In search for novel agents in therapy of *Plasmodium falciparum*

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Parts of the results of this study have been published in advance by permission of the Mathematisch-Naturwissenschaftlichen Fakultät, presented by supervisor of this study.

Publications

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Erklärung:

Hiermit erkläre ich, die vorliegende Arbeit einschließlich beigefügter Zeichnungen und Darstellungen selbstständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben. Alle Stellen der Arbeit, die dem Wortlaut oder dem Sinn nach anderen Werken entnommen sind, habe ich unter Angabe der Quelle kenntlich gemacht.

Bonn, den

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Salem Ramadan Sarite

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IV. List of Abbreviations

Ad-GFP	Adenovirus expressing GFP
AdIL-12	Adenovirus expressing IL-12
AdOVA	Adenovirus expressing OVA
APC	antigen presenting cell
BSA	bovine serum albumine
bp	base pairs
ConA	Concavalin A
CM	cerebral malaria
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	Dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	Desoxyribonukleosidtriphosphate
dpi	days post infection
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated-cell-sorter
FCS	fatal calf serum
GC	germinal center
ICAM-1	intercellular adhesion molecule-1
IFN	Interferon
IFN-γ	interferon gamma
IL	interleukin
iRBC	infected red blood cell
i.p.	intraperitoneal

i.v.	intravenous
LT-α	Lymphotoxin alpha
LPS	lipopolysaccharide
MHC	Major Histocompatibility Complex
µg	microgram
µM	micromolar
mM	millimolar
ml	milliliter
NaCl	Sodium chloride
ng	nanogram
NK-cells	Natural Killer cells
NO	Nitric oxide
NES	nuclear export signal
OD	Optical density
OVA	Ova Albumin
PbANKA	*Plasmodium berghei* ANKA
Pf/NF-54	*Plasmodium falciparum*/NF-54
Pf/k1	*Plasmodium falciparum*/k1
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
p.i.	Post infection
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
rpm	rotation per minute
TBE	TRIS-Borat-EDTA

TMB	3,3`, 5,5`Tetramethylenbenzidine
TNF	Tumour necrosis factor
TRIS	Tris-(hydroxymethyl)-aminomethan
Tween®	Polyoxyethylenesorbitanmonolaurate
uiRBCs	uninfected red blood cells
VCAM-1	vascular adhesion molecule-1
v/v	volume per volume
WT	$C57Bl/_6$ Wild type
w/v	weight per volume

V. Summary

Malaria is an infectious disease transmitted by the bite of the female *Anopheles* mosquito. It's the third most infectious cause of mortality, is prolific in more than 40% of the world's population and causes more than one million deaths each year, especially in Africa, in addition of more than 300 million acute illnesses. Malaria is caused by an intracellular protozoan of the genus *Plasmodium* belonged to the *Phylum* of *Apicomplexa*. The four species of *Plasmodium* are i) *Plasmodium vivax,* ii) *Plasmodium ovale,* iii) *Plasmodium malariae, and* iv) *Plasmodium falciparum.*

P. falciparum is by far the most prevalent species and causes most problems as a result of its virulence and drug resistance. It has a rapid rate of asexual reproduction in the host and an ability to sequester in small blood vessels, with high risk for development of cerebral malaria. Over the last few years the situation has become even more complex because malaria parasites have been developing unacceptable levels of resistance to one drug after another. Hence, the increasing resistance of the malaria parasite has enforced new strategies of finding new drug targets.

The eukaryotic translation initiation factor 5A is an intriguing protein, because it is the only cellular protein known to contain the unique amino acid hypusine, a modification that appears to be required for cell proliferation. The eukaryotic translation initiation factor 5A and its activating enzymes are novel targets in HIV (AIDS) and cancer therapy. In principle two different strategies of inhibition of the eukaryotic translation initiation factor 5A formation exist: One strategy is inhibition of the eukaryotic translation initiation factor 5A modification by means of the deoxyhypusine synthase protein and a second one focuses on the deoxyhypusine hydroxylase inhibition.

Natural and synthetic phenazines have attracted considerable attention because of their interesting biological activities, including broad-spectrum antibiotic, anti-malarial, trypanocidal along with anti hepatitis C viral replication activities.

Phenazines have also been described as dual inhibitors of topoisomerase enzymes class I and II. In addition to that, phenazines can play an important role as antioxidants by interfering with the oxidative burst.

This study was aimed to investigate the in vitro anti-malarial affectivity of the deoxyhypusine synthase inhibitor, the deoxyhypusine hydroxylase inhibitors, the polyamintransport inhibitors, the phenazines analogues and the dihydroartemisinin analogues. And to confirm in vivo the results obtained in vitro.

The *Plasmodium* culture systems have been extensively used for screening new drugs, to study the mode of entry of parasite into erythrocytes, to isolate and characterize strains and clones, and to identify immunogenic and genome of parasite. However, in this study the microscopic screening method has been chosen to test the in vitro and in vivo antimalarial activity for several kinds of inhibitors.

Among the compounds studied in here, nineteen new compounds exhibited in vitro anti-malarial properties, due to different mode of action.

Plasmodium berghei ANKA was used in this study to confirm in vivo the obtained in vitro results. *Plasmodium berghei* ANKA murine malaria has many features in common with the human disease and is thus an accepted model for certain important aspects of clinical malaria.

In conclusion, inhibiting the deoxyhypusine synthase seems to have more efficiency than inhibiting the deoxyhypusine hydroxylase in inhibiting the proliferation of the *Plasmodium berghei* ANKA. Inhibition of polyamine synthesis in vitro showed a promising approach to the identification of anti-protozoan drugs with chemotherapeutic potential.

Some phenazines exhibited anti-proinflammatory property, as they were able to interact with the circulated tumour necrosis factor and lymphotoxine alpha, and reduce the plasma levels of these proinflammatory cytokines. The high plasma tumour necrosis factor levels

cannot be the only element responsible for the symptoms unique to cerebral *Plasmodium berghei* ANKA malaria.

1. Introduction

1.1 Malaria

Malaria is an infectious disease transmitted by the bite of the female *Anopheles* mosquito (Fig. 1.1). It is the third most infectious cause of mortality, it is prolific in more than 40% of the world's population and causes more than one million deaths each year (WHO,2006), especially in Africa, in addition of more than 300 million acute illnesses. Around 90% of these deaths occur in young children. According to the WHO in every 30 seconds one African child is killed by Malaria (WHO, 2005).

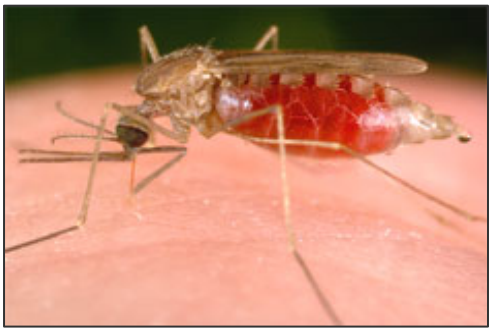

Fig. 1.1: A female of *Anopheles* mosquito feeding on the human host
Image from: http://www.cdc.gov/malaria/biology/mosquito/index.htm#images

Malaria, along with HIV/AIDS and Tuberculosis, is one of the major public health challenges facing the poorest countries of the world, particularly in terms of morbidity, mortality and deleterious economic consequences (Hunt et al., 2006).

1.2 Geographical distribution

The disease is found mainly throughout the tropical and sub-tropical regions of the world (Fig. 1.2). Temperature is particularly critical. For example, at temperatures below 20°C, *Plasmodium falciparum* (which causes severe malaria) cannot complete its growth cycle in the *Anopheles* mosquito, and thus cannot be transmitted (http://www.cdc.gov/malaria).

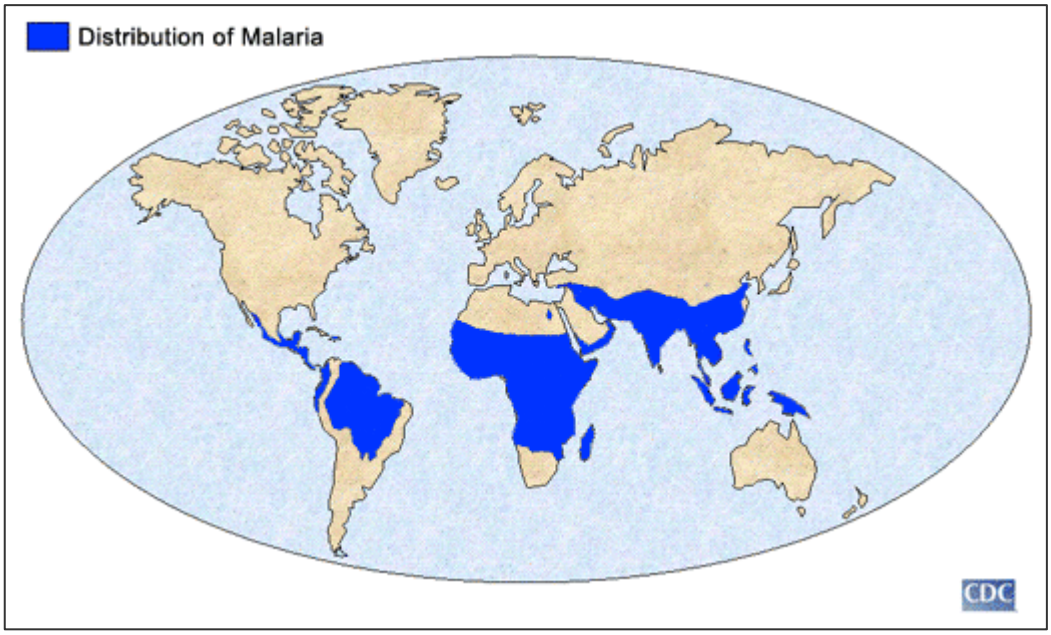

Fig. 1.2: Malaria world distribution
Image from: http://www.cdc.gov/malaria/distribution_epi/distribution.htm

However, *P. vivax* a rarely lethal infection that is widespread in Central and South America, Asia and Oceania can exist as a latent infection in the hepatocytes of the liver, re-emerging after many months to several years (Mangoni et al., 2003). Though it causes morbidity and may be an important cause of low birth weight in pregnancy, it is associated with relatively fewer severe complications (Nosten et al., 1999; Breman, 2001). Though commonly transmitted, infections due to *P. ovale* are rare as immunity is established early and parasitaemia remains low, thus long term latent infections like *P. vivax* are established. It is found principally in Africa causing less than 0.5% of malaria infections (Breman, 2001). *P. malariae* is found worldwide but with a very patchy distribution. It can cause renal complications or chronic nephropathy and if left untreated patients will remain parasitaemic though asymptomatic for years (Hendrickse et al., 1972; Barsoum, 2000). Whereas, *P. falciparum* is the most highly virulent species and causes almost all of the 1.7-2.5 million deaths worldwide from malaria (Bray and Sinden, 1979; Aikawa, 1988; Ringwald et al., 1993).

1.3 Classification of the human *Plasmodium*

Malaria is caused by an intracellular protozoan of the genus *Plasmodium* belonged to the *Phylum* of *Apicomplexa*. The four species of *Plasmodium* are (Neva and Brown, 1994):

i) *Plasmodium vivax*

ii) *Plasmodium ovale*

iii) *Plasmodium malariae*

iv) *Plasmodium falciparum*

1.4 The life cycle of malaria parasite

The life cycle is complex, consisting of an exogenous sexual stage in the *Anopheles* mosquito and an asexual reproduction stage in the mammalian host (Fig. 1.3).

The parasitic life cycle is categorised into four phases as follows (Knell, 1991; Foley, 1998).

Phase 1:

Fertilisation- a female mosquito, *Anopheles*, withdraws blood from an infected host that contains intraerythrocytic parasites. The parasites develop into sexual stage gametocytes in eight to ten days, which undergo fertilisation in the gut of the insect. The resulting invasive ookinete burrows into the stomach wall of the mosquito (sexual phase).

Phase 2:

Sporogony- development of the oocyst continues until the mature cyst bursts. Free sporozoites migrate into the salivary glands of the mosquito, ready to be transmitted to another individual (asexual phase).

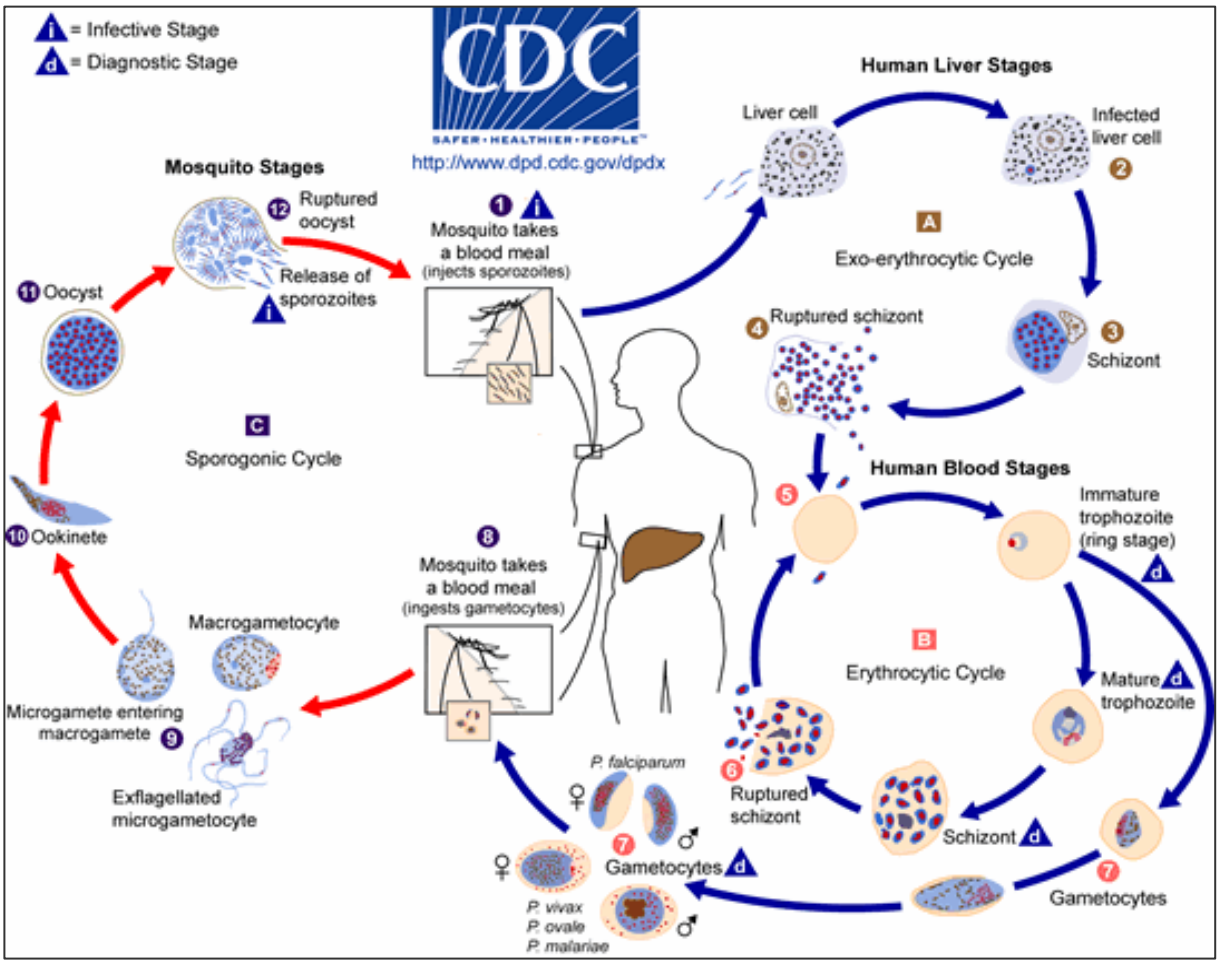

Fig. 1.3: malaria parasite life cycle.

Phase 3:

Hepatic schizogony- the now infected female mosquito feeds again, both withdrawing blood from the human host while injecting sporozoite-containing saliva into the capillaries of the skin. The sporozoites invade liver cells and multiply to form merozoites. After five days the infected liver cell bursts, releasing merozoites into the blood stream (asexual phase).

Phase 4:

Erythrocyte schizogony- Merozoites enter red blood cells where they develop through ring, trophozoite and schizont stages. The human erythrocytes provide the parasite with a safe environment, where it can grow and mature. The parasite divides to produce 16 - 20

daughter merozoites during a forty-eight hour cycle. When mature, the red blood cell bursts, releasing merozoites to invade new red blood cells. The cycle then continues (asexual phase).

1.5 Clinical symptoms

About one third of infections with *Plasmodiums* are forms of mild malaria. These ones are caused by *P. vivax* , *P. ovale* and *P. malariae*. Symptoms include fatigue caused by fever, recurrent chills, and haemolytic anaemia. Two third of the cases are forms of severe malaria caused by *P. falciparum*. Intense headaches, pain in the joints, splenomegaly and hepatomegaly are accompanied by complications like cerebral malaria, intravasal haemolysis (bilious fever), glomerulonephritis caused by immune complexes, leuko- and thrompopenia, hypoglycaemia (especially children) and oedema of lung (Renz-Polster *et al.*, 2004). Differential characteristics of clinical significance are outlined in (table 1.1) (Neva and Brown, 1994; Zeibig, 1997; John et al., 2006). An elegant and exhaustive review on human CM pathology has been published (Turner, G. 1997).

Table 1.1: Clinical differentiation of the human malarias

	P. vivax	***P. ovale***	***P. malariae***	***P. falciparum***
Other names	benign tertian	benign tertian or ovale	quartan	malignant tertian aestivoautumnal
Incubation period (days)	14 (8-27)	15 (9-17)	15-30	12 (8-25)
Eeythrocytic cycle (Hours)	48	48	72	48
Persistent Exoerythro-cytic stage	Yes	Yes	No	No
Parasitemia (mm^3)				
Average	20000	9000	6000	50000-500000
Maximum	50000	30000	20000	up to 2500000
Duration of untreated Infection (Year)	1.5-4	probably 1.5-4	1-30	0.5-2
Anemia	++	+	++	++++
Other Complications			renal	cerebral
Relative age of cells infected RBCs	young and immature	young and immature	mature	may infect of all ages

1.5.1 Cerebral Malaria (CM)

CM is severe malaria which occurs when *P. falciparum* infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The pathogenic mechanisms underlying CM and why a small percentage of patients develop CM are not fully understood, but the accumulation of large numbers of parasites in specific sites such as the brain or placenta (MacPherson et al., 1985; Aikawa, 1988; Rogerson et al., 1995; Ricke et al., 2000), associated with adverse clinical outcomes, suggests that organ-specific accumulation of parasites is important (see also section 1.6.1). The manifestations of severe malaria include cerebral malaria, pulmonary edema, and acute renal failure, severe anemia, and/or bleeding.

Acidosis and hypoglycemia are the most common metabolic complications. Any of these complications can develop rapidly and progress to death within hours or days (WHO, 2000). A wordiness explanation on severe malaria can be reviewed in (Trampuz, 2003).

1.6 *Plasmodium berghei* ANKA in rodents, a model for human malaria

Plasmodium berghei ANKA (PbANKA) murine malaria has many features in common with the human disease and is thus an accepted model for certain important aspects of clinical malaria (Miller et al., 2002). It manifests a cytokins-dependent encephalopathy associated with up-regulation of adhesions on the cerebral microvascular endothelium and attendant neurological complications (Grau et al., 1989; Jennings et al., 1997; de Souza and Riley, 2002). Hence, this strain providing a good model to estimate survival and antimalarial efficacy in reducing parasitemia. On the other hand, several animal models have been established in which animals are infected with erythrocytes parasitized by various types of *Plasmodium*. Although these animal models do not exactly reproduce the human disease, they nevertheless exhibit some similarities to human CM. Consecutively; PbANKA is known to induce CM in C57BL/6 mice (Jennings et al., 1997). Susceptible and resistant mouse strains to (PbANKA) induced CM are named in table (1.2).

Table 1.2: Mouse strain susceptibility to (PbANKA) **induced CM (Lou et al., 2001).**

Mice strain	Ability to develop CM
C57 BL/6	CM-susceptible
CBA	CM-susceptible
NRMI	CM-susceptible
(BALB/c x C57BL/6) F1	CM-susceptible
BALB/c	CM-resistant
C3H	CM-resistant
A/J	CM-resistant

1.6.1 Cerebral Malaria in mouse experimental model

Several hypotheses have been developed to explain the pathogenesis of cerebral malaria. Sequestration of PRBC, the production of proinflammatory cytokines, and their downstream reactions such as mechanical blockage, aschemia, acidosis, hemorrhages, and nitric oxide production have been implicated in the pathogenesis (Clark and Rockett, 1994; Berendt et al., 1994). Platelet deposition appears to be a major contributor to death, given that platelets accumulate in microvessels and that antiplatelet therapy can improve outcome (Grau and Lou, 1993). Likewise, in another study *P. falciparum* adhesive phenotype is platelet mediated clumping of parasitized red blood cells (PRBCs), which have been associated with disease severity in Kenyan children (Pain et al., 2001). However, various hypotheses have been proposed (For review see (Lou et al., 2001; Hunt and Grau, 2003)), and some progress has been made using *in vitro* as well as *in vivo* models, the mechanisms of CM pathogenesis remain incompletely understood and are the subject of a continuing debate (Berendt et al. ,1994; Clark and Rockett, 1994; Grau and Kossodo, 1994). Pathogenesis of CM is illustrated in Fig. 1.4.

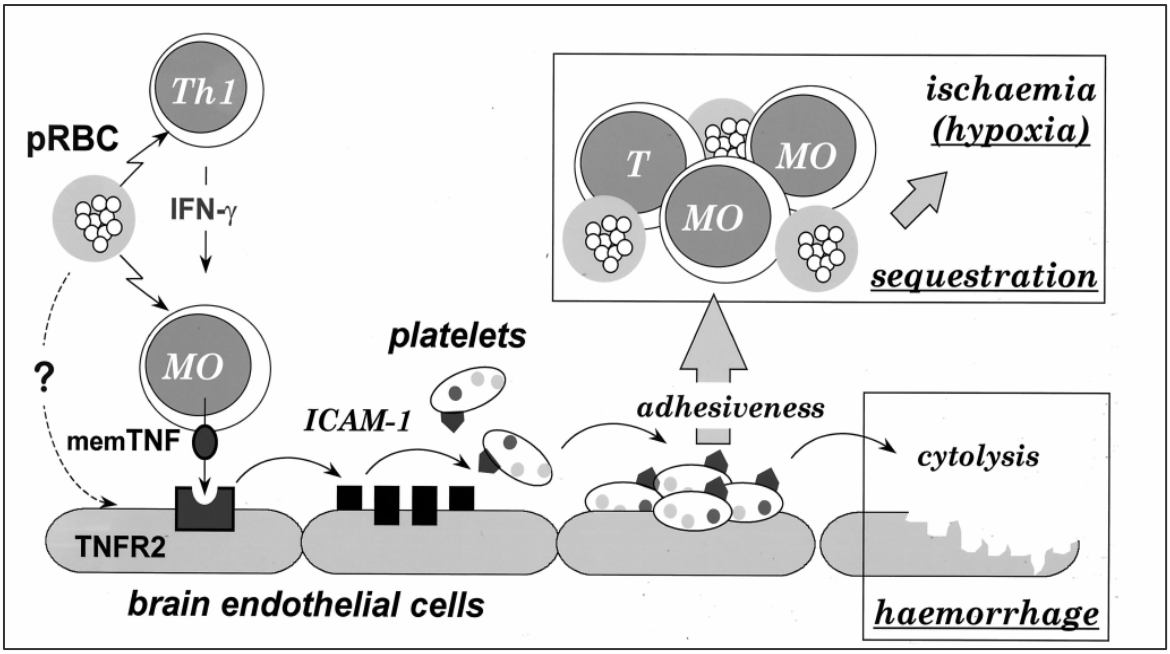

Fig. 1.4: Pathogenesis of CM: Importance of other blood cells in the modulation of pRBC binding in the pathogenesis of CM. The malarial parasite (pRBC) stimulates the host immune response, notably an expansion of Th1 clones, leading to overproduction of IFN-γ. Apart from upregulating some potential receptors, such as CD36, IFN- γ stimulates monocytes to produce soluble TNF (solTNF) and to express higher levels of the transmembrane form of the cytokine (memTNF). Both forms, but particularly the memTNF via an interaction with TNFR2 expressed in increased amounts, cause an upregulation of ICAM-1 on brain endothelial cells. In turn, high levels of ICAM-1 cause platelets to adhere and fuse to brain endothelial cells, with at least two important functional consequences: an increased adhesiveness for pRBC (via CD36) and leukocytes (via LFA-1, P-selectin, etc.), responsible for vessel obstruction, ischemia and possible neuronal dysfunction, and a potentiation of endothelial killing by TNF, leading to vessel disruption and brain hemorrhages. **Image and legend are taken from (Lou et al., 2001).**

Whereas:
- ICAM-1= Intra-cellular Adhesion Molecule-1.
- INF-γ= Inter feron-γ.
- solTNF= Soluble Tumor Necrosis Factor.
- memTNF= membrane Tumor Nocrosis Factor.
- LFA-1= leukocytes-associated antigen-1.
- TNFR2= Tumor Necrosis Factor receptors-2

1.7 The host immune response to the *Plasmodium* infection

The immune system provides different mechanisms to protect organisms against pathogens, most of which are infectious agents. Consequently, the course of *Plasmodium falciparum* malaria is characterized by a complex interaction of host immune responses and parasite survival strategies (Winkler et al., 1998).

In *Plasmodium* infection, it has been suggested that the balance between Th1 (IL-2, IFN-γ and TNF) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) immune response determines the degree of parasitemia, level of anaemia, clinical severity, presentation and/or outcome through direct or indirect reactions of cytokines and other physiologically active substances (Ho et al., 1995). More over, in *Plasmodium* infection the cell-mediated-immunity plays a key role, whereas the humoral (B cell driven) immune response is less important (Yanez et al., 1996), and the lack of naturally acquired immunity to malaria (premunition) is known to contribute to the severity of the infection (as reviewed by Hviid, 2005).

1.7.1 Cytokines

Cytokines are proteins produced and released by monocytes, macrophages, epithelium, fibroblasts, smooth muscles cells (Belec et al., 1997; Abbas, 2000). Cytokines have a role in both the innate and the humoral *in vivo* immune response (Abbas, 2000), and are often classified as “pro” and “anti” inflammatory (Pedersen et al., 1998; Ostrowski et al., 1999). The pro-inflammatory cytokines include IL-1, IL-6, TNF and INF-γ while some of the anti-inflammatory cytokines are IL2-, IL-4 and IL-10 (Abbas, 2000; Balkwill, 2000). In malaria, cytokines may be the key determinants of severity and outcome (Urquhart, 1994; Winkler et al., 1998). E.g. TNF, IL-1 and IL-6 were further substantially higher in children with severe malaria than in uncomplicated malaria (Wassmer et al., 2003; Vogetseder et al., 2004).

1.7.1.1 Tumour necrosis factor (TNF)

TNF mediate innate immunity and it is the primary mediator of acute inflammation (Abbas 2000). TNF is released in large amounts from monocytes and macrophages when gram-negative bacteria are encountered (LPS stimulation). Besides monocytes, neutrophils, activated lymphocytes, NK cells, endothelial cells and smooth muscle cells are sources of TNF production (Oppenheim and Feldmann, 2000). TNF acts in a dependent concentration manner, where low quantities stimulate local inflammation with neutrophil and macrophage infiltration and moderate quantities cause systemic effects such as fever. If the plasma concentration of TNF becomes very high, the result will be septic shock (Abbas 2000; Janeway et al., 2001). Some of the *in vitro* effects of TNF are increased adhesion molecule expression on endothelium (Blue et al., 1993), release of chemotactic substances and apoptosis (Monney et al. 1998; Flad et al. 1999). Numerous studies provide evidence for the key role of TNF in the pathogenesis of CM, and a clear relationship has been established between plasma concentrations of TNF levels and cerebral pathology (Grau, et al., 1989; Molyneux, et al., 1993). Furthermore, TNF has been reported to play a central role in malarial fevers and parasitemia (Mosmann and Coffman, 1989; Mordmuller et al., 1997). However, in other studies, high plasma concentrations of TNF were found to be associated with disease severity but not specifically with CM (Shaffer, 1991; Molyneux, 1991).

1.7.1.2 Lymphotoxine-α (LT-α)

LT-α, which is produced by T and B cells (Janeway et al., 2001) and natural killer cells (NKC) (Gruss and Dower, 1995), plays a pivotal role in the development of secondary lymphoid organs, which serve as sites of contact between antigen-presenting cells (APCs) and immune effectors T and B lymphocytes (Spahn et al., 2005).

Recently, it was shown that LT-$\alpha^{-/-}$ mice are protected against CM induced by *P. berghei* ANKA, whereas $TNF^{-/-}$ mice are not (Engwerda et al., 2002). In addition, CLARK et al., (2007) confirmed that LT-α, but not TNF, was necessary for early IFNγ production and the regulation of IFNγ production later in *P. chabaudi chabaudi AS* infection. However, similar to TNF, LT-α thought to be also a risk factor for the development of septic shock (Waterer and Wunderink, 2003). Indeed, LT-α and TNF belong to the same family, and interact with a common receptor, and could act together during the pathogenesis (Hunt and Grau, 2003). Moreover, LT upregulates TNF expression (Owen-schaub et al., 1989) and both cytokines, TNF and LT-α, share a pair of receptors (tumor necrosis factor receptors I and II), and signalling via these pathways can result in cell activation, proliferation, or apoptosis, depending on the cell lineage, metabolic state of the cell, and kinetics of ligand binding (Gruss and Dower, 1995).

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1.8 Antimalarial drugs

The first developed antimalarial was quinine, prepared from *Cinchona bark* (Guerra, 1977).

After which, the two classes of drugs, antifolates and quinoline-containing drugs, have traditionally been used as a classic approach to antimalarial chemotherapy (Table 1.2), (Go, 2003; Macreadie, 2000). The most successful compound is chloroquine (CQ), which was first introduced in the 1940s. Its effectiveness and low risk have resulted into this compound being widely used. In consequence, CQ is subject to malarial resistance and is now ineffective in some areas (Foley and Tilley, 1998). Parasite resistance of quinine and other antimalarials like proguanil, has prompted the development of new and effective drugs against existing targets (Macreadie, 2000).

Table 1.3: The four categories of antimalarial compounds and their effect on the life cycle of the malaria parasite group (White, 1998; Maselli, 2006):

Group	Compound types	Stage of life cycle affected	Action on cells	Action on malarial pigment
1	Chloroquine Mepacrine 4-minoquinolines	Schizogony All asexual stages	--	Rapid coarse clumping
2	Quinine Mefloquine Primaquine 8-Aminoquinolines	All stages except mature gametocytes of P. falciparum	- Degeneration of nuclei -Vacuolation of cytoplasm	Slow, fine clumping
3	Antifolates: - Proguanil - Pyrimethamine - Sulphonamides	Schizogony	Maturation arrest producing large non-viable parasites	--
4	Sesquiterpenes: -Artemisinine	Schizogony	--	--

In ontrast, the resistance to some antimalarial drugs in the field, such as artmisinin, has not been reported yet. Neurotoxicity has been reported in animal studies, particularly with very high doses of intramuscular artemotil and artemether, but has not been substantiated in humans (Vugt et al., 2000; Hien et al., 2003). Similarly, evidence of death of embryos and morphological abnormalities in early pregnancy has been demonstrated in animal studies (WHO, 2003).

The characteristics of current treatment options entailing both single drug and combination therapies are listed in Table 1.3 (Bloland, 2001; Lindberg, 2004). (For reviewing the antimalarial drugs Pathway, mechanism and the target molecule see Fidock et al., 2004).

Table 1.4: Summary of the characteristics of current anti-malarial drugs.

Drug	Resistance	Side effects	Costs/dose (US$)
Chloroquine	Yes	+	0.070
Sulfadoxinepyriamine	Yes	++	0.083
Quinine	Yes	++	1.350
Amodiaquine	Yes	+++	0.150
Artesunate	None*	++	2.160
Mefloquine	Yes	++	3.220
Halofantrine	None	+++	4.750
Atovacuone	Yes	+	35.00

*** Afonso et al., (2006) pointed to the probability of developing resistance to this class of drugs (see section 1.8.1)**

1.8.1 Drug resistance in *P. falciparum*

Drug resistance can be defined as the ability of a cell or an organism to survive in the presence of higher concentration of a drug that normally destroys cells or organisms of the same species or prevent their multiplication. The development of resistance to antimalarial drugs poses one of the greatest threats to malaria control and is the main cause of recent increases in malaria morbidity and mortality (Stepniewska, 2007). Overall, resistance to antimalarials has been documented for *P. falciparum, P. vivax* and, recently, *P. malariae.*

In *P. falciparum*, resistance has been observed to almost all currently used antimalarials (amodiaquine, CQ, mefloquine, quinine and sulfadoxine–pyrimethamine) except for artemisinin and its derivatives (WHO, 2006). Unfortunately, resistance is now widely disseminated throughout malaria-endemic regions (Fig. 1.5), (Fidock et al, 2004).

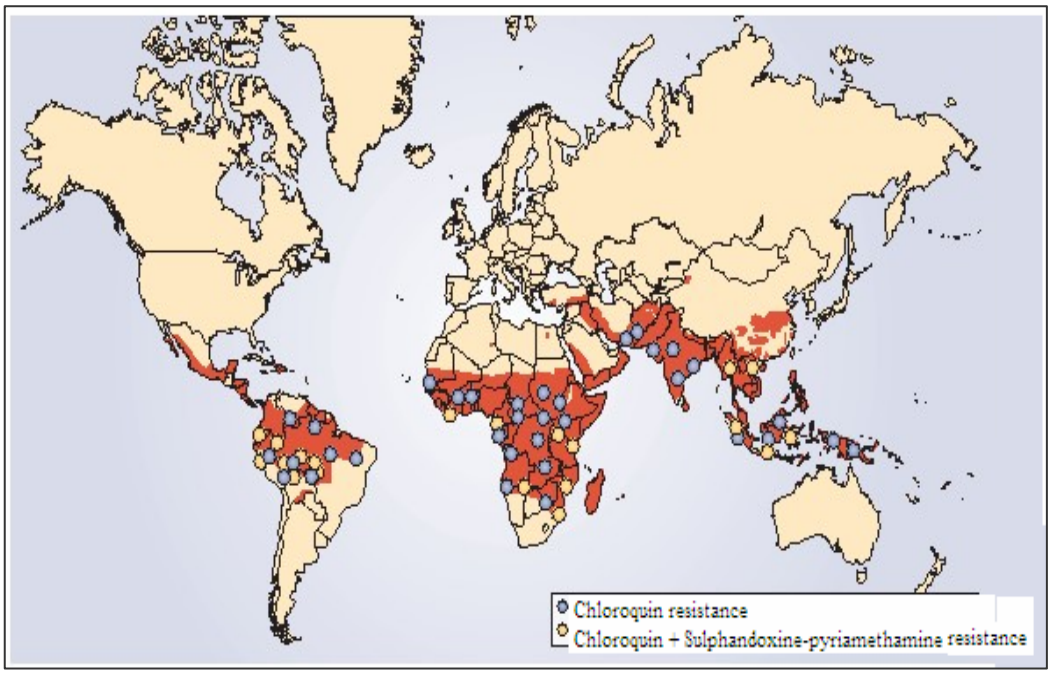

Fig. 1.5: The global distribution of malaria, showing areas where *P. falciparum* resistance to the most commonly used antimalarial drugs, chloroquine and sulphadoxine-pyrimethamine, has been documented. (malaria-endemic regions coloured in red) (Fidock et al., 2004).

The genes involved in CQ and sulfadoxine- pyrimethamine (SP) resistance are known (Djimde et al., 2001; Hayton and Su, 2004), these are *P. falciparum* multi-drug resistance-1 (pfmdr1) and *P. falciparum* chloroquine resistance transporter (pfcrt), encoding membrane transporter proteins, which are localized in the membrane of the parasite's food vacuole (Cowman et al., 1991; Fidock et al., 2000). In addition, the molecular basis for resistance to antifolates, such as SP has been well characterized. *P. falciparum* resistance to SP is primarily conferred by successive single-point mutations in parasite dhfr, the gene that encodes the target enzyme dihydrofolate reductase (DHFR), and by additional

mutations in dhps, which encodes for the enzyme dihydropteroate synthetase (DHPS) (Triglia et al., 1997).

In fact, part of the reason for the failure to control malaria is the emergence and spread of resistance to first-line antimalarial drugs and the cross-resistance between drugs of the same class, e.g. mefloquine and quinine (Brasseur et al. 1992), as well as cross-resistance between the members of the limited number of drug families available (Olliaro, 1996). Likewise, Craig et al. (1993) reported a cross-resistance to halofantrine and artemisinin. However, Cross resistance between artemisinin and other antimalarials may limit its use as a replacement drug (Doury et al., 1992). Even though resistance of *P. falciparum* to artemisinin and its derivatives has not been reported in human right now (WHO, 2006), there are some evidences indicate that this resistance may happen in any time. Recently, Afonso et al., (2006) reported murine malaria parasite that is genetically stable and transmissible resistance to artemisinin and artesunate. Although there is some other reports published on development of the rodent malaria parasite strains resistant to the drug (Klayman, 1985; Chawira, 1986).

Taking together, drug resistance in *P. falciparum* has become extremely important and accomplished fact, which should be faced. This fact has enforced new strategies of finding new drug targets.

1.9 Eukaryotic translation initiation factor 5A (eIF5A)

The eukaryotic translation initiation factor 5A (eIF5A) is an intriguing protein, because it is the only cellular protein known to contain the unique amino acid hypusine, a modification that appears to be required for cell proliferation (Park et al., 1997; Chen and Jeo, 1999).

The unusual amino acid hypusine (see Fig. 1.6) is a posttranslational modification of the eIF- 5A and necessary for eIF-5A activity (Park et al., 1993). Hypusine is formed in two steps by deoxyhypusine synthase (DHS) [EC 1.1.1.249] and by deoxyhypusine hydroxylase (DHH) [EC1.14.99.29] (Abbruzzese et al., 1988). DHS transfers an aminobutyl moiety from the triamine spermidine to a specific lysine residue in the eIF-5A precursor protein to give deoxyhypusine (Park et al., 1988) and subsequently DHH hydroxylates this molecule completing the hypusine biosynthesis.

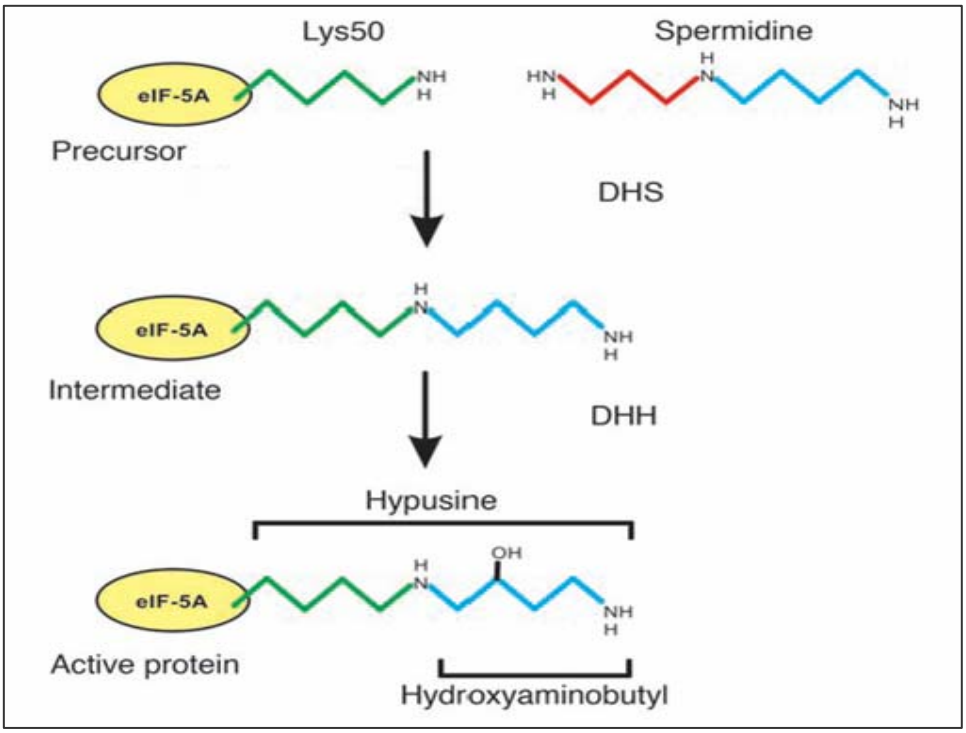

Fig. 1.6: Schematic representation of the pathway of eIF-5A biosynthesis. Hypusine is required for the biological activity of the eIF5A protein. The conversion of a specific lysine residue (Lys50) into the unusual amino acid hypusine occurs in 2 posttranslational reactions that are mediated by the enzymes DHS and DHH. Image and legend are taken from (Hauber et al., 2005).

Previous studies have shown that mature eIF-5A formation in *Plasmodia* can be blocked by inhibition of DHS by means of 1,7-diaminoheptane *in vitro* (Njuguna et al., 2006). Furthermore, the eIF-5A is an essential cofactor of the viral regulatory protein Rev which is important for HIV-1 replication. (Bevec et al., 1996) Consequently the direct inhibition of AdoMetDC, DHS and DHH with small molecules has been already shown to block the Rev activity and, thus, the virus replication (Andrus et al., 1998; Hart et al., 2002; Hauber et al., 2005; Schäfer et al., 2006) indicating that inhibition of hypusine (especially DHS and DHH) is a preferred strategy for the development of anti-HIV drugs. Moreover, the factor interacts with the general nuclear export receptor CRM1 (see section 1.12), during their transportation from the nucleus to the cytoplasm (Rosorius et al., 1999). The eIF5A protein expression was normally very low but inducible with T lymphocyte-specific stimuli in human peripheral blood mononuclear cells (PBMCs) of healthy individuals, and a significant up-regulation of eIF5A mRNA was found in the PBMCs of HIV-1 infected patients (Bevec et al., 1994). The eIF5A expression is also significantly increased during dendritic cell maturation (Kruse et al., 2000). More recently, Li et al., (2004) have demonstrated, for the first time that a new biological activity for eIF5A as the regulator of p53, and eIF5A and syntenin could engage in a specific interaction both *in vitro* and *in vivo*, and function collaboratively to regulate p53 activity.

1.9.1 Strategies in prevention of eIF5A formation

EIF5A and its activating enzymes are novel targets in HIV (AIDS) and cancer therapy. In principle two different strategies of inhibition of eIF-5A formation exist: One strategy is inhibition of eIF5A modification by means of DHS protein and a second one focuses on DHH inhibition (Kaiser et al., 2006).

1.9.2 Deoxyhypusine synthase (DHS) inhibitor

Blocking the biological activity of eIF5A by means of its first modification step has recently been demonstrated to be an alternative in HIV-1 treatment since eIF5A is an important cofactor in HIV-1 replication (Sommer et al., 2004). CNI-1493 had an IC_{50} value between 1.5 to 2.5 μM *in vitro* in HIV-resistant lines. Therefore the identification of new DHS inhibitors could be one step in the development of new HIV-therapies. However, CNI-1493 (*N*,*N*′-bis[3,5-bis[1 (aminoimino-methyl) hydrazonoethyl]phenyl] decanediamide tetrahydrochlo-ride) is an experimental drug that is already in phase II clinical trials for Crohn disease (Hauber et al., 2005). In addition, DHS is a target of experimental low-molecular weight drugs such as the tetravalent guanylhydrazone CNI-1493, which is considered as a novel and potent inhibitor of DHS, since 1.0 μM of CNI-1493 inhibited the hypusine formation in eIF5A *in vivo* (Hauber et al., 2005).

Moreover, CNI-1493, on the other hand, has been shown to inhibit synthesis of TNF in monocytes/macrophages by somehow suppressing translation efficiency (Cohen et al., 1996). In addition, this compound has been reported to prevent the phosphorylation of p38 MAPK, which has been demonstrated to be crucial in the posttranscriptional regulation in the synthesis of some proinflammatory cytokines (Lee et al., 1995). Further more, evidence suggests that discrete signal transduction pathways; whose intermediates include cdc42, MEKK, MKK3/MKK4, and p38 MAP kinase; regulate the transcriptional and translational regulatory events (Fig. 1.7), (Coso et al., 1995; Derijard et al., 1995; Cohen et al., 1996). However, a major effect of CNI-1493 in suppression of TNF synthesis, mediated by a dose-dependent inhibition of the translation of TNF mRNA (Cohen et al., 1996; Cohen et al., 1997). Secondarily, the synthesis of other cytokines and chemokines is also inhibited, including that of IL-1β, macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β (Bianchi et al., 1996) and suppresses the production of TNF and IL-1 even in the presence of gamma interferon (IFN-g) (Bianchi et al., 1996).

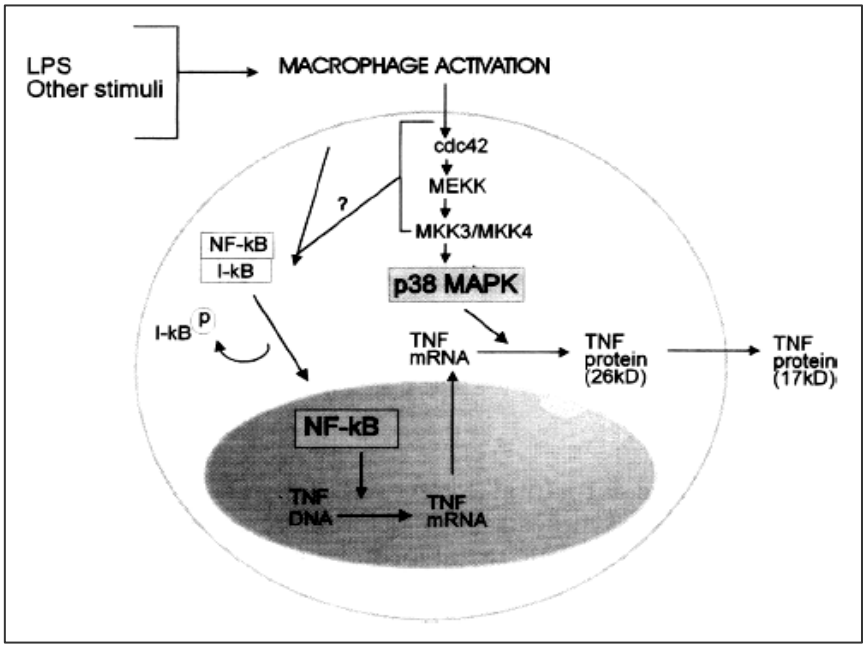

Fig. 1.7: Schematic illustration of putative signal transduction pathways regulating the macrophage activation-mediated production of TNF. MAPK= mitogen-activated protein (MAP) kinase. LPS= Lipopolysaccaride. (Image from Cohen et al., 1996)

Additionally, administration of CNI-1493 in a murine model of polymicrobial sepsis significantly reduced levels of TNF in serum and increased survival rates (Villa et al., 1997). The same observation was made by Granert et al., (2000) where CNI-1493 have been used at 5 mg/kg, given i.p., by which survival rates were increased by 75% for infant rats with systemic and CNS inflammation induced with *Haemophilus influenzae* type b. Moreover, treatment with CNI-1493 resulted in a profound inhibition of lipopolysaccharide (LPS)- induced production of TNF, IL-1, IL-6, and IL-8 (Bjork et al., 1997).

Thereby it would be of interest to assay the anti-malarial activity of CNI-1493, and its ability to altred the outcomes of CM.

1.9.3 Deoxyhypusine hydroxylase (DHH) inhibitors

In the same line with DHS, the eIF5A formation can be prevented by inhibition of DHH. Findings in rat testis have demonstrated that the DHH can be inhibited by the antifungal drug ciclopiroxolamine. Ciclopiroxolamine showed antiangiogenetic effects in human vascular endothelial cells (HUVEC) and antiproliferative effects in the chick aortic arch sprouting assay (Clement et al., 2002). Moreover, ciclopiroxolamine inhibits the *in vitro* proliferation of the chloroquine sensitive (CQS) NF-54 *P. falciparum* strain with an IC_{50} value of 8.2 μM. These findings proved the hypusine biosynthesis pathway to be a target for antimicrobial therapy (Kaiser et al., 2006) and therefore prompted us to asses the anti-malarial ability of the compounds, which will be able to target the DHH. However, different saturated and non-saturated dipyridine substituted mono- and dieters DHH inhibitors were used in this study and tested in CQS Pf/NF-54 strain.

1.9.4 Polyamintransport inhibitors

Targeting of enzymes of the polyamine pathway (see Fig. 1.8), like the ornithine decarboxylase (ODC) [EC 4.1.1.17], adenosylmethionine decarboxylase (AdoMetDC) [4.1.1.50], and the spermidine synthase (SPDS) [EC 2.5.1.16] turned out to be valuable for both anti-parasitic chemotherapy and prevention (Müller et al., 2000; Haider et al., 2005; Casero et al., 2007). E.g. difluoromethylornithine (DFMO) which is a specific inhibitor of ODC blocks the erythrocytic schizogony of *P. falciparum* in culture, and recent results have shown that spermidine synthase from the malaria parasite can be inhibited by trans-4-methylcyclohexylamine with an inhibitory effect on cell proliferation (Kaiser et al., 2001; Das Gupta et al., 2005). In contrast to the enzyme of the parasite, the drug inhibits the mammalian enzyme without any antiproliferative effect. In malaria parasite, during erythrocytic schizogony, *P. falciparum* proliferates rapidly within host cells, leading to 12 to 18 new merozoites every 48hrs.

It has been shown for many organisms that growth and differentiation processes depend on adequate intracellular concentrations of the polyamines puterscine, spermidine, and spermine (Marton and Pegg, 1995; Seiler, 2003). As a consequence, depletion of cellular polyamine levels has antiproliferation effects on cells, including *P. falciparum* (Marton and Pegg, 1995; Müller, 2001; Seiler, 2003). Therefore, the anti-malarial effects of the polyamintransport inhibitors were investigated in this study.

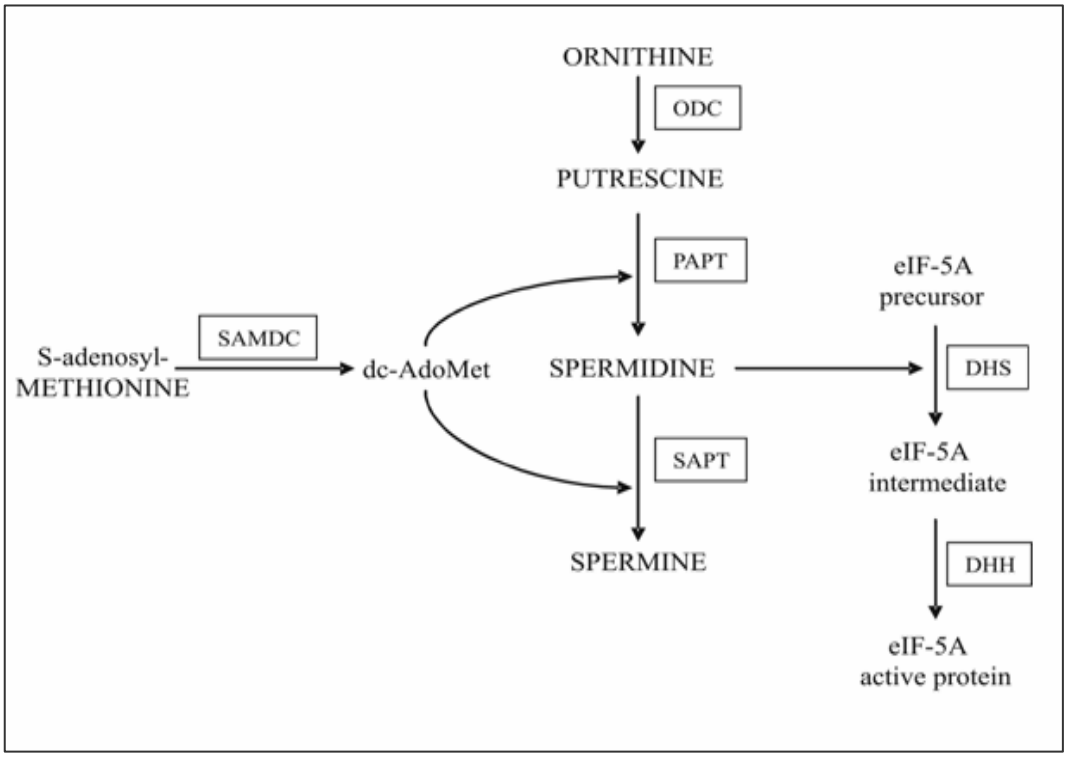

Fig. 1.8: Schematic representation of the polyamine and eIF-5A biosynthesis pathway.

Putrescine is formed by decarboxylation of ornithine, a reaction that is catalyzed by ornithine decarboxylase (ODC). Spermidine is synthesized by the action of putrescine aminopropyl transferase (PAPT). The aminopropyl group is derived from decarboxylated-S-adenosylmethionine (dc-AdoMet), which is provided by S-adenosylmethionine decarboxylase (SAMDC). Spermine is formed by addition of an aminopropyl group to spermidine by enzymatic action of spermidine aminopropyl transferase (SAPT). The biological activity of the eukaryotic initiation factor 5A (eIF-5A) depends on its hypusine modification, a spermidine-dependent posttranslational reaction that is catalyzed by subsequent action of the two enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydroxylase (DHH). **(Image and legend are taken from Schäfer et al., 2006)**

1.10 Phenazine derivatives

Phenazines natural products are isolated as secondary metabolites primarily from *Pseudomonas*, *Streptomyces*, and few other genera from soil or marine habitats (Laursen and Nielsen, 2004). These natural products with their interesting biological effects have prompted the development of synthetic derivatives with tailor-made properties similar to or even better than those of the natural compound.

Newbouldai laevis SEEM. Or "Boundary Tree" is a medium sized angiosperm in the *Bignoniaceae* family. It is native to tropical Africa, and grows to a height of about 10 meter (Okeka, 2003). The species *N. laevis* is widely used in Africa folk medicine for the treatment of several diseases such as an astringent in diarrhea and dysentery. It is also employed in the treatment against worms, malaria, sexually transmitted disease, and in the reduction of dental caries (Eyong et al., 2005). However, little is known of its anti-malarial properties despite the fact that this species is widely used by local healers to treat malaria (Eyong et al., 2006).

In general, natural and synthetic phenazines have attracted considerable attention because of their interesting biological activities (Emoto et al., 2000), including broad-spectrum antibiotic (Abken et al., 1998), antimalarial (Makgatho et al., 2000; Andrade-Neto et al., 2004), trypanocidal (Neves-Pinto et al., 2002) along with anti hepatitis C viral replication activities (Wang et al., 2000). Phenazines have also been described as dual inhibitors of topoisomerase I and II (Wang et al., 2002). Moreover, phenazines, such as pyocyanine, are electron carriers (Massey and Singer, 1957) and can inhibit T-lymphocyte proliferation in mice (Mühlradt et al., 1986). In addition to that, phenazines can play an important role as antioxidants by interfering with the oxidative burst (or the reactive oxygen species (ROS)) (for reviews, see Badwey and Karnovsky, 1980; Babior, 1984; Laursen and Nielsen, 2004).

1.10.1 Oxidative stress may play a key feature of cerebral malaria

The term oxidative stress describes the increased occurrence of ROS in the extracellular and intracellular environment (Halliwell, 1993). ROS includes superoxide anion (O_2^-) , Hydroxyl radical (OH°), Hydrogen peroxide (H_2O_2), and Hypochlorous acid (HOCL); components which can induce oxidative damage in biological systems (Halliwell, 1993; Halliwell and Gutteridge, 1998; El-Benna et al., 2005). In essence, oxidative stress results from an imbalance between the production of reactive oxygen species and antioxidative defence (Finkel and Holbrook, 2000). However, in malaria the effects of ROS can be both beneficial and pathological, depending on the amount and place of production (Postma et al., 2004). IFN-γ synergizes with lipopolysacharride and TNF to enhance the superoxide anion radical production by phagocytes (Berton et al., 1986; Cassatella et al., 1990) that accompanies phagocytosis of microorganisms. This superoxide production requires a protein complex known as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), which contains the heterodimeric cytochrome b-$_{245/558}$, composed of an a-subunit (gp^{22phox}) and a b-subunit (gp^{91phox}) (Parkos et al., 1987). Following activation of NADPH oxidase, large quantities of superoxide are produced, and are rapidly converted into hydrogen peroxide and other (ROS) that are essential for effective phagocyte microbicidal activity. Besides this role as end-stage effector molecules, ROS are recognized as mediators of cell apoptosis (Buttke and Sandstrom, 1994) and regulators of gene expression (Baeuerle et al., 1996; Finkel, 1998). However, excessive or inappropriate release of ROS may contribute to tissue injury (Jackson and Cochrane, 1988), such as endothelial cell damage (Volk et al., 1995). Thus, activated phagocytes that attach to brain endothelial cells in murine CM (Neill and Hunt, 1992) might cause oxidative damage to the endothelium and compromise the blood-brain barrier. Interestingly, Paraquat, a radical generator, causes cerebral lesions similar to those seen in CM (Grant et al., 1980).

On the other hand, administration of butylated hydroxyanisole, an antioxidant, can prevent the development of CM in PbANKA-infected mice (Thumwood et al., 1989). In fact, earlier studies with free radical scavengers suggested a role for ROS in the pathogenesis of CM (Thumwood et al., 1989), and therefore, drugs which prevent host pathology, such as the cerebral complications, might be life saving. Thus, using the phenazines derivatives, as known ROS scavengers (Laursen and Nielsen, 2004), may be useful for that.

1.11 Dihydroartemisinin analogues

During the asexual development of *P. falciparum* in human RBCs the parasite digests about 75% of the host cell haemoglobin (Hb) but is unable to catabolize the heme liberated (Francis et al., 1997). To protect the parasite membranes against the toxic effects of free heme, heme is polymerized to hemozoin (malarial pigment) (Slater et al., 1991) or broken down non-enzymatically (Ginsburg et al., 1998; Papalexis et al., 2001). However, the heme polymerization pathway is specific to the malarial parasite and offers a potential biochemical target for the design of antimalarials (Pandey and Chauhan, 1998). Hong et al. (1994) has shown that artemisinin taken up by the malarial parasite growing *in vitro* was selectively concentrated in the parasite food vacuole and was associated with hemozoin. Artemisinin also interacts with heme, forming covalent adducts (Meshnick et al., 1991; Hong et al., 1994).

Actually, the presence of the peroxide bridge is essential for artemisinin's antimalarial activity as a reduced form of the compound, deoxyartemisinin, lacks the antimalarial activity (Klayman, 1985; Woerdenbag et al., 1994). Artemisinin are hardly soluble in water or oil and therefore formulations other than oral and rectal are not in clinical use (Golenser et al., 2006). However, since the peroxide bridge of the compound is stable under certain chemical reactions, several oil- and water-soluble derivatives of artemisinin have been synthesized.

These include dihydroartemisinin, artemether, and artesunate. (Chemical structures of artemisinin and dihydroartemisinin are shown in appendix No. 7). Moreover, *in vitro* studies have suggested an uptake of artemisinin by both healthy and malaria infected red blood cells (Pan et al., 1989; Asawamahasakda, 1994). It is known that artemisinin binds to hem, either in haemoglobin (inside red blood cells) or hemozoin (stored heme within the malaria parasites) and through an iron-mediated cleavage of the peroxide bridge artemisinin free radicals are formed. These free radicals are destructive to different parasite membranes; including mitochondria, rough endoplasmic reticulum, and plasma membranes, thereby killing them (Maeno et al., 1993; Asawamahasakda, 1994; Cumming, 1997). The proposed mechanism of action of artemisinin and related endoperoxide antimalarials is shown in fig. 1.9 (Pandey et al., 1999).

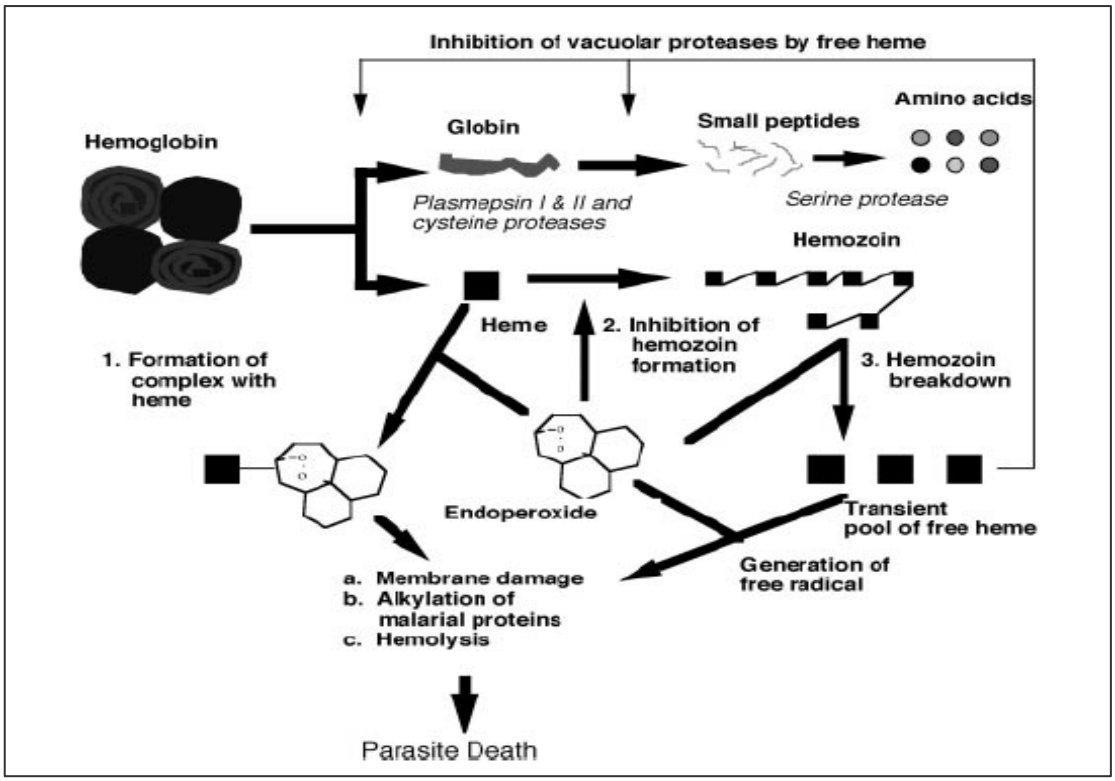

Fig. 1.9: A schematic diagram describing the proposed blood schizontocidal mechanism of action of artemisinin and related endoperoxide antimalarials (Pandey et al., 1999).

