalling pathways converge to trigger eryptosis: (a) formation of prostaglandin E2 leads to activation of Ca\(^{2+}\) permeable cation channels, and (b) the phospholipase-A2 mediated release of platelet-activating factor activates a sphingomyelinase, leading to formation of ceramide. Increased cytosolic Ca\(^{2+}\) activity and enhanced ceramide levels lead to membrane scrambling with subsequent phosphatidylserine exposure. Moreover, Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive K\(^+\) channels, leading to cellular KCl loss and cell shrinkage. In addition, Ca\(^{2+}\) stimulates the protease calpain, resulting in degradation of the cytoskeleton (108, 146).

1. 3. 2. 1. Role of prostaglandins in stimulation of erythrocyte cation channels
Intriguing evidence points to a role of prostaglandins in the regulation of eryptosis. Hyperosmotic shock and Cl\(^-\)-removal trigger the release of prostaglandin E2 (PGE2). PGE2 in turn activates the cation channels, increases the cytosolic Ca\(^{2+}\) concentration, and stimulates phosphatidylserine exposure at the erythrocyte surface. The activation of the cation channels by Cl\(^-\)-removal is abolished by the cyclooxygenase inhibitor diclofenac. Moreover, phospholipase-A2 inhibitors quinacrine and palmitoyl trifluoromethyl ketone and cyclooxygenase inhibitors acetylsalicylic acid and diclofenac blunt the increase of phosphatidylserine exposure following Cl\(^-\) removal. PGE2 further activates the Ca\(^{2+}\) dependent cysteine endopeptidase calpain, an effect, however, apparently not required for stimulation of phosphatidylserine exposure but playing a role in the degradation of the cytoskeleton (141).

1. 3. 2. 2. Role of Ca\(^{2+}\) sensitive K\(^+\) channels (Gardos channels) in eryptosis
Calcium ions entering erythrocytes do not only activate the scramblase but in addition stimulates the Ca\(^{2+}\) sensitive “Gardos” K\(^+\) channels in erythrocytes (147). The activation of the channels leads to hyperpolarization of the cell membrane driving Cl\(^-\) in parallel to K\(^+\) into the extracellular space. The cellular loss of KCl favours cell shrinkage. Moreover, the
cellular loss of $K^+$ presumably participates in the triggering of “eryptosis”. Increase of extracellular $K^+$ or pharmacological inhibition of the Gardos channels by clotrimazole or charybdotoxin do not only blunt the cell shrinkage but also decrease the phosphatidylinerine exposure following exposure to ionomycin. Presumably, cellular loss of $K^+$ somehow stimulates “eryptosis” as has been shown for apoptosis of nucleated cells (148). As PGE2 increases cytosolic $Ca^{2+}$ activity, it similarly activates the $Ca^{2+}$ sensitive “Gardos” $K^+$ channels with subsequent cell shrinkage (149).

1. 3. 2. 3. Role of protein kinase C in eryptosis
Erythrocyte energy depletion enhances phosphorylation of membrane proteins by protein kinase C (PKC), leads to subsequent phosphatidylinerine exposure at the cell surface and triggers cell shrinkage, effects mimicked by stimulation of protein kinase C (PKC) with phorbol esters or inhibition of protein phosphatases with okadaic acid (150). PKC activation has previously been shown to stimulate erythrocyte $Ca^{2+}$ entry and phosphatidylinerine exposure (151). PKC is a family of serine/threonine-specific protein kinases consisting of 10 members and requiring $Ca^{2+}$, diacylglycerol, and a phospholipid for activation. PKC isoenzymes play an essential role in the regulation of diverse cellular functions including proliferation, differentiation, and apoptosis. Human erythrocytes express PKC isoenzymes mediating the phosphorylation of cytoskeletal proteins, such as band 4.1, 4.9, and adducing the human Na+/H+ exchanger (NHE 1) (151, 152).

1. 3. 2. 4. Role of oxidative stress and caspase activation in eryptosis
Oxidative stress resulting from exposure to tert-butylhydroperoxide or peroxynitrite, for instance, is a major cause of erythrocyte injury (153, 154). It has been shown to activate aspartyl and cysteinyl proteases (155). Caspases have been shown to be expressed in erythrocytes, to cleave the anion exchanger band 3 in vitro (155), and to stimulate phosphatidylinerine exposure of erythrocytes (156). Conversely, eryptosis after ionomycin or hyperosmotic shock does not require activation of caspases (157). Besides its effect on caspases, oxidative stress or defects of antioxidative defence, enhance $Ca^{2+}$ entry via activation of the cation channels and thus stimulate eryptosis at least partially through channel activation (158). Oxidation of erythrocytes leads to an increase of the cation permeability of the membrane, Oxidation with tert-butylhydroperoxide also enhanced erythrocyte annexin-V binding as a measure of phosphatidylinerine exposure by some six folds. Interestingly, not all erythrocytes of one population showed the same sensitivity
against oxidative stress, and only one third of the population was shown to be annexin-V positive (141). Oxidative stress further activates erythrocyte Cl⁻ channels (159), which are required for erythrocyte shrinkage and thus also participate in the triggering of eryptosis (143). Antioxidants, such as vitamin E (160, 161), glutathione (162), or the semi-synthetic flavonoid 7-monohydroxyethylrutoside may protect erythrocytes from oxidative stress and thus presumably from eryptosis (163).

1. 3. 2. 5. Role of platelet-activating factor (PAF) and stimulation of sphingomyelinase in eryptosis

Erythrocyte shrinkage triggers the formation of platelet-activating factor (PAF) which is involved in the regulation of inflammation, thrombosis, atherogenesis, and cardiovascular function (164). PAF then stimulates a sphingomyelinase, leading to the breakdown of sphingomyelin and release of ceramide from erythrocytes (122). Osmotic shock thus leads to the appearance of ceramide at the erythrocyte surface. At least partially because of ceramide formation, PAF triggers scrambling of the cell membrane with phosphatidylserine exposure at the erythrocyte surface. C6- ceramide as well as treatment with purified, bacterial sphingomyelinase similarly triggers phosphatidylserine scrambling (165). Moreover, eryptosis after osmotic shock is blunted by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin. PAF further activates Ca²⁺-sensitive K⁺ channels (Gardos channels) in the erythrocyte cell membrane (166). Conversely, PAF is released from erythrocyte progenitor cells on increase of cytosolic Ca²⁺ activity (114). The signalling through PAF does, however, not necessarily require elevated cytosolic Ca²⁺ concentrations, and enhanced PAF levels at least partially account for Ca²⁺-independent eryptosis (164).

1. 3. 3. Eryptosis inhibitors

Erythropoietin is a potent inhibitor of erythrocytic progenitor cells apoptosis, and also inhibits the suicidal death of mature erythrocytes. The hormone is effective through inhibition of the Ca²⁺-permeable cation channels. The anti-eryptotic effect of erythropoietin presumably accounts for its ability to increase the life span of circulating cells. Dopamine, isoproterenol, and epinephrine similarly inhibit the Ca²⁺-permeable cation channels and thus interfere with eryptosis. The effect is presumably not relevant for physiologic regulation, as the concentrations of catecholamines required exceed those encountered in vivo. However, animal experiments revealed that the hematotoxicity
(including red blood cell counts) of cyclophosphamide has been reduced by simultaneous dopamine treatment. Thus, administration of dopamine at pharmacologic doses may well be therapeutically applicable for the suppression of eryptosis (167).

1.3.4. Clinical conditions associated with eryptosis

A variety of clinical conditions decrease the life span of mature erythrocytes by facilitating eryptosis. Increased sensitivity of sickle cells and of glucose 6-phosphate dehydrogenase–deficient cells to osmotic shock, oxidative stress and glucose depletion has been observed previously. The enhanced susceptibility to eryptosis is believed to decrease the erythrocyte life span in those disorders (168).

Similarly, iron-deficient erythrocytes are more sensitive to eryptosis. The enhanced eryptosis is at least partially the result of enhanced cation channel activity. Presumably, the decreased volume of iron-deficient erythrocytes decreases the threshold for the activation of the channel. Interestingly, the enhanced exposure of phosphatidylserine on the surface of iron-deficient erythrocytes coincides with a substantial decrease of the life span of iron-deficient erythrocytes (169). Eryptosis is further triggered by exposure of erythrocytes to lead or mercury, or by treatment with plasma from patients with recurrent hemolytic-uremic syndrome. Thus, the typical anemia after lead intoxication is at least partially due to enhanced eryptosis. Oxidant injury of erythrocytes and/or erythrocyte precursors may further occur in HIV (162).

Even though it is a pathophysiologic mechanism, eryptosis serves an important physiologic function (i.e., the prevention of hemolysis). Energy depletion, defective Na⁺/K⁺ATPase, or enhanced leakiness of the cell membrane eventually leads to cellular gain of Na⁺ and Cl⁻ with osmotically obliged water, resulting in subsequent cell swelling (170). Initially, the entry of Na⁺ is compensated by cellular loss of K⁺; the decrease of the K⁺ equilibrium potential leads, however, to gradual depolarization, which favours the entry of Cl⁻. The cell swelling jeopardizes the integrity of the cell membrane. Rupture of the cell leads to release of hemoglobin, which may be filtered at the glomeruli of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules, and thus lead to renal failure. The phosphatidylserine exposure at the cell surface allows the macrophages to recognize defective erythrocytes and to clear them from circulating blood before hemolysis. Moreover, the activation of the Gardos K⁺ channel delays swelling and disruption of
defective erythrocytes, thus expanding the time allowed for macrophages to clear the injured erythrocytes from circulating blood (167).

1. 3. 5. Eryptosis in malaria
1. 3. 5. 1. Stimulation of membrane transport in *P. falciparum* infected erythrocytes and activation of ion channels

A prerequisite for intracellular survival of pathogens is an adequate supply of nutrients and disposal of waste products (171). Additional transport systems for nutrient supply and disposal of waste products are needed particularly during infection of erythrocytes with *Plasmodium species*. Before infection, the substrate turnover of erythrocytes is low. Since they do not synthesize proteins, DNA or membrane components, they have no need for amino acids, nucleic acids, lipids or most of the vitamins. The replicating *Plasmodium*, however, has extensive requirements for all of these nutrients and, to fuel the replication process, *Plasmodium* parasites consume large amounts of glucose. An infected erythrocyte takes up 40–100 times more glucose than a non-infected cell and releases the corresponding amounts of lactic acid (172). To gain access to the necessary nutrients and to dispose the waste products, *Plasmodium* induces the so-called new permeability pathway (NPP). This pathway mediates the transport of electrolytes, sugars, nucleic acids, membrane components and other substances across the erythrocyte membrane. In addition to nutrient uptake and lactate disposal the NPP contributes to the strategy of the pathogen to counteract swelling of the host cell by decreasing the colloid-osmotic pressure in the host cytosol. The parasite digests haemoglobin in excess (i.e. more than needed for its protein biosynthesis) and exports the haemoglobin-derived amino acids via the NPP thus preventing host cell swelling and lysis (173). NPP further allows the entry of Na\(^+\) and Ca\(^{2+}\) into the host cell, thus adjusting the electrolyte composition of the host cytosol to the needs of the parasite. Starting >15 h post-infection, K\(^+\) in the host cytosol is replaced increasingly by Na\(^+\). The parasite most probably requires an outwardly directed K\(^+\) and an inwardly directed Na\(^+\) gradient across its plasma membrane since decreasing these gradients experimentally *in vitro* abolishes parasite growth. In addition, absence of extracellular Ca\(^{2+}\) disrupts the intraerythrocyte survival of the pathogen (174). The necessity of the NPP for *Plasmodium*’s survival is illustrated by the fact that several inhibitors of this pathway eventually kill the pathogen within the erythrocyte (175). On the other hand, the
Plasmodium species imposes oxidative stress on host cells, and this has been proposed to be required for the activation of the cation channels (135).

