GENETIC AND BIOCHEMICAL STRATEGIES TO BLOCK THE TRANSMISSION CYCLE OF THE MALARIA PARASITE

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ABSTRACT

A transmission-blocking strategy will prevent the spread of malaria and has the potential to reduce morbidity and death from this disease. Reducing parasite transmission would also prolong the useful life of antimalarial drugs or vaccines by preventing the spread of parasites that become resistant to these therapies. In this thesis, I evaluated molecular and pharmacological methods to block the transmission of the malaria parasite from the definitive host, the mosquito, to the intermediate host.

Inhibitors of proteins that are involved in mosquito invasive stages could decrease the transmission potential of each mosquito. Disruption of the calmodulin-dependent protein kinase related protein (*carp*) gene in *Plasmodium berghei*, decreased the ability of ookinetes to invade the mosquito midgut, and those sporozoites that were produced were defective in their ability to traverse mosquito salivary glands. This indicates that a putative signaling pathway involving PbCARP regulates the level of infection in the mosquito by mediating invasion of mosquito tissues.

A compound that targets both asexual and transmission stages would be an ideal antimalarial. I showed that treatment of malaria with the AT-binding drug, centanamycin inhibited blood-stage infections in mice and arrested parasite development within the midguts of mosquitoes, such that sporozite development was effectively blocked. The mechanism of parasite death is associated with modification of *Plasmodium* genomic DNA. Damage to parasite DNA during

blood-stage infection persisted from the vertebrate to the mosquito host and provides a novel biochemical strategy to block malaria transmission.

Radiation or genetic attenuation of sporozoites are two approaches for whole organism vaccines that are protective against malaria. Using centanamycin, I demonstrated that attenuated sporozoites could also induce protective immunity in mice. The chemical attenuation of sporozoites using centanamycin impaired parasite function both *in vitro* and *in vivo*. Inoculation of mice with sporozoites that were treated *in vitro* with centanamycin failed to produce a blood stage infection, and mice injected with a single immunizing dose were protected against subsequent challenge with wild type sporozoites. This demonstrates that chemical attenuation of sporozoites is a viable alternative for the production of an effective liver stage vaccine for malaria.

RÉSUMÉ

Le paludisme est une maladie parasitaire causée par le *Plasmodium* qui tue chaque année plusieurs millions de personnes. Une stratégie bloquant la transmission du parasite entre le moustique et l'homme pourrait en bloquer la propagation et réduire la morbidité et mortalité associées. Cela pourrait aussi diminuer la dissémination de parasites devenus résistants aux médicaments antimalariques. Durant ma thèse, j'ai utilisé des méthodes moléculaires et pharmacologiques qui m'ont permis de bloquer la transmission du parasite.

Certains inhibiteurs de protéines impliqués dans l'invasion du moustique pourraient diminuer l'efficacité de transmission. En enlevant, chez *Plasmodium berghei,* le gène qui code pour *carp*, une protéine ressemblant à la protéine kinase calmoduline-dépendante, j'ai observé que les ookinètes envahissaient moins facilement le tube digestif et que les sporozoites produits traversaient les glandes salivaires de manière décrue. Cela indique qu'une probable voie de signalisation impliquant *carp* régule le niveau d'infection en empêchant l'invasion des tissues du moustique.

Un composé chimique qui aurait pour cible les étapes asexuelle et de transmission serait un antimalarique idéal. J'ai montré que le traitement de la malaria avec la centanamycine, un composé qui s'attache aux bases A et T, empêche le stade sanguin de l'infection chez la souris et bloque la croissance du parasite dans le tube digestif du moustique et donc le développement des sporozoites. La centanamycine induit des modifications létales de l'ADN du

Plasmodium qui persistent de l'hôte vertébré au moustique, ce qui constitue une nouvelle stratégie biochimique pour bloquer la transmission de la malaria.

L'irradiation et la modification génétique de sporozoites sont deux approches protectives contre la malaria utilisant comme vaccin un organisme entier atténué. J'ai pu démontrer que des sporozoites atténués chimiquement avec la centanamycine avaient une fonction anormale *in vitro* et *in vivo*, mais étaient capables d'induire une immunité protective chez la souris. En effet, l'inoculation de souris avec des sporozoites prétraités avec la centanamycin ne provoque pas de stade sanguin et les souris immunisées avec une seule dose sont protégées contre des infections ultérieures par des sporozoites non-traités. Ces résultats démontrent que l'atténuation chimique des sporozoites serait une alternative efficace pour la production d'un vaccin antimalarique.

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CONTRIBUTIONS OF AUTHORS

The experimental design, laboratory work (other than the *P. falciparum* and *P.c. adami* experiments in Chapter 4), data analysis, and writing contained in this thesis were performed by the Candidate.

S. K. Yanow designed the initial *P. falciparum* and *P. c. adami* experiments to test centanamycin. She provided advice on the work presented in Chapters 3, 4, and 5, and wrote (in conjunction with the Candidate) the article presented in Chapter 4.

T.W. Spithill, as well as A. Rodriguez, are included as senior authors on all papers. They both played supervisory roles in these projects, contributed to the design and interpretation of experiments, and with the Candidate, contributed to the writing of the articles in Chapters 3, 4, 5.

M. Lee provided centanamycin and is a senior collaborating colleague in this project. He was included as an author on the papers presented in Chapters 4 and 5.

G. Pradel completed electron microscopy on the sample mosquito midguts provided by the Candidate and is an author on the paper presented in Chapter 4.

T. Ono assisted in experimental design of the knockout construct used in Chapter 3. All experimental work was performed by the Candidate.

A. Sato synthesized centanamycin, and has been included as an author of Chapter 4.

TABLE OF CONTENTS

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

CHAPTER 1:

Introduction

The development of an effective control strategy for malaria is critical to curb the devastation caused by the millions of clinical cases and deaths from this parasite each year. The disease also has tremendous social and economic impacts in endemic countries [1, 2]. Malaria infection is initiated when an infected *Anopheles* mosquito injects *Plasmodium* sporozoites into a mammalian host. The sporozoites make their way from the skin to the blood stream, and finally to the liver. In the liver they eventually invade hepatocytes and transform into exoerythrocytic forms (EEFs) that replicate to produce schizonts containing thousands of merozoites [3]. These merozoites are released into the circulation of the host and subsequently invade erythrocytes. The blood stages are responsible for producing the symptoms of the disease. Many attempts have been made to try to control the transmission of the disease using various techniques, which include transfection technology to better understand the biology of the parasite, the development of novel antimalarial compounds, and the development of an effective vaccine.

Targeting the transmission stages of the malaria parasite will require a focus on the sexual stages within the intermediate host, or within the definitive host, the mosquito. A transmission-blocking strategy will prevent the spread of malaria [4, 5] and would have the potential to reduce the burden of disease and death [6, 7]. Targeting the parasite in this manner would also greatly prolong the useful life of antimalarials or vaccines by preventing the spread of parasites that become resistant to those approaches [8, 9].

The malaria parasite also experiences a severe population bottleneck within the mosquito. The *Plasmodium falciparum* parasite population is reduced from millions of blood stage parasites within an infected patient to only one or a few oocysts in the infected mosquito [10, 11]. It is here, before replication of the parasite within the oocyst to produce infective sporozoites, that the possibility of using a genetic or biochemical technique to block transmission is the most feasible.

Genetic manipulation of malaria parasites is a relatively new, but still complicated technique [12]. The development of gene knockout strategies has been pivotal in advancing our understanding of *Plasmodium.* Many genes essential for the development of the parasite within the mosquito have been manipulated [13-20]. Some of these genes include protein kinases that are crucial for the signaling pathways in this stage of the life cycle [20, 21]. Protein kinases that belong to the calmodulin-dependent protein kinases, including the calcium-dependent protein kinases have been at the forefront of the studies on signaling during malaria transmission [16, 17]. Specific inhibitors directed against these kinases could prevent the transmission of the parasite by blocking these signaling cascades.

Blocking the transmission of the parasite using antimalarial chemotherapies has proven to be an elusive concept. The discovery of novel antimalarials, which could block transmission and halt the worsening problem of resistance to current chemotherapies is essential. Given the high AT content of malaria DNA (~80%, [22, 23]), AT sequence-specific DNA alkylating agents could be a novel approach to this challenge. These compounds will bind in the

minor groove of DNA and alkylate on a specific recognition sequence. Treatment of the intermediate host while circulating gametocytes are present in the blood, may damage the gametocyte and affect the ability of the parasite to replicate in the mosquito, thus decreasing the number of sporozoites in the midgut and salivary glands and the transmission potential of the mosquito.

Recently, the viability of the use of a whole-organism sporozoite vaccine has gained popularity [24, 25]. Sporozoites that have been attenuated through gamma irradiation or using genetic manipulation by knocking out an essential gene have been shown to be viable alternatives in the search for a malaria vaccine [26]. These attenuated sporozoites produce protective immunity within the intermediate host and prevent blood stage infection, thus eliminating the possibility of symptoms in the host, and preventing the possibility of transmission of parasites to a subsequent host.

Given the number of clinical cases and deaths each year from malaria, there is an urgent need for new chemotherapies and control strategies, especially those directed against the transmission stages of the parasite. Here, I will explore three strategies to block the transmission of the malaria parasite using *in vitro* and *in vivo* models. These strategies may provide new methodologies that can be applied to eliminate the threat of this deadly parasitic infection.

Study objectives

The objectives of this study are to evaluate strategies to block the transmission of the malaria parasite from the definitive host, the mosquito, to the

intermediate host using molecular and pharmacological methods. My specific goals will be to:

(1) Characterize the function of a novel CaMK-related protein (CARP) predicted to be involved in transmission to the mosquito.

(2) To investigate the transmission-blocking activity of centanamycin, an AT sequence-specific DNA alkylating agent.

(3) To evaluate the *in vitro* chemical attenuation of sporozoites (CAS) using centanamycin.

CHAPTER 2.

Review of Pertinent Literature

Life Cycle of *Plasmodium*

Malaria is one of the most prevalent infectious diseases worldwide, affecting up to 500 million people with up to 2 million people dying from the disease each year [1]. The disease also has tremendous social and economic impacts in endemic countries [2]. The complexity of the life cycle contributes to the success of the parasite. *Plasmodium*, part of the phylum Apicomplexa, has arguably one of the most complex life cycles, taking the parasite through development in the definitive host in the mosquito, to the multiple rounds of replication in the liver of the intermediate host, to obligate, intracellular, asexual replication in the erythrocytes, which is the stage responsible for the symptoms of the disease (Fig. 1) [3].

Malaria infection starts when an infected *Anopheles* mosquito probes for a blood meal and injects sporozoites into the skin of the host. The sporozoites enter the blood stream and are transported to the liver. The sporozoites invade hepatocytes and develop into exoerythrocytic forms (EEFs). Each EEF enters into multiple rounds of replication to produce thousands of merozoites. When the infected hepatocyte ruptures, the merozoites are released and rapidly invade erythrocytes. Here each parasite undergoes schizogony and propagates the asexual cycle of the infection. Some merozoites develop into gametocytes instead of producing schizonts. The mechanism governing this switch is not well understood, but molecular and environmental factors are both thought to play a

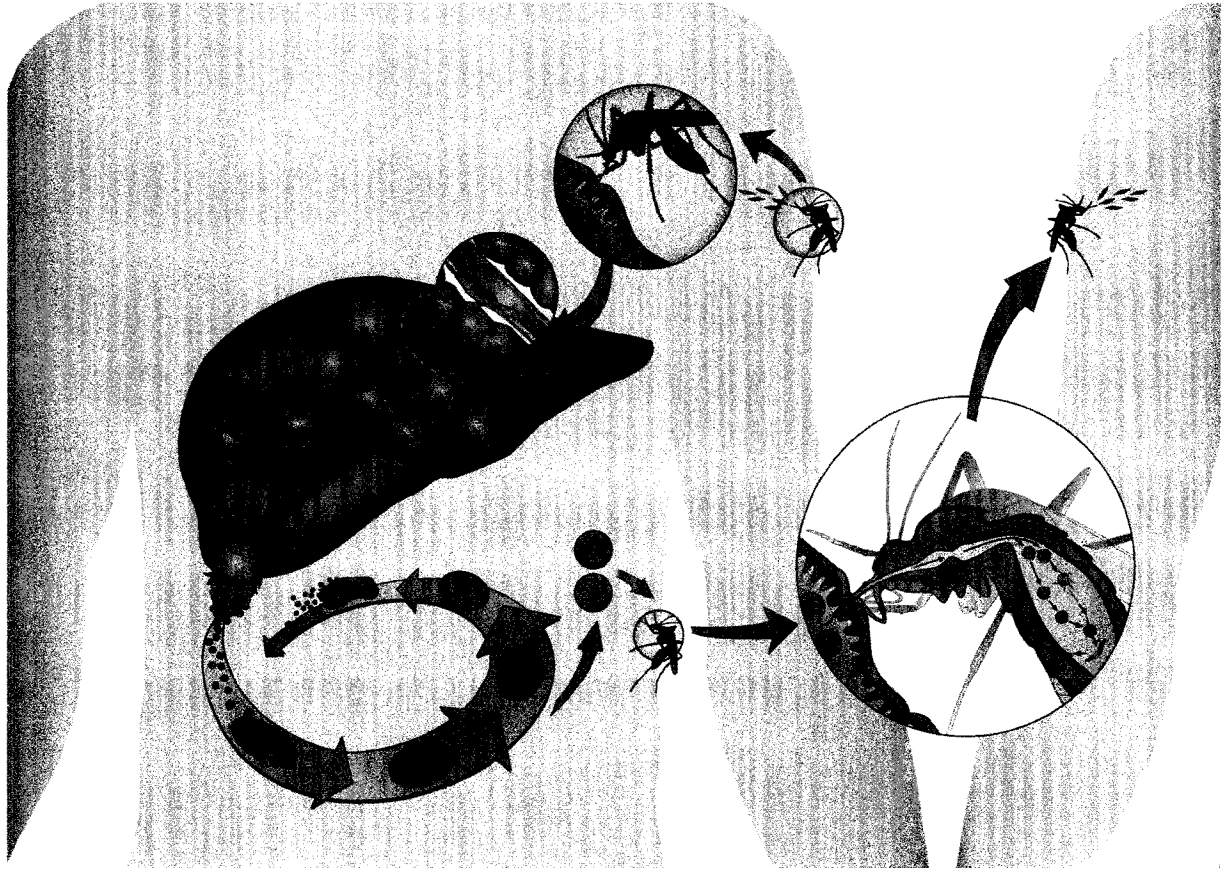

Figure 2-1. Life cycle of *Plasmodium* parasites. The complex life cycle alternates between proliferative and non-proliferative stages. Four species of *Plasmodium* infect humans (*P. falciparum, P. vivax, P malarie*, and *P. ovale*), while three of the most prominent rodent species include *P. berghei*, *P. yoelii*, and *P. chabaudi*. (Reproduced with permission from M. Mota, Instituto de Medicina Molecular, Unidade de Malária, Universidade de Lisboa, Portugal.)

role [27, 28]. Gametocytes are ingested by mosquitoes in a subsequent blood meal. Inside the mosquito midgut, the gametocytes differentiate into gametes, with the male (micro-) gametocyte undergoing 3 rounds of mitosis within 15 minutes. The gametes fuse to form zygotes, which then transform into the invasive ookinetes. Ookinetes migrate to the space between the midgut epithelium and the basal lamina of the mosquito where they differentiate into the oocyst. Thousands of sporozoites develop from the multinucleated oocysts and are released into the hemolymph of the mosquito. The sporozoites make their way to the salivary glands where they remain viable, but do not replicate [29, 30].

Investigations into the biology of sporozoites have long lagged behind those in the blood stages, mainly because of the inherent inaccessibility of this stage. Sporozoites must be isolated from infected mosquitoes using manual dissection, and are really only obtained in relatively small numbers. Despite these limitations, the understanding of parasite biology in terms of *Plasmodium* sporozoite development, migration and host cell infection is expanding.

Sporozoite gene and protein expression

Considerable progress has been made in recent years in the identification of genes and proteins expressed in the sporozoite stages. These studies have been greatly aided by the publication of the genome sequences of different human, rodent, and primate species of *Plasmodium* [22, 23, 31, 32]. Oligonucleotide microarrays have been used to identify many genes that are transcriptionally active in salivary gland sporozoites, and these studies have been able to differentiate those genes from those upregulated in the blood stages [33, 34].

Proteomic analysis of the stage-specificity of malaria proteins revealed that the sporozoite proteome is very unique, with almost half of the proteins found upregulated in the stage expressed only in sporozoites [35]. Many of the proteins identified in proteomic analysis of sporozoites are cell surface and organelle proteins [35]. These proteins may be involved in motility, invasion, and the generation of the parasitiphorous vacuole, and therefore may be localized to the micronemes and rhoptries of the sporozoite [23]. Interestingly, sporozoite expressed genes are distributed throughout the 14 chromosomes of the parasite, and they do not necessarily cluster in active regions. A select few sporozoite genes have been found in recent decades to be pivotal to transmission of the parasite.

Circumsporozoite Protein

The circumsporozoite protein (CSP) is a distinctive molecule of *Plasmodium* that is not found in other Apicomplexa. Encoded by a single copy gene, it covers the entire surface of sporozoites [36, 37]. The protein is also found on the plasma membrane of early EEFs and in the cytoplasm of infected hepatocytes [38, 39]. When salivary gland sporozoites are incubated with antibodies to CSP, they undergo a characteristic change. A thick precipitate is formed on the surface of the sporozoite and it is gradually shed from the posterior end of the parasite; this is known as the circumsporozoite protein (CSP) reaction. The reaction is a demonstration of the continuous secretion of CSP from the anterior end of the parasite, the incorporation on the plasma membrane, and its release at the posterior end [40, 41]. When sporozoites are placed on glass

surfaces, the released CSP appears in circular trails and is embedded in vesicle-like particles, although the composition of these particles is unknown [42]. Due to the abundance, localization to the surface of the sporozoite, immunogenicity, and key role in parasite invasion of hepatocytes, CSP is one of the leading candidate proteins that could be used in the development of a malaria pre-erythrocytic vaccine [43].

Thrombospondin-Related Anonymous Protein

The thrombospondin-related anonymous protein (TRAP) is a member of a family of proteins, which have also been identified in other apicomplexan parasites [44-46]. Two members of the family have been identified in *Plasmodium*: TRAP in sporozoites and CSP- and TRAP-related protein (CTRP) in ookinetes [14, 47]. The TRAP family members connect the parasite surface receptors with the molecular motor that drives parasite motility and cell invasion. TRAP is localized to the micronemes and plasma membrane of sporozoites. When the *trap* gene is deleted using transfection, *P. berghei* parasites develop normally within oocysts, but the free sporozoites are immobile and do not infect hepatocytes [48, 49]. Ookinetes that lack the *ctrp* gene have impaired motility and do not invade the mosquito midgut [14, 50, 51].

Sporozoite development

The diploid zygote that is formed in the midgut of the mosquito transforms into the motile and invasive ookinete that undergoes meiosis. This leads to the formation of four haploid genomes within a single nucleus [20]. The ookinete

traverses several midgut epithelial cells, migrates through the intercellular space, to the basal lamina where it transforms to the oocyst [30]. The nucleus of the oocyst is polyploid, and after about 10 days, the genome rapidly divides to produce up to 8000 nuclei. Thousands of sporozoites bud from the sporoblast surface. There is little information about the molecular events that regulate sporogenesis, but a partial deletion of CSP produces sporozoites of which half are shortened and deformed [52, 53].

Gliding motility

The invasive sporozoite stages of *Plasmodium* are motile and can enter cells quickly. Sporozoites glide on solid substrates at a rate of about 1 to 10 μm/s. The sporozoites exhibit a circular gliding pattern that is driven by an actin-myosin motor [44, 54, 55]. The intracellular motor is composed of MyoA and is linked to the substrate surface by TRAP [56]. The molecular motor pulls TRAP backward leading to forward movement, as extracellular TRAP is attached to a fixed substrate, although the nature of the substrate in the mosquito is unknown [57]. If TRAP encounters cellular receptors in the mosquito, a moving junction is formed between the host cell membrane and the parasite, and the parasite invades the host cell as is true for *Toxoplasma gondii* [58]. CSP is also involved in gliding motility. Large amounts of CSP are continuously released at the posterior end of the parasite while gliding [59], although the involvement of CSP with the actin-myosin motor has not been established.

In addition to sporozoites, ookinetes seem to rely on an actin-myosin motor. The protein CTRP is essential for ookinete motility suggesting a design

that is similar to the motility demonstrated in sporozoites [14]. A calcium-dependent protein kinase, CDPK3, was recently shown to have an important function in regulating motility of the ookinete in the mosquito midgut. Parasites lacking CDPK3 failed to engage the mosquito midgut epithelium due to a reduction in their ability to glide [60].

Invasion of mosquito salivary glands

Midgut oocysts release sporozoites into the hemocoel of the mosquito about 10 to 14 days after the initial blood meal. Sporozoites released into the hemocoel will invade the distal lateral lobes of the salivary glands [61, 62]. A random peptide library was recently used to identify one peptide (SM1) that binds to the luminal side of the mosquito midgut and the distal lobes of the salivary glands. This peptide had a significant effect on oocyst formation and sporozoite invasion of the salivary glands in mosquitoes [63]. Salivary gland invasion is thought to involve both CSP and TRAP. CSP is thought to be involved in the initial attachment of the sporozoites to the salivary glands. This phenomenon is evidenced by the observation that recombinant CSP protein binds only to the median and distal lobes of the salivary glands [64]. Sporozoites that lack the *trap* gene are unable to glide and therefore unable to invade the salivary glands [48]. Interestingly, *trap*$^-$ sporozoites actually adhere to the salivary glands. Mutations in various regions of the *trap* gene lead to varying reductions in the number of sporozoites that are able to invade the salivary glands [13, 65]. Once the sporozoites invade the salivary glands, they demonstrate considerable

movement within the salivary ducts and very rapid ejection with the saliva when the mosquito probes for a blood meal [66].

The differences between oocyst and salivary gland sporozoites

Sporozoites released from oocysts and salivary gland sporozoites must be able to invade numerous tissue types, including several cell types within the mosquito host. Oocyst and salivary gland sporozoites show very different phenotypes [41, 67]. Salivary gland sporozoites are highly infectious to the mammalian host, migrate in a typical circular gliding pattern, and can elicit strong protective immune responses. Oocyst sporozoites are essentially noninfectious to the mammalian host, do not show the typical circular gliding motility, and do not produce protective immunity in mice [41]. Differences in gene expression between these two sporozoite stages were shown with differential expression profiling [68]. Thirty genes were upregulated in the salivary gland sporozoites, which were termed UIS. These proteins are predicted to encode molecules with various functions, including kinases, phosphatases, and transcriptional regulators. A number of the UIS proteins have predicted transmembrane domains [68]. All of these genes, with the exception of one, were not expressed in blood stages. This suggests that these genes are all involved either in the interactions with the salivary gland or mammalian tissues and possibly in EEF formation. Perhaps theses changes in gene expression could be induced in oocysts by some signal after contact with the salivary glands.

Sporozoite migration and invasion of hepatocytes

When mosquitoes probe the skin of the host, they deposit *Plasmodium* sporozoites. Given that salivation stops when the mosquito locates and begins to take in blood, it has been shown that most sporozoites are deposited into the dermis and not directly into circulation [69]. Removal or heat-treatment of the mosquito injection site can actually prevent malaria infection in animals [70, 71]. Quantitative PCR has shown that the mean number of sporozoites injected by a single mosquito is 123, but ranges from no sporozoites to 1297 [72]. When sporozoites are injected directly into the blood stream via intravenous injection, the parasites migrate to the liver within minutes [73]. When the injection of parasites occurs intradermally, either artificially or by mosquito bite, sporozoites have been shown to leave the site between 1 and 3 hours after injection [69]. Some of these parasites (approximately 20%) have been shown to leave the injection site via the lymph nodes [69, 74].

After injection into the skin, sporozoites must make their way to the hepatocyte. The mechanism that the parasite uses to locate the liver are unknown, although they have been shown to migrate up to 5 times faster through non-hepatic cells compared to hepatocytes [75]. So far, the only host cell molecules shown to bind sporozoites are highly sulfated heparan sulfate proteoglycans (HSPGs), although the nature of the receptor is unknown [76]. Evidence has shown that an interaction between HSPGs and CSP arrests sporozoites in the liver [76, 77]. The sporozoite must then shed the CSP bound to the host cell and traverse the sinusoidal lining, perhaps through Kupffer cells [78, 79] or between

endothelial cells [80]. The events that take place during invasion of hepatocytes are unknown.

