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Parasitological Evaluation of SC81458 and SC83288, a Novel Class of Drugs against *Plasmodium Falciparum*

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ABBREVIATIONS

3D	3 Dimensional
ACT	Artemisinin-based combination therapy
ADMET	absorption, distribution, metabolism, excretion and toxicity
AG	*Aktiengesellschaft*
ATP	Adenosine triphosphate
cm	centimeter
CQR	Chloroquine resistance
CRT	Chloroquine resistance transporter
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
E	Glutamic acid
Fig.	Figure
GmbH	*Gesellschaft mit beschränkter Haftung*
hrs	hour
H_2O	Water
IC_{50}	half maximal inhibitory concentration
IC_{90}	90% of maximal inhibitory concentration
IC_{99}	99% of maximal inhibitory concentration
L	Leucine
m	Milli
M	Molar or Methionine
m	Meter
MDR	Multi-drug resistance
min	Minute
MMV	Medicines for Malaria Venture
n	Nano
ND	Not determined
NMR	Nuclear magnetic resonance
NPP	New Permeation Pathway
O_2	Oxygen

^{o}C	Celcium degrees
P.	*Plasmodium*
PCT	Parasite clearance time
Pf	*Plasmodium falciparum*
pH	Potential hydrogenii
PK	Pharmacokinetics
PMR	Parasite multiplication rate
PPM	Parasite Plasma Membrane
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane
QSAR	Quantitative structure-activity relationship
RPMI	Rosewell Park Memorial Institute
RT	Room Temperature or Reverse Transcriptase
SAR	Structure-activity relationship
SBDD	Structure based drug design
sec	Second
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
v/v	volume to volume
vol	Volume
w/w	weight to volume
WHO	World Health Organization
X-ray	Roentgen ray
µ	Micro

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Summary

Drug resistance is one of the most important reasons for the increase in rates of malarial morbidity and mortality. However, even now, there is no effective anti-malarial vaccine available. Presently, development of anti-malarials acting by novel pathways is the best option to control this disease. The biphenyl urea compound, the lead compound in this study, is a new structural class of potent anti-malarial agents. We obtained two subclasses of active compounds, termed "sulfnamidomethylen" and "diarylsulfone" during the optimisation to the lead compound with computer assistance. The dose-response curves of the two subclasses of compounds indicated that they exert their anti-malarial action by different mechanisms. The curve of the diarylsulfone showed a biphasic property, while the curve of the sulfnamidomethylen is single phasic and very steep. Then we optimized the sulfnamidomethylen compound based on SAR analysis for more potential anti-malarial activity. SC81458 was the first candidate compound we obtained, which showed an IC_{50}-value of 5.5 nM, and IC_{99}-value of 15.0 nM against Dd2. Then parasitological evaluation showed that SC81458 exhibits potent *in vitro* activity against a wide range of *Plasmodium falciparum* strains, with the IC_{50}-values at the magnitude of nano molar. The study of the property of SC81458 against drug resistance selection indicated that there were no Dd2 parasites recovered within eight weeks when 10^8 parasites are exposed to SC81458 at the concentration of 100 nM continuously. The stage-specific susceptibility and growth inhibition time course study of SC81458 showed that the trophozoites and schizonts are more sensitive to SC81458, showing a 6 hrs IC_{50}-value of 36.9 nM and 53.7 nM, respectively, while the rings exhibited a 6 hrs IC_{50}-value of 5124.3 nM. SC81458 can totally kill the trophozoites within 30 minutes at a concentration of less than 1 μM, a non-toxic concentration to mammalian cells. Furthermore, the *in vivo* test in the humanized mouse model showed that SC81458 exhibits an excellent *in vivo* activity against both drug sensitive (3D7) and resistant (W2) *P. falciparum* strains. However, SC81458 showed poor bioavailability. Then SC81458 was further optimized for better bioavailability. The backup compound SC83288 was obtained, which showed better bioavailability (10%) and excellent anti-*P. falciparum* activity (slightly better than SC81458). The parasitological evaluation of SC83288 indicated that SC83288 exhibits the same anti-*Plasmodium* property as SC81458 does, which suggested that the two compounds should exert their action by the same mechanism.

Zusammenfassung

Medikamentenresistenz ist einer der wichtigsten Gründe für die erhöhte Rate an Malariaerkrankungen und -sterblichkeit. Auch heute gibt es keine effektive Impfung gegen Malaria. Zur Zeit ist die Entwicklung neuer Antimalariamedikamente die beste Möglichkeit zur Kontrolle dieser Krankheit. Die Biphenyl Urea Verbindung, die bedeutendste Verbindung dieser Studie, ist eine neue strukturelle Klasse von wirksamen Antimalariamitteln. Wir haben während der Optimierung der aktiven Verbindung mittels Computeranalyse die zwei Subklassen „Sulfonamidomethylen" und "Diarylsulfon" gefunden. Die dosisabhängigen Kurven dieser beiden Subklassen der Verbindung legen nahe, dass ihrer Antimalariaaktivität unterschiedliche Mechanismen zugrunde liegen. Die Kurve des Diarylsulfon ist zweiphasig, während die Kurve von Sulfonamidomethylen einphasig und sehr steil ist. Wir haben die Sulfonamidomethylen-Verbindung SAR-abhängig optimiert um eine höhere Antimalariaaktivität zu erreichen. SC81458 war der erste erhaltene Kandidatnenwirkstoff und zeigte IC_{50}-Werte von 5,5 nM und IC_{99}-Werte von 15,0 nM gegenüber Dd2. Parasitotlogische Evaluationen zeigten dann, dass SC81458 eine wirksame *in vivo* Aktivität mit IC_{50}-Werten im nanomolarem Bereich gegen eine große Anzahl von *Plasmodium falciparum* Stämmen aufweist. In einer Untersuchung über die Wirksamkeit von SC81458 gegen das Auftreten von Medikamentenresistenz konnte nachgewiesen werden, dass bei der kontinuierlichen Verabreichung von 100 nM SC81458 innerhalb von acht Wochen keine Dd2 Parasiten überleben. Weiterhin konnten bei der Messung der stadienspezifischen Empfindlichkeit und der zeitabhängigen Wachstumsinhibitions gezeigt werden, dass Trophozoiten und Schizonten sensitiver als Ringe gegenüber SC81458 reagieren. So weisen Trophozoiten und Schizonten nach sechs Stunden einen IC_{50}-Wert von 36,9 nM beziehungsweise 53,7 nM auf, während für Ringe ein IC_{50}-Wert von 5124,3 nM ermittelt wurde. SC81458 kann Trophozoiten innerhalb von 30 Minuten bei einer Konzentration von 1 µM, die für Säugetierzellen nicht toxisch ist, vollständig abtöten. Darüber hinaus konnte *in vivo* Tests in humanisierten Mäusemodellen nachgewiesen werden, dass SC81458 eine hervorragende *in vivo* Aktivität gegenüber medikamenten-senstiven (3D7) und –resistenten Stämmen (W2) besitzt. SC81458 weist eine schlechte Bioverfügbarkeit auf. Daher wurde SC81458 weiter optimiert, um eine bessere Bioverfügbarkeit zu erreichen. Das Backup der Verbindung SC83288 wurde verwendet und zeigte eine bessere Bioverfügbarkeit (10%) und exzellente Aktivität gegenüber Stämmen von *P. falciparum* (etwas besser als SC81458). Die parasitologische Evaluation von SC83288 hat gezeigt, dass SC83288 die gleiche antiplasmodiale Eigenschaft wie SC81458 besitzt. Das bedeutet, dass beiden Verbindungen der gleiche Wirkungsmechanismus zugrunde liegen sollte.

1 Introduction

1.1 Malaria

Malaria is an ancient and deadly infectious disease of humankind, which is caused by *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*). Nowadays, malaria is the most serious tropical disease around the world. As WHO reported in 2008, there were about 3 billion people threatened by malaria in 109 countries and territories from South America to the Indian peninsula (WHO, 2008). There were around 247 million malaria cases worldwide in 2006, of which 91% or 230 million (175-300 million) were due to *P. falciparum* (WHO, 2008). The vast majority of cases (86%) were in the African region, followed by South- East Asia (9%) and Eastern Mediterranean regions (3%). The percentage of cases due to *P. falciparum* exceeded 75% in most African countries but only in a few countries outside Africa (WHO, 2008). In 2004, malaria was one of the leading causes of death worldwide from a single infectious agent (WHO). The disease causes a serious loss of wealth at the same time as killing lives. Malaria causes poverty by impeding economic growth, and poverty promotes malaria transmission at the same time. With the efforts of many years around the whole world, the burden of malaria in some countries is now decreasing. However, malaria still presents a terrible burden to public health and malaria control measures still require significant improvement. WHO has identified four phases on the path to malaria elimination. By July 2008, the 109 countries/territories affected by malaria were classified as follows: control (82 countries/territories), pre-elimination (11 countries/territories), elimination (10 countries/territories), and the prevention of reintroduction (6 countries/territories).

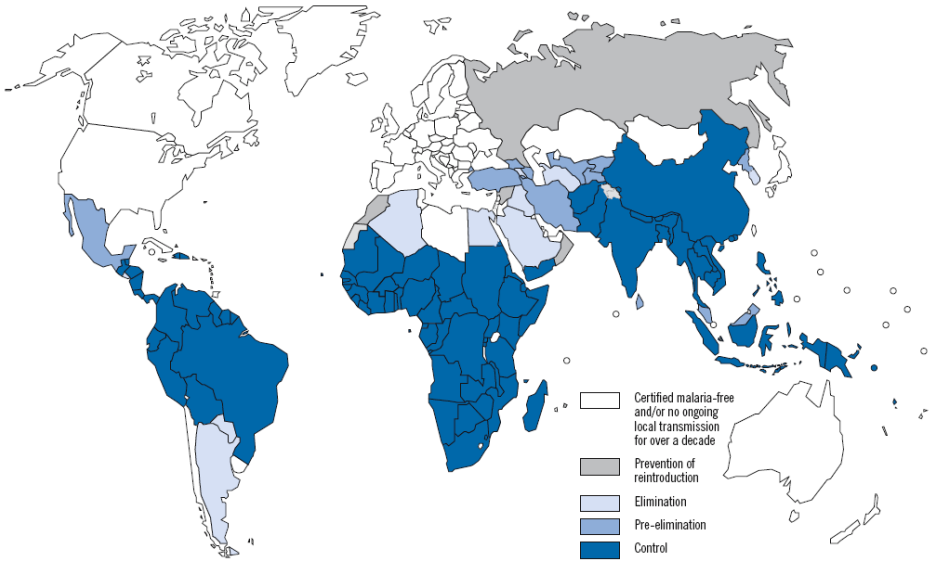


Fig 1.1 Malaria-free countries and malaria-endemic countries in phases of control, pre-elimination, elimination and prevention of reintroduction, end 2007 (WHO, 2008).

1.1.1 Life cycle

The malaria parasite exhibits an extremely complex life cycle involving a cold-blooded insect vector (mosquito) and a warm-blooded vertebrate host (human). All four *Plasmodium* species exhibit a similar life cycle with only minor variations.

During the blood meal of a malaria-infected female *Anopheles* mosquito, sporozoites from its salivary glands are injected into the human host along with anticoagulant-containing saliva. *Plasmodium* is extremely well-adapted to its hosts. In human, a malaria infection can be established by a small number of sporozoites (15-40 on average) (Frischknecht et al., 2004; Rosenberg et al., 1990). Malaria in humans develops via two phases: an exoerythrocytic (hepatic) and an erythrocytic phase. Sporozoites move away with the blood stream from the site of injection to the liver, and then infect liver cells. In hepatocytes, each sporozoite undergoes extensive replication within a parasite-derived vacuole, essentially walling off the parasite from the liver cell cytoplasm. When sporozoites mature into schizonts, the infected liver cells rupture and release merozoites. Merosomes appear to play a role in this step to ensure safe release of the merozoites directly into the blood circulation (Sturm et al., 2006). The time taken to complete this phase is called the prepatient period and varies between different *plasmodium* species. (*P. falciparum* 8-25 days, *P. vivax* 8-27 days, *P. ovale* 9-17 days and *P. malariae* 15-30 days). In the case of *P. vivax* and *P. ovale*, some of the sporozoites

entering hepatocytes do not develop into exo-erythrocytic schizonts directly, but instead form hypnozoites. These small parasite forms (4-5 µm in diameter) can remain dormant in the liver for years. At a given point in time, although the triggering signal is still unknown, the hypnozoites develop into exo-erythrocytic schizonts, producing thousands of merozoites, thereby causing relapses of the disease (Cogswell, 1992).

Merozoites invade the erythrocytes within minutes after their release and start a second round of asexual replication. The process of erythrocytes invasion by the parasite includes a series of well-defined and rapid events at the red blood cell (RBC) surface. First, the merozoite interacts with the RBC surface and reorients the apical region towards the membrane. The contents of the apical organelles (rhoptries and micronemes) are expelled and a moving junction is formed between the merozoite and RBC membrane. Finally, the parasite enters a vacuole formed by invagination of the RBC membrane and membranes secreted from the parasite. This process is completed in a few minutes. However, a huge membranes system is synthesized during the short process, which is thought as a potential anti-malarial drug target (Ancelin et al., 2003). Three organelles are present on the invasive (apical) end of the parasite (rhoptries, micronemes and dense granules). Receptors that mediate invasion of RBCs by merozoites and invasion of liver by sporozoites are localized in micronemes, on the cell surface, and in rhoptries. Accumulating evidence suggests that these molecules are the major targets of neutralizing immune responses (Mahanty et al., 2003). The erythrocyte invasion requires a series of highly specific interactions and it is considered to be a very attractive target for drug development. Inside the erythrocyte, the parasite develops through different stages (Bannister et al., 2000). *P. falciparum* differs from other human malarial species in that parasitized erythrocytes do not remain in the circulating blood for their entire life cycle. After 24-32 hours, when young parasites mature from the ring to the trophozoite stage, parasitized erythrocytes adhere to endothelial cells in the microcirculation of various organs (termed sequestration). Trophozoites mature into schizonts, which eventually rupture and release 16-32 daughter merozoites that invade fresh erythrocytes to perpetuate the asexual life cycle (Baruch, 1999; Chen et al., 2000; Newbold et al., 1999). The release of merozoites coincides with the sharp increase in body temperature during the progression of the disease. This procedure occurs quite synchronously and merozoites are released approximately the same time of the day which explains the periodicity of the fever paroxysms experienced by the patient (Tuteja, 2007). *P. malariae* exhibits a 72 hrs periodicity, whereas the other three species exhibit 48 hrs cycles. As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes. The gametocytes are large parasites which fill up the

erythrocyte, but only contain one nucleus. Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and escape from the host erythrocyte. Factors which participate in the induction of gametogenesis include: a drop in temperature, an increase in carbon dioxide, and mosquito metabolites. Microgametes, formed by a process known as exflagellation, are flagellated forms which will fertilize the macrogamete leading to a zygote. The zygote develops into a motile ookinete which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the hemocoel (i.e., body cavity) of the mosquito. The sporozoites migrate to and invade the salivary glands, where the sporozoites are injected to a new host during blood feeding. The infective bite of the mosquito will start a new life cycle (Tuteja, 2007).

1.1.2 Clinical manifestations

The patients suffering from malaria showed a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and even death. Actually, malaria is a curable disease if diagnosed and treated promptly and correctly, even for severe malaria. The clinical manifestation with the development of malaria consists of a cold stage (sensation of cold, shivering), a hot stage (fever, headaches, vomiting; seizures in young children) and finally a sweating stage (sweats, return to normal temperature, tiredness). The blood stage is responsible for the pathology associated with malaria. The intermittent fever paroxyms are due to the synchronous lysis of the infected erythrocytes. *P. malariae* exhibits a 72 hrs periodicity, whereas *P. falciparum*, *P. vivax*, and *P. ovale* exhibit 48 hrs cycles. Severe malaria occurs when *P. falciparum* infection is complicated by serious organ failures or abnormalities in the patient's blood or metabolism. Seizures, hypoglycemia, and severe anemia are common manifestations of severe malaria in children, whereas jaundice, pulmonary edema, and acute renal failure are more common in adults. The mortality despite treatment rises to 15–20%. Cytoadherence of infected erythrocytes to the brain microvasculature, mediated by the members of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family (Baruch et al., 1995) is thought to contribute to cerebral malaria and coma (Kirchgatter and Del Portillo, 2005; Warrell et al., 1990). Meanwhile, lactic acidosis can also be a cause (English et al., 1996). Lowered oxygen delivery caused by severe anemia and impaired tissue perfusion are thought to be responsible for respiratory distress (Marsh and Snow, 1997). The clinical

outcome of malaria is complex and depends on many host and parasite factors, which makes a better understanding of the disease itself and the biology of the parasite imperative.

1.2 Anti-malarial drugs

1.2.1 Anti-malarial drugs in clinical use

Currently used anti-malarials stem from seven drug classes: 4-Aminoquinolines, arylaminoalcohols, 8-aminoquinolines, artemisinins, antifolates, inhibitors of the respiratory chain, and antibiotics (Schlitzer, 2008). Although continued attempts to develop a vaccine for malaria are ongoing, drugs continue to be the only treatment option today.

1.2.1.1 Arylaminoalcohols

This class of anti-malarial drugs includes quinine, mefloquine, halofantrine and lumefantrine. The mechanism of action of them seems to be different from that of 4-aminoquinolines. However, it still remains not clear. Arylaminoalcohols seem to interfere with the heme digestion. As reported, the amplification of *pf*mdr1 gene contributes to arylaminoalcohol resistance, which codes for a transport protein (*P. falciparum* multidrug resistance 1, *Pf*MDR1) located in the membrane of the digestive vacuole.

1.2.1.1.1 Quinine

Quinine is the first known effective anti-malarial agent, which is the active ingredient of *Cinchona bark*, a tree found in South America. Quinine acts as a blood schizonticide although it also has gametocytocidal activity against *P. vivax* and *P. malariae*. Because it is a weak base, quinine is concentrated in the food vacuoles of parasites. The action mechanism of quinine still not clear. It is said to act by inhibiting heme polymerase, thereby allowing accumulation of its cytotoxic substrate, heme. However, the research of Chou and Fitch indicated that quinine showed no inhibition activity against heme polymerase (Chou and Fitch, 1993). Quinine is less effective and more toxic than chloroquine. The typical syndrome of quinine side effects is called cinchonism, which consists of ringing in the ears, headache, nausea and disturbed vision. Tinnitus, decreased auditory acuity and vertigo are caused by quinine through functional impairment of the eighth nerve. Visual symptoms include blurred vision, disturbed colour perception, photophobia, diplopia, night blindness, and rarely, even blindness. Gastrointestinal symptoms consist of nausea, vomiting, abdominal pain and diarrhoea. Rashes, sweating,

angioedema may be seen. Excitement, confusion, delirium are also seen in some patients. Coma, respiratory arrest, hypotension, and death can occur with over dosage. Quinine can also cause renal failure. Massive hemolysis and hemoglobinuria can occur, especially in pregnancy or on repeated use. Hypoprothrombinemia, agranulocytosis are also reported. Quinine has little effect on the heart in therapeutic doses and hence regular cardiac monitoring is not needed. However it can cause hypotension in the event of overdose. Quinine reduces the excitability of the motor end plate and thus antagonises the actions of physostigmine. It can cause respiratory distress and dysphagia in patients of myasthenia gravis. Quinine stimulates insulin secretion and in therapeutic doses it can cause hypoglycemia. This can be more severe in patients with severe infection and in pregnancy. Hypoglycemia in malaria may go unnoticed and could even cause death. Therefore, it is advisable to monitor blood glucose levels at least once in 4-6 hrs while quinine is administered, especially in severe infection and in pregnancy. Hypersensitivity in the form of rashes, angioedema, visual and auditory symptoms is indications for stopping the treatment. It is contraindicated in patients with tinnitus and optic neuritis. It should be used with caution in patients with atrial fibrillation. Hemolysis is indication for immediately stopping the drug. It is also contraindicated in patients suffering from myasthenia gravis. Quinine still is used in the treatment of severe *falciparum* malaria in areas with known resistance to chloroquine.

