EFFECT OF METALLOPROTEASE INHIBITORS ON INVASION OF RED BLOOD CELL BY *PLASMODIUM FALCIPARUM*

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ABSTRACT

The entry of malaria parasites into their host's red blood cells (rbc) initiates the intra-erythrocytic asexual cycle that is responsible for the clinical manifestations of malaria. The process by which a merozoite invades rbc is crucial to the survival of the parasite and ensures maintenance of the blood stage infection. However, the molecular mechanism of rbc invasion by the malaria parasite is still poorly understood. Upon invading the host red cell, the merozoite surface coat is removed implicating that surface proteins proteolysis is essential for invasion to occur. Many malaria proteases have been implicated in the invasion process, but their specific types remain unclear.

To demonstrate the involvement of metalloprotease in the process of *Plasmodium falciparum* merozoite entry into host red blood cells, schizont-infected rbcs and parasitophorous vacuolar membrane-enclosed merozoite structures were treated with 1,10-phenanthroline, a metal chelator, resulting in a reduction of invasion with the IC_{50} values of 24.7±7.2 and 28.9±7.7 μM, respectively. The absence of an accumulation of schizont stages after treatment with 1,10-phenanthroline indicated that the inhibitory effect was not due to suppression of merozoite release from rbcs, but on the invasion step. GM6001, a well-known inhibitor of the mammalian matrix and disintegrin metalloprotease family, was less effective with an IC_{50} of 112±20 μM. Although neither metalloprotease activity nor gene homologs could be detected by gelatin zymography and similarity search, zinc-binding motif search in the *Plasmodium* genome database suggested the possibility of the malaria parasite making use of metalloprotease during invasion.

To probe for the mechanism regulating this metalloprotease, the effects of the combination of inhibitors were employed. Staurosporine, inhibitor of protein kinase with broad spectrum activity, had an additive effect with 1,10-phenanthroline and GM6001, suggesting that metalloproteases (MMP-like and/or metal requiring enzymes) and protein kinase(s) were likely to act independently. Although these data are insufficient to establish a signaling pathway involvement during merozoite invasion, nevertheless this study points to the importance of metal-requiring proteases in the process of invasion of host rbc by the malaria parasite.

KEYWORDS: *PLASMODIUM FALCIPARUM* / RED BLOOD CELL INVASION / METALLOPROTEASE / 1,10-PHENANTHROLINE

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บทบาทของ metalloprotease inhibitors ต่อการเข้าเซลล์เม็ดเลือดแดงของเชื้อ พลาสโมเดียม ฟัลซิพารั่ม
(EFFECT OF METALLOPROTEASE INHIBITORS ON INVASION OF RED BLOOD CELL BY *PLASMODIUM FALCIPARUM*)

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บทคัดย่อ

การเข้าสู่เซลล์เม็ดเลือดแดงของเชื้อมาลาเรียเป็นการเริ่มต้นของวงชีวิตระยะไม่มีเพศในเม็ดเลือดแดง ซึ่งทำให้เกิดอาการทางคลินิกร่วมด้วย กระบวนการที่เชื้อมาลาเรียระยะ merozoite ใช้ในการเข้าเซลล์นี้มีความสำคัญต่อการดำรงชีวิตและการติดเชื้อมาลาเรียในเม็ดเลือดแดง อย่างไรก็ตามกลไกในระดับโมเลกุลยังไม่สามารถอธิบายให้แน่ชัดได้ ในระหว่างการเข้าสู่เซลล์เม็ดเลือดแดง มีการหลุดลอกของผิวชั้นนอกของ merozotie แสดงว่า proteolysis มีความเกี่ยวข้องและสำคัญต่อการเข้าสู่เซลล์เม็ดเลือดแดงของเชื้อมาลาเรีย เป็นที่ทราบกันว่าเอนไซม์ protease ของเชื้อมาลาเรียมีส่วนร่วมในกระบวนการนี้ทว่าชนิดของว่าเอนไซม์เหล่านี้ยังไม่เป็นที่แน่ชัด

จากการทดสอบ *P. falciparum* ระยะ schizont และ PEMS ด้วย 1,10-phenanthroline ซึ่งเป็น metal chelator และตัวยับยั้งของ metalloprotease พบว่าสามารถยับยั้งการเข้าเซลล์เม็ดเลือดแดงของ *P.falciparum* ได้50%ที่ความเข้มข้น 24.7±7.2 μM และ 28.9±7.7 μM ตามลำดับ ในการศึกษาเพิ่มเติมถึงขั้นตอนที่ถูกยับยั้งในระหว่างการเข้าสู่เซลล์ ไม่พบว่ามีการสะสมของเชื้อมาลาเรียระยะ schizont แสดงว่า 1,10-phenanthroline มีผลจำเพาะต่อการเข้าเซลล์ใหม่เท่านั้น แต่ไม่รบกวนการออกจากเม็ดเลือดแดง ส่วน GM6001 ซึ่งเป็นตัวยับยั้งการทำงานของ metalloprotease ในกลุ่ม matrix และ disintegrin ของสัตว์เลี้ยงลูกด้วยนม มีประสิทธิภาพด้อยกว่า โดยยับยั้งการเข้าเซลล์เม็ดเลือดแดงของ *P.falciparum* ได้50%ที่ความเข้มข้น 112±20 μM อย่างไรก็ตาม ในการศึกษานี้ไม่สามารถตรวจพบทั้ง metalloprotease activity และ gene homologues แต่เมื่อค้นหาด้วย zinc binding motif ในยีโนมของ *Plasmodium* พบว่ามีความเป็นไปได้ที่ metalloprotease มีความเกี่ยวข้องกับการเข้าสู่เซลล์เม็ดเลือดแดงของเชื้อมาลาเรีย

นอกจากนั้นได้ทำการศึกษาเพิ่มเติมถึงกลไกที่ควบคุมการทำงานของ metalloprotease เมื่อนำ staurosporine ซึ่งเป็นตัวยับยั้งการทำงานของ protein kinase มาทดสอบร่วมกับ 1,10-phenanthroline หรือ GM6001 ไม่พบการต้านยาร่วมกันของเชื้อมาลาเรีย นอกจากนี้ เมื่อนำ 1,10-phenanthroline มาทดสอบร่วมกับ GM6001 ไม่พบการต้านยาร่วมกันของเชื้อมาลาเรีย ผลที่ได้จากการทดลองนี้เป็นข้อบ่งชี้ว่าการทำงานของ metalloprotease และ protein kinase ไม่น่าจะมีความเกี่ยวข้องกัน ถึงแม้ว่าข้อมูลเหล่านี้ยังไม่เพียงพอที่จะสามารถชี้แนะ signaling pathway ได้ ผลจากการวิจัยในครั้งนี้บ่งชี้ว่า metalloprotease มีส่วนร่วมที่สำคัญในการเข้าสู่เซลล์เม็ดเลือดแดงของเชื้อมาลาเรีย

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LIST OF ABBREVIATIONS

ABRA	acidic basic repeat antigen
ADAM	a disintegrin and metalloprotease
AMA1	apical merozoite antigen1
CDPK	calmodulin-like domain protein kinase
DAG	diacylglycerol
DBL-EBP	Duffy binding like-erythrocyte binding proteins
EBA175	erythrocyte binding antigen 175 kDa
FIC	fractional inhibitory concentration
GPI	glycosylphosphatidylinositol
MAEBL	paralogues of both AMA-1 and DBL-EBP
MCP-1	merozoite capping protein 1
MLCK	myosin light chain kinase
MMP	matrix metalloprotease
MSP1	merozoite surface protein1
PEMS	PVM-enclosed merozoite structures
PI-PLC	phosphatidylinositol-phospholipase C
PKC	protein kinase C
PKG	protein kinase G
PTK	protein tyrosine kinase
PVM	parasitophorous vacuole membrane
RAP-1	Rhoptry-associated protein 1
Rbc	erythrocyte
RESA	ring-infected erythrocyte surface antigen
Rhop-1	rhoptry proteins-1
RIMA	ring membrane antigen
SDS-PAGE	sodium dodesyl sulphate-polyacrylamide gel electrophoresis
SERA	serine repeat antigen
SUB-1/2	subtilisin-1/2
SVMP	snake venom metalloprotease

CHAPTER 1
INTRODUCTION

Plasmodium falciparum is the causative agent of the most virulent form of human malaria and up to 2.7 million deaths annually have been attributed to this pathogen (1). Entry of malaria parasite into its host erythrocyte (rbc) initiates the intra-erythrocytic asexual cycle that is central to the pathogenesis of this devastating pathogen. The process by which a merozoite invades rbc is crucial to the survival of the parasite and ensures maintenance of the blood stage infection. Interference with this process would prevent disease as merozoites are short-lived outside the host cell.

Merozoite function is to gain entry to a new red cell. Once inside a new red cell it changes markedly, grows, and divides. After 48 hr, the products of this division are released as sixteen or so new merozoites. Rbc invasion by merozoite comprises of several sequential steps: initial attachment of any part of the merozoite to rbc membrane, reorientation to allow the apical end of the parasite to contact with rbc membrane, release of the contents of the apical organelles, junction formation, membrane invagination, and finally parasite entry (2, 3). However, the molecular mechanisms by which these processes occur remain poorly understood.

The surface of the merozoite is covered by fibrilar material comprising a surface coat (2, 4). During the invasion process, the distribution of the merozoite surface coat alters. The surface coat is absent on the portion of the merozoite within the rbc invagination, whereas the part remaining outside the rbc appears to be similar to that seen on free merozoite (4). Removal of the surface coat must involve selective enzymatic cleavage of the major surface components. This course of event could be mediated by protease(s) located on the merozoite surface, activated upon attachment of the merozoite apical end to the rbc membrane.

Several parasite proteolytic enzymes have been identified, some of which, because of their location, represent possible candidates for a role in merozoite

invasion. A membrane-bound calcium-dependent merozoite serine protease has been reported to mediated multiple proteolytic cleavages of *P. falciparum* merozoite surface protein-1 (MSP-1), an abundant protein on the surface of the invading merozoite, and the shedding of apical membrane antigen-1 (PfAMA-1), a micronemal protein, prior to erythrocyte entry (5, 6). A set of serine protease inhibitors can prevent shedding of both proteins from purified merozoites as demonstrated by Western blot analysis (6). Other serine protease activities likely to be involved in erythrocyte invasion have been reported in *P. falciparum* and *P. chabaudi* merozoite (7, 8).

Besides serine proteases, cysteine protease also plays a role in invasion. Using a chemical proteomic screen, falcipain-1, a cysteine protease, was shown to be active during the invasive merozoite stage (9). Immunofluorescence revealed that the enzyme is located at the apical end of the merozoite that is distinct from rhoptries and micronemes (9). However, a recent report has shown that cysteine protease inhibitors, including YA29-Eps(S,S), do not inhibit falcipain-1 knock out merozoite invasion but blocked hemoglobin hydrolysis in trophozoites, with a subsequent block in rupture of erythrocytes by mature schizonts (10).

In mammal, proteolytic processing of the cell surface protein ectodomain is often sensitive to metalloprotease inhibitors (11, 12). A model for processing of the membrane proteins by this group of membrane-anchored metalloprotease requires both the enzyme and its substrate to be in the same membrane to achieve proteolysis (13).

Therefore, there is possibility that the malaria merozoite could use this kind of membrane bound protease during invasion. We hypothesized that the activity of malaria merozoite surface metalloprotease is essential for merozoite invasion into rbc. Binding of merozoite to erythrocyte activates metalloprotease, thus removing the surface coat and allowing merozoite entry to rbc.

OBJECTIVES

To investigate the involvement of membrane anchor metalloprotease on merozoite invasion of erythrocyte. Strategies used are as follows.

1 To demonstrate the presence and involvement of metalloprotease in rbc invasion of malaria merozoite by using inhibitor study, detection of enzymatic activity, and data mining of *Plasmodium* genome.

2 To assess the possible mechanism of *P. falciparum* rbc invasion by looking at the effect of combination of metalloprotease inhibitor and protein kinase inhibitor.

CHAPTER 2
LITERATURE REVIEW

2.1 Malaria life cycle

Plasmodium falciparum is the causative agent of the most virulent form of human malaria and up to 2.7 million deaths annually have been attributed to this pathogen (1). Malaria is caused by unicellular protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* produce the disease in man: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*.

The life cycle of malaria parasite is complex. The infection begins with the intravenous inoculation of sporozoites by infected Anophelene mosquitoes. The sporozoites circulate for a short time in the blood stream, then invade hepatocytes, where they develop into exoerythrocytic schizonts during the next 5 to 15 days. An exoerythrocytic schizont contains 10,000-30,000 merozoites, which are released and invade the rbcs. Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. The entire invasion process takes about 30 seconds. The merozoite develops within the erythrocyte through ring, trophozoite and schizont stages (erythrocytic schizogony). The parasite modifies its host cell in several ways to enhance its survival. The schizont matures to produce 8-32 invasive merozoites which are released from the ruptured rbc. The whole asexual cycle takes about 48 hr. After rupture of rbc, these merozoites penetrate new rbcs, and the multiplication cycle starts again. This multiplication results in a rapid increase in the number of infected erythrocytes. Clinical malaria (pathogenic process) results from cyclic asexual replication of the blood-stage parasite in circulating erythrocytes of the human host. Erythrocytic schizogony is the stage of the life cycle that has received most attention because it is responsible for pathology and also the most amenable to study, as it is comparatively easy to obtain biological material from blood stage cultures.

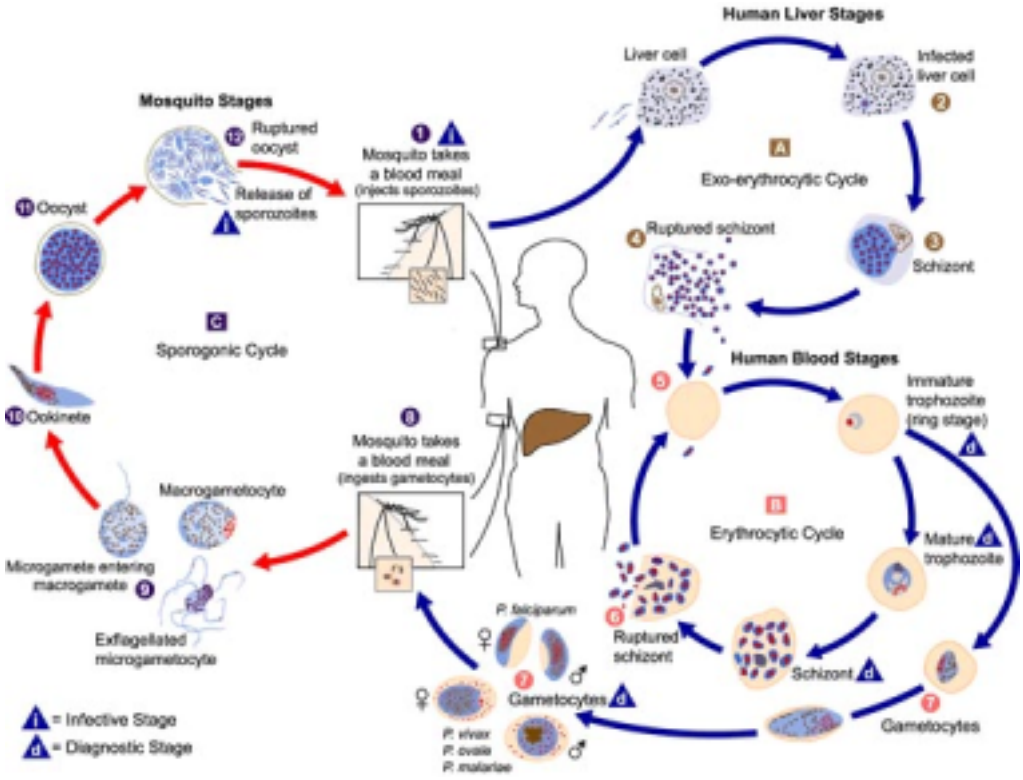


Figure 1 Malaria life cycle (http://www.cdc.gov/malaria/biology/life_cycle.html).

Only a small proportion of merozoites invade erythrocytes and then develop to gametocytes. Another mosquito arriving to feed on the blood may take up these gametocytes into its gut, where exflagellation of male microgametocytes occurs and the female macrogametocytes are fertilized to form a zygote. The zygote then elongates into a motile ookinete. The ookinete travels through epithelial cell of the midgut and then transforms into an oocyst beneath the basement membrane of the midgut epithelium. Over the next week or more, depending on the *Plasmodium* species and ambient temperature, a single oocyst forms more than 10,000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate there. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again.

2.2 Invasion of erythrocyte by malarial merozoite

The major function of the merozoite is the invasion of new rbcs. The process of invasion is quite rapid and has been described in ultrastructural studies, but the molecules involved and the biochemical events that occur are still poorly understood. It is a multistep process comprising of cell recognition, attachment, cell membrane invagination and parasite entry (3) (Figure 2). Studies have predominantly focused on *P. knowlesi*, because of the ease of obtaining relatively pure preparations of viable merozoites, but it seems likely that the invasion process of all species is similar. These studies have led to a general model of invasion being proposed, consisting of phases of recognition and attachment, reorientation and entry. Some species, such as *P. falciparum* are able to invade rbcs of all stages, whereas others, such as *P. vivax*, preferentially invade reticulocytes.

The *Plasmodium* merozoite is very small, 1.5 μm in length and 2 μm in diameter (2, 3), but it contains all things necessary to invade and establish itself in a new rbc. At the apical end of the egg-shaped merozoite are three sets membrane-bound secretory organelles. They are twin pear-shaped rhoptries, the more numerous but smaller micronemes and small rounded vesicles called dense granules. Rhoptries and micronemes are part of the apical complex and are connected via a common duct that extends to the apical membrane, whereas dense granules are round vesicles

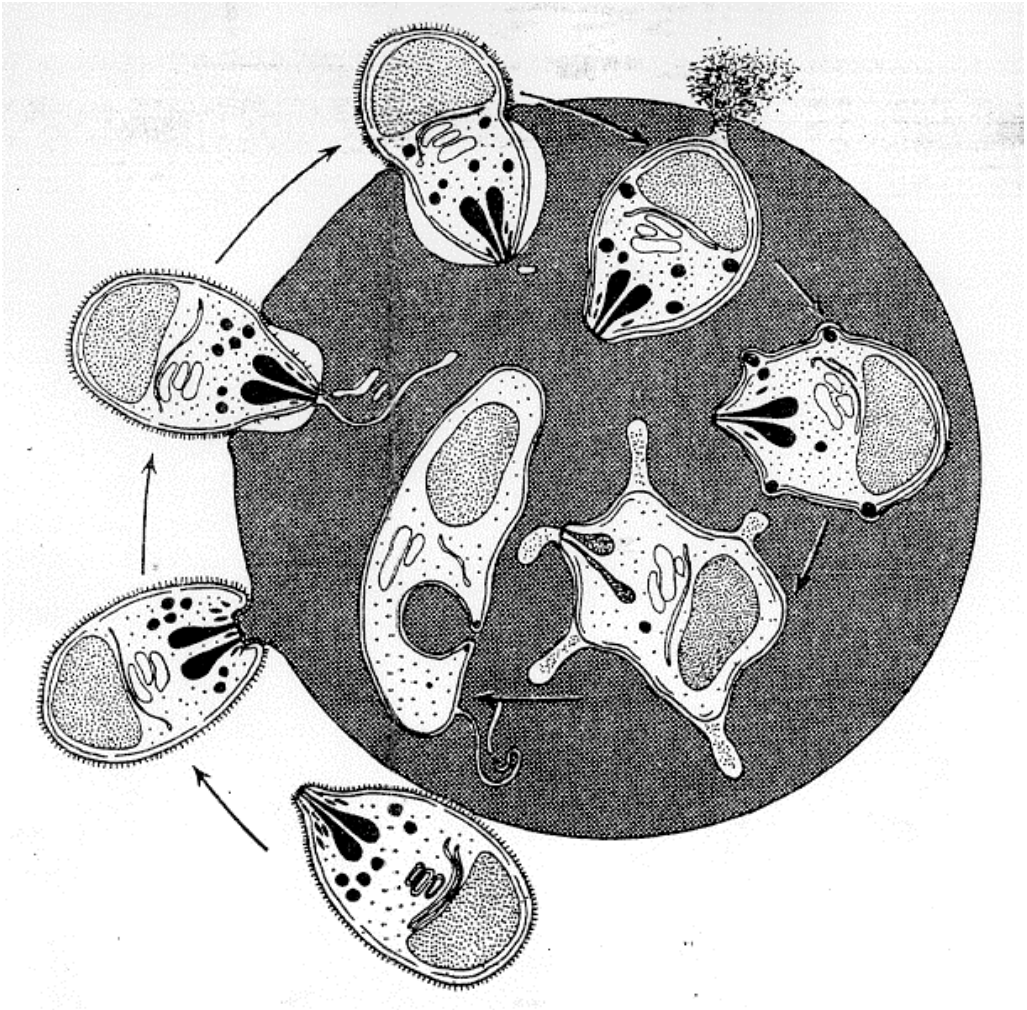


Figure 2 Schematic diagram of the erythrocyte entry by malaria (adopted from 3).

located more deeply within the merozoite cytoplasm (14). The contents of these organelles appear to play a role in the binding and entry of the merozoite into the host cell. The nucleus lies at the other end, and a plastid and a mitochondrion lie along one side of the merozoite, near a band of two or three microtubules. Apically, three dense cytoskeleton rings (polar ring) brace the apical prominence. A flat sac of membrane underlies most of the merozoite surface membrane, forming with it the merozoite's pellicle, which lines the whole cell except most apically. The merozoite also contains numerous free ribosomes. Surrounding the free merozoite, there is a 20 nm thick bristly adhesive coat. The surface coat is lost during the invasion process presumably as a result of proteolytic cleavage (Figure 3).