Dihydroartemisinin was shown to bind to peripheral membrane proteins located on the cytoplasmic face of red blood cells (Asawamahasakda, 1994). The ether and ester derivatives are metabolized to dihydroartemisinin, which accounts for most of the clinical effect of these derivatives after intake. Overviews on synthesis of artemisinin and the mechanism of action are given elsewhere (Webster and Lehnert, 1994, Wu and Li, 1995; Cumming, 1998; Meshnick, 1998; Van Agtmael et al., 1999; Pandey et al., 1999; Golenser et al., 2006).

Despite the high efficacy of artemisinin and artemisinin-derived drugs, the synthesis of these analogues from the extracts of the original plant *Artemisia annua* with all its drawbacks and costs is highly problematic (the growing of *Artemisia annua* is possible only in limited geographic areas, namely in the South Chinese and Vietnamese uplands, and the yield of extraction is low, about 0.4 % (Chan et al., 1995)).

This has drawn attention to the design and full synthesis of analogues, structurally simple and peroxides as inexpensive antimalaria drugs that share the benefits of artemisinin high efficacy without its disadvantages. This is considered now by the WHO (World Health Organization) as a crucial matter. Therefore, it is important to test new analogues of this class.

1.12 Nuclear export signal inhibitor (Leptomycin B)

Leptomycin B (LepB) was originally discovered as a potent anti-fungal antibiotic from *Streptomyces sp.* (Hamamoto et al., 1983). In addition, LepB a metabolite that disrupts the interaction of nuclear export signals (NESs) with the chromosome reign maintenance-1 (CRM-1), also called Xpo1 in yeast, receptor by binding to a cysteine residue localized in the central domain of the receptor, constitutes a useful reagent for studying the CRM-1-mediated protein nuclear export (Kudo et al., 1998; Kudo et al., 1999).

The main function of chromosomal region maintenance (CRM-1) is to export proteins from the nucleus (Fomerod et al., 1997) by binding to a specific leucine-rich nuclear export signal, (leucine-rich NES), of exported proteins and introduce them to the export machinery of the nuclear pores (Ullman et al., 1997). However, collected data showed that LepB is a potent anti-tumor agent against murine experimental tumors (Yoshida et al., 1990, Komiyama et al., 1985). Studies in Scizosaccharomyces *pombe* identified the cellular target of LepB as the CRM1/exportin 1 protein (Nishi et al., 1994), which is critical for the export of RNA and proteins containing a nuclear export sequence (NES). In the case of RNA export, CRM1 binds to ribonuclear proteins containing NES motif (Watanabe et al., 1999; Kuersten et al., 2001). For example, the export of Rev-response element-containing human immunodeficiency virus (HIV) RNA is inhibited by LepB (Wolff et al., 1997). Moreover, the nuclear export of mRNAs of TNF, LT-α, and other cytokines were shown to be influenced by LepB. Treating the activated Jurkat T cells with LepB resulted in reducing the cytoplasmic mRNA of TNF (30.5 folds), LT- α (3.6 folds), and several other productions including IL-2 (Schütz et al., 2006). In addition, Zeiner et al., (2003), has reported that, the spliced leader (SL) RNA of *Trypanosoma brucei* was sensitive to LepB treatment (≤ 1µg/mL), which implied the involvement of Xpo1 (or CRM-1) in SL RNA nuclear export; whereas *Leishmania tarentola* was insensitive to LepB treatment, which demonstrated by growth curves and unaltered morphology. However, the ability of LepB to inhibit nuclear export has made it a useful tool in the study of the subcellular localization of many regulatory proteins (Asscher et al., 2001). Therefore, the LepB ability to reduce the plasma TNF and LT-α level, as well its *in vitro* efficacy on Pf/NF-54, have been investigated in this study.

The objectives of this Study

As stated above, drug resistance in *P. falciparum* has become extremely important and accomplished fact, which should be faced. This fact has enforced new strategies of finding new drugs targets. On the other hand, these new anti-malarials must meet the requirements of rapid efficacy, minimal toxicity, low cost and should be efficacious against drug-resistant strains. However, the major objective in this study was to investigate the in vitro inhibition of five targets taken from HIV-1, antimicrobial, cancer agents, *P. falciparum* and other parasites (such as *Trypanosoma brucei*), based on studies have primarily been aimed at evaluating and proving the importance of the selected targets. Therefore this study was aimed to investigate the in vitro anti-malarial affectivity of the following:

1: The deoxyhypusine synthase inhibitor.

2: The deoxyhypusine hydroxylase inhibitors.

3: Polyamintransport inhibitors.

4: The phenazines analogues.

5: The dihydroartemisinin analogues.

6: To confirm in vivo the results obtained in vitro, for the substances which exhibit anti-malarial efficacy, by using the murine *Plasmodium* model, *Plasmodium berghei* ANKA (chloroquine sensitive strain), as a model for human malaria.

7: To investigate the ability of CNI-1493, Leptomycin B, and other obtained inhibitors in saving the infected C57Bl/6 mice of developing cerebral malaria, by targeting tumour necrosis factor and lymphotoxine-α.

8: The substances which exhibit in vivo anti-malarial efficacy, on *Plasmodium berghei* ANKA will under go further in vivo investigations by using the chloroquine resistant *plasmodium* strain (*Plasmodium yoelii nigeriensis*), and to prove the anti-malarial mode of action for the in vivo selected substances.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Agarose:	Top Vision™ LE GQ Agarose MBI Fermentas, Germany
Ammoniumchlorid:	ROTH, Germany
Ampicilin:	SIGMA-aldrich, Germany
Antibiotic-Antimycotic solution	SIGMA-aldrich, Germany
Biocarbonat (NaHCO3):	
Bromphenolblau:	SIGMA-aldrich, Germany
1-Brom-3-chloro-propan:	SIGMA-aldrich, Germany
BSA:	Roth, Karlsruhe, Germany
TMB:	ROTH, Germany
Circle Grow:	Q-BIOgene, UK
Chloroquin diphosphate sate:	SIGMA-aldrich, Germany
Dihydroartmmisinin:	SIGMA-aldrich, Germany
Dimethylsulfoxid (DMSO):	SIGMA-aldrich, Germany
DEPC-water:	Ambion, UK
Ethanol:	MERCK, Germany
Ethidiumbromid:	Biomol, Germany
FCS:	PAA, Österreich
Ficoll®:	SIGMA-aldrich, Germany
Gentamycin:	CAMBREX, USA
Giemsas Azur-Eosin- Methyleneblue-solution:	Merck, Germany
Hepesbuffer:	MP Biomedicals, inc..

Hypoxanthin, minimum 99%:	SIGMA-aldrich, Germany
IPTG:	SIGMA-aldrich, Germany
Isoforene:	ABBOT, USA
Isopropanol:	MERCK, Germany
Glycerol:	SIGMA-aldrich, Germany
Potassium dihydrogen phosphat:	MERCK, Germany
LB Agar:	SIGMA-aldrich, Germany
Leptomycin B	LC Laboratoreis, USA.
L-Glutamin:	PAA, Österreich
Methanol:	MERCK, Germany
Sodium dihydrogen phosphat:	MERCK, Germany
Sodium chlorid:	ROTH, Germany
Sodium hydroxid:	MERCK, Germany
RPMI-1640 media with L-glutamine	
for plasmodium cultivation:	SIGMA-aldrich, Germany
RPMI 1640-Medium	
for macrophages cultivation:	PAA, Austria
Percoll®:	SIGMA-aldrich, Germany
Buffer tablets 1/l:	MERCK, Germany
Hydrochlororic acid:	SIGMA, Germany
Potassium hydrogen sulfat:	MERCK, Germany
SOC-RPMI-Medium	Invitrogen, Germany
Sorbitol:	SIGMA-aldrich, Germany
Streptavidin-POD:	Boehringer, Germany
TRIS-hydrochlorid:	ROTH, Germany

Trizol®:	Invitrogen, UK
TWEEN®20:	SIGMA-aldrich, Germany
Hydrogen peroxide 30%:	SIGMA-aldrich, Germany
X-GAL:	ROTH, Germany
Xylencyanol FF:	MERCK, Germany

2.1.2 Inhibitors used in this study

2.1.2.1 deoxyhypusine synthase (DHS) inhibitor:

This inhibitor was kindly given by Dr.Iliona Hauber, Institute for experimental virology and immunology-Hamburg-Germany.

NO.	**Code-No.**	MW
1	CNI-1493	892.2

(For chemical structure see appendix No. 1)

2.1.2.2 Deoxyhypusine hydroxydase (DHH) inhibitors:

This group of inhibitors synthesized by the team of Prof. Ulrike Holzgrabe, Institute of Pharmacy and food chemistry-Würzburg-Germany.

NO.	**Code-No.**	**MW**	**NO.**	**Code-No.**	**MW**
1	UL3B1P	458.52	2	inhi. 3	345.4
3	inhi. 6	411	4	inhi. 7	350
5	SL28	450.29	6	SL33	427.46
7	UL3d1P	457.49	8	SL18	413.43
9	SL14	409.45	10	JK14N	407.47
11	UL4P	460.49	12	UL3c1P	455,47
13	JK7E	575.68	14	JK8-2	525.52
15	JK12-ML2	574.63	16	JK11-2	497.47
17	UL3c3P	511.58	18	TG 17	547.53
19	TG 25	401.47	20	TG 26	561.55
21	TG 27	577.55	22	TG 28	581.97

(For chemical structures see appendix No. 2)

Pertain to deoxyhypusine hydroxydase (DHH) inhibitors

23	FD 59	273.38	24	UD 73	335.45
25	UD 114	321.43	26	UD 146	363.46
27	UD 170	357.5	28	UD 172	315.42
29	UD 178	287.36	30	UD 186	273.34
31	UD 190	329.45	32	UD 191	301.39
33	UD 196	391.52	34	UD 197	405.54
35	UD 198	419.57	36	UD 208	391.52
37	UD 212	363.46	38	UD 218	422.49
39	UD 219	331.42	40	UD 221	526.64
41	UD 222	571.64	42	UD PIP	245.33
43	EHW437	441.8	44	EHW612	232.33
45	MKK4	427.8	46	TG014	401.47

(For chemical structures see appendix No. 2)

More chemical information can be reviewed in (Saeftel et al., 2006)

2.1.2.3 Polyamintransport inhibitors:

This group of inhibitors synthesized by Dr. Otto Phanstiel, Chemistry department, university of central Florida, USA.

NO.	Code-No.	MW
1	NK-1-33	458.4
2	NK-1-63	351

(For chemical structures see appendix No. 3)

2.1.2.4 Phenazines derivatives:

This group of inhibitors synthesized by the team of Prof. K. Krohn, Department of chemistry, university of Paderborn-Germany.

NO.	Code-No.	MW	NO.	Code-No.	MW
1	EK1	314.38	2	EK1.1	383.27
3	EK2	224.21	4	EK2.2	293.1
5	EK3	280.32	6	EK3.1	349.21
7	EK4	279.33	8	EK4.1	349.21
9	EK7.1	315.15	10	EK8	294.35

(For chemical structures see appendix No.4)

Pertain to Phenazines derivatives

NO.	Code-No.	MW	NO.	Code-No.	MW
11	EK8.1	363.24	12	EK1.2	315.37
13	EK132-a	242.27	14	EK132-b	242.27
15	PR1	372.42	16	PR2	260.29
17	PR3	196.2	18	PR4	196.2
19	PR5	210.23	20	PR6	210.23
21	NL-13	242.27	22	NL-33	410.37
23	EK2.3	225.2	24	EK3.3	281.31
25	EK-1-4	364	26	DP-2	372.42
27	DP-3	246.26	28	MP-1	230.26
29	Er-0-0	428.65	30	OZ-36-24	270.05
31	OZ-30-5	426.3	32	OZ-39-5-18	454.3
33	OZ-12-13-1	424.3	34	PR-1-CL	440.05
35	PR-1-Py	373.12	36	PR-1-Pym	422.08
37	SDP-1	618.21	38	F-7092-5	304.06
39	F-7092-3	270.05	40	F-7092-8	254.06
41	F-6710-1	272.07	42	Dai7177 1	300.26
43	Dai7177 2	284.26	44	DP-2-Cl	440
45	DP-2-Py	373	46	TEA-9	345
47	TEA-5	257	48	TEA-2	325
49	TEA-4	241	50	PR-1-OQ	300
51	DP-2-OQ	300	52	PR-1-Gn	338
53	PR-1-Pro	366	54	PR-1-Cyc	378
55	DP-2-Pro	366	56	Ph-Bz-1	288
57	Anth-2	208	58	Anth-2-Ph	280
59	Anth-1	182	60	Anth-1-Ph	254
61	PR-OX-1	330.3	62	PR-Br-2	527.9
63	PR-Br-1	307.9	64	PR-CoH-1	460.1
65	PR-So3-1	843.7	66	Q-Br-1	300.8
67	QMQ-Br-1	250.9	68	QM-Br-1	316.9
69	QMQ-2F-1	435	70	QQ-2F-2	447.5
71	QQ-Br-1	238	72	LP-2	240

(For chemical structures see appendix No.4)

More chemical information can be reviewed in (Eyong et al., 2006)

2.1.2.5 Dihydroartmisinin analogues

This group of inhibitors synthesized by Prof. Dr. Axel G. Griesbeck, Department of chemistry, university Köln - Germany.

NO.	**Code-No.**
1	AGG55
2	AGG56
3	AGG57
4	AGG58
5	AGG59
6	Troxi.Ad
7	CST

(For chemical structures see appendix No.5)

2.1.2.6 Nuclear export signal (NES) inhibitor:

Leptomycin B (LepB), was obtained from LC Laboratories® USA, (the chemical structure is shown in appendix No.6).

2.1.3 Polymerase chain reaction (PCR) Materials

DNAse	AMBION, UK
DNAse Inhibitor	AMBION, UK
dNTPs	Quiagen Omniscript kit, Quiagen, Germany
HotStarTaq® DNA polymerase	Quiagen, Germany
Magnesium chloride ($MgCl_2$)	Quiagen, Germany
Oligo-dTs	Invitrogen, Germany
Reverse Transcriptase (RT)	Quiagen Omniscript kit, Quiagen, Germany
RNase Inhibitor	PeqLab, Germany
Sybr Green®	ROCHE, Germany
10xPuffer	Quiagen Omniscript kit, Quiagen, Germany

2.1.4 Primers

β-Aktin-FW	$_{5'}$AGAGGGAAATCGTGCGTGAC$_{3'}$
β-Aktin-RV	$_{5'}$CAATAGTGATGACCTGGCCGT$_{3'}$

From Quiagen, Germany.

mLTA_F1	GGACAGAGACCTGGACATGAA
mLTA_R1	CACCTTTGTGTCTGGGACCTA

From Operon-molecules for life, Germany.

TNFFW	TAGCCCATGTTGTAGCAAACC
TNFRV	ATGAGGTACAGGCCCTCTGAT

From Operon-molecules for life, Germany.

2.1.5 *Plasmodium* strains used for in vitro assay

Plasmodium falciparum/NF-54 (Pf/NF-54), Chloroquin sensitive strain (CQS), was maintained as cryo-frozen stock of infected red blood cells (iRBCs) cultured in vitro.

Plasmodium falciparum/K1 (Pf/K1), Chloroquin resistance strain (CQR), was maintained as cryo-frozen stock of iRBCs cultured in vitro.

2.1.6 *Plasmodium* strain for in vivo drug assay

Plasmodium berghei ANKA (PbANKA), Chloroquin sensitive strain (CQS), was maintained as cryo-frozen stock of iRBCs, prepared from donor mice infected with sporozoites.

2.1.7 Animals

Males and females (6-9 weeks old) of C57Bl/$_6$-Wild type mice and BALB/c mice were obtained from our own animal breeding facility or the House of Experimental Therapy (HET).

2.2 Instruments

Cell culture plates	CELLSTAR®, 96-well flat bottom tissue culture plates, greiner bio-one, Germany
Centrifuges	Mikro 20" Hettich, Tuttlingen, Germany Universal 32R centrifuge, Germany
Ceramic beads	Percellys Keramik-Kit 1,4mm, Peqlab Universal 32R centrifuge, Germany
Cuvettes	Eppendorf, Germany
Disposable pipettes	COPAN INNOVATION
EDTA-Reactions-test tube	Kabe, Germany
Eppendorf-Reaction-test tube	Eppendorf, Germany
Electrophoreses system	BioRad Power Pac HC
ELISA-plates	Microlon, 96 Well GREINER bio-one, Germany
FACS CANTO	Becton Dickinson GmbH, Germany
Free RNA&DNA Water	Millipore Direct-Q 3 UV
Haematocrit Centrifuge	QBC, BECTON-DICKINSON, Germany
Heating block	Eppendorf Thermomixer comfort, ROTH, Germany
Immersion oil	FLUKA, Germany
Incubator	Hereaeus B5050, Germany

Incubator	BINDER, Germany
Micro Haematocrit capillary test tube, L= 75 ± 1.00mm & d = 1.15 ± 0.05 mm	
	Brand, Wertheim, Germany
Microscop	ZEISS Axioscope 135, ZEISS, Germany
Mini-incubator chamber	California, USA.
Needles	Sterican one time-Injection size 0.90x40mm
	Sterican one time-Injection size 0.40x20mm
	B/BRAUN, Germany
Object slides	ENGELBRECHT, Germany
PCR-Thermocycler:	MWG Primus, Germany
Photometer	EPPENDORF, Germany
Rotor gene 6000	Corbett Research, Australia
Spectra Max340 Microwellreader	Molecular Devices, Sunnyvale, California, USA
Syrings	1mL, B/BRAUN, Germany
Tissue culture dishes 60/15mm	Greiner bio-one, Germany

2.3 Media and Buffers

2.3.1 RPMI-1640 media for *P. falciparum* strains in-vitro cultivation:

Two liters of aq.dest.water containing 20.8 g RPMI-1640 media, 11.9 g Hepesbuffer and 100mg Hypoxanthin, minimum 99%, were mixed by a magnetic mixture for 30 min, ph adjusted to 7 with NaoH. The prepared media was sterilized by media filter 0.20µM + GF (Sartorius), and then stored at 8º C.

2.3.2 Complete malaria culture media (Complete MCM):

87.3 ml of the prepared RPMI 1640 culture medium was supplemented with 2.7ml biocarbonat buffer and 10ml serum. 1% antibiotic antimycotic solution (SIGMA-aldrich) was used when it is necessary.

2.3.3 Biocarbonat buffer (NaHCO3):

One hundred mL of aq.dest.water contain 7.5mg NaHCO3, mixed by a magnetic mixture for 1 hour and then sterilized by media filter 0.20µM + GF. Aliquots of 50ml were stored at 8º C in falcon test tubes.

2.3.4 Freezing solution for the *plasmodium* cryopreseving composed of:

28% glycerol, 3% sorbitol and 0.65% NaCl

For 250ml: Mix 180ml of 4.2% sorbitol in 0.9% NaCl with 70 ml glycerol.

Filter, sterilize and then store at 8º C.

2.3.5 Thawing solution containing:

3.5% of sterilized NaCl

2.3.6 RPMI 1640 Medium for macrophages cultivation composed of:

(500 ml) RPMI 1640

500 µl of 50 µg/ml Gentamycin

5 ml of 2 mM L-Glutamin

50 ml of 5 % FCS

2.3.7 Phosphate-buffered saline solution (PBS 20x): 1 Litre containing**:**

160g NaCl

23,6g NA_2HPO_4

4,0g KH_2PO_4

4,0g KCl

Adjust to pH 7.4

2.3.8 PBS/BSA 1%: 1g BSA in 100ml 1xPBS

2.3.9 TBE-buffer 10x: each 1 Litre (pH 8) containing

0,89 M TRIS

0,89 M Borsäure

20 mM EDTA

2.3.10 Giemsa Stain:

One ml Giemsa Azure-Eosin-Methyleneblue-solution in 19 ml buffer solution pH 7.2.

2.3.11 Trypan-blue Stain:

Trypan-blue (SIGMA-aldrich, Germany), was diluted 1:5 in 1xPBS to be used for cells viability assay.

2.3.12 Buffers for Enzyme Linked Immunosorbent Assay (ELISA):

Coating buffer:	0.1 M Na2HPO4 in Milli-Q water, pH 9.0
Washing buffer:	1 M PBS, 0.05% Tween
Blocking solution:	1 M PBS with 1% BSA
Substrate buffer:	0.1 M NaH2PO4. H2O, pH 5.5
Substrate:	60 mg 3,3`,5,5`Tetramethylenbenzidine (Roth, Karlsruhe, Germany) dissolved in 10ml DMSO
Substrate solution:	10 ml substrate buffer 200 µl substrate 2 µl H2O2
Stop solution:	2 M H2SO4

ELISA's antibodies are shown in table 2.1

Table 2.1: Antibodies used for detection of cytokines by ELISA

Cytokine	Coating antibody	Detection antibody	Recombinant protein	Source
IFN-γ	R4-6A2, 2µg/ml	XMG1.2, 1µg/ml	19301T	BD Pharmingen, Germany
IL-10	JES5-2A5, 2µg/ml	SXC-1, 1µg/ml	19281T	BD Pharmingen, Germany
TNF	840143, 0.8µg/ml	840144, 150ng/ml	840145	R&D Systems, UK
LT-α	168717	JIQ03, biotinylated antibody	749-TB	R&D Systems, UK

2.4 Methods

2.4.1 *P. falciparum* strains in vitro cultivation method

2.4.1.1 Serum preparation

Human serum was obtained from blood bank (Uni-clinics of Bonn University). The serum was inactivated in a water bed at 56ºC with disinfectant (EtOH) for 60 min. Each 4-5 packets were mixed in 500mL glass bottle and aliquots of 50 ml were stored at -20ºC in falcon test tubes.

2.4.1.2 Blood washing (Human blood group A^+ and O^+)

Blood samples, obtained from blood bank (Uni-clinics of Bonn university), were collected in 10 ml heparinized tubes (citrate solution tubes, SARSTEDT). These samples were centrifuged for 5 min at 3000 rpm. The supernatant serum was discarded and the rest was washed, 2-3 times, with RPMI-1640 by centrifugation for 5 min at 3000 rpm. 2-3 ml of serum were added to the finally obtained Red blood cells (RBCs) in each sample which were then stored at 8ºC.

2.4.1.3 Parasite cultivation

NF-54 and K1 strains of *P. falciparum*, were picked up from the liquid nitrogen, thawed in thawing solution by adding 1:1 to the cryotube of the parasite. The thawed parasites were centrifuged at 3000rpm for 5 min, the supernatant was discarded and the iRBCs were adding to 0.8ml of uninfected RBCs (uiRBCs) in 5 cm Petri dishes and cultured by standard method of Trager and Jensen (1976), under the guide lines of (Schlichtherle et al., 2000).

Each culture dish for the routine culture contains 5ml of complete MCM and 0.8 - 1 ml RBCs, about 10% hematocrit. The culture dishes were placed in a Modular incubator chamber (Fig.2.1) in an atmosphere of 90% N_2, 5% O_2 and 5% Co_2 at 37 ºC. Antibiotic-Antimycotic solution was used when it is necessary. Cultures with parasitaemias (varied 3% to 5%) were used for *in vitro* drug assay.

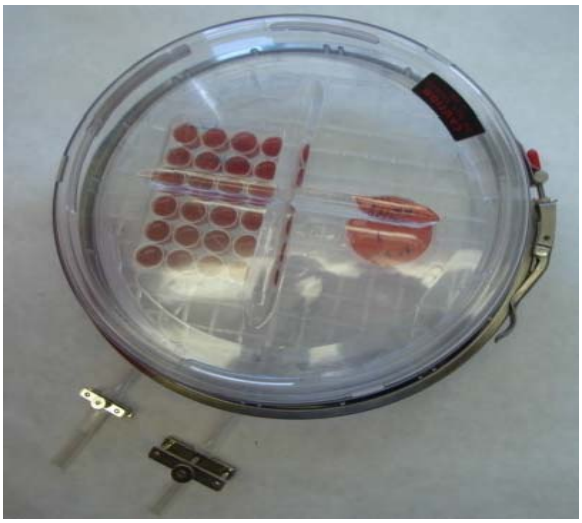

Fig.2.1: Modular incubator chamber

Mini-incubator for the *P. falciparum* cultivation.

2.4.2 In vitro drug efficacy assay method

The drug effect assay method used in this study was based on the Giemsa stained slide method. This method relies on a morphological criterion of response and reports a single concentration as the end point i.e. concentration of a drug in the first sample showing complete inhibition of growth. This measurement is classically known as the Minimum Inhibitory Concentration (MIC), method which is suitable for distinguishing susceptible and resistant isolates (Desjardins, 1984)

The initial parasitemia used in the drug efficacy test was adjusted to obtain parasitemia of about o.5%, by adding uninfected erythrocytes, to be used in the drug efficacy test. Four

days screening test (96hrs) was used to assay the drug effect on the growth of *P. falciparum*. Equal numbers of parasites were seeded in 24-well plates, at about 0.5% parasitemia and 8% hematocrit. Four wells for each test, each well contains 200µl infected erythrocytes and 2ml complete MCM with inhibitor (test) or without (compound-free control). The medium was changed every 24hrs. Thin blood smears Giemsa-stained slides (Fig.2.2) were prepared from each well, and parasite counts (parasitemia) and morphological evaluation were determined microscopically, daily on day 0 and on the following 4 days. The morphological evaluation of parasite maturation was done, under 100x oil immersion objective, by counting the percentage of infected cells in at least 1000 erythrocytes in each slide. The drug ability to inhibit the parasite growth (reduce the parasitemia) compared with the mean value of the compound-free control (taken as 100%), was calculated by the following equation:

$$\text{The inhibition percent} = 100 - \frac{\text{Mean parasitemia in the tested}}{\text{Mean parasitemia in the compound-free control}} \times 100$$

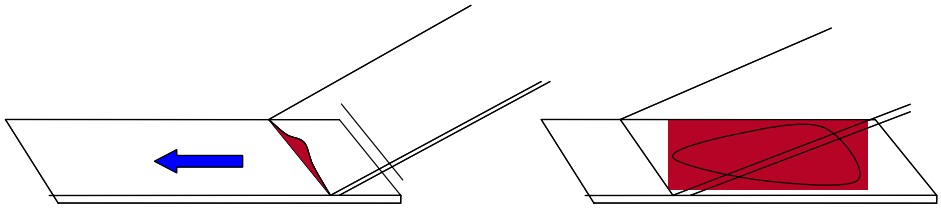

Fig. 2.2: Thin bloods smear preparation

2.4.2.1 Calculation of 50% inhibition concentration (IC_{50}):

The dose (IC_{50}) of each drug or each drug combination required to achieve a 50% inhibition of malarial activity. IC_{50} assay was used in this study to allow direct comparison of results obtained from different inhibitors on parasite growth. The selected time point of IC_{50} calculation was 72 hours post-treatment, for two reasons, 1: As the 72hrs are a part of two life cycles of the cultivated plasmodium (Basco, 2004), where the parasite gets enough time to show a clear response to the used inhibitor. 2: The inhibitor will be able to show a clear efficacy on the parasitemia. Some inhibitors showed an effect at 24hrs post-treatment, but the parasite was able to resist this effect and parasitemia started to appear at 48hrs post-treatment (A typical image shown in fig. 2.3), (differences in the calculated IC_{50}s are shown in Fig.2.4 and Fig.2.5).

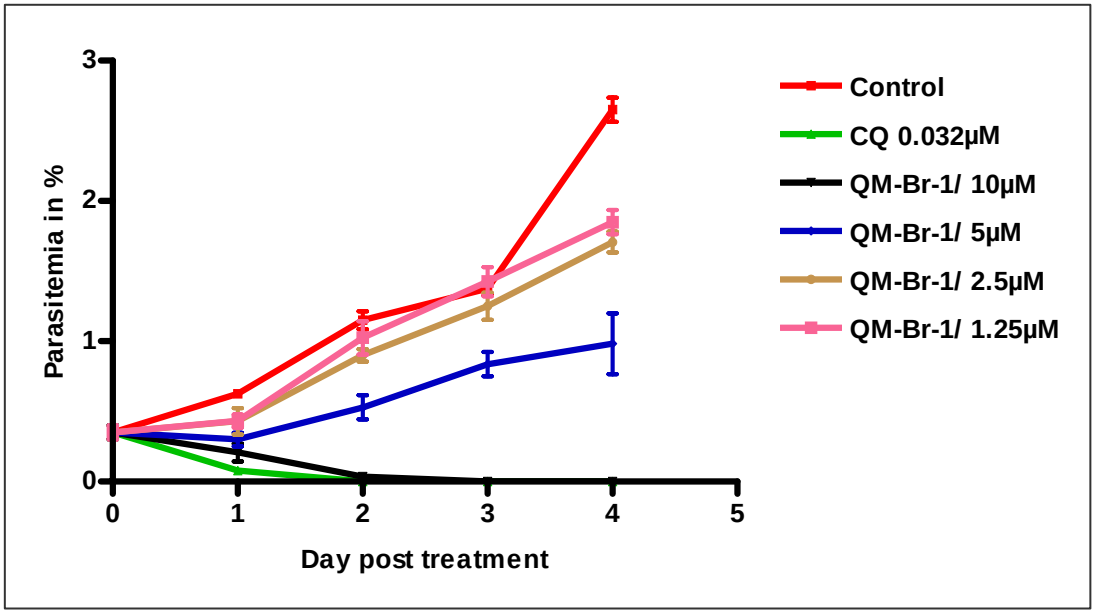

Fig. 2.3: Parasitemia grows up on day2 post treatment after Pf/NF-54 strain in vitro exposured to QM-Br-1.

Data obtained from the inhibitor-dependent concentration growth curve were computed into plots with linear regression analysis from y axis (inhibition %) to x axis (inhibitor

concentration µM), (Singh et al., 1997; Kaiser et al., 2001), by using Microsoft office Excel 2003 (Fig.2.4 and 2.5).

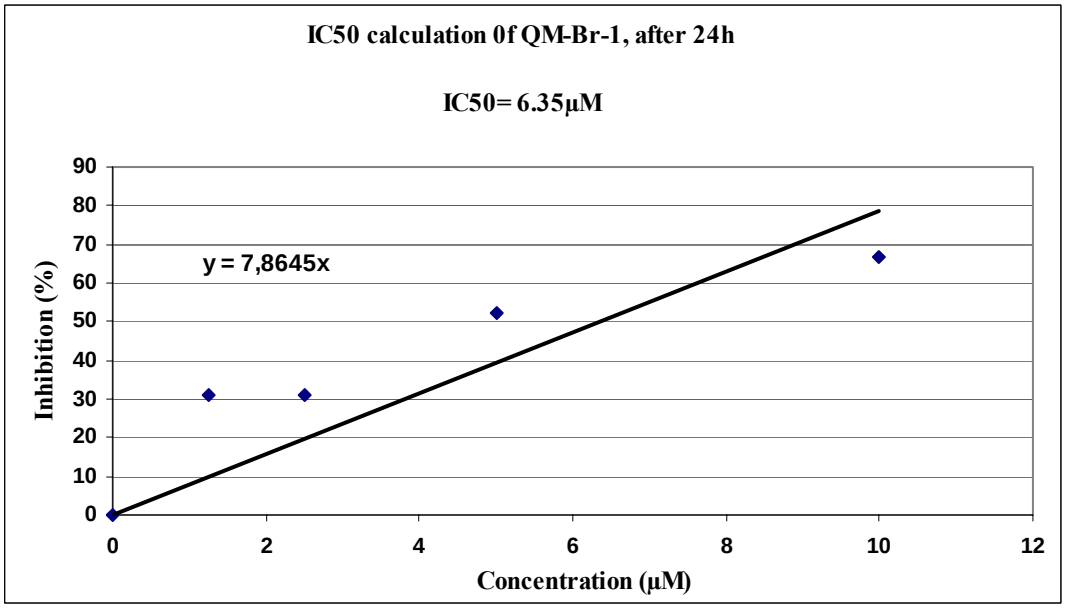

Fig. 2.4: The calculated IC_{50} of QM-Br-1, after 24hrs incubation with Pf/NF-54 strain

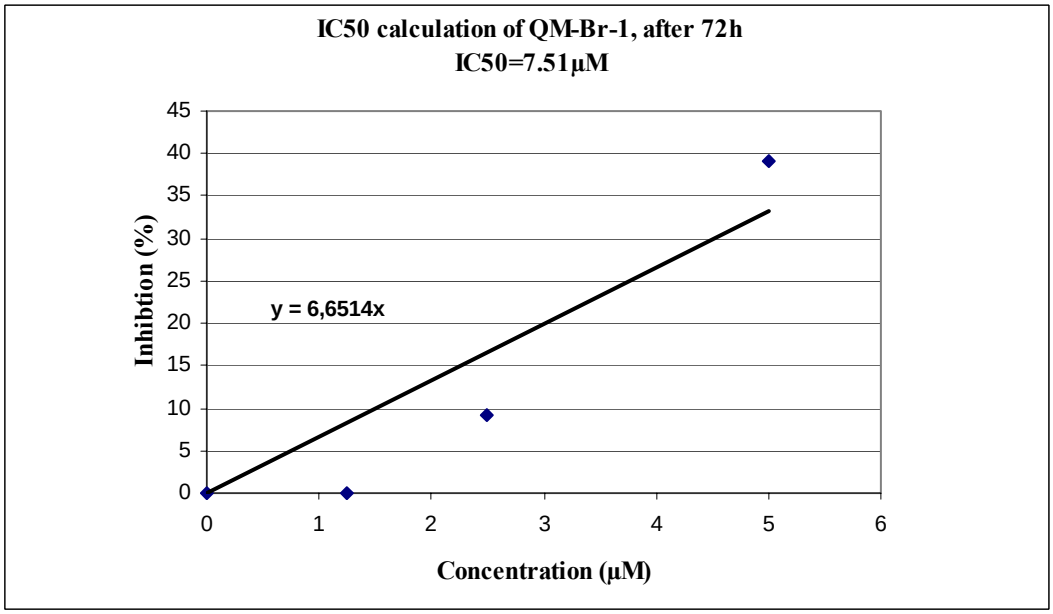

Fig. 2.5: The calculated IC_{50} of QM-Br-1, after 72hrs incubation with Pf/NF-54 strain.

2.4.2.2 Standard drugs for the in vitro assay

Chloroquin diphosphate and dihydroartmmisinin have been used as a reference drugs in parallel of the tested inhibitors.

2.4.3 In vivo methods

2.4.3.1 In vivo infection with iRBCs

PbANKA was used for the infection were about 150µl of iRBC stock was intraperitoneally (i.p.) injected into donor mice of the same strain as animals in the experiment. 3-4 days after infection, parasitemia was determined. If parasitemia reached levels of 4-10%, blood samples were taken by orbital puncture after short term anaesthesia. Infected solution was prepared with sterile 1xPBS. Each mouse received intravenously (i.v.) 200 µl of 1xPBS /erythrocyte solution with approximately $5x10^4$ iRBCs to infect C57Bl/6 WT mouse and approximately $1x10^5$ iRBCs to infect BALB/c mouse. Infected mice were named PbANKA infected mice. The parasite was detected on a Giemsa-stained thin-blood smear, by collecting blood via the mouse tail vein. Parasitemias were quantified as the percentage of the infected red blood cells (iRBCs)/1000RBCs/slide.

2.4.3.2 CNI-1493 in vivo cytotoxicity assay (in vivo killing assay)

2.4.3.2.1 Adenovirus infection

Adenovirus expressing ovalbumin (AdOVA) induce an increase, as a short peak, in IL-12, (Furumoto et al., 2000; Miller et al., 2002), shortly after administration, and increased ovalbumin (OVA)-specific cytotxic T lymphocyte cells (Wingender et al., 2006). To investigate whether the administration of 4mg/kg, CNI-1493, will suppress CTL activity in vivo or not. $1x10^8$ pfu/mouse of AdOVA were given i.v., (i.v. immunonization) to two groups of uninfected C57Bl/6 WT mice; CNI-1493 pre-treated group (treated i.v. on -5, -3

and -1 day before AdOVA infection) and non-treated group. Recombinant Adenovirus type 5 expressing OVA (AdOVA) were kindly provided by the Institute for Molecular Medicine and Experimental Immunology (IMMEI), Bonn, Germany.

2.4.3.2.2 Target cells administration

58L target cells were labelled with 1 μM CFSE to allow visualization via flow cytometry and labelled with OVA antigen-specific class I peptides, these target cells are recognized by CTLS and being lysed by antigen-specific CTLs of immunized animals. Internalization of adenovirus (Ad) into target cells is mediated by two steps: The fiber knob of Ad particles initially attaches to coxsackie-adenovirus receptor (CAR) on the cell surface (Bergelson et al., 1997), and then $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins which interact with the Arg-Gly-Asp motif in the Ad-penton base and hence facilitate internalization of the virion (Wickham et al., 1993).

$1x10^7$/mouse from the 58L target cells were given i.v. five days after immunization. Five hours later the spleen was taken out and homogenised in 1xPBS. The ratio of lysed target cells to was determined by flow cytometry. Immunized mice should have reduced numbers of target cells, whereas naïve animals should have stable numbers of target cells.

58L target cells, generated from splenocytes from syngenic donors, were kindly provided by the Institute for Molecular Medicine and Experimental Immunology (IMMEI), Bonn, Germany. The ratio of lysed target cells was determined by the following equation:

$$\% \text{ of spicific lysis} = 100 - \left(100x \frac{\left[\frac{\text{CFSE high}}{\text{CFSE low}} \right] \text{Immunized mouse}}{\left[\frac{\text{CFSE high}}{\text{CFSE low}} \right] \text{Non-Immunized mouse}} \right)$$

Where as CFSE: CarboxyFluoroscein Succinimidyl Ester

2.4.3.3 Drugs for in vivo administration

After being dissolved in DMSO and then in 1xPBS, unless otherwise stated, the drugs were administrated intraperitoneal (i.p.), intravenous (i.v.) or orally (by using an especial oesophagus-tube) in a volume of 200µl/mouse, unless stated otherwise.

Whereas the reference drug, Chloroquin diphosphate (CQ) was dissolved in 1x PBS and administered i.p. at 25mg/kg body weight of the mouse in a volume of 200µl/mouse.

2.4.3.4 Blood plasma collections

Plasma is the liquid portion of blood, which consists of water, proteins (including cytokine, albumins and globulins), electrolytes, nutrients and hormones. About 80µl blood were collected from the mouse tail vein, directly in a capillary tube and then transferred into 1ml haematology test tube (containing EDTA) maintained on ice. Afterwards, the collected blood samples were centrifuged at 8000rpm for 5min, and stored in 0,5 ml Eppendorf-test tube at-80 °C, for later analysis of cytokines.

In order to avoid interference with the infection process due to the possible effect of daily bleeding on immune reactions, the blood samples were taken from each mouse only on the second day, unless stated otherwise. Each group was sub-dividing in to two groups where blood plasma was collected from the first sup-group on the odd days after infection and from the second sup-group on the even days.

2.4.4 Cell culture methods

2.4.4.1 Macrophages purification method:

Macrophages were purified from spleens of C57Bl/6 WT mice. Spleens were collected under sterile conditions in individual tubes containing 5ml 1xPBS, then homogenized on steal filter and then centrifuged at 1300 rpm for 5 min. The supernatant was discarded and loosening the remaining erythrocytes were loosened by adding of 14 ml NH4Cl and left in RT for 15 min. The loosening solution was removed and cells washed two times with PBS. The White Blood cells (WBCs) were re-suspended in PBS and passed for filtration through a 30µm pre-separated filter (Milteny Biotech, Bergish Gladbach, Germany) to remove cell aggregates and large particles. The number of WBCs per ml of blood was determined by using Neubauer's counting chamber. Macrophages were purified by positive selection with magnetically labeled CD11b MicroBeads using the column of MACS® system (Milteny Biotech, Bergish Gladbach-Germany). The cell suspension (positive fraction) was centrifuged and the cell number was determined, by using Neubauer's counting chamber under the light microscope. Using this method of purification, macrophages purity determined by Cytospin was found to be 90%. Cells viability were assessed by trypan blue exclusion test, pre and post cultivation.

2.4.4.2 Macrophages cultivation method

Spleenic macrophages, of day3 post-infection (d3pi), of PbANKA infected C57Bl/6 mice were plated $5x10^4$ cell/well, either with 200µl media alone or with the presence of increasing concentrations of Leptomycin B and Lipopolysuccaride (LPS) 100µg/ml, in 96-well flat bottom tissue culture plates (Greiner bio-one), for 24 hrs at 37°C under 5% CO_2. The suspension was removed and stored at -20°C, till the time for ELISA assay.

2.4.4.3 Cytospin preparation method:

Cytospin was prepared after the adding $2x10^5$ of the spleen cells to 1ml of 1% PBS. 200µl of the cell suspension was centrifuged against a glass slide covered with a special filter paper, using a universal 32R centrifuge (Hettich zentrifugen), for 5 min at 800 rpm. These filter papers allowed the absorption of the solution and therefore the flattened adherence of the cells to the surface of the slide. The slides were dried overnight and stained with May-Grünwald stain for 3 min and then with Giemsa stain for 10 min.

2.4.5 Immunological methods

2.4.5.1 Sandwich Enzyme Linked Immunosorbent Assay (ELISA) method:

Cytokine profiles over the time in the blood plasma were determined by specific sandwich ELISA using microlon high bonding microtiter plates. Antibody pairs and recombinant proteins used as standards can be observed in (Table 2.1). The plates were incubated over night at 4°C with 50 µl coating antibody diluted in coating buffer. After two washings with washing buffer, the plates were incubated with 150µl/well blocking solution for one hour at room temperature and washed again. 100µl/well of plasma samples and a series of standards diluted in 0.1% BSA-PBS, were added and incubated over night a 4°C.

After five washes, 100µl biotinylated detection antibodies diluted in 0.1%BSA-PBS were added to each well. After 60 min of incubation, plates were washed five times, and incubated with 100µl streptavidin-peroxidase complex (1:5000) for 45 minutes at room temperature. After five washes 100µl substrate solution were added to each well. The reaction was stopped after colour development by adding 100µl stop solution. Plates were read at 450 nm using a Spectra Max 340 Microwell reader. Concentration of the samples was determined from the standard curve.

2.4.5.2 Direct Enzyme Linked Immunosorbent Assay (D. ELISA) method:

Direct ELISA used to determine the LT-α profile in the mice blood plasma, the ELISA plates are coated with 100µl of plasma samples, which diluted 1:20 in 1xPBS, or series of standards, diluted in 1xPBS, and then incubated over night at 4°C. Afterwards, the plates were incubated with 150µl/well blocking solution for one hour at room temperature (RT), then washed with the washing buffer and incubated again with the antibody for 3 hours at RT. The remaining steps of the procedure are similar to those of the sandwich ELISA method.

2.4.6 Molecular biology methods

2.4.6.1 RNA isolation

Brains of pbANKA infected C57Bl/6 WT mice were collected, frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated with Trizol® where the frozen mice brains were homogenised in percellys ceramik-kit-1,4mm-2ml-tube, which filled with 1ml Trizol® and treated in the Percellys 24 Homogeniser for 2 cycles/30s in 6500 U/pm, whereby all cell components are contained in a suspension. Subsequently, the suspension transferred to 1.5ml Eppendorf-tube vibrated with 100µl Bromchloropropan (BCP), incubated 10 min at RT, and then centrifuged at 12000g for 20 min in 4°C. By this time, RNA should have been separated from proteins, DNA and fabrics. The supernatant aqueous phase, which contains RNA, was shifted and vibrated with 500 µl Trizol® +100µl BCP and then incubated for 5 min at RT and centrifuged at 12000g for 15 min in 4°C. Again the upper aqueous phase removed and filled up to the RNA precipitation with isopropanol and incubated for 1 hour in -20°C and then centrifuged at 12000g for 15 min in 4°C. Afterwards, the RNA pellet washed 2x with ethanol (75%) and centrifuged each

time at 12000g in 4°C, for 10 and 5 min, respectively. Ethanol was removed out of the projection and the pellets dried for 5min at 37°C on a heating block.

Finally RNA pellets were dissolved in 50µl DEPC water (millipore Direct q 3 UV) and RNA concentrations were determined photo-metrically at a wavelength of 260nm (OD_{260}) and 280nm (OD_{280}) where the degree of the impurities determines.

The optical density (OD) of the samples is directly proportional to their RNA content.

The RNA content was determined out of: OD_{260} x dilution x 40 = RNA content (µg/ml).

Reference = 50µl DEPC water.

Sample = 1µL RNA + 50µL DEPC water => RNA content in (µg/µL).

To digest the existed DNA in the sample, 10µg RNA/sample adjusted into 0.5mL Eppendorf-tube with 1/10 volume 10xbuffer and 1µg DNase blended for 30 min at 37°C on the heating block. That digestion was stopped by adding 1/10Volumen DNase inactivator, followed with mixing and centrifuged at 10000 for 1.5min. Thus, the projection was transferred in new Eppendorf-tube and the absolute mRNA content determined by the photometer.

2.4.6.2 mRNA transcription into DNA complements for the comparative RT-PCR

The extracted mRNA from mice brains must be rewritten in cDNA for the quantification RT-PCR, which was done according to the manufacturer's manual (see table 2.2), in a reaction volume of 20µL.

To start the transcription: 1µg RNA (=x) + (13.67-xµl) H_2O in 0.2µl PCR-Tubes.

Extracted mRNA used as a template to prepare the single stranded cDNA which has been done by using PCR thermal Cycler (RT-AAAAA.Cyc. programme), the probes were incubated for 1 hour in 37°C and subsequently, the reaction was stopped for 5min in 95°C and afterwards down-cooled to 4°C. Finally, cDNA was diluted 1:3 with DEPC water and used for the quantitative analysis by means of real-time PCR.

Table 2.2: The reagents contents for transcription from mRNA to cDNA.

Substance:	Volum in µL/probe:
10xbuffer	2
dNTP	2
Oligo Nucleotide	1
Reverse Transcriptase (RT)	1
RNase-Inhibitor	0.33

2.4.6.3 Establishment of an external standard for LT-α -Gene of C57BL/6WT mice

In order to test the specificity of the murine LT-α Primer, amplification products of these primers have to be sequenced. A normal-PCR out of cDNA probes of brains of pbANKA infected C57Bl/6 WT mice were accomplished, in order to receive the LT-α amplified product in adequate amount for farther investigation, the normal-PCR was set as follows:

Table 2.3: Setup of LT-α PCR (the reaction volume was 20µL)

Substance:	Volume in µL/probe:
H_2O	12.2
10xPuffer	2
MgCl (25mM)	1.2
dNTP (40mM)	0.1
LT-α $_{FW\ 5\mu M}$	1.2
LT-α $_{Rv\ 5\mu M}$	1.2
HotStarTaq® (250 U)	0.1
cDNA	2

After which treated in PCR thermal Cycler (Real test. Cyc. programme).

2.4.6.4 Agarose gel electophoresis

To control the PCR product, a gel electrophoresis, 1% agarose gel in 0.5× TBE buffer + 3µL Ethidiumbromid, was used to investigate the amplification of the expected 183 bp DNA sequence of LT-α gene. The gel was polymerized 30 min. Subsequently, loaded 10µL PCR product and 2µL loading buffer/lane. As a reference control 5µL 50 base pairs (bp) DNA marker was used. The electrophoresis chamber filled with 0.5% TBE buffer and operated for 45 min with 125 V and 3 mA. (The results can be seen in appendix No. 9).

2.4.6.5 Cloning of LT-α PCR product:

The cloning of PCR product was made by using pCR®4-TOPO® cloning kit for sequencing (Invitrogen, Germany), and performed according to transformation of one Shot® Chemically Competent *E.coli* cells. The reagent contents of the TOPO® cloning reaction are described in table 2.4.

Table 2.4: Reagent contents of the TOPO® cloning reaction for cloning of LT-α amplified product

Reagent	Volum in µL:
Salt solution	1
Sterile Water	2
pCR®4TOPO®-Vector	1
PCR-Product	2

The cloning took place at RT and incubation period of 20 min. At expiration of the incubation the vector which was stored in -20°C into chemically competent *E.coli*, transferred on ice for the transformation.

The transformation performed by using a victor in Top10 and DH5α™-T1^R competent *E.coli* cells, 4µl of the prepared TOPO® cloning reaction was add into 50µl of the one Shot® Chemically Competent *E.coli* and mixed gently. The mixture was incubated for 5

min at RT, and then seeded on a pre-warmed LB agar-plates containing 50µg/ml ampicillin which were incubated at 37°C overnight for culture.

2.4.6.6 Selection of transformed cells by antibiotic resistance

The agar plates contain ampicillin, an ß-Lactam-antibiotic effective against the gram-negative organisms, to be used as a selective substance**.** The transformed vector contains a gene for the resistance against ampicillin, and thus, recombinant *E. coli* colonies (white colonies – deficient of β- Lactam) were picked with a sterilised platinum wire loop and screened by PCR using the same primer pairs as in the original PCR amplification (Table 2.3). The loop contains *E. coli* cells, was placed directly into the PCR reaction tube. The PCR programme was the same as for the original LT-α amplification. The PCR products were run on 1 % agarose gel and stained with ethidium bromide to identify positive clones.

2.4.6.7 Plasmid DNA preparation and purification

Individual *E. coli* clones with LT-α inserts, positively cloned, were inoculated into 5 mL, of Circle Grow® + 10 µg/L ampicillin, medium and incubated at 37^{o}C with vigorous shaking (300rpm/min) overnight. The cells were harvested the next morning by centrifugation at 5000 rpm for 1 minute. The cell pellets were re-suspended in 250µL solution A from the Plasmid Midi Two Kit and plasmid DNA was isolated as described in the manufacturer's protocol, Invisorb® Spin Plasmid Mini Two for plasmid DNA isolation (Invitek, Germany).

The LT-α gene was confirmed by Eurofins MWG Company, and by BLAST, and found to be the write gene with a mass of 183 bp.

The LT-α plasmid concentrations of DNA copies were determined by the following equation:

$$\frac{6 \times 10^{23}\ (\text{Copies/mol}) \times \text{Plasmid concentration (g/µl)}}{\text{Molecular weight}_{MW}\ (\text{g/mol})} = \text{Copies/µl}$$

The molecular weigh of double strand DNA (dsDNA) was determined by the following equation:

No. of Base pairs (insert+plasmid) x 660Dalton/Base pairs = MW dsDNA

2.4.6.8 The cDNA measurements

To determine the concentration of mRNA for TNF and LT-α, in the different groups, the transcripted cDNA was used to compare between the concentrations in the mice probes by using the quantitative Real Time PCR, in a total reaction volume of 20µL. (For reagents contents used in this work (see tables 2.5, 2.6 , 2.7 and table 2.8 for the used programme to setup a RT-PCR). To calculate the DNA comparative concentrations a known standard concentration was used, for each tested cytokine, as a positive control (Table 2.9).

Table 2.5: Reagents contents to setup a RT-PCR for mouse LT-α.

Substance	Volume/Probe
H_2O	12,8 µl
10xPuffer	2,0 µl
MgCl(25mM)	0,4µl
dNTP(40mM)	0,1 µl
mLTA_F1, $_{5\mu M}$	1,2 µl
mLTA_R1, $_{5\mu M}$	1,2 µl
SYBR® Green$_{1:1000}$	0,2 µl
HotStarTaq®	0,1 µl
cDNA	2 µl

Table 2.6: Reagents contents to setup a RT-PCR for mouse TNF.

Substance	Volume/Probe
H_2O	11,6 µl
10xPuffer	2,0 µl
MgCl(25mM)	1,6µl
dNTP(40mM)	0,1 µl
TNFFW$_{5\mu M}$	1,2 µl
TNFRv$_{5\mu M}$	1,2 µl
SYBR® Green$_{1:1000}$	0,2 µl
HotStarTaq®	0,1 µl
cDNA	2 µl

Table 2.7: Reagents contents to setup a RT-PCR for mouse ßActin (reference gene).

Substance	Volume/Probe
H_2O	10,8 µl
10xPuffer	2,0 µl
MgCl(25mM)	2,4µl
dNTP(40mM)	0,1 µl
P- β-Aktin $_{FW5\mu M}$	1,2 µl
P- β-Aktin $_{Rv5\mu M}$	1,2 µl
SYBR® Green$_{1:1000}$	0,2 µl
HotStarTaq®	0,1 µl
cDNA	2 µl

Table 2.8: RT-PCR program:

No. Of cycles	Temperature/Time	Step
1x	95°C/15sec.	Denature, Activating the HotStarTaq®
95x	94°C/10sec.	Anneal Primers
	58°C/20sec.	Elongation, SybGr Detection
	72°C/20sec.	Denature the DNA-Double strand
	72°C-1°C/each step 4sec.	Final elongation

-The DNA comparative concentrations were calculated by using this equation:

$$\frac{\text{Amplification control (const.)}^{\text{Take off control (const.)}}}{\text{Amplification sample}^{\text{Take off sample}}} = \text{Comparative concentration}$$

-The mRNA copies/probe was calculated by using ß actin as a reference gene.

Table 2.9: Concentrations of positive controls

Standard/dilution µg/µL	Copies/µl
β-Actin / 10^{-5}	1,82 x 10^{5}
LT- α / 1,63x10^{-5}	3,58 x 10^{6}
TNF / 10^{-5}	744000

2.4.7 Statistical analysis

The graph PAD Prism 4 (graph PAD 4.00 software, San Diego California, the USA) was used for statistical analyses of the obtained data and for plotting of the results.

All parasitemias, cytokines levels and mRNA copies are expressed as mean values ± SEM (standard error of the mean). Data obtained from more than two groups were analyzed by using the One Way ANOVA, a Kruskal Wallis test and a Dunn´s multiple comparison test.

Data obtained from two groups were analyzed by using Mann Whitney test or the two-tailed unpaired *t* test, considering confidence intervals of 95% and F test was performed to compare the variance. The survival curves were accomplished by using Logrank Test. Whereas, Chi-square was used to analyze the differences in the parasite's erythrocytic stages in the tested groups compared to that in the control. P-values of ≤ 0.05 were considered to be significant.

3. Results

3.1 In vitro results

In vitro screens for activity constitute a key component for anti-malarial drug screening. It is based on the ability to culture *P. falciparum* in human erythrocytes in vitro. The development of techniques for continuous cultivation of *P. falciparum* is a reliable source, for continuous stock culture of parasite, as a part of drug screening and long term assessment (Kalra et al., 2006). In addition, this assay technique is a simple method to determine the susceptibility of *P. falciparum* to various drugs.

3.1.1 General investigations

3.1.1.1 In vitro effects of chloroquine (CQ)

To evaluate the validity of the drug susceptibility assay, which is used in this study, the IC_{50}s of CQ for (CQS strain) Pf/NF-54 and (CQR strain) Pf/K1, were determined to be compared with previously published values. CQ showed an IC_{50} of 0.0138µM on Pf/NF-54 strain, after 24 hrs post-treatment (Fig. 3.1).

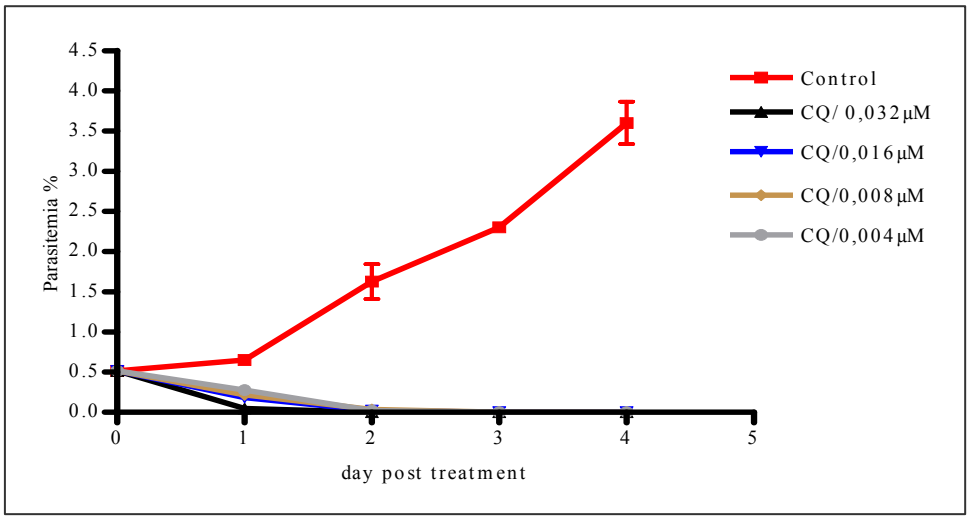

Fig. 3.1: In vitro effects of CQ on Pf/NF-54.

On Pf/K1 strain the CQ showed an IC_{50} of 0.468µM, after 24 hrs post treatment (Fig. 3.2).

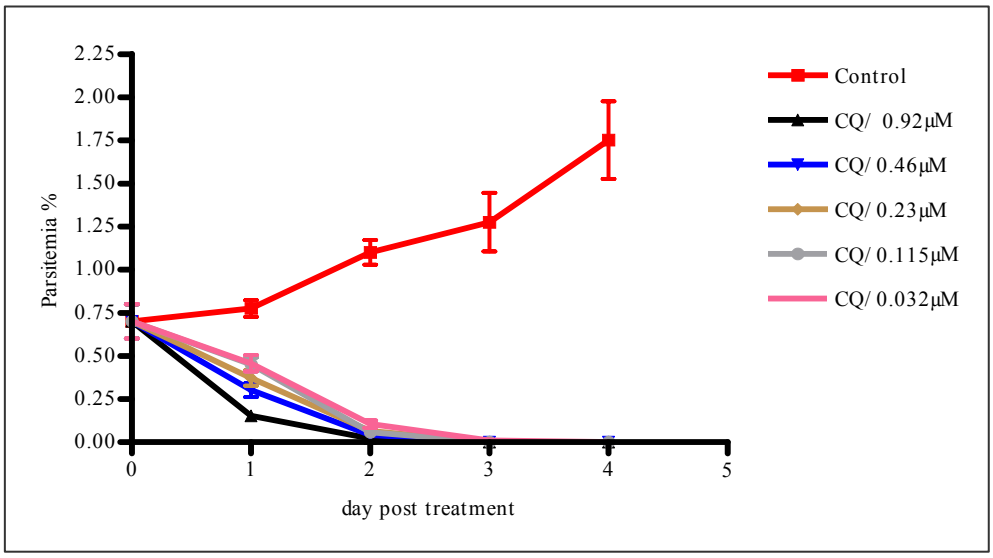

Fig. 3.2: In vitro effects of the CQ on Pf/K1.

3.1.1.2 In vitro effects of dihydroartemisinin (DHA)

On the same reason, evaluating the validity of the drug susceptibility assay; IC_{50} of DHA was also determined on Pf/NF-54 strain (Fig. 3.3). DHA showed an IC_{50} of 0.565ng/ml on the Pf/NF-54 strain, after 24 hrs post-treatment.

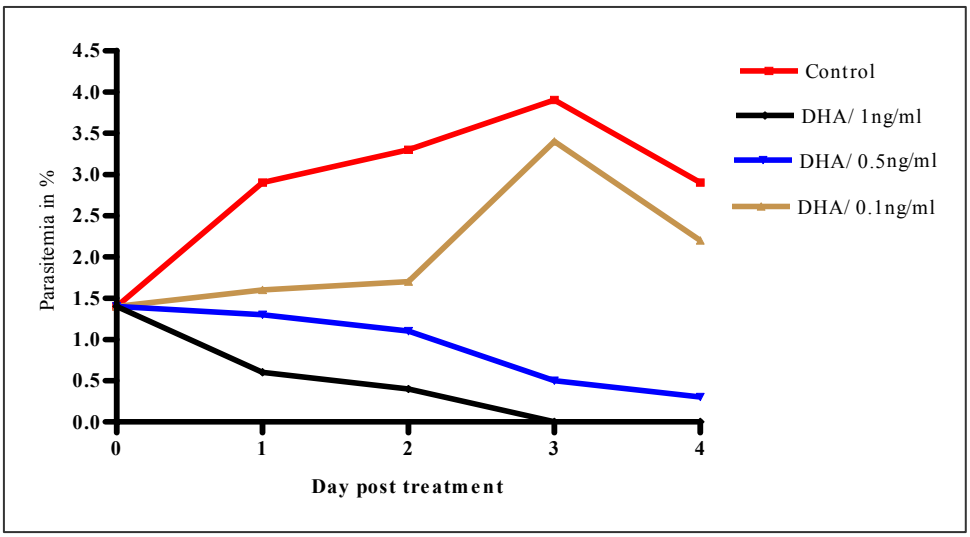

Fig.3.3: In vitro effects of Dihydroartemisinin (DHA) on Pf/NF-54.

3.1.1.3 In vitro effects of dimethyl sulfoxide (DMSO)

Since most of the inhibitors were dissolved in DMSO, the influence of this solvent on parasite growth was determined by using concentrations ranging from 0.25% to 4% (vol/vol), (Fig. 3.4).

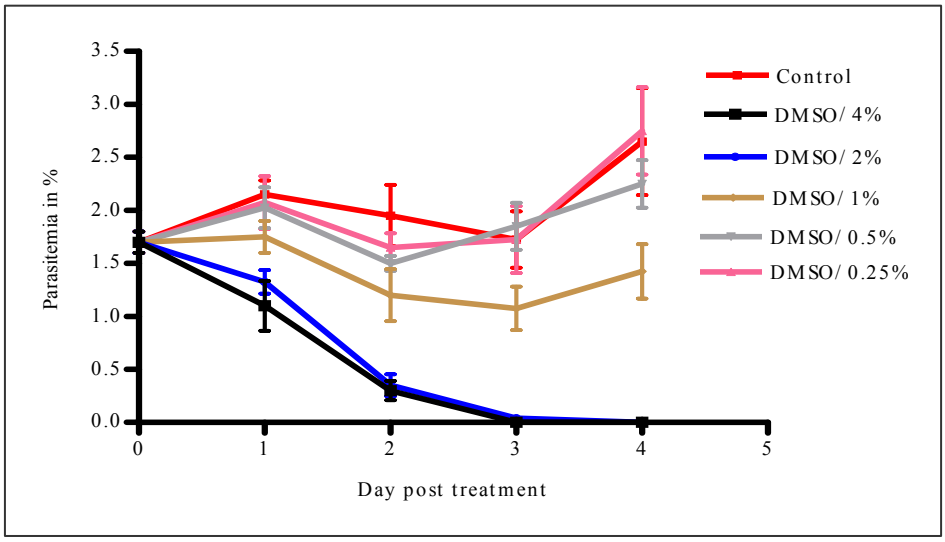

Fig. 3.4: The In vitro responses of Pf/NF-54 to various concentrations of Dimethyl Sulfoxide (DMSO).

