Chapter 6

Effect of Vasicine, Vasicinone and Embelin on Some Important Targets of Plasmodium falciparum

6.1 INTRODUCTION

Plasmodium falciparum, the most dangerous human malaria parasite, has 14 chromosomes, approximately 5,300 protein-encoding genes – almost two thirds of which appear to be unique to the organism – and about 208 genes known to be involved in the evasion of the host immune system. This genomic information can be used to exploit new chemotherapeutic targets, as well as antigens for potential vaccines. With scientific advancements, some promising targets are developed (Figure 6.1) e.g. malarial parasite protease inhibition, histidine rich protein (HRP2) inhibition, fatty acid biosynthesis (FAB) inhibition, plasmodium protein kinase inhibition, protein farnesyltrasferase inhibition for the malarial parasite control. With these tools in hand, phytomedicine research has a good chance of contributing to the development of new drugs for combating malaria (Pall & Shukla, 2003). Identifying compounds that bind to and modulate biological targets implicated in disease pathways is a fundamental step in the drug discovery process (Houston et al., 2008). This prompted us to investigate whether these isolated compounds have any effect on different targets of malarial parasite and to establish the probable mechanism of action.

![Figure 6.1 New drug targets and site of antimalarial drug action (Ridley, 2003).]
6.2 EFFECT OF VASICINE, VASICINONE AND EMBELIN ON SOME IMPORTANT TARGETS OF \textit{PLASMODIUM FALCIPARUM}

Most of the established drugs for malaria has developed resistance for malarial parasite due to mutation in the respective targets. So newer compounds acting on valid targets for \textit{P. falciparum} are likely to be effective. Therefore we investigated the effect of vasicine, vasicinone and embelin on some of the important targets of \textit{P. falciparum}.

6.2.1 Effect of vasicine, vasicinone and embelin on heme

During its 48-hour cycle of invasion, growth and release from an infected erythrocyte, the malaria parasite degrades up to 80% of the haemoglobin in the host cell and releases heme which is toxic to parasite (Wellems and Plowe, 2001). Neutralisation of heme occurs mainly by haemozoin formation. Inhibition of hemozoin formation took place through different routes viz drug binding to the heme or inhibition of GSH dependent heme degradation (Figure 6.2). Quinoline type of antimalarial drugs are acting on heme and lack of enzyme target for these quinoline type drugs is probably the chief reason why resistance development to these drugs is relatively slow (Francis et al., 1997). Hence we investigated effect of vasicine, vasicinone and embelin on heme.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.2.png}
\caption{General pathway for hemoglobin metabolism in \textit{Plasmodium falciparum} (Tripathi et al., 2004)}
\end{figure}
Effect of vasicine, vasicinone and embelin on some important targets

6.2.1.1 Effect of vasicine, vasicinone and embelin on hemozoin formation inhibition

Introduction

Asexual reproduction of the malaria parasite within the host erythrocytes, commonly referred as intraerythrocytic schizogony, is the main cause of clinical manifestations of malaria infection. During development and proliferation in host erythrocytes, the malarial parasite degrades hemoglobin for use as a major source of amino acids (Slomianny et al., 1982). Digestion of hemoglobin by the malaria parasite occurs through a sequential metabolic processes (Gluzman et al., 1994; Coombs et al., 2001). Haem (ferriprotoporphyrin IX) is a by-product of haemoglobin digestion, a process which takes place in an acidic compartment of intraerythrocyte-stage malaria parasites (Goldberg et al., 1990).

Free heme is oxidatively active and toxic to both the host cells and the malarial parasites, and it causes parasite death. Heme could cause extensive damage to membranes and inhibit a variety of enzymes resulting in the death of the parasite (Pandey et al., 1998). Due to the absence of heme oxygenase, the parasite is unable to cleave heme into an open-chain tetrapyrrole, which is necessary for cellular excretion (Eckman et al., 1997). To protect itself, the malarial parasite detoxifies free heme via neutralization with histidine-rich protein (HRP-2) (Sullivan et al., 1996; Huy et., 2003), degradation with reduced glutathione (Atamna et al., 1995; Huy et al., 2002), or crystallization into β-haematin (BH) also known as hemozoin (HZ), which is a water-insoluble malarial pigment produced in the food vacuole (Sullivan et al., 1996; Francis et al., 1997). Malaria pigment hemozoin formation occurs by assembly of heme: one carboxylic oxygen of the propionate side chain of one haem molecule is coordinated to the central iron of an adjacent haem subunit to form oxygen-iron bonds (Slater et al., 1992; Scott Bohle & Helms, 1993).

Polymerisation of soluble haematin has been shown to occur spontaneously in vitro at acidic pH (Egan et al., 1994; Dorn et al., 1995). Several factors such as thermos (Egan et al., 1999), HRP-2 (Sullivan et al., 1996; Huy et., 2003), lipid (Dorn et al., 1995; Tripathi et al., 2001), and preformed BH (β-haematin) (Sullivan, 2002) have been reported to be responsible for promoting BH formation. However, the thermal BH crystallization is not natural and is a nonphysiological process. Some strains of Plasmodium lack HRPs but still form hemozoin (Sullivan, 2002), suggesting that HRPs are sufficient but not completely necessary for hemozoin formation. Therefore, two
most important and natural initiators of BH formation are probably lipids and preformed BH. Several studies used preformed BH (Dorn et al., 1995; Chong & Sullivan, 2003; Dorn et al., 1998) and lipids extracted from parasites (Fitch et al., 1999) or infected plasma (Tripathi et al., 2001) as an initiator of BH formation assay as a high-throughput screening of new antimalarial compounds. Using lipids extracted from parasites or infected plasma is laborious and requires many steps for preparation.

In the present study the isolated compounds were screened for the inhibition of hemozoin formation.