1.3.5.2. Induction of eryptosis by P. falciparum infection

The opening of cation channels in the host cell by P. falciparum is expected to increase the cytosolic Ca\(^{2+}\) activity and thus to induce eryptosis. However, most of the Ca\(^{2+}\) entering the parasitized erythrocyte is either extruded across the host cell membrane or taken up by the pathogen so that cytosolic free Ca\(^{2+}\) activity remains low (176). Nevertheless, some in vitro studies have shown infection with P. falciparum increase the number of erythrocytes that expose phosphatidylserine at the outer membrane leaflet, (177) while another study could not detect breakdown of the phospholipid asymmetry. Infection with P. falciparum increases the formation of ceramide (122) that in turn increases the Ca\(^{2+}\) sensitivity of the erythrocyte scramblase and may account for the stimulation of the scramblase even at low or only slightly enhanced cytosolic Ca\(^{2+}\) concentrations. Beyond that, Ca\(^{2+}\) independent mechanisms may participate in the triggering of eryptosis. As observed in nucleated cells the cellular loss of K\(^{+}\) via the induced cation channel is expected to augment the activation of the erythrocyte scramblase and thus adds to the stimulation of the phosphatidylserine exposure (178).

1.3.5.3. Role of eryptosis in parasitized erythrocytes

Human erythrocytes infected with trophozoite and shizont stages of P. falciparum may adhere to endothelial cells lining the post-capillary venules. Adhesion of the infected erythrocyte to the endothelium involves receptors in the endothelial membrane such as CD36, secreted proteins such as thrombospondin, parasite-encoded molecules such as P. falciparum erythrocyte membrane 1 (PfEMP1) and a modified erythrocyte anion exchanger (AE1, band 3) (177). In particular, exposure of phosphatidylserine at the outer membrane leaflet contributes to erythrocyte-endothelium adhesion via binding to thrombospondin and CD36 receptor. Thus “eryptosis” of parasitized erythrocytes may contribute to tissue sequestration, which is thought to avoid splenic clearance of the parasitized erythrocyte and to favour parasite development in a low oxygen pressure microenvironment. On the other hand “eryptosis” may result in clearance of parasitized erythrocytes. As macrophages are equipped with receptors specific for phosphatidylserine, erythrocytes exposing phosphatidylserine at their surface will be recognized, bound, engulfed and degraded rapidly. Thus, infected erythrocytes are liable to be eliminated as
soon as they expose phosphatidylserine. The removal of the infected erythrocyte would of course clear the pathogen as well, since it would be similarly degraded by lysosomal enzymes of the macrophages (179, 180).

1.4. Rodent malaria parasites as models for human malaria

A wide range of investigations using rodent parasites have contributed to development and shaping of basic concepts in research of human diseases. Rodent malaria parasites are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to human malaria in most essential aspects of structure, physiology and life cycle (181). Metabolic pathways are conserved between rodent and human malaria parasites. No gross differences in metabolic pathways have been reported in the various malaria parasites. The similarity in sensitivity of these parasites to anti-malarial drugs and other specific inhibitors emphasises the similarities in their metabolic processes. The life cycles and the different developmental stages of all rodent and human malaria parasites are largely comparable (182, 183). Rodent parasites are recognized as valuable model parasites for the investigation of the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing. The relationships of rodent parasites to other human malaria parasites in brief are:

• The basic biology of rodent and human parasites is similar.
• The genome organization and genetics is conserved between rodent and human parasites (184).
• Housekeeping genes and biochemical processes are conserved between rodent and human parasites.
• Methodologies for genetic modification are available for both types of parasites.
• Rodent hosts with extensively characterised genetic backgrounds and transgenic lines are valuable and available tools for immunological studies.
• The structure and function of vaccine candidate target antigens are conserved between rodent and human parasites.
• The manipulation of the complete lifecycle of rodent parasites, including mosquito infections is simple and safe.
• In vitro culture techniques for large-scale production and manipulation of different life cycle stages are available. For example, in vitro cultures of liver and mosquito stages provide tools to investigate the less accessible parts of the life cycle in the human
• The molecular basis of drug-sensitivity and resistance show similar characteristics in rodent and human parasites.
• Rodent parasites allow in vivo investigations of parasite-host interactions and in vivo drug testing.

The malaria parasites *P. berghei* which infects hamsters, rats and mice, is one of the many species of malaria parasites that infect mammals other than humans. A susceptible mosquito vector for *P.berghei*, which is widely used in the laboratory, is *Anopheles stephensi* (182, 184).

1. 5. Pharmacological agents
1. 5. 1. Gum Arabic

Gum Arabic (GA) is a water-soluble dietary fibre derived from the dried gummy exudates of the stems and branches of *Acacia Senegal* (185). Chemically, GA is a polysaccharide of (1-3) linked β-D-galactopyranosyl units with branches of two to five units in length attached by (1-6) links to the main chain. Both the main chain and side chains contain α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-glucuronopyranosyl, and 4-O-methyl-β-D-glucuronopyranosyl units. Gum arabic is readily soluble in water without increasing viscosity (186).

Gum Arabic is a highly heterogeneous material, but was separated into three major fractions by hydrophobic affinity chromatography (187). Most of the gum (88.4% of total), an arabinogalactan (AG), had a very low protein content (0.35%) and a molecular mass of 3.8 - 105 Da. The second fraction (10.4% of total), an arabinogalactan-protein complex (AGP), contained 11.8% protein and had a molecular mass of 1.45 - 106 Da. The third fraction (1.2% of total gum), referred to as a low molecular weight glycoprotein (GP), had a protein content of 47.3%, and a molecular mass of 2.5 - 105 Da (GPC data). The major amino acids present in the protein of AG and AGP were hydroxyproline, serine and proline, whereas in GP, aspartic acid is the most abundant (188).

Gum Arabic is primarily indigestible to both humans and animals. It is not degraded in the small intestine, but fermented in the large intestine by microorganisms to short-chain fatty acids, particularly butyric and propionic acids. Such degradation products are absorbed in the human colon and subsequently metabolized. In an experiment using an enrichment
culture of pig cecal bacteria, showed that a *Prevotella ruminicola*-like bacterium was the predominant organism that is most likely to be responsible for the fermentation of GA to butyrate and propionate (189, 190).

Gum Arabic is widely used in both the pharmaceutical and food industries as an emulsifier and stabilizer of various products for human consumption. Examples include tablets or food applications such as puddings and fillings, frostings, candy, and chewing gum. The United States Food and Drug Administration recognize it as one of the safest dietary fibers (189, 191).

Pharmacologically, GA has been claimed to act as an anti-oxidant, and to protect against experimental hepatic, renal and cardiac toxicities in rats. GA has been claimed to alleviate the adverse effects of chronic renal failure in humans. This could not be corroborated experimentally in rats (183). Reports on the effects of GA on lipid metabolism in humans and rats are contradicting, but mostly suggest that GA ingestion can reduce plasma cholesterol concentrations. GA has proabsorptive properties and can be used in diarrhoea. It enhances dental remineralization, and has some antimicrobial activity, suggesting a possible use in dentistry. GA has been shown to have an adverse effect on electrolyte balance and vitamin D in mice, and to cause hypersensitivity in humans (183).

1. 5. 2. Amphotericin B

Amphotericin B is a polyene antifungal agent with an *in vitro* activity against a wide variety of fungal pathogens. Amphotericin B exerts its antifungal effect by disruption of fungal cell wall synthesis because of its ability to bind to sterols, primarily ergosterol. This affinity may also account for its toxic effects against selected mammalian cells. Amphotericin B may be fungistatic or fungicidal, depending upon drug concentration and sensitivity of the pathogen (192).

Oral preparations of amphotericin B are used to treat oral thrush; these are virtually nontoxic. The main i.v. use is in systemic fungal infections (eg. in immunocompromised patients), and in visceral *Leishmaniasis. Aspergillosis, Cryptococcus* infections (eg. meningitis) and *Candidiasis* are treated with amphotericin B. It is also used empirically in febrile immunocompromised patients who do not respond to broad-spectrum antibiotics (193).
1. 5. 2. 1. Mode of action
As with other polyene antifungals, amphotericin B associates with ergosterol, a membrane chemical of fungi, forming a pore that leads to $K^+$ leakage and fungal cell death. Recently, however, researchers found evidence that pore formation is not necessarily linked to cell death. The actual mechanism of action may be more complex and multi-faceted. Amphotericin B is believed to interact with membrane sterols (ergosterol) to produce an aggregate that forms a trans-membrane channel. Intermolecular hydrogen bonding interactions among hydroxyl, carboxyl and amino groups stabilize the channel in its open form, destroying activity and allowing the cytoplasmic contents to leak out (194).

1. 5. 2. 2. Side effects
Very often a most serious acute reaction after the infusion (1 to 3 hours later) is noted consisting of fever, shaking chills, hypotension, anorexia, nausea, vomiting, headache, dyspnea, and tachypnea. This reaction sometimes subsides with later applications of the drug and may in part be due to histamine liberation. An increase in prostaglandin-synthesis may also play a role. Often the most difficult decision has to be made, whether the fever is disease or drug-related. In order to decrease the likelihood and severity of the symptoms, initial doses should be low and increased slowly. The liposomal preparation obviously has a lower incidence of the syndrome. Acetaminophen, pethidine, diphenhydramine and/or hydrocortisone have all been used to treat or prevent the syndrome, but the prophylactic use of these drugs should be limited (195).

Nephrotoxicity (kidney damage) is a major issue and can be severe and/or irreversible. It is much milder when amphotericin B is delivered in liposomes. Electrolyte imbalances (eg. hypokalemia and hypocalcemia) may also occur (196).

1. 6. Rational of the study
Targeting the pathogen with antimalarial drugs has only been partially successful because of the spread of drug-resistant parasites and the optimal use of effective drugs has always been a major concern. Hence all possibilities to combat this disease must be explored. The course of the disease is not only a function of the pathogen but is heavily influenced by properties of the host. Hence, studies must be carried out to fight infection by altering host physiology which decimates the problem of drug resistance and if successful, these
approaches may prove extremely useful, particularly in the treatment of resistant pathogens. Targeting at the accelerated clearance of the infected host cells is expected to provide protection against malaria.

Pharmacological induction of phosphatidylserine exposure of ring stage-infected erythrocytes reportedly accelerates their clearance. Thus, manoeuvres accelerating eryptosis may result in premature clearance of the intraerythrocytic parasite and partially enhance the survival of *Plasmodium berghei*-infected mice. 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.

In this study we are interested in investigating the role of phosphatidylserine (PS) exposure and eryptosis which may allow the pharmacological manipulation of erythrocyte survival and hence of the malaria disease course. This may be achieved by determining the effect of eryptosis inducing drugs on the proliferation and survival of the *Plasmodium* pathogen.
1. 7. The objectives of the study

1. 7. 1. General objective

The aim of the present study is to investigate the effect of amphotericin B or gum Arabic administration on eryptosis of infected cells and their influence on the course of malaria in *P. berghei* infected mice.

1. 7. 2. Specific objectives

1. To determine the effect of amphotericin B or butyrate on *in vitro* growth and DNA amplification of *P. falciparum* infected erythrocytes.

2. To determine the effect of amphotericin B or butyrate on the cytosolic Ca\(^{2+}\) activity of *P. falciparum* infected and non-infected erythrocytes.

3. To determine the effect of amphotericin B or butyrate on the phosphatidylinerine exposure on *P. falciparum* infected and non-infected erythrocytes.

4. To determine the effect of amphotericin B or gum Arabic administration on eryptosis of infected cells and their influence on the course of malaria in *P. berghei* infected mice.

5. To determine the significance of phosphatidylinerine exposure in clearance and elimination of infected erythrocytes.
2. Materials and methods

2.1. Animals
Experiments were carried out on 4 to 5-month-old wild-type SV129/mJ mice of either sex (n = 6 male, 8 female for each one of the three series) in gum Arabic and amphotericin B experiments. The animals were housed under controlled environmental conditions (22-24°C, 50-70% humidity, and a 12-h light/dark cycle). Throughout the study, mice had free access to standard pelleted food (C1310, Altromin, Heidenau, Germany) and tap water or GA solution, as indicated. All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals, and were approved by local authorities in Eberhard Karls University of Tübingen, Germany. (Registration number PY 2/06). Mouse erythrocytes were drawn from animals by retroorbital venopuncture or by incision of the tail vein.