The DMSO showed an IC_{50} of 1.14% on the Pf/NF-54 strain, after 72 hrs post-treatment (which is the time point chosen to evaluate the in vitro IC_{50} of the studied substances). However in this study, the concentration of DMSO was taken into consideration, by supplementing the complete malaria culture media (complete MCM) with DMSO in the control test whenever DMSO is used as a dissolvent.

3.1.2 In vitro results of deoxyhypusine synthase (DHS) inhibitor

CNI-1493 considered as a novel and potent inhibitor of DHS (Hauber et al., 2005) and depends on its property as a DHS inhibitor; Pf/NF-54 was exposed to several concentrations of CNI-1493, which ranged from 100 - 800 µM (Fig. 3.5). The choice of these concentrations is dependent on previous experiments of CNI-1493 (Data not shown). After 72 hrs of incubation CNI-1493 showed an IC_{50} of 135.79µM. A relation between CNI-1493 concentrations and the parasite's erythrocytic stages can be seen in figure (3.6).

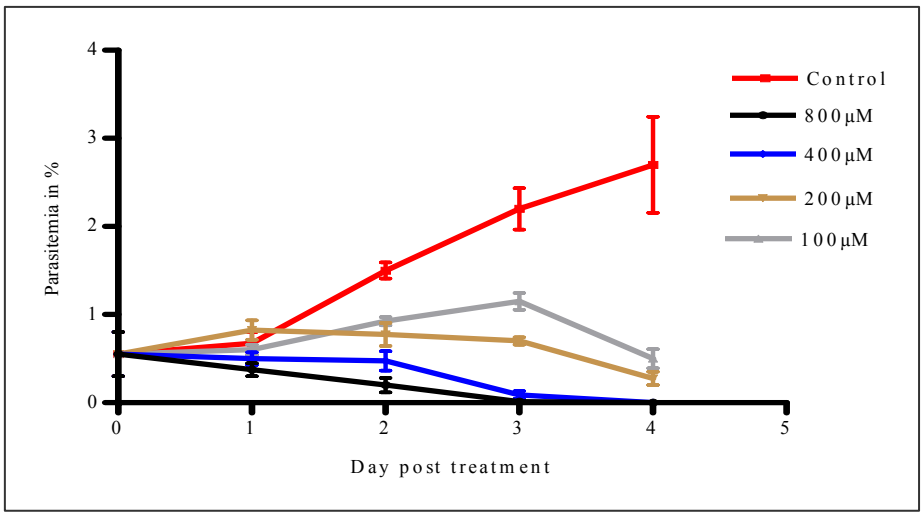

Fig.3.5: In vitro effects of CNI-1493 on Pf/NF-54. DMSO=0.42% in CNI-1493/800µM and control.

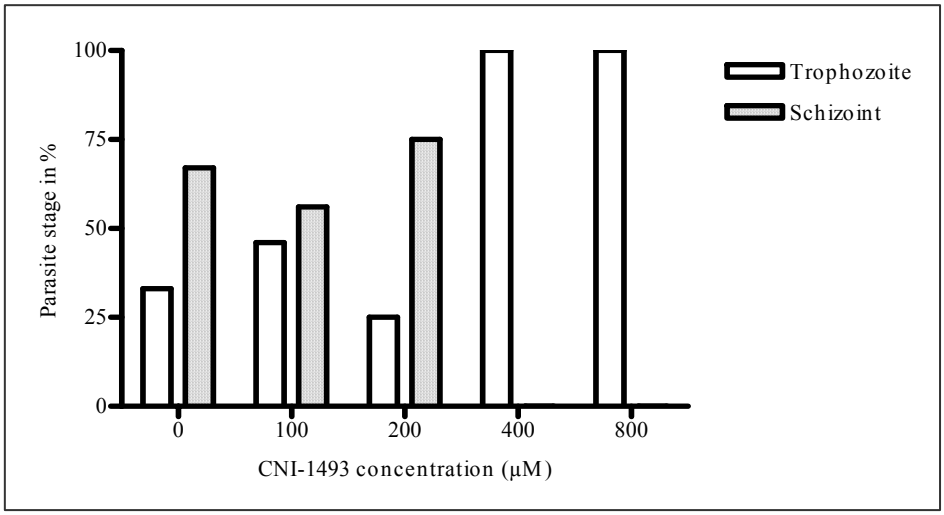

Fig. 3.6: Pf/NF-54 erythrocytic stages in relation to CNI-1493 concentrations on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 400 and 800µM, $P < 0.0500$

3.1.3 In vitro results of deoxyhypusine hydroxylase (DHH) inhibitors

It has been reported previously that Ciclopiroxolamine, a DHH inhibitor, showed in vitro anti-malarial efficacy with an IC_{50} value of 8.2 µM on Pf/NF-54 strain. However, this IC_{50} was employed to select the concentrations which will be used to test the in vitro efficacy of the DHH inhibitors on Pf/NF-54 strain. The first DHH inhibitor was UL3B1P, which exhibited in vitro antimalarial efficacy (Fig. 3.7) with an IC_{50} of (10.88 µM). A relation between UL3B1P concentrations and the parasite's erythrocytic stages can be observed in figure (3.8).

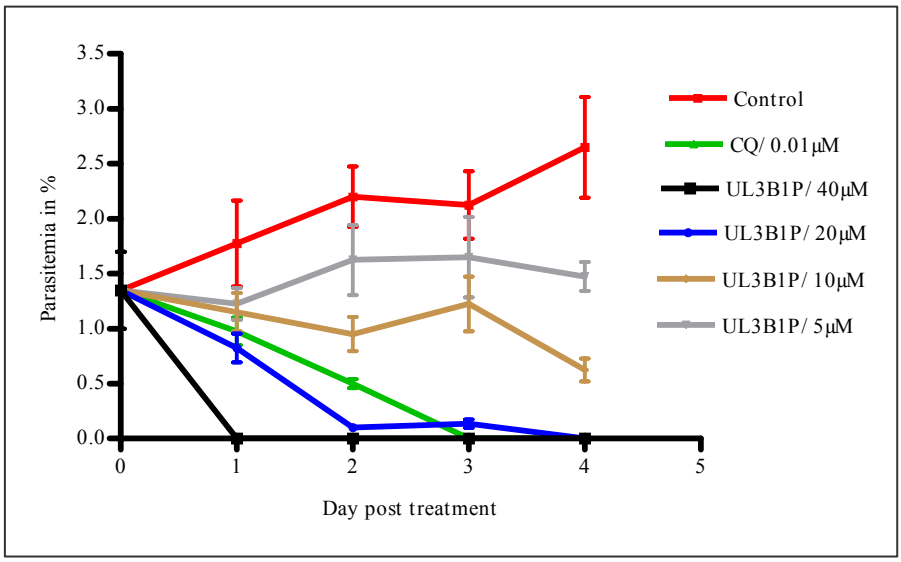

Fig.3.7: In vitro effects of UL3B1P on Pf/NF-54. DMSO =0.091% in both UL3B1P/40µM and control.

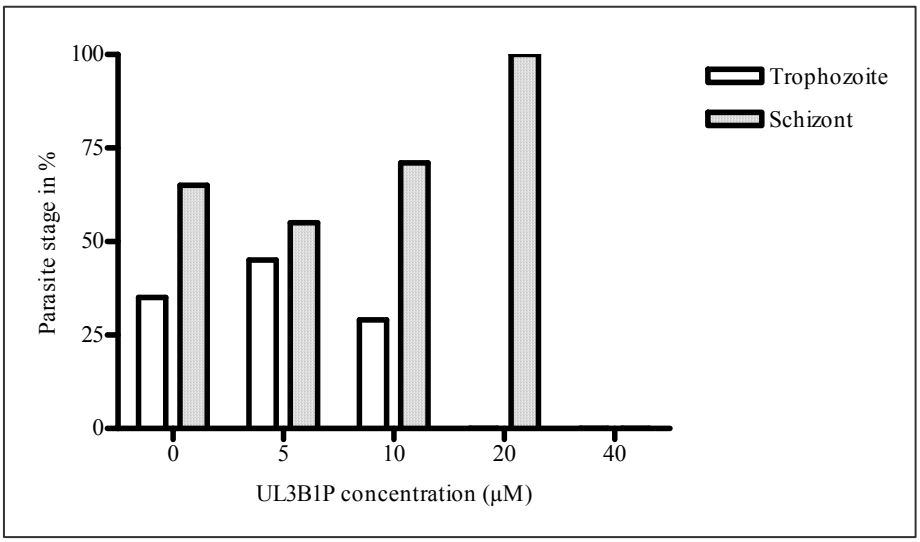

Fig. 3.8: Pf/NF-54 erythrocytic stages in relation to UL3B1P concentrations on d3 post-treatment
-Chi-square for differences in the number of trophozoit stage: Control vs 20µM, $P < 0.0500$

3.1.3.1 In vitro results of three DHH inhibitors

In this group, three of DHH inhibitors were identified: Inhib.3, Inhib.6, and Inhib.7. They were tested under the same conditions, and concentrations. However, Inhib.3 (fig. 3.9) gave an IC_{50} of (12.03 µM); whereas it seemed that Inhib.6 (fig. 3.11) and Inhib.7 (Fig. 3.12) have no *in vitro* effective on Pf/NF-54 strain. Concentrations of Inhib.3 in relation to the parasitic stages are illustrated in figure (3.10).

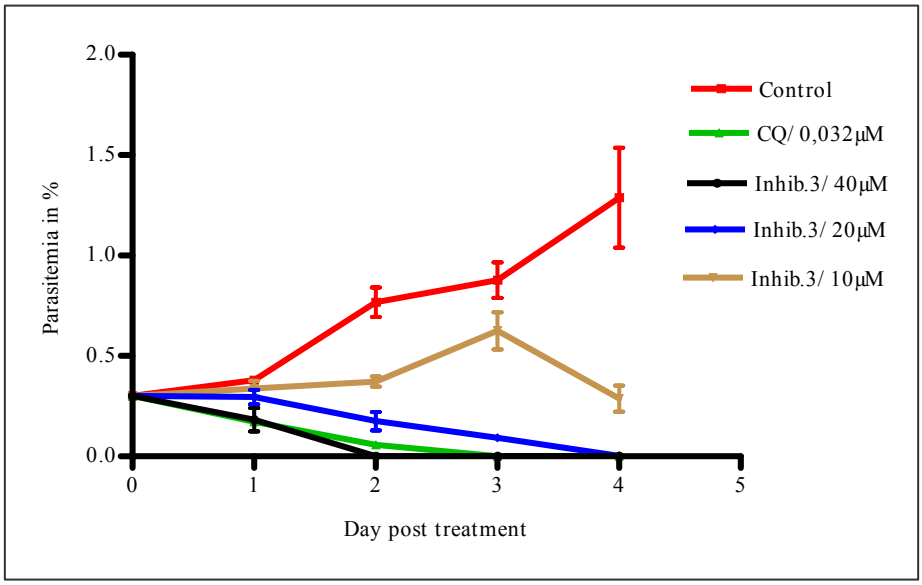

Fig. 3.9: In vitro effects of Inhib.3 on Pf/NF-54. DMSO= 0.17% in Inhib.3/40µM and control.

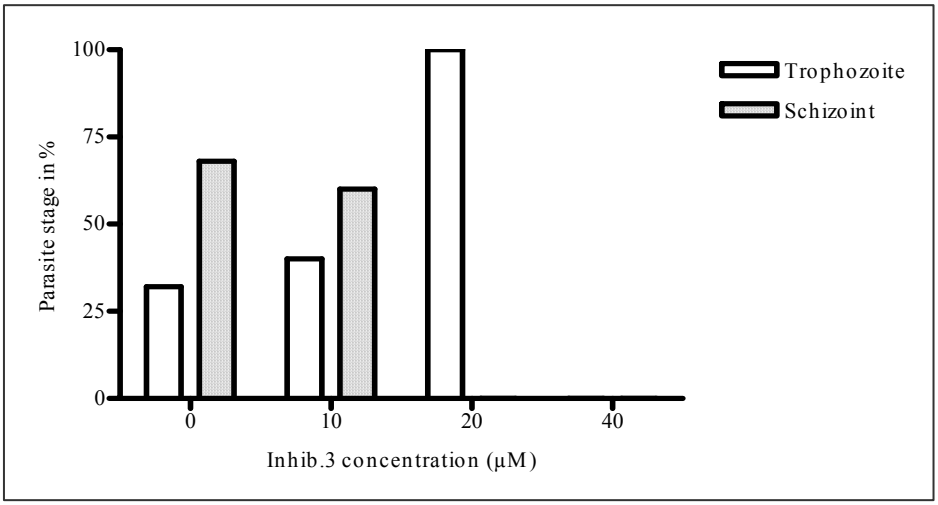

Fig. 3.10: Pf/NF-54 erythrocytic stages in relation to Inhib.3 concentrations on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 20µM, $P < 0.0001$

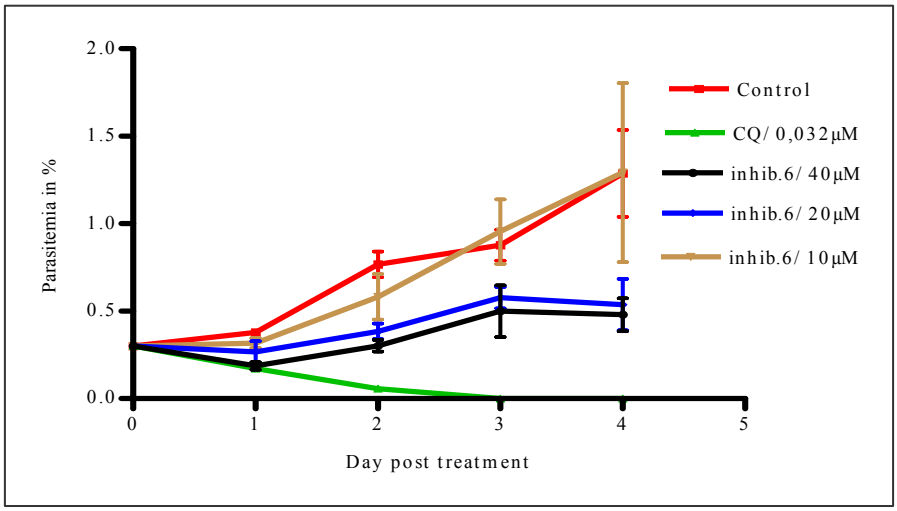

Fig. 3.11: In vitro effects of Inhib.6 on Pf/NF-54. DMSO= 0.16% in inhib.6/40µM.

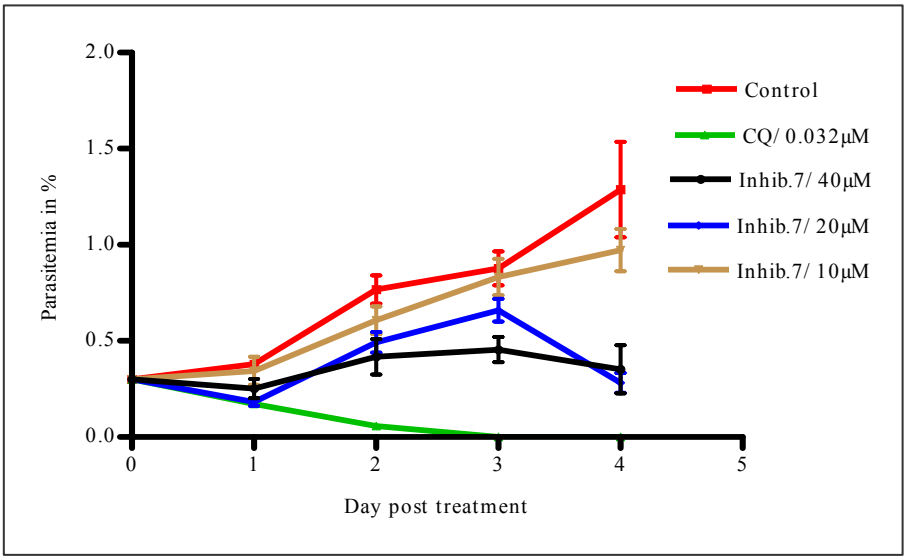

Fig. 3.12: In vitro effects of Inhib.7 on PF/NF-54. DMSO= 0.06% in Inhib.7/40µM.

3.1.3.2 In vitro results of thirteen DHH inhibitors

In this time thirteen inhibitors underwent laboratory test where they were dissolved in DMSO. The control test contains 0.11% of DMSO as high as the JK7E, which has the highest DMSO concentration in this group. Figure (3.13) illustrates the antimalarial ability of each. However, just three inhibitors out of thirteen, (JK12-ML2, JK7E, and JK-8-2), were selected for further *in vitro* investigation.

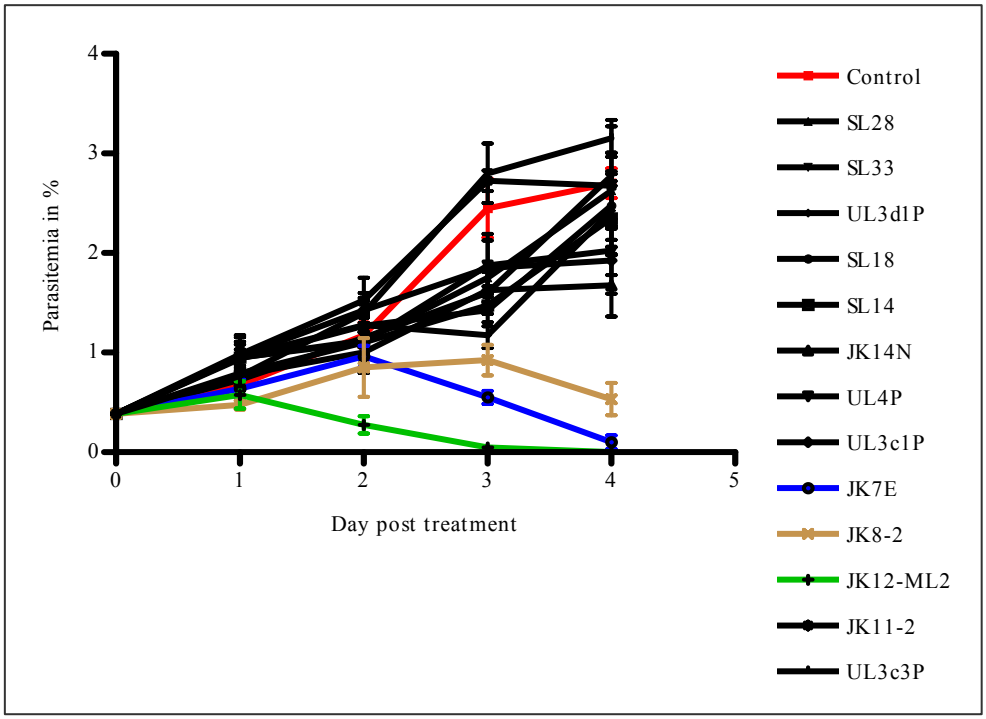

Fig. 3.13: In vitro effects of 13 DHH inhibitors on Pf/NF-54 at 20µM of each

Pf/NF-54 was in vitro exposed to several dilutions of JK-8-2 (fig. 3.14), JK12-ML2 (fig. 3.15), and JK7E (fig. 3.16). The three of them exhibit in vitro anti-malarial activity with IC_{50}s of 11.03 µM, 11.91 µM, and 10.06 µM respectively.

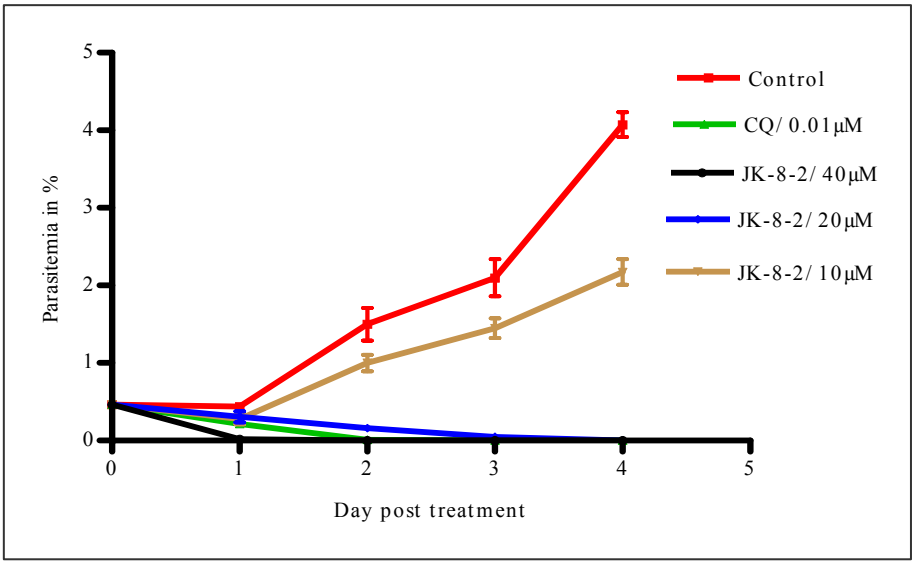

Fig. 3.14: In vitro effects of JK-8-2 on Pf/NF-54. DMSO= 0.21% in 40µM and control.

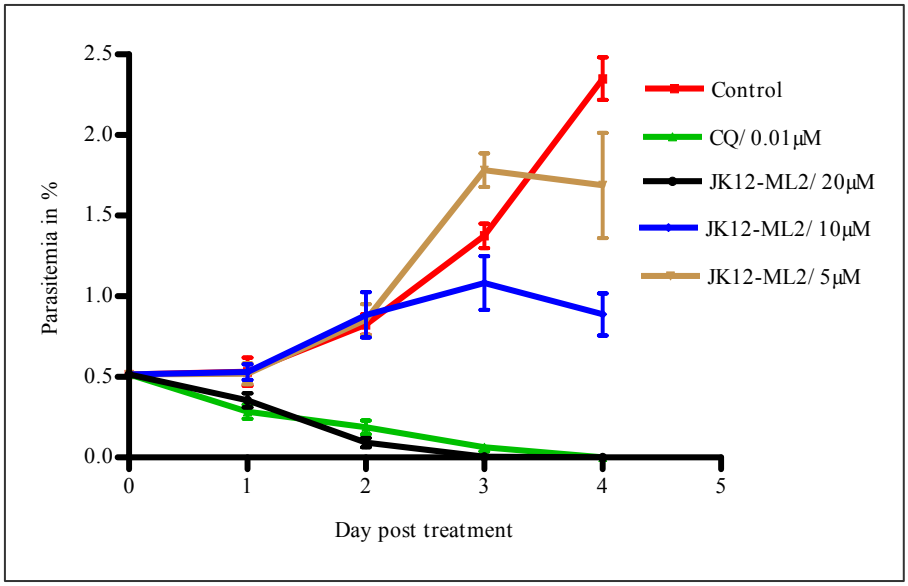

Fig. 3.15: In vitro effects of JK12-ML2 on Pf/NF-54. DMSO= 0.11% in 40µM and control.

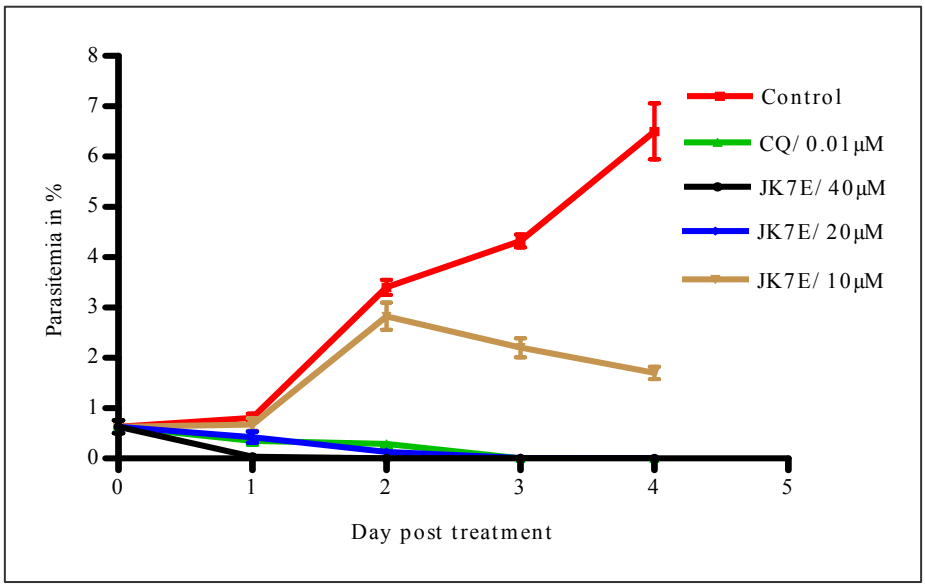

Fig. 3.16: In vitro effects of JK7E on Pf/NF-54. DMSO= 0.24% in 40µM and control.

In addition, the in vitro affectivity of JK-8-2, JK12-ML2, and JK7E on the erythrocytic stages of Pf/NF-54 strain can be observed in (fig. 3.17), (fig. 3.18), and (fig. 3.19) respectively.

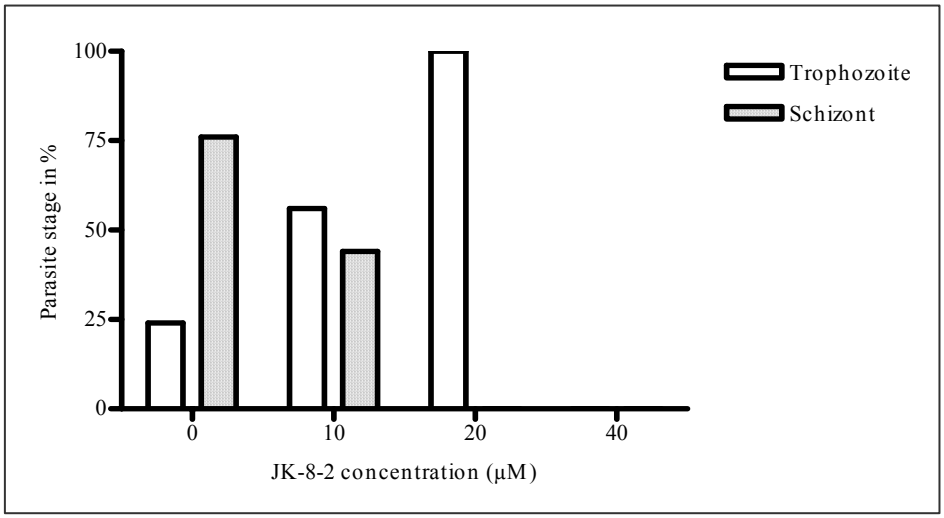

Fig. 3.17: Pf/NF-54 erythrocytic stages in relation to JK-8-2 concentrations on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 10 and 20µM, $P < 0.0500$

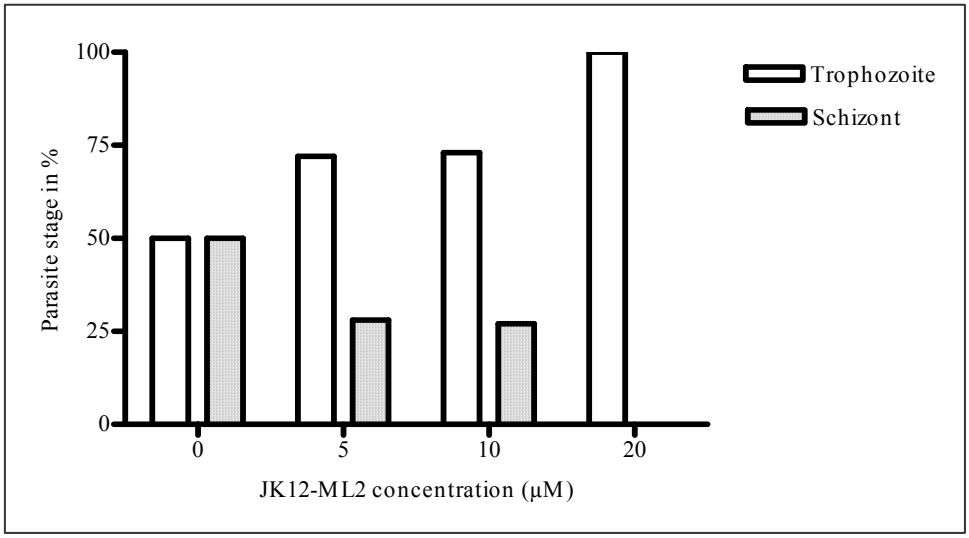

Fig. 3.18: Pf/NF-54 erythrocytic stages in relation to JK12-ML2 concentrations on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 5, 10 and 20µM, P < 0.0500

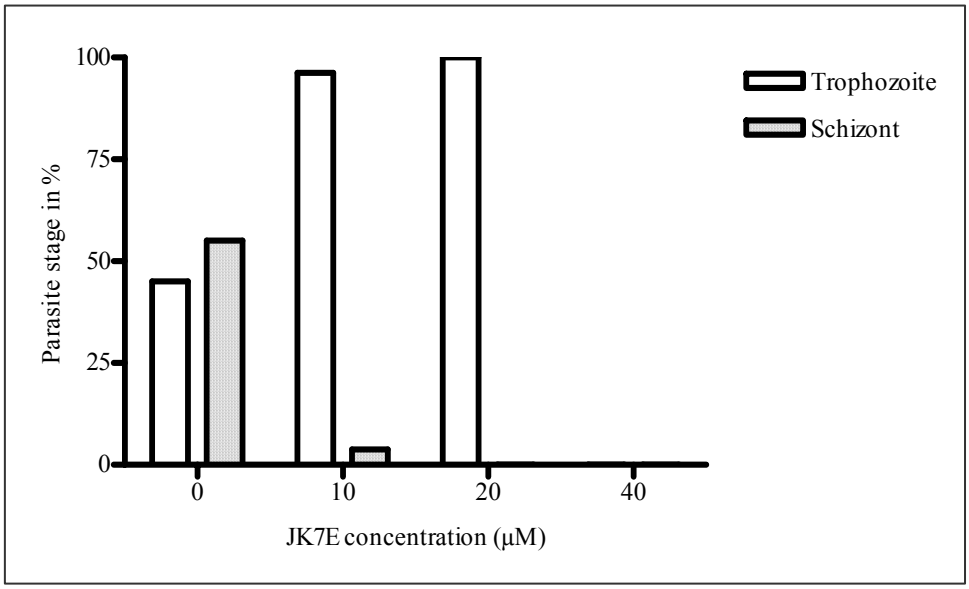

Fig. 3.19: Pf/NF-54 erythrocytic stages in relation to JK7E concentrations on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 10 and 20µM, P < 0.0500

3.1.3.3 In vitro results of twenty five DHH inhibitors

In this group twenty five DHH inhibitors have been tested for in vitro antimalarial activity. The inhibitors were dissolved in DMSO. As UD222 contains the highest DMSO concentration (0.11%), the control was supplied with the same amount of DMSO.

20µM of each was presented in the complete MCM and their ability to reduce the parasitemia of Pf/NF-54 strain, were examined microscopically (fig. 3.20).

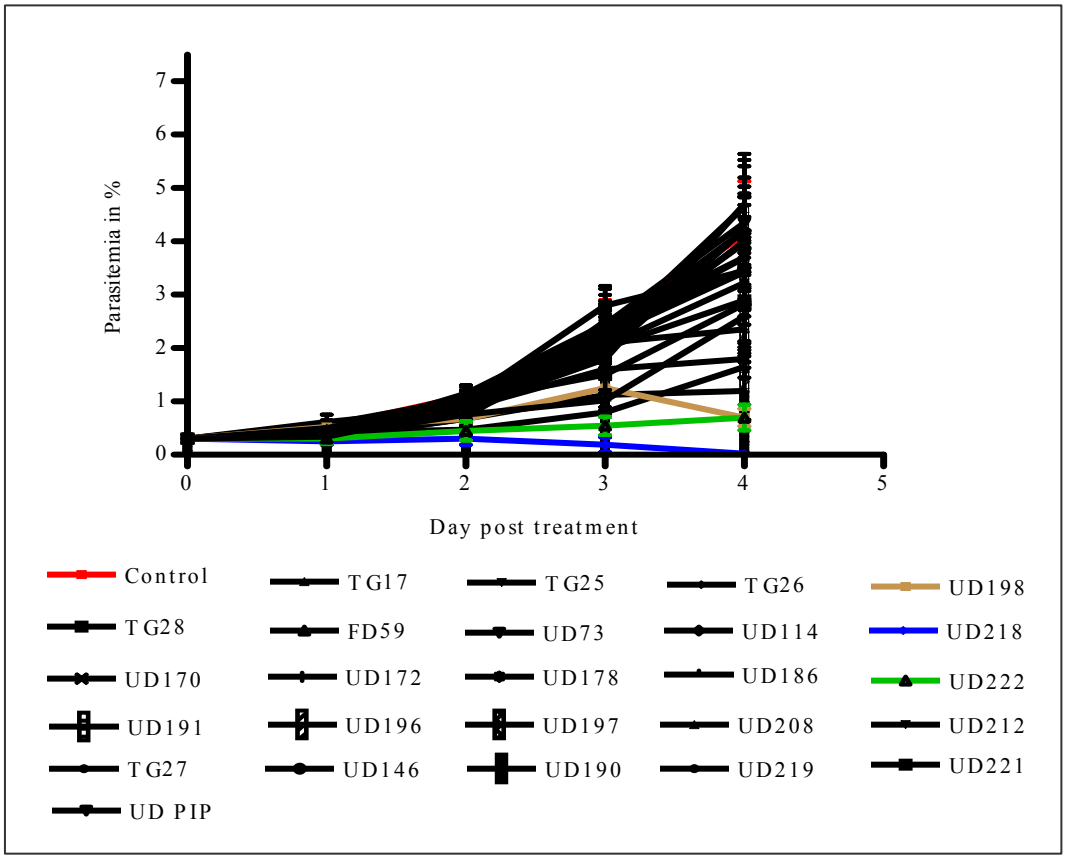

Fig. 3.20: In vitro effects of 25 DHH inhibitors on Pf/NF-54 at 20µM of each.
DMSO= 0.11% in UD222 and control.

Also, Out of this group just three inhibitors exhibit antimalarial in vitro efficacy, UD198, UD218, and UD222. Therefore, they were selected for IC_{50}s investigation.

The IC_{50} of UD198 was not calculated, because it did not exhibit in vitro antimalarial activity (fig. 3.21).

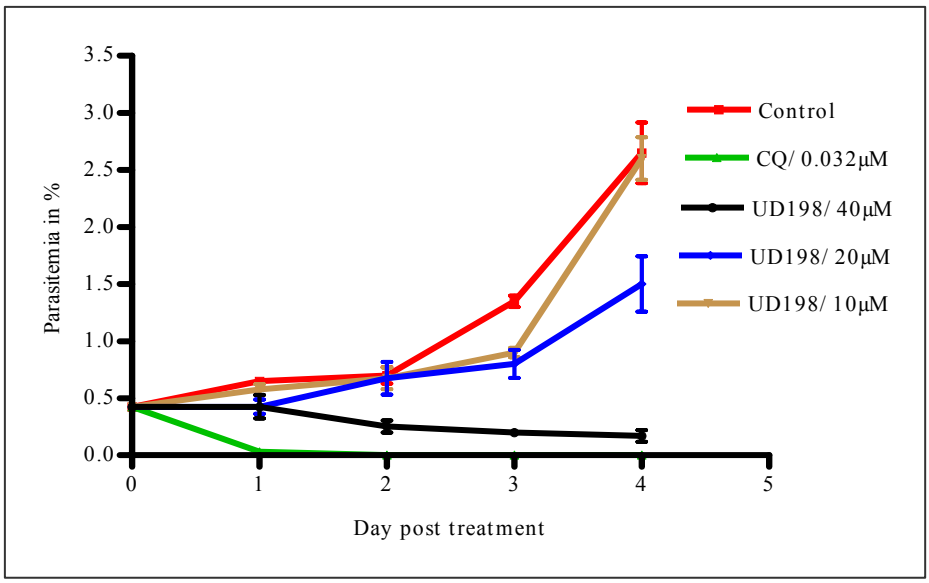

Fig. 3.21: In vitro effects of UD198 on Pf/NF-54. DMSO= 0.16% in 40µM and control.

As shown in figure (3.22), UD218 gave an IC_{50} of 14.57µM, its concentrations in relation to Pf/NF-54 strain erythrocytic stages is illuminated in figure (3.23).

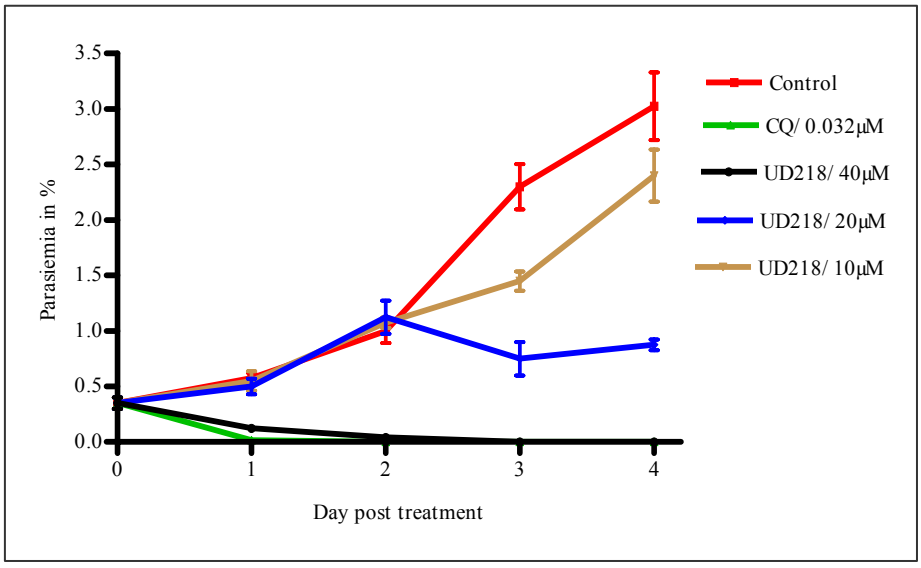

Fig. 3.22: In vitro effects of UD218 on Pf/NF-54. DMSO= 0.16% in 40µM and control.

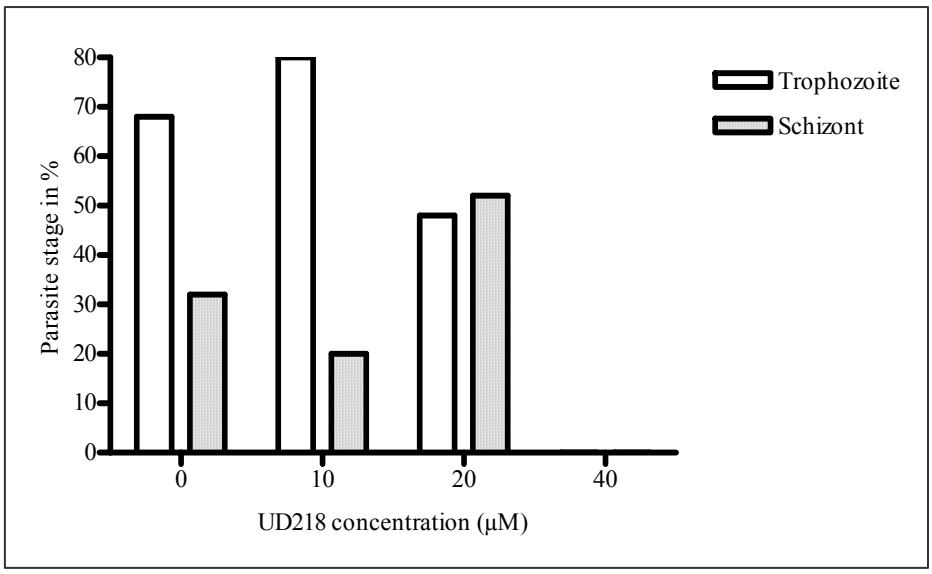

Fig. 3.23: Pf/NF-54 erythrocytic stages in relation to UD218 concentrations on d3 post-treatment
-Chi-square for differences in trophozoit stage: Control vs 20µM, $P < 0.0042$

Like UD198, UD222 exhibited no *in vitro* antimalarial activity on Pf/NF-54 strain at the used concentrations (Fig. 3.24).

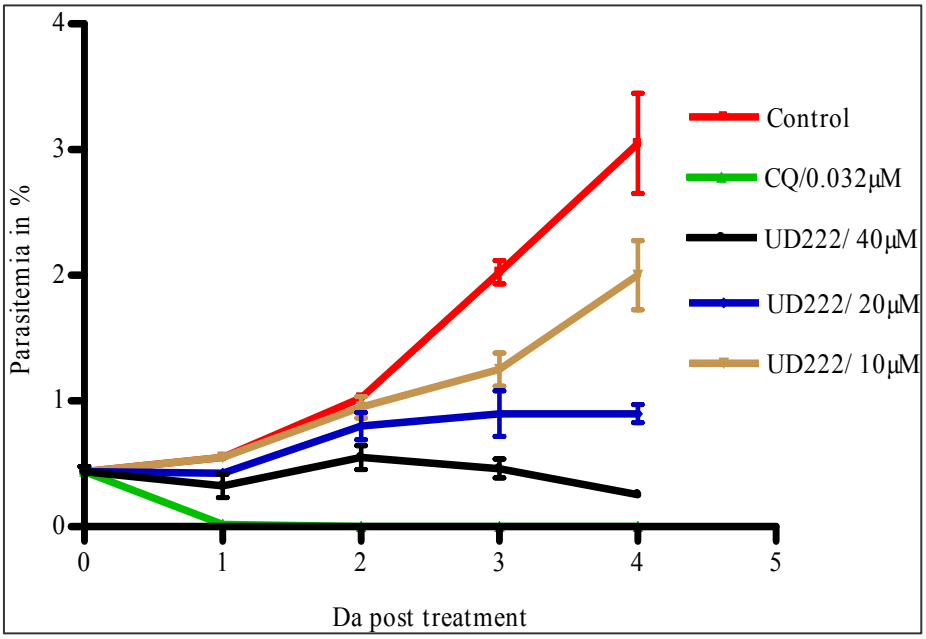

Fig. 3.24: In vitro effects of UD222 on Pf/NF-54. DMSO= 0.22% in 40µM and control.

3.1.3.4 In vitro results of four DHH inhibitors

Three of the received inhibitors were highly soluble in culture media or PBS, whereas TG014 was soluble just in DMSO. However, 10µM of each substance was prepared in the complete MCM and their ability to inhibit the in vitro proliferation of Pf/NF-54 was checked (fig. 3.25).

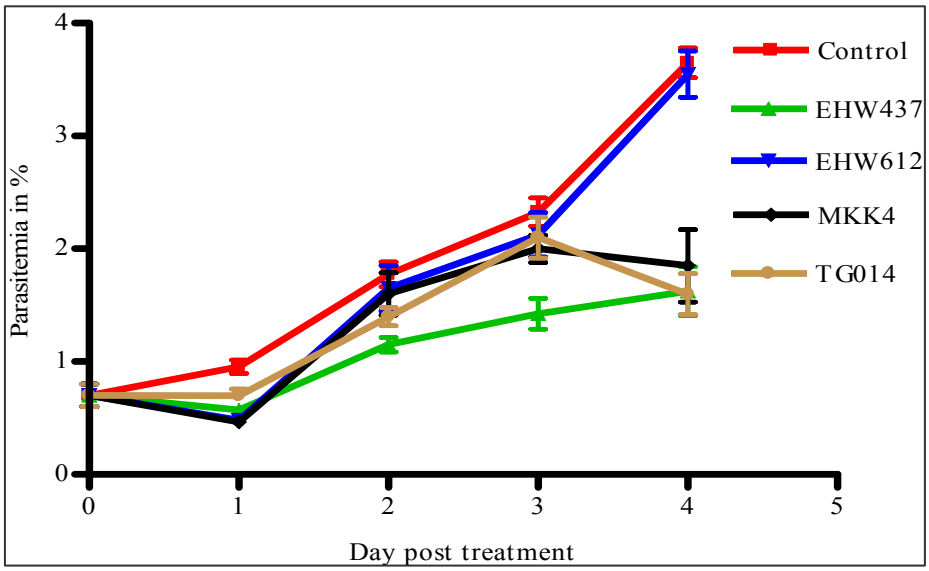

Fig. 3.25: In Vitro effects of four DHH inhibitors on Pf/NF-54 at 10µM of each.
DMSO in TG014= 0.04%.

The tested inhibitors did not show in vitro anti-malarial efficacy when used at 10 µM, but because of their high solubility in media and PBS, they underwent further in vitro investigations by increasing their concentrations. However, just EHW437 showed in vitro ability to reduce the parasitemia of Pf/NF-54 with an IC_{50} of 8.29µM (fig. 3.26), and (fig. 3.27) shows the relation between EHW437 concentrations and the parasite's erythrocytic stages. Whereas the other three substances were considered as non-active substances, at the used concentrations (10, 20, and 40 µM) (Data not shown).

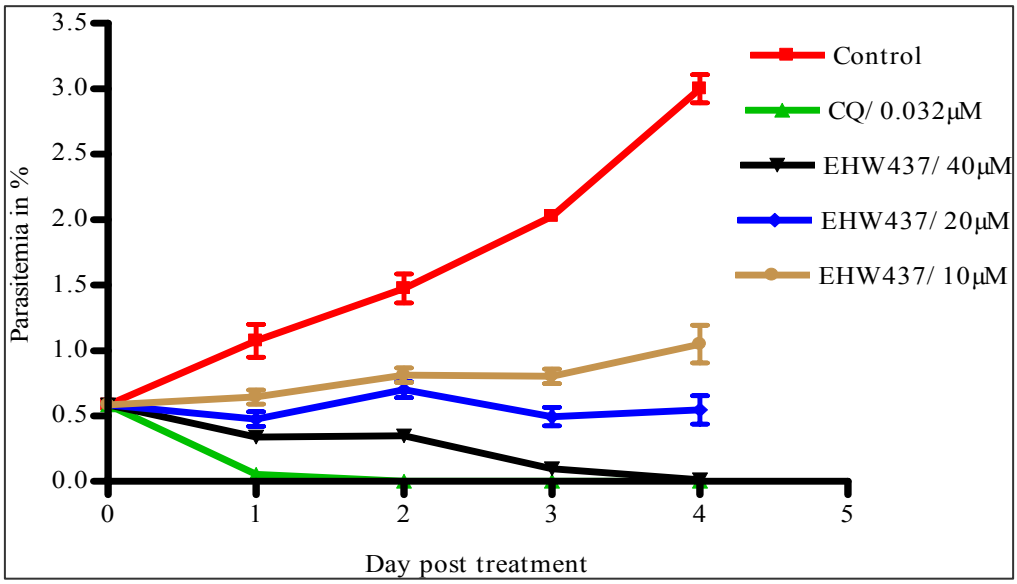

Fig. 3.26: In vitro effects of EHW437 on Pf/NF-54 strain.

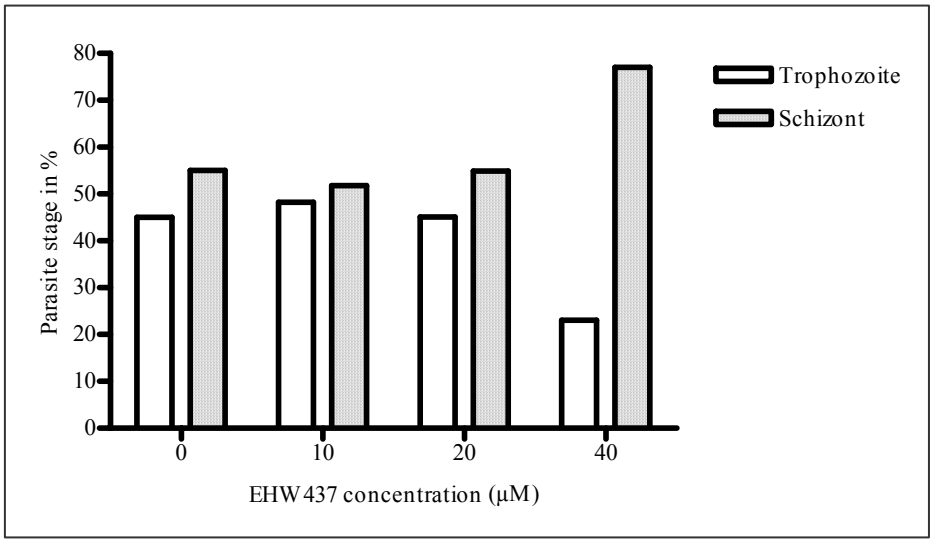

Fig. 3.27: Pf/NF-54 erythrocytic stages in relation to EHW437 concentrations on d3 post-treatment

3.1.4 In vitro results of polyamintransport inhibitors

Two inhibitors of this class underwent in vitro estimation for their anti-malarial activity. In the first series (10, 20, and 40µM) of NK-1-33 and NK-1-63 exhibited high antimalarial in vitro activities (Data not shown). Therefore the used concentrations were reduced to allow the calculations of the in vitro IC_{50}s.

Both inhibitors were highly soluble in water, and they were presented to Pf/NF-54 in complete MCM. NK-1-33 (fig. 3.28) showed an IC_{50} of 4µM, whereas, NK-1-63 showed a potent in vitro anti-malarial activity. The evaluated IC_{50} was found to be 0.63 µM (fig. 3.29).

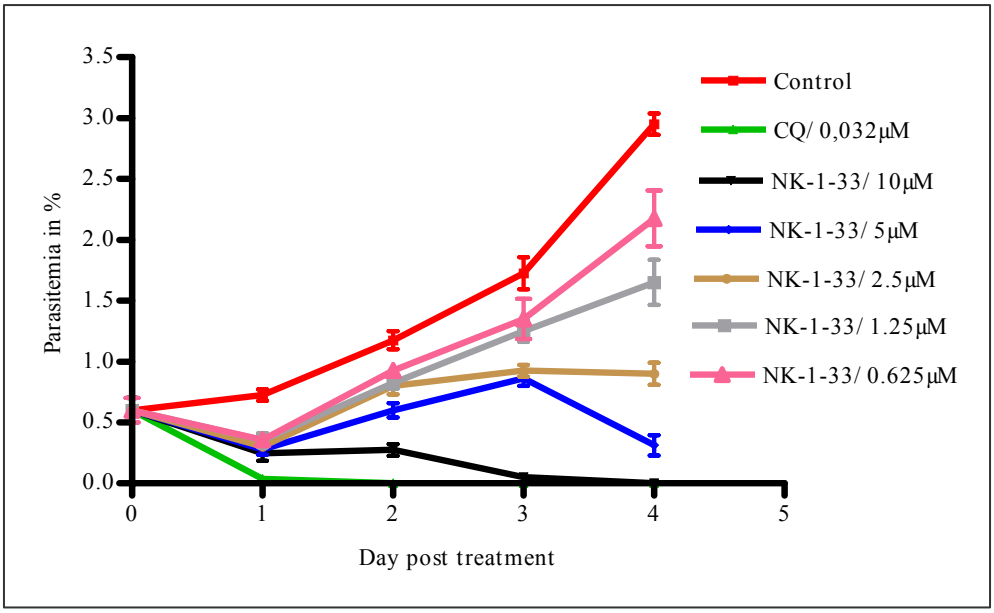

Fig. 3.28: In Vitro effects of NK-1-33 on P.f/NF-54

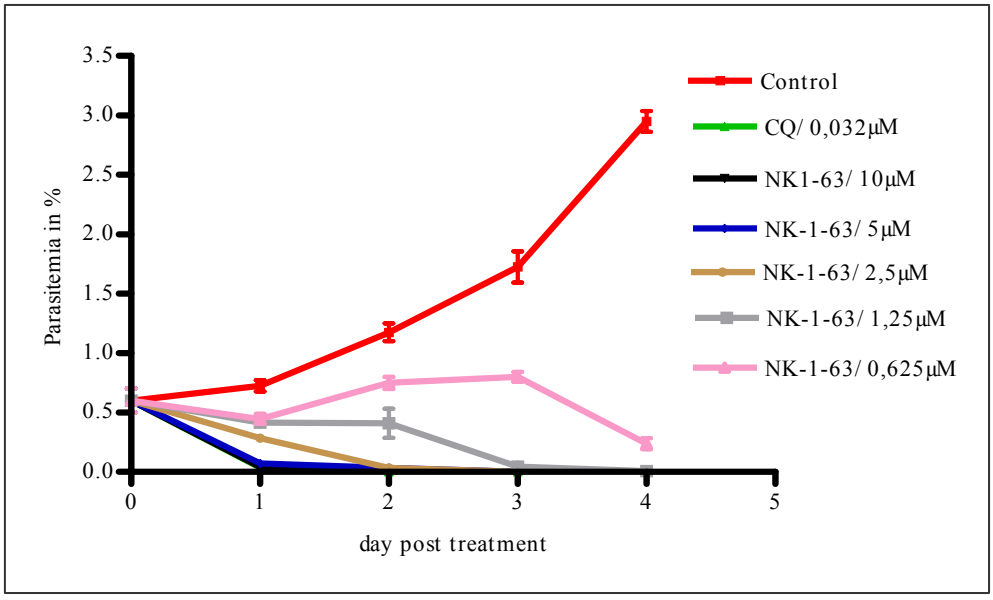

Fig. 3.29: In Vitro effects of NK-1-63 on P.f/NF-54

NK-1-33 and NK-1-63 efficacy on the parasite erythrocytic stages can be observed in figures (3. 30) and (3.31) respectively.

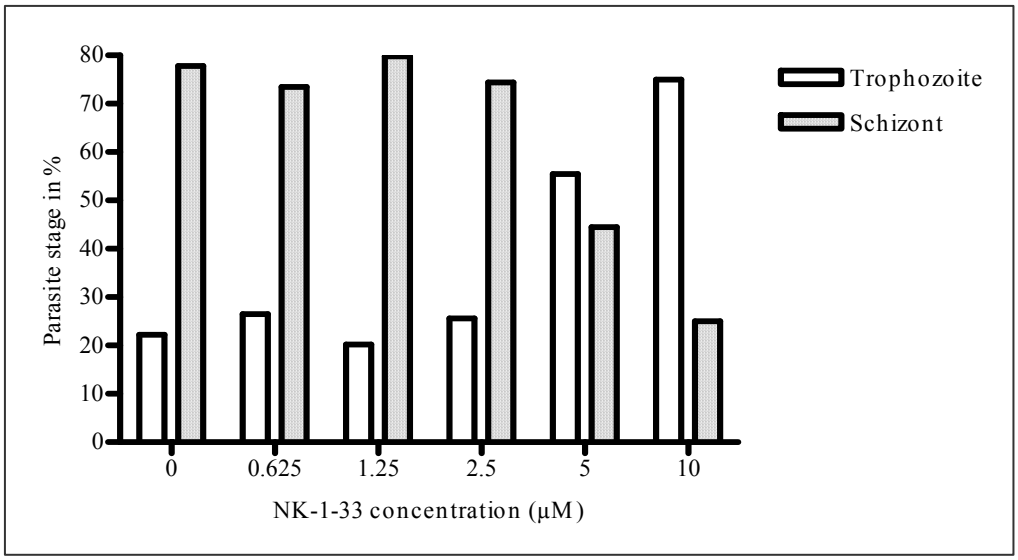

Fig. 3.30: Pf/NF-54 erythrocytic stages in relation to NK-1-33 concentrations on d3 post-treatment
-Chi-square for differences in Schizont: Control vs 5 and 10µM, P < 0.0500

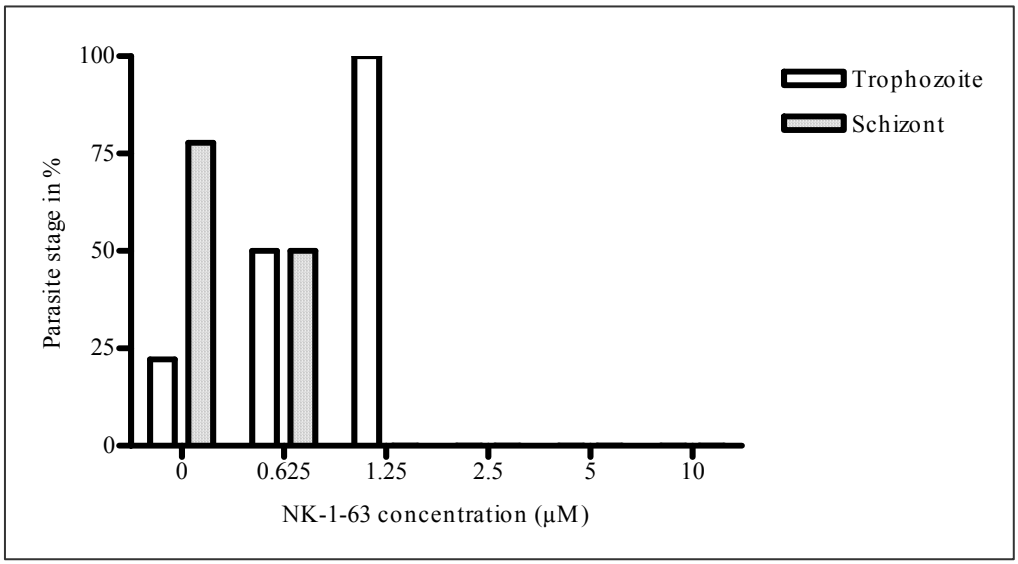

Fig. 3.31: Pf/NF-54 erythrocytic stages in relation to NK-1-63 concentrations on d3 post-treatment
-Chi-square for differences in Schizont: Control vs 0.625 and 1.25µM, P < 0.0500

3.1.5 In vitro results of phenazines derivatives

As presented in sections (1.10 and 1.10.1), phenazines have been described as dual inhibitors of topoisomerase enzymes, class I and II (Wang et al., 2002). On the other hand they are known to be ROS scavengers (Laursen and Nielsen, 2004). Depending on these two properties, phenazines analogues were used in this study.

3.1.5.1 In vitro results of twenty four phenazines derivatives

All the members of this group have been dissolved in DMSO and presented as 10µM in complet MCM to Pf/NF-54 (Fig. 3.32).

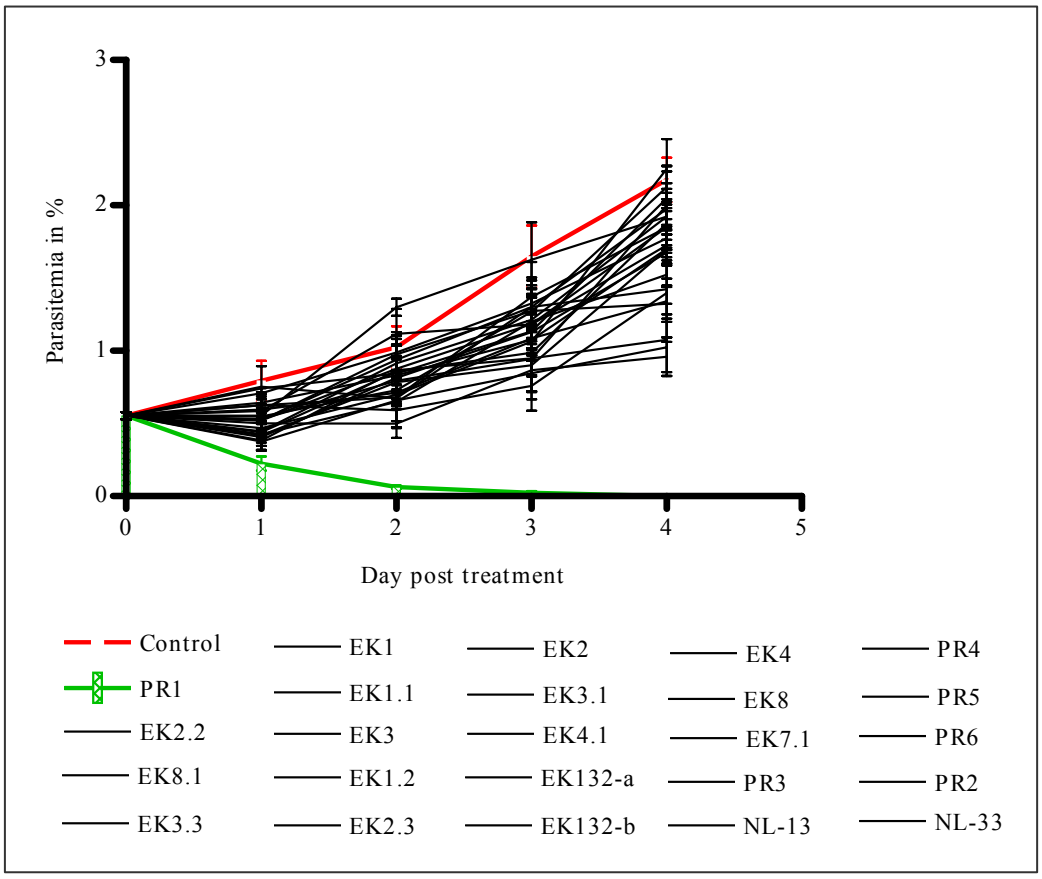

Fig. 3.32: In vitro effects of 24 new phenazins on Pf/NF-54. DMSO= 0.63 in control and EK7-1.

Only PR-1 was selected for further in vitro investigations.

Pf/NF-54 was exposed to increased concentrations of PR-1, which exhibited in vitro anti-malarial activity with an IC_{50} of 4.11 µM (fig.3.33). The sensitivity of Pf/NF-54 erythrocytic stages to different concentrations of PR-1 is illustrated in figure (3.34).