**Materials and methods**

**Preparation of reagents**

**Preparation of heme solution (8 mM)**

5.2 mg of haemin chloride (Sigma chemical Co., USA) was dissolved in 1 ml of DMSO.

**Preparation of 8 M acetate buffer (pH 5)**

A. Acetic acid (8 M) – 457.52 ml of glacial acetic acid dilute to 1000 ml with double distilled water

B. Sodium acetate (8 M) – 656.24 g of sodium acetate was dissolved in 1000 ml of double distilled water

14.8 ml of solution A was mixed with 35.2 ml of solution B volume made up to 100 ml with double distilled water and the pH adjusted to 5.0 with 0.1 N HCl or 0.1 N NaOH.

**Preparation sodium hydroxide (0.1 M)**

0.4 g of sodium hydroxide was dissolved in 100 ml of double distilled water.

**Standard drug**

Clotrimazole with different molar equivalents (0.12-5.0) to haemin was prepared by dissolving compounds separately in DMSO.

**Test solution**

Vasicine, vasicinone and embelin with different molar equivalents (0.12-5.0) to haemin were prepared by dissolving compounds separately in DMSO.

**Experiment protocol**

**Assay of hemozoin formation inhibition**
50 µl of an 8 mM solution of hemin chloride was dissolved in DMSO and compounds dissolved in DMSO, in doses ranging from 0.12 to 5 molar equivalents to haemin chloride. In control 50 µl distilled water was added. By adding 100 µl of 8 M acetate buffer (pH 5) the hemozoin formation was initiated and the plates were incubated at 37ºC for 18 h. After centrifugation, the soluble fraction of unprecipitated material was collected. 200 µl of DMSO was added to resuspend the remaining pellet in order to remove unreacted haematin. The plates were centrifuged again, the DMSO soluble fraction was collected and the residual pellet, consisting of pure precipitate of β-haematin, was dissolved in 200 µl of 0.1 M NaOH. 75 µl of it was transferred to new tubes and diluted four times by adding 0.1 M NaOH. The amount of haematin was determined by measuring the absorbance at 414 nm using UV spectrophotometer (Shimadzu UV 2450) with software UV Probe V 2.1 (Shimadzu, Japan). Clotrimazole was used as positive control.

The percentage inhibition of hemozoin formation was calculated using the formula given below:

\[
\text{Percentage inhibition} = 100 - \frac{A_{\text{test}}}{A_{\text{sample}}} \times 100
\]

The percentage inhibition of hemozoin by the standard drug clotrimazole was compared with the control. Method was standardized and validated. Isolated compound/s were tested in this model to establish their mechanism of action.

**Critical Points**
- Concentration and pH of acetate buffer
- Incubation time
- Concentration of inhibitor

**Results**

Of the three compounds tested, embelin was found to inhibit the formation of hemozoin *in vitro* and the activity was comparable to that of the positive control clotrimazole. At 5 molar equivalents to haemin, embelin showed 85.21 %, vasicine showed 32.7 % and vasicinone showed only 9.68 % inhibition (compared with positive control clotrimazole 94.64 % inhibition).
As embelin showed maximum β-haematin formation inhibition at a concentration of 5 molar equivalents to haemin, we evaluated its activity at different concentrations (Table 6.1)

Table 6.1 Effect of embelin on β-haematin formation

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>% Inhibition</th>
<th>IC_{50} (mMeq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.21 ± 1.04</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>25.26 ± 0.86</td>
<td>11.66</td>
</tr>
<tr>
<td>10</td>
<td>41.03 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>60.84 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>85.21 ± 0.78</td>
<td></td>
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</table>

* Mean ± SEM, n = 3

Discussion

In the present work it has been demonstrated that the in vitro formation of polymeric non-toxic hemozoin by the parasite was prevented by embelin. Furthermore, there is a direct relationship between intra-erythrocytic anti-malarial activity and inhibition of β-haematin formation. Mechanism of action of clotrimazole, a potent antifungal agent, for antiplasmodial activity has already been established (Huy et al., 2002). These findings could provide rational for the hypothesis that haem could be the drug receptor, at least in part, for embelin as antimalarial agent. This hemozoin formation inhibition may be occurred through various routes e.g. binding of heme to the drug or GHS dependent heme degradation. Hence we evaluated effect of embelin in embelin-heme interaction assay and inhibition of GSH dependent heme degradation assay.

6.2.1.2 Drug-Heme Interaction Assay

Introduction

It was reported that quinoline-containing drugs inhibit heme polymerization and can form a complex with heme (Chou et al., 1980; Fitch and Chevli, 1981; Constantinidis and Satterlee, 1988; Blauer, 1988). Extensive studies showed that chloroquine forms a stable complex with two μ-oxo heme dimers (Dorn et al., 1998; Egan et al., 1996; Ridley et al., 1997; Slater, 1993; Vippagunta et al., 2000). The chloroquine-heme complex can be incorporated into the growing hemozoin polymer, blocking its extension (Sullivan et al., 1996), and leading to an increase in the transient half-life of μ-oxo dimers heme in its non polymerized form (Chou and Fitch, 1993). This drug
induced accumulation of μ-oxo dimers may disrupt the normal lattice of reciprocal dimers, leading to structural disorganization and deterioration of the acidic vacuole. As a result, hemoglobin digestion is impaired and the ring maturation inhibited (Orjih et al., 1997).

Again, the inhibition of hemozoin formation may develop oxidative stress due to the accumulation of free heme, which can generate highly reactive hydroxyl radical (·OH), and the malaria parasite is susceptible to oxidative stress (Kumar and Bandopadhyay, 2005; Kumar et al., 2007). Therefore, the enhancement of oxidative stress to the parasite by any means is a promising strategy in developing new antimalarial agents.

In the present study, we investigated whether hemozoin formation inhibition occurred by binding of embelin to heme.