2.2. Parasites
Plasmodium falciparum BINH (197) and Plasmodium berghei ANKA (198) were a kind gift from the Institute of Tropical Medicine in Tuebingen, Germany. All chemicals and equipments used in this study are shown in appendix 1.

2.3. Infection of mice
For infection of mice Plasmodium berghei ANKA-parasitized murine erythrocytes (1x10^6) were injected intraperitoneally into wild-type mice (199, 200).

2.4. Gum Arabic treatment
Gum Arabic in powder form was provided as a generous gift from Dar Savanna, Ltd., Khartoum, Sudan (www.ssgums.com). It is a 100% natural extract powder produced mechanically from the wildly grown Acacia senegal tree, with a particle size of <210 µm. Its quality conforms to the food and pharmaceutical requirements of the Food and Agriculture Organization of the United Nations (FAO), the British Pharmacopoiea (BP), the United States Pharmacopoiea (USP), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Experiments were carried out on wild-type SV129/mJ mice of either sex (n = 6 male, 8 female for each one of the three series). Starting from the day of infection, animals were provided with 10% (w/w) GA dissolved in tap water (100 g/L). Preparations were
refreshed every day during the treatment. The effects of GA treatment were investigated starting from day 8 after the infection. The intake corresponded to a dose of approximately 10 g/kg body weight/day. Blood was collected from the mice 8 days after infection by incision of the tail. Parasitemia was determined by Syto-16 staining in FACS analysis or using Giemsa stain.

2.5. Amphotericin B administration
Experiments were carried out on six male mice for control and other six mice for Plasmodium berghei infected group in each one of the three series of the experiment. Amphotericin B (1.4 mg/kg body weight for three successive days) was administered subcutaneously from the eighth day of infection. Blood was collected from the mice 8 days after infection by the incision of the tail. Parasitemia was determined by Syto-16 staining in FACS analysis and Giemsa stain.

2.6. Preparation of human erythrocytes
Human erythrocytes were drawn from healthy volunteers. They were used after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). The buffy coat and upper 10-20% of the red blood cells were discarded; the remaining pellet was used for experiments after washing three times in phosphate buffer saline. The RBCs were 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 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl₂ or RPMI 1640 medium. Blood donors gave their informed consent and the study was approved by the Ethical Committee of Eberhard Karls University of Tübingen, Germany (project number: 184/2006V). (Appendix-2 protocol 1)

2.7. Investigations
2.7.1. In vitro culture of Plasmodium falciparum infected human erythrocytes
For infection of human erythrocytes, the P. falciparum strain BinH (197) was grown in vitro (135) in human erythrocytes. Cultures were maintained continuously by routine passage in fresh and stored human erythrocytes. Parasites were cultured as described earlier (201, 202) 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 dilution factors (Df) for splitting the infected RBCs suspension in order to maintain the *P. falciparum in vitro* culture were calculated as follows:

\[
Df = \frac{mP \times V(RBC)}{dP \times dV(RBC)}
\]

Fresh blood was added according to the following formula:

\[
dV(RBC) = \frac{(V(RBC))}{Df}
\]

Where mP: measured parasitemia; V(RBC): volume of packed erythrocytes in the culture flask; dP: desired parasitemia; dV(RBC): desired volume of packed erythrocytes in the flask. The volume of the medium was adjusted according to the degree of parasitemia and the time until the next medium change (203) (Appendix-2 protocol 2).

**2.7. 1. 1. Freezing and sawing of parasites**

From time to time samples with high, *i.e.* ≥ 20 % parasitemia, predominantly containing ring stages were deep frozen in liquid nitrogen for stock purposes. The cell pellet obtained after centrifugation (450g / 8 min at RT) was mixed with an equal volume of freezing solution, sterile transferred to a 2.0 ml cryotube vial and directly deep frozen in liquid nitrogen. For defreezing the parasites from cryotubes containing infected RBCs (> 20 % parasitemia) were thawed quickly at 37°C in a water bath (3-5 min). The contents were transferred to a 15 ml falcon tube and centrifuged (450g / 4 min at room temperature). The supernatant was discarded. A volume of sterile filtered 3.5 % NaCl solution equal to cell pellet volume (V(RBC)) was added at a rate of 1 - 2 drops per second while gently shaking the tube. Finally the liquid was mixed with a pipette. After centrifugation at 450g for 4 min the supernatant was discarded. The RBC pellet was carefully suspended in original RPMI 1640 medium (2*V (RBC)). Washing was repeated twice. The remaining cell pellet was re-suspended in complete medium and transferred to a culture flask. Fresh RBCs were added (amount depending on V(RBC)). The culture flask was filled with 90% N₂/5% O₂/ 5% CO₂ (Appendix-2 protocol 3).
2. 7. 1. 2. Isosmotic sorbitol synchronization of *P. falciparum* infected human erythrocytes

The *P. falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment (197). Briefly, the infected RBCs (>5% parasitemia) were spun down at 600×g and re-suspended 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 sub-cultured for further experiments (Appendix-2 protocol 4).

2. 7. 1. 3. *In vitro* *P. falciparum* growth 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 (159) (Appendix-2 protocol 5). The erythrocytes were grown in the presence or absence of different concentrations of sodium butyrate or amphotericin B. The parasitemia of human erythrocytes were 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 ± 15 nm. FACS analysis proved a more sensitive technique for determining parasitemia than either Giemsa or Field’s rapid staining. (Appendix-2 protocol 6). In mice the course of parasitemia (=percentage of infected cells) was determined in Giemsa stained blood films made from tail blood. The thick blood film was air-dried and fixed with methanol. 2% Giemsa solution (Sigma) was added for 30 min. The slide was rinsed with water and again dried. Then, the slides were analysed under a Leica CM E light microscope (100 X, oil immersion) (204) (Appendix-2 protocol 7).
2. 7. 2. Intraerythrocytic DNA amplification of *P. falciparum*

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 different concentrations of sodium butyrate or amphotericin B. Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

2. 7. 3. Annexin binding experiments (Determination of phosphatidylserine exposure)

Suspensions with non-infected RBCs were stained with annexin V-FLUOS. Suspensions with *P. falciparum* infected RBCs were stained with annexin V-568 and/or with the DNA dye Syto16 to assess PS exposure in the outer leaflet of the RBC membrane and the percentage of infected RBCs, respectively (205). (Appendix-2 protocol 8)

For annexin binding, RBCs were washed, re-suspended in annexin-binding buffer, stained with annexin V-568 (dilution 1:50) or annexin V-FLUOS (dilution 1:100), and incubated for 20 min at RT in Ca\(^{2+}\)-free annexin binding buffer. AnnexinV binding to negatively charged phospholipids (with a high specificity for PS) is Ca\(^{2+}\)-dependent. Therefore an annexin binding buffer containing Ca\(^{2+}\) was used. Syto16 (final concentration of 30 nM) was co-incubated in the annexin binding buffer for 20 min at RT for double-staining purposes. Samples were diluted 1:5 with annexin binding buffer just before FACS analysis. Cells were analyzed by flow cytometry. Fluorescence 1, FL-1 (detector for an emission wavelength of 530 ± 15 nm) was either an indicator of annexin V-FLUOS fluorescence intensity (excitation: 450 - 500 nm, emission: 515 -565 nm) (simple staining for annexin); or of Syto16 fluorescence (maximum excitation wavelength: 488 nm for DNA, 494 nm for RNA, maximum emission wavelength: 518 nm for DNA, 525 nm for RNA). Syto16 fluorescence was taken to reflect the degree of parasitemia. Fluorescence 2, FL-2 (detector for an emission wavelength of 585 ± 21 nm) or FL-1 was used to determine the annexin fluorescence of Annexin V-568 or Annexin Fluos, respectively, i.e. the percentage of PS exposing cells. Annexin V-568 has an absorption spectrum from 450 – 650 nm, a maximum at 578 nm, an emission spectrum from 560 nm up to > 700 nm with a maximum at 603 nm. Double staining necessitated compensation for overlapping emission spectra by
electronic subtraction of any unwanted spectral “spillover” from the signal of interest. Simple stained infected erythrocytes (with Syto16 or annexin V-568) containing dye in the relevant concentrations were used as compensation controls for this multicolor study.

To induce annexin binding according to Lang et al. (116), non-infected RBCs were incubated for 1 h at 37 °C with the Ca\(^{2+}\) ionophore ionomycin (0 and 1 \(\mu\)M, respectively) or oxidized by t-butylhydroperoxide (t-BHP, 1 mM for 15 min followed by 24h of post-incubation) in a modified NaCl test solution consisting of (in mM): 125 NaCl, 5 KCl, 5 D(+)-glucose, 1 CaCl\(_2\), 1 MgSO\(_4\), 32 HEPES titrated to pH 7.4 with NaOH.

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

Fluo 3 acetoxymethyl AM ester was used as the fluorescent indicator. Fluo-3 (1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy] -2-(2-amino-5-methylphenoxy) ethane-N,N,N’,N’-tetra-acetic acid, \(C_{51}H_{50}C_{12}N_{2}O_{23} =1129.85\)) is a fluorescent cheater excited by visible light (\(~488\)nm), and emits a yellowish green fluorescence (\(~525\)nm) when bound to a calcium ion (198). The intensity of fluorescence depends on the free calcium concentration. Fluo-3 does not fluoresce unless bound to calcium ions. Intracellular Ca\(^{2+}\) measurements were performed as described previously by Andrews et al., (2002) (206). 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-µl 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 re-suspended in 1 ml of Ringer solution (0.16% hematocrit) containing serial concentrations of amphotericin B or sodium butyrate 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 (115).

2. 7. 5. Measurement of hemolysis
After incubation of infected erythrocytes for 48 hours at 37°C, the samples were centrifuged (3 min at 400 g, RT), and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis (207, 208) (Appendix-2 protocol 9).

2. 7. 6. In vivo proliferation of P. berghei ANKA
For infection of mice P. berghei ANKA-parasitized mouse erythrocytes (1x10^6) were injected intraperitoneally (209) into sex- and age-matched mice fed on control diet and with free access to drinking water or 10% (w/w) GA dissolved in tap water (100 g/L) or amphotericin B (1.4 mg/kg body weight for three successive days after day 8) administered subcutaneously. Parasitemia was determined daily from the 8th day of infection by flow cytometry. The blood samples were stained with Giemsa stain and the DNA/RNA-specific fluorescence dye Syto-16 and the Syto-16 fluorescence was analysed with a FACS Calibur cytometer (Becton Dickinson, Heidelberg, Germany) at fluorescence channel 1 (FL1 488 nm excitation and 530 nm emission as described above).

2. 7. 7. Measurement of the in vivo clearance of fluorescence-labelled erythrocytes
Fluorescence-labelled erythrocytes from mice were obtained by staining erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). The labelling solution was prepared by addition of adequate amounts of a CFSE stock solution (10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 µM. Then, the cells were incubated with labelling solution for 30 min at 37°C. The cells were pelleted at 1000 x g for 5 min, washed and re-suspended in fresh, pre-warmed PBS. The fluorescence-labelled erythrocytes were injected into the tail veins of mice. At indicated time points, blood was taken from the mice and CFSE-dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. The percentage of CFSE-positive erythrocytes was calculated in % of the whole population (169).
2. 7. 8. Measurement of the fluorescence-labelled erythrocytes in the spleens of mice
The fluorescence-labelled erythrocytes were injected into the tail veins of mice. After 2 hours the mice were sacrificed and the spleens were dissected and carefully mashed through a net. Finally, CFSE-dependent fluorescence intensity of the erythrocytes in spleen suspensions was measured in the fluorescence channel FL-1 (209).

2. 7. 9. Data analysis and statistics
Data are expressed as means ± SEM, and (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. Statistical analysis was performed with GraphPad InStat version 3.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).
3. Results

3.1. Influence of amphotericin B on the course of malaria

Effect of amphotericin B on \textit{in vitro} growth of \textit{P. falciparum} in human erythrocytes.

\textit{In vitro} growth of \textit{P. falciparum} in human erythrocytes was significantly decreased at amphotericin B started from concentration of 0.01 µM (Fig. 3.3). Within 48 hours the percentage of infected erythrocytes increased from 4 to 23%. Increased amphotericin B concentration decreases percentage parasitemia in a dosage dependant manner, an effect reaching statistical significance at $\geq 0.01$ µM amphotericin B concentrations ($p< 0.05$).

