Anti-Plasmodium Activity of Small Imidazolium- and Triazolium-based Compounds

by

Eva Patricia Rodriguez

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology
University of Toronto

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2011

ABSTRACT

In response to growing levels of resistance to currently used antimalarials, there is an urgent need to develop drugs that exhibit novel mechanisms to kill *Plasmodium* parasites. The objective of this study was to examine the antiparasitic activity of newly synthesized compounds based on imidazolium and triazolium rings. According to our structure/activity relationship studies the key components appear to be their positively charged rings and hydrophobic side groups, and bivalent compounds, which incorporate two positively charged rings, show even greater potency than monovalent compounds. Depending on the concentration used, our compounds appear to primarily inhibit intracellular parasite development or invasion into red blood cells. Selected compounds have been tested *in vivo* using a *P. berghei* ANKA murine model. Together, our findings demonstrate that small imidazolium- and triazolium-based compounds display both *in vitro* and *in vivo* activity through a novel mechanism of action that may involve inhibition of erythrocyte invasion.
DEDICATION

I would like to dedicate this thesis to my parents, Victor and Margarita, my sister, Julie, and my grandmother, Julia. Thank you for all of your love and for supporting me in everything I do.
ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Ian Crandall, for being a wonderful teacher and for all of his guidance and support throughout my graduate studies. I would also like to thank our collaborators, Dr. Walter Szarek and Dr. Jason Vlahakis for the synthesis of the compounds without which this thesis would not be possible. I would like to offer my gratitude to my committee members, Dr. Yvonne Yau, Dr. Scott Gray-Owen, and Dr. James Brunton for all of their guidance and insightful questions and comments. I would like to thank Tassnim Moradipour for her contribution to the viability assays. Finally, I would like to thank all members of the lab for making it an amazing place for me to learn and grow and for all of their help over the past few years, with a special thank you to Dr. Constance Finney for help with flow cytometry, Dr. Kodjo Ayi for everything he does to ensure we have healthy parasite cultures, Dr. Ziyue Lu and Kathleen Zhong for helping me learn to work with mice, and Laura Erdman, Dr. Michael Hawkes, and Dr. Hani Kim for their incredible mentorship.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ACT</td>
<td>artemisinin-based combination therapy</td>
</tr>
<tr>
<td>ADME</td>
<td>absorption, distribution, metabolism, and elimination</td>
</tr>
<tr>
<td>AE1</td>
<td>anion exchange protein 1</td>
</tr>
<tr>
<td>AMA-1</td>
<td>apical membrane antigen-1</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBA</td>
<td>erythrocyte binding antigen</td>
</tr>
<tr>
<td>EBL</td>
<td>erythrocyte binding ligand</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>glideosome-associated protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IMC</td>
<td>inner membrane complex</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal injection</td>
</tr>
<tr>
<td>ITN</td>
<td>insecticide-treated net</td>
</tr>
<tr>
<td>MSP</td>
<td>merozoite surface protein</td>
</tr>
<tr>
<td>MTRAP</td>
<td>merozoite thrombospondin-related anonymous protein</td>
</tr>
<tr>
<td>NBT</td>
<td>nitrotetrazolium blue chloride</td>
</tr>
<tr>
<td>nRBCs</td>
<td>uninfected red blood cells</td>
</tr>
<tr>
<td>pLDH</td>
<td><em>Plasmodium</em> lactate dehydrogenase</td>
</tr>
<tr>
<td>pRBCs</td>
<td>parasitized red blood cells</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCD</td>
<td>sulfated cyclodextrin</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TV</td>
<td>tetrazolium violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
PART I

LITERATURE REVIEW
SECTION 1.1 – MALARIA

1.1.1 The disease

Malaria is a parasitic infectious disease that causes a massive public health burden; it is endemic in 108 countries around the globe (Figure 1) and presents a health risk for 50% of the world’s population (World Health Organization, 2010). In 2008 malaria affected close to 250 million people and caused nearly 1 million deaths (World Health Organization, 2009). Eighty-five percent of these cases and 89% of these deaths occurred in Africa (World Health Organization, 2009). In particular, African children under 5 years of age are among the most vulnerable population adversely affected by this disease as they account for the majority of the annual deaths attributed to malaria (85% in 2008) (World Health Organization, 2009). In addition to its negative impacts on human health, this parasitic infection also contributes to economic loss in malaria endemic regions, reducing gross domestic product by upwards of 1.3% (World Health Organization, 2010); thus, malaria is a factor in perpetuating the economic disparity between malaria-free and malaria-endemic countries (World Health Organization, 2010).

Malaria is a disease caused by *Plasmodium* species and a person may become infected when they are bitten by a female anopheline mosquito that is carrying these parasites (Cowman and Crabb, 2006). The five *Plasmodium* species that affect humans are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, with *P. falciparum* infection being responsible for the deadliest form of the disease (World Health Organization, 2010). The onset of symptoms tends to occur within 10-15 days of being bitten by the infected mosquito and early symptoms include fevers, chills, headaches, and vomiting (World Health Organization, 2010). This uncomplicated malaria can be managed by oral antimalarial drugs (Ashley et al., 2006). However, if appropriate
antimalarial treatment is not obtained promptly, the disease may progress to severe malaria. A patient with severe malaria may experience complications such as severe anemia; acute respiratory failure; hypoglycemia; renal failure; pulmonary edema; or cerebral malaria (Ndam and Deloron, 2007), a special case of severe disease characterized by seizures and an unrousable coma.

A key part of the pathogenesis of *P. falciparum* infection is attributed to the ability of parasitized RBCs to adhere to blood vessel endothelial cells (Ndam and Deloron, 2007); this is thought to physically block blood flow to vital organs and impair the delivery of oxygen to these organs (Erdman et al., 2008). The pathogenesis of severe malaria is also thought to involve an exaggerated or dysregulated host inflammatory response (Erdman et al., 2008). Toll-like receptor (TLR) pathways have long since been implicated in the inflammatory response to malaria; *P. falciparum* glycosylphosphatidylinositol (GPI) is known to bind TLR2 and TLR4 and stimulate macrophages to produce cytokines, while parasite DNA with hemozoin binds to TLR9 (Erdman et al., 2008). Although TLRs are often thought to be central to the malaria inflammatory response, this inflammatory response has also been shown to be mediated by: Nod-like receptors (Ockenhouse et al., 2006); uric acid production by the host (Orengo et al., 2008); the proinflammatory complement component C5a (Roestenberg et al., 2007); the release of proinflammatory histamine from mast cells (Beghdadi et al, 2008); as well as the induction of heme oxygenase-1 and subsequent production of carbon monoxide which serve to decrease inflammation (Pamplona et al., 2007).

Children and pregnant women are the most susceptible to developing severe malaria. Individuals who are raised in malaria-endemic regions eventually become immune to severe forms of the disease and are protected from death attributed to malaria (Langhorne et al., 2008).
However, young children are vulnerable because they have not had the opportunity to develop this immunity. Pregnant women in malaria endemic areas are at risk of developing severe anemia and delivering low birth weight babies (Ndam and Deloron, 2007). In mothers with *P. falciparum* malaria, the low birth weight appears to be attributed to a disruption in the exchange of substances across the placenta resulting from the placental sequestration of parasitized RBCs (Ndam and Deloron, 2007); however, the delivery of low birth weight babies by mothers infected with *P. vivax*, which is not known to sequester in the placenta, suggests that an increase in proinflammatory cytokines in the placenta may also be a key factor contributing to low birth weight (Nosten et al., 1999). Pregnant women living in malaria endemic areas with high transmission should receive intermittent preventative treatment to avoid the adverse outcomes of placental associated malaria (World Health Organization, 2009).

Once an individual has progressed to severe malaria they should be taken to a health facility where they can receive intravenous or intramuscular treatment with artemisinin derivatives or quinine (discussed in Section 1.2) (World Health Organization, 2009). However, even with treatment, the rate of mortality is still high. For example, in cases of treated cerebral malaria, 20% of non-pregnant adults, 15% of children, and 50% of pregnant women still die (Ashley et al., 2006). To date, there is no commercially available vaccine to protect against malaria. The most promising candidate appears to be the RTS,S vaccine that began its development in the early 1980s (Casares et al., 2010). Although this vaccine does not offer complete protection, it has been shown to offer some degree of protection against clinical and severe disease in children (Alonso et al., 2004; Alonso et al., 2005; Bejon et al., 2008); RTS,S is now being tested in a phase 3 clinical trial that has enrolled upwards of 16,000 children living in sub-Saharan Africa (Casares et al., 2010).
Current efforts to control malaria combine the use of insecticide-treated nets (ITNs) with indoor residual spraying and antimalarial drug therapy (World Health Organization, 2009). Recent years have seen an increased global interest in the fight against malaria partnered with a surge in international funding to support initiatives to control malaria; this includes increasing access to existing ITNs and antimalarial drugs, as well as sponsoring research into the development of novel insecticides to target the vector, better diagnostic tests to detect the infection, a vaccine to prevent the disease, and novel therapies to treat it. As it is only by combining these different approaches that we will truly be able to maximize our chances of eliminating and eventually eradicating malaria.
Figure 1. Malaria endemicity around the world

Reproduced with permission from the World Health Organization.

http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_ITHRiskMap.JPG
1.1.2 The parasite life cycle

The complete life cycle of the *Plasmodium* parasite can be viewed in Figure 2. Before an infected *Anopheles* mosquito takes a blood meal, it injects the host with saliva containing anticoagulants and simultaneously injects parasite sporozoites (Tuteja, 2007). These sporozoites go on to invade hepatocytes (Cowman and Crabb, 2006) when parasite ligands bind negatively charged sugars on the hepatocytes. The sporozoites grow and replicate their DNA to become multinucleated schizonts that give rise to tens of thousands of merozoites (Wiesner et al., 2003). When these merozoites are released from the hepatocytes into the bloodstream they quickly invade red blood cells (RBCs) (Cowman and Crabb, 2006); this marks the beginning of the blood stage of the disease (Cowman and Crabb, 2006), the stage that is responsible for the clinical symptoms of malaria (Wiesner et al., 2003). It is important to note that in both *P. vivax* and *P. ovale* infections, some of the sporozoites that invade hepatocytes develop into dormant forms called hypnozoites that can cause relapses weeks to months after the initial infection (Ashley et al., 2006; World Health Organization, 2010).

In the blood, the time taken to complete one replication cycle depends on the *Plasmodium* species causing the infection. While *P. falciparum*, *P. vivax*, and *P. ovale* display a 48-hour asexual life cycle, *P. malariae* and *P. knowlesi* exhibit a 72-hour and a 24-hour asexual life cycle respectively (Bray and Garnham, 1982; Chin et al., 1965). The following is a description of the 48-hour parasite life cycle, although the same stages exist in all *Plasmodium* species. Once the parasite has successfully invaded a RBC, it spends its first 20-24 hours in the ring stage (Ben Mamoun et al., 2001), a stage that is named so because of the ring-like appearance of Giemsa-stained parasites when examined microscopically. During this stage the parasite feeds on haemoglobin and plasma nutrients, synthesizes ring-stage molecules and
modifies the RBC membrane (Bannister and Mitchell, 2003). The next stage of the parasite life cycle is the trophozoite stage that occurs between 24 and 36 hours following invasion (Baumeister et al., 2009). At this point in its development the parasite is feeding, growing, and modifying the RBC membrane more than at any other stage (Bannister and Mitchell, 2003). In \textit{P. falciparum} infection the addition of \textit{P. falciparum} erythrocyte membrane protein 1 to the surface of the red cell causes parasitized RBCs to stick to the endothelial cells of blood vessels, avoiding the clearance of these infected cells by the spleen (Bannister and Mitchell, 2003). As the parasite feeds on haemoglobin it produces toxic heme by-products that it polymerizes into nontoxic haemozoin (Francis et al., 1997). Trophozoites grow and replicate their DNA to develop into schizonts (Ben Mamoun et al., 2001). Once schizonts rupture they release as many as 16 to 32 new merozoites that then go on to invade new RBCs and repeat the cycle, exponentially increasing the parasite biomass (Koussis et al., 2009). In addition, in order to maintain the transmission of malaria, some of the merozoites that invade RBCs will develop into female or male gametocytes, sexual forms of the parasite (Tuteja, 2007); gametocytes are taken up by \textit{Anopheles} mosquitoes during a blood meal and they form male and female gametes which fuse to produce a zygote in the mosquito’s midgut, ultimately leading to the production of new sporozoites (Cowman and Crabb, 2006).
Figure 2. The life cycle of the *Plasmodium* parasite

Adapted from Good, M.F., 2005. Vaccine-induced immunity to malaria parasites and the need for novel strategies. Trends Parasitol 21, 29-34.
SECTION 1.2 – ANTIMALARIALS

1.2.1 The need for new drugs

To date there are a number of existing drugs to treat malaria, however the spread of resistance to these established antimalarials is of critical concern. The widespread resistance to chloroquine and sulfadoxine-pyrimethamine, the two lowest cost antimalarials, is illustrated in Figure 3. Today, the WHO recommends that cases of uncomplicated *P. falciparum* malaria be treated using artemisinin-based combination therapy (ACT) and that *P. vivax* malaria be treated with chloroquine, or ACT wherever *P. vivax* is known to be chloroquine resistant (World Health Organization, 2009); however, the fear is that the resistance to artemisinin-based therapy that was recently reported at the Thai-Cambodia border may soon become widespread. Thus, there is an urgent need to develop new antimalarial agents with novel mechanisms of action. In addition, as the hardest hit populations include young children, pregnant women, and those living in poverty, these new drugs must be safe enough to be used in intermittent preventative treatment in pregnant women and inexpensive to ensure that they are accessible to resource poor populations. Before delving into the background on our antimalarial compounds I will provide a review of the established antimalarials.