Materials and methods

Preparation of reagents

Preparation of heme solution (100 µM)

1.30 g of haemin chloride was dissolved in 100 ml of DMSO.

Preparation of HEPES buffer (0.2 M)

4.7 g of HEPES buffer was dissolved in 10 ml of double distilled water.

Test solution

29.43 mg of embelin was dissolved in 100 ml DMSO.

Experimental protocol

To examine the interaction of embelin and heme, a differential absorbance technique (Job’s plot) was performed to determine the spectral changes as described by Auparakkitanon et al. (2006). Stock solutions of heme and embelin (100 µM) were prepared. A series of solutions containing embelin and heme were prepared in DMSO (40%; vol/vol) buffered by 20 mM HEPES (pH 7.4). The total final combined concentration of heme and drug in the mixtures was constant at 10 µM. After incubation of the mixtures at room temperature for 30 min, the absorbance was recorded at 386 nm. The differential absorbance was obtained by subtracting the total absorbance at 386 nm of heme and drug from the absorbance of the heme-embelin mixture.
Effect of vasicine, vasicinone and embelin on some important targets........

Critical control points

- Preparation of stock solution of heme.
- Combined concentration of embelin and heme.

Results and discussion

Chloroquine, the most widely used antimalarial, binds to heme (Sullivan et al., 1996; Chou et al., 1980) with a 1:2 stoichiometry (Leed et al., 2002) and prevents its detoxification, leading to heme toxicity and death of the parasite. Similarly, artemisinin has been shown to form heme adducts that cannot be detoxified (Kannan et al., 2002). However, there is currently no evidence for chloroquine that resistance arises from a modification of the drug target heme. According to Fidock et al (2000) the resistance appears to arise mainly from mutations in a *Plasmodium* transmembrane protein, PfCRT, mutant forms of which seem to lower the accumulation of chloroquine in the parasitised cell (Sidhu et al., 2002). Therefore, new drugs acting on the chloroquine target i.e. heme have every possibility of being active, even in chloroquine-resistant parasites, provided that they can circumvent the effects of PfCRT.

The dimer units are intermediate forms of heme dimers linked together by reciprocal iron-propionate bonds, which are known to be involved in the formation of hemozoin in the parasite food vacuole (Pagolo et al., 2000). It was also observed that the compounds bind primarily to the µ-oxo dimer form of haematin rather than the monomeric form.

The inhibition of hemozoin formation by antimalarial drugs may be mediated by binding to heme (Fitch et al., 2004). Here we investigated the interaction of embelin with heme by using a continuous variation technique (Job’s plot), as described in Materials and Methods (Section 5.2.1.2.2). UV-Visible spectrum of a heme solution (50μM) at pH 7.4 in 40% DMSO was taken from 300-600 nm. It showed a broad Soret band from 370 to 390 nm resembling dimer. Upon the addition of embelin, the spectrum change was accompanied by a significant decrease in the intensity of dimeric heme at the Soret band indicating an interaction between embelin and heme. The interaction of heme with embelin was monitored in the Soret region of the UV-visible spectrum. The changes in the absorbance at 386 nm, as a function of embelin concentration, are presented in (Figure 6.3). It was reported that the presence of hydroxyl groups in chloroquine bind iron of heme and lead to the formation of π–π
adducts, which inhibited hemozoin formation (Basilico et al., 1997). Similarly hydroxyl groups present in embelin may bind iron of heme.

The loss of Soret band intensity may result from the formation of \( \pi-\pi \) complexes (Shelnutt et al., 1983) or aggregation and precipitation of heme (White et al., 1978). To investigate both possibilities, a dilution test was performed with a solution containing embelin and heme in a molar ratio of 10 to 1. Dilution of this sample was found to obey the Beer-Lambert law in the concentration range of 1 to 10 \( \mu \text{M} \) heme. This observation indicates that further heme aggregation and/or precipitation does not occur under our test conditions. The interaction of heme and embelin in stoichiometries was further explored by Job’s plot (Huang et al., 1982). The differential absorbance of heme-embelin mixture at 386 nm was recorded and plotted as shown in (Figure 6.4). This gave us quantitative estimate of the number of heme molecules required for the binding of embelin. For this two solutions of equal concentration were prepared. The first solution contained embelin in DMSO while the second solution contains heme. The solutions were mixed in various ratios, while keeping the total volume of the mixtures constant, to vary the relative mole fractions of the heme and embelin. For each heme-embelin mixture the absorbance was measured at 386 nm which measures the concentration of the heme protein. Formation of the complex is maximized when
embelin and heme are present in a 2:3 ratio (Figure 6.4). The results of Job plots for the heme indicate that embelin bind with heme with a 2:3 stoichiometry.

![Heme binding assay](image)

### Figure 6.4 Job's plot of embelin binding to heme

#### 6.2.1.3 Inhibition of GSH-dependent heme degradation by embelin

**Introduction**

Malaria-infected RBCs are distinguished from normal cells by their high levels of heme. The parasite ingests the cytosol of its host cell which is essentially composed of hemoglobin (Olliaro and Goldberg, 1995; Francis et al., 1997). In the cytosol, reduced glutathione (GSH) binds to heme via its thiol and degrades heme (Atamna and Ginsburg, 1995; Ginsburg et al., 1998; Platel et al., 1999). In binding to heme, GSH competes with the membrane since heme being hydrophobic in nature has affinity for the membrane. However, millimolar concentrations of GSH in the malarial cytoplasm is enough to bind heme despite the low affinity ($K_D = 2.8 \times 10^{-3} \text{ M}$) (Atamna and Ginsburg, 1997; Luersen et al., 2000). This indicates that GSH could protect malarial parasite from toxic heme released from hemoglobin.

The major degradation pathway of heme is not the hemozoin formation, since around 70% of non-crystallized heme exits in the food vacuole and is subsequently catabolized by GSH, leading to the formation of oxidized glutathione (Ginsburg et al., 1998). Hence, a drug that can inhibit the interaction between GSH and heme could have potential antimalarial properties.