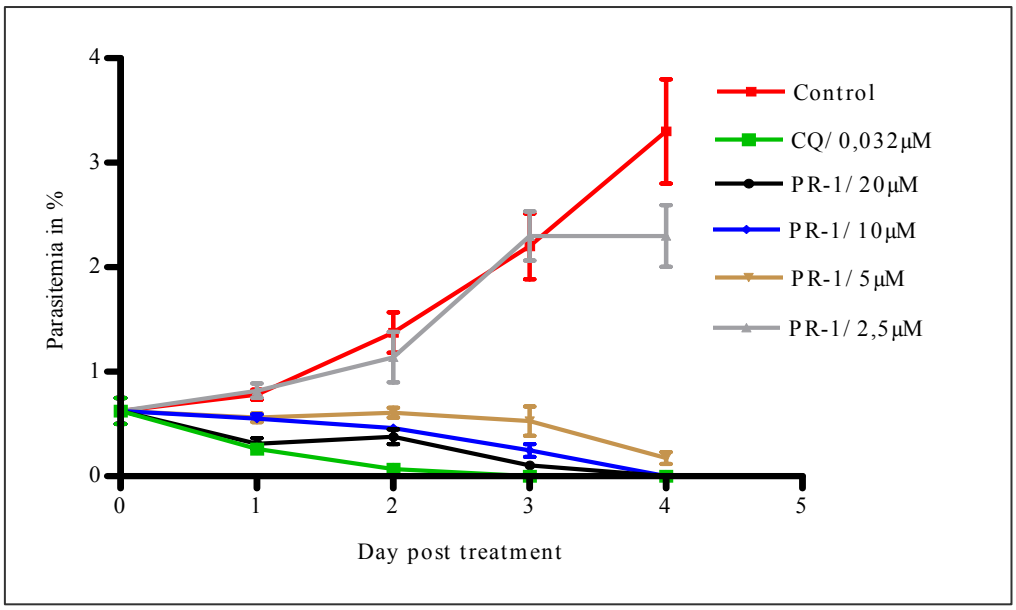

Fig. 3.33: In vitro effects of PR-1 on Pf/NF-54. DMSO= 0.15% in PR-1/20µM and control.

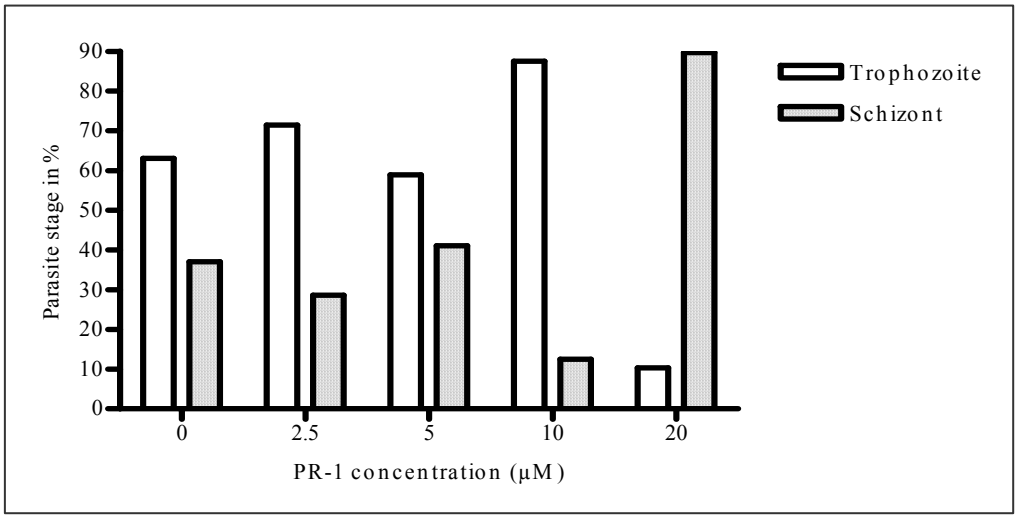

Fig. 3.34: Pf/NF-54 erythrocytic stages in relation to PR-1 concentrations on d3 post-treatment
-Chi-square for differences in Schizont: Control vs 10 and 20µM, $P < 0.0500$

3.1.5.2 In vitro results of four phenazines derivatives

To investigate the in vitro anti-malarial abilty of these four phenazines, 20µM of each substance were supplemented to the cultured Pf/NF-54 strain as shown in (fig. 3.35).

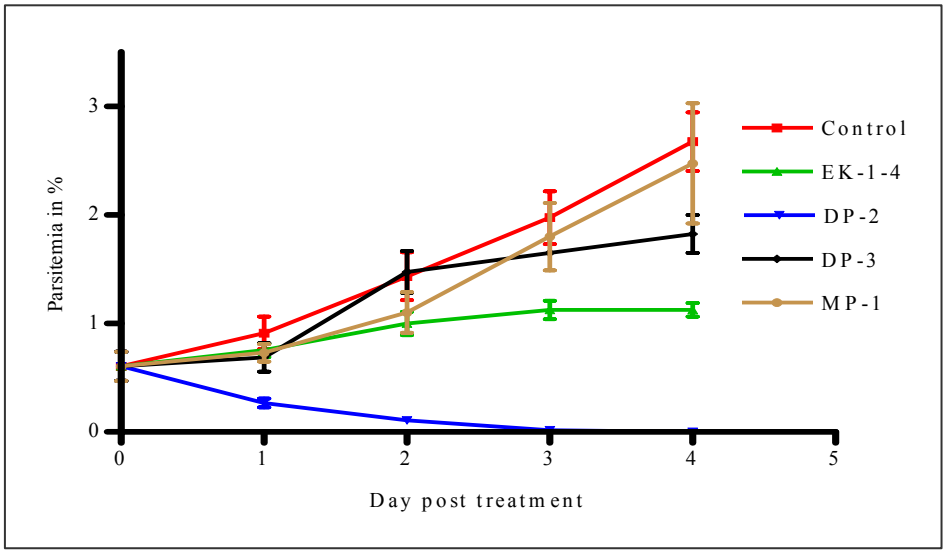

Fig. 3.35: In vitro effects of 4 phenazines on Pf/NF-54 at 20µM of each.
DMSO= 0.37% in DP-2 and control

DP-2 was first tested in the same concentrations used in PR-1 evaluation (2.5 to 20µM), but it was found to be high active (Data not shown). Therefore, the DP-2 concentrations were decreased to be ranged from 0.18 to 3µM (fig. 3.36). The evaluated IC_{50} of DP-2 was 0,88 µM on Pf/NF-54.

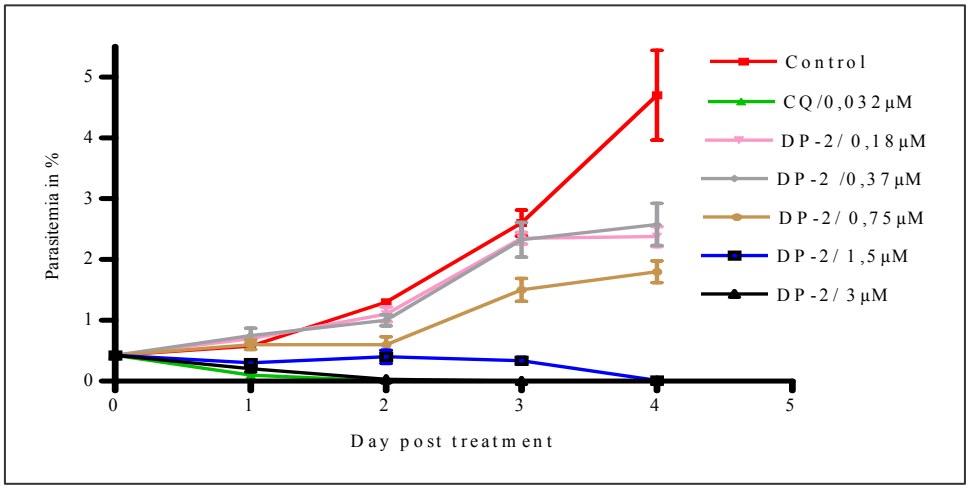

Fig. 3.36: In vitro effects of DP-2 on Pf/NF-54. DMSO= 0.055% in DP-2/3µM and control.

Figure (3.37) illustrated a relation of DP-2 concentrations to the parasite erythrocytic stages.

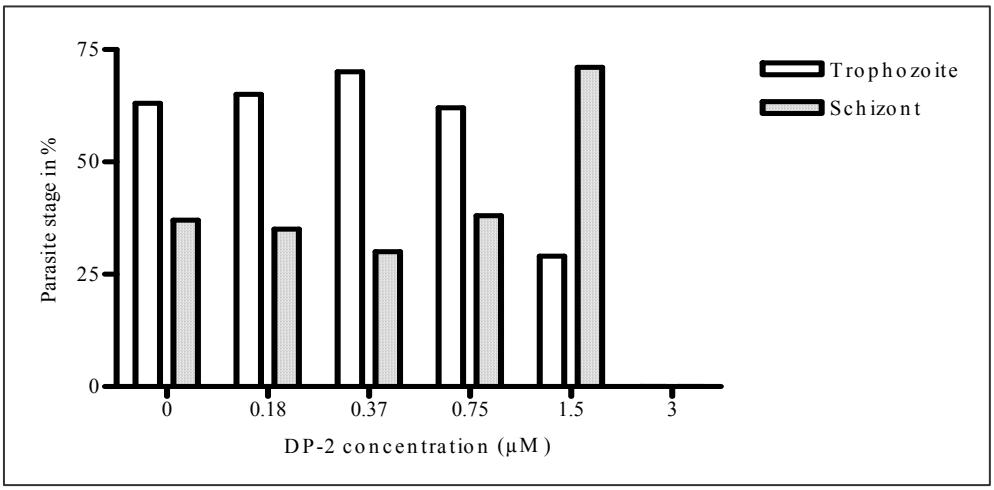

Fig. 3.37: Pf/NF-54 erythrocytic stages in relation to DP-2 concentrations on d3 post-treatment
-Chi-square for differences in Schizont stage: Control vs 1.5µM, P < 0.0001

The DP-2 anti-malarial in vitro activity was also assessed on the CQR strain, Pf/K1. DP-2 showed in vitro ability to influence the parasitemia of Pf/K1 strain with an IC_{50} of 0.799µM (fig.3.38), which is in the same IC_{50} range on CQS strain Pf/NF-54.

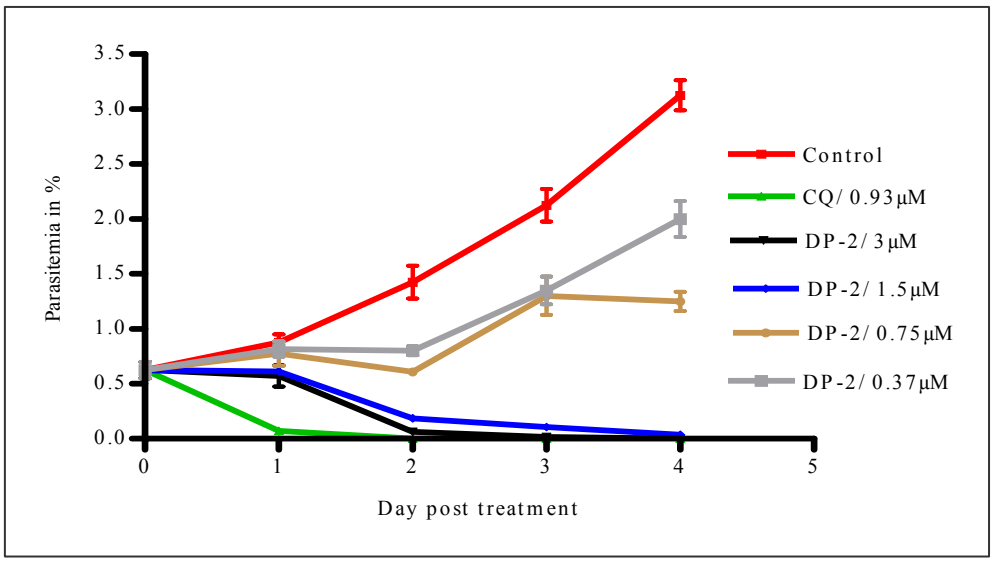

Fig. 3.38: In vitro effects of DP-2 on Pf/ K1. DMSO= 0.055% in DP-2/3µM and control.

The sensitivity of erythrocytic stages of Pf/K1 to different DP-2 concentrations can be observed in figure (3.39).

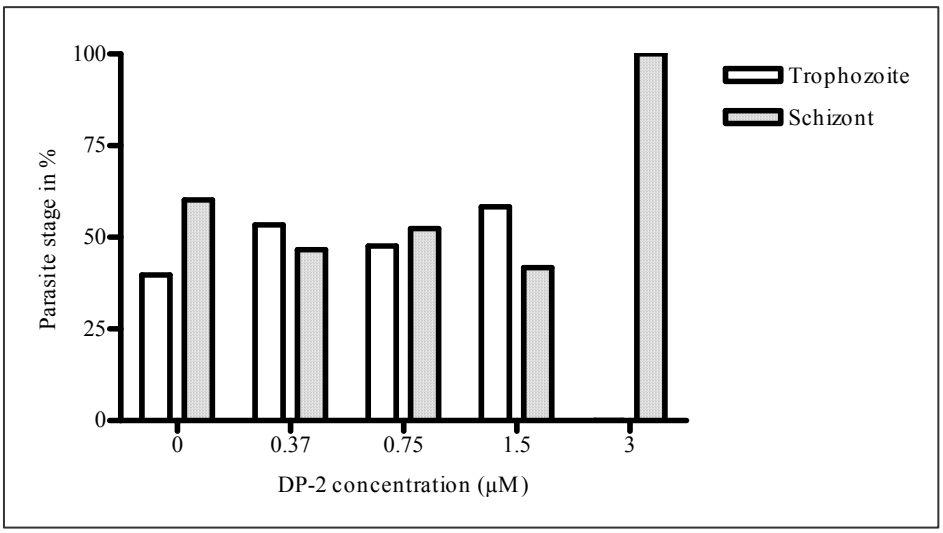

Fig. 3.39: Pf/K1 erythrocytic stages in relation to DP-2 concentration on d3 post-treatment

3.1.5.3 In vitro results of five phenazines derivatives

The in vitro assay of these group showed that they are inactive substances at 20µM, as shown in figure (3.40). Therefore, none of them was selected for further investigations.

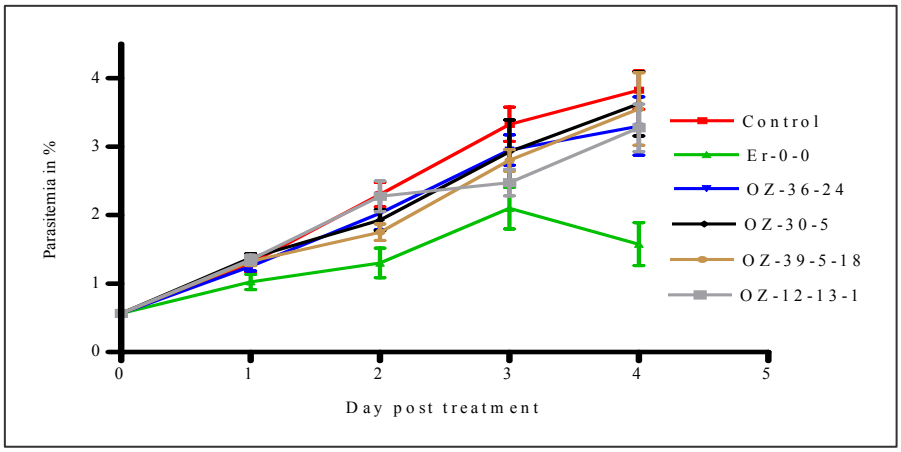

Fig. 3.40: In vitro effects of 5 new phenazines on Pf/NF-54 at 20µM of each.
DMSO= 0.18% in OZ-12-13-1and control.

3.1.5.4 In vitro results of ten phenazines derivatives

As during the in vivo assay of PR-1 we have faced a solubility problem, PR-1 was modified by connecting it to some other substances, including chlore, to improve its solubility. However, the members of this group were dissolved in DMSO and prepared at 20µM in the complete MCM. Two of them, PR-1-cl and PR-1-py, showed in vitro antimalarial ability on Pf/NF-54 (Fig. 3.41), and therefore underwent IC_{50} investigations.

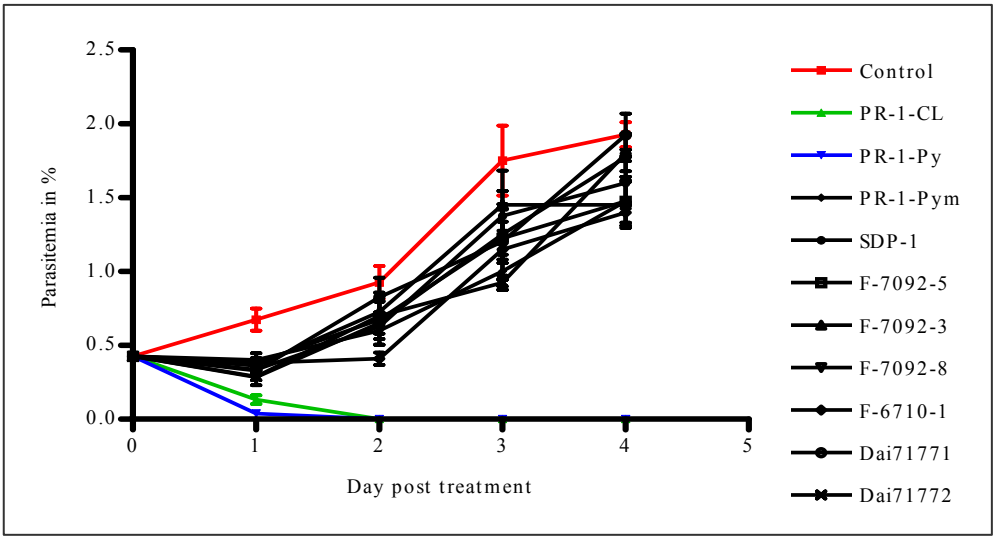

Fig. 3.41: In vitro effects of ten phenazines on Pf/NF-54 at 20µM of each.
DMSO= 0.41% in SDP-1and control.

Like the mother substance, PR-1-CL and PR-1-Py exhibited in vitro anti-malarial activity with similar IC50s, 5,05 µM for PR-1-cl and 5,23µM for PR-1-Py, as shown in Figures (3.42 and 3.43) respectively.

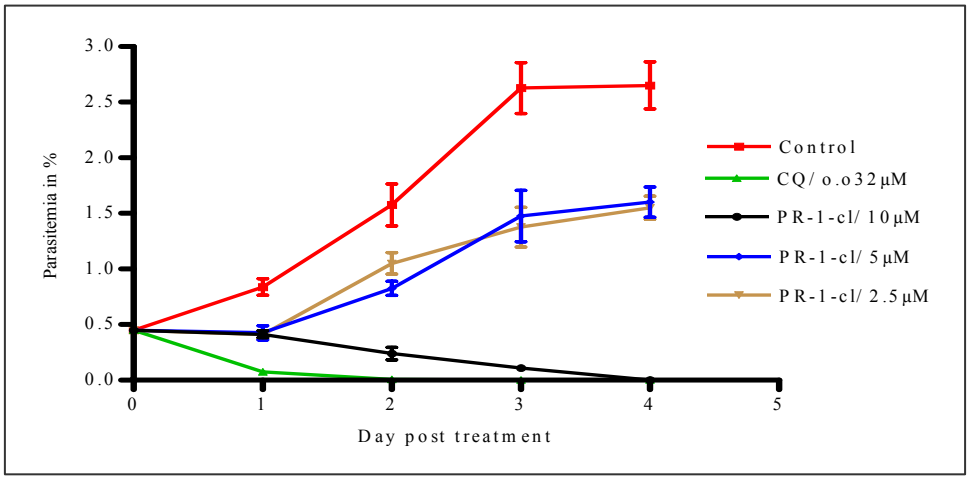

Fig: 3.42: In vitro effects of PR-1-cL on Pf/NF-54. DMSO= 0.044% in PR-1-cL/10µM and control.

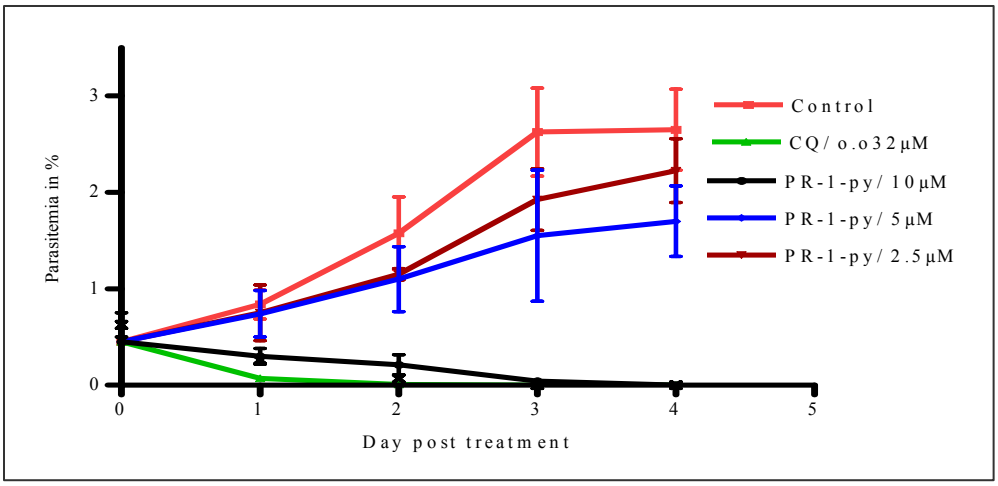

Fig. 3.43: In vitro effects of PR-1-py on Pf/NF-54. DMSO= 0.044% in PR-1-py/10µM and control.

The sensitivity of Pf/NF-54 erythrocytic stages to PR-1-cL can be observed in figure (3.44).

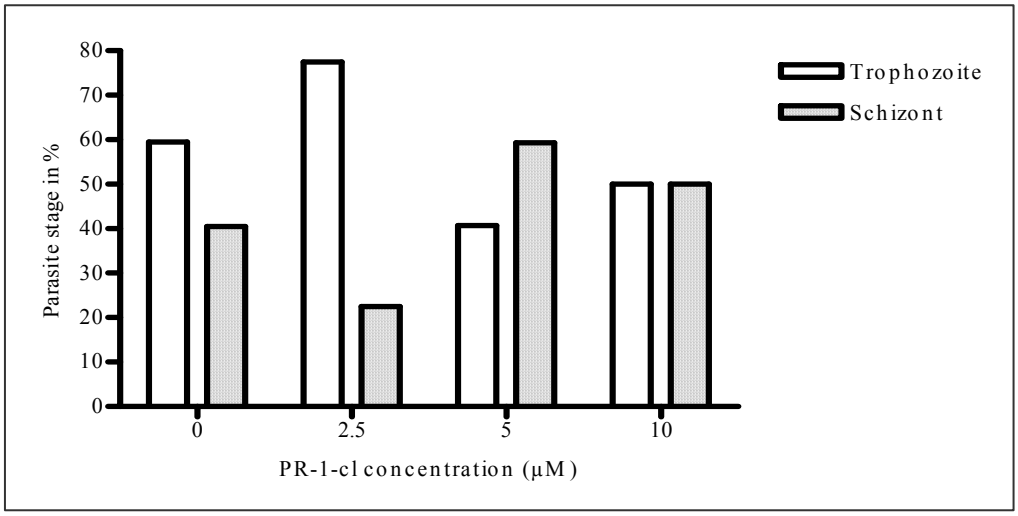

Fig. 3.44: Pf/NF-54 erythrocytic stages in relation to PR-1-cL concentration on d3 post-treatment
-Chi-square for differences in Schizont stage: Control vs 2.5 and 5µM, $P < 0.0500$

And figure (3.45) illustrated the sensitivity of Pf/NF-54 erythrocytic stages to PR-1-Py.

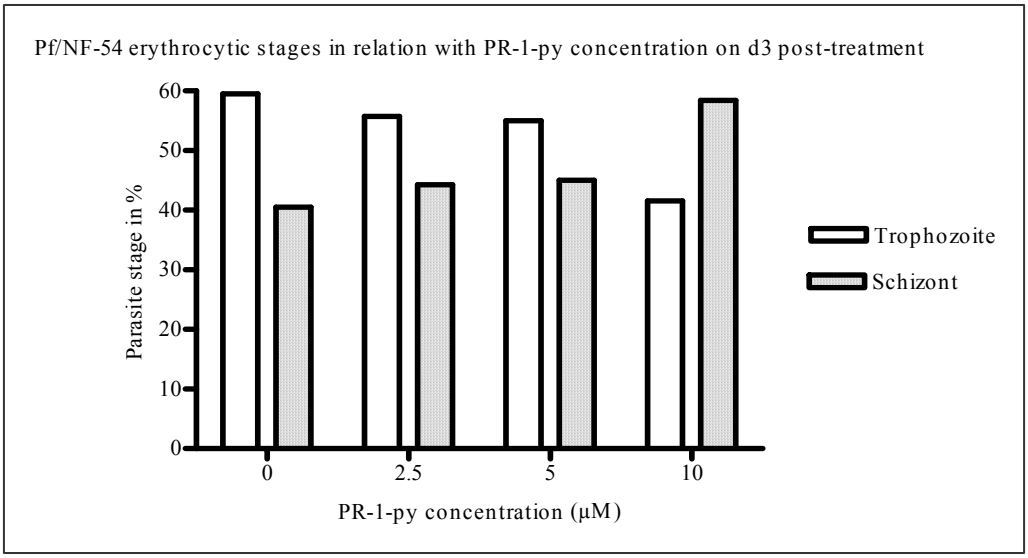

Fig. 3.45: Pf/NF-54 erythrocytic stages in relation to PR-1-Py concentration on d3 post-treatment

3.1.5.5 In vitro results of two phenazines derivatives

Like PR-1, DP-2 has faced solubility problem when tested in vivo, therefore DP-2 was connected to some chemicals (including chlor) to improve its solubility. The two new delivered substances were dissolved in DMSO and Pf/NF-54 was exposed to the same concentrations which used in DP-2, the mother substance, to assay the in vitro antimalarial activity of DP-2-cl and DP-2-Py. DP-2-cl shows no in vitro antimalarial activity (fig. 3.46).

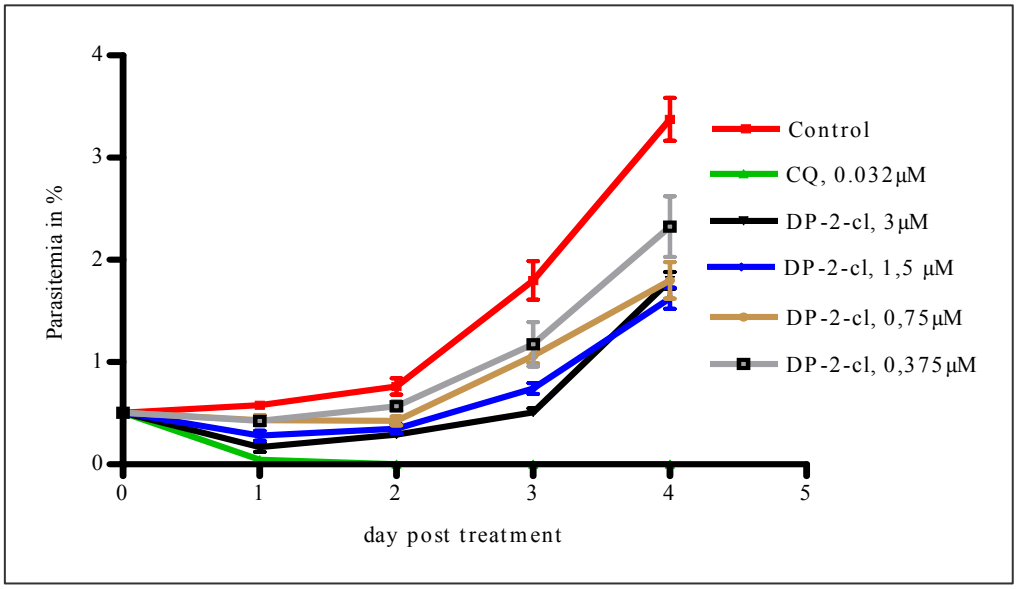

Fig: 3.46: In Vitro effects of DP-2-cl on PF/NF-54. DMSO= 0.013% in DP-2-cl/3µM and control.

Whereas, like the mother substance DP-2-py exhibited in vitro anti-malarial activity with an IC_{50} of 0.51 µM (fig. 3.47). A relation between the Pf/NF-54 erythrocytic stages and the DP-2-py can be observed in figure (3.48).

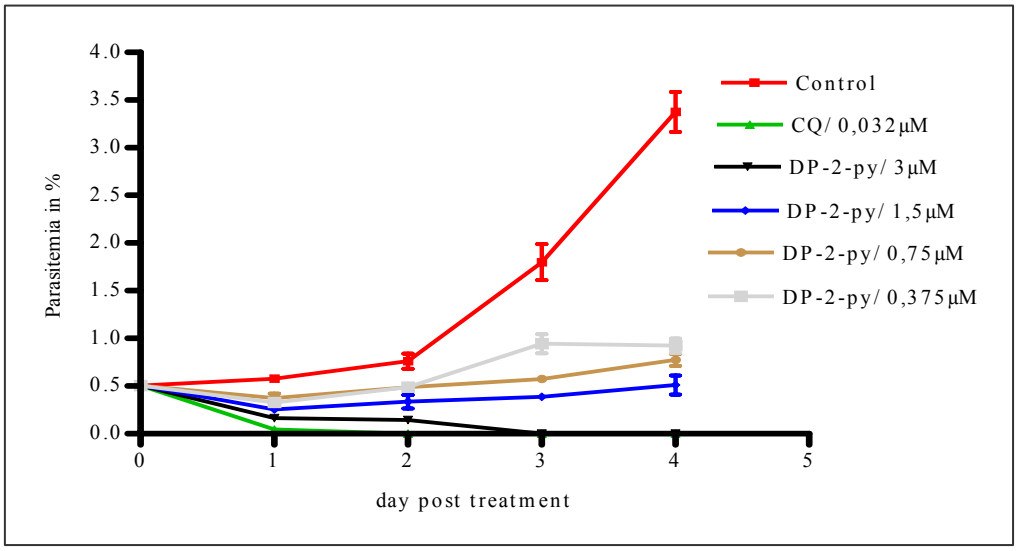

Fig: 3.47: In Vitro effects of DP-2-py on Pf/NF-54. DMSO= 0.013% in DP-2-py/3µM and control.

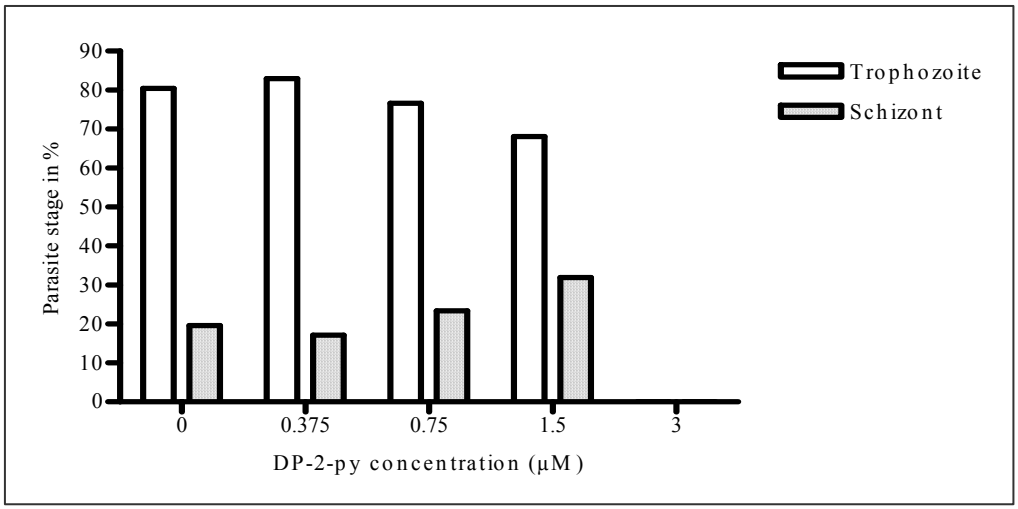

Fig. 3.48: Pf/NF-54 erythrocytic stages in relation to DP-2-Py concentration on d3 post-treatment

The in vitro anti-malarial activity of DP-2-py was assessed on Pf/K1, CQR strain. Unlike its assay on Pf/NF-54 strain, DP-2-py was less affect on Pf/K1 strain (fig. 3.49), indicating that Pf/K1 is resistant to this substance.

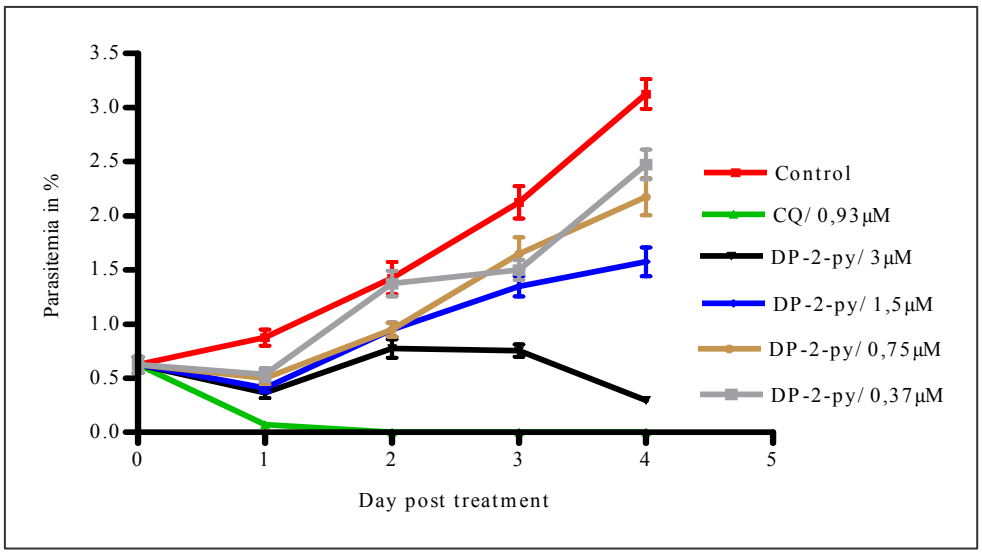

Fig: 3.49: In vitro effects of DP-2-py on Pf/K1. DMSO= 0.011% in DP-2-py/3µM and control.

3.1.5.6 In vitro results of fifteen phenazines derivatives

The received group were dissolved in DMSO and presented to PF/NF-54 strain at 3µM of each (fig. 3 50). Two of fifteen substances exhibited semi-affectivity, TEA-5 and Anth-2. Therefore, they were re-assessed for IC_{50}s evaluation by using higher concentrations than that in the first assay. However, TEA-5 and Anth-2 exhibited inability to reduce the parasitemia of Pf/NF-54 at concentrations of 2, 4, and 8µM (Data not shown), therefore they were dropped down for any further investigations.

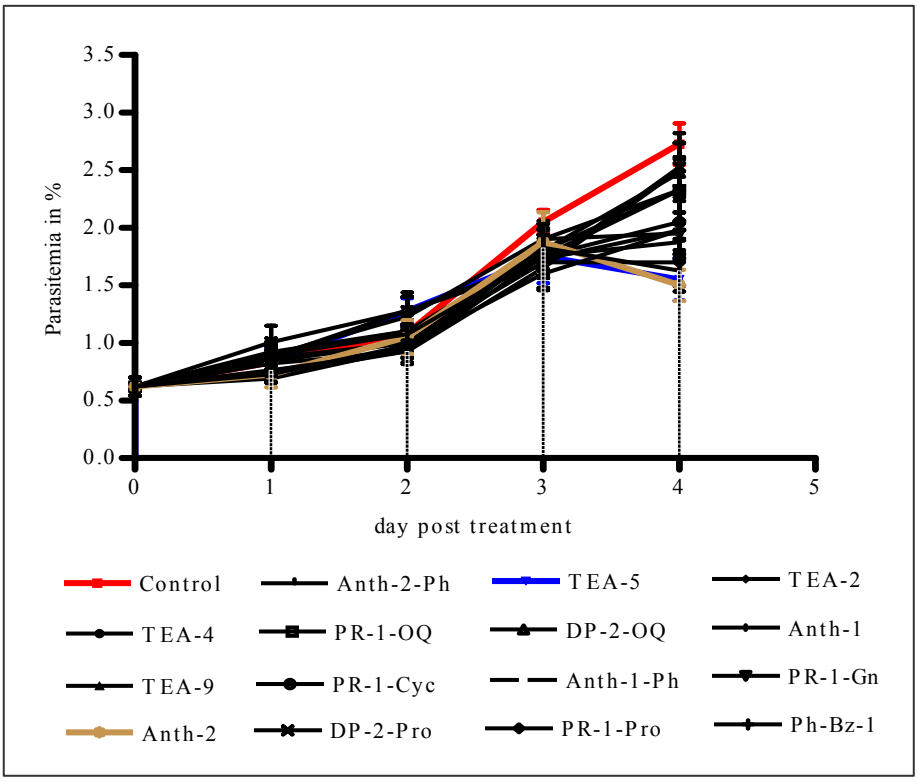

Fig: 3.50: In vitro effects of fifteen phenazines on Pf/NF-54 strain at 3µM of each DMSO= 0.032% in TE-2 and control.

3.1.5.7 In vitro results of twolefe phenazines derivatives

As the previous groups, the phenazines derivatives of this group were dissolved in DMSO and presented to Pf/NF-54 in 10µM of each (fig. 3.51).

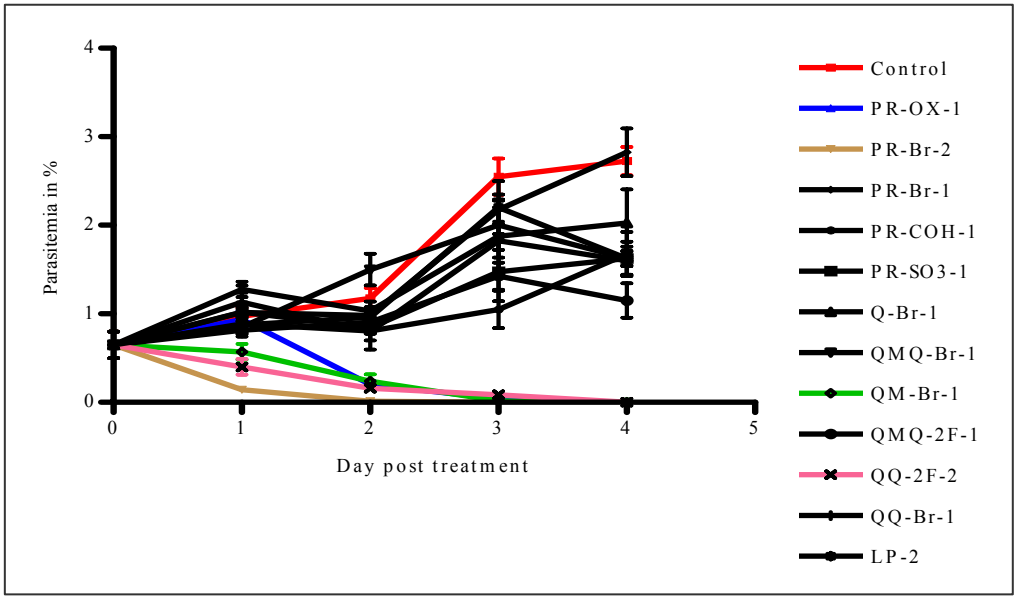

Fig. 3.51: In vitro effects of (12) phenazines on Pf/NF-54 strain at 10µM of each. DMSO= 0.081% in PR-So_3-1and control.

PR-OX-1, PR-Br-2, QM-Br-1, and QQ-2F-2 were selected for further in vitro investigations by using Pf/NF-54 strain. PR-OX-1 showed an IC_{50} of 3.73µM (fig.3.52), its concentrations in relation to the parasite's erythrocytic stages are illustrated in figure (3.53), where the schizont stage seems to be more sensitive to PR-OX-1 than trophozoite stage.

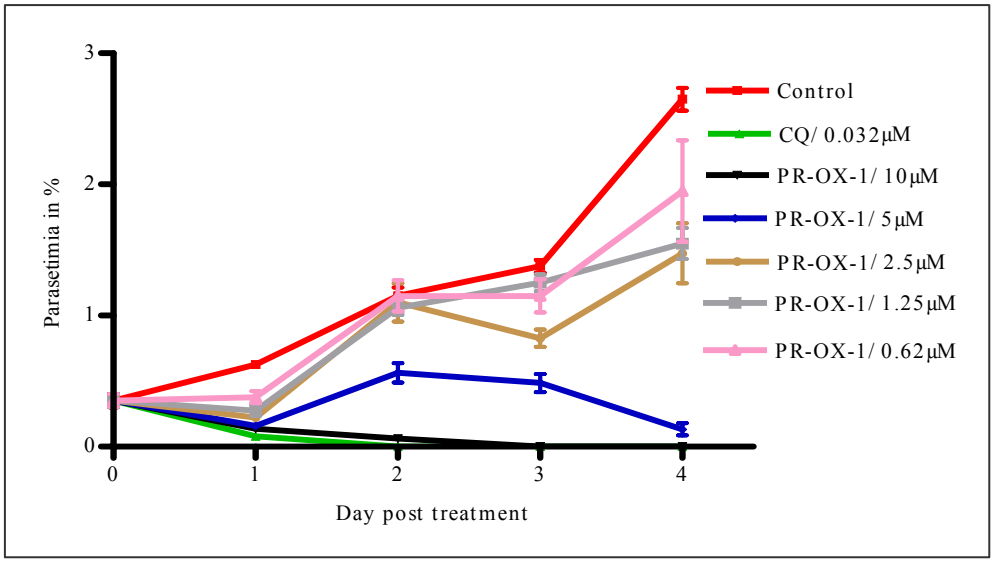

Fig. 3.52: In vitro effects of PR-OX-1 on P.f/NF-54. DMSO= 0.044% in control.

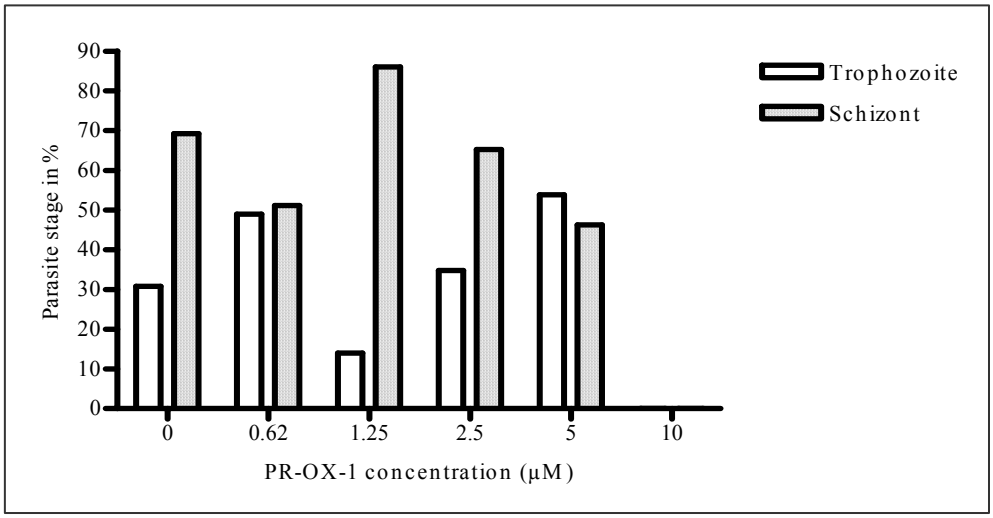

Fig. 3.53: Pf/NF-54 erythrocytic stages in relation to PR-OX-1 concentration on d3 post-treatment
-Chi-square for differences in Schizont stage: Control vs 1.25 and 5µM, $P < 0.0500$

The highest in vitro affectivity, in this group, was obtained from PR-Br-2 which gave an IC50 of 0.643µM (fig. 3.54).

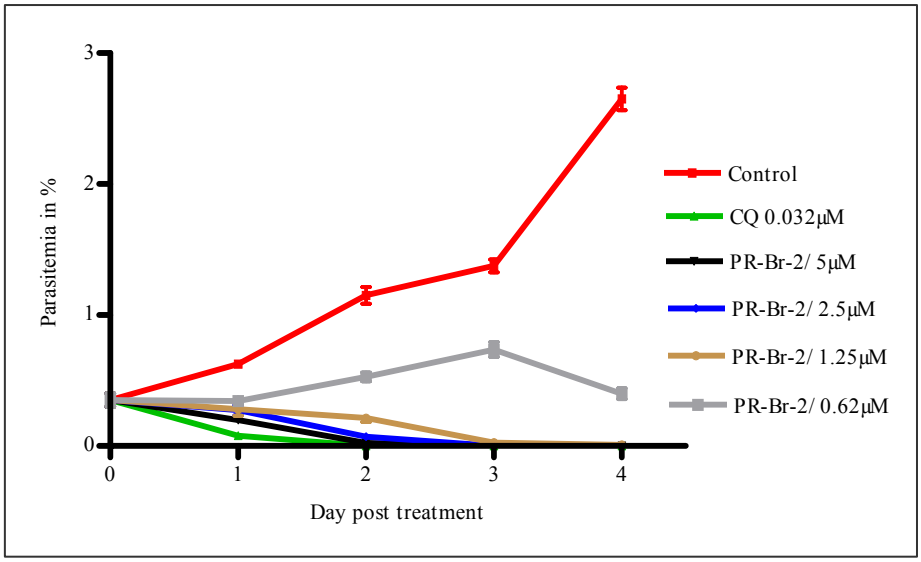

Fig. 3.54: In Vitro effects of PR-Br-2 on P.f/NF-54. DMSO= 0.044% in control.

In addition, like the PR-OX-1, the schizont stage is more sensitive to PR-Br-2 than the trophozoite (fig. 3.55).

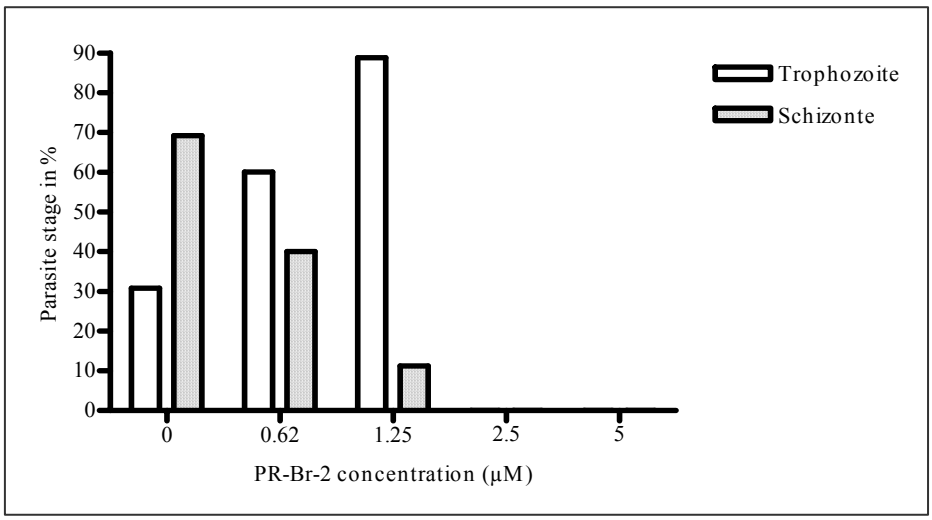

Fig. 3.55: Pf/NF-54 erythrocytic stages in relation to PR-Br-2 concentration on d3 post-treatment
-Chi-square for differences in the number of schizont stage: Control vs 0.62 and 1.25µM, $P < 0.0500$

QM-Br-1 gave an IC_{50} of 7.54µM as shown in figure (3.56), which indicates the highest IC_{50} obtained of the members of this group,

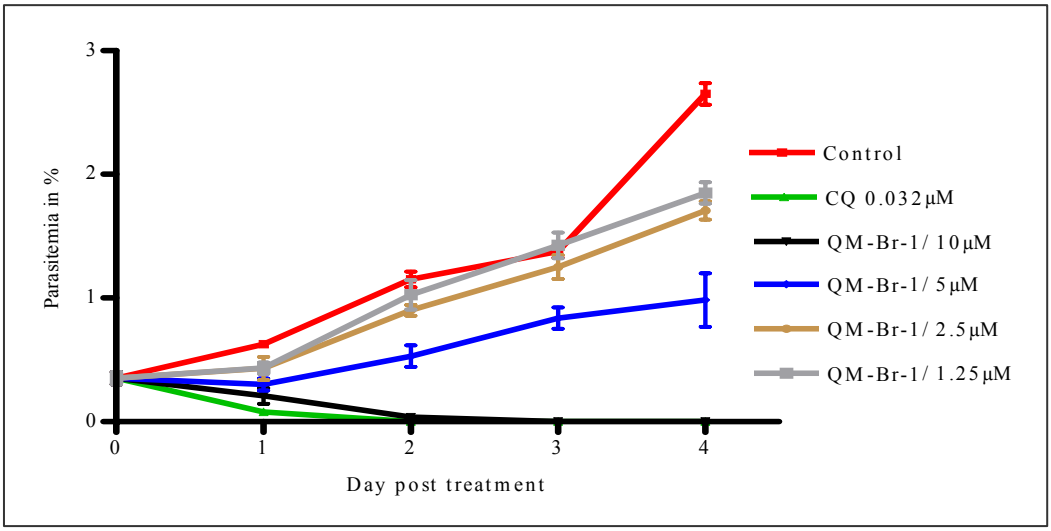

Fig. 3.56: In Vitro effects of QM-Br-1 on P.f/NF-54. DMSO= 0.044% in control.

Unlike PR-OX-1 and PR-Br-2, schizonte stage of Pf/NF-54 seems to be less sensitive to QM-Br-1 as shown in figure (3.57)

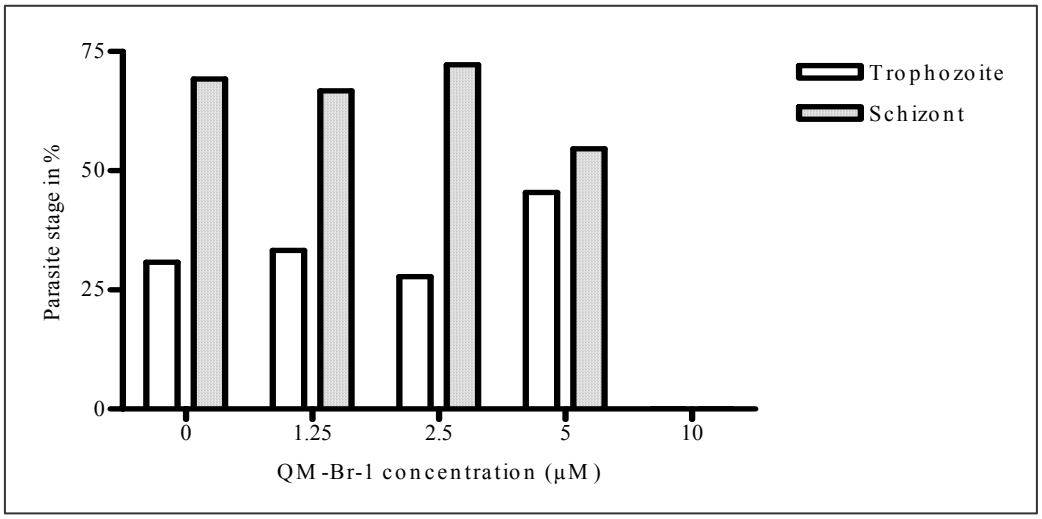

Fig. 3.57: Pf/NF-54 erythrocytic stages in relation to QM-Br-1 concentration on d3 post-treatment

For QQ-2F-2 the evaluated IC_{50} was 3.0µM (fig. 3.58), which is in the same range of PR-OX-1.

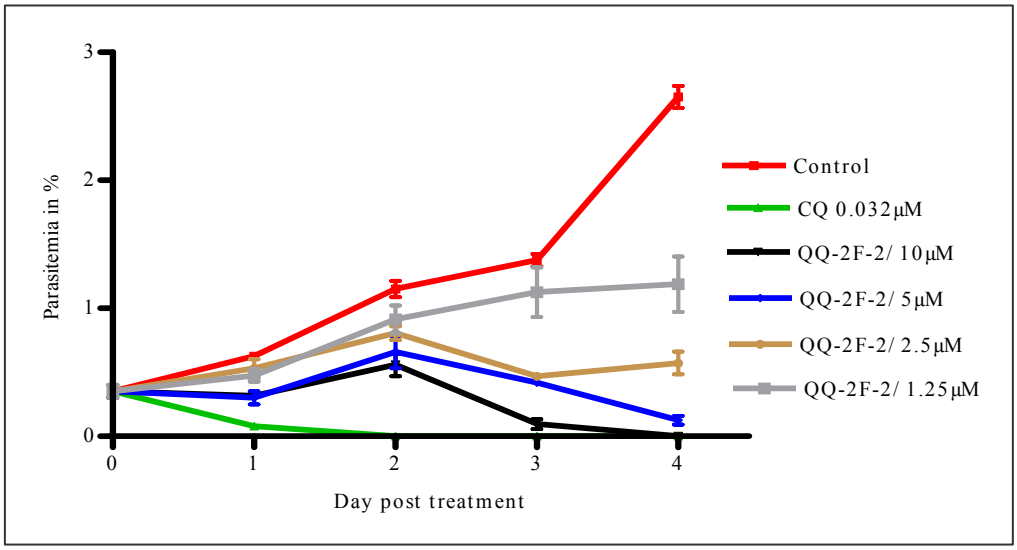

Fig. 3.58: In vitro effects of QQ-2F-2 on P.f/NF-54. DMSO= 0.044% in QQ-2F-2/10µM and control.

QQ-2F-2 showed a little different, to its group members, in its effecting on the trophozoite stage rather than the schizont stage (fig. 3.59).

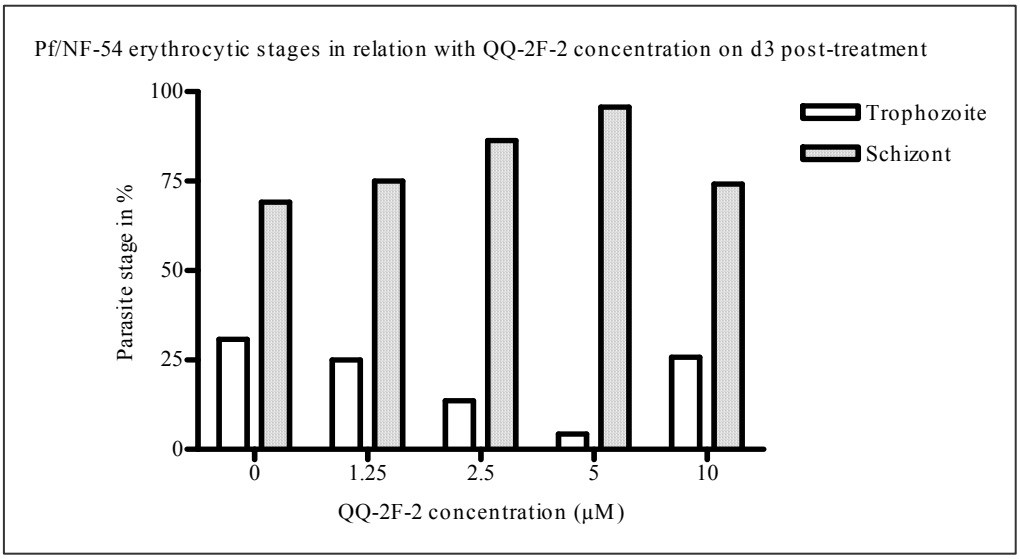

Fig. 3.59: Pf/NF-54 erythrocytic stages in relation to QQ-2F-2 concentration on d3 post-treatment
-Chi-square for differences in the number of trophozoit stage: Control vs 2.5µM, P= 0.0102

3.1.6 In vitro results of dihydroartmisinin (DHA) analogues

DHA is an active metabolite of artesunate, an anti-malarial commonly used in Thailand for the treatment of *P. falciparum* infection (Russell et al., 2003). However, the heme polymerization pathway is specific to the malarial parasite, and therefore offers a potential biochemical target for the design of anti-malarials (Pandey and Chauhan, 1998). In this study, seven substances of DHA analogues underwent in vitro assay for their anti-malarial affectivity.

3.1.6.1 In vitro results of five DHA analogues

Five DHA analogues underwent in vitro assay (fig. 3.60), all were dissolved in DMSO and Pf/NF-54 strain was exposed to 200ng/ml of each. After all, just AGG55 were selected for further in vitro investigations.

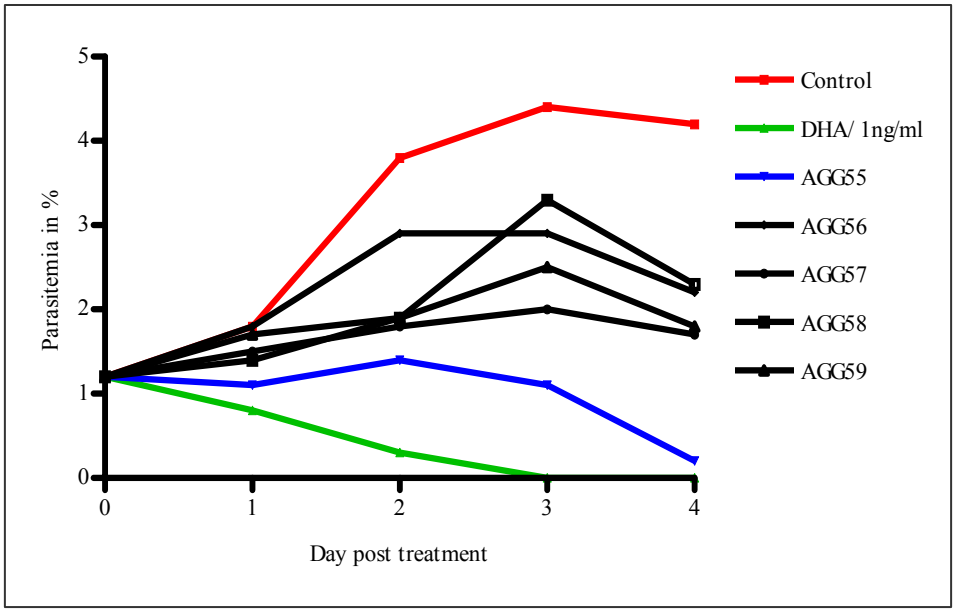

Fig. 3.60: *In vitro* effects of five DHA analogues on Pf/NF-54 at 200ng/ml of each.
DMSO=0.004% in AGG56 and control.

Series concentrations of AGG55 were prepared in complete MCM and then they were in vitro presented to Pf/NF-54 (fig. 3.61). Unfortunately, no in vitro efficiency was notable out of this analogue in the used concentrations.

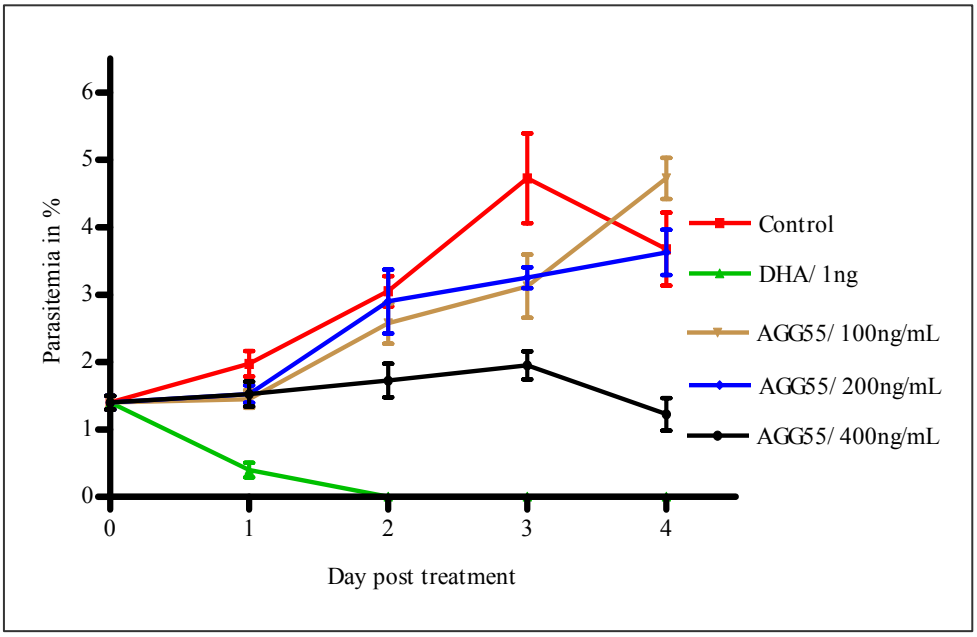

Fig. 3.61: *In vitro* effects of AGG55 on Pf/Nf-54. DMSO=0.008% in AGG56 and control

3.1.6.2 In vitro results of two DHA analogues

After the non-success with the first group of the DHA analogues, and to increase the possible affectivity of the new analogues, trioxan was connected to one of the two new analogues (Trio.Ad and SCT), as it is known that trioxan itself has an antimalarial activity (Liu et al., 1993; Haynes and Vonwiller, 1997). However, to investigate the *in vitro* activity of Trio-Ad analogue, it was in vitro presented to Pf/NF-54 in various concentrations ranged form 25 to 400ng/ml, but no in vitro activity could be observed (Data no shown), after which the concentrations were increased to reach 1000ng/ml.

Like the first group of DHA analogues, Trio.Ad and SCT showed a very low efficacy on Pf/NF-54, as shown in (fig. 3.62 and fig. 3.63) respectively.