Merozoites released into the bloodstream following schizont rupture come into contact with rbcs moving through the microcirculation. There may be a preference fornearby rbcs immobilized in rosettes surrounding the rupturing schizont, although the evidence for this is not yet clear-cut. Invasion is a remarkably rapid event, going to completion within some 30 seconds of initial interaction with the erythrocyte (15). The initial contact, accidental in nature in the flowing bloodstream, may occur via any part of the merozoite surface, and is mediated by the merozoite coat filaments, and is not strong, specific nor irreversible. Merozoites are frequently observed to break off contact and abut fresh rbcs.

The initial factor underlying recognition between merozoite and rbc may occur between the merozoite surface coat filament and rbc surface. This requires appropriate receptor-interactions (16, 17). Merozoite surface protein-1, with a glycosylphosphatidylinositol anchor (MSP-1) could be involved in the initial recognition of the erythrocyte. In this process merozoite surface proteins (eg. MSP-1 (18)) or apically secreted (eg. AMA-1 (19)) proteins may play a role which remains unclear. MSP-1, which is uniformly distributed on the merozoite surface, may serve as the ligand that mediates the initial, reversible contact with the erythrocyte surface coat. It is suggested that AMA-1 may be involved in molecular interactions that mediate the initial steps of erythrocyte invasion such as apical reorientation; however, the precise function of AMA-1 remains to be determined. Antibodies that recognize MSP-1 and AMA-1 can block invasion (19, 20).

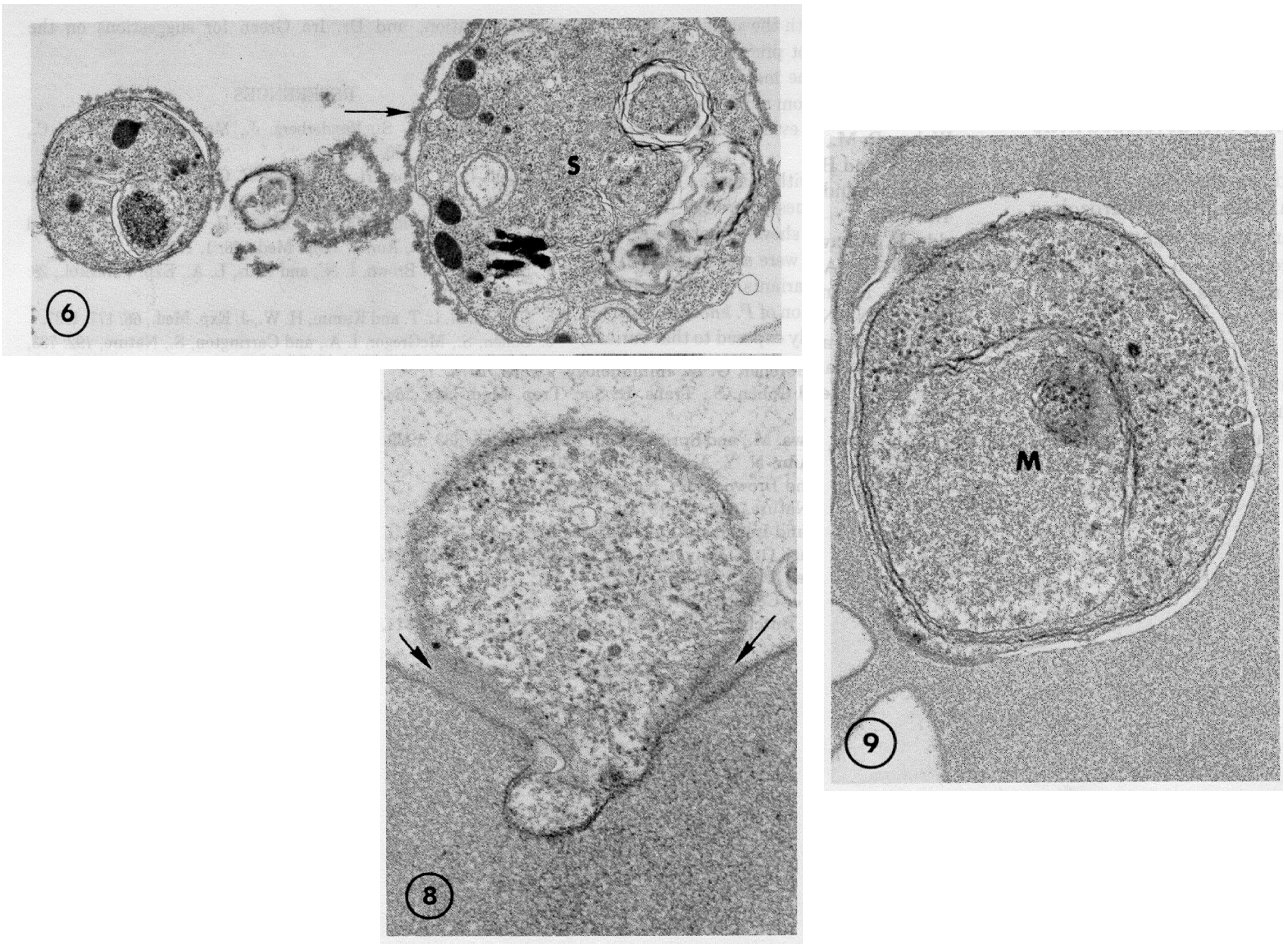


Figure 3 Alteration of merozoite surface coat during invasion. Upper left, free merozoite with prominent surface coat (arrow), lower left, merozoite during erythrocyte invasion, notice that surface coat appears to be push back, and right, merozoite recently invaded erythrocyte, surface coat is absent (adopted from 4).

P. falciparum invades erythrocytes by using multiple receptor–ligand interactions defined as invasion pathways (16, 21). A family of erythrocyte binding proteins have been identified in *P. falciparum*, consisting of EBA-175, BAEBL/EBA-140, JESEBL/EBA-181 and EBL-1, that share a similar cysteine-rich binding domain as *P. vivax*/*P. knowlesi* DBP and belong to the Duffy-binding-like (DBL) superfamily (22). EBA-175 was the first *P. falciparum* ligand identified that binds to erythrocytes with high affinity (16). It binds to glycophorin A, the major glycoprotein of the erythrocyte, and this interaction mediates an invasion pathway for merozoite entry into erythrocytes (16, 23). Disruption of *eba-175* gene results in parasites switching to sialic acid-independent invasion pathway (24). BAEBL/EBA-140 binds to glycophorin C and other receptors depending on mutation in BAEBL/EBA-140 (22, 25-26). JESEBL/EBA-181 binds to trypsinized erythrocytes and targeted disruption of this gene has no effect on invasion phenotype of the parasite (27-28). EBL-1 was identified based on sequence homology and is also probably involved in erythrocyte receptor recognition, playing either a synergistic or an alternative role in the invasion process (29).

A second family of high molecular weight erythrocyte binding proteins designated as the RBL (reticulocyte binding-like) family have been identified in *P. vivax*, with homologs present in *P. falciparum* and *P. yoelii*. It is speculated that the *P. falciparum* RBL homologs (PfRh) may be involved in recruitment of high affinity receptors like EBA-175 by signaling release of the micronemal proteins (30). The *P. falciparum* RBL family includes PfRh1, PfRh2a/b, PfRh3 and PfRh4 (30). PfRh1 binds to erythrocyte in a trypsin-resistant, sialic acid-dependent manner and thus, can not bind to glycophorin A, B, C, D or receptor X (31). Recently, targeted gene disruption of the *PfRh1* gene in *P. falciparum* showed that the encoded protein is required for sialic acid-dependent invasion of human erythrocytes. The parasites are able to invade normally; however, they utilize a pattern of ligand–receptor interactions that are more neuraminidase-resistant (32). PfRh2a/2b were knocked out by targeted gene disruption in the clone 3D7 (33). The transfected parasites lacking PfRh2b showed a significantly altered invasion phenotype with enzymatically treated erythrocytes compared to the wild type clone, whereas the knockout parasite lacking PfRh2a showed no apparent difference to the wild type (33). The PfRh2b knockout

parasites invaded erythrocytes, sequentially treated with two enzymes, neuraminidase and trypsin, at a lower efficiency but better than the wild type parasites for trypsin or trypsin and chymotrypsin treatment. From this, the PfRh2b ligand has been suggested to mediate invasion through a chymotrypsin sensitive, trypsin/neuraminidase resistant receptor, receptor Z (33). PfRh4 is more similar to PvRBP-1 than PvRBP-2 and is located in the micronemes as shown by immunofluorescence studies (34). Erythrocyte-binding activity of PfRh4 has not been demonstrated. PfRh3 is a pseudogene that is transcribed but not translated due to a frameshift at the 5' end of the gene (35).

It is not clear what molecular event triggers the merozoite reorientation but once it commences, the interaction between merozoite and rbc becomes much stronger until it is functionally irreversible. Actual entry of merozoite into the red cell has only been observed with the merozoite in this apically-attached position. Between the merozoite apex and the rbc surface, in the area of molecular interaction between the merozoite adhesins and the rbcs membrane receptors, a so-called junction is formed, and a dense undercoating of the rbc membrane can be observed in this region. The formation of a tight junction between the invading merozoite and the erythrocyte is an irreversible step that commits the parasite to invasion. The junction is visible in electron micrograph as an electron-dense thickening under the inner leaflet of the erythrocyte membrane bilayer (36).

The junction, which initially caps the apical end of the invading merozoite, transforms into a circumferential ring that moves around the surface of the parasite from the apical to the posterior end so that it is always present as a ring around the orifice of the expanding vacuole. When merozoite is completely inside the vacuole, the orifice closes behind the parasite as an iris diaphragm, the vacuolar and erythrocytic membrane fuse, and the merozoite is surrounded by a parasitophorous vacuolar membrane (PVM) (2). A 60 kD merozoite capping protein1 (MCP1) with an oxidoreductase domain has been identified at the tight junction (37). During invasion, MCP-1 moved along from the apical end to the posterior end of the merozoite, suggesting the role for this protein in facilitating attachment or movement of the junction along the parasite surface coat. Cytochalasin B or D, inhibitor of actin filament function blocks the merozoite invasion step into erythrocyte (36). From these

results an actin-based motility system, probably within the parasite, appears to play an important role in the movement of junction during merozoite invasion into erythrocyte.

Invasion is accompanied by exocytosis of the contents of the various secretory organelles of which several proteins have been identified (see Figure 4 and Table 1). How this process is controlled is not understood, but it may occur in a defined sequence, in which one event occurs as a consequence of the previous step, or as a cascade catalyzed by a single trigger. This process is incompletely characterised in *Plasmodium*, but the weight of evidence from studies in this and other apicomplexan parasites, particularly *Toxoplasma gondii* (38), suggests that microneme secretion takes place at an early stage in the invasion pathway, and perhaps even initiates junction formation. This is followed by rhoptry release both at and immediately following junction formation, as well as during movement into the parasitophorous vacuole (39, 40), followed by release of the contents of the dense granules. In contrast to rhoptry and microneme discharge which occurs exclusively at the apical prominence, dense granule release involves movement of the bodies to the anterior lateral surface of the cell where they fuse with the pellicle (3, 41). In addition, though dense granule release can apparently take place during the latter stages of the invasion process (41), most seems to occur following completion of invasion.

During the invasion process, the distribution of the surface coat alters. Before invasion, merozoite is covered with a surface coat of 20 nm thickness (2) (Figure 3). The surface coat or glycocalyx found on merozoite is removed from the invading end (2). It appears to accumulate posterior to the moving junction, and is eventually released into the extracellular milieu. A line of attachment is maintained along the outer rim of the pits and the surface coat is absent from the portion of merozoite within the parasitophorous vacuole, while those remaining outside the erythrocyte appear to be similar to that seen on free merozoites (4). Thus modification of cell surface protein by proteolytic cleavage appears to be essential for invasion to occur.

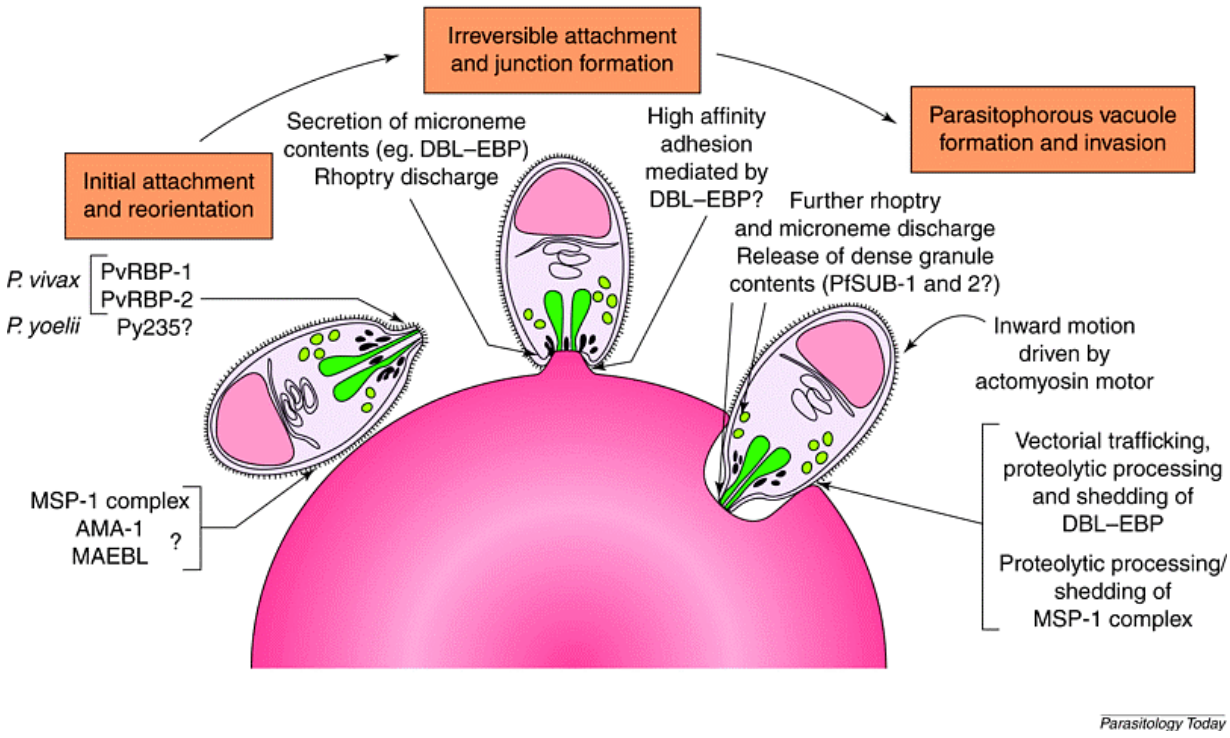


Figure 4 Interaction between merozoite and host red cell during invasion. Shematic depicting the early stages of erythrocyte invasion by the malaria merozoite, and the putative roles of the various organelles and some proteins. The initial, low-affinity interaction with the host rbc may involve adhesive ligands stably resident on the merozoite surface such as MSP-1 and other components of the associated protein complex. Alternatively, it may involve AMA-1, which is secreted onto the parasite surface in a truncated form from a primary location in rhoptries, or MAEBL. Reorientation of the bound parasite may be favoured by the presence of higher-avidity ligands clustered around the apical prominence, such as PvRBP1 and 2 in *P. vivax* (or functionally equivalent molecules in *P. falciparum* and *P. yoelii*). Upon reorientation, tight attachment and junction formation may be initiated by discharge of micronemal components such as EBA-175 and other DBL-EBPs. The effects of anterior to posterior trafficking and/or proteolytic shedding of these and other rbc-binding proteins, linked to the action of a subpellicular actomyosin motor, may then aid in driving the parasite into the nascent parasitophorous vacuole. Abbreviations: AMA-1, apical membrane antigen-1; DBL–EBP, Duffy-binding-like erythrocyte-binding protein; EBA-175, 175 kDa erythrocyte-binding antigen; MSP-1, merozoite surface protein-1; PfSUB-1, *P. falciparum* subtilisin-like protease-1; PvRBP, reticulocyte-binding protein of *P. vivax*; Py235, *P. yoelii* 235 kDa rhoptry protein. (adopted from 42).

Table 1 Apical organelle proteins of *Plasmodium falciparum.*.

Protein	Size (kDa)	Features	References
Rhoptry			
P225	225	Integral membrane protein?	(44)
HMW			
Rhop-1	140	Rbc binding protein	(45)
Rhop-2	130	Rbc binding protein	(45)
Rhop-3	110	Rbc binding protein	(45)
LMW			
RAP-1	80	Associated with rbc membrane?	(46)
RAP-2	42	-	(47)
RAP-3	37	-	(48)
Serine protease (gp76)	76	GPI-anchored serine protease, PI-PLC cleaved to activate protease activity, cleaves band 3 and glycophorin A	(7, 49)
Pf 60.1	60	Rbc binding protein?	(50)
MAEBL	47/39/30	Transmembrane	(51)
MCP-1	60	Oxidoreductase domain	(52)
Dense granule			
RESA	155	PVM; binds rbc cytoskeleton	(53)
RIMA	14	PVM	(53)
SUB-1	80	Subtilase ; invasion	(54)
SUB-2	150	Subtilase ; invasion	(55, 56)
Microneme			
EBA-175	175	Transmembrane, then cleaved for release, binds glycophorin A during invasion	(16)
AMA-1	80	Transmembrane, translocates and is processing during invasion, associates with the PV	(6)

(Adopted from 43)

2.3 *Plasmodium* proteases mediating erythrocyte rupture and invasion

Malarial proteases are involved in at least three distinct aspects of red cell invasion: modification of parasite proteins involved in host cell recognition and entry; restructuring of the surface and cytoskeleton of host cell itself, during and following invasion; and parasite release from the host cell.

Indeed several parasite proteolytic enzymes have been identified, some of which, because of their location, represent possible candidates for a role in merozoite invasion. A membrane bound calcium-dependent merozoite serine protease has been reported to mediated multiple proteolytic cleavages of merozoite surface protein-1 (MSP-1), an abundant protein on the surface of the invading merozoite, prior to erythrocyte entry (5). This protease has been later reported to be a serine protease that can mediate MSP-1 secondary processing and shedding of *P. falciparum* apical membrane antigen-1 (PfAMA-1), a micronemal protein (6). A set of serine protease inhibitors can prevent shedding of both proteins on purified merozoites as demonstrated by Western blot analysis (6). Apart from proteolytic cleavage of parasite surface proteins, the release of components from micronemes is also crucial for malaria invasion. These proteins translocate onto parasite surface and are subsequently shed by proteolysis. These two events, if being interrupted, will compromise the invasion process.

Two other plasmodial serine proteases have recently been described as well. These subtilisin-like proteases, Pfsub-1 (54) and Pfsub-2 (55, 56), are both located in dense granules, secretory organelles released from merozoites at the time of erythrocyte invasion. These findings suggest that the proteases mediate invasion by action upon host or parasite proteins. It has been proposed that they process key parasite proteins (viz., merozoite surface protein-1), but the specific substrates of the proteases are not yet described.

Another serine protease activity likely to involve in erythrocyte invasion has been characterized in *P. falciparum* and *P. chabaudi* merozoite (7, 8). Treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) can cleave an inactive glycosylphosphatidylinositol (GPI) -linked serine protease from intact merozoites or isolated schizont membranes and release it in an active form (7).

The acidic basic repeat antigen (ABRA) shows sequence similarity with chymotrypsin proteases (57), and may thus be involved in the proteolytic activities essential for schizont rupture and/or invasion of merozoites. ABRA is a highly conserved protein of 101 kD, which shows essentially the same location in the infected erythrocyte as SERA (58). ABRA localises to the parasitophorous vacuole during schizont maturation, and at schizont rupture is released as a soluble protein into the culture medium (58).

Besides serine proteases, cysteine proteases also plays quite prominent role in invasion. Using a chemical proteomic screen, falcipain-1, a cysteine protease, was shown to be active during the invasive merozoite stage (9). Falcipain-1 specific inhibitor, YA29-Eps(S,S), identified by screening of chemical libraries, blocked parasite invasion of host erythrocytes (9). Immunofluorescence revealed that the enzyme is located at the apical end of the merozoite that is distinct from rhoptries and micronemes (9). However, a recent report showed that cysteine protease inhibitors, including YA29-Eps(S,S), do not inhibit falcipain-1 knock out merozoite invasion, rather they blocked hemoglobin hydrolysis in trophozoites, with a subsequent block in rupture of erythrocytes by mature schizonts (10).

A 68 kD cysteine protease was identified in schizonts and merozoites and localized to the merozoite apex, suggesting that it may be released from the rhoptry organelle during invasion (59). A cysteine protease of mature schizonts and a serine protease of merozoites were identified in highly synchronized parasites (60). Another protease, inhibited by both cysteine and serine protease inhibitors, hydrolyzed the erythrocyte cytoskeletal proteins spectrin and band 4.1 (61).

The serine repeat antigen (SERA) (62, 63) and the related protein SERP H (64), both expressed in mature schizonts, have important similarities in their sequences with cysteine proteases. SERA is present in the parasitophorous vacuole as a 126 kD protein which is processed at about the time of schizont rupture to generate fragments found in the culture supernatant and associated with the merozoite surface. SERA processing is mediated by at least three distinct proteases, based on sensitivity to serine protease inhibitor DFP, cysteine protease inhibitor E64, leupeptin and iodoacetamide (65).