Most of the established antimalarials fit into the following four categories, as reviewed in Wiesner et al., 2003: 1) the 4-amino quinolines (e.g. chloroquine, amodiaquine) and aryl aminoalcohols (e.g. mefloquine, lumefantrine, quinine), which appear to inhibit the parasite’s ability to safely deal with toxic heme moieties released from the breakdown of haemoglobin; 2) the antifolates (e.g. sulfadoxine, pyrimethamine, proguanil) that disrupt the parasite’s folate metabolism (Gregson and Plowe, 2005); 3) artemisinin and its active derivatives (artemether, artesunate, artemotil, dihydroartemisinin) which are endoperoxides that are thought to interact
with reduced heme and modify parasite enzymes and lipids (Taylor et al., 2004); and 4) the antibiotics that interfere with parasite RNA and inhibit protein synthesis (e.g. tetracycline, doxycycline, clindamycin) (Wiesner et al., 2003). Table 1 contains descriptions of the currently available antimalarial drugs (modified from the WHO’s “Guidelines for the Treatment of Malaria-- 2nd edition.”).
Figure 3. Widespread resistance to chloroquine and sulfadoxine-pyrimethamine

Table 1. Established antimalarials

Contains descriptions of the currently available antimalarial drugs modified from the WHO’s “Guidelines for the Treatment of Malaria-- 2nd edition”. Chemical structures are reproduced with permission from the WHO.

<table>
<thead>
<tr>
<th>Name, Chemical structure, Chemical classification</th>
<th>Possible mechanism</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>• Inhibits the parasite’s ability to safely deal with toxic heme moieties released from the breakdown of haemoglobin</td>
<td>• Widespread resistance of <em>P. falciparum</em> to chloroquine (however this drug remains useful to treat <em>P. vivax</em>, <em>P. malariae</em>, and <em>P. ovale</em>). • Resistance appears to be due to genetic mutations in transporters PfCRT and PfMDR that work by decreasing the concentration of chloroquine inside the food vacuole</td>
</tr>
<tr>
<td><img src="image1" alt="Chloroquine" /></td>
<td>4-amino quinoline</td>
<td></td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>• Appears to be working by a similar mechanism as chloroquine</td>
<td>• Amodiaquine is a structural analogue of chloroquine • Although there is cross-resistance with chloroquine, it may still be effective against some strains of chloroquine-resistant <em>P. falciparum</em></td>
</tr>
<tr>
<td><img src="image2" alt="Amodiaquine" /></td>
<td>4-amino quinoline</td>
<td></td>
</tr>
<tr>
<td>Sulfadoxine</td>
<td>• Interferes with the synthesis of folic acid by competing with p-aminobenzoic acid, thereby inhibiting the enzyme dihydropteroate synthase</td>
<td>• Used in combination with pyrimethamine</td>
</tr>
<tr>
<td><img src="image3" alt="Sulfadoxine" /></td>
<td>Sulphonamide</td>
<td></td>
</tr>
</tbody>
</table>
| **Pyrimethamine** | **•** Blocks the parasite’s production of nucleic acids by inhibiting dihydrofolate reductase  
**•** Kills schizonts | **•** Used in combination with sulphonamides such as sulfadoxine for treatment of malaria or dapsone for prophylactic therapy  
**•** Despite rapid emergence of resistance to pyrimethamine, it continues to be effective at killing *P. falciparum, P. vivax, P. malariae,* and *P. ovale* |
| **diaminopyrimidine** | | |
| **Mefloquine** | **•** Structurally related to quinine | **•** Effective against *P. falciparum, P. vivax, P. malariae,* and *P. ovale* |
| **4-methanolquinoline** | | |
| **Artemisinin** | **•** Kills parasites in their ring stage, trophozoite stage, and in their schizont stage  
**•** Also effective at killing *P. falciparum* gametocytes  
**•** Artemisinin and its derivatives interfere with the activity of the calcium-dependent PfATPase 6 | **•** Derived from the Qinghao plant, *Artemisia annua*  
**•** Effective against all species of *Plasmodium*  
**•** Use of artemisinin has mostly been replaced with the use of its more active derivatives artemether, artesunate, artemotil, and dihydroartemisinin  
**•** The WHO recommends that artemisinin derivatives be used in combination therapies rather than monotherapies to slow down the emergence of resistance to these drugs |
| **sesquiterpene lactone** | | |
| **Artemether** | • Same as artemisinin | • Its active metabolite is dihydroartemisinin  
• Can be used in combination therapy with lumefantrine |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Artemether" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Artesunate</strong></td>
<td>• Same as artemisinin</td>
<td>• Mostly metabolized into the active dihydroartemisinin</td>
</tr>
<tr>
<td><img src="image2" alt="Artesunate" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Artemotil</strong></td>
<td>• Same as artemisinin</td>
<td>• Its active metabolite is dihydroartemisinin</td>
</tr>
<tr>
<td><img src="image3" alt="Artemotil" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dihydroartemisinin</strong></td>
<td>• Same as artemisinin</td>
<td>• It is an active metabolic product of artemisinin, artemether, artemesunate, and artemotil, and can also be taken as a drug directly</td>
</tr>
<tr>
<td><img src="image4" alt="Dihydroartemisinin" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lumefantrine</strong></td>
<td>• Its mechanism of action is similar to that of mefloquine and quinine</td>
<td>• Used in combination with artemether</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><img src="image" alt="Lumefantrine" /></td>
<td>aryl aminoalcohol</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Primaquine</strong></th>
<th>• Kills liver-stage parasites of all <em>Plasmodium</em> species as well as <em>P. falciparum</em> gametocytes</th>
<th>• Used in combination with antimalarials that kill blood-stage parasites in the treatment of <em>P. vivax</em> and <em>P. ovale</em> malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Primaquine" /></td>
<td>8-aminoquinoline</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Atovaquone</strong></th>
<th>• Kills blood-stage parasites of all <em>Plasmodium</em> species</th>
<th>• Used in combination with proguanil</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Atovaquone" /></td>
<td>hydroxynaphtoquinone</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Proguanil</strong></th>
<th>• Inhibits parasite’s dihydrofolate reductase</th>
<th>• Certain individuals (3% of Caucasians and Africans and 20% of Asians) have lower levels of proguanil that is metabolised into cycloguanil</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Proguanil" /></td>
<td><em>a biguanide and its active metabolite is cycloguanil</em></td>
<td>• Used in combination therapy with atovaquone</td>
</tr>
<tr>
<td>Drug</td>
<td>Mechanism</td>
<td>Classification</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td><strong>Quinine</strong></td>
<td>• Mainly kills mature trophozoites&lt;br&gt;• Also kills <em>P. vivax</em>, <em>P. malariae</em>, and <em>P. ovale</em> gametocytes&lt;br&gt;• Hypothesized to inhibit the parasite’s ability to safely deal with toxic heme moieties released from the breakdown of haemoglobin</td>
<td>• It is a natural compound derived from the Cinchona tree</td>
</tr>
<tr>
<td><strong>Tetracycline</strong></td>
<td>• Interferes with protein synthesis by inhibiting the binding of aminoacyl-tRNA</td>
<td>• An antibiotic</td>
</tr>
<tr>
<td><strong>Doxycycline</strong></td>
<td>• Interferes with protein synthesis by inhibiting the binding of aminoacyl-tRNA</td>
<td>• A derivative of tetracycline&lt;br&gt;• An antibiotic</td>
</tr>
<tr>
<td><strong>Clindamycin</strong></td>
<td>• Kills parasites by interfering with protein synthesis&lt;br&gt;• It shares a similar mechanism of action with macrolides, which block the elongation step of protein synthesis by binding to 23S rRNA</td>
<td>• A chlorinated derivative of the antibiotic lincomycin</td>
</tr>
</tbody>
</table>
1.2.2 Developing new antimalarials

A review of the established antimalarials makes it clear that many of these drugs have similar methods of action. However, instead of focusing our efforts on developing analogues of existing drugs, we must generate antimalarial agents that utilize new mechanisms of action, with the aims of avoiding cross resistance and providing synergy with established drugs. As will be described later, our group is investigating compounds that appear to inhibit the invasion of parasites into the RBC. Examples of new classes of antimalarials being explored by other groups include inhibitors of fatty acid synthesis (Perozzo et al., 2002), inhibitors of choline uptake (Calas et al., 2000), inhibitors of glycolysis (Razakantoanina et al., 2000; Choi et al., 2007), and inhibitors of a *Plasmodium* heat shock protein (Shahinas et al., 2010). In terms of parasite stages that could be targeted, there are currently few drugs that kill gametocytes, hypnozoites, and ring-stage parasites, and there is yet to be a commercially available drug that targets merozoites.

In addition to the problem of resistance, some of the established antimalarials have safety issues associated with them or properties that may decrease compliance. For example, although mefloquine continues to be effective in many parts of the world, it is relatively expensive and its side effects, which include seizures, acute psychosis, and anxiety neurosis (Ashley et al., 2006), can make certain individuals think twice before taking it. There are also problems associated with our dependence on artemisinin and its derivatives. We currently rely on the extraction of artemisinin from *Artemisia annua* for the semisynthetic production of artemisinin derivatives (Taylor et al., 2004). However, the low content of artemisinin that is actually present in each plant, between 0.01 to 0.8% of *A. annua*’s dry weight, makes the production of artemisinin as a drug expensive (Liu et al., 2010). It is important that new antimalarials be safe and inexpensive to produce so that they can be affordable to the populations that need them the most (Gelb,
Another problem associated with certain antimalarials is the length of treatment; for instance, a recent study focusing on compliance to the 7-day primaquine treatment for *P. vivax* among patients living along the Iquitos-Nauta road in the Peruvian Amazon found that compliance was high during the initial three days of treatment but decreased as symptoms disappeared (Grietens et al., 2010). It is generally thought that the optimal antimalarial drugs would require at most three days of treatment, either once or twice a day, to be effective (Gelb, 2007); this would be one way to avoid problems associated with compliance and the development of drug resistance.
SECTION 1.3 INVASION

1.3.1 The invasion of parasites into red blood cells

Invasion of parasites into RBCs is a multistep process. Once merozoites are released from either hepatocytes or RBCs they rapidly recognize and adhere to the surface of a new RBC. Research on *P. falciparum* has shown that merozoites are able to recognize new RBCs within 1 minute following schizont rupture (Gilson and Crabb, 2009). This initial attachment to the RBC is thought to be of low affinity and reversible in order to allow the merozoite to reorient itself until its apical end is in contact with the RBC surface (Cowman and Crabb, 2006). Once apical contact is made tight junctions are formed between the parasite and host membranes, bringing these membranes into closer contact (Cowman and Crabb, 2006). The tight junctions then move from the parasite’s apical end through to its posterior end as the parasite’s actin-myosin motor propels the parasite into the RBC (Keeley and Soldati, 2004). Gilson and Crabb (2009) have shown that once *P. falciparum* merozoites have recognized a new RBC, the parasite is able to invade this new host cell in about 28 seconds (Gilson and Crabb, 2009).

Relatively little is known about the mechanism by which the parasite initially recognizes and attaches to the RBC (Galinski et al., 2005), however one of the candidate proteins thought to play an important role in the initial attachment step of invasion is the parasite’s Merozoite Surface Protein-1. This protein will be discussed in detail in the following subsection. Another protein widely believed to be involved in RBC invasion is the parasite’s Apical Membrane Antigen-1 (AMA-1) protein. AMA-1 is concentrated on the apical end of the merozoite and antibodies targeting AMA-1 are capable of blocking invasion (Triglia et al., 2000; Mitchell et al., 2004). A study carried out by Mitchell et al. (2004) has shown that AMA-1 may be involved in
the reorientation step of invasion and that treatment with antibodies to AMA-1 inhibits parasite entry into RBCs without affecting the tethering of the merozoite to the RBC membrane.