Once the sporozoite invades the hepatocyte, it develops and multiplies within the cell as an EEF, generating thousands of new parasites (merozoites). Little is known about the development of these EEFs. Recently, several genes involved in EEF development in the liver were reported. These include UIS3 [81] and UIS4 [82], P36p (a protein localized to the micronemes) [83], and P52 and P36 [84]. Although the nature of the involvement of these proteins in parasite invasion or EEF development is unknown, the deletion of the genes encoding these proteins results in EEF formation, but the parasites are deficient in replication. Further investigation of the molecular and cellular interactions governing sporozoite biology are needed to identify proteins and signaling events involved in motility, cell invasion and development of the transmission stages of *Plasmodium*.

Protein kinases

A major regulatory mechanism of most cellular processes occurs through signal transduction mechanisms, which may involve protein phosphorylation by protein kinases [85]. As such, the *Plasmodium* genome has been predicted to encode protein kinases. Recently, two independent studies identified 65 [86] and 99 [87] genes encoding proteins that contained predicted eukaryotic protein kinase (ePK) domains. Most *Plasmodium* sequences that contain ePK-related domains can be distributed into established families: although several form distinct phylogenetic branches that do not cluster with any of these groups [87].

Recent studies indicate that the *Plasmodium* kinome contains 3 classes of enzymes: (i) orthologues to the kinases from higher eukaryotes; (ii) enzymes that can be assigned to a particular group, but that do not have a clear eukaryotic orthologue; and (iii) enzymes that do not belong to any established ePK group [86, 87]. As yet, only a few essential functions of *Plasmodium* protein kinases have been positively identified.

Protein kinases involved in the transmission stages of malaria

Transfection techniques using the mouse malaria model, *Plasmodium berghei*, allow for the determination of essential functions in the transmission stages of the disease [12]. Gene knockout clones can be generated using the asexual stages, before transmission to mosquitoes to determine the mutant phenotype. These reverse genetics studies have identified essential functions for four protein kinases in the transmission stages of *P. berghei.*

Calcium-dependent protein kinases regulate male gamete formation and ookinete motility

The *Plasmodium* family of calcium-dependent protein kinases (CDPKs) is characterized by an N-terminal Serine/Threonine (Ser/Thr) kinase domain that is linked by an auto-inhibitory region to a C-terminal calmodulin-like domain, which is comprised of 4 calcium-binding EF-hand domains [86, 87]. Both CDPK3 and CDPK4 are expressed mostly during the gametocyte and ookinete stages [86, 87]. CDPK3 knockout parasites form ookinetes, but they lack the ability to glide and fail to infect the midgut epithelium of the mosquito midgut

[60, 88]. Conversely, CDPK4 is an essential protein in the male gametocyte, where it is responsible for progression through the cell cycle [17]. Microgametocytes that lack the *cdpk4* gene cannot replicate their genome such that parasites lacking the enzyme do not release gametes and fail to infect mosquitoes.

Male gamete release is also controlled by a MAP kinase

An atypical mitogen-activated protein (MAP) kinase, Map-2, was also shown to be involved in male gamete formation [18]. This protein is only expressed in male gametocytes [19]. Three independent studies using the *P. berghei* rodent malaria model determined that disruption of the *map-2* gene prevents transmission of the parasite by disrupting exflagellation in the male gamete [19, 89, 90]. The development of the *map-2*$^-$ male gametocytes is blocked after replication and mitosis, shortly before wild type gametes become motile [19].

Cell cycle progression in the zygote is controlled by a NIMA-related kinase

When malaria parasites are ingested by the mosquito, the gametes fuse and meiosis occurs [19]. An essential function for Nek-4 was established just prior to replication in the zygote [19, 20]. Nek-4 is a member of the never in mitosis/Aspergillus (NIMA)-related protein family present in *Plasmodium*. The Nek-4 enzyme is expressed exclusively in the female gametocyte [19]. Male gametocytes (microgametocytes) lacking the *nek-4* gene fuse with the female *nek-*

4- parasites, but replication fails to occur. These Nek enzymes are present in all eukaryotes and have important functions in controlling mitosis and meiosis [91].

Calcium/calmodulin-dependent protein kinase (CaM-kinase)

Cellular processes as diverse as contraction, secretion and gene expression have been shown to be controlled in various organisms by calcium/calmodulin-dependent protein kinase (CaM kinase) [92]. CaM kinase is a ubiquitous enzyme that has been found in all eukaryotes examined [93]. Second messengers, such as calcium, interact with target molecules to initiate a signaling cascade that leads to a change in cellular function. Elevation of calcium concentrations in the cytosol of cells can occur by the release from intracellular stores or by an influx by way of regulated ion channels and transporters. There are many intracellular-binding calcium proteins, with calmodulin being the major molecule [94]. Calmodulin binds four calcium ions [94]. CaM kinase requires calcium-bound calmodulin for activation, but is able to phosphorylate and alter the function of a variety of substrates [93].

Several CaM kinases have been predicted in the *Plasmodium* genome [86, 87]. Some of these kinases belong to the family of CDPKs discussed earlier, however a few kinases form a group that do not contain the four calcium-binding EF-hand motifs characteristic of the CaM kinases [86]. Inhibition of these CaM kinases using modulators of intracellular calcium and calmodulin antagonists blocks the differentiation of zygotes to ookinetes using *Plasmodium gallinaceum* [95].

Plasmodium kinase inhibitors block transmission to mosquitoes

Recent studies on *Plasmodium* protein kinases have begun to explore the possibility of generating selective protein kinase inhibitors for antimalarial and transmission-blocking chemotherapies [96]. These inhibitors could target a single protein kinase that could potentially have essential regulatory functions in both asexual and sexual stages. As with other therapies, this approach may be vulnerable to the development of parasite drug resistance, but could be used in combination with other antimalarial agents as is now recommended by WHO [97, 98]. A protein kinase inhibitor could also potentially be designed so that it hits several targets in several parasite stages, such as a family of protein kinases that are upregulated in any of the malaria life cycle distinct stages. Finally, an inhibitor of these enzymes could target the transmission cycle alone, by killing the gametocytes themselves, or by targeting parasite development in the mosquito after the blood meal.

Targeting the parasites once they have been taken up by the mosquito has clear advantages. Malaria experiences a severe population bottleneck within the mosquito. The parasite population is reduced from about 10^{12} blood stage parasites in an infected patient at 2% parasitemia to only one or a few oocysts in the infected mosquito [10, 11]. In addition, since only a few parasites would be under selective pressure, the possibility of the emergence of drug resistance would be much less likely [11].

Transmission-blocking drugs

Increasing parasite resistance to standard antimalarial drugs is a widespread problem, adding to the morbidity and mortality of the disease. Chloroquine and sulfadoxine-pyrimethamine are two antimalarials to which the malaria parasite has been able to develop resistance [99, 100]. The transmission dynamics of the disease has been shown to play a key role in the spread of drug resistance alleles [10, 11]. The primary aim of antimalarial chemotherapy must be to eliminate the asexual parasites in a symptomatic individual [101]. However, to decrease overall parasite transmission and to try to curb the spread of resistant alleles, the number of infective gametocytes or infectivity in mosquitoes must be controlled at the population level [11]. Many drugs, including chloroquine and sulfadoxine-pyrimethamine fail to kill mature gametocytes [11, 102, 103]. Interestingly, mature gametocytes can persist in circulation for weeks, and it has been shown that as parasites become resistant to drugs such as sulfadoxine-pyrimethamine, gametocyte numbers and infectivity have been shown to increase [104, 105].

It is now widely recognized that new antimalarials should have at least three key properties in order to try to limit the threat of drug resistance. The drugs should hit multiple targets in the parasite, making it less likely that resistance would emerge [106]. The antimalarial should be cleared rapidly to try to minimize the possibility that new parasites are exposed to subtherapeutic levels of the drug [107]. Finally, the antimalarial should block the transmission of the disease.

Comparison of transmission-blocking activities of drugs in a rodent malaria model

Many commonly used antimalarials do not affect the number of parasites that are taken up by the mosquito. Many different antimalarial treatments have been initially studied using rodent malaria models as a first step to identify potential transmission-blocking properties. One model, *P. berghei*, strain ANKA, can be combined with a laboratory strain of the mosquito, *Anopheles stephensi,* to test the effectiveness of potential transmission-blocking drugs. Primaquine is a commonly used antimalarial that has been shown to be partially effective against gametocytes. Interestingly, doses of the drug as high as 100 mg/kg given 90 minutes before feeding mosquitoes, have no effect on the number of oocysts on the midguts of the mosquitoes 12 days later [108]. In contrast, atovaquone, which was given to mosquitoes through a blood meal, was able to reduce the number of oocysts by 46.1 % at a dose of 10 mg/kg and by 71 % at 100 mg/kg. The higher dose of 100 mg/kg also reduced the number of infective sporozoites in the salivary glands by 22 % [109]. Other experiments try to simulate what may be occurring in the field. For example, sera from humans that receive a drug could be mixed with healthy blood and fed to *P. berghei* - infected mosquitos through membrane feeds. When the transmission-blocking activity of Malarone is examined using this experimental design, the number of oocysts is reduced by approximately 43 % [110].

These studies highlight the difficulties associated with the ability of various antimalarials, effective against the asexual blood stages, to block the transmission stages as well. Considerable efforts should be concentrated on

interrupting the transmission cycle to help in the efforts to both limit the spread of existing drug-resistant parasites, and helping to prevent the development of drug resistance [111, 112]. As such, new approaches to drug design must be employed to reduce symptomatic parasitemia in the human host, and also block transmission to the mosquito. Novel antimalarials should employ new strategies to target the parasite.

Sequence-specific DNA binding agents

Given the widespread resistance to current chemotherapies, and the need to develop novel antimalarials to combat the disease, unique methods to target the parasite must be explored. Recent studies, many initiated after the genome of *Plasmodium falciparum* was first published [22], have explored possible ways to target the genome of the parasite, for example using knockout technologies [12, 19]. One approach, which was designed to exploit the extraordinary AT-richness of the *Plasmodium* genome (~80%, [22]), is the use of AT-specific DNA binding agents.

Mechanisms of action of DNA alkylating agents

Alkylation occurs when an alkyl group is transferred from one molecule to another [113]. Alkylating agents are widely used in chemistry, as the alkyl group is one of the most common organic molecules. Alkylating agents react with the nucleotides. AT- alkylating agents specifically involve reactions with the purine, adenine and the pyrimidine, thymine. The AT-specific alkylating agents add additional alkyl groups onto molecules. This in turn inhibits the correct utilization

of the bases and causes one of three situations, depending on the organism and the mechanism of the compound. The first occurs after the alkylating agents attach alkyl groups to DNA bases. The alteration results in the fragmentation of DNA by repair enzymes in their attempts to replace the alkylated bases [114]. Alkylated bases prevent DNA synthesis and RNA transcription from the affected DNA [113]. Alkylating agents can also cause DNA damage by the formation of cross-bridges. In this process, two bases are linked together by a compound that has two binding sites. The bridges can be formed within a single molecule of DNA or a cross-bridge may connect two different DNA molecules. This cross-linking prevents DNA from being separated for transcription [115]. A third mechanism of action of alkylating agents causes the mispairing of the nucleotides leading to mutations [115].

The mechanism by which organisms maintain genomic stability when subjected to DNA damaging agents is through DNA repair. The function of DNA repair is to remove potentially deleterious lesions through either damage reversal or damage excision [116]. Damage excision processes can be classified as nucleotide excision repair (NER), characterized by dual incisions in the phosphodiester backbone, 5' and 3' to the damaged site [117], or by the base excision repair (BER) process, where damaged bases are excised by the cleavage of the N-glycosidic bond [117]. The BER pathway has been the only characterized repair pathway in *P. falciparum* [118, 119].

Activity of DNA-binding agents against *Plasmodium*

The mechanism of the AT-specific DNA binding agents and the high AT content of malaria DNA has led to a number of different AT-specific DNA binding

agents being tested against *Plasmodium* (Table 1). Some of these compounds have exhibited significant activity against the parasite. Pentamidine, along with other diamidine compounds, has been used for decades to treat human protozoal infections [120]. Pentamidine is not membrane-permeable, so its action relies on parasite-specific uptake mechanisms, such as active transporters [121]. When pentamidine was tested against *P. falciparum* cultures *in vitro*, the half maximal inhibitory concentration (IC_{50}) was 58.4 nM (Table 1). Interestingly, two other AT-specific DNA binding agents, adozelesin and bizelesin, exhibit an IC_{50} of 800 - 5000 fold less than pentamidine (0.07 and 0.01 nM respectively, Table 1). Adozelesin forms adducts on DNA, whereas bizelesin forms crosslinks between strands. Although both show potent anitmalarial activity *in vitro*, both are limited by myelotoxicity.

Interestingly, although the hypothesis that the AT DNA-binding drugs could be used as antimalarials, based on the AT content of the parasite's DNA, has existed for decades, the theory has been validated only twice using bioinformatics [122, 138]. Recently, an *in silico* approach was used to analyze the genome of *P. falciparum* to define the potential use of adozelesin and bizelesin as antimalarials [122]. Adozelesin and bizelesin recognize selective sequences within DNA. Adozelesin recognizes $(A/T)_3A$, while bizelesin prefers $T(A/T)_4A$ (Table 1, [122]). When bioinformatic analysis was used to calculate the frequency of binding sites in the *Plasmodium* genome compared to human genome, it was found that the *P. falciparum* genome contained 3.9 and 7 fold more binding sites for adozelesin and bizelesin respectively [122]. These sites seem to be located in super AT-rich islands, located on each chromosome, which constitute a region of about 1-4 kbp in length and are

Table 2-1. Anti-plasmodial activities of A/T–specific DNA binding agents.

Compound	Sequence motif[A]	Bond type[B]	Mode of DNA binding	IC_{50} against *P.f.*	Drug discovery stage	Limitations of therapeutic use	Refs
Adozelesin	$(A/T)_3A$	Covalent	Adduct formation	0.07 nM	Phase II trial for cancer; not pursued Preclinical testing in animal models of malaria	Myelotoxicity	[122, 123]
Bizelesin	$T(A/T)_4A$	Covalent	Interstrand crosslinking	0.01 nM	Phase I trial for cancer *In vitro* testing for malaria	Myelotoxicity	[122, 124, 125]
Centanamycin	AAAAA	Covalent	Adduct formation	1.8 nM	Preclinical testing in animal models of malaria	Not determined	[123, 126]
Furamidine (DB75)	AAAA and ATTA	Non-covalent	Unknown[C]	15.5 nM	*In vitro* testing for malaria Pro-drug DB289 pursued	Lacks oral bioavailability	[127-130]
Pafuramidine (DB289; pro-drug of Furamidine)	Not applicable	Not applicable	Not applicable	11400 nM	Phase IIb trial for malaria treatment Phase II trial for malaria prevention	Ineffective at once daily, 3-day regimen for malaria therapy	[128-134]
Pentamidine	AATT	Non-covalent	DNA Intercalation[C]	58.4 nM	Phase II proof-of-concept study for malaria Clinically used for treatment of *T.b.g.*, antimony-resistant Leishmaniasis and AIDS-related *P. jiroveci* pneumonia	High toxicity, poor oral bioavailability	[127-129, 135]

Distamycin A	AAATTT	Non-covalent	Reversible	618 nM	*In vitro* testing for malaria	High toxicity	[136, 137]
Netropsin	AATT	Non-covalent	Reversible	3000 nM	*In vitro* testing for malaria	High toxicity	[136]
DAPI	AATT	Non-covalent	Reversible	9.7 nM	*In vitro* testing for malaria	High toxicity	[136]
Hoechst 33258	AATT	Non-covalent	Reversible	35.6 nM	*In vitro* testing for malaria	High toxicity	[136]

[A] "Pu" denotes a purine, "N" denotes any nucleotide.

[B] All compounds bind preferentially in the minor groove of DNA.

[C] The mode of action of these diamidine compounds still remains to be elucidated, but it has been suggested that the intracellular targets include mitochondrial respiration, hemoglobin degradation and DNA replication.

nearly 100 % AT. These predictions imply that the potent antimalarial effects of adozelesin and bizelesin could be at least partially due to the high AT content of malaria DNA. This class of drugs should be considered as potential antimalarial treatments to try to control the disease.

Whole organism malaria vaccines

Control of the widespread devastation of malaria has been one of the biggest medical challenges confronting mankind. An effective vaccine against *P. falciparum* is a goal which has not been realized as of yet, but is seen as a requirement for the global eradication of malaria. With the advances in molecular biology and increased knowledge of the parasite, a range of novel vaccine candidates has been developed [43, 139, 140]. However, the majority of these vaccine candidates have failed to provide high levels of protective immunity in humans [141]. One vaccine, which was first reported using bird malaria in 1949 [142] and then in rodent models over half a century ago, is still in the forefront as the most promising vaccine candidate, albeit with some development challenges. This whole-organism sporozoite vaccine had been shown to induce sterile protection in humans after vaccination [143].

Radiation-attenuated sporozoites (RAS)

A study completed in 1967 showed that immunization of mice with sporozoites attenuated using gamma irradiation could provide protection against subsequent challenge with infectious sporozoites [144]. Consideration of this type of whole-organism vaccine has been recently revived. This type of

vaccination strategy is optimal in terms of controlling individual and population risk, since the vaccine prevents the sporozoite from developing into the blood stages [145]. It has the advantage of acting before the onset of pathology that is associated with the intraerythrocytic stage of the disease.

Radiation-attenuated sporozoites (RAS), like normal sporozoites, penetrate the hepatocytes of the inoculated host and begin intracellular development [26, 83]. Sporozoites can be delivered by irradiated mosquitoes or through intravenous (i.v.) injection if the sporozoites were dissected first and then irradiated [144]. Once these RAS start development, they normally progress only to the trophozoite stage of EEF development, and, for the most part, do not progress through nuclear division [146, 147]. The EEFs that result from RAS are able to persist at least 48 hours, and perhaps longer [146, 147]. This arrest of development within the liver is thought to be responsible for the generation of protective immunity within the host. The immune responses to RAS are thought to be mediated by both $CD8^+$ T cells and by antibodies, which target infected hepatocytes and sporozoite surface proteins, respectively [145, 148, 149].

Although the overall evidence seems to indicate that whole organism sporozoite vaccines are a viable method for vaccination against malaria, technical and safety concerns need to be addressed. These include mass production of sterile parasites, proper storage by cryopreservation to maintain low infectivity and high immunogenicity, and the safety of a mosquito-derived vaccine with the risk of coadministration of unknown pathogens [26, 143]. The most pivotal concern with RAS is the correct irradiation dosage. Too much irradiation produces sporozoites that cannot invade hepatocytes and will therefore not

produce an immune response, whereas too little irradiation will produce breakthrough infections [150]. Finally, the duration of protection provided by RAS is less than 1 year [24], which suggests that in addition to the initial immunization, booster doses of RAS will be need to be administered, and close monitoring of boosting by natural infections will have to be completed. Sanaria, Inc., a US-based privately held company in Rockville, MD, has made the development of a RAS vaccine its major portfolio and is poised to address some of the issues associated with the development of the vaccine [143]. Given the major consideration of the delivery of sporozoites with the correct irradiation dosage, other avenues of attenuation have been explored.

Genetically-attenuated sporozoites (GAS)

Genetically-attenuated sporozoites (GAS) are sporozoites that, like RAS, undergo arrested development in the liver and stimulate a protective immune response against challenge infections in rodent malaria models [26, 81-84]. GAS differ from RAS in that the attenuation is due to the deletion of specific genes that are essential for hepatocyte development. These include the deletion of UIS3 [81] P36p [83], and simultaneous deletion of UIS3/UIS4 [82] genes in *P. berghei*, as well as deletions of UIS3 and UIS4 [151], and simultaneous deletion of P52 and P36 genes [84] in *P. yoelii*. These GAS infect hepatocytes and progress to EEF development, but EEFs disappear more quickly (within 24 hours) than RAS [83]. The complete protection provided by GAS constituted a proof-of-concept that genetically modified whole sporozoites might, like RAS, be useful malaria control tools. Some safety concerns still exist, however, with the risk of breakthrough

infections from reverted parasites or if the liver stage development of the parasite can still be completed without a disrupted gene. These concerns could be addressed by using new transfection methods, such as negative selection [152]. The production of the vaccine is still hampered by the same technical and logistical problems as exist for RAS. As well, *P. falciparum* GAS have yet to be developed. In addition, there is the added mistrust of the population for genetically modified products, which is a serious issue that has to be considered.

The question of the feasibility of widespread immunization with RAS or GAS still remains. Given the success of the generation of protective immunity by RAS in over 90% of immunized individuals for at least 10.5 months against multiple isolates of *P. falciparum*, and the need for a malaria vaccine, it seems obvious that further development of this approach is warranted [143]. The development of an effective vaccine against one of the deadliest parasitic diseases is of utmost importance.

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CHAPTER 3:

A putative *Plasmodium berghei* CaMK-related protein (CARP) mediates the levels of infection in the midgut and salivary glands of the mosquito

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Running title: *carp*$^-$ parasites are reduced in mosquito infection.

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Key words: malaria, *Plasmodium berghei*, calmodulin-dependent protein kinase, transmission, ookinete, oocyst, sporozoites, invasion, epithelium, midgut, salivary gland

Abbreviations: area under the curve (AUC), cycle threshold (C*t*), intraperitoneal (i.p.), intravenous (i.v.), post-feeding (p.f.), post-infection (p.i.), transmission electron microscopy (TEM)

ABSTRACT

The completion of the *Plasmodium* (malaria) life cycle in the mosquito, requires the parasite to traverse both the midgut and salivary gland epithelium. Here we show that a putative calmodulin-dependent protein kinase related protein (CARP) is predicted to be an atypical protein kinase that is conserved across many species of *Plasmodium*. Targeted disruption of the putative *carp* gene in *P. berghei*, a rodent model of malaria, decreased the ability of ookinetes to invade the mosquito midgut, and those sporozoites that were produced were defective in their ability to traverse mosquito salivary glands. Sporozoites that emerged from oocysts were not attached to the salivary glands themselves, but were found circulating in the mosquito hemocoel. Our findings indicate that a putative signaling pathway involving PbCARP regulates the level of infection in the mosquito by mediating invasion of mosquito tissues.

INTRODUCTION

The transmission of the malaria parasite from the bite of an *Anopheles* mosquito is responsible for the millions of clinical cases and deaths from malaria each year [1]. The complex life cycle of the parasite requires an intricate network of signaling events to enable the parasite to invade multiple cell types in both the invertebrate and mammalian hosts [2, 3]. Two invasive life cycle stages that occur exclusively in the mosquito, the ookinete and the sporozoite, require invasion into the mosquito midgut and salivary gland epithelium, respectively. Ookinetes cross the midgut epithelium within 24 hours of a blood meal and produce oocysts. Sporozoites are released from the oocysts into the hemocoel

about 18 days later and invade the salivary glands by passing through the epithelium, completing the development of the parasite in the mosquito.

The mechanism used by the parasite to invade mosquito epithelial tissues is unknown but is thought to involve a signaling pathway. Recently, a peptide that is involved in attachment of the parasite to the salivary gland and midgut (SM1) was identified that binds specifically to the two epithelia traversed by the parasite in the mosquito [4]. Transgenic mosquitoes that overexpressed this peptide in the midgut epithelium were less susceptible to *P. berghei* infection, and contained fewer sporozoites in the salivary glands [5]. These interactions between parasite proteins and receptors on the mosquito epithelium may initiate a downstream signaling cascade to facilitate parasite invasion.

Protein kinases, such as calmodulin-dependent protein kinases (CaMK), are involved in many cellular processes, including invasion of cells [6, 7]. In *Plasmodium*, the CaMK family includes calcium-dependent protein kinases (CPDKs), as well as a distinct group that does not contain EF-hand motifs (required for calcium-binding) [3]. One calcium-dependent protein kinase, CDPK3, is required for ookinete motility in *P. berghei*, which in turn is required for efficient mosquito midgut invasion [8, 9]. Other parasites, including *Toxoplasma gondii* and *Cryptosporidium parvum,* also utilize CDPK-like proteins in invasive stages [7, 10]. This suggests that the CaMK family may play an important role in cell invasion in many parasite species.

Here we show using a *P. berghei* knock-out mutant of a putative CaMK-related protein (CARP) is involved in mediating cell invasion in the midgut and salivary glands of the mosquito. Ookinetes deficient in this protein do not

efficiently invade the midgut epithelium, resulting in a reduction in the number of midgut sporozoites. Sporozoites that are released from oocysts fail to invade salivary gland epithelium and are predominantly found circulating in mosquito hemocoel. We suggest that the putative PbCARP may be involved in a signaling pathway that mediates the parasite's ability to invade these mosquito tissues.

MATERIALS AND METHODS

Multiple sequence alignment

The orthologs of the various putative *Plasmodium carp* genes were predicted using OrthoMCL [11]. Multiple sequence alignments of the first 500 amino acids of *Plasmodium falciparum* CARP, *P. berghei* CARP, *P. chabaudi* CARP, and *P. yoelii* CARP were performed using Clustal W [12] on the SDSC Biology Workbench server (http://workbench.sdsc.edu) and a consensus sequence was determined. To construct the predicted catalytic domain alignment of PfCARP and PbCARP, an amino acid BLAST search of both domains was conducted to first retrieve those kinases that showed homology. The human testis-specific serine/threonine kinase (hTSSK) was selected as the most similar protein using OrthoMCL and a multiple sequence alignment was constructed using Clustal W.