A putative *P. falciparum* zinc-metallopeptidase has been identified by gene cloning (66). It belongs to the M1 family of aminopeptidases whose members differ widely in substrate specificity. Polyclonal antibodies raised to a synthetic peptide deduced from the gene sequence were found to react with a 96 kD and 68 kD protein present in a soluble schizont extract (66). Its expression in trophozoites and schizonts has been experimentally confirmed by Western blotting (67). Detection by immunofluorescence revealed spots in free merozoite but not in early ring, making it an interesting candidate for inhibition studies of the invasion process (67).

There is a substantial body of evidences indicating that merozoite release from the mature schizont requires protease activity. Although the identity of proteases involved in the degradation of the parasitophorous vacuole membrane and the infected rbc membrane of mature schizonts is still being uncovered, treatment of schizonts with some protease inhibitors has been shown to have a substantial or a partial inhibitory effect on merozoite release (68, 69). A combination of leupeptin, chymostatin, pepstatin and antipain dramatically affect schizont release, resulting in the accumulation of aggregates of merozoite (70). More recently, rbcs incubated in the presence of the cysteine protease inhibitor, l-transepoxyl-succinyl-leucylamido-(4-guanidino)butane (E64) resulted in accumulation of extraerythrocytic merozoites enclosed within the PVM, suggesting that proteolysis is required for this phase of the release process (69).

In a recent study, host urokinase was shown to bind to the surface of *P. falciparum*-infected erythrocytes, and the depletion of urokinase from parasite culture medium inhibited erythrocyte rupture by mature schizonts (71). This inhibition was reversed by exogenous urokinase.

2.4 Proteases involved in ectodomain shedding

Many proteins leads a dual existence as both membrane-bound and soluble isoforms. In general, soluble and membrane-bound isoforms of the same protein can be generated by one of the two mechanisms: first, by separate biosynthetic pathways, either by alternative pre-mRNA splicing of a common transcript or by transcription of closely related but distinct genes; and second, by posttranslational release of the

extracellular domain of membrane proteins by hydrolytic cleavage of the membrane anchor. This hydrolysis involves either a protease or a phospholipase, depending on the type of membrane anchor on the protein.

The endogenous proteolysis release of integral transmembrane proteins is limited to those of Type I, which has a cleaved N-terminal signal sequence and a C-terminal membrane anchoring sequence, and Type II which has uncleaved signal sequence doubling as membrane anchor. The cleavage site is generally located close to the membrane surface such that the bulk of the protein is released into the extracellular milieu, often in a fully functional form. In the majority of cases it is likely that the cleavage occurs at a single, unique site defined by the specificity of the secretase and the topology of the protein substrate (12). Most but not all are membrane-anchored metalloproteases (12).

The release of the extracellular domain through limited proteolysis is recognized as a general mechanism to regulate the function of transmembrane protein. This type of limited proteolysis is currently known as ectodomain shedding. Ectodomain shedding can potentially regulate most cellular functions mediated by transmembrane proteins. It occurs at or near the cell surface and is a regulated process (13).

2.4.1 Metalloprotease disintegrins/ADAMs

They are mammalian proteases belongs to the reprolysin family which have homologous protease or protease-like domains to the snake venom metalloprotease (SVMP) subfamily. All members of this subfamily also have a pro-domain, a disintegrin-like domain and a cysteine-rich domain. Additionally, some also have transmembrane and cytoplasmic domains. Some members do not have functioning protease domains.

ADAMs are proteins containing both a disingrin and a metalloprotease domain (72). They form a large group of cell surface proteins that combine features of both cell surface adhesion molecules and proteases. The name ADAM describes the two domains that ADAMs share with their closest relatives, the PIII class of snake venom metalloproteases (SVMP), and that have, to date, been shown to be functional.

ADAMs have also been referred to as MDCs (metalloprotease/disintegrin/cysteine-rich), cellular disintegrins, disintegrin-metalloproteases, and metalloprotease-disintegrins. All ADAMs display a common domain organization and, as a group, possess four potential functions, namely proteolysis, adhesion, signaling and fusion (Figure. 5).

The ADAM proteases fall within the adamalysin/reprolysin subfamily of the metzincin superfamily of Zn^{2+}-dependent metalloproteases. The other members of the subfamily are the SVMPs. ADAMs and SVMPs share an extended catalytic site sequence, HEXGHXXGXXHD (in the single letter code for amino acids), a mechanism of activation involving proteolytic removal of the Pro domain, as well as other structural features. Some SVMPs and ADAMs have been reported to cleave (*in vitro*) both extracellular matrix (ECM) molecules as well as cell surface protein. And, like all Zn^{2+}-dependent metalloproteinases, ADAM proteases are inhibited by EDTA and 1,10 phenanthroline both of which chelate Zn^{2+} ions.

There is some cross inhibition by antagonists that block members of the matrix metalloproteases (MMPs), a distinct subfamily of the metzincins. The endogenous inhibitor of MMPs, tissue inhibitor of metalloprotease (TIMP-1 and TIMP3), can inhibit ADAM10 and ADAM17. Generic functions proposed for ADAM proteases include matrix degradation, cell migration, and localized shedding of various proteins, including cytokines and growth factors, from membrane-anchored precursors (11, 73, 74).

Hydroxamic acid-derived inhibitors have been routinely used to characterize the shedding of molecules analyzed to date, and invariably, these inhibitors are shown to prevent ectodomain shedding (11, 74). Some are specific for ADAM, MMP or both.

Processing of membrane protein by the ADAMs and others requires both the membrane-anchored enzyme and its substrate to be present in cis on the same cell (13) (Figure 5). Upon cell activation, the attachments change and the proteases and substrates become coclustered and can interact. Alternatively, the signaling cascade can modify the cytoplasmic domains of the protease or substrate, producing a conformational change that either activates the enzyme or makes the cleavage site available (13).

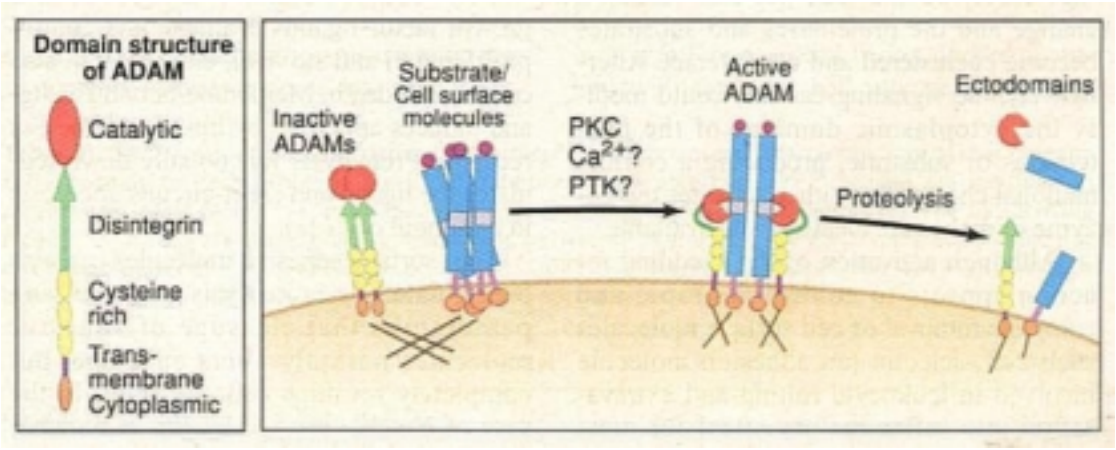


Figure 5 Ectodomain shedding model (13). The proteases and substrates are anchored apart in the plane of the membrane. Upon activation, they are brought together and proteolysis takes place, leading to free ectodomains.

2.4.2 1,10-Phenanthroline and GM6001

1,10-Phenanthroline (Figure 6), a nitrogen-containing chelating agent, is regarded as a general metalloprotease inhibitor. Generally, chelating agents inhibit metalloenzymes either by removing the metal or by forming a complex with it *in situ* (75).

GM6001 is an inhibitor of both matrix (MMP) and disintegrin (ADAM) metalloprotease family (76, 77). GM6001 is hydroxamic acid-derived inhibitor, and its structure is shown in Figure 7.

2.5 Mechanism of regulating apicomplexa host cell invasion

During invasion of an erythrocyte by malarial merozoite, a complex sequence of interactions occurs between the two cells. Although components of signal transduction pathway in malaria parasites have been identified (78), the major biochemical mechanisms underlying invasion are not understood. Calcium-mediated signaling is recognized to involve with malaria and *Toxoplasma* apicomplexan host cell invasion. In addition, it is likely that this process could rely on phosphorylation/dephosphorylation since these protein modifications are common for signaling regulation. However, only few data from malaria are available to connect these molecules into a given biochemical pathway, thus signaling data from *Toxoplasma* invasion events, which is related to malaria, are also covered.

Calcium ions have long been known to play a key role in many cell events. Intracellular Ca^{2+} is an important second messenger in eukaryotic cells, mediating the cell's response to external stimuli as well as playing a pivotal role in the control of many intracellular process. These Ca^{2+}-regulated processes are mediated by calmodulin, which acts as a multipurpose intracellular receptor for Ca^{2+} and calcium/calmodulin complexes control a wide range of cellular processes. Host cell invasion of *Plasmodium* and *Toxoplasma* are likely to be partially regulated by calcium-dependent transduction pathway.

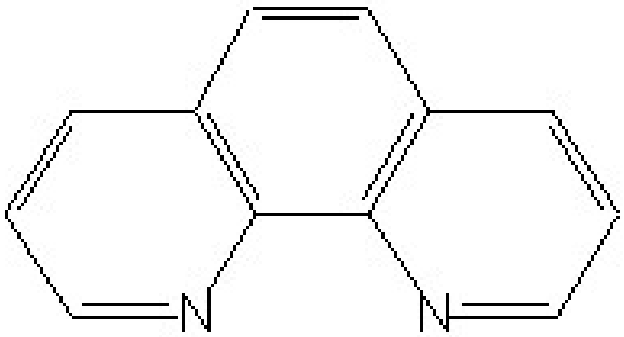


Figure 6 Structure of 1,10-Phenanthroline.

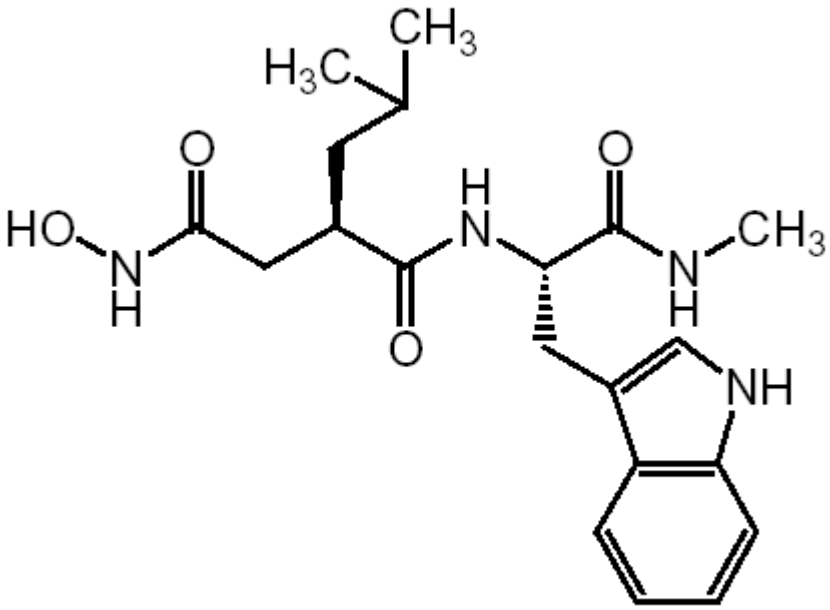


Figure 7 Structure of GM6001.

Calcium is required for human malaria parasite invasion of rbc (79). Addition of EGTA and EDTA chelators to schizont stage parasites can reduce invasion (80). Calmodulin is located at the apical region of both free and intraerythrocytic merozoites and it is the region that attaches to the rbc as the first step in invasion (81, 82). Calmodulin antagonists have been shown to have effect on invasion (82). This calcium/calmodulin involvement has also been implicated during *Toxoplasma* invasion of its host cell (83, 84).

Apical attachment and the formation of the junction zone triggers discharge from the rhoptries and micronemes, located at the apical end of merozoite. A calcium-dependent second messenger system may be involved in the secretion of the malarial rhoptry-microneme contents (81). The discharge of *Toxoplasma* adhesins by micronemes is known to occur in response to a rise in intracellular calcium (85). Calcium dependent protease is also implicated in malaria parasite. MSP-1, the well characterized major surface protein of *Plasmodium*, is proteolytically processed before and during invasion. The final processing event appears to be due to the action of membrane-bound calcium-dependent serine protease (5, 86).

Parasite protein kinases have been implicated in the invasion through previous studies with inhibitors. Staurosporine, an inhibitor of protein kinase, is a potent inhibitor of *Plasmodium knowlesi* (87) and *Plasmodium falciparum* (88) erythrocyte invasion. Staurosporine arrests invasion at the step which is ultrastructurally similar to the arrest caused by cytochalasins B and D. Merozoite attachment, apical reorientation and junction formation appear to proceed normally, but the attached merozoite does not internalize (87). This suggests that protein phosphorylation within the merozoite plays a critical role in the internalization step of invasion.

Staurosporine (Figure 8) is a potent inhibitor of several different serine/threonine and tyrosine protein kinases (89). Since this block was shown to be partially overcome in the presence of the serine-threonine phosphatase inhibitor, okadaic acid (OA), and OA has little effect on protein tyrosine phosphatase, therefore, it implies the role of serine/threonine phosphatase and protein kinases in merozoite invasion. A serine/threonine protein phosphatase type 1(PP1) exists in *P. falciparum* mature schizont infected cells (90). However, exposure to several phosphatase inhibitors could inhibit parasite growth but not invasion (90). Study on *Toxoplasma*,

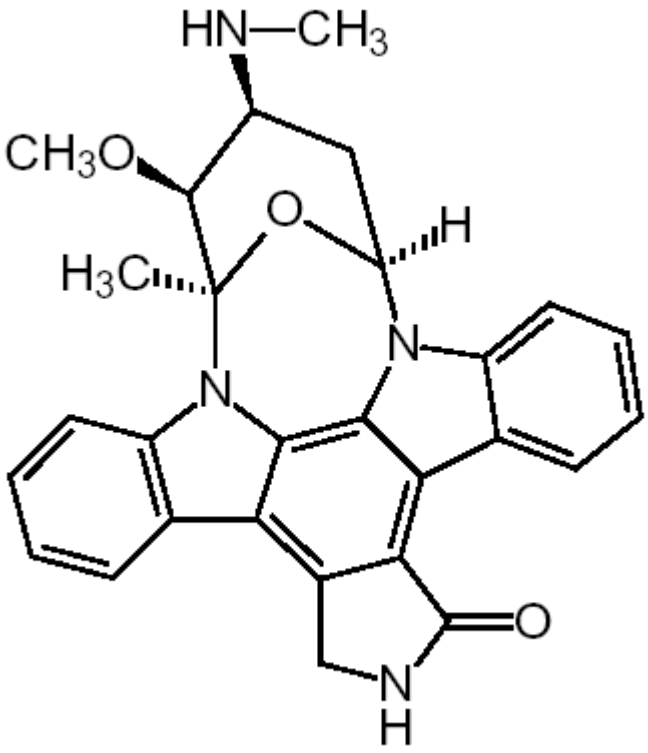


Figure 8 Structure of staurosporine.

unlike the malaria parasite, has identified TgPP1 activity involved in tachyzoite invasion of host cell (91).

After apical end contact to the host cell, there is sequence of exocytosis from secretory organelles. Previous inhibitor studies in *T. gondii* implicated serine/threonine protein kinases in the concerted regulation of microneme secretion, gliding motility, and host cell invasion. Staurosporine inhibits discharge of the micronemal protein, MIC2, which mediates host cell attachment and invasion in *Toxoplasma* (85). Recent study reveals that a cGMP-dependent serine/threonine protein kinase of *T. gondii* is involved with host cell invasion. Inhibiting of microneme secretion, host cell attachment and host cell invasion of the coccidian *Eimeria* sporozoite and *Toxoplasma* tachyzoite have been demonstrated by using a selective inhibitor of PKG, trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine (92).

However, *Toxoplasma* microneme exocytosis is calcium dependent as mentioned previously (85). Artificially induced increases in intracelluar calcium in the absence of host cell trigger microneme discharge (83). The mechanism by which PKG or other serine/threonine kinase regulate microneme secretion is unknown, probably involved with communication of calcium-dependent pathway. However, artificially raising calcium concentration could not overcome kinase inhibitor inhibitory effect (76). As a result, both investigators suggested that PKG or staurosporine blocked serine/threonine kinase acts downstream of calcium in the signal transduction pathway that triggers microneme secretion or perhaps act independently (85, 92).

Calcium dependent protein kinase is also required for *Toxoplasma* tachyzoite attachment to host cell (93). The kinase belongs to calmodulin-like domain protein kinase (CDPK) family, which are activated by calcium in the absence of calmodulin or phospholipids. TgCDPK1 was inhibited by KT5926, an inhibitor of calcium-dependent protein kinases including calmodulin kinase and myosin light chain kinase (MLCK). Three protein phosphorylated by TgCDPK1 were identified but how these phosphorylation contribute to parasite entry is unclear. The block by KT5926 is possibly involved with microneme secretion.

Additionally, other serine/threonine protein kinase also plays a role during *Toxoplasma* invasion. Tachyzoite invasiveness could also require a mitogen-activated protein kinase as suggested by the use of different kinase inhibitor (94).

Protein tyrosine kinase (PTK) activity in *Plasmodium falciparum* has been reported (95) although the genome of *P. falciparum* appears not to possess genes encoding conventional receptor-linked tyrosine kinase (96). There is a stage specific increase in the PTK activity, in the order ring < trophozoite < schizont (95). Conversion of the schizont stage to ring stage via release of merozoites is associated with a decrease in PTK activity. It is unclear if this PTK may play role during parasite invasion of rbc.

Phospholipase is involved in malaria invasion. Parasite derived GPI anchored gp76 serine protease was cleaved with phospholipase C and it became activated (7). The active, solubilized protease is required for merozoite entry into the erythrocyte. As this reaction release diacylglycerol-DAG (a known activator of protein kinase C), it is of interest to know whether it is functionally linked to the staurosporine-sensitive step that is essential for completion of invasion process (87).

A parasite phosphoprotein is hydrolysed in schizonts, this cleavage is inhibited by cysteine protease inhibitor (97). During this period, the parasite protein kinase activity also decreases suggesting that this hydrolysed product may be a protein kinase physical regulator (97).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Malaria parasite

Plasmodium falciparum K1 (98) was maintained under continuous culture *in vitro* using the candle-jar method of Trager and Jensen (99).

3.1.2 Materials for cultivation of malaria parasites

1. RPMI 1640 powder, Formular No. 430-1800, was product of GIBCO Laboratories.

2. Human erythrocytes (O, Rh^+) from local blood donors were kept at 4 ºC in blood bags containing acid citrate dextrose (ACD).

3. Human serum (group A, B, AB, or O) was stored at –20 ºC.

4. Tissue culture plastic petri dish (100x20 mm and 60x15 mm) was purchased from Corning.

5. Gentamycin (80 mg/2 ml) was purchased from the Government Pharmaceutical Organization, Thailand.

6. 0.45 μM Millipore filter was from Whatman.

3.1.3 Materials for inhibitor testing

1. 1,10-Phenanthroline was purchased from Sigma.

2. Bathophenanthrolinedisulfonic acid disodium salt(Sigma), was kindly provided by Assoc. Prof. Prapin Wilairat, Department of Chemistry, Faculty of Science, Mahidol University.

3. E64 (l-transepoxyl-succinyl-leucylamido-(4-guanidino)butane), a cysteine protease inhibitor was purchased from Sigma.

4. Staurosporine was purchased from Calbiochem.

5. GM6001, metalloprotease inhibitor was purchase from Calbiochem.

6. 96-wells microculture plastic plates were purchase from Nunc.

7. [^{3}H]-hypoxanthine (specific activity of 21 Ci/mmol) was purchased from Amersham.

8. Glass filter paper grade 943 AH was from Whatman.

9. Minicell harvester was from Nunc.

10. Liquid scintillation counter LS1801 was from Beckman.

3.1.4 Chemicals

All chemicals used in this study were reagent grade and listed alphabetically as follows. For special reagents, the name and the distributor will be detailed at the first mention of each reagent either in sentence or in parenthesis.