1.2.1.1.2 Mefloquine

Mefloquine was developed as a novel anti-malarial drug against multidrug resistant *falciparum* (Trenholme et al., 1975) , which was born during the Vietnam war to protect the American soldiers from malaria (Horton, 1988). Mefloquine has been found to produce swelling of the *P. falciparum* food vacuoles. It was believed to act by forming toxic complexes with free heme that damage membranes and interact with other plasmodial components. It showed an activity against the blood forms of *falciparum* malaria, including the chloroquine resistant types. The side effects of mefloquine consist of nausea, vomiting, abdominal pain and dizziness. Less frequently it can cause nightmares, sleeping disturbances, dizziness, ataxia, sinus bradycardia, sinus arrhythmia, postural hypotension, and an 'acute brain syndrome' consisting of fatigue, asthenia, seizures and psychosis. Drug resistance to mefloquine was developed very rapidly by the *falciparum* parasites (Horton, 1988; Ridley, 2002).

1.2.1.1.3 Halofantrine

Halofantrine was developed in the 1960s by the Walter Reed Army Institute of Research (Horton, 1988). More than 250 000 compounds were tested in this project. Two important compounds emerged after the screen – mefloquine and halofantrine. Both of them are structurally related to quinine. Its mechanism of action still is not known, which may be different from quinine, and mefloquine (Horton, 1988). This synthetic anti-malarial is effective against multi drug resistant (including mefloquine resistant) *P. falciparum* malaria. As reported, halofantrine could cause cardiotoxicity (Monlun et al., 1995).

1.2.1.1.4 Lumefantrine

Lumefantrine, discovered in the Peoples' Republic of China, is an arylamino alcohol in the same general group as mefloquine and halofantrine. It is active against all human malaria parasites, which has been used clinically in China for several years. Even the multidrug resistant *P. falciparum* strains also are sensitive to lumefantrine. Lumefantrine usually is used combinated with artemether. This combination is currently used under the brand name Riametm or Co- Artem™, which is the first fixed combination of an artemisinin derivative and a second unrelated anti-malarial compound. The anti-malarial activity of lumefantrine is lower than halofantrine. However, lumefantrine do not cause any cardiac side effect as halofantrine does. It is a highly lipophilic drug. The bioavailability of lumefantrine critically depends on coadministration with fats.

1.2.1.2 4-Aminoquinolines

4-Aminoquinolines anti-malarial drugs include chloroquineand amodiaquine, which have been used for clinical malaria therapy more than 60 years. Resistance against 4-aminoquinolines results from a mutation of K76T in the gene of a transport protein (chloroquine resistance transporter, CRT) located in the membrane of the digestive vacuole. It pumps 4-aminoquinolines outside from the digestive vacuole and reduce the concentration of drugs inside the digestive vacuole.

1.2.1.2.1 Chloroquine

Chloroquine is the prototype anti-malarial drug with a long clinical use history. It was used as an anti-malarial drug for decades in all malaria-endemic areas and is still used as a first-line treatment in central America and parts of Africa for *P. falciparum* malaria. Even now, chloroquine is still the anti-malarial agent that is cheapest, time tested, easiest to administer and has fewest side effects. It showed a very significant effect against erythrocytic forms of *P.*

vivax, *P. ovale* and *P. malaria*e, sensitive strains of *P. falciparum* and gametocytes of *P. vivax*. It was widely used to treat all types of malarial infections. The most spectacular characteristic of chloroquine is its capacity to concentrate itself from nanomolar levels outside the parasite to millimolar levels in the digestive vacuole of the intraerythrocytic trophozoite. However, the action mechanism of chloroquine still is not clear even now. The most common idea on this is that chloroquine could accumulate within the food vacuoles of the parasite, where the haemoglobin is digested into its component peptides. The peptides are exported from the DV and leave behind ferriprotoporphyrin IX (FPIX), also known as haem, which is potentially toxic to the parasite. The toxicity of hame is circumvented by incorporation of FPIX dimers into an inert, crystalline substance, hemozoin. Chloroquine is thought to form complexes with toxic hame moietie and interfere with the detoxification mechanism. (Ginsburg et al., 1999; Pagola et al., 2000; Sullivan et al., 1998). The side effects of chloroquine include dizziness, headache, diplopia, disturbed visual accomodation, dysphagia, nausea, malaise, pruritus of palms, visual hallucinations, confusion, and occasionally frank psychosis. These side effects do not warrant stoppage of treatment. Chloroquine resistance (CQR) is a important reason for the wide spread of malaria, which was first reported in Southeast Asia and South America (Payne, 1987; Ridley, 2002). In the late 1950's, resistance to chloroquine was noted on the Thai-Cambodian border and in Colombia. All endemic areas in South America were affected by 1980 and almost all in Asia and Oceania by 1989. In Africa, chloroquine resistance was first documented in the east in 1978. Resistance spread to the central and southern parts of the continent before arriving in west Africa in 1983. By 1989, chloroquine resistance was widespread in sub-Saharan Africa (Wongsrichanalai et al., 2002). Sulfadoxine-pyrimethamine or a combination of chloroquine and sulfadoxine-pyrimethamine were used as first-line anti-malarial drug in chloroquine resistance region for many years (Wongsrichanalai et al., 2002). As reported recently, the chloroquine resistance mutation in the parasite had nearly disappeared after the stop of the use of chloroquine in this region, and studies of adults hinted that chloroquine could clear the parasite again (Vogel, 2006).

1.2.1.2.2 Amodiaquine

Amodiaquine is structurally related to chloroquine and has been widely used as anti-malarial drug around for 60 years. In the mid-1990s, amodiaquine was pushed by WHO in the treatment of malaria. As reported, amodiaquine showed good safety and efficacy in children with uncomplicated malaria when it is used alone or in combination with artesunate or with sulfadoxine-pyrimethamine (D'Alessandro and ter Kuile, 2006). Amodiaquine is a low cost

anti-malarial drug, which means it could be widely used in poor countries. Amodiaquine use has been limited since the mid 1980s after it was linked causally to the occurrence of occasional agranulocytosis in adult travellers taking the drug prophylactically. But because amodiaquine retains a high degree of efficacy against all but the most highly chloroquine-resistant strains, there has been a recent increase in its use. Actually, artesunate-amodiaquine is used as first-line treatment for children with uncomplicated malaria in some African countries. Amodiaquine can cause annoying pruritus, agranulocytosis and liver damage.

1.2.1.3 8-Aminoquinolines

1.2.1.3.1 Primaquine

Primaquine, the only 8-aminoquinoline currently in use, is a highly effective anti-malarial agent, which showed activity against the gametocytes of all plasmodia specially. Thereby, it can prevent spread of the disease to the mosquito from the patient. Primaquine is also effective against the dormant tissue forms of *P. vivax* and *P. ovale* malaria. Until now, primaquine is still the only anti-malarial drug licensed for the radical cure (or antirelapse therapy) of *P. vivax* infections. Primaquine showed an excellent activity against the asexual blood forms of the parasite and therefore it is always used in combination with a blood schizonticide. Furthermore, primaquine has shown to be effective in the chemoprophylaxis of *P. falciparum* infections. The action mechanism of primaquine is not well understood. It may exert its anti malaria action by generating reactive oxygen species (Thornalley et al., 1983) or by disrupting intracellular transport processes such as secretion and receptor recycling (Hiebsch et al., 1991). Primaquine may cause occasional epigastric distress and abdominal cramps at larger dose, which can be minimised by taking the drug with a meal. In some cases, mild anemia, cyanosis and methemoglobinemia may also be seen. Severe methemoglobinemia can occur rarely in patients with deficiency of NADH methemoglobin reductase. Granulocytopenia and agranulocytosis are rare complications. Patients with deficiency of Glucose 6-phosphate dehydrogenase will develop hemolytic anemia on taking usual doses of primaquine (Alving et al., 1956). This problem is restricted to certain sections of the population. The drug should be stopped when signs of hemolysis and anemia are observed.

1.2.1.4 Artemisinines

Li Shizhen, a great Chinese herbalist of 400 years ago, wrote in his *Ben Cao Gang Mu* that chills and fever of malaria can be combated by qing hao (Artemisia annua shrub) preparations, which has been used for centuries in traditional Chinese medicine to cure fevers. Artemisinin was first isolated in 1971 by Chinese chemists. Artemisinin is called as Qing Hao Su in Chinese, which means the active element of qin hao. Artemisinin and semisynthetic derivatives, including artesunate, artemether and dihydroartemisinin, are short-acting anti-malarial agents that kill parasites more rapidly than conventional anti-malarials, and are active against both the sexual and asexual stages of the parasite cycle. Furthermore, artemisinins kill all species of *plasmodium* that infect humans. Artemisinin fever clearance time is shortened to 32 hrs as compared with 2-3 days with older agents (German and Aweeka, 2008). Artemisinin derivatives are the most important new class of anti-malarials and are in widespread use. Artemisinin is chemically unrelated to existing anti-malarial agents and thus no traditional drug resistant parasite strain cross-resistant to artemisinin. However, the *in vivo* half lives of theses drugs are very short. Recrudescence of parasites is seen in patients undergoing short course treatment, unless it is combined with another long acting drug, such as mefloquine. The action mechanism of artemisinin still is not clear. There many different hypothesises on it. As reported, artemisinin may exert its effect by interfering with the plasmodial hemoglobin catabolic pathway and inhibition of heme polymerization (Pandey et al., 1999). Another study indicated that the activity of artemisinin might be propagated by interactions between the endoperoxide bridge of the drug and heme-iron, produced during hemoglobin degradation inside the DV. This iron has been proposed to generate free radicals that alkylate and oxidize proteins and lipids within infected RBCs (Kannan et al., 2005). This hypothesis is consistent with the finding that artemisinin activity can be potentiated by oxygen and oxidizing agents and attenuated by reducing agents. However, the study on the stage activity of artemisinin indicated that artemisinins are effective against ring-stage parasites, which do not seem to have high concentrations of heme. One proposed that artemisinins are activated via reductive cleavage of the peroxide bond by intracellular iron–sulfur redox centers, which are common to *Plasmodium* enzymes, and that alkylation of these enzymes could result in parasite death. The binding analysis between parasite lysates and radiolabeled artemisinin identified several interacting proteins, indicating that parasite death might result from the alkylation and inactivation of parasite proteins. The potential protein target of artemisinins include cysteine proteases, proteins of the electron transport chain, translationally controlled tumor protein and

*Pf*ATP6, a SERCA-type Ca^{2+}ATPase. *Pf*ATP6 has received the most attention because its activity in transfected *X. laevis* oocytes is abolished by artemisinin but is unaffected by other anti-malarials. As further reported, an L263E point mutation in *Pf*ATP6 can abolish inhibition by artemisinins. The study on allelic exchange indicated that L263E mutants exhibit a higher IC_{50}-value than the wild-type control. However, additional work indicated that artemisinin derivatives cause early disruption of the parasite DV and do not seem to affect the structure of the endoplasmic reticulum, where *Pf*ATP6 is presumably located. With the work on the modification of artemisinin, a series of artemisinin derivatives showed excellent anti-malarial activity and better ADMET properties. There were few side effects in experimental animals when some derivatives were used at a very high dose, which include neurotoxicity and electro-cardiographic and haematological abnormalities (Brewer et al., 1994). Artemisinin-based combination therapies (ACTs) are a new generation of anti-malarial drugs, which have been used for around a decade.

1.2.1.5 Antifolates

1.2.1.5.1 Chloroguanide (Proguanil)

Chlorproguanil is a biguanide prodrug that is metabolized to chlorcycloguanil, an specific inhibitor of the *P. falciparum* DHFR, by which it exerts its anti-malarial action (Hastings and Sibley, 2002).This drug was developed by British anti-malarial research in 1945. It has causal prophylactic and suppressive activity against *P. falciparum* and cures the acute infection. It is also effective in suppressing the clinical attacks of *vivax* malaria. However it is slower compared to 4-aminoquinolines. At the prophylactic doses, it produces occasional nausea and diarrhoea.

1.2.1.5.2 Dapsone

Dapsone is a sulfa drug, killing malarial parasites by inhibiting the enzyme dihydropteroate synthase (DHPS) (Triglia et al., 1997) with an elimination half-life of about 30 hrs. It has been combined with chlorproguanil (which has an elimination half-life of about 20 hrs). Dapsone can cause methaemoglobinaemia and haemolysis in individuals with glucose-6-phosphate dehydrogenase deficiency. This limited the use of dapsone in the treatment of malaria.

1.2.1.5.3 Pyrimethamine and Sulphadoxine

Both of the two drugs are antifolate anti-malarial drugs. They will be discussed in the "combination therapy" section later.

1.2.1.6 Antibiotics

1.2.1.6.1 Tetracyclines

Tetracyclines are a group of effective anti-malarials, which act relatively slowly. They are always used in combination with a faster acting drug like quine to treat malaria and for anti-malarial chemoprophylaxis (Dahl et al., 2006). One of the first antibiotics to come into use in human beings, these drugs have stood the test of time and are continuing to be useful in treating a broad range of infections, including malaria. Tetracyclines are bacteriostatic agents, supposedly acting by inhibiting protein synthesis by binding to the 30 S ribosome subunit. The anti-malarial mechanism of action of tetracyclines remains undefined. They maybe exert their anti-malarial action by inhibiting the production of proteins encoded by the apicoplast genome, leading to a subsequent loss of apicoplast function. So tetracyclines exhibit a delayed but potent anti-malarial effect, especially in the treatment of drug-resistant *P. falciparum* malaria. (Dahl et al., 2006). The adverse effects mainly include gastrointestinal irritation, nausea, vomiting, diarrhoea, photosensitivity, hepato-toxicity, aggravation of uremia, hypersensitivity reactions, staining of the teeth if used in young children and pregnant women etc. They are contraindicated in children below the age of 8 years and in pregnant women because of their adverse effects on bones and teeth.

1.2.1.7 Inhibitors of the respiratory chain

1.2.1.7.1 Atovaquone

As a synthetic hydroxynaphthoquinone developed in the early 1980s, atovaquone showed potential activity against *Plasmodium* (as well as *Toxoplasma* and *Pneumocystis carinii*). It is a structural analogue of coenzyme Q and exerts its anti-malarial action by collapsing the organellar membrane potential, thus arresting parasite respiration and essential pyrimidine biosynthesis. This effect of atovaquone was caused by its inhibition activity against cytochrome *b* in complex III of the chain. The human mitochondria employ a CoQ10 complex (i.e. one carrying 10 isoprene units on the aromatic ring), which differs from the CoQ8 type found in the parasite. So atovaquone can kill *Plasmodium* parasites *in vivo* without affecting human mitochondria. However, cytochrome *bc*1 mitochondrial complex, the target of

atovaquone, is subject to a high frequency of pre-existing mutations, which means atovaquone is very easy to be resistant by parasites through mutation in the target gene. As reported, one in three patients treated with atovaquone monotherapy in Thailand already carried parasites resistant to the drug (Vaidya, 2001). Atovaquone may cause rash, fever, vomiting, diarrhoea and head ache. Safety in pregnancy, lactation, children, and elderly is yet to be established.

1.2.2 Anti-malarial drugs combination in clinical use

Combination therapy with anti-malarial drugs is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and different biochemical targets in the parasite (WHO, 2006). Combination chemotherapy showed excellent efficacy in the treatment of tuberculosis, HIV disease and cancer. The reasons for the combination of anti-malarial drugs mainly lie in three aspects. Firstly, drug combination can decrease the risk of multidrug resistant parasites arising during therapy, which are the biggest therapeutic challenge to health care in most malaria-endemic areas. Multidrug resistance has been reported from most parts of the world and traditional monotherapy is either ineffective or less effective. Therefore, rational anti-malarial combination chemotherapy is widely advocated. Theoretically, if drug resistance develops by spontaneous point mutation or gene amplification, the frequency of resistance to both of the two drugs with different modes of action should be a product of the two mutation frequencies. As the drug resistance frequencies very low in malaria parasites, it seems unlikely that a parasite could develop spontaneously resistant to two unrelated drugs. Secondly, drug combination can increase anti-malarial efficacy. Thirdly, drug combination can shorten duration of treatment, hence increasing compliance. Furthermore, the pharmacokinetic properties of the combinated drugs should also match well. Otherwise, the anti-malarial efficacy of combination drugs would not be increased significantly, even serious advers effect occurred. The successful ACTs are as follows:

1.2.2.1 Artemisinin-based combination therapies (ACTs)

Artemisinin-based combination therapies (ACTs) are the new generation of anti-malarial drugs, which have been used around for a decade. Artemisinin showed an excellent activity against malarial parasites and can kill the parasites *in vivo* rapidly. However, the *in vivo* half life of artemisinin is really short, which means the more risk of parasite recrudescence. So artemisinins are combined with one of several longer-acting drugs, such as amodiaquine, mefloquine, sulfadoxine/pyrimethamine and lumefantrine, which can eliminate the residual

malarial parasites (German and Aweeka, 2008). Artemisinin-based combination therapies are currently recommended by WHO for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2008). The clinical use of ACTs showed that they can improve cure rates, reduce the development of resistance and they might decrease transmission of drug-resistant parasites. The mainly used ACTs are as follows:

1.2.2.1.1 Artemether-Lumefantrine (Coartem™)

Lumefantrine is structurally similar to mefloquine and halofantrine, and no neurotoxicity and cardiotoxicity was seen during animal toxicology studies in preclinical development. Coartem is now registered widely as an approved anti-malarial drug, which is pushed by WHO in tropical Africa. The rapid clinical parasite clearance and the theoretically excellent property against drug resistance selection make this combination an attractive treatment for malaria, despite its cost. It is the only fixed-dose artemisinin-containing formulation registered after internationally recognised guidelines. It seemed safe and well tolerated in children as well as in adults. There is considerable mismatch in elimination half-lives of artemether and lumefantrine. Efficacy of the six-dose regimen is adequate, which has given satisfactory cure rates, but inferior to the combination of artesunate-mefloquine in Thailand.

1.2.2.1.2 Artesunate-mefloquine

A 3-day regimen of artesunate-mefloquine has been the preferred treatment for malaria in Thailand. It is safe, well tolerated and highly effective. In Thailand, malaria incidence and mefloquine resistance have decreased since this combination has come into use. Disadvantages of this regimen include its price and the pharmacokinetic mismatch.

1.2.2.1.3 Artesunate-Amodiaquine

It showed a better efficacy than amodiaquine alone and is well tolerated. However, the pharmacokinetic properties of the two drugs do not match well. WHO recommends this regimen for treatment of uncomplicated *falciparum* malaria in African children. Indeed, in some African countries, artesunate-amodiaquine is considered as first-line treatment for children with uncomplicated malaria.

1.2.2.1.4 Dihydroartemisinin–piperaquine (Artekin™)

Piperaquine, a bisquinoline anti-malarial drug, is developed by the group from China and France in the 1960s. The combination of dihydroartemisinin-piperaquine was developed as

Artekin™ to provide a cheap and reasonably well-tolerated, short-course treatment for *P. falciparum*, which showed a high cure rate against drug-resistant parasites. It has been widely used in controlled clinical trials in South-East Asia, with excellent clinical efficacy against chloroquine-resistant parasites. However, there is considerable mismatch in elimination half-lives of piperaquine (23 days) and dihydroartemisinin (1 hrs), which might develop resistance rapidly.

Theoretically, artemisinin-containing combinations might not only improve cure rates but also delay the development of resistance. Furthermore, artemisinin-containing combinations might also decrease the transmission of drug-resistant parasites by reducing the number of gametocytes carried by patients. Based on available safety and efficacy, ACTs should be pushed as active anti-malarial drugs. Widespread use of these drugs could roll back malaria. However, the cost of ACTs treatment is more expensive than conventional anti-malarial drugs, which may be a hindrance in poor communities.