Effect of amphotericin B on \textit{P. falciparum} DNA amplification

Intraerythrocytic DNA amplification in erythrocytes was not significantly influenced by amphotericin B at the concentrations employed but only at the highest one (1µM). (Fig. 3.4).

Effects of amphotericin B on erythrocytes hemolysis.

At the applied concentrations amphotericin B did not disrupt the integrity of the cell membrane and did not induce significant hemolysis (Fig. 3.5).

**Fluo 3 fluorescence intensity in control, ionophore ionomycin and amphotericin B treated erythrocytes**

Erythrocyte shrinkage induces activation of Ca$^{2+}$-permeable non-selective cation channels. Thus erythrocytes were loaded with the Ca$^{2+}$-sensitive fluorescence dye fluo-3 (2 µM fluo-3 AM) to determine the effect of amphotericin B on cytosolic Ca$^{2+}$ activity. Cells were incubated in the absence or presence of amphotericin B (1 µM for 30 min) or the Ca$^{2+}$ ionophore ionomycin (1 µM), and fluo-3 fluorescence intensity was monitored using FACS. As illustrated in Fig. 3.6, amphotericin B increased fluo-3 fluorescence intensity. As a positive control, the Ca$^{2+}$ ionophore ionomycin (1 µM) similarly enhanced the fluo-3 fluorescence intensity. The data strongly suggest that the observed amphotericin B effect on fluo-3-loaded erythrocytes reflect an increase in cytosolic free Ca$^{2+}$ concentration.

**Fluo 3 fluorescence intensity in control and amphotericin B treated erythrocytes**

Incubation of erythrocytes in Ringer solution with amphotericin B for 48 h indicates significant difference from the respective value of Fluo 3 fluorescence intensity in the absence of amphotericin B (Fig. 3.7). Fluo3 fluorescence intensity increased by increased
amphotericin B concentration. It was found to be 19.73 (arb. units) in the absence of amphotericin B, increases in Fluo3 fluorescence reaching statistical significance at ≥ 0.1 µM amphotericin B concentration (p< 0.01).

Effects of amphotericin B on phosphatidylserine exposure of *P. falciparum* infected and non-infected erythrocytes.

To identify phosphatidylserine exposing erythrocytes, annexin V binding has been determined in FACS analysis. The percentage of annexin-binding erythrocytes was low in fresh erythrocytes (1.24 ± 0.24%, n = 6). Exposure of uninfected erythrocytes to amphotericin B-free Ringer solution increased the percentage of annexin binding cells within 24 h to 4.14 ± 0.50% (n = 6) (Fig. 3. 8 A). Infection of erythrocytes with *P. falciparum* significantly enhanced the percentage of annexin binding erythrocytes. Phosphatidylserine exposure of infected erythrocytes was augmented by amphotericin B, an effect reaching statistical significance at 0.01 µM amphotericin B (Fig. 3. 8 B).

Effects of amphotericin B on forward scatter of infected and non-infected erythrocytes.

Infection of human erythrocytes with *P. falciparum* induces a biphasic change of the host erythrocyte volume. In the early trophozoite stage infected cells loose KCl and water leading to cell shrinkage. In the present study, ring-stage-synchronized erythrocytes when grown for 48h re-invaded new erythrocytes in which the parasite at the early trophozoite stages. Accordingly, infected cells had a lower forward scatter than uninfected erythrocytes. Amphotericin B leads to farther significant decrease in infected erythrocyte volume illustrated in Fig. 3. 9 A & B, which shows decreased forward scatter.

*In vivo* proliferation of *P. berghei* in control and amphotericin B treated mice.

Further experiments were performed on the impact of amphotericin B treatment on the course of malaria infection *in vivo*. To this end mice were infected with *P. berghei*. As shown in Fig. 3. 10, parasitemia in control and amphotericin B mice was (2.61 ± 0.18 and 2.33 ± 0.12%) respectively 8 days after infection but gradually increased in the next 12 days. In untreated animals, the percentage of parasitized erythrocytes increased up to 50.36 ± 3.98%. The addition of 0.7 mg/kg b.w. amphotericin B had only a slight effect on parasitemia. Thus, the concentration of amphotericin B was increased to 1.4 mg/kg b.w., which indeed significantly blunted the increase of parasitemia compared to non treated animals to 39 ± 2.46 % (mean ± SEM, n = 6), (p<0.05) (Fig. 3. 11).
Clearance of amphotericin treated erythrocytes from circulating blood

A next series of experiments explored, whether the enhanced phosphatidylserine exposure accelerates clearance of erythrocytes from circulating blood in vivo. For this purpose mice were either left untreated or treated with 1.4 mg/Kg b w amphotericin B intraperitoneally, their erythrocytes were labelled with the dye CFSE and injected into the tail vein. As illustrated in Fig. 3. 12 A, the clearance of erythrocytes in amphotericin B treated mice was slightly, but significantly faster than erythrocyte clearance in untreated mice. On the other hand, the fraction of labelled erythrocytes recovered in the spleen was significantly enhanced by amphotericin B treatment (Fig. 3. 12 B).

The effect of amphotericin B on survival of P. berghei infected mice.
The effect of amphotericin B on the parasitemia was paralleled by enhanced survival of the amphotericin B treated animals. Whereas all untreated animals died within 27 days after the infection, 80% of the amphotericin B treated animals survived the infection for more than 27 days (Fig. 3. 13). Thus, amphotericin B treatment indeed significantly modified the course of malaria.

3. 2. Influence of butyrate and gum Arabic on the course of malaria

Effect of butyrate on in vitro growth of P. falciparum in human erythrocytes.
The influence of butyrate on the in vitro growth of the parasite was analyzed. P. falciparum-infected erythrocytes were cultured in human erythrocytes and synchronized to ring stage by sorbitol treatment. Within 48 hours the percentage of infected erythrocytes increased from 5 to 26 %. Increased butyrate concentration decreases percentage parasitemia in a dosage dependant manner, an effect reaching statistical significance at ≥ 0.5 mM butyrate concentration (Fig. 3. 14) (p< 0.05).

Effect of butyrate on P. falciparum DNA amplification.
To test for intraerythrocytic DNA amplification infected erythrocytes were ring-stage synchronized and cultured for further 24h. As a result of increased butyrate concentration, statistically significant decrease in DNA amplification was observed only at the highest concentration (10 mM) (Fig. 3. 15).
Effect of butyrate on erythrocytes cytosolic Ca$^{2+}$ concentration.

Activation of cation channels is expected to increase cytosolic Ca$^{2+}$ activity. Fluo3 fluorescence was employed to test, whether butyrate modifies the cytosolic Ca$^{2+}$ concentration. As illustrated in Fig. 3. 16, 48 hours incubation of erythrocytes in the presence of different concentrations of amphotericin B was followed by a significant increase in Fluo3 fluorescence. Accordingly, exposure to amphotericin B increases cytosolic Ca$^{2+}$ activity of human erythrocytes.

Effects of butyrate on phosphatidylserine exposure of infected and non-infected erythrocytes.

To determine the effect of infection and butyrate on suicidal erythrocyte death (eryptosis), the percentage of phosphatidylserine-exposing erythrocytes was estimated by measurement of annexin V-binding in FACS analysis. Prior to the infection the percentage of annexin V-binding erythrocytes was low (1.2 ± 0.11%, n = 6). Within 24 hours, infection with *P. falciparum* markedly increased annexin V-binding of infected erythrocytes compared to non-infected mean ± SEM, (26.5 ± 4.3 and 15.9 ± 3.2) respectively (p≤0.05; paired ANOVA). Both in the absence and presence of butyrate the percentage of annexin V-binding was significantly higher in infected than non-infected erythrocytes. The phosphatidylserine exposure of infected erythrocytes was significantly increased by increased butyrate concentration, an effect reaching statistical significance at 5 mM butyrate (p≤0.05; paired ANOVA) (Fig. 3. 17).

Effects of butyrate on forward scatter of infected and non-infected erythrocytes.

At early stages of the parasite development, infection of erythrocytes decreased erythrocyte forward scatter, pointing to cell shrinkage. At late stages of the parasite development (trophozoites), infection increased the forward scatter, pointing to cell swelling. Butyrate decreased the forward scatter of the early and late stage infected erythrocytes. Fig. 3. 18 A & B shows significant difference (p < 0.05) from absence of butyrate at early stages, and also indicates significant difference to non-infected erythrocytes (p < 0.05).

*In vivo* effect of gum Arabic on parasitemia of *P. berghei* infected mice.

To determine the *in vivo* efficacy of gum Arabic, mice were infected with *P. berghei* with or without gum Arabic treatment. Gum Arabic (10 % in drinking water) was administered
from the 8th day of infection, when parasitemia was still low in control and infected mice, mean ± SEM, (3.44 ± 0.63 and 5.09 ± 0.21%) respectively, no significant deferens was observed. The percentage of infected erythrocytes increased gradually in mice without or with gum Arabic treatment reaching 43.6 ± 4.39 and 58.08 ± 3.03 respectively at day 21 (Fig. 3.19). However, the percentage of parasitized erythrocytes was slightly lower in gum Arabic-treated mice than in mice without gum Arabic treatment, an effect reaching statistical significance between day 16 to day 20 of infection (p≤0.05; paired ANOVA) (Fig. 3.19).

Effects of gum Arabic on survival of *P. berghei* infected mice.
The treatment with gum Arabic further influenced the survival of *P. berghei*-infected mice. As shown in Fig. 3. 20, all of the gum treated animal survived up to day 21 after the infection, were the untreated animals number decreased by 30 %. All of the untreated animals died within 26 days after the infection. In contrast as many as 70% of the gum Arabic-treated animals survived the infection for more than 26 days.
Fig. 3. In vitro growth of *Plasmodium falciparum* in human erythrocytes as a function of amphotericin B concentration.