Parasite maintenance and transmission to mosquitoes

Plasmodium berghei ANKA wild-type (clone C_1) and transgenic lines were maintained in Swiss-Webster mice (approximately 4 weeks old; NIH). *Anopheles stephensii* mosquitoes were maintained at 70% humidity and 22°C and infected

with parasites essentially as described [13]. Infected midguts and/or salivary glands were dissected on ice on the days post-feeding (p.f.) as indicated. Hemocoel was dissected using volume displacement (perfusion) essentially as described [14]. Whole, dissected salivary glands were treated with trypsin to remove any sporozoites that did not invade the salivary gland as described [15]. The number of sporozoites for a given experiment was determined using an appropriate dilution of sporozoites in DMEM and counted on a hemocytometer. Ookinete-infected midguts were dissected by removing the midguts from mosquitoes fed 24 h earlier on infected blood. The blood meal was removed and the midgut was washed extensively using PBS.

Deletion of the *carp* gene and genotype analysis

A targeting vector for *pbcarp* was constructed in plasmid pL0001 (plasmid b3; b3D.DT^H.^D), in which polylinker sites flank a *T. gondii dhfr/ts* expression cassette transferring resistance to pyrimethamine. A 457 bp fragment of the 5' UTR sequence was PCR amplified from *P. berghei* gDNA using the primers G1FKp (5'-agaaggtaacc cacatcataataatagcaaactgcac – 3'; restriction site underlined) and G1RH3 (ttagaagctt ttccagacgggttaatatcaaaa – 3'). The fragment was inserted into the KpnI and HindIII restriction sites upstream of the *dhfr/ts* cassette of pL0001. A 800 bp fragment of the 3' UTR sequence was PCR amplified from the same gDNA using the primers G2FBam (ttatggatcctgaaaatagataaaaattcaatcatgg – 3'; restriction site underlined) and G2RXb (aaaatctagatttttcacct – 3'). The fragment was inserted into the BamHI and XbaI restriction sites downstream of the *dhfr/ts* cassette of the vector. The

replacement construct was excised as a KpnI/SacII fragment and used for the electroporation of cultured *P. berghei* ANKA wt (clone C_1) schizonts essentially as described [16]. Two independent rounds of limiting dilution cloning of drug-resistant parasites were completed, and genotyping of Clone 1 (L_{10}) and Clone 2 (O_1) was carried out. Diagnostic PCR was used to amplify a 250 bp fragment of the *carp* gene using the primers ORF F (5' – ccttttgctatgcatggtgat – 3') and ORF R (5' – cattaaaatggagggcttgc – 3'). To confirm 5' integration, a 837 bp fragment of the 5' region was amplified using two primers: the forward primer (Pb837; 5'-caacaaaccaattgcatggac – 3') amplified a region upstream of the flanking sequence used to integrate the plasmid along with a reverse primer (Pb103; 5' – taattatatgttattttatttccac – 3') located 3' of the flanking sequence, but 5' to the *dhfr/ts* sequence (Fig 2A). To confirm 3' integration, a 1549 bp fragment of the 3' region was amplified using two primers: the forward primer (Pb106a; 5' – tgatgcacatgcatgtaaatagc – 3') amplified a region located 3' of the *dhfr/ts* sequence along with a reverse primer (Pb1549; 5' – cattgtatgccgtttggttc – 3') that amplified the region downstream of the flanking sequence used to integrate the plasmid (Fig 3-2A). A negative control of gDNA alone was also analysed with each PCR reaction. Southern blot analysis was carried out using the manufacturer's instructions (Roche). A labeled probe was PCR-amplified using the ORF F/ORF R primer set described above. Northern blot analysis on a standard formaldehyde gel was completed using the DIG kit (Roche) by analyzing 5 μg of total RNA extracted from sporozoites of wt or the transfected clones. A sample of the same, labeled probe from the Southern blot was used to label the northern blot.

Antibody production and western blot analysis

A synthetic peptide (*N* - LFENEKNGLIYPVLNDPGQAIYF - *C*; Biosynthesis Inc.) derived from the predicted catalytic region of PbCARP (residues 301 to 324) was used for immunization of rabbits (Cocalico Biologicals Inc; see Fig. 1). For western blotting, 7.5×10^4 sporozoites were dissected from each clone and separated on a 8% SDS-PAGE gel. Rabbit antisera (from naïve and immunized rabbits) was used at a 1:300 dilution for western blotting using the ECL western blotting kit (Amersham).

Extraction of gDNA from mosquito midguts and salivary glands

Midguts (ookinete or oocyst stage) and salivary glands (n = 10) from each clone were dissected from mosquitoes as described above, and placed in 500 μL oocyst lysis buffer (100 mM NaCl, 25 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.8), 0.5% Sarkosyl, and 1 mg/mL proteinase K). The mixture was incubated overnight in a 56°C water bath. Genomic DNA was then isolated using a phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 100 μL of TE.

Real-time PCR

Genomic DNA isolated from each clone was diluted equally to the indicated concentrations before running the assay. Real-time PCR reactions were performed in triplicate as described [17]. For midgut amplifications, DNA was diluted 1:4 and for salivary gland reactions, the DNA was diluted 1:14. For each 25 μl

reaction, 5 μl of the diluted gDNA was added. The following oligonucleotides were used: *P. berghei 18S rRNA* gene: 5'-ggcaacaacaggtctgtg-3' and 5'-gtacaaagggcagggacg-3'; *P. berghei ama-1* gene: 5'– accggtgatcagtcagtgagaagt-3' and 5'- gctacaatatcttggaccc-3'; *Pbcarp*: 5' – ccttttgctatgcatggtgat – 3' and 5' – cattaaaatggagggcttgc – 3'. The percent amplification efficiency was calculated using the formula % AE = $2^{\Delta Ct}$ * 100 as described [18, 19], except the % AE was calculated using the wt as unmodified and *carp*$^{-}$ clone as modified templates.

Statistical analysis

All statistical analyses were completed using Prism (v. 4.0a). Where the Area Under the Curve (AUC) was calculated, the average AUC was calculated for each cycle, using software default parameters, and then the average of all AUC was calculated. Where differences in AUC and quantitative PCR were assessed, normality was tested using the Kolmogorov-Smirnov Goodness-of-Fit Test. Data with a p value > 0.10 were considered normal. The differences were then tested using an ANOVA with a Tukey's Multiple Comparison post-hoc test. Assays showing significant differences were noted.

RESULTS

Bioinformatic analysis of the putative CARP

Using bioinformatics, we have analysed a *Plasmodium* protein (PFC0485w) and propose to label it a putative CaMK-related protein (CARP)

based on its signature motifs as described below. *Plasmodium falciparum* CARP (*Pfcarp*) is located on chromosome 3. Expression data from this gene shows low level expression throughout the asexual stages, and highest levels in the gametocytes (PlasmoDB, release 5.3 and [20]). The first 500 amino acids containing the N terminus and predicted kinase domain of the various putative *Plasmodium* CARP proteins show high homology between species: *Plasmodium berghei* CARP (*Pbcarp*; PB001650.02.0) is 93% identical to the counterpart in *P. yoelii* (*Pycarp*; PY02490), while PfCARP is only 57% and 60% identical to PbCARP and PyCARP, respectively (Fig. 3-1A). All four *Plasmodium* genes contain two exons, with the predicted protein kinase region in the first exon. The predicted size of the proteins vary with PfCARP, PyCARP and PcCARP (PC000082.02.0) comprising proteins of 2515, 2700, and 2435 amino acids, respectively (reannotation of PcCARP completed by Dr. Andrew Berry, Sanger), while a truncated PbCARP is annotated at 503 amino acids (PlasmoDB release 5.3).

Various searches were completed to identify a human orthologue of the *Plasmodium* CARP. None of the full length CARP sequences could be assigned clear othology to other Ser/Thr protein kinases, but kinases sharing sequence identity with the predicted catalytic domain of CARP were predicted. One of these kinases, the human testis-specific serine kinase (hTSSK; [21]) has been described as a CaMK-related protein kinase that is classified as atypical, as they

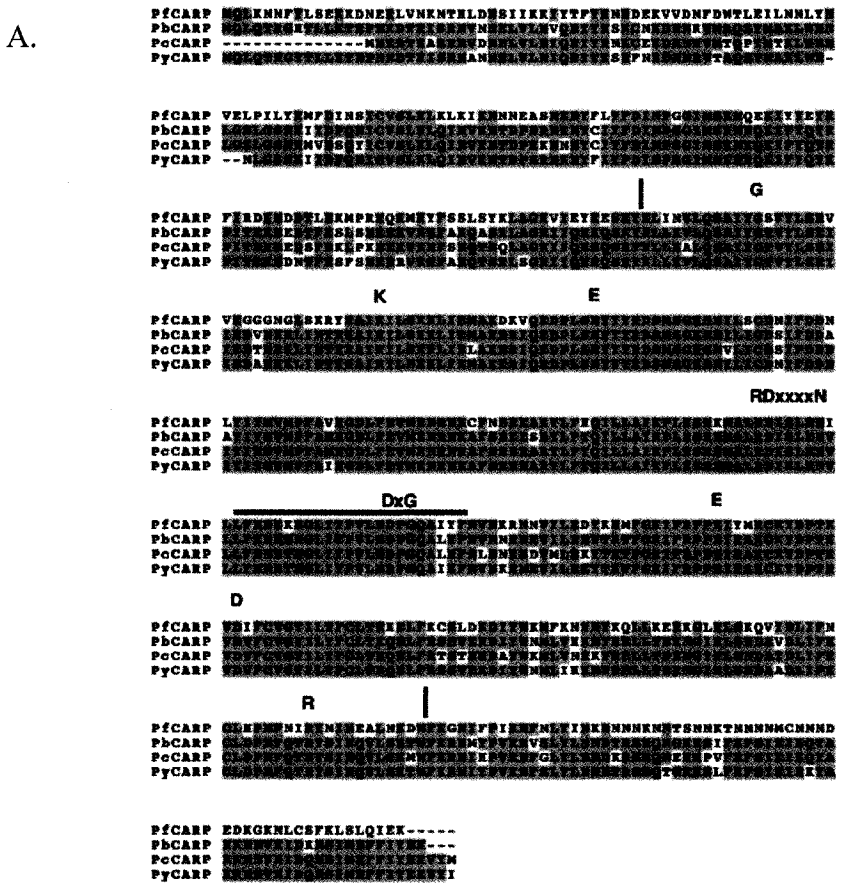

Figure 3-1. Alignments of Serine/Threonine kinases

The first 500 amino acids of the putative CaMK-related proteins found in four *Plasmodium* species are aligned (A). Amino acids found in two of the four aligned sequences are shaded to show identity. The *vertical bars* at residue 161 and 441 indicate the N- and C- terminal boundaries of the catalytic domain respectively. The sequence of the peptide used for immunization is indicated by the *horizontal bar*. Residues that are largely conserved in Ser/Thr protein kinases are indicated *above* the PfCARP sequence. The catalytic domains of PfCARP and PbCARP were aligned with human TSSK1 as an example of a related CamK protein kinase (B).

contain only a single glycine in the glycine loop motif. TSSK1 shares limited identity to CARP. The catalytic domains of hTSSK (261 amino acids) shares 26% identity with PfCARP and 25% identity with PbCARP (281 amino acids) (Fig. 3-1B). The catalytic domains of PfCARP and PbCARP share 75% identity.

Pbcarp is processed at the RNA and protein levels

To characterize the function of CARP, we used *P. berghei* for transfection studies in mice [22] since mosquitoes can be fed on these animals as a model for malaria transmission. To disrupt the putative *Pbcarp* gene, the 503 amino acids predicted to compose the full-length protein were replaced with a pyrimethamine-resistant allele of the *dhfr/ts* M2/M3 gene from *T. gondii* (Fig. 3-2A). Following drug selection, the targeting construct was shown to have integrated into the genome of the parasite. This was confirmed in two clones, designated clone 1 (Fig 3-2B, 3-2C) and clone 2 (Fig 3-2C), isolated independently from the transfected population using PCR and Southern blot analysis.

The predicted sequence of *pbcarp* is annotated to be 1,509 bp (PlasmoDB, release 5.3) after splicing. When mixed blood stage RNA was isolated from wild-type parasites and both *Pbcarp*$^-$ clones, a Northern blot revealed a broad RNA band at approximately 5,300 bp that was absent from in the knock-out clones (Fig. 3-3A). The size of this RNA is more consistent with the predicted size of *carp* from other *Plasmodium* species that have lengths of approximately 7,548 bp (*pfcarp*). When protein was extracted from the sporozoite stages of wild-type and *Pbcarp*$^-$ clones and analysed by western blotting, an antibody directed against a *Pfcarp* peptide from the predicted active site kinase domain detected a single,

strong band in the wild type parasites but not in the *Pbcarp*$^-$ clones (Fig 3-3B). The band corresponds to a protein of approximately 92 kDa, which is smaller than the protein predicted for PfCARP (295.5 kDa), but larger than that predicted for PbCARP (59.2 kDa). This may indicate that there is an error in the annotation of the *Pbcarp* gene: this issue is discussed below.

Pbcarp$^-$ parasites produce fewer ookinetes that are deficient in midgut endothelium invasion

We next characterized the role of PbCARP in both the blood stage and mosquito stages of infection. Asexual growth was unaffected in both *Pbcarp*$^-$ clones and they gave rise to wild-type numbers of gametocytes (data not shown). Mosquito infection, however, was different between the wild-type and *pbcarp*$^-$ parasites. We used a real-time PCR assay to assess ookinete infection in the mosquito midgut. Twenty-four hours after feeding on *Pbcarp*$^-$ clones and wild-type parasites, the midguts of mosquitoes were assessed for parasite infection using specific primers for *Pb18S rRNA* and *Pbama-1*, along with primers to *Pbcarp* as a negative control. The amplification efficiency [17-19] of clones 1 and 2 was significantly reduced 64% and 71% at the *Pb18S rRNA* locus and 58% and 65% at the *ama-1* locus (Fig. 3-4). There was no amplification of *carp*, as expected (Fig. 3-4). This indicates that fewer *Pbcarp*$^-$ ookinetes were present in mosquito midguts 24 h after infection.

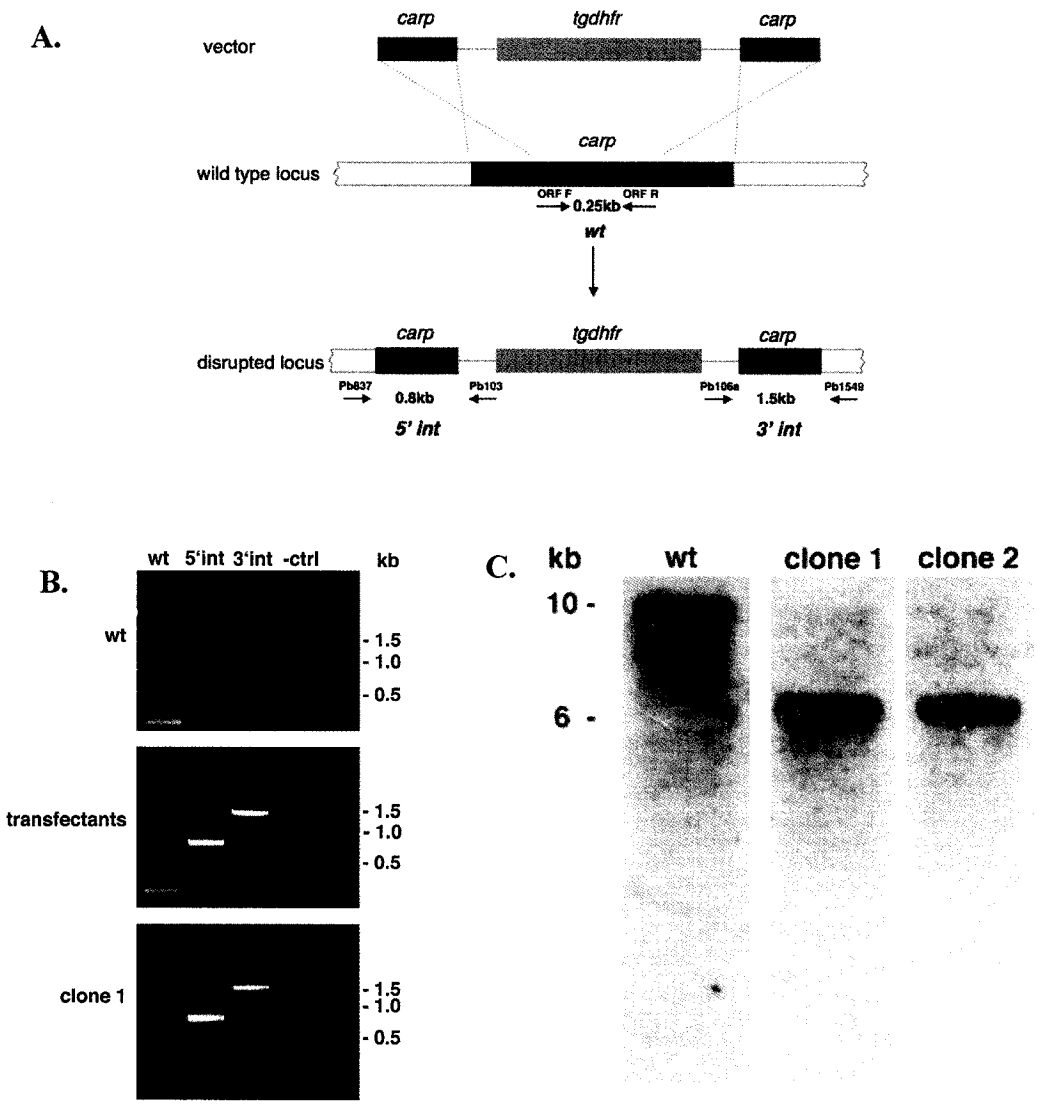

Figure 3-2. Targeted disruption of the *carp* gene in *P. berghei*
Schematic representation of the *pbcarp* locus and the gene-targeting construct used for gene replacement by double homologous recombination, along with the resulting disrupted locus (A). Diagnostic PCR verifying the disruption of the *pbcarp* locus (B). Representative PCR is shown using primers to the wt (clone C_1) open reading frame (labeled "*wt*" in A), a PCR of the mixed wt and transfected parasites which contain gDNA that show products from the wt and 5' and 3' integrated primers (labeled "*5'int*" and "*3'int*" in A). Finally, gDNA from a representative disrupted clone (clone 1) shows only the 5 and 3' integrated products. A negative control (gDNA alone) was also included. Southern blot analysis of BglII-digested DNA, showing the intact *carp* locus in the wild type population, and a shifted, smaller band associated with both the clone 1 and 2 disrupted locus (C).

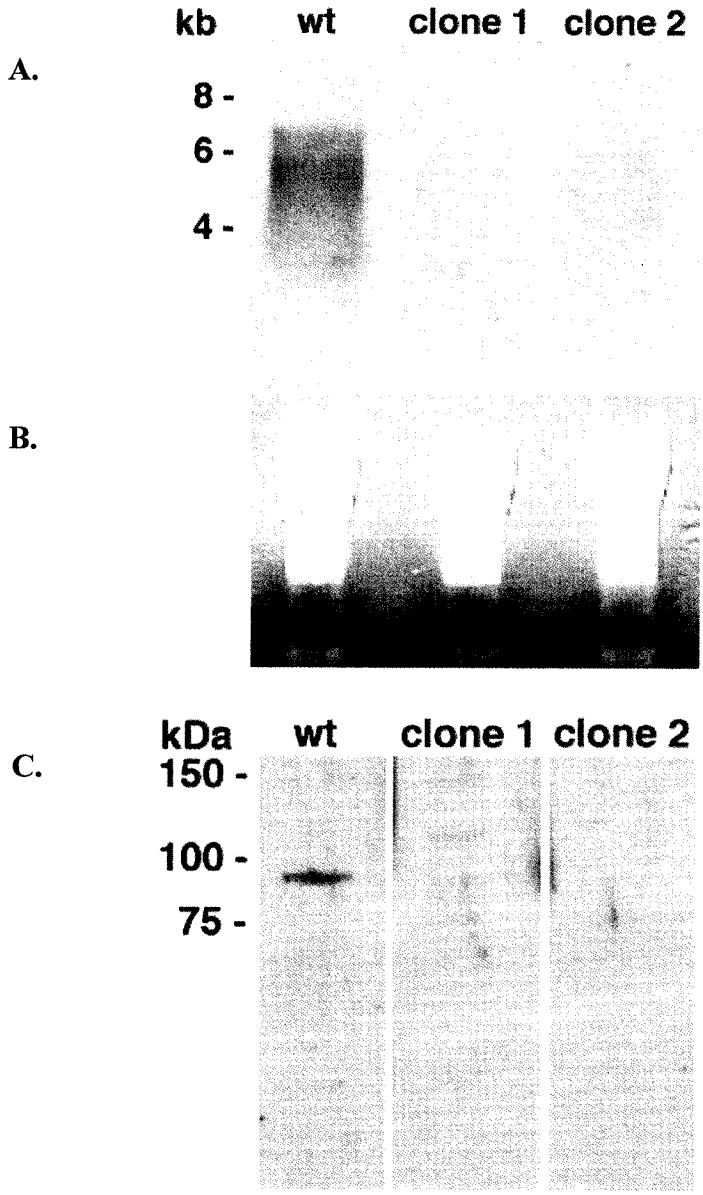

Figure 3-3. CARP is processed post-transcriptionally and is further processed at the protein level

Total RNA was extracted from mixed blood stage wt and *carp*$^-$ parasites and 5 μg of RNA from each clone was used for northern blot analysis (A). The ethidium bromide-stained agarose gel showing equal loading of RNA is also shown. Western blot analysis was completed on protein extracts from 7.5×10^4 wt and *carp*$^-$ sporozoites (B). The blot was probed with the anti-peptide rabbit polyclonal serum raised against a conserved kinase domain of CARP (see Fig. 3-1A).

This assay was also used to determine the oocyst production in the mosquito midguts. Similar to the results at the ookinete stage, the amplification efficiency of DNA extracted from mosquito midguts on day 18 p.f. showed approximately an 80% reduction in parasite gDNA compared with wild-type parasites (Fig. 3-5A). Midgut infections were also assessed by sporozoite counts every second day from day 10 to 24 post-feeding (p.f.). Overall, the number of sporozoites in the midguts of the *pbcarp*$^-$ clone 1 and clone 2 was reduced 44% and 51%, respectively, throughout the course of infection (Fig. 3-5B), although this decrease was not significant due to the inherent variation in infection levels from cycle to cycle. These results suggest that the reduction in midgut sporozoites may arise from a defect in ookinete production and indicates a role for PbCARP prior to sporoblast formation in the endothelium of the mosquito.

Pbcarp$^-$ sporozoites fail to invade mosquito salivary glands

The number of salivary gland sporozoites in wild type and *Pbcarp-* clones was assessed at the same time as midgut infections. A significant reduction in the number of salivary gland sporozoites was observed for both clones (75.7% for clone 1 and 76.2% for clone 2; Fig. 3-6A). This was further confirmed by real-time PCR on DNA from salivary glands collected at day 18 p.f. As observed by sporozoite counts, there was a 75% reduction in amplification efficiency from salivary glands of *pbcarp*$^-$ parasites. (Fig. 3-6B). These results suggest that

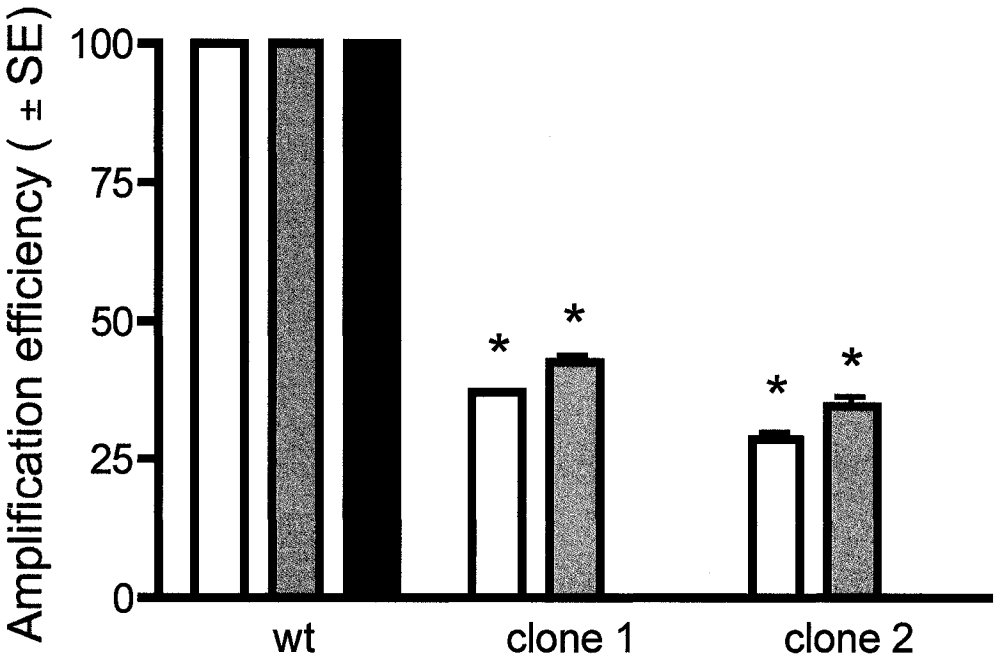

Figure 3-4. *carp*⁻ ookinetes are deficient in invasion of the midgut
Anopheles stephensi mosquitoes were fed directly on mice infected with wt or *carp*⁻ (both clone 1 and 2) parasites on day 0. At 24 h p.f., midguts were dissected from 10 mosquitoes from each group. The blood meal was dissected and the midgut was washed extensively. Genomic DNA was extracted from the pooled midguts from each group. Malaria infection was determined using real-time PCR and primers directed towards *pb18s rRNA* (white bars), *pbama-1* (grey bars) and *pbcarp* (black bars). The amplification efficiency was significantly reduced ($p<0.0001$, ANOVA, $n=10$) in both clones of *carp*⁻ ookinetes compared to wt.