We have a better understanding of the formation of tight junctions during invasion. The Duffy binding protein is a parasite ligand involved in tight junction formation during *P. vivax* invasion, and it is known to bind the Duffy blood group antigen found on RBCs (Gaur et al., 2004); until recently, it was widely thought that *P. vivax* relies on this single host receptor for tight junction formation and that individuals who are Duffy blood group antigen negative are protected from *P. vivax* infection (Miller et al., 1976). However, the identification of Duffy-negative individuals infected with *P. vivax* has shown that this is no longer the case (Menard et al., 2010) and researchers working with *P. vivax* now face the challenge of identifying the molecules involved in this *P. vivax* invasion of Duffy-negative RBCs.

*P. falciparum*, on the other hand, has long been known to use multiple host receptors for the formation of tight junctions. Proteins that appear to be involved in *P. falciparum*’s secondary attachment include proteins from the Duffy binding-like protein family: Erythrocyte binding antigen-175 (EBA-175) (Camus and Hadley, 1985), EBA-140 (Maier et al., 2003), EBA-181 (Gilberger et al., 2003), and erythrocyte binding ligand-1 (EBL-1) (Peterson and Wellems, 2000). EBA-175 binds glycophorin A (Orlandi et al., 1992), EBA-140 binds glycophorin C (Maier et al., 2003), and EBL-1 binds glycophorin B (Mayer et al., 2009). The existence of multiple possibilities to form parasite ligand/RBC receptor tight junctions appears to be a way for the parasite to increase its chances of invasion in response to host deletions of entry receptors (Mayer et al., 2006). Thus, although certain RBC polymorphisms such as the glycophorin A-negative phenotype may decrease the probability of parasite invasion (Miller et al., 2002), they
do not provide full protection against infection because of the parasite’s ability to use redundant pathways to invade.

Once a tight junction has formed, the motor that propels the *Plasmodium* merozoite into the RBC requires: 1) the interaction of filamentous actin with myosin; 2) a way of linking the actin-myosin component to the extracellular adhesins; and 3) a way of anchoring the actin-myosin component inside the parasite. The current model of the merozoite motor is outlined in a review by Cowman and Crabb (2006). Filamentous actin interacts with myosin A bound to myosin light chains. This actin-myosin component is linked to extracellular adhesins (currently unidentified) via a plasma membrane protein called merozoite thrombospondin-related anonymous protein (MTRAP). Specifically, filamentous actin is linked to MTRAP through the molecule aldolase. The actin-myosin component is also anchored to the parasite’s inner membrane complex (IMC); briefly, myosin A binds to myosin light chains which in turn bind to the IMC in the parasite through glideosome-associated protein (GAP) 50 and GAP45.

### 1.3.2 Merozoite Surface Proteins

The Merozoite Surface Protein (MSP) family consists of 10 proteins found on the surface of merozoites. MSP-1, MSP-2, MSP-4, MSP-5, MSP-8 and MSP-10 are attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor (Gaur et al., 2004). While MSP-3, MSP-6, MSP-7, and MSP-9 are known to be soluble proteins; these MSPs either associate with other membrane proteins or have weak interactions with the merozoite surface itself (Rodriguez et al., 2008). MSP-1 is the major protein on the merozoite surface and is key to the survival of the parasite (O'Donnell et al., 2000). This protein is also the most well studied member of the MSP family and as mentioned earlier, it is believed to play a role in the initial attachment step of invasion. MSP-1 is synthesized as an approximately 200kDa precursor and later processed into
four fragments: MSP-183, MSP-130, MSP-138, and MSP-142 (Holder et al., 1992). MSP-142 is further processed into MSP-133 and MSP-119, which is the only MSP-1 fragment that is attached to the membrane by a GPI anchor and stays on the surface of the merozoite until the parasite is inside the RBC (Blackman et al., 1990). MSP-119 has been shown to be an important player in invasion, since antibodies that target it inhibit parasite invasion (O'Donnell et al., 2000).

Although invasion studies of MSP-1 would suggest that antibodies to MSP-1 should provide protection against malaria, the degree of protection they confer are lower than might be expected. A meta-analysis by Fowkes et al. reported that individuals with MSP-119 IgG showed an 18% decreased risk of symptomatic disease, while those with MSP-142 IgG showed a 24% decreased risk of symptomatic disease (Fowkes et al., 2010).

A 36 kDa component of MSP-6 and a 22 kDa component of MSP-7 are both known to associate non-covalently with the portion of the MSP-1 protein complex that is shed from the merozoite surface during invasion (Pachebat et al., 2001; Trucco et al., 2001). However, MSP-9 has recently been shown to bind to MSP-142, suggesting that these MSPs may form a co-ligand during RBC invasion (Kariuki et al., 2005). It is currently thought that the epidermal growth factor like-domains present in some of these MSPs are key to their role in invasion (Rodriguez et al., 2008). However, there are still many gaps in our knowledge of MSPs. For instance, despite studies showing that individuals with MSP-3 IgG exhibited a 54% decreased risk of symptomatic disease (Fowkes et al., 2010), we are still unaware of the function of this MSP.

1.3.3 Anion Exchange Protein 1:

Anion Exchange Protein 1 (AE1) is hypothesized to be the host receptor that binds MSP-1 (Goel et al., 2003) and MSP-9 (Li et al., 2004). AE1 is the most common protein in a RBC membrane with 1 million copies per cell and it is highly conserved among species (Okoye and
Bennett, 1985). AE1’s main function is its role in the transport of CO₂ throughout the body, as it exchanges chloride ions for bicarbonate ions across the red cell membrane. There are several lines of evidence indicating that the RBC’s AE1 protein plays a key role in invasion including: 1) antibody that blocked *P. knowlesi* invasion immunoprecipitated AE1 (Miller et al., 1983); 2) liposomes incorporating AE1 inhibited *P. falciparum* invasion into RBCs (Okoye and Bennett, 1985); 3) antibodies targeting AE1 extracellular epitopes inhibited *P. falciparum* invasion (Clough et al., 1995); and 4) peptides of the AE1 receptor region also blocked *P. falciparum* invasion into RBCs (Goel et al., 2003). In 2003, Goel et al. were able to show that AE1 binds a domain present in MSP-1₁₉ and in 2004 this group also demonstrated that AE1 binds to MSP-9 (Goel et al., 2003; Li et al., 2004).
SECTION 1.4 – BLOCKING INVASION

1.4.1 Sulfated cyclodextrins

Negatively charged sulfated glycoconjugates such as heparin and dextran sulfate have previously been reported to inhibit invasion of merozoites into RBCs (Xiao et al., 1996; Clark et al., 1997). Work at our laboratory has shown that sulfated cyclodextrins (SCDs, as seen in Figure 4) inhibit the invasion of \textit{P. falciparum} merozoites into RBC by interacting specifically with a conserved domain on AE1 (Crandall et al., 2007). In addition to their antiplasmodial activity in cultures of \textit{P. falciparum}, these SCDs are also able to suppress the development of parasitemia in a \textit{P. berghei} mouse model of malaria (Crandall et al., 2007), suggesting that they are inhibiting a parasite ligand-host receptor interaction that is common in the RBC invasion of at least two \textit{Plasmodium} species. However, the high copy number of AE1 proteins, as well as the possibility of negatively affecting the functioning of AE1, makes it impractical to develop an antimalarial drug that works by targeting the host’s AE1. A more practical and potentially safer approach would be to develop a drug that targets a parasite ligand.

1.4.2 Tetrazolium salts

By a chance observation, the SCDs were seen to bind to nitrotetrazolium blue chloride (NBT, as seen in Figure 4) (Cui et al., 2008). Competitive binding assays have suggested that this interaction is in fact irreversible. The high affinity binding of SCDs to NBT may be explained by their electrostatic attraction, as SCDs are negatively charged compounds, while NBTs contain electron deficient rings. Since SCDs inhibit invasion by binding to a RBC receptor, the tight binding of SCDs to NBT suggested that NBT may in turn bind to the corresponding parasite ligand. This led to the screening of commercially available tetrazolium salts and small molecules with a tetrazolium core synthesized by our collaborators so that their
anti-*Plasmodium* properties could be evaluated (Cui et al., 2008). Tetrazolium salts are compounds with the cationic form of the tetrazole ring (composed of one carbon and four nitrogens, see Figure 4). The tetrazolium compounds tested were found to display antiparasitic activity in *P. falciparum* cultures, while showing much lower inhibitory activity in Chinese hamster ovary (CHO) cells, mammalian cells that are widely used for toxicity testing in academia.

Experiments were then carried out with a model tetrazolium compound, tetrazolium violet (TV, which has a tetrazolium ring attached to two phenyl groups and one naphthyl group, as seen in Table 2), in order to examine the mechanism of action of the tetrazolium compounds. In order to determine whether TV affects a parasite component or a RBC component, Crandall and Szarek (2009) treated either uninfected RBCs or infected RBCs with the compound, washed it out, combined the cells and examined if there was an effect on parasite viability 48 hours later. While pre-treating uninfected RBCs with TV had no effect on parasite viability, pre-treating infected RBCs resulted in decreased parasite viability. This suggested that TV works by binding a parasite component. TV was then tested in a parasite development assay in order to determine what stage of the parasite life cycle is being targeted. Parasites were synchronized at the ring stage, treated with various concentrations of TV, and monitored over a period of 4 days. Crandall and Szarek (2009) observed that micromolar concentrations of TV noticeably affect intracellular development, while nanomolar concentrations affect invasion without obvious effects on intracellular development. Lastly, flow cytometry was also carried out on TV-treated parasite cultures in the attempt to quantify the effect of TV on successful merozoite invasion. However these experiments were in their early stages, requiring optimization as well as confirmation that we were truly quantifying merozoite invasion.
Overall, it appears that our SCD compounds and our tetrazolium-based compounds are both inhibiting the same process by either binding a host receptor (as seen with the SCDs) or by binding the corresponding parasite ligand (as seen with the tetrazolium salts). Although the tetrazolium salts posses potent antiplasmodial activity in culture and have the desired property of targeting parasite ligands instead of host receptors, their observed instability makes tetrazolium salts less than ideal drug candidates. Thus, our collaborators created a series of compounds based on imidazolium and triazolium cores, compounds that are structurally related to tetrazoliums, with the added advantage of remaining stable when exposed to light and room temperature (Vlahakis et al., 2010). Since the tetrazolium salts bind a parasite ligand, our expectation was that imidazolium and triazolium compounds should also bind this same parasite ligand. The work that is described in the subsequent chapters was carried out with the following overall aims: 1) To determine if imidazolium and triazolium salts show antiplasmodial activity in vitro, as seen with the tetrazolium salts, 2) To determine if imidazolium and triazolium salts show antimalarial activity in vivo, as seen with the SCDs, and 3) To examine if imidazolium and triazolium salts use the same method of action as that of the tetrazolium salts.
Figure 4. Sulfated cyclodextrins and tetrazolium salts


Tetrazolium salts are compounds with the cationic form of the tetrazole ring.
PART II

THE ANTIPLASMODIAL ACTIVITY OF MONOVALENT IMIDAZOLIUM COMPOUNDS
SECTION 2.1 - INTRODUCTION

*Imidazolium salts as potential antimalarial candidates*

Previous work at our laboratory, carried out in collaboration with medicinal chemists at Queen’s University, has shown that tetrazolium compounds are capable of displaying potent antiplasmodial activity (Cui et al., 2008). In addition, work with our model tetrazolium compound, TV (a tetrazolium ring attached to two phenyl groups and one naphthyl group, as seen in Table 2) suggests that these tetrazolium-based compounds are working by inhibiting the invasion of merozoites into the RBC (Crandall and Szarek, 2009). However, in response to the observed instability of the tetrazolium compounds (they are reduced to their corresponding formazans when stored at room temperature and not protected from exposure to light), our collaborators created a series of compounds based on imidazolium cores (Vlahakis et al., 2010).

Imidazolium salts are compounds with the cationic form of the imidazole ring (composed of three carbons and two nitrogens, as seen in Figure 5) and they possess the desired property of chemical stability. The positive charge observed in the imidazolium ring results from the substitution of functional groups onto imidazole’s nitrogen atoms. Since imidazolium salts are structurally related to tetrazolium salts (imidazoliums contain two nitrogens in their ring, while tetrazoliums contain four nitrogens in their ring) and they retain the positively charged ring that appears to be key to the activity of the tetrazoliums (see Figure 4 and Figure 5), we hypothesized that the imidazolium compounds would show the same antiplasmodial activity seen with the tetrazolium compounds and that they would employ the same mechanism of action. Our collaborators have also synthesized imidazole compounds in order to compare the effect of charge on antiplasmodial activity. A description of the synthesis of the monovalent imidazole and imidazolium compounds can be found in Vlahakis et al., 2010.
QT69, a compound consisting of an imidazolium ring with benzyl and β-naphthylmethyl groups attached to its nitrogen atoms (Table 2), was used as a model compound to study the mechanism of action of the imidazolium salts. QT69 was chosen as it contains similar side groups to TV, our model tetrazolium compound. Our working hypothesis is that the positively charged rings in tetrazolium and imidazolium salts allow them to interact with a putative negatively charged parasite ligand involved in merozoite invasion. This hypothesis is based on the structure/activity relationship studies conducted with our series of tetrazolium compounds. Although the majority of the tetrazolium compounds tested in Cui et al. (2008) do show potent antiplasmodial activity, the presence of a positively charged tetrazolium core does not always guarantee potency or selectivity. The presence of side groups capable of interacting with a hydrophobic environment also appears to be important.