Arithmetic means ± SEM (n = 8) of *in vitro* growth of *Plasmodium falciparum* in human erythrocytes as a function of amphotericin B concentration. *, **Indicates significant difference (p < 0.05; p < 0.01) from absence of amphotericin B.
Fig. 3.4: DNA amplification of *Plasmodium falciparum* in human infected erythrocytes as a function of amphotericin B concentration (arithmetic means ± SEM, n = 8). *Indicates significant difference (p < 0.05;) from absence of amphotericin B.
Fig. 3. 5: Effect of amphotericin B on erythrocyte membrane integrity. Arithmetic means ± SEM (n= 4) of the percentage of haemolysed erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) amphotericin B at the indicated concentrations.
Fig. 3. 6: Effect of amphotericin B and ionomycin cytosolic fluo-3 fluorescence intensity in non-infected erythrocytes. Mean $\text{Ca}^{2+}$-sensitive fluo-3 fluorescence (arithmetic means ± SE; $n = 6$) of erythrocytes incubated either in Ringer solution (Control) or in Ringer solution containing amphotericin B (1 µM) or ionomycin (1 µM). * Indicates significant difference ($p < 0.05$) from control.
Fig. 3. Effect of amphotericin B on cytosolic Ca\textsuperscript{2+} concentration in non-infected erythrocytes. Arithmetic means ± SEM (n = 6) of the geo. means of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) different concentrations of amphotericin B. ** (p<0.01) indicates significant difference from the respective value in the absence of amphotericin B.
Fig. 3. A & B: Annexin binding of non-infected and *Plasmodium falciparum* infected erythrocytes from control and amphotericin B treated samples. A) Arithmetic means ± SEM (n = 4) of annexin binding to erythrocytes in freshly drawn blood (open bar) and 24 hour after incubation in RPMI 1640 medium containing 0.05 µM amphotericin B (closed bar) #Indicates significant difference between two groups (p ≤ 0.01). B) Arithmetic means ± SEM (n = 4) of annexin binding of infected (closed bar) and non-infected (open bar) erythrocytes before and after treatment with different concentrations of amphotericin B. *Indicates significant difference (p < 0.05) to non-infected erythrocytes.
Fig. 3. 9 A & B: Effects of amphotericin B on forward scatter of *Plasmodium falciparum* infected and noninfected erythrocytes. **A).** Arithmetic means ± SEM (n = 6) forward scatter of the early stage-infected erythrocytes (closed symbols) and non-infected (open symbols) erythrocytes as a function of the butyrate concentration. * indicates significant difference (p≤0.05; ANOVA) from absence of butyrate, # indicate significant difference (p≤0.05; ANOVA) from non-infected erythrocytes. **B).** Arithmetic means ± SEM (n = 6) forward scatter of late stage-infected erythrocytes (closed symbols) and non-infected (open symbols) erythrocytes as a function of the butyrate concentration. * indicates significant difference (p≤0.05; ANOVA) from absence of butyrate. # indicates significant difference to non-infected erythrocytes.
Fig. 3. 10: Effect of amphotericin B treatment on parasitemia of *Plasmodium berghei* infected mice: Original histograms of parasitemia-dependent Syto 16 fluorescence in untreated animals (upper panels) and animals treated from day 8 until day 20 with 1.4 mg amphotericin B intraperitoneally (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*. 
Fig. 3. 11: Effect of amphotericin B on parasitemia of *P. berghei* infected mice. Arithmetic means ± SEM (n = 6) of parasitemia in mice without treatment (open circles) or treated with amphotericin B (closed circles) as a function of days after infection with *P. berghei*. * indicates significant difference (p ≤ 0.05) from iron replete animals.
Fig. 3. 12 A & B: *In vivo* clearance of fluorescence labelled erythrocytes from circulating blood. Disappearance of CFSE labelled non infected and infected mouse erythrocytes from circulating blood following injection into the tail vein of untreated and amphotericin B (1.4 mg i.p.) treated mice. A): Arithmetic means ± SEM (n = 6) of the percentage of CFSE-labelled non-infected erythrocytes in circulating blood of untreated (open symbols) or amphotericin B (closed symbols) treated animals. * indicates significant difference (p<0.05) from untreated animals. B): Abundance of CFSE labelled untreated (open bar) or amphotericin B treated (closed bar) erythrocytes in spleen. Arithmetic means ± SEM (n=6). *indicates significant difference from the untreated animals.
Fig. 3.13: Effect of amphotericin B on survival of *P. berghei* infected mice. Arithmetic means ± SEM (n = 6) represent survival of mice without treatment (open circles) or treated with amphotericin B (closed squares) as a function of days after infection with *P. berghei*. 
**Fig. 3.14:** *In vitro* growth of *P. falciparum* in human erythrocytes as a function of butyrate concentration (arithmetic means ± SEM, n = 8). *, ** indicates significant difference (p ≤ 0.05, p ≤ 0.01) from absence of butyrate.
Fig. 3. 15: Intraerythrocytic DNA amplification of *P. falciparum* in human erythrocytes as a function of butyrate concentration (arithmetic means ± SEM, n = 6). * indicates significant difference (p≤0.05) from absence of butyrate.
Fig. 3.16: Effect of butyrate on cytosolic Ca^{2+} activity in non-infected erythrocytes. Arithmetic means ± SEM (n = 6) of the geometric means of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) different concentrations of butyrate. * (p<0.05) indicates significant difference from the respective value in the absence of butyrate.
Fig. 3.17: Effects of butyrate on phosphatidylserine exposure of infected and non-infected erythrocytes. Arithmetic means ± SEM (n = 6) of annexin V-binding of infected (closed bars) and non-infected (open bars) erythrocytes following infection of human erythrocytes with *P. falciparum* at 0 mM (left bars) and 10 mM butyrate. * indicates significant difference (p ≤ 0.05; paired ANOVA) from non-infected erythrocytes, # *Indicates significant difference (p < 0.05) from absence of butyrate.
Fig. 3. 18 A & B: Effects of butyrate on forward scatter of infected and non-infected erythrocytes. 

A): Arithmetic means ± SEM (n = 4) of forward scatter of the early stage-infected erythrocytes (closed symbols) and non-infected (open symbols) erythrocytes as a function of the butyrate concentration. *Indicates significant difference (p < 0.05) from absence of butyrate, #Indicates significant difference to non-infected erythrocytes. 

B): Arithmetic means ± SEM (n = 4) of forward scatter of late stage-infected erythrocytes (closed symbols) and non-infected (open symbols) erythrocytes as a function of the butyrate concentration. *Indicates significant difference (p < 0.05) from absence of butyrate, #Indicates significant difference to non-infected erythrocytes.
Fig. 3. 19: Parasitemia of *Plasmodium berghei* infected mice. Arithmetic means ± SEM of parasitemia in mice without treatment (open circles, n = 8) or with 10 mg/kg B. W. of gum Arabic (closed circles, n = 6) as a function of days after infection with *Plasmodium berghei*. *Indicates significant difference from the untreated animals.
Fig. 3. 20: Effect of gum Arabic treatment on survival of *Plasmodium berghei* infected mice. Survival of mice without treatment (open circles) or with 10% gum Arabic in drinking water (closed squares) as a function of days after infection with *Plasmodium berghei*. 
4. Discussion

Over the past centuries malaria, has been the major cause of morbidity and mortality among the mankind. As the time passed the pathogens coevolved with their hosts, simultaneously manipulating the hosts defence mechanisms by intruding and mimicking various host related metabolic and signalling 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 kind of controlling the diseases has lead to the development of parasitic resistance and consequent relapse of once-contained infectious diseases amidst the host. This study intended for a novel drug discovery 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 involved 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.

Malaria pathogen, *Plasmodium*, enters erythrocytes and thus escapes recognition by the immune system. The pathogen induces oxidative stress to the host erythrocyte, which triggers eryptosis, the suicidal death of erythrocytes (167, 178). Eryptosis is characterized by cell shrinkage, membrane blebbing and cell membrane phospholipids 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 subsequent infection of other erythrocytes by *Plasmodia*, hence, the premature eryptosis may protect against malaria. Accordingly, any therapeutic intervention accelerating suicidal death of infected erythrocytes has the potential to foster elimination of infected erythrocytes, delay the development of parasitemia and favourably influence the course of malaria.

Observations of this study indicate that *Plasmodium* infected erythrocytes can be targeted for induction of eryptosis *in vitro* and *in vivo*. This accelerated eryptosis of *Plasmodium*-infected erythrocytes results in rapid clearance of infected erythrocytes and confers protection against severe course of malaria. *Plasmodium* imposes oxidative stress to the host cell leading to cation channel activation and Ca$^{2+}$ entry (210, 211). Thus, infection was
expected to trigger phosphatidylserine exposure. Infection of erythrocytes with Plasmodium was indeed found to trigger eryptosis (135). If the stimulation of eryptosis remains an abated, it may proceed too fast for the pathogen to allow intraerythrocytic maturation.

4.1. Influence of amphotericin B on the course of malaria

According to the present observations, amphotericin B in different concentration exerted an inhibitory effect on intraerythrocytic P. falciparum proliferation in vitro (Fig. 3.3). Moreover, presence of amphotericin B further decreased the intraerythrocytic DNA amplification of the parasite, an effect reaching statistical significance only at the highest concentration in the experiment (Fig. 3.4). In theory, the positive effect of amphotericin B treatment could have been due to amphotericin B induced lysis of the trophozoite stage of P. falciparum (212). However, at the applied concentrations in this study, amphotericin B did not disrupt the integrity of the cell membrane and did not induce significant hemolysis (Fig. 3.5). The concentrations required to elicit eryptosis are well within the range of those reached under treatment with amphotericin (213, 214). Eryptosis clearly exceeds hemolysis and thus, the observed phosphatidylserine exposure is not secondary to hemolysis. Higher levels of hemolysis have been reported earlier following exposure of erythrocytes to amphotericin B formulations (215).

P. falciparum survival depends on the activation of several ion channels in the erythrocyte cell membrane, as they allow the uptake of nutrients, Na\(^+\) and Ca\(^{2+}\) and the disposal of waste products (172). Increases in cytosolic Ca\(^{2+}\) activity is one of the major stimulators of eryptosis, which leads to cell membrane vesiculation, stimulates cell membrane scrambling and activates the cysteine endopeptidase calpain, an enzyme degrading the cytoskeleton and thus causing cell membrane blebbing (216). Ca\(^{2+}\) may enter through non-selective cation channels (132). The molecular identity of those channels is incompletely understood but may include transient receptor potential channels (TRPC6). The cation channels are activated by osmotic shock, oxidative stress and Cl\(^-\) removal (134).

In this study to determine the effect of amphotericin B on cytosolic Ca\(^{2+}\) activity, cells were incubated in the absence or presence of amphotericin B or the Ca\(^{2+}\) ionophore
ionomycin, and fluo-3 fluorescence intensity was monitored using FACS. As illustrated in Fig. 3.6, amphotericin B indeed increased fluo-3 fluorescence intensity. As a positive control, the Ca\textsuperscript{2+} ionophore ionomycin similarly enhanced the fluo-3 fluorescence intensity. The data strongly suggest that the observed amphotericin B effect on fluo-3-loaded erythrocytes reflect an increase in cytosolic free Ca\textsuperscript{2+} concentration. Moreover, amphotericin B farther increased fluo-3 fluorescence intensity in a dose dependant manner (Fig. 3.7). These results were in line with studies by Lang et al, which demonstrated the effect of amphotericin B on cytosolic free Ca\textsuperscript{2+} (205, 208).

An increase in cytosolic Ca\textsuperscript{2+}-activity is expected to stimulate cell membrane scrambling with phosphatidylserine exposure, which could be quantified by determination of annexin V-binding. As shown in Fig. 3.8A, the percentage of phosphatidylserine-exposing erythrocytes was low in the absence of amphotericin B. Exposure of erythrocytes for 48 hours to Ringer solution containing amphotericin B (0.1 µM) was followed by a significant increase in annexin V-binding. Phosphatidylserine exposure was used as indicator of apoptosis (217). In this study amphotericin B was found to increase the percentage of annexin V binding which indicate phosphatidylserine exposure. As shown in Fig. 3.8B. Furthermore, the effect was particularly prominent in infected erythrocytes. Thus, amphotericin B preferably triggered phosphatidylserine exposure of infected cells.

Infection of human erythrocytes with P. falciparum induces a biphasic change of the host erythrocyte volume. In the early trophozoite stage infected cells loose KCl and water leading to cell shrinkage. An increase in cytosolic Ca\textsuperscript{2+} activity is further expected to induce cell shrinkage, which should be reflected by a decrease of forward scatter in FACS analysis. Amphotericin B further decreased forward scatter pointing to cell shrinkage, the effect which is more prominent in early stages P. falciparum infected erythrocytes (Fig. 3.9A & B). The present experiments confirm the ability of amphotericin B to induce eryptosis (207). The decrease in forward scatter during eryptosis is paralleled by a decrease of packed cell volume and thus indeed reflects cell shrinkage (218). It was early shown that, the decrease in forward scatter is reversed by increased extracellular K\textsuperscript{+} concentration, which dissipates the chemical driving force for K\textsuperscript{+} exit. The decrease in forward scatter is further blunted by inhibitors of Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels. Thus the decrease in forward...
scatter following entry of Ca\textsuperscript{2+} is most likely due to activation of Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels, with subsequent exit of K\textsuperscript{+}, hyperpolarization, and exit of Cl\textsuperscript{-} and H\textsubscript{2}O (144). The ability of amphotericin B to trigger scrambling of the cell membrane is shared by several amphiphiles (219).

Obviously, increasing cytosolic Ca\textsuperscript{2+} concentrations leads to erythrocyte hyperpolarization, and activation of Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels (Gardos channels). The parallel stimulation of erythrocyte scramblase and Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels by an increase of cytosolic Ca\textsuperscript{2+} activity presumably serves to circumvent hemolysis of defective erythrocytes. Leakage of the cell membrane or impairment of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is expected to result in gain of NaCl, cell swelling, and, eventually, cell lysis (220). Even in normal erythrocytes, cytosolic Cl\textsuperscript{-} activity is high, and the potential difference across the cell membrane is low. Thus the gain of Na\textsuperscript{+} and loss of K\textsuperscript{+} must be excessive to compromise volume constancy. Prior to that the leaky cell membrane increases cytosolic Ca\textsuperscript{2+} activity which activates the erythrocyte scramblase, leading to breakdown of phosphatidylserine asymmetry. The phosphatidylserine-exposing erythrocytes are detected by the macrophages and, thus, cleared from the circulation before hemolysis. The parallel activation of the Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels, hyperpolarization, and cellular loss of K\textsuperscript{+} and Cl\textsuperscript{-} counteract cell swelling and, thus, may serve to prevent premature lysis of the erythrocytes (114).

