THE P2X7-PANNEXIN COMPLEX IS INVOLVED IN PLASMODIUM FALCIPARUM MEROZOITE EGRESS FROM ERYTHROCYTES

A Dissertation in Integrative Biosciences

by

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ABSTRACT

The malaria parasite is a devastating pathogen, killing nearly a million people annually. Attempts to prevent the spread of this pathogen are hindered by its emerging resistance to chemotherapeutic drugs. Thus efforts to combat malaria require an aggressive and continuing search for new or improved approaches to limit the growth and spread of the parasite. Successful intervention mandates that we fully investigate the complex mechanisms that permit the parasite to successfully infect, develop, multiply, and escape from its host.

Like many intracellular pathogens, the process of parasite egress from the infected host cell is of utmost importance to Plasmodium falciparum. This thesis reviews the players involved in P. falciparum’s escape from infected erythrocytes and investigates potential mediators of membrane pore-formation that initiate the end stages of the escape process. I hypothesize that membrane pore formation, an important step in the escape program, occurs through a P2X7-pannexin-mediated event. Based on the results of the experimental work performed, I propose an egress model that explains how P. falciparum might initiate pore formation during merozoite egress from erythrocytes.
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine DiPhosphate</td>
</tr>
<tr>
<td>ADPr</td>
<td>ADP ribosylation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic ADP Ribose</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine MonoPhosphate</td>
</tr>
<tr>
<td>CBEV</td>
<td>Curling, Buckling, Eversion, Vesiculation</td>
</tr>
<tr>
<td>CBX</td>
<td>CurBenoXolone</td>
</tr>
<tr>
<td>CDPK</td>
<td>Calcium Dependent Protein Kinase</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanosine MonoPhosphate</td>
</tr>
<tr>
<td>CSP</td>
<td>CircumSporozoite Protein</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DAG</td>
<td>DiAcylGlycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>DiMethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>DPAP</td>
<td>DiPeptidyl Amino Peptidases</td>
</tr>
<tr>
<td>ECP</td>
<td>Egress Cysteine Protease protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylyl Cyclase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>HC</td>
<td>Host Cell</td>
</tr>
<tr>
<td>HCM</td>
<td>Host Cell Membrane</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitor Concentration</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triPhosphate</td>
</tr>
<tr>
<td>KD</td>
<td>Knock Down</td>
</tr>
<tr>
<td>KO</td>
<td>Knock Out</td>
</tr>
<tr>
<td>LISP</td>
<td>Liver Specific Protein</td>
</tr>
<tr>
<td>MAC/PF</td>
<td>Membrane Attack Complex/PerForin</td>
</tr>
<tr>
<td>MAEBL</td>
<td>MAalaria Erythrocyte Binding Ligand</td>
</tr>
<tr>
<td>MAOP</td>
<td>Membrane Attack Ookinite Protein</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>MilliLiter</td>
</tr>
<tr>
<td>MQ</td>
<td>MefloQuine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MS</td>
<td>Merozoite Stage</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite Surface Protein</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleotid Cyclases</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>PBCD</td>
<td>ProBeneCID</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PDE</td>
<td>PhosphoDiEsterase</td>
</tr>
<tr>
<td>PFA</td>
<td>ParaFormAldehyde</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphoinositol 4,5-bisPhosphate</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein Kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipid C</td>
</tr>
<tr>
<td>Pnx</td>
<td>Pannexin</td>
</tr>
<tr>
<td>PLP</td>
<td>Perforin-Like Protein</td>
</tr>
<tr>
<td>PPLP</td>
<td>Plasmodium Perforin-Like Protein</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous Vacuole</td>
</tr>
<tr>
<td>PVM</td>
<td>Parasitophorous Vacuolar Membrane</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RBCm</td>
<td>Red Blood Cell membrane</td>
</tr>
<tr>
<td>RFI</td>
<td>Relative Fluorescent Intensity</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RS</td>
<td>Ring Stage</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SERA</td>
<td>Serine Repeat Antigen</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3</td>
</tr>
<tr>
<td>SPECT</td>
<td>Sporozoite Protein Essential for Cell Traversal</td>
</tr>
<tr>
<td>SS</td>
<td>Schizont Stage</td>
</tr>
<tr>
<td>SUB</td>
<td>SUBtilisin-like protease protein</td>
</tr>
<tr>
<td>TC</td>
<td>Tissue Culture</td>
</tr>
<tr>
<td>Tg</td>
<td>Toxoplasma gondii</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombospondin Related Anonymous Protein</td>
</tr>
<tr>
<td>TS</td>
<td>Trophozoite Stage</td>
</tr>
<tr>
<td>ul</td>
<td>microliter</td>
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</table>
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Thank you all and I sincerely hope for the opportunity to assist others with their dreams and goals in life, and pay forward those positive experiences that each of you have shared with me.
Chapter 1

*Plasmodium falciparum*: lifecycle and components

of the egress process

Review of *Plasmodium falciparum* lifecycle

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Their phylum is apicomplexa, a member of the alveolate superphylum that includes the animal pathogens (*Toxoplasma*, *Cryptosporidium*, *Theileria*, *Eimeria*, and *Babesia*), the free living ciliate protozoa (*Paramecium* and *Tetrahymena*) and the dinoflagellates.¹ There are more than 120 malaria parasite species that infect many types of vertebrates ranging from birds (*P. relicturn*, *P. gallinaceum*, *P. durum*, *P. lophurum*, and *P. catemerium*) to reptiles (*P. agamae* and *P. lacertiliae*) and amphibians (*P. bufonis* and *P. catesbiana*) to mammals. In all cases, the female Anopheles mosquito is the obligatory vector. In vertebrate hosts and mosquito vectors, the parasite undergoes asexual and sexual multiplications, respectively. Five species of the parasite can infect humans – those are *P. vivax* (the causative agent of tertian malaria), *P. malariae* (quartan malaria), *P. falciparum* (falciparum malaria), *P. ovale* (ovale malaria), and *P. knowlesi*. The latter, while specific to monkeys, has recently been adapted to infect humans.²³ Of these, *P. falciparum* is the most virulent and is responsible for most of the deaths due to malaria, especially in Africa. On the other hand, *P. vivax* is the most prevalent yet relatively rarely fatal.
It is found in almost all tropical and subtropical regions of the world, particularly Southeast Asia, the Indian subcontinent, the Middle East, and South America.

The malaria parasite faces numerous challenges during its growth and development in both the sexual and asexual stages of its lifecycle. As a gametocyte in the vertebrate blood, it must survive the harsh conditions of the mosquito midgut and quickly differentiate, mate and develop into a zygote. To escape this toxic environment rich in digestive enzymes, the motile ookinete must enter the midgut epithelium and avoid the rapidly responding innate immune system. This is achieved in part through oocyst formation. Once released from the oocyst the sporozoites migrates through the haemocel to invade the mosquito salivary glands, in preparation for injection into the mammalian host. Following its escape from its mosquito host, the differentiated sporozoite must adapt to changes in the environment of the mammalian host. The parasite develops from sporozoite to merozoite (and in some instances gametocyte) and adapts to unique environments ranging from the dermis to the liver and blood. Among the most daunting task during these transitions is avoidance of both the innate and adaptive immune systems. At each step in their lifecycle following nuclear expansion or development, the parasite must escape from its host-derived membrane shelter. Failure to escape is a death sentence from which there is no reprieve.

**Gametocytogenesis and sporogony**

All malaria parasite species have similar life cycles both in the vertebrate and mosquito vector, henceforth, the following description is confined to *P. falciparum.*
In order to survive and propagate, *P. falciparum* must return from the vertebrate host to the mosquito vector for completion of its life cycle. This phase, also known as the sexual cycle, begins with the uptake of differentiated male and female gametocytes within the blood meal from hosts. (Figure 1.1)

**Figure 1.1 Plasmodium falciparum lifecycle**
Illustration showing the sexual and asexual life stages of *Plasmodium falciparum*. Mosquitoes inject sporozoites which migrate to the liver (exoerythrocytic phase) where the parasites undergo sporogony, developing into merozoites. Merozoites escape the liver to infect erythrocytes (erythrocytic phase). Both phases are considered part of the asexual stage. Once in the erythrocytic phase the parasite cycles from ring stage to trophozoite stage to schizont stage and finally back to the free merozoite stage. Most merozoites will repeat this cycle but a small fraction will differentiate into male and female gametocytes, remaining in the G₀ stage until they are taken up by the female mosquito. After uptake by the mosquito, the gametocytes with develop into gametes and mate (sexual reproduction). The resulting zygotes will differentiate into an ookinete, cross the epithelial lining of the mosquito midgut and bud into an oocysts. Oocysts develop into sporozoites (sporogenesis), escape from the oocysts, and migrate to the mosquito salivary glands in preparation for entering into the mammalian host. Access into the mammalian host is gained when the mosquito injects it probiscus into the host to draw a blood meal. Saliva from the mosquito, which contains the parasite in sporozoites form, enters the host.
Following male exflagellation, the gametes mate in the insect’s mid-gut to form a zygote that eventually develops into an ookinete. Roughly 0.2% of the total parasites successfully reach this developmental stage.

The ookinete penetrates the peritrophic matrix and midgut epithelium, budding from the basal lamina of the midgut epithelium into an oocyst. (Figure 1.2)

![Figure 1.2 P. falciparum escape from mosquito basal lamina](image)

Illustration of an oocyst budding from the mosquito basal lamina lining of the midgut epithelium. The details of the egress process from the oocyst are unknown but thought to involve proteases, kinases, and perforin-like molecules. Diagram from Friedrich et al., 2012.

The oocyst wall is composed of a two-layered structure, an inner wall of parasite origin and an outer wall derived from the basal lamina of the insect midgut epithelium. The budding process, named sporogenesis, is defined by sporoblast partitioning to develop thousands of sporozoites enclosed by the basal lamina. From 2% to 20% of the ookinetes successfully reach the mature oocyst stage. The oocyst ruptures, releasing thousands of sporozoites into the mosquito
Unlike egress from hepatocytes and erythrocytes, oocyst rupture does not arise from previously invaded cells, but rather from an ex-vagination of the migut epithelium’s basal lamina. Little is known about oocyst rupture, but *P. falciparum* perforin-like proteins (PPLP 3 and 5) and egress cysteine protease 1 (ECP1), are thought to play a role in the process. Knock-out (KO) lines of ECP1 exhibited no phenotype during early sporogony or blood stages but sporozoites failed to egress from oocysts. The released sporozoites migrate through the haemocoel towards the mosquito’s salivary glands. Invasion of mosquito salivary glands involves the parasite circumsporozoite protein (CSP), thrombospondin-related anonymous protein (TRAP), and membrane antigen/erythrocyte binding-like protein (MAEBEL), which assists in sporozoite development, motility, salivary gland recognition and invasion. These proteins are excreted by the parasite’s microneme and/or rhoptry organelle.

**Exoerythrocytic Liver Stage**

To enter the vertebrate host, mosquitoes inject their probiscus into the skin to obtain a blood meal and in the process roughly 35% of the injected sporozoites gain access to the blood stream. (Figure 1.3)
Sporozoites entering the blood through injection by the mosquito mark the beginning of the exoerythrocytic phase, one of two phases in the asexual stage. Secreted pore-forming proteins and proteases are important for sporozoite gliding motion and cell traversal in the dermis. These processes also allow the parasite to escape the dermis and enter the bloodstream en-route to the liver. After exiting the dermis, sporozoites enter the blood circulation and migrate to the liver. They traverse the liver sinusoid to access hepatocytes. The liver sinusoid is a fenestrated blood
vessel through which the oxygen-rich hepatic artery and nutrient-rich portal vein flow. The sinusoidal barrier is composed of fenestrated endothelial cells and Kupffer cells and is separated from hepatocytes by the space of Disse. Current thinking holds that sporozoites invade, traverse, and exit Kupffer cells, entering the space of Disse through fenestrations in the endothelial cell layer. Pore forming proteins called perforins are involved in this process. Sporozoites freely migrate through several hepatocytes before taking up residence within a hepatocyte and beginning the liver stage growth. The process, from dermis to hepatocytes, has commonly been reported to take minutes. Recent findings however challenge those reports, indicating that it can take anywhere from one to three hours.

The parasite develops inside the hepatocyte and cytokinesis is completed before daughter cell formation takes place. Upon completion of development, parasitophorous vacuolar membrane (PVM) breakdown involves serine repeat antigen (SERA) proteins and liver specific protein (LISP) 1, a protein of unknown function. The host mitochondria disintegrates, and the host membrane is reorganized to become the merosome’s limiting membrane, filled with individual parasites called merozoites. (Figure 1.4)
Figure 1.4  Merosome egress from hepatocyte
Illustration of a merosome budding out from a dying hepatocyte. The mechanism of the egress process is unknown, but is thought to involve proteases, kinases, and perforin molecules. The merosome will leave the hepatocyte and migrate through the heart towards the lungs where the limiting membrane will disintegrate to release infectious merozoites into the blood stream. Diagram from Graewe et al., 2012.