Both in cultures of *P. falciparum* (Ginsburg et al., 1998) and in *P. berghei* infected mice (Dubois et al., 1995; Platel et al., 1999), the reduction of GSH leads to sensitization of
the parasites to chloroquine. This perception of the involvement of GSH in the antimalarial mode of action of chloroquine has directed us to test the inhibition of GSH dependent heme degradation of embelin.

Materials and methods

Preparation of reagents

Preparation of heme solution (100 µM)

1.30 g of haemin chloride was dissolved in 100 ml of DMSO.

Preparation of HEPES buffer (0.2 M)

4.7 g of HEPES buffer was dissolved in 10 ml of double distilled water.

Preparation of GSH solution (10 mM)

61.46 mg of GSH was dissolved in 10 ml of HEPES buffer.

Standard solution

34.48 mg of clotrimazole (CLT) (positive control) was dissolved in 10 ml DMSO.

Test solution

29.43 mg of embelin was dissolved in 10 ml DMSO.

Experimental protocol

Heme degradation by GSH was monitored by measuring spectral change as described by Atamna and Ginsburg, (1997). Stock solutions of heme, embelin and clotrimazole (100 µM) were prepared in DMSO. Fresh GSH stock solution (200 mM) was prepared in HEPES buffer (0.2 M, pH 7.4). Heme (10 µM) and GSH (final concentration of 10 mM) were mixed in HEPES buffer (pH 7.4) and incubated at 37°C. Absorption spectra (300–600 nm) were recorded at 6 min intervals after mixing, using the same spectrophotometer.

In presence of clotrimazole (10 µM) and embelin (10 µM), the time-dependent spectral measurements were obtained by same procedure. Heme (10 µM), GSH (10 mM), and either CLT or embelin (10 µM) were mixed in 0.2 M HEPES buffer (pH 7.4) and incubated at 37 °C. In the control experiment, DMSO (final concentration, 0.2% (v/v) was added to the mixture of heme (10 µM) and GSH (10 mM) instead of CLT and embelin. The time-dependent change of absorbance at 386 nm was recorded as an indicator of heme degradation.
Critical control points

- Preparation of stock solution of heme.
- Time points.

Results

The absorption spectrum of heme (10 µM) in DMSO exhibited broad soret band at 386 nm dimer form. The maximal absorption of the soret of heme (10 µM) was shifted to broader soret band between 360 to 370 nm after adding GSH (10 mM), probably due to the formation of GSH-heme complex. (Figure 6.5) shows that the soret absorption of heme complexed with GSH declined rapidly indicating the degradation of heme by GSH. During 30 min this peak decreased to approximately half, without further decrease. Absorbance did not decline to zero, as the degradation product also absorbs, to some extent, at 386 nm. In the case of clotrimazole and embelin the soret band at 386 nm was not formed. Addition of clotrimazole to GSH-heme resulted in the shift of peak to 366, 413 and 564 nm, which may be due to altered effects of glutathione. Huy et al., (2002) showed that absorption maxima for heme-GSH-clotrimazole complex were 416, 533 and 564 nm. After the addition of embelin to GSH-heme complex, there is a shift of peak to 327 and 396 nm, which may be due to the altered effect of GSH on heme.

![Figure 6.5: Effect of clotrimazole and embelin on spectral changes occurring during the reaction of heme with GSH](image-url)
The GSH-dependent degradation of heme (10 µM) in the presence of CLT (10 µM) or embelin (10 µM) was monitored as a decrease of absorbance at 386 nm. The results shown in (Figure 6.6) indicate that embelin inhibited GSH-dependent heme degradation and its activity is comparable to CLT, the positive control used.

6.2.1.4 Conclusion

We have provided experimental evidence showing that embelin acts as an antimalarial with a mechanism of action similar to that of the well-known 4-aminoquinoline chloroquine, in inhibiting hemozoin formation in vitro (a process which closely parallels hemozoin formation within the parasite food vacuole), forms a heme-embelin complex and inhibited glutathione-dependent degradation of heme but at a lower concentration than seen with chloroquine.

6.2.2 Effect of vasicine, vasicinone and embelin on protein kinase inhibition

6.2.2.1 Introduction

Cyclin-dependent protein kinases (CDPKs) are essential for the regulation of the eukaryotic cell cycle, and several enzymes of this family have been identified in P. falciparum (Leonie Harmse et al., 2001). P. falciparum homologues of several essential cell cycle genes have been identified (Doerig et al., 1999, Kappes et al., 1999). The first kinase of this family to be identified was PfPK5 (Plasmodium falciparum Protein Kinase 5), a putative homologue of CDPK1 (Ross MacDonald et al., 1994). In vitro, PfPK5 kinase activity can be increased by three orders of magnitude by the addition of
a newly described *P. falciparum* cyclin homologue or by certain mammalian cyclins (Le Roch K et al., 2000). A second CDPK-related kinase, PfPK6, shares structural properties with both CDPK and mitogen-activated protein kinases and does not require cyclin binding for kinase activity *in vitro* (Bracchi-Richard et al., 2000). The functions of these kinases in the life cycle of the parasite remain undetermined, mainly as a consequence of the difficulties associated with genetic manipulation of the parasite. Despite being sufficiently conserved to allow their classification within the CDPK family, the parasite’s CDPK-related kinases nevertheless display significant divergences when compared to the mammalian enzymes. In the catalytic domain, 40–60% of the residues are different between the mammalian and plasmodial enzymes, and the latter display unique structural features such as insertions or terminal extensions. Furthermore, there is evidence that the regulation of the activity of the *P. falciparum* enzymes differ from that of mammalian CDPKs. For instance, there is no known homologue of PfPK6 in mammalian cells, and PfPK5 is able to autophosphorylate in the presence of a cyclin, a peculiarity that has not been documented in the CDPKs from higher eukaryotes (Bracchi-Richard et al., 2000). Such considerations suggest that the enzymes from *P. falciparum* may have different susceptibilities to small molecule kinase inhibitors (Kappes et al., 1999). A second essential feature for a molecule to be considered as a potential drug target is that it must be distinguishable from its counterpart in the human host. The CDPKs proved very valuable in this respect – mammals do not have this type of protein kinase. These differences can be utilized to explore specific plasmodial protein kinase inhibitor.