Name of chemical	**Company**
Acrylamide	Sigma
Ammonium persulfate	Sigma
Bathophenanthrolinedisulfonic acid disodium salt	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Calcium chloride ($CaCl_2.2H_2O$)	Baker
Serva Blue R (Coomassie Blue R)	Serva
Di-sodium hydrogen phosphate (Na_2HPO_4)	Merck
Dimethylsulfoxide (DMSO)	Sigma
Garamycin	Schering
Gelatin	Sigma
Glycerol	Carlo Erba
Glycine	Sigma

HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])	Sigma
Propidium iodide	Sigma
2,4 diphenyloxazole (PPO)	Sigma
1,4-bis[2-(5-Phenyloxazolyl)]benzene (POPOP)	Sigma
Potassium dihydrogenphosphate (KH_2PO_4)	Riedel-de Haen
Di-potassium hydrogenphosphate (K_2HPO_4)	Merck
Saponin	Sigma
Sodium bicarbonate ($NaHCO_3$)	Baker
Sodium carbonate (Na_2CO_3)	Fluka
Sodium chloride	Sigma
Sodium dihydrogenphosphate ($NaH_2PO_4.2H_2O$)	Riedel-de Haen
Sodium dodecyl sulfate (SDS)	Sigma
D-Sorbitol	Fluka Bio Chemika
TEMED (N,N,N',N'-Tetramethylethylene diamine)	Sigma
Toluene	Shell Chemicals
Tris base	Sigma
Triton-X 100	Sigma
Zinc chloride	Sigma

3.1.5 Instruments

Name of instrument	**Company**
Centrifuge (for malaria culture), model PR-2	IEC International
Centrifuge	SORVALL RC 2B Plus
Microcentrifuge	SORVALL Biofuge
Flow cytometer	Becton Dickinson
Gel electrophoresis system	Hoeffer
Power supply, model 200/2.0	Biorad

3.2 METHODS

3.2.1 *In vitro* cultivation of *P. falciparum*

3.2.1.1 Preparation of stock materials for cultivation

3.2.1.1.1 Preparation of stock medium

A solution of RPMI 1640 medium (10.4 g initially dissolved in 800 ml of double-distilled water) and 5.94 g of HEPES were mixed together to give the final concentration of 25 mM HEPES. After pH adjustment to 6.75, double-distilled water was added to the medium solution at a final volume of 960 ml. An antibiotic, gentamicin sulfate (garamycin), was added to the medium solution at a final concentration of 40 μg/ml to inhibit bacterial contamination. Finally, the medium solution was sterilized by filtration through a 0.45 μm Millipore filter and then aseptically aliquoted into 100 ml sterilized bottles and stored at 4^{o}C for use within 1 month.

3.2.1.1.2 Sodium bicarbonate, 5% (w/v)

Five grams of sodium bicarbonate were dissolved in 100 ml of double-distilled water. After sterilization by filtration through a 0.45 μM Millipore filter, 10 ml aliquots were made and kept at 4^{o}C for use within 1 month.

3.2.1.1.3 D-sorbitol, 5% (w/v)

5% D-sorbitol was prepared in the manner similar to that described for 5% sodium bicarbonate.

3.2.1.1.4 Serum

Fresh type A, B, AB or O, Rh^+ human serum was obtained from clotted blood. Three hundred ml of blood drawn from a donor, who has never been afflicted with malarial infection and does not live in endemic area of malaria, were allowed to clot for a few hr at room temperature before being subjected to complete coagulation at 37°C for a further one hour. The clotted blood was stored overnight at 4°C allowing clot retraction. Serum was isolated from the clot by centrifugation at 2,000 rpm (IEC model) at 4°C for 10 min and then transferred in 10 ml aliquots and stored at –20°C for use up to 3 months. All steps were performed aseptically.

3.2.1.1.5 Uninfected erythrocytes

Three hundred ml of O type Rh^+ of human blood group were obtained from a donor into a sterile bag containing citrate phosphate dextrose (CPD), which has the formula shown below, as anticoagulant. Then 50 ml aliquots were removed and stored at 4°C for use within 1 month after collection.

Citrate phosphate dextrose (CPD)

Citric acid (anhydrous)	0.327 g
Sodium citrate (anhydrous)	2.63 g
Dextrose (anhydrous)	2.32 g
Sodium dihydrogenphosphate	0.22 g

Distilled water to 100 ml

Blood : CPD = 10 : 1.4 (v/v)

3.2.1.1.6 Culture medium

A. Incomplete medium

For washing of uninfected red cells, 100 ml of stock medium were supplemented with 4.2 ml of 5% sodium bicarbonate to yield a medium with final concentration of 0.2% (w/v) sodium bicarbonate. The mixture solution turned from

yellow to orange indicating a shift in pH 6.75 to 7.4, and was kept at 4^{o}C for use within 2 days or until it turned red. The limit of storage was reached when the medium mixture turned red indicating the shift to alkaline pH resulting from loss of carbon dioxide, after which the mixture was discarded.

B. Complete medium

For cultivation, stock medium was added with sodium bicarbonate as described above. In addition, the medium mixture was supplemented with 10 ml of A, B, AB, or O type, Rh^{+} human serum to give a final concentration of 10% serum and kept at 4^{o}C for use within 1 week.

3.2.1.2 Cultivation technique

The culture method described was according to Trager and Jensen method (83).

3.2.1.2.1 Preparation of uninfected erythrocytes

A desired volume of blood, usually 10 ml, was transferred into 50 ml centrifuge tube and centrifuged at 2,000 rpm (IEC model) at 4^{o}C for 10 min. After removal of plasma and buffy coat, packed red cells were washed twice by adding of at least an equal volume of incomplete medium and centrifuged as above. The cells were resuspened to 50% (v/v) with the complete medium.

3.2.1.2.2 Preparation of parasitized erythrocytes

P. falciparum-infected red cells may come from either culture material or frozen storage. In case of continuing from the established culture, the culture material was placed in 50 ml centrifuge tube and centrifuged at 2,000 rpm (IEC model) for 5 min. The supernatant fluid was decanted, and the remaining red cells were resuspended in an equal volume of complete medium. Thin films were made to

determine the level of parasitemia by counting the number of viable parasites per 1,000 erythrocytes. The parasitemia was adjusted to 0.5% by adding freshly washed erythrocytes. To each 1 ml of this infected blood suspension were added 25 ml of complete medium to give a final cell suspension of 2%, which was dispensed into 100x20 mm plastic culture petri dishes (Corning), which were then placed in a desiccator containing a white candle. The candle was lit and the cover of the desiccator was tightly sealed with silicone grease with the stopcock still open until the candle flame went out. Then the stopcock was immediately closed producing an atmosphere of relatively low O_2 and high CO_2 content (17% O_2, 3% CO_2, 80% N_2) and the desiccator was placed in a 37^{o}C incubator. If the source of the parasites was from frozen samples, the procedure described in Section 3.2.1.2.6 was followed.

Every 4 days, fresh red cells were routinely added to the culture plates providing the parasitemia did not exceed 6-8%. If this was not done, pH shift and glucose consumption rate will exceed the capacity of the medium resulting in parasite death.

In order to get enough schizont stage parasites for invasion assay, the final cell suspension percentage has to be kept as low as possible. Therefore, subculturing of the parasites and addition of 50 % rbc suspension was done when we obtained trophozoite-containing infected red cells. When parasitemia of highly synchronized parasite was about 10-15 % or more, culture medium was changed once daily, and the amount of medium used varied dependent on how high the parasitemia was.

3.2.1.2.3 Change of culture medium

The old culture medium had to be changed every 24 hr by gently tipping the dish and aspirating off the old medium with sterile pasteur pipettes. Disturbance of settled red cells was kept to a minimum and as much fluid as possible was removed without removing the cells. Ten ml of new complete medium were added back to the culture dish, the solution was gently resuspended and replaced in the candle jar. Thin smear was made by first removing the medium from the settled red cells and then spreading a small amount of cells on a slide. The thin smear was then quickly dried, fixed with methanol and stained with 5% Giemsa's stain at pH 7.2 for 10 min.

3.2.1.2.4 Synchronous culture

To obtain the ring stage parasites, an asynchronous culture was centrifuged at 2,000 rpm (IEC model) for 10 min, the supernatant fluid was removed and then the infected cells were resuspended with 5 volumes of 5% sorbitol, and left standing at 25^{o}C for 5 min. After centrifuging at 2,000 rpm (IEC model) for 10 min, the supernatant fluid was removed to leave only ring-stage parasite-infected red cells, washed with incomplete medium and then was further used as culture material.

3.2.1.2.5 Parasitemia determination

The level of parasitemia was determinded by counting the number of viable parasites per 1,000 erythrocytes on a thin blood film after Giemsa's staining. Counting area chosen should have good distribution of erythrocytes. Preparation of Giemsa's stain, phosphate buffer and staining are described below.

A. Giemsa's stain

Giemsa powder	0.6 g
Glycerol	50 ml
Absolute methanol, acetone free	50 ml

A small amount of dry stain was placed in a mortar. A small volume of glycerol was then added and ground thoroughly. The mixture was poured into a clean dry flask. The above procedure was repeated until all the powder has been ground with glycerol. The mortar and pestle were rinsed with the remaining glycerol and poured into the flask. The glycerol-dye mixture was placed in a 55-60^{o}C water bath for 6-8 h with periodic shaking. A portion of methanol was then added to the glycerol-dye mixture after the latter had been removed from the water bath and allowed to cool down to room temperature and stored in a stoppered flask. The stain was allowed to age for about 2 weeks at 37^{o}C and then filtered into a dry bottle.

B. Phosphate buffer, pH 7.2

To give a fine picture of nucleus (red color) and cytoplasm (blue color) of *P. falciparum* in the blood film, 0.067 M phosphate buffer, pH 7.2, was used. This was prepared by dissolving 9.47 g of Na_2HPO_4 and 9.38 g of KH_2PO_4 in 1 litre of distilled water.

C. Staining

A 10% solution of Giemsa's stain in phosphate buffer, pH 7.2, was used to stain the blood film about 10 min.

3.2.1.2.6 Cryopreservation of malarial parasites

1. Freezing

A. The freezing solution

One hundred and eighty ml of 4.2% sorbitol in 0.9% NaCl solution were added to 70 ml of glycerol to give a 28% glycerol, 3% sorbitol, 0.65% NaCl solution. The solution was then sterilized by Millipore filtration.

B. The freezing procedure

The ring-stage parasite-infected red cells were centrifuged at 2,000 rpm (IEC centrifuge) for 10 min. The supernatant fluid was decanted and the remaining red cells were resuspended in an equal volume of freezing solution, taking care to mix well the freezing solution before use because the glycerol is dense and remains at the bottom of the tube. Aliquots of 0.5 ml of the above suspension were distributed in small screw cap vials and quickly frozen by immersion in a dry ice-alcohol bath and then transferred to a liquid nitrogen refrigerator (Union Carbide, XR-16 type).

2. Thawing

A. Hypertonic saline

This was a 3.5% NaCl solution that was sterilized by autoclaving.

B. Thawing and cultivation procedure

The screw cap of vial was slightly loosened and set upright in water. The thawed suspension was transferred to a centrifuge tube, centrifuged at 2,000 rpm (IEC model) for 10 min and the supernatant fluid removed. The cells were resuspended in an equal volume of hypertonic saline and centrifuged at 2,000 rpm (IEC model) for 10 min, and the supernatant fluid again was removed. The cells were then resuspended in an equal volume of incomplete medium, centrifuged at 2,000 rpm (IEC model) for 10 min and the supernatant fluid was removed. This step was repeated once more. The cells were finally resuspended in an equal volume of complete medium to give 50% cell suspension as culture material.

3.2.2 Concentration of schizont-infected red cells

To directly demonstrate the effect of inhibitor on merozoite invasion to rbc, synchronized schizont-stage parasitized rbc were used and this was achieved by Percoll gradient technique. Schizont-infected red cells could be concentrated by a Percoll density gradient centrifugation technique as described by Tharavanij *et al*. (100) with some modification. Percoll (Pharmacia Chemicals) was diluted with 1X phosphate buffer saline (PBS) pH 7.4 to obtain 40, 50, and 60 % Percoll. Four ml each of diluted Percoll solution were layered with decreasing density from the bottom to the top of 50 ml centrifuge tube.

For each experiment, about 6-8 100x20 mm petri dishes (Corning) of parasites which has been synchronized, were cultured to high parasitemia of about 10-20 % or more at mature stage (Section 3.2.1.2.2). The cells were collected, centrifuged, and the supernatant was removed and the cell sediment was restored to a 50% hematocrit with incomplete medium. The cells suspension was layered on top of freshly prepared discontinuous Percoll gradients composed of successive layers of 4 ml each of 40%, 50%, and 60% Percoll and centrifuged at 2000 rpm, 4^{o}C for 20 min. Approximately 80-90% of the mature schizont-infected red cells, with density similar to that of 40% Percoll, remained at the interphase of 40% Percoll in the brown layer, whereas the sedimented red cells contained other stages of infected cells and noninfected cells. Mostly trophozoites and early schizonts were localized in the layer of 50% Percoll.

The brown layer at the interphase was carefully removed to another centrifuge tube and washed for three times with incomplete medium. Thin smears were made prior to and after Percoll gradient centrifugation, stained with Giemsa and examined by light microscopy. The cells were then diluted with complete medium to the desired volume for further invasion experiment.

3.2.3 PEMS preparation

At least three steps are involved in this invasion assay, that is schizont development, merozoite release and invasion. To single out merozoite invasion step, the other steps needs to be excluded. Hence, PEMS, an intact schizont with no rbc membrane, was used in comparison with schizont.

PEMS were prepared according to the method of Salmon *et al*. (69). Highly synchronized parasitized cells were grown, until middle-stage schizonts were obtained, each containing between five and seven nuclei. Synchronous cultures were treated with 10 μM of freshly prepared E64 dissolved in distilled water at 37°C for approximately 6-8 hr to obtain PEMS. To purify PEMS, cells from E64-treated cultures (8 of 100x15 mm plates, 12-15% parasitemia) were collected by centrifugation at 2,000 rpm (IEC model) for 10 min and then separated on a cushion of freshly prepared 45% (v/v) Percoll in PBS pH 7.4 by centrifugation (2,000 rpm, 20 min). The brown layer of PEMS was collected from the top of the cushion, pelleted, washed in incomplete medium for 3 times before use in further experiment.

PEMS obtained was added to uninfected red cells to give a starting parasitemia of 3-5% at hematocrit of 1.5% for invasion assay**.** The culture was left for invasion for 15-20 hr and then assayed by counting the number of ring stage parasites per 1,000 cells of each blood smear.

For gelatin zymography assay, washed PEMS were treated with 3 volumes of PBS pH 7.4 containing 0.15% (w/v) saponin for 15 min in water bath at 37 °C with regular agitation. Then PEMS were washed with PBS pH 7.4 and centrifuged at 1,2000 rpm (Sorvall) for 10 min several times until the supernatant colour became clear. The pellet obtained was aliquoted, 10 μl each, into a microcentrifuge tube and frozen at –80 °C

3.2.4 Invasion and growth inhibition assay by the [^{3}H]-hypoxanthine incorporation method

The susceptibility of the parasites to various inhibitors were determined by [^{3}H]-hypoxanthine incorporation method modified from that described by Desjardins *et al.* (101).

Inhibitors were initially dissolved in suitable solvent to get a stock concentration and diluted with incomplete medium to appropriate concentrations just before use. The effect of solvents were also tested and designated as "controls".

The synchronized schizont stage cultures were placed in 15 ml centrifuge tubes and centrifuged at 2,000 rpm (IEC) for 10 min. The supernatant was discarded and the packed cells were resuspended in an equal volume of complete medium. Thin films were made to determine the level of parasitemia by counting the number of viable parasites per 1,000 erythrocytes. The parasitemia was adjusted to give a desired final concentration of 1.5% hematocrit with 1% parasitemia by adding 50% (v/v) uninfected erythrocytes.

To each well of a 96-well plate, 25 µl aliquots of various inhibitor concentrations were added, followed by 200 µl of the synchronized parasitized erythrocyte suspension to give a final volume of 225 µl/well. The plates were placed in a candle jar, and incubated at 37 ^{o}C under 17% O_2, 3% CO_2 and 80% N_2 for 16 hr and then washed twice with complete medium and further reincubated for 8 hr. 25 µl of 10 µCi/ml ^{3}H-hypoxanthine (specific activity of 21 Ci/mmol) in complete medium were added to yield 0.5 µCi/well. The plates were then placed in a candle jar and incubated further at 37 ^{o}C for 16 hr. Control cells were exposed to the incomplete medium in the absence of inhibitor.

After that the cells in each well were harvested onto glass filter paper (Whatman grade 943 AH) by using a minicell harvester. The filters were dried in a hot air oven for 1-2 h before each disk was placed in a 1.5 ml. microtube. A 0.5 ml aliquot of liquid scintillation fluid (0.35%(w/v) PPO and 0.05%(w/v) POPOP in toluene) was added into each tube. The microtubes were then placed in plastic vials for measurement of radioactivity (count per minute) by using a liquid scintillation counter (Beckman LS1801). The 50% inhibitory concentration (IC_{50}) value, which is defined

as the drug concentration required for 50% reduction of the [^{3}H]-hypoxanthine uptake by parasites as compared to control, was determined from the dose-response curve. The results shown represent mean ± S.E.M of percentage of ^{3}H-hypoxanthine incorporation relative to control from three independent experiments, each carried out in triplicates.

3.2.5 Invasion assay by microscopic determination

Experiments were set up in 96 well plate, three replicates for each dilution of inhibitor. Schizont stage parasites were concentrated by Percoll cushion (section 3.2.2) and then further used for invasion assay. Twenty-five microliters of each of the various concentrations of inhibitors and that of incomplete medium, representing control, were added to each well of 96 well plate. Then, 200 µl of synchronized schizont stage parasitized red cell suspension with 1.5-2 % parasitemia and 1.5 % hematocrit were added. Then parasites were incubated with inhibitor for 17-18 hr, and erythrocytes were collected from the culture. Thin smears were made and stained with Giemsa. The proportion of infected red cells was measured by counting of 1,000 erythrocytes/slide. Percent inhibition was calculated as follows:

$$\text{Percent inhibition} = \frac{\text{No. of ring and trophozoite of control} - \text{No. of ring \& trophozoite of test}}{\text{No. of ring \& trophozoite of control}} \times 100$$

Inhibition result is expressed as IC_{50}, the concentration that inhibits invasion of rbc by 50%. The IC_{50} values were obtained from the dose-response curves.

3.2.6 Invasion assay by flow cytometry

At the end of treatment of malaria culture with inhibitor incubated in 96 well plate, the cells of each well were transferred to Eppendorf microcentrifuge tubes. The cells were then washed for 3 times by adding 0.5 ml of PBS, mixed, and centrifugued for 10 sec at 9,000 rpm (SORVALL Microfuge) to remove the supernatant. Subsequently, 1 ml of 0.25% glutaraldehyde diluted with PBS was added and the

sample was mixed vigorously by a vortex mixer. Fixation was done at 4 ºC for at least 15 min (or the fixed sample was sealed and stored at 4 ºC until the analysis was performed). On the day of analysis, 0.25% glutaraldehyde solution was carefully removed and cells washed with 1 ml of PBS for 3 times. One microlitre of packed cells was transferred to polystyrene FACS tube (Falcon) containing 20 µg/ml of propidium iodide in 350 µl of PBS. Cells were stained in the dark at room temperature for at least 30 min before flow cytometry analysis.

The propidium iodide-stained cells were excited at 488 nm. Erythrocytes were gated on the basis of their logarithmic amplification of the forward scatter and 90º light scatter signals. Red fluorescence of propidium iodide was measured through 585 nm band pass filter. A total of 30,000 rbc are analysed. Percent parasitemia and fluorescence intensity were obtained from an integrated fluorescense histogram between the infected and the control uninfected samples. The IC_{50} values (50% inhibition of invasion compared to control) were obtained from the drug dose-response curves.

3.2.7 Invasion inhibition with release control study

To clarify that the reduction in number of ring was due to inhibition of invasion and not due to merozoite release, invasion inhibition by 1,10-phenanthroline was compared with the inhibition of merozoite release by E64. Ten µM of E64, a cysteine protease inhibitor, can inhibit merozoite release, thus it was used as merozoite release control (9).

Schizont-infected rbc purified by Percoll (5% parasitemia, 2% hematocrit) were treated with various concentrations of E64 and 1,10-phenanthroline. After incubation for 13-15 hr, thin smears were prepared and counted for 1000 cells/slide. Three independent experiments were performed at each concentration in triplicate.

3.2.8 Determination of inhibitor combination effect on malaria invasion

3.2.8.1 Inhibitor preparation

Inhibitors were initially dissolved in suitable solvent to get a stock concentration. For 1,10-phenanthroline, it was solubilized with phosphate buffer (PB) pH 7.2 to get a stock concentration of 9 mM and kept at 4°C. Staurosporine and GM6001 was solubilized with DMSO at 2.14 mM and 12.87 mM, respectively. Staurosporine was kept at 4°C whereas GM6001 was kept at –20 ºC. Due to its light sensitivity so staurosporine was handled with minimum light contact. The effects of DMSO and incomplete medium were also tested and designated as solvent control and control.

3.2.8.2 Assay of inhibitor combination effect on malaria invasion

Serial dilutions of 1,10-phenanthroline, GM6001 and staurosporine were prepared separately. Equal volumes of the two drugs with different concentrations were mixed together to obtain a set of drug with various concentration combination. 25 µl aliquot of each mixture was put in 96 well plate in triplicate. Two hundred microliters of 2% parasitemia and 1.5% hematocrit parasitized cell suspension were added and incubated for 17-18 hr at 37 ºC. Thin smears were prepared for light microscopic examination.

Computer-generated concentration-response curves were analyzed by nonlinear regression and 50% inhibitory concentrations were calculated (IC_{50}) for each inhibitor, both alone and in combination. Fractional inhibitory concentrations (FIC) were calculated.

$$FIC = \frac{IC_{50} \text{ of drug of interest in presence of another drug}}{IC_{50} \text{ of drug of interest alone}}$$

The FIC index is simply a mathematical representation of whether the IC_{50} of drug of interested is reduced, unchanged, or increased in the presence of the second drug. A FIC index of 1.0 would represent independence, whereas indices >1.0 would indicate antagonism and indices <1.0 would indicate synergism.