1.2.2.2 Atovaquone-Proguanil (Malarone™)

Atovaquone acts by inhibiting the parasite's mitochondrial complex *bc*1 and disrupting the membrane potential. Because of the high frequency of resistant mutants associated with atovaquone monotherapy, it was combined with proguanil (a biguanide acting on DHFR) based on promising results of *in vitro* experiments, which showed synergistic activity (Canfield et al., 1995). This combination, registrated as Malarone™, is approved for both treatment and prophylaxis of malaria. It showed high efficacy in the treatment of uncomplicated malaria caused by *Plasmodium falciparum*, even by chloroquine-resistant or multidrug-resistant strains. As atovaquone is fat-soluble, the daily dose should be taken with food or milk.

1.2.2.3 Pyrimethamine-Sulphadoxine (Fansidar™)

Fansidar is a very useful adjunct in the treatment of uncomplicated, chloroquine resistant, *P. falciparum* malaria. Pyrimethamine exerts its killing action by the inhibition to dihydrofolate reductase of malaria parasites and thereby blocks the biosynthesis of purines and pyrimidines, which are essential for DNA synthesis and cell multiplication. Then the parasites were killed in schizont stage in erythrocytes and liver. It has been used as a prophylactic agent against malaria since 50 years ago. Sulfadoxine inhibits the utilisation of para-aminobenzoic acid in the synthesis of dihydropteroic acid. The combination of pyrimethamine and sulfadoxine offers two step synergistic blockade of plasmodial division. Other than the action mechanism, the

ADMET properties of the two components also match well. Pyrimethamine has good oral bioavailability. It's slowly but completely absorbed after oral administration and is eliminated slowly, with a plasma half-life of 80-95 hours. Sulfadoxine is rapidly absorbed from the gut and is bound to plasma proteins. This drug is metabolised in the liver and is excreted in the urine. Sulfadoxine is a long acting sulfonamide with a half-life of 7-9 days. After the emergence of chloroquine resistance, sulfadoxine-pyrimethamine was the most commonly used anti-malarial drug in almost all endemic areas between the 1960s and the 1980s. Now, with the emergence of resistance to this drug, it is mainly used in Africa. The dominant reasons for the continue use of this drug are that it is a single-dose treatment and it's cheaper than the alternatives. Pyrimethamine can cause occasional skin rashes and depression of hematopoiesis. Excessive doses can produce megaloblastic anemia. Sulfonamides can cause agranulocytosis, aplastic anemia, hypersensitivity reactions, liver dysfunction, anorexia, vomiting and acute hemolytic anemia. This drug is contraindicated in patients with known hypersensitivity to sulfa, infants below 2 months of age, patients with advanced renal disease and first and last trimesters of pregnancy.

The reasons why combination regimens do not always work

Firstly, the genome of *P. falciparum* is more complexe than bacteria, which means it is easier to produce drug resistance mutants. Furthermore, the eukaryotic *falciparum* genome contains more complex mechanisms that might mediate drug resistance than does the bacterium. For example, there are so many drug resistance transporters in *P. falciparum*, such as *Pf*MDR1 and *Pf*CRT. And there must be still a lot of unknown drug resistance transporters in the *P. falciparum* parasites. They pump out the anti-malarial drugs and cause the concentration decrease of drugs around their targets. Even one transporter can pump diverse anti-malarial drugs, such as *Pf*MDR1, a well known transporter, which can transport aminoquinolines (chloroquine and amodiaquine and mefloquine and halofantrine) and confer the resistance to those drugs. Furthermore, the drug-resistance-inducing property of combination therapy depends on the area of the parasite's genome targeted by the drugs. Combination therapy would show a poor effectiveness when the targets genes of the combined drugs are subjected to high frequency mutation areas. And multidrug-resistant parasites often develop resistance to unrelated compounds at rates far higher than those noted for parasites still sensitive to most drugs (Rathod et al., 1997). Actually, there is no combination regimen ideal in all aspects, including matched action mechanisms and pharmacokinetic properties, toxicity tolerance, drug resistance induce property, drug-drug interaction and *in vivo* half life.

1.2.3 Drugs in clinical studies for future chemotherapy

1.2.3.1 Tafenoquine

The 8-aminoquinoline tafenoquine, a more lipophilic derivative of primaquine, showed excellent activity against blood and liver stage parasites. It has also been used for the elimination of *P. vivax* hypnozoites and the therapy of acute *vivax* malaria. Tafenoquine showed a clinical protective efficacy of 86–100%. The action mechanism of tafenoquine still remains unclear. It exhibits primaquine-like effect on the respiratory chain and quinine-like activity on heme polymerization. This is consistent with the activity against asexual blood stages. As reported, tafenoquine still cause hemolysis in glucose-6-phosphate-dehydrogenase-deficient humans. However, it is better tolerated than primaquine.

1.2.3.2 AQ-13

AQ-13 is a chloroquine derivative with a shortened side chain. However, it is active against chloroquine-resistant parasites. There still is a significant correlation between the susceptibility of different isolates toward AQ-13 and CQ, indicating the cross-resistance between CQ and AQ-13. At high doses, AQ-13 exhibited more toxicity in rats than chloroquine.

1.2.3.3 Ferroquine

Ferroquine showed activity against different chloroquine-sensitive and chloroquine-resistant strains, which bears a ferrocenyl moiety in the side chain. The lipophilic ferrocenyl moiety may be responsible for the activity against chloroquine-resistant strains, which make ferroquine does not fit into the substrate binding site of the chloroquine resistance transporter. As reported, this drug is about to enter clinical development .

1.2.3.4 T3

After the invasion into erythrocyte, parasites produce a huge amount of membrane system through the phospholipids metabolism pathway. This class of bis-cationic compounds was developed targeting this pathway. T3, an excellent bis-cationic candidate compound, exhibited IC_{50}-values between 2.3 and 6.3 nM against different chloroquine-sensitive and chloroquine-resistant parasite strains and an ED_{50} of 0.44 μM when administered i.p. in rodent model. It is about to enter clinical development. However, T3 showed a poor bioavailability. As reported, an *in vivo* active prodrug has been developed, with a bioavailability of about 16% in rats.

1.2.3.5 Fosmidomycin

Fosmidomycin is an excellent inhibitor of *plasmodium falciparum* 1-desoxy-Dxylulose-5-phosphate reductoisomerase (DXR), which is responsible for mevalonate-independent isoprenoid biosynthesis. This pathway is essential for the parasites and does not exist in humans, which means it is a potential target for anti-malarial drug development. Fosmidomycin showed *in vitro* anti-malarial activity against four laboratory strains with IC_{50}-values of 390–940 nM. There is no cross-resistance between fosmidomycin and other conventional anti-malarials. Fosmidomycin is a rapid acting anti-malarial agent, with a PCT50 of 21 hrs and a PCT90 of 28 hrs in clinical patients. The cure rate of 100% was observed at day seven. However, the recrudescence rate in non-immune patients was unacceptably high when fosmidomycin was used alone. Now, the combination of fosmidomycin with clindamycin has entered clinical phase development.

1.2.3.6 Isoquine

Isoquine was discovered by reseachers from the University of Liverpool through optimization of amodiaquine. The toxicity of amodiaquine, such as hepatotoxicity and agranulocytosis, was bypassed but the antiparasitic activity was retained. Isoquine is scheduled to enter Phase I trials in March 2006.

1.2.4 Anti-malarial drugs combination in clinical studies

As no presently used combination regimen is ideal in all aspects, novel combinations of anti-malarial drugs are being developed. Some of them have entered the clinical trial phase.

1.2.4.1 Chlorproguanil-dapsone-artesunate (Lapdap™)

The combination of chlorproguanil and dapsone was developed as an effective and cheap anti-malarial agent in 2003, which used as substitution for sulfadoxine-pyrimethamine in Africa. Chlorproguanil-dapsone-artesunate (CDA) is developed based on the ACTs to slow down the spread of drug resistance. The addition of artesunate causes rapid *in vivo* parasite clearance, thus reduce the parasite biomass sufficiently to allow the antifolate to clear away the remainder and delay the emergence of resistance to the combination. Clinical trials of CDA entered Phase III in 2005.

1.2.4.2 Fosmidomycin-clindamycin

Fosmidomycin, which can clinically cure *P. falciparum* infection, exerts its anti-malarial activity by the inhibition of 1-deoxy-D-xylulose 5-phosphate reductoisomerase. This enzyme, which is absent in humans, is essential for the biosynthesis of isoprenoid in malarial parasites. The parasites were arrested at the late schizont stage when they were exposed *in vitro* to fosmidomycin for a full replication cycle (Lell et al., 2003). The combination of fosmidomycin-clindamycin showed a synergistic effect in the treatment of malaria. Rapid parasite clearance and 100% cure rates were achieved together in a clinical study. (Borrmann et al., 2004).

1.2.4.3 Pyronaridine-artesunate (PANDA)

Pyronaridine, a member of Mannich-base class schizontocides, has been used in clinical malaria therapy nearly 20 years in China. The development project of PANDA was supported by MMV for the treatment of acute uncomplicated malaria. The work on Phase I trial was completed in 2004. Then the project went into Phase II trial in 2005 and the work on Phase-III trial started in 2006.

There are still many other combination regimens, which have been clinically used or trialed. However, the data does not encourage the use of those combinations on grounds of poor effectiveness, serious side effects, emergence of cross-resistance to those combinations before their use or expensive prices. They mainly include: sulfadoxine-pyrimethamine-chloroquine (poor effectiveness), sulfadoxine-pyrimethamine-mefloquine (poor synergistically efficacy, pharmacokinetic properties not match well, high price), sulfadoxine-pyrimethamine-amodiaquine (serious side effects), sulfadoxine-pyrimethamine-quinine (poor synergistically efficacy), chlorproguanil-dapsone (Cross resistance emergenced before the use, serious side effects), quinine-tetracycline (serious side effects), quinine-clindamycin (serious side effects), Artesunate-sulfadoxine-pyrimethamine (poor effectiveness).

1.3 The reason for the widespread of malaria

1.3.1 No effective vaccine available against malaria

Vaccine is an effective way to control infectious diseases. Remarkable examples are the eradication of smallpox, the virtual eradication of polio, and the striking reduction in the

prevalence of measles in the western hemisphere (Mahanty et al., 2003). As reported, there are special antibodies against malaria in the Sera from humans living in hyperendemic regions, which can prevent red cell invasion by targeting antigens on merozoites (Mahanty et al., 2003). Antibodies in purified immunoglobulins, which are passive transfered from 'naturally immune' individuals into partially immune children, were found to produce rapid clearance of parasites in recipient children even when the antibodies did not block growth *in vitro* (Mahanty et al., 2003). Protective immunity could be inducted by defined antigens in animal models. Those researched indicated that it's possible to develop a safe and efficacious malaria vaccines, which has attracted so much attention around the world for many years. However, even now, there is still no effective vaccine available against malaria, which is one of the most important reasons for the widespread of malaria. There are so many scientific and technical challenges for malaria vaccine development. Firstly, the *Plasmodium* is a highly complex parasite. Its genome encodes an estimated 5,600 proteins in the whole multi-stage life cycle. The parasite expresses a different repertoire of antigens in different stages, and multiple antigens exhibit remarkable polymorphisms. Both stage-specific expression and polymorphisms of antigen genes deployed in vaccine may profoundly influence the human immunity against malaria. This leads to the main hindrance to malaria vaccine development. Even now, no ideal target antigen is established, which showed a stable property among different stages and strains. Secondly, the antigens of malaria parasites inside RBC exposure to antibody occur only between release from one RBC and invasion of another. This provides a short window of opportunity for antibodies to bind and neutralize merozoites. Therefore, considerable effort is being devoted to the testing and development of new adjuvants for asexual blood stage antigens to enhance the delivery of antigen to immune system and get high antibody level. (Guinovart and Alonso, 2007; Mahanty et al., 2003). Thirdly, in humans, sera from immune individuals have high titers of antibodies against erythrocyte membrane protein 1 (*Pf*EMP1) in addition to other surface and internal merozoite antigens, so investigators have speculated that *Pf*EMP1 is a target for antibody-mediated immunity (Mahanty et al., 2003). However, *Pf*EMP1 is encoded by a large and diverse var gene family, which plays a key role in clonal antigenic variation. Immune pressure results in quick variation of *Pf*EMP1.

1.3.2 Drug resistance

An important reason for continued innovation on anti-infectious drugs is the development of drug resistance. As a serious infectious disease, malaria is also challenging the effective

clinical therapy by the emergence of drug resistance. Studies on malaria drug resistance indicated that parasite clones resistant to some traditional anti-malarial agents acquire resistance to new ones at a high frequency (accelerated resistance to multiple drugs, ARMD). Drug resistance causes the incidence of malaria infection continues to increase at a significant rate. There are multiple factors that contribute to malarial-drug resistance, both from parasites and anti-malarial drugs.

1.3.2.1 Parasite originated drug resistance elements: transporter and enzyme

The genetic events in malaria parasites responsible for drug resistance are spontaneous and rare. Those events must not impact the viability of parasite. The drug resistance related genetic events include gene mutation and increase of gene copy number. The genes involved in the events mainly are responsible for the encoding of drug target or drug transporter. In some cases, a single genetic event may be sufficient to create drug resistance.

1.3.2.1.1 Drug resistance related *Plasmodium* transporters

There are two digestive vacuole membrane localized transporters mainly contribute to the drug resistance of *P. falciparum* parasite, chloroquine resistance transporter (*Pf*CRT) and Pglycoprotein homologue 1 (Pgh1). The mutation of the two genes could confer resistance to many anti-malarial drugs presently used, such as chloroquine, mefloquine, quinine and halofantrine. The study on isolates from endemic areas of *falciparum* malaria showed that there is a typically positive correlation between mutations in either or both the two transporter genes and *in vitro* drug resistance. Several other transporters may also be related to drug resistance. Theoretical analysis has indicated that as many as nine putative transporter genes (including *pf*mdr1 and *pf*crt) showed significant associations with decreased CQ sensitivity, with linkage disequilibria suggesting there may be multiple interactions between these genes (Mu et al ., 2003).

1.3.2.1.1.1 *Pf*CRT

The *pfcrt* gene is located on chromosome 7 and encodes a 424 amino acid (48.6 kDa) protein. The *Pf*CRT protein contains ten predicted transmembrane domains and is located on the membrane of the digestive vacuole, where the weak base chloroquine concentrates in its

diprotonated form and binds with hematin (a dimeric form of oxidized heme). *Pf*CRT is a member of the drug-metabolite transporter superfamily. It was identified as a candidate gene for CQR by the analysis of a genetic cross between a chloroquine-resistant clone (Dd2, Indochina) and a chloroquine-sensitive clone (HB3, Honduras) (Wellems *et al* ., 1990). The specific polymorphisms in *pf*crt are tightly correlated with chloroquine resistance. Transfection studies have shown that *pfcrt* mutations confer verapamil reversible chloroquine resistance *in vitro* and reveal their important role in resistance to quinine. The K76T mutation is crucial for chloroquine resistance. The other mutations may serve to compensate for a loss of endogenous function associated with the K76T mutation (Valderramos and Fidock, 2006).

1.3.2.1.1.2 *Pf*MDR1

The protein Pgh1 localizes to the digestive vacuole membrane of *P. falciparum*. It is an analog to the P-glycoprotein (P-gp) over expressed in cancer cells. P-gp functions as pumps expelling cytotoxic drugs (ATP-binding cassette transporters) in drug resistant tumor cells. As reported, Pgh1 overexpression is associated with increased CQ susceptibility in strain-dependent manner. The mutations in Pgh1 is thought to be responsible for conferring resistance to anti-malarials like mefloquine, quinine, halofantrine, chloroquine and artemisinins, which can cause impaired drug uptake by the parasite vacuole.

1.3.2.1.1.3 *Plasmodium falciparum* CG2

*Pf*CG2, whose gene closely linked to the chloroquine resistance locus in chromosome 7, is a putative drug resistance related protein. *Pf*CG2 is a peripheral membrane protein and is exposed to the erythrocyte cytosol at the outer face of the PVM. There is no significant evidence that *Pf*CG2 involve in chloroquine resistance directly. However, the polymorphisms of *pf*cg2 are associated with chloroquine resistance.

There are still some other putative transporter genes, located on different chromosomes, were associated with chloroquine and quinine resistance. (Valderramos and Fidock, 2006)

1.3.2.1.2 Drug resistance related enzymes

There several enzymes are related with the drug resistance of malaria. The mutation, single nucleotide polymorphisms or over expression could confer drug resistance to malarial parasites.

1.3.2.1.2.1 *Plasmodium falciparum* dihydrofolate reductase (PfDHFR)

*Pf*DHFR is the target of anti-malarial drugs proguanil (cycloguanil) and pyrimethamine. Single point mutation in *pf*dhfr is sufficient to create resistance and there are multiple foci showing high mutation frequency, explaining the rapid emergence of drug resistance. The S108N substitution is the principal mutation associated with resistance to pyrimethamine or cycloguanil in Africa and in South-east Asia. Substitution S108T is also found in South America. The most frequent additive mutations are N51I and C59R. The mean IC_{50}-value of cycloguanil increases with the number of mutations. Higher level pyrimethamine resistance is associated with multiple mutations at the target site. However, multiple mutations diminish the efficacy of the enzyme on dihydrofolate and thus suggest that additional mutations are unfavourable to the parasites in the absence of drug pressure.

1.3.2.1.2.2 *Plasmodium falciparum* dihydropteroate synthase (PfDHPS)

*Pf*DHPS is a key enzyme in the folate pathway of *Plasmodium falciparum* parasite. Sulfadoxine exerts its anti malaria action by the inhibition to this enzyme. The resistance to sulfadoxine is developed by mutations in *Pf*DHPS. The crucial mutations lie at positions 436, 437, 540, 581 and 613.

1.3.2.1.2.3 *Plasmodium falciparum* ATP6 (*Pf*ATP6)

Artemisinin specifically inhibit *Pf*ATP6, a SERCA-type ATPase of *Plasmodium falciparum*. Now *Pf*ATP6 is a candidate gene that has been associated with parasite response to artemisinin. As reported, a single amino acid in transmembrane segment 3 of SERCAs can determine susceptibility to artemisinin. The mutation in Leu263 in *Pf*ATP6 is crucial for the interaction between *Pf*ATP6 and artemisinin (Uhlemann et al., 2005). However, the association of mutations in *Pf*ATP6 with artemisinin resistance requires further clinical confirmation.

Cross resistance: Multi-drug resistance for malaria refers to one parasite strain resists to several anti-malarial drugs with diverse structures, which can be simultaneous or cross-resistance. Multi-drug resistance is caused by the large-scale and simultaneous use of several anti-malarials, which causes strong selective pressure. The parasites, with a high level of chloroquine resistance, are generally resistant to amodiaquine as well. The same is probably true for mefloquine and halofantrine. The cross-resistance between anti-malarials with diverse structures is a phenomenon linked to the common or shared aspects of their modes of action and probably of their resistance mechanisms, such as the cross-resistance between cycloguanil and pyrimethamine in Africa. Both cycloguanil and pyrimethamine exert their anti malaria

action by the inhibition against the same target, *Pf*DHFR. The resistance to the two drugs is also conferred by the same mutations in *Pf*DHFR. So the parasite strain, which is resistant to pyrimethamine, also be cross-resistant to cycloguanil, and *vice versa*. So it seems essential to combine the anti-malarial drugs without cross resistance tendency for treatment and for prophylaxis in order to limit the extension of resistant genotypes.

1.3.2.2 Drug originated drug resistance elements

Some properties of anti-malarial drugs are closely related to the emergence of drug resistance, which are crucial for the success of anti-malarial drug development.