The mechanism by which this occurs is currently unclear, but it is thought to involve proteases. The hepatocyte death process shares features with autophagy in that phosphatidyl serine is not switched, caspases are not involved, and DNA is unprocessed.\textsuperscript{15} Calcium (Ca\textsuperscript{2+}) is released by the host, but sequestered by the parasite to prevent apoptosis. Death of the infected hepatocyte occurs by a process similar to oncosis, a programmed form of caspase-independent necrosis.\textsuperscript{16}

The merosomes are released into the sinusoidal lumen, pass through the heart and accumulate in the lungs, where they break-up, releasing from 4,000 to 30,000 merozoites per merosome into the bloodstream of the pulmonary capillaries.\textsuperscript{10,15} The mechanism of merozoite egress from merosomes is unknown. However, not excluding an active process, researchers speculate that it may result from the sheer stress of blood flow through the lung capillaries and heart.\textsuperscript{5}
Completion of the exoerythrocytic phase marks the first egress event in the asexual stage of the parasite’s lifecycle.

**Erythrocytic Stage**

Once in the bloodstream the merozoites are infectious and will begin the next phase of their journey inside the host erythrocyte. They attach, re-orient and invade red blood cells (RBCs). The erythrocytic phase can cycle indefinitely within the mammalian host by progressing unidirectionally through stages morphologically distinguished as the mononucleated ring and trophozoite stages, the multinucleated schizont stage, and the infectious merozoite stage. (Figure 1.5)
Figure 1.5 Erythrocytic cycle
Illustration of events following escape from liver hepatocytes. Merozoites will continue to cycle almost indefinitely from merozoite (free infectious form) to ring stage (newly invaded parasite), to trophozoite stage (mononucleated mid-stage), to schizont stage (multinucleated mature stage). Egress from schizont-enclosed RBCs releases infectious merozoites for re-infection of erythrocytes. The egress process, the focus of this study, is preceded by pore-formation and thought to involve perforin molecules. Diagram from Winzeler et al., 2008.

Merozoites egress from schizonts following completion of schizont development and re-invade uninfected RBCs to form rings. During *P. falciparum* egress, roughly 10 to 30 merozoites are released per schizont every 48 hrs.

During late trophozoite development, a small fraction of zoites avoid schizont development and the recurring erythrocytic stage. They instead develop into gametocytes in preparation for gametocytogenesis and sexual reproduction inside the mosquito midgut. The gametocyte is the only stage that can initiate infection inside the mosquito host. Like the merozoite, the gametocyte lies within the parasitophorous vacuole (PV) and is shielded from the erythrocyte cytoplasm by the PV membrane (PVM). Gametocyte maturation is classified into five morphological stages (stages I–V), and gender specificity is established in late trophozoite/early schizont stages. Gametocytogenesis begins with the uptake of gametes by the female mosquito during feeding. External stimuli (e.g. temperature drop, xanthurenic acid, pH changes) activates sexual reproduction of the parasite inside the mosquito midgut. Prior to activation, both sexes of gametocytes are arrested in the G0 phase of the cell cycle. Once activated, genome replication and nuclear division only occur in the male gametocytes. Up-regulation of translationally silent mRNAs is observed in female gametocytes. In preparation for sexual reproduction, both male
and female gametocytes must egress from the RBC membrane (RBCm). Failure to escape the host membrane leads to parasite death, a common theme throughout the parasite’s lifecycle.

**Components of the egress process**

To continue propagation, the parasite must escape from both the PVM and RBCm. Therefore, prevention of the egress process halts the logarithmic growth of the malaria parasite. Thus understanding the order of membrane destabilization and the proteases involved was of great interest to researchers. Morphological investigations describe egress as occurring in a sequence starting with curling and buckling of the host cell membrane (HCM) to eversion and vesiculation. (Figure 1.6)
Figure 1.6 The CBEV process
Illustration of events occurring after pore-formation but before merozoite release into the media or blood. This is considered the active process of RBC membrane rupture and release of merozoites. Curling, buckling, eversion and vesiculation (CBEV) describe the chronological events occurring to the RBC membrane as observed through microscope and video analysis. Diagram from Lew et al., 2011.
The process has been termed CBEV, representing each event, respectively. While documenting the above mentioned CBEV process, researchers noted that just prior to buckling an initial pore was formed. It has also been suggested that proteases and perforins were involved in the egress process. Evidence in support of poration came from observations during PVM swelling. Investigators noted that application of sealant P1107 during PVM swelling prevented pore formation, while protease inhibitors failed to prevent pore formation. They also noted that osmiophilic pressure did not cause pore formation since pores were observed in infected RBC that were not swollen. However, although perforins were suspected of causing the initial pore, it is still unclear if they are involved in parasite egress, invasion, or both.

**Proteases and egress**

Initial investigation of the egress process utilized protease inhibitors, with contradictory results as to whether the PVM or HCM was first degraded. Studies by Chandramohanadas et al. clarified the issue with construction of a GFP-expressing parasite line that localized to the PVM cytosol. By this time it was suspected that cysteine proteases targeted the RBCm. Identifying the specific protease and the order in which the membranes were degraded was the primary focus of egress investigations. In conjunction with membrane-permeable protease inhibitor E64d (cysteine protease inhibitor) and EGTA-AM (calcium chelator), they showed that the PVM degraded first, leaking GFP into the RBC cytosol. They noted that under these conditions, the RBCm was intact and without cytoskeleton reconfiguration. The results indicated that calcium and cysteine proteases are involved in RBCm breakdown, not the PVM breakdown, and that the PVM loses integrity before the RBCm. Based on these results as well as those from other researchers, they determined that *P. falciparum* egress from the RBC occurs via an inside-out
process. Further investigations to discover the protease(s) acting on the RBCm led researchers to the findings that although plasmepsin 1-4 (aspartic proteases) and falcipain 1-3 (cysteine proteases) could digest RBCm proteins, they were not major players in the egress process since KO of each did not affect the ability of the parasite to egress from the RBCm. In addition, the proteases of the digestive vacuole were not present in the PV or RBC cytoplasm before or during the egress process. Lastly, the parasite’s endogenous calpain, essential for cell cycle progression, was not found to participate in the egress process.

Attention focused on serine repeat antigen 5 (SERA5), one of nine SERA cysteine proteases in P. falciparum. Of the nine SERA proteases, only SERA 5 (ser type) and SERA-6 (cys type) are essential for parasite survival as shown by genetic knock-out studies. SERA is secreted into the PV as a 126 kDa protein during the trophozoite and schizont stages. Prior to egress, this protease is processed into 47kDa (p47), 50kDa, (p50), 18kDa (p18), and 6kDa (p6) fragments. Cleavage of p126 to yield the p47 and p18 fragments is sensitive to the serine protease inhibitor diisopropyl fluorophosphate yet resistant to many other protease inhibitors (serine, aspartic, cysteine and metalloprotease). Further processing of the remaining fragment, from p56 to p50, is sensitive to a range of cysteine protease inhibitors. Both serine and cysteine protease inhibitors effectively inhibit parasite egress from the RBCm. The p50 fragment contains the enzymatic activity of SERA5. Characterization of SERA5 showed that the prodomain (T391 to D577) of the p50 central domain (T391 to N828) fragment contained the regulatory activity for the enzyme domain (E578 to N828). Although Alam et al. (2012) were unable to conclude the prodomain’s mechanism of action (directly acting on enzyme or indirectly by sequestering its target), they did show in vitro that the prodomain and heptapeptide sequence arrested schizont
egress and that the heptapeptide sequence was able to bind the enzyme domain.\textsuperscript{42} Further investigation found that a serine protease (SUB1) processed SERA5 from the p126 protein down to the p47, p18 and p56 fragments.\textsuperscript{29} \textit{P. falciparum} SUB1 (PfSUB1) is synthesized as a precursor protein and secreted in vesicles from the exonemes. Inactive PfSUB1 is trafficked to vesicles containing a cysteine protease (DPAP3) and DPAP3 cleaves PfSUB1 for activation. Chemical inhibition of DPAP3 or PfSUB1 prevents parasite egress and gene deletion attempts were unsuccessful.\textsuperscript{31} Before the RBCm ruptures, mature PfSUB1 is released into the PV, where it directly mediates processing of SERA5.\textsuperscript{31} To date, the cysteine protease responsible for converting the p56 fragment to the p50 and p6 fragments remains unidentified, however it has been determined that SERA5 does not have autocatalytic activity. Speculative candidates, although not yet investigated, could be DPAP3 and human calpain 1. The combined evidence implicates SERA5 as a potential protease candidate involved in the parasite egress process, since mature SERA5 was required for the process to proceed. However, there is no evidence showing that SERA5 or p50 directly participates in PVM or RBCm degradation and the actual target of SERA5 has not been found. Evidence shows that invasion of erythrocytes by released merozoites involves proteins discharged from secretory organelles called micronemes and rhoptries, whose secretion is insensitive to E64\textsuperscript{43} but sensitive to serine protease inhibitors\textsuperscript{44}, implicating SUB1 in SERA5 processing and possibly in microneme/rhoptry secretion. To complicate matters further, researchers found that chemical inhibition of human calpain I prevents parasite egress and the action of this enzyme can directly degrade the RBCm protein matrix.\textsuperscript{38} Human RBCs contain soluble but inactive calpain I, which becomes membrane bound and active upon calcium binding.\textsuperscript{38} Although it is clear that proteases are necessary for egress, they are not sufficient.\textsuperscript{45} The implications are that proteases are required to destabilize the PVM and RBCm through their
degradative action, but such action is not sufficient to cause rupture of the membranes. They may also be required to activate the micronemes for secretion as well as proteins within the micronemes (MSP, perforin, etc.) in addition to their role in PVM and RBCm destabilization. Recent investigations have found that other factors (calcium, kinases, and potentially perforin proteins) are also required for egress.

**Perforins and egress**

In mammals, perforins are mainly associated with cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and have homology to complement proteins. They are stored inside cytotoxic granules with a group of serine proteases called granzymes. Perforins are synthesized in the endoplasmic reticulum (ER), proteolytically processed by cysteine proteases within the cytolytic granules, bind their inhibitor calreticulin at low pH, and become active at neutral pH. Upon activation, they bind phospholipids and multimerize in a calcium dependent manner, creating 5 to 20 nm diameter membrane pores. The mechanism of action is uncertain but it is hypothesized that perforins and proteases are endocytosed by the target cell. A rise in endosomal pH and calcium activates perforins and proteases within the endosome. Perforins form pores in the endosome to aid in the release of proteases from the endosome into the target cell’s cytoplasm. This highly regulated process is thought to help protect the CTL or NK cell from proteolytic damage.

In *T. gondii*, a protozoan similar to the malaria parasites, perforin-like protein1 (TgPLP1) rapidly compromises the integrity of membranes encasing the parasite and localizes to the micronemes.
Kafsack et al. (2009) were able to show that TgPLP1 was necessary for disruption of the PVM without affecting microneme secretion. (Figure 1.7)

Figure 1.7 T. gondii and P. falciparum schizont egress
Illustration depicting parasite egress from their host cell. Pacman-like figures represent proteases. Equal signs represent perforin molecules. In P. falciparum, there is evidence that perforin-like protein (PLP) molecules act on the PVM but evidence indicating that they additionally act on the RBCm is not apparent. In T. gondii, PLP molecules acting on the host plasma membrane is still under investigation. The egress mechanism is thought to be similar in both parasites. Diagram from Roiko et al., 2009.