The treatment of *P. berghei* infected mice with amphotericin B significantly decreased the development of *in vivo* parasitemia (Fig. 3.10 & 3.11). The effect is presumably due to premature phosphatidylserine exposure of infected cells. *In vitro*, phosphatidylserine exposure does not lead to sequestration and clearance of erythrocytes and thus does not significantly modify the increase of parasitemia. Amphotericin B treated phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. In nucleated cells amphotericin B has been demonstrated to enhance the cation conductance in the plasma membrane (221) with subsequent Na\textsuperscript{+} entry and increase in the intracellular Ca\textsuperscript{2+} concentration (222). It has further been shown to trigger a signaling cascade involving TLR-2, Btk, PLC, PKC, c-Src and NF-kappaB (223, 224).

A next series of experiments explored, whether the enhanced phosphatidylserine exposure accelerates clearance of erythrocytes from circulating blood *in vivo*. For this purpose mice
were either left untreated or treated with 1.4 mg/Kg b. w. amphotericin B., their erythrocytes were labelled with the dye CFSE and re-injected into the tail vein. As illustrated in Fig. 3. 12 A, the clearance of erythrocytes in amphotericin B treated mice was slightly, but significantly faster than erythrocyte clearance in untreated mice. Most importantly, the decline was particularly fast in infected CFSE-labelled erythrocytes (Fig. 3. 12 A). On the other hand, the majority of cleared labelled cells were accumulated in the spleen (Fig. 3. 12 B).

Decreased in vivo parasitemia following amphotericin B treatment may be expected to affect the survival of infected animals. Similar to previous report (212), none of the untreated mice survived the infection with P. berghei (Fig. 3. 13). In contrast, 80 % of the amphotericin B treated mice were still alive 28 days after the infection illustrating that amphotericin B treatment indeed favourably modifies the course of malaria.

4. 2 Influence of butyrate and gum Arabic on the course of malaria

In the other part of this study butyrate in different concentrations inhibited the proliferation of P. falciparum in a dosage dependant manner (Fig. 3. 14). This effect is most probably due to the activity of butyrate as histone deacetylase inhibitor reported by Davie 2003.(225). Andrews et al reported the antimalarial activity of histone deacetylase inhibitors and cyto-differentiating agents in vitro and in vivo (226). Histones are nuclear core proteins act as spools around which DNA winds, they play an important role in transcriptional regulation through the continuous acetylation/deacetylation of the ε-amino group of specific lysine residues (225). Evert et al (2006) reported that butyrate alter gene expression and arrest cell proliferation by inhibition of the chromatin-remodelling activity of histone deacetylases (HDAC) (227). Interestingly, butyrate inhibitory action of HDAC activity was found to affect the expression of only 2% of genes (225). The findings of this study were in parallel with that, as butyrate exerted very mild effects on P. falciparum DNA amplification only at highest concentrations (10 mM) (Fig. 3. 15). Butyrate farther increased fluo-3 fluorescence intensity in a dose dependant manner (Fig. 3. 16). Increased fluo 3 fluorescence intensity was found to be directly proportional to cytosolic Ca²⁺ concentration (207).
On the other hand, the presence of butyrate significantly increased the percentage of annexin V binding erythrocytes. The effect was particularly prominent in infected erythrocytes (Fig. 3. 17). Thus, butyrate preferably triggered phosphatidylserine exposure of infected cells. The specific binding of annexin V to phosphatidylserine has greatly facilitated the measurement of the exposure of small amounts of this phospholipid on the outer membrane of cells. Annexin V can be labelled with a fluorescent compound; this will make it possible to use a flow cytometer to estimate the percentage of erythrocytes with phosphatidylserine exposed on their outer surface. Several lines of evidence reported by a number of investigators indicated that exposure of phosphatidylserine on the cell surface is a general feature of apoptosis and suicidal erythrocyte death (eryptosis) (228, 229). As shown earlier (179, 230), eryptosis is further stimulated by \textit{P. falciparum} infection. Intraerythrocytic \textit{Plasmodia} impose oxidative stress to the host cell, which in turn activates the Ca$^{2+}$ permeable cation channels (231). The following Ca$^{2+}$ entry leads to stimulation of cell membrane scrambling (232).

Butyrate further decreased forward scatter pointing to cell shrinkage (Fig. 3. 18), which was presumably due to increase of cytosolic Ca$^{2+}$ activity, activation of Ca$^{2+}$ sensitive K$^+$ (GARDOS) channels (144), K$^+$ exit, hyperpolarization of the erythrocyte membrane potential and Cl$^-$ exit (143). The exit of KCl is followed by osmotically obliged water and thus leads to cell shrinkage (170). The volume regulatory cation channels are not only expressed in erythrocytes but in several nucleated cells (233). As an increase of cytosolic Ca$^{2+}$ could similarly induce apoptotic cell death in nucleated cells, activation of the volume regulated cation channels could similarly participate in the triggering of apoptosis in anucleated cells exposed to an osmotic shock (234, 235).

The present study reveals a completely novel effect of gum Arabic on the course of malaria. Following infection with \textit{P. berghei} the increase of parasitemia was significantly less rapid in gum Arabic treated mice than in untreated mice (Fig. 3. 19). Moreover, gum Arabic significantly increased the survival of \textit{P. berghei} infected mice. Similar to what has been shown earlier (210), untreated mice all die from infection with \textit{P. berghei} at day 26. In contrast, 70% of the gum Arabic treated mice survived up to day 26 following infection (Fig. 3. 20). Thus, gum Arabic treatment favourably modifies the course of malaria.
The present observations provide some hints to mechanisms underlying the beneficial effect of gum Arabic treatment. It is well-known that gum Arabic is fermented by intestinal bacteria, leading to the formation of various degradation products such as short-chain fatty acids as butyrate which is important (236). In a recent study, serum butyrate concentrations were increased after treatment with gum Arabic in healthy subjects (237). Moreover, butyrate compounds have been shown to up regulate the formation of fetal haemoglobin (238), which may in turn confer some protection against a severe course of malaria. Specifically, fetal haemoglobin has been shown to delay the haemoglobin degradation and thus to impede the intraerythrocyte growth of *Plasmodium* (239).

Gum Arabic could have affected parasitemia and host survival by increasing butyrate levels, which is known to delay the intraerythrocyte growth of the parasite by its is effects as inhibitor of histone deacetylase activity, or alternatively by increasing the erythrocyte content of fetal haemoglobin, which is also known to delay the intraerythrocyte growth of the parasite (239). Gum Arabic, butyrate and/or fetal hemoglobin may affect parasitemia and host survival by accelerating the eryptosis of infected erythrocytes (240). Phosphatidylserine-exposing erythrocytes are phagocytosed and thus rapidly cleared from circulating blood (241).

One of the important results of this study is the prominent effects of amphotericin B and butyrate/or gum Arabic in increasing erythrocytes cytosolic Ca\(^{2+}\). Increased cytosolic Ca\(^{2+}\) is crucial factor for stimulation of eryptosis (175). Eryptosis is triggered by redox-sensitive, Ca\(^{2+}\) permeable, non-selective cation channels, activation of the Gardos K\(^{+}\) channel and cell shrinkage.

Infection with *P. falciparum* is expected to increase the cytosolic Ca\(^{2+}\) activity and thus to induce eryptosis as a result of non selective cation channels stimulation. Most probably, the parasite utilizes these Ca\(^{2+}\) permeable non-selective cation channels to adapt the ionic composition of the host cytosol to its needs(175). However, most of the Ca\(^{2+}\) entering the parasitized erythrocyte is either extruded across the host cell membrane or taken up by the pathogen “buffering” so that cytosolic Ca\(^{2+}\) activity remains low. Buffering of the cytosolic Ca\(^{2+}\) by the parasite probably prevents Gardos K\(^{+}\) channel activation and substantial host cell shrinkage during early parasite development. Hence, in addition to its
effect on parasite growth, the infection-induced Ca\(^{2+}\)-permeable NSC conductance mediates at least in part the reported transiently elevated free Ca\(^{2+}\) concentration in the host cytosol Ca\(^{2+}\) (127, 242). Possibly in synergy with oxidative stress, the elevated cytosolic Ca\(^{2+}\) in turn triggers breakdown of the phospholipid asymmetry of the host cell membrane at mature parasite stages (243). Phosphatidylserine scrambling occurs especially under conditions of high parasitemia, which is associated with increased oxidative stress and ATP depletion (244).

Phosphatidylserine is present only in small amounts in the outer leaflet of fresh erythrocytes (245). This is consistent with the low annexin-binding, i.e. in principle phosphatidylserine exposure, observed in non-infected control co-cultured erythrocytes in the present study. Phosphatidylserine exposure increases progressively on erythrocytes when treated with the Ca\(^{2+}\) ionophore ionomycin (122). This is consistent with the present measurements: erythrocytes treated with the Ca\(^{2+}\) ionophore ionomycin showed a high degree of phosphatidylserine exposing erythrocytes. The phosphatidylserine exposure observed in ionomycin-treated erythrocytes might be the result of the synergistic action of the two known eryptosis signaling pathways: the increase in cytosolic Ca\(^{2+}\) per se activates the scramblase and inhibits the translocase. Moreover, the Gardos effect activates the sphingomyelinase that results in ceramide formation, which further stimulates the scramblase.

Early the exposure of phosphatidylserine at the outer surface of the cell membrane is presumably followed by binding to phosphatidylserine receptors on macrophages and subsequent phagocytosis of the affected erythrocyte. The lysosomal degradation may eventually eliminate the pathogen (246). Macrophages that phagocytose ring-stage infected erythrocytes are less intoxicated by hemozoin as opposed to those phagocytose late-stage infected erythrocytes. *Plasmodium* parasites detoxify the free heme of digested hemoglobin as polymerized ß-hematin (the pigment hemozoin) in their food vacuoles. Ingestion of late-trophozoite infected erythrocytes with a high amount of hemozoin by phagocytes alters the function of macrophages, monocytes and dendritic cells (247). Moreover, hemozoin-macrophage interactions impair migration of macrophages and contribute to the cytokine-mediated pathology of malaria (248, 249).
Erythrocytes with haemoglobinopathies or G6PD-deficiency are highly prone to enter eryptosis (158) and pharmacological induction of phosphatidylserine exposure of ring stage-infected erythrocytes reportedly accelerates their clearance. Thus, manoeuvres accelerating eryptosis by administration of amphotericin B or gum Arabic may result in premature clearance of the intraerythrocytic parasite. As a matter of fact, iron deficiency, lead, chlorpromazine, inhibition of NO synthase by L-NAME, azathioprine, amiodarone, and aurothiомalate (179, 180, 200, 250, 251), 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.