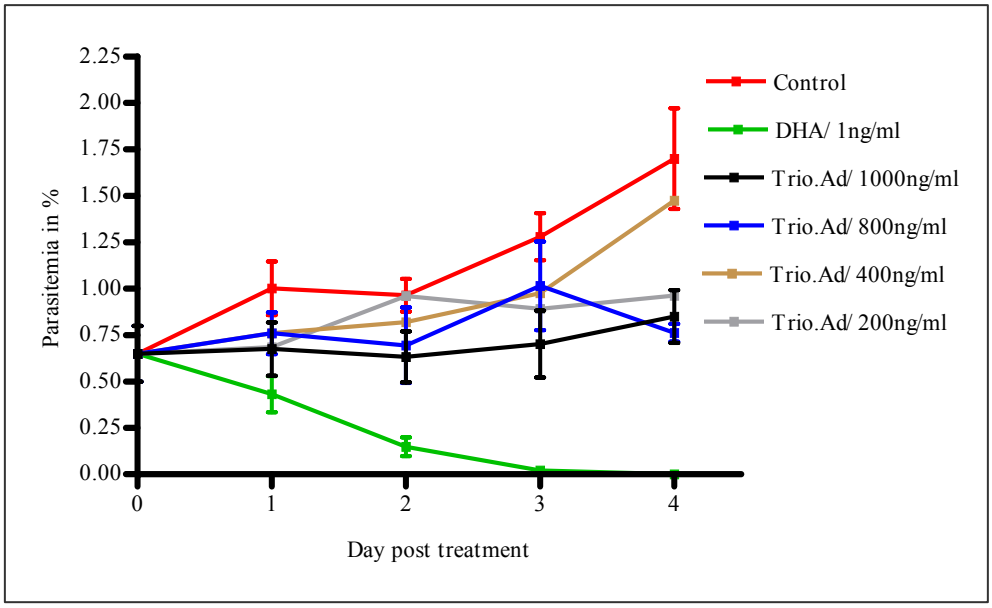

Fig. 3.62: In vitro effects of Trio-Ad on Pf/NF-54. DMSO=0.007% in AGG56 and control

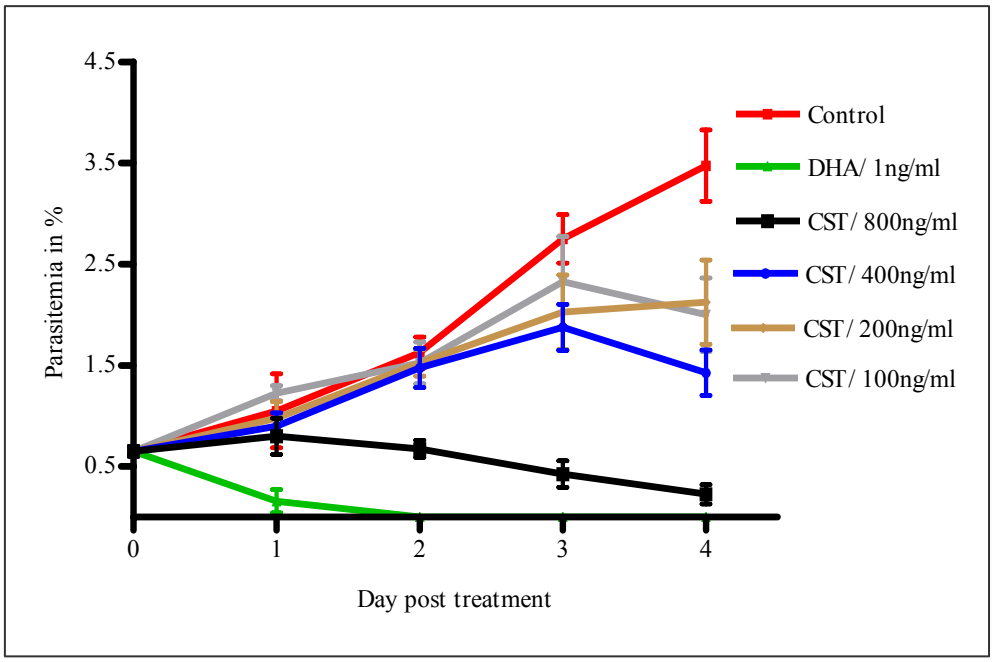

Fig. 3.63: In vitro effects of CST on Pf/NF-54. DMSO=0.001% in AGG56 and control

Since, the IC_{50}s of DHA and its derivatives are ranged from 0.5 to 1.0 ng/ml (Ittarat et al., 2003) and since the studied analogues showed almost no anti-malarial affectivity, even in high doses. Therefore, this class of inhibitors was dropped down for any further investigations.

3.1.6 In vitro results of nucliar export signals (NES) inhibtor

Studies in Scizosaccharomyces *pombe* identified the cellular target of LepB as the CRM-1/exportin 1 protein (Nishi et al., 1994), which is critical for the export of RNA and proteins containing a nuclear export sequence (NES). Whereas, the finding that eIF5A is a cellular cofactor of human immunodeficiency virus type 1, Rev, and HTLV-1 Rex transactivator proteins in mRNA export suggests that it can have additional activities. Moreover, the factor interacts with the general nuclear export receptor CRM-1 during their transportation from the nucleus to the cytoplasm (Rosorius et al., 1999). Furthermore, Zeiner et al., (2003) has reported that the spliced leader (SL) RNA of *Trypanosoma brucei* was sensitive to LepB treatment (≤ 1µg/mL), which implied the involvement 0f CRM-1) in SL RNA nuclear export of *T. brucei*. However, the ability of LepB to inhibit nuclear export has made it a useful tool in the study of the subcellular localization of many regulatory proteins (Asscher et al., 2001). Therefore, the *in vitro* ability of LepB in reducing the macrophages TNF production and its ability to influence the parasitemia of Pf/NF-54, have been in vitro investigated in this study.

Firstly, the in vitro ability of LepB to reduce TNF level was assessed where the LPS stimulated murine macrophages were treated with series concentrations of LepB, ranged from 0.62 to 40 nM. The results obtained by sandwich ELISA from the macrophages culture media after 24hrs, have shown high reductions in TNF levels when compared to the control non-LepB treated-LPS stimulated macrophages (Data not shown). However, with this result it was difficult to distinguish, whether these reductions were due to LepB toxicity on the macrophages, or due to the ability of LepB to interact with mRNA export of macrophages TNF. Thereby, the LepB concentrations have been modified to be ranged from 0.039 to 1.25nM where TNF levels were found to be influenced by increasing the LepB concentrations (fig. 3.64).

In addition, the validity of murine macrophages was confirmed by trypanblue stain pre- and post-LepB treatment.

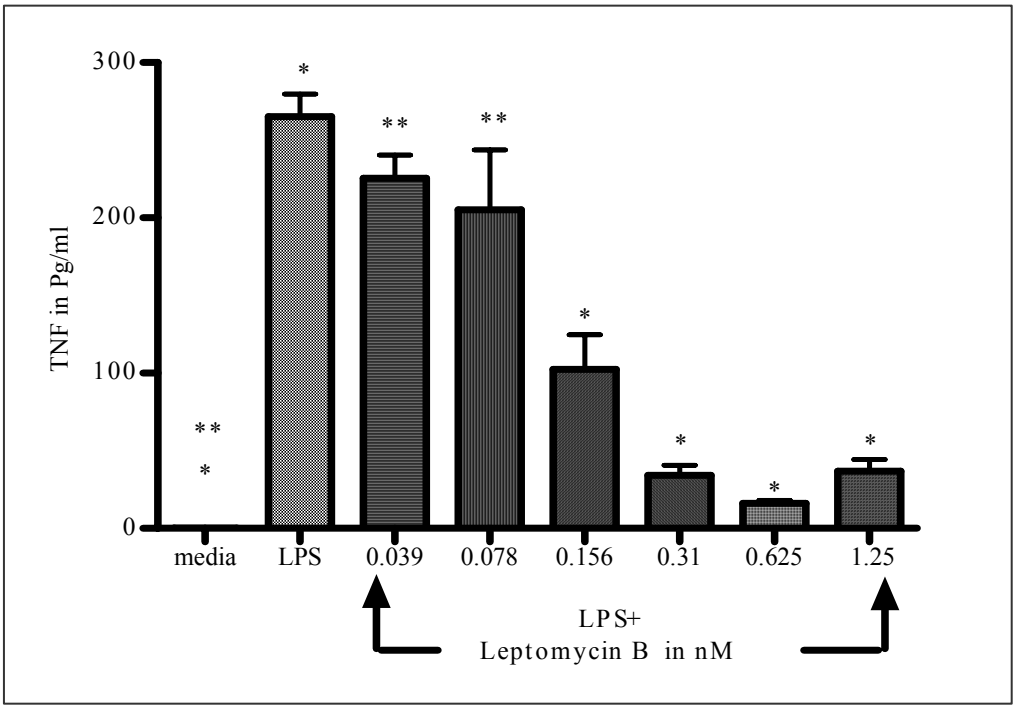

Fig.3.64: TNF levels by stimulated macrophages in cell culture.
Spleenic macrophages out of PbANKA infected C57Bl/6 mice d3p.i.
Macrophages=5x10^4 cell/well in 24hrs culture, LPS 100 ng/ml (LPS= Lipopolysaccaride)
Cell validity (by trypanblue stain): 0h= 97%, after 24h=97% at media LPS and all Lep. B concentrations
Tukey's Multiple Comparison Test: * LPS vs media, Lep. B 0.15, 0.31, 0.62 and 1.25nμ P< 0.001.
** Media vs LepB 0.039 and 0.078 and 1.25nM P< 0.05.

On the other hand, the in vitro anti-malarial ability of LepB has been evaluated on Pf/NF-54. In the first assay LepB was used in low concentrations 5 to 40 nM supplemented in Complete MCM. But no anti-malarial activity could be observed in these concentrations (Data not shown). After which, and since *Trypanosoma brucei* was sensitive to LepB treatment (≤ 1μg/mL), which demonstrated by growth curves and altered morphology (Zeiner et al., 2003). Therefore, LepB concentrations ranged between 0.57 to 3μM were selected to be assessed against Pf/NF-54. Again, no in vitro antimalarial ability could be observed of LepB (fig. 3.65).

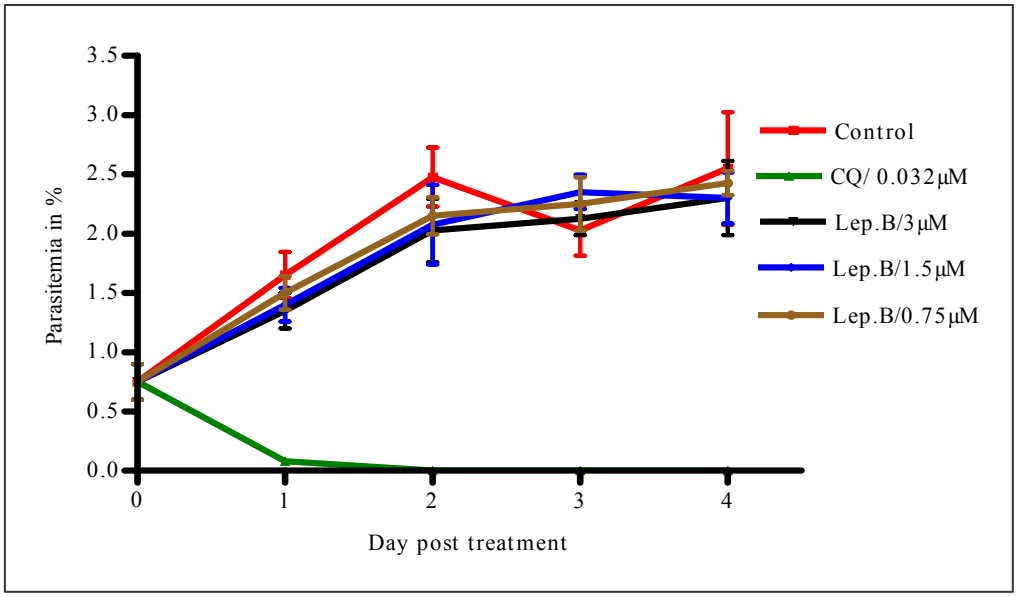

Fig. 3.65: In Vitro effects of Leptomycin B on Pf/NF-54.

The results presented here showing an in vitro lack of effect of the antibiotic LepB to influence the parasitemia of Pf/NF-54 strain, when used at ≤ 3µM.

3.2 In vivo results

The inhibitors which exhibited in vitro anti-malarial activities were selected to investigate whether they could influence the parasite proliferation in vivo in a mouse model by using *Plasmodium berghei* ANKA (PbANKA); a rodent malaria parasite (Lou et al., 2001).

However in this study, PbANKA infected C57Bl/6 mice were considered to have cerebral malaria (CM) if they have neurological signs (ataxia, paralysis, deviation of the head, and convulsions), (Amani et al., 1998; Golenser et al., 2006), in a period ranged of day6 to day 10 post-infection (d6 to d10 p.i.). Whereas BALB/c mice considered as CM-resistance mice (Lou et al., 2001). The infected dose was $5x10^4$ iRBCs to infect C57Bl/6 WT mouse and $1x10^5$ iRBCs to infect BALB/c mouse.

3.2.1 In vivo results of deoxyhypusine synthase (DHS) inhibitor

CNI-1493 considered as a novel and potent inhibitor of DHS (Hauber et al., 2005) and has been shown to inhibit synthesis of TNF in monocytes/macrophages by somehow suppressing translation efficiency ((Cohen et al., 1996)). However, the evaluated in vitro IC_{50} of CNI-1393 was 135.79µM on Pf/NF-54; CQS strain. Therefore it confirmed in vivo the CNI-1493 in vitro results however, a major draw back has faced the in vivo CNI-1493 evaluation that was representing in the toxicity of this substance. BALB/c mice were injected i.v. with 50mg/kg of CNI-1493 died immediately; we thought that was not because of the CNI-1493 direct toxicity, but may be because of the high viscosity of this substance, in this dose. In addition, two BALB/c mice died (of 3 mice), after three times i.v. injections of 40mg/kg (once a day). On the other hand, i.p. administration of 40mg/kg CNI-1493 led to losing in the mice body weight (Data not shown). This draw back had limited the dose of CNI-1493 to be tested in vivo. Conversely, 1mg/kg of CNI-1493 showed no efficacy on the parasitemia of PbANKA when given to C57Bl/6 mice 9 times i.v. (fig.3.66), it has not even saved the mice from developing CM (fig. 3 67).

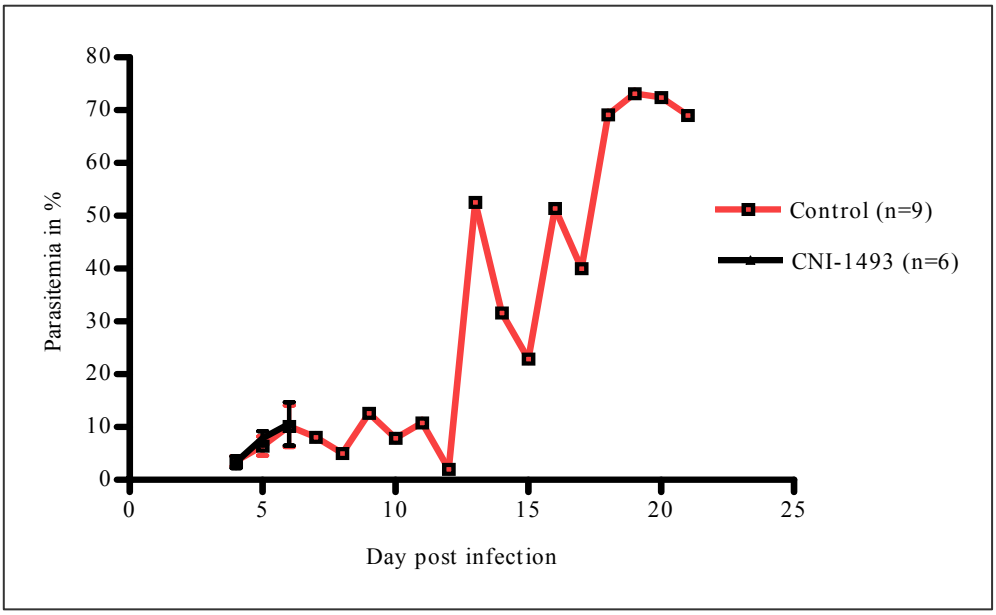

Fig. 3.66: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with 1mg/kg of CNI-1493.
Treated 9 times: -2, -1 day and from d1 to d7 post-infection

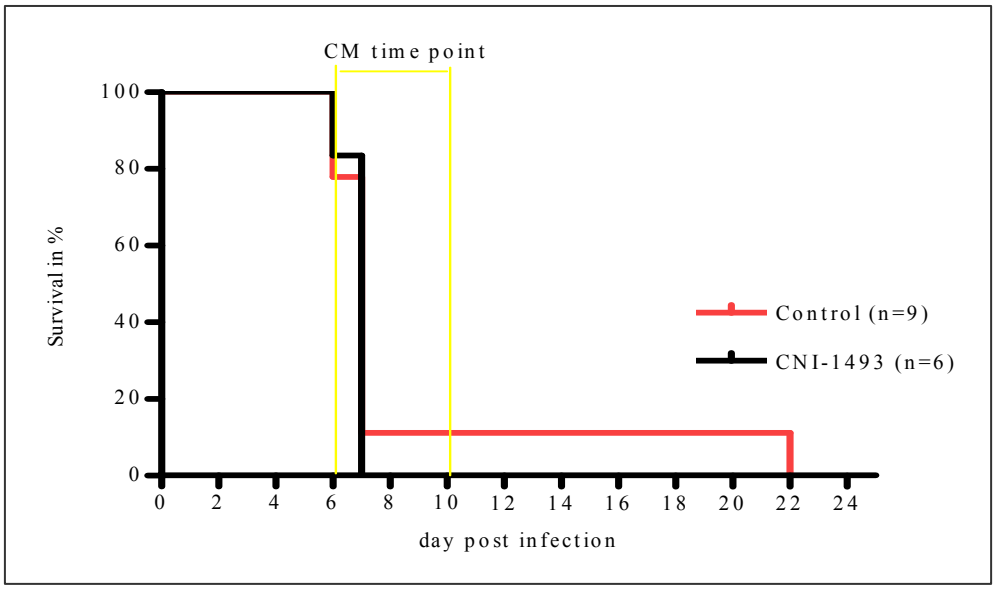

Fig. 3.67: Survival of PbANKA infected C57/Bl/6 mice traeted iv with 1mg/kg of CNI-1493.
Treated 9 times: day -2 and -1, and from d1 to d7 post-infection. Logrank Test for survival: NS

After which, the dose of CNI-1493 was increased to be 4mg/kg, to find out whether it could save the mice from CM if administrated one time. The CNI-1493 mice groups were divided into two groups; a group was injected on d3 p.i. and the other group on d5 p.i. (fig. 3.68). However, the injections on these two time points did not influence the parasitemia of PbANKA (Data not shown). Thus CNI-1493 has not saved the mice From CM when injected on d5 p.i.

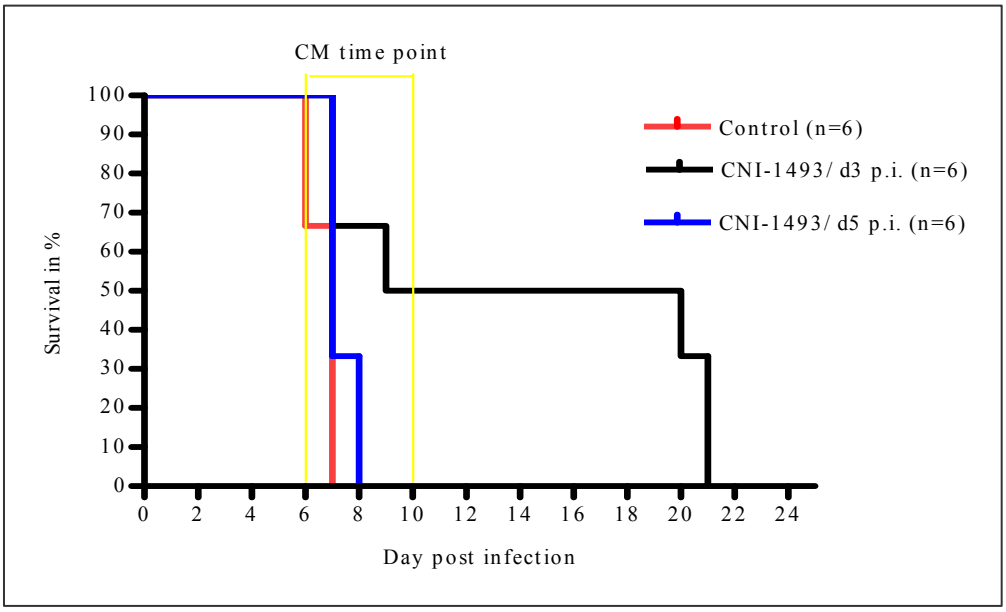

Fig. 3.68: Survival of PbANKA infected C57Bl/6 mice, treated i.v. one time with 4mg/gk of CNI-1493.
Logrank Test for survival: CNI-1493 on d3p.i. vs control, P value= 0.0129.
CNI-1493 on d3p.i. vs on d5p.i., P value=0.0313.

But CNI-1493, given on d3 p.i. saved 50% of the infected mice, in which CNI-1493 showed significant statistical differences in the mice survival versus (vs) the control group and vs CNI-1493 on d5 p.i.. Therefore this experiment (fig. 3.68) was repeated in the same procedure to prove the differences in the survival time.

Unlike the first experiment, no statistical differences in the repeated experiment as 5 of 7 mice developed CM in both groups (CNI-1493 on d3 and d5 p.i.), versus 7 of 7 in the control group. In addition, the parasitemia of PbANKA hasn't been influenced by CNI-1493 in both groups (Data not shown).

3.2.1.1 In vivo results of 12mg/kg of CNI-1493

To find out, if 12mg/kg of CNI-1493 will reduce the parasitemia of PbANKA in C57Bl/6 mice, considered CNI-1493 effects on CM. 12mg/kg of CNI-1493 was prepared in 1xPBS (after primary dissolving in DMSO) and given i.v. from d1 to d9 p.i. (fig. 3.69).

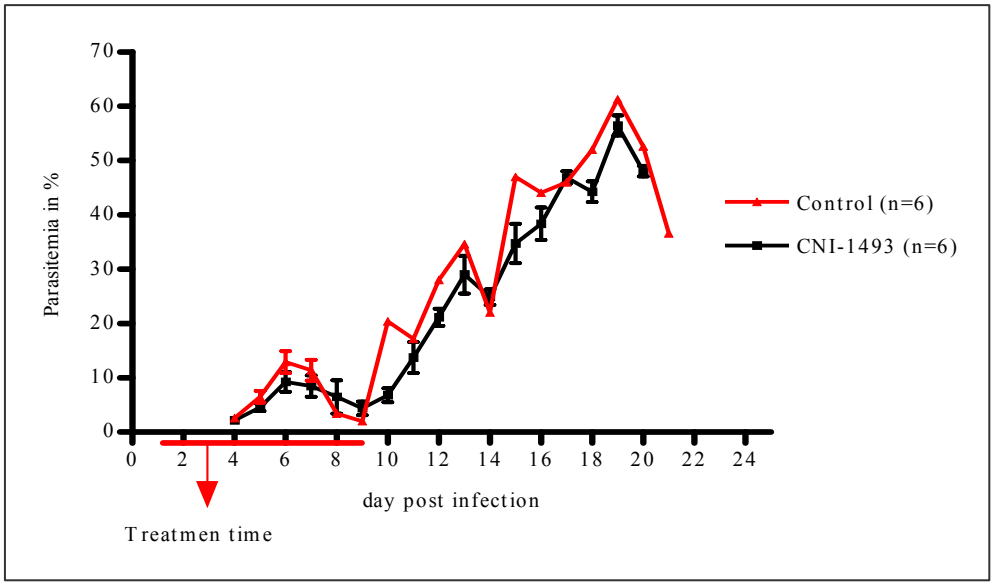

Fig. 3.69: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with 12mg/kg of CNI-1493. Treated 9 times: from d1 to d9 post-infection. Mann Whitney test for differences in parasitemia: NS. DMSO= 0.62% in groups, CNI-1493 and control.

CNI-1493, at 12mg/kg, showed inability to reduce the parasitemia in the treated group.

On the other hand, there were no statistical differences in the developing of CM between CNI-1493 treated and control group (fig.3.70). Indicating, that CNI-1493 is unable to save the infected mice of developing CM if given post-infection.

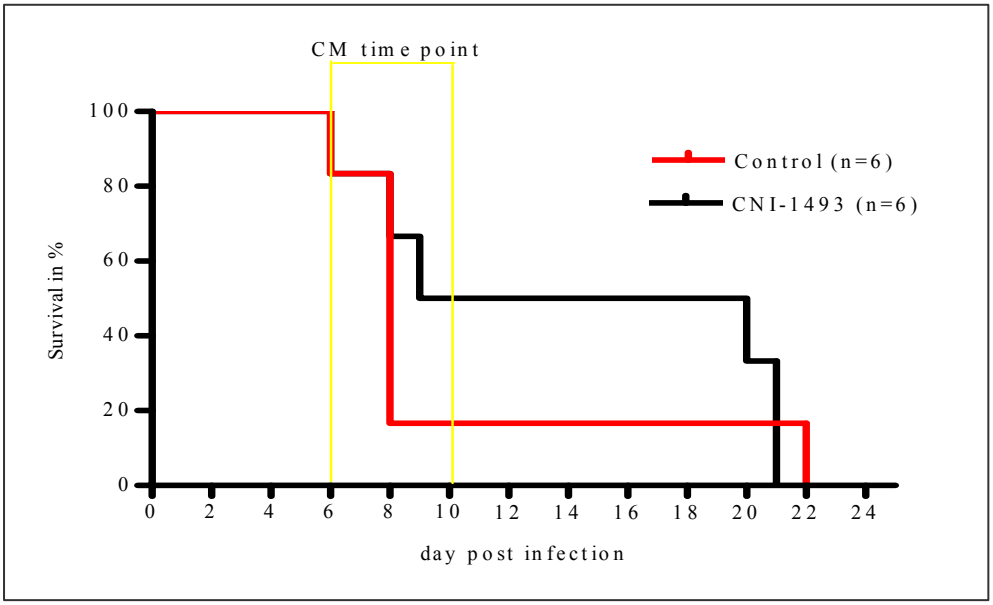

Fig. 3.70: Survival of PbANKA infected C57Bl/6 mice traeted i.v. with 12mg/kg of CNI-1493.
Treated 9 times: from d1 to d9 post-infection. Logrank Test for survival: NS.

3.2.1.2 In vivo results of 4mg/kg of CNI-1493

The above findings of CNI-1493, found to be in agreement with likewise previous studies, by Curfs (1993) and Belnoue (2002) where they reported that, depletion of monocytes/ macrophages by treatment with a liposome containing dichloromethylene diphosphonate, if administered before the day of infection, but not later, prevents the development of CM.

So if this is the case with CNI-1493, it should prevent CM if given pre-infection. And since, we have already given 1mg/kg CNI-1493 two days pre-infection, with no income for CM (fig. 3.67). On the other hand, according to our previous observations that giving CNI-1493 i.v. every day, makes the tissue of the mice tail kind of hard tissue, which makes the i.v. injections very difficult to be managed. Therefore, 4mg/kg of CNI-1493 was given i.v. pre-infection once every two days. In parallel of that, two control groups; i) injected

control where the mice received 1xPBS+DMSO, on the same time points of CNI-1493. ii) non-injected control group (fig.3.71).

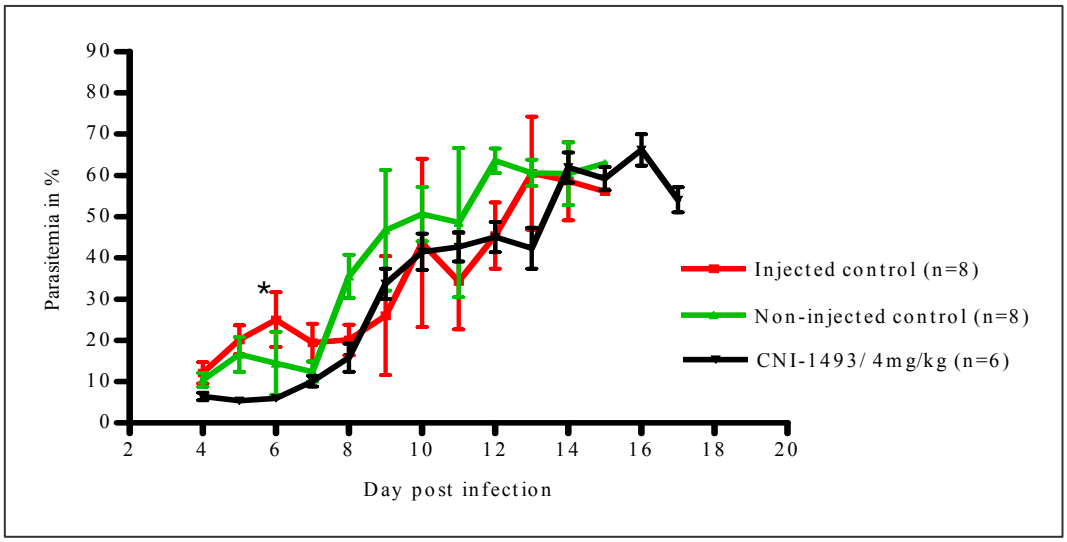

Fig. 3.71: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with CNI-1493.
-DMSO% in both injected control and CNI-1493 group =0,6% in 1xPBS.
-CNI-1493 and Injected control have been injected each 2 days: -5, -3, -1, d1, d3, d5, d7 and d9 p.i.
*Kruskal-Wallis test for differences in parasitemia on d5 and d6 p.i.: CNI-1493 vs Injected control $P < 0.05$

In this system of injections CNI-1493 showed a limited ability to reduce the parasitemia of PbANKA. On the other hand, and as expected, CNI-1493 protected the C57Bl/6 mice of developing CM if given pre-infection (fig. 3.72).

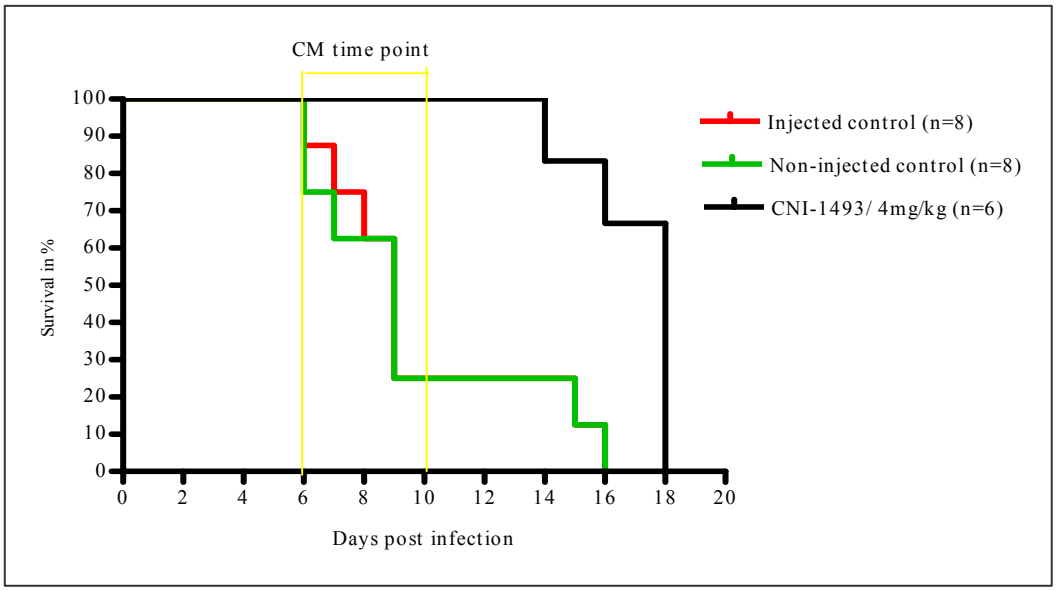

Fig. 3.72: Survival of PbANKA infected C57Bl/6 mice.
-CNI-1493 and Injected control have been injected each 2 days: -5, -3, -1, d1, d3, d5, d7 and d9 p.i.
-Logrank Test for survival: CNI-1493 vs injected control P value=0.0029.
-CNI-1493 vs Non-injected control P value=0.0028.

In addition to observing the parasitemia and CM, the levels of cytokines expression (IL-10, and IFN-γ) during the infection course were assessed in the mice plasma by sandwich ELISA assay. Each group of the experimental mice was sub-divided into two sub-groups for plasma collections. From the first sub-group, plasma was collected on the odd days post-infection, whereas from the second sub-group plasma was collected on the even days post-infection. However, plasma IL-10 of both sub-groups can be observed in figures (3.73 and 3.74). A clear pike of IL-10 on d3 and 4 p.i., which also shows IL-10 reductions in CNI-1493 treated group.

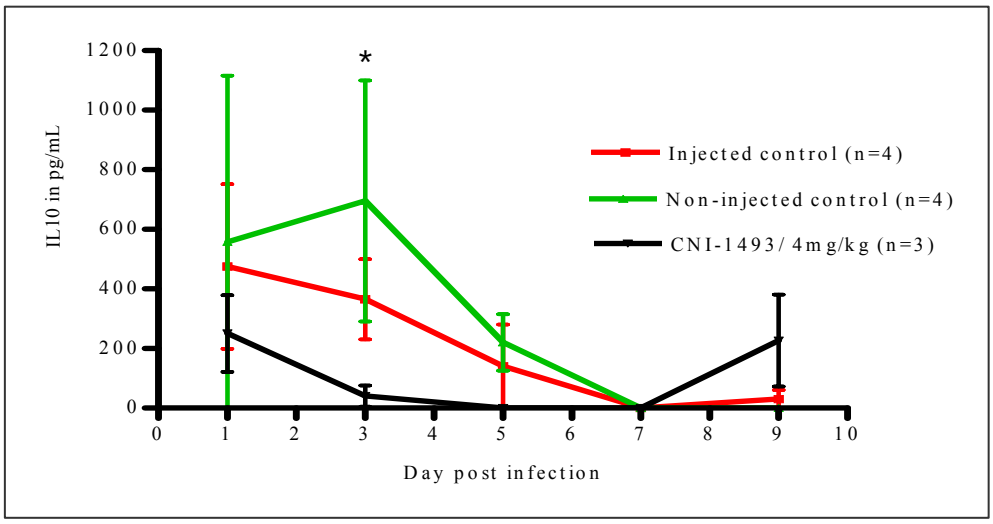

Fig. 3.73: IL-10 in plasma of PbANKA infected C57Bl/6 mice on the odd days post-infection.
*F test to compare variances on d3 p.i., CNI-1493 vs Non-injected control: P= 0,0113

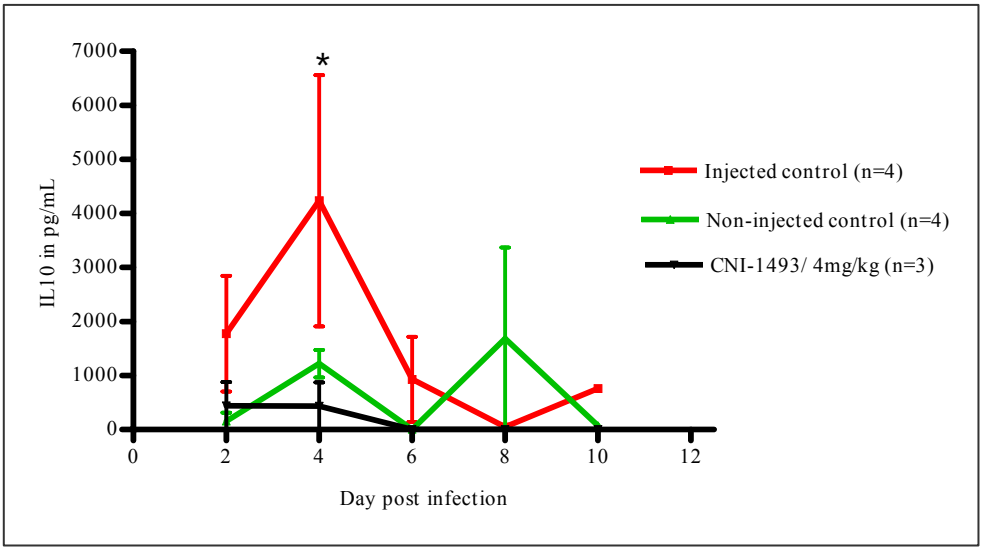

Fig. 3.74: IL-10 in plasma of PbANKA infected C57Bl/6 mice on the even days post-infection.
*F test to compare variances on d4 p.i., Injected control vs Non-injected control: P= **0,0042**

As IFN-γ has also been shown to have an important role in the pathogenesis of CM (Angwerda et al., 2005), the plasma levels of IFN-γ was assessed by sandwich ELISA assay. The IFN-γ bike was found to be on d3 and d4 p.i., as shown in figures (3.75 and 3.76).

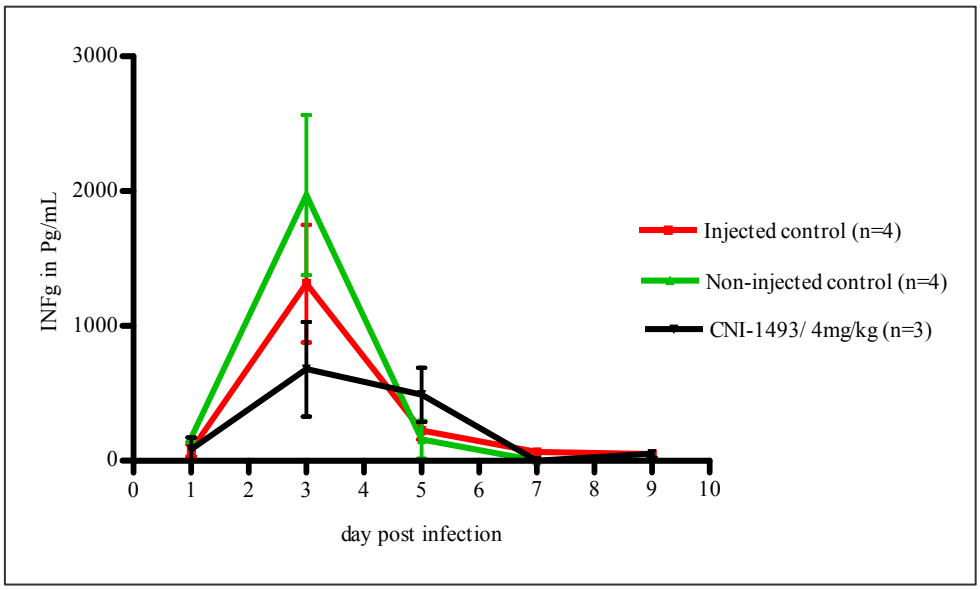

Fig. 3.75: IFN-γ in plasma of PbANKA infected C57Bl/6 mice on the odd days post-infection.

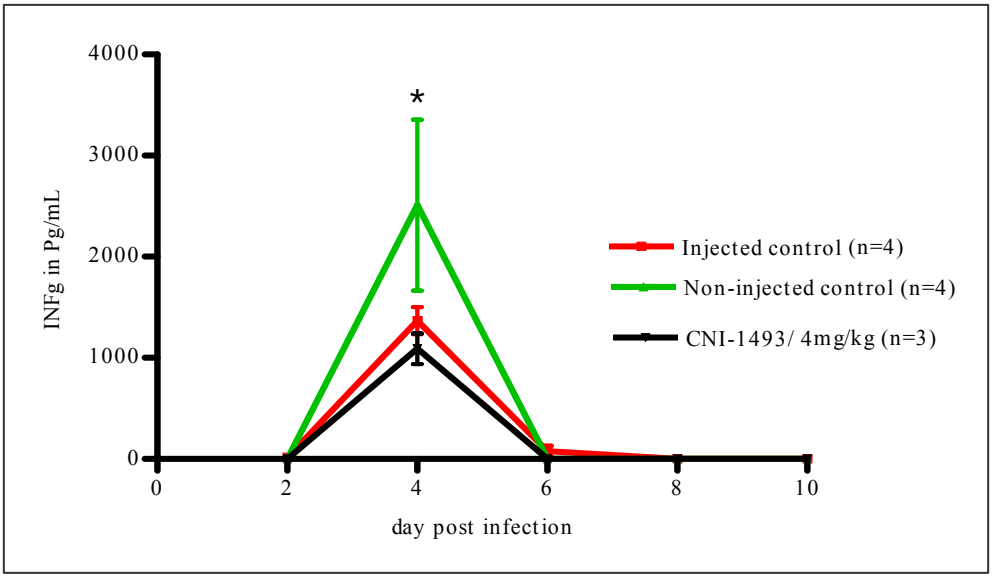

Fig. 3.76: IFN-γ in plasma of PbANKA infected C57Bl/6 mice on the even days post-infection.
*F test to compare variances on d4 p.i., CNI-1493 vs Non-injected control: P= 0,0482

Since, treatment with 4mg/kg of CNI-1493 pre-PbANKA infection has saved the C57Bl/6 mice from CM, it was important to find out whether this dose of CNI-1493, if given post-infection, will save the mice of CM or not. For that, PbANKA infected C57Bl/6 mice were prepared as the following: i) Control group injected with 1xPBS+DMSO, ii) CNI-1493 injected pre-infection, and iii) CNI-1493 injected post-infection; as shown in figure (3.77), where CNI-1493 showed also a limited ability to reduce the parasitemia.

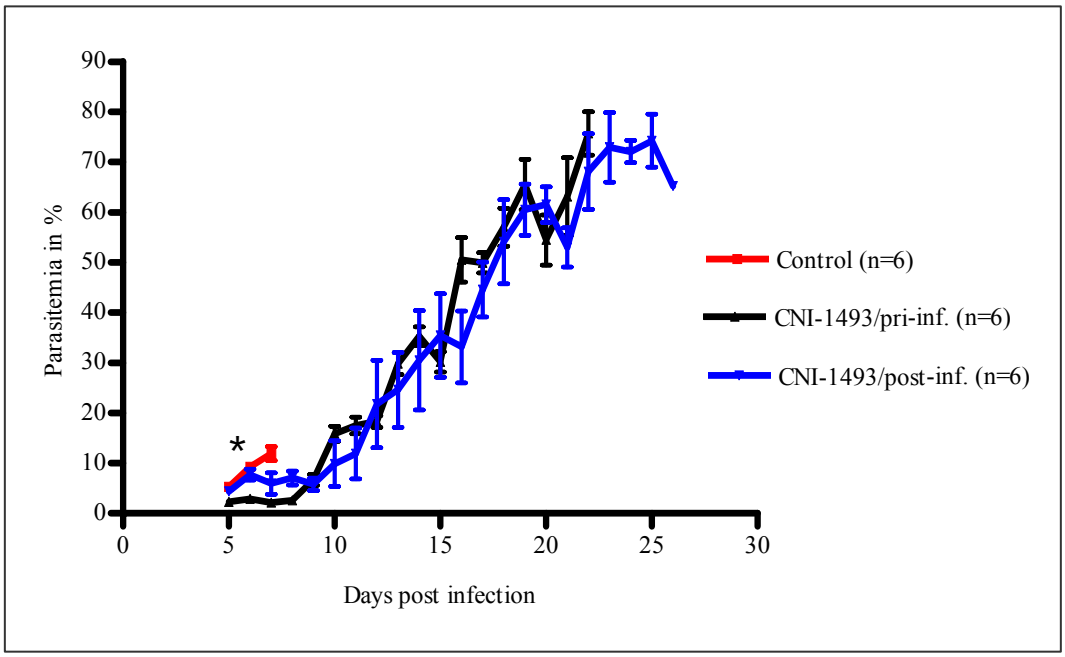

Fig. 3.77: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
-Treated each 2 days: pre-infection: on -3 and -1 day, d1, d3 and d5 post infection.
Post-infection: on d1, d3 and d5 post infection.
* Mann Whitney test for differences in parasitemia on d5 p.i.:
CNI-1493 pre-inf. vs Control, P= 0.0022.
CNI/1493 pre/inf. vs CNI/1493 post-inf., P= 0,0043
-DMSO= 0.61% in CNI-1493 treated groups and control group.

CNI-1493 in 4mg/kg presented again its ability to save the mice from CM if given pre-infection (fig. 3.78). Whereas, CNI-149370% prevented the CM in about 70% of the infected mice if given post-infection. Even though, 3 mice of the survival mice in the post-infection treated group had CM symptoms on d6, 7 and 8 p.i., and after all they clarified the symptoms and became healthy again, which could happen, some time, even to the non-treated groups (previous observations).

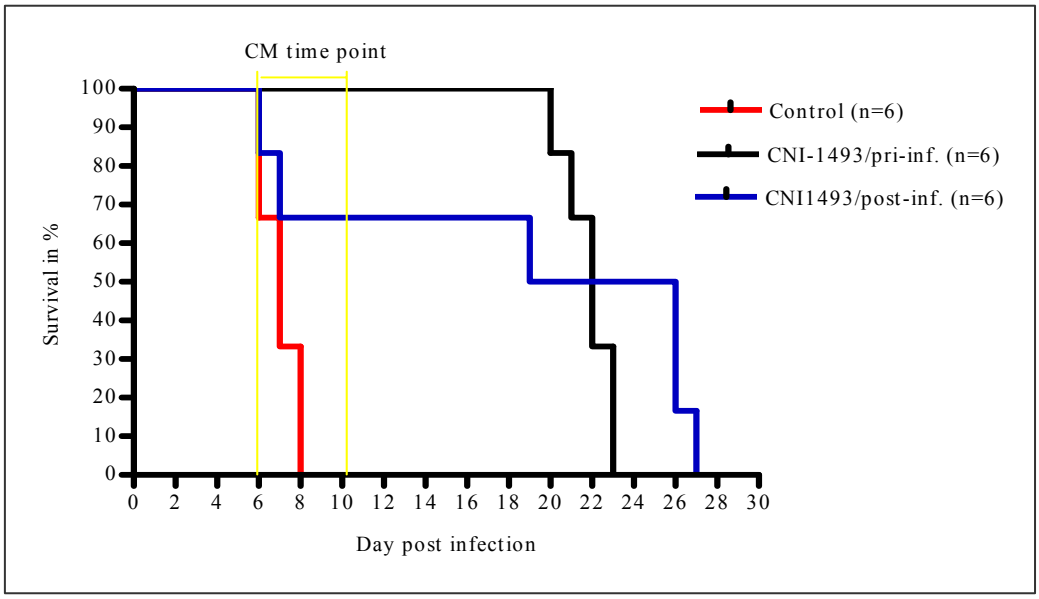

Fig. 3.78: Survival of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
Logrank Test for surivival: CNI pre-infection vs control: P= 0.0007.
CNI pre-infection vs CNI post-infection: P= 0.4240.
CNI post infection vs Control: P= 0.0429.

However, this experiment was repeated on C75Bl/6 mice, in the same way of CNI-1493 administration (pre and post- infection), to prove the survival for CM time point, especially for the CNI-1493 post-infection treated group.

The result reveal, again, that CNI-1493 saved the mice from CM (100% saved n=8), if given pre-infection, and failed to save the whole group if given post-infection (50% saved n=6). In contrast, in the control group 65.5% died on CM (n=8), also the CNI-1493 treated group showed the same limitation in the parasitemia reduction.

3.2.1.3 Are reductions in parasitemia related to CNI-1493 treatment?

To answer this question, two experiments have been performed at the same time and under the same conditions (the mice batch, the same preparation of the dose of PbANKA infection, and the same CNI-1493 preparation). The first experiment underwent the same CNI-1493 procedure (like in fig. 3.77), treated pre and post-infection with the same end point of the treatment (d5 p.i.), (fig. 3.79). Whereas the other, or the second experiment,

under went the same procedure except the end point of CNI-1493 treatment, which ended in d19 p.i. (fig. 3.80).

As shown in figure (3.79), parasitemia levels in CNI-1493 treated groups were significantly different (P< 0.05), on d 6 p.i. compared to that in the control, which was in the same previous behaviour of CNI-1493. In addition, the plasma levels of TNF and LT-α have been assessed in this experiment (the results will be presented later).

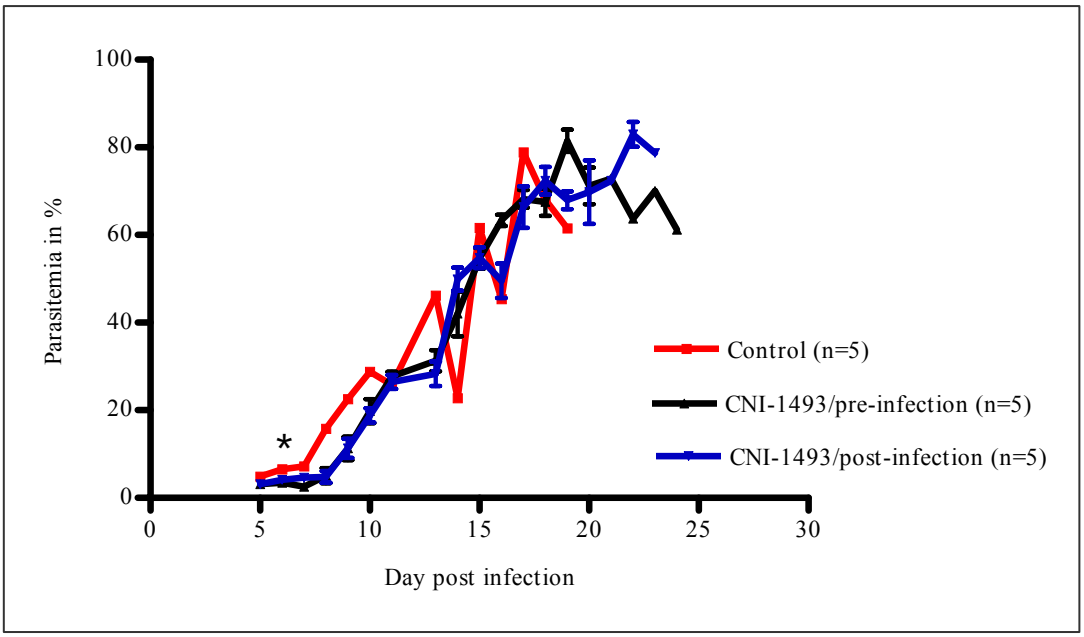

Fig. 3.79: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
-Treated each 2 days on: Pre-infection group: -3, -1, d1, d3 and d5 p.i.
Post-infection group: d1, d3 and d5 p.i.
* Mann Whitney test for differences in parasitemia on d6 p.i.: Control vs CNI-1493/Pre-infection, P= 0,0357
Control vs CNI-1493/Post-infection, P= 0,0357
-DMSO= 0.61% in CNI-1493 treated groups and control group.

In the other experiment, where the mice received CNI-1493 pre-infection, and then to d19 p.i., in addition to save the mice of CM (fig. 3.81); CNI-1493 influenced the parasitemia and exhibited similar to the previous experiments (fig. 3.80).

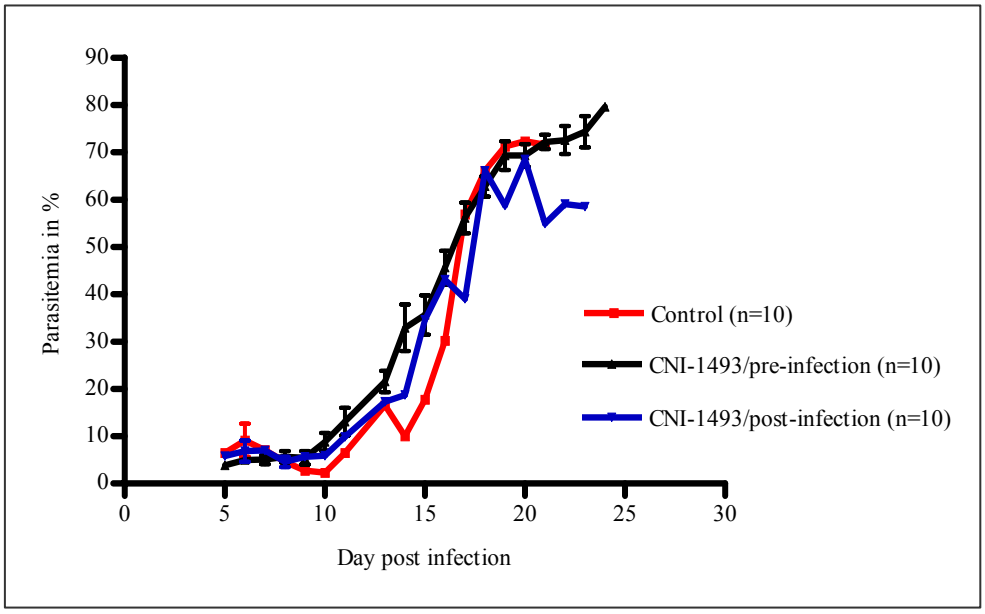

Fig. 3.80: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
-Treated on: Pre-infection group: -3, -1, d1, 3, 5, 7, 9, 11, 13, 15, 17 and d19 p.i.
Post-inf. group: d1, 3, 5, 7, 9, 11, 13, 15, 17 and d19 p.i.
* Mann Whitney test for differences in parasitemia on d5 p.i.: CNI-1493/pre-infection vs Control P= 0,0029.
-DMSO= 0.61% in CNI-1493 treated groups and control group.

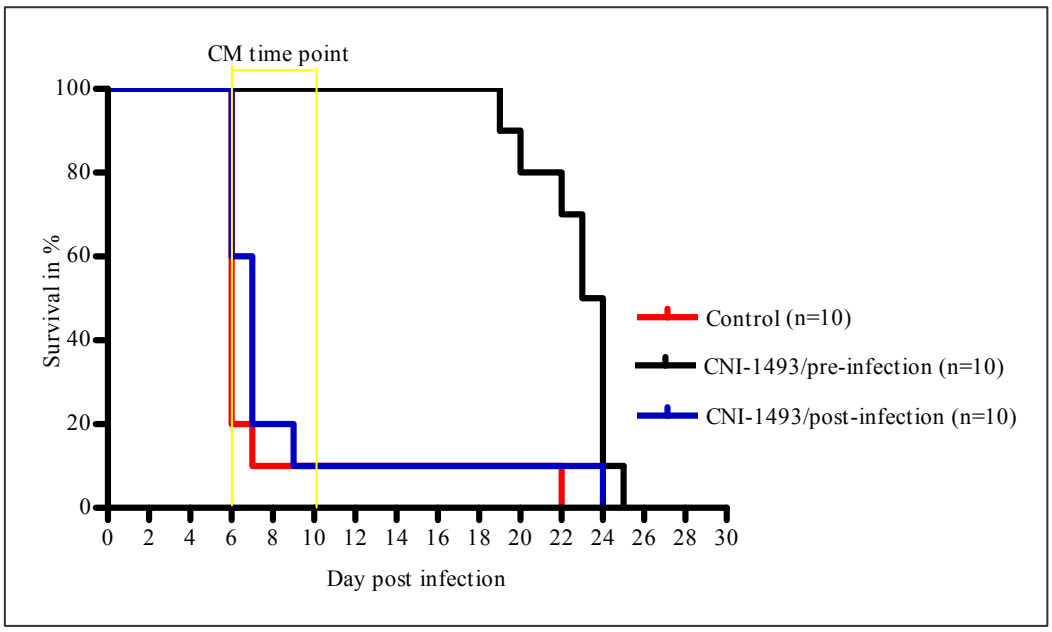

Fig. 3.81: Survival of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg of CNI-1493.
-Logrank Test for survival: Pre-inf. vs Control P< 0.0001
pre-inf. vs post-inf. P=0.0012

Coming back to the main aim of these last two experiments and to answer the question; are reductions in parasitemia related to CNI-1493 treatment? For that, the parasitemias in both experiments, which was obtained from treating with CNI-1493 pre-infection, underwent statistical evaluation (fig. 3.82).

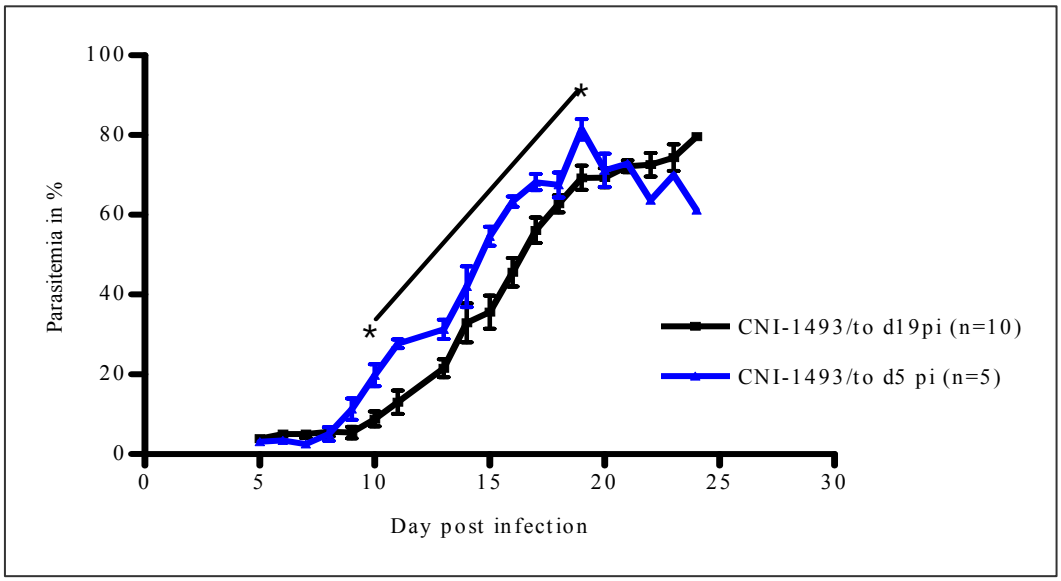

Fig. 3.82: Parasitemia in PbANKA infected C57Bl/6 mice treated iv with 4mg/kg CNI-1493.
-Treated on: CNI-1493 to d5p.i.: -3, -1, d1, 3, and d 5p.i.
CNI-1493 to d19p.i.: -3, -1, d1, 3, 5, 7, 9, 11, 13, 15, 17 and d19 p.i.
*Mann Whitney test for differences in parasitemia: CNI-1493 to d19 p.i. vs CNI-1493 to d5 p.i.: on d10, 11, 13, 15, 16, 17, and 19 p.i.: $P < 0.05$

The both groups exhibited differences in parasitemia but not in total survival (fig. 3.83).

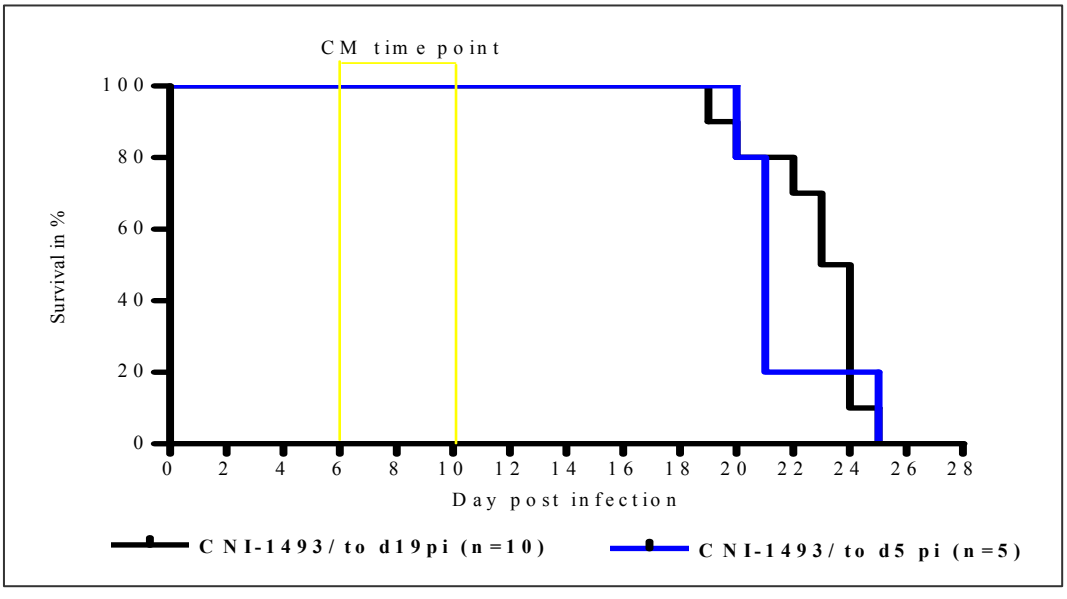

Fig. 3.83: Survival of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg of CNI-1493.
-Treated on: Pre-inf. to d5pi: -3, -1, d1, 3, and d 5pi.
Pre-inf. to d19pi: -3, -1, d1, 3, 5, 7, 9, 11, 13, 15, 17 and d19 pi.

Overall, a decrease in the parasitemia rate can be observed on day 10 to day 19 post-infection (as shown in fig. 3.82), which indicate a limitation in CNI-1493 ability to influence the parasitemia of PbANKA. This limited affectivity is companied with no ability to increase the total survival time points of the infected C57Bl/6 mice.

3.2.1.4 The ability of CNI-1493 to interact the plasma TNF and LT-α

Cytokines expression (TNF and LT-α) were assessed during PbANKA infection in the blood plasma of CNI-1493 treated groups (pre and post-infection), the parasitemia of which presented in figure (3.79). Plasma was daily collected from each mouse and TNF assessed by sandwich ELISA assay, whereas LT-α by Direct ELISA assay. The TNF bike could be easily recognized on d4 p.i. as shown in (fig. 3.84). The plasma TNF level exhibited reduction of 5.2 times on d4 p.i., in the CNI-14 pre-infection treated group and 2.1 times in CNI-1493 post-infection group, if compared to the control group.

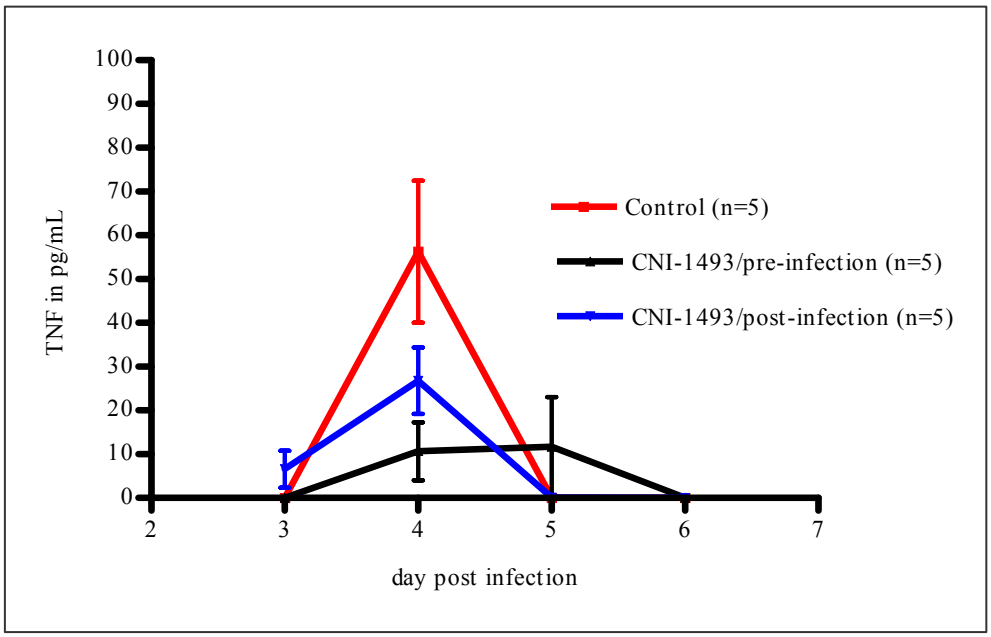

Fig. 3.84: Plasma TNF of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
-Treated on: CNI-1493 Pre-infection group: -3, -1, d1, d3 and d5 pi.
CNI-1493 Post-infection group:d1, d3 and d5 pi.