1.3.2.2.1 *In vivo* elimination half-life

The response of malaria to treatment is determined by blood concentrations of anti-malarial drugs. Most of the drugs are absorbed more quickly than they are eliminated, which gives rise to the characteristic concentration-time profile: a maximum plasma concentration, achieved within a few hours of drug administration, followed by an exponential decline at a rate governed by the specific elimination rate constant. The residual drug concentration in the blood of the host will be sufficient to prevent reinfection until its concentration falls below the minimum parasiticidal concentration for a particular parasite genotype. Elimination half life is a very important pharmacokinetic parameter for all drugs, and refers to the time it takes for a drug to lose half of its pharmacological activity. Anti-malarial drugs with a long elimination half-life have two therapeutic advantages. Firstly, this kind of drugs can provide long-term protection against reinfection, such as pyrimethamine-sulphadoxine. Prolonged anti-malarial drug activity *in vivo* is an advantage to the patient who is recovering from malaria in an area of moderate or high transmission, because further disease episodes are prevented. Secondly, long half-life drugs require a fewer administrations, which reduces the risk of underdosage and some of the problems of compliance. However, a slow eliminated drug would persist in the patient for a very long time at low concentration, which means the residual drug constitutes a potent selective force for the emergence of drug resistance. For the development of pyrimethamine-sulphadoxine resistance, there is a very important model consisting of two separated phases. In phase A, intermediate levels of susceptibility parasite strain is spreading and replacing the original sensitive forms while drug resistant strain remains at a low level. Phase B starts once parasites are selected that can escape from drug action. Drug treatment is clinically successful during phase A, and health workers may be unaware of the substantial changes in parasite population genetic structure that predicate the onset of phase B. The very

rapid elimination of artemisinin, its derivatives and their active metabolites means that even fully susceptible parasites are affected by residual drug for times only marginally greater than the period of treatment. The drug effect is either maximal or zero. Artemisinin-based combination therapies have been widely used as the first line anti malaria treatments around for a decade. However, even now, widespread clinical artemisinin still not emerged. So drug *in vivo* elimination half-life is a critical factor for the development of drug resistance.

1.3.2.2.2 The gene stability of drug target

The gene stability of a drug target is closely related to the development of resistance to this drug. If the gene of an anti-malarial drug target showed a high frequency of pre-existing mutations, this drug would show a poor property against drug resistance selection as it is very easy for the parasite to develop resistance through mutation in a unstable target gene, such as *pf*dhfr.

1.4 The targets used for the development of novel anti-malarial drugs

With the emergence of widespread drug resistance, novel anti-malarial drug development is the best way to override this problem. The novel anti-malarial drug will interact with a new target and exert their action by a different mechanism comparing with the presently used anti-malarial drugs. Novel drug targets identification is crucial for the development of new anti-malarial drugs.

1.4.1 The drug targets of *Plasmodium Falciparum* in biochemical pathways

Several parasite specific and essential biochemical pathways have been exploited for novel drug target identification. The *Plasmodium Falciparum* parasite should be killed selectively by the block to those pathways. With the development of *in silico* target screen among metabolic enzymes, more and more metabolic enzymes are proposed to be novel drug targets.

1.4.1.1 The *Plasmodium Falciparum* specific biochemical pathways in the apicoplast

The apicoplast in malaria parasite contains a range of metabolic pathways and housekeeping

processes, which are essential to the parasites and differ radically to those of the human host. Thus, those pathways are thought to be potential anti malaria drug targets, such as fatty acid, isoprenoid and heme synthesis. Presently, the most efficous anti-malarials targeting apicoplast are the antibiotics, such as clindamycin, tetracycline and azithromycin. Fatty acid synthesis is a major function of the apicoplast, which is crucial for the synthesis of cell and organellar membranes. There is an inherent difference between the fatty acid biosynthesis pathways of the parasite (type II) and the human host (type I), thus making them a promising target for the development of anti-malarials. Another promising drug target pathway is isoprenoid biosynthesis, which is essential in blood-stage. The significant evidence is the excellent activity of fosmidomycin, an inhibitor of the isoprenoid enzyme 1-deoxy-D-xylulose 5-phosphate (DOXP) reductase. Some of vitamin biosynthesis pathways are also thought as novel targets for anti malaria drugs development, which occurs in *P. falciparum* but is absent in humans, such as the pyridoxal phosphate pathway and the biosynthesis pathway of coenzyme from the precursor pantothenate (vitamin B5).

1.4.1.2 The *Plasmodium Falciparum* specific metabolic pathways in the mitochondrial

Electron transport and protein synthesis are the major functions of mitochondria in *Plasmodium Falciparum*, which are also potential targets for anti-malarial chemotherapy. *Plasmodium* mitochondria use a different homolog of CoQ comparing their mammalian host, which offers a unique chemotherapeutic target for anti-malarial drug development. Atovaquone is an excellent anti-malarial drug, which was developed based on this target (complex III). It exerts its anti-malarial action by inhibiting electron transport and collapsing mitochondrial membrane potential, which is required for a number of parasite biochemical processes. Another potential drug target in *Plasmodium Falciparum* mitochondrial is Dihydroorotate dehydrogenase (DHODH). *Plasmodium* in humans can capture and use the host's purines but not their pyrimidines, which means the *de novo* biosynthesis pathway is essential for the parasite. DHODH is a key flavin mononucleotide-dependent mitochondrial enzyme, which utilizes coenzyme Q as the final electron acceptor. As reported, several potent inhibitors of *Pf*DHODH have been developed, which display IC_{50}-values in the range 20-600 nM. Those compounds showed a 200-20 000-fold selectivity for the malarial over the human enzyme. Another drug target in mitochondrial is the quinol oxidation site (Qo) of the parasite mitochondrion *bc*1 complex, whose inhibitors can cause collapse of the mitochondrion

membrane potential and cell death. The inhibitors display a selectivity against the parasite enzyme that is 5000-fold higher than for human liver *bc*1.

1.4.1.3 Choline biosynthesis pathway

Intra-erythrocytic malaria parasites have to synthesize an abundance of phospholipids, which increases by as much as 500% in infected erythrocytes, for the development of the parasitophorous vacuole, cytosol and multiple subcellular compartments. At the same time, a large amount of phospholipids are used for the growth and divide of malaria parasites. The major of phospholipids in the membranes of plasmodial is phosphatidylcholine (PC), about 45% of the total. Thus *de novo* phosphatidylcholine biosynthesis pathway is characterized as an excellent target for anti-malarial drug development, which is absent from mature human host erythrocytes. Choline transport is a rate-limiting step in this pathway. Choline analogs were developed based on the idea to inhibit choline transport. At the same time, choline analogs also could exert an effect on parasite phospholipid biosynthesis. As reported, choline analogs showed an IC_{50}-value against *P. falciparum* parasites in asexual blood stages at single-digit nanomolar concentrations. The *in vivo* test in rodents and non-human primates infected at high parasitemias showed that choline analogs exhibit a fully curative anti-malarial activity with short-course treatments. The chemical structure and modes of action are distinct from current anti-malarial agents, which could help delay the development of resistance. Now, this exciting new class of compounds have entered clinical trial (Schlitzer, 2008).

1.4.1.4 *Plasmodium Falciparum* special proteases

Malarial proteases play crucial roles in the parasite life cycle, which makes them potential anti-malarial targets. They act as a key factor not only for the degradation of a myriad of hemoglobin, but also in the invasion and rupture of erythrocytes (McKerrow et al., 1993; Rosenthal, 2002). Hemoglobin (Hb) digestion is apparently mediated by many food vacuole aspartic proteases, cysteine proteases, metalloprotease and dipeptidyl aminopeptidase 1 (DPAP1), by which the parasite captures the nutrient for its own protein synthesis (Sahu et al., 2008). So those enzymes have long been defined as potential drug targets. As reported, inhibitors of falcipains 2 and 3 could prevent *in vitro P. falciparum* growth at low nanomolar concentrations. *In vivo* test in a new immunocompromised mouse showed that these inhibitors can cure *P. falciparum* infections propagated in human RBCs. However, the research on plasmepsin enzymes showed that parasites can survive, albeit with reduced growth rate,

without all four plasmepsins of the DV (Fidock et al., 2008). Cysteine protease inhibitor E-64 could inhibit schizont rupture in *P. falciparum* and inhibitors of serine/cysteine proteases could inhibit merozoite invasion by three *Plasmodium* species significantly (Francis et al., 1997; Wang and Wu, 2004). So proteases would be promising targets for clinical therapeutics.

1.4.1.5 *Plasmodium Falciparum* Carbonic Anhydrase (CA) inhibitors

P. falciparum carbonic anhydrase plays a crucial role in the interconversion of carbon dioxide and bicarbonate at neutral pH and biosynthesis of pyrimidines, which is thought as a potential anti-malarial drug target. As reported, the inhibitor of *P. falciparum* Carbonic Anhydrase (*Pf*CA) showed eccellent anti-malarial activity, with the IC_{50}-value at the range of micro molar (A.A. Joshi 2006).

1.4.1.6 Targeting the shikimate pathway

Aromatic amino acids are essential in the diet of malaria parasite, which are synthesized by the shikimate pathway. However, this pathway is not present in human host. This pathway offers some potential anti-malarial drug targets. The enzymes in the shikimate pathway are localized in the cytosol of *P. falciparum* parasite. As reported, the analogs of shikimate and inhibitors of the enzymes in this pathway are able to inhibit *P. falciparum* growth.

1.4.1.7 Microtubule inhibitors

Microtubular systems are the principal components of mitotic spindles, which are essential for cell division and play a major role in cell motility. They contribute to the maintenance of cell shape and integrity and the organization of subcellular compartments. They also act as tracks for intracellular transport of vesicles and organelles. Now, the microtubular systems of malarial are thought as potential drug targets. Several inhibitors of tubulins, the principal proteins of microtubules, showed potential anti-malarial activity. However, the selectivity of those inhibitors need to be improved.

1.4.1.8 Targeting the redox system

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to clear the reactive intermediates or easily repair the resulting damage. It can cause toxic effects and damage all components of the cell, including proteins,

lipids, and DNA, to which Plasmodia is very sensitive. In intra-erythrocytic-stage malaria, parasites encounter reactive oxygen species (ROS) from either themselves or the host. Malaria parasites have their own biological system to prevent oxidative stress, including a series of antioxidant enzymes. The key enzymes of *P. falciparum* involved in redox metabolism are glutathione reductase, GPx, thioredoxin reductase, and thioredoxin peroxiredoxin. The parasite thioredoxin reductase differs from the host enzyme, which is thought as potential drug target. The inhibitors of thioredoxin reductase showed *in vitro* anti-malarial activity. Another potential target in this pathway is Plasmodial GST, which acts as a ligand for parasitotoxic hemin and is highly abundant in the parasite. The crystal structure of *P. falciparum* GST differs considerably from the human enzyme. So the specific inhibitors of GST should have promising future for development.

1.4.1.9 Targeting the methionine cycle, methylation, and polyamines

The methionine cycle in parasites has recently received attention as it is the essential biochemical pathway to provide substrates for methylation of DNA, RNA, proteins, phospholipids and for the synthesis of polyamines, which means the methionine cycle is involved in many key processes in the life cycle of parasite. Some enzymes and metabolites in this pathway are unique to parasites, which would offer potential targets for malaria chemotherapy. Polyamines play a crucial role in many key processes such as cell growth, differentiation, and macromolecular synthesis. Thus, the enzymes in the polyamine biosynthesis pathway were thought as potential targets for anti-malarial drugs development.

1.4.1.10 Targeting nucleic acid metabolism

Nucleic acid metabolism pathways are essential for the growth of malaria parasites. As those pathways differ between *P. falciparum* and the human host, they are thought as promising drug targets. *Plasmodium* in humans can capture and use the host's purines but not their pyrimidines. Plasmodia synthesize pyrimidines by *de novo* biosynthetic pathways. The human host cells synthesize purines *de novo* and either salvage or synthesize pyrimidine by a *de novo* pathway. Hypoxanthine-guanine phosphoribosyltransferase (*Pf*HGPRT) and purine nucleotide phosphorylase (*Pf*PNP), two key enzymes of purine synthesis pathway in *P. falciparum* parasites, are thought as potential drug targets. As reported, inhibitor of *Pf*PNP showed anti-malarial activity. Pyrimidine biosynthesis also presents an attractive drug target in malaria

parasites because of the absence of a pyrimidine salvage pathway. The key enzymes in this pathway include carbamoyl phosphate synthase, aspartate transcarbamylase, dihydroorotase, dihydroorotate dehydrogenase (*Pf*DHODH), orotate phosphoribosyl transferase and orotidine 5-phosphate decarboxylase.

1.4.1.11 Targeting folate metabolism

Folate metabolism pathway is essential for the synthesis of nucleotides required for DNA replication and the synthesis of the amino acids glycine and methionine. It also plays a very important role in the metabolism of histidine, glutamic acid and serine and protein synthesis in mitochondria through formylation of methionine. So folate metabolism pathway is one of the most common pathways that have proved to be a valuable target for decades. There are two classes of drugs targeting this pathway: inhibitors of dihydropteroate synthase (DHPS) (known as class I antifolates) and inhibitors of dihydrofolate reductase (DHFR) (the class II antifolates). Anti-malarials such as proguanil, chlorproguanil, clociquanil and pyrimethamine exert their activities by inhibiting dihydrofolate reductase (DHFR). Dapsone is a sulfa drug, which kills malarial parasites by inhibitting dihydropteroate synthase (DHPS) (Triglia et al., 1997). The combination of the two classes of drugs is synergistic in clinical, hence their use in combination in the treatment of malaria. Dapsone was combined with pyrimethamine as Maloprim. Lapdap is a combination of chlorproguanil and dapsone.

1.4.1.12 Glycolysis

Malaria parasites are microaerophic homolactate fermenters and rely on glycolysis for ATP production. So the glycolytic pathway is a vital pathway for malaria parasites, which may be a novel target. There are 11 enzymes to convert glucose to lactic acid in glycolytic pathway, among which three enzymes are considered to be rate-limiting: hexokinase, phosphofructokinase and pyruvate kinase. The three enzymes may be novel targets for the development of anti-malarials. *P.falciparum* Lactate dehydrogenase (*Pf*LDH) is a key enzyme in the glycolytic pathway of *P. falciparum* and has several unique amino acids, related to other LDHs, at the active site, making it an attractive target for anti-malarial agents. As reported, one class of *Pf*LDH inhibitors have recently been shown to have anti-malarial activity both *in vitro* and *in vivo* (Sahu et al., 2008).

1.4.1.13 Targeting Hematin or the Hemozoin Crystal

Heme, hematin or hemozoin are believed to be the targets of many anti-malarials, such as chloroquine, quine, mefloquine and artemisinin. Heme has been implicated as the target of endoperoxide anti-malarials, such as artemesinin, which have been proposed to form radical adducts with heme that act against the parasite. Hematin is thought as the target of chloroquine and other quinoline anti-malarials. As reported, those drugs act by preventing the detoxification of hematin, which are normally converted to hemozoin or malaria pigment. The accumulation of hematin causes toxicity to parasites. Chloroquine has variously been proposed to inhibit hemozoin formation via direct interaction with hematin. The research indicated that this pathway is an important target for novel anti-malarial drugs development.

There are still many other metabolic enzymes, which are viable targets for novel anti-malarial drugs development, such as *P. falciparum* Peptide Deformylase (*Pf*PDF), Erythrocyte G Protein, helicases and Farnesyl transferase.

1.4.2 Other drug targets of *Plasmodium Falciparum*

With the exception of the metabolic enzymes, there are still many other types of drug targets in malaria parasites, including transporters and permeability pathways.

1.4.2.1 New permeability pathways (NPPs)

Molecular traffic across the host erythrocyte membrane undergoes dramatic changes with respect to intensity and the nature of permeating solutes. The induced permeability pathways are known as new permeability pathways (NPPs). They occurs 12-16 hrs post erythrocyte invasion by merozoite (Becker and Kirk, 2004). NPPs are involved in the increasing permeability of host erythrocyte membranes to a wide range of low molecular weight solutes. They are thought to provide the major entry of some essential nutrients (pantothenate) required by the parasites and mediate the efflux from the infected cell of various metabolic wastes, such as lactic acid and amino acids (Saliba et al., 1998). The pathways are broadly anion selective, but with a significant permeability to both organic and inorganic cations (Becker and Kirk, 2004). The properties of the parasite-induced transport systems are significantly different from those in normal human cells. Therefore, these transport systems are proposed to be targets for anti-malarial chemotherapy. The drugs targeting on this pathway will exert their activities by inhibiting the transport and hence depriving the parasite of nutrients essential for its

development or by selectively entering the parasite through these induced transporter routes and hence cannot enter normal mammalian cells. NPPs are inhibited by a range of classical anion transport blockers and various derivatives, some of which are effective at nanomolar concentrations. There are many new anti-malarial candidate compounds targeting NPPs. The most potent NPP inhibitors are based on furosemide and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), amongst which the most effective one exhibited an IC_{50}-value around 100 nM.

1.4.2.2 Parasite transporters

There are some essential transporters in malaria parasites involved in nutrient acquisition from the host, which are also under investigation as candidate targets. As reported, this kind of parasite-specific transporters include *Pf*FBT1, *Pf*FBT2, *Pf*HT and the *P. falciparum* V-type H^+-ATPase. The inhibition to these transporters will block some necessary metabolism pathways of parasites and cause the death of them.

1.5 Aim of this study

The aim of this study is to identify novel anti-malarial drug candidates, with distinct chemical structures from current anti-malarial agents. The candidate compounds would showed excellent activities against *P. falciparum* blood stage and exert their anti-malarial action by a novel mechanism, which could be used as efficacious anti-malarial drugs in the future to override the widespread drug resistance.

2 Material and method

2.1 Materials

2.1.1 Equipment

Analytical scales	Sartorius, Göttingen
Autoclave	Tuttnauer Systec 2540, Wettenberg
automated 12-channel pipette aid	Eppendorf, Germany
Cell harvester	Packard, USA
Centrifuges:	
J2-MC	Beckman, Krefeld
RC5BPlus	Sorvall, Langenselbold
Megafuge 2.0R	Heraeus Instruments, Hanau
Megafuge 1.0R	Heraeus Instruments, Hanau
Biofuge fresco	Heraeus Instruments, Hanau
Biofuge pico	Heraeus Instruments, Hanau
CCD Camera	Princeton Instruments, USA
Computer-software:	
Adobe Photoshop® 5.0	Adobe Systems Inc, USA
Chromas	Technelysium.com.au/chromas
ClustalW	http://www.ebi.ac.uk/Tools/clustalw/
EndNote 8.0.2	ISI Research Soft, CA, USA
MS Powerpoint	Microsoft Corporation, CA USA
MS Word 98	Microsoft Corporation, CA USA
Sigmaplot 9.0	Systat Software, Inc. USA
Internet Explorer	Microsoft Communications Corp., USA
Freezer -80ºC, UF85-300S	Heraeus GmbH, Hanau
Freezers -20ºC	Liebherr, Biberach
Icemachine AF 30	Scotsman, Milano, Italy
Incubator (*P. falciparum*)	Heraeus Instruments

Liquid nitrogen tank	Air Liquide, Ludwigshafen
Magnetic stirrer	Heidolph, Schwabach
Microscopes:	Zeiss, Jena
Microwave oven	AEG, Nürnberg
pH-meter pH 537	WTW, Weilheim
Microtiter plate scintillation counter	Packard, USA
Printer hp LaserJet 1300	Hewlett Packard, Heidelberg
Quartz cuvettes	Hellma, Müllheim
Spectrophotometer UVIKON 923	Kontron Instruments,
Sterile work bench Herasafe	Heraeus Instruments, Hanau
Stop watch	Roth, Karlsruhe
UV-lamp Typ N-6 L	Benda Laborgeräte und Ultraviolettstrahler
UV-table UV-Transilluminator	Gibco BRL, Karlsruhe
Vortex Genie 2	Roth, Karlsruhe
Water bath Julabo 7A	Julabo, Seelbach

2.1.2 Disposables

Aluminium foil	Roth, Karlsruhe
Centrifugation tubes, Polypropylen-12/75	Greiner Bio-one, Frickenhausen
Centrifugation tubes, Polystyren-6, 0/38 mm	Greiner Bio-one, Frickenhausen
Clingfilm Saran	Dow Chemical Company, Schwalbach
Coverslides	Roth, Karlsruhe
Cryovials	Nalgene®, Wiesbaden
Cuvettes	Sarstedt, Nümbrecht
Electroporation cuvettes	Biorad, München
Eppendorf tubes	Sarstedt, Nümbrecht
Falcon tubes (15 ml; 50 ml)	Corning incorporation, Bodenheim
Gloves	Hartmann, Heidenheim
Immersion oil	Zeiss, Jena
Object slides	Marienfeld, Lauda-Königshofen
Parafilm	American International CanTM, USA
Plastic pipettes	Corning incorporation, Bodenheim

Sterile filters (0,2 μm)	Corning incorporation, Bodenheim
96-well sterile culture plates(sterile, F-botton with lid)	Greiner bio-one
Unifilter plate GF/C-96/50	Perkin Elmer LAS, The Netherlands

2.1.3 Buffers, media and solutions

Cell culture media	10% Human serum 0.2 μg/ml Gentamycin 0.1 mM Hypoxanthine in RPMI 25 mM HEPES L-Glutamine (Gibco)
Freezing Solution	28% Glycerol (v/v) 3% D-Sorbitol 0,65% NaCl in ddH_2O filter sterilize
[^{3}H]-hypoxantine 10-30 Ci/mmol, 1mCi/ml	Perkin Elmer
Scintillation cocktail, Microscint	Perkin Elmer
Sorbitol Solution	5% (w/v) D-sorbitol in ddH_2O filter sterilize
Thawing solution I	12% NaCl autoclave
Thawing solution II	1.6% NaCl autoclave
Thawing solution III	0.9% NaCl / 0.2% glucose filter sterilize

2.1.4 Chemicals

The screened anti-malarial compounds were provided by 4SC. The structures of the compounds were checked by NMR, and the purity was analyzed by HPLC, which were more than 99%. The other chemicals used in this study are from the firms Merck, Sigma, Serva and Applichem and were ordered directly or through the Chemical facility of the Heidelberg Medical faculty.

