Design and Synthesis of Malarial Aspartic Protease Inhibitors

KAROLINA ERSMARK
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Abstract

Malaria is one of the major public health problems in the world. Approximately 500 million people are afflicted and almost 3 million people die from the disease each year. Of the four causative species *Plasmodium falciparum* is the most lethal. Due to the rapid spread of parasite resistance there is an urgent need for new antimalarial drugs with novel mechanisms of action. Several promising targets for drug intervention have been revealed.

This thesis addresses the parasitic aspartic proteases termed plasmepsins (Plm), which are considered crucial to the hemoglobin catabolism essential for parasite survival. The overall aim was to identify inhibitors of the *P. falciparum* Plm I, II, and IV. More specific objectives were to attain activity against *P. falciparum* in infected erythrocytes and selectivity versus the most homologous human aspartic protease cathepsin D (Cat D). To guide the design process the linear interaction energy (LIE) method was employed in combination with molecular dynamics.

Initial investigations of the stereochemical requirements for inhibition resulted in identification of an L-mannitol derived scaffold encompassing a 1,2-dihydroxyethylene transition state isostere with affinity for Plm II. Further modifications of this scaffold provided inhibitors of all three target plasmepsins (Plm I, II, and IV). Apart from the stereochemical analysis three major kinds of manipulation were explored: a) P1/P1′ and P2/P2′ side chain alterations, b) replacement of amide bonds by diacylhydrazine, 1,3,4-oxadiazole, and 1,2,4-triazole, and c) macrocyclization. Several inhibitors of Plm I and II with *K*<sub>i</sub> values below 10 nM were discovered and one Plm IV selective inhibitor comprising two oxadiazole rings was found which represents the most potent non-peptide Plm IV inhibitor (*K*<sub>i</sub> = 35 nM) reported to date. Some of the identified plasmepsin inhibitors demonstrated significant activity against *P. falciparum* in infected erythrocytes and all inhibitors showed a considerable selectivity for the plasmepsins over the human Cat D.

**Keywords:** malaria, plasmepsin, aspartic protease, protease inhibitor, macrocycle

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<th>Definition</th>
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<tbody>
<tr>
<td>ADME</td>
<td>administration distribution metabolism elimination</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<tr>
<td>Asn or N</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp or D</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>9-BBN</td>
<td>9-borabicyclo[3.3.1]nonyl</td>
</tr>
<tr>
<td>DDT</td>
<td>1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DHPS</td>
<td>dihydropteroate synthetase</td>
</tr>
<tr>
<td>DME</td>
<td>1,2-dimethoxyethane</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DOXP</td>
<td>D-1-deoxy-D-xylulose 5-phosphate</td>
</tr>
<tr>
<td>DPAP1</td>
<td>dipeptidyl aminopeptidase 1</td>
</tr>
<tr>
<td>Gly or G</td>
<td>glycine</td>
</tr>
<tr>
<td>HAP</td>
<td>histo-aspartic protease</td>
</tr>
<tr>
<td>HGPR</td>
<td>hypoxanthine-guanin phosphoribosyl</td>
</tr>
<tr>
<td>HMBC</td>
<td>multiple-bond heteronuclear multiple-quantum coherence</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>inhibitor concentration resulting in 50% inhibition</td>
</tr>
<tr>
<td>Ile or I</td>
<td>isoleucine</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>inhibition constant/dissociation constant for inhibitor (I) - enzyme (E) binding; $Kᵢ = [E][I]/[EI]$</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography- mass spectrometry</td>
</tr>
<tr>
<td>Leu or L</td>
<td>leucine</td>
</tr>
<tr>
<td>LIE</td>
<td>linear interaction energy</td>
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<tr>
<td>MD</td>
<td>molecular dynamics</td>
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<tr>
<td>Met or M</td>
<td>methionine</td>
</tr>
<tr>
<td>MIM</td>
<td>multilateral initiative on malaria</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
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<tr>
<td>Papp</td>
<td>apparent permeability coefficient</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>Pd₂(dba)₃</td>
<td>tris(dibenzylideneacetone)dipalladium</td>
</tr>
<tr>
<td>Phe or F</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>Plm</td>
<td>plasmepsin</td>
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</tbody>
</table>
Pro or P  proline
RBM  roll back malaria partnership
RCM  ring-closing metathesis
Scissile bond  the amide bond cleaved by the protease
Ser or S  serine
TDR  the special program in training and research in tropical diseases
Tf  trifluoromethanesulfonyl
THF  tetrahydrofuran
Thr or T  threonine
Tyr or Y  tyrosine
UV  ultraviolet
Val or V  valine
“Wat”  water molecule
WHO  world health organization
1 Introduction