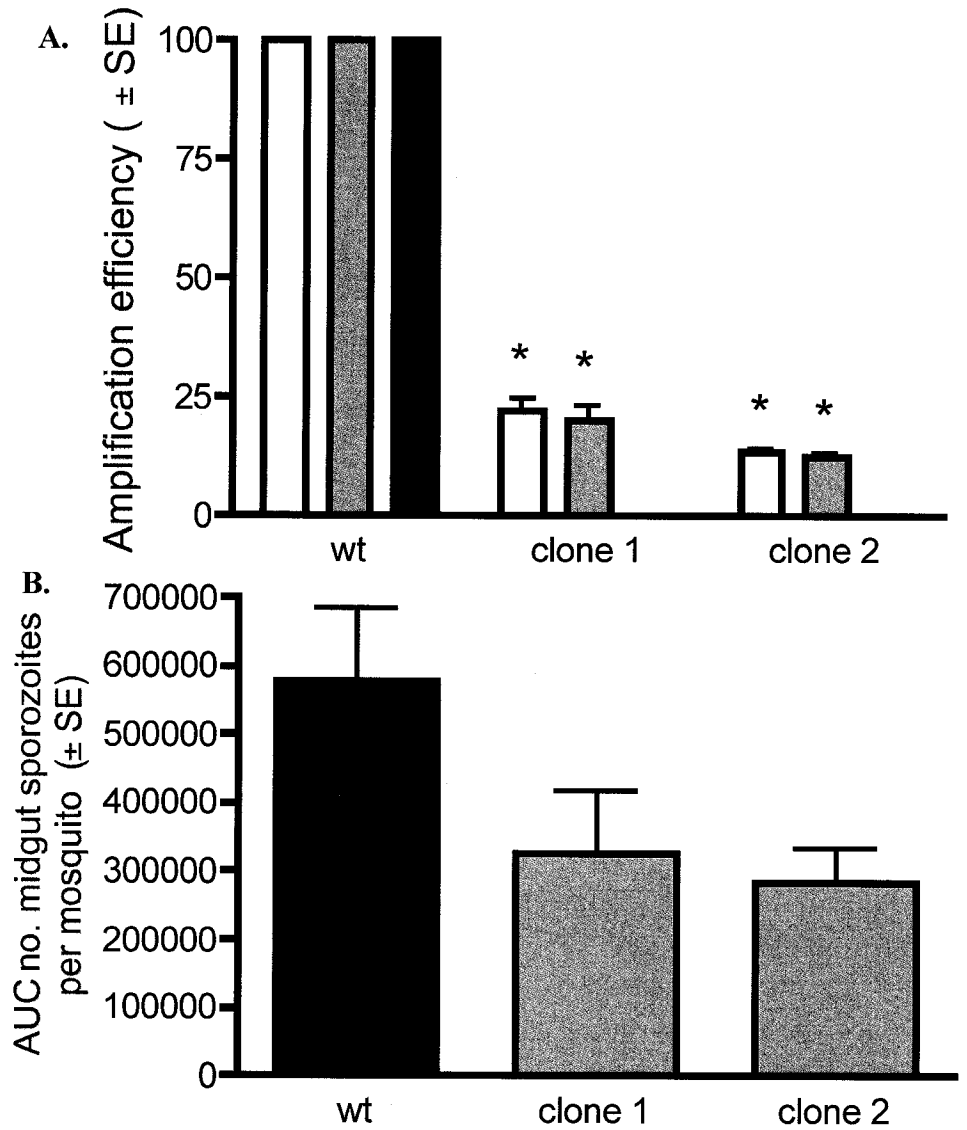

Figure 3-5. The number of sporozoites is reduced in the mosquito midgut in *carp*⁻ clones throughout mosquito infection

Anopheles stephensi mosquitoes were fed directly on mice infected with wt or *carp*⁻ (both clone 1 and 2) parasites on day 0. On day 18 p.f., mosquito midguts were dissected from 10 mosquitoes each from each group and assayed using the real-time PCR technique as shown in Fig. 4. There was a significant reduction in amplification efficiency in (A) mosquito midguts ($p<0.0001$, ANOVA, $n=10$). Results represent two independent experiments with 10 mosquitoes per group per clone. Every second day from day 10 to day 24 p.f., midguts were dissected from 10 mosquitoes from each group. The number of sporozoites per mosquito was determined and the total number of sporozoites (expressed as area under the curve) calculated for the duration of the infection. A reduction in the number of sporozoites in the midguts of mosquitoes fed on *carp*⁻ parasites persisted throughout the mosquito infection (B). Shown is the mean area under the curve for the number of sporozoites per mosquito in four independent experiments using individual batches of mosquitoes, with 100 mosquitoes dissected per region.

PbCARP may be involved in multiple stages of parasite development in the mosquito.

Given the greater reduction of sporozoites in the salivary gland compared with the midgut, it is possible that *Pbcarp*$^-$ sporozoites were unable to invade the salivary gland. To test this, whole salivary glands were treated with trypsin prior to manipulation to release sporozoites that had adhered to the outside of the glands. Sporozoites that were attached are located in the supernatant of treated, whole salivary glands, and the numbers were quantified. The number of sporozoites attached to the salivary glands in *Pbcarp*$^-$ sporozoites was 57.5% and 81.8% reduced at day 24 p.f. compared to wild-type parasites (Fig. 3-7A). Although the variation in counts was high, it seemed that the sporozoites in the KO clones were not attached to the glands.

An alternative hypothesis is that the *Pbcarp*$^-$ sporozoites fail to migrate from the midgut epithelium to the salivary gland. Sporozoites emerging from the oocyst would be released into the circulation in the mosquito hemocoel, which sporozoites use to transit to the salivary glands. The number of *Pbcarp*$^-$ sporozoites circulating in mosquito hemocoel was approximately equal to wild-type numbers (Fig 3-7B). Given the fact that the number of midgut sporozoites is reduced, one would also expect that the number of hemocoel sporozoites would be as reduced, but the numbers are approximately equal, indicating that many sporozoites are trapped and accumulate over time within this cavity. Based on these results, we propose that, in addition to affecting ookinete numbers, putative PbCARP affects the number of sporozoites in the mosquito midgut; those sporozoites,

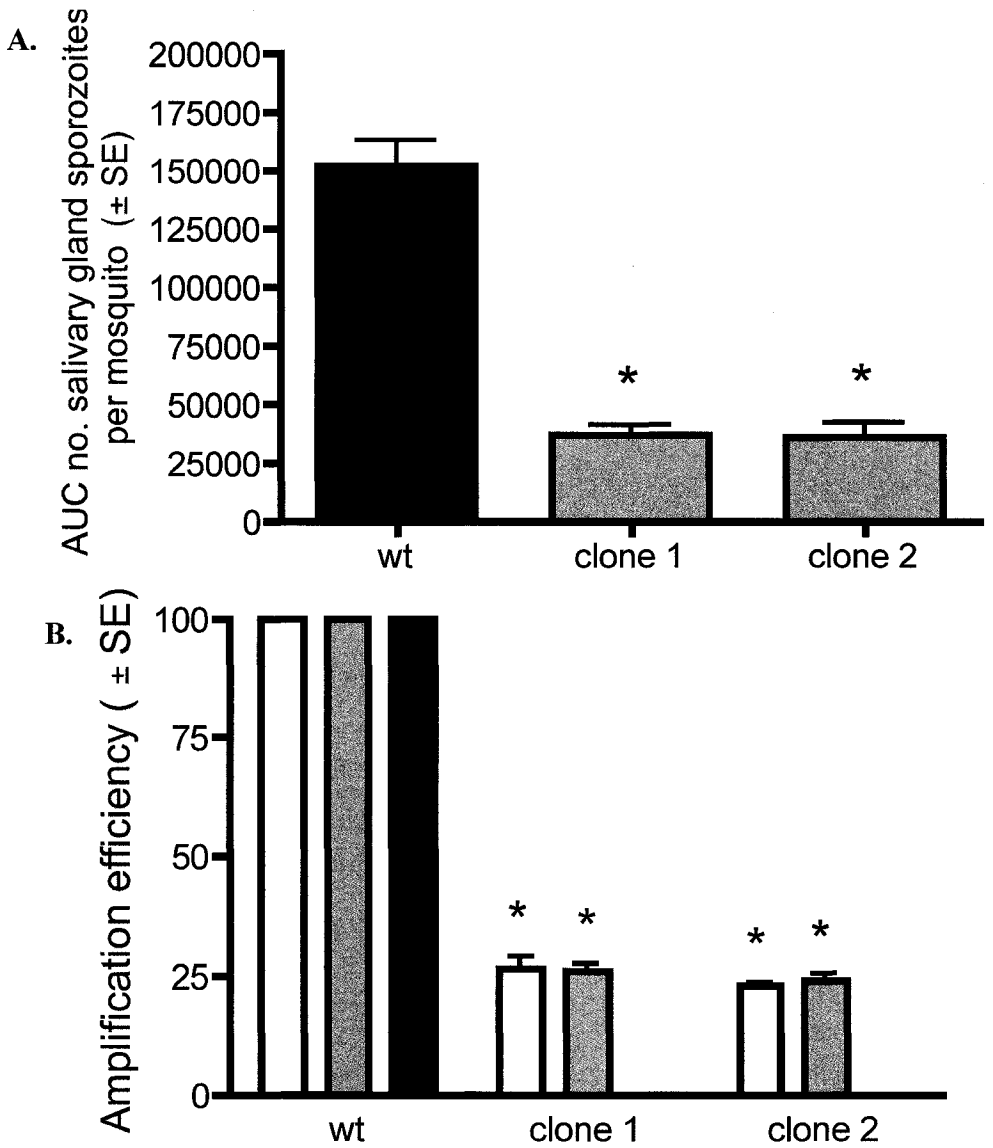

Figure 3-6. The number of sporozoites is significantly reduced in the mosquito salivary glands in *carp*⁻ clones throughout mosquito infection
Anopheles stephensi mosquitoes were fed directly on mice infected with wt or *carp*⁻ (both clone 1 and 2) parasites on day 0. Every second day from day 10 to day 24 p.f., midguts were dissected from 10 mosquitoes from each group. The number of sporozoites per mosquito was determined and the total number of sporozoites (expressed as area under the curve) calculated for the duration of the infection. A significant reduction of *carp*⁻ salivary gland sporozoites was present throughout the observed infection ($p<0.0001$, ANOVA, $n = 400$) (A). Shown is the mean area under the curve for the number of sporozoites per mosquito in four independent experiments using individual batches of mosquitoes, with 100 mosquitoes dissected per region. On day 18 p.f., mosquito salivary glands were dissected from 10 mosquitoes each from each group and assayed using the real-time PCR technique. There was a significant reduction in amplification efficiency in (B) mosquito salivary glands ($p<0.0001$, ANOVA, $n=10$). Results represent two independent experiments with 10 mosquitoes per group per clone.

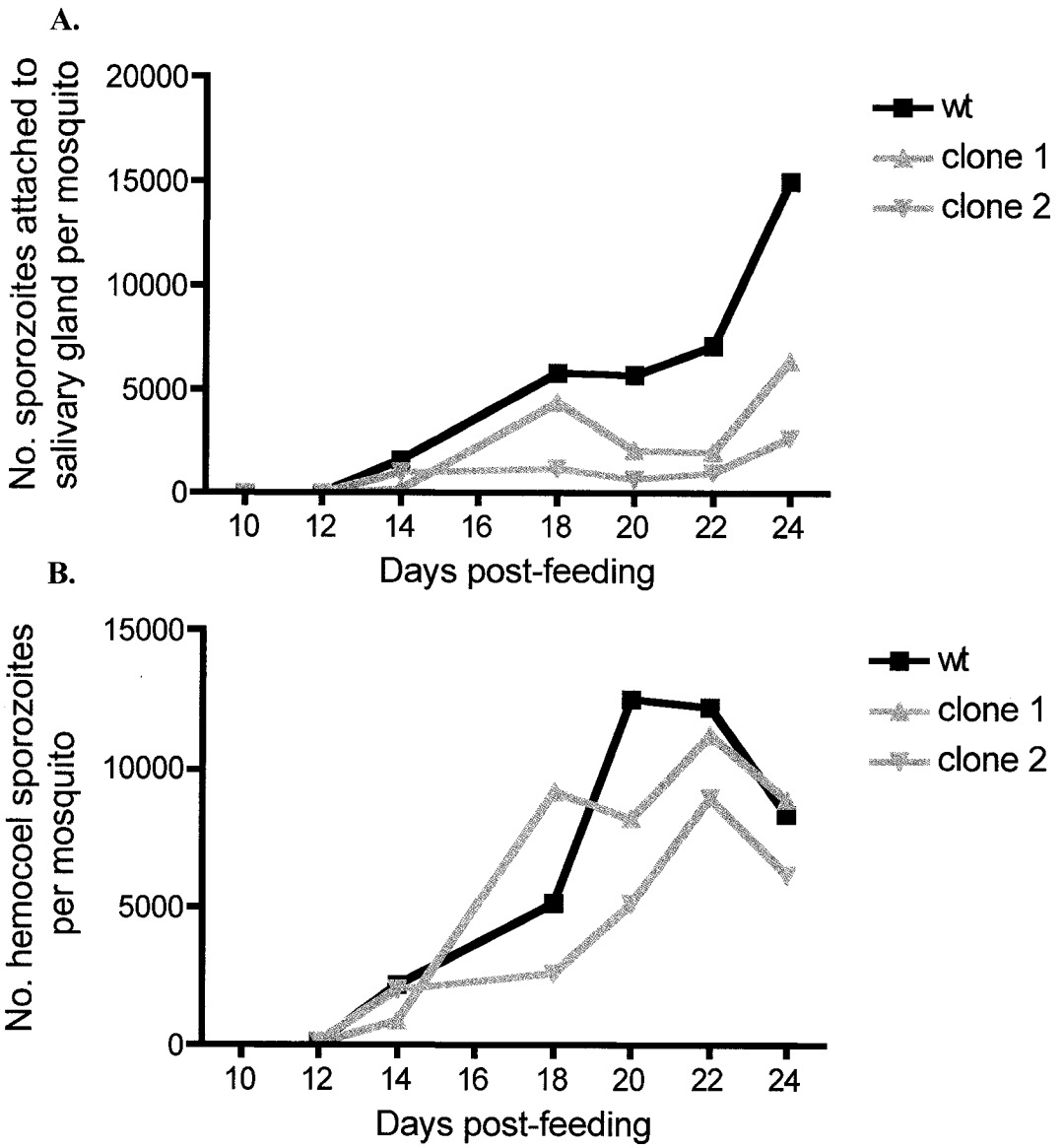

Figure 3-7. Sporozoites released from the midgut are not attached to salivary glands, but are circulating in mosquito hemocoel in *carp*⁻ parasites
Anopheles stephensi mosquitoes were fed directly on mice infected with wt or *carp*⁻ (both clone 1 and 2) parasites on day 0. On day 10 to day 24 p.f., hemocoel was dissected from 10 mosquitoes from each group. On the same days, whole salivary glands were dissected from the same number of mosquitoes and treated with trypsin to detach sporozoites on the outside of the glands. The number of sporozoites per mosquito was determined and the area under the curve calculated for the duration of the infection. Fewer sporozoites were attached to mosquito salivary glands in parasites lacking the *carp*⁻ locus (A). The number of hemocoel sporozoites per mosquito was equivalent in both the wt and *carp*⁻ parasites. Shown are the sporozoite kinetics of a representative experiment with 100 mosquitoes dissected per region. Three independent experiments were completed, but one representative experiment is shown.

which successfully develop are able to migrate to the mosquito hemocoel but are less able to infect mosquito salivary glands than wild-type parasites.

DISCUSSION

Calmodulin (CaM) is a ubiquitous, calcium-binding protein that can bind and regulate a multitude of different protein targets, thereby affecting many different cellular functions [23]. Calmodulin-dependent protein kinases (CaMK) are primarily regulated by a change in Ca^{2+} concentrations. In this study, we show that a putative CaMK-related protein (PbCARP) has an important function in mediating the level of *P. berghei* infection in *A. stephensii* mosquitoes. The analysis of the *carp*$^{-}$ deletion mutants shows that this protein is not essential for asexual and sexual intraerythrocytic development. However, putative PbCARP seems to be involved in mediating the invasion of ookinetes in the midgut epithelium of the mosquito, thereby affecting the number of sporozoites that are released from oocysts. In addition, sporozoites that are released into the mosquito hemocoel have a reduced ability to invade the salivary glands. The results suggest that putative PbCARP increases the potential transmissibility of malaria by each mosquito to another mammalian host.

Assigning clear orthology of putative PbCARP to a human protein kinase is difficult, given the phylogenetic distances between the species. Based on bioinformatic analysis, putative PbCARP is most closely related to the human enzyme TSSK1. Human TSSK1 is almost exclusively expressed in the testes, and a role for this enzyme in the fusion of the sperm and oocyte, along with the breakdown of the nuclear envelope, has been proposed [21]. Interestingly, this is

consistent with our data suggesting that putative PbCARP has a role in cell invasion. As is true with TSSK, putative PbCARP shows sequence similarity with the CaMK family, although it contains no EF-hand motifs, and has a conserved catalytic aspartate. The protein is predicted to be an atypical kinase with a single glycine in the glycine loop motif. Although numerous attempts were made to clone and express a heterologous, active form of CARP in *E. coli*, none was successful (data not shown). Expression of *Plasmodium* proteins has proven to be challenging at best, with estimates that only 6.3% of attempts at heterologous expression were successful [24].

The *P. berghei* CARP was expressed as a protein of about 90 kDa, which is larger than the predicted sequence (59.2 kDa). This may indicate that the native protein is post-translationally modified in some way or that there is an error in the annotation of the putative *Pbcarp* gene: if PbCARP actually encodes a larger protein similar to the PfCARP gene (295.5kDa), these results may suggest that the protein is processed at the protein level. Post-translational and/or post-transcriptional processing is a well-characterized observation in protein kinases, sometimes occurring in a tissue-specific manner [25, 26]. If the native putative PbCARP protein is actually larger than 90kDa, this putative processing could play a role in suppressing kinase activity until the ookinete is formed and is ready to invade the mosquito endothelium. Further work is needed to resolve this question such as generating antibodies to the predicted C terminal region of putative PbCARP to determine whether a larger precursor form of putative PbCARP is expressed.

Mechanisms are required for invasion of the ookinete into the midgut, exit of the sporozoites from the midgut and sporozoite entry into the salivary glands. Given the quantitative reduction in *Pbcarp*$^{-}$ parasites at all of these stages, putative PbCARP may be involved in the invasion of both midgut and salivary gland epithelial tissues, although complementation of the knock-out parasites may be required to clearly demonstrate this phenotype. Some evidence indicates that parasite interactions with the two types of tissue are specific [4, 27], but little is known about the parasite molecules that interact with these tissues. A small peptide, called SM1 has been shown to bind to the luminal side of the midgut epithelium and the distal lobes of the salivary glands and interfere with parasite infection in mosquitoes. SM1 presumably blocks a specific interaction between a receptor on the parasite and a binding site on the midgut epithelium and the distal lobes of the salivary glands. Interestingly, this peptide did not bind to the midgut surface in the hemocoel [4, 5]. Development of transgenic mosquitoes that express certain inhibitory molecules or that have certain ligands disrupted has been proposed as a method to control malaria transmission by replacing wild type mosquitoes with transgenic mosquitoes that are refractory to infection [28]. The identification of putative CARP as a molecule determining parasite development in the mosquito is a further step toward characterizing protein interactions that could identify compounds with transmission-blocking potential.

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CONNECTING STATEMENT

In Chapter 3, a novel protein, a putative calmodulin-dependent protein kinase related protein (CARP) was shown to be involved the transmission of the rodent malaria parasite, *Plasmodium berghei*. Of particular interest was the observation that the levels of sporozoite infection in the mosquito could be manipulated using genetic modification. The next Chapter investigates the question of whether a pharmacogenomics approach can be used to interfere with transmission of the malaria parasite to mosquitoes. In collaboration with S.K. Yanow, I investigated the activity of the AT-binding drug, centanamycin against blood stages of murine malaria and assessed the ability of this drug to block transmission to mosquitoes.

CHAPTER 4:

Potent antimalarial and transmission-blocking activities of a novel DNA binding agent

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Running title: Antimalarial activity of centanamycin

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Abbreviations: *Plasmodium* (*P.*), *chabaudi* (*c.*), post-infection (p.i.), intraperitoneal (i.p.), post-feeding (p.f.), transmission electron microscopy (TEM), cycle threshold (C*t*), amplification efficiency (AE)

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Footnotes

(1) The authors affirm they have no commercial or other association that might pose a conflict of interest.

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ABSTRACT

Most treatments for malaria target the blood-stage of infection in the human host while few can also block transmission of the parasite to the mosquito. We show that compound centanamycin is very effective against blood-stage malaria infections *in vitro* and *in vivo* and has profound effects on sexual differentiation of the parasites in mosquitoes. Following drug treatment, parasite development is arrested within the midguts of mosquitoes, failing to produce the infective forms that migrate to the salivary glands. The mechanism of parasite death is associated with modification of *Plasmodium* genomic DNA. We detect DNA damage in parasites isolated from mice 24 h after treatment with centanamycin and, importantly, this DNA damage is also detected in parasites within mosquitoes 10 days after feeding on these mice. This demonstrates that damage to parasite DNA during blood-stage infection persists from the vertebrate to the mosquito host and provides a novel biochemical strategy to block malaria transmission.

Key words: Malaria, chemotherapy, immunity, *Plasmodium falciparum*, *P. chabaudi, P. berghei*, transmission-blocking, DNA damage, sporozoite, oocyst, centanamycin

INTRODUCTION

Malaria is one of the leading parasitic infections worldwide and is responsible for the deaths of 1-2 million people annually, mostly young children [1]. The disease is caused by an intracellular parasite (*Plasmodium*) that infects human hepatocytes and red blood cells, and is transmitted by a mosquito vector. Current therapies generally target the blood-stage of infection to alleviate symptoms within the human host. However, most of

these antimalarials are ineffective against the sexual forms of the parasite [2]. The capability of antimalarials to block the transmissive stages of the parasite life cycle is a particularly important aspect of malaria control, especially in areas of high transmission [2]. Not only can transmission-blocking compounds block the spread of disease, but they also have the potential to reduce the prevalence of drug-resistant parasites in endemic areas.

One common feature of all *Plasmodium* species is the high proportion of adenine and thymine nucleotides within their genomic DNA (about 80% A/T) [3-5]. A/T-specific agents have been tested for antimalarial activity *in vitro* and *in vivo*, but most of these drugs are too toxic for clinical development [6-10]. Exceptionally, treatment with the drug DB289, a prodrug for the A/T DNA binding compound DB75, resulted in a 96% cure rate against *P. vivax* and uncomplicated *P. falciparum* infections in human Phase II clinical trials [11].

We investigated the antimalarial activity of centanamycin, a rationally designed compound inspired from (+)-duocarmycin SA that lacks a stereocenter [12]. Centanamycin binds covalently to adenine-N3 in the motif (A/T)AAA, displays potent anticancer activity *in vitro* and *in vivo*, and is not overtly toxic to C57/BL6 mice at doses as high as 15 mg/kg [12]. Centanamycin represents a new generation of A/T-specific alkylating agents that exhibits the greatest potential for clinical application as a new antimalarial.

MATERIALS AND METHODS

Compounds

Centanamycin (1.5 mg/ml) was prepared in a PET/glucose solution as described [12].