2.1.5 *Plasmodium falciparum* strains

Dd2 (the laboratory line Dd2 originated from Thailand), 3D7 (clone from NF54, which was isolated from a patient who lived near the airport in Amsterdam), D10 (originated from Papua New Guinea), K1 (originally isolated from a Thai patient), 7G8 (isolated from Brazil), FCR3 (originated from the Gambia, West Africa), Thai19 (originated from Thailand).

2.2 Method

2.2.1 Cell culture

2.2.1.1 *In vitro* culture of *Plasmodium falciparum*

Erythrocytic forms of *P. falciparum* Strains (Dd2, 3D7, D10, FCR3, K1, 7G8, Thai19) were maintained in continuous *in vitro* culture as described previously (Trager and Jensen, 1976). The parasites were cultured in A^+ human erythrocytes at a hematocrit of approximately 5% in Petri dishes of either 10 cm or 25 cm diameter containing 15 ml or 35 ml, respectively, of HEPES-buffered RPMI medium supplemented with 10% human serum type A^+ ,20µg/ml of gentamicin and 100 µM of hypoxanthine. The parasite cultures were incubated in an atmosphere of 3% CO_2, 5 % O_2, 92 % N_2, and 95% humidity at 37°C. Every two to three days, the parasitemia determined by Giemsa-stained blood smears and medium was exchanged. By the time the parasitemia had reached 5-10%, the culture was split in order to avoid parasite death due to high levels of toxic metabolites in the medium.

2.2.1.2 Preparation of human serum and erythrocytes

Human group/type A^+ erythrocytes concentrate and A^+ human serum were obtained from the blood bank of the German Red Cross, Heidelberg. The human serum was prepared as follows. It was aliquoted into 50 ml falcon tubes. In each falcon tube, 800 µl of sterile 1 M $CaCl_2$ was added, and then incubated first at 37°C for 30 mins and overnight at 4°C. The next day the tubes were centrifuged at 4000 rpm for 30 mins to pellet the fibrin and incubated at 56°C for 30 mins to inactivate the complements in serum. Thereafter the serum was kept at -20°C. The erythrocyte concentrate was aliquoted into 50 ml Falcon tubes. 10 ml RPMI medium was added and the tubes were centrifuged (2300 rpm, 4 min) and then kept for two to three weeks at 4°C.

2.2.1.3 Staining of *P. falciparum* with Giemsa

To enable determination of parasitemia and parasite developmental stage, thin smears of the culture were made as follows: 50 µl of culture were spread evenly onto the surface of a clean microscope slide, air dried, fixed in 100% methanol for 30 sec, and again air dried. Fixed cells were stained for 5-10 min in a solution of 10% Giemsa, washed with water and allowed to dry.

2.2.1.4 Determining parasitaemia

The parasitemia is the percentage of *P. falciparum* infected erythrocytes. Giemsa-stained blood smears were examined on a light microscope under oil immersion using a 100x objective. The number of both infected and uninfected erythrocytes was counted in ten consecutive fields, with multiple infections assessed as single parasites. The parasitemia was then calculated according to the following formula:
(Number of parasites / Number of erythrocytes) * 100= parasitemia (%).

2.2.1.5 Parasite synchronization at Ring stage with sorbitol

Sorbitol (5%) treatment at 37 °C causes osmotic lysis of late stage trophozoites (Lambros and Vanderberg, 1979). This selective lysis by osmotic shock is possible due to an induced transport pathway in the red cell membrane that is permeable to sorbitol. This transport pathway present in late stage trophozoites, while absent in ring stage parasites. Culture media was removed from the culture dish, and the blood resuspended in 10 ml of filter-sterilized, pre-warmed 5% D-sorbitol solution and transferred to a Falcon tube. After a 10 min incubation at 37°C the suspension was then centrifuged (1900 rpm, 2 min, RT). The pellet was washed with 10 ml complete culture medium two times, before re-suspended in 15 ml of media and returned to the culture dish. For a tightly synchronized culture, this procedure was repeated for three times successively at the transition from schizonts to ring stage, which means only the first few rings survived. The age difference is confined to few hours.

2.2.1.6 Freezing parasites

A culture used for freezing should be at ring stage with a parasitemia higher than 5%. Infected erythrocytes were centrifuged at 1900 rpm for 2 min. The supernatant then removed, then the pellet re-suspended in equal volume of sterile freezing solution. The suspension was gently mixed, filled into a cryotube at a volume of about 1 ml and snap frozen in an ethanol/ dry ice

slurry for 15 min. The frozen parasites can stay for several months at -80°C or for longer storage in liquid nitrogen at -196 °C.

2.2.1.7 Thawing parasites

The cryotube was removed from the liquid nitrogen tank and thawed in a 37°C water bath for 2 min before being transferred to a 15 ml Falcon tube. Add 200 µl of 12% NaCl to 1 ml of blood, and stand for 3 min at RT. Then a further 7 ml of 1.6% NaCl was then added at a rate of 4 to 6 drops per minute. Then the suspension was centrifuged at 1900 rpm for 2 minutes. The supernatant was then removed. 7ml of 0.9% NaCl /0.2% glucose was added at a rate of 4 to 6 drops per minute. The suspension was then centrifuged as before, the supernatant removed and the parasite pellet resuspended in 15 ml of complete parasite medium and transferred to a Petri dish. 0.5 ml of fresh blood was then added, and the dish transferred to the parasite incubator. The parasite culture was left in the incubator for two days before the medium would be changed and the parasitemia determined. All solutions were pre-warmed to 37°C before starting the thawing procedure.

2.2.2 Compound screen

2.2.2.1 Compound preparation for screening

The anti-malarial compounds from 4SC were dissolved in DMSO at the concentration of 100 mM. And, they were diluted to 100 µM by the medium before screening.

2.2.2.2 [^{3}H]-hypoxanthine incorporation inhibition based anti-malarial compounds screening

Drug activity *in vitro* was determined based on the inhibition of the incorporation of [^{3}H]-hypoxanthine into parasite nucleic acids according to the method of Desjardins et al. (Desjardins et al., 1979), at 2.5% final hematocrit and 0.5% parasitemia. The detail is as follows:

A culture of highly synchronized rings are diluted in RPMI 1640 medium supplemented with 10% human serum type A+ (supplemented 20 µg/ml of gentamicin and 3 µM of hypoxanthine) to yield a hematocrit of 2.5% and parasitemia of 0.25-0.5% .

The compound to be tested is dissolved in DMSO at the concentration of 100 mM and kept at -20 °C. From this a working stock solution of 100 µM is prepared in the culture media described above.

X	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Fig. 2.1: Layout of the microtiter plate

- add 100 µl of culture medium (see above) to all wells.
- add 50 µl the compound to be tested (from the 100 µM working solution) to wells B1-B2 (drug number 1), duplicative determinations
- follow the same procedure for the other wells
  - 50 µl of 0.6 mM drug 2 to B3-B4
  - 50 µl of 0.6 mM drug 3 to B5-B6
  - 50 µl of 0.6 mM drug 4 to B7-B8
  - 50 µl of 0.6 mM drug 5 to B9-B10
  - 50 µl of 0.6 mM drug 6 to B11-B12
- mix using the multi-channel-pipette.
- take 50 µl from row B and transfer it to row C.
- take 50 µl from row C and transfer it to row D.
- follow the same procedure for rows E to H.
- remove 50 µl from row H and discard it. This will result in 1:3 dilution steps.
- add 100 µl of the Dd2 culture (2.5% hematocrite, 0.3-0.5% parasitemia ring stages) to each well.
- Incubate for 48 hrs at 37°C in the gassed incubator.

- Add 50 µl of the [^{3}H]- hypoxanthine-solution to each well:
    - Each well will receive 0.5 µCi [^{3}H]-hypoxanthine. The hypoxanthine solution is prepared as follows: Take the appropriate amount of labeled [^{3}H]-hypoxanthine, evaporate the solvent and then resuspend [^{3}H]-hypoxanthine in the appropriate volume of culture medium at a final concentration of 0.5 µCi / 50 µl.
- Incubate for 24 hrs more at 37°C in the gassed incubator
- Harvest cell on glass-filter plates, using the cell harvester.
- Dry glass-filter at 60 °C for 60 min.
- Add 20 µl of scintillation cocktail and determine the incorporated radioactivity using the microtiter plate scintillation counter.
- Save the data on a disk and transfer them to Sigma Plot for further analysis.
- Subtract the amount of radioactivity associated with the uninfected erythrocyte control
- Graph the percent growth as a function of the compound concentration.
- Fit the data points using a Hill function.

2.2.3 Parasitological evaluation of SC81458

2.2.3.1 *In vitro* antiplasmodial assay

The anti-malarial activity of SC81458 *in vitro* was determined as before. The activity of SC81458 comparing with chloroquine, quinine, mefloquine, halofantrine, quinidine, pyrimethamine, amodiaquin, artemisinin against Dd2, 3D7, D10, FCR3, 7G8, K1 and Thai19 were determinated.

2.2.3.2 Stage-specific susceptibility

The stage-specific susceptibility of SC81458 against *P. falciparum* was evaluated according to the method of Henri J. Vial, Sharon Wein with some modifications (Vial et al., 2004). Briefly, Dd2 was highly synchronized by three successive sorbitol lysis treatments. SC81458 was added at various concentrations to synchronized parasites in culture at rings (2-4 hrs post invasion), trophozoites (24-26 hrs post invasion) and schizonts (35-37 hrs post invasion). Incubation was continued for 6 hrs, cells were washed twice and resuspended in fresh complete

medium. [^{3}H]-Hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs and parasitemia was evaluated for each stage and expressed as a percentage of the control (without drug).

2.2.3.3 Time course of *Plasmodium falciparum* growth inhibition

The time course of *Plasmodium falciparum* growth inhibition of SC81458 against *P. falciparum* was evaluated according to the method of Henri J. Vial, Sharon Wein with some modifications (Vial et al., 2004). Briefly, Dd2 were highly synchronized by three successive sorbitol lysis. SC81458 was added at 100 nM, 500 nM, 1 μM, 5 μM, 10 μM to synchronized cultures at rings (2-4 hrs post invasion), trophozoites (24-26 hrs post invasion) and schizonts (35-37 hrs post invasion), respectively. After incubation for the times indicated, cells were washed twice and resuspended in fresh complete medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia were expressed as a percentage of the control (without drug).

2.2.3.4 Drug resistance induce property to Dd2

Drug resistance inducibility of SC81458 to Dd2 was evaluated according to the method of Rathod, et al (Rathod et al., 1997). Briefly, Dd2 were challenged by SC81458 at the concentration of 100 nM with different beginning parasite number (10^8,10^7,10^6,10^5) for eight weeks. At the same time, 5-fluoroorotate (100 nM) was used as drug resistance positive control. A small portion of the culture material was smeared onto a slide per week and the slide was stained with Giemsa in order to observe the appearance of recovered parasites.

3 Results

3.1 Cell based compounds screen and optimization

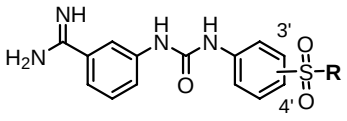
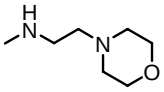
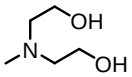
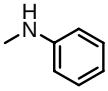
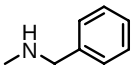
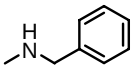
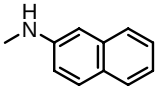
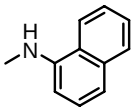
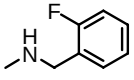
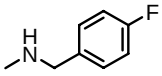
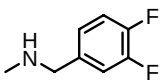


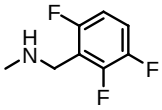
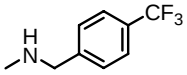
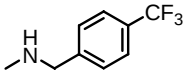
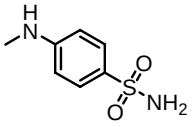
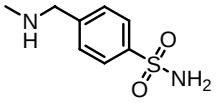
Fig. 3.1: The structure of sulfonamido-phenyl-ureido benzamidine compounds.

R stands for side chains variation shown in Table 3.1 showed the IC_{50}-values of different benzamidine derivatives against *P. falciparum* blood stages (Figure 3.1). All the sulfonamido-phenyl-ureido benzamidine compounds were designed and synthesized by 4SC AG, Munich. IC_{50}-values were determined, using a standard cell proliferation assay, in which *P. falciparum* blood stages (Dd2) were exposed to the drug for 72 hrs. The data obtained were analyzed and the half maximal inhibitory concentrations, IC_{50}-values, determined. As table 3.1 indicated, the compounds displayed a broad range of IC_{50}-values ranging from nano molar to micro molar. The replacement of simple aliphatic by aromatic groups showed a significant increase in activity (compare compound 13 with compounds 6-11). However, simple aniline derivatives were not active (see compound 12, 15, 16 and 24) and the methylene spacer of the benzyl moiety played a fundamental role for the high anti-*P. falciparum* activity (see compound 13, 17-23 and 25). In general, 4'-substitution gave more active compounds when compared with 3' substitutions (compare compound 13 with 14, 20 with 21; 22 with 23). Substitution of the phenyl ring with one or more fluoro atoms helped to increase the activity, and the tri-substitution with fluoro atoms produced a very active compound (see compound 20). The best activity (IC_{50} = 10 nM) was obtained with compound 25, where a bulky para-sulfonamino group was introduced. The removal of the amidino group or its complete substitution with an amine, amide or ester group abolished the anti-*P. falciparum* activity. Sulfonamido substitutions on the east side of the diarylurea were preferred to simpler carbonyl-amino, amino-sulfonyl and amino-carbonyl groups.

Table 3.1: Anti-*P. falciparum* activity of different sulfonamido-phenyl-ureido benzamidine derivatives.

The chemical structure of the parental compound is shown in Fig 3.1. ND, not determined, which was caused by a poor solubility of the compound in the medium.

Compound	Sulfon position	R-	Activity *vs* Dd2 IC_{50} (nM)
6	4’	$-NH_2$	49900
7	4’	$-NH-CH_3$	4400
8	4’	$-NH-CH_2-CH_2-CH_3$	4750
9	4’	$-NH-CH_2-CH_2-OH$	1100
10	4’		ND
11	3’		> 5000
12	4’		9000
13	4’		450
14	3’		> 5000
15	4’		8200
16	4’		17000
17	4’		620
18	4’		320
19	4’		1110
20	4’		17

21	3’		220
22	4’		390
23	3’		710
24	4’		19000
25	4’		10

As Table 3.1 showed, these compounds vary in the position of the sulfon and the R substitutions. The results of the SAR study indicated that the novel class of anti-malarial compounds was diarylurea, with a benzamidine group on the west side of the diaryl urea and a sulfonamido-benzyl substitution on the east side. This class of compounds was further characterized, particularly with the determination of their physicochemical and ADMET properties to find the ideal candidate for development as potential anti-*P. falciparum* drug and provide necessary data for further SAR analysis.

3.2 Anti-malarial mechanism: comparative study between sulfnamidomethylen and diarylsulfone compounds

Sulfonamido-phenyl-ureido benzamidine compounds were further optimized for better anti-*P. falciparum* activity with the direction of SAR analysis. We analyzed two subclasses: the “sulfnamidomethylen” and the “diarylsulfone”. As the two classes of compounds came from the same lead compound, the structures of them were very similar. However, we were not sure that the two classes of novel anti-*P. falciparum* compounds exerted their action by the same mechanism. The SAR analysis for further optimization was based on the assumption that all the analyzed compounds exert their action by the same mechanism. So we compared the anti-*P. falciparum* mechanism between the two compound classes, which was crucial for further SAR analysis.

The dose-response curves revealed important information, such as the shape and the steepness (Hill number). As Fig 3.2 shows, the dose-response curve of diarylsulfone compound showed a

biphasic property, while the dose-response curve of sulfnamidomethylen was single phasic and very steep. The dose-response curves of the two classes of compounds indicated that they exert their anti-*P. falciparum* action by different mechanisms.

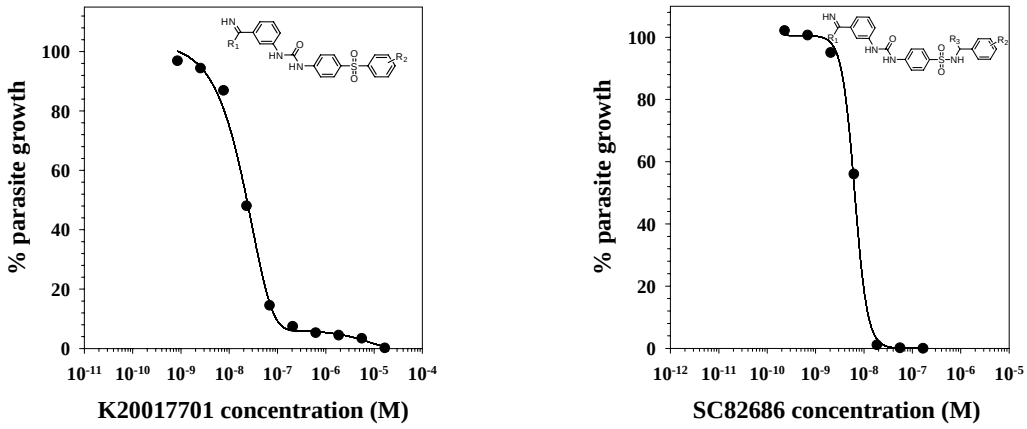


Fig. 3.2: The dose-response curves of K20017701 and SC82686.

K20017701 and SC82686 are representative example compounds of the diarylsulfone and sulfnamidomethylen subclasses, respectively.

The data from the microscopic examination was consistent with the dose-response curve of diarylsulfone, which revealed that, at low drug concentrations, parasites were not killed but rather growth-arrested. Full killing only occurred at much higher concentration. We speculate that these compounds might interact with two targets: with high affinity against a target that would block parasite growth (observed at low concentrations) and with a lower affinity against a target that would kill the parasites (observed at high concentrations).