1.1 Malaria

Malaria is one of the earliest known diseases. The name originates from the Italian “mala aria”, which means bad air; a suitable name since it was thought to arise from exhalation of swamps. The true cause of the disease became clear first in 1880, when the French researcher Laveran (Nobel Prize in Medicine 1907) discovered the malaria parasite in human blood. Some years later (1897), the English physician Ross (Nobel Prize in Medicine 1902) and the Italian zoologist Grassi (1898) demonstrated that the parasite was injected into the human bloodstream through the bite of an infected female mosquito.

Malaria parasites belong to the protozoan subkingdom of the class Sporozoa. Four species of the Plasmodium genus are responsible for human malaria: P. vivax, P. ovale, P. malariae, and P. falciparum. On an evolutionary basis P. vivax, P. ovale, and P. malariae are closely related to the simian malarias, whereas P. falciparum is thought to be of a more recent origin closely related to the malarias of birds.

The natural vectors of the malaria parasites are female Anopheles mosquitoes. Of the approximately 400 species about 60 transmit malaria under natural conditions.

The clinical picture of malaria varies with each species. However, the usual symptoms are chills and fever at more or less pronounced intervals. Due to development of so-called severe malaria, infection caused by P. falciparum is the only one normally lethal. Severe malaria is a complex multisystem disorder involving adherence of parasites to blood vessel endothelial cells and severe anemia.

1.1.1 The Burden of Malaria

Malaria today is a disease of poverty and underdeveloped countries. As a consequence of several factors, e.g. limited availability of medical care and lack of adequate diagnostic tools in most endemic areas, assessment of the malarial burden is difficult. Recent estimates of the global incidence vary
between 300 and 500 million clinical cases annually, of which 1-3 million are fatal.7,10 Most of the deaths are among young children under the age of five.7 In Africa, where 90% of all malaria mortalities occur,10 the disease is directly responsible for one in five childhood deaths.7,11

It is estimated that over 40% of the world’s population lives in malaria endemic areas, which are mainly centered throughout the tropics and the subtropical regions (Fig. 1).12 The global distribution as well as the endemicity are largely dependent on the type of mosquito, the parasite species and the climate.12

Figure 1. Malaria risk areas. (Reproduced with permission from WHO.)13

Several organized efforts to control the transmission of the disease have been made throughout history.14 Two major approaches have been employed: killing of the parasite and killing of the parasite vector. In addition, various attempts to develop antimalarial vaccines can be added to the arsenal of control efforts.15 The first large multilateral initiative was the WHO Malaria Eradication Program (1955-1969), which aimed at the total eradication of malaria mainly by vector control (particularly by using DDT).14 This effort failed but achieved regional eradication in Southern Europe and some countries in North Africa and the Middle East. Subsequent important initiatives were the creation of the Special Program in Training and Research in Tropical Diseases (TDR) in 1975, the Multilateral Initiative on Malaria (MIM) in 1997, the Roll Back Malaria Partnership (RBM) launched in 1998 by the WHO, and the Global Fund, created in 2002.14 Despite all efforts to reduce the burden of the disease the number of malaria cases is constantly increasing.7,16 This is primarily due to resistance of the
mosquito to insecticides and, even more important, a rapidly growing resistance of the malaria parasite to the available drugs. However, the recent completion of the *Plasmodium falciparum* genome project and the *Anopheles gambiae* genome project has offered new hope for future malaria control.