TgPLP1 KO parasites were egress-deficient. In P. falciparum, five genes have been identified that encode membrane attack complex/pore forming (MAC/PF)-like domains and secretory sequences. P. falciparum perforin-like proteins (PPLP3, -4, and -5) are expressed in the ookinete. PPLP3 (also known as Membrane Attack Ookinete Protein or MAOP) and PPLP5 function in ookinete cell traversal of the mosquito midgut epithelium. PPLP3 localizes to the micronemes. According to Roiko et al. (2009) PPLP1, 3, and 5, function in cell traversal. However, cell traversal requires temporary membrane disruption which could be interpreted as prerequisite for either egress or invasion. PPLP1 (also known as Sporozoite Microneme Protein Essential for Cell Traversal 2 or SPECT2), is important for sporozoite cell traversal of epithelial cells in the dermis and liver hepatocytes. It is specifically expressed in salivary gland
The function of PPLP4 is unknown and gene deletions have been unsuccessful. Evidence suggests that PPLP1, 3, 4, and 5 function in invasion rather than egress. In this regard, whether these proteins assist with the entrance or exit from their traversed target remains to be clarified. The protein, PPLP2, is expressed in the gametocytes of P. berghei and P. falciparum and shown to be necessary for egress of the male gametocyte from the host erythrocyte. In P. berghei, PPLP2 is not detected in wild-type (WT) female gametocytes, and PPLP2 KO females were noted to have escaped the RBCm. In addition, when crossed with WT males, they were competent to form zygotes. This indicates that another process or unidentified protein plays a role in female gametocyte egress from RBCs. PPLP2 is expressed in male gametocytes and KO male gametocytes fail to egress. Although egress inhibition can be rescued for the PPLP2 KO male gametocytes using saponin or equinotoxin, there is no evidence that they are competent for invasion or reproduction. It is unknown if PPLP2 is required for the rescued-PPLP2 KO male gametocytes during invasion/mating with WT or PPLP2 KO female gametocytes. In P. falciparum, unlike P. berghei, PPLP2 is found in both male and female gametocytes.

Recently, transcripts of PPLP1, 2, 3, and 5 were detected in Plasmodium blood stages and PPLP1 and -2 proteins were detected by Western blotting. Immunofluorescent (IFL) experiments showed that pre-secretion, PPLP1 and 2 localized to the micronemes, and in late schizonts, after secretion, they were found to be associated with both the PVM and RBCm in a Ca\(^{2+}\)-dependent manner. However, these finding are in contrast with other transcript and fluorescent labeling studies. The identification of a perforin protein associated with schizont egress from RBCs remains to be confirmed, given the discrepancies. Since P. falciparum micronemes are necessary
for RBC invasion, it is possible that a schizont associated PPLP found in the merozoite microneme, like PPLP1 and 3, may function in RBC invasion. In this regard, further studies into the localization of PPLP1 and 2 and the fecundity of male KO parasites would be very insightful.

Kinases and egress

Although proteases are necessary but not sufficient for egress, and perforins may represent the factor causing pore formation that leads to rupture of the PVM and RBCm, it was noticed that kinase activity is required for the function of proteases and perforins in the egress process. To date, approximately 100 kinases have been identified in the *P. falciparum* genome. Of these, at least two kinases have been implicated in schizont egress; *P. falciparum* protein kinase G (PfPKG) and *P. falciparum* calcium-dependent kinase 5 (PfCDPK5). The signaling pathway for each kinase can be thought of as Ca\(^{2+}\)-dependent (CDPK) or Ca\(^{2+}\)-independent kinase (PKG). (Figure 1.8)
Figure 1.8 *P. falciparum* egress signaling pathway from RBCs
Schematic illustration showing the potential signaling pathway involved in parasite egress. External signals can activate three possible pathways, two of which are calcium-dependent. Pathway 1 involves receptor-mediated activation of ADPR cyclase. ADPR cyclase uses NAD to produce cADPR which binds ryanodine receptors for calcium secretion. Pathway 2 involves PLC-mediated cleavage of PIP into DAG and IP\(_3\). IP\(_3\) binds IP\(_3\)R for calcium release, while DAG binds PKC. PKC activation commences upon DAG and calcium binding (not shown). Pathway 3 involves receptor-mediated activation of guanylyl cyclase (GC) to produce cGMP. PKG binds GC directly for activation. Signals from both PKG and CDPK have been shown to activate microneme secretion for invasion or egress processes. Diagram from Billker *et al.*, 2009.

PKG signaling occurs through G\(_s\), G\(_i\), or G\(_q\), which are coupled to a receptor or channel through its G\(_\beta\)/G\(_\gamma\) subunits. G\(_s\) and G\(_i\) link the G-protein coupled receptor (GPCR) to adenylyl cyclase (AC) or guanylyl cyclase (GC) for cyclic nucleotide signaling (cAMP and cGMP) and Ca\(^{2+}\) secretion via ryanodine receptors.\(^{57,58}\) However in *P. falciparum* and *T. gondii*, PKG binds cGMP directly for kinase activation, abrogating the need for receptor-mediated Ca\(^{2+}\) secretion.\(^{59}\) G\(_q\), on the other-hand, links the GPCR to the PIP-PLC pathway, creating IP\(_3\) and DAG for Ca\(^{2+}\) secretion and PKC activation.\(^{58}\) PfCDPK5 relays Ca\(^{2+}\) signals to intracellular targets and like PfPKG, does not require GPCR signaling. PfCDPK is a member of a family of kinases originally discovered in plants. There are five PfCDPK genes (1 – 5) in *P. falciparum*.\(^{60}\) These calcium-dependent kinases are of special interest to researchers because they are not found in mammals, making them attractive therapeutic targets.\(^{61}\)

In *P. berghei*, there are four nucleotide cyclases (NC), four phosphodiesterases (PDE), and one PKG gene with orthologues in *P. falciparum*.\(^{62}\) However, the upstream receptors initiating cGMP intracellular signaling are unknown, and the GCs are membrane-bound rather than soluble.\(^{59}\) PfPKG itself was recently found to be soluble and cytosolic with low overlap
localization with the ER. In *T. gondii*, the microneme is at least one target of PKG, and TgPKG inhibition blocks microneme secretion as well as parasite egress. In *P. falciparum*, PfPKG participates in initiation of gametocytogenesis, ookinete development and motility, merosome release during the exoerythrocytic phase, and merozoite egress from RBCs. With regards to schizont/merozoite egress from RBCs, chemical inhibition of PfPKG with Compound 1, a trisubstituted pyrrole pyridine, blocked proteolytic processing of PfMSP1 without affecting PfSUB1 activity directly, indicating a block in microneme and/or exoneme secretion. This result is in contrast to Pf CDPK5 knockdown (KD) studies, where processing of PfMSP1, SERA5, and PfSUB1 was unaffected and PfSUB1 discharge and activity were normal. In addition, mechanically disrupted schizonts from PfCDPK5 KD mutants were viable while PfPKG chemically inhibited schizonts were not invasive following mechanical disruption. However, like PfPKG-inhibited parasites, PfCDPK5-inhibited parasites failed to egress. Interestingly, kinase inhibitor KT5926 inhibits microneme secretion in *P. falciparum* and has little reactivity against PfPKG. Although KT5926 was shown to target PfCDPK1 and its activity against PfCDPK5 is unknown, the findings indicate that microneme secretion is regulated by at least two different kinase pathways. *T. gondii* studies support the finding that at least two kinases regulate microneme secretion. Both TgPKG and TgCDPK1 are implicated in regulating the secretion of micronemes by independent pathways.

**Summary and Hypothesis**

To date, it is well established that proteases are necessary but not sufficient for the egress process. Proteases function to destabilize the PVM and RBCm and process/activate other key proteins involved in the process. Proteases do not directly cause the initial pore formation. Pore
formation theory is slowly gaining acceptance with increasing evidence that perforins may be key to initial pore formation. However, the precise role of perforins in *P. falciparum* biology is unclear. Do they function in egress, invasion, or both processes? What mechanisms govern their expression, processing, and activity? With regards to schizont egress from RBCs, the role of a perforin-like molecule has not been demonstrated even though their existence has been established in the other stages as well as in related organisms. Currently there is no evidence directly linking *P. falciparum* perforin-like proteins to pore-formation. This leaves open the possibility that an alternative mechanism of pore formation is required to facilitate parasite egress from RBCs during the schizont phase. This thesis, in part, describes an alternative hypothesis for schizont egress and provides evidence indicating that a P2X7-pannexin complex might fulfill this niche. Since synchronized *Plasmodium* cultures are required to study egress processes, this thesis also describes the development and application of a new methodology for obtaining a highly synchronized *P. falciparum* culture for the purpose of investigating late stage processes, such as egress.
Chapter 2

An alternative protocol for *P. falciparum* culture synchronization and a new method for synchrony confirmation

**Background**

The malaria parasite possesses a complex life cycle that can be separated into two main phases, the mosquito phase and the mammalian phase. Under appropriate conditions, the parasite cycles in the human erythrocyte from merozoite stage (MS) to ring stage (RS), trophozoite stage (TS), and schizont stage (SS).\(^{65,66}\) Failure of schizonts to egress from the erythrocytes or invade new ones, leads to parasite death.\(^{67}\) Although parasite egress and invasion are crucial for the parasite’s survival, the underlying mechanisms are not fully understood. Investigation of these events usually requires that the parasites within the culture are at equivalent developmental stages or “synchronized”.\(^{68}\) The degree of synchrony required can vary depending on the target under investigation and goals of the study.

One of the most common procedures used to obtain synchronized cultures is Percoll density centrifugation. The original isopycnic Percoll method, established in 1981 for *Plasmodium berghei* and 1983 for *P. falciparum*, was unable to tightly synchronize parasite stages because it could not separate late trophozoites and early schizonts from late schizonts.\(^{69-70}\) The sorbitol synchronization method, developed in 1979, relied on lysing trophozoites and schizonts while allowing for continued growth of the RS.\(^{71}\) Several rounds of sorbitol treatment were necessary and, even then, a tight synchronization was difficult to achieve by this procedure. To overcome these limitations, a combination of two methods was established in 1985 by Kutner *et al.*; the
isolation of matured parasites by centrifugation on Percoll cushions, and sorbitol treatment of schizont stages.\textsuperscript{72} They reasoned that sorbitol altered the buoyant density of mature stages due to increased expression of permeability pathways, allowing a higher degree of separation in a Percoll gradient. Recently, Radfar \textit{et al.} modified this protocol by alternating the Percoll and sorbitol treatments during specific stages to achieve tight synchronization at high parasitaemia.\textsuperscript{73}

The repeated Percoll/sorbitol treatments needed to synchronize a culture, as well as the difficulty in achieving tight synchronization following such treatments, prompted a search for a method that did not poison the culture, reasonably defined the zero-hour start point for RS, tightly synchronized the culture, and was easy and reproducible. Here, an alternative Percoll synchronization protocol is described and evidence is provided showing that the use of the SyBr Green I assay is valid for measuring RS parasites and culture synchrony.

\textbf{Experimental Procedures}

\textbf{Parasite culture and preparations}

Pf-RPMI solution was made from RPMI 1640 supplemented with 25 mM Hepes, 0.37 mM Hypoxanthine, 25 mM sodium bicarbonate, and gentamycin to 2.5 μg/ml. Plasmodium complete culture medium was composed of Pf-RPMI supplemented with 5 g/l Albumax II (Invitrogen). Both solutions were filter-sterilized before use. \textit{P. falciparum} (3D7 strain) was cultured in complete medium at 1% haematocrit at 37\textdegree C in a 5% CO\textsubscript{2}/3% O\textsubscript{2}/92% N\textsubscript{2} gas mixture as described previously.\textsuperscript{74} Human red blood cells (RBCs) were washed three times, stored at 4-8\textdegree C, and used within three weeks. The haematocrit was measured from a packed RBC volume that
was centrifuged in a swing-bucket rotor at 2,000-x g for 5 min at room temperature. Schizonts were isolated on Percoll cushions centrifuged at room temperature in a swing-bucket rotor at 2,000 x-g for 5 min. For RS parasite analysis, aliquots were layered on the top of 70% Percoll in 1.6 ml tubes and centrifuged at 4,000 x-g for 5 min. Pellets were washed once with RPMI 1640 medium and immediately frozen at -20°C until used.

Synchronization of parasite culture

For culture synchronization, parasites were cultured to at least 10% parasitaemia in T-75 flasks containing 50 ml medium at 1% haematocrit. When most of the parasites matured to SS, the culture was loaded on a 20% over 60% Percoll cushion and centrifuged for 5 min at 2,000 x-g. Schizonts from the top of 60% Percoll was collected (without washing) and re-suspended in complete medium containing 2% RBCs at a final Percoll concentration of less than 6%. This culture was incubated at 37°C for 3 hr in a 5% CO₂/3% O₂/92% N₂ gas mixture, then separated on a 20% over 70% Percoll cushions. The pellet contained newly infected RBCs or rings (S1), while the layer on top of 70% Percoll contained schizonts. The schizonts were returned to culture as above and the process was repeated two additional times, thereby producing additional batches of highly synchronized RS cultures (herein called S2 and S3). For a detailed protocol, see Figure 2.1.
**4hr-6hr Pf synchronization protocol**

### Semi-synchronization steps (T75 flask)

To grow culture to high parasitemia (10% or higher) and partially synchronous, proceed as follows:

1. Change media by aspirating at the 24hr-30hr stage (early to mid TS).
2. Isolate schizonts using 20%/60% percoll step-gradient (2x 15ml tubes).