To further compare the anti-*P. falciparum* mechanism of the two subclasses, a cross-resistance study between them was performed based on a sulfnamidomethylen resistant Dd2 strain. The sulfnamidomethylen resistant Dd2 strain, recovered from a long term exposure to a low concentration of SC81458, showed cross-resistance to active sulfnamidomethylen compounds (data not shown).

As Fig. 3.3 indicates, there was no significant difference between the sensitivity of K20017701 against SC81458 sensitive and resistant Dd2. However, the IC_{50}-value of SC81458 to the resistant Dd2 strain is 30 folds of that to parental Dd2. So the two subclasses of compound most likely kill Dd2 parasites by different mechanisms.

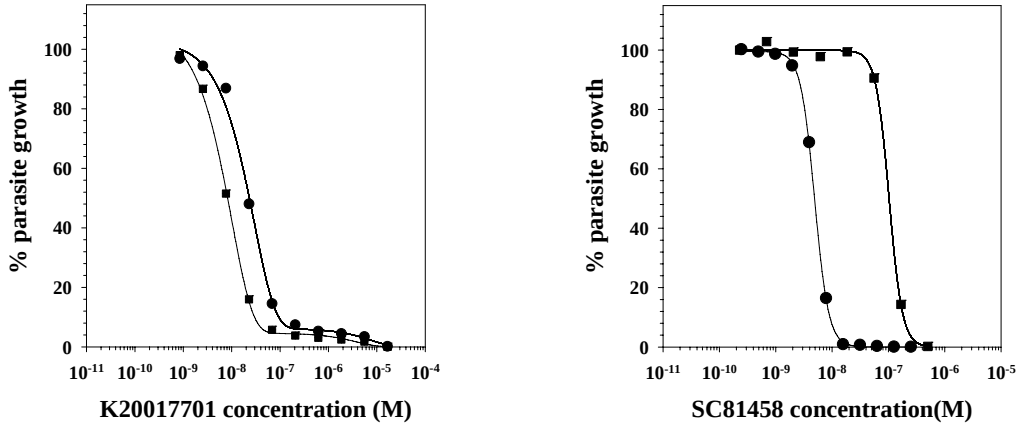


Fig. 3.3: The dose-response curves of K20017701 and SC81458 to Dd2 (cycle) and Dd2 recovered from 50 nM SC81458 (Square).

Dd2 cultures (10^8 infected erythrocytes/10ml/plate) were exposed to 50 nM of SC81458. Parasites were cultured by standard methods in the presence of 2% hematocrit with medium changes three times per week. In addition, cultures were split 1:2 with fresh erythrocytes once a week. Slides were prepared from the plate with each medium change and stained with Giemsa to test for the emergence of parasites. Dd2 parasite recovered 38 days post-challenge. The strain was transfered to drug free medium and assayed using the standard cell proliferation assay.

The dose-response curve of diarylsulfone revealed a biphasic dose-response curve. As Figure 3.4 indicated, similarly complex dose-response curves were observed when this compound was test against ring/trophorzoite and trophozoite stage parasites. The nature of the complex dose-response curves was not investigated in this study. A possible interpretation might be that this class of compounds interacts with multiple parasite factors. No further optimization was conducted on this subclass of compounds.

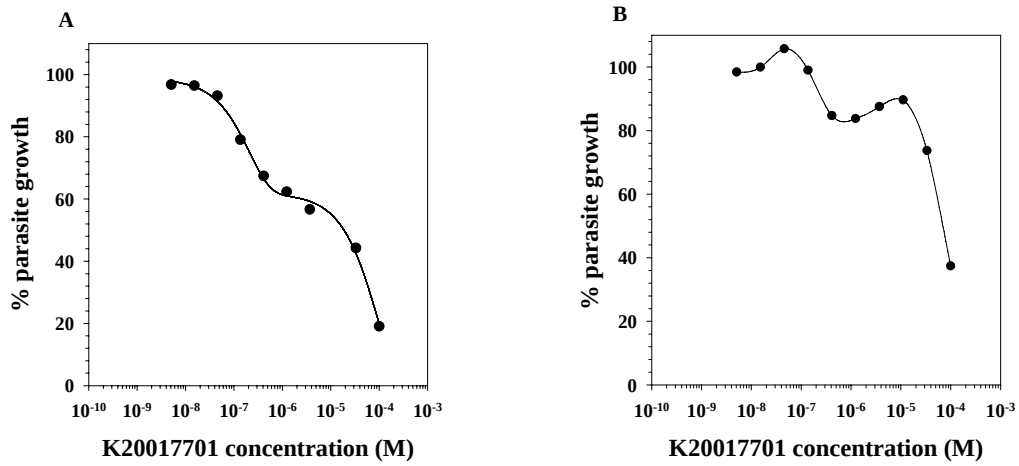


Fig. 3.4: Action of diarylsulfone against ring / trophozoite and trophozoite stages of *P. falciparum*. Proliferation assay using ring-stage (A) parasites (5 hrs post invasion) that were exposed to diarylsulfone (K20017701) for 22 hrs before [^{3}H]-hypoxanthine addition. Incubation with [^{3}H]-hypoxanthine for 3 hrs and the cells were subsequently analyzed for hypoxanthine incorporation. Proliferation assay using trophozoites (26 hrs post invasion) that were exposed to K20017701 for 6 hrs before [^{3}H]-hypoxanthine addition. Incubation with [^{3}H]-hypoxanthine was for 3 hrs and the cells were subsequently analyzed for hypoxanthine incorporation.

3.3 The structure-activity relationship of SC81458

SC81458 was obtained by further optimization of sulfnamidomethylen compounds, taking into consideration the *in vitro* anti-*P. falciparum* activity and the ADMET properties.

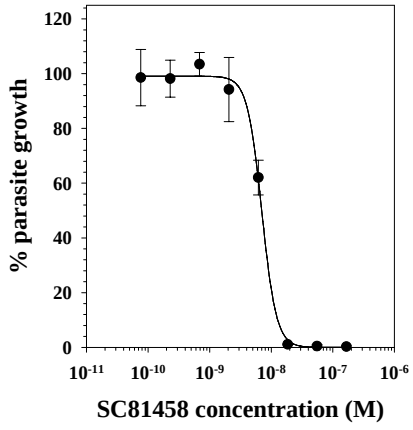


Fig. 3.5: The dose-response curve of SC81458 against *P. falciparum*.

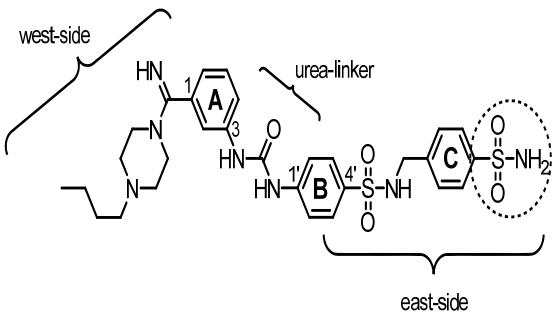
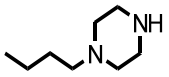
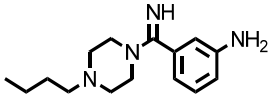
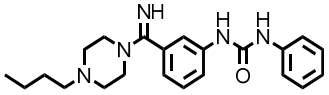
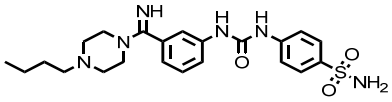
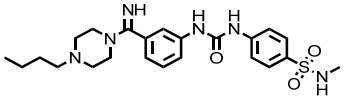
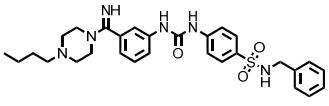
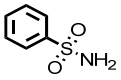


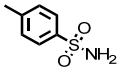
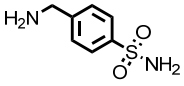
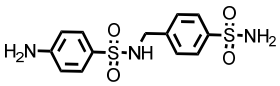
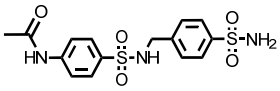
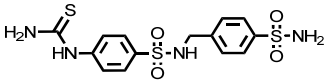
Fig. 3.6: The structure of SC81458

To further understand the structural determinants of SC81458, a structure-activity relationship study was performed by 4SC, which was based on the data of the IC_{50}-determinations and ADMET evaluation of a series of compounds. All the compounds used in the SC81458 SAR study were designed and provided by 4SC. SAR analysis on SC81458 would provide crucial information for further optimization. As Fig 3.6 indicates, the structure of the compound was divided into three regions and each one of them was investigated, introducing tailored structural variations. Four different blocks of compounds were synthesized and analyzed: short fragments, variation to the sulfonamidomethylen group at the east-side, variations at the urea bridge and variations at the functionalized amidine group at the west-side. All compounds were tested using a standard cell proliferation assay (Desjardins et al., 1979). The results are shown in tables 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7.

As table. 3.2 indicates, the presence of the amidino function on the west side was necessary for the anti-*P. falciparum* activity of SC81458, and no fragment starting from the east side showed any anti-*P. falciparum* activity even at a high concentration (16 µM). These data suggested that both east and west side of SC81458 are functionally important for the anti-*plasmodium* activity of this compound. A shorter fragment of SC81458, even with a smaller loss of molecular structure, did not exhibit good *in vitro* anti-*P. falciparum* activity.

Table 3.2: The IC_{50}-values of shorten side chains of starting from either the east or the west side against *P. falciparum*.

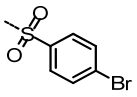
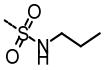
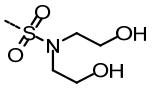
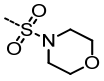
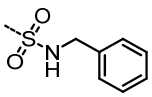
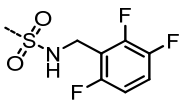
N°	Fragment Structure	IC_{50} (µM)
West		
1		> 16
2		ND
3		ND
4		4.56
5		2.17
6		0.594
east		
7		> 16

8		> 16
9		> 16
10		> 16
11		> 16
12		> 16

ND, not determined, which was caused by poor solubility of the compound in the medium.

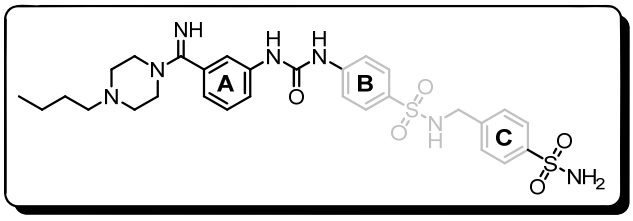
As table 3.3 indicates, the presence of a polar oxygen atom on the east-side of the molecule is detrimental to the activity (see compound 15, 16 and 17). Additionally, the dose-response curves of these compounds never showed the same steepness as that of the parent compound SC81458 (data not shown).

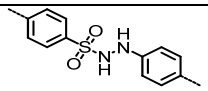
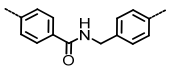
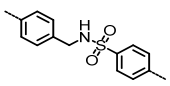
Table 3.3: Activity of east side analogues of SC81458 *vs P.falciparum* .

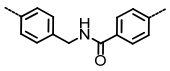
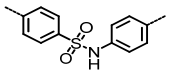
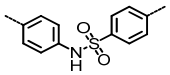
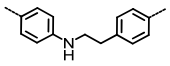
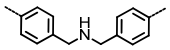
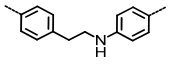
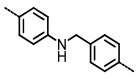
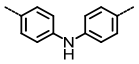
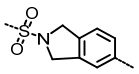
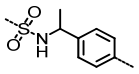
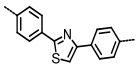
N°	Structure (-R)	IC_{50} (µM)
13		1.15
14		0.597
4		4.56
5		2.17
15		1.76
16		6.25
17		2.57
6		0.594
18		0.544

The sulfonamidomethylen linker of SC81458 is involved in the metabolic stability of the compound (4SC unpublished data). In an effort to improve the metabolic stability of SC81458, several structural analogues were synthesized with different tethering groups linking ring B and C. As table 3.4 indicates, none of the compounds reached the activity of the parent compound; even the most conservative replacements (see compounds 19, 20, 23). The fundamental role of the sulfone group is shown by compound 20 with an IC_{50}-value higher than 2 µM. Reversal of the tether (long, short or carbonyl) produced compounds which were ca. 40-fold less potent (see compounds 21, 22, 24). The use of alkyl-amino linkers of different length gave compounds with very little actives (see compounds 25, 26, 27, 28). The nitrogen alone linking the two rings was not much active as well (see compound 29). The best compound was the sterically hindered isoindoline derivative (see compound 30) with an IC_{50}-value of 73 nM. The branched compound 31 (racemic mixture) was less active. From these selected examples it is clear that a replacement of the sulfonamidomethylen linker can be performed only at the cost of the high anti-*P. falciparum* activity.

Table 3.4: Anti-*P.falciparum* activity of SC81458 analogues, with different tethering groups linking ring B and C.

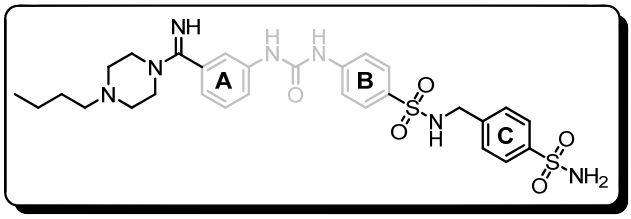


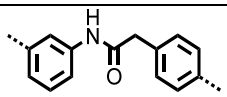
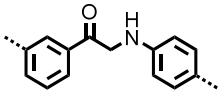
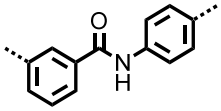
N°	Structure of the tether	IC_{50} (µM)
19		1
20		2.17
21		0.168

22		0.732
23		0.266
24		0.217
25		2.18
26		ND
27		1.44
28		2.53
29		4.1
30		0.073
31		0.224
32		1.71

Few analogues were synthesized to verify the influence of the urea linker between phenyl rings A and B to the anti-*P. falciparum* activity of the parent compound SC81458. As table 3.5 indicates, a simple replacement of a –NH– group with the isosteric $–CH_2–$ group (see compound 33) caused a significant decrease of anti-*P. falciparum* activity (174-fold less active than SC81458), confirming the important role of this hydrogen-bond donor group. Meanwhile, a shorter analogue (see compound 35) or scrambling the carbonyl and amino group (see compound 34) were also not tolerated.

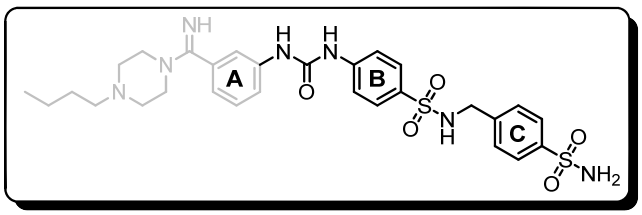
Table 3.5: Activity of the urea modified analogues of SC81458 *vs P.falciparum*.

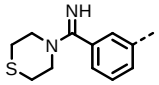
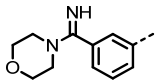
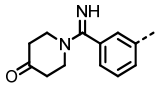
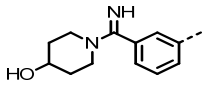
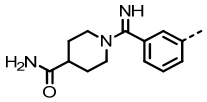
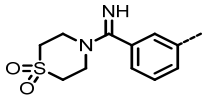
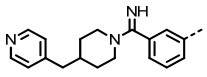


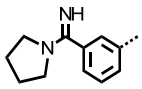
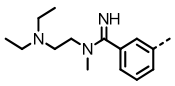
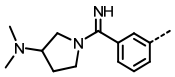
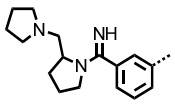
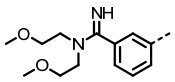
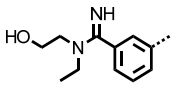
N°	Structure of the tether	IC_{50} (µM)
33		0.87
34		7.16
35		0.372

A series of SC81458 west side analogues were designed and synthesized by 4SC to study the SAR of the west side of SC81458. As table 3.6 indicates, no compounds showed the same anti-*P.falciparum* as SC81458, which suggests that the presence of additional nitrogens seems to be crucial for a high anti-*P.falciparum* activity.

Table 3.6: Anti-*P. falciparum* activity of the west side analogues of SC81458.

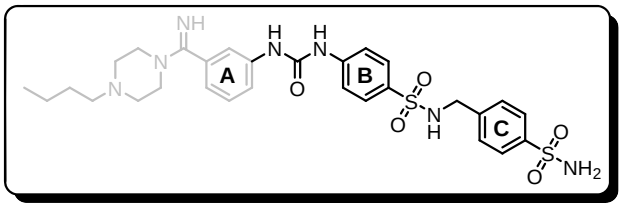


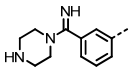
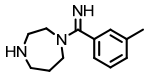
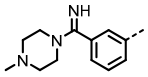
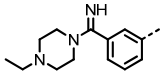
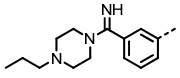
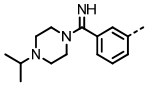
N°	Benzamidine	IC_{50} (µM)
	SC81458	0.0055
36		0.39
37		ND
38		0.278
39		0.165
40		0.149
41		0.041
42		0.995

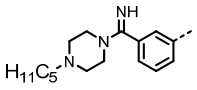
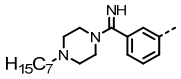
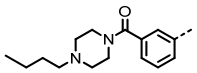
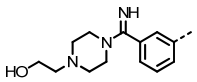
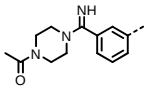
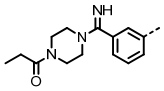
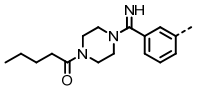
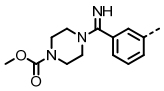
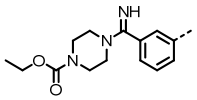
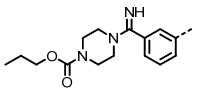
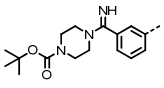
43		0.176
44		0.089
45		0.146
46		0.034
47		0.496
48		0.201

The SAR study on the west side of SC81458 focused on the piperazine-containing west side analogues for more detailed information. As expected, some of the analogues exhibited excellent anti-*P.falciparum* activity, even more potent than SC81458 (table 3.7). The results indicated that the size of the carbon chain and the amidine are closely associated with the anti-*P. falciparum* activities of this series of compounds. Furthermore, acylation or carbamoylation of the piperazine nitrogen analogs exhibited comparable or even more potent anti-*P. falciparum* activities than the parent compound (see compound 60, 62, 63).

Table 3.7: Anti-*P. falciparum* activity of the piperazine-containing west side analogues of SC81458.



N°	Piperazine structure	IC_{50} (nM)
	SC81458	5.5
49		75
50		75
51		277
52		25
53		7
54		38

55		13
56		196
57		5420
58		100
59		25
60		3.8
61		25
62		2.8
63		4.4
64		12
65		606

These data together with the ADMET properties (provided by 4SC) were used for the SAR study of SC81458, which eventually led to the discovery of SC83288. The discovery of SC83288, a sulfnamidomethylen compound with better bioavailability and anti-*P. falciparum*

activity than SC81458, is an excellent example for SAR successfully directing drug optimization.

3.4 Parasitological evaluation of SC81458

We found many compounds that showed *in vitro* anti-*P. falciparum* activity. However, it does not mean all of them would become anti-*P. falciparum* drugs. Actually, only very few of the active anti-*P. falciparum* compounds are being selected as candidate compounds for future development. Above all, the active compound should showed good ADMET properties. Otherwise, it is impossible for this compound to become a candidate compound for further study. Furthermore, the active compound must exhibit good parasitological properties, such as failure to induce drug resistance under *in vitro* conditions, activity against several parasite stages and a fast killing speed etc. The work of 4SC indicated that SC81458 exhibit ideal ADMET properties. The parasitological evaluation of SC81458 was performed as follows.

An excellent anti-*P. falciparum* drug should be efficious against a broad range of *P. falciparum* strains. As Fig 3.5 indicates, SC81458 showed excellent *in vitro* activity against blood stages of the *P. falciparum* clone Dd2.