### 1.2 The Parasite Life Cycle

The life cycle of malaria parasites is complex and consists of several distinct phases. Two basic cycles, an *asexual cycle* in man and a *sexual cycle* in the female *Anopheles* mosquito, constitute the total life cycle (Fig. 2). In man the asexual cycle can be further divided into a *liver stage* or a *pre-erythrocytic stage* and an *erythrocytic stage* (Fig. 2).

**Figure 2.** The life cycle of the malaria parasite in the human host and the mosquito vector.
During the bite of an infected mosquito the malaria parasite is injected into the human host in the form of a sporozoite. The sporozoites migrate through the bloodstream to the liver, where they invade the hepatocytes. Inside the hepatocyte the sporozoite is converted to a trophozoite, which in turn divides into several schizonts. A membrane and a cytoplasm encapsulate each schizont forming a merozoite. The merozoites rupture the hepatocyte and are released back into the bloodstream. The development and multiplication of the parasite in the hepatocytes is called the pre-erythrocytic stage. This stage is asymptomatic and takes 5-16 days depending on the species. \textit{P. vivax} and \textit{P. ovale} are able to remain in this stage as dormant hypnozoites, capable of producing relapses years after the initial infection.\textsuperscript{5}

The subsequent erythrocytic stage begins with the merozoite invasion of an erythrocyte. Generally, the erythrocytic stage is similar to the pre-erythrocytic. After invasion, the merozoite transforms into the ring stage, which grows and matures to a trophozoite. The division of the mature trophozoite to schizonts in the erythrocytic stage is termed schizogony. A membrane and a cytoplasm surround each schizont and the merozoites formed rupture the host cell and invade new erythrocytes. Some of the merozoites do not divide but develop into microgametocytes and macrogametocytes, which degenerate within 6-12 hours if they are not taken up by another mosquito. The lysis of the erythrocytes is responsible for the fever paroxysms and the time intervals between cell lysis (fever), invasion of new erythrocytes, and their lysis (new fever attack) are different for each of the four parasite species.

The sexual reproduction cycle begins when a gametocyte is ingested during the bite of a mosquito. Depending on their sex, the gametocytes are transformed into male or female gametes in the gut of the mosquito. Gametes of the opposite sex merge to form diploid zygotes, which develop into mobile ookinetes. The ookinetes move to the mid-gut surface where they are converted into oocysts. Several hundreds of sporozoites are formed within the oocyst and liberation enables transport of the infectious sporozoites to the salivary glands. The mosquito is now able to transmit the infection during the next human bite, continuing the parasite life cycle.

1.3 Hemoglobin Metabolism

The parasite degrades most of the host cell hemoglobin during the morphologically separate phases inside the erythrocyte (ring stage, trophozoite stage, and schizont stage).\textsuperscript{24} The metabolic activity varies between the different phases and is most pronounced during the trypozoite stage.\textsuperscript{25}

Hemoglobin is primarily ingested by means of a system formed between the two membranes separating the parasite from the erythrocyte cytoplasm,
called the cytostome (Fig. 3). Vesicles budding from the cytostome transport hemoglobin to a specialized acidic food vacuole (pH ~5) where degradation takes place (Fig. 3).

Figure 3. Electron micrograph of a P. falciparum trophozoite inside an erythrocyte. (Reproduced with permission from the authors of ref. 27.)