Parasite growth inhibition assays

P. falciparum was cultured in human erythrocytes at 3-5% hematocrit in complete media and *in vitro* growth assays were performed essentially as described [9, 13]. Centanamycin was tested at six concentrations, in triplicate, in three independent experiments.

Administration of centanamycin to infected mice

Procedures for *P.c. adami* animal experiments were approved by the Macdonald Campus Animal Care Committee of McGill University while procedures for *P. berghei* animal experiments were approved by New York University School of Medicine Institutional Animal Care and Use Committee. Groups of female BALB/c mice (18-20 g) were injected i.p. on day 0 with rodent malaria parasites from an infected donor mouse [14]. Mice were infected with 0.5-1 x 10^5 parasites. Mice were injected i.p. with centanamycin or vehicle alone on day 4 or 5 post-infection (p.i.). Parasitemia was monitored from thin blood films as described [9]. Total parasite burden was calculated per mouse from the cumulative parasitemia throughout the trial [14, 15]. The 4-day suppression test was used as described previously [16]. Experiments were performed twice with four mice per group per experiment.

Transmission of drug-treated parasites to mosquitoes

Anopheles stephensi mosquitoes were raised and infected with *P. berghei* ANKA $PbGFP_{CON}$ as described [17]. Eight separate cages of 25-30 mosquitoes were each fed on 8 mice infected with $PbGFP_{CON}$ at 5 d p.i. Twenty-four hours after drug treatment, 8 more cages of mosquitoes were fed on the separate mice. Mosquito midguts were dissected in PBS at 10 d post-feeding (p.f.), mounted on a glass slide and the total oocysts counted using a Nikon Eclipse E600 fluorescence microscope with the Nikon Digital Camera DXM1200 and ACT-1 v.2.70 acquisition software.

Electron microscopy

Infected mosquito midguts were dissected at day 10 p. f. and fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS for 5 days. Specimens were post fixed in 1% osmium tetroxide and 1.5% $K_3Fe(CN)_6$ in PBS for 2 h at RT, followed by incubation in 0.5% uranyl acetate for 1 h. Midguts were dehydrated in increasing concentrations of ethanol and incubated for 1 h in propylene oxide, followed by incubation for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences). Specimens were embedded in Epon at 60°C for 2 d. Post-staining of sections was done with 1% uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope and scanned images were processed using Adobe Photoshop 6.0 software.

Extraction of parasite and mosquito genomic DNA

Infected mice were sacrificed and whole blood collected. Parasite DNA was isolated [18] using the QIAamp DNA Blood Mini Kit (Qiagen). DNA from infected mosquitoes was

isolated as described [19, 20]. Dissected midguts were incubated in 1 ml of oocyst lysis buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, pH 8.8, 0.5 % Sarkosyl, 1 mg/ml proteinase K) at 56°C overnight. Genomic DNA was extracted using phenol/chloroform, ethanol precipitated and dissolved in TE.

Treatment of genomic DNA with centanamycin *in vitro*

Reactions consisted of 2 μg of *P. berghei* ANKA genomic DNA, centanamycin (20 μM) or DMSO, in a 20 μl volume containing 0.1X SSPE as described [21].

Real-time PCR

Genomic DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Real-time PCR reactions were performed as described [9] using 62.5 pg and 500 pg of DNA isolated from infected mice and mosquitoes, respectively, per 25 μl reaction. The following oligonucleotides were used: *P. berghei 18S rRNA* gene: 5'-ggcaacaacaggtctgtg-3' and 5'-gtacaaagggcagggacg-3'; *P. berghei ama-1* gene: 5'–accggtgatcagtcagtgagaagt-3' and 5'-gctacaatatcttggaccc-3'; GFP gene: 5'-cctgtccttttaccagacaacca-3' and 5'-ggtctctcttttcgttgggatct-3'; mouse *β-actin* gene: 5'-gtgggccgctctaggcacca-3' and 5'- cggttggccttagggttcaggggg-3' [22].

The percent amplification efficiency was calculated using the formula % AE = $2^{\Delta Ct} \times 100$ as described [23]. For DNA extracted from oocysts, the C*t*'s for each parasite gene were first normalized to the amount of GFP DNA in the parasites and the % AE was calculated between the unmodified and modified templates using the formula $2^{(-\Delta\Delta Ct)} \times 100$.

RESULTS

Centanamycin is effective against drug-sensitive and drug-resistant strains of P. falciparum

We tested the antimalarial activity of centanamycin (Fig. 4-1A) against *P. falciparum in vitro*. The IC_{50} for centanamycin was 1.8 nM for the chloroquine-sensitive 3D7 strain (10 fold lower than for chloroquine), and similar values were obtained with two chloroquine-resistant strains, FCR3 and 7G8 (data not shown), indicating that centanamycin has potent cytotoxic effects on *P. falciparum in vitro.*

A single injection of centanamycin reduces parasitemia in mice infected with malaria

We assessed the effects of centanamycin on malaria blood-stage infection *in vivo*. Mice were infected with the avirulent murine malaria strain *P. chabaudi adami* DK, followed by a single i.p. injection of centanamycin or the vehicle on day 4 p.i. (Fig. 4-1B). In contrast with controls, the parasitemia became subpatent in mice that received a 15 mg/kg dose of centanamycin from 24 h after drug injection and throughout the 13 day trial. In a dose-response experiment (Fig. 4-1C), 5 mg/kg centanamycin suppressed the parasite infection until day 11 p.i., when a low level parasitemia was detected; with 1.5 mg/kg centanamycin, peak parasitemia was delayed by two days relative to the controls and the total parasite burden was reduced by 48 %. These results suggest that the ED_{50} for centanamycin is about 1.5 mg/kg in BALB/c mice infected with *P. c. adami* DK.

To determine whether centanamycin could suppress an established infection, mice with a starting parasitemia of 7.8 - 13.6 % were treated with a single injection of 5 mg/kg centanamycin or the vehicle. Twenty-four hours later, the mean parasitemia in the drug-

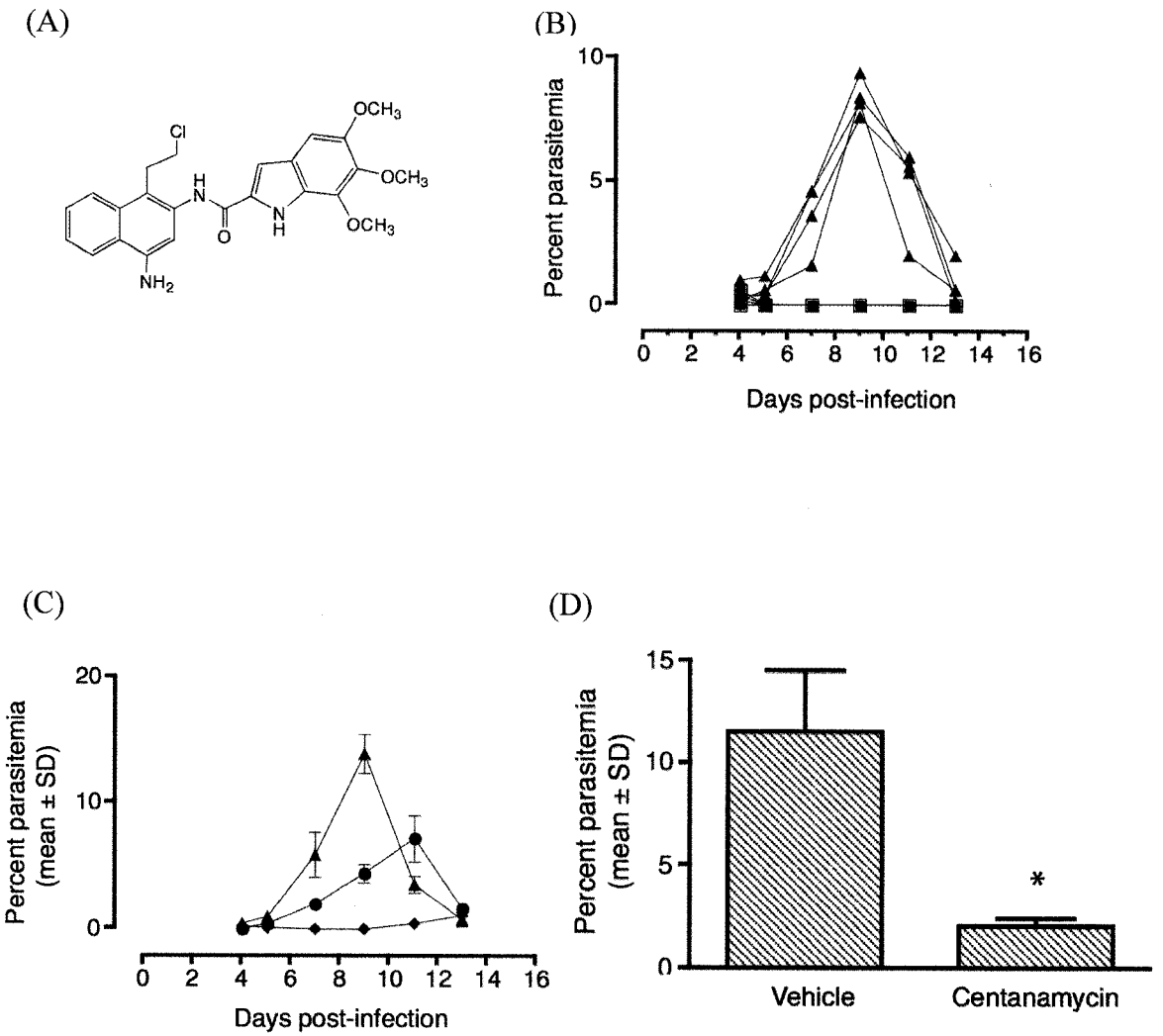

Figure 4-1. Centanamycin rapidly suppresses avirulent malaria infection *in vivo*. (A) Structure of centanamycin. (B, C) Parasitemia of mice infected with *P. c. adami* DK and treated with vehicle (B,C; filled triangles), 15 mg/kg (B; filled squares), 5 mg/kg (C; filled diamonds), or 1.5 mg/kg (C; filled circles) centanamycin on day 4 p.i. (D) Parasitemia of mice 24 h after a single i.p. injection of the vehicle or 5 mg/kg centanamycin at day 11 p.i. with *P. c. adami* DK. * Indicates significant difference, $p = 0.006$, Student's *t*-test, $n = 3$.

treated animals was reduced by 83 % compared with controls (Fig. 4-1D; p = 0.006, Student's *t*-test, n = 3), demonstrating that a single subcurative dose of centanamycin administered at peak infection rapidly reduces the level of parasitemia within 24 h.

The effects of centanamycin were evaluated in mice infected with the highly virulent rodent malaria strain *P. c. adami* DS. Infected mice received a single i.p. injection on day 5 p.i. of either the vehicle or 15 mg/kg centanamycin (Fig. 4-2A). Control mice rapidly developed a malaria infection and by day 10 p.i., the mice either died or were euthanized. Mice receiving centanamycin cleared the initial infection and the parasitemia remained subpatent for at least 6 days. A recrudescent parasitemia was observed in the drug-treated mice between 12 and 26 days p.i. but the parasite burden was low. These mice successfully cleared the parasites by day 26 p.i. and no signs of malaria-associated pathologies (ruffled fur, lethargy, anemia) were noted.

Treatment with centanamycin induces protective immunity to homologous challenge

The observation that drug-treated mice were able to resolve the recrudescent parasitemia in a rodent malaria model suggested they developed protective immunity. To test this directly, drug-treated mice were re-challenged with a second *P. c. adami* DS infection 34 days after the initial parasite infection (Fig. 4-2B). Naïve control mice developed a high level of parasites in the blood. In all four drug-treated mice, no parasites were detected over a 14-day time period suggesting that a reduction of parasitemia following antimalarial treatment enables mice to acquire immunity. In a recent study, immunity was induced following repeated subpatent infections with *P. c. chabaudi* that were drug-cleared with atovaquone-proguanil [24]. Treatment of human volunteers infected with

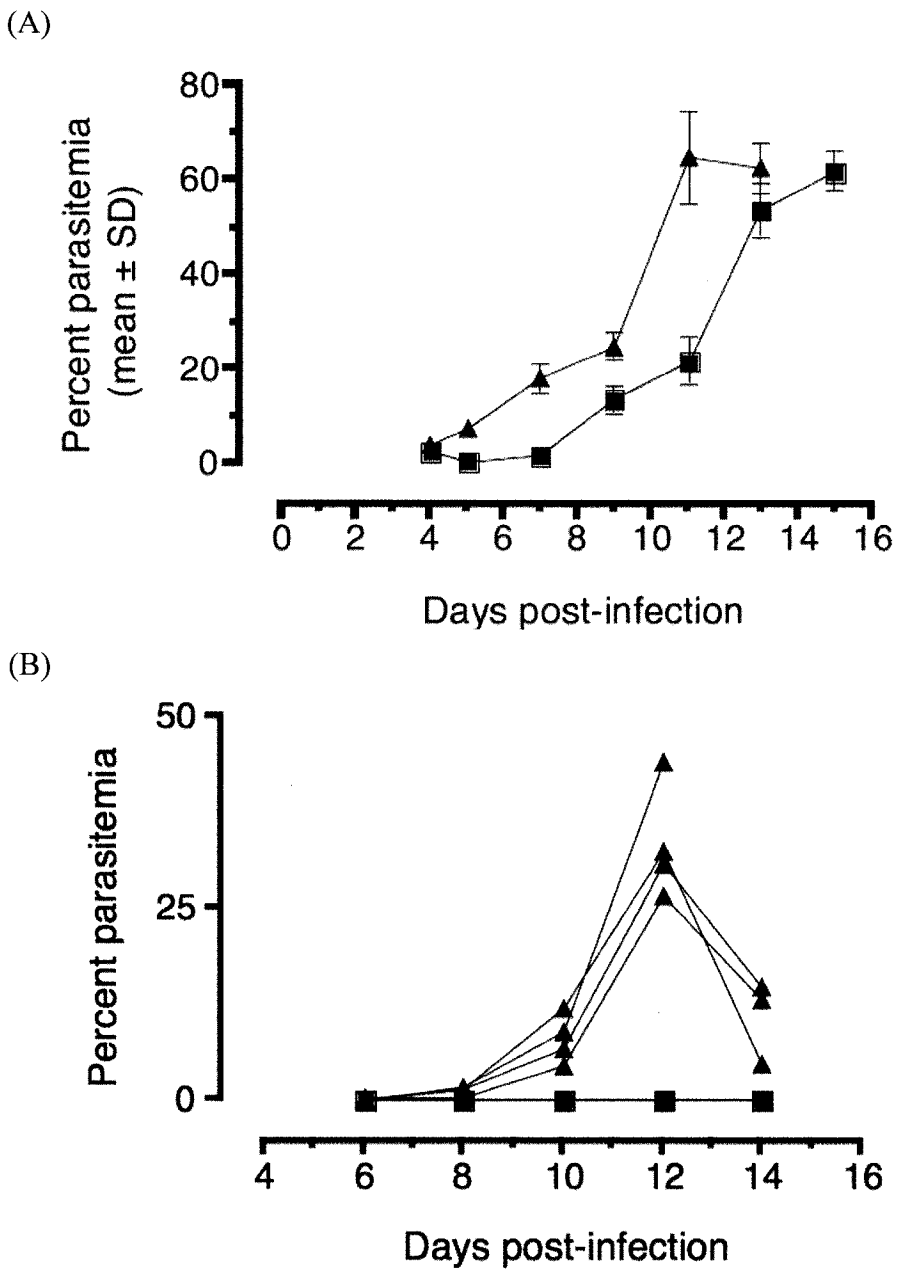

Figure 4-2. Increased survival and immunity in centanamycin-treated mice infected with a virulent strain of rodent malaria.
(A) Parasitemia of mice infected with *P. c. adami* DS and treated with vehicle (filled triangles) or 15 mg/kg centanamycin (filled squares) on day 5 p.i. (B) Drug-treated mice from (A) were challenged with a second *P. c. adami* DS infection at day 34 p.i. Parasitemia of drug-treated mice (filled squares) and naïve mice (filled triangles) was monitored over 14 days.

low levels of *P. falciparum* using various regimens of atovaquone-proguanil, chloroquine and malarone, gave protection from homologous challenge [25] and intermittent treatment of infants with sulfadoxine-pyrimethamine facilitates development of immunity to malaria [26].

Centanamycin promotes survival of mice infected with P. berghei ANKA

Using *P. berghei* ANKA, a single injection of vehicle or 10 mg/kg centanamycin on day 4 p.i. effectively suppressed the infection within 24 h, reducing the parasitemia of the drug-treated animals by 90% compared with controls (Fig. 4-3A). However, this effect was not sustained and the drug-treated mice developed an infection 3 days later than controls. Given the virulence of *P. berghei* ANKA strain, we evaluated the activity of centanamycin using the 4-day suppression test[16] and a moderate dose of 5 mg/kg. The parasitemia on the day after treatment termination is an indicator of drug efficacy [2]. Centanamycin resulted in a 100% reduction in *P. berghei* parasitemia on day 5 after infection (data not shown). This effective dose is similar to the ED_{90} values for chloroquine, amodiaquine and primaquine in the 4-day test [15]. In two animal trials, all mice that received centanamycin survived until at least day 20 p.i. with no signs of toxicity; 3/8 mice did not develop parasitemia for 45 days and were considered cured (Fig. 4-3B). In contrast, all control animals succumbed to the infection by day 15 p.i. Our results show that centanamycin is effective at rapidly suppressing blood-stage malaria infections from three different rodent malaria models. Furthermore, the efficacy of the compound can be enhanced using multiple dosing protocols against the most virulent rodent parasites.

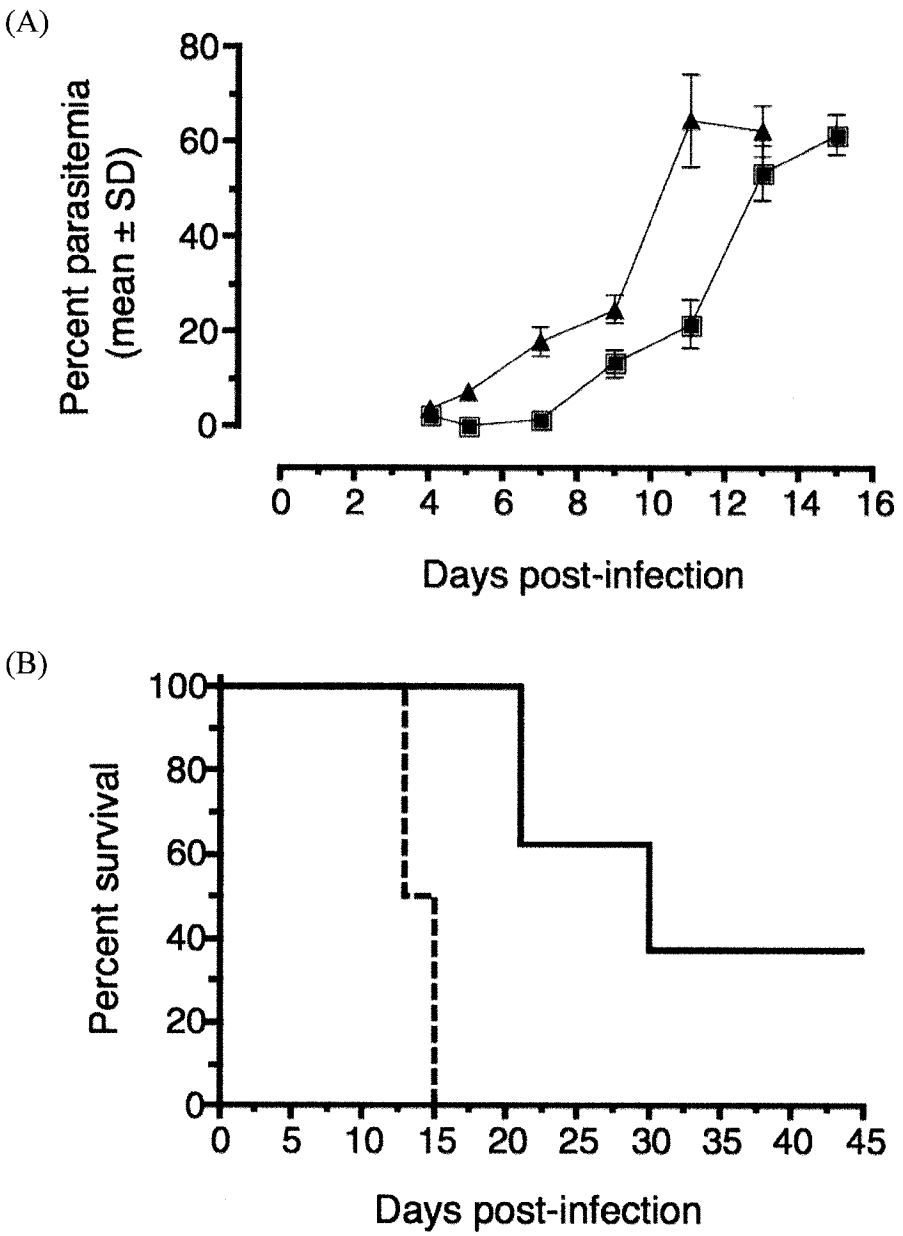

Figure 4-3. Effects of a single and multiple dose regimen of centanamycin in mice infected with *P. berghei* ANKA.

(A) Parasitemia of mice infected with *P. berghei* ANKA and treated with vehicle (filled triangles) or 10 mg/kg centanamycin (filled squares) on day 4 p.i. (B) Survival curve of *P. berghei* infected mice treated 2, 24, 48, and 72 hours p.i. with 5 mg/kg centanamycin (solid line) or vehicle (stippled line).

Asexual, but not sexual blood-stage parasites, are sensitive to centanamycin

Many antimalarial drugs, including chloroquine [27, 28], quinine [29], and pyrimethamine-sulfadoxine [30], have little effect on the transmission of *Plasmodium* to the mosquito. To evaluate the effects of centanamycin on transmission, separate sets of mosquitoes were fed on mice infected with a *P. berghei* ANKA strain expressing GFP [31], both before and 24 h after treatment with centanamycin or the vehicle. Parasitemias and gametocytemias were comparable in both groups of mice before drug administration (data not shown). Four days after infection, mice received a single injection of 10 mg/kg centanamycin or the vehicle. Twenty-four hours later, a significant reduction (76 %) in mean blood-stage parasitemia was observed in drug-treated mice compared with controls (Fig. 4-4A; $p < 0.0001$, Student *t*-test, $n = 4$). In contrast, gametocytemias of control and drug-treated groups were not significantly different (Fig. 4-4B) suggesting that centanamycin had no immediate effect on gametocytes.

Aberrant oocyst development in mosquitoes fed on drug-treated mice

Plasmodium infection results in the formation of oocysts in the midgut of mosquitoes, where the sporozoite stage of the parasite develops. The number of *P. berghei* oocysts on the midguts of mosquitoes fed on the two groups of mice was counted on day 10 p.f. The average number of oocysts in mosquitoes fed on drug-treated mice was reduced by 83 % (Fig. 4-5A; $p = 0.012$, Student's *t*-test, $n = 100$) compared with controls. Interestingly, in addition to the reduced number, oocysts in mosquitoes fed on drug-treated mice were often misshapen and much smaller than those formed on the midguts of mosquitoes fed on control mice (Fig. 4-5B). To characterize these morphological defects further,

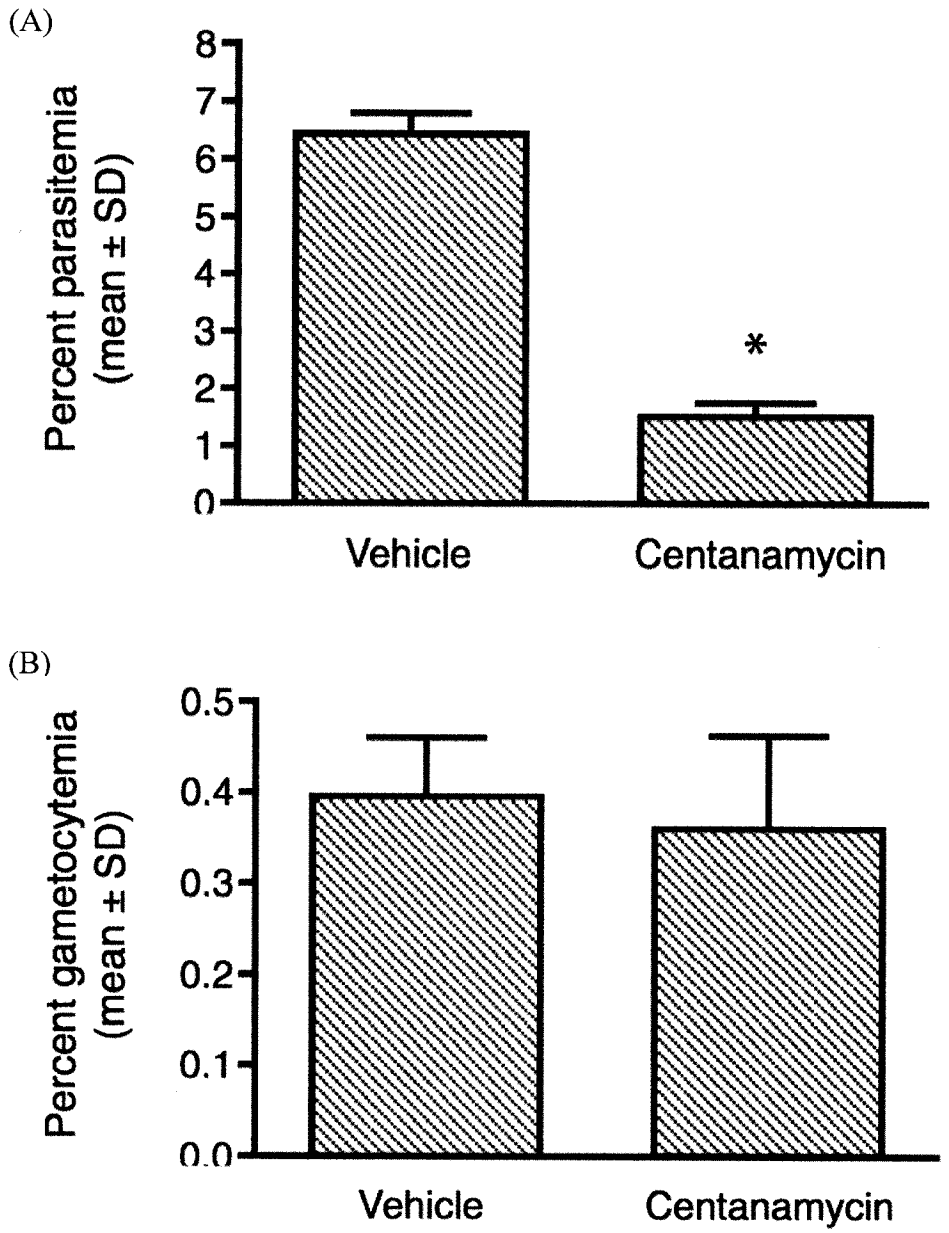

Figure 4-4. Asexual parasites, but not gametocytes, are sensitive to centanamycin in mice.