We have previously determined that tetrazoliums can interact very tightly with SCDs (Cui et al., 2008). SCDs, in turn, bind to the AE1 receptors on the surface of RBCs which are predicted to play an important role in merozoite invasion (Crandall et al., 2007). Since our tetrazolium salts interact with compounds that bind a conserved domain on the AE1 receptor, we predict that our tetrazolium and imidazolium compounds are mimicking this conserved AE1 domain. Furthermore, as AE1 is believed to bind the *Plasmodium* proteins MSP-1 and MSP-9 during invasion (Goel et al., 2003; Li et al., 2004), we predict that our compounds may be interacting with conserved domains on these parasite ligands.
Imidazolium salts are compounds with the cationic form of the imidazole ring (composed of three carbons and two nitrogens).

**Figure 5. Imidazoles and imidazolium salts**

Imidazolium salts are compounds with the cationic form of the imidazole ring (composed of three carbons and two nitrogens).
SECTION 2.2 – AIMS AND HYPOTHESES

Overall Aim: To investigate the antiplasmodial effect of monovalent imidazolium salt compounds.

Hypothesis 1: Monovalent imidazolium salts will show both antiplasmodial activity \textit{in vitro} and antimalarial activity \textit{in vivo}.

Hypothesis 2: Monovalent imidazolium salts inhibit merozoite invasion into RBCs.
SECTION 2.3 – MATERIALS AND METHODS

Compounds:

The synthesis of the test compounds is described in Vlahakis et al. (2010). All compounds were dissolved to a stock concentration of 10mg/mL in DMSO prior to use.

Cell culture:

*P. falciparum* lines 3D7 and ItG were grown in O+ RBCs obtained from volunteers and maintained according to the methods outlined in Trager and Jensen (1976). RPMI 1640 was supplemented with 10% human serum (obtained from the Chemo Day Care Department of the Princess Margaret Hospital, Toronto, Canada, under ethical consent), 6 g/L Hepes, 1.8 g/L sodium bicarbonate, 50 uM hypoxanthine, and 10 g/L gentamicin.

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were grown at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 25 mM Hepes, and gentamicin.

RAW cells (mouse leukemic monocyte macrophage) were grown at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 25 mM Hepes, and gentamicin.

Viability assays:

Viability assays were performed on *P. falciparum* 3D7 parasite cultures as described in Cui et al. (2008); a serial dilution was created for each test compound using a 96 well plate, cells were exposed for a period of 72 hours, and a *Plasmodium* lactate dehydrogenase (pLDH) enzyme assay was carried out to determine the susceptibility of *P. falciparum* cultures to individual compounds. The effect of QT69 on the viability of *P. berghei* was determined by obtaining blood from an infected mouse by heart puncture, washing the blood in RPMI, plating the red cells in a 96 well plate and allowing the parasites to develop for a further 48 hours in the
presence of varying amounts of QT69. Parasite growth was quantified by adding SYBR Green I (Smilkstein et al., 2004) and determining the relative fluorescence present in a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany). The toxicity of individual compounds in mammalian cells was measured in CHO cells and RAW cells using the method of Campling as modified by Cui et al. (2008). IC50 values in parasite cultures, CHO cell cultures, and RAW cell cultures were calculated using the computer program SigmaPlot (Jandel Scientific) to carry out non-linear regression analysis of the dose-response curves. All IC50 values represent the mean of 4 determinations.

**Examining the effect of compounds on RBCs versus parasites:**

ItG *P. falciparum* parasites were synchronized as mature forms using a Percoll density gradient (Rivadeneira et al., 1983). Isolated parasitized RBCs (pRBCs) and uninfected RBCs were separately treated with 5ug/mL QT69, which represents 10 times the IC50 value of QT69 in *P. falciparum* cultures, for a period of 3 hours. Following this period the compound was washed out by pelleting the cells and resuspending them in 1mL fresh media for a total of 3 washes. Treated pRBCs were then combined with untreated RBCs, and untreated parasites were combined with treated RBCs. Cultures were incubated for a period of 48 hours, after which a pLDH assay was conducted to test for the effect of QT69 on parasite viability. Controls consisting of: 1) an untreated parasite culture; 2) a parasite culture treated with 100nM chloroquine; and 3) a parasite culture treated with 5ug/mL QT69 during the 48-hour incubation period, were included.
**Parasite development assay:**

ItG *P. falciparum* parasites were synchronized at the ring stage of development using sorbitol treatment, which lyses RBCs containing mature forms of the parasite (Lambros and Vanderberg, 1979) and were then set up in 6-well plates at a hematocrit of 1% and a parasitemia of 3.5%. Parasite cultures were exposed to concentrations of QT69 ranging from 0 to 675 uM over a period of four days. The treated cultures were sampled daily to produce blood films that would allow us to determine their stage of development and parasitemia. Blood films were methanol-fixed, Giemsa stained, and examined using light microscopy. Parasitemia counts were generated by examining a minimum of 250 RBCs.

**Flow cytometry to detect merozoite population:**

Samples of an uninfected RBC culture, a parasitized RBC culture, and a concentrated merozoite culture were run through a FACS Calibur flow cytometer to examine the merozoite population. The concentrated merozoite control was prepared by isolating mature forms using a Percoll gradient (Lambros and Vanderberg, 1979) and allowing schizonts to rupture at 37°C in 95% N₂, 3%CO₂, and 2% O₂ over 4 hours. A ‘putative merozoite’ population was detected by examining graphs of forward scatter versus side scatter using the FlowJo software program.

A separate sample of ItG *P. falciparum* culture was stained with anti-glycophorin A to examine if the ‘putative merozoite’ population is not simply RBC fragments. Specifically, 20 uL samples of parasite culture was stained with 5 uL Anti-glycophorin A (neat), 5 uL isotype control, or 5 uL PBS, and incubated for 20 minutes in the dark. 200 uL PBS was then added to each sample and samples were left at room temperature for 15 minutes prior to being read. A sample of untreated uninfected RBCs was included as a control. Samples were left unfixed and
were run in a FACS Calibur flow cytometer in a level II BSC. Data was analyzed using the FlowJo software program.

**Flow cytometry to examine the effect of QT69 on merozoites:**

A Percoll density gradient (Rivadeneira et al., 1983) was used to synchronize parasites at the mature stage of development on day 1. Various concentrations of QT69 (1.025 uM, 2.05 uM, 4.1 uM, 8.2 uM, 16.4 uM, and 65.6 uM) were added to ItG parasite cultures (2% parasitemia and 1% hematocrit). On day 2, two samples were taken from each treatment condition so that one could be stained with SYBR Green I (final volume of 200 uL 0.5x SYBR Green I for 30 min at 4°C) and the second could be used as the SYBR negative control that was incubated in FACS buffer (PBS containing 2% FBS, 0.005 M EDTA, 0.02% Azide). Samples were washed once in 1 mL of FACS buffer following staining (spin at 2500 g for 20 min at 4°C). Controls included untreated uninfected RBCs, untreated parasitized RBCs, chloroquine-treated parasite cultures (100 nM), and SCD-treated cultures (50 ug/mL). Samples were left unfixed and were run in a FACS Calibur flow cytometer in a level II BSC. Data was analyzed using the FlowJo software program.

**Evaluation of in vivo activity of QT69:**

6-8 week old Balb/c female mice were purchased from Charles River and were allowed unrestricted access to food and water. Mice were infected with 10⁶ RBCs parasitized with *P. berghei* ANKA on day 0. This inoculum abundance, a standard in the field, results in a course of infection with a long enough window for therapeutic interventions to be examined. In the current mouse model of malaria, parasites are expected to be detectable with Giemsa staining on days 4-5 and parasitemia is expected to increase daily as *P. berghei* displays a 24 hour parasite cycle. Balb/c mice do not clear the infection on their own nor do they develop cerebral malaria.
If untreated, parasitemia continues to increase until mice meet the requirements for humane euthanasia (as outlined by the University Health Network’s Animal Resource Centre).

Once evidence of parasitemia was present in the mice (i.e. examination of blood smear samples showed at least one parasite was present in each mouse), groups of mice (5/group) received either 1% (v/v) DMSO in RPMI 1640, 18 ug QT69, or 18 ug QT72 via an i.p. injection, once a day for three days. Blood films were prepared by obtaining a 5 uL sample of blood from the tail vein of each mouse daily in order to monitor parasitemia.
SECTION 2.4 – RESULTS

Structure and activity:

Table 2 compares the activities of tetrazolium- and imidazolium-based compounds on *P. falciparum* and CHO cell cultures with the aim of defining the active motif common to both tetrazolium and imidazolium salts. TV, a tetrazolium ring bound to two phenyl groups and a naphthyl group is active against *P. falciparum* cultures at an IC$_{50}$ value of 0.15 ± 0.05 uM while showing an IC$_{50}$ value of 15 ± 3 uM in CHO cells (Cui et al., 2008); tetrazolium red, composed of a tetrazolium ring and three phenyl groups, has IC$_{50}$ values of 0.3 ± 0.2 uM in *P. falciparum* cultures and 71 ± 15 uM in CHO cells (Cui et al., 2008). Of key importance, the neutral reduced form of TR, designated QT67, has an IC$_{50}$ value in *P. falciparum* of 666 uM and is selectively toxic to CHO cells (IC$_{50}$ = 6 ± 2uM). To examine if the substitution of a tetrazolium ring with an imidazolium ring would render similar antiparasitic activity, we compared the activity of TV with that of QT69, a compound consisting of an imidazolium ring with similar side groups to TV. QT69 produced an IC$_{50}$ value of 0.9 ± 0.2 uM in *P. falciparum* cultures and an IC$_{50}$ value of 108 ± 6 uM in CHO cells. The presence of both good activity and parasite specificity for this structure suggests that keeping similar side groups and substituting an imidazolium ring for a tetrazolium ring retains the same level of antiplasmodial activity. This supports our hypothesis that a positively charged ring is a key component for antiplasmodial activity.

Hydrophobic side groups also appear to be a requirement for anti-*Plasmodium* activity; the relatively high IC$_{50}$ value of 543 ± 66 uM in parasite culture observed for QT70, a compound with a positively charged imidazolium ring lacking hydrophobic side groups, indicates that a single charged imidazolium ring is not sufficient for good activity and specificity; hydrophobic side groups are also necessary. The presence of electronegative halogen atoms on the side groups
of the molecules, which should increase the positive charge on the central ring, does not appear to prevent the molecules from being active and specific as compounds QT72, QT75 and QT83 also demonstrated good activity and parasite specificity.
Table 2. Activities of tetrazolium- and imidazolium-based compounds in *P. falciparum* and CHO cell cultures.