**NOTE:** Do not wash schizonts and minimize recovered percoll volumes to less than 2mls.

3. Resuspend schizonts in 50mls complete media containing 1% RBCs.
4. Repeat as necessary until a semi-synchronized high parasitemia is obtained (10% or higher).

### Synchronization steps

5. Isolate schizonts (step 2), and resuspend them in 25mls complete media containing 2% RBCs.
6. Incubate for 3hrs (upright standing position), then run over a 20%/70% percoll step-gradient.
7. Save the 70% layer (schizonts) and the pellet (rings).
8. Schizonts: Repeat steps 5-7 for a total of three times (never wash or pellet schizonts).
9. Rings: wash rings with ~12mls-14mls RPMI and pellet.

### Optimization steps

10. Resuspend rings in 25mls complete media (pre-warmed).

**NOTE:** Document start-time and packed cell volume (PCV). Make smears and take 0.5ml aliquot. Adjust aliquot to 1% hematocrit, pellet, and freeze. Determine the RFI value of all 3 samples using the SyBr Green assay. All samples are 8hr synchronized but at different parasitemia.

11. Incubate for 7-9hrs, then run over a 40%/70% percoll step-gradient and save pellets.
12. Wash pellet with 12mls-14mls RPMI, pellet and resuspend in 50mls complete media.
13. Incubate 20hrs-24hrs, aspirate media, then replenish with 50mls complete media.

**NOTE:** Harvest 46hrs-48hrs from step 10 (documented start time) as follows:

14. Aspirate media leaving 18-20mls in the flask, then run over a 20%/60% percoll step-gradient.
15. Save schizonts, which are now 4-6hr synchronized. Test synchrony and/or use for experiments.

### Additional NOTES:

- 80% Percoll may be used in place of 70% Percoll for slightly higher RS purity
- All spins are 2000 x G for 6min @ RT in a swinging bucket rotor.
- All percoll volumes during extraction are <2mls.
- All incubations are in CO₂ incubator @ 37°C and media is warmed to 37°C before use.
- Never wash schizonts and keep recovered percoll volumes to a minimum (i.e. less than 5% V/V).

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**Figure 2.1 Synchronization protocol**

A detailed description of the synchronization protocol developed and used in this study.

To determine the degree of synchrony for these parasite batches (S1, S2, or S3) or for use in egress and invasion experiments, the synchronized RS cultures must develop into schizonts. Therefore each synchronized RS pellet isolated above was re-suspended in 20 ml complete medium and the time was documented as start time (equals 0 hr). The RS sample was then incubated for 8 hr as above, pelleted by overlaying onto a 40% over 70% Percoll cushions, and
washed with RPMI 1640. This important Percoll step removes trophozoites that may have escaped the initial separation process. To ensure that the high parasitaemia culture is not depleted of nutrients, pellets were re-suspended in 50 ml of complete medium, incubated for 20 hr, then aspirated (not pelleted) and replaced with 50 ml of fresh complete medium. The incubation continued such that the total incubation time from the above-mentioned documentation time was 48 hr (i.e. another 20 hr). At harvest the synchronized schizonts were re-isolated using a 20% over 70% Percoll cushions and used immediately.

A standard Percoll/sorbitol synchronization method was also used for comparison. Briefly, schizonts were isolated over a 40% over 60% Percoll cushions and allowed to infect RBCs for a period of 6 to 12 hr. The resulting RS were subjected to 5% sorbitol treatment for 5 min at 37°C. This process was repeated two to three times. Following the final sorbitol treatment of schizonts, the culture was washed and re-suspended in complete medium containing 1% RBC. Parasites not treated with sorbitol were also cultured as a control for comparison (herein described as mock culture or MS1).

Confirmation of culture synchrony
To determine the degree of culture synchrony, schizonts were isolated from a 20% over 60% Percoll cushions, re-suspended in at least 20 volumes of complete medium at 1% haematocrit, then incubated at 37°C. One ml aliquots were removed at 0, 3, 6, 9 hr and each was overlaid onto a 70% Percoll cushion (0.5 ml) and centrifuged to recover RS parasites in the pellets. The pellets were washed with RPMI 1640 and frozen for storage. They were later thawed and processed for SyBr Green I analysis. For the detailed protocol, see Figure 2.2.
Confirmation of culture synchrony

1. Isolate SS over a 20%/60% percoll step-gradient, minimizing percoll recovery.
2. Resuspend isolate in at least 20 volumes of pre-warmed complete media containing 1% RBCs, and incubate at 37°C in CO2 incubator.
3. Remove 1ml aliquots every 2hrs-3hrs starting from 0hrs and ending @ 9hrs-12hrs.
4. Run 0.5ml aliquots over 70% percoll (0.5mls) in 1.6ml eppendorf tubes and spin @ 4000 x G for 6min at rt.
5. Wash RS pellet with 1.5mls of RPMI and freeze for later analysis.
6. When ready to process, thaw samples by resuspending in SyBr Green resuspension buffer.
7. Process via the SyBr Green detection method and analyze results.

Figure 2.2 Synchrony confirmation protocol
A detailed description of the synchrony validation method used for this study.

SyBr Green assay

The SyBr Green I assay was carried out as previously described with the following modifications\textsuperscript{75-81}: all parasite pellets obtained from 0.5 ml culture aliquots were thawed in 0.5 ml re-suspension buffer (25 mM Tris, pH 7.5, 10 mM EDTA, 20 μg/ml RNase A), making the final haematocrit of 1%. Aliquots of 100 μl were mixed with 100 μl of ice-cold SyBr Green I solution (25 mM Tris, pH 7.5, 10 mM EDTA, 0.01% saponin, 0.1% Triton X-100, 0.2 μl/ml SYBR Green I) in a 96-well black plate (Corning #3916). The plate was incubated at room temperature for 1 hr on a rotating platform. The relative fluorescent intensity (RFI) readings were measured (excitation 485 nm/emission 520 nm) using a Fluostar Optima Microplate Reader (BMG Labtech).

Results and Discussion

Parasite synchronization by Percoll density centrifugation
The synchronization method described here takes advantage of Percoll’s ability to fractionate the RS from matured parasites. When parasite cultures are centrifuged on 60% Percoll cushions, the SS population is retained on the top layer and used to initiate new infections. Purified SS vary in maturity, and the time of merozoite egress from infected RBCs (iRBCs) could last up to 9 hr following the initial Percoll isolation. Thus, by repeating the above process additional times at defined intervals, most of the released merozoites are captured as synchronized rings in subsequent isolations.

The time window for the incubation can range from 1 hr to several hours, depending on the desired degree of synchrony. It should be noted that reducing the time interval, substantially decreases the resulting parasitemia of the synchronized culture. A 3-hr incubation time interval was used on schizonts to illustrate this principle (Figure 2.3).
Figure 2.3  Conceptual rationale: alternative synchronization method
Theoretical outline depicting the progress of a synchronized *Plasmodium* culture. The illustration depicts a 3hr period for each set of Percoll captured RS. The long arrows point from the culture status (i.e. bell shaped graph) towards time (i.e. 0 hr, 3 hr, 6hr, etc.), denoting the development of the culture. As time progresses, the synchronized culture will develop from TS to SS then RS. The beginning of lysis marks time 0 hr for this illustration. At 3 hr, the fraction of SS that develop into RS is noted as S1. This S1 fraction is collected by 70% Percoll centrifugation. By 6 hr, the fraction of SS that develops into RS is noted as S2 and is subsequently harvested by 70% Percoll centrifugation. The last fraction of SS to develop into RS is S3 and it occurs at 9 hr. Each fraction (S1 through S3) has its own 0 hr start time which is 3 hr after its predecessor. Each fraction will begin the egress/invasion process 48 hr from the time it is harvested. Each fraction is 4-6 hr synchronized with fraction S2 having the highest parasitemia (Figure 2.4).
For cultures used in these experiments, the second fraction (3 to 6 hr) had the highest number of rings and resulted in the highest parasitemia (Figure 2.4).

**Figure 2.4 Parasite concentration from 3hr-synchronized cultures**
Cultures were synchronized as detailed in the Methods section. Aliquots of each synchronized 3 hr period (S1, S2, and S3) were taken to determine which preparation possessed the highest parasite concentration as measured by the SyBr Green assay. S1 represent the 0-3 hr aliquot. S2 and S3 represent the 3-6 hr and 6-9 hr aliquots respectively. Results are averages from two independent cultures each ran in triplicate. RFU, relative fluorescence unit.

Parasites isolated as outlined above maintained a highly synchronous 48-hr developmental cycle and began the egress/invasion process at 48-hr from the time they were harvested.

In addition to the ease of application, another attractive advantage of this method is that the initial capture of schizonts (20% over 60% Percoll cushions) needs only to occur at an approximate time to the onset of egress. For example, if the initial schizont capture is too early (i.e. mid-stage schizonts rather than late-stage schizonts), a S1 population of rings (0 to 3 hr) might not be recovered within the 3-hr egress/invasion period. Therefore, the recovered parasitaemia
may be too low for use in experiments. However, subsequent schizont isolations will produce synchronized rings populations (i.e. S2 at 3 to 6 hr, and S3 at 6 to 9 hr) each having a defined 0 hr start time and at higher parasitaemia.

Confirmation of culture synchronization

To determine the degree of synchrony, a SyBr Green based assay was adopted. First, periods within the parasite’s lifecycle when the relative fluorescent intensity (RFI) values did not increase during the culture proceedings were identified. This allowed for the determination of which parasite stages could be quantified using the SyBr Green assay. Earlier studies showed that parasite DNA replication does not occur until early TS, (i.e. ~28 hr after parasite invasion).\textsuperscript{82} Measurements from SyBr Green I fluorescence showed that RFI did not increase until at least 24 hr after invasion, which corresponds to RS (Figure 2.5 and Figure 2.6). The lack of an RFI increase during ring development justifies the use of the SyBr Green I for both detection and quantification of RS parasites. Incubation of the lysates with RNase A showed that RNA did not have any noticeable effect on the RFI values.
Figure 2.5  **SyBr Green analysis of parasite lifecycle**
The culture was initiated by incubating mature schizonts with RBCs in a complete medium at 1% haematocrit. Aliquots were taken at the indicated time points (0 to -94 hr) and centrifuged on 40% Percoll cushions to remove haemozoin and cell debris. Cell pellets were then used in the SyBr Green I assay either with or without RNase A treatment to determine the effects of RNA transcripts on relative fluorescence intensity (RFI) readings. Three biological replicates were run in triplicate and averaged, and the standard deviations calculated.

![Graph showing RFI over time with RNase A treatment](image)

**Figure 2.6  SyBr Green lifecycle analysis: the alternative Percoll method**
This figure is similar to Figure 2.5 except the culture was synchronized using the Percoll synchronization method and we assayed for Total DNA versus Live Cell DNA rather than cells.

![Graph showing RFU over time for Total DNA and Live cell DNA](image)
with or without RNase A. Synchronized 3D7 schizonts were isolated and allowed to infect uRBCs. Culture was run over a 40% and 60% Percoll cushion to recover only schizonts. Schizonts were washed with RPMI, pelleted, then resuspended in complete media containing 1% RBCs and returned to culture. At the indicated time points following egress, aliquots were either harvested with (live cell DNA) or without (total DNA) a 40% Percoll centrifugation. Collected aliquots were frozen and after thawing, a SyBr Green assay was performed.

Next, to verify culture synchrony, late SS from synchronized cultures were purified and incubated with fresh RBCs. Aliquots were removed every 3 hr and centrifuged over a 70% Percoll cushion. The resulting pellets, which contained newly formed RS parasites, were used in the SyBr Green I assay. The RFI increase of the pelleted aliquots correlates with an increase in newly formed RS, indicating egress and invasion has occurred. Likewise, once egress and invasion ceases, the RFI values will stop increasing because new rings are not formed. The period of time it takes for the RFI values to stop increasing determines the degree of culture synchrony. Figure 2.7 confirms the synchrony of a culture synchronized using the method described here.

Figure 2.7  Synchrony confirmation from Percoll synchronization method
The synchrony of two S2 aliquots (3 to -6 hr, see also Figure 4) was confirmed by incubating the RS samples in complete media until they reached SS. The 0 hr denotes the calculated start of egress/invasion. The matured SS parasites were isolated over a 20%/60% Percoll cushion at the calculated egress time (S2-2) or 3 hr before the calculated egress time (S2-1) and re-suspended in complete media containing 1% RBC. Aliquots were taken (0.5 ml) at the indicated time points and ran over a 70% Percoll cushion, washed, and frozen. Samples were later re-suspended to a 1% haematocrit using resuspension buffer (0.5 ml) and 0.1 ml aliquots were assayed in triplicate using the SyBr Green assay format.