6.2.2.2 Materials and Methods

**Kinase assay components**

- 20 mM Tris pH7.5
- 20 mM MgCl₂ (kinase cofactor)
- 2 mM MnCl₂ (kinase cofactor)
- 10 mM Sodium fluoride (phosphatase inhibitor)
- 10 mM β–glycerophosphate (phosphatase inhibitor)
- 10 µM cold ATP
- γ³²P ATP 0.25 µl/reaction

**Kinase assay conditions**
Effect of vasicine, vasicinone and embelin on some important targets

− 30 min at 30°C
− Stop the assay with 7.5 µl of 4 x SDS-PAGE loading buffer and boil for 5 min.
− Run 15 µl of each sample on a 12% SDS-PAGE gel until the bromophenol blue leaves the gel.

Experiment protocol

Kinase inhibition assay

1 µg of recombinant kinase (PK6, PK7 and Pf130085) was assayed in 100 mM tris pH 7.5, 100mM MgCl2, 10mM MnCl2, 50mM NaF, 50mM β−glycerophosphate, with 5µg of appropriate substrate. Myelin basic protein was used as a substrate for PK6 whereas casein was phosphorylated by PK7 and Pf130085. Enzymes were incubated with either 100 µM inhibitor or an equivalent volume of DMSO for 15 minutes prior to the initiation of the reaction. The reactions were initiated by the addition of ATP (10 µM), 5% of which was γ(32P)ATP. Reaction mixtures were incubated at 30°C for 30 minutes and samples were separated by SDS-PAGE. Activity was determined by auto-radiography.

6.2.2.3 Results

Kinase inhibitors are widely employed as biological reagents and as leads for drug design, but their use is often complicated by their lack of specificity. Therefore, it was important to evaluate the selectivity of the protein kinase inhibitory activity of compounds against a panel of P. falciparum protein kinases including PfPK6, PfPK7, and Pf130085. In the present study none of the three isolated compounds showed activity on this target at 100 µM.

6.2.3 Effect of vasicine, vasicinone and embelin on plasmepsin II and IV

6.2.3.1 Introduction

There are 10 aspartic proteases or plasmepsins (PLM) reported in the genome of Plasmodium falciparum, viz., PLM I, II, and IV-X, and histo-aspartic protease (HAP) (Coombs et al., 2001). Four of them, PLM I, PLM II, HAP, and PLM IV have been localized in the food vacuole of the parasite, and have been shown to be involved in hemoglobin degradation (Banerjee et al., 2002).

Considerable advancements were made in the development of potent plasmepsin inhibitors (Silva et al., 1996; Jiang et al., 2001; Asojo et al., 2002; Asojo et al., 2003;
Nezami et al., 2003; Boss et al., 2003; Dahlgren et al., 2003; Ersmark et al., 2003; Johansson et al., 2004). Boss et al (2003) showed that the most attractive target amongst the aspartic proteases is plasmepsin II. PLM I and PLM II encode proteins with sequence homology to mammalian aspartic proteases such as cathepsin D (Cat D) and renin, and these are 73 % identical to each other. A peptidomimetic inhibitor of PLM I blocks hemoglobin degradation in culture and kill the parasite, validating this pathway as a target for antimalarial drug development (Francis et al., 1994). Structural differences in the active sites of PLM II and Cat D were used to rationalize differential inhibition of these enzymes and potent and selective inhibitors of PM II also inhibit the growth of *P. falciparum* in cell culture strongly supports the concept that inhibition of plasmepsins is a viable strategy for antimalarial therapy (Silva et al., 1996).

### 6.2.3.2 Materials and methods

Recombinant pro-PLM II and IV were purified from BL21-(DE3) pLysS *E. coli* (Invitrogen, Milano) according to the method of Hill et al., (1994) with slight modifications (Dell'Agli et al., 2006).

**Experiment protocol**

**PLMs inhibition assays**

Protein concentration was determined according to the method of Bradford (1976). Proteins were diluted to the final concentration of 0.5 mg/ml in 50% glycerol and stored at -20°C. Pro-PLM II and IV were activated by addition of one tenth volume of 100 mM sodium acetate buffer pH 4.7 by incubation at 37°C for 90 min. The enzyme activity of PLM II and IV was evaluated spectrophotometrically at 300 nm as described by (Hill et al., 1994). Vasicine, vasicinone and embelin were tested at 25 µM. Embelin showed good inhibition as compared to vasicine and vasicinone, further embelin was tested at 5 – 25 µM. The assays were conducted twice in triplicate. Inhibition curves and IC₅₀ values were calculated by a non-linear regression for sigmoidal curves using Graph Pad Prism 4.