Shown here is a subset of our tetrazolium- and imidazolium-based compounds along with their corresponding IC$_{50}$ values in *P. falciparum* and CHO cell cultures. Values represent the mean of 4 independent determinations with the standard error of the mean indicated. Data is from Cui et al. (2008) and Vlahakis et al. (2010). This particular subset of compounds was chosen because it allows us to compare the effect of 1) changing the core structure from a tetrazolium to an imidazolium, 2) reducing the nitrogen ring, 3) removing the hydrophobic side groups, and 4) adding electronegative halogen atoms on the side groups of the molecules.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th><em>P. falciparum</em> IC$_{50}$ (μM)</th>
<th>CHO IC$_{50}$ (μM)</th>
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</thead>
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<td>Tetrazolium red</td>
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<td>Tetrazolium violet</td>
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<td>0.15 ± 0.05</td>
<td>15 ± 3</td>
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<td>QT67</td>
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<td>666± 0</td>
<td>6± 2</td>
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<td>QT70</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>543 ± 66</td>
<td>894 ± 19</td>
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<td>QT69</td>
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<td>0.9 ± 0.2</td>
<td>108 ± 6</td>
</tr>
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<td>171.9 ± 0.1</td>
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<td>1.05 ± 0.06</td>
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**QT69 targets a parasite component:**

Since SCDs bind to RBCs and tetrazolium salts bind to SCDs, we originally hypothesized that tetrazoliums would bind to the corresponding parasite ligand. Using our model tetrazolium compound we have previously shown that TV appears to inhibit merozoite invasion by binding a parasite ligand (Crandall and Szarek, 2009). We hypothesize that imidazoliums bind to the same parasite ligand and thus we predicted that QT69, an imidazolium compound that is structurally related to TV, would also interact with a parasite component. In order to distinguish between a potential effect on a parasite component versus a potential effect on a RBC component we treated either uninfected RBCs or infected RBCs with the compound, washed it out, combined treated infected RBCs with untreated RBCs, combined untreated parasites with treated RBCs and examined if there was an effect on parasite viability 48 hours later (Figure 6). While pre-treating uninfected RBCs with QT69 had no effect on parasite viability, pre-treating infected RBCs resulted in decreased parasite viability. This supports our hypothesis that imidazolium compounds function by targeting a parasite component. In cultures treated with QT69 for a 48-hour period parasite viability is comparable to that of the chloroquine-treated control. This confirms QT69’s effectiveness as an anti-*Plasmodium* compound. It is worth noting that the three-hour pRBC treatment results in intermediate antiplasmodial activity compared to the 48-hour treatment. One possible explanation for this is that some of the treated schizonts contained parasite ligands that were unavailable for binding during the three-hour treatment. It is reasonable to predict that our imidazoliums are able to cross cell membranes, as tetrazoliums, which are used in MTT assays, must cross cell membranes in order to access the mitochondria and become reduced.
**Figure 6. QT69 targets a parasite component**

ItG *P. falciparum* parasite cultures were synchronized as matures and isolated parasitized RBCs (pRBCs) were treated with 5ug/mL QT69 for a period of 3 hours. Uninfected RBCs were separately treated with 5 ug/mL QT69 for 3 hours. Following this 3-hour incubation period the compound was washed out (3 washes) and cells were resuspended in fresh media. Treated pRBCs were then combined with untreated RBCs, and untreated parasites were combined with treated RBCs. Cultures were incubated for a period of 48 hours, after which a pLDH assay was conducted to test for the effect of QT69 on parasite viability. Controls consist of an untreated parasite culture, a parasite culture treated with 100 nM chloroquine, and a parasite culture treated with 5 ug/mL QT69 during the 48-hour incubation period. Results are the mean of 4 determinations with the standard error indicated by error bars.
**QT69 inhibits parasite maturation and parasite invasion in vitro:**

The structural similarity of QT69 to TV suggested that QT69 works by inhibiting invasion. In order to distinguish possible effects on invasion from potential effects on intracellular parasite maturation we carried out a parasite development assay. Briefly, the parasites were synchronized as rings on day 1 at a parasitemia of 3.5%, and their ability to go through their normal developmental stages when exposed to varying concentrations of QT69 was determined. Our test is based on the WHO’s in vitro micro-test (Mark III), which involves tracking the development of parasites treated with an antimalarial in order to assess for emerging resistance to that particular drug (World Health Organization, 2001). *P. falciparum* parasites growing at 37°C do not enter stationary phases during development; thus, parasites that fail to cycle between ring and mature stages from one day to the next are dead.

The results of our parasite development assay experiment are shown in Figure 7. In our control well parasites developed into healthy mature forms on day 2. On day 3 we saw that these untreated cultures contained healthy ring forms as well as an increase in parasitemia, as a result of a reinvasion event between days 2 and 3. These parasites then developed into the healthy mature forms seen on day 4. If we look at our QT69-treated cultures on day 2 we see that parasites exposed to concentrations at or above 21.1 μM have failed to mature, indicating an effect on intracellular parasite development. While cultures treated with less than or equal to 10.5 μM QT69 contained healthy-looking mature stage parasites on day 2. However, on day 3 parasite cultures exposed to QT69 concentrations between 1.3 μM and 10.5 μM do not show the expected increase in parasitemia seen in the untreated controls. We have also noticed that cultures treated with these concentrations of QT69 contain immature parasites stuck to the
outside of RBCs. Together these results are consistent with the idea that at these lower concentrations our compounds are working by inhibiting the invasion of parasites into RBCs.
Figure 7. QT69 can inhibit intracellular development and RBC invasion

Shown here are representative images and parasitemia values of *P. falciparum* ItG cultures treated with various concentrations of QT69 (0, 0.3, 0.7, 1.3, 2.6, 5.3, 10.5, 21.1, 42.2, and 84.4 uM) over 4 days (2 replication cycles).
The detection of a putative merozoite population using flow cytometry:

Since our light microscopy data suggests that our imidazolium compounds may be inhibiting invasion, we evaluated flow cytometry to attempt to quantify the number of merozoites that do not successfully invade RBCs when parasites are treated with our compounds. Thus, we first needed to determine if we could detect a merozoite population using flow cytometry. We know that *Plasmodium*’s invasion efficiency is not 100% because neither our parasite cultures nor our animal models show 16-32 fold increases in parasitemia between reinvasion cycles; rises in parasitemia are much more modest. Thus, one would expect all parasite cultures to contain a population of free merozoites. Figure 8A shows a comparison of the forward scatter versus side scatter plots for uninfected RBC cultures, parasitized RBC cultures, and a concentrated merozoite culture; forward scatter is a measure of cell size, while side scatter is a measure of cellular complexity, and plots of forward versus side scatter allow us to distinguish between different cell types in a mixed sample. Gate i represents the population of putative merozoites that is present in parasite cultures and concentrated merozoite cultures but is absent in samples of uninfected RBCs. Gate ii represents the population of RBCs that is present in samples of parasite cultures and samples of uninfected RBCs; this RBC population was also present to a lesser extent in our concentrated merozoite cultures because these samples did contain mature forms that had not yet burst as well as a few uninfected RBCs (Figure 8B).
Figure 8. Red blood cell population and putative merozoite population

A) Forward scatter vs. side scatter plots for uninfected RBC cultures, parasitized RBC cultures, and a concentrated merozoite culture; gates shown for putative merozoite population (i) and RBC population (ii). B) Representative image of Giemsa-stained concentrated merozoite culture. Small black dots are free merozoites (1.0-1.2um), large gray cells are uninfected RBCs (6-8um), and large black cells are RBCs infected with mature stage parasites.
**Putative merozoite population is not composed mainly of RBC fragments:**

The small size of the material in the putative merozoite gate suggests that this population is composed of free merozoites. However, there exists the possibility that this material could instead be composed of RBC fragments released by the rupture of schizont-infected RBCs. In order to rule out the possibility that the putative merozoite population is actually composed of RBC fragments we carried out an experiment in which we stained parasite culture samples with anti-glycophorin A conjugated to fluorescein isothiocyanate (FITC). Glycophorin A is a RBC marker, thus we examined if our population of interest, the putative merozoite population, stained positive for glycophorin A. However, compared to the isotype control, only 15.6% of events in the putative merozoite gate stained positively for glycophorin A, indicating that 84.4% of that population is not of RBC origin (Figure 9). Thus, we conclude that this material is of parasite origin, which is consistent with their being free merozoites. Our results also suggest that merozoites do not contain attachments to RBC membrane fragments following the rupture of schizont-infected RBCs.
Figure 9. Putative merozoite population is composed mainly of glycophorin A negative material

*P. falciparum* culture was either left unstained, stained with isotype, or stained with anti-glycophorin A conjugated to FITC. The events in the putative merozoite gate were separated into glycophorin A positive and glycophorin A negative. The values in the i gate represent the percentage of glycophorin A positive events in the putative merozoite gate.
The effects of QT69 on the merozoite population:

Now that we have shown that it is possible to quantify the free merozoite population we aimed to determine if the absence of a rise in parasitemia following a reinvasion event was due to i) a reduction in the number of released merozoites or ii) an inability of merozoites to invade the RBC. Thus, flow cytometry was used to quantify the number of merozoites that do not successfully invade RBCs when parasite cultures are treated with QT69. The count of free merozoites for a particular treatment was generated by counting the number of events staining positive for SYBR-Green in the SYBR-Green stained sample minus the SYBR-Green positive events in the unstained sample.

In figure 10 we have a graph showing a measure of free merozoites 24 hours post-treatment of mature stage parasite cultures with various concentrations of QT69. At this time, we expect that any merozoite that did not successfully invade a new RBC and is not stuck to the outside of a RBC would be detected in the free merozoite gate. Because invasion efficiency is not 100% in our parasite cultures, we do see free merozoites in the untreated culture. Treatment with SCDs should lead to an increase in the number of free merozoites, however this was not the case in this experiment. This result was unexpected, as we have previously shown that SCDs interfere with invasion by inhibiting the interaction of merozoites with the surface of the RBC; as such, we are unable to draw conclusions regarding QT69’s ability to increase the number of free merozoites. Since chloroquine is a drug that kills both early and late stage parasites, treatment with chloroquine was expected to decrease the number of released merozoites. As predicted, treatment with chloroquine does decrease the number of free merozoites compared to the untreated control. In our experimental conditions, treatment with 65.6 uM and 16.4 uM QT69 results in a decrease in the number of free merozoite compared to the untreated control; while,
treatment with 8.2 uM, 4.1 uM, 2.1 uM, and 1.0 uM QT69 does not decrease the number of free merozoites. The finding that treatment with 65.6 uM and 16.4 uM QT69 reduced the number of released merozoites was not surprising, as these concentrations appear to inhibit the parasite’s intracellular development. However, the finding that treatment with concentrations hypothesized to primarily affect invasion (8.2 uM, 4.1 uM, 2.1 uM, and 1.0 uM QT69) does not result in a reduction of free merozoites suggests that the decreased invasion rate observed in our parasite development assay is not attributed to a reduction in the number of merozoites that are available to invade. These results support our hypothesis that monovalent imidazolium salts work by interfering with the merozoite’s ability to invade RBCs.
Figure 10. Preliminary data suggest that QT69 is able to inhibit parasite replication at a step following merozoite release.

Shown here are counts of free merozoites per 10,000 RBCs obtained one day following treatment of mature parasites with various concentrations of QT69. Uninfected RBCs, untreated parasite cultures, sulfated cyclodextrin-treated parasite cultures, and chloroquine-treated cultures were included as controls.
**QT69 inhibits parasite replication in vivo:**

We have previously observed that our model tetrazolium salt, TV, was also active in *ex vivo* assays employing the murine malaria parasites *P. berghei* and *P. chabaudi* chabaudi (Cui et al., 2008). However due to the likely reductive inactivation of TV in a living animal we were unable to evaluate this compound in a mouse model of malaria. In contrast, the stable nature of imidazolium salts meant that we could potentially test the effectiveness of QT69 in an *in vivo* mouse model. Prior to the animal challenge we tested QT69 in a mouse *ex vivo* assay (i.e. blood from a parasitized mouse was temporarily cultured in the presence of compound) using the mouse malaria parasite *P. berghei*. The observed IC$_{50}$ value of 12 ± 3µM, was higher than the 0.9 ± 0.2 µM seen with *P. falciparum*, however it suggested that QT69 would still display good antiparasitic activity in mice. This finding supports the hypothesis that our compounds are targeting a ligand that is conserved across different *Plasmodium* species; thus, we expected that compounds showing potent antiplasmodial activity in *P. falciparum* cultures should also show good antiparasitic activity in an *in vivo* mouse model of malaria. A comparison of QT69’s IC$_{50}$ value in *P. berghei* with its IC$_{50}$ value in CHO cells (120 µM) was consistent with the compound having a degree of specificity for *Plasmodium*.

It is worth noting that compound development was being driven by *in vitro* inhibitor potential, rather than by favourable drug properties. Thus, *in vivo* testing was undertaken as a proof of concept experiment to examine if compounds that are active in parasite culture are actually effective in mice. To determine if QT69 treatment is able to reduce the parasite load in a murine model of malaria, mice infected with *P. berghei* were given one dose of the compound daily for a period of three days. A comparison of parasitemia counts over the course of the infection (Figure 11) reveals that compared to the untreated controls, treatment with QT69 is
effective at suppressing parasitemia (p=0.028). Another imidazolium compound, QT72 (see Table 2), was administered to a separate arm of the challenge experiment, however the results obtained were no different from those of the untreated control (p=0.734), indicating that the specific side groups present in QT69 directly influenced the observed outcome.