(A) The percent parasitemia and (B) gametocytemia of two groups of *P. berghei* ANKA-infected mice 24 h after treatment with 10 mg/kg centanamycin or vehicle. * Indicates significant difference, $p < 0.0001$, Student *t*-test, $n = 4$.

transmission electron microscopy (TEM) was carried out on fixed midguts at day 10 p.f. In oocysts from mosquitoes fed on control mice (Fig. 4-5C, *left*), sporoblast formation and sporozoite budding were observed. In contrast, oocysts from mosquitoes fed on drug-treated mice were small and highly vacuolated (Fig. 4-5C, *middle* and *right*). The cytoplasm was homogenous and no sporoblasts were detected, indicative of severe defects in the development of these oocysts.

Parasites exposed to centanamycin in mice fail to produce sporozoites in mosquitoes

To determine whether these oocysts were viable and capable of producing sporozoites, the number of *P. berghei* sporozoites in the mosquito midguts was counted on day 18 p.f. In mosquitoes fed on drug-treated mice, the number of midgut sporozoites per mosquito was reduced by ≥ 99 % relative to controls (Fig. 4-6A; $p = 0.0001$, Student's *t*-test, $n = 4$). A similar reduction in the number of sporozoites was observed in the salivary glands (Fig. 4-6B; $p = 0.001$, Student's *t*-test, $n = 4$). These results suggest that the drug-treated parasites failed to develop from the oocyst to the sporozoite stage.

Antimalarial activity of centanamycin is associated with parasite DNA damage

Studies in mammalian cells have demonstrated that centanamycin covalently binds to DNA, resulting in the formation of drug adducts within a specific A/T motif [12]. *In vitro*, centanamycin:DNA adducts have been shown to block *Taq* polymerase and prevent amplification by PCR of plasmid DNA treated with centanamycin [12, 32]. We adapted this assay for real-time PCR [9] to detect adducts in *P. berghei* ANKA genomic DNA treated with centanamycin *in vitro.* Amplicons were selected from regions of the

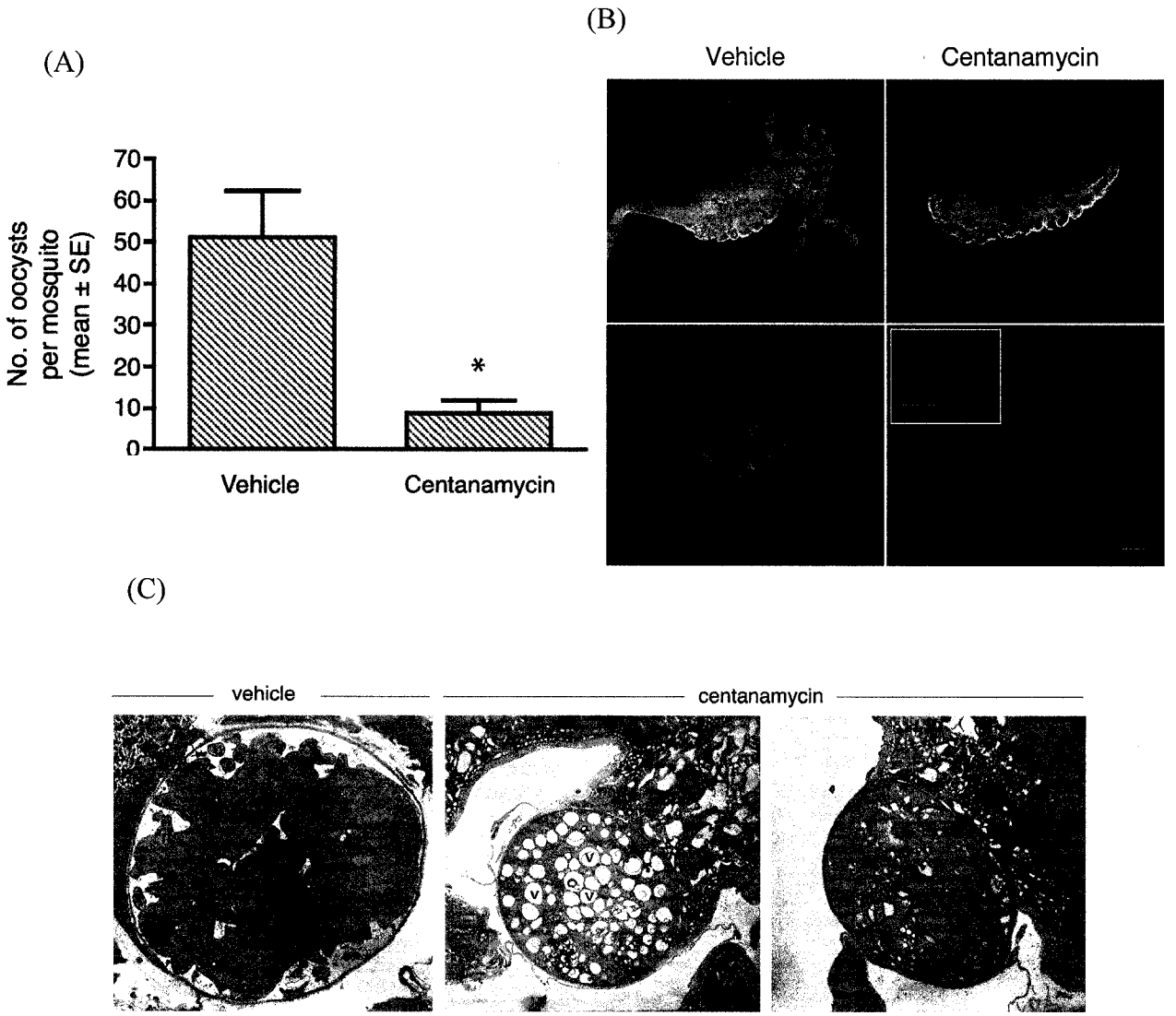

Figure 4-5. Treatment of mice with centanamycin arrests development of *P. berghei* ANKA oocysts in mosquito midguts.

(A) The mean number of oocysts in the midgut of mosquitoes fed on mice 24 h after treatment with 10 mg/kg centanamycin or the vehicle. * Indicates significant difference, p = 0.012, Student's *t*-test, n = 100. (B) Photographs showing light microscopic (upper quadrants) and fluorescent images (lower quadrants) of representative midguts from mosquitoes fed on control (*left*) and centanamycin-treated (*right*) animals infected with GFP-expressing *P. berghei* ANKA. Inset shows a magnification of the oocysts on the midgut from the mosquito fed on the drug-treated animal. Scale bar represents 200 μm. (C, *left*) Representative TEM of an oocyst on the midgut of a mosquito fed on vehicle-treated control mice. The oocyst cytoplasm has subdivided into sporoblasts (spr) while budding (b) and free sporozoites (sp) are observed. Nuclei (n) can be seen within the sporozoites. (C, *middle and right*) TEM of representative oocysts on the midgut of mosquitoes fed on mice treated with centanamycin for 24 h. The oocysts are misshapen; nuclei are sometimes present (n), but the cytoplasm is highly vacuolated (v) and contains no sporoblasts. Scale bar represents 2 μm.

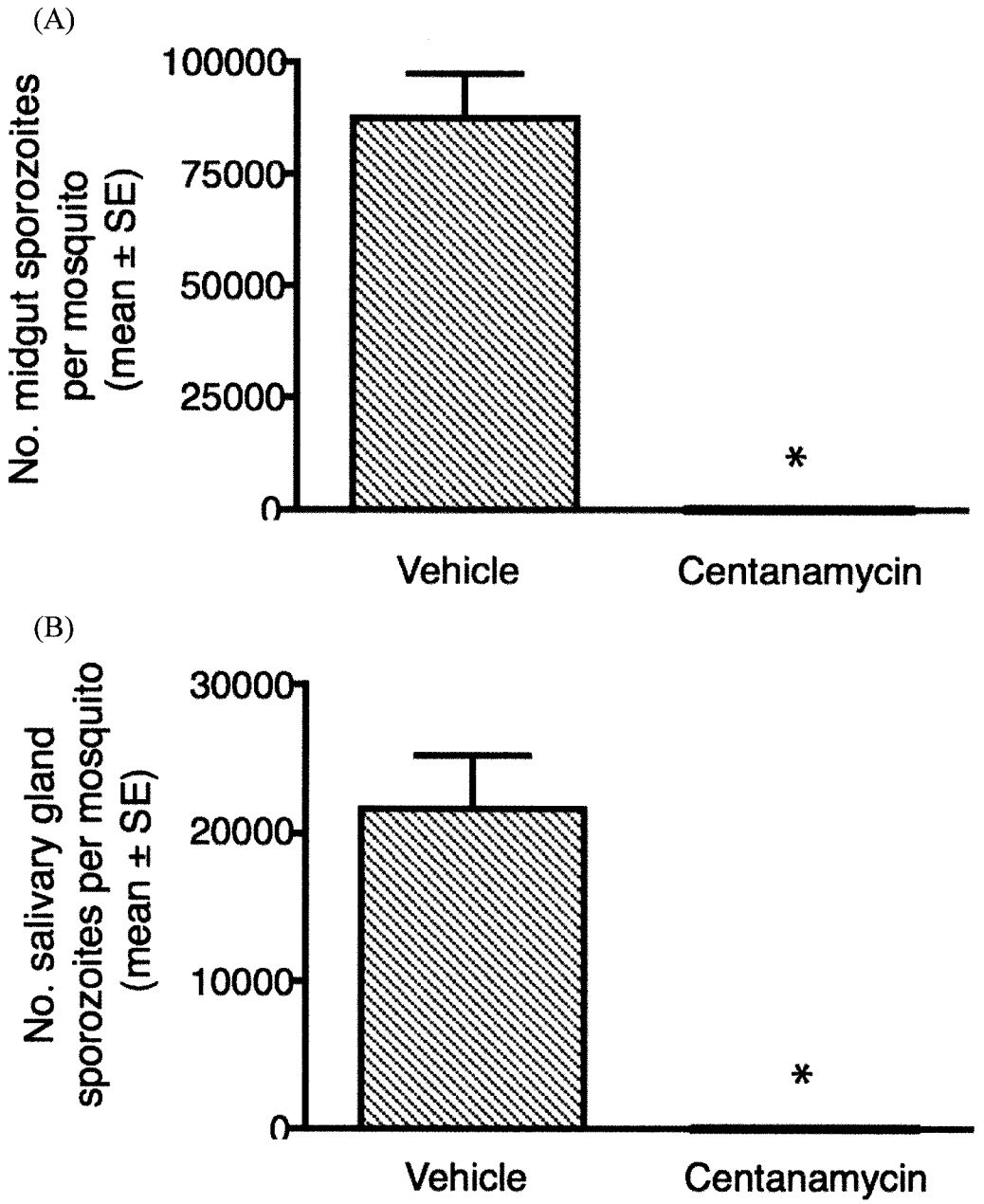

Figure 4-6. Centanamycin treatment in mice inhibits sporozoite production in mosquitoes.

(A) The mean number of midgut sporozoites per mosquito on day 18 p.f. (B) The mean number of salivary gland sporozoites per mosquito on day 18 p.f. * Indicates significant difference: p = 0.001, Student's *t*-test, n = 4 (A); p = 0.0001, Student's *t*-test, n = 4 (B).

Plasmodium 18S rRNA and *ama-1* genes that contain four and ten (A/T)AAA motifs, respectively. The C*t* values are expressed as the percent amplification efficiency (AE) of the drug-treated DNA relative to the control DNA as described previously [23]. The mean C*t* values for two *Plasmodium*-specific loci (*18S rRNA*, *ama-1*) were higher in drug-treated DNA compared with control DNA, corresponding to a decrease of 77 % in the amplification efficiency (AE) at each locus (Fig. 4-7A).

We next isolated parasite DNA from peripheral blood of mice infected with GFP-expressing *P. berghei* ANKA, following treatment with centanamycin (10 mg/kg) for 24 h. We observed 43 % and 54 % reduction in the AE of for the parasite genes *18S* and *ama-1*, respectively (Fig. 4-7B). No decrease in AE was observed following amplification of the mouse *β-actin* gene (data not shown): this amplicon does not contain any (A/T)AAA sites and served as a negative control. These results suggest that parasite DNA is modified *in vivo* by centanamycin and correlates with its rapid effects on blood-stage parasitemia.

Given the extensive defects in parasite development within the mosquito, we tested whether the DNA damage induced in mice treated with centanamycin was sustained within the mosquitoes fed on these mice. Genomic DNA was extracted from mosquito midguts 10 days p.f. on *P. berghei* infected mice treated with centanamycin. The AE was reduced by 62 % for both the *Plasmodium 18S* and *ama-1* loci in DNA isolated from mosquitoes fed on drug-treated relative to control mice (Fig. 4-7C). These findings demonstrate that the DNA damage inflicted on the parasites within the mammalian host is stable and persists for at least 10 days within the mosquito host.

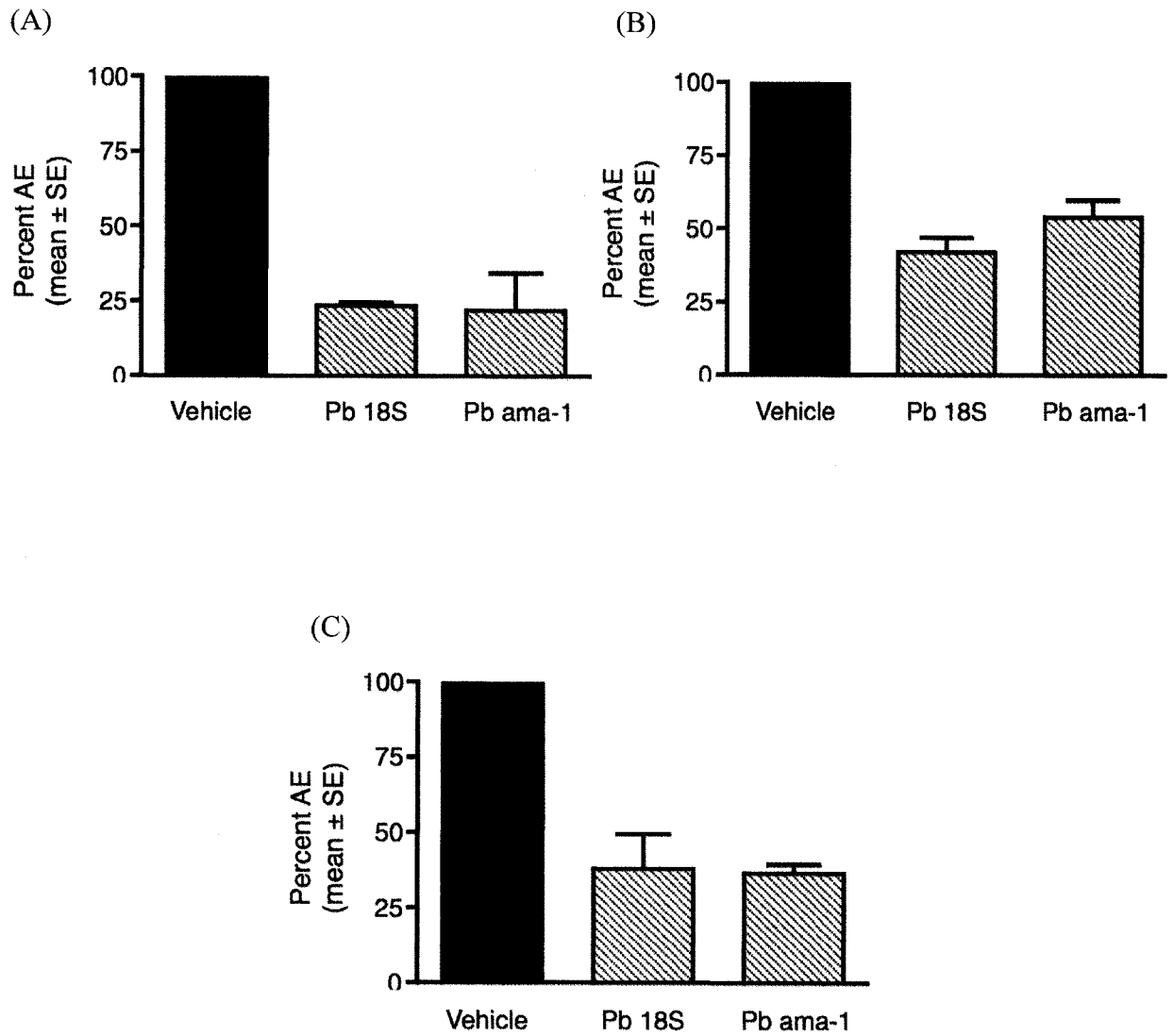

Figure 4-7. Parasite genomic DNA is modified by centanamycin *in vitro* and *in vivo*. (A) Percent AE of genomic DNA isolated from mice infected with *P. berghei* ANKA following treatment with vehicle or centanamycin *in vitro*. Real-time PCR was used to amplify regions of the *Plasmodium 18S rRNA* and *ama-1* genes. (B) Percent AE of genomic DNA isolated from infected mice 24 h after treatment *in vivo* with 10 mg/kg centanamycin relative to controls. (C) Percent AE of genomic DNA isolated from oocysts on mosquito midguts 10 days p.f. on the mice in (B).

DISCUSSION

Here, we describe the potent antimalarial activity of the DNA-binding compound centanamycin against *P. falciparum in vitro* and murine malaria *in vivo*. In all three rodent malaria models, a rapid reduction in parasitemia was observed one day following a single treatment with centanamycin. A critical observation is that the effects of centanamycin on blood-stage parasites also dramatically compromised the viability of the mosquito stages derived from these treated parasites, resulting in a 99% reduction in sporozoite production. Primaquine is a commonly used antimalarial with gametocytocidal properties, but has no effect on the numbers of oocysts in mosquito midguts, even at doses as high as 100 mg/kg [33]. Atovaquone, administered to mosquitoes from a blood meal at a dose of 100 mg/kg, reduced the number of oocysts by 71 % whereas the number of sporozoites was reduced by only 22 % [34]. Treatment with centanamycin, therefore, is comparatively much more effective as a transmission-blocking compound.

We propose that the mechanism underlying parasite death, in the mouse and the mosquito hosts, involves covalent modification of *Plasmodium* genomic DNA. An important consideration for the use of DNA damaging agents in humans is the genotoxicity of the compound. This is particularly important for the treatment of malaria, which most severely afflicts children who are often continuously at risk of re-infection. Results from *in vitro* genotoxicity studies in human lymphocytes showed no statistically significant chromosome aberration with 80 nM centanamycin, following 4 hr or 21 h exposure (unpublished results). Centanamycin is not toxic to cultured murine bone marrow cells at a concentration of 8.4 nM [12] and is cleared from murine plasma in 4 h when administered by an i.v. injection (unpublished results). Given the rapid parasite

clearance by centanamycin, exposure of human cells to this drug may be minimal since only a single dose of drug may be needed, as observed here in rodent malaria models. However, this concern must be addressed by in-depth pharmacokinetic and mutagenicity studies following single and multiple exposures to centanamycin.

Both centanamycin and its parent compound adozelesin, bind to sequences within genomic DNA that share the motif (A/T)AAA [21]. The covalent interaction of centanamycin with these A/T binding sites can exert a wide range of deleterious effects on genomic stability, disrupting essential processes such as DNA replication and gene transcription. Using an *in silico* approach, Woynarowski *et al.*, have recently shown this motif to be 3.9 times more frequent within the genome of *P. falciparum* compared to the human genome and is distributed across all 14 malaria chromosomes [35]. These findings strengthen the rationale for developing A/T-specific antimalarial drugs since these compounds exploit the preferential availability and distribution of binding sites within the *Plasmodium* genome compared to the host genome.

Given its low toxicity, potent antimalarial activity and unique mode of action, we propose that centanamycin be developed as an important component of combination therapies to reduce parasitemia in the human host and also diminish the level of transmission in malarious regions. Due to its rapid effects after a single dose *in vivo*, the drug may be useful for treatment of severe life-threatening malaria infections.

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CONNECTING STATEMENT

In Chapter 4, I showed that the novel sequence specific DNA binding agent, centanamycin, arrests parasite development within the midguts of mosquitoes, resulting in a failure to produce the infective forms that migrate to the salivary glands. Radiation and genetic attenuation of *Plasmodium* sporozoites have been used as approaches for whole-organism vaccines against malaria. This next Chapter explores the possibility of using centanamycin-treatment of sporozoites *in vitro* as a way of producing a chemically attenuated sporozoite (CAS) vaccine. I evaluated the activity of the drug directly against sporozoites *in vitro* to determine whether centanamycin would interfere with sporozoite biology. In particular, I examined the effect of centanamycin exposure on the ability of sporozoites to establish liver and blood stage infections and whether vaccination with CAS infections would protect against challenge with wild type sporozoites.

CHAPTER 5:

Chemical attenuation of *Plasmodium berghei* sporozoites induces sterile immunity in mice

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Running title: Sterile immunity generated from CAS.

Abbreviations: chemically attenuated sporozoites (CAS), cycle threshold (C*t*), exoerythrocytic forms (EEFs), genetically attenuated sporozoites (GAS), intraperitoneal (i.p.), intravenous (i.v.), post-feeding (p.f.), post-infection (p.i.), radiation-attenuated sporozoites (RAS)

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ABSTRACT

Radiation or genetic attenuation of *Plasmodium* sporozoites are two approaches for whole organism vaccines that are protective against malaria. We evaluated chemical attenuation of sporozoites as an alternative vaccine strategy. Sporozoites were treated with the DNA sequence-specific alkylating agent centanamycin, a compound that significantly affects blood stage parasitemia and transmission of murine malaria and also inhibits *P. falciparum* growth *in vitro*. Here, we show that treatment of *P. berghei* sporozoites with centanamycin impaired parasite function both *in vitro* and *in vivo*.

Infectivity of hepatocytes by sporozoites *in vitro* was significantly reduced and treated parasites showed arrested liver stage development. Inoculation of mice with sporozoites that were treated *in vitro* with centanamycin failed to produce blood stage infections. Furthermore, BALB/c and C57/BL6 mice injected with treated sporozoites were protected against subsequent challenge with wild type sporozoites. Our findings demonstrate that chemically attenuated sporozoites (CAS) could be a viable alternative for the production of an effective liver stage vaccine for malaria.

INTRODUCTION

The development of an effective vaccine is critical to curb the significant health, social and economic impacts caused annually by malaria in endemic countries [1]. Malaria infection involves injection of *Plasmodium* sporozoites from a mosquito into humans . The sporozoites migrate to the liver, invade hepatocytes and transform into exoerythrocytic forms (EEFs) that replicate to produce schizonts containing thousands of merozoites [2]. These merozoites are released into the host blood stream and invade erythrocytes. The blood stages of malaria are responsible for producing the symptoms of the disease. Many attempts have been made in recent years to develop effective sub-unit vaccines composed of recombinant *Plasmodium* antigens. Due to the complexity of *Plasmodium*, these vaccines have only been partially effective [3-5].