### 6.2.3.3 Results and discussion

Hemoglobin degradation is mediated by the action of several vacuolar digestive enzymes. The general aspartic proteases inhibitor pepstatin has been shown to prevent the parasite from breaking down host cell haemoglobin by inhibiting this initial cleavage event. This implicates an aspartic protease as the enzyme responsible for
initiating the pathway (Goldberg et al., 1991). The plasmepsins produced by the *Plasmodium* parasite are aspartic proteases which perform a crucial role in providing nutrients for the red blood cell stages of the malaria parasite and have been recognized as attractive targets for the design of novel chemotherapeutic compounds for the control of malaria (Berry, 1997). In an attempt to find out the mechanism of action of vasicine, vasicinone and embelin, these three compounds have been screened against PLM II (Table 6.2). Vasicinone reduced PLM II activity in a concentration dependent manner ($IC_{50}$ $13.3 \pm 0.56 \mu M$), while it was inactive against PLM IV.

| Table 6.2: Effect of vasicine, vasicinone and embelin on PLM II. |
|-----------------------------|------------------|
| **Compound**                | **IC$_{50}$**    |
| Vasicine                    | NA               |
| Vasicinone                  | $13.3 \pm 0.56 \mu M$ |
| Embelin                     | NA               |
| PS777621                    | 100 nM           |

* Mean ± SEM, n = 3; NA: Not active

Vasicine and embelin were found to be inactive against PLM II and PLM IV at 25 $\mu M$ concentration. From the results it is evident that, neither parasite growth inhibition nor hemozoin formation is correlated to PLM inhibition. This may be due to the blockage of other proteolytic enzymes working in combination with PLM inhibition.

### 6.2.4 Effect of vasicine, vasicinone and embelin on histidine rich protein-2 (HRP-2)

#### 6.2.4.1 Introduction

Histidine rich proteins (HRP-2 and HRP-3) are the heme polymerases. These proteins were shown to be present in the food vacuole, and to unambiguously polymerize heme (Sullivan Jr et al., 1996). Also synthetic peptides corresponding to a repetitive sequence of HRP-2 bind heme and inhibit hemozoin formation *in vitro* (Pandey et al., 1997). The similarity of the hexapeptide, Ala-His-His-Ala-Ala-Asp, a 33 times repetitive sequence of HRP-2, to the heme binding site of histidine rich glycoprotein (HRG), Gly-His-His-Pro-His-Gly, was also supportive of the hypothesis that HRP-2 polymerizes heme (Sullivan Jr et al., 1996; Pandey et al., 1997). Further, Choe et al. (1999) demonstrated that HRP-2 can bind heme via bis-histidyl coordination.
HRP-2, a 30 kDa protein which is largely made up of repeats involving three amino acids: histidine (34%), alanine (37%) and aspartic acid (10%) (Wellems and Howard, 1986). Compounds that either inhibit heme binding to HRP-2 or inhibit polymerization of heme bound to HRP-2 should be toxic to the parasite. Inhibition of heme binding could occur in two ways. Histidine-iron center coordination is the dominant mechanism of interaction between the amino acid and heme. The protein also contains aspartate allowing for ionic/coordination interactions between the carboxylate side chain and the heme metal center (Sullivan Jr et al., 1996). Thus HRP-2 has been established as a catalyst for haemozoin formation, and haem metabolism has emerged as a potential drug target in the malaria parasite (Padmanban and Rangarajan et al., 2000).

6.2.4.2 Materials and methods

Culture plates

Stock solutions (1 mg/mL) of vasicine, vasicinone and embelin were prepared in 70% ethanol. These were diluted with distilled water to obtain the desired test concentrations of vasicine, vasicinone and embelin (0.78-35 µg/mL). Serial two-fold dilutions (seven concentrations and one drug-free control well) of the drugs (25 µL/well) were dispensed in duplicate into standard 96-well microculture plates (Costar 3599; Costar, Cambridge, MA) by a semiautomated microdilution technique. The plates were dried overnight in an incubator at 37°C and stored at 4°C.

Culture

Using a multichannel pipette, 200 µL of cell medium mixture (CMM) was dispensed into each well of the predosed culture plates (including around 10 drug-free wells that serve as additional controls). The plates were then incubated in a candle jar at 37°C for 72 hours. After the first 24 hours of incubation, the contents of four control wells were harvested, transferred into a microcentrifuge tube, and stored at -20°C. This sample may be used in the ELISA to subtract the background (i.e., parasite growth within the first 24 hours). After the end of the 72-hour incubation time, thick and thin blood films were prepared from one of the control wells to check for adequate parasite growth and reinvasion. The culture plates were then transferred into a simple household freezer (approximately -15°C) and frozen and thawed twice.

Enzyme-linked immunosorbent assay
Commercial ELISA kits were used to quantify the amount of HRP2 (Malaria Ag CELISA; Cellabs Pty. Ltd., Brookvale, New South Wales, Australia). However, basically any ELISA specific to HRP2 may be used (by using a simple generic HRP2 double-site sandwich ELISA, the overall cost for the ELISA may be drastically reduced). The culture samples were diluted directly on the ELISA plates with distilled water according to their starting parasite densities to obtain the equivalent of approximately 0.01-0.1% (ideally 0.05%; e.g., if the initial parasite density in the culture was 0.25%, one part (20 µL) of the hemolyzed CMM was added to four parts (80 µL) of water to obtain 100 µL of sample with the equivalent of a 0.05% parasitemia). This was done by first adding the water to the ELISA plates and then transferring and carefully mixing the hemolyzed CMM in each well using a multichannel pipette. In addition to the samples from the predosed wells, 100 µL of the control sample frozen after 24 hours and diluted in the same way were added to two wells to determine background HRP2 concentrations. The ELISA plates were then incubated at room temperature for one hour. Subsequently, the plates were washed three times with washing solution, and 100 µL of the diluted antibody conjugate were added to each well. After further incubation for one hour, the plates were washed three times and 100 µL of the diluted tetramethylbenzidine chromogen were added to each well. The plates were then incubated for an additional 15 minutes in the dark, and 50 µL of the stopping solution were added. Spectrophotometric analysis was performed using a small, field-suitable ELISA plate reader (Tecan Sunrise Absorbance Reader; Tecan Austria GmbH, Groedig, Austria) at an absorbance maximum of 450 nm.