A validated system to predict in vivo toxicity of small compounds does not exist, however after the administration of QT69 to mice we also examined the toxicity of QT69 in RAW (mouse leukemic monocyte macrophage) cells, hypothesizing that an assay utilizing RAW cells may provide a better indicator of toxicity in mice than an assay using CHO cells. QT69 produced an IC_{50} value in RAW cells of 12 ± 2 uM which is directly comparable to the IC_{50} value of 12 ± 3uM in P. berghei. However, since P. berghei infected mice receiving QT69 treatment did not visibly appear more ill than infected untreated control animals, this suggests that toxicity in RAW cells is not predictive of toxicity in mice.
Figure 11. QT69 is effective at suppressing parasitemia in vivo

Groups of 5 female Balb/c mice were infected with $10^6$ P. berghei parasites i.p. and the parasitemia was estimated daily by examining a Giemsa-stained blood film. Once parasites were observed mice were infused once daily with 1% (v/v) DMSO in RPMI 1640 (●), with 18 ug/day of QT72 (○), or 18 ug/day of QT 69 (▼) for three days, as indicated by the arrows. Average parasitemias are shown with standard error of the mean for each experimental group indicated by bars.
SECTION 2.5 - DISCUSSION

We have previously reported that tetrazolium-based inhibitors are effective agents against *P. falciparum* in culture (Cui et al., 2008) and we have acquired data suggesting that these compounds block parasite invasion into erythrocytes (Crandall and Szarek, 2009). However, tetrazolium salts are unstable and can be reduced to their corresponding neutral formazan. Thus, we have turned our focus to studying imidazolium-based compounds and we have recently shown that imidazolium salts are also capable of showing antiparasitic activity *in vitro* (Vlahakis et al., 2010). We hypothesize that the key to the success of the tetrazolium- and imidazolium-based inhibitors is their electron deficient ring with hydrophobic side groups. Our comparison of the activities of tetrazolium- and imidazolium-based compounds in both *P. falciparum* and CHO cell cultures (Table 2) supports this hypothesis. One possible explanation for why positively charged rings are more effective than uncharged compounds is that only compounds with positive charges are able to bind a putative negatively-charged parasite ligand.

Previous work at our laboratory used TV, a tetrazolium ring attached to two phenyl groups and one naphthyl group, as a model compound to determine how tetrazolium-based inhibitors exert their antiplasmodial activity. The current study was undertaken to examine if imidazolium salts use the same method of action as that of tetrazolium compounds and we used QT69, an imidazolium ring with similar side groups to TV, as our model imidazolium compound. We were unable to use an exact analog of TV for comparison, as such a compound was not available. Although the side groups on QT69 and TV are not identical, they are similar enough that both compounds display potent antiplasmodial activity that is 100-fold greater in *P. falciparum* than in CHO cells. Our data is consistent with the hypothesis that QT69, like TV, works by affecting a parasite component and that it can inhibit either intracellular development
or merozoite invasion into RBCs depending on the concentration that is used. Our parasite developmental assay suggests that while concentrations at or below 10.5 uM QT69 do not appear to affect parasite maturation, they do result in a decreased invasion rate coincidental with detection of parasites stuck to the outside of RBCs. In order to exclude the possibility that the decrease in invasion was due to a reduction in the number of merozoites released from schizont stage parasites, we compared the number of free merozoites in treated and untreated cultures. We found that treatment of parasite cultures with 1.0-8.2 uM QT69 does not result in a decreased number of free merozoites. This finding lends support to our hypothesis that the imidazolium compounds are capable of inhibiting invasion of merozoites into RBCs. However, it does not provide us with information about why this inhibition is taking place. Although we hypothesize that our compounds inhibit invasion of merozoites into RBCs by interfering with a parasite ligand-host receptor interaction, we must still rule out the possibility that treatment with our compounds leads to the production of abnormal merozoites.

It is possible that the higher concentrations of QT69 that inhibit the parasite’s intracellular development are doing so by interfering with lipid synthesis, as is the case with the thiazolium compounds described by Hamze et al. (2005). Since thiazolium and imidazolium compounds have structural similarities (thiazoliums have one nitrogen, one sulfur, and three carbons in their ring instead of two nitrogens and three carbons), they may work by similar mechanisms. Hamze’s group has also determined that one of the keys to the activity of their compounds appears to be positively charged rings (Salom-Roig et al., 2005). They hypothesize that their compounds, designed to be analogues of choline, interfere with the synthesis of phosphatidylcholine. Lipid synthesis is important for both parasite growth and parasite replication, when the parasite must produce enough lipid membrane for each of the new
merozoites. Hamze et al. have not reported that their compounds may have a possible effect on invasion. Although it is possible that imidazoliums may have similar effects as thiazoliums at their higher concentrations, our imidazolium compounds do not appear to be inhibiting intracellular development at concentrations close to their IC$_{50}$ in parasite culture, as is seen with the thiazolium compounds. If we were inhibiting lipid synthesis when parasites are exposed to these lower concentrations of imidazolium compounds we would not expect to see normal development at lower concentrations that kill cultures. Furthermore, a study by Higuti et al. (1983) has reported that triphenyltetrazolium and its derivatives (such as tetrazolium red in Table 2) are capable of interfering with energy transduction in rat liver mitochondria. This may explain the toxic effects observed in CHO cells treated with tetrazolium and imidazolium salts despite lacking ligands involved in invasion.

The results of our *in vivo* mouse trials have demonstrated that imidazolium salt compounds that are effective *in vitro* can also be effective *in vivo* (Figure 11); QT69 kills parasites *in vitro* and suppresses parasitemia *in vivo*. However, we also observed that related compounds displaying similar effectiveness against *P. falciparum* *in vitro* can have varying degrees of effectiveness *in vivo*. The cause of the discrepancy seen between the effect of QT69 and QT72 is unknown, but it is possible that differences in the side groups of these compounds may lead to differences in the way the compounds are absorbed, distributed, metabolised, and eliminated in the body. In retrospect, the fact that *P. berghei* infected mice receiving QT69 treatment did not visibly appear more ill than infected untreated control animals suggests that our hypothesis that RAW cells could potentially predict toxicity in mammalian cells was incorrect; thus we continue to use CHO cells in our mammalian cell toxicity assays. Although we observed a beneficial effect when mice are treated with QT69, this treatment did not cure the mice of their
infection. Possible explanations as to why a cure was not achieved include, but are not restricted to, the following reasons: perhaps the parasites became resistant to our compounds, perhaps the dose tested was too low, or potentially there are aspects of the compound’s absorption, distribution, metabolism, or excretion that decrease the amount of active compound that the parasites are exposed to.

In conclusion, we have shown that our imidazolium compounds have both \textit{in vitro} and \textit{in vivo} activity and we suspect that they may be working through a novel mechanism of action. In addition to affecting intracellular parasite development, our model imidazolium compound, QT69, also appears to be inhibiting merozoite invasion into RBCs. Our focus now turned to improving the activity of the compounds \textit{in vivo}. Thus, we next searched for compounds with increased potency and specificity that could form tighter interactions with parasite ligands.
PART III

THE ANTIPLASMODIAL ACTIVITY OF BIVALENT IMIDAZOLIUM AND TRIAZOLIUM COMPOUNDS
SECTION 3.1 - INTRODUCTION

We have previously reported that monovalent tetrazolium and imidazolium compounds are effective at killing *P. falciparum* parasites in culture and that they are able to inhibit the invasion of merozoites into the RBC (Cui et al., 2008; Crandall and Szarek, 2009; Vlahakis et al., 2010; Section 2). We have also shown that a monovalent imidazolium compound is capable of suppressing parasitemia in an *in vivo* mouse model of malaria (Section 2) - however, we were not able to cure mice of their malarial infection. Thus, we aimed to generate compounds with increased potency and specificity. Structure/activity relationship studies of our monovalent tetrazolium and imidazolium-based compounds suggest that the key to their activity lies in their positively charged rings and their hydrophobic side groups (Cui et al., 2008; Vlahakis et al., 2010; Section 2).

In the field of medicinal chemistry one technique for improving the potency of a drug is to put two of the active molecules together in the same molecule. Thus, our collaborators at Queen’s University synthesized bivalent versions of monovalent imidazolium and triazolium compounds, i.e. they synthesized molecules containing two imidazolium or triazolium rings with a spacer between them. Imidazolium salts are compounds with the cationic form of the imidazole ring (composed of three carbons and two nitrogens, as seen in Figure 5) and triazolium salts are compounds with the cationic form of the triazole ring (composed of two carbons and three nitrogens; in Table 3, QT125 contains triazole rings, while QT124 contains triazolium rings). The positive charge observed in the imidazolium and triazolium rings results from the substitution of functional groups onto the nitrogen atoms of imidazoles or the carbon and nitrogen atoms of triazoles.
We carried out a structure/activity relationship study on our bivalent imidazolium and triazolium compounds in order to address the following questions: 1) Does the spacing between the rings matter? 2) Does the presence of a charge on the ring matter? 3) Does the identity of the spacer matter? and 4) Can you substitute triazoliums for imidazoliums and retain activity? We examined the effect of spacing between the rings by comparing the antiplasmodial activity of compounds containing alkyl spacers of different lengths. The effect of charge on activity was determined by synthesizing bivalent imidazole and triazole compounds and comparing their effectiveness to that of their corresponding bivalent imidazolium and triazolium compounds. Finally, we were able to determine if the identity of the spacer separating the two rings has any effect by comparing the antiplasmodial activity of compounds with different types of spacers.

We hypothesized that bivalent compounds containing two positively charged rings may interact with two putative parasite ligands. Thus, we predicted that alterations in the spacing between the rings would have an effect on antiparasitic activity. We expected there to be an optimal spacer length which allows the two rings to interact with two ligands and increases the potency of the compounds beyond what we observed with our monovalent series. On the other hand, if the spacing between the two rings is too short or too long we predicted that the activity of the bivalent compounds would be similar to that of our monovalent compounds. Regarding the type of spacer being used, if altering the nature of the hydrophobic spacer has no effect on activity this may imply that the potency of the compound is primarily dependent on the presence of positively charged rings and any hydrophobic side groups. However, a decrease in activity resulting from a change in spacer type may indicate that the identity of the spacer is key and that undesirable spacers may be interfering with the interaction between electron deficient rings and their ligands. Furthermore, we have used QT124 (Table 3), composed of two triazolium rings
separated by a 12-carbon alkyl chain, as our model bivalent compound in order to study the
method of action of our bivalent compounds. Although it is possible that bivalent and
monovalent molecules kill parasite cultures using different methods, we hypothesize that they
are employing the same method of action.
SECTION 3.2 – AIMS AND HYPOTHESES

Overall Aim: To investigate the antiplasmodial and antimalarial effect of bivalent imidazolium and triazolium salt compounds and compare them to that of our monovalent compounds.

Hypothesis 1: Molecules with two active centres will make for more potent and specific inhibitors.

Hypothesis 2: Bivalent compounds will retain the mechanism of action of our monovalent compounds.
SECTION 3.3 - MATERIALS AND METHODS

Compounds:

The synthesis of the compounds was carried out by our collaborators at Queen’s University and is described in Vlahakis et al. (2010) and the specific synthesis of individual bivalent compounds is described in a manuscript about to be submitted to Bioorganic and Medicinal Chemistry. All compounds were dissolved to a stock concentration of 10 mg/mL in DMSO prior to use.

Cell culture:

Cell cultures were maintained as described in section 2.3.

Viability assays:

Viability assays were carried out as described in section 2.3.

Parasite developmental assay:

A Percoll density gradient was used to synchronize parasites at the mature stage of development one day prior to the start of the parasite developmental assay. On day 1 various concentrations of QT124 (40 nM, 83 nM, and 664 nM) were added to 3D7 *P. falciparum* cultures (2% parasitemia and 1% hematocrit). Samples were collected once a day on days 1, 2, 3, and 4. In addition to the sample that was taken to make a Giemsa-stained blood smear to count parasitemia, two samples of each condition were taken daily: one sample was stained with SYBR Green I (final volume of 200 uL 1x SYBR Green I for 30 min at 4°C) and the third sample was used as the SYBR Green negative control that was incubated in FACS buffer (PBS containing 2% FBS, 0.005 M EDTA, 0.02% Azide). Samples were washed once in 1 mL FACS buffer following staining (spin at 2500g for 20 min at 4°C). Controls included untreated uninfected RBCs, untreated parasitized RBCs, chloroquine-treated parasite cultures (100 nM), and SCD-
treated cultures (50 ug/mL). Samples were left unfixed and were run in a FACS Calibur flow cytometer in a level II BSC. Data was analyzed using the FlowJo software program.