The reason for hemoglobin degradation has been the subject of debate, and various hypotheses have been put forward. Since the Plasmodium parasite has a limited capacity for de novo amino acid synthesis it has been suggested that the hemoglobin derived residues are of vital importance for the protein biosynthesis of the parasite. The amino acids from hemoglobin proteolysis also appear to be available for energy metabolism. Excess amino acids are generated by this degradation raising the additional possibility of hemoglobin catabolism being in fact a necessary strategy to prevent premature erythrocyte lysis. Regardless of the reason, studies using protease inhibitors have proven hemoglobin degradation to be essential for parasite survival.

Several enzymes have been demonstrated to be involved in hemoglobin proteolysis. In P. falciparum these are aspartic proteases (plasmepsin (Plm) I, II, IV and the closely related histo-aspartic protease (HAP)), cysteine proteases (falcipain-1, -2, and -3), a metalloprotease (falcilysin), and the recently discovered dipeptidyl aminopeptidase 1 (DPAP1). The degradation process appears to follow an ordered pathway. However, it has been difficult to determine the precise sequence of events, especially whether a plasmepsin or a falcipain catalyzes the initial cleavage. The general pathway is outlined in Figure 4. Initial cleavage between Phe33 and Leu34 in the hinge region of the domain responsible for holding the oxygen bound tetramer together, unravels the protein exposing it to further cleavage. Subsequent cleavage into smaller peptides can be accomplished by both plasmepsins and falcipains. The metalloprotease falcilysin is only able to cleave small peptides, up to 20 amino acids, delivering even shorter oligopeptides. DPAP1 was recently discovered to cleave off dipeptides from
hemoglobin derived oligopeptides in the food vacuole. Final hydrolysis to free amino acids is thought to be carried out in the cytoplasm by aminopeptidases.

During hemoglobin degradation, free heme is released and almost entirely oxidized from the ferrous (+2) state to the ferric (+3) hematin. Both heme and hematin are potentially toxic to the parasite. To counter this, the parasite has evolved a detoxification system resulting in polymerization of hematin to the inert crystalline substance hemozoin (Fig. 4). Hemozoin, also known as the malaria pigment, is microscopically visible as a characteristic of the disease. Polymerase activity has been observed in connection with hemozoin formation. However, other studies demonstrate non-enzymatical polymerization. One suggestion is that a polymerase initiates the process, which thereafter continues spontaneously.

**Figure 4.** The general pathway for hemoglobin metabolism in the *P. falciparum* food vacuole.

1.4 Antimalarial Drugs

The discovery of drugs to combat malaria has to a large extent been serendipitous, and the mechanism of action of many agents is incompletely or totally unknown.

A common way to classify the different antimalarial drugs is in terms of their activity in different stages of the parasite life cycle.

1. Causal prophylaxis
   
   A causal prophylactic is an agent that has a lethal effect on the parasites in the pre-erythrocytic stages and thereby prevents the development of symp-
toms. Examples of causal prophylactic agents include primaquine, pyrimethamine, proguanil, dapsone, and doxycycline. The term true causal prophylaxis refers to the killing of sporozoites before they infect the hepatocytes. No such drug is available today, although true causal prophylaxis may be achieved in the future with vaccines.¹⁵

II Suppressive treatment
Suppressive treatment means inhibition of the erythrocytic stage keeping the individual free from symptoms by early treatment. Drugs used for this purpose include chloroquine and mefloquine. This class, together with the causal prophylactic agents, belongs to the drugs used for chemoprophylaxis when traveling to malaria endemic areas.

III Clinical cure
Agents in this category are also called blood schizonticides as they interrupt erythrocytic schizogony and terminate the clinical attack. This is the largest group including the quinolin-methanols (e.g. quinine and mefloquine), the 4-aminoquinolines (e.g. chloroquine), the phenanthrenes (e.g. halofantrine), the antifolates (e.g. pyrimethamine, proguanil, dapsone, and sulfadoxine), the artemisinin group (e.g. dihydroartemisinin, artesunate, and artemether) and some antibiotics (e.g. tetracycline and doxycycline).