**Conclusion**
In conclusion amphotericin B and gum Arabic stimulate the erythrocytic machinery responsible for the eryptosis following infection with *Plasmodium*. The acceleration of eryptosis precedes the full intraerythrocytic maturation of the pathogen, thus prevents 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.
Recommendation

1. 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. Amphotericin B and gum Arabic was found to blunts the parasitaemia and leads to a favourable course of malaria. Further studies are needed to explore the additional mechanisms behind these beneficial antimalarial activity.
References


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Appendix I

Laboratory chemicals

Acetic acid (100%, glacial) Merck Eurolab GmbH
Adenine Sigma, Deisenhofen
Agarose Carl Roth, Karlsruhe
Azure B Sigma-Aldrich, Taufkirchen
Albumax II Gibco-Invitrogen, Karlsruhe
Annexin V-FLUOS Boehringer, Mannheim
Annexin V-568 Roche, Mannheim
Amphtericin B Sigma, Deisenhofen
\( t \)-butylhydroperoxide (8 M) Sigma, Deisenhofen
CaCl\(_2\) Sigma, Deisenhofen
Cardiolipin (from bovine heart) Sigma, Munich
Citrate-Phosphate-Dextrose-Stabiliser Baxter S.A. (Fenwal), Maurepas, France
DETAPAC (C\(_{14}\)H\(_{23}\)N\(_3\)O\(_{10}\)) Sigma (Fluka), Munich
Dextrose monohydrate (D (+) glucose) Sigma, Deisenhofen
DL-Dithiothreitol (DTT) minimum 99 % Sigma, Deisenhofen
2,4 –Dichloroisocoumarin Biomol GmbH, Hamburg
Dipotassium hydrogen phosphate Merck KGaA, Darmstadt
Dimethylsulfoxid (DMSO) Sigma, Deisenhofen
Disodium hydrogen phosphate anhydrous Merck KgA, Darmstadt
EDTA Sigma, Steinheim
Ethanol Merck Eurolab, Darmstadt
Ethidium bromide (10 mg/ml) Carl Roth, Karlsruhe
Fluo-3/AM Calbiochem, Bad Soden
Gas mixture (90% N\(_2\) / 5% O\(_2\) / 5% CO\(_2\) ) Hoepfner, Reutlingen; Mast, Tuebingen
Gentamicin sulphate Gibco InVitrogen, Karlsruhe
Glutamine Gibco InVitrogen, Karlsruhe
Giemsa staining solution     Sigma, Deisenhofen
Glycerol                     Sigma-Aldrich, Taufkirchen
Gum Arabic                   Dar Savana, Sudan
HCL                           Merck Eurolab, Darmstadt
HEPES                        Sigma, Deisenhofen
HEPES/NaOH 1M solution       Sigma, Deisenhofen
Hypoxanthine                 Sigma, Deisenhofen
Ionomycin                    Sigma, Munich
Immersion oil Type A          Cargille Laboratories, Cedar Grove, NJ, USA
Inosine                      Sigma-Aldrich, Taufkirchen
Instamed RPMI 1640 with Glutamine Gibco Invitrogen, Karlsruhe
Isopropanol                  Merck Eurolab, Darmstadt
KCl                           Sigma, Deisenhofen
Mannitol                     Sigma, Deisenhofen
Methanol                     Merck KgaA, Darmstadt
Methylene blue               Sigma-Aldrich, Taufkirchen
Multi Twist Top Vials 1.7 ml  Roth, Karlsruhe
Multi Twist Top Vial Caps     Roth, Karlsruhe
NaCl                          Sigma, Deisenhofen
Na-gluconate                 Sigma, Deisenhofen
NaHCO3                        Sigma, Deisenhofen
NaOH                          Sigma Diagnostics, St Louis, MO, USA
Na-vanadate                  Sigma-Deisenhofen
Nitrogen (Gas)                Mast, Tuebingen
NMDG-Cl                       Sigma, Deisenhofen
n-octylglucopyranoside (C_{14}H_{28}O_{6}) Sigma (Fluka), Munich
PBS                           Gibco BRL, Karlsruhe
Potassium dihydrogen phosphate Merck Eurolab, Darmstadt
Propidium iodide
Sigma, Munich

SAG-Mannitol solution
Baxter S.A. (Fenwal), Maurepas, France

Sodium butyrate
Sigma, Deisenhofen

Spiroepoxide
Alexis, Gruenberg

Sphingomyelinase (50 U)
Biomol, Hamburg

1-Stearoyl-2-arachidonoyl-sn-glycerol
Alexis, Lausen, Switzerland

Syto16 (1mM in DMSO)
Invitrogen (Molecular Probes), Karlsruhe

Sorbitol
Sigma, Deisenhofen

Stock materials

6-, 12-, 24- & 96-well plates
Greiner Bio-One, Frickenhausen

Bottle-top filters for 0.125 l
Millipore, Schwalbach

Bottle-top filters for 0.25 and 0.5 l
Carl Roth, Karlsruhe

CryoTube Vials 2.0 ml
Greiner Bio-One, Frickenhausen

EDTA tubes
Monovette, Sarstedt

FACS tubes
Greiner Bio-One, Frickenhausen

Falcon tubes
Greiner Bio-One, Frickenhausen

Microcentrifuge Filters Ultrafree-MC,
NMWL 5,000 Dalton, PLCC

cellulosic membrane
Sigma, Taufkirchen

MµlTI Twist Top Vials (1.7 ml;
10 x 45 mm polypropylene)
Roth, Karlsruhe

MµlTI Twist Top Vial Caps
Roth, Karlsruhe

Syringe sterile filter (0.22 µm)
Millipore, Schwalbach

Scintillation vials
PerkinElmer (LAS), Rodgau-Juegesheim

Tissue culture flasks
Nunc, Wiesbaden

Transfer pipettes, polyethylene, extended
fine tip, large bulb, Bulb: 3 ml, sterile
Sigma, Deisenhofen

Tissue culture flasks
Greiner Bio-One, Frickenhausen
Devices

Adapter for gas dosing unit EC for 24 Pasteur pipettes (V826.612.000) VLM, Leopoldshöhe
Autoclave (with outlet air bacterial filter) Systec Labor Systemtechnik, Wettenberg
8-channel-multi-pipette Research proEppendorf, Hamburg
Bath sonicator (Transsonic 310) Elma, Singen
Biofuge pico Heraeus Kendro laboratory products, Hanau
β-scintillation counter Wallac 1409 PerkinElmer (LAS), Rodgau-Juegesheim
Concentrator 5301 Eppendorf, Hamburg
Dissolved Oxygen Meter (DO-5509) Lutron, Copersburg, PA, USA
Horizontal DMZ puller Zeitz, Augsburg
Evaporator EVA-EC1-24-S (V832.000.002) VLM, Leopoldshöhe
FACS Calibur Becton Dickinson, Heidelberg
Hamilton microliter syringe(100 µl, 250 µl) Carl Roth, Karlsruhe
Hera cell incubator 37 °C Kendro Laboratory Products, Langenselbold
Heraeus Sepatech Centrifuge Kendro laboratory products, Hanau
Light microscope Leica CM E Leica, Solms
Rotina35 centrifuge Hettich GmbH & Co KG, Tuttlingen
Safety cabinet class II (Hera Safe) Kendro Laboratory Products, Langenselbold
Scanning Spectrophotometer Thermo Electron, Bremen
Thermomixer 5436 Eppendorf, Hamburg

Media, buffers and solutions

Solutions for preparing human erythrocytes for storage

**Citrate-Phosphate-Dextrose-Stabilizer** (g/l aqua ad injectabilia)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate acide – monohydrate</td>
<td>3.27</td>
</tr>
<tr>
<td>Sodium citrate – monohydrate</td>
<td>26.30</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate – dehydrate</td>
<td>2.50</td>
</tr>
<tr>
<td>Dextrose monohydrate</td>
<td>25.50</td>
</tr>
</tbody>
</table>
Citrate-Phosphate-Dextrose-Stabilizer (70 ml) and SAG-Mannitol (SAGM) solution (110 ml) were obtained sterile in pockets connected by thin tubes, separated by a leukocyte filter, ready for a blood donation of 500 ml.

**Sterile saline solution** (in mM):

- NaCl: 150
- KCl: 5
- CaCl$_2$: 1.4
- MgCl$_2$: 1
- HEPES/NaOH pH 7.4: 10
- Glucose: 10

The saline solution was prepared according to Egee *et al.* [Egee, 2002 484/4]. The pH was adjusted to 7.4 and the osmolarity to 320 ± 5 mOsm (kg H$_2$O)$^{-1}$ before sterile filtering the saline solution through a filter unit of 0.22 µm pore size.

**Media and solutions for the maintenance of *P. falciparum* in vitro culture**

**Supplemented RPMI 1640 medium**

- HEPES/NaOH: 25 mM
- Gentamicin sulphate: 20 µg/ml
- Glutamine: 2 mM
- Hypoxanthine: 200 µM
- Albumax II™: 0.5 %

1 M sterile filtered CaCl$_2$ (0.22 µm pore size) was used for preparing human serum from plasma.
**Albumax stock solution 10 x**

(in g/l):

- Instamed RPMI 1640 with Glutamine
- NaHCO3: 10.43
- HEPES: 5.96
- D (+) glucose: 2
- Hypoxanthine: 0.2
- Albumax II™: 50
- Gentamicin sulphate: 0.01

The pH was adjusted to 7.1 - 7.4. The sterile filtered Albumax stock solution was stored in 50 ml aliquots at -20 or – 80 °C.

**Freezing solution** (in %)

- Glycerol: 28
- Sorbitol: 3
- NaCl: 0.65

The freezing solution was sterile filtered (through 0.22 µm pore size) and stored at 4 °C.

**Defreezing solution** (in %)

- NaCl: 3.5

The sterile filtered defreezing solution (through 0.22 µm pore size) was stored at room temperature (RT). The solution was warmed up to 37 °C before use.

**Solutions to analyze blood smears of *P. falciparum* infected RBCs**

- Dipotassium hydrogenphosphate: 1 M
- Potassium dihydrogenphosphate: 1 M

Potassium phosphate buffer of pH 7.2 0.1 M was prepared according to Sambrook et al. {Sambrook, 1989 1205 /id}. GIEMSA staining solution was freshly prepared from the purchased GIEMSA staining solution by dilution 1:10 with 0.1 M potassium phosphate buffer pH 7.2, filtered over a paper filter.
**Solutions for *P. falciparum in vitro* growth assays**

All solutions were sterile filtered (through 0.22 µm pore size) and added to the supplemented RPMI1640 media. Changes in media concentration resulting from the addition of solutions were tolerated up to a concentration loss of 10%. In cases where the addition of the stock solution to the RPMI medium would have diluted the supplemented RPMI medium by more than 10%, the relevant chemical was added directly to the supplemented medium, which was then sterile-filtered again.

For the test solutions the different stock solutions or chemicals were diluted with sterile distilled water to obtain the final concentrations shown below.

**Stock solutions (1 M):**

- CaCl₂
- KCl
- NaCl
- Na-glutamate
- NMDG-Cl titrated with HCl to pH 7.4
- D (+) glucose
- HEPES (titrated with TRIS (Trizma base) or with NaOH to pH 7.4)

**Synchronization solution**

- Sorbitol isosmotic (5%) 290
- HEPES / NaOH pH 7.4 10
- Glucose 5

When erythrocytes were to be used further, e.g. for western blots (membrane proteins) or for measuring $^{45}$Ca²⁺ uptake, the 5% sorbitol solution was supplemented with HEPES and glucose. This was done to prevent infected RBCs from being stressed or oxidized through glucose depletion.

**Ca²⁺- Ringer**

- NaCl 140
- KCl 5
- HEPES 10
- MgCl₂ 1
- CaCl₂ 1
Glucose  5

**Hyperosmolar Ca$^{2+}$- Ringer** (in mM)
NaCl  140
KCl   5
HEPES 10
MgCl2 1
CaCl2 1
Glucose 5
Sucrose 650

**NaCl test solution** (in mM)
NaCl  120
HEPES / TRIS (titrated with TRIS to pH 7.4) 30
KCl  5
Glucose 5
CaCl2 1.7

**Solutions for fluorescence assisted cell sorting (FACS) analysis**

**Annexin binding buffer** (in mM)
NaCl  140
HEPES / NaOH pH 7.4 10
CaCl2 5

**PBS** (in M)
NaCl  0.14
PO$_4$ Buffer pH 7.4 0.01
KCl  0.0003

Each tablet of 5 g PBS had to be dissolved in 500 ml distilled water, giving a final pH 7.45.

**Syto16** was diluted before use in a dark environment in PBS or annexin binding buffer to a final concentration of 10 nM - 1 µM.
Appendix II

Protocol (1): Preparation of human and mouse erythrocytes (Washing of RBCs)

1. Take fresh heparinised blood from the transfusion centre.
2. Transfer the heparinised blood into the 50 ml falcon tube and centrifuge it at 2600 rpm for 4 min without any break.
3. Separate the plasma from the cells in the falcon tube.
4. Now add double volume of RBC wash to the cells in the falcon tube and gently mix it.
5. Again centrifuge the mixture at 2600 rpm for 4 min.
6. Repeat 4th and 5th steps for three times…
7. Finally store the washed RBC in the 4°C for further experimental procedures.