**Evaluation of in vivo activity of bivalent compounds:**

In order to examine the effectiveness as well as the toxicity of the bivalent compounds QT109, QT119 and QT124, these compounds were administered to both infected and uninfected mice. 6-8 week old Balb/c female mice were purchased from Charles River and were allowed unrestricted access to food and water. Half of the mice were infected i.p. with $10^6$ red blood cells parasitized with *P. berghei* ANKA on day 0. Once evidence of parasitemia was present in the infected mice, infected and uninfected groups of mice (5/group) received 1% (v/v) DMSO in RPMI 1640, 60 ug of QT109, 9 ug of QT119, 7.5 ug of QT124, or 450 ug of chloroquine via an i.p. injection, once a day for three days. Blood films were prepared by obtaining a 5 uL sample of blood from the tail vein of each mouse daily in order to monitor parasitemia.
SECTION 3.4 - RESULTS

Structure and activity:

We have determined that by synthesizing bivalent compounds, we can achieve greater potency and specificity than has been observed with our most active monovalent compounds (Bivalent compound structures examined are listed in Table 3). Figure 12 allows us to compare the effect of varying the length of the carbon spacer that separates the imidazole or imidazolium rings. In the present series, the IC_{50} values of bivalent imidazoles do not differ greatly between *P. falciparum* and CHO cell cultures. Increases in the length of the alkyl chain between two imidazole rings results in a drop in the IC_{50} in *P. falciparum* culture that is matched by a drop in the IC_{50} value in CHO cells. However, increases in the length of the alkyl chain between two imidazolium rings results in different effects in *P. falciparum* and CHO cells (Figure 12). In CHO cells, this increase in spacer length resulted in a modest decrease in IC_{50} values. In *P. falciparum* culture, compounds with alkyl spacers consisting of 4, 6, and 8 carbons (as seen in compounds QT86, QT87, and QT71) had activity comparable to that of our monovalent imidazolium compounds (Figure 12; Vlahakis et al., 2010). However, carbon spacers of at least 10 carbons in length resulted in increased potency over our monovalent compounds (Figure 12; Vlahakis et al., 2010). Thus, the length of the carbon spacer strongly influences the antiparasitic activity of the compound, which is consistent with the idea that bivalent imidazolium compounds may be interacting with two negatively charged parasite ligands and that a minimum spacing between the active rings is required for increased avidity.

Figure 12 also compares the activity of pairs of structurally related bivalent compounds in which one has positively charged rings and the other has neutral rings, in both *P. falciparum* and CHO cell cultures. As predicted, bivalent compounds with positively charged rings are more...
active than those with neutral rings when tested in *P. falciparum* culture (Figure 12). This supports the hypothesis that charge is important to the activity of our bivalent compounds. On the other hand, there was relatively little difference in activity between bivalent imidazole and imidazolium compounds when treating CHO cells (Figure 12). Lastly, we compared the activity of bivalent imidazolium compounds with the following types of spacers: a phenyl, a biphenyl, a methylbiphenylmethyl, and a 1,4-dialkoxyphenyl spacer (QT114, QT98, QT113, and QT123 respectively, as seen in Table 3). While the shorter phenyl spacer did not show good specificity for *P. falciparum* cultures over CHO cells, compounds with a biphenyl, a methylbiphenylmethyl, and a 1,4-dialkoxyphenyl spacer did display high levels of antiparasitic potency and specificity (Table 3). This suggests that in the current series of compounds the length of the hydrophobic carbon spacer between two positively charged rings may be more important than the nature of the spacer itself.
Table 3. Activities of bivalent compounds in *P. falciparum* and CHO cell cultures. For each indicated structure the IC₅₀ value was determined in both *P. falciparum* and CHO cell cultures. Values represent the mean of 4 independent determinations with the standard error of the mean indicated.

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<td>Standard Deviation</td>
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Figure 12. Effect of the alkyl spacer and charge on the activity of compounds in *P. falciparum* and CHO cell cultures. Compounds were synthesized either as bivalent imidazolium salts, or as neutral bivalent imidazole structures (see compounds in Table 3). The compounds were then evaluated for activity in *P. falciparum* and CHO cell cultures using the pLDH assay and MTT assay respectively. When neutral imidazole compounds (top left panel) were assayed, a moderate increase in activity was observed as the alkyl chain length increased, however little, or no, selective toxicity was observed for *P. falciparum* cultures. When the experiment was repeated using similar bivalent imidazolium salts (top right panel) it was observed that increasing the length of the alkyl chain between the imidazolium moieties resulted in a moderate increase in toxicity in CHO cultures throughout the range, however, a significant increase in activity is observed in *P. falciparum* cultures when an alkyl chain of more than 8 carbon atoms was present. When the data were plotted to allow a direct comparison of the effect of the imidazole and imidazolium compounds in *P. falciparum* cultures alone (bottom left panel) it was observed that the presence of electron-deficient imidazolium rings was only a significant determinant of activity at alkyl spacer lengths greater than 8 carbon atoms. Conversely, bivalent compounds containing either imidazole or imidazolium rings were found to have similar activities in CHO cell cultures (bottom right panel).
*QT124 inhibits merozoite invasion into RBCs without interfering with adhesion to the RBC membrane:*

The parasite developmental assay was carried out using QT124, our model bivalent compound, in order to determine what stage(s) of the parasite life cycle is/are being affected by our compounds. Figure 13A displays the parasitemia counts on days 1-4 for all treatment conditions tested in the parasite developmental assay, as measured using light microscopy. As expected, uninfected RBC cultures have parasitemia values of zero on all 4 days. Untreated parasite cultures show an expected increase in parasitemia from about 2% to 16% between days 2 and 3, the interval in which we would expect a reinvasion event to occur. Further, these untreated parasite cultures show no increase in parasitemia between days 1 and 2 or between days 3 and 4, as these intervals correspond to stages of intracellular development. SCD treatment, which prevents merozoite entry by blocking the RBC receptor, and chloroquine treatment, which kills ring and mature forms of the parasite, both prevent an increase in parasitemia over the 4 days. Treatments with 40 nM, 83 nM, and 664 nM QT124 were all expected to kill parasite cultures, as these three concentrations are well above the IC$_{50}$ value of QT124 in *P. falciparum*; however I hypothesized that these concentrations may differ in their method of action, as we have previously seen that monovalent imidazolium compounds appear to target different stages of the parasite life cycle depending on the concentrations used. As expected, all these concentrations were found to decrease parasitemia compared to the untreated control.

Figure 13C contains representative images of Giemsa-stained parasite cultures in different treatment conditions on days 2, 3, and 4 and offers insight about the method of action of QT124 that is not obvious from comparing parasitemias. Treatment with 40 nM and 83 nM QT124 show healthy looking matures on day 2, while treatment with 664 nM QT124 shows
unhealthy looking parasites on day 2, suggesting that at concentrations as high as 664 nM QT124 we are affecting intracellular parasite maturation. Treatment with 664 nM QT124 subsequently resulted in a lack of immature parasites on day 3 and the absence of parasites on day 4. In contrast, treatment with 40 nM and 83 nM QT124 did result in visible ring forms on day 3. However, many of these ring forms appeared to be extracellular, bound to the outside of the RBC. Although we observed what looked like a mixture of intracellular and extracellular ring forms and despite the healthy appearance of these ring forms, treatment with 40 nM and 83 nM QT124 resulted in the absence of mature forms on day 4. The presence of extracellular ring forms together with the observation that these same concentrations do not appear to affect intracellular development from rings to matures between days 1 and 2 are consistent with the idea that treatment with 40 nM and 83 nM QT124 is affecting the ability of merozoites to properly invade.

Flow cytometry may be used to distinguish between, and quantify populations of, uninfected RBCs, infected RBCs, and putative merozoites in a sample; thus, we are using this technique to examine if there is an effect of our model bivalent compound, QT124, on the number of free merozoites, in order to determine if our bivalent compounds retain the mechanism of our monovalent compounds. The number of free merozoites in each treatment condition was compared to that in untreated parasite cultures; since invasion efficiency is not 100% in our parasite cultures, we do see free merozoites in untreated cultures. Figure 13B shows a graph of free merozoites on day 3. On day 3, we expect that any merozoite that did not successfully invade a new RBC and is not stuck to the outside of a RBC would be detected in the free merozoite gate. The estimate of free merozoites was generated by counting the number of SYBR Green positive events in the SBYR Green stained samples and subtracting the number of
SYBR Green positive events in the unstained controls. We then expressed this value as the number of free merozoites per 1000 RBCs. Treatment with SCDs should lead to an increase in the number of free merozoites, however this was not the case in this experiment. This result was unexpected, as we have previously shown that SCDs interfere with invasion by inhibiting the interaction of merozoites with the surface of the RBC; as such, we are unable to draw conclusions regarding QT124’s ability to increase the number of free merozoites. Since chloroquine is a drug that kills both early and late stage parasites, treatment with chloroquine resulted in a decreased number of free merozoites. Treatment with 664 nM QT124 results in a decrease in the number of free merozoites compared to the untreated control; this finding suggests that at higher concentrations QT124 functions by inhibiting intracellular development. While treatment with 40 nM and 83 nM QT124 does not decrease the number of free merozoites. This suggests that the decrease in successful invasion rate observed when parasites are treated with 40 nM and 83 nM QT124 (Figure 13A) is not due to a reduction in the number of released merozoites and supports our hypothesis that at certain concentrations QT124 is primarily working by inhibiting the invasion of merozoites into RBCs.
Figure 13. The effect of QT124 on parasitemia and merozoite counts

*P. falciparum* parasite cultures synchronized at the ring stage of development were treated with various concentrations of QT124 (40 nM, 80 nM, 664 nM) on day 1 and sampled daily over 4 days; control conditions include uninfected RBCs, untreated cultures, sulfated cyclodextrin-treated cultures, and chloroquine-treated cultures. Panel A: parasitemia counts were generated by visual examination of Giemsa-stained blood smears. Panel B: counts of free merozoites per 1000 RBCs on day 3 of the experiment were generated using flow cytometry. Panel C: photographs are representative images of Giemsa-stained parasite cultures in different treatment conditions on days 2, 3, and 4.
**QT124 inhibits parasite replication in vivo:**

While we have shown that a monovalent imidazolium compound is capable of having antimalarial activity in an *in vivo* mouse model (Section 2), seeing the improved potency and specificity of our bivalent imidazolium and triazolium compounds in *P. falciparum* culture we hypothesized that they may also achieve improved activity in mice. Figure 14 contains the results of our *in vivo* mouse trial, comparing average parasitemias of the different treatment groups over the course of the infection. Treatment with QT124 was clearly effective at suppressing parasitemia, an effect that was found to be statistically significant with *p*=0.008. Although not as pronounced, treatment with QT109 also slows down the rise in parasitemia (*p*=0.034). While, treatment with QT119 trends towards a suppression of parasitemia but is not found to be statistically significant (*p*=0.38); it is possible that an effect is not detected due to the high variability in the parasitemia of the untreated controls. QT109, QT119 and QT124 were chosen to be tested because they all possess the qualities we predict are important to successful inhibitors: they are bivalent structures with positively charged rings separated by 12-13 carbon chains. QT109 has two positively charged imidazolium rings decorated with methyl groups separated by a 13 carbon chain. QT119 has two positively charged imidazolium rings decorated with propyl groups separated by a 12 carbon chain. And QT124 has two positively charged triazolium rings decorated with methyl groups separated by a 12 carbon chain. The ability of these compounds to suppress parasitemia, especially that of QT124, supports our hypothesis that bivalent compounds with positively charged rings would display antimalarial activity *in vivo*.

In addition, since none of the uninfected controls showed signs of illness this indicates that on their own our compounds are not toxic at the concentrations being used. During our mouse trial we observed that although both QT124 and QT109 helped suppress parasitemia, they
had very different effects on the apparent health of the mice. While infected QT124-treated mice looked healthier than infected controls, infected QT109-treated mice looked sicker than infected controls. As mentioned before, uninfected mice receiving QT109 looked healthy. We speculate that although QT109 does not seem to have any adverse effects in healthy mice it may have adverse effects on infected mice by negatively affecting their immune system. The best test of health is usually weight, which is data we do not have.
Figure 14. Bivalent compounds are effective at suppressing parasitemia *in vivo*

Groups of 5 female Balb/c mice were infected with $10^6$ *P. berghei* parasites i.p. and the parasitemia was estimated daily by examining a Giemsa-stained blood film. Once parasites were observed mice were infused once daily with 1% (v/v) DMSO in RPMI 1640 (●), with 60 ug/day of QT109 (▼), 9 ug/day of QT119 (▼), 7.5 ug/day of QT124 (■), or 450 ug/day of chloroquine (○) for three days, as indicated by the arrows. Average parasitemias are shown with standard error of the mean for each experimental group indicated by bars.
SECTION 3.5 - DISCUSSION

We have previously reported that the key to the antiplasmodial activity of monovalent tetrazolium and imidazolium compounds lies in their positively charged rings and hydrophobic side groups (Cui et al., 2008; Vlahakis et al., 2010). Compounds containing these properties are selectively active against cultures of *P. falciparum* over cultures of CHO cells. Our comparison of the antiplasmodial activity of bivalent imidazole and imidazolium compounds has demonstrated that positively charged rings are also important in the selective action of bivalent compounds. In fact, some of our bivalent compounds exhibited greater potency than our previously studied monovalent compounds. In addition to studying the effect of charge on antiplasmodial activity, we also examined the effect of alkyl spacer length. We found that the activity of bivalent imidazolium compounds with alkyl spacers comprised of 8 or less carbons was comparable to that of the monovalent imidazolium compounds. However, alkyl spacers of 10 or more carbons greatly increased the potency and selectivity of these bivalent compounds. It is possible that bivalent compounds are more effective than monovalent compounds because bivalent compounds are able to simultaneously bind to two ligands, increasing the avidity of the molecule for its parasite ligand. In addition, in the current compound series, it appears that the length of the hydrophobic spacer between two positively charged rings is more important than the identity of the spacer itself. It is possible that the hydrophobic portion of the compound plays a role in the interaction with the plasma membrane.