IV Radical cure
Radical cure refers to the eradication of not only the erythrocytic parasites but also those in the pre-erythrocytic stage such as hypnozoites. Only primaquine has this action.

V Prevention of transmission
These agents prevent transmission via the mosquito by destroying the gametocytes (e.g. primaquine, proguanil, the artemisinins, and pyrimethamine).

Another way to group antimalarial drugs is according to their chemical structure (Fig. 5).⁴⁷
- Quinoline-Methanols
  This class of agents originates from the cinchona bark alkaloids. The two major agents are quinine and mefloquine.

  For hundreds of years quinine was the only known effective treatment for malaria.⁴⁸ Today, the advent of drug resistance has made its importance return mainly for the treatment of severe malaria. Mefloquine is a relatively expensive drug commonly used as a prophylactic for travelers to chloroquine-resistant areas.

  The mechanism of action of this group has been the focus of much research but is still not fully understood.⁴⁷ The most accepted hypothesis is interference with the detoxification of heme to hemozoine.⁴⁷

- 4-Aminoquinolines
  Chloroquine is the main 4-aminoquinoline used clinically. At first it was thought to be too toxic for human use, but this was reconsidered during the Second World War. Until a decade ago, chloroquine was the first-line treat-
ment in most parts of the world. Today, the extensive spread of parasite resistance has severely limited its use.

Several hypotheses have been proposed to explain the mechanism of action. As for the quinoline-methanols the most probable mechanism is interference with hemozoin formation, probably by heme/hematin-binding resulting in parasite death by heme/hematin poisoning.\textsuperscript{17,49}

- **8-Aminoquinolines**

Primaquine, derived from methylene blue,\textsuperscript{48} is so far the only drug on the market that can effect a radical cure by killing the hypnozoites. Alternative 8-aminoquinolines (e.g. tafenoquine)\textsuperscript{50} are under clinical development.\textsuperscript{51} The mechanism of action is unknown but is proposed to involve an effect on parasite mitochondria.\textsuperscript{46,47}

![Figure 5. Examples of antimalarial drugs from structurally different classes.](image)

- **Phenanthrenes**

This class was found to be active as antimalarials during the drug discovery efforts of the Second World War. However, due to the efficiency of chloroquine, halofantrine was not marketed until 1988. Adverse cardiac effects and high price have limited its use.\textsuperscript{17,52} Halofantrine has a blood schizonticidal effect, but the mechanism of action is still unknown.\textsuperscript{47,51}

- **Artimisinins -Sesquiterpene lactones**

These compounds are related to artemisinin, a sesquiterpene derived from the herb *Artemisia annua*, which has been used historically in China as a treatment for malaria.\textsuperscript{53} In addition to the natural artemisinin, semisynthetic derivatives have been increasingly employed during the past 20 years.\textsuperscript{54} The antimalarial action is mediated by free radicals and involves covalent linkage of artemisinin to parasite membranes, proteins, and heme.\textsuperscript{47,54,55}
• **Antifolates**
  This class can be further divided into two separate groups depending on their activity on the parasite’s folate pathway: inhibitors of DHFR, e.g. pyrimethamine and proguanil, and inhibitors of DHPS including the sulfonamides, e.g. sulfadoxine, and the sulfones, e.g. dapsone.\textsuperscript{47} A combination of these two groups, sulfadoxine-pyrimethamine (SP) is currently the first-line treatment in many parts of Africa.\textsuperscript{17}

• **Antibiotics/Tetracyclines**
  With the increase in drug resistance, the use of some antibiotics has been re-evaluated.\textsuperscript{47} The most commonly used antibiotics are tetracycline and doxycycline. These are generally used in combination with other drugs.\textsuperscript{17}

### 1.4.1 Parasite Drug Resistance

To date resistance has emerged towards all classes of antimalarial drugs except for the artemisinins.\textsuperscript{56} Despite not yet having been encountered in the field, it is believed that artemisinin resistance will develop in the near future as it has been observed in the murine \textit{P. yoelii}.\textsuperscript{57}