High levels of LT-α were detected in the plasma of PbANKA infected C57Bl/6 mice. However, the LT-α level exhibited reductions of 1.2 times on d4 p.i. in the CNI-1493 pre-infection treated group, whereas no reductions could be observed in CNI-1493 post infection treated group (fig. 3.85).

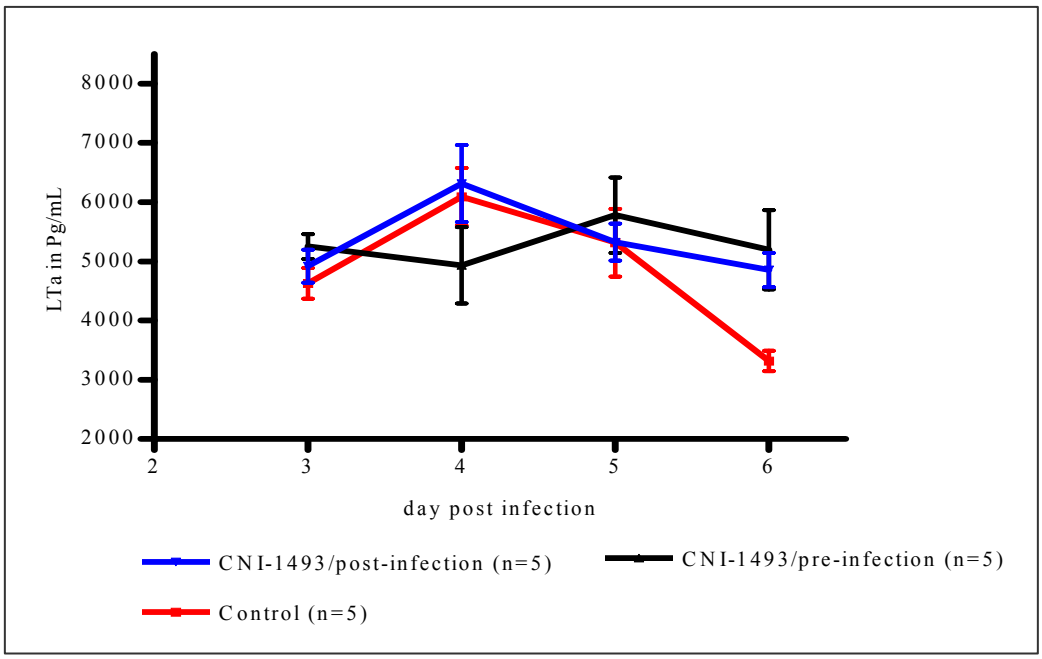

Fig. 3.85: Plasma LTa of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493.
-Treated on: CNI-1493 Pre-infection group: -3, -1, d1, d3 and d5 pi.
CNI-1493 Post-infection group:d1, d3 and d5 pi.

In another meaning on d4 p.i. in comparison to the non-treated Control, 81.1% of the plasma TNF was suppressed by CNI-1493 if given pre-infection, whereas 52.3% if given post-infection. For the plasma LT-α, 19% was suppressed if CNI-1493 given pre-infection; and CNI-1493 failed to suppress the plasma LT-α if given post infection. This could be an important indicator, pointed to the LT-α as an important cytokine in developing CM, especially in our system; where CNI-1493 could not save C57Bl/6 mice from CM if given post-infection.

3.2.1.5 mRNA of TNF and LT- α in the brains of CNI-1493 treated mice

Levels of mRNA for both TNF and LT- α were assessed by RT-PCR, in the brains of C57Bl/6 mice, during the course of PbANKA infection; in presence of CNI-1493 (pre and post-infection) or absent of CNI-1493 (Control). The brains have been collected on d6 and d7 post-PbANKA infection. The increasing in mRNA levels was compared with those levels in uninfected control in addition between each others. mRNA levels are expressed as copy numbers/µL. Whereas, he copy numbers have been calculated by using ß-Actin as a reference gene.

The result showed up-regulation in both cytokines, TNF (Fig. 3.86) and LT- α (Fig. 3.87), in the brains of PbANKA infected C57Bl/6 mice.

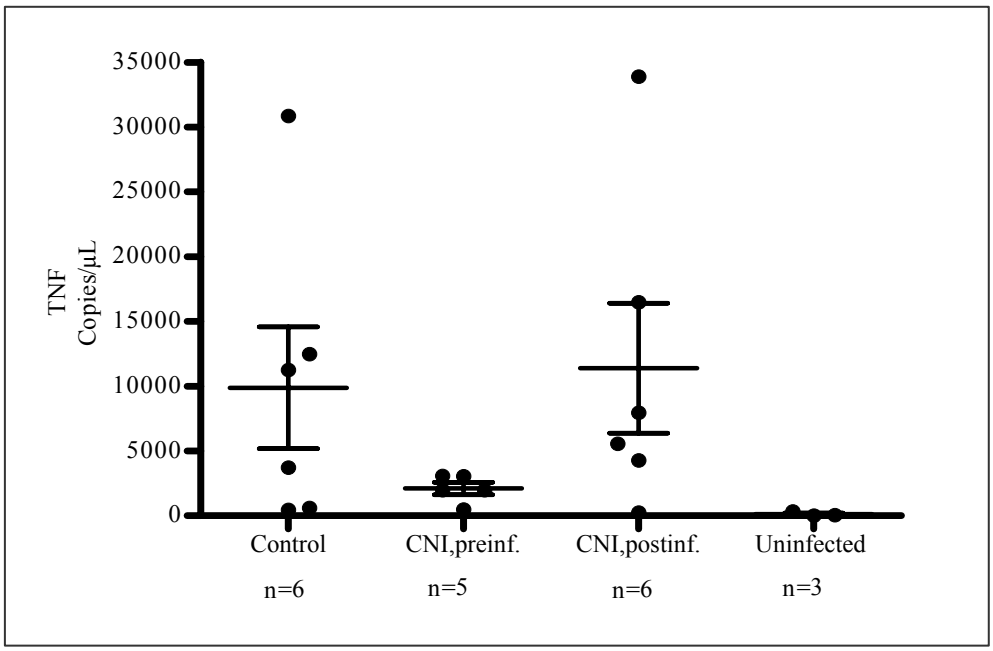

Fig. 3.86: mRNA copies of TNF in the brains of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493. -Treated on: CNI-1493 pre-infection: -5, -3, -1, 1, 3 and d5 p.i.
CNI-1493 post-infection: 1, 3 and d5 p.i.
-Mann Whitney test for statistical differences: Control vs uninfected: P= 0.0238
CNI-1493 preinf. vs uninfected: P= 0.0357
CNI-1493 postinf. vs uninfected: P= 0,0476
-F test to compare variance: Control vs CNI-1493 preinf.: P= 0,0004
CNI-1493 preinf. vs CNI-1493 postinf.: P= 0,0003
Control vs CNI-1493 postinf: NS.
-DMSO= 0.61% in CNI-1493 treated groups and control group.

Compared to the non-treated control, 78.6% of TNF mRNA in the brains was suppressed by CNI-1493, if given pre-infection.

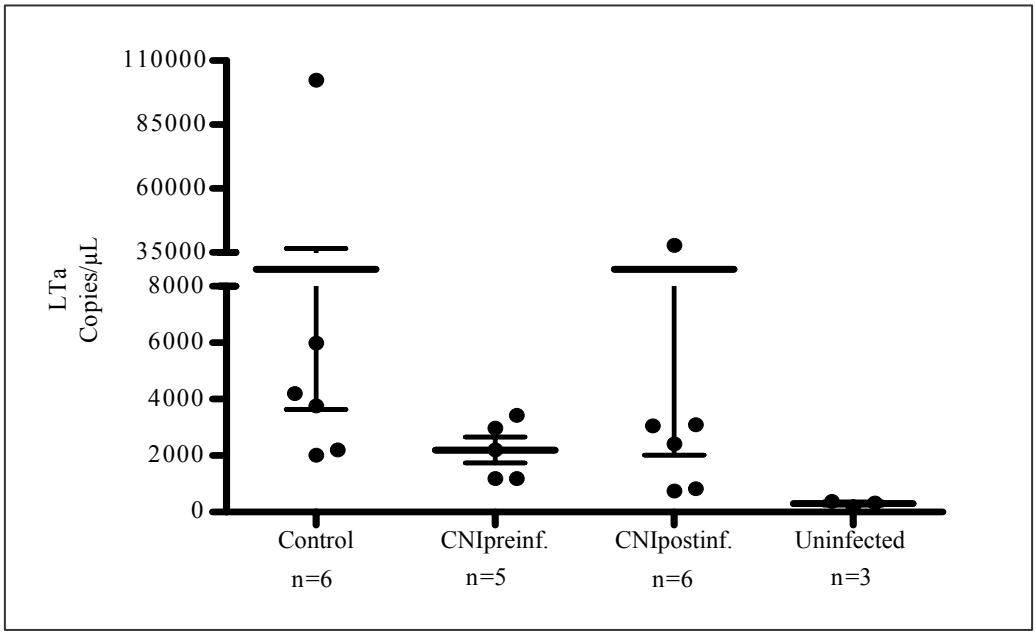

Fig. 3.87: mRNA copies of LT-α in the brains of PbANKA infected C57Bl/6 mice treated i.v. with 4mg/kg CNI-1493. -Treated on: CNI-1493 pre-infection: -5, -3, -1, 1, 3 and d5 p.i.
CNI-1493 post-infection: 1, 3 and d5 p.i.
-Mann Whitney test for statistical differences: infected control vs uninfected P= 0,0238
CNI-1493 preinf. vs uninfected P= 0,0357
CNI-1493 postinf. vs uninfected P= 0,0238
-F test to compare variances: CNI-1493 preinf. vs CNI-1493 postinf. P= 0,0001
CNI-1493 preinf. vs Control P< 0.0001
CNI-1493 postinf. vs Control P= 0,0446
-DMSO= 0.61% in CNI-1493 treated groups and control group.

Compared to the non-treated control, 89.1% of LT- α mRNA in the brains was suppressed by CNI-1493, if given pre-infection and 60.1% if given post-infection.

3.2.1.6 CNI-1493 in Vivo cytotoxicity assay

As shown above, CNI-1493 inhibits development of cerebral malaria if administrated pre-infection. Therefore suppressive effects of CNI-1493 on cytotoxic T cells were investigated. However, 4mg/kg CNI-1493 was given i.v., 5 days pre-Ad-OVA infection (d-5). The generation of OVA specific cytotoxic T cells (CTLs) in CNI-1493 treated or untreated mice was evaluated by flow cytometry. The OVA specific lysis of target cells in naïve C57Bl/6 mice inoculated with AdOVA was 70.9% whereas in the CNI-1493 treated mice (treated pre-AdOVA infection) the specific lysis was 27% of the OVA specific target

cells (Fig. 3.88), which indicated that CNI-1493 suppressed about 43.9% in the function of the specific CTLs generated in the spleen.

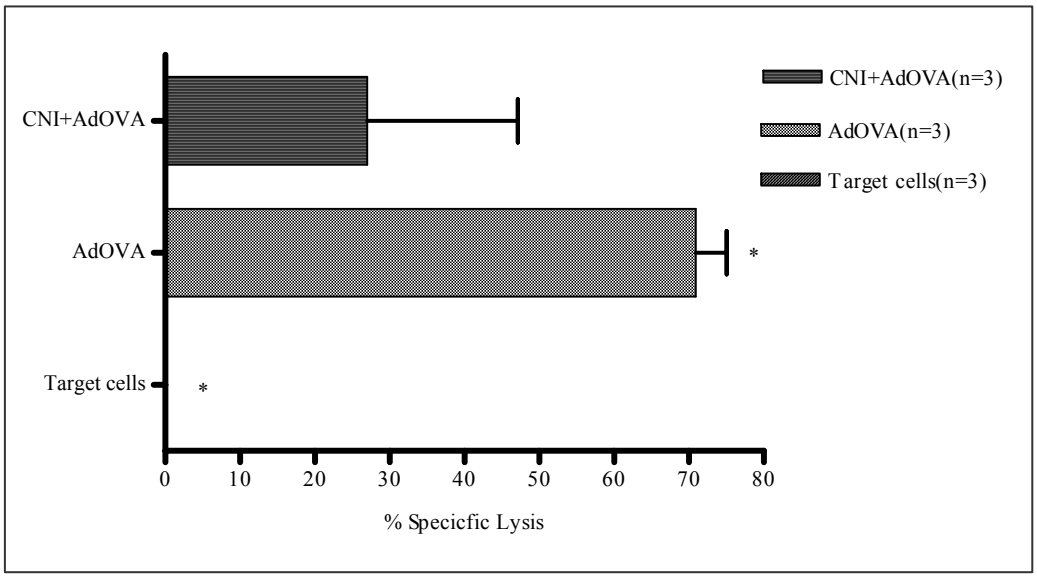

Fig.3.88: In vivo cytotoxicity assay of CNI-1493. Mean lysis of target cells by antigen specific T cells of AdOVA infected mice is 70.9 %. CNI-1493 administration pre-infection non-significantly reduced the cytotoxic activity of antigen specific T cells in the spleen. 4mg/kg of CNI-1493 were given i.v. to C57Bl/6 mice on d -5, -3 and d-1 pre AdOVA administration. Each mouse received $1x10^7$ of target cells, which given iv on d 5 post AdOVA administration. Splenocytes were harvested 4 h after target cells administration. *Target cells vs AdOVA, $P < 0.05$ by ANOVA.

3.2.2 In vivo results of deoxyhypusine hydroxylase (DHH) inhibitors

A major drawback has faced the in vivo assay of DHH inhibitors, due to its poor solubility in both water and DMSO it was difficult to be administered i.v. into the mice. This drawback became clearer when they were being mixed in 1xPBS, after primary dissolved in DMSO. To overcome this problem, several dissolvents have been used (see appendix No. 8). Unfortunately, none of the tested dissolvents was helpful. Therefore, DHH inhibitors were primarily dissolved in DMSO and administered i.p. in 1xPBS, (except one time i.v. (see fig. 3.95)), by using a big size needle (0.90x40mm). In addition, up to 20% DMSO in 1xPBS was proved to be inactive in vivo on the PbANKA (Data not shown). However, to confirm in vivo the in vitro results obtained from DHH inhibitors; several experiments were performed to score a good in vivo assay of the in vitro selected inhibitors.

3.2.2.1 In vivo results of UL3B1P

Concentrations of 20 and 40 mg/kg were injected i.p. in BALB/C mice from d6 to d9 p.i., to assay the ability of UL3B1P in reducing the parasitemia, by using the four days test, as a standard in vivo assay to estimate the antimalarial efficacy (Peters et al., 1975; Kalra et al., 2006). However, the outcome of injecting these doses of UL3B1P, within four days duration, showed no in vivo efficacy of this inhibitor (Data not shown). After which a concentration of 80mg/kg of UL3B1P was injected i.p. in BALB/C mice, by keeping on the same four days in vivo assay (Fig. 3.89), considered the mice survival time in (fig. 3.90). UL3B1P was first dissolved in DMSO and then mixed with 1xPBS to reach 80mg/kg in 200µl. In a final DMSO concentration of 10%, the same DMSO amount was injected into the control group. In addition, chloroquine (CQ) was administered i.p. at 25mg/kg in the same mice batch as a positive treated control group.

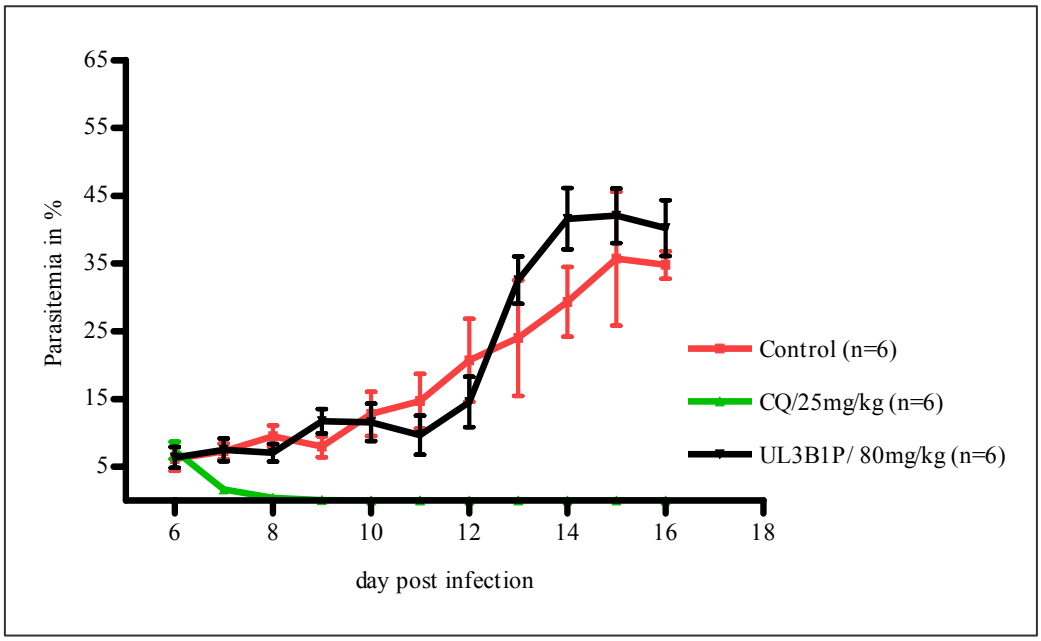

Fig.3.89: Parasitemia in PbANKA infected BALB/C mice treated i.p. with UL3B1P 4 days test: treated from d6 to d9 p.i.

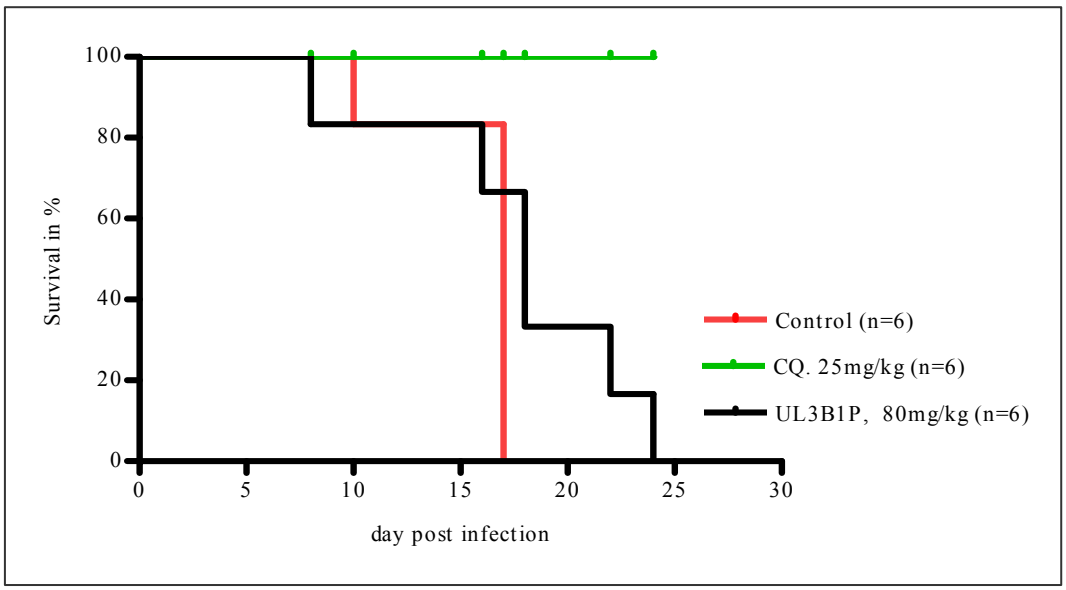

Fig.3.90: Survival of PbANKA infected BALB/C mice treated 4 days with UL3B1P

Still no in vivo activity could be observed of UL3B1P when 80mg/kg was used. And since no previous in vivo data of this class of inhibitors was available and because of the incomplete solubility, the primary results forced us to increase the UL3B1P dose to 120mg/kg, from d5 to d8 p.i.; but also no antimalarial activity could be observed (Data not shown). This fixed negative result with series concentrations of UL3B1P, brought the suggestions that, the UL3B1P uptake might be very low and to overcome the less uptakes, which were mistrust because of the low solubility, UL3B1P was injected i.p., at 300 mg/kg a little early, from d4 to d7 p.i., to give this substance more chance to show its efficacy. As shown in figure (3.91) UL3B1P exhibited a limited ability to reduce the parasitemia of PbANKA on d8 p.i. where by this time 20% of the UL3B1P treated mice were dead as shown in (3.92).

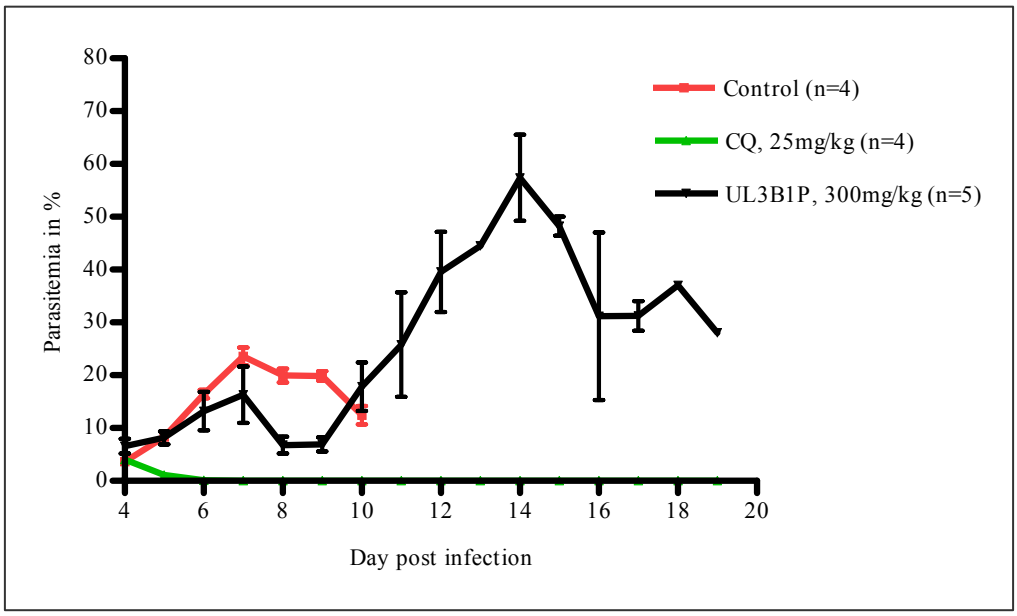

Fig. 3.91: Parasitemia in PbANKA infected BALB/c mice treated i.p. with UL3B1P.
- four days test: treated from d4 to d7 p.i., DMSO=10% in both tested and control group.
-Mann Whitney test for parasitemia differences on d8 p.i.: Control vs UL3B1P/300mg/kg: P= 0,0286.

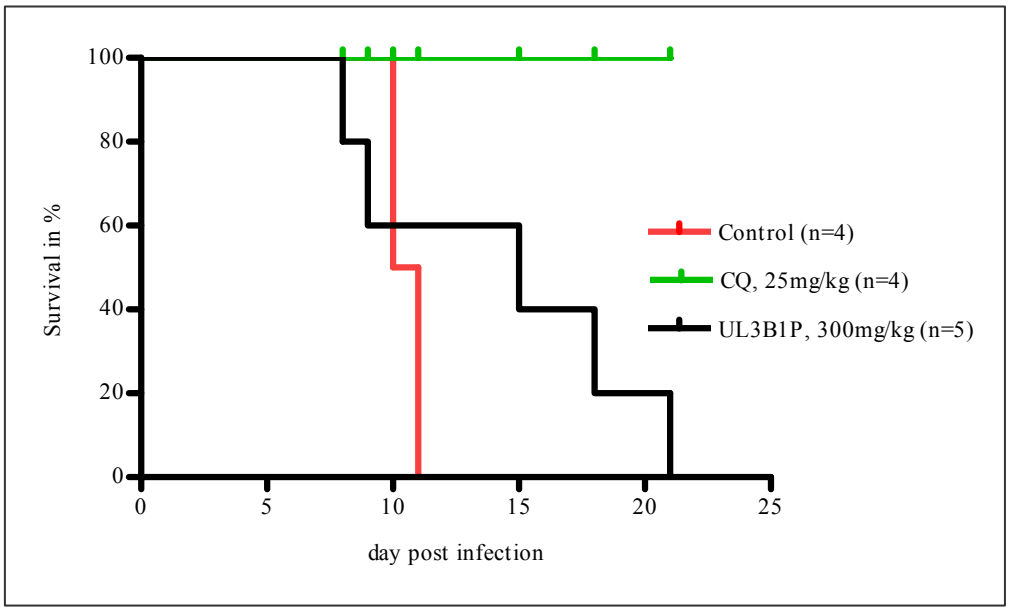

Fig. 3.92: Survival of PbANKA infected BALB/c mice treated i.p. with UL3B1P.
-Logrank Test for survival: UL3B1P vs Control:NS

3.2.2.2 The effects of UL3B1P on CM

In this experiment C57Bl/6 WT mice were used to assay the ability of UL3B1P in interacting with CM, and saving the infected mice of this syndrome. It has been reported that inhibition of eIF5A might lead to a reduced synthesis of TNF in mice (Taylor et al., 2004). Therefore, UL3B1P gave enough time to show any activity in this direction. In addition, it was given in a long term to assay its outcome on parasitemia. However, UL3B1P was prepared at 300mg/kg and i.p. injected two days before the infection (-2d) up to d7 p.i. (fig. 3.93). The result obtained showed no efficacy of UL3B1P, neither on parasitemia, CM syndrome nor on the total survival time of the treated group (fig. 3.94).

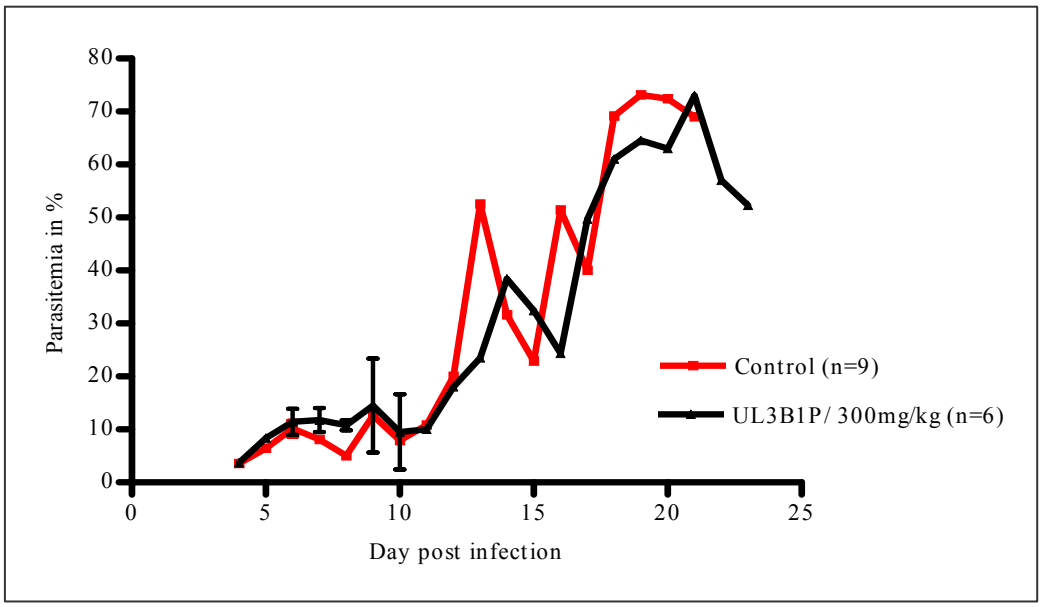

Fig. 3.93: Parasitemia in PbANKA infected C57Bl/6 mice treated i.p. with UL3B1P
Treated 9 times: -2d, -1, d1 to d7 post-infection.

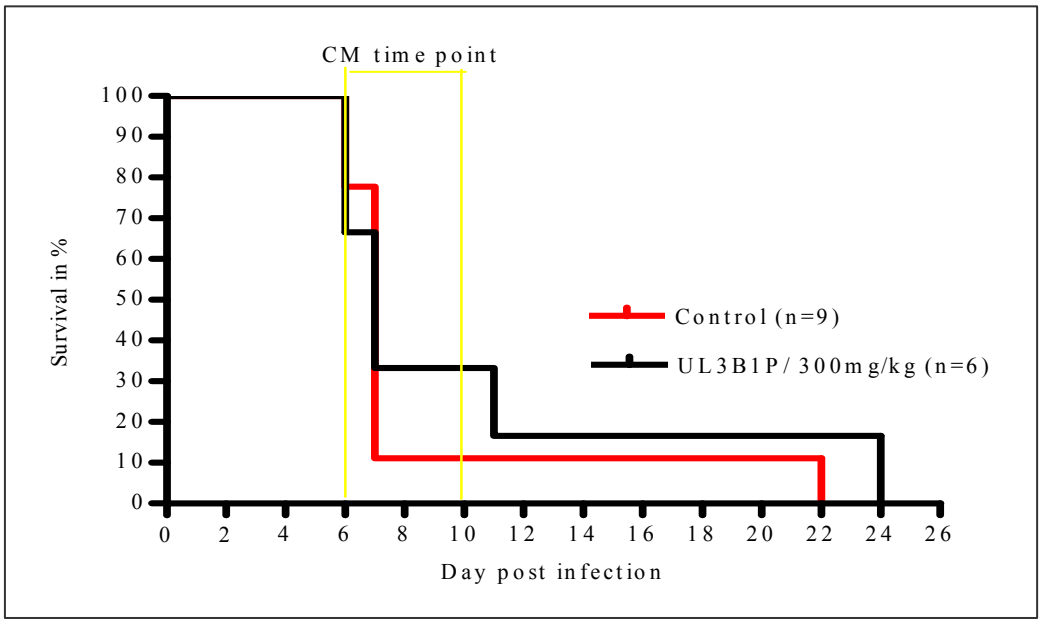

Fig. 3.94: Survival of PbANKA infected C57Bl/6 mice treated i.p. with UL3B1P
Treated 9 times: -2, -1 day and from d1 to d7 post-infection
Logrank Test for survival:NS

3.2.2.3 The in vivo efficacy of UL3B1P when i.v. injected

To overcome the solubility problem, a mixture solution of DMSO, glycerol and 1xPBS was used to allow the i.v. injection of UL3B1P. Therefore, 80 mg/kg of UL3B1P was dissolved in DMSO; on the other hand glycerol 1xPBS solution was prepared and then mixed to gather with the DMSO containing 80 mg/kg. The final concentrations are 10% DMSO and 9% glycerol in 1xPBS. However, the anti-malarial activity and the toxicity of the above solution (10% DMSO and 9% glycerol in 1xPBS) were proved in mice before using it with UL3B1P, and found to be safe with no anti-malarial activity or even observed side effects neither on the mice nor on CM pathogenesis (Data not show).

No overt signs of toxicity or abnormal behaviour were observed when 80mg/kg of UL3B1P injected i.v. into mice, but UL3B1P could not alter the parasitemia progression (Fig. 3.95) or the survival time of the infected mice (Fig. 3.96).

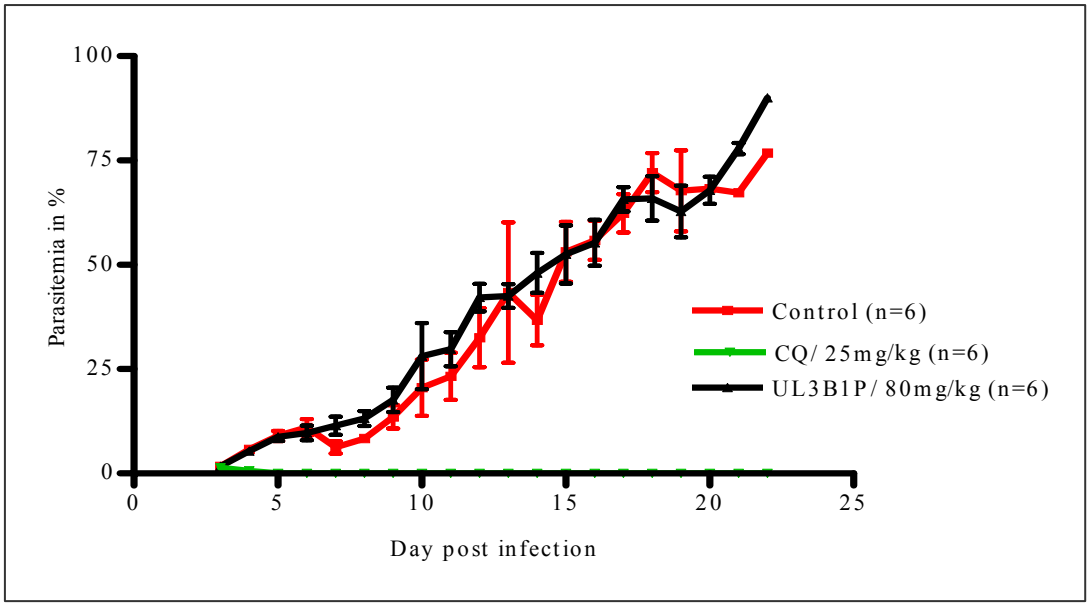

Fig.3.95: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with UL3B1P.
-Treated from d3 to d9 p.i.

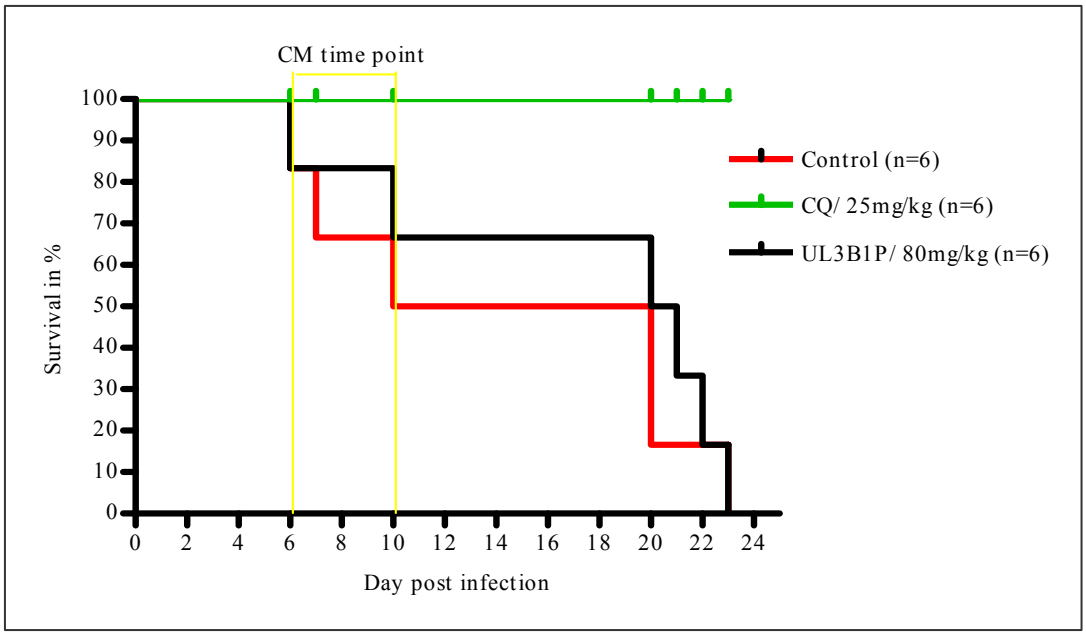

Fig.3.96: Survival of PbANKA infected C57Bl/6 mice treated i.v. with UL3B1P
-Treated from d3 to d9 p.i.
- Logrank Test for survival: UL3B1P/80mg/gk vs Control: NS.

3.2.2.4 In vivo results of three DHH inhibitors

JK-8-2, JK12-ML2, and JK7E, which gave *in vitro* IC_{50}s in the same range; they under went *in vivo* assay at the same time by using BALB/c mice for their anti-malarial activity. Each substance was prepared in a dose of 300 mg/kg in DMSO and 1xPBS (200µl/mouse)

to be injected i.p., in a long term test (10 days). The efficacy of the three substances on parasitemia and on survival period can be observed in figures (3.97 and 3.98).

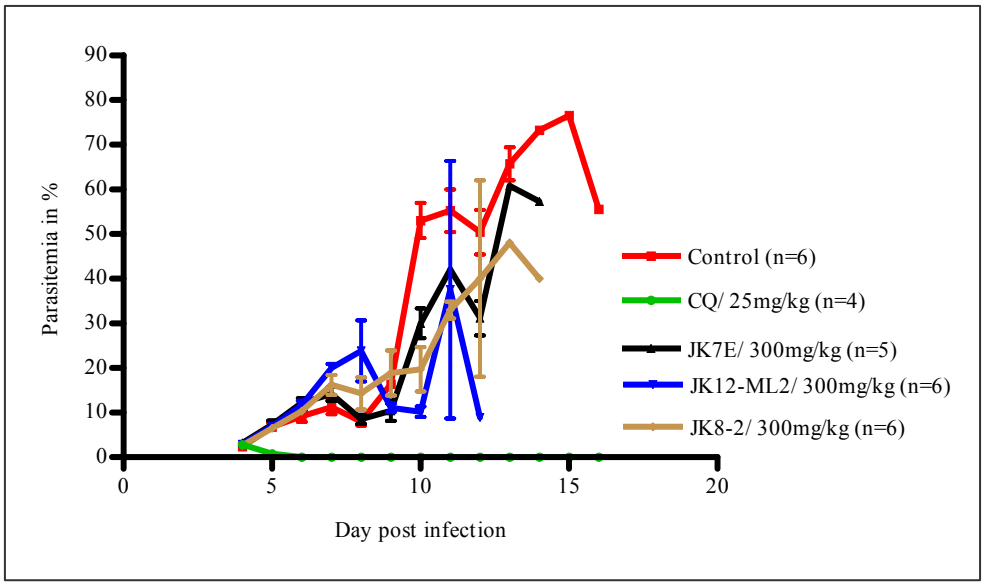

Fig.3.97: Parasitemia in PbANKA infected BALB/c mice treated i.p. with DHH inhibitors.
- Treated 10 times: from d4 to d13 p.i.
-DMSO% in the tested groups and control= 13.5%.

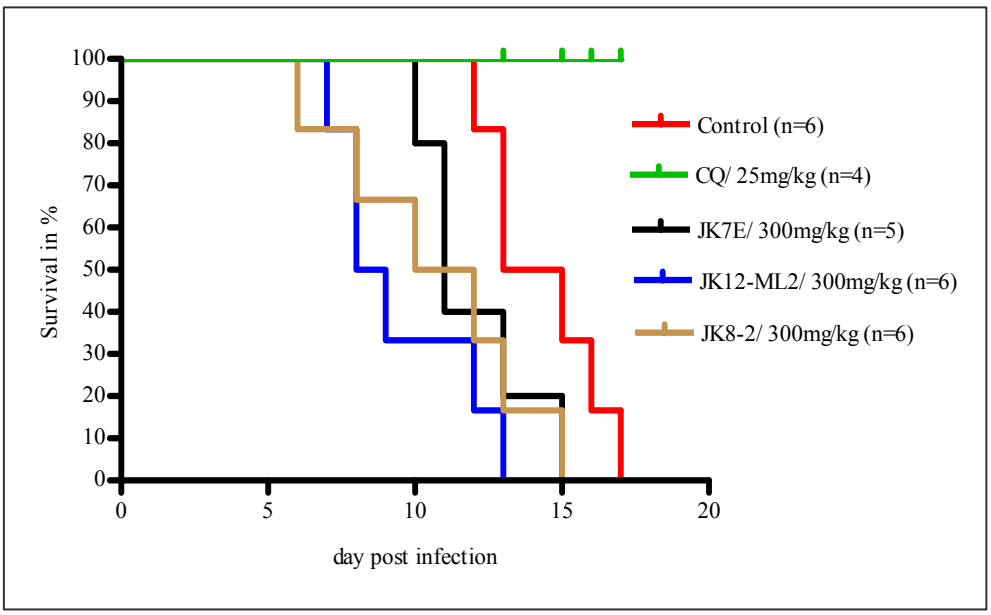

Fig.3.98: Survival of PbANKA infected BALB/c mice treated i.p. with DHH inhibitors.
-Logrank Test for survival differences: Control vs Jk12-ML2, P= 0,0076
Control vs Jk8-2, P= 0,0497

As BALB/c mice known to be CM-resistant strain (Lou et al., 2001), it was unexpected that the treated BALB/c mice died within the CM time point (6 to 10 days p.i.).

3.2.2.4 In vivo results of JK-8-2 orally administered

Also to overcome the solubility problem, the administration route of this class was again changed and this time to be orally, as the preferred route for the clinical deployment of antimalarial is oral (Peters et al., 2002). However, 200 mg/kg of JK-8-2 was prepared in 1xPBS and given orally to C57Bl/6 mice, to find out any efficacy on parasitemia, shown in (fig. 3.99) and CM, shown in (fig. 3.100), which illustrate that, the outcome of giving this substance orally could not influence the PbANKA infection.

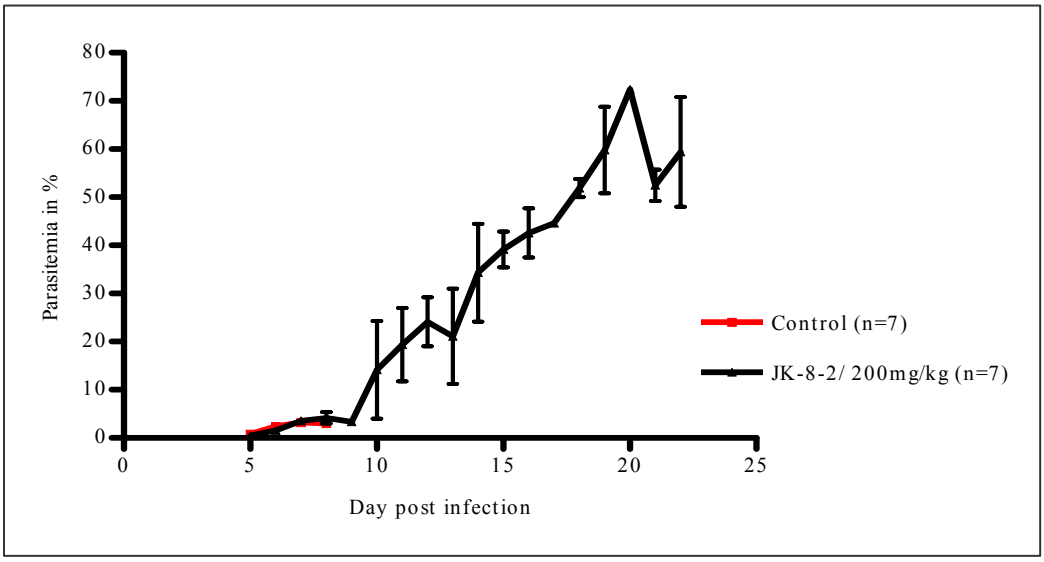

Fig.3.99: Parasitemia in PbANKA infected C57Bl/6 mice treated orally with JK-8-2.
-Treated from d1 to d6 pi.

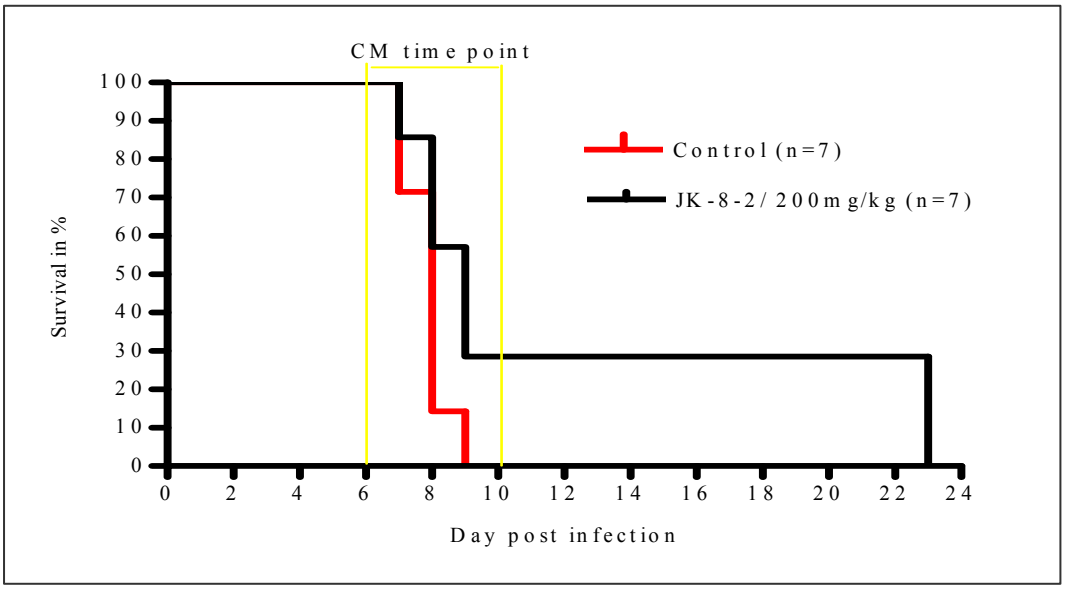

Fig.3.100: Survival of PbANKA infected C57Bl/6 mice treated orally with JK-8-2.
-Treated from d1 to d6 pi.

3.2.3 In vivo results of polyamintransport inhibitor

The polyamintransport inhibitor, NK-1-63, gave an interesting in vitro IC_{50} (0.63 µM), with its advantage as a water soluble substance, and the in vivo result of this inhibitor was expected to be great. But unfortunately, this substance found to be toxic when administered in to the mice, whether i.v. or i.p., at a concentration of as low as 10 mg/kg (Data of the toxicity assay are not shown). Therefore, NK-1-63 was prepared, directly in 1xPBS, at 5mg/kg (which was found to be safe for the mice), and given i.v. for 5 days as shown in (fig. 3.101).

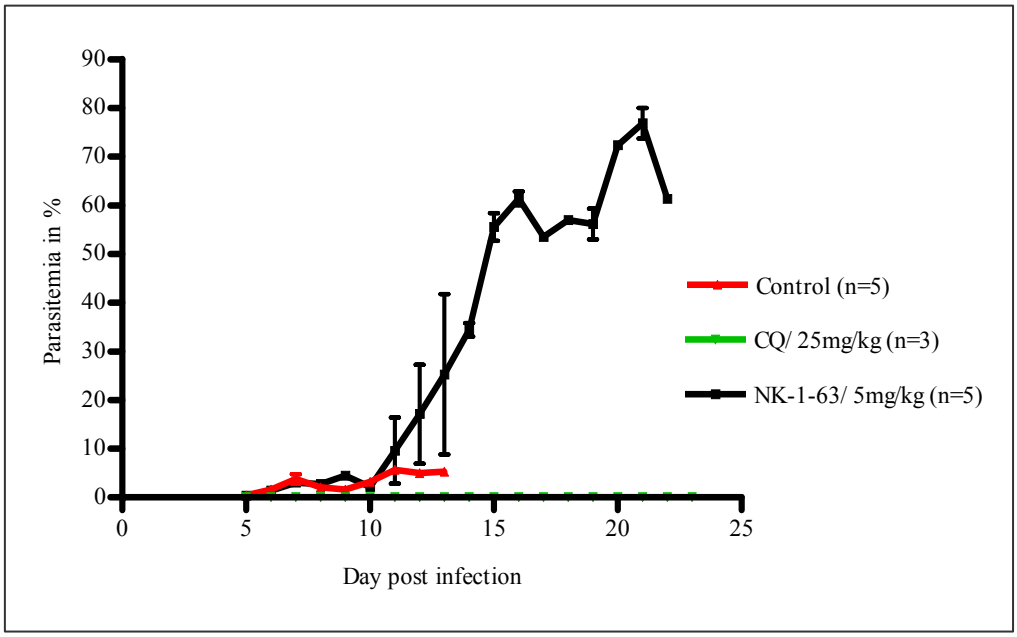

Fig.3.101: Parasitemia in PbANKA infected C57Bl/6 mice treated iv with NK-1-63.
Treated from d2 to d6 p.i..

The results obtained indicated no in vivo efficacy of 5mg/kg of NK-1-63, not even modulating the CM time point of the treated group (fig. 3.102).

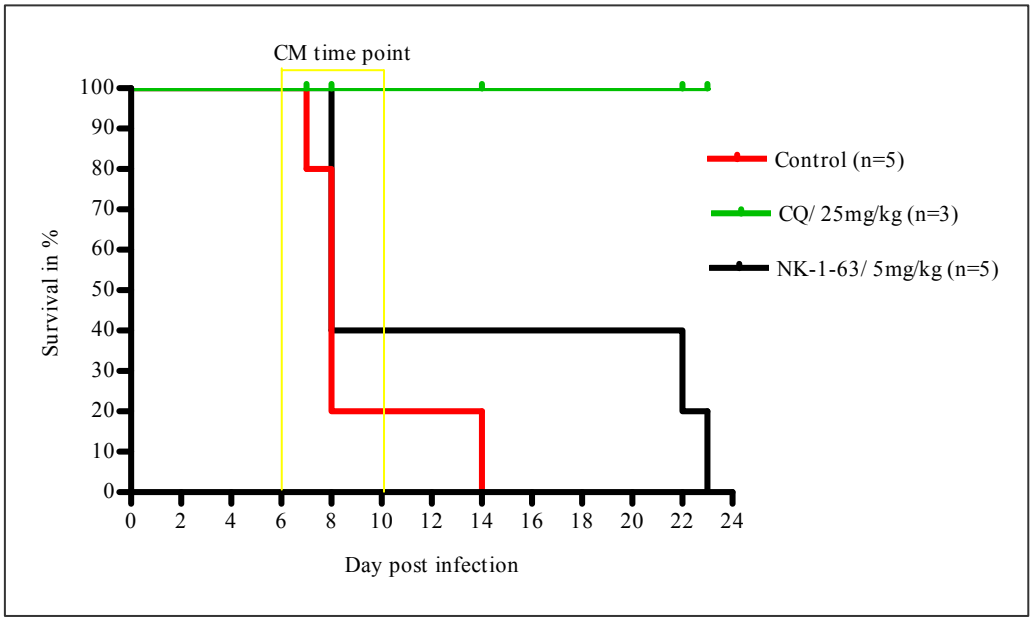

Fig.3.102: Survival PbANKA infected C57Bl/6 mice treated i.v. with NK-1-63.
-Treated from d2 to d6 p.i.
-Logrank Test for survival: NK-1-63/5mg/kg vs Control P> 0.05

3.2.4 In vivo results of phenazines derivatives

Like the DHH inhibitors, the in vivo assay of phenazines derivatives also faced solubility problems.

3.2.4.1 In vivo results of PR-1 given intra-peritoneal (i.p.)

200mg/kg of PR-1 was prepared in DMSO (40%) and 1xPBS to be given i.p. to PbANKA infected BALB/c mice from d3 to d9 p.i.. Even in this high DMSO percentage PR-1 was incompletely soluble. However, the results obtained from administrating 200mg/kg of PR-1 can be observed in figures (3.103 and 3.104).

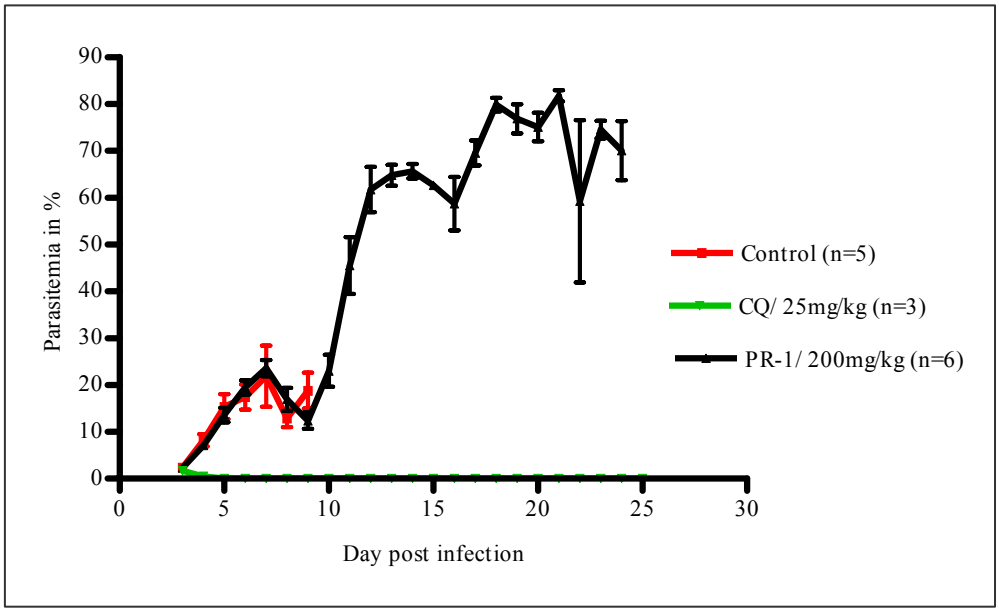

Fig.3.103: Parasitemia in PbANKA infected BALB/c mice treated i.p. with PR-1.
-Treated from d3 to d9 p.i.

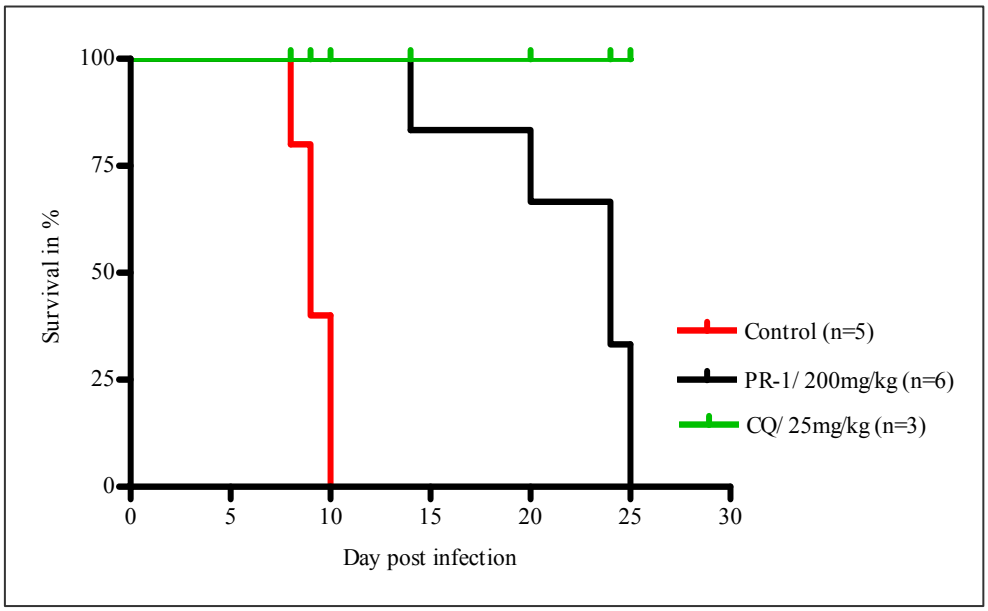

Fig.3.104: Survival of PbANKA infected BALB/c mice treated i.p. with PR-1
-Logrank Test for survival: PR-1/200mg/kg vs Control, P= 0,0011.

3.2.4.2 In vivo results of PR-1 and DP-2 given intra-vinous (i.v.)

PR-1 and DP-2 were prepared at 80mg/kg of each, for i.v. administration by using the DMSO, glycerol, and 1xPBS solution. The parasitemia progression can be observed in figure (3.105).

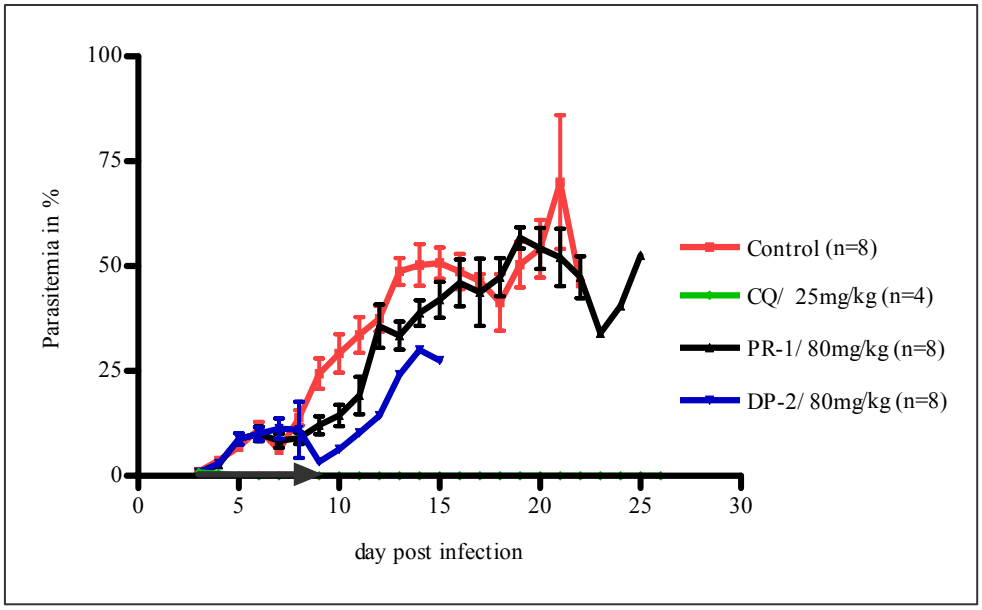

Fig.3.105: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with PR-1 &DP-2
-Treated from d3 to d9 p.i.
-PR-1&DP-2 dissolved in DMSO and mixed with glycerol PBS solution (10% DMSO and 9% glycerol).
-Mann Whitney test for differences in parasitemia on d8, 9, and d10 p.i.:
Control vs PR-1/80mg/kg: P< 0.0500.

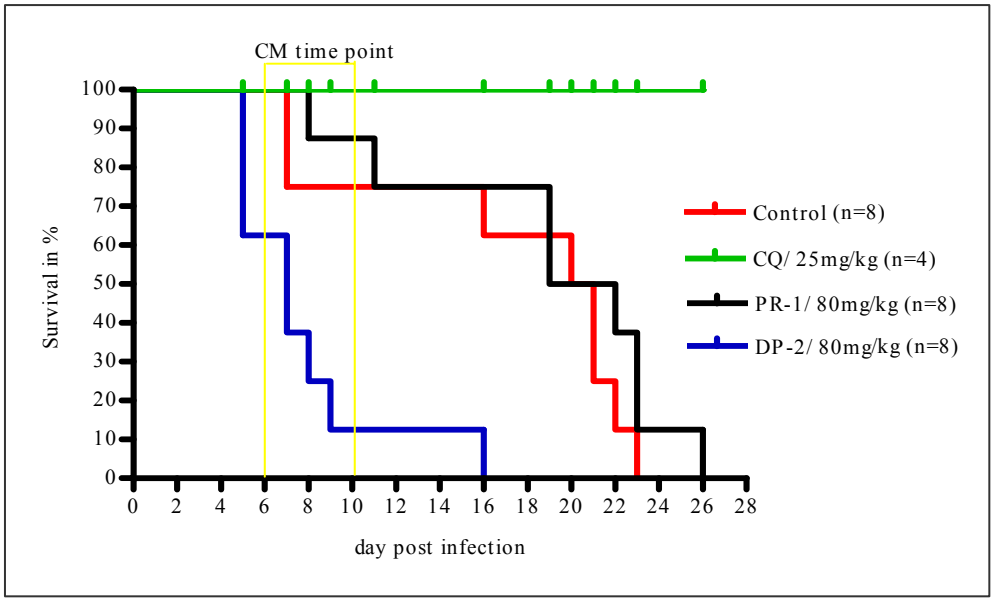

Fig.3.106: Survival of PbANKA infected C57Bl/6 mice treated i.v. with PR-1&DP-2.
Logrank Test for survival: Control vs DP-2, P = 0,0021 and PR-1 vs Control: NS.

As shown in the survival curve (fig .3.106) the death of DP-2 treated group started early, even earlier than the CM time point. This mice group were suffering from breathing difficulties accompanied with inactivity in the movements; some mice were suffering from symptoms which were similar to CM symptoms but with no paralysis.

3.2.4.3 In vivo results of DP-2 given orally

To overcome the solubility problem of the phenazines derivatives, DP-2 was prepared in 1xPBS at 200mg/kg to be given orally. The administration was done by using special oesophagus tube, on a time period from d1 to d6 p.i. (fig 3.107)

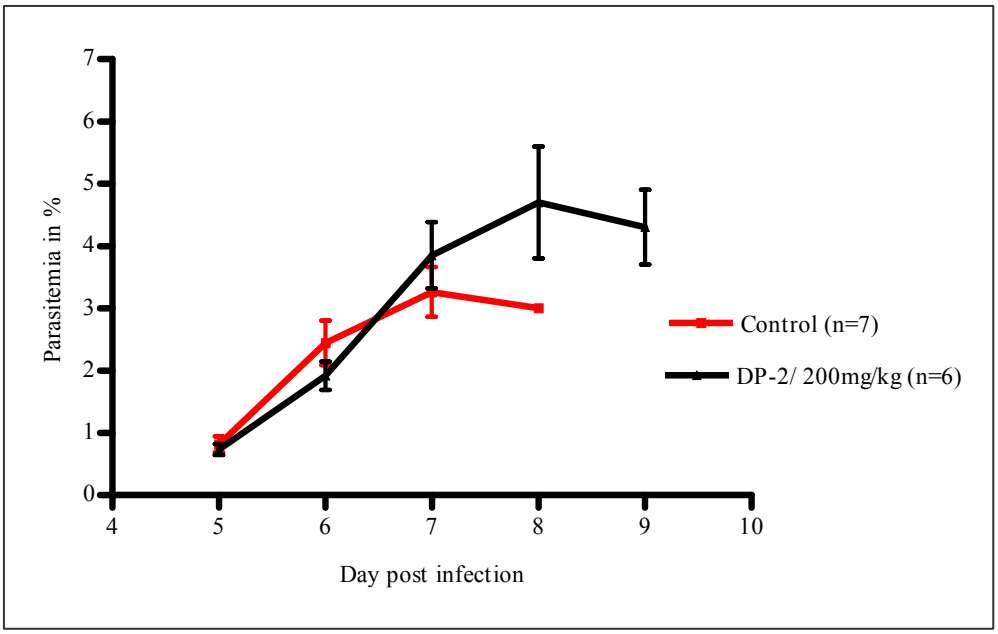

Fig.3.107: Parasitemia in PbANKA infected C57Bl/6 mice treated orally with DP-2.
-Treated from d1 to d6 p.i.