Table 3.8: The IC_{50}-values (nM) of SC81458 and the other licensed anti-*P. falciparum* drugs against different *P. falciparum* strains. All data were from three independent experiments.

P.falciparum strains	SC81458	Chloroquine	Quinine	Mefloquine	Halofantrine	Quinidine	Pyrimethamine	Amodiaquin	Artemisnin
Dd2	5,5±1,3	70,5±12,1	124,1±25,2	1,3±0,1	16,8±4,7	37,4±7,8	>15000	6,8±1,4	5,0±0,3
D10	9,1±3,5	11,4±3,2	58,2±5,1	3,5±0,1	31,7±4,6	15,8±1,2	10,5±1,6	7,1±1,5	8,5±0,4
3D7	12,5±3,9	2,9±0,7	13,7±2,1	3,7±0,6	19,8±2,2	14,2±1,1	15,8±2,8	7,8±1,1	7,7±0,3
FCR3	13,1±7,9	25,3±4,2	182,2±27,4	1,2±0,2	7,0±1,5	32,4±6,4	31,3±7,8	7,6±0,4	5,0±0,9
K1	9,4±2,9	69,7±14,1	46,2±4,1	1,2±0,1	5,1±1,4	11,8±2,7	>15000	9,2±0,9	3,3±0,8
Thai19	7,4±1,3	74,1±17,1	62,2±10,0	1,1±0,1	4,3±0,7	21,5±5,1	>15000	8,5±0,8	2,8±0,5
7G8	18,4±4,5	30,2±7,3	69,1±17,3	0,2±0,1	2,7±0,5	15,9±3,9	>15000	7,4±1,9	1,1±0,3

As Tab. 3.8 shows, SC81458 exhibited excellent *in vitro* anti-*P. falciparum* activities against seven *P. falciparum* strains, which were derived from different endemic areas and which showed different drug sensitivities to established anti-malarial drugs. The IC_{50}-values ranged from a few nanomolar to less than 20 nM. The multi-drug resistant strains, such as Dd2, K1 and Thai19, were significantly more sensitive to SC81458 than were the drug-sensitive strains investigated ($P< 0.05$). The results indicated that SC81458 killed a wide range of *P. falciparum* strains, irrespectively of their drug resistance phenotypes.

3.4.1 Stage-specific activity of SC81458

To further study the anti-*P. falciparum* activity of SC81458, I evaluated the activity of SC81458 against different *P. falciparum* blood stages. Highly synchronized cultures of Dd2 containing rings (2-4 hrs post invasion), trophozoites (24-26 hrs post invasion) and schizonts (35-37 hrs post invasion) were exposed to different concentrations of the compound for six hrs. The parasites were subsequently washed twice and transferred to drug-free medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. The IC_{50}-value of SC81458 against the different *P. falciparum* blood stages was used to evaluate the stage-specific susceptibility of this compound. Under these conditions, SC81458 exerted the highest activity against trophozoites, with an IC_{50}-value of 36.9 nM (Fig. 3.7A). A comparable, but slightly lower activity was observed against schizonts, with an IC_{50}-value of 53.7 nM (Fig. 3.7). The activity against ring stages was only moderate, with an IC_{50}-value of around 5 µM (Fig 3.7). The data indicated that SC81458 exhibits a stage-specific activity against *P. falciparum*.

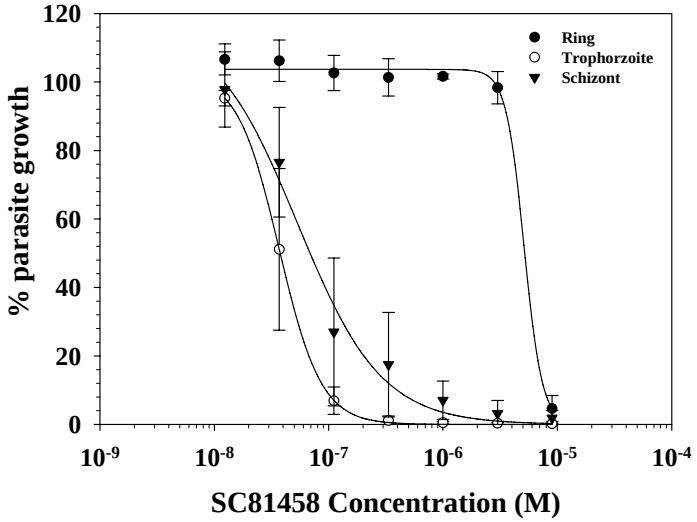


Fig. 3.7: Stage Specific activity of SC81458 against *P. falciparum*

SC81458 was added at various concentrations to synchronized parasites in culture at ring stage (2-4 hrs), trophozoite stage (24-26 hrs) and schizont stage (35-37 hrs). Incubation was continued for 6 hrs, cells were washed twice and resuspended in fresh complete medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia were evaluated for each stage and expressed as a percentage of the control (without drug). The data represent the means ± SEM of three independent determinations.

3.4.2 Time course of growth inhibition

The killing speed of an anti-*P. falciparum* drug is a very important property for the drug, since it is closely related to the clinical efficacy. Furthermore, it also relates to the emergence of drug resistance. A drug, such as artemisinin, that quickly kills the parasites, quickly decreases the number of the *in vivo* parasite, i. e. only few or even no parasites have a long term exposure to the drug. In turn, the possibility for the emergence of drug resistance would be reduced. Additionally, killing speed and PK property are crucial parameters of drug dosage. To assess how quickly SC81458 is able to kill the parasite, we exposed highly synchronized cultures of Dd2 containing rings (2-4 hrs post invasion), trophozoites (24-26 hrs post invasion) and schizonts (35-37 hrs post invasion) to different concentrations of SC81458 (ranging from 0.1 to 10 µM). The parasites were washed twice and transferred to drug-free medium at different time point. Then [^{3}H]-hypoxanthine was added at 54 hrs (counted post invasion of the first replication cycle) to monitor parasite viability. As depicted in Fig. 3.8 A, B and C, SC81458 killed trophozoites and schizonts within hours after exposure. For example, at a concentration of 500 nM, 100 % of the trophozoites and 100 % of the schizonts were dead within 1 hr and 5 hrs, respectively. At 100 nM, the lowest concentration tested, almost 80 % of the trophozoites and 40 % of the schizonts were killed within the first hour.

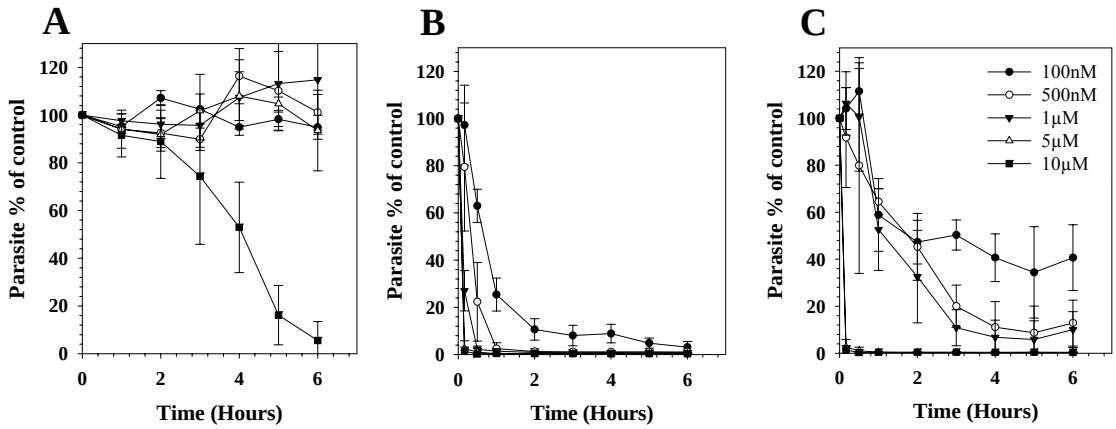


Fig. 3.8: The killing speed of SC81458 against *P. falciparum* at different blood stages.

SC81458 was added at 100 nM, 500 nM, 1 µM, 5 µM, 10 µM to synchronized cultures at ring stage (2-4 hrs), trophozoite stage (24-26 hrs) and schizont stage (35-37 hrs), respectively. After incubation for the times indicated, cells were washed twice and resuspended in fresh complete medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia were expressed as a percentage of the control (without drug). The data represent the means ± SEM of three independent determinations.

The parameter of $t_{1/2}$ is a constant to evaluate the killing speed of anti-infectious drugs, which means the time it takes for an anti-infectious drug to kill half of the pathogens at a certain concentration. The $t_{1/2}$ could be used as a standard to compare the killing speed between different drugs. As expected, the t1/2 curves declined with increasing concentrations of SC81458.

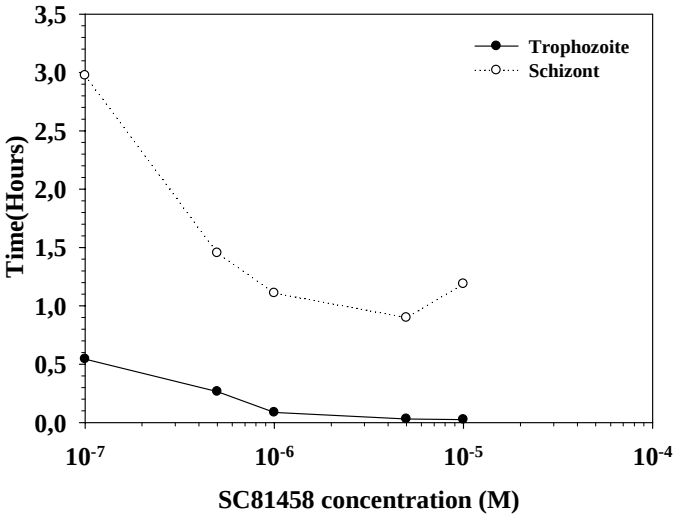


Fig. 3.9: The Killing $t_{1/2}$ of SC81458 against *P. falciparum* at different stages.

SC81458 was added at 100 nM, 500 nM, 1 µM, 5 µM, 10 µM to synchronized cultures at trophozoite stage (24-26 hrs) and schizont stage (35-37 hrs). After incubation for the times indicated, cells were washed twice and resuspended in fresh complete medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia was expressed as a percentage of the control (without drug). Then the data were fit to exponential decrease equation to calculate $t_{1/2}$. All data were from three independent experiments.

The result of the time course study is consistent with the stage specific study (Fig 3.7 and Fig 3.8).

3.4.3 Drug resistance inducing property of Dd2

The emergence of drug resistance means a decrease or even loss of responsitiveness. Therefore, drug candidate should not be able to induce drug resistance. The drug resistance property of SC81458 was determinated based on the standard protocol of Rathod et al (Rathod et al., 1997). Different inoculating number of Dd2 parasites were exposed to SC81458 (100 nM) for eight weeks. At the same time, 5-fluoroorotate was used as a positive control. 5-fluoroorotate was chosen as a positive control owing to the fact that its IC_{50}-value is comparable to that of

SC81458 (approximately 5 nM). Parasites were cultured by standard methods in the presence of 2% hematocrit with medium changes three times per week. In addition, cultures were split 1:2 with fresh erythrocytes once a week. Slides were prepared from every flask with each medium change and stained with Giemsa to test for the emergence of parasites. Ten parasites in one plate (3 replicates) were used as the control for parasite culture. As Tab 3.9 indicates, all the parasite culture controls grew up within three weeks in the absence of drugs (data not shown). Parasites recovered in the drug-resistance positive control plates, where 10^8 parasites were exposed to 5-fluoroorotate at the concentration of 100 nM. No parasites recovered in all the plates exposed to SC81458, which indicated Dd2 could not develop *in vitro* resistance to SC81458 at the concentration of 100 nM.

Table 3.9: Drug resistance inducing property of Dd2.

Inocculating number of parasite	**10^5**	**10^6**	**10^7**	**10^8**
SC81458 (100 nM / 8 weeks)	---	---	---	---
5-Fluoroorotate (100 nM / 8 weeks)	---	---	---	+++

3.5 The further optimization of SC81458 for better activity and ADMET properties

SC81458 showed many excellent anti-*P. falciparum* drug properties. However, the bioavailability of SC81458 was found to be poor (4SC unpublished data). As part of new round of SAR analysis, 4SC synthesized a series of derivatives, one of the derivatives, compound SC83288, turned out to have a very potent anti-*P. falciparum* activity, with an IC_{50}-value of 3.8 nM (Fig 3.10). As described for SC81458, I evaluated the stage-specific activity, the killing speed, the *in vitro* activity against a panel of different *P falciparum* strains and the possibility of inducing resistance under *in vitro* culture condition (This experiment is still ongoing).

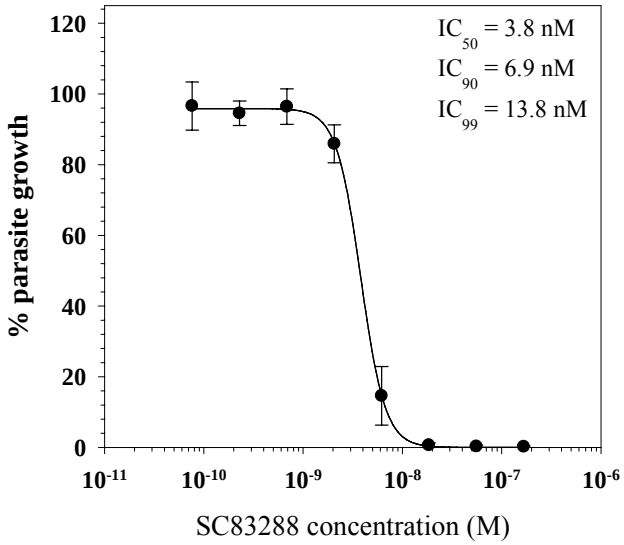


Fig. 3.10: The dose-response curve of SC83288 against *P. falciparum*. The data represent the means ± SEM of three independent determinations.

As Tab. 3.10 indicates, the seven *P. falciparum* strains examined were sensitive to SC83288, with IC_{50}-values ranging from 3 nM to 10 nM.

Table. 3.10: The activity of SC83288 against different *P. falciparum* strains. All data were from three independent determinations.

***P.falciparum* strains**	**IC_{50}-values**
Dd2	3,8 ± 0,5
D10	5,3 ± 0,5
3D7	8,2 ±2,0
FCR3	6,4 ± 0,5
K1	4,9 ±0,8
Thai19	3,9 ± 0,8
7G8	10,2 ±0,5

Both SC81458 and SC83288 belong to the same subclass of sulfnamidomethylen compounds. SC83288 revealed the same stage specific activity as did SC81458, i.e., trophozoites and schizonts were more sensitive to SC83288 than were rings, with IC_{50}-values of 23.8 nM, 124.8 nM, and 2968.3 nM, respectively. In terms of the killing speed, SC83288 killed 90% of the trophozoite and the schizonts within 6 hrs at a concentration of 100 nM and 500 nM, respectively. The killing speed against rings was lower. 60-80% of the rings were killed after 6 hrs at a concentration of 10 μM.

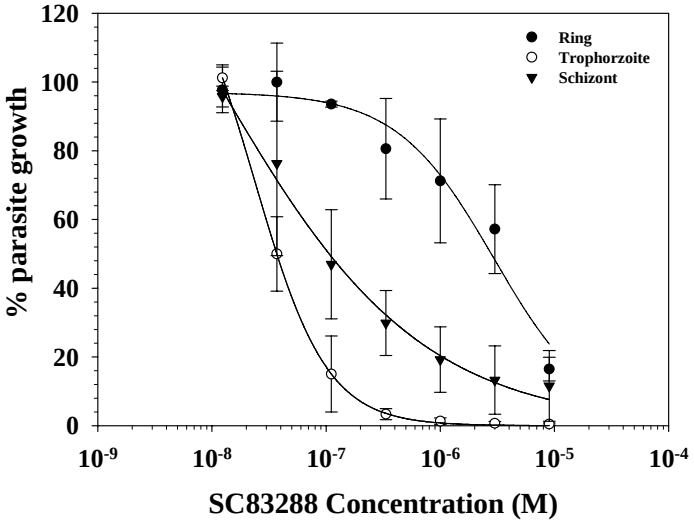


Fig. 3.11: Stage Specific evaluation of SC83288 to Dd2.

SC83288 was added at various concentrations to synchronized parasites in culture at ring stage (2-4 hrs), trophozoite stage (24-26 hrs) and schizont stage (35-37 hrs). Incubation was continued for 6 hrs, cells were washed twice and resuspended in fresh complete medium.[^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia were evaluated for each stage and expressed as a percentage of the control (without drug). The data represent the means ± SEM of three independent determinations.

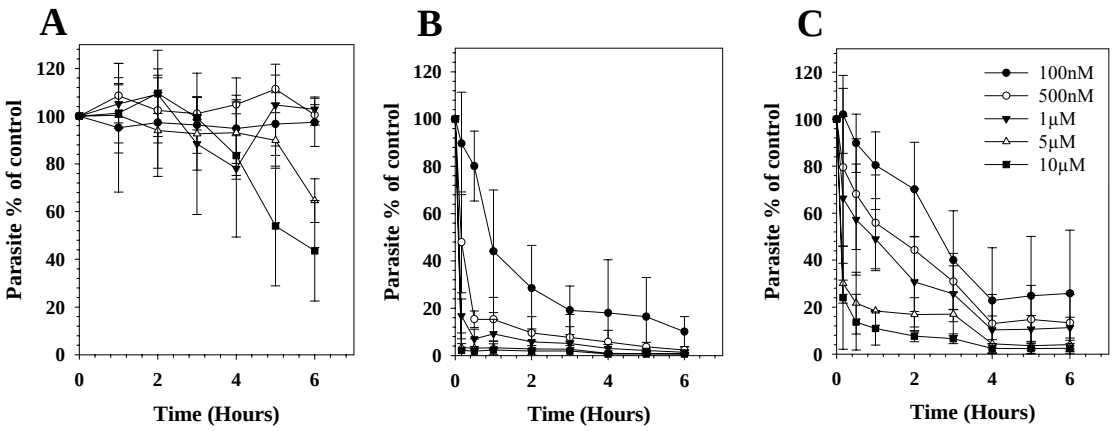


Fig. 3.12: The killing speed of SC83288 at different concentrations against *P. falciparum* at ring stages.

SC83288 was added at 100 nM, 500 nM, 1 µM, 5 µM, 10 µM to synchronized cultures at ring stage (2-4 hrs), trophozoite stage (24-26 hrs) and schizont stage (35-37 hrs), respectively. After incubation for the times indicated, cells were washed twice and resuspended in fresh complete medium. [^{3}H]-hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia were expressed as a percentage of the control (without drug). The data represent the means ± SEM of three independent determinations.

The $t_{1/2}$-values obtained for SC83288 were comparable to those of SC81458.

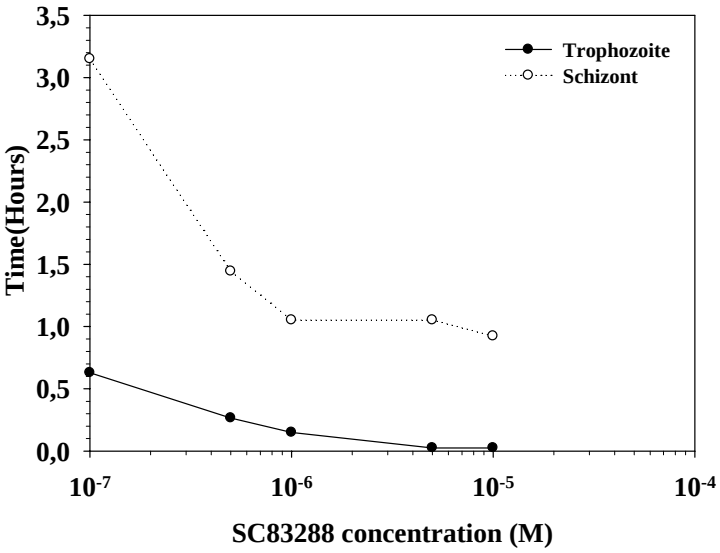


Fig. 3.13: The Killing $t_{1/2}$ of SC83288 against *P. falciparum* at different stages.