Among the human parasite species resistance has primarily been documented for \textit{P. falciparum} and \textit{P. vivax}, the two species accounting for more than 95% of all malaria cases.\textsuperscript{18} Additionally, multidrug-resistant strains of \textit{P. falciparum} are emerging in several parts of the world.\textsuperscript{18}

The molecular mechanisms behind resistance depend on the chemical class of the drug and its mechanism of action.\textsuperscript{56} Generally, resistance arises from mutations in genes encoding the parasite drug target or influx/efflux pumps that affect the concentration of the drug at the target.\textsuperscript{56} Resistance to chloroquine is thought to be multigenic resulting in a reduced access to heme/hematin, but the details have still not been fully elucidated.\textsuperscript{56,58,59}

From observations in the past, resistance to any new therapeutic agent can be expected. Strategies to lengthen drug lifetime are combination therapies and the use of old drugs where they remain effective.\textsuperscript{60} Several fixed combinations are under development and some have been approved for clinical use.\textsuperscript{54}

### 1.4.2 New Approaches to Antimalarial Drug Development

Two major approaches have been employed in the search for new antimalarial drugs. The most widely used is the development of chemically related analogs to the existing antimalarial agents.\textsuperscript{61-63} For example, a structurally less complicated and synthetically more easily accessible trioxalane derivative, related to artimisinin, has recently advanced to clinical trials.\textsuperscript{64}

The other approach is identification of novel drug targets and the design of chemical entities active on these targets.\textsuperscript{63} The newly released data from the sequenced \textit{P. falciparum} genome is expected to be very useful in this
A number of potential targets for drug intervention have emerged.\textsuperscript{54,61,63,65} The targets can be broadly classified into three categories: 1) targets involved in the hemoglobin metabolism, e.g. proteases such as the falcipains and the plasmepsins; 2) targets responsible for macromolecular and metabolite synthesis, e.g. DOXP reductoisomerase, farnesyl transferase, parasite HGPR transferase, and lactate dehydrogenase; and 3) targets engaged in membrane transport and signaling, e.g. the choline transporter and the protein kinases. Apart from rational design, new lead compounds are also being sought after from natural products and library screening.\textsuperscript{61,65-67}

The present work addresses the hemoglobin metabolism and is especially focused on inhibition of the plasmepsins, which belong to the aspartic protease family. The special aims are presented in Chapter 2.

1.5 Aspartic Proteases

Proteases, also referred to as peptidases or proteinases, are enzymes that catalyze the hydrolysis of amide bonds linking amino acids in peptides and proteins. Approximately 2\% of the genes in all kinds of organisms encode proteases.\textsuperscript{68} These enzymes are involved in a number of essential processes both in humans and pathogens, making them attractive as drug targets.\textsuperscript{69} In particular, the success achieved with protease inhibitors in the battle against HIV/AIDS has accelerated interest in these enzymes for drug design.

The\textsuperscript{MEROPS} database provides a hierarchical classification of proteases into subclasses, clans, and families.\textsuperscript{69} Depending on the structural element involved in the catalysis proteases are divided into four major subclasses: aspartic, cysteine, metallo-, and serine proteases.\textsuperscript{69} Structural homology further distinguishes each subclass into families and similar families are grouped into clans.\textsuperscript{69} Most of the aspartic proteases (including the plasmepsins and cathepsin D) are members of the pepsin family only found in eukaryotes which, together with the viral retropepsins (including the HIV-1 protease), constitute a clan.\textsuperscript{70}