Aliquots from preparation S2-2 (Figure 2.7) were taken at the start of the estimated egress time. Aliquots from preparation S2-1 were taken 3 hr before the onset of egress. Notice the delay in the onset of egress when aliquots were removed 3 hr before the start of the egress period. The delay indicates that the detection method is functioning properly. Rings are not formed since egress has not occurred, thus no RFI increase is observed. Figure 2.7 illustrates how the SyBr Green I method can determine the synchrony of two cultures. Both cultures (S2-1 and S2-2 preparations) are synchronized within a range of 4 to 6 hr. There was no significant increase in RFI readings prior to 0 hr and after 6 hr (S2-1) or before 3 hr and after 9 hr (S2-2). Although preparation S2-2 shows a slight RFI increase during the 0 to 3 hr period, this increase was compared to the average increase from 0 to 9 hr using a one-tailed t-test, assuming unequal variances. The average RFI increase from 0 to 3 hr was negligible compared to the RFI increase from 0 to 9 hr ($P = 0.0002$), indicating a 4 to 6 hr synchrony period. This procedure can be used to verify the synchrony of any culture regardless of synchronization method.

Comparing synchronization procedures

Figure 2.8 shows the comparison for the degrees of synchrony between Percoll synchronized samples (S1: 0 to 3 hr and S2: 3 to 6 hr) to that obtained from: (1) unsynchronized schizonts isolated from a one-step procedure of 20% over 60% Percoll cushion (US1); (2) a culture
synchronized by the Percoll/sorbitol treatment method (JS1); and (3) a mock synchronized control culture treated similar to JS1 above except without sorbitol treatment (MS1).

Figure 2.8 Synchrony comparison of culture synchronization methods
For JS1, parasites were synchronized as outlined in Miao et al. [10]. MS1 parasites were treated similarly to JS1 except sorbitol treatment was omitted. S1 and S2 preparations represent two fractions of the Percoll synchronization method described here. For the unsynchronized culture (US1), schizonts were isolated from a 20% over 60% Percoll cushion. Following synchronization, schizonts were cultured as previously described in 1% haematocrit, and aliquots were taken every 3 hr (starting at 0 hr up to 12 hr). Samples were frozen, thawed, and subjected to SyBr Green I assay. RFI units are listed on the left of the graph. All data points indicate average and standard deviations from triplicate samples.

The results show that the unsynchronized parasites isolated on 60% Percoll (US1) continued its RFI increase beyond 9 hr. In contrast, the first two preparations (S1 and S2) were synchronized within the range of 4 to 6 hr. The Percoll/sorbitol synchronized culture produced a 9 hr synchrony period (JS1), while the mock culture (MS1) closely mimicked preparations S1 and S2. These results indicate that sorbitol treatment may not actually improve culture synchrony.
**Conclusion**

An alternative method for the synchronization of *P. falciparum* cultures was developed, which demonstrates that Percoll density centrifugation alone is sufficient to synchronize the parasite cultures. Furthermore, procedures were established that tests the degree of culture synchrony. This procedure is objective, easy to perform, and can validate the degree of synchrony regardless of synchronization method employed. These two techniques will be used to study the late-stage developmental processes associated with *P. falciparum* egress, as detailed in chapter 3.
Chapter 3

The P2X7-pannexin complex is involved in the egress of *Plasmodium falciparum* merozoites from erythrocytes.

**Background**

The development of the blood-stage malaria parasites during their 48 hr life cycle involves four distinct morphological stages: ring, trophozoite, schizont, and merozoite. During invasion of the red blood cell (RBC) merozoites create a vacuolar membrane barrier and reside inside the vacuole within the erythrocyte’s cytoplasm. At this stage, parasites inside the parasitophorous vacuolar membrane (PVM) appear as rings, develop into trophozoites, and then differentiate into schizonts inside the PVM by producing daughter merozoites. (Figure 3.1).

![Figure 3.1 Erythrocytic stages and merozoite organelle localization](image)

**Figure 3.1 Erythrocytic stages and merozoite organelle localization**
Illustration detailing the erythrocytic stages and the apical location of organelles in *Plasmodium falciparum* merozoites.

For propagation, the daughter merozoites must egress from their host RBCs and invade new RBCs. Failure to complete either of these two processes results in parasite death. Therefore
egress and invasion are crucial for parasite survival. The mechanisms underlying these processes are complex and not fully understood.

Reports indicate that increases in calcium (Ca\(^{2+}\)) concentration are crucial to the \textit{P. falciparum} egress process. Ca\(^{2+}\) regulates the activities of a diverse set of parasite and host molecules involved in egress such as the cysteine protease calpain I, the calcium-dependent protein kinases CDPK, and the membrane attack complex/pore-forming molecule perforin.\(^{84-86}\) Ca\(^{2+}\) is also thought to regulate the secretion of molecules from the micronemes and exonemes, all housed within the parasite and necessary for egress and invasion.\(^{87-89}\) Gazarini detailed the Ca\(^{2+}\) concentration gradients within the infected RBC, showing that Ca\(^{2+}\) in the parasite cytoplasm (low nM) and RBC cytoplasm (~100 nM) were roughly 10- to 1000-fold lower than that in the parasitophorous vacuole (PV) cytosol (low \(\mu\text{M}\)), which is ~1000-fold lower than the extracellular Ca\(^{2+}\) concentration (low mM).\(^{90}\) These findings show that upon infection, a calcium gradient is established between the PV and parasite cytosol that differ roughly 1000 fold. (Figure 3.2).
In addition, several intracellular Ca\(^{2+}\) stores have been identified (i.e. endoplasmic reticulum (ER), food vacuole, mitochondrion, acidocalcisomes).\(^9\) The established Ca\(^{2+}\) gradient and intracellular Ca\(^{2+}\) storage facilities allow the parasite to regulate numerous functions necessary for its survival, including egress from the host RBC.\(^8\)

The mechanism of *P. falciparum* egress from RBCs during the schizont phase is unclear. It is known that both parasite and host proteases are required, but not sufficient.\(^8\) Proteases are
required for degrading proteins associated with the PVM and RBC membrane (RBCm) in preparation for escape. They may also be needed for proteolytic activation of other molecules involved in the process. Evidence indicates that a key step of the egress process is pore-formation.\textsuperscript{92,93} Once the limiting membranes have been weakened by proteases, PV swelling and RBC crenation follows.\textsuperscript{92} Shortly thereafter, pore-formation marks the physical start of egress, which is rapidly followed by the curling, buckling, eversion, and vesiculation (CBEV) process.\textsuperscript{93} Recently, Glushakova \textit{et al.} presented evidence indicating that secretion of intracellular calcium stored in the parasite’s ER is sufficient to support egress.\textsuperscript{94} Based on their findings, they concluded that 2 hrs prior to egress, the events leading up to pore-formation are dependent upon intracellular rather than extracellular Ca\textsuperscript{2+}. Although their work establishes a model wherein the parasite regulates egress through calcium secretions from intracellular stores, it is unclear how a key element, pore-formation, is initiated. Current theories indicate that perforins are directly involved in pore formation.\textsuperscript{95} Perforins depend on calcium to bind membranes and signaling molecules, and to self-aggregate.\textsuperscript{96} They are pH dependent, processed by proteases, regulated by kinases, and shown to create 100Å pores in membranes upon activation.\textsuperscript{96} With regards to \textit{P. falciparum}, inactive perforins localize to the micronemes, an organelle strongly associated with parasite invasion.\textsuperscript{97-100} The role of perforins in \textit{P. falciparum} has not yet been fully characterized. Therefore some confusion exists as to whether they are involved with egress, invasion, or both processes. More importantly, a PPLP-like molecule associated with schizont egress has yet to be fully characterized. Several important yet unaddressed questions concerning the role of perforins in \textit{P. falciparum} remain, such as: 1) How the parasite secretes an active pore-forming molecule that will not attack its own plasma membrane? 2) Is the perforin molecule activated in the cytosol or inside a membrane? 3) What are the activation requirements? 4) Does it associate
with the PVM, RBCm or both? 5) How is it targeted to these limiting membranes? Until these questions are addressed and the PPLP molecules are fully characterized, the search for factor(s) that establish pore-formation should continue. In this study, an alternative model of pore-formation for *P. falciparum* schizont egress from RBCs is hypothesized. We contend that the P2X7-pannexin complex mediates pore-formation, resulting in parasite egress from RBCs.

P2X7 is a purinergic receptor expressed on RBCs. Purinergic receptors are a growing family of plasma membrane molecules classified as the P1 and P2 purinergic receptors. The terms receptor and channel are used interchangeably when referring to purinergic molecules. P1 receptors are G protein-coupled receptors that bind adenosine and whose family consists of the A1, A2a, A2b, and A3 adenosine receptors. To date, only the P1-A2b has been found and studied in RBCs. It was shown to participate in regulatory volume decrease in the mature erythrocyte. The P2 receptors bind nucleotides and are divided into two categories, P2Y and P2X. P2Y receptors are also G protein-coupled seven-pass transmembrane channels. The P2X receptors are heteromeric (with exception of the homomeric P2X7) ATP-gated nonselective ion channels with cytoplasmic N- and C-termini and two transmembrane domains. These receptor types are also known as metabotropic and ionotropic receptors respectively. Currently, there are eight known P2Y receptors (P2Y1-2, 4, 6, 11-14) and seven P2X receptors (P2X1-7). P2 receptors can be found on cells of both hematopoietic and non-hematopoietic origin, participating in numerous cellular events associated with inflammation, hypoxia, apoptosis, Alzheimer’s disease, cancer and microbial infections. In RBCs, mRNA for P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-13, P2X1, P2X4, and P2X7 has been observed, however only the proteins for P2Y1, P2Y13, P2X1, P2X2, P2X4 and P2X7 have been detected.
The human P2X7 gene encodes a 595 amino acid protein and is composed of 13 exons, with exon 12 and 13 coding for the C-terminal tail of this molecule.\textsuperscript{113} It localizes to chromosome 12q24 and is methylated at CpG motifs located within the gene. This type of epigenetic regulation is thought to govern its tissue-specific expression, as well as its reduced expression in cancer cells.\textsuperscript{114,115} P2X7 is N-linked glycosylated on 5 residues (residues 187, 202, 213, 241, and 284) and mutations in N187 result in reduced activity due to altered trafficking.\textsuperscript{116} The N-terminal (27aa) and C-terminal (250aa) ends are cytoplasmic, with the C-terminus participating in signaling processes and pore-formation.\textsuperscript{117} The proline rich region (residues 441 – 460) can bind proteins containing a Src homology 3 (SH3) domain and the C-terminal has been reported to bind laminin, actin, integrins, PI4 kinase, and receptor protein phosphatase.\textsuperscript{118,119} The receptor also possesses several potential phosphorylation sites. Only Y343 and Y550 exhibited reduced pore formation when mutated.\textsuperscript{119} Deletion of the C-terminal tail and point mutations at P451L or E496A of P2X7 also prevents pore-formation.\textsuperscript{113,120-124} (Figure 3.3).
Figure 3.3 P2X7 schematic diagram
Schematic diagram of P2X7 taken from Adriouch 2008. Conserved arginine residues (yellow diamonds), cysteine residues (red circles), and potential glycosylation sites (green triangles).

The P2X7 channel regulates cation fluxes, such as calcium and sodium, and is activated by NAD and ATP via independent and distinct mechanisms. The extracellular ATP binding domain of P2X7 spans amino acids 47-329 and includes 10 conserved cysteine residues thought to engage in intrachain disulfide bonding. The extracellular modification site for adenosine
diphosphate ribosylation (ADPr) is at R125. Unlike other P2X receptors, P2X7 is unique in that it possesses a long C-terminal tail, is homomeric, has greater affinity for Bz-ATP over ATP, shows selectivity for divalent cations over monovalent cations, and participates in large pore-formation (900 Da) upon prolonged stimulation. Brief P2X7 stimulation opens the channel, but does not activate pore formation. While the C-terminal tail is necessary for pore-formation, it is not sufficient, as determined by P2X2 chimeric studies.