Recently, there has been renewed interest in the attenuated, whole organism vaccine strategy [6-8]. The whole organism approach has historically used radiation-attenuated sporozoites (RAS) [9], to provide sterile immunity experimentally in both

mice and humans. These RAS invade hepatocytes in a susceptible host and begin to develop into EEFs, but the majority of parasites fail to undergo nuclear division and do not progress to the merozoite form [10, 11]. Using mice, RAS dosing regimens that generate protective immunity have varied, although most require a prime-boost schedule [12-15]. A meta-analysis of 10 years of immunization of human volunteers using irradiated *Plasmodium falciparum* sporozoites showed a dose-response in terms of the immunization dose required for protection [6, 7]. One key issue with RAS has been the delivery of the correct irradiation dosage to ensure adequate attenuation of the parasite [6, 16-18]. A strategy to overcome this issue has been to generate genetically attenuated sporozoites (GAS) in which parasite strains are deleted for genes essential to sporozoite function. Since the publication of the *Plasmodium* genome [19], there have been several reports using this strategy in rodent models of malaria. These include the deletion of *uis3* [20], *uis4* [21], *P36p* [14], and simultaneous deletion of *uis3/uis4* [22] genes in *P. berghei*, as well as deletions of *uis3* and *uis4* [23], and simultaneous deletion of *P52* and *P36* genes [24] in *P. yoelii*. These GAS resemble RAS in terms of invasion of host hepatocytes and arrested development, but GAS-infected hepatocytes disappear almost completely after 24-36 h in culture [14, 20, 21, 23], while RAS persist for longer times in the arrested form [10, 25]. As with RAS, most GAS need to be delivered in a multiple dosing strategy in order to induce sterile immunity.

We have developed a new strategy to generate attenuated parasites based on the *in vitro* chemical treatment of sporozoites. We have previously reported the antimalarial activity of A/T-specific DNA binding agents that exploit the A/T richness of the

Plasmodium genome [26] and showed that the compound centanamycin has a significant effect on both blood stages and on transmission of malaria to mosquitoes [27]. Here, we used centanamycin to attenuate *P. berghei* sporozoites *in vitro*. Chemically attenuated sporozoites (CAS) showed a significant reduction in hepatocyte infection and, in those hepatocytes that were infected, EEFs were greatly reduced in size. We show that CAS do not generate blood-stage infections in mice and immunization of BALB/c and C57/BL6 mice with CAS produces sterile immunity against challenge with wild type sporozoites.

MATERIALS AND METHODS

Treatment of sporozoites with centanamycin

Anopheles stephensii mosquitoes were maintained at 70 % humidity and 22°C and infected with *P. berghei* ANKA wild-type parasites as described [28]. Infected mosquito salivary glands were dissected at or about day 18 post-feeding (p.f.) and kept on ice. Sporozoites were quantified by microscopic counting in a hemocytometer. Centanamycin (2 M) was prepared in a PET (polyethylene glycol 400, ethanol, Tween 80)/glucose solution [29]. Each group of sporozoites was treated with 2 mM centanamycin diluted in DMEM, while control groups received the same volume of vehicle. Incubations were performed at room temperature for 30, 60, or 90 min the sporozoites were centrifuged at 21,000 × *g* for 5 min at room temperature and resuspended in the appropriate medium for each assay.

Analysis of viability and infectivity of treated sporozoites *in vitro*

Mouse hepatoma cells (Hepa 1-6) [30] were grown in DMEM medium with 10% FBS with 1% PSG (penicillin, streptomycin, gentamicin) at 37°C and 5% CO_2. Hepa 1-6 cells (2×10^5) were seeded on glass coverslips in 24-well plates 24 h prior to testing.

Membrane integrity

Sporozoites were incubated with 10 g/mL propidium iodide for 5 min at room temperature after incubation with the vehicle or centanamycin. Sporozoites were washed 3 times and resuspended in 10 L DMEM, then wet mounted on a microscope slide and covered with a glass coverslip. The number of fluorescent sporozoites was quantified using a Nikon Eclipse E600 microscope. As a control, freshly dissected sporozoites were labeled and quantified soon after dissection to ensure viability of the dissected sporozoites. Sporozoites that were heat killed at 65°C for 15 min served as a control. Incubations were performed in triplicate in two independent experiments, with 100 sporozoites counted per well.

Gliding motility

Glass, 8-chambered Lab-Teks (Nalgene) were coated with 5 g/mL 3D11, a monoclonal antibody directed against the repeat region of *P. berghei* circumsporozoite protein [31], in PBS overnight at room temperature. The 3D11 antibody is used to capture shed circumsporozoite protein. The wells were washed 3 times with PBS. For each well 2×10^4 sporozoites were treated as outlined above. Sporozoites were centrifuged, the

medium was replaced with DMEM containing 3 % BSA and incubated at 37°C in 5 % CO_2 for 1 h, after which the sporozoites were fixed with 4 % paraformaldehyde at 4°C overnight. Each well was washed with PBS and blocked with 1% BSA in PBS. The biotinylated 3D11 monoclonal antibody [32] was added, followed by streptavidin-fluorescein isothiocyanate (Sigma) for 1 h at 37°C. The percent gliding motility was quantified by counting both the number of sporozoites with trails, as well as the number of circles each trail contained using a Nikon Eclipse E600 microscope. Incubations were performed in triplicate in two independent experiments, with 100 sporozoites counted per chamber.

Invasion of hepatoma cell line in vitro

Sporozoites (5×10^4) were treated as above, resuspended in cell medium and added to each well containing semiconfluent Hepa 1-6 cells. Plates were incubated for 1 h at 37°C, fixed in 4 % paraformaldehyde at 4°C and stained using 3D11 and a double staining technique [33]. Intracellular and extracellular sporozoites were differentially stained and counted using a Nikon Eclipse E600 microscope. Incubations were performed in triplicate in two independent experiments, with 100 sporozoites counted per well.

Liver stage development in vitro

Sporozoites (5×10^4) were treated as above, resuspended in cell medium and added to each well of semiconfluent Hepa 1-6 cells. Plates were incubated for 42 h at 37°C to allow

for EEF development, then fixed in 4 % paraformaldehyde at 4°C overnight and washed with PBS. Each coverslip was blocked and permeabilized in a solution of 10 % goat serum, 1 % BSA, 100 mM glycine, 0.05 % NaN_3 (pH 7) and 0.2 % saponin for 30 min at room temperature. The coverslips were then incubated with 2E6 (a monoclonal antibody that recognizes *Plasmodium* HSP70) [34] for 1 h at room temperature, washed with PBS, then incubated with anti-mouse fluorescein isothiocyanate conjugated antibodies (Sigma) for 1 h. Coverslips were washed with PBS, mounted on microscope slides and the number of EEFs counted on a Nikon Eclipse E600 microscope. Images were taken with a Leica TCS SP2 AOBS confocal microscope using Leica LCS Software (v. 5). Incubations were performed in triplicate in two independent experiments, with 50 random fields counted per well.

Liver stage development *in vivo*

Procedures for animal experiments were approved by New York University School of Medicine Institutional Animal Care and Use Committee. Eight-week old female C57/BL6 mice were injected intravenously (i.v.) with 2×10^4 sporozoites treated with centanamycin for 30 min and resuspended in DMEM. Livers were harvested from infected mice 40 h later, as well as from one uninfected mouse. Total RNA was isolated using TRIzol (Invitrogen) and cDNA synthesized according to the manufacturer's instructions (Applied Biosystems). Malaria infection was quantified using quantitative real-time PCR with primers specific for *P. berghei* 18S rRNA [35, 36]. Tenfold dilutions of a plasmid construct containing the 18S rRNA sequence were used to create a standard

curve. Two independent experiments were performed and each sample was analysed in triplicate with 4 mice per treatment group.

Blood stage development from treated sporozoites

Eight-week old female BALB/c and C57/BL6 mice were injected i.v. with 5×10^4 or 2×10^4 CAS as indicated, which had been treated with centanamycin for 30 min and resuspended in DMEM. Parasitemia was evaluated from day 3 p.i., onwards by Giemsa-stained thin blood smears. Percent parasitemia was calculated from 1000 cells per slide. Animals were evaluated for 7, 10, or 21 days post-injection of treated sporozoites injection as indicated.

Challenge of mice with wild-type sporozoites

The same groups of mice that were injected with CAS to determine blood stage development were challenged by i.v. injection using 5×10^3 untreated, wild type *P. berghei* ANKA sporozoites (for the BALB/c mice), and 1×10^3 or 1×10^4 untreated wild type sporozoites (for the C57/BL6 mice) 21, 14, or 10 days after the immunization regimen. Age-matched, naïve mice were injected with the same number of sporozoites as infection controls to assess infectivity of the untreated sporozoites. Parasitemia was evaluated from day 3 p.i., onwards by Giemsa-stained thin blood smears. Percent parasitemia was calculated from 1000 cells per slide. Animals were evaluated for at least 30 days post-challenge.

Statistical analysis

All statistical analyses were completed using Prism (v. 4.0a). Where differences in toxicity, gliding motility, invasion, EEF formation and quantitative PCR were assessed, normality was tested using the Kolmogorov-Smirnov Goodness-of-Fit Test. Data with a p value > 0.10 was considered normal. The differences were then tested using an ANOVA with a Tukey's Multiple Comparison post-hoc test. Only those assays showing significant differences were noted.

RESULTS

Chemical attenuation does not result in sporozoite death

Whole organism malaria vaccine strategies depend on the parasite being replication-deficient, yet still capable of invading host cells and producing antigens to induce an immune response. Incubation of sporozoites for 30, 60 and 90 minutes with centanamycin *in vitro* did not result in decreased viability of sporozoites relative to controls (Fig. 5-1). Propidium iodide, which is membrane impermeable, is frequently used to stain viable cells in a population. Similar labeling with propidium iodide was observed in control and centanamycin-treated sporozoites suggesting that centanamycin did not affect membrane integrity. As incubation times increased from 30 to 90 min, the number of non-viable sporozoites increased similarly in the drug-treated population as compared with controls. This decrease in the viability of sporozoites is expected after dissection from mosquito salivary glands [37].

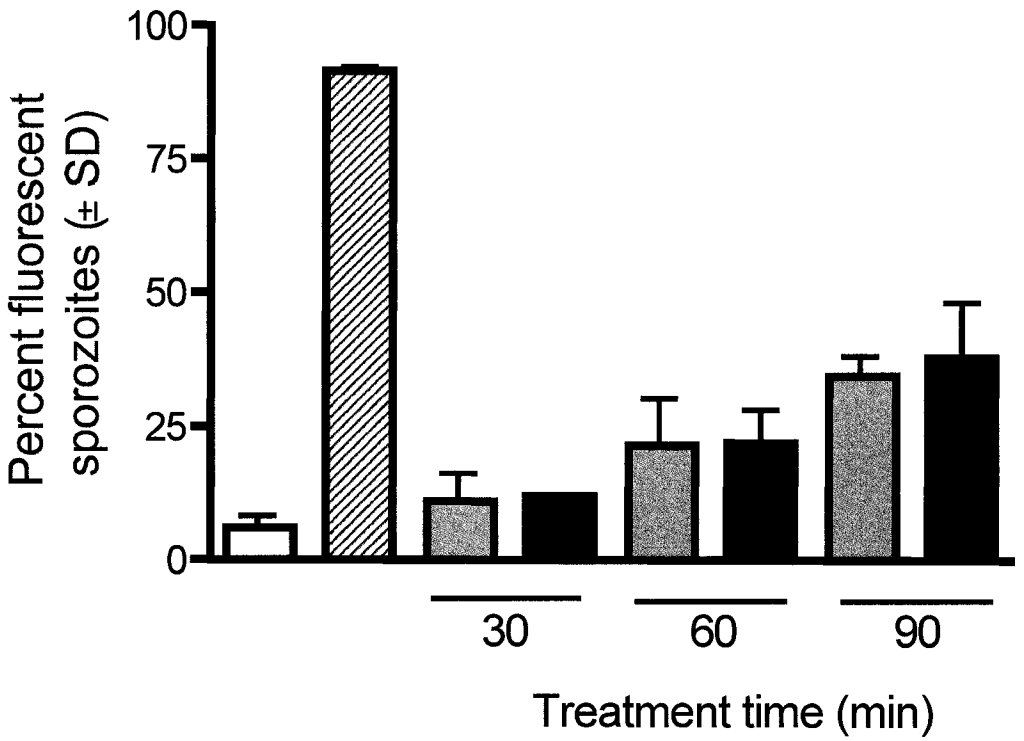

Figure 5-1. Treatment of sporozoites with centanamycin *in vitro* does not affect sporozoite membrane integrity.
P. berghei sporozoites were incubated with vehicle (gray bars) or 2 mM of centanamycin (black bars) for 30, 60, or 90 minutes before the addition of propidium iodide. Control sporozoites were either tested immediately following dissection (white bar) or heat killed (striped bar) for 15 min at 65°C before counting. For each sample, 100 sporozoites were counted in two separate experiments and the average percent staining with propidium iodide is shown.

Gliding motility and invasion of hepatocytes by drug-treated sporozoites

Gliding motility is a feature of *Plasmodium* sporozoites and is required for invasion of hepatocytes [38]. Treatment of sporozoites for 30, 60 or 90 minutes with centanamycin reduced the motility of sporozoites in a time-dependent manner (Fig. 5-2A). While the motility of vehicle-treated sporozoites also decreased, the motility of centanamycin-treated sporozoites was approximately 24% less than that of vehicle-treated sporozoites throughout the time course. Those sporozoites that remained motile after treatment with centanamycin produced trails of similar quality as vehicle-treated sporozoites (Fig. 5-2B). Gliding motility in RAS and all GAS reported so far is not different from wild type sporozoites [14, 20, 21, 24, 39].

RAS are able to invade hepatocytes with the same efficiency as wild type sporozoites, even if their development is later impaired [11, 21, 40]. We therefore analyzed the ability of centanamycin-treated sporozoites to invade hepatocytes (Hepa 1-6) *in vitro*. Treated sporozoites invaded hepatocytes efficiently (Fig. 5-3) although they did demonstrate an apparent mean reduction in invasion. However, this difference was only significant at the 60 minute treatment time when the average of all three experiments was considered. The cause for the reduced invasion rates is not known, but since sporozoite motility is required for invasion [38], it could be a consequence of the decrease motility observed in CAS.

EEF development is impaired after drug treatment

EEF formation is a critical phase in the *Plasmodium* life cycle, responsible for the

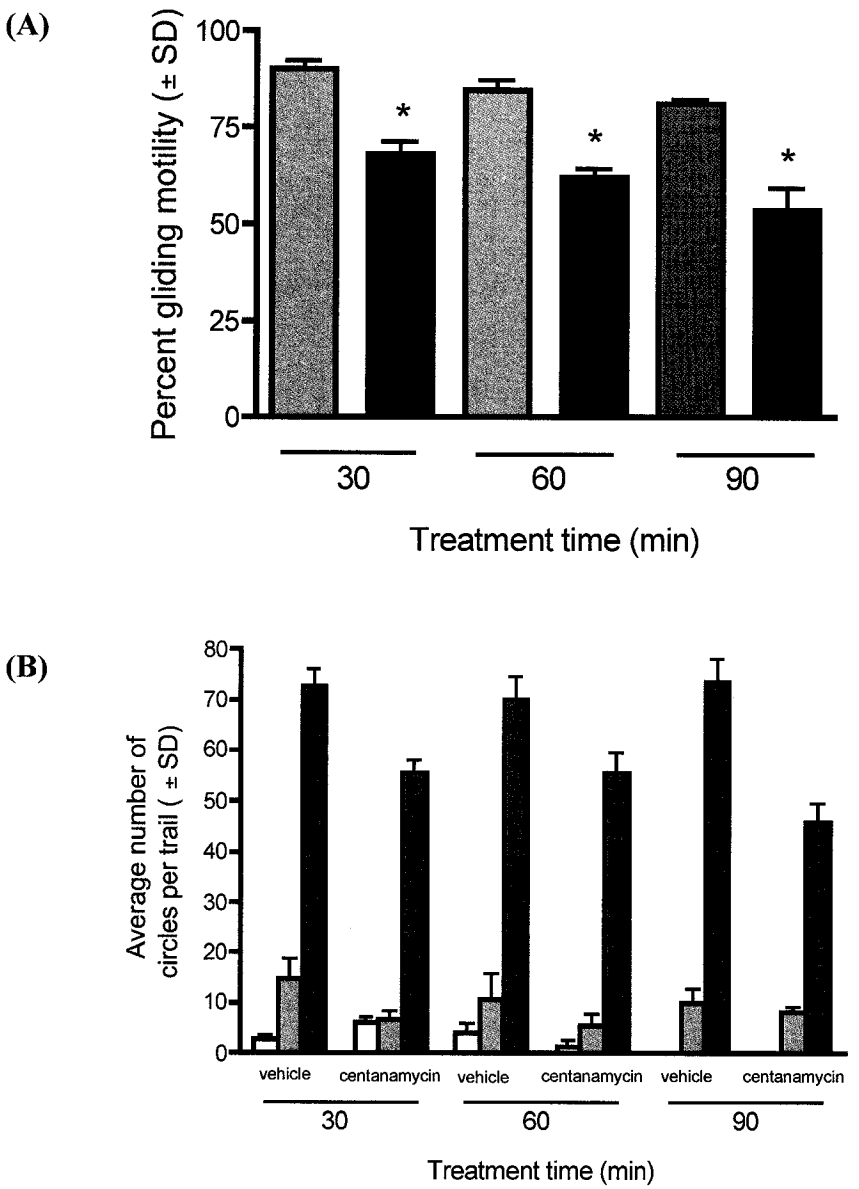

Figure 5-2. Gliding motility of sporozoites treated with centanamycin *in vitro*.
P. berghei sporozoites were incubated with vehicle or 2 mM of centanamycin for 30, 60, or 90 minutes, incubated for 1 h at 37°C to allow parasites to move, then assessed for gliding motility. (A) A significant reduction in the percentage of centanamycin-treated sporozoites (black bars) that exhibited gliding motility was observed at all time points relative to controls (gray bars; $p < 0.0001$, ANOVA, n=100). The quality of the trails denoted as the number of circles each sporozoite generated (B) is also shown (1 trail = white bars, 2-10 trails = light gray, and >10 trails = dark gray). Incubations were performed in triplicate in two independent experiments, 100 sporozoites were counted per well.

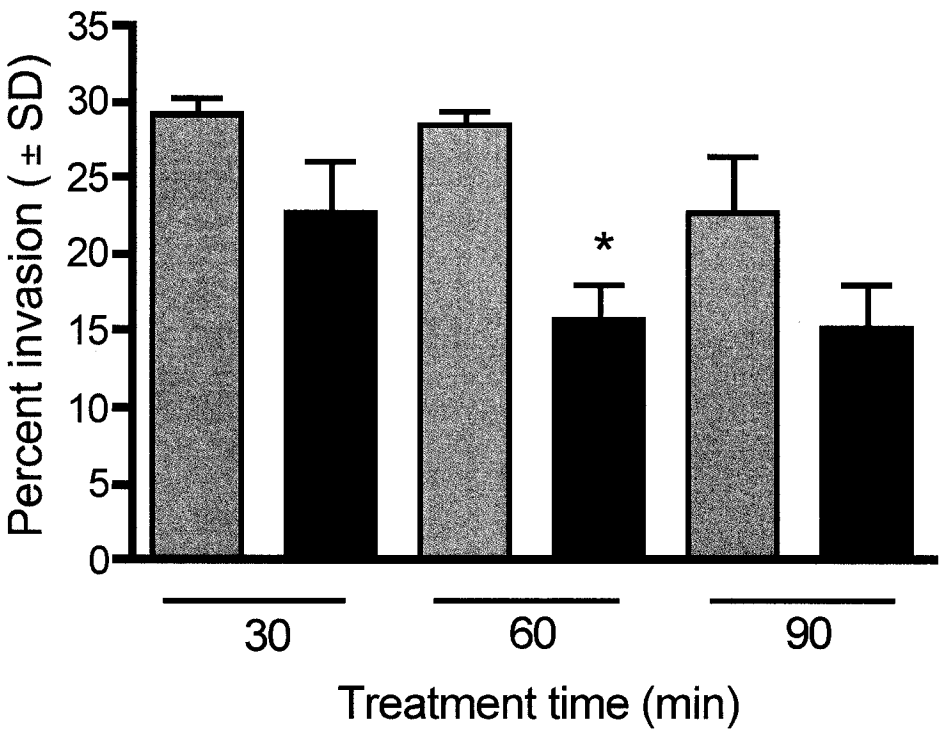

Figure 5-3. Invasion of hepatoma cells *in vitro* is not significantly reduced after treatment with centanamycin.
P. berghei sporozoites were incubated with vehicle (gray bars) or 2 mM of centanamycin (black bars) for 30, 60, or 90 minutes. Sporozoites were stained with 3D11 followed by secondary antibodies both before and after permeabilization to determine the number of sporozoites that invaded the cells. A significant reduction in invasion was observed only after the 60-minute treatment ($p=0.0065$, ANOVA, $n=100$). Incubations were performed in triplicate in two independent experiments and 100 sporozoites were counted per well.

generation of thousands of merozoites that will infect erythrocytes. Hepatocytes infected with wild type sporozoites develop EEFs that increase in size reaching approximately 10 μm after 48 h of culture [30]. Treatment with centanamycin for 30, 60 and 90 min significantly reduced the number of EEFs formed *in vitro* after 42 h (Fig. 5-4A), although a low number of EEFs could still be observed even after the 90 minute treatment. This is in sharp contrast to GAS, which in general, do not produce EEFs persisting beyond 24-36 h [14, 20, 21, 24], but it is more similar to RAS, where EEFs persist at least until 48 h [10, 17, 25]. While the vehicle-treated EEFs were homogeneous in size, centanamycin-treated EEFs were always smaller (Fig. 5-4B). These results show that centanamycin treatment significantly affects EEF formation *in vitro*.

Centanamycin-treated sporozoites fail to establish infection in mice

Since the number of EEFs formed in culture was significantly reduced when sporozoites were treated with centanamycin, we analyzed the ability of sporozoites treated for 30 minutes to establish a liver stage infection in C57/BL6 mice. Treated sporozoites (2×10^4) were injected into mice i.v. and parasites were allowed to develop for 40 h. Using real-time PCR to quantify the level of infection in the liver, vehicle-treated sporozoites produced nearly 6 orders of magnitude more copies of *Pb* 18S rRNA than those mice injected with centanamycin-treated sporozoites (Fig. 5-5). The number of copies in mice that received the drug-treated sporozoites was not significantly different compared to an uninfected control mouse.

Since the CAS reduced the extent of liver stage infection we tested the potential of

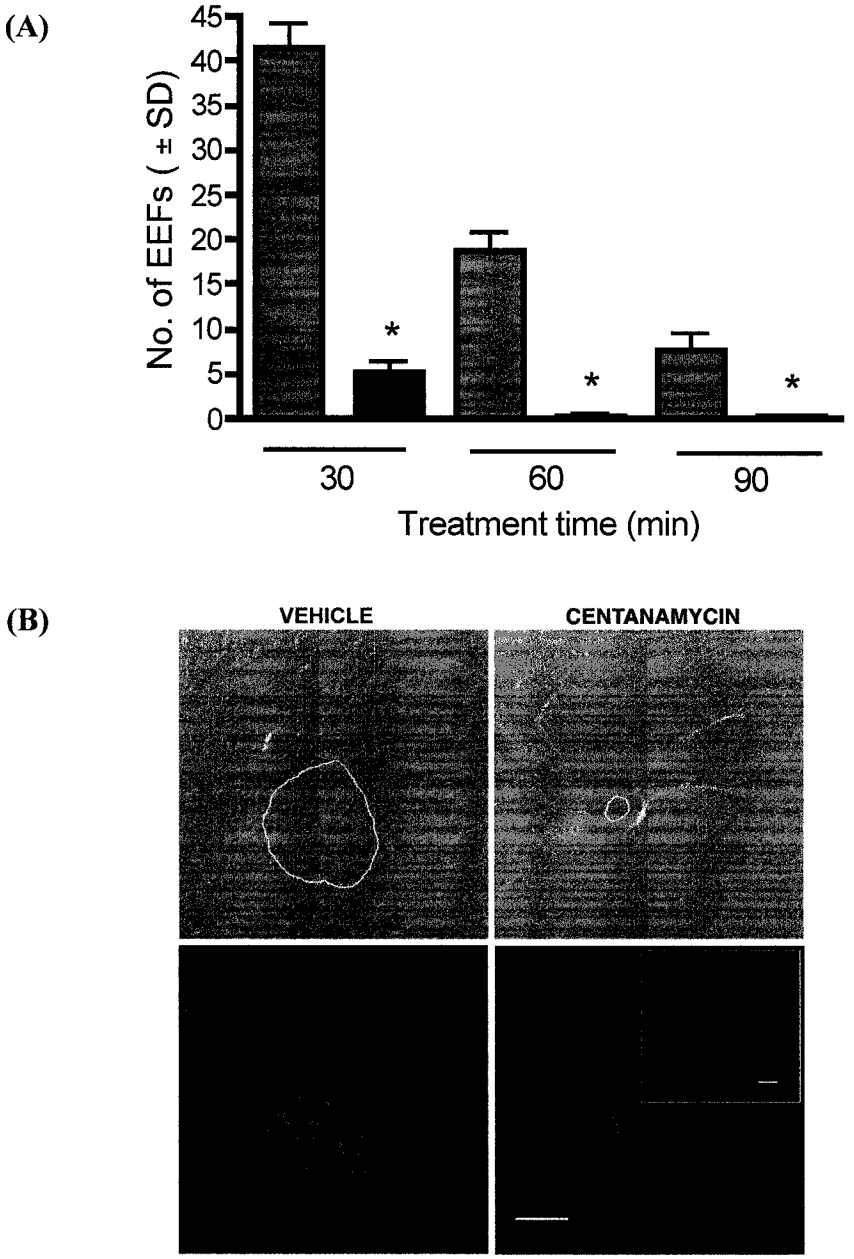

Figure 5-4. Treatment of sporozoites with centanamycin decreases the formation of EEFs in hepatoma cells.