The results obtained showed no affectivity of the orally DP-2 in reducing the PbANKA parasitemia. Also, DP-2 could not modulate the CM time point of the PbANKA infected C57Bl/6 mice as shown in (fig. 3.108).

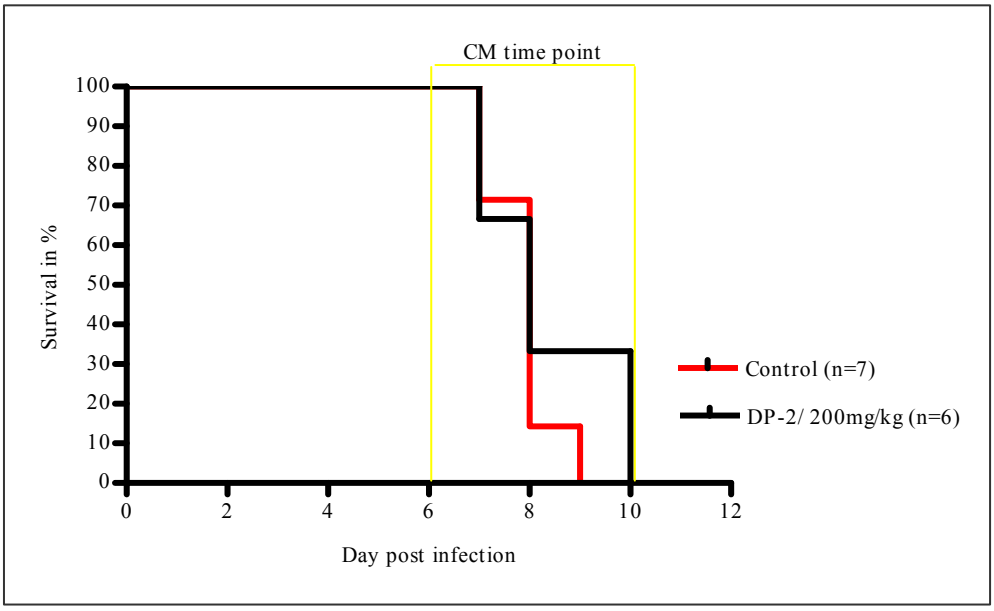

Fig.3.108: Survival of PbANKA infected C57Bl/6 mice treated orally with DP-2.
-Treated from d1 to d6 pi.

3.2.4.4 In vivo results of PR-1-cl and PR-1-py

PR-1-cl and PR-1-py were prepared in DMSO, glycerol, and 1xPBS solution to be given i.v. in a dose of 100mg/kg; but unfortunately, it was difficult to manage the i.v. injections, due to the same solubility problem. However, both phenazines derivatives were i.p. administered and showed inability to reduce the parasitemia (fig. 3.109).

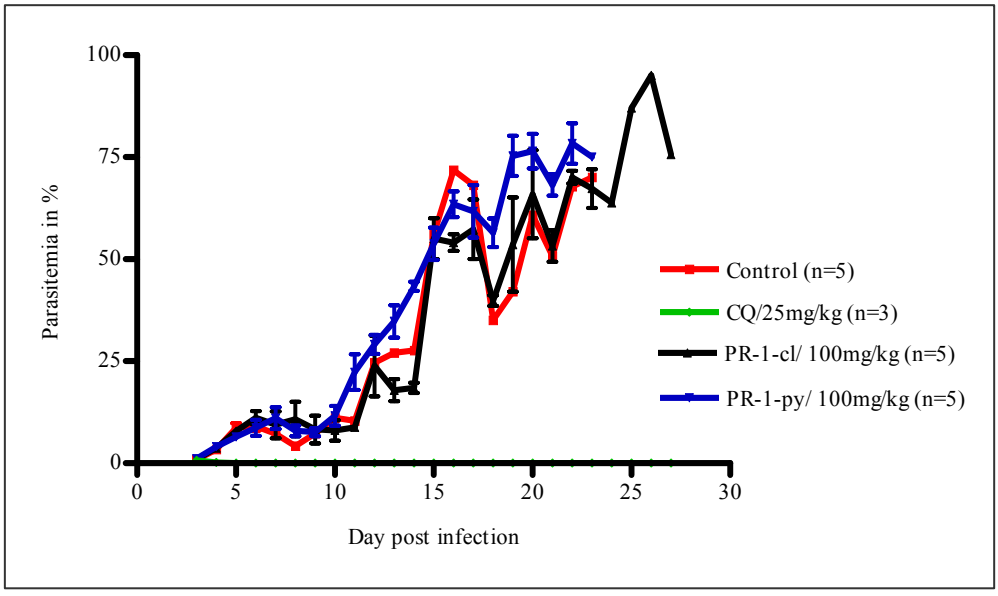

Fig.3.109: parasitemia in PbANKA infected C57Bl/6 mice treated i.p. with PR-1-Cl & PR-1-Py.
-Treated from d3 to d9 p.i.
-PR-1-cl&PR-1py dissolved in DMSO and mixed with glycerol PBS solution: 10% DMSO and 9% glycerol.

But PR-1-py showed an ability to save the PbANKA infected C57Bl/6 mice of CM, as shown in figure (3.110).

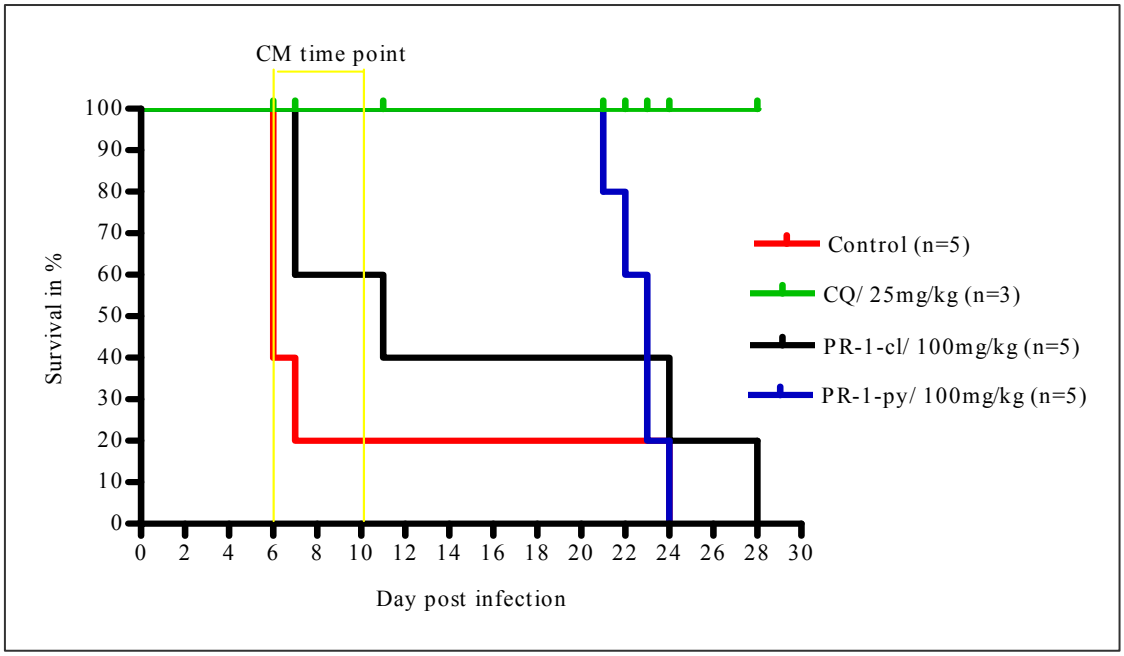

Fig.3.110: Survival of PbANKA infected C57Bl/6 mice traeted i.p. with PR-1-Cl & PR-1- py.
- Logrank test for survival during CM time point: PR-1-py vs Control, P= 0,0144
-Logrank test for total survival: PR-1-cl vs Control: NS and PR-1-py vs Control: NS

3.2.4.5 In vivo results of PR-Br-2

The ability of PR-1-py to save PbANKA infected C57Bl/6 mice of CM has encouraged us to find out the ability of phenazines derivatives to reduce the plasma TNF and LT-α, as important cytokines in developing CM. Therefore, this experiment planned to evaluate the plasma levels of TNF and LT-α in the PR-Br-2 treated mice, additionally to its affectivity on parasitemia. PR-Br-2 shows no activity against the parasitemia of PbANKA (fig. 3.111). As the mice groups in this experiment were bled for plasma collections, the survival time points were not considered in the results of this phenazine.

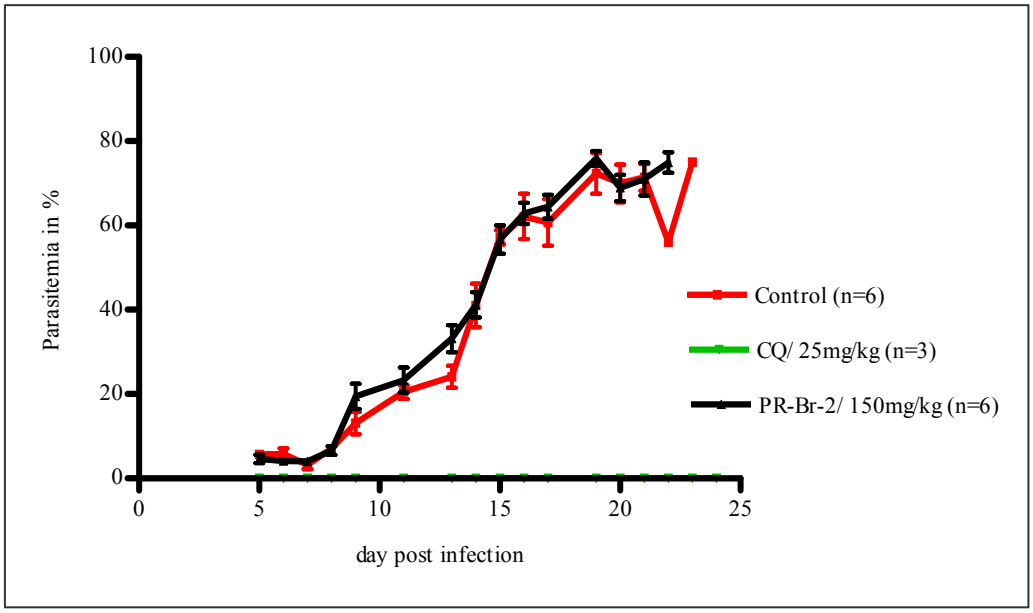

Fig.3.111: Parasitemia in PbANKA infected C57Bl/6 mice treated i.p. with PR-Br-2.
-Treated 6 times, from d1 to d6 pi)
-DMSO= 2% in PR-Br-2 and control group.

Taking the control group as 100%, on d4 and d5 p.i. 100% of plasma TNF was reduced by injecting PR-Br-2 (fig. 3.112). This reduction is similar to that obtained from CQ treated group.

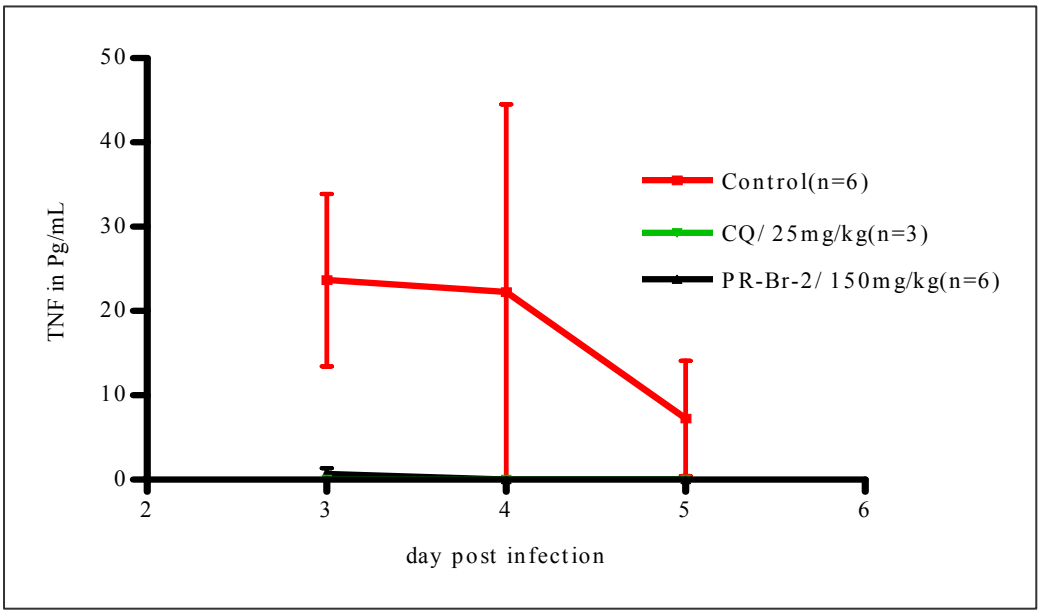

Fig.3.112: PlasmaTNF of PbANKA infected C57Bl/6 mice treated i.p. with PR-Br-2.
-Treated 6 times from d1 to d6 p.i.

In the same line PR-Br-2 shows an ability to reduce the plasma LT-α (fig. 3.113). PR-Br-2 on d4 and d5 p.i. reduced 19.8% and 25.4%, respectively, of the plasma LT-α. Whereas in CQ treated group 7.1% and 47.5% was reduced on d4 and d5 p.i. Indicating that even in the CQ treated group there is still LT-α presented in plasma, might be as a part of the host immuneresponse against PbANKA infection.

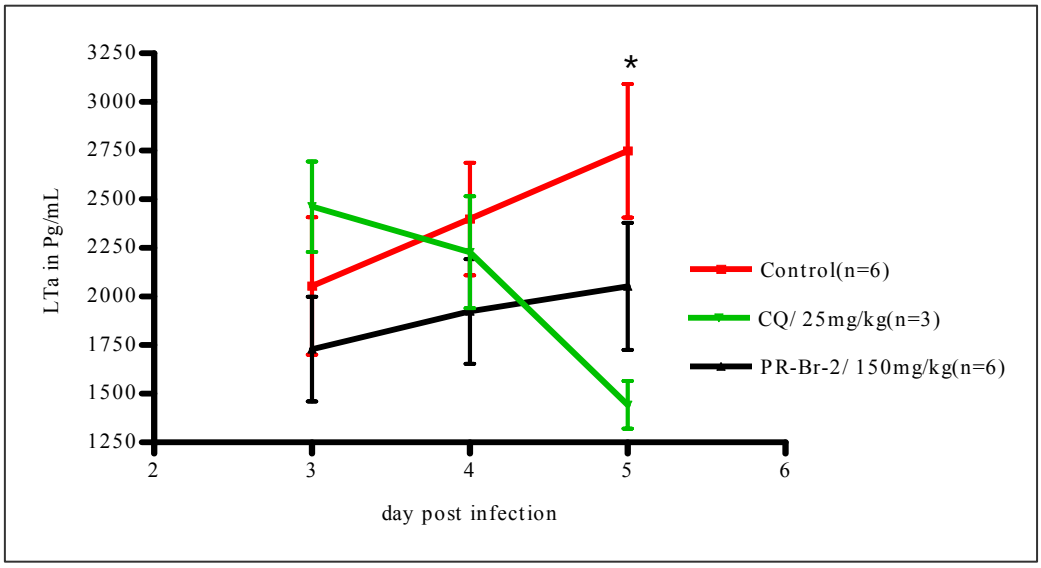

Fig.3.113: Plasma LTa of P.bA infected C57Bl/6 mice treated i.p. with PR-Br-2.
-Treated 6 times from d1 to d6 p.i.
-Mann Whitney test on d5 p.i.: Control vs CQ, P= 0.0370

3.2.5 In vivo results of nuclear export signal (NES) inhibitor

To confirm in vivo, the in vitro significantly TNF reductions in the LepB treated macrophages; LepB was prepared at 20nM in 200µl of 1xPBS and injected i.v. into two PbANKA infected C57Bl/6 mice groups: G1/which was named LepB, pre-inf; treated 6 hrs pre-infection and then from d1 to d7 p.i. (n=7) and G2/ which was named LepB, post-inf; treated from d3 to d7 p.i. (n=7); in addition of a control group (n=8). LepB in 20nM has not influenced the parasitemia levels in both groups (Data not shown). To evaluate TNF and LT-α in plasma, plasma was collected via the mouse tail. Each group of the experimental mice was sub-divided into two sub-groups for plasma collections. From the first sub-group, plasma was collected on the odd days post-infection whereas the second

sub-group on the even days post-infection. However, plasma TNF levels of both sub-groups can be observed in figures (3.115 and 3.116).

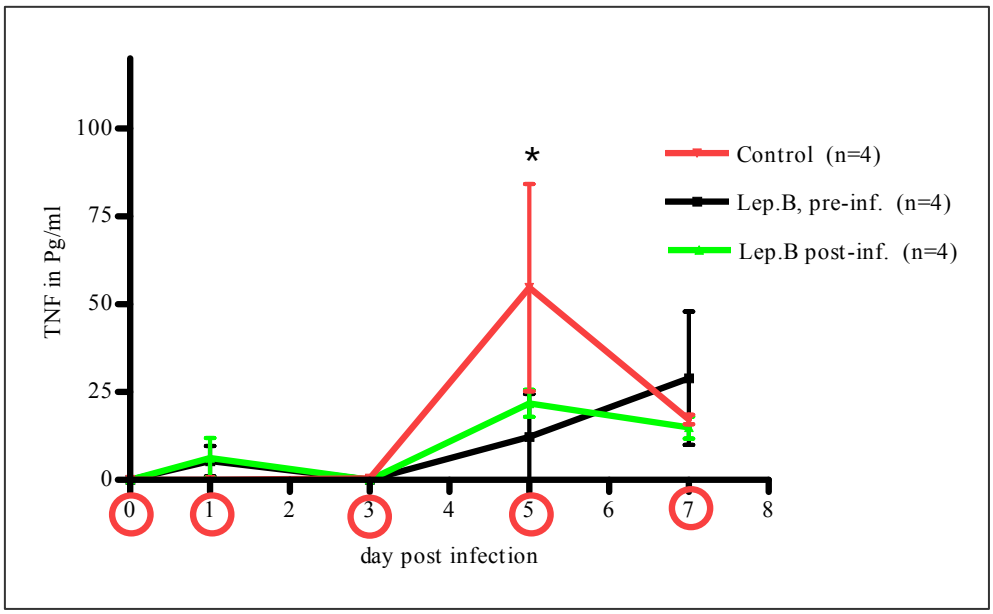

Fig.3.115: Plasma TNF of infected C57Bl/6 mice treated i.v. with Lep.B 20nM.
-Plasma collected on the odd days post-infection.
*F test to compare variances: Control vs LepB post-inf., P= 0.0073

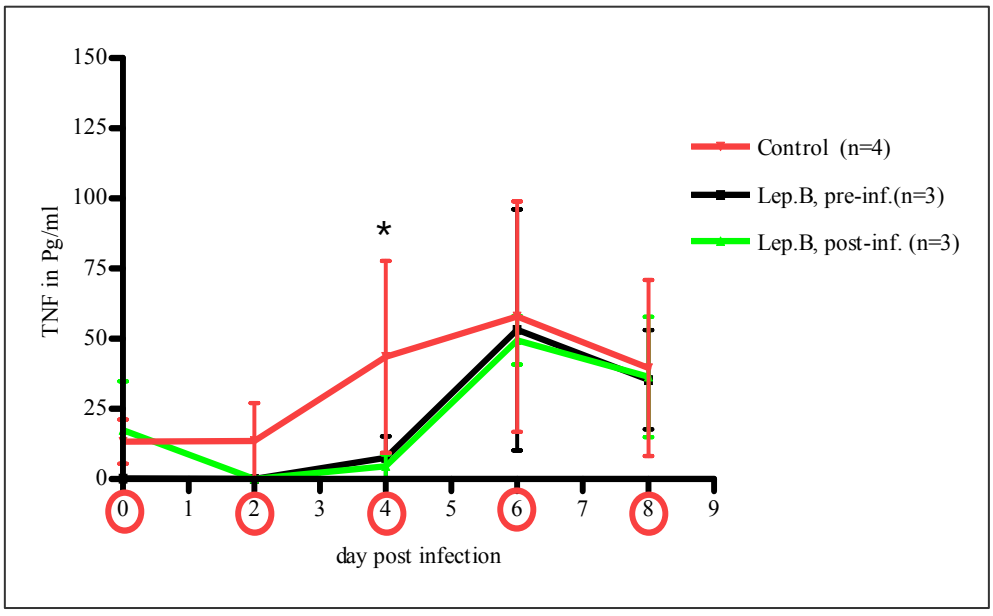

Fig.3.116: Plasma TNF of PbANKA infected C57Bl/6 mice treated i.v. with 20nM LepB.
-Plasma collected on the even days post-infection.
*F test to compare variances: Control vs LepB post-inf., P= 0.0232

LepB if given 6hrs pre-infection shows an ability to reduce 82.6% of the plasma TNF on d4 p.i., and 89.6% if given post-infection, whereas on d5 p.i. shows 77.7% and 60.2% pre-

and post-infection, respectively. The LepB ability to reduce the plasma LT-α seems to be less than that of TNF, as on d4 p.i. its ability to reduce the plasma LT-α was 44.1% if given 6hrs pre-infection and 31.7% if given post-infection (fig. 3.118), whereas on d5 p.i. the reductions were 9.5% in pre-infection treated group and no reductions observed in the post-infection LepB treated group (fig. 3.117).

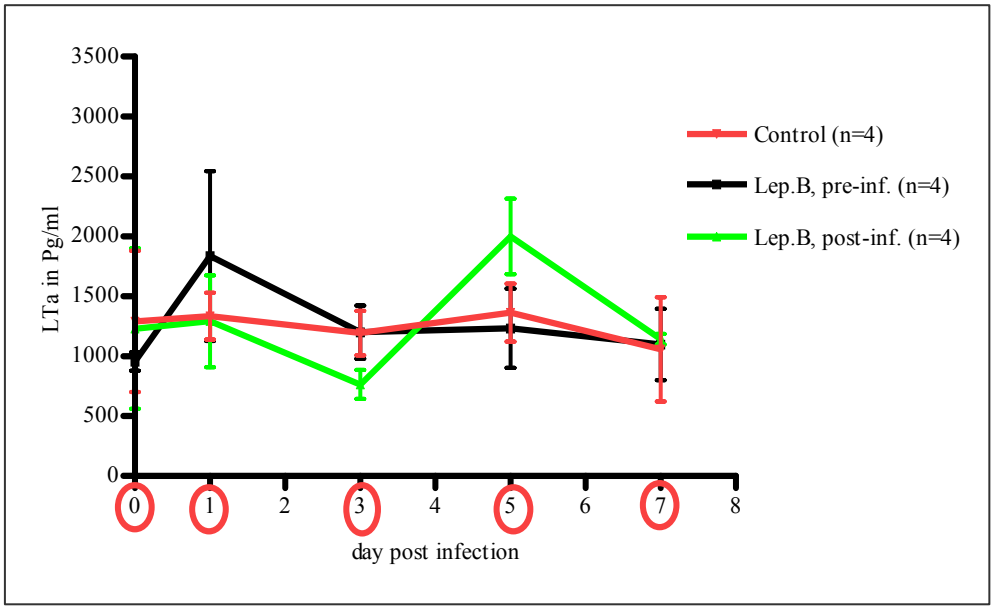

Fig.3.117: Plasma LT- α of infected C57Bl/6 mice treated i.v. with LepB 20nM.
-Plasma collected on the odd days post-infection.

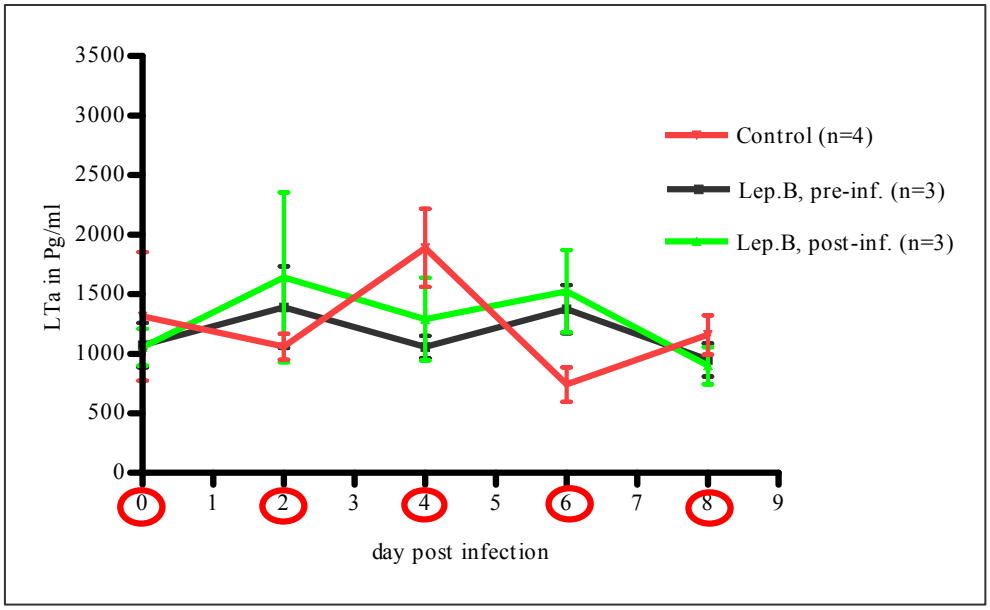

Fig.3.118: Plasma LT-α of infected C57Bl/6 mice treated i.v. with LepB 20nM.
-Plasma collected on the even days post-infection.

As presented above, LepB has the ability to reduce the TNF in vivo in a range of 60.2% to 89.6% on d4 and 5 p.i. and an ability to reduce the plasma LT-α of 0% to 44.1% on the same time points; there fore it was of interest to find out the real ability of LepB to save the PbANKA infected C57Bl/6 mice, without being under any bleeding stress. Therefore LepB was administered i.v. in the same way as in the last experiment (pre and post-infection), in a concentration of 1µM in 200 µL 1xPBS.

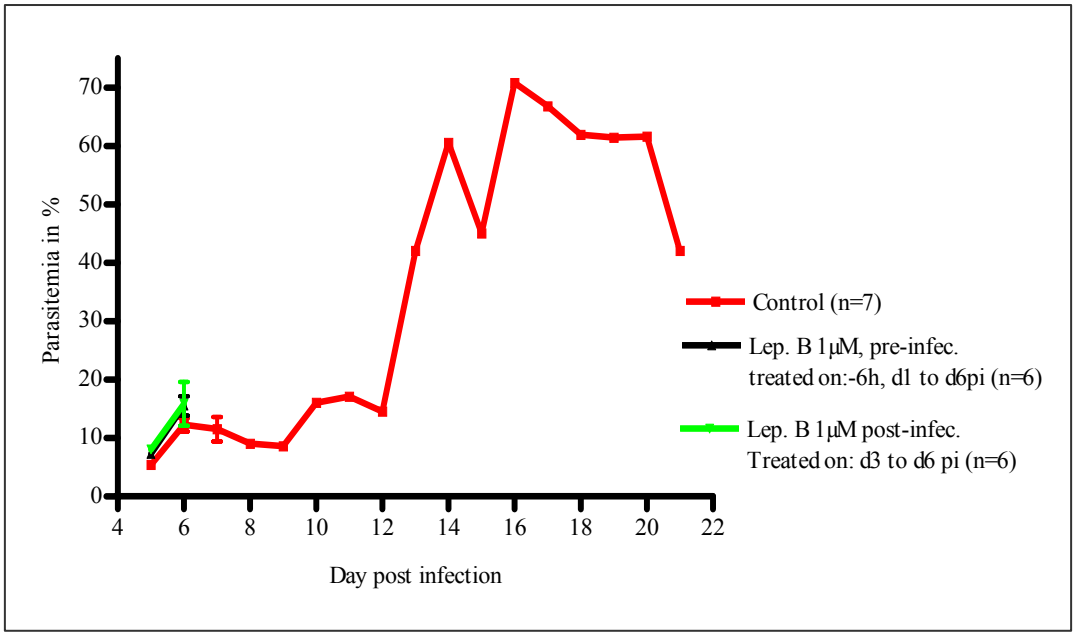

Fig.3.119: Parasitemia in PbANKA infected C57Bl/6 mice treated i.v. with LepB.

The experimental plan was to inject the mice with LepB to d7 p.i., but because the mice in both treated groups have died on d6 p.i.; the treatment was adjusted to d6 p.i. as a last day. However, as shown in figure (3.119) 1µM of LepB does not modulate the PbANKA parasitemia.

1µM of LepB could not even save the infected C57Bl/6 mice from CM as shown in (fig. 3.120).

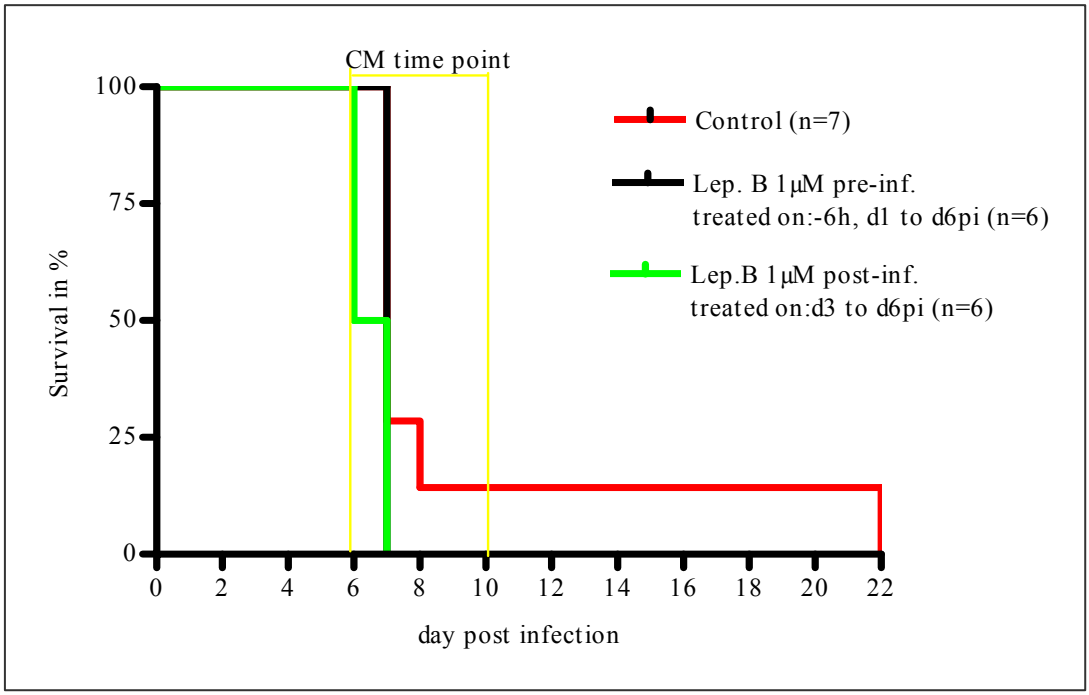

Fig.3.120: Survival of C57Bl/6 mice treated i.v. with LepB.

4. Discussion

4.1 The in vitro results

Plasmodium culture systems have been extensively used for screening new drugs, to study the mode of entry of parasite into erythrocytes, to isolate and characterize strains and clones, and to identify immunogenic and genome of parasite. However, in this study the microscopic screening method has been chosen to test the in vitro and in vivo antimalarial activity for several kinds of inhibitors.

4.1.1 In vitro IC_{50}s of the chloroquine (CQ)

To evaluate the validity of the drug susceptibility assay used in this study, the IC_{50}s of CQ for the CQS strain Pf/NF-54 and the CQR strain Pf/K1 have been determined to be compared with previously published values. An IC_{50} of 0.0138 μM was determined for the Pf/NF-54 strain in this study. An equal IC_{50}, 15.1 ± 3.1 nM, was determined for this strain (Smeijsters et al., 1996) and similar with (Davis, et al., 1992). For Pf/K1 strain, an IC_{50} of 0.468μM was determined in this study. An equal IC_{50}, 459 ± 74 nM, was determined for this strain by Smeijsters et al. (1996) and up to greater than 640 nM (Alin et al., 1992) by microscopic evaluation. A closely related value of 367 ± 136 nM (Nateghpour et al., 1993) determined by [3H]hypoxanthine incorporation has been reported, although values as low as 221 ± 57 nM (Ekong et al., 1991), by the same method, have been reported for this strain.

4.1.2 In vitro IC_{50} of the Dihydroartemisinin (DHA)

The IC_{50} determined for DHA on Pf/NF-54 strain was 0.565ng/mL. A closely related value of 0.47ng/mL on *P. vivax,* has been determined by Russell et al., (2003). In general, DHA has in vitro IC50s ranged 0.5-1.0 ng/mL on the malaria parasite (Ittarat et al., 2003).

However, determination of IC50s of CQ and DHA for the Pf/NF-54 and Pf/K1 strains confirmed that the method used in this study is suitable for the antimalarial drug susceptibility assay.

4.1.3 The time point for the in vitro IC_{50} calculation

An accurate IC_{50}s of the tested substances could not be established after a 48 hrs incubation period, but accurate determination from a linear regression analysis curve was possible after a 72 hrs incubation period. Such a prolonged incubation period is also applied in the extended microscopic drug susceptibility test elsewhere (Thaithong et al., 1983)

4.1.4 In vitro results obtained from deoxyhypusine synthase (DHS) inhibitor

The essential function of DHS in eukaryotic cell proliferation has been demonstrated by inhibitor experiments (Lee and Folk, 1998). In addition, DHS is a target of experimental low-molecular weight drugs such as the tetravalent guanylhydrazone CNI-1493, which is considered as a novel and potent inhibitor of DHS (Hauber et al., 2005). Besides the approach on the molecular level of the new inhibitor semapimod, formerly CNI-1493 which has already been applied for treatment of Morbus Crohn disease (Atkins et al., 2001), has been shown to inhibit DHS in a dose dependent manner. In case of Morbus Crohn disease it suppresses formation of antiinflammatory cytokines like TNF-α by MAP kinase inhibition.

CNI-1493 had IC_{50} values of 1.5 to 2.5 µM in vitro in HIV-resistant lines (Sommer et al., 2004) and since 1.0 µM of CNI-1493 inhibited the hypusine formation in eIF5A in vivo (Hauber et al., 2005). The hoped in vitro IC_{50} of CNI-1493 on PF/NF-54 strain was in a low-molecular weight. But contrary to the expectations CNI-1493 showed a high in vitro IC_{50} (135.79µM) on the chloroquine sensitive strain (fig.3.5). This high IC_{50} reflect the specificity of malaria parasite when compared to other illness causative agents, such as HIV-resistant lines. However, considering that the use of DHS inhibitors in mammalian cells resulted in the loss of cell viability (Chen et al., 1996). it would be of interest to investigate whether CNI-1493 had reduced the parasitemia of Pf/NF-54 by reaching its target (DHS) or it was affecting the viability of the cultured red blood cells, which also lead to reduce the parasitemia (Chotivanich et al., 2000).

Nevertheless, CNI-1493 on the other hand showed an interesting affectivity on the schizont stage of Pf/NF-54 (fig. 3.6). This affectivity representing the disappearance of schizont stages when high CNI-1493 concentrations presented. Thus, CNI-1493 affecting the schizont stage, which is an important erythrocytic stage for malaria proliferation (Marton and Pegg, 1995; Seiler, 2003).

4.1.5 In vitro results obtained from deoxyhypusine hydroxylase (DHH) inhibitors

Findings in rat testis have demonstrated that the DHH can be inhibited by the antifungal drug ciclopiroxolamine. Ciclopiroxolamine inhibits the in vitro proliferation of the chloroquine sensitive (CQS) NF-54 *P. falciparum* strain with an IC_{50} value of 8.2 µM (Clement et al., 2002). However, the saturated and non-saturated dipyridine substituted mono- and dieters DHH inhibitors which is used in this study showed varied in vitro anti-malarial efficacy, since 7 inhibitors of 46 exhibited in vitro anti-malarial activity on Pf/NF-54 strain in IC_{50}s ranged between 8.29µM, obtained from EHW437 (fig.3.26),

to 14.57μM which was obtained from UD218 (fig. 3.22). Additionally, it was not clear which parasite's erythrocytic stage was more sensitive to this class, whereas UL3B1P (fig. 3.8) showed more efficiency on the trophozoite stage, there were some others that were more efficient on the schizont stage than trophozoite; such as JK8-2 (fig. 3.17), JK-12-ML2 (fig. 3.18), and JK7E (fig. 3.19), in addition of both stages they were sensitive to UD218 (fig.3.23). However, within this class of inhibitors seven substances were reported as anti-malarial inhibitors in vitro.

4.1.6 In vitro results obtained from polyamintransport inhibitors

In malaria parasite, during erythrocytic schizogony, *P. falciparum* proliferates rapidly within host cells, leading to 12 to 18 new merozoites every 48 hrs. It has been shown for many organisms that growth and differentiation processes depend on adequate intracellular concentrations of the polyamines puterscine, spermidine, and spermine (Marton and Pegg, 1995; Seiler, 2003).

In this study, NK-1-33 and NK-1-63, in addition to their advantage as high soluble in water; they also exhibited in vitro antimalarial efficacy with IC_{50}s of 4μM for NK-1-33 and 0.63 μM for NK-1-63. The IC_{50} of NK-1-63 could be considered as a potent in vitro anti-malarial activity with a low-molecular weight of $IC_{50.}$

On the other hand, the schizont stage seems to be more sensitive to this class of inhibitors that was represented in the systemic reduction in the schizonts percentage in presence of different concentrations of NK-1-33 (fig. 3.30) and in the case of NK-1-63 the schizont stage disappeared in presence of 1.25μM (fig. 3.31). These observations were concurrent with previous observations obtained by using several ornithine decarboxylase inhibitors (Whaun and Brown, 1985).

The in vitro results of NK-1-33 and NK-1-63 revealed that inhibition of polyamine synthesis is a promising approach to the identification of anti-protozoan drugs with chemotherapeutic potential, which have also been concluded elsewhere (Das Gupta et al., 2005). Considering that, the polyamine biosynthetic enzymes of the parasites seem to exhibit features that are significantly different from those of the human host (Heby et al., 2003).

4.1.7 In vitro results obtained from phenazines derivatives

Phenazines have been described as dual inhibitors of topoisomerase I and II that play vital roles in DNA replication and transcription (Wang et al., 2002); topo I catalyzes the passage of DNA strands through a transient single strand break, while topo II catalyzes the passage of DNA double strands through a transient double strand break. In addition, phenazines could play an important role as antioxidants by interfering with the reactive oxygen species (ROS) (Badwey and Karnovsky, 1980; Babior, 1984; Laursen and Nielsen, 2004). Consequently, phenazines analogues could act in between these two mechanisms to show their anti-malarial efficiency.

In the present study seventy two phenazines underwent for in vitro assay for their anti-malarial activity, nine of them exhibited in vitro anti-malarial efficiency in IC_{50}s ranged 0.51 μM to 7.54 μM. The lowest IC_{50} was obtained from DP-2-py (fig. 3.47), whereas the highest IC_{50} within this class was obtained from QM-Br-1 (fig. 3.56). These IC_{50}s concentrations showed to be lower than those previously related to anti-malarial activity (De Andrade-Neto, et al,2004), where seven phenazines derivatives have been tested, four had significant in vitro anti-malarial activities against *P. falciparum*, the IC_{50} varied from 1.67 to 9.44 μM. In addition, DP-2 exhibited in vitro anti-malarial activity even on Pf/K1 strain (CQR) with an IC_{50} of 0.799μM, which is in the same range with that on Pf/NF-54 strain (CQS), (0.88 μM).

Most of the in vitro effective phenazines derivatives showed efficiency on both parasitic erythrocytic stages, whereas the schizont stage was more sensitive to two of them, PR-OX-1 (fig. 3.53) and PR-Br-2 (fig.3.55).

However, accumulated data indicates that intraerythrocytic forms of malaria parasites are exposed to oxidative stress (Golenser and Chevion, 1989; Hunt and Stocker, 1990; Becker et al., 2004) and that increasing oxidative stress is inimical to their growth and survival (Hunt and Stocker, 1990; Vennerstrom and Eaton, 1988). In addition, ROS-generating systems kill murine malaria parasites in vitro (Dockrell and Playfair, 1983; Dockrell and Playfair, 1984; Buffinton et al., 1986) and in vivo (Clark and Hunt, 1983; Clark et al., 1984) and kill *Plasmodium falciparum* in vitro (Clark et al., 1983; Wozencraft et al., 1984; Berman et al., 1991; Marva et al., 1991).

Moreover, there is substantial evidence that malaria parasites inside erythrocytes exert an oxidative stress within the PRBC (Golenser and Chevion, 1989; Hunt and Stocker, et al., 1990). It has been suggested that the parasite generates ROS through one or more of the following pathways: i) an electron transport chain (Deslauriers et al., 1987), ii) degradation of hemoglobin and cytosolic proteins (Atamna and Ginsburg, 1993), or iii) redox reactions of hemin (Har-El et al., 1993), (as reviewed by Potter et al., 2005).

On the other hand, the parasite is protected against this oxidative stress by a number of host- or parasite-encoded enzymes (Hunt and Stocker, et al., 1990; Becker et al., 2004), by vitamin C (Iheanacho et al., 1993), by vitamin E (Eaton et al., 1976), and by glutathione (Atamna and Ginsburg, 1997, Becker et al., 2003). Parasite antioxidant defence is believed to include export of oxidized glutathione to the erythrocyte cytosol (Atamna and Ginsburg, 1997). In addition the parasite not only needs to protect itself against ROS but it also needs to insure that the host erythrocyte is not damaged before the parasite completes its erythrocytic schizogony.

Moreover, against the phenazines property as antioxidants, the artemisinin and its derivatives enhanced their anti-malarial properties by generating free radicals, causing the reduction of red blood cell antioxidants and glutathione (Ittarat et al., 2003).

Based on these facts, inhibiting ROS generation by using the phenazines derivatives will not inhibit the proliferation of the malaria parasite. Therefore, and since phenazines have been ascribed as dual inhibitors of topoisomerase I and II enzymes (Wang et al., 2002); we believe that the anti-malarial efficacy of the phenazines derivatives, which is reported in this study as anti-malarial inhibitors in vitro, could be due to their ability as dual inhibitors of topoisomerase I and II enzymes rather than due to their ability as antioxidants by interfering with the reactive oxygen species, unless there is another unproved mode of action.

4.1.8 In vitro results obtained from dihydroartemisinin analogues

Artemisinins became a crucial part of most recommended regimens because they work against otherwise-resistant parasites (Kreidenweiss et al., 2006). In addition, DHA kills malaria parasites by generating free radicals, causing the reduction of red blood cell antioxidants and glutathione (Ittarat et al., 2003). Thus, the endoperoxide anti-malarials interrupt the haemoglobin catabolism system of the malarial parasite by causing inhibition of haemoglobin degradation as well as polymerization of heme to hemozoin (Pandey et al., 1999). Artemisinin and its derivatives have a rapid anti-malarial effect, decreasing the number of parasite faster than any other known drug (White, 1994).

The in vitro assay developed during this study used a wide range of drug concentration (from 100 to 1000ng/ml) to determine, very broadly, the levels of anti-malarial susceptibility of the DHA analogues, but even in this wide range, there was no in vitro activity observed in this group of DHA analogues (fig. 3.60 to fig.3.63).

However, whenever testing analogues of such class for their anti-malarials susceptibility, certain facts should be taken into account, which would help in assessing these substances to be evaluated, these facts are: i) Endoperoxide anti-malarials are fast acting drugs, which exert their anti-malarial effect within an hour of administration (Geary et al., 1989), ii) These drugs have a highly selective effect on different stages of the malarial parasite and mainly affect the mature stages of the parasite (late trophozoites), which are heavily laden with hemozoin pigment, iii) IC_{50}s of DHA and its derivatives are ranged from 0.5 to 1.0 ng/mL (Ittarat et al., 2003), and lastly iv) Artemisinin is ineffective against those parasite strains that do not produce the malaria pigment (Peters et al., 1986). On the other hand, decreasing the efficient doses is a desirable goal in order to decrease the risk of resistance and also from an economic point of view.

Relying on the above, all of the tested dihydroartemisinin analogues in this study are unfit to be anti-malarials agents.

In addition, it is a point of interest to examine the differences between CQS strains and CQR strains in the ability to produce the malarial pigment. That would help in choosing the right strain to test this class of anti-malarials and may add more information on the mechanism of action.

4.1.9 In vitro results obtained from Leptomycin B (LepB)

Nucleocytoplasmic transport of proteins occurs by active or passive mechanisms, depending on the requirements for energy, the size of the molecule, and the presence of specific sequences, which are recognised by receptors for nuclear import or export (Conti and Izaurralde, 2001). However, the nuclear export of mRNAs of TNF, LT-α, and other cytokines were shown to be influenced by LepB.

As treating the activated Jurkat T cells with LepB resulted in reducing the cytoplasmic mRNA of TNF (30.5 folds), LT- α (3.6 folds), and several other productions (Schütz et al., 2006).

In this study, LepB was used in vitro to treat the LPS activated murine macrophages to reduce their TNF productions, which were detected in the macrophages culture media by sandwich ELISA assay. The results obtained reveal that LepB significantly reduced the TNF productions when used in a dose of 0.156 nM, this dose was related to 2.59 folds of TNF reduction, whereas dose of 0.31 nM of LepB was responsible to reduce 7.77 folds of the macrophages TNF production (fig. 3.64). These findings similar to that obtained from Schütz et al., (2006) in the ability of LepB to inhibit the TNF productions, considering the different ways of assessing TNF productions.

In contrast, LepB showed no in vitro anti-malarial activity on the chloroquine sensitive strain Pf/NF-54 when used in 3μM (fig. 3.65). Similar LepB inactivity have been observed on *Leishmania tarentola,* when Zeiner et al., (2003) reported that, the spliced leader (SL) RNA of *Trypanosoma brucei* was sensitive to LepB treatment, which implied the involvement of Xpo1 (or CRM-1) in SL RNA nuclear export; whereas *Leishmania tarentola* was insensitive to LepB treatment, which demonstrated by growth curves and unaltered morphology. This finding, again, reflect the specificity of malaria parasite when compared to other parasites, such as *Trypanosoma.*

4.2 The in vivo results

PbANKA was used in this study to confirm in vivo, the obtained in vitro results. PbANKA murine malaria has many features in common with the human disease and is thus an accepted model for certain important aspects of clinical malaria (Miller et al., 2002). Particularly in the development stages, the murine disease is accepted to reflect the cytokine-dependent inflammatory cascade contributing to cerebral and systemic involvement in humans (Hansen et al., 2003).

However, C57Bl/6 mice genetically predisposed toward TH1-dominated responses (Scott et al., 1989) are susceptible to the murine cerebral malaria syndrome, whereas BALB/c mice with a genetically determined bias toward TH2 responses (Scott et al., 1989) are resistant to the murine cerebral malaria syndrome (de Kossodo and Grau, 1993).

In this study C57Bl/6 mice that developed CM showed neurological signs between days 6 and 10 p.i. and had parasitemia about 5 to 20%, in consistent with likewise systems (Hanum et al., 2003; Golenser et al., 2006).

4.2.1 In vivo results obtained from deoxyhypusine synthase inhibitor

4.2.1.1 Effects of CNI-1493 on parasitemia

CNI-1493 showed a very limited susceptibility to reduce the parasitemia of PbANKA (fig. 3.77, 3.79, 3.80, and 3.82), this in vivo limitation in CNI-1493 efficacy might be due to some reasons; i) The low concentration which has been used in this study; which was as a result of CNI-1493 toxicity (or high viscosity) if used in higher concentrations. ii) The real anti-malarial activity of CNI-1493 may be very low, especially if we considered the high IC_{50} (135.79µM) that was obtained from the in vitro assay. In addition to that CNI-1493 if used in a high dose, the drug seems to inhibit the mammalian enzyme with antiproliferative effect; that was responsible in losing the mice weight which we observed when CNI-1493 was given at 40 mg/kg to the uninfected mice.

Likewise, the loss of cell viability in mammalian cells have been reported by Chen et al., (1996) when 1.7-diaminoheptane-derivatives were used as DHS inhibitors. Thus, CNI-1493 might change the cells viability of the treated mice.

Nevertheless, using CNI-1493 in vivo pointed to the importance of its target (DHS) in the parasite proliferation, which was clear when CNI-1493 was used in a long term of treatment (fig. 3.82), as the parasitemia was significantly reduced along with the CNI-1493 treatment. Therefore, inhibiting the malaria eIF5A in vivo by means of DHS protein seems to be a sufficient strategy in inhibiting the parasite proliferation.

However, to overcome these expectations another DHS inhibitor should be used, this new inhibitor should have a very low anti-malarial in vitro IC_{50}, on the other hand it should allow different concentrations to be used in vivo.

4.2.1.2 Effects of CNI-1493 on cerebral malaria

CNI-1493 is a compound that inhibits activation of proinflammatory cytokine production in macrophages (Bianchi, M., et al. 1996). The mechanism of action is by preventing the phosphorylation of p38 MAP kinase, which is required for the translation of the mRNA for TNF and other proinflammatory mediators (Cohen, P. S., et al. 1997). Thereby, CNI-1493 providing the molecular background for its action as inhibitor of cytokine translation in the macrophage (Cohen et al., 1997).

However, CNI-1493 was used in this study as macrophages/ monocytes TNF inhibitor.

CNI-1493 showed varied results depending on the time point where CNI-1493 was used. CNI-1493 on d4 post-PbANKA infection showed an ability to reduce 81.1% of the plasma TNF if given pre-infection and to reduce 52.3% if given post-infection (fig. 3.84). Whereas, for the plasma LT-α, CNI-1493 reduced 19% of LT-α production if given pre-infection, which was observed in the infected mice group with no CM. In that time CNI-1493 failed to suppress the plasma LT-α, if given post-infection (fig. 3.85), which was

observed in the infected mice group with CM.

Since the circulating TNF was reduced by CNI-1493 in both groups, if given pre- or post-infestation (without and with CM), this could be an important indicator which points to the circulating LT-α as an important cytokine in developing CM, since it was observed to be reduced just in that group with no CM.

Recent findings of Engwerda et al., (2002) in *P. berghei* ANKA model of CM suggest that it is not the circulating TNF itself that is responsible for developing CM but it is the circulating LT-α, which can bind to the TNF receptor II (p75) (Bazzoni and Beutler, 1996) and is recognized by most anti-TNF antibodies (Sheehan et al., 1989). In addition, Jacobs et al., (1996) have reported that treatment of resistant B6 mice early during *P. chabaudi* AS infection with anti-TNF antibody results in 50% mortality but has no effect on the course of parasitemia. They also suggested that higher doses of anti-TNF polyclonal antibodies would have resulted in 100% mortality of these mice. Therefore they have suggested that TNF has a protective role during blood-stage of *P. chabaudi* AS malaria when it is produced in the spleen and the liver early during infection. Likewise, high plasma concentrations of TNF were found to be associated with disease severity but not specifically with CM (Shaffer et al., 1991; Molyneux et al., 1991).

The levels of mRNA of TNF and LT- α were also influenced by CNI-1493 if given pre-infection (fig. 3.86 and 3.87). Whereas giving CNI-1493 post-infection showed susceptibility to reduce the levels of LT-α mRNA in the brains of the infected mice, but not TNF mRNA levels (fig. 3.86). Thus, this data together with that obtained from the plasma assay, give the evidence that TNF and LT-α levels during the murine malaria infection are different if assessed in different sites and different times. Jacobs et al., (1996) have concluded that, the paradoxical role of TNF, that is, protection versus pathology during malaria, may depend not only on the amount of TNF released but also on the time and site of its expression and the presence of other cytokines regulating its production.

This conclusion might come up with the LT-α production during the murine malaria infection, as our results revealed that LT-α was produced in different levels depend on the site where its produced and the time of the infection.

In addition, the in vivo results of this study indicated that treatment with CNI-1493 reduced mortality of CM by 100%, in the experimental mice model, if given pre-infection (Fig. 3.72, 3.78, and 3.81). Whereas, failed to protect them from CM, if given post-infection. This loss of cytokine-suppressive activity suggests that CNI-1493 inhibits macrophages activation at an early stage in the intracellular signaling pathway, which was also concluded by Cohen et al., (1996).

On the other hand our findings indicated that CNI-1493 was unable to act as a specific TNF inhibitor, since about 18.9% to 47.7% of the TNF was still present in plasma of the infected mice; especially if we considered that CNI-1493 was not reported as a specific TNF inhibitor but as a general proinflammatory cytokines inhibitor (including IL-1, IL-6, and IL-8, and TNF), which had been reported by (Bianchi et al., 1996; Bjork et al., 1997).

The above findings were found to be in agreement with previous ones; where CNI-1493 did not interfere with LPS-induced up-regulation of TNF mRNA, which predicted that CNI-1493 would not suppress the early activation of NF-$_{KB}$, a transcriptional activator of TNF (Goldfeld and Doyle, 1990; Jongeneel, 1992; Trede et al., 1995). Likewise, the suppression of TNF was not complete, since CNI-1493-treated macrophages can still produce about 10% of the amount of TNF (Bianchi et al., 1996; Bjork et al., 1997). In addition, Bianchi et al,. (1996) performed a time course study to assess the effects of CNI-1493 added to monocytes at different stages of LPS and IFN-g induced activation. TNF protein was completely suppressed when CNI-1493 was added to quiescent monocytes before stimulation with LPS and IFN-g, but when added to monocytes just before or after their activation by LPS and IFN-g, CNI-1493 was less effective in suppressing TNF produ-

ction.

Moreover, depletion of monocytes/macrophages by treatment with a liposome containing dichloromethylene diphosphonate, if administered before the day of infection, but not later, prevents the development of CM (Curfs 1993; Belnoue 2002). Thereby, the role of macrophages is most likely related to the release of cytokines early in the infection (Engwerda et al. 2005).

In the same line, blocks of TNF by using anti-TNF monoclonal antibody (Van Hensbroek et al. 1996) or pentoxifylline (Di Perri et al. 1995) failed to improve survival of African children with CM, they do suggest that TNF may have a limited role in the terminal stages of CM pathology. However, while it is possible to overcome CM by treatment with anti-malarial drugs (Turner, 1997), in some patients, as well as in experimental mouse models of CM, bur there is an irreversible stage after which the patient dies, despite massive anti-parasitic treatment. Similarly, in the mice at a later stage when they demonstrate the first signs of CM, it is impossible to save them (Golenser et al., 2006).

The displayed data fit with our findings by using CNI-1493. Given the impression that, blockage of TNF of the activated macrophages by CNI-1493 to protect the mice of CM is useless, taking into account that the normal treatment will usually take place after the infection not pre-infection.

4.2.1.3 CNI-1493 in Vivo cytotoxicity assay

Dendritic cells (DCs) provide a critical link between the innat and adaptive immune response, and they are specialized for the uptake, processing, and presentation of pathogen-derived antigens to T cells. They are the only antigen-presenting cells (APCs) that can activate naïve T cells (Banchereau and Steinman, 1998). This is a major characteristic of DCs, and they are thus essential for the initiation of an immune response against a new antigen (Banchereau and Steinman, 1998).

AdOVA induce an increase, as a short peak, in IL-12, (Furumoto et al., 2000; Miller et al., 2002), shortly after administration, and increased ovalbumin (OVA)-specific cytotxic T lymphocyte cells (Wingender et al., 2006). However, the ability of CNI-1493 to suppress CTL activity in vivo was investigated by immunized the C57Bl/6 mice with AdOVA. CNI-1493 showed a non-significant ability to suppress the function of the specific CTLs, which generated in the spleen (Fig. 3.88).

4.2.2 In vivo results obtained from deoxyhypusine hydroxylase inhibitors

Deoxyhypusine hydroxylase (DHH) the metalloenzyme catalyzing the final step in hypusine biosynthesis (fig. 1.6), which is required for the biological activity of the eIF5A protein.

In this study DHH inhibitors were used in vivo depend on the in vitro selections. However, using UL3B1P in vivo in varied concentrations, ranged from 20 to 300mg/kg, revealed that there is no anti-malarial activity observed of this substance when used i.p. against PbANKA infected (Fig. 3.89 and 3.91). It was also clear that UL3B1P does not have any kind of ability to reduce the parasitemia (fig. 3.95) or even to save the mice from CM (fig. 3.96), when administered as a solution i.v. at 80mg/kg. Delivered as a solution UL3B1P may be rapidly removed from the circulation by tissues up taking, which might be similar to that described when halofantrine delivered as a solution (Krishna et al., 1993; Karbwang

and Bangchang, 1994).

In the cerebral malaria direction, it has been reported that inhibition of eIF5A might lead to a reduced synthesis of TNF in mice (Taylor et al., 2004). Therefore, UL3B1P was used to find out its outcome on the cerebral malaria (fig. 3.94); however using UL3B1P in this direction could not prevent the cerebral malaria syndrome.

Hence we have not studied the direct effects of UL3B1P on the TNF productions; it could be that UL3B1P has reduced TNF productions in the infected mice but it was impossible to save the mice from CM, may be, because of the less importance of TNF in developing CM (as we observed with CNI-1493 and LepB, sections 4.2.1.2 and 4.2.5). It is also possible that UL3B1P has no effects on TNF productions.

In addition, using JK-8-2, JK12-ML2, and JK7E i.p. at 300mg/kg could not confirm any in vivo anti-malarial activity of this class of inhibitors (fig. 3.97 and 3.98). More than that, JK-8-2 when administered orally failed to reflect any anti-malarial activity (fig. 3.99 and 3.100).

Overall, the DHH inhibitors used in this study exhibits in vitro inhibitory activity on *P. falciparum* growth but lacks the in vivo activity in mice. That could be due to several reasons: i) may be the DHH inhibitors used has no in vivo anti-malarial properties which may be due to the target enzyme itself (different pathways), ii) it could be also that the observed in vitro anti-malarial activity of this class was indirect activity by affecting the viability of the cultured red blood cells, which also lead to reduce the parasitemia (Chotivanich et al., 2000). Likewise it has previously been described that lupeol exhibits inhibitory activity on *P. falciparum* growth in vitro but lacks in vivo activity in mice infected with *P. berghei* (Alves et al., 1997). In addition to that, various factors change during an infection; one of these is an increase of various plasma proteins, which may influence the drug behaviour.

However, the amount of active compound seemed to be insufficient to kill the parasites. Therefore, the in vivo low anti-malarial activity of DHH inhibitors may be a result of slow uptake or rapid elimination of the active metabolites to an intracellular compartmentalization, or inactivation of the compound in vivo as that have been reported for other molecules (Zani et al., 1997). After all, the DHH inhibitors which is used in this study take the same route taken by ciclopiroxolamine; which inhibits the in vitro proliferation of the chloroquine sensitive (CQS) NF-54 *P. falciparum* strain with an IC_{50} value of 8.2 μM (Clement et al., 2002) and it was ineffective in vivo in a rodent malaria model.

In a final conclusion, targeting DHH in vivo showed less importance than targeting DHS in inhibiting the cell proliferation of the malaria parasite.

However, the increase of the water solubility of the DHH inhibitors has to be kept in mind, because this is an important prerequisite for high in vivo efficacy. On the other hand, the future work for this class should concentrate on optimization of pharmacokinetic parameters, such as absorption, distribution, metabolism, elimination and toxicity.

4.2.3 In vivo results obtained from polyamintransport inhibitors

The polyamintransport inhibitors studied here (NK-1-33 and NK-1-63) showed very interesting in vitro anti-plasmodial activities, but on the other hand, it is toxic in mice. It was not possible to increase the drug in vivo concentration due to the high toxicity of this class.

The same drawback has faced the mimosine, which showed in vitro IC_{50}s of 32μM for QCS and 39μM for QCR of the *P. falciparum* strains (Kaiser et al., 2006).

However, selecting the toxic substances may due to the limitations of in vitro methods; where even the toxic compounds will get select (Kalra et al., 2006).

Nevertheless, the in vitro anti-malarial activities of NK-133 and NK-163 might be due to an active principle(s) responsible for that, and that may need to bio-guided fractionation of these inhibitors, which may lead to new derivatives with improvement in their therapeutic index or pharmacological profiles.

4.2.4 In vivo results obtained from phenazines derivatives

4.2.4.1 Effects of phenazines derivatives on the parasitemia

The in vitro selected phenazines underwent for in vivo assay for their anti-malarial activity against PbANKA. However, PR-1 was used at 200mg/kg i.p. and injected for 7 times (fig. 3.103). No significant differences in parasitemia between the PR-1 treated group and the control group during the time course of infection till d9 post-infection. Suggesting, that there are no in vivo effects of PR-1 when demonstrate i.p., may be that was due to the incomplete solubility of this phenazine. In addition, in this experiment it was very clear that the control group of BALB/c mice died very early d8 to d10 p.i. (PR-1/200mg/kg vs Control, P= 0,0011) (fig. 3.104), correlated with about 20% parasitemia, which is not normal for the BALB/c mice (CM resistance mice). As non-susceptible strains of mice do not develop CM but die after about 21d p.i. from severe anaemia and hyperparasitemia (Kossodo and Grau, 1993). Similar dose and long term of treatment (200mg/kg for 7 days) have been used by De Andrade-Neto et al., (2004) where seven phenazines derivatives were tested; four had significant in vitro anti- malarial activities against *P. falciparum*, IC_{50} varied from 1.67 to 9.44 µM. The two most active ones were also tested in vivo against *P. berghie* in mice, 3-sulfonic acid-ß-lapochone- derived phenazine was the most active causing up to 98% inhibition of parasitemia. In PR-1 and DP-2 when administered i.v. at 80mg/kg of each, exhibited contradictory behaviour as for PR-1 there were significantly differences in parasitemia during d8, d9 and d10 p.i. if compared to the control group (fig. 3.105), giving the impression that PR-1 has limited in vivo effects when used i.v., whereas

DP-2 killed the mice earlier than the control group (will be discussed in section 4.2.4.2).

In addition DP-2 exhibited no in vivo anti-malarial activity even if used orally at 200mg/kg (fig. 3.107). Similar results were obtained from PR-1-cl and PR-1-py (fig. 3.109) and from PR-Br-2 (fig. 3.111).