Pannexin hemi-channels are required for P2X7-mediated pore-formation. Pannexins are nonjunctional hemichannels with four transmembrane regions, two extracellular loop domains each containing two Cys residues, and one cytoplasmic loop domain as well as cytoplasmic N- and C-terminal domains. It is a hexameric protein that is assembled and trafficked through the ER/Golgi network prior to plasma membrane localization in a wide variety of cell types. There are 3 pannexin genes (Pnx1-3) in the human genome all exhibiting gene and tissue-specific expression. Pannexin 2 is associated with the brain and pannexin 3 is found predominately in the skin and connective tissues. Pannexin 1 has ubiquitous distribution. Pannexins are associated with initiation/propagation of Ca\(^{2+}\) waves, vasodilation, and signal transduction, and participate in biological functions such as tumorigenesis, Ca\(^{2+}\) homeostasis, and immune responses. Of particular interest for this investigation, pannexins complex with the P2X7 receptor and upon prolonged receptor stimulation can form a pore large enough for a 900 Da molecule to pass. Equally important is the finding that pannexins are activated by intracellular Ca\(^{2+}\) and can release ATP or NAD upon activation. Like P2X7, inhibition of Pannexins prevent pore-formation following prolonged P2X7 channel activation. In a microglial point-mutation model, P2X7 ion channel conductance and pore formation were shown
to be separable. The report by Iglesias et al. (2008) further indicates that a Src tyrosine kinase phosphorylates the Pannexin channel following P2X7 activation, an intermediate step in the pore-formation mechanism. Interestingly, it was recently found that a calcium-dependent protein kinase (PfCDPK5) regulates P. falciparum egress from erythrocytes. It is currently unknown if PfCDPK5 targets Pannexins and/or is activated by P2X7 conductance.

Although it remains to be determined which process - channel opening, pore formation, or both - is key to parasite escape from erythrocytes, investigations into the cytolytic nature of P2X7 have established that both processes require receptor activation by either ATP or NAD. Most importantly, RBCs possess both P2X7 receptors and Pannexin hemi-channels. RBCs do not possess connexin channels. Because P2X7 and Pannexin are linked to pore-formation, inhibition of either should inhibit pore-formation in a P. falciparum egress model. Therefore, if pore-formation from the P2X7-pannexin complex is involved in egress, then inhibition of the complex, or of either component, should also prevent parasite egress.

Materials and Methods

Test compounds and media

Table 3.1 contains a chart of test compounds, their concentrations, the suppliers used in this investigation, and literature references. All stock solutions were made according to the manufacturer’s recommendations.
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Table 3.1 Study compounds
List of the drugs/chemicals used in this study, to include abbreviated names, inhibitor concentrations, their supplier, and literature references.

Pf-RPMI solution was made from RPMI 1640 supplemented with 25 mM Hepes, 0.37 mM Hypoxanthine, 25 mM sodium bicarbonate, and gentamycin to 2.5 µg/ml. Plasmodium complete culture medium was composed of Pf-RPMI supplemented with 5 g/l Albumax II (Invitrogen). Both solutions were filter-sterilized before use.
Parasite culture, synchronization, and synchrony confirmation

*P. falciparum* 3D7 parasites were cultured in a complete medium at 1% hematocrit at 37°C in a 5% CO₂/3% O₂/balanced N₂ gas mixture, herein described as standard conditions. Parasite synchronization and synchrony confirmation were performed as reported earlier.

Assessment of parasitemia by light microscopy

Culture aliquots (0.5 ml) were pelleted over a 40% Percoll cushion, washed once with Pf-RPMI, and re-pelleted. Slides were prepared by resuspending the pelleted aliquots in approximately 20 µl Pf-RPMI, smearing 2–5 µl onto slides, and air drying. Dried slides were then fixed for roughly one minute in methanol and transferred into a 5% to 10% Giemsa stain solution (pH 7.1) for 5 min at room temperature (RT). Finally, slides were rinsed briefly with water, quick dried, and viewed using a light microscope with a 100x oil immersion lens.

**SyBr Green I assay**

The SyBr Green I assay was carried out as previously described. Briefly, frozen parasite pellets obtained from culture aliquots were thawed in re-suspension buffer (25 mM Tris, pH 7.5, 10 mM EDTA, 20 µg/ml RNase A), to make the final hematocrit at 1%. Aliquots were mixed with ice cold SyBr Green I solution (25 mM Tris, pH 7.5, 10 mM EDTA, 0.01% saponin, 0.1% Triton X-100, 0.2 µl/ml SYBR Green I) in a 96-well black plate (Corning #3916). The plate was incubated at RT for 1 hr on a rotating platform. The relative fluorescent intensity (RFI) readings were measured (excitation 485 nm/emission 520 nm) using a Fluostar Optima Microplate Reader (BMG Labtech).
Drug titration assay

Drugs were resuspended in phosphate buffer solution (PBS) or dimethyl sulfoxide (DMSO) per manufacturer’s instructions. Working stocks and aliquots were made in Pf-RPMI and frozen. *P. falciparum* cultures were grown to a high density parasitemia (at least 10%) and synchronized according to the method of Childs et al. When ready, schizont stage parasites were isolated and re-suspended in complete media containing 2% freshly washed RBCs.

Literature searches provided the highest drug concentration used in any experiment and ~100 times this concentration was used as the starting concentration. Drugs were serially 1:2 diluted into a 48-well tissue culture plate using complete media as the diluent. Half a milliliter of the isolated schizont suspension, prepared as above, was aliquoted into each well of the 48-well culture plate containing 0.5 ml of serially diluted drug. Drug was omitted from the last well of each series to serve as a control. Plates were incubated for 10 hr at 37°C (standard conditions). Parasites from the wells were harvested by gently recovering each culture and placing it onto the top of 0.5 ml of a 70% Percoll cushion in 1.6 ml Eppendorf tubes. Tubes were spun at 5000 x g for 5 min at RT to recover any potential rings. Each pellet was washed once with 1 ml Pf-RPMI, re-pelleted, frozen, and then thawed for SyBr Green I analysis. Experiments are averages of at least three biological replicates unless otherwise noted.

Egress inhibition assay

The assay was performed similar to the procedure used for drug titrations except the drug concentrations used were adjusted to 1 - 3x the calculated IC\textsubscript{100} value. Briefly, highly synchronized schizonts at high parasitemia were isolated and re-suspended in 10 ml complete
media containing 1% RBCs and the selected drug. The T25 flasks were then incubated in the standing position under standard conditions. To process, 1 ml aliquots were removed at the designated time for up to 10-12 hr, layered on top of 0.5 ml of a 70% Percoll cushion, pelleted, washed with Pf-RPMI, re-pelleted, and frozen as indicated in the drug titration assay. SyBr Green I analysis was performed to quantify ring parasites. Giemsa stain analysis was performed to confirm egress inhibition.

**Egress inhibition rescue assay**

The rescue assay was performed similar to the egress inhibition assay except that 1.4 ml aliquots of the cultures were incubated in 1.6 ml Eppendorf tubes for 2 - 4 hr and then gently pelleted (200 x g for 2 min at RT) to remove the test compound. Pellets were resuspended, without washing, in 1.4 ml complete media and returned to the incubator, lid-side down. After completion of the 10 - 12 hr total incubation time (i.e. 6 - 10 hr additional incubation), processing followed the procedure for the egress inhibition assay. SyBr Green I and Giemsa stain analyses were performed as outlined above.

**Invasion assay**

RBCs were incubated in Pf-RPMI containing the drug of interest for 2 hr at RT on a rotating platform. Prior to use, they were pelleted and resuspended in complete media (without washing) to a 2% hematocrit. Schizonts were isolated from a 60% Percoll cushion, washed briefly with Pf-RPMI, and resuspended in complete media. An equal volume of compound-treated RBC suspension (2% hematocrit) and schizont culture was mixed such that the final hematocrit was 1%. Cultures were incubated using standard conditions. At completion, aliquots were processed
according to the egress inhibition assay and then subjected to the SyBr Green I and Giemsa stain analysis to quantify rings and verify parasite invasion.

**Drug analysis software and statistics**

Graphpad Prism5 and Microsoft Excel were used to plot the graphs and calculate the IC values. One-way ANOVA was performed on the Egress Inhibition experiments with Tukey’s Multiple Comparison Tests. Two-way ANOVA was performed on the Rescue from Egress Inhibition experiments with Bonferroni posttests.

**Results and Discussion**

**Drug Titrations and Egress Inhibition**

**Analysis of the controls**

The amount of drug required to inhibit parasite egress was determined by modifying the standard *P. falciparum* drug titration assay to a 10 - 12 hr incubation period at the late schizont stages. Normally, this technique is applied for ~72 hr to the whole schizogony cycle, beginning at the ring stage. The ability of the modified approach to determine the IC values of compounds known to inhibit parasite egress was initially tested. The “known inhibitor controls” were the calcium sequesters EDTA and EGTA; the protease inhibitors leupeptin, calpain, E64, and E64d; and the kinase inhibitor Staurosporine. The tyrosine-specific kinase inhibitor PP2 was also included since it has been shown to prevent the P2X7-mediated pore-formation of pannexin, a process not investigated in *P. falciparum.*
Inhibition charts given in Figure 3.4 show egress inhibition by the known controls and established the IC values necessary for further investigations.

![Inhibition charts](image)

**Figure 3.4 Compound Titration plots of ‘known’ inhibitor controls**

Titration results of ‘known’ chemical inhibitors at various concentrations were incubated with mature synchronized schizonts for ~12 hrs under standard conditions, then harvested and processed as outline in the Materials and Methods. Protease inhibitors were leupeptin, calpain inhibitor I, E64, and E64d. Kinase inhibitors were Kn-62, PP2, and staurosporine. The calcium chelators were EDTA and EGTA.

The results were repeated at the adjusted IC\textsubscript{100} values and the results were displayed as relative fluorescent intensity values (Figure 3.5).
Figure 3.5  Egress inhibition chart
Synchronized late-stage schizonts were used and incubated for 12 hr then processed. The “no drug control” (C+ 0hrs) indicates relative parasitemia before drug addition under the indicated processing conditions. Test compounds and the “no drug control (C+12hrs)”, indicates relative parasitemia after 12 hr incubation using similar processing conditions. RFI is the Relative Fluorescent Intensity obtained from SyBr Green I Recordings. Drug abbreviations, IC values, and drug references are outlined in Table 3.1. Three biological replicates were each run at least in duplicate. Graphs represent average readings with standard deviations.

RFI units were later converted into “percent of control” (POC) using the chart from Figure 3.6. Figure 3.6 was constructed using 1:2 serial dilutions of ring stage parasites in a 1% hematocrit. OD$_{520}$ readings were recorded and a best fit line from 12.5% to 100% produced a $R^2$ value of 0.98. The chart also shows that the POC results and their corresponding standard deviations are within the 95% confidence band (dotted lines). For easier conversion, Table 3.2 shows pre-selected RFI values along the “best fit” line of Figure 3.6 and their corresponding POC values.
Synchronized late-stage schizonts were serially diluted (1:2) from 100% to roughly 0.1% and allowed to infect RBCs at a 1% hematocrit for 10hrs under standard culture conditions. After incubation, ring stage parasites were harvested by centrifugation over a 40%/70% Percoll step-cushion. Ring parasites were washed with RPMI and assayed using the SyBr Green I assay as previously described. Only the first four dilutions (100% to 12.5%) were used to produce the above chart as this produced a line with the highest R-squared value and covered the range of values for the experimental data. The chart represents averages from five biological replicates and was constructed using the PRISM software.
Table 3.2 RFI/POC conversion table

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Table values were PRISM software generated and represent calculations of RFI values for each given POC value taken from Figure 3.5A.
Conversion of the RFI values into POC values was necessary for statistical analysis (ANOVA with Tukey or Bonferroni post-test) of the data since RFI values are not linear. The converted RFI values for Figure 3.5 are shown in Figure 3.7 as POC values.

![Figure 3.7](image)

**Figure 3.7  POC values for Egress Inhibition**
Chart represents conversion of Figure 3.5 into POC values using Figure 3.6 and Table 3.2.