Protocol (2) Infection of washed RBCs with *Plasmodium falciparum*

Check the parasitemia of the culture flask in the FACS machine using Syto 16

1. Take into account what percent of parasitemia is needed for experimentation.
2. Split the culture into the new sterile flasks
   I. Remove the supernatant from the old flask so that the dead RBC and the metabolic wastes of the pathogen may be cleared off.
   II. Gently tilt the flask and measure the pellet volume in the flask (M).
3. Follow the practical protocol

Calculations:

\[
N = \left( \frac{\text{Percentage parasitemia of old culture flask}}{\text{Vol of old media RBC}} \right) \times \left( \frac{\text{Vol of new media with RBC}}{\text{Required percentage parasitemia}} \right)
\]

Then,

\[
\text{amount of fresh blood to be added (FB)} = (500\mu l - 500\mu l/N)
\]

\[
\text{OB} = 500\mu l - \text{FB}
\]

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

\[
= \text{OB/500} \mu l \times M
\]
**Procedure:**

1. In the laminar flow hood, label sterile culture flasks clearly.
2. Add 25 ml RPMI complete into the labelled culture flasks.
3. Add calculated amount of fresh blood to each labelled flask.
4. Add the calculated amount of old blood from the old culture flasks into the new culture flasks.
5. Gently tilt the flasks.
6. Gas the culture flasks with 5% CO$_2$, 5% O$_2$ and 90% N$_2$ for 1 min.
7. Finally incubate the culture flasks at 37°C.

**Protocol (3): Thawing parasite and culture**

1. Take a 15 ml sterile falcon tube.
2. Adjust the water bath for 37°C.
3. Take one parasites cryotube from the liquid nitrogen and thaw at water bath for 5 min.
4. Spray the disinfectant on the thawed tube.
5. Gently uncap the tube, with the help of a pipette collect the thawed blood and add it into the 15ml falcon tube.
6. Discard the thawed tube.
7. Centrifuge the 15 ml falcon tube with thawed blood at 1800 rpm for 4 min.
8. Remove the supernatant from the falcon tube. (if there is no supernatant remove the blood till there is at most 500µl of it left in falcon tube)
9. Add equal amount of sterile 3.5% sodium chloride solution drop/sec into the falcon tube. (This kills all other cells but keeps alive the parasites.)
10. Mix gently and then centrifuge at 1800 rpm for 4 min.
11. Wash the pellet with RPMI normal twice to remove the traces of sodium chloride and maintain the tonicity of the solution.
12. Estimate the pellet and remove the supernatant, now add almost excess double the volume of RPMI normal to the volume of pellet and mix gently.
13. Centrifuge the falcon at 1800 rpm for 4 min.
14. Remove the supernatant.
15. Suspend the pellet in 3 ml of RPMI complete medium.
16. Transfer 12 ml of the RPMI complete medium into the cultured flasks.
17. Add 180µl of fresh blood to the culture flask.
18. Add the pellet to the cultured flask.
19. Gas the culture with 5% CO₂, 5% O₂ and 90% N₂ for 1 min. and keep in the BOD for incubation.

Protocol (4): Isosmotic sorbitol synchronization of *P. falciparum* infected human erythrocytes for *in vitro* growth assay or DNA amplification and double staining

Select low parasitemia culture (3-5%) for *in vitro* growth assay or a high parasitemia culture (20-30%) for DNA amplification and double staining techniques. (double staining to detect PS exposure).

1. Remove the supernatant from the selected culture flask
2. Collect the blood in the sterile 50 ml falcon tube.
3. Centrifuge the falcon tube at 2000 rpm for 4 min.
4. Remove the supernatant an estimate the volume of the pellet.
5. Add 5ml of sorbitol to 500µl of pellet. (Synchronisation) only the ring stages and non-infected RBC are left out.
6. Transfer the pellet sorbitol mixture in to 25 ml falcon tube.
7. Put on the Wave Tec machine for synchronisation up to 20 min.
8. Again centrifuge the falcons at 2000 rpm for 4 min.
9. Remove the supernatant and add 10 ml of normal RPMI-1640 medium to remove the remaining sorbitol (the tube containing only ring stages and non-infected RBC).
11. Transfer the pellet into the small eppendorf tube and wash again the ring stages and non-infected erythrocytes with normal RPMI-1640 medium and centrifuge at 2000 rpm for 2 min.
12. Remove the supernatants from the eppendorf tube and mix the pellet gently.
13. Keep the eppendorf tube in the refrigerator at 4°C.
14. Now the pellet is ready for DNA amplification and double staining.
Protocol (5) In vitro growth assay

Selection of low parasitemia culture (3-5%) for in vitro growth assay technique.

1. Remove the supernatant from the selected culture flask.
2. Collect the blood in the sterile 50 ml falcon tube.
3. Centrifuge the falcon tube at 2000 rpm for 4 min.
4. Remove the supernatant and estimate the volume of the pellet.
5. Add 5ml of sorbitol to 500µl of pellet. (Synchronisation) only the ring stages and non-infected RBC are left out.
6. Transfer the pellet sorbitol mixture in to 25 ml falcon tube.
7. Put on the Wave Tec for synchronisation up to 20 min.
8. Again centrifuge the falcons at 2000 rpm for 4 min.
9. Remove the supernatant and add 10 ml of normal RPMI-1640 to the falcon and wash the ring stage.
11. Transfer the pellet into the small eppendorf tube and wash again the erythrocytes with normal RPMI-1640 and centrifuge at 2000 rpm for 2 min.
12. Remove the supernatants in the eppendorf tube.
13. Keep the eppendorf tube in the refrigerator at 4°C.

Procedure:

1. Collect the low parasitemia blood pellet and store in the refrigerator in 4°C.
2. Label the required 12ml PP-tubes according the drug concentrations.
3. Put 2 ml of complete RPMI into each labelled PP-tubes.
4. Add respective drug concentrations in to the RPMI containing labelled PP-tubes.
5. Vortex the falcons for homogenous mixture of the drug in the RPMI.
6. Add 20 µl of low parasitemia blood pellet to the above falcons and mix gently with a pipette.
7. Take a sterile 96 well plate and label it.
8. Put 200µl of the RPMI+ high parasitemia blood pellet + drug into each well. Avoid the bubble formation in the wells.
9. Incubate for 48 hrs in the candle jar in the BOD.
10. After 48 hrs, take slowly the incubated 96 well plate and gently mix the culture in each well.
11. Take 50µl of the culture from wells of each concentration and put in labelled FACS falcon tubes.
12. Now add 200µl of PBS + EDTA with Syto-16 to each falcon tube and incubate for 20 min.
13. Analyse the sample with FACS machine.

Protocol (6): Measurement of percentage parasitemia by Syto-16
1. Preparer the required amount of BPS containing Syto 16 by the ratio of 1000:1 (the volume of BPS required for each tube is 250 µl.)
2. Label the FACS (Fluorescence Activated Cell Sorting) tubes.
3. Take small amount (~20 µl) of infected blood from the cultured flasks.
4. Add Syto-16 to PBS with Syto 16 to FACS tube containing infected blood and vortex.
5. Incubate the FACS tubes in the BOD (Biochemical Oxygen Demand) 37°C for 30 min.
6. Estimate the percent parasitemia in each cultured flasks with the help of FACS machine.

Protocol (7): Giems staining technique
1. Put a drop of sample on a clean and sterile slide with the help of transferring pipette.
2. Make a thin film smear on the slide with the help of another slide
3. Air dry the smear on the slide.
4. fix the smear in methanol for 1 min.(by sprinkling few drops on the smear)
5. Dry again the slide and immerse the slide in Giems + May Grinwald (10+90 ml) mixture for 30 min.
6. wash the slide gently under running tap water and observe under the microscope at 100X
Protocol (8): Annexin binding experiments (Determination of phosphatidylserine exposure by double staining technique)

1. Collect the high parasitemia blood pellet and store in the refrigerator in 4°C.
2. Label the required 12 ml PP-tubes according to the drug concentrations required.
3. Put 2 ml of complete RPMI into each labelled falcon.
4. Add respective drug concentrations in to the RPMI containing labelled PP-tubes.
5. Vortex the falcons for homogenous mixture of the drug in the RPMI.
6. Add 20µl of high parasitemia blood pellet to the above PP-tubes and mix gently with a pipette.
7. Take a sterile 96 well plate and label it.
8. Put 200µl of the RPMI+ high parasitemia blood pellet+ drug into each well. Avoid the bubble formation in the wells.
9. Incubate for 24 hrs in the candle jar in the BOD.
10. After 24 hrs, take slowly the incubated 96 well plate and gently mix the culture in each well.
11. Take 50µl of the culture from any of the two wells from each concentration and put in labelled eppendorf tubes.
12. Add 200µl of annexin wash buffer to each eppendorf tube and mix well.
13. Centrifuge at 2000 rpm for 5 min.
14. Remove the supernatant with help of a vacuum pump and retain the pellet.
15. Add 5µl of annexin APC to each pellet and mix.
16. Add 100 µl of annexin wash buffer with syto-16 to each eppendorf tube and incubate for 20 min in the incubator.
17. Transfer the incubated mixture into small falcons and add 100µl of annexin wash buffer to each small falcon before analysing with FACS.
18. Prepare some extra single stain samples for each syto-16 and annexin for adjusting the settings for FACS.
Protocol (9): Haemolysis test

1. Prepare 6 samples each containing 12.5 µl of pellet containing 5% of hematocrit. (total pellet required for 6 samples is 75 µl)
2. Add 200 µl of ringer to each sample in sterile eppendorf. (total ringer required is 1200 µl)
3. Incubate the samples in the incubator for 24 hrs at 37 °C.
4. After incubation gently tilt the samples and centrifuge for 5 min at 2000 rpm.
5. Collect the supernatant and transfer it into cuvettes.
6. Add 550µl of transferring reagent to each sample.
7. Prepare a standard curve using control samples.
   a. After removing the supernatant from the eppendorf add 200µl of millipore water and gently mix.
   b. Transfer it into another eppendorf and add 200 µl of millipore water.
   c. Make the required concentrations to establish a standard curve.
8. Measure the absorbance of the samples in cuvettes at 546 nm using spectrophotometer.

Protocol (10): Preparation of RPMI complete medium

Requirements:
- Glutamine (2 mM) 5 ml
- HEPES/ NaOH pH 7.4 (25 mM) 12.5 ml
- Gentamycin (20µg/ml) 0.5ml
- Albumax (0.5%) 50 ml

1. Take fresh RPMI-1640 from the cold room, falcon of 50 ml albumax 10X from refrigerator and 5 ml of glutamine from -20°C, allow to worm in the water bath to 37°C.
2. Take glutamine from the -20 °C refrigerator.
3. In the laminar flow hood, gently mix albumax and glutamine with the worm RPMI-1640.
4. Then add 12.5 ml HEPES and 0.5 ml Gentamycin
5. Mix well.
6. Store now the RPMI complete medium in 4°C for further experimental procedures.
Protocol (11): Preparation of RBCs wash solution

Requirements:

- 150 mM NaCl (54.44) 8.166 g
- 5 mM KCl (74.55) 0.3727 g
- 1.4 mM CaCl₂ (110.98) 0.15537 g
- 1 mM MgCl₂ (95.21) 0.09521 g
- 10 mM HEPES (238.30) 2.38 g
- 10 mM Glucose (198.17) 0.9817 g

1. Weigh accurately all the required chemicals and put in a sterile 1000 ml bottle. Then add millipore distilled water. (mix with magnetic stirrer to homogenise the solution)
2. Adjust the pH of the solution upto 7.4, initial pH will be up to 5.34 then add 1M NaOH to make it 7.4.
3. Then check the osmolarity.
4. Calibrate the osmolity with 290 and 1000 then check the osmolity of the RBC wash solution. It must be within 290-310. (If slightly above 310 add normal distilled water to adjust.)
5. After preparation of the homogenous solution filter it with millipore (0.22µM) using vacuum in sterile conditions.
6. Now store it in clean and dry place.