The gastric aspartic protease pepsin was one of the first enzymes to be crystallized in the 1930s.\textsuperscript{71} However, the first fully characterized aspartic protease sequence was not available until 1973.\textsuperscript{72} Aspartic proteases consist of two domains (the pepsin family having one bilobed molecule) defining the active site, where each domain contributes one aspartic acid residue to the catalytic dyad.\textsuperscript{73} A conserved network of hydrogen bonds, termed the “fireman’s grip”,\textsuperscript{74} stabilizes the catalytic site structure and a $\beta$-hairpin turn, also known as the “flap”, covers the binding cleft with the ability to interact with substrates and inhibitors.\textsuperscript{73} Depending on the type of aspartic protease different numbers of conserved water molecules have been found to stabilize the enzyme geometry.\textsuperscript{75} Additional features of aspartic proteases are low
optimal pH and sensitivity to inhibition by *Streptomyces*-derived pepstatin.76,77

1.5.1 The Catalytic Mechanism

The catalytic mechanism of aspartic proteases has been extensively studied by kinetic methods, isotope labeling, theoretical calculations, and X-ray crystallography, as described in several reviews.73,78-80 Although the common consensus is a general acid-base mechanism some aspects are still not fully elucidated. A schematic representation of the mechanism of action is outlined in Figure 6.

![Figure 6. Schematic mechanism of action of aspartic proteases.](image)

In all aspartic proteases of the pepsin and retroviral families a catalytic water molecule has been found to be hydrogen bound between the two active site aspartates.75 This water, activated by the aspartic acids, makes a nucleophilic attack on the substrate amide carbonyl, generating a tetrahedral intermediate. Due to enzyme stabilization this intermediate is much lower in energy compared to the tetrahedral intermediate of a non-enzyme-catalyzed reaction, resulting in a considerably higher reaction rate.81 Protonation of the amide nitrogen results in a collapse of the tetrahedral intermediate and departure of the products. The protonation states of the two catalytic aspartic acids have been widely debated.73,78,79 One suggestion has been the existence of a low-barrier hydrogen bond where one proton is equally shared between the two aspartates.80

The substrate binds to the enzyme in its extended β-strand conformation.73,82 Selectivity between different proteases can occur as the protease recognizes a specific combination of amino acids (usually <10 residues) and upon binding these amino acids form complimentary interactions with the subsites of the enzyme.83,84 It has been proposed that subsite binding facilitates the distortion of the amide bond during the formation of the tetrahedral intermediate, which further lowers the activation energy.73 Schechter and Berger have established a nomenclature to designate substrate residues and the corresponding enzyme subsites based on their position relative to the
scissile amide bond (Fig. 7). Residues (P) in the direction from the scissile bond toward the C-terminal are denoted prime, while residues in the N-terminal direction are nonprimed. The same notation is applicable to the subsites (S) they occupy.

![Figure 7. Schechter and Berger’s nomenclature for substrate residues (P1-Pn/P1’-Pn’) and their corresponding binding sites (S1-Sn/S1’-Sn’).](image)

1.5.2 Inhibition Strategies

Proteases can be inhibited by several mechanisms including the formation of covalent and/or noncovalent bonds. Inhibition can be either reversible or irreversible, and the inhibitor can be directed towards the active site or elsewhere on the enzyme (allosteric inhibitors).

In fact, nature itself produces protease inhibitors. Natural proteinaceous inhibitors are classified in the MEROPS database in a similar way to the proteases. Historically, new inhibitors have been discovered either by chance or by screening of natural and synthetic compounds. Over the years, through a better understanding of biochemical processes, rational design has emerged as a complement to these techniques.

The general design strategy for inhibitors of aspartic proteases utilizes the enzyme stabilization of the tetrahedral intermediate. As a consequence of stabilization, compounds that mimic the intermediate bind much more tightly than the substrates. These compounds are imprecisely called transition-state analogs, even though most of them are actually analogs of high-energy intermediates.