SC83288 was added at 100 nM, 500 nM, 1 µM, 5 µM, 10 µM to synchronized cultures at Trophozoite stage (24-26 hrs) and Schizont stage (35-37 hrs). After incubation for the times indicated, cells were washed twice and resuspended in fresh complete medium. [^{3}H] hypoxanthine was added at 54 hrs to monitor parasite viability. Reactions were stopped at 78 hrs, and parasitemia was expressed as a percentage of the control (without drug). Then the data were fit to exponential decrease equation to calculate $t_{1/2}$.

As Fig 3.14 indicated, the SC81458 resistant Dd2 strain also showed cross-resistance to SC83288, the IC_{50}-value increased almost 15 fold as compared to parental Dd2 strain. It is possible that SC83288 interacts with the same target as does SC81458. Furthermore, the positive correlation (r^2=0.91) between the IC_{50}-values of SC81458 and SC83288 against the seven *P. falciparum* strain suggests that the two compounds exert their action by the same mechanism (Fig. 3.15).

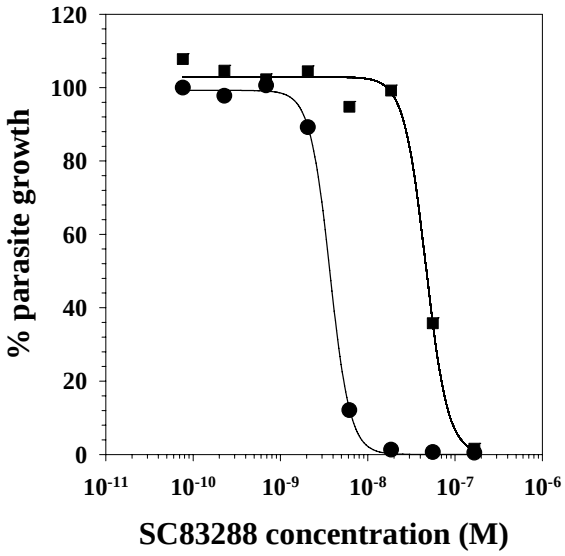


Fig. 3.14: The dose-response curve of SC83288 against *P. falciparum* (Ring) and SC81458 resistant Dd2 (Square).

Dd2 cultures (10^8 infected erythrocytes/10ml/plate) were exposed to 50 nM of SC81458. Parasites were cultured by standard methods in the presence of 2% hematocrit with medium changes three times per week. In addition, cultures were split 1:2 with fresh erythrocytes once a week. Slides were prepared from the plate with each medium change and stained with Giemsa to test for the emergence of parasites. Dd2 parasite recovered 38 days post-challenge. The strain was transfered to drug free medium and assayed using the standard cell proliferation assay.

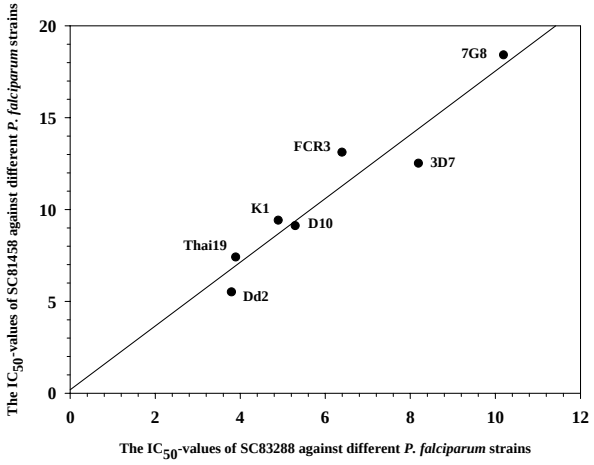


Fig. 3.15: The correlation between the IC_{50}-values of SC83288 and SC81458 against different *P. falciparum* strains.

4 Discussion

My data show that SC81458 and SC83288 represent a new class of compounds with potent activity against *P. falciparum* blood stages. The compounds are highly active against trophozoites and schizonts, with IC_{50}-values in the lower nano-molar range. Moreover, they are able to kill the parasite within less than one hour of exposure.

SC81458 and SC83288 were developed from the same lead compound of biphenyl urea. During the initial phase of our project, a large number of virtual compounds were designed as inhibitors of the *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) based on the structure of *Pf*LDH and screened virtually by 4SC using docking experiments (Leban et al., 2004), which was based on the consideration and calculation of the interaction energy, hydrogen bonding and solvation energy changes. Presently, target-based drug design is an important approach for anti-*P. falciparum* drug development. *Pf*LDH, structurally and functionally different from the human host isozymes, is an essential enzymes of *Plasmodium falciparum*. Furthermore, the crystal structure of *Pf*LDH is available. Therefore, *Pf*LDH is a potential drug target for the development of novel anti-malarials.

The *in silico* approach identified the biphenyl urea compounds as a novel structural class of potent anti-malarial agents. However, the inhibition activity of this class of compounds against the *Pf*LDH enzyme was very low, which means this class of compounds kill *P. falciparum* parasites through another unknown mechanism (Leban et al., 2004). Therefore, we changed our strategy and used cell based compound screening for further development. This approach tests the anti-malarial activity of compounds by the killing action to the parasites directly. Actually, many anti-malarial drugs, such as mefloquine, halofantrine and artemisnin, were developed by this approach. As the screen was based on the cell level, the target of the biphenyl urea compounds remained unknown when we obtained them. So it is impossible for us to get any information on the target.

The SAR study on diarylureas compounds was driven to guide further optimization, which would establish effective predictive models based on the experimental data (Fig. 3.1) and the information on essential chemical requirements for biological activity. This work was based on the assumption that all the tested analogues interact with the same target in the parasite. The substituent parameters from physical chemistry were used to correlate chemical properties with biological activity to provide important directions for the next round of optimization. Until now, we have finished 14 rounds of screen and optimization, with more than 300 compounds tested (data not shown). With the assistance of the SAR

study, we obtained the first generation of diarylureas antiparasitic compounds, a class of sulfonamido-phenyl-ureido benzamidine compounds. With further optimization of the sulfonamido-phenyl-ureido benzamidine compound, two subclasses of active compounds, sulfnamidomethylen and diarylsulfone, were obtained.

As Fig 3.2 indicates, the two subclasses of novel anti-*P. falciparum* compounds exerted their action by different mechanisms. The dose-response curves of diarylsulfone compounds showed a biphasic behavior, which means these compounds might interact with different targets or interact with the same target with different forms of post-translational modification during the whole blood stages. The binding of the diarylsulfone compound with a high affinity target (or high affinity target form) would block parasite growth and the binding with lower affinity (or low affinity target form) would kill the parasites. However, the results of Fig 3.3 indicated that there are different targets even in the same blood stage. Furthermore, the sulfnamidomethylen resistant Dd2 did not show any resistance to the diarylsulfone compounds (Fig. 3.3), which indicated that the two subclasses of compounds kill parasites by different mechanisms. The single phasic property of the dose-response curve of sulfnamidomethylen indicated that this class of compounds interact with a single target in all the erythrocytic phase stages. The sulfnamidomethylen compound was further optimized for more potent anti-malarial activity and better pharmacokinetic properties. Finally, we obtained SC81458 and SC83288, which showed the best anti-*P. falciparum* activity (Fig 3.5 and 3.10).

SC81458, which was first obtained, showed excellent *in vitro* anti-*P. falciparum* activity and good ADMET properties. Then a very accurate SAR study on SC81458 was performed by 4SC, which focused on the relationship between chemical structures and ADMET properties / anti-*Plasmodium* activity of this compound. Based on the SAR study on diarylureas compounds, the structure of SC81458 was divided into three regions (Fig. 3.6) and each one of them was investigated, introducing tailored structural variations. Few structural features of SC81458 were maintained unaltered; for example the phenyl rings A and B with the 1, 3 and 1', 4' functionalized positions. As tables 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7. indicated, the anti-*P. falciparum* activities of four different blocks of compounds were determined, which include short fragments, variation to the sulfonamidomethylen group at the east-side, variations at the urea bridge and variations at the functionalized amidine group at the west-side. Furthermore, the ADMET properties of all the compounds above were evaluated. Then, all the data on ADMET and anti-*Plasmodium* activity were combined together for SAR analysis. The results indicated that the rings in SC81458 are

fundamental to preserve the high activity. The functionalized positions of the rings are also essential for the high activity. Both the east and west side of SC81458 are necessary for anti-*plasmodium* activity. Even a smaller loss of the molecular structure would cause a decrease in anti-*P. falciparum* activity (table 3.2). The east-side ending sulphonamide was maintained as substituent group of phenyl ring C, which is necessary to confer the characteristic steepness to the dose-response curves of this class of compounds. Analogues bearing different substituents at this position showed more flat dose-response curves than SC81458. The size of the carbon chain and the amidine in the west side are closely associated with the anti-*P. falciparum* activities of the analogues. Acylation or carbamoylation of the piperazine nitrogen analogs even exhibited more potent anti-*P. falciparum* activities than SC81458 (table 3.7). Furthermore, the sulfonamidomethylen linker of SC81458 is involved in the metabolic stability of the compound (4SC unpublished data), which is closely related to the PK property of SC81458. The PK data of SC81458 (not shown) indicated that the metabolic stability of this compound should be improved in the further optimization for better *in vivo* activity. However, the results indicated that all the alterations of the sulfonamidomethylen linker would cause a loss of high anti-*P. falciparum* activity to SC81458 (table 3.4). The SAR study of SC81458 provided crucial direction for further optimization of the sulfnamidomethylen compound. The successful discovery of SC83288, which showed significantly improved bioavailability and slightly better anti-*Plasmodium* activity than SC81458 does, is powerful evidence for the direct effect of the SAR study. Bioavailability is an important ADMET property of a drug, which is used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation. Good bioavailability is crucial for a promising anti-*Plasmodium* candidate drug as the drug is expected to be orally administered in a poor country. The discovery of SC83288 caused important progress in the development of the sulfnamidomethylen compound.

As a novel class of anti-*Plasmodium* compounds, SC81458 and SC83288 exhibited excellent activities against a wide range of *P. falciparum* strains. As table 3.8 and 3.10 showed, seven *P. falciparum* strains isolated from different epidemic regions were used for the *in vitro* activity evaluation, which included Dd2, 3D7, D10, K1, FCR3, 7G8 and Thai19. The seven *P. falciparum* strains exhibited different sensitivities to the commonly used anti-*P. falciparum* drugs, including chloroquine, quinine, mefloquine, halofantrine, quinidine, pyrimethamine, amodiaquin, artemisnin (table 3.8). However, all the IC_{50}-values of SC81458 and SC83288 against the seven *P. falciparum* strains are in the same nano

molar range. Dd2, K1 and Thai19 are of high-level chloroquine resistance among the seven strains. However, the three chloroquine resistant strains were significantly more sensitive to SC81458 and SC83288 than were the other chloroquine sensitive strains ($P<0.05$). Thus, sulfnamidomethylen compound may become a promising substitute for some of the presently used drugs against which resistance has emerged. Furthermore, there is a strong correlation between the IC_{50}-values of SC81458 and SC83288 against the seven *P. falciparum* strains, suggesting that the two compounds interact with the same target in the seven strains and that the difference in IC_{50}-values between the two compounds is caused by a different binding affinity to the target. Meanwhile, there is no significant correlation between the IC_{50}-values of sulfnamidomethylen compounds and the other drugs tested, suggesting sulfnamidomethylen compounds exert their anti-*P. falciparum* action by a novel mechanism

A good anti-malarial drug should cure this disease with once or twice a day dosing within a maximum of three-day therapy, which means the drug should be able to kill the parasites in different blood stages very quickly. Otherwise, some parasites might escape from the therapy and reach the next life cycle, which would cause a failure of three-day therapy. As Fig 3.7 and 3.11 indicated, the trophozoites and schizonts are very sensitive stage to the two compounds, with 6 hrs IC_{50}-values around 30 nM and 100 nM, respectively. However, the rings exhibit the IC_{50}-values against the two compounds in a micromolar range. The result of the time course study is consistent with the stage specific study (Fig 3.8 and Fig 3.12). The killing speed of SC81458 and SC83288 against the trophozoites is the fastest among the three stages, which is close to artemisinin dose (data not shown). Additionally, the killing speed against schizont stage is faster than that against ring stage. The rings can be killed by more than 60% in 6 hrs by SC81458 or SC83288 only at the concentration of 10μM. However, the lowest concentration of SC81458 or SC83288 is able to kill schizont stages by more than 90% in 6 hrs between 100 and 500nM, and even less than 100nM in trophozoite stages. The same phenomenon is seen for chloroquine. Actually, this is a very common phenomenon for most anti-malarial drugs as trophozoites and schizonts are more metabolically active than rings (Tamez et al., 2005). Only few drugs, such as artemisinin and TE3 (Schlitzer, 2008; Vial et al., 2004), showed activity against all the blood stages of *Plasmodium falciparum*. However, no anti-malarial drug could be used alone due to drug-resistance. We can design a combination for SC81458 or SC83288 based on the data of stage-specificity. The combined drugs would show activity against all the blood stages of *Plasmodium falciparum* and exhibit more efficacy against malaria.

The quick clearance of SC81458 and SC83288 against the trophozoites and schizonts indicates that the number of parasite will decrease significantly in one life cycle or less time. There would be few of or even no parasites exposed to the anti-*Plasmodium* drug, which means a less opportunity for the parasites to develop resistance to this drug. Furthermore, the time course of *Plasmodium falciparum* growth inhibition and the stage-specific susceptibility study would also provide important data for future clinical use of the two compounds, such as the dosage regimen and the combination protocol with the other anti-malarial drugs. We can design a rational combination protocol for SC81458 or SC83288 with the other licensed anti-malarial drugs based on the data above. *P. falciparum* parasites at both trophozoite and schizont stages are sensitive to SC81458 or SC83288, which suggests that the two candidate drugs should be combined with a rings-sensitive drug, such as artemisinins or T3. Then the combination would show better killing speed against the parasites in different blood stages, which is crucial against the drug resistance selection.

As death by severe malaria usually occurs within 48 hours of presentation, equal to the time for one asexual cycle of the blood-stage infection, so the anti-*P. falciparum* drugs must kill the current generation of *P. falciparum* malaria parasites and decrease the amount of parasites emergent in the next cycle. Parasite killing by anti-malarial drugs is a first-order process, which leads to a log-linear reduction in parasite numbers with time. Uninhibited blood-stage multiplication at 100% efficiency results in a parasite multiplication rate (PMR) equal to the median number of viable merozoites liberated by rupturing schizonts. *In vivo* efficiencies may exceed 50% in nonimmune patients, resulting in PMRs of approximately 10 per asexual cycle. Anti-malarial drugs convert this positive value to a negative value, resulting in PMRs that range between 10^{-1} and 10^{-4} per cycle. These negative PMRs are also termed parasite killing rates or parasite reduction ratios, which depend on the killing speed and the concentration of the anti-malarial drug and the time of parasite exposure to the drug. We can model the clinical action for the sulfnamidomethylen compound. With clinical presentation, the parasites start a new life cycle in the body of severe malarial patients. Then the patient presents to a clinician and is prescibed SC81458 or SC83288. As this class of compound is less toxic, the *in vivo* concentration would reach the magnitude of micro molar. When the drug was absorbed by the body, the present stage of the parasite should be late ring or trophzoite, even schizont. As Fig 3.7 and 3.12 indicates, the sensitive stages of the *P. falciparum* to the compound are trophozoite and schizont stage, with a 6 hours exposure IC_{50}-value of less than 100nM,

which means the parasite stage matched with the stage-specificity of drug action. Otherwise, most of the parasites go through to the next cycle and the parasite number will increase more than ten-fold, which will cause the death of the patient. Even if the patient take the drug very late in this life cycle (late schizont stage), the drug still could kill more than 80 percent in 4 hours at a concentration of 500 nM. So the patient should be alive 48 hours post clinical presentation. The quick action of such an anti-malarial drug means more hope for the survival of the severe malaria patient. For the uncomplicated malaria patient, inhibition of parasite multiplication has greater importance, as this prevents the progression to severe disease and leads to resolution of fever and other symptoms. Meanwhile, the combination of the sulfnamidomethylen with artemisinins or other rings sensitive drugs should provide more efficacious therapy against malaria. However, much work should be performed for the use of the sulfnamidomethylen in combination with other drugs, such as PK, and toxicity evaluation on the combined drugs.

The property against drug resistance selection is one of the most crucial properties of an anti-malarial drug, which could be conferred by special enzymes or transporters. Most resurgences of malaria are caused by drug resistance. Widespread drug resistance is also an important reason for the increase of malarial infection. Artemisinin and chloroquine are excellent in this aspect. However, pyrimethamine and sulfadoxine were poor against drug resistance selection. The emergence of drug resistance means the decrease or even loss of anti-malarial efficacy of a drug. So this property is a fatal factor for the future development of a novel anti-malarial compound. As table 3.9 indicates, SC81458 can totally clear Dd2 with a number ranging from 10^5 to 10^8 at the concentration of 100nM without parasite recovery or drug resistance emerging within eight weeks. Even in drug free medium, no parasite could be seen in the following two weeks. As reported, many of the presently used anti-*P. falciparum* drugs such as quinine, chloroquine, amodiaquine, pyrimethamine, sulfadoxine, atovaquone and artemisinin derivatives are compromised by resistance. The mechanisms behind malaria drug resistance mainly include drug target mutation, drug transporter up-regulation or mutation (Fidock et al., 2000; Sanchez et al., 2005) and mutations/up-regulation of enzymes metabolizing the drug (Uhlemann et al., 2005; Yuvaniyama et al., 2003). Additionally, cross-resistance between different drugs is occasionally present. Thus, rational combination of different drugs is an important strategy to overcome drug resistance. So the next step is to combine the sulfnamidomethylen with other anti-*P. falciparum* drug based on the data of stage specificity, drug target and PK property.

In vivo efficacy is the one of the most important aspects in the pre-clinical phase of drug development, which can decide if the project will go ahead, backtrack or even be canceled. The data from the *in vitro* study could not provide all the information for the *in vivo* action of the drugs, such as the binding of drugs with plasma protein, which will influence the *in vivo* efficacy. There is sometimes a significant difference between *in vitro* and *in vivo* activities for some drugs. The *in vivo* study of the sulfnamidomethylen showed that SC81458 is active against *P. vinckei* at 30 mg/kg (ip) (data not shown). The parasitemia decreased more than 90% in comparison with controls after four days daily ip at 30 mg/kg. However, SC81458 showed no *in vivo* efficacy against the murine parasite *P. berghei* at a concentration of 30 mg/kg (ip) (data not shown), which indicated that there is a species specificity for the anti-*P. falciparum* activity of SC81458. Further *in vivo* efficacy evaluation was performed in humanized mice (multiple dose study), which were infected with the human parasite *P. falciparum*, 3D7 and W2 (CQ sensible and resistant, respectively) (data not shown). The results indicated that SC81458 showed an excellent *in vivo* therapy efficacy in the humanized mice model. Both the two strains of parasites were totally clear within 3 days by once-daily ip (5 mg/kg) injection. Parasite clearance even occurred within 2 days at a dosage of 10 mg/kg. A good anti-malarial drug should be able to cure this disease within three days with dosing once or twice a day. It is very crucial for the delay of the drug resistance development. Furthermore, the sulfnamidomethylen is very easy to be synthesized at a low cost, which is nontrivial for anti-malarial drugs as these drugs would be used in many developing countries.

As a novel anti-malarial candidate drug, the sulfnamidomethylen exhibited many excellent properties, such as excellent *in vivo* activity with quick action, good ADMET properties, a broad spectrum, excellent property against drug resistance selection and low cost. Next, we should design a rational combination for the sulfnamidomethylen based on the data of PK, action mechanism, toxicity and the stage-specific properties.

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