Our parasite development assay suggests that QT124 is able to inhibit merozoite invasion into RBCs without affecting intracellular maturation. Visual inspection of cultures treated with 40 nM and 83 nM QT124 reveals a number of parasites that are adherent to the membrane of RBCs following a reinvasion event; this suggests that QT124 is able to inhibit invasion without
interfering with the parasite’s ability to bind the RBC membrane. The most interesting finding is perhaps the observation that although there appears to be an increase in parasitemia following a reinvasion event when parasites are treated with 40 nM QT124, none of these parasites are able to mature. As it can be difficult to differentiate between intracellular and extracellular parasites using light microscopy, one possible explanation is that all of the observed ring forms are actually extracellular and thus cannot mature properly outside of the RBC. Although some may argue that we are inhibiting parasite maturation, the observation that parasites are able to mature from ring forms to mature forms from day 1 to day 2 is inconsistent with this explanation. Instead, our results support our hypothesis that the compounds are interfering with a specific ligand receptor interaction that is necessary for successful invasion. Flow cytometry analysis was subsequently carried out to examine if the decreased invasion rates following QT124 treatment were due to a reduction in the number of released merozoites. Our findings indicate that at the lower concentrations, e.g. treatment with 40 nM QT124 and 83 nM QT124, the decrease in invasion is not due to a reduction in free merozoites. This finding is consistent with QT124 having its effect at a step following merozoite release and prior to merozoite invasion.

The final class of electron deficient ring structure is pyridinium salts and their activities were not examined in this thesis. However, a study carried out by Yoshikawa et al. (2008) examining the antiplasmodial effect of pyridinium dicationic compounds lends support to our hypothesis that our bivalent imidazolium and triazolium compounds are negatively affecting merozoite invasion into the RBC. Pyridiniums are structurally related to imidazoliums, triazoliums, and tetrazoliums (instead of two, three, or four nitrogens in the ring, pyridiniums contain one nitrogen in their ring) and they retain the positive charge in their ring that appears to be key for antiplasmodial activity. Similar to our findings with bivalent imidazolium and
triazolium compounds, Yoshikawa’s group reports that their bivalent pyridinium compounds also exhibit antiparasitic effects at a point between the schizont and ring stage (Yoshikawa et al., 2008).

We hypothesize that our imidazolium and triazolium compounds are binding to a conserved parasite ligand that is involved in invasion. If our compounds are inhibiting the initial attachment step of invasion, they may be interacting with MSP-1, or MSP-9, both of which contain negatively charged components that may be binding to our positively charged compounds. As we have previously shown that tetrazolium compounds bind negatively charged sulfated glycans, it is possible that our triazolium and imidazolium compounds are interacting with the GPI anchor on MSP-1, which contains phosphorylated glycans. If our compounds bind MSP-9 they may be interacting with the glutamate rich, and thus negatively charged, regions of this protein. Alternatively, our compounds may be inhibiting the reorientation step of invasion, in which case they may be binding to AMA-1. Mitchell et al. (2004) have shown that treatment with antibodies to AMA-1 inhibits parasite entry into RBCs without affecting the tethering of the merozoite to the RBC membrane. Thus, the observation that parasite cultures treated with a bivalent triazolium compound display lower rates of invasion and parasites bound to the RBC surface is consistent with the hypothesis that our compounds are interfering with the role of AMA-1. Although it is possible to speculate regarding potential targets, at this point in our investigations, we do not yet know if the parasite component being affected is involved in either the initial attachment step of invasion, the reorientation step, the formation of tight junctions, or the actin-myosin motor that propels merozoites inside the RBC. One potential strategy to identify the target may be to add radioisotope labels to our compounds and examine what they bind to.
Based on the findings we have presented we conclude that bivalent imidazolium and triazolium compounds are capable of inhibiting parasite multiplication both in vitro and in vivo through a novel mechanism of action that may involve the inhibition of erythrocyte invasion. One of the weaknesses of the current state of our knowledgebase of antimalarial drugs is that we are still unaware of how many of the currently used antimalarials are actually working (Grimberg et al., 2009). Since RBC invasion is a necessary step in the life cycle of Plasmodium parasites, we can take advantage of this by developing drugs that specifically target merozoite invasion. To date, there is still much that we do not know about this process (Gaur et al., 2004). However, our compounds appear to be interfering with invasion, thus, through the continued investigation into the mechanism of action of these compounds we are potentially one step closer to both a new class of antimalarials as well as a better understanding of Plasmodium parasite biology.
PART IV

CONCLUSIONS AND FUTURE DIRECTIONS
SECTION 4.1 - STUDYING MEROZOITE INVASION

One of the aims of the present body of work was to determine which stage of the parasite’s life cycle is being affected by our imidazolium and triazolium compounds. Our findings suggest that depending on the concentration used the compounds appear to either inhibit intraerythrocytic development or merozoite invasion into RBCs. As there are currently no therapies to target the invasion step of the *Plasmodium* life cycle we are most interested in the anti-invasion capabilities of these compounds. Thus, I have outlined a set of future experiments that would allow us to gain a better understanding of the effect of our inhibitors.

*Isolating viable merozoites:*

Although we hypothesize that our compounds inhibit invasion of merozoites into RBCs by interfering with a parasite ligand-host receptor interaction, there exists the possibility that treatment with our compounds is resulting in the production of abnormal merozoites that are unable to invade. In order to test the hypothesis that our compounds are inhibiting merozoite invasion at a step following the release of merozoites from infected RBCs, I would carry out the following experiment: I would isolate free merozoites, incubate them with the test compounds and normal RBCs, and then compare the invasion rates of treated and untreated merozoites. Until recently attempts to isolate viable *P. falciparum* merozoites had been unsuccessful (Narum et al., 2008). *P. falciparum* merozoites quickly lose their invasiveness, with a half-life of about 5 minutes at 37°C and 15 minutes at room temperature (Boyle et al., 2010). However, a newly published technique by Boyle et al. (2010) describes a way to isolate viable merozoites that involves the selective use of protease inhibitors, which presumably prevents schizont rupture and merozoite release. This new technique may revolutionize the study of *P. falciparum* merozoite
invasion by making it easier to examine the molecules and mechanisms involved in the process of invasion in addition to facilitating the testing of potential invasion inhibitors.

**MSP-1 antibodies and flow cytometry:**

Although our current flow cytometry experiments which are designed to measure the effect of our compounds on merozoites are carried out using SYBR Green I to label parasites, it would also be useful to confirm our results using an MSP-1 antibody that labels merozoites. This would allow us to verify that the putative merozoite population is truly composed of merozoites, and it may allow us to use flow cytometry to differentiate between infected RBCs and RBCs with merozoites that are stuck to their surface. Although I did carry out immunocytochemistry and flow cytometry using a polyclonal rabbit antibody to MSP-1 (AbCAM) in conjunction with a fluorescent secondary antibody to stain concentrated merozoite samples, I did not detect any fluorescence. Thus, we must find an MSP-1 antibody that will allow us to detect merozoites.

**Lipophilic probe experiments:**

When examining our compound-treated cultures to determine parasitemia following a reinvasion event, it can be difficult to differentiate between intracellular and extracellular parasites. An approach that may facilitate the distinction of parasites that are on the outside of the RBC from parasites that have successfully invaded is to label RBCs with a lipophilic probe, label parasites with a DNA stain, and then examine the cultures using confocal microscopy following one reinvasion event (Ward et al., 1994). Infected RBCs should stain positive for DNA (of parasite origin) in addition to showing positive staining for the lipophilic probe within the RBC, as the parasite will have incorporated parts of the RBC membrane into its parasitophorous vacuole membrane. On the other hand, RBCs containing parasites adhered to their surface are not expected to show any staining for the lipophilic probe within the RBC.
**Electron microscope examination:**

Since we hypothesize that our compounds are inhibiting invasion at a time following schizont rupture, future experiments could be carried out to investigate which part of the multi-step invasion process may possibly be affected. One approach would be to examine the adherence of treated merozoites to RBCs using an electron microscope, as was done by Mitchell et al. (2004) in order to investigate the role of AMA-1 in merozoite invasion. At present it is possible to predict three possible outcomes of such an experiment: 1) merozoites bound to the RBC surface by their non-apical end; 2) merozoites bound to the RBC by their apical end but lacking the formation of tight junctions; and 3) merozoites bound to the RBC by their apical end with the presence of tight junctions. Observation of 1 would be consistent with our compounds affecting invasion prior to the formation of tight junctions, observation of 2 would be consistent with our compounds inhibiting the formation of tight junctions, and observation of 3 would be consistent with our compounds inhibiting the function of the actin-myosin motor.
SECTION 4.2 – IN VIVO STUDIES

Varying the treatment regime:

Our in vivo studies carried out to date have shown that imidazolium and triazolium compounds are capable of suppressing parasitemia in a mouse model of malaria (Sections 1 and 2). Although treatment with our compounds has not resulted in the cure of infected mice, the observation that compounds retain their antiparasitic activity in a living system suggests that it may be possible to see improved outcomes by changing the treatment regime we employ. For instance, we begin treatment the day parasites become visible in the blood, however other in vivo studies of antiplasmodial compounds begin treating mice the day following infection. Such was the case with the in vivo studies of the bis-thiazolium compounds and these compounds did result in the cure of mice infected with Plasmodium (Caldarelli et al., 2010). In the future we may begin administration of the compounds the day following the infection of mice with the parasite, or even prior to infection in order to assess the use of these compounds in prophylactic treatment. We may also choose to extend our course of therapy from three days to one week or to increase the frequency of the daily injections. Since we are appear to be targeting an event that occurs for a brief period every 24 hours we require that the parasites be continuously exposed to effective concentrations of the compound.

Survival studies:

We could also carry out studies to examine if treatment with our compounds offers mice a survival advantage. This could be done using a model of malaria in which we infect C57BL/6 mice with P. berghei. These mice are susceptible to cerebral malaria and thus they would allow us to determine if treatment with our compounds could potentially protect against this severe form of the disease and result in increased survival.
**Combination therapies:**

Furthermore, we could also test our compounds in combination therapy. Combination therapy is now preferred over monotherapies as it delays the spread of drug resistance. The key to the advantage of combination therapy is that the probability of the parasite developing mutations that confer resistance to multiple drugs is smaller than the chance of the parasite developing a mutation to the individual drugs (White, 1998). We should thus assess for potential synergistic effects between our compounds and established antimalarials.
SECTION 4.3 – FROM LEAD COMPOUNDS TO DRUG DEVELOPMENT

Although our compounds demonstrate both in vitro and in vivo antiparasitic activity, it is important to note that the road to the development of successful therapeutics is long and full of obstacles. To start, we will have to test if our compounds have favorable absorption, distribution, metabolism, and elimination (ADME) properties that will allow them to become effective drugs. For instance, drugs should preferably be bioavailable when taken orally, as this will make it practical for individuals to use outside of the hospital. Poor ADME properties often prevent the development of lead compounds into drugs (Butina et al., 2002). For example, the thiazolium compounds described previously were not capable of being developed into successful drugs as they were not bioavailable (Personal communication with Ian Bathhurst-head scientist at Medicines for Malaria Venture).

Another important consideration in the development of new antimalarials is their cost. Since malaria is a disease that disproportionately affects individuals living in developing countries we need to ensure that we are focusing our efforts on the generation of treatments that will actually be affordable to those who need them the most. One of the highlights of our compounds is that imidazolium and triazolium salts are not expensive to produce. An additional benefit is that there are no known stereo isomers resulting from chiral centers in the current series of imidazolium and triazolium compounds; this is an advantage as sometimes isomers can have different activities, including higher levels of toxicity.

As mentioned previously, we are in need of antimalarials that use novel mechanisms of action to kill the offending pathogen. We report here that our compounds interfere with parasite replication and that they may be inhibiting the invasion step into RBCs, a stage of the parasite life cycle that is not currently targeted by existing antimalarials. Through our rational drug
development approach we have selected for compounds with increased antiparasitic potency and specificity. The optimization of our inhibitors is now complete. The next step is to take our lead compounds and develop them into potent drugs.
PART V

REFERENCES


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