Three combinations of inhibitors were studied; 1,10-phenanthroline and staurosporine, GM6001 and staurosporine, and 1,10-phenanthroline and GM6001.

3.2.9 Determination of metalloprotease activity by gelatin zymography

Zymography was used to show metalloproteinase-like activity in PEMS. It is a common method for assaying protease activity by looking at an in-gel activity in gelatin SDS-PAGE. If there is (metallo) protease activity a clear band will be seen after Coomassie blue staining. This protocol was based on detection of secreted MMP 2 and MMP 9 of cancer cell with some modification (102).

Protein determination of PEMS obtained from Section 3.2.3 was done by Bradford assay before performing SDS-PAGE electrophoresis.

Frozen stored PEMS was thawed at room temperature and solubilized in sample buffer in the absence of reducing agent (5X SDS sample buffer: 0.25 Tris-HCl pH 6.8, 40 % glycerol, 6 % SDS, 0.08 % bromophenol blue) and electrophoresed in SDS-PAGE copolymerized with 0.1% gelatin. The stacking gel contained 4 % acrylamide: bis-acrylamide (29:1), 125 mM Tris-HCl pH 6.8, 10 % SDS, 0.1 % ammonium persulfate, and 0.1 % TEMED, and the separating gel contained 12% acrylamide: bis-acrylamide (29:1),1 mg/ml gelatin, 375 mM Tris-HCl pH 8.8, 0.1 % SDS, 0.02 % ammonium persulfate, and 0.04% TEMED. Electrophoresis was performed at 200 volts, 4ºC in SDS-PAGE running buffer pH 8.3 (0.025 M Tris base, 0.192 M glycine, 0.1 % SDS). After electrophoresis, the gel was washed twice for 30 min in 2.5 % Triton X-100 at room temperature to remove SDS from the gel. The gel was incubated in a reaction buffer containing 0.05 M Tris-HCl pH 7.5, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, and 1% Triton X-100 at 37 ºC for 72 hr. Metalloprotease activity was visualized as clear zone against the blue background after 0.5 % Coomassie brilliant blue staining. Molecular weight was determined by using pre-stained standard

markers (Bio-Rad). Complete medium containing 10% serum and erythrocyte lysate were used as control.

3.2.10 Similarity and zinc-binding motif search

The protein sequences in the annotated *P. falciparum* genome were subjected to search against the Merops protease database, which has a catalog and a structure-based classification of proteases.

Nonredundant query sequences of characterized and predicted proteases from various organisms were obtained from the Merops database (http://www.merops.ac.uk, release 6.60 of 29 Mar 2004). BLASTP searches with default setting were targeted to the annotated *P. falciparum* genome that was published in the PlasmoDB (http//plasmodb.org/; (103)).

The presence of zinc binding signature, HEXXH motif, can be indicative of metalloproteases. Consensus pattern for zinc-binding region signature of neutral zinc metallopeptidases, PS00142 obtained from Prosite (http://www.expasy.org/prosite) is [GSTALIVN]-x(2)-H-E-[LIVMFYW]-{DEHRKP}-H-x-[LIVMFYWGSPQ]. Motif search of the *P. falciparum* annotated protein sequences (http://www.plasmodb.org, release 4.2) with this pattern was done.

CHAPTER 4
RESULTS

Malaria parasite proteases have been shown to play important role for its host red cell invasion; modification of parasite proteins involved in host cell recognition and entry; restructuring of the surface and cytoskeleton of host cell itself, during and following invasion in order to allow parasite release from the host cell (104). During invasion the merozoite surface coat is removed (2). This proteolytic cleavage appears to be mediated by the parasite surface protease.

Proteolysis of the extracellular part of membrane proteins occurs in mammalian eukaryotes and is mostly mediated by membrane-anchored metalloproteases (11, 12). This type of membrane-protease may be responsible for shedding of merozoite surface coat. Thus, the study on malaria metalloprotease involvement with erythrocyte invasion and its possible biochemical regulation was conducted.

We have attempted to develop an invasion assay by using [^{3}H] hypoxanthine incorporation method and flow cytometry; however, microscopic examination was still the method of choice. Invasion assay has been done by applying metalloprotease inhibitor; 1,10-phenanthroline and GM6001, to schizont stage parasites and monitoring invasion efficiency by counting the number of ring-infected red cells by microscopic examination. To distinguish schizont release and merozoite invasion, PEMS, merozoites contained within parasitophorous vacuole membrane (PVM), were also tested against metalloprotease inhibitor.

As the reduction number of ring forms may be due to the inhibition of merozoite release but not of invasion, the invasion inhibition by metalloprotease inhibitor was compared with the inhibition of merozoite release by E64. E64, a cysteine protease inhibitor can inhibit merozoite release (9), thus it was used as merozoite release control. It would be another evidence if the enzymatic activity could be demonstrated *in vitro*. Thus, zymogram SDS-PAGE electrophoresis having

gelatin as a substrate for metalloprotease was performed. Homologs of malaria metalloprotease-like were also looked for by searching the *Plasmodium* genome database. Malaria parasite protein kinase appears to play a key role in merozoite invasion as demonstrated by the use of protein kinase inhibitors (87, 88). Therefore, regulation mechanism of this metalloprotease by as yet to be identified protein kinase was investigated by protein kinase and metalloprotease inhibitor combination assay.

4.1 Malaria metalloprotease involvement with erythrocyte invasion

4.1.1 Attempt to develop invasion assay

We have tried set up an invasion assay by [^{3}H] hypoxanthine incorporation method and flow cytometry. To do this, the 50% inhibitory concentration from microscopic examination was used as a standard. The two methods will be accepted and used as invasion assay if the 50% inhibitory concentrations are similar to that obtained from microscopic examination assay.

Invasion assay by [^{3}H] hypoxanthine incorporation method was carried out as described in section 3.2.4. Inhibitor was removed from the culture after a certain period to see an effect on invasion only. Schizonts were treated with inhibitor for several hr and washed out at ring stage. After reincubated in inhibitor-free medium, [^{3}H] hypoxanthine was added at trophozoite stage, and then the reduction of the incorporation of [^{3}H] hypoxanthine by the malaria parasite was measured at schizont stage. IC_{50} of 1,10-phenanthroline by [^{3}H] hypoxanthine incorporation was 5.3 ± 1.5 μM (Figure 9) whereas that of 1,10-phenanthroline IC_{50} obtained from microscopic examination was 24.7 ± 7.2 μM (Figure 10). Thus, the [^{3}H] hypoxanthine incorporation assay appeared not to be appropriate for the evaluation of inhibitor effect on parasite invasion.

Besides [^{3}H] hypoxanthine incorporation assay, we also tried to set up the other method, flow cytometry. Schizonts were treated with inhibitor for 20 hr and then rbc containing ring stage parasites were collected, glutaraldehyde fixed, and parasitemia determined by propidium iodide fluorescence intensity. IC_{50} for schizont treatment obtained by flow cytometric method was 7.6 μM (Figure 11) but from the same set of

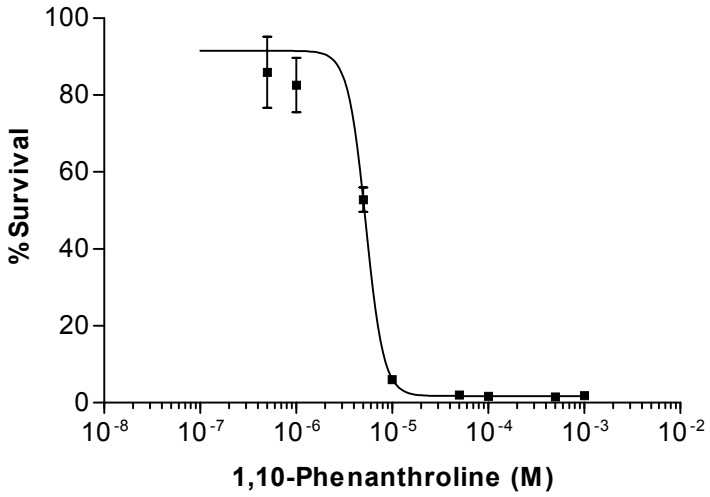


Figure 9 IC_{50} for the inhibition of rbc invasion. Dose–response curve for invasion inhibition was generated by using the [^{3}H]hypoxanthine incorporation assay. Middle to late stage schizonts at 1% parasitemia and 1.5% hematocrit were treated with inhibitor for 16 hr before subjected to washing with complete medium twice. After reincubation for 8 hr, [^{3}H] hypoxanthine were added and incubation continued for 16 hr before harvesting. Each point represents mean $\pm$ SEM. IC_{50} = 5.3 ± 1.5 μM.

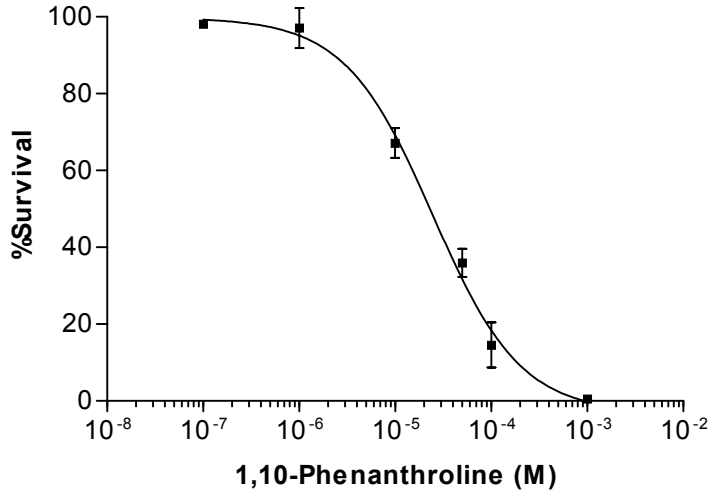


Figure 10 Inhibition of *P. falciparum* merozoite invasion of erythrocytes by 1,10-phenanthroline. Schizonts were incubated with 1,10-phenanthroline for 15-20 hr and the number of ring forms were counted from Giemsa-stained thin films. The inhibition curve was drawn using a GraphPad Prism program. Each point represents mean $\pm$ SEM. IC_{50} = 24.7 ± 7.2 μM.

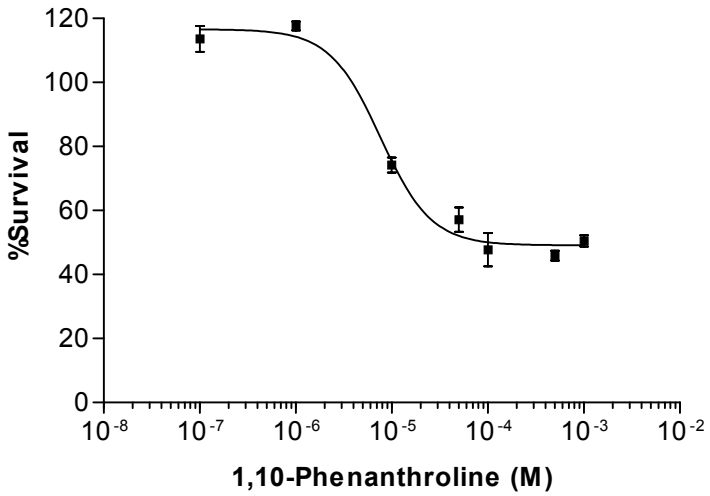


Figure 11 Inhibition of *P. falciparum* merozoite invasion of erythrocytes by 1,10-phenanthroline monitored by flow cytometry. Schizonts were incubated with 1,10-phenanthroline for 20 hr. Ring form parasites were collected and washed before being glutaraldehyde fixed, propidium iodide stained, and subjected to flow cytometric analysis. Each point represents mean $\pm$ SEM. $IC_{50} = 7.6 \pm 3.2$ μM.

experiment analyzed by microscopic examination the IC_{50} value was 22.8 μM. This discrepency indicated that flow cytometry method was not suitable for invasion assay.

Summary of IC_{50} for invasion obtained from three different methods are shown in Table 2.

Table 2 Summary of inhibitory effect of 1,10-phenanthroline on erythrocyte invasion of *P. falciparum* tested by [^{3}H] hypoxanthine incorporation method, flow cytometry and microscopic examination.

	[^{3}H] Hypoxanthine incorporation	**Flow cytometry**	**Microscopic examination**
IC_{50} (μM)	5.3 ± 1.5	7.6 ± 3.2	24.7 ± 7.2

IC_{50} = concentration of inhibitor (μM) that inhibit the incorporation of [^{3}H] hypoxanthine or invasion by 50% of control.

4.1.2 Microscopic examination assay

To directly demonstrate the involvement of metalloprotease-like activity in merozoite invasion of rbc, metalloprotease inhibitor was applied to synchronized schizont stage parasite for a certain period, then the number of ring forms was determined by microscopy. The reduction in the number of ring stage parasites indicates inhibition of invasion. The results of inhibition of *P. falciparum* merozoite invasion of erythrocytes by 1,10-phenanthroline are shown in Figure 10. IC_{50} of 1,10-phenanthroline from microscopic assay was higher than that of [^{3}H] hypoxanthine incorporation and flow cytometry methods. Although the method was more labor intensive, it was more reliable and indicated the presence of metalloprotease (and other metal requiring enzymes) involved with rbc invasion of malaria parasites. Bathophenanthrolinedisulfonic acid, which is more water-soluble than 1,10-phenanthroline, inhibited invasion with IC_{50} value of 330 ± 34 μM. The non-chelating analog of 1,10-phenanthroline, 7,8-benzoquinoline, could not inhibit malaria parasite invasion at 1 mM, the highest concentration tested.

To address whether a specific group of metalloprotease, in particular the cell surface type, disintegrin and metalloprotease (ADAM) and/or matrix matalloprotease (MMP) families, was involved, GM6001, a broad spectrum metalloprotease inhibitor for MMP and some ADAM members, was used in the invasion inhibition assay. Inhibition of *P. falciparum* merozoite invasion of red blood cells by GM6001 is shown in Figure 12. The low inhibitory effect of GM6001 as seen from high IC_{50} value (112 ± 20 μM) indicated that the malaria metalloprotease may be different from that of human.

At least three steps are involved in this invasion assay, that is schizont development, merozoite release and invasion. To single out merozoite invasion step, the other steps needs to be excluded. Hence, PEMS, an intact schizont with no rbc membrane, was used in comparison with schizont.

IC_{50} of 1,10-phenanthroline for PEMS-derived merozoite invasion was 28.9 ± 7.7 μM as shown in Figure 13, which is not significantly different from that for schizont-derived merozoites, indicating that the metal chelator affected only the invasion step and not schizont maturation nor merozoite release.

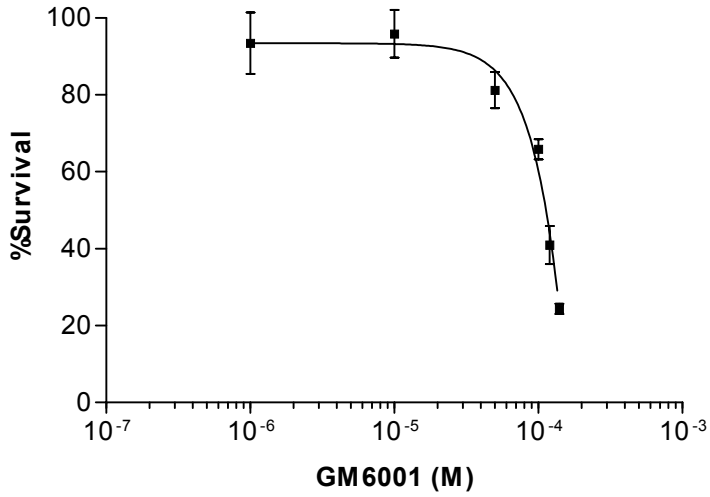


Figure 12 Inhibition of *P. falciparum* merozoite invasion of erythrocytes by GM6001. Schizonts were incubated with GM6001 for 15-20 hr and the number of ring forms were counted from Giemsa-stained thin films. The inhibition curve was drawn using a GraphPad Prism program. Each point represents mean $\pm$ SEM. IC_{50} = 112 ± 20 μM.

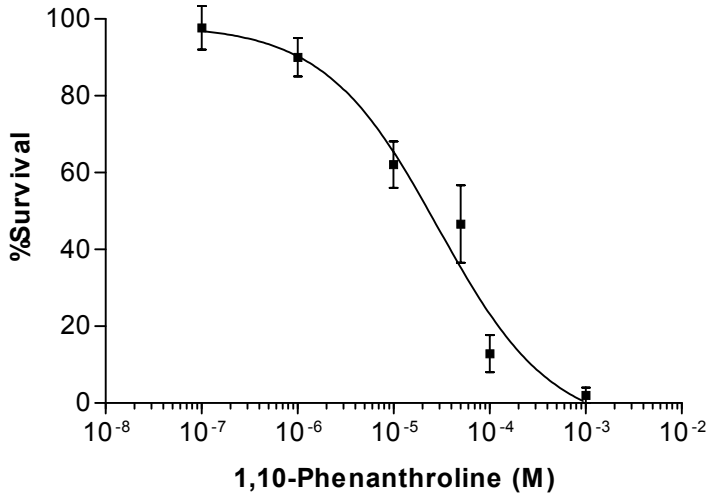


Figure 13 Inhibition of PEMS-derived *P. falciparum* merozoite invasion of erythrocytes by 1,10-phenanthroline. PEMS were incubated with 1,10-phenanthroline for 15-20 hr and the number of ring forms were counted from Giemsa-stained thin films. The inhibition curve was drawn using a GraphPad Prism program. Each point represents mean $\pm$ SEM. $IC_{50} = 28.9 \pm 7.7$ μM.

A summary of IC_{50} values for invasion obtained from treatment by different metalloprotease inhibitors is shown in Table 3.

Table 3 Inhibitory effect of metalloprotease inhibitors on erythrocyte invasion of *P. falciparum* as evaluated by microscopic method.

Inhibitor	**IC_{50} (μM)**
1,10-Phenanthroline (schizont-derived)	24.7 ± 7.2
1,10-Phenanthroline (PEMS-derived)	28.9 ± 7.7
Bathophenanthrolinedisulfonic acid	330 ± 34
GM6001	112 ± 20

4.1.3 Invasion inhibition with release control study

To clarify that the reduction in number of ring is due to inhibition of invasion but not of merozoite release, this invasion inhibition by 1,10-phenanthroline was compared with the inhibition of merozoite release by E64. E64, a cysteine protease inhibitor can inhibit merozoite release, and thus it was used as merozoite release control (9). Treatments with both E64 and 1,10-phenanthroline reduced the number of rings. Treatment with E64 but not with 1,10-phenanthroline resulted in partial accumulation of schizont (Figure 14) consistent with the role of E64 in preventing merozoite release. This suggested that 1,10-phenanthroline was likely to have an effect on invasion, not release.

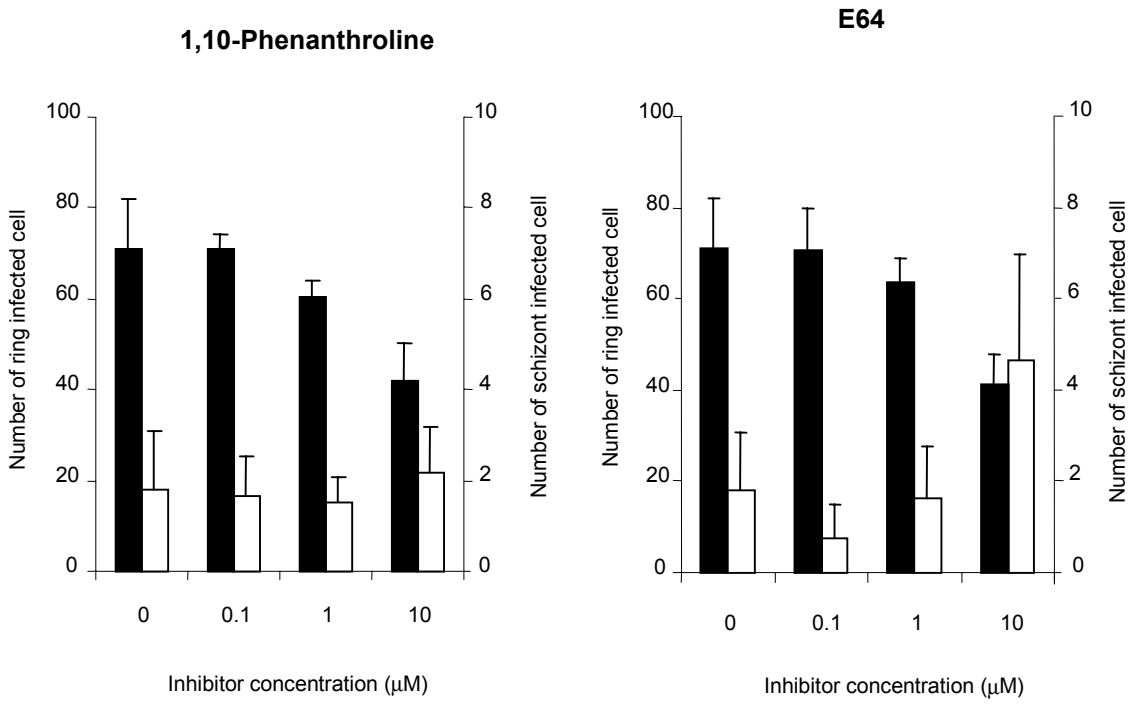


Figure 14 Comparison of 1,10-phenanthroline and E64 treatment on merozoite release and invasion. Schizonts (5% parasitemia) obtained 40 hr after synchronized culture were treated with 1,10-phenanthroline and E64 at the indicated concentrations for 13-15 hr. The numbers of ring- and schizont-infected erythrocytes in1000 cells were counted from Giemsa-stained thin films. Solid bar: ring; open bar: schizont. Each experiment was conducted three times in triplicate and the error bar indicates SD.