6.2.4.3 Results and discussion

HRP-2 drug sensitivity test, incubation period 72 hours, allows testing of slow-acting drugs such as antifolate antimalarials and antibiotics. The main criterion for the success of the HRP-2 test is the reinvasion of the parasites into previously uninfected red blood cells after the first cycle is completed. This may easily be assessed in a thin blood film prepared after 72 hours of culture. Even most samples that showed no schizont maturation after 24 hours were successfully tested in the HRP-2 assay (Noedl et al., 2002). Another major advantage of using HRP-2 levels as an indicator of parasite growth lies in the stability of the protein (Ceritii et al., 1999).
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Table 6.3 Effect of vasicine, vasicinone and embelin on HRP-2

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; µM</th>
<th>IC&lt;sub&gt;90&lt;/sub&gt; µM</th>
<th>IC&lt;sub&gt;95&lt;/sub&gt; µM</th>
<th>IC&lt;sub&gt;99&lt;/sub&gt; µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasicinone</td>
<td>8.04</td>
<td>11.38</td>
<td>11.84</td>
<td>12.21</td>
</tr>
<tr>
<td>Embelin</td>
<td>24.48</td>
<td>33.46</td>
<td>33.47</td>
<td>35.26</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.051</td>
<td>0.148</td>
<td>0.181</td>
<td>0.228</td>
</tr>
</tbody>
</table>

In histidine rich protein (HRP-2) inhibition assay vasicine, vasicinone and embelin exhibited IC<sub>50</sub> value as 8.62 µM, 8.04 µM, and 24.48 µM respectively as compared to CQ of IC<sub>50</sub> with 0.051 µM (Table 6.3).

6.2.5 Effect of vasicine, vasicinone and embelin on invasion of red blood cells

6.2.5.1 Introduction

RBC invasion by merozoite comprises of several sequential steps which include 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 (Aikawa et al., 1978). 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.

The surface of the merozoite is covered by fibrillar material comprising a surface coat (Aikawa et al., 1978). 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 (Miller et al., 1975). Removal of the surface coat must involve selective enzymatic cleavage of the major surface components. This course of events could be mediated by protease(s) located on the merozoite surface, activated upon attachment of the merozoite’s apical end to the RBC membrane (Kitjaroentham et al., 2006).

Inhibition of invasion was observed in resealed erythrocyte ghosts upon chemical or immunochemical cross-linking of spectrin (McColm et al., 1980). Inhibition of invasion was observed upon treatment of erythrocytes with membrane-active drugs such as colchicine and vinblastine, but very high (millimolar) concentrations were used
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(McColm et al., 1980). Later, compounds possibly acting as cholesterol mimics were shown to inhibit proliferation of malaria parasites \textit{in vitro} in a low-micromolar concentration range (Ziegler et al., 2002). This showed that there is a link between the erythrocyte membrane properties and the ability of erythrocytes to function as hosts for the \textit{Plasmodium} parasites, which could possibly be exploited for a novel antimalarial therapy.

The isolated compounds vasicine, vasicinone and embelin were evaluated for the inhibition of invasion of RBC by the malaria parasite.

6.2.5.2 Materials and methods

\textit{P. falciparum FCK2}

Chloroquine sensitive \textit{P. falciparum} FCK2 was obtained from the Jawaharlal Nehru Centre for Scientific and Advanced research, Bangalore.

\textit{Staining}

Giemsa’s stain: Merck limited, Mumbai.

\textit{Experimental protocol}

Synchronous cultures of \textit{P. falciparum} FCK2, routinely maintained according to the method of Trager and Jensen (1976), were obtained by treatment with 5\% sorbitol (Lambros and Vanderberg, 1979). Parasitized RBC with late stage trophozoites were used in the invasion assay. 1 mg/ml solution of test samples were prepared in DMSO prior to dilutions with culture medium to the required concentrations. Invasion assay was performed by exposing 200 µl aliquot of 1.5\% RBC suspension with 2–5\% parasitemia to 25 µl of culture medium containing inhibitor or culture medium (as negative control) in 24-well tissue culture plate. After 15–20 h incubation, thin smears were made and stained with Giemsa. Percent parasitemia was determined by counting 1000 cells/slide.

6.2.5.3 Results and discussion

Three steps are involved in the invasion process of parasites in fresh RBCs, namely schizont development, merozoite release, and merozoite invasion. In the present study late stage trophozoites were incubated with vasicine, vasicinone and embelin for 15–20 h and the number of ring forms and schizonts were counted from Giemsa-stained thin films. Vasicine showed 70.17 \% inhibition of merozoite invasion of human RBC at 25
µg/ml while it showed 77.18 % inhibition of schizont maturation at 25 µg/ml. Embelin showed 73.65 % inhibition of merozoite invasion of human RBC at 25 µg/ml while it showed 71.44 % inhibition of schizont maturation at 25 µg/ml (Figure 6.10). From the above experiments it was observed that vasicine and embelin showed good activity in inhibiting schizont maturation and merozoite invasion of RBC. Both the compounds have the potential to block parasite development.
6.3 SUMMARISED DISCUSSION

Anti-infective drugs rely for their efficacy and specificity on their ability to interfere with aspects of metabolism that differ significantly from the human host. *Plasmodium* infects host erythrocytes during their life cycle that gives rise to the symptoms of
malaria. Parasite survival in this environment requires several metabolic adaptations that render it susceptible to chemotherapy. This is evident both in the mechanism of action of existing drugs and in excess of potential drug targets which were being identified after the malaria genome project (Macreadie et al., 2000; Padmanaban and Rangarajan, 2001; Ridley, 2002; Carucci et al., 1998; Verma and Sharma, 2003; Verma and Sharma, 2003; Gardner et al., 2002; Carlton and Carucci, 2002; Kissinger et al., 2002). Additionally, the sequence of the mammalian host and vector genomes (Land, 2003; Mongin et al., 2004; Lander et al., 2001) can also prove to be very useful in identifying differences between plasmodial and human gene sequence.