P. berghei sporozoites were incubated with vehicle (gray bars) or 2 mM of centanamycin (black bars) for 30, 60, or 90 minutes. Sporozoites were added to Hepa1-6 cells for 42 h at 37°C. (A) The number of EEFs was significantly reduced in all treatment groups ($p < 0.0001$, ANOVA). (B) Phase contrast (upper quadrants) and fluorescent images (lower quadrants) of representative EEFs from vehicle (left) and centanamycin (right) treated sporozoites. The outline of the EEF is shown in white in the upper quadrants. Scale bar represents 10 μm, while scale bar on the inset image represents 1 μm.

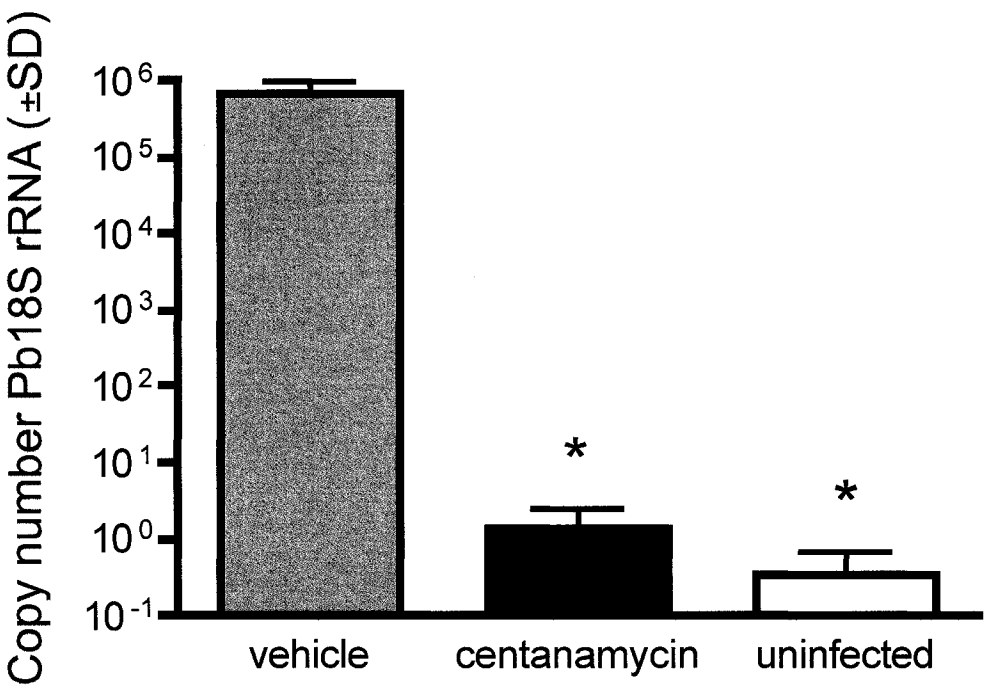

Figure 5-5. Sporozoites treated with centanamycin for 30 min fail to establish a significant liver-stage infection.
P. berghei sporozoites were incubated with vehicle or 2 mM centanamycin for 30 minutes, washed then injected i.v. into C57/BL6 mice. Forty hours later, mice were sacrificed and total liver RNA was extracted. An uninfected mouse served as a negative control. Malaria infection was determined by quantitative RT-PCR. Infection is expressed as the number of copies of *P. berghei* 18S rRNA. Treatment of sporozoites with centanamycin resulted in a significant reduction ($p < 0.0001$, ANOVA) in 18S rRNA levels compared with the vehicle-treated controls. Results represent one of two independent experiments with 4 mice per treatment group.

centanamycin-treated sporozoites to block a blood stage infection in both BALB/c and highly susceptible C57/BL6 mice. Mice were infected with 2×10^4 or 5×10^4 sporozoites that were treated with vehicle or centanamycin for only 30 minutes. BALB/c mice that received the vehicle-treated sporozoites developed hyperparasitemia and were euthanized by day 15 p.i. In contrast, mice that received the centanamycin-treated sporozoites never developed a patent parasitemia during the 21 days of observation (Fig. 5-6). C57/BL6 mice that received the vehicle-treated sporozoites were euthanized after developing symptoms of cerebral malaria by day 8 p.i., while those mice that received the centanamycin-treated sporozoites never developed a patent parasitemia during the 14 days of observation (data not shown). This is in agreement with the *in vitro* data suggesting that EEFs derived from centanamycin-treated sporozoites do not differentiate into infectious merozoites.

Immunization with treated sporozoites protects mice against subsequent infection.

Some RAS and GAS immunization schemes generate sterile protection in mice against subsequent challenge with wild type, non-attenuated sporozoites. We used mice that received dose(s) of non-infectious, chemically-attenuated sporozoites (CAS) described above and challenged them with a dose of 5×10^3 or 1×10^3 untreated sporozoites to evaluate the efficacy of CAS as a vaccine. BALB/c mice that received the CAS did not develop any detectable parasitemia over 30 days of observation, while naïve mice injected with the same challenge dose developed high parasitemias and were euthanized by day 16 p.i. (Table 5-1). C57/BL6 mice had a 2 day delay in developing

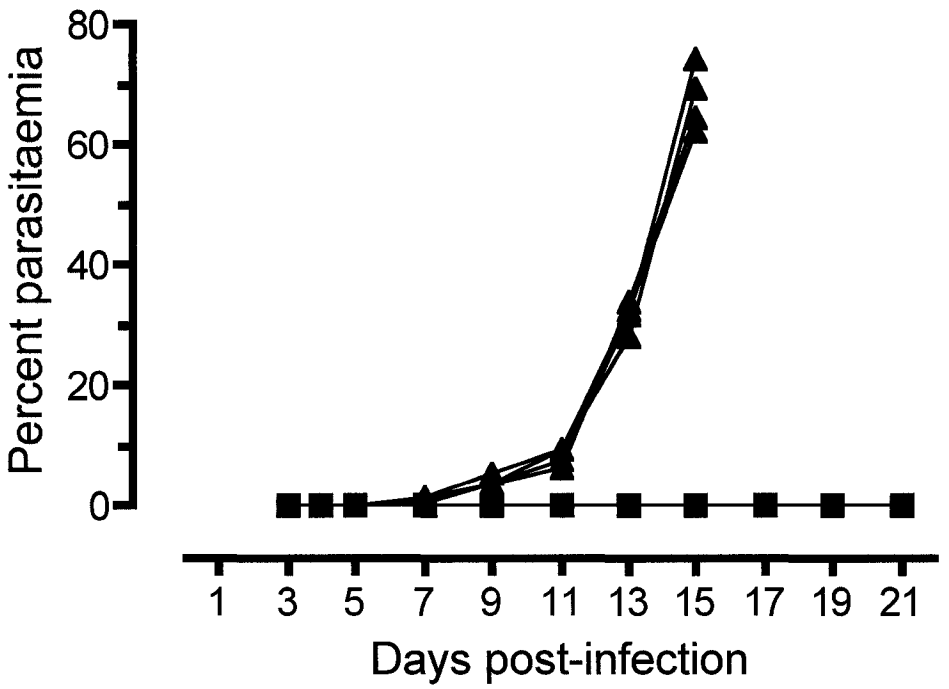

Figure 5-6. Treatment of sporozoites for 30 minutes *in vitro* before injection of mice prevents blood stage infection.
P. berghei sporozoites were incubated with vehicle or 2 mM centanamycin for 30 minutes before the injection of washed sporozoites into BALB/c mice. Development of detectable blood stage parasites was followed over 21 days p.i. Mice that received vehicle-treated sporozoites developed parasites on day 4 p.i. and were euthanized by day 15 p.i. Experiments were performed twice with 4 mice per group. One representative experiment is shown.

detectable parasitemia with the challenge dose compared to naïve mice with a single immunization. A multiple immunization schedule of 5×10^4, and 2 doses of 2×10^4 CAS each 7 days apart, was used to generate sterile immunity with a challenge dose of 10^4 untreated sporozoites in C57/BL6 mice. This dosing schedule also produced sterile immunity using RAS sporozoites from the same mosquito batches. The genetic restriction observed, where sterile immunization requires more doses of attenuated sporozoites in C57/B6 compared to BALB/c mice has been observed before [41] and it is probably a consequence of the high susceptibility to infection of C57/B6 mice [42] that renders this strain of mice more difficult to protect. Most GAS and RAS vaccine schedules require some combination of multiple dosing periods in order to consistently obtain complete protection. CAS fully protected BALB/c mice in two independent experiments using a single dosing schedule. Interestingly, mice that were challenged once again with 5×10^3 sporozoites 30 days after the initial challenge dose, did not develop detectable blood stage parasitemias (data not shown).

DISCUSSION

Our results show that chemically attenuated *P. berghei* sporozoites (CAS) are completely arrested at the liver stage, fail to produce blood stage parasites in mice and induce sterile protection in BALB/c following vaccination. An earlier study attempted a chemical attenuation strategy by treating *P. berghei* NK65 sporozoites with high doses (0.8 mg/mL) of chloroquine [43]. It was found that 5 immunizing doses with 2.5×10^4 sporozoites treated with 0.8 mg/mL of chloroquine for 60 minutes produced 78.6%

Table 5-1. Protection of mice immunized with CAS against challenge with wild-type sporozoites

Group	Mouse strain	Immunization [a] RAS/CAS × 10^3	Challenge [b]	Day of challenge	Sterile immunity?	Prepatent period (days)	No. protected (no. challenged)		
							Control [c]	CAS	RAS
1	BALB/c	20	5,000	21	yes	N/A	0 (4)	4 (4)	ND[d]
2	BALB/c	20	5,000	21	yes	N/A	0 (4)	4 (4)	ND
3	C57/BL6	20	5,000	14	no	5[e]	0 (4)	0 (4)	ND
4	C57/BL6	50/20/20	10,000	10	yes	N/A	0 (8)	4 (4)	4 (4)

[a] Groups of mice were immunized with *P. berghei* ANKA control (vehicle-treated) sporozoites, CAS, or RAS as indicated, isolated from different mosquito batches and where multiple immunizations were completed, were immunized at 7 day intervals.

[b] Groups of mice were challenged with *P. berghei* ANKA wild-type sporozoites isolated from different mosquito batches.

[c] Naïve, age-matched mice were used at the time of all immunizations and challenges.

[d] Indicates that treatment group was not included.

[e] Naïve, age-matched mice developed a patent parasitemia 3 days post-infection.

protection in mice, although the viability of the sporozoites was not reported. This malaria vaccine strategy, as well as the RAS vaccine approach, was then abandoned in favor of the subunit vaccine approach [7].

With the renewed interest in whole organism vaccines [7, 8], we evaluated the chemical attenuation of parasites using the DNA sequence-specific alkylating agent, centanamycin, and characterized its effects on sporozoites both *in vitro* and *in vivo*. Centanamycin has been shown to block *P. falciparum* blood stage growth *in vitro*, inhibit blood stage infections with *P.c. adami* and *P. berghei* in mice as well as significantly reduce the transmission potential of *P. berghei,* with a 99% reduction in sporozoite production [27]. Our studies show that treatment of *P. berghei* ANKA sporozoites with centanamycin for 30, 60 or 90 minutes *in vitro* does not affect membrane integrity. We found a moderate decrease in gliding motility of treated sporozoites that probably caused the small decrease found in hepatocyte invasion *in vitro*. There is a marked decrease in hepatocyte invasion by control sporozoites after 60 and 90 minutes *in vitro*. This decrease is normally observed when sporozoites are not immediately used for infection assays after dissection from mosquitoes. Sporozoite infectivity is progressively lost after dissection from salivary glands [44] The small decrease in hepatocyte invasion by treated sporozoites did not seem to affect the capacity of treated sporozoites to induce protective responses in mice.

Hepatocyte invasion by sporozoites is an important step in eliciting an immune response to the parasite. Inactivated sporozoites that are not able to infect hepatocytes have consistently failed to induce protective immune responses [16, 45, 46], although

they can efficiently prime the immune system [47]. Conversely, malaria infected hepatocytes and their extracts induce significant protection when injected into rats or mice [17, 48]. Previous experiments suggested that when immunizing with RAS, the protection against a challenge dose of sporozoites is dependent upon the persistence of irradiated sporozoites in the liver [16, 17]. However, more recent data indicate that GAS do not require persistence in the liver to induce protective immunity [14, 20, 21, 23]. Our study shows that CAS do produce liver stages *in vitro*, albeit at significantly lower levels (>85% reduction) compared to control sporozoites, and these liver stages were much smaller than controls. Whereas RAS and GAS both invade liver cells and transform into the rounded trophozoite stage, they generally do not enter schizogony [18]. GAS-infected hepatocytes normally do not persist longer than 24-36h [14, 20, 21, 23] compared to RAS-infected hepatocytes, which persist much longer [10, 17, 25]. Our study showed that CAS persist in cultured hepatocytes for at least 42 h. Taken together, these results suggest that CAS is an effective attenuation strategy that can produce the infective liver stages needed to elicit an immune response.

A dose of 2×10^4 CAS in BALB/c and C57/Bl6 mice failed to establish a blood stage infection and the CAS- vaccinated BALB/c mice demonstrated protective immunity when challenged with a dose of 5×10^3 untreated, wild type sporozoites. A multiple dose regimen was employed to produce sterile immunity in C57/BL6 mice. In general, both RAS and GAS require higher initial doses of *P. berghei* ANKA sporozoites in BALB/c mice (at least 2×10^4) to provide complete protection against lower or similar challenge doses of wild type parasites (1×10^3) [14, 15, 49]. This suggests that CAS

could be an efficient approach for producing a whole organism malaria vaccine.

Immune responses against both RAS and GAS are complex and involve both cell-mediated and humoral immunity [22, 23, 48, 50, 51]. In addition, some RAS and GAS seem to induce long-lasting, cross-species protection [12, 15, 45]. Attenuation of irradiated sporozoites presumably occurs due to double strand breaks in the DNA that lead to a block in liver stage development. Each sporozoite would contain a number of strand breaks randomly distributed in its DNA. In the case of CAS treated with centanamycin, the attenuated sporozoites would contain a set of adducts covalently bound to adenine nucleotides [29]. This compound, like other A/T-specific binding compounds, recognizes selective DNA sequences and the potential number of adducts can be defined bioinformatically [52]. Given that both the CAS and RAS approaches disrupt the integrity of the parasite DNA, it is possible that the immune response generated by RAS and CAS would be similar but further studies are necessary to confirm this point.

Many issues have been raised concerning the feasibility of both GAS and RAS as whole organism sporozoite vaccines, including the mass production of sterile parasites, proper storage to maintain viability and the safety of a mosquito-derived vaccine [6, 7, 53]. Both types of attenuation have individual inherent weaknesses [7, 8]. In the case of RAS, the over-attenuation of sporozoites has been shown to block liver stage development at the trophozoite stage [11, 16] and generate poor protection [16], suggesting the dosage of irradiation is pivotal to the success of each lot of RAS. Uniform exposure of parasites to the radiation source is essential to prevent escape sporozoites that could generate a malaria infection following vaccination. [6, 18]. In contrast, the risk

of 'breakthrough infections' with GAS is low due to the gene knockout strategy employed [14, 21, 54]. Yet, the widespread distrust of genetically modified products, especially for a vaccine that will be inoculated into humans and invade host cells, may complicate efforts to utilize GAS in the field. Our proposed CAS vaccine has the advantage that the chemical attenuation process can be strictly controlled, leading to a vaccine that is reproducibly attenuated. In addition, centanamycin is washed from treated sporozoites before injection into mice to rid CAS from free centanamycin, and the drug present in treated sporozoites is covalently bound to parasite DNA. Genotoxicity studies using centanamycin show that human lymphocytes show no statistically significant chromosome aberration with 80 nM of the drug after 4 or 21 h exposure [27]. As well, centanamycin is not toxic at 8.4 nM to murine bone marrow cells *in in vitro*. Given the residual levels that may be present in the treated sporozoites, the level of free centanamycin should pose no risk to the host, although this concern must be addressed by in-depth pharmacokinetic and mutagenicity studies. Given that centanamycin shows similar covalent DNA sequence specificity to adozelesin, and the frequency of binding sites for adozelesin has been estimated at 440 sites per kilobase of genomic *Plasmodium* DNA [52], treatment of sporozoites with centanamycin could potentially saturate these binding sites for maximal effect on the parasite. Since the generation of viable, cryopreserved sporozoites is currently being optimized [7], chemical attenuation using centanamycin could be considered as an additional strategy for the production of whole organism vaccines against malaria. . More generally, our results suggest that chemical attenuation with drugs such as centanamycin may be a feasible approach for generating

live attenuated vaccines for other major parasites with AT-rich DNA such as *Theileria* (19, 51).

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CHAPTER 6:

Summary and Conclusions

This study has evaluated strategies to block the transmission of the malaria parasite from the mosquito to the intermediate host using genetic and biochemical methods. The mosquito stages represent a severe bottleneck in the malaria life cycle. Targeting the transmission stages of malaria will not only prevent the spread of the disease, but will also prolong the life of antimalarials and vaccines by preventing the spread of parasites that become resistant to these control measures. Here, I have evaluated three strategies using novel approaches for blocking the transmission of malaria to the mosquito as well as to the mammalian host. In the first approach, transfection was used to delete a predicted protein kinase gene (*carp*), which I have shown to be involved in ookinete invasion of the mosquito midgut epithelium as well as the invasion by midgut sporozoites of salivary glands. A second approach used centanamycin, a DNA-alkylating agent, to treat malaria-infected mice which once fed on mosquitoes, showed a >99 % reduction in the number of sporozoites was observed. Finally, a third approach used centanamycin as a method to chemically attenuate sporozoites *in vitro*, which, once injected into mice provided sterile immunity to a challenge dose of sporozoites.

Implications of the results and future directions

PbCARP mediates the levels of infection in midguts and salivary glands of mosquitoes

I showed that a putative CaMK-related protein (PbCARP) has an important function in mediating the level of *P. berghei* infection in *A. stephensii* mosquitoes. This predicted kinase was chosen based on the unique kinase domain that it possessed and data showing that the RNA from this gene was upregulated in the gametocyte stages: I considered that the putative kinase may be involved in gametocyte function or the transmission of malaria. Although expression of the recombinant putative kinase was not achieved in *E. coli*, which may have been used to demonstrate kinase activity, deletion of the rodent orthologue of the predicted *P. falciparum* putative kinase was successful. The analysis of the *carp*$^-$ deletion mutant showed that this protein seemed to be involved in mediating the successful invasion of ookinetes in the midgut epithelium of the mosquito, thereby affecting the number of sporozoites that are released from oocysts. In addition, sporozoites that were released into the mosquito hemocoel had a reduced ability to invade the salivary glands. The results suggested that the putative PbCARP increases the potential transmissibility of malaria by each mosquito to another mammalian host. Consequently, this protein could be a potential transmission-reducing drug target. The rationale for targeting this putative protein kinase lies in the biochemical divergence in the properties of the kinase relative to orthologues kinases in humans: this biochemical difference could potentially be exploited for specific inhibition of the putative enzyme of the parasite.

Development of an inhibitor would require more extensive investigation of CARP function, but inhibitor design could be based on the structure of the putative kinase active site. For example, the inhibitor could target unique inactive conformations of the protein, thereby stabilizing the protein in an inactive form, or selectively inhibit the putative kinase by targeting amino acid differences in the kinase active site. This approach has been used in treating cancer, where at least five kinase drugs have been approved. The inhibitor could be included in a combination therapy whereby one drug targets asexual stages, but the kinase inhibitor could be taken up by the mosquito with its blood meal to reduce sporozoite loads in infected mosquitoes. Some challenges associated with this approach might be the potential cross-reactivity of the inhibitors in humans and the stability of the inhibitor before ingestion by the mosquito. Ideally, this combination therapy would reduce blood-stage malaria in the infected individual, and meet the growing need of new transmission-blocking strategies.

Centanamycin is a potent transmission-blocking antimalarial

Also described in this thesis was the potent antimalarial activity of the DNA-binding compound centanamycin against *P. falciparum in vitro* and murine malaria *in vivo*. In all three rodent malaria models, a rapid reduction in parasitemia was observed one day following a single treatment with centanamycin. A critical observation was that the effects of centanamycin on blood-stage parasites also dramatically compromised the viability of the mosquito stages derived from these treated parasites, resulting in a 99% reduction in sporozoite production. A critical possible drawback to the clinical use of this

compound would be the potential risk of toxicity in humans, especially children and pregnant women. This is particularly important given that individuals are continuously at risk for re-infection, and consequently are required to take regular doses of the drug. Clinical studies to examine the possible uses of the drug as a chemotherapeutic drug that could be given on multiple occasions, or as a method to treat severe, life-threatening malaria infections must be completed. Despite these concerns, given its potent antimalarial activity and unique mode of action, it is proposed that centanamycin or similar non-toxic orthologues should be developed as an important component of therapies, including combination therapies to reduce parasitemia in the human host and also diminish the level of transmission. These studies could include more extensive genotoxicity, pharmacodynamic, and pharmacokinetic experiments, along with the use of primate models to test the efficacy of the drug against human malaria *in vivo*.

Centanamycin-treated sporozoites are a protective malaria vaccine

Finally, this study showed that *in vitro* treatment of *P. berghei* sporozoites with centanamycin, termed chemically attenuated sporozoites (CAS), were completely arrested at the liver stage, failed to produce blood stage parasites in mice, and induced sterile protection in mice following vaccination with CAS. This same phenomenon has been demonstrated with radiation-attenuated sporozoites (RAS) and genetically-attenuated sporozoites (GAS). Several challenges need to be addressed with both of these approaches before clinical application is feasible: these include the dose of radiation and the possibility of breakthrough infections

in the case of RAS and acceptance of a genetically modified injected malaria vaccine in malarious regions in the case of GAS. I showed that treatment of *P. berghei* ANKA sporozoites with centanamycin for various times *in vitro* did not affect membrane integrity. There was a moderate decrease in gliding motility of treated sporozoites that probably caused a small decrease found in hepatocyte invasion *in vitro*. CAS produced liver stages *in vitro*, albeit at significantly lower levels compared to sporozoites treated with a vehicle control. Importantly, those CAS liver stages found in culture were much smaller and the developing parasites appeared abnormal. A dose of 2×10^4 CAS in BALB/c mice failed to establish a blood stage infection. More importantly, these same mice demonstrated protective immunity when challenged with a dose of 5×10^3 untreated, wild type sporozoites. Since the generation of protective immunity was demonstrated using CAS, chemical attenuation using centanamycin should be considered as an additional strategy along with RAS and GAS for the production of whole organism vaccines against malaria, but challenges still remain. These include the mass production of enough sterile parasites to deliver the vaccine, along with the proper storage to maintain parasite viability during vaccine delivery to the field. These challenges have been investigated and strategies are in place to overcome these obstacles, mainly led by the private company, Sanaria, Inc which has been able to secure both national and international funding to develop a RAS vaccine. Specifically, CAS also has some drawbacks, including consistent drug uptake by the sporozoites, the possibility of drug carry-over into humans, the cost-effective synthesis of the compound, and measures for quality control. Even with these

constraints, my data suggests that a CAS vaccine could potentially be used with the same production facility, offering an alternative strategy to the use of radiation for attenuating sporozoites.

In conclusion, it was possible during this thesis, to characterize the function of a putativeCARP involved in the ookinete and sporozoite level of infection of the mosquito, to show that centanamycin is a transmission-blocking compound, and to demonstrate that centanamycin can effectively attenuate sporozoites *in vitro* and produce sterile immunity in mice.

APPENDIX A:

Approval Forms and Certificates