4.1.4 Gelatin zymography

Zymography was used to detect metalloprotease-like activity in PEMS. It is a common method for assaying protease activity by looking at an in-gel activity in gelatin SDS-PAGE. If there is protease activity the clear band will be seen after Coomassie blue staining.

An example of gelatin zymogram is shown in Figure 15. Metalloprotease activity (in presence of $ZnCl_2$) was seen in complete medium that contained serum and rbc lysate but not in PEMS. Several attempts were made to modify the experimental condition, however, we could not demonstrate the metalloprotease activity of malaria parasites under the experimental conditions used. This may be the result from low sensitivity of the system tested and/or the low amount of malaria metalloprotease used.

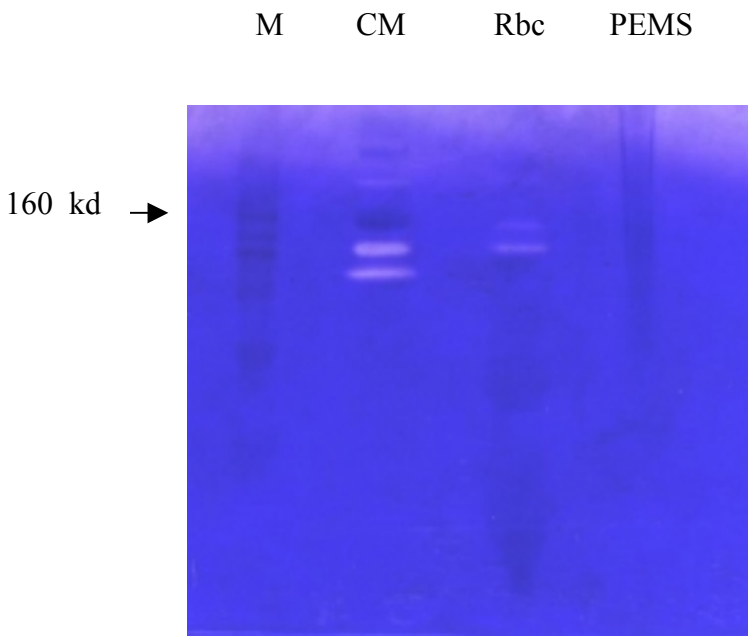


Figure 15 Gelatin zymogram. PEMS are solubilized in SDS loading buffer lacking β-mercaptoethanol and electrophoresed through a 12% polyacrylamide gel copolymerized with 0.1% gelatin. After electrophoresis at 4°C, the SDS was removed from the gel by washing twice for 30 min in 2.5 % Triton X-100 at room temperature. The gel was then incubated in a reaction buffer containing 0.05 M Tris-HCl pH 7.5, 0.01 M $CaCl_2$, 1 μM $ZnCl_2$, and 1% Triton X-100 at 37 ºC for 72 hr. Metalloprotease activity was visualized as clear zone against the blue background after 0.5 % Coomassie brilliant blue staining. Molecular weight was determined by using pre-staine standard marker (Bio-Rad).

M, Molecular weight marker; CM, complete medium containing 10% serum; Rbc, erythrocyte lysate.

4.1.5 Homology search of malaria genome databases for possible candidates of metalloprotease

To find homologs of membrane-anchored metalloproteases of interest i.e., ADAMs, MMP families including meprins, the metalloprotease protein sequences from Merops databases Release 6.60 of 29 Mar 2004 were subjected to search against the annotated *P. falciparum* genome.

A total of 295 nonredundant query sequences of characterized and predicted proteases were obtained from the Merops database (http://www.merops.ac.uk, release 6.60 of 29 Mar 2004) which has a catalog and a structure-based classification of proteases. The BLASTP searches with default setting were targeted to the annotated *P. falciparum* genome that was published in the PlasmoDB (http//plasmodb.org/; (103)).

The results of BLASTP search for ADAM and MMP family are shown in Table 4 and 5, respectively. From 295 sequences, only 36 sequences showed significance matching E-score at the level of N x 10^{-4} or less as shown in Table 6.

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk). MERxxxx = Merops identifier; gp = gene product; TrEMBL= obtained from TrEMBL; no label = presented in UniProt database (the universal protein resource).

Metalloprotease family subclasses	**Species**	**Annotated Malaria Gene ID**	**Score**	**E-score**
ADAM1	*Bos taurus*	MAL8P1.153	33	0.29
	Cavia porcellus	PF08_0013	36	0.043
	Gorilla gorilla	MAL13P1.316	43	4e-04
	Homo sapiens	MAL13P1.316	46	5e-05
	Macaca mulatta alpha I	MAL7P1.146	33	0.32
	Macaca mulatta alpha II	MAL7P1.146	33	0.29
	Oryctolagus cuniculus	PF10_0367	34	0.19
	Papio hamadryas (alpha I)	MAL7P1.146	33	0.30
	Papio hamadryas (alpha II)	MAL7P1.146	34	0.13
	Pongo pygmaeus	MAL13P1.316	44	2e-04
	Rattus norvegicus	PFC1045c	41	0.001
	Saguinus oedipus (alpha I)	PFI0975c	34	0.17
	Saguinus oedipus (alpha II)	PFE0120c	32	0.48
	Tupaia glis	PFD0965w	33	0.18
ADAM2 Non-peptidase	*Bos taurus*	PF10_0367	39	0.003
	Cavia porcellus	PF07_0139	39	0.003
	Homo sapiens	PFC1045c	37	0.023
	Macaca fascicularis	PF10_0320	37	0.023
	Macaca mulatta	PF10_0320	37	0.023
	Mus musculus	PFC1045c	35	0.088
	Oryctolagus cuniculus	PFL1445w	38	0.008
	Rattus norvegicus	PFC1045c	34	0.11
	Sus scrofa	PFI1615w	35	0.067
ADAM3 Non-peptidase	*Homo sapiens* (ADAM 3A protein)	PFD0200c	34	0.16
	Homo sapiens (ADAM 3B protein)	PF13_0235	27	4.4

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	**Species**	**Annotated Malaria Gene ID**	**Score**	**E-score**
	Mus musculus	PF14_0588	39	0.007
	Rattus norvegicus	MAL6P1.86	35	0.067
ADAM4 Non-peptidase	*Mus musculus*	PFD0090c	31	1.0
ADAM5 Non-peptidase	*Cavia porcellus*	PFI0975c	46	5e-05
	Macaca fuscata (MER01141)	PFI0210c	33	0.34
	Macaca fuscata (MER01142)	PF14_0143	34	0.12
	Mus musculus	PFI0975c	38	0.008
	Rattus norvegicus	PF13_0064	33	0.15
ADAM6 Non-peptidase	*Cavia porcellus*	PFI0975c	41	0.001
	Macaca fascicularis	PF14_0172	33	0.14
	Macaca fascicularis (ADAM 6a)	PF07_0086	32	0.58
	Macaca fascicularis (ADAM 6b)	PF10_0192	33	0.33
	Oryctolagus cuniculus (ADAM 6d)	PF13_0168	41	9e-04
	Oryctolagus cuniculus (ADAM6e)	PFI0975c	34	0.15
	Rattus norvegicus	PFI0975c	41	0.002
	Mus musculus	PFI0975c	45	1e-04
	Mus musculus (gp product)	PFI0975c	44	1e-04
ADAM7 Non-peptidase	*Homo sapiens*	MAL6P1.316	33	0.27
	Macaca fascicularis	PF11_0508	33	0.27
	Mus musculus	PFC1045c	40	0.002

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Rattus norvegicus	PFC1045c	39	0.005
ADAM8	*Brachydanio rerio*	MAL13P1.316	33	0.32
	Homo sapiens	PFA0170c	32	0.86
	Mus musculus	PFC1045c	32	0.66
		PFA0170c	32	0.66
	Rattus norvegicus	PF10_0367	31	1.7
ADAM9	*Homo sapiens*	PFC1045c	34	0.22
	Mus musculus	PFC1045c	35	0.10
	Rattus norvegicus	PF11_0007	29	2.8
	Xenopus laevis	PF14_0194	44	2e-04
ADAM10	*Bos taurus*	PFC1045c	37	0.023
	Gallus gallus	PF10_0234	37	0.018
	Homo sapiens	PFC1045c	36	0.028
	Mus musculus	PFC1045c	36	0.052
	Rattus norvegicus	PFC1045c	37	0.016
	Xenopus laevis	PF13_0298	34	0.15
ADAM11 Non-peptidase	*Homo sapiens*	PF14_0175	29	6.9
	Mus musculus	PF14_0175	29	7.0
ADAM12	*Coturnix coturnix*	PFC1045c	32	0.73
	Homo sapiens	PF11_0259	35	0.11
	Mus musculus	PF11_0521	32	0.55
ADAM13	*Xenopus laevis*	PF13_0364	31	1.6
ADAM15	*Homo sapiens*	PFB0340c	29	4.3
	Mus musculus	PFL2405c	29	7.4
ADAM17	*Cricetulus griseus*	PF14_0094	38	0.011
	Drosophila melanogaster	MAL8P1.124	35	0.079
	Mus musculus	PF14_0094	37	0.020
	Rattus norvegicus	PF14_0094	36	0.033

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Homo sapiens	MAL13P1.316	52	9e-07
ADAM18 Non-peptidase	*Homo sapiens*	PF14_0114	36	0.039
	Macaca fascicularis	PFC0425w	33	0.34
	Rattus norvegicus	PF14_0175	33	0.20
ADAM19	*Coturnix coturnix*	PFB0010w	30	3.6
	Homo sapiens	MAL6P1.86	31	1.3
	Mus musculus	PF14_0175	35	0.11
ADAM20	*Homo sapiens*	PFC1045c	34	0.15
ADAM21	*Homo sapiens*	PFC1045c	33	0.33
ADAM22 Non-peptidase	*Homo sapiens* (Merops)	MAL7P1.155	26	2.6
	Homo sapiens (TrEMBL)	PFC1045c	38	0.017
	Mus musculus	PFC1045c	34	0.18
ADAM23 Non-peptidase	*Homo sapiens*	PFC1045c	43	5e-04
	Mus musculus	PFC1045c	42	6e-04
	Rattus norvegicus	PFC0795w	35	0.060
ADAM24	*Mus musculus*	PF08_0013	34	0.16
	Rattus norvegicus	PFC1045c	42	7e-04
ADAM25	*Mus musculus*	PFI0980w	31	1.8
ADAM26	*Mus musculus*	PFC1045c	41	0.001
	Rattus norvegicus	PF11_0220	43	2e-04
ADAM27 Non-peptidase	*Mus musculus*	PFL1375w	32	0.43
ADAM28	*Homo sapiens*	MAL13P1.316	35	0.071
	Macaca fascicularis	MAL7P1.119	41	0.002
	Mus musculus	MAL6P1.24	36	0.042
	Rattus norvegicus	PFC1045c	37	0.022

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	**Species**	**Annotated Malaria Gene ID**	**Score**	**E-score**
ADAM29 Non-peptidase	*Homo sapiens*	MAL13P1.316	36	0.034
ADAM30	*Homo sapiens*	PFA0620c	43	2e-04
		PF11_0049	42	6e-04
ADAM31	*Mus musculus*	MAL7P1.167	31	1.7
	Rattus norvegicus	PF11_0127	41	0.001
ADAM32 Non-peptidase	*Homo sapiens*	PF14_0712	31	1.4
	Mus musculus	MAL8P1.124	38	0.008
	Rattus norvegicus	PF14_0084	33	0.096
ADAM33	*Homo sapiens*	PF07_0019	29	4.3
	Mus musculus	PFC1045c	31	1.9
	Rattus norvegicus	PF07_0019	31	2.1
ADAM36	*Mus musculus*	PFC1045c	38	0.013
ADAM38	*Mus musculus*	PFI0975c	36	0.041
ADAM39	*Mus musculus*	PFC1045c	43	4e-04
ADAMTS1	*Caenorhabditis elegans*	PFC0640w	78	2e-14
		PF13_0201	44	4e-04
		PFL0870w	44	4e-04
	Equus caballus	PFC0640w	44	2e-04
	Homo sapiens	PFC0640w	43	3e-04
	Mus musculus	PFC0640w	45	1e-04
	Rattus norvegicus	PFC0640w	47	2e-05
ADAMTS3 g.p	*Homo sapiens*	PFC0640w	48	2e-05
ADAMTS4 g.p	*Bos taurus*	No hits found		
	Cavia porcellus	PF08_0141	27	4.7
	Equus caballus	No hits found		
	Homo sapiens	PFC0640w	29	4.4
	Mus musculus	PFC0640w	29	7.5

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	**Species**	**Annotated Malaria Gene ID**	**Score**	**E-score**
	Rattus norvegicus	PFC0640w	28	6.2
ADAMTS5	*Bos taurus*	No hits found		
	Homo sapiens	MAL8P1.143	32	0.57
	Mus musculus	PFL0870w	35	0.11
	Oryctolagus cuniculus	PFL0870w	32	0.25
	Rattus norvegicus	PFL0870w	37	0.031
ADAMTS6	*Homo sapiens*	PFC0640w	33	0.31
ADAMTS7	*Homo sapiens*	PFL0870w	33	0.47
	Rattus norvegicus	PFC0640w	45	2e-04
ADAMTS8	*Homo sapiens*	PFC0640w	41	0.002
	Mus musculus	PFC0640w	44	2e-04
	Rattus norvegicus	PFC0640w	34	0.15
ADAMTS9	*Homo sapiens*	PFC0640w	87	6e-17
	Rattus norvegicus	PFC0640w	57	3e-08
ADAMTS10	*Homo sapiens*	PF11_0395	35	0.13
	Mus musculus	PFC0640w	38	0.020
	Rattus norvegicus	PFC0640w	35	0.15
ADAMTS12	*Homo sapiens*	PFC0640w	45	1e-04
	Mus musculus	PFC0640w	45	2e-04
	Rattus norvegicus	PFC0640w	39	0.008
ADAMTS13	*Homo sapiens*	PFC0640w	31	2.0
ADAMTS14	*Homo sapiens*	PFC0640w	39	0.006
ADAMTS15	*Homo sapiens*	PFC0640w	36	0.067
	Rattus norvegicus	PF13_0201	37	0.030
ADAMTS16	*Homo sapiens*	PFA0200w	46	7e-05
	Mus musculus	PFA0200w	43	5e-04
ADAMTS17	*Homo sapiens*	PFC0640w	50	5e-06
ADAMTS18	*Homo sapiens*	PFC0640w	49	8e-06
	Mus musculus	MAL6P1.116	29	3.3

Table 4 Homology search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the ADAM and ADAM-TS metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
ADAMTS19	*Homo sapiens*	PFC0640w	67	3e-11
	Mus musculus	PFC0640w	66	8e-11
ADAMTS20	*Homo sapiens*	PFC0640w	64	5e-10
	Mus musculus	PFC0640w	66	1e-10

Table 5 Homology search search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the MMP, MT-MMP and meprins metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
MMP1	*Bos taurus*	PF14_0060	31	0.80
	Equus caballus	PF14_0060	31	0.61
	Homo sapiens	PF13_0274	29	4.1
	Oryctolagus cuniculus	MAL8P1.73	31	1.1
	Ovis aries	MAL8P1.73	26	5.3
	Rana catesbeiana	MAL8P1.103	29	3.2
	Sus scrofa	MAL8P1.73	33	0.16
MMP2	*Bos taurus*	MAL7P1.92	31	0.90
	Canis familiaris	MAL7P1.92	29	4.3
	Equus caballus	MAL6P1.284	31	0.89
	Gallus gallus	MAL13P1.174	31	1.5
	Homo sapiens	MAL6P1.284	31	0.89
	Mus musculus	MAL7P1.92	31	1.5
	Oncorhynchus mykiss	PF14_0267	29	3.4
	Oryctolagus cuniculus	MAL7P1.92	28	7.8
	Oryzias latipes	PF14_0513	30	2.6
	Ovis aries	PFC0710w	30	0.89
	Paralichthys olivaceus	PFE1570c	32	0.40
	Rattus norvegicus	MAL7P1.92	31	1.5
	Sus scrofa	MAL7P1.92	31	0.90
	Xenopus laevis	MAL7P1.92	34	0.18
MMP3	*Bos taurus*	No hits found		
	Equus caballus	PF11_0371	31	1.1
	Felis catus	PFL0935c	28	5.7
	Homo sapiens	PFL1345c	31	1.1
	Mus musculus	PF07_0118	29	2.4
	Oryctolagus cuniculus	PF11_0415	29	3.3
MMP7	*Bos taurus*	PF10_0047	25	9.8
	Felis catus	No hits found		
	Homo sapiens	PF11_0483	36	0.013

Table 5 Homology search search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the MMP, MT-MMP and meprins metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Mus musculus	PF07_0017	30	0.72
	Ovis aries	PFB0345c	26	4.4
	Rattus norvegicus	PFL1240c	33	0.11
	Sus scrofa	PFL1620w	32	0.19
MMP8	*Cavia porcellus*	MAL7P1.167	25	4.7
	Homo sapiens	PFI1270w	32	0.35
	Mesocricetus auratus	No hits found		
	Mus musculus	MAL8P1.13	32	0.27
	Rattus norvegicus	PFC0005w	30	1.8
MMP9	*Ambystoma mexicanum*	PF10_0027	28	2.0
	Bos taurus	PF11_0467	29	3.7
	Canis familiaris	PF11_0007	29	3.7
	Canis familiaris	PF11_0362	30	0.33
	Cynops pyrrhogaster	PFI0500w	29	6.1
	Cyprinus carpio	PFA0445w	34	0.11
	Gallus gallus	PFA0445w	29	4.7
	Homo sapiens	PFA0445w	30	2.8
	Mus musculus	PF11_0362	30	2.2
	Oncorhynchus mykiss	PF11_0362	31	1.6
	Oryctolagus cuniculus	PFA0445w	31	1.7
	Oryzias latipes	PF11_0362	31	1.2
	Paralichthys olivaceus	PFA0445w	29	3.5
	Rattus norvegicus	PF11_0362	29	6.4
	Xenopus laevis	PFA0445w	30	2.7
MMP10	*Bos taurus*	PF14_0110	31	0.15
	Homo sapiens	PF11_0326	30	1.8
	Mus musculus	PFI0105c	34	0.12
	Rattus norvegicus	PF07_0118	31	1.1
MMP11	*Ambystoma mexicanum*	No hits found		

Table 5 Homology search search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the MMP, MT-MMP and meprins metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Homo sapiens	No hits found		
	Mus musculus	PF14_0159	28	7.4
	Oryzias latipes	PFL0250w	27	9.7
	Rattus norvegicus	No hits found		
	Xenopus laevis	PFD0900w	32	0.28
MMP12	*Homo sapiens*	PFI1120c	32	0.47
	Mus musculus	PFD1165w	32	0.37
	Oryctolagus cuniculus	MAL8P1.122	31	1.0
	Rattus norvegicus	PFD1165w	30	1.4
MMP13	*Ambystoma mexicanum*	PFI1365w	26	4.7
	Bos taurus (UniProt:O77656)	PF14_0541	31	0.81
	Bos taurus (GB:AF135235)	PF14_0541	28	1.7
	Brachydanio rerio	PFL1930w	27	4.6
	Canis familiaris	PF14_0541	31	0.30
	Cavia porcellus	PF11_0092	27	1.1
	Cynops pyrrhogaster	PFL2405c	31	0.81
	Equus caballus	PF14_0541	30	1.4
	Gallus gallus	No hits found		
	Homo sapiens	PF14_0541	31	0.81
	Mesocricetus auratus	PF14_0541	31	0.13
	Mus musculus	PF14_0541	30	1.4
	Oncorhynchus mykiss	PF14_0303	29	4.1
	Oryctolagus cuniculus	PF14_0541	31	0.65
	Rattus norvegicus	PF14_0541	31	0.64
	Sus scrofa	PF14_0541	31	0.078
	Xenopus laevis (UniProt:Q10835)	PFL2405c	31	1.1

Table 5 Homology search search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the MMP, MT-MMP and meprins metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Xenopus laevis (GB:U41824)	PFL2405c	32	0.36
MMP14/MT1-MMP	*Bos taurus*	PF13_0003	29	2.6
	Brachydanio rerio	PF13_0003	29	2.6
	Canis familiaris	PFD0110w	26	1.3
	Capra hircus	PF11_0392	29	3.0
	Cricetulus griseus	PF11_0392	29	3.9
	Homo sapiens	PF13_0003	29	2.7
	Mus musculus	PF13_0003	29	2.9
	Oryctolagus cuniculus	PF11_0392	29	3.9
	Ovis aries	PF13_0003	29	1.1
	Rattus norvegicus	PF13_0003	29	3.0
	Sus scrofa	PF11_0392	29	3.4
MMP15/MT2-MMP	*Homo sapiens*	PFB0110w	29	5.4
	Mus musculus	PFL0935c	33	0.30
MMP16/MT3-MMP	*Gallus gallus*	No hits found		
	Homo sapiens	PF14_0359	31	1.2
	Mus musculus	PF14_0359	29	2.8
	Rattus norvegicus	PF14_0359	29	3.2
	Scyliorhinus torazame	PFL1535w	29	3.7
MMP17/MT4-MMP	*Homo sapiens*	MAL6P1.39	29	5.4
	Mus musculus	MAL6P1.39	29	4.5
MMP18	*Xenopus laevis*	PFB0345c	30	1.4
MMP19	*Homo sapiens*	PFE0930w	28	6.4
	Mus musculus	PF10_0326	28	7.9
	Rattus norvegicus	No hits found		
MMP20	*Bos taurus*	MAL7P1.155	32	0.37
	Cynops pyrrhogaster	PFI1100w	30	1.5
	Homo sapiens	MAL7P1.155	34	0.095

Table 5 Homology search search by BLASTP with default setting of annotated *P. falciparum* (http//plasmodb.org) using the MMP, MT-MMP and meprins metalloprotease protein sequences from Merops databases Release 6.60 (http://merops.sanger.ac.uk) (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
	Mus musculus	MAL7P1.155	39	0.002
	Rattus norvegicus	PF13_0239	28	4.7
	Sus scrofa	PF14_0172	33	0.13
MMP21	*Cynops pyrrhogaster*	PF11_0268	33	0.24
	Homo sapiens	PFL0255c	33	0.17
	Mus musculus	PF11_0275	32	0.37
	Rattus norvegicus	PF11_0275	32	0.46
	Xenopus laevis	PFI0240c	36	0.024
MMP22	*Gallus gallus*	PF14_0066	31	0.81
	Homo sapiens	PFL0115w	31	1.2
	Rattus norvegicus	PF14_0690	29	3.2
	Tupaia belangeri	PFL0115w	34	0.13
MMP23	*Homo sapiens*	No hits found		
	Mus musculus	No hits found		
	Rattus norvegicus	No hits found		
MMP25/MT6-MMP	*Homo sapiens*	PF13_0153	28	6.5
MMP26	*Homo sapiens*	PF11_0452	30	0.73
	Macaca mulatta	PF14_0402	28	0.73
MMP28	*Homo sapiens*	No hits found		
	Mus musculus	No hits found		
	Rattus norvegicus	No hits found		
Meprin alpha	*Homo sapiens*	PF10_0045	30	3.0
	Mus musculus	PFI1410c	33	0.26
	Rattus norvegicus	PF07_0107	30	3.0
Meprin beta	*Homo sapiens*	PF14_0412	32	0.19
	Mus musculus	PF14_0412	36	0.025
	Rattus norvegicus	PFC0940c	39	0.003
	Sus scrofa	PF10_0174	25	6.3

Table 6 Selected annotated malaria protein sequences showed significant matching E-score at the level of N x 10^{-4} or less.