Drug targets of *Plasmodium falciparum* are related to the functions of distinct *Plasmodium falciparum* organelles. Potential chemotherapeutic targets in the malaria parasite can be broadly classified into three categories: those involved in processes occurring in the digestive food vacuole (the site of extensive haemoglobin degradation), the apicoplast (enzymes involved in macromolecular and metabolite synthesis), and the mitochondrion (those responsible for membrane processes and signalling). The processes occurring in the digestive vacuole include haemoglobin digestion, redox processes and free radical formation, and reactions accompanying haem release followed by its polymerization into haemozoin. Many enzymes in macromolecular and metabolite synthesis are promising potential targets, some of which have been established in *Plasmodium* and other microorganisms, such as dihydrofolate reductase. Proteins responsible for membrane processes, including trafficking and drug transport and signalling, are potentially important also to identify compounds to be used in combination with antimalarial drugs to combat resistance (Olliaro and Yongyuth, 1999; Ridley, 2002). Various inhibitors of proteases, choline uptake, glycolysis type II fatty acid synthesis and farnesyl transferase display promising antiplasmodial activity *in vitro* and in animal models. However, as similar target molecules are present in humans, enzyme inhibitors with high degree of selectivity for the malarial enzymes should be developed (Wiesner et al., 2003).

Still there is a large gap between target identification and validation on the one hand and lead discovery and optimization on the other hand. Biochemical studies of the targets and structural studies of the interaction between target and candidate inhibitor, is the good strategy to fill this gap. Such interaction studies can also be useful in search of new targets of known antimalarial drugs. This, in turn, could yield new insights into
the search for new compounds, which can avert the problem of resistance to existing drugs. Understanding the mode of action of and mechanism of resistance to drugs is central to optimising their use, and discovering new therapeutics with novel targets (Olliaro, 2001). However, some of the compounds were identified by random screening against whole parasite cultures and in vivo testing, but their mechanism of action has not been elucidated (Olliaro and Yongythe, 1999).

Therefore in the present study the effect of the three isolated compounds, vasicine, vasicinone and embelin has been investigated on some important targets of Plasmodium falciparum to establish the probable mechanism of action.

After every 48 h development of the schizont leads to formation of multiple merozoites, which on rupture of the infected host cell, invade other erythrocytes. There is a link between the erythrocyte membrane properties and the ability of erythrocytes to function as hosts for the Plasmodium parasites and this was exploited for antimalarial therapy (Ziegler et al. 2002). From the results it was observed that vasicine and embelin showed good activity in inhibiting schizont maturation and merozoite invasion of RBC. Both the compounds have the potential to block parasite development.

There is a direct relationship between intra-erythrocytic anti-malarial activity and inhibition of hemozoin formation (Pandey et al., 1998). Out of three compounds screened for hemozoin formation inhibition, embelin showed very good inhibition with IC50 value of 11.66 mMeq at 5 molar equivalents of heme.

The inhibition of heme crystallization (hemozoin formation) by antimalarial drugs is mediated by binding to heme (Fitch et al., 2004) and around 70% of non-crystallized heme exists in the food vacuole and is subsequently catabolized by GSH (Ginsburg et al., 1998). Hence embelin was evaluated for its ability to bind the heme and inhibition of GSH dependent heme degradation. The absorption spectrum of heme in DMSO exhibited broad soret band at 386 nm resembling dimer form. There is a decrease in the absorbance at 386 nm, with increase in embelin concentration. The interaction of heme with embelin was monitored in the Soret region of the UV-visible spectrum. The interaction of heme and drugs in stoichiometries was further explored by Job’s plot (Huang et al., 1982). Formation of the complex is maximized when embelin and heme are present in a 2:3 ratio. The maximal absorption of the soret of heme (10 μM) was shifted to broader soret band between 360 to 370 nm after adding GSH (10 mM),
Effect of vasicine, vasicinone and embelin on some important targets.

probably due to the formation of GSH-heme complex. After addition of embelin to GSH-heme complex, there is a shift of peak to 327 and 396 nm, may be due to altered effect of GSH on heme.

In the catalytic domain of protein kinase, 40–60% of the residues are different between the mammalian and plasmodial enzymes, and the latter display unique structural features such as insertions or terminal extensions. These considerations suggest that the enzymes from \textit{P. falciparum} have different susceptibilities to kinase inhibitors helped to develop kinase inhibitors against protein kinase enzymes from \textit{P. falciparum}. (Kappes et al., 1999). Vasicine, vasicinone and embelin did not inhibit any of the protein kinase enzyme.

The plasmepsins produced by the \textit{Plasmodium} parasite are aspartic proteases and have been recognized as attractive targets for the design of novel chemotherapeutic compounds for the control of malaria (Olliaro and Goldberg, 1995). Vasicinone exhibited IC\textsubscript{50} value of 13.3 µM in plasmepsin II inhibition while vasicine and embelin did not show any inhibition up to 25 µM. None of the compounds showed Plasmepsin IV inhibition.

HRP-2 is largely made up of repeats involving three amino acids: histidine (34%), alanine (37%) and aspartic acid (10%) (Wellems and Howard, 1986). HRP-2 has been established as a catalyst for haemozoin formation, and haem metabolism has emerged as a potential drug target in the malaria parasite (Padmanban and Rangarajan et al., 2000). Vasicine, vasicinone and embelin showed moderate inhibition of histidine rich protein (HRP2) with IC\textsubscript{50} value of 8.62 µM, 8.04 µM, and 24.48 µM respectively as compared to CQ with IC\textsubscript{50} with 0.051 µM.

6.4 CONCLUSION

Embelin was found to target lysosomal food vacuole by demonstrating significant effect on respective heme. Since embelin was found to act on two different targets of the parasite development, chances of development of resistance are less in such cases. Vasicine and vasicinone doesn’t exhibited significant activity on none of the targets.
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