To determine egress inhibiton, the parasitemia of the test compound after 12hrs incubation with schizont parasites must be significantly lower than the parasitemia of the no-drug control (with or without DMSO) after 12hrs incubation. Incubation of schizont parasites with the “known” controls (EDTA, Leupeptin, Calpain I, Staurosporine, and PP2) did not differ significantly from the 0 hr background control (P > 0.05). The analysis indicates that ring stage parasites did not develop or were not recovered. Although PP2 has previously been shown to be associated with P2X7 inhibition in other model systems, it is a tyrosine specific kinase inhibitor whose effects in Plasmodium, unlike the general kinase inhibitor staurosporine, has not been studied. Because kinases have previously been shown to participate in parasite egress, PP2 will be considered a “known” control in this study. As expected, the 12 hr “no-drug” and
DMSO controls were significantly higher than the background readings, indicating ring stage development for these two controls (P < 0.001). Although the DMSO control inhibited parasite development, the extent of inhibition was less than the known control compounds that required DMSO for re-suspension (i.e. Calpain I, staurosporine, and PP2). SyBr Green I analysis from Figure 3.5 and ANOVA calculations (Table 3.3) show that all the known control compounds significantly inhibited parasite development (P < 0.05) when compared to the “12 hr no drug” or DMSO controls. Giemsa stains were also performed to distinguish between egress and invasion inhibition (Figure 3.8 and 3.9). All inhibitions were of the egress type rather than the invasion type, since numerous intact schizonts and a lack of free merozoites were observed. All of the known controls showed egress inhibition as expected. These results confirm previously published reports regarding the effects of Ca\(^{2+}\) chelators and protease and kinase inhibitors on parasite egress.
Figure 3.8 Egress inhibition: Modified photographs
Schizonts were incubated for 12 hr in the presence of compound/drug, or without compound/drug for the no drug control. Following incubation, cultures were processed and aliquots were smeared onto glass slides for Giemsa staining and microscopic analysis. Photos were modified to highlight ring and schizont parasites. Red dots denote ring stage parasites. Yellow squares denote schizonts. The original pictures (without modification) are given in Figure 3.9. Drug abbreviations are outlined in Table 3.1.
Paraformaldehyde (PFA) and heparin treatments were included as background or invasion inhibitor controls, respectively. PFA crosslinks proteins, preventing merozoite egress from schizonts. Heparin, on the other hand, is reported to be an invasion inhibitor. The POC chart and Giemsa stain analysis from Figures 3.7 and 3.8 confirm that after 12 hr incubation, the “no drug” control contained many rings (red dots) and very few schizonts (yellow squares). The PFA egress control showed the opposite, many schizonts and very few rings. Surprisingly, the heparin invasion control paralleled the PFA control, acting more like an egress inhibitor rather than an
invasion inhibitor. Although we expected to observe few schizonts, many free merozoites, and few rings, the Giemsa stain analysis for heparin instead showed many schizonts and few free merozoites or rings, indicating egress inhibition. Under these conditions, we could not determine (due to the block in egress) if heparin could also block invasion. To further investigate heparin’s ability to inhibit invasion, an efficient methodology for isolation of viable free-merozoites would need to be established so that any heparin block could be analyzed specifically in terms of invasion.

Taken together, these results provide evidence that the method is functioning properly. The negative controls (“no-drug treatment” and DMSO controls) showed significant egress when compared to background (P < 0.05). All the positive controls (the known inhibitors as well as the PFA background control) significantly inhibited egress as shown by comparisons with the background and 12 hr continuous compound treatment (P < 0.05). When the known controls were compared to the relevant negative controls, significant egress inhibition was observed (P < 0.001). In summation, these results substantiate the validity of this method and allow for further investigation into the effects of unknown compounds (P2X7 and pannexin inhibitors) on parasite egress.

**Effect of P2X7 inhibitors**

We next determined the IC\textsubscript{100} values of P2X7 inhibitors. Table 3.1 lists the IC\textsubscript{100} values. Figure 3.10 and 3.11 shows the chart used to determine Table 3.1 values.
Figure 3.10 Compound titration chart: P2X7 inhibitors
Synchronized schizonts were incubated with 1:2 serial dilutions of kinase inhibitors for 12 hr in complete media under standard culture conditions. Following harvest, aliquots were processed to recover ring stages. Rings were assayed by SyBr Green I measurements and values were plotted to show RFI (OD_{520}) versus concentration (Log X), yielding a drug inhibition curve. IC values were calculated using GraphPad 5. Results are averages from at least two biological replicates each ran in duplicate.

Kn-62 is reported to inhibit the P2X7 receptor, and like PP2, is also a known kinase inhibitor.\textsuperscript{130,142} In \textit{P. falciparum}, this compound was reported to be an invasion inhibitor.\textsuperscript{143} Because others have shown that kinase inhibitors can inhibit egress, we tested whether Kn-62 is also an egress inhibitor. Additional P2X7 inhibitors were included in these tests since they are reported to be P2X7-specific and are not kinase inhibitors. Figures 3.5, 3.7, and 3.8 shows that while many rings and few schizont are observed in the “12 hr no-drug” control, all of the P2X7
inhibitors showed the opposite effects, many schizont and few rings. We found that Kn-62 reduced parasitemia, as did the other more selective P2X7 inhibitors (i.e AZ11, A740, A804). The Kn-62 results were expected since the control kinase inhibitors staurosporine and PP2 also inhibited parasite egress. When DMSO-suspended test compounds (Kn-62 and all the P2X7 inhibitors) were compared with the DMSO control, they showed greater inhibition than the DMSO control and their standard deviations did not overlap with the DMSO control. However, only egress inhibition by AZ11 was significantly lower (P < 0.05) than the DMSO control and not significantly different from the 0 hr or PFA background controls (P > 0.05). The data indicates that P2X7 inhibitor AZ11 participates in the inhibition of schizont egress from RBCs. In addition, while we are unable to conclude that the other P2X7 inhibitors affected the egress process, we can state that AZ11 provides evidence supporting a role for P2X7 in the Plasmodium egress process.

Effect of pannexin inhibitors

Pannexins are hemi-gap junction channels. They are tightly associated with P2X7 receptor activation and are present on the surface of RBCs. Because the evidence supports a potential role for P2X7 in the egress process, and the kinase inhibitor PP2 prevents P2X7-mediated pannexin-dependent large pore formation in other model systems, it is possible that this pathway of pore formation is a key element of the P. falciparum egress process. Results from pannexin inhibitor mefloquine (MQ) did not show significant egress inhibition (P > 0.05) from the DMSO control. However, pannexin inhibitors probenecid (PBCD) and carbenoxolone (CBX) did show strong inhibition (Figures 3.5 and 3.7) when compared to the 0 hr background and 12hr no-drug controls, and they were not re-suspended in DMSO. The ANOVA analysis of Figure
3.7, summarized in Table 3.3, show that both CBX and PBCD were significantly different from the 12 hr no-drug control (P < 0.001).

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Table 3.3 One-way ANOVA of Egress Inhibition Data
Summary analysis of one-way ANOVA with Tukey post-tests. The P-value and significance (denoted by *) are given. Significant P-values are denoted as P < 0.05 (*), P < 0.01 (**), and P < 0.001 (**). Non-significant values are listed as P > 0.05 (NS).

CBX and PBCD were similar to or not significantly different from the 0 hr background or PFA control (P > 0.05). These results indicate that both CBX and PBCD inhibit parasite egress, presumably through their effects on the pannexin hemichannels. SyBr Green I analysis (Figure 3.5) and microscopy data confirmed the results (Figure 3.8). Several schizonts and few rings or merozoites were observed using Giemsa stain analysis on cultures tested with these compounds.
Figure 3.11 Compound titration chart: pannexin inhibitors
Synchronized schizonts were incubated with 1:2 serial dilutions of pannexin inhibitors for 12 hr in complete media under standard culture conditions. Following harvest, aliquots were processed to recover ring stages. Rings were assayed by SyBr Green I measurements and values were plotted to show RFI (OD_{520}) versus concentration (Log X), yielding a drug inhibition curve. IC values were calculated using GraphPad 5. Results are averages from at least two biological replicates each ran in duplicate.

Rescue of parasite from egress inhibition

Rescue analysis for controls

Although the data indicate that P2X7 and pannexin inhibitors interfere with parasite egress, rescue experiments were performed to determine if the observed inhibition phenotype was due to parasite death. The rescue experiments were designed to show that compound/toxin removal after 2 hr or 4 hr incubation with schizont parasites, might relieve egress inhibition and return
ring stage parasitemia to untreated control levels. If egress inhibition is not relieved after compound removal, then parasites are likely dead, resulting in reduced ring stage parasitemia. To determine if rescue has occurred, the parasitemia from the 2 hr or 4 hr rescue treatments must be significantly higher than the parasitemia from the same cultures at 12 hr continuous treatment (12hr no rescue). The results are summarized in Table 3.4.

Table 3.4 Two-way ANOVA of Rescue from Egress Inhibition
Summary analysis of two-way ANOVA with Bonferroni post-tests. The P-value and significance (denoted by *) are given. Significant P-values are denoted as P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***). Non-significant values are listed as P > 0.05 (NS).

The experimental design takes advantage of the tightly synchronized parasite population that will egress within a very narrow window. To minimize variations, all parasites for each biological run were derived from a single synchronized batch that was divided into aliquots for individual compound treatment.

In these experiments, the inhibiting compounds PFA and Ox-ATP served as controls that cannot be rescued. PFA additionally kept dead schizonts intact. Ox-ATP, on the other-hand served as an
irreversible non-specific modifier of many proteins, including the P2X7 receptor, and should continue to inhibit egress upon rescue. Figures 3.12 and 3.13 shows that for these control compounds, the 2 hr and 4 hr rescue time points produced parasitemia that were significantly lower than the no-drug control (P < 0.001). More importantly, these rescue time points never produced a parasitemia that was significantly higher than background (P > 0.05). As expected, SyBr Green I analysis showed that the PFA and Ox-ATP controls were unable to be rescued under the conditions of these experiments, likely due to their irreversible modification of parasite and host proteins. Likewise, Giemsa stains (Figures 3.14 and 3.16) of the PFA-treated culture showed many schizonts and no rings. Mechanical disruption (trituration) or chemical digestion (tetanolysin) of the schizonts did not recover any ring parasites (data not shown).

![Figure 3.12](image)

**Figure 3.12** POC values for Rescue from Egress Inhibition
Chart represents conversion of Figure 3.13 into POC values using Figure 3.6 and Table 3.2.
Figures 3.12 and 3.13 shows parasites from the “known” control inhibitors (i.e. EDTA, protease and kinase inhibitors) were rescued from egress inhibition, yielding numerous ring stage parasites. Giemsa stain analysis confirmed the presence of rings (Figure 3.14 and 3.16).

The parasitemia for the known control inhibitors at the 2 hr rescue (2hr-R) and 4 hr rescue (4hr-R) was significantly higher than background (0hr-B) or PFA treatment (P < 0.05). Only leupeptin at the 4hr-R did not vary significantly from the 0hr-B (P > 0.05). However, the leupeptin data is counterbalanced by the Calpain I inhibitor data where significant recovery was observed at the 4 hr rescue time point (P < 0.05). When rescue time points (2 hr and 4 hr) were compared with the no-rescue time point (12 hr no-R) for the known inhibitors (except leupeptin), the parasitemia was significantly higher for the rescue time points, indicating parasite recovery from compound inhibition (P < 0.001 except Calpain I, where P < 0.01 and P < 0.05, respectively). Unlike the 2 hr and 4 hr rescue conditions, incubation of the “known control” compounds with parasites for 12 hr without rescue, showed substantial egress inhibition that did not differ significantly from background (P > 0.05). These data provide evidence that parasites can be rescued from egress inhibition and more importantly, that the parasites are not killed by the compound under these treatment conditions.

Additional support for parasite rescue from inhibition comes from comparative analysis with the negative controls. When the positive controls (known control compounds) were compared to their respective negative controls (i.e. no-drug or no-drug + DMSO), we found substantial inhibition of the known controls within the 12 hr no-rescue time point (P < 0.001). As expected, during rescue conditions (2hr-R and 4hr-R) the results were not significantly different (P > 0.05) from the negative controls at the 12 hr time point (no rescue). This shows that the parasites were
able to recover following compound treatment for 2 hr or 4 hr, and recovery is evidence that the parasites are not killed by these treatment conditions. Once again, only leupeptin showed a significantly reduced parasitemia for these time points. Surprisingly, when leupeptin was compared to the PFA background control at the 2 hr rescue time point, the parasitemia was significantly higher (P < 0.05), indicating parasite rescue. The data shows that parasites could be rescued from leupeptin inhibition but recovery was time dependent and substantially less than the no-drug treatment conditions. One possible explanation for this finding is that unlike Calpain I, leupeptin is a serine and cysteine protease inhibitor. Since the same inhibitor profile is not observed with cysteine protease inhibitor Calpain I, it is possible that the additional targeting of serine-type proteins by leupeptin could lead to the inhibition of processes that cannot be efficiently rescued. In summary, these results support that the experiment is functioning properly. They substantiate the validity of the rescue method, allowing for further investigation into the effects of P2X7 and pannexin inhibitors on parasite egress.
Figure 3.13 Rescue from Egress Inhibition chart
Cultures were treated similar to the 12 hr egress inhibition experiments (12 hr with drug). Test compounds were removed at the 2 hr and 4 hr rescue time points as indicated in the Materials and methods. Incubation continued in complete media without drug until the 12 hr period was completed. The 0 hr time-point (blue bar) indicates the relative parasitemia before drug addition. Red, green, and purple bars represent 12 hr with drug, 2 hr with drug, and 4 hr with drug, respectively. Yellow dots over some of the graphs indicate one biological run with three technical triplicates. Samples without the yellow dots represent at least two biological replicates each ran with three technical triplicates. Graphs represent average readings with standard deviations. Drug abbreviations, IC values, and drug references are outlined in Table 3.1.
Figure 3.14 Rescue from Egress Inhibition: control (modified)
Representative pictures of the control slides (no compound/drug treatment) taken between 0 hr and 12 hr. Three representative slides are given for each time point. Photos were modified to highlight ring and schizont parasites clearly. Red dots denote ring stage parasites. Yellow squares denote schizonts. The original pictures (without modification) are given in Figure 3.15.
Figure 3.15 Rescue from Egress Inhibition: control (unmodified)
Unmodified version of figure 3.14.
Figure 3.16 Rescue from Egress Inhibition: test samples (modified)
Representative pictures of drug treated schizonts, with [(+) rescue] and without [(-) rescue], with smears taken upon completion of the 12 hr incubation period. Rescue was performed 2 hr following drug treatment with completion of the 12 hr incubation period after drug removal. Photographs were modified to highlight ring and schizont parasites clearly, with red dots denoting ring stages and yellow squares denoting schizont stages. The original pictures (without modification) are given in Figure 3.17.
Figure 3.17 Rescue from Egress inhibition: test samples (unmodified)
Unmodified version of figure 3.16.