Metalloprotease family subclasses	**Species**	**Annotated Malaria Gene ID**	**Score**	**E-score**
ADAM1	*Gorilla gorilla*	MAL13P1.316	43	4e-04
	Homo sapiens	MAL13P1.316	46	5e-05
	Pongo pygmaeus	MAL13P1.316	44	2e-04
ADAM5 Non-peptidase	*Cavia porcellus*	PFI0975c	46	5e-05
ADAM6 Non-peptidase	*Oryctolagus cuniculus* (ADAM 6d)	PF13_0168	41	9e-04
	Mus musculus	PFI0975c	45	1e-04
	Mus musculus (gp product)	PFI0975c	44	1e-04
ADAM9	*Xenopus laevis*	PF14_0194	44	2e-04
ADAM17	*Homo sapiens*	MAL13P1.316	52	9e-07
ADAM23 Non-peptidase	*Homo sapiens*	PFC1045c	43	5e-04
	Mus musculus	PFC1045c	42	6e-04
ADAM24	*Rattus norvegicus*	PFC1045c	42	7e-04
ADAM26	*Rattus norvegicus*	PF11_0220	43	2e-04
ADAM30	*Homo sapiens*	PFA0620c	43	2e-04
		PF11_0049	42	6e-04
ADAM39	*Mus musculus*	PFC1045c	43	4e-04
ADAMTS1	*Caenorhabditis elegans*	PFC0640w	78	2e-14
		PF13_0201	44	4e-04
		PFL0870w	44	4e-04
	Equus caballus	PFC0640w	44	2e-04
	Homo sapiens	PFC0640w	43	3e-04
	Mus musculus	PFC0640w	45	1e-04
	Rattus norvegicus	PFC0640w	47	2e-05
ADAMTS3 g.p	*Homo sapiens*	PFC0640w	48	2e-05
	Homo sapiens	MAL13P1.316	52	9e-07
ADAMTS7	*Rattus norvegicus*	PFC0640w	45	2e-04
ADAMTS8	*Mus musculus*	PFC0640w	44	2e-04
ADAMTS9	*Homo sapiens*	PFC0640w	87	6e-17
	Rattus norvegicus	PFC0640w	57	3e-08

Table 6 Selected annotated malaria protein sequences showed significant matching E-score at the level of N x 10^{-4} or less (continued).

Metalloprotease family subclasses	Species	Annotated Malaria Gene ID	Score	E-score
ADAMTS12	*Homo sapiens*	PFC0640w	45	1e-04
	Mus musculus	PFC0640w	45	2e-04
ADAMTS16	*Homo sapiens*	PFA0200w	46	7e-05
	Mus musculus	PFA0200w	43	5e-04
ADAMTS17	*Homo sapiens*	PFC0640w	50	5e-06
ADAMTS18	*Homo sapiens*	PFC0640w	49	8e-06
ADAMTS19	*Homo sapiens*	PFC0640w	67	3e-11
	Mus musculus	PFC0640w	66	8e-11
ADAMTS20	*Homo sapiens*	PFC0640w	64	5e-10
	Mus musculus	PFC0640w	66	1e-10

4.1.6 Zinc-binding motif search for malaria metalloprotease candidate genes

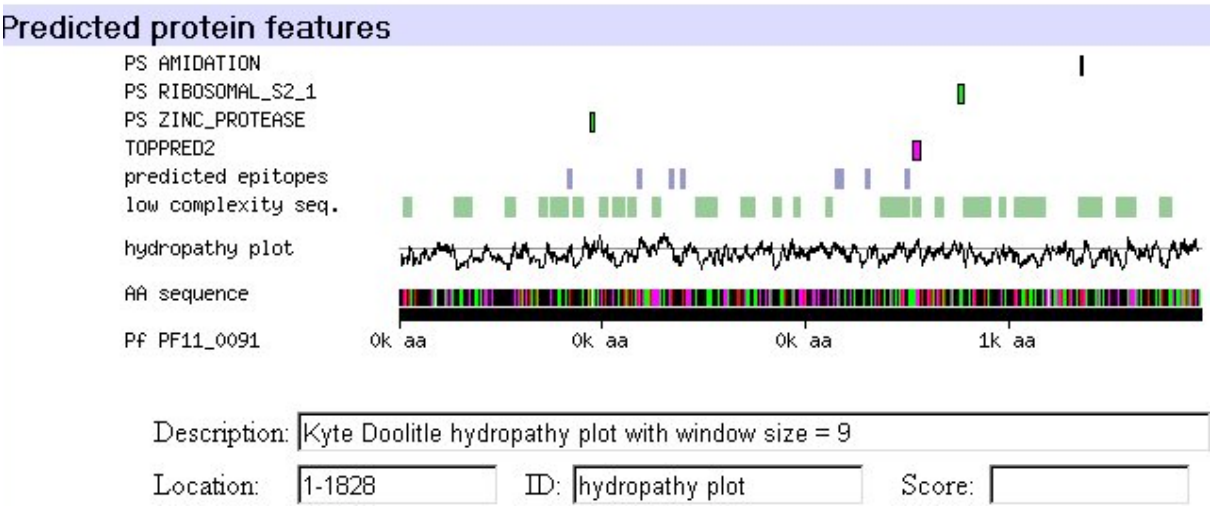
The presence of zinc binding signature, HEXXH motif can be indicative of metalloproteases. Consensus pattern for zinc-binding region signature of neutral zinc metallopeptidases, PS00142 obtained from Prosite (http://www.expasy.org/prosite) is [GSTALIVN]-x(2)-H-E-[LIVMFYW]-{DEHRKP}-H-x-[LIVMFYWGSPQ]. Motif search of the *P. falciparum* annotated protein sequences (http://www.plasmodb.org, release 4.2) with this pattern gave 19 matching sequences. Their annotated gene names and functions are listed in Table 7. Location of zinc-binding region on PF11_0091 amino acid sequence resulting from PS00142 search is given in Figure 16A.

However, out of 19 sequences, we found that 3 annotated genes, PF10_0205, PF11_0091 and PF14_0480 having this signature and were highly expressed during schizont developmental stage. Time course analysis of PF10_0205 mRNA expression showed that it was maximally expressed at 48 hr post invasion whereas those of PF11_0091 and PF14_0480 had the highest expression at 35-42 hr post invasion followed by a gradual decrease (Figure 17) (105). Proteomics data revealed that PF11_0091 was present in the invasive merozoite stage and was predicted to have transmembrane region (Figure 16). These data indicated that the malaria parasite possibly employed metalloprotease during host cell invasion.

Table 7 Zinc binding motif (PS00142 from Prosite, http://www.expasy.org/prosite) search result of annotated *P. falciparum* (http://plasmodb.org, release 4.2). Annotated genes marked with asterisk (*) showed maximum expression at schizont stage.

Annotated Malaria Gene ID	Function
PFC0790w	Hypothetical protein
PFD0425w	Hypothetical protein
PFD0070c	Rifin
MAL6P1.151	Transportin
MAL8P1.144	Hypothetical protein
PF10_0224	Putative dynein heavy chain
PF10_0205*	Hypothetical protein
PF10_0092	Hypothetical protein
PF10_0058	Hypothetical protein
PF11_0091*	Hypothetical protein
PFL1315w	Hypothetical protein
PF14_0396	Hypothetical protein
PF14_0692	Hypothetical protein
PF14_0480*	Hypothetical protein
MAL13P1.56	M1-family aminopeptidase
PF13_0028	Hypothetical protein
MAL13P1.184	Putative endopeptidase
MAL13P1.191	Hypothetical protein
PF13_0260	Hypothetical protein

A.



B.

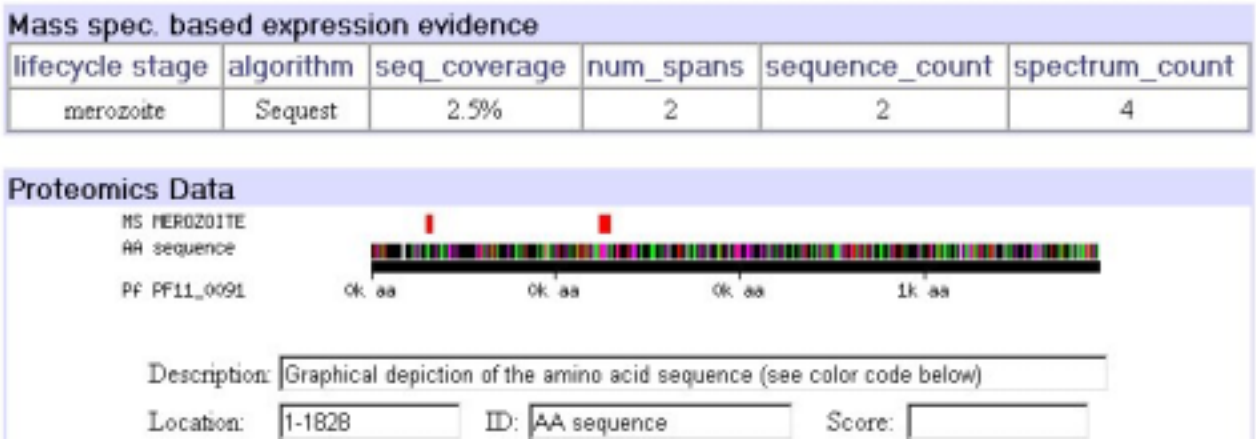
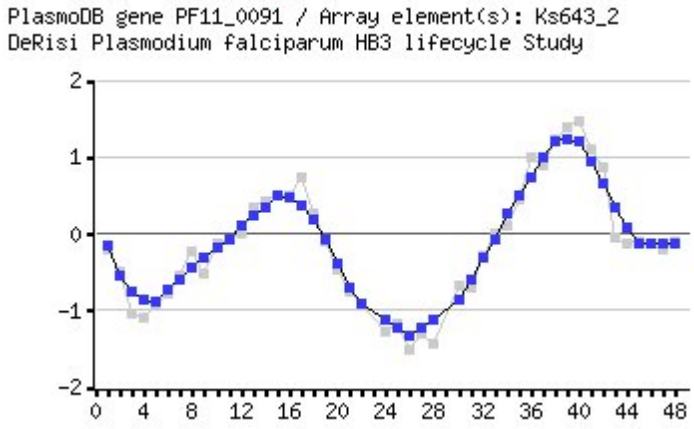
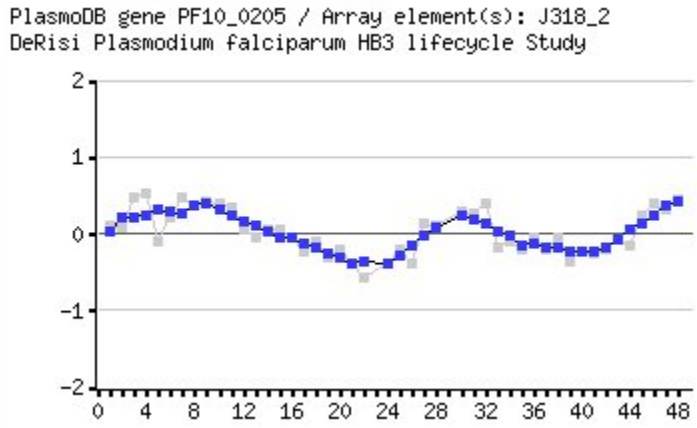


Figure 16 A. Zinc-binding region motif in PF11_0091 is found among other predicted protein features (http://www.plasmodb.org, release 4.2). It is designated as PS ZINC_PROTEASE of which has been found in other 18 annotated amino acid sequences listed in Table 5. This protein also has transmembrane region as predicted by TOPPRED2. B. This protein is present in merozoite stage malaria parasite as shown by mass spectrophotometry based expression evidence.

A.



B.



C.

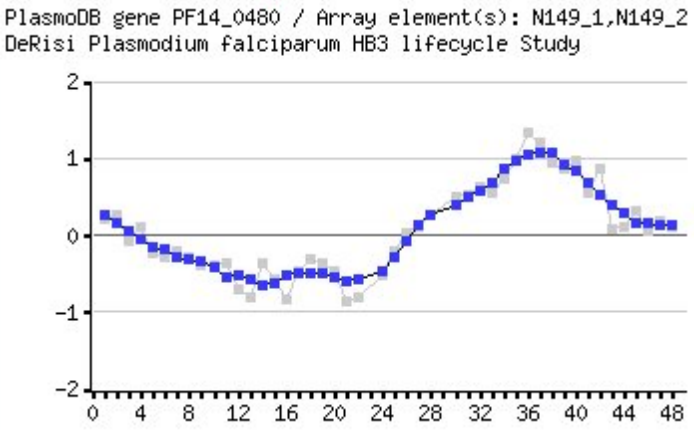


Figure 17 Time course analysis of (A) PF11_0091, (B) PF10_0205, and (C) PF14_0480 mRNA expression (105) (http://www.plasmodb.org, release 4.2). X-axis represents time in hr after adding synchronized culture of HB3 parasites to fresh blood. Black plot represents averaged smoothed normalized log base(2) of Cy5/Cy3 while gray plot is an averaged normalized log base(2) of Cy5/Cy3 for each transcript.

4.2 Study on the regulation mechanism of this metalloprotease using inhibitor combination test

Complete isobologram was not performed but only the effects of staurosporine on invasion inhibitory effect of 1,10-phenanthroline, staurosporine on GM6001 inhibition, and GM6001 on 1,10-phenanthroline inhibition were tested. The curves were drawn in fractional inhibitory concentration (FIC) units. The FIC is a mathematical representation of whether the effect of one inhibitor is reduced, unchanged, or increased in the presence of the second inhibitor.

4.2.1 1,10-Phenanthroline and staurosporine combination

Staurosporine has been reported to inhibit malaria merozoite invasion of rbc (87). It is a broad spectrum protein kinase inhibitor including PKA, MLCK, CAM kinase and PKC. Shedding by ADAMs is often in response to PKC activation (13). Therefore, the effect of these two combined inhibitors were tested.

Although staurosporine at low concentrations, appeared to antagonize 1,10-phenanthroline, this is not significant statistically. At higher concentrations, the effects were additive (Figure 18).

4.2.2 Staurosporine and GM6001 combination

As we used GM6001 to define a more specific group of metalloproteases, it was worth trying its combination with protein kinase inhibitor, staurosporine. The combined effects of staurosporine and GM6001 were additive (Figure 19).

4.2.3 1,10-Phenanthroline and GM6001 combination

Both are metalloprotease inhibitors, the former being a commonly used chelator and the latter a synthesized peptide having broad spectrum inhibitory effect but with more specificity to MMPs and ADAMs groups. Low concentrations of GM6001 showed a synergistic effect with 1,10-phenanthroline (Figure 20).

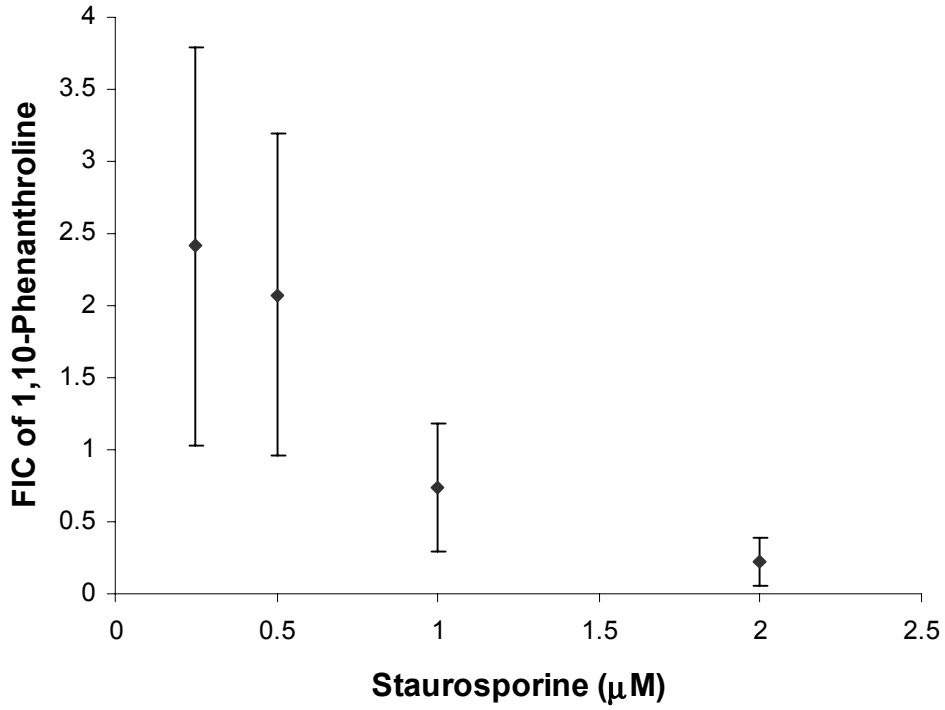


Figure 18 Effect of the combination of staurosporine and 1,10-phenanthroline on *P. falciparum* invasion. Synchronous schizont at 2% parasitemia and 1.5% hematocrit were treated with both inhibitors for 17-18 hr at 37 ºC. Thin smears were prepared and light microscopic examinations were done. IC_{50} of 1,10-phenanthroline alone and in the presence of staurosporine at sub-inhibitory concentrations (0.25, 0.5, 1, and 2 μ M) were determined. IC_{50} of staurosporine is 2 μM. Fractional inhibitory concentrations (FIC) were plotted.

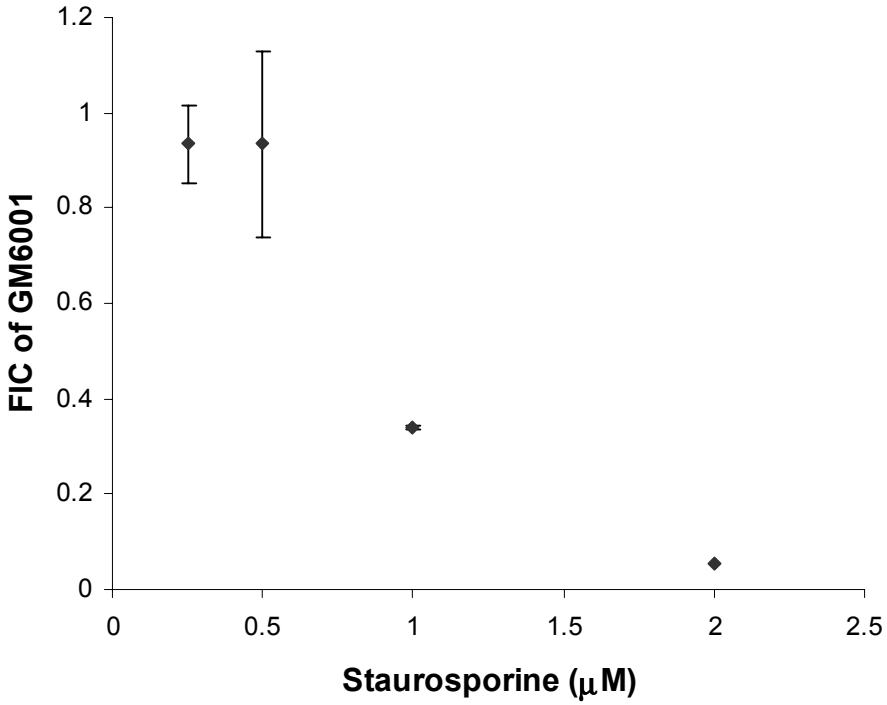


Figure 19 Effect of the combination of staurosporine and GM6001 on *P. falciparum* invasion. Synchronous schizont at 2% parasitemia and 1.5% hematocrit were treated with both inhibitors for 17-18 hr at 37 ºC. Thin smears were prepared and light microscopic examinations were done. IC_{50} of GM6001 alone and in the presence of staurosporine at sub-inhibitory concentrations (0.25, 0.5, 1, and 2 μM) were determined. IC_{50} of staurosporine is 2 μM. Fractional inhibitory concentrations (FIC) were plotted.