Although, PR-1 gave an in vitro IC_{50} of 4.11 μM but showed limited activity against the parasite in vivo. A possible explanation for the insufficient activity against *plasmodium* could be due to the incomplete solubility, which make the amount of the drug not long enough available or even too low to exhibit anti-malarial effects. It could also be, that uptake of the used drugs, in PbANKA infected erythrocytes is reduced compared to uptake in Pf/NF-54 infected erythrocytes and that therefore insufficient levels of drug in the parasites were obtained. However, to find out why phenazines derivatives exhibits anti-plasmodial activity in Pf/NF-54 in vitro and why this is not the case when administered in vivo, a pharmacokinetic study may be needed, in the hope to find a reasonable explanation. As discussed in section (4.1.7) we do not think that the phenazines anti-malarial in vitro effects was because of their property as ROS scavengers, but may due to their features as dual inhibitors of topoisomerase I and II enzymes (Wang et al., 2002). However, to enhance the phenazines anti-malarial properties, we think that, they should undergo an in vitro assay for their efficacy on the topoisomerase enzymes, that would benefit in selecting just the high active analogues for further investigations for their anti-malarial activity; in this way much time and effort could be saved. On the other hand, further assays are needed to check the proposed mechanistic hypothesis of this class of the phenazines derivatives.

4.2.4.2 Effects of phenazines derivatives on CM

By far the most important multienzyme complex involved in the generation of ROS in signal transduction pathways is the NADPH oxidase. This plasma membrane-associated oxidase has been implicated as the source of most growth factor- and/or cytokine-stimulated oxidant production (Griendling et al., 1994; Thannickal and Fanburg, 1995). It is suggested that TNF. generates ROS through the mitochondrial respiratory chain during activation of mitochondrial apoptotic pathways (Sidoti-de et al., 1998; Wissing et al., 1998).

There is a considerable body of evidence consistent with the concept that phagocyte-derived ROS are involved in host immunity against malaria infection (Hunt and Stocker, 1990). Thus, ROS may be involved in the pathogenesis of CM, especially since the brain is particularly vulnerable to oxidative damage (Floyd and Carney, 1992). Additionally, ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of the cellular metabolism with the potential to cause damage to lipids, proteins, and DNA (Freeman and Crapo, 1982).

Since, one of the important properties of the phenazines is that they have been ascribed as ROS scavengers (Laursen and Nielsen, 2004) by targeting the NADPH oxidase enzyme, phenazines derivatives were used in this study to assay their ability to save the infected C57Bl/6 mice from developing CM depend on the phenazines properties, as ROS scavengers.

However, using the phenazines derivatives in this study could not confirm positively the involvement of ROS generation in the murine CM syndrome. From the derivatives which were tested in vivo; just one phenazine, PR-1-py (used i.p.), showed the ability to save 100% of the infected C57Bl/6 mice during the cerebral malaria time points (fig. 3.110).

On the other hand PR-1-cl in the same experiment (fig. 3.110) was unable to save the treated mice, which gave a strong indicator that these derivatives are acting in somehow different ways. In addition, saving the infected mice from CM by PR-1-py it could not be due to scavenging ROS productions, because the other phenazines are ROS scavengers too, and they were unable to prevent CM.

Vice versa when DP-2 was used i.v. in 80mg/kg (fig. 3.106), it showed a different behaviour, as it killed the treated mice earlier than that in the non-treated control (Control vs DP-2, P= 0.0021). However, Roos et al., (1996) has described that NADPH oxidase enzyme has a vital important role, that illustrated by a human genetic disorder called chronic granulomatous disease (CGD), which is associated with life-threatening bacteria and fungal infections. Additionally, DP-2 when used orally (200mg/gk) it has no efficacy on CM, as the treated mice died of CM as the control group did (fig. 3108).

Interestingly, one phenazine, PR-Br-2, showed high affectivity in inhibiting the plasma TNF (fig. 3.112) when 100% of plasma TNF was reduced on d4 and d5 p.i., whereas 25.4% of the plasma LT-α was reduced on d 5 p.i. by using PR-Br-2; this could be due to the ability of PR-Br-2 to reduce ROS generation and thus reduced the plasma TNF and LT-α productions, as it is known that ROS could behave as signalling molecules that activate the expression of the proinflammatory cytokines genes (Baeuerle et al., 1996; Kaul and Forman, 1996), considering the absence of any direct information that indicating the relation between LT-α and ROS and the ability of ROS to induce this cytokine, or the ability of LT-α to induce ROS productions. Whereas it has been reported that TNF stimulates superoxide anion (O_2^-) production in vascular smooth muscle cells by a p^{22phox}-based NADH oxidase and appears to upregulate p^{22phox} gene expression in these cells (De Keulenaer et al., 1998).

In addition PR-Br-2 reduced plasma TNF and LT- α, could also indicate that, this phenazine may have direct anti-inflammatory effects. Similar effects have been reported by using Curcumin which has anti-oxidant and anti-inflammatory effects (Yadav et al., 2005). However, the ability of PR-Br-2, as anti-inflammatory, might be due to its unique behaviour and we could not generalize this behaviour to the other phenazines which have been tested in this study, because of the different behaviours that was observed within the tested phenazines, even due to the same analogue (e.g. DP-2), when used in different ways of administration.

So, the question remains, how could PR-1-py save the infected mice of CM, whereas PR-1-cl could not? To get the right answer, further investigations should be done on PR-1-py and PR-1-cl. However, one possible explanation that may PR-1-py has anti-inflammatory effects and it was able to interact directly with TNF and LT-α, as PR-Br-2 did. Considering that Sanni et al., (1999) showed that $gp^{91phox-/-}$ mice lacking the respiratory oxidative burst are as susceptible to cerebral symptoms as their wild- type counterparts.

In addition, Latifu et al., (1999) suggested that phagocyte-derived ROS are not involved in the pathogenesis of murine CM. The cause of death in murine CM is probably not due to excessive production of ROS in the brain parenchyma leading to protein oxidation, lipid peroxidation, or DNA damage.

4.2.5 In vivo results obtained from the nuclear export signal (NES) inhibitor

To confirm in vivo, the in vitro significantly TNF reductions in the LepB treated macrophages, LepB was used in vivo to prove that this compound is able to reduce the plasma TNF productions in both in vitro and in vivo and to find out the LepB effects on CM outcomes. On the other hand to find out the in vivo effects of LepB on the plasma LT-α. LepB (20nM) was injected into two different time points (pre and post-PbANKA infection). As it was proved by Schütz et al., (2006) that LepB could act in vitro within 3.5 hrs; hence, the time 6hrs pre-infection, which was used to treat LepB in vivo, was based on reasonable guesses. However, the in vivo results obtained revealed that LepB is able to influence the plasma TNF if treated pre or post-infection with percentage of reductions ranged from 60.2% to 89.6% on d4 and 5 p.i. (fig. 3.115 and 3.116). These high percentages of reductions were on the other hand companied with inability of LepB to prevent the CM syndrome as shown in figure (3.120).

In the same time where TNF was significantly reduced on d5 p.i. (fig. 3.115), LepB when given post-infection was unable to inhibit the plasma LT-α productions on the same day post-infection (fig. 3.117), whereas LepB was able to reduce just 9.47% of the plasma LT-α. This result together with that obtained from CNI-1493 supporting the importance of the circulating LT-α in developing CM in concurrent with likewise findings (Shaffer et al., 1991; Molyneux et al., 1991; Engwerda et al., 2002). Also the high ability of LepB in reducing the plasma TNF and its low ability to reduce the plasma LT-α, this result however tallies with that reported by Schütz et al., (2006) where they reported that treating the activated Jurkat T cells with LepB resulted in reducing the cytoplasmic mRNA of TNF (30.5 folds), LT- α (3.6 folds). On the other hand, d5 p.i. seems to be the most important time point for the circulating LT-α production. This observation is supported by using the chloroquine treatment, where the plasma LT-α was significantly reduced on d5 p.i. in the chloroquine treated group (fig. 3.113).

However, using LepB in vivo revealed that a small amount of plasma TNF could still be present in the plasma. This fact may actually have more of a physiological effect and allow the immune system to respond to the infectious agent by blunting the pathological concentrations of TNF that occur, as it was reported by Wassmer et al., (2005).

4.3 Cerebral malarial overall

CM is incompletely understood. Two dominant hypotheses have been proposed and hotly debated: i) the "sequestration" hypothesis, which suggests that the adherence of parasitized red blood cells (PRBCs) to the cerebral microvascular endothelium obstructs blood flow and causes downstream hypoxia in regions of the brain, thereby compromising the central nervous system (CNS) function (Berendt et al., 1994); ii) the "cytokine" hypothesis, which proposes that effector molecules produced by the immune system during its response against the malarian parasite adversely affect CNS function (Clark and Rockett, 1994). It has been also suggested that both mechanisms are involved, with the presence of intravascular PRBCs in the brain serving to focus the production of immune system effector, with adverse consequences for CNS function (Clark and Cowden, 1999).

Accumulating evidence indicates that in addition to parasite virulence factors, the host's immunological response (e.g., proinflammatory cytokines and chemokines) also participates in malaria pathophysiology, including tissue cell damage, microvascular obstruction, severe anemia, and cerebral malaria (Urquhart, 1994; Clark et al., 1994; Hunt and Grau, 2003).

In the present study, the effects of CNI-1493, UL3B1P, PR-Br-2 and LepB on the pathogenic sequelae of cytokine release in an experimental model of C57Bl/6 mice infected with PbANKA, have been evaluated by targeting TNF and LT-α, and what one could conclude of that are:

-Malaria is a complex syndrome, so it is unlike that one single process it is responsible for pathogenasis.

-Prevent the cerebral malaria syndrome by targeting the proinflammatory cytokines seems to be not the ideal way, as they are very important productions as a part of the host immune defence against the malaria parasite. Taken into account that, the induction of TNF producing T cell is of particular interest in malaria because TNF can inhibit both liver-and blood-stage parasites (Nussler et al., 1991; Jacobs et al., 1996; Lau et al., 2001) and synergizes with IFN-γ to induce nitric oxide and kill parasite (Jacobs et al., 1996).

-It seems clear that high plasma TNF levels cannot be the only element responsible for the symptoms unique to cerebral PbANKA malaria. Similar conclusions have been reached in studies of murine malaria (Clark et al., 1990) and *P. faciparum* malaria (Hunt et al., 2003).

-Also it seems to be that circulating LT-α more important than the circulating TNF in developing cerebral malaria syndrome.

In summary, *P. falciparum* is by far the most prevalent species and causes most problems as a result of its virulence and drug resistance. It has a rapid rate of asexual reproduction in the host and an ability to sequester in small blood vessels, with high risk for development of cerebral malaria (Winstanley, 2000). Over the last few years the situation has become even more complex because malaria parasites have been developing unacceptable levels of resistance to one drug after another. Hence, the increasing resistance of the malaria parasite has enforced new strategies of finding new drug targets (Saeftel, et al., 2006).

The standard procedures for screening the anti-malarial properties, of new compounds, are the use of in vitro cultures of *Plasmodium falciparum* and of mice infected with one of rodent *Plasmodium spp* (Peters et al., 2002), but the in vivo methods for screening anti-malarial compounds should depend on the compounds effective in in vitro screening tests (i.e. those with $IC_{50} < 1$ μM) are taken up for evaluation (Kalra et al., 2006).

In addition, the *Plasmod-ium berghei* 4-day suppression test should be used as a preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice (Trager and Jensen, 1976; Kalra et al., 2006).

Among the compounds studied in here, 19 new compounds exhibited in vitro anti-malarial properties, due to different mode of action. However, testing the in vitro selected compounds in vivo in the *P. berghei* mouse model showed no significant effect could be demonstrated on the parasite, which reflect the specificity of malaria parasite when compared to other illness causative agents, such as HIV-resistant lines, bacteria, and other protozoan agents; because the malaria parasite changes through several life stages even while in the human host, and because the parasite has developed a series of strategies that allow it to misdirect the drugs efficacy, mainly by the size and genetic complexity of the parasite. This genetic complexity allows the parasite to develop its resistance against the anti-malarial drugs used.

Finally, the fact that the activity of new anti-malarial drugs should be independently of the sensitivities of *P. falciparum* strains to chloroquine, this fact is particularly important for new drugs that will be used in areas where malaria is endemic and where chloroquine resistance is widespread (White et al., 1999; Mutabingwa et al., 2005).

5. Conclusion and recommendations

The most important points concluded from this study and the recommendations are as follows:

5.1. CNI-1493 (*N*,*N*′-bis[3,5-bis[1 (aminoimino-methyl) hydrazonoethyl]phenyl] decanedi-amide tetrahydrochlo-ride) can not be considered as anti-malarial agent.

5.2. Inhibiting the deoxyhypusine synthase seems to have more efficiency than inhibiting the deoxyhypusine hydroxylase in inhibiting the proliferation of the *Plasmodium berghei* ANKA.

5.3. Inhibition of polyamine synthesis in vitro showed a promising approach to the identification of anti-protozoan drugs with chemotherapeutic potential.

5.4. Some phenazines exhibited anti-proinflammatory property.

5.5. High plasma tumour necrosis factor levels cannot be the only element responsible for the symptoms unique to cerebral *Plasmodium berghei* ANKA malaria.

5.6. The in vivo methods for screening anti-malarial compounds should depend on the compounds effective in in vitro screening tests, and just the substances those with $IC_{50} < 1$ μM should be taken up for in vivo evaluation.

5.7. The four days suppression assay should be used as a preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mouse survival time in treated and untreated mice.

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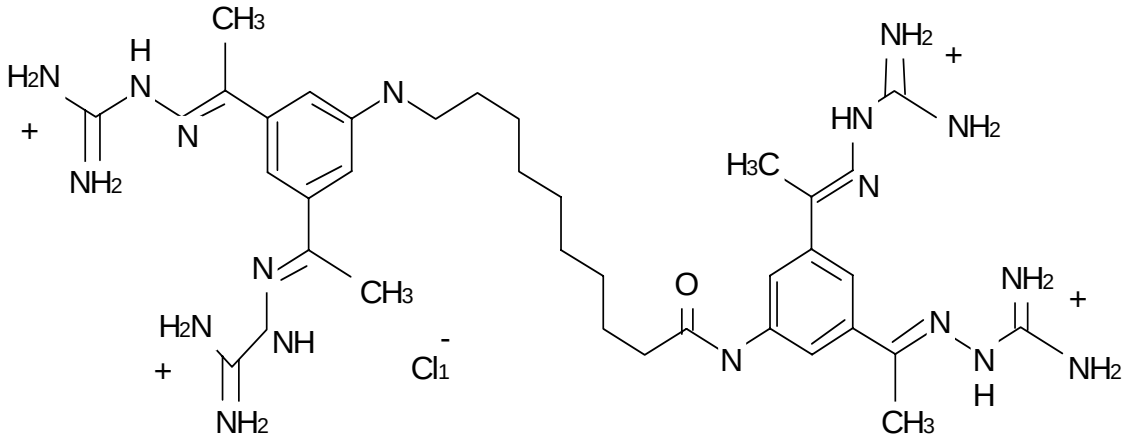
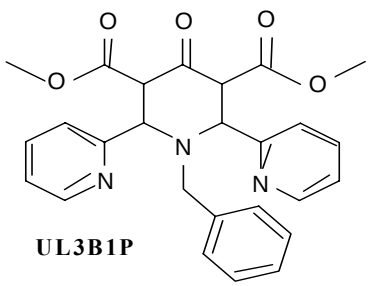
Appendix 1: The chemical structure of CNI-1493.



CNI-1493

Appendix 2: The chemical structures of deoxyhypusine hydroxydase inhibitors.



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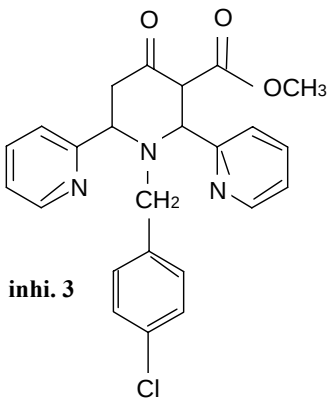

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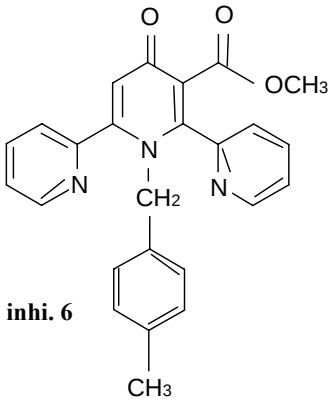

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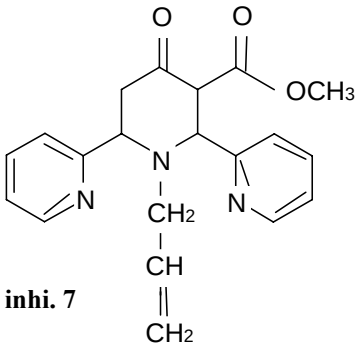

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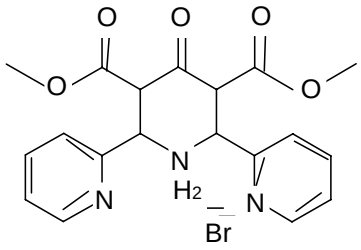

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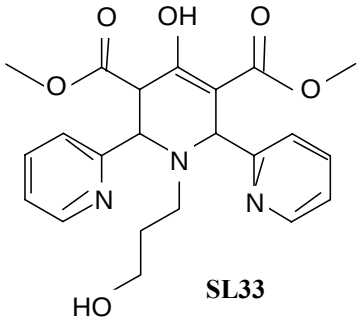

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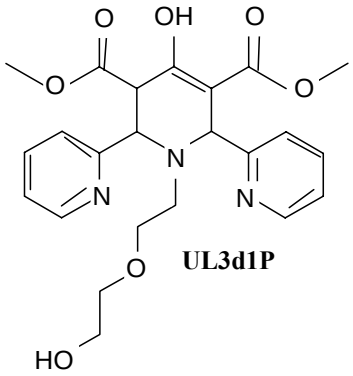

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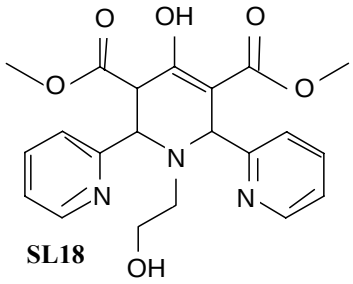

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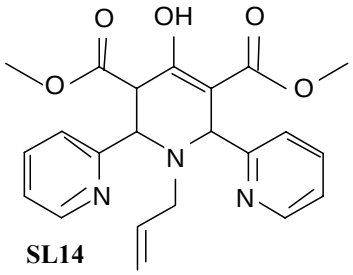

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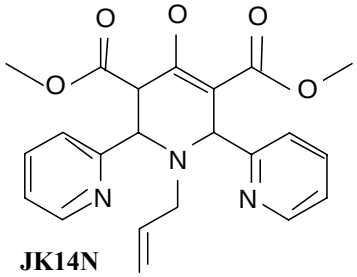

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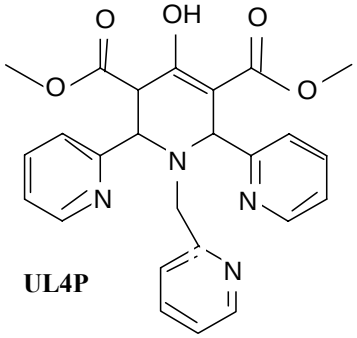

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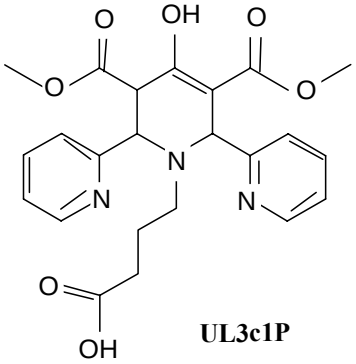

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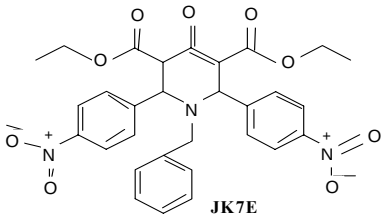

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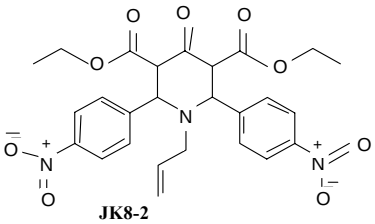

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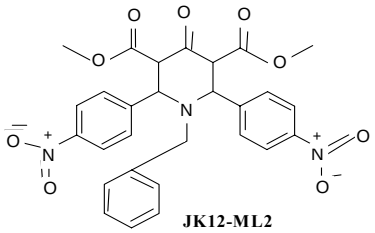

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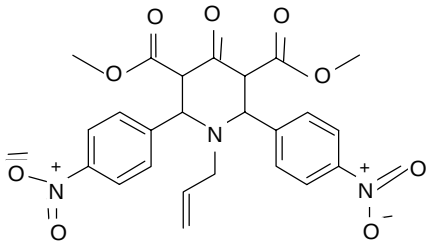

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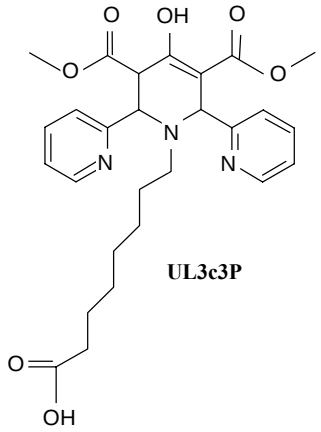

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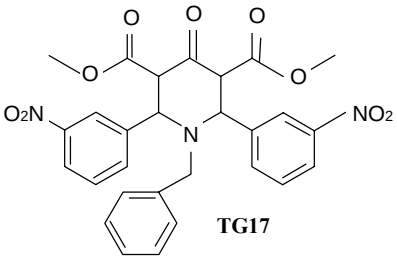

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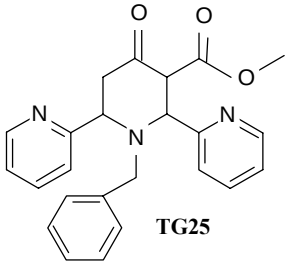

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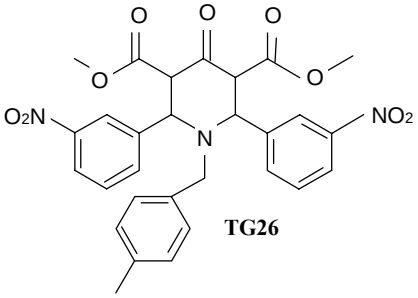

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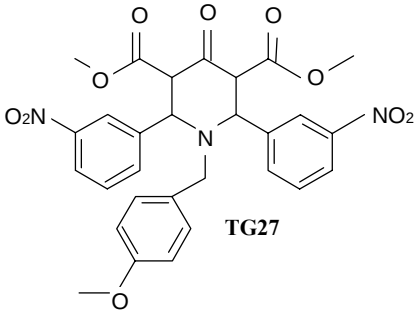

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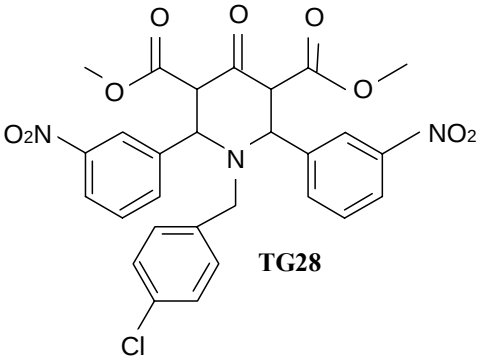

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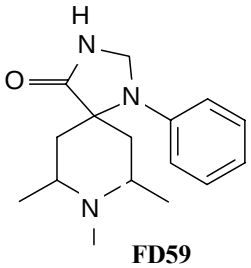

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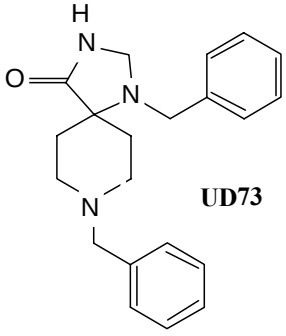

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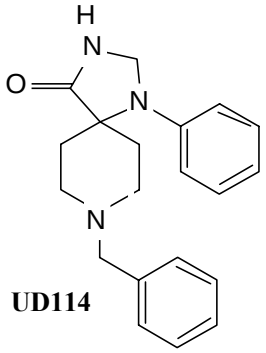

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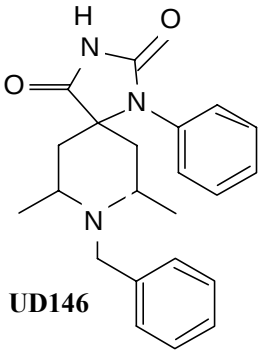

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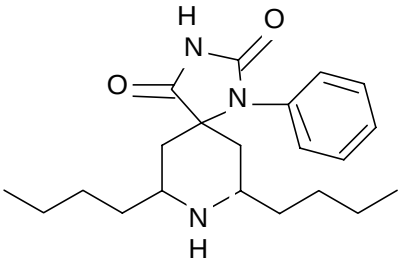

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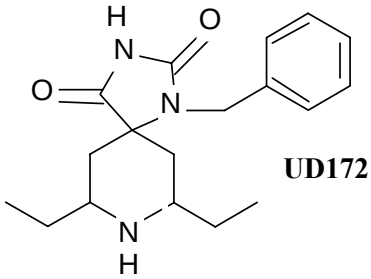

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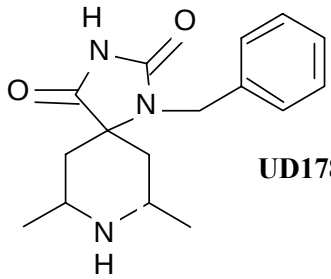

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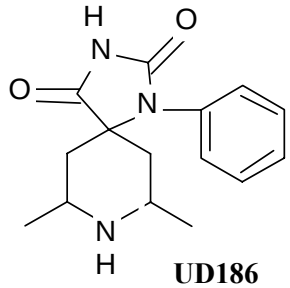

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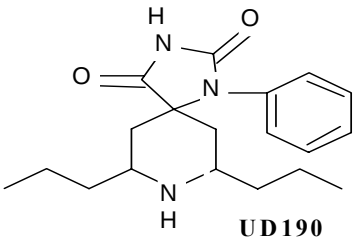

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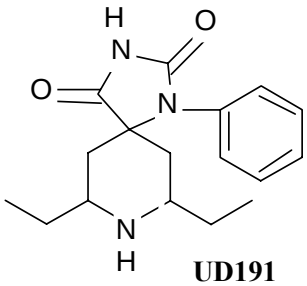

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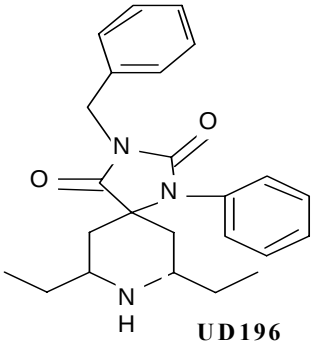

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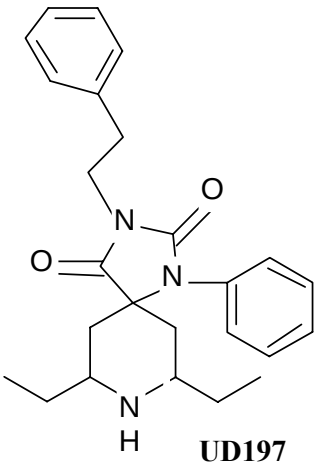

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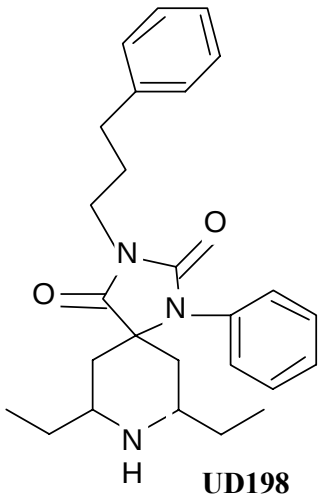

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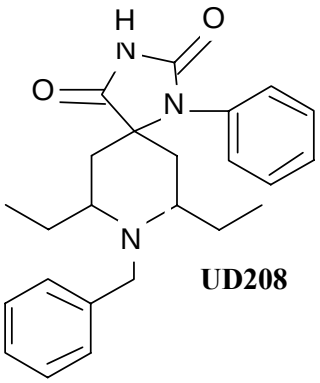

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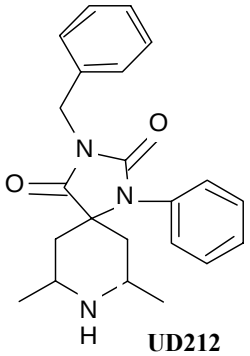

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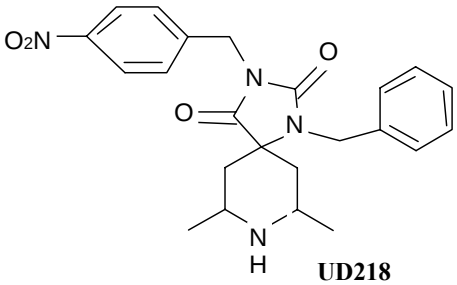

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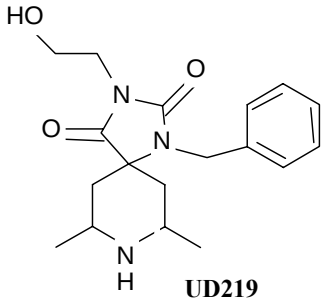

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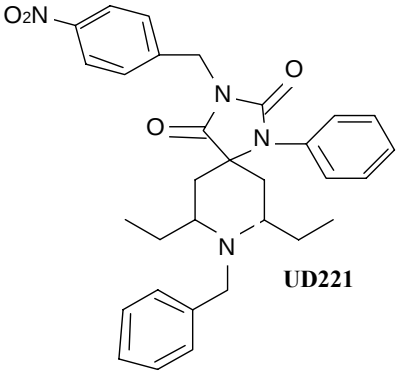

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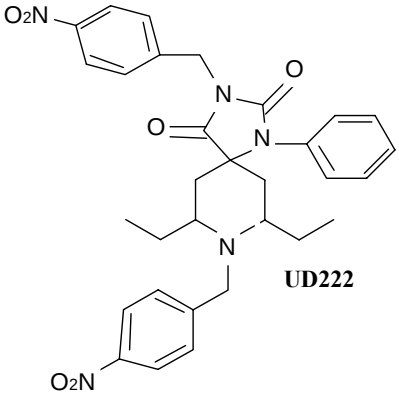

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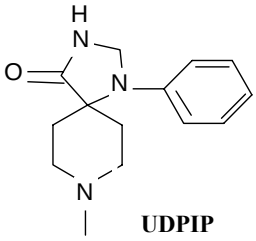

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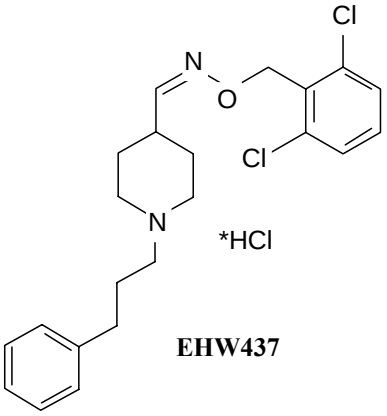

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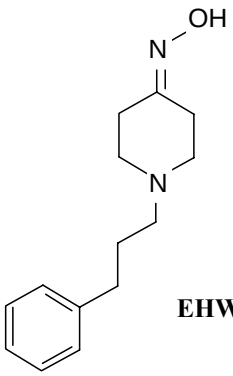

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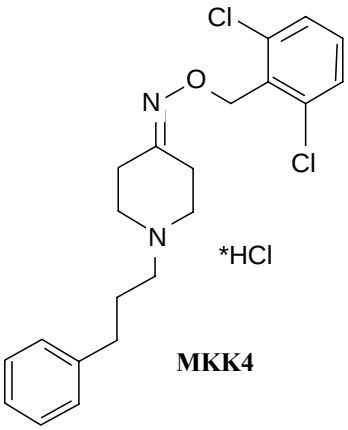

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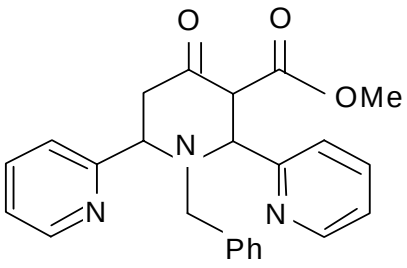
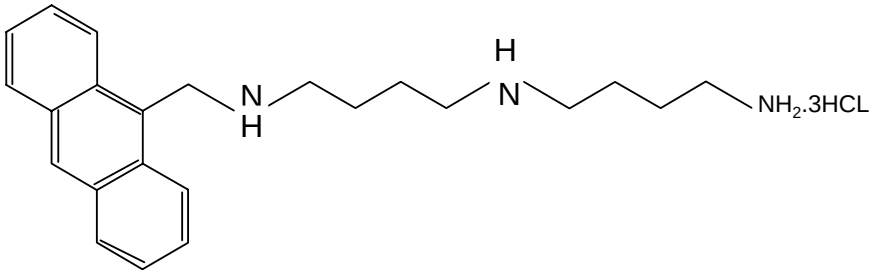
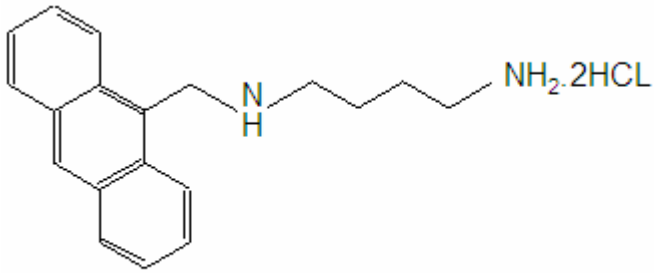


TG014

Appendix 3: The chemical structures of polyamintransport inhibitors.

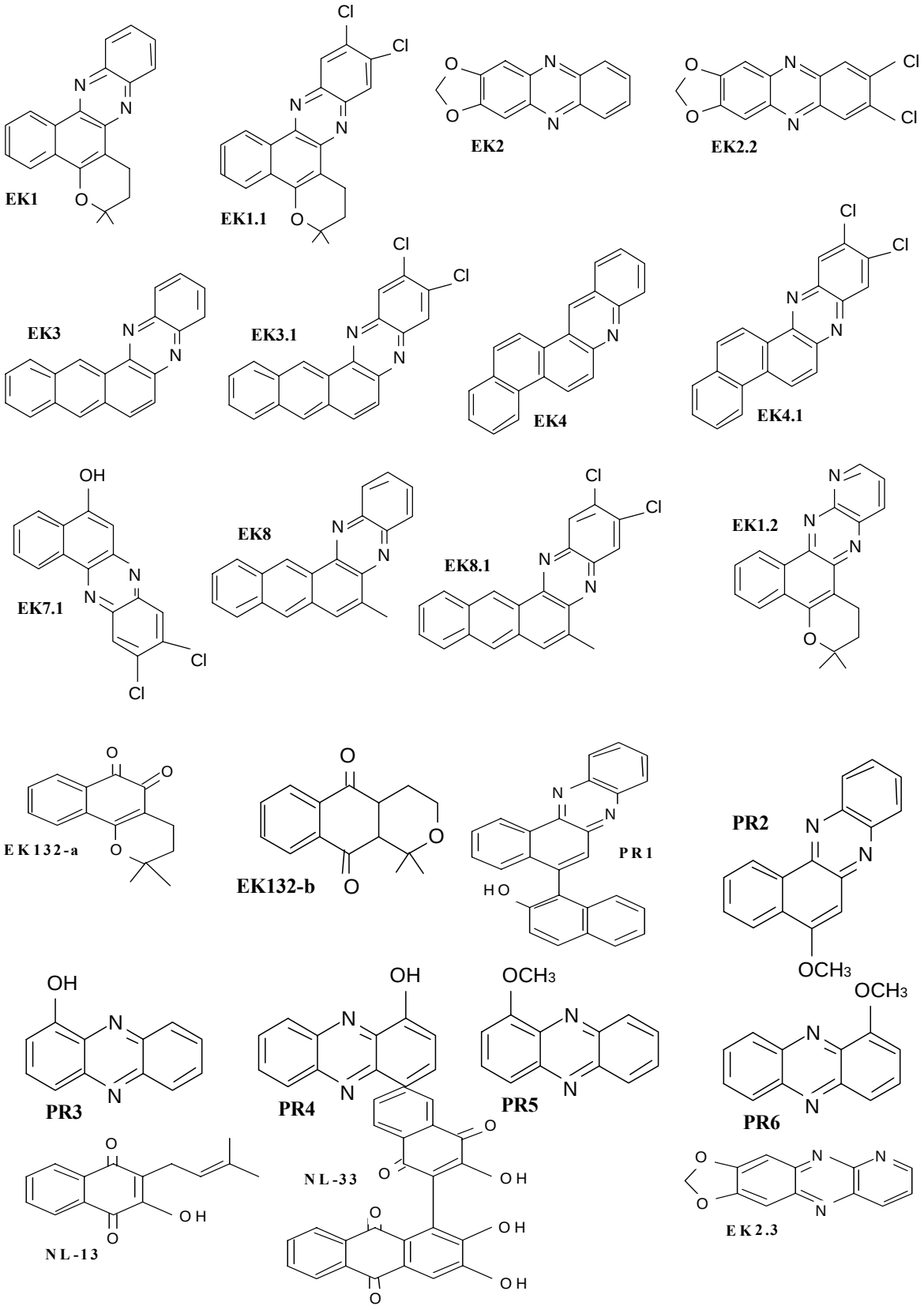


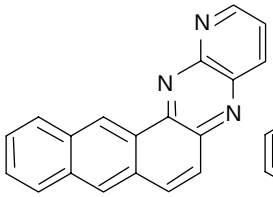
NK-1-33

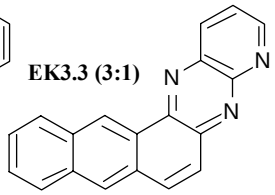


NK-1-63

Appendix 4: The chemical structures of phenazines derivatives.



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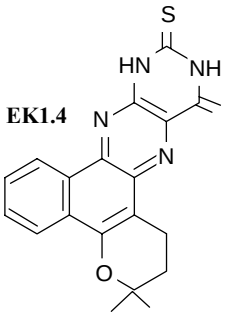
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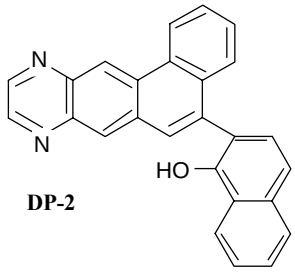
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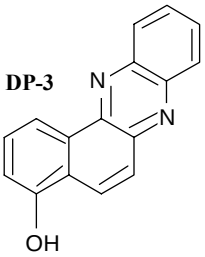
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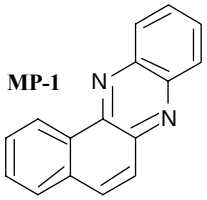
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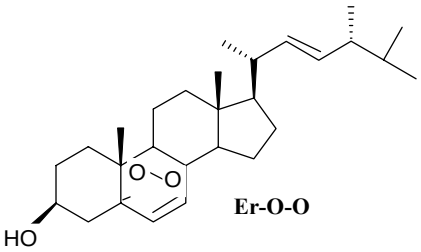
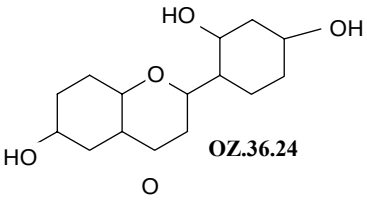

Er-O-O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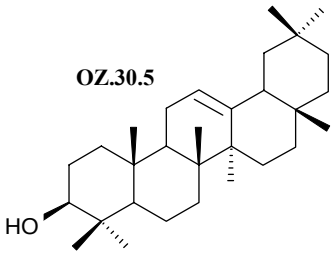
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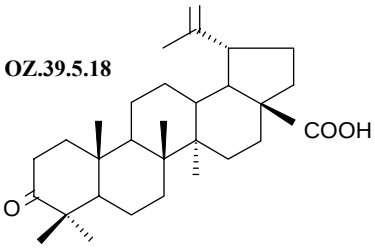
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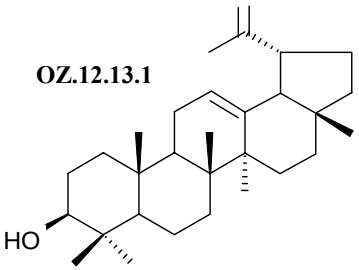
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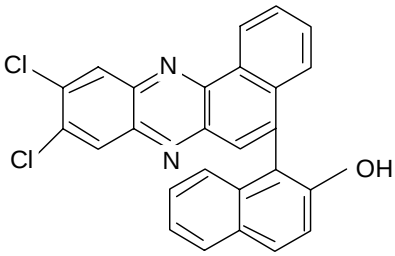
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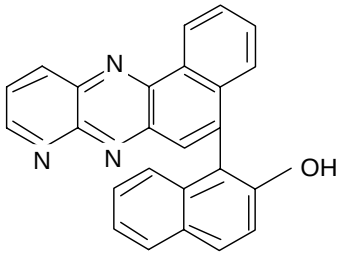
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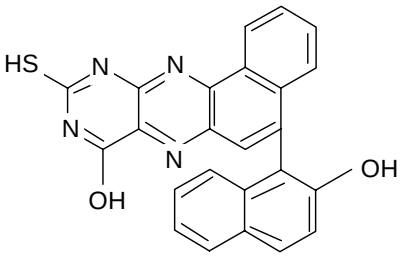
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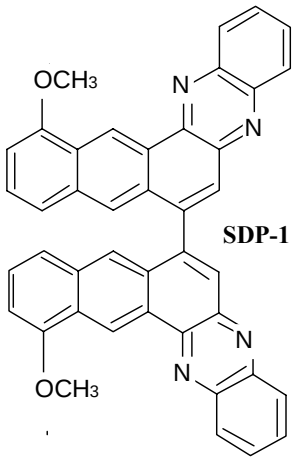
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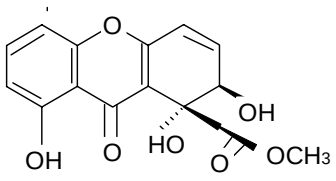
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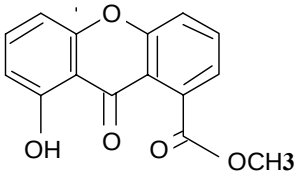
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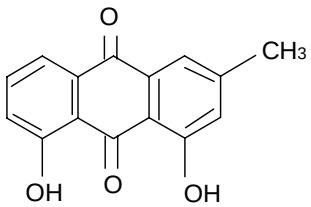
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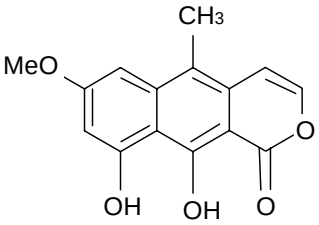

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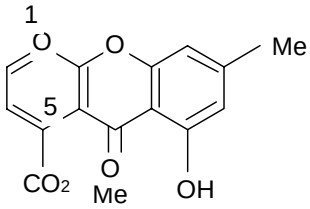

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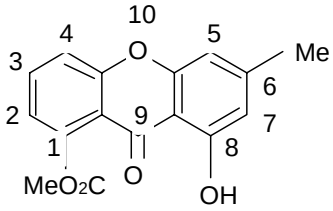

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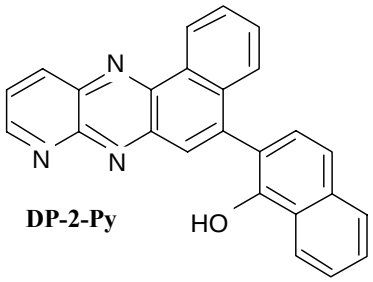

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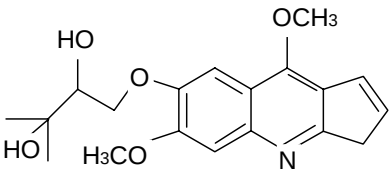

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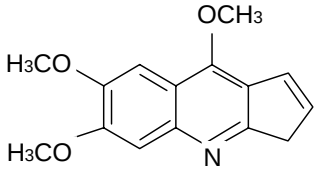

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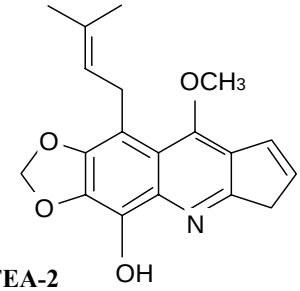

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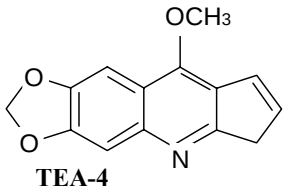

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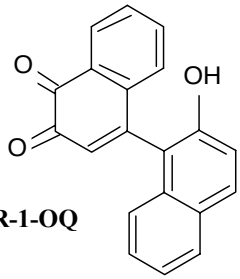

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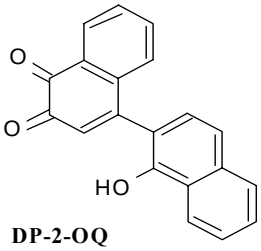

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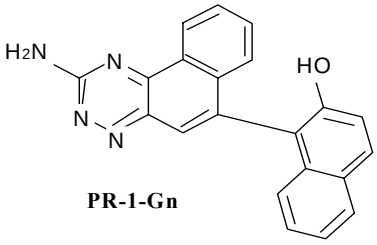

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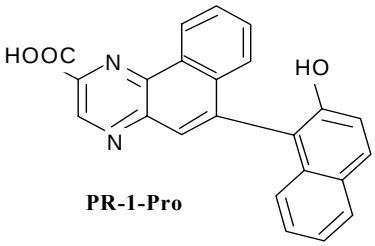

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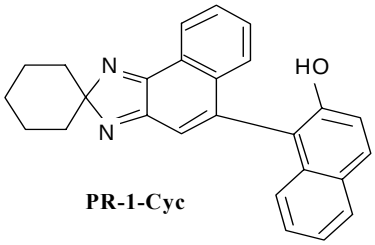

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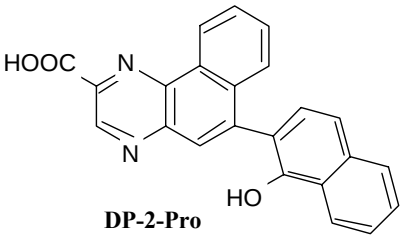

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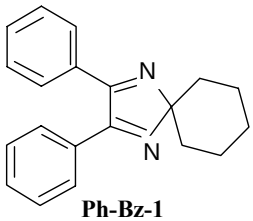

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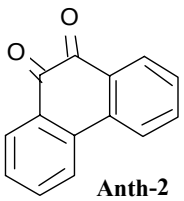

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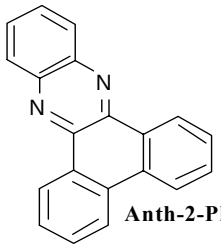

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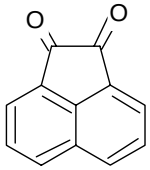

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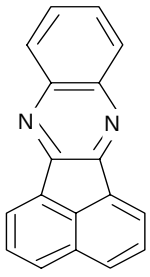

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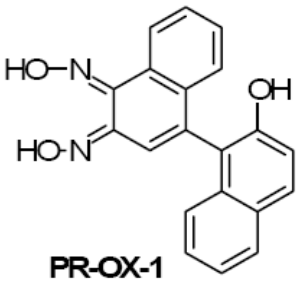

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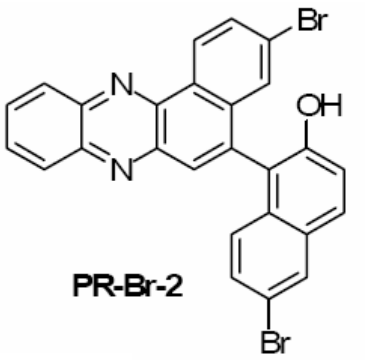

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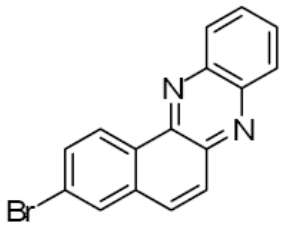

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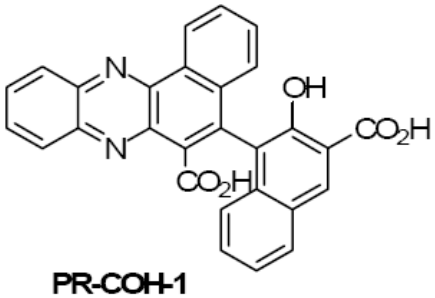

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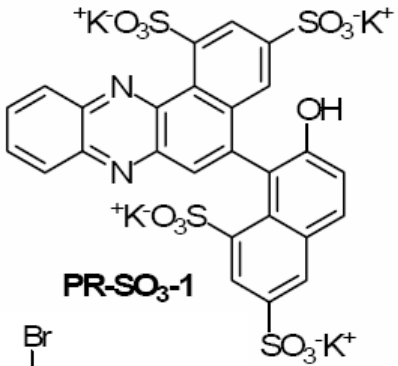

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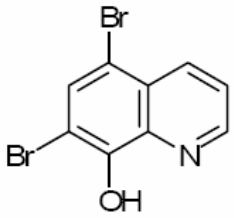

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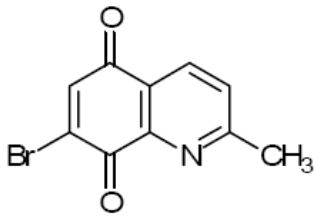

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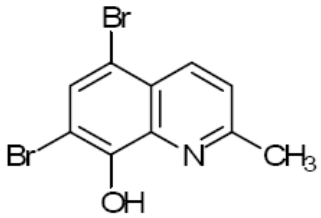

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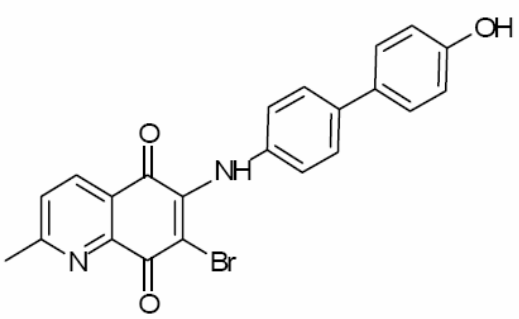

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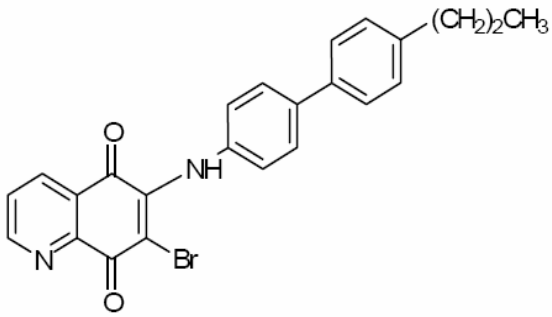

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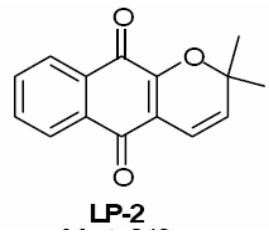

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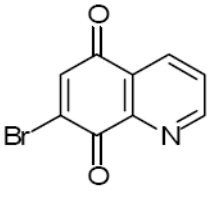

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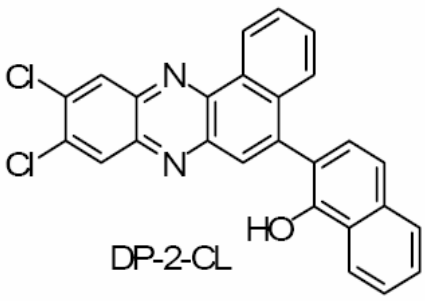
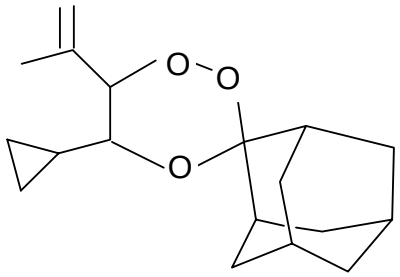


DP-2-CL

Appendix 5: The chemical structures of dihydroartmisinin analogues.



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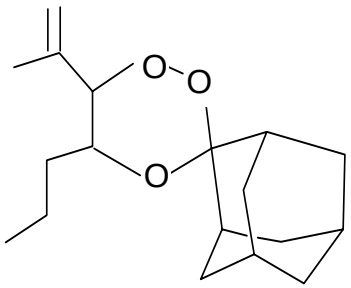

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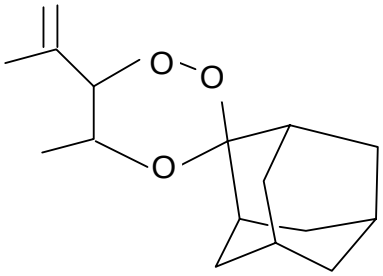

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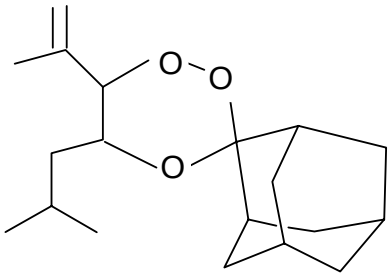

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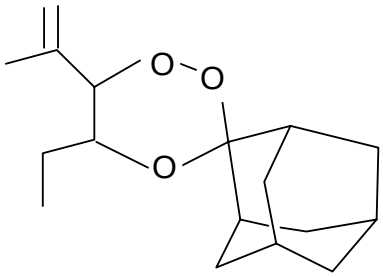

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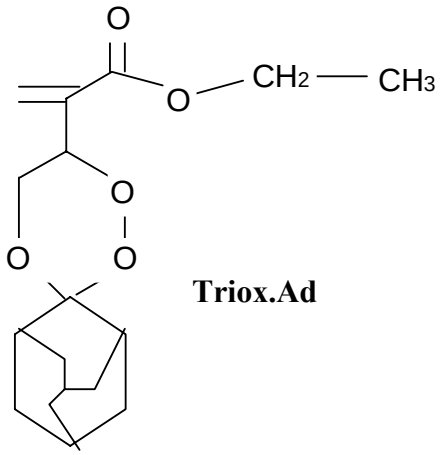
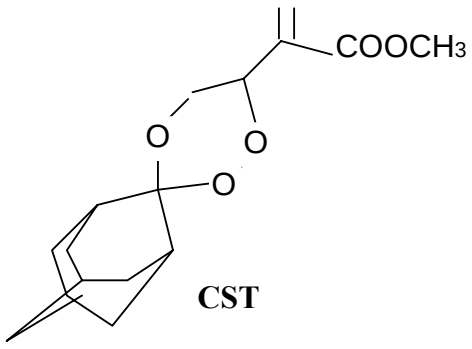
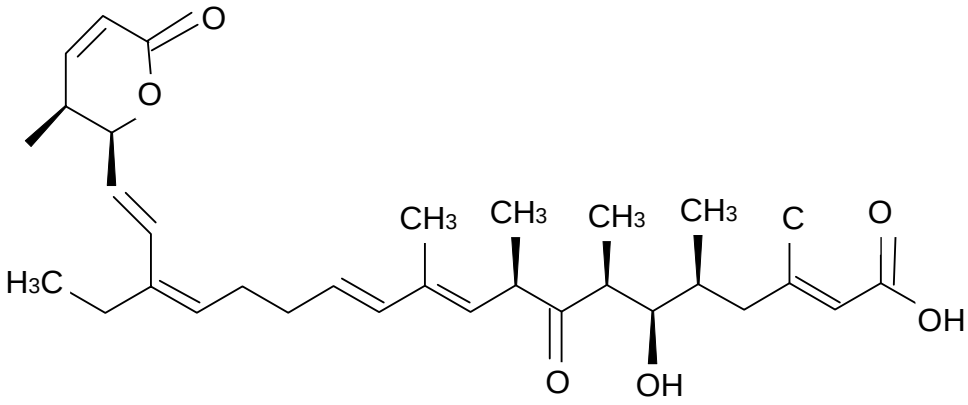


Triox.Ad



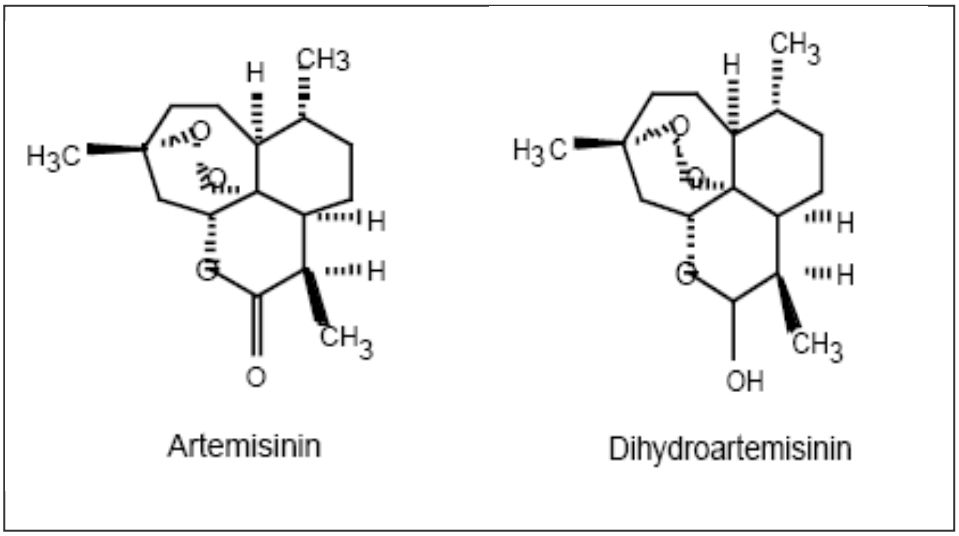
CST

Appendix 6: The hemical structures of Leptomycin B.



Leptomycin B

Appendix 7: The chemical structures of artemisinin and Dihydroartemisinin.



Appendix 8: A list of the dissolvents which used to solve the solubility problem:

-Dimethyl Sulfoxide (DMSO) at room temperature (RT) and at 37c° overnight.

-Isopropyl myristate, at RT and 37c° overnight.

-Hydroxypropyl-ß-Cyclodextrin 10%, at RT and 37c° overnight.

-D-Mannitol 10%, at RT and 37c° overnight.

-Pluronic F-68, at RT and 37c° overnight.

-Propylenglycol, at RT and 37c° overnight.

-Sodium deoxycholate 10%, at RT and 37c° overnight.

-Lipoveös, at RT.

Appendix 9: Results of the agarose gel electophoresis.

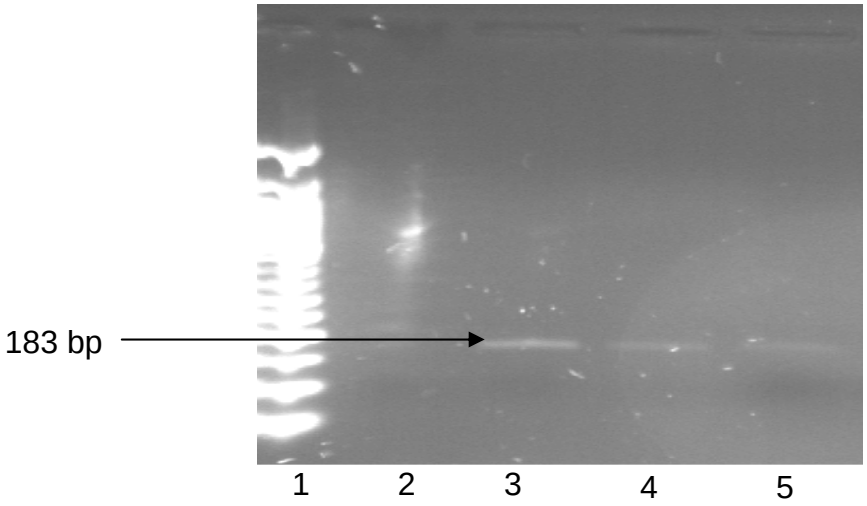


Fig. 9.1: Controling the PCR product: The sequenced DNA of LT-α resolved in 1% agarose gel electrophoresis of polymerase chain reaction (PCR). Lane 1: A 50 base pair (bp) molecular weight marker. Lane 2: cDNA probe of brain of uninfected C57Bl/6 WT mouse. lane 3, 4 and 5: cDNA probes of brains of pbANKA infected C57Bl/6 WT mice. Lane 3 was selected to be cloned as shown in (fig. 9.2.). (See also section 2.4.6.4)

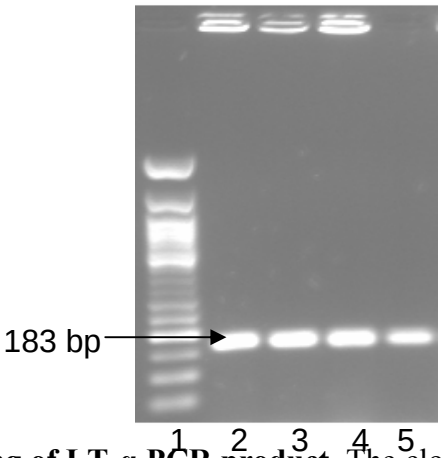


Fig. 9.2: Cloning of LT-α PCR product. The cloning was made by using pCR®4-TOPO® cloning kit as described in section (2.4.6.5). The cloned LT-α PCR product resolved in 1% agarose gel electrophoresis of polymerase chain reaction (PCR). Lane 1: A 50 base pair (bp) molecular weight marker. Lane 2, 3, 4 and 5: The colned LT-α gene.

Publications

EYONG, K. O., G. N. FOLEFOC, V. KUETE, V. P. BENG, K. KROHN, H. HUSSAIN, A. E. NKENGFACK, M. SAEFTEL, S. R. SARITE and A. HOERAUF (2006). "Newbouldiaquinone A: A naphthoquinone-anthraquinone ether coupled pigment, as a potential antimicrobial and antimalarial agent from Newbouldia laevis." Phytochemistry **67**(6): 605-9.

SAEFTEL, M., R. S. SARITE, T. NJUGUNA, U. HOLZGRABE, D. ULMER, A. HOERAUF and A. KAISER (2006). "Piperidones with activity against *Plasmodium falciparum*." Parasitol Res **99**(3): 281-6.