Rescue analysis for P2X7 and pannexin

The P2X7 inhibitors (except AZ11) did not produce an egress inhibition profile that was statistically lower than the DMSO control. Therefore we were unable to determine with certainty if those compounds inhibited egress. However, based on the SyBr Green I data, a low level of egress inhibition does appear to be present. Assuming there is a low level of egress inhibition, we assessed whether rescue experiments could be performed with those compounds. Analysis of rescue conditions showed that the 2hr time point could be rescued. The 2hr-R parasitemia was significantly higher than both background parasitemia and the 12hr time point (P < 0.001 and P < 0.01, respectively). Although the evidence indicates that the parasites were rescued from inhibition, the recovered parasitemia was not significantly different from the DMSO control (P >
Thus, while we could not determine the cause of inhibition (DMSO or compound), the data showed that parasites are not dead at the 2hr-R time point and can be rescued from egress inhibition.

We next looked at the rescue parasitemia from treatment with the AZ11 compound. While AZ11 rescue was higher than background (P < 0.001), it was not significantly higher than the 12hr time point (P > 0.05), indicating that these parasites could not be rescued from inhibition. Thus, the parasitemia following 2hr or 4hr treatment with AZ11 is statistically equivalent to the parasitemia following AZ11 treatment for 12hrs. In this case, while the data shows that AZ11 inhibits parasite egress, it also kills the parasites. In summary, based on the data derived from these experiments, we are unable to determine whether P2X7 participates in *Plasmodium* egress from RBCs.

With regards to pannexin, MQ showed clear evidence of rescue (P < 0.001 at 2hr-R and P < 0.01 at 4hr-R), however the parasitemia did not differ significantly from the DMSO control following the 12hr incubation (P > 0.05). The interpretation is that DMSO, rather than MQ, caused the observed egress inhibition. Although the MQ inhibition data was disappointing, the CBX and PBCD data showed strong egress inhibition as mentioned earlier (Figures 3.12 and 3.13). More importantly, both compounds gave a parasitemia statistically higher at the 2hr-R and 4hr-R time points compared to the 12hr no-R time point (P < 0.001). This provides supporting evidence that parasite cell death is not the immediate cause of pannexin-mediated egress inhibition. Although both egress inhibition and rescue from egress inhibition is apparent from the pannexin data,
further studies are required to verify that pore-formation from the P2X7-pannexin complex directly results in parasite egress from RBCs.

Invasion inhibition

Analysis of invasion inhibition

None of the compounds, except PFA, inhibited invasion under the experimental conditions (Figure 3.18). The Giemsa stain analysis confirmed the presence of rings for most of the drug-treated RBCs (Figure 3.19).

**Figure 3.18 Invasion inhibition chart**

Uninfected erythrocytes were incubated with test compound for 2 hr, then pelleted and resuspended with synchronized schizonts in complete media. Following 12 hr incubation, samples were harvested, processed, and analyzed to determine the number of rings present, an indirect measurement of invasion. Samples were run three times each in duplicate, except AZ11, MQ, and DMSO, which were ran two times each in duplicate. Graphs represent average readings with standard deviations. Drug abbreviations, IC values, and drug references are outlined in Table 3.1.
**Figure 3.19 Invasion inhibition: Modified photographs**
Red dots denote ring stage parasites. Yellow squares denote schizonts. Note the lack of yellow squares and abundance of red dots (except PFA which shows the opposite results). Pictures correlate with results of Figure 3.15. The original pictures (without modification) are given in Figure 3.20.

**Figure 3.20 Invasion inhibition: Unmodified photographs**
Unmodified version of figure 3.19.

As expected, the PFA control prevented invasion by presumably crosslinking receptors or important surface proteins on the RBC, making them unavailable for parasite use during the invasion process. This result equally indicates that proteins, lipids and/or other factors associated with the RBC, are required for parasite invasion. Surprisingly, Ox-ATP, a non-specific and
irreversible covalent modifier of proteins such as P2X7, did not inhibit invasion under these conditions. Ox-ATP has been shown to irreversibly inactivate P2X7. In contrast to a recent publication, this result provides strong evidence that P2X7 receptors on uninfected RBCs are not required for parasite invasion. In light of the Ox-ATP result, it is not surprising that none of the P2X7 compounds inhibited invasion, given that most are reversible inhibitors. Therefore this finding is supportive yet inconclusive. Experiments showing that egressed/free merozoites are able to invade RBC in the presence of these test compounds would strengthen the findings. However, methods to isolate large quantities of live synchronized merozoites are a major obstacle for directly testing the ability of target compounds to inhibit invasion.

**Discussion**

In this dissertation, an *in vitro* method was established to determine if selected drugs were capable of inhibiting *P. falciparum* egress and invasion. The method proposed in this study investigated the survival of compound-treated schizonts during their development to rings, targeting the egress to invasion transition. This approach is feasible and quick because it is possible to easily isolate, identify, and measure rings and schizonts using a combination of Percoll density centrifugation, SyBr Green I analysis, and Giemsa staining.

The validity of the method was confirmed using compounds known to inhibit parasite egress. These compounds inhibited egress and their effects were able to be rescued. As expected, the controls PFA and Ox-ATP provided a clear representation of complete and irreversible egress
inhibition. Two surprising findings are the heparin and Ox-ATP results. Heparin was previously reported to be an inhibitor of parasite invasion and few reports have identified its role as an egress inhibitor, as shown in this study.\textsuperscript{146-149} The surprising heparin results are best explained from evidence indicating that heparin inhibits IP3 receptor signaling.\textsuperscript{87,150} IP3-dependent Ca\textsuperscript{2+} release is an established signaling pathway in \textit{P. falciparum} and it is possible that the released Ca\textsuperscript{2+} affects some steps of the egress program.\textsuperscript{151} Further studies are necessary to establish a connection between IP3 signaling and parasite egress. Ox-ATP is an irreversible covalent modifier of many proteins including P2X7, but its use to treat uninfected RBCs did not prevent parasite invasion. The findings indicate that P2X7 may not be involved in parasite invasion as previously reported.\textsuperscript{143}

Using the methodologies reported herein, we investigated the participation of the P2X7 receptor as a potential egress-associated cation transporter. The results were unable to show that the P2X7 receptor participates in parasite egress. However, they were able to show that the P2X7 receptor is not involved in parasite invasion of RBCs. This determination is based on the finding that the covalent modifications of surface proteins (including P2X7) on the uninfected RBC by Ox-ATP did not prevent merozoite invasion. However, Ox-ATP was shown to irreversibly inhibit parasite egress when incubated with infected RBCs. Whether P2X7 is the exclusive calcium transporter for egress related processes, remains to be determined. Because P2X7 activation is tightly associated with pannexin-mediated large pore formation and RBCs possess both P2X7 and pannexin channels, the role of pannexin was also investigated. The pannexin results indicate a role in parasite egress. Two of the three pannexin inhibitors showed decreased egress ability when tested on matured schizonts. When viewed in its totality, the implications are that during
the late stages of the egress program, pannexin and possibly P2X7 are activated to form a pore, and inhibition of either protein prevents pore-formation, thereby inhibiting egress.

The signal that initiates the onset of the egress program in *P. falciparum* is unknown. In *T. gondii*, abscisic acid (ABA) induces parasite egress from host cells through cyclic ADP ribose (cADPR)/ryanodine signaling. ABA is produced by the parasite and its signaling can be chemically disrupted with fluridone, negatively affecting the egress program.¹⁵² It is unknown if ABA signaling functions in *P. falciparum*. The IP₃ signaling pathway is well established in *P. falciparum* and may offer some insight. While the heparin results reported herein indicates that the IP₃ signaling pathway may be connected to the *P. falciparum* egress program, the upstream receptor and signal mediating the PLC activity are still unknown. Alternatively the cADPr/ryanodine pathway for Ca²⁺ secretion could operate as the signaling pathway. This speculation is supported by evidence that NAD precursors and possibly NADase prevents schizont egress from RBCs. Regardless of the pathway used, the resulting Ca²⁺ increase would initiate microneme secretion, protease processing and kinase activity. This in turn would result in PVM swelling and its subsequent lysis. While not established, the lysis of the PVM could involve perforin molecules. The release of PV contents into the RBC cytosol would increase the Ca²⁺ concentration of the RBC cytosol, activating both Calpain I and pannexin. RBC Calpain I would degrade the RBC cytoskeleton and RBC pannexin would secrete ATP outside the cell and into the waiting arms of the associated P2X7 receptor. The increased intracellular Ca²⁺, which activates pannexin to secrete ATP, and the activation of P2X7 by the secreted ATP, would lead to pore-formation. We speculate that a transient activation of pannexin or P2X7 is not sufficient for pore-formation. The simultaneous activity of both channels is required for pore-formation.
Once the pore is formed, the CBEV process\textsuperscript{93} rapidly follows, completing the proposed model for schizont egress from RBCs.

**Conclusion**

In conclusion, a method to determine a drug's ability to inhibit egress events was established and verified. As a result, previously unknown drugs were identified as interfering with parasite egress, their targets presumably playing a role in the egress process. In addition, a model is presented, explaining how the P2X7-Pannexin complex represents the pore-forming constituents for merozoite egress from RBCs. The methodology and findings presented in this study add important investigative tools and insights that will advance the understanding of the egress process and help identify new therapeutic targets against malaria parasites.
Chapter 4

Summary Conclusions

In this study, it was hypothesized that the P2X7 ion channel and pannexin hemichannels participate in Plasmodium falciparum egress from erythrocytes by forming the initial membrane opening just prior to the CBEV stages of egress. The use of several toxin and inhibitors to test the hypothesis required the establishment of an efficient synchronization methodology, as presented in Chapter 2. In preparation for egress studies, Chapter 2 provides a quick and simple method for both parasite synchronization and confirming the degree of synchrony. Chapter 2 also shows that the number of ring parasite can be quantified by SyBr Green I, efficiently distinguishing between schizont and ring stages, the characteristic determinant of parasite egress in these studies. The developed methodologies facilitated testing of the hypothesis and allowed for efficient processing of more than 15 compounds and inhibitors.

Using the methods that we developed, several “known” inhibitors of egress were tested to confirm the experimental design. Calcium mobilization and the activities of class specific proteases and kinases were identified as the “known” egress inhibitors. Their application prevented parasite egress. As a result, inhibitors of the target P2X7 and pannexin molecules were tested. The P2X7 (AZ11) and pannexin (PBCD and CBX) inhibitors showed significant egress inhibition. Rescue experiments were performed to show that egress inhibition was not the result
of parasite death. Although AZ11 could not be rescued, two of the three pannexin compounds were able to be rescued. This result confirms that parasites were not killed during compound treatment. Lastly evidence was provided showing that the test compounds did not inhibit parasite/merozoite invasion of RBCs.

In summation, this dissertation provides a methodology for parasite synchronization, confirms the degree of parasite synchronization, and indirectly measures schizont stage egress. Although we were unable to show that P2X7 receptors participate in parasite egress, these results do not confirm their exclusion. However, we did find that pannexin channels participate in schizont egress from erythrocytes. To understand how pannexin and possibly P2X7 channels might participate in the egress process, a model is presented detailing the potential role these molecules play in initiating pore-formation. In conclusion, while additional investigation is needed to confirm various aspects of the egress mechanism, this dissertation provides both insight and evidence of *P. falciparum* late stage egress processes which, if confirmed, could help design egress blocking chemotherapeutics for efficient control of parasite infection in mammals.
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Abstract: Monahan, SJ., Salgaller,ML., Lodge,PA., McLean,JG., Childs,RA., Boynton,AL., Murphy,GP.: (1998) Generation of tumor-specific CTLs from prostate cancer patients by using peripheral blood lymphocytes stimulated with allogeneic prostate tumor cell lines. 89th Annual meeting of the American Association for Cancer Research, March 28-April 1, New Orleans, LA.