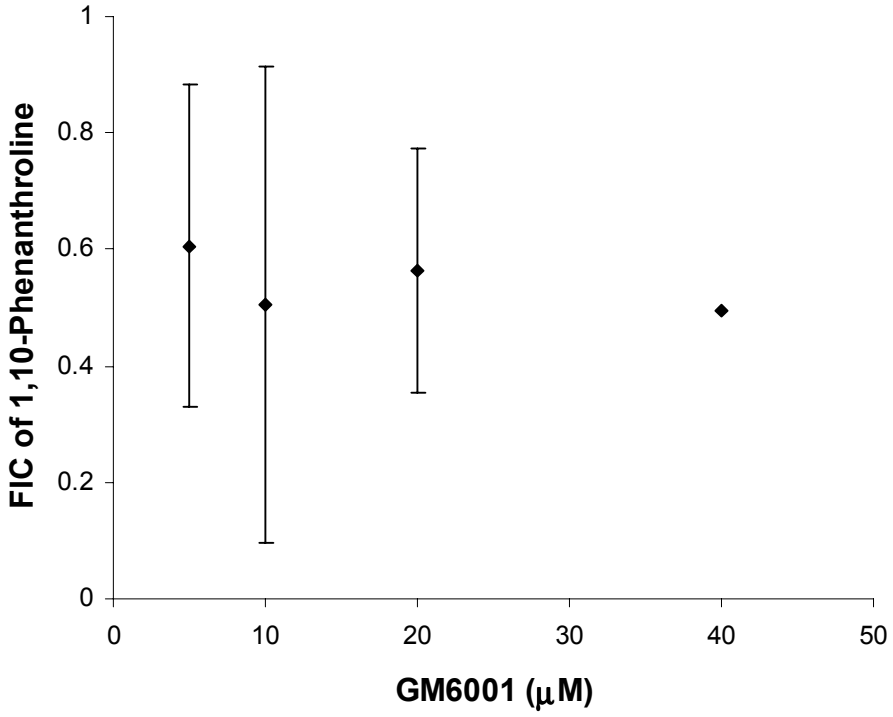


Figure 20 Effect of the combination of GM6001 and 1,10-phenanthroline on *P. falciparum* invasion. Synchronous schizont at 2% parasitemia and 1.5% hematocrit were treated with both inhibitors for 17-18 hr at 37 ºC. Thin smears were prepared and light microscopic examinations were done. IC_{50} of 1,10-phenanthroline alone and in the presence of GM6001 at sub-inhibitory concentrations (5, 10, 20, and 40 μM) were determined. IC_{50} of GM6001 is 112 μM. Fractional inhibitory concentrations (FIC) were plotted.

CHAPTER 5
DISCUSSION

Invasion of the host red cell is important for malaria parasite survival. During invasion of an erythrocyte by a malarial merozoite, a complex sequence of interaction occurs between the two cells. These compose of recognition, reorientation, attachment, junction formation and parasite entry to host cell. However, the molecular mechanism of red cell invasion by the malaria parasite is still a poorly understood process.

Upon invading erythrocyte, the merozoite surface coat is removed. Thus, modification of cell surface proteins by proteolytic cleavage appears to be essential for invasion to occur. Of particular interest is how the merozoite sheds the extracellular domain of such membrane proteins during invasion. This has led to the suggestion that parasite surface coat removal may mediated by the parasite surface protease of which is activated upon the entry of the parasite.

Several parasite proteases have been identified, some of which, because of their location, represent possible candidates for a role in merozoite invasion. These include a membrane-bound calcium-dependent merozoite serine protease capable of mediating multiple proteolytic cleavages of *P. falciparum* merozoite protein, MSP-1 on the surface coat of the invading merozoite, and the shedding of apical membrane antigen-1 (PfAMA-1), a micronemal protein, prior to erythrocyte entry (5, 6), and a GPI-anchored serine protease activated by PI-PLC cleavage (7).

Cell surface metalloproteases constitute the major type for proteolytic processing of the ectodomain (extracellular domain) of membrane proteins (12). A model for processing of the membrane proteins by this group of membrane-anchored metalloprotease usually requires both the enzyme and its substrate to be in the same membrane to achieve proteolysis (13). This system exists in mammalian eukaryotes. It is possible that the parasite membrane-anchored protease may belong to this class of protease that is responsible for shedding of the merozoite surface coat**.** In the present

study the involvement of *P. falciparum* merozoite metalloprotease in erythrocyte invasion and its possible biochemical regulation have been investigated.

5.1 Development of invasion assay

Previous attempts have been made to set up an invasion assay by using [^{3}H]-hypoxanthine incorporation. Reinvasion inhibition assay using this [^{3}H]-hypoxanthine incorporation method for invasion has been reported (106). The experiment was started from incubation of trophozoite stage parasites with drug for 20-22 hr, drug was removed, and hypoxanthine added for 20-22 hr before harvesting. IC_{50} obtained represents invasion inhibition (106).

On the other hand, we have started from schizonts treated with inhibitor, then washing out the inhibitor at ring stage and measuring the reduction of the incorporation of [^{3}H] hypoxanthine at schizont stage. IC_{50} of inhibition of invasion by 1,10-phenanthroline using the [^{3}H] hypoxanthine incorporation method was 5.3 ± 1.5 μM whereas a value of 24.7 ± 7.2 μM was obtained from microscopic counting. The lower value IC_{50} value obtained by the [^{3}H]hypoxanthine incorporation method indicated that the chelator was already affecting ring stage viability and/or that the chelator still was retained within the infected rbc and thus affected parasite development. Thus, the [^{3}H] hypoxanthine incorporation assay appeared not to be appropriate for the evaluation of inhibitor effect on parasite invasion.

Flow cytometric analysis of asexual erythrocytic malaria parasites has been reported (107). The advantages of flow cytometry are the speed of measurement, reproducibility, and the ability to process large numbers of parasites samples. However, in our hands there was a discrepancy between IC_{50} obtained from the same set of experiment using microscopic examination (22.8 μM) and FACS analysis (7.6 μ M). Percent parasitemia as determined by flow cytometry did not correspond with percentage established from microscopy of Giemsa-stained blood films. This could result from long time storage of glutaraldehyde-fixed cells that can cause an increase in background fluorescense of uninfected rbc, resulting in overlap of fluorescence signal with infected rbc (107).

Thus, visual counting of Giemsa-stained thin films by microscopy was selected as our method of choice in experiments to determine the effect of metalloprotease inhibitor on efficiency of malaria merozoite invasion of erythrocyte.

5.2 Involvement of metalloprotease in erythrocyte invasion of malaria parasite

Application of specific protease inhibitor on schizonts to ring development has long been used for identifying the role of proteases in invasion of rbc by malaria merozoites (68, 108). In this study, the presence of malaria metalloprotease involved with erythrocyte invasion has been demonstrated by adding metalloprotease inhibitors to schizont stage parasites. The reduction of the number of ring stage parasites indicates inhibition of invasion. This has been monitored by visual counting of the number of parasites under a light microscope. Other methods used were *in vitro* detection of protease activity by zymography and searching of protease homologs by data mining.

The metalloprotease inhibitors, 1,10-phenanthroline and GM6001 were able to inhibit invasion at IC_{50} of 24.7 ± 7.2 and 112 ± 20 μM, respectively. However, there is a major problem in this type of invasion assay; the reduction may not only be due to the invasion step since at least three processes are involved, namely, schizont-to-merozoite development, merozoite release and invasion. To single out the merozoite invasion step, the exclusion of its release is required. Treatment of middle-stage schizonts for 13-15 hr with E64, a cysteine protease inhibitor, can inhibit merozoite release resulting in an accumulation of schizonts (9). Therefore it was used as a control for release inhibition assay. In contrast to E64, 1,10-phenanthroline reduced number of rings without any effect on schizont accumulation. This demonstrated that 1,10-phenanthroline has no effect on merozoite release. Thus, the reduction of ring numbers should result from its inhibitory effect on invasion.

To confirm this, middle stage schizonts (5-7 nuclei) were treated with E64, a cysteine protease inhibitor, for 6-8 hr, so that merozoites could be obtained as a cluster within a parasitophorous vacuole membrane (PVM), called PVM-enclosed merozoite structure (PEMS) (69). This PEMS was then used for invasion assay and for *in vitro*

detection of protease activity. IC_{50} of 1,10-phenanthroline for PEMS-derived merozoite invasion was 28.9 ± 7.7 μM, which was not significantly different from that for schizont-derived merozoites, indicating that the metal chelator affected only the invasion step and not schizont maturation nor merozoite release.

Besides inhibitor studies, an alternative approach would be to demonstrate protease activity *in vitro*. A protein substrate assay can be a sensitive and powerful tool for studying protease. Zymography using co-polymerizing gelatin as a protein substrate is a common method to show the protease activity. *P. falciparum* malaria proteases from different stages have been detected by this method (60) as well as the heat shock metalloprotease activity of *P. vivax* (109). Therefore, gelatin zymography was used to detect metalloprotease activity from PEMS. However, our zymogram system could not detect metalloprotease activity in PEMS. This lack of success may result from its inactivity at neutral pH, irreversible denaturation by SDS during electrophoresis, inability to degrade the gelatin substrate, lack of an essential factor in incubation solution and/or an inadequate quantity of parasites assayed. And possibly, if the enzyme is activated upon cell contact, therefore the activity will not detected by this assay. Another explanation is that during the isolation of parasites for zymography, which involved use of buffer containing the detergent saponin, cytoplasmic enzymes may have been washed out from the parasite preparations.

On the other hand, there are evidences for MMP-like activity in schizont stage malaria parasite by zymography (110). Among models trying to explain their findings, they have suggested that proteolysis of the antiinflammatory cytokine transforming growth factor-beta could be mediated by a single bifunctional molecule such as a member of the disintegrin and MMP domain TSP type-1 zinc MMP family of enzymes (110).

We have also tried looking for malaria metalloprotease genes by BLASTP for membrane-anchored types including matrix (MMP) and disintegrin (ADAM) metalloproteases and zinc-binding motif search in the *Plasmodium* genome database (http://www.plasmodb.org). However, we found neither malaria metalloprotease-like gene nor its homolog from similarity search. Annotated sequences listed in Table 6 were explored for homologies. However, no similarity was found in protease domain (data not shown).

There are evidences showing that parasites closely related to *Plasmodium* contain metalloprotease. A calcium-dependent neutral metalloprotease secreted from the apicomplexan parasite *Toxoplasma gondii* detected by gelatin zymogram has been recently reported (111). This protease activity was observed only in the excretory/secretory proteins not in the somatic extracts of tachyzoites. This suggests that excretion/secretion is a critical event for the activation of enzymes (111). It is possible that its biological function may involve with invasion process of Apicomplexa that is accompanied by the sequential secretion of parasite organelles.

In contrast to similarity search, motif search of annotated protein sequences containing zinc-binding region suggested a possibility of malaria making use of metalloprotease during host cell invasion. All 19 sequences containing this motif were not included within those of homology search results. However, our result is not similar to a recent data-mining of *P. falciparum* genome which has identified 92 putative proteases, including 17 newly-found metalloprotease homologs (112). We were unable to identify falcilysin by this search and only 3 sequences, PF10_0058, MAL13P1.56 and MAL13P1.184 are similar to that reported by Wu *et al*. PF11_0091 is a good candidate for metalloprotease and other metal-requiring protein from its striking characteristics. Apart from having zinc-binding domain and maximum expression at schizont developmental stage, this protein is present in invasive merozoite stage as well. However, this remains as prediction until proven by experiments for its enzymatic activity and its involvement of rbc invasion.

These results indicate that merozoite may use metalloprotease(s) during its invasion of host cells. Inhibition by GM6001 has implicated the role of a specific group of metalloproteases and this could be of different from that inhibited by 1,10-phenanthroline. Inhibition by 1,10-phenanthroline, although reflecting metalloprotease in general, may involve other metal-requiring enzymes, and this cannot as yet be excluded. These metal-requiring enzymes appear to be localized intracellularly due to the reduced inhibitory effect of bathophenanthroline in comparison to that of 1,10-phenanthroline.

5.3 Combination effect of the inhibitor on parasite invasion

To probe for the mechanism regulating this metalloprotease the effects of the combination of inhibitors were employed. Understanding the mechanisms regulating parasite invasion could thus be useful in the design of new therapeutic strategies aimed at blocking invasion.

5.3.1 Staurosporine and 1,10-phenanthroline combination

The sensitivity of invasion to kinase inhibitors indicates an essential role for these activities in merozoite release as well as in the reinvasion process (87, 88). Staurosporine, inhibitor of protein kinase with broad spectrum activity, had additive effect (at high concentrations) to 1,10-phenanthroline indicating that the two inhibitors acted independently. Targets for 1,10-phenanthroline could be metalloprotease or other metal requiring enzymes as previously discussed. However, staurosporine at low concentrations, had antagonistic effect to 1,10-phenanthroline indicating that metal-requiring enzyme activity had become less sensitive to the metal chelator. One possible explanation is that phosphorylation is involved in the role of some of the metal-requiring enzymes in invasion.

As reviewed in Chapter II Literature Review, staurosporine (Figure 8) is a potent inhibitor of several different serine/threonine and tyrosine protein kinases (89). Similar invasion inhibition, but more specifically defined effect of staurosporine to microneme secretion step of *Toxoplasma gondii* invasion, has been demonstrated (85). Micronemes are the first secretory organelles to be discharged during *Toxoplasma gondii* invasion. Because of the close relationship between *Toxoplasma* and *Plasmodium*, it is expected that fundamental findings in *Toxoplasma gondii* will be applicable to the malaria parasites. From this point of view, possible protein kinase(s) inhibitable by staurosporine are cGMP-dependent serine/threonine protein kinase (92) and calmodulin-like domain protein kinase (CDPK) (93). Protein kinase C (PKC) is also likely to be another target as suggested by Ward *et al.* (87) working on the role of protein kinase and staurosporine inhibition in malaria merozoite invasion. There is no experimental evidence for the presence of malaria PKC but recent literature of the

intraerythrocytic developmental cycle of malaria transcriptome reveals that six PKC homologs and three Ca^{2+}-dependent protein kinases among 12 serine/threonine protein kinases that are expressed in the schizont stage parasites (105). Guanylyl cyclase peptides were detectable by proteome analysis of gametocytes, and trophozoites in *Plasmodium* genome database (http://plasmodb.org/).

Additionally, staurosporine is able to inhibit protein tyrosine kinase (PTK) (89). PTK activity has been shown in *P. falciparum* (95) although the genome of *P. falciparum* appears not to possess genes encoding conventional receptor-linked tyrosine kinase (96). There is a stage specific increase in the activity, with schizont stage parasites having maximum level of PTK. Conversion of the schizont stage to ring stage via release of merozoites was associated with a decrease in PTK activity (95).

5.3.2 Staurosporine and GM6001 combination

Combination of staurosporine and GM6001 resulted in additive effect. It seems that they acted independently. The metalloprotease sensitive to GM6001 could be of a type different from that inhibited by 1,10-phenanthroline. It could be a MMP-like enzyme because GM6001 is a specific inhibitor of both disintegrin (ADAM) and matrix (MMP) metalloprotease (76, 77). Recent literature has reported the presence of malaria MMP-like enzyme inhibitable by 1,10-phenanthroline and dual MMP-2/MMP-9 inhibitor in schizont lysate by gelatin zymography (110). It is worth noting that we could not detect this MMP by amino acid sequence similarity search as discussed above. The parasite *Toxoplasma gondii* has a secreted calcium-dependent neutral metalloprotease (111). This protease activity was observed only in the excretory/secretory proteins suggesting the importance of this event for the activation of these enzymes (111).

5.3.3 1,10-Phenanthroline and GM6001 combination

This combination of the two metalloprotease inhibitors gave additive effect. This indicated that there are two different types of metalloprotease working

independently in malaria invasion. One may belong to MMP-like family (GM6001-inhibited) and the other is another type of metalloprotease or a metal-requiring enzyme (1,10-phenanthroline-inhibited).

In summary, these studies on inhibitor combinations indicated that there are protein kinase(s) and metalloprotease (MMP-like or metal-requiring enzymes) operating during rbc invasion by the merozoite. However the data are only indicative and the genes of these enzymes need to be isolated, expressed and their protein products investigated for their sensitivity to the inhibitors. Furthermore, gene knockout experiments will have to be conducted to validate their potential roles as targets for future antimalarial drugs development.

CHAPTER 6
CONCLUSION

Invasion of the host red cell is important for malaria parasite survival. However, the molecular mechanism of red cell invasion by the malaria parasite is still a poorly understood process. Upon invading erythrocyte, the merozoite surface coat is removed. Thus, modification of cell surface proteins by proteolytic cleavage appears to be essential for invasion to occur. This has led to the suggestion that parasite surface coat removal may mediated by the parasite surface protease which is activated upon the entry of the parasite.

Several parasite proteases represent possible candidates that are involved in merozoite invasion. These includes a membrane-bound calcium-dependent merozoite serine protease capable of mediating multiple proteolytic cleavages of *P. falciparum* merozoite protein, MSP-1 on the surface coat of the invading merozoite, and the shedding of apical membrane antigen-1 (PfAMA-1), a micronemal protein, prior to erythrocyte entry (5, 6) and a GPI-anchored serine protease activated by PI-PLC cleavage (7).

Involvement of metalloprotease has been demonstrated by testing the effects of metalloprotease inhibitors on invasion of both schizont- and PEMS-derived merozoites. Release inhibition assay was also performed by using E64, a cysteine protease inhibitor, to discriminate whether the effect of inhibitor was on release or invasion of free merozoites. Attempts were also made to show *in vitro* metalloprotease activity and to find its homologue in the *Plasmodium* genome. Mechanism regulating this metalloprotease has been demonstrated by the combination of inhibitors to see whether signal transduction component (s) or pathway was possibly involved with rbc invasion by malaria merozoite.

The metalloprotease inhibitors, 1,10-phenanthroline and GM6001, were able to inhibit invasion at IC_{50} of 24.7 ± 7.2 and 112 ± 20 μM. IC_{50} of 1,10-phenanthroline

for PEMS-derived merozoite invasion was 28.9 ± 7.7 μM, which was not significantly different from that for schizont-derived merozoites, indicating that the metal chelator affected only the invasion step and not schizont maturation nor merozoite release. 1,10-phenanthroline had no effect on merozoite release and its inhibitory effect was on invasion. GM6001 has implicated the role of a specific group of metalloproteases and this could be of different from that inhibited by 1,10-phenanthroline. Inhibition by 1,10-phenanthroline, although reflecting metalloprotease in general, may involve other metal-requiring enzymes, and this cannot as yet be excluded.

However, malaria metalloprotease-like activity was not detected. Possible explanations include limited amount of parasites available, susceptibility of the enzyme to denaturation during electrophoresis, and the narrow substrate specificity. If the enzyme has to be activated upon cell contact, therefore, it will not be detected by our system. However, malaria MMP-like activity has recently been shown by zymography in serum-free preparations of schizont-infected erythrocytes (110). There is also evidence of secreted calcium-dependent neutral metalloprotease in *Toxoplasma gondii* (111). Its biological function may involve with the invasion process as Apicomplexa invasion process is accompanied by secretion of the parasite organelles.

Motif search of annotated protein sequences containing zinc-binding region suggested that malaria parasite makes use of metalloprotease during host cell invasion. PF11_0091 characteristics of having zinc-binding domain, maximum expression at schizont developmental stage, and its detection in merozoite stage, makes it a good candidate for metalloprotease. However, this remains as prediction until proven by experiments demonstrating its enzymatic activity and involvement in rbc invasion.

Mechanism regulating this metalloprotease has been demonstrated by inhibitor combination studies to see whether signaling component (s) and/or pathway possibly was involved with rbc invasion by malaria merozoite. Staurosporine, inhibitor of protein kinase with broad spectrum activity, viz. cGMP-dependent serine/threonine protein kinase, CDPK, PKC and PTK, had an additive effect with 1,10-phenanthroline. and GM6001, indicating that the metalloproteases and signaling kinases were acting independently.

Combination 1,10-phenanthroline and GM6001 also gave an additive effect, indicating that there may be two different types of metalloprotease working in malaria

invasion, one metalloprotease belonging to MMP-like family (GM6001-inhibited) and the other another type of metalloprotease or metal-requiring enzyme (1,10-phenanthroline-inhibited).

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