A transition-state isostere is defined as a functional group that can mimic the tetrahedral intermediate of amide bond hydrolysis but is stable to enzymatic cleavage. Analysis of protease-inhibitor crystal structures shows that the catalytic water molecule of the native enzyme has been displaced by the transition-state mimicking moiety (Fig. 8). It has been suggested that this water release adds to the favorable binding energy, as it results in a gain in entropy. A number of chemical functionalities and structural units have been employed as non-cleavable transition-state isosteres, some of which are illustrated in Figure 8.
To generate an inhibitor specific to a certain protease, substrate-based design is usually employed to the remaining parts of the molecule. However, this tends to result in inhibitors, which are peptide-like in structure and therefore seldom clinically useful. The most important obstacle in the use of peptidic compounds as drugs is their rapid degradation by specific and nonspecific proteases resulting in low bioavailabilities and short half-lives.\cite{91}

Apart from peptide-likeness other structural features influence the clinical usefulness of a compound. Lipinski set out a “rule of 5” to guide molecular properties favorable for oral absorption.\cite{92} This rule states that good absorption is more likely when there are ≤ 5 H-bond donors, the molecular weight is < 500 Da, Log P < 5, and there are ≤ 10 H-bond acceptors. A compound that fulfills three of these four criteria is considered to be in agreement with Lipinski’s “rule of 5”. Recently, Veber proposed additional rules linked to oral bioavailability.\cite{93} His observations on rat suggested that an orally bioavailable compound should have ≤ 10 rotatable bonds and a polar surface area ≤ 140 Å² (or ≤ 12 H-bond donors and acceptors). Several other properties and chemical elements of importance for pharmacokinetics and “drug-likeness” have been discussed and reviewed in the literature.\cite{94,98}

Non-peptide inhibitors of aspartic proteases have been identified via high-throughput screening of compound libraries.\cite{99,100} These inhibitors bind in a
mechanism-based fashion with a hydrogen bonding functionality acting as a transition state isostere, as deduced from crystal structures. However, the other parts of the molecule bind in a totally different fashion from the substrate-based inhibitors. The active site topography of the protease stabilized by peptide-derived and non-peptide-derived inhibitors reveals major conformational differences. Previously unobserved enzyme conformations have been revealed by these non-peptide inhibitors. This unexpected flexibility offers new hope for finding novel, clinically useful inhibitors.

1.5.3 Ligand Binding

Historically, different models have been used to rationalize ligand-enzyme binding (Fig. 9). Originally, the events of ligand binding were described by the “lock and key” rationale where the enzyme is thought of as a rigid lock and the ligand is symbolized by the key. Later on, Koshland proposed the more flexible “induced fit” model. This model suggests that the substrate induces conformational distortion of the active site leading to a fit. More recently, a third hypothesis based on “conformational ensembles” has been presented. The enzyme is assumed to exist in numerous equilibrating conformations and the ligand stabilizes only the conformation in which the binding site is formed, shifting the equilibrium towards this conformation.

![Figure 9. Models proposed for ligand-enzyme binding](image)

1.6 The Plasmepsins

The *Plasmodium* aspartic proteases are termed plasmepsins. At least ten genes encoding aspartic proteases have been revealed in the *P. falciparum* genome (including Plm I, II, IV, V, VI, VII, VIII, IX, X, and the closely
related HAP).\textsuperscript{42} It has been suggested that the plasmepsin family is smaller in the other three human \textit{Plasmodium} species.\textsuperscript{105} The precise role of each plasmepsin in parasite metabolism is not clear. Expression of Plm I, II, IV, V, IX, X and HAP occurs in the erythrocytic stage, while Plm VI, VII, and VIII are expressed in the exo-erythrocytic stages.\textsuperscript{35}

To date, the most extensively studied plasmepsins are those involved in hemoglobin metabolism inside the parasite food vacuole. In \textit{P. falciparum} these are Plm I, II, IV and HAP.\textsuperscript{35} In the other human species only the ortholog of \textit{P. falciparum} Plm IV has been observed in the food vacuole.\textsuperscript{105} For almost a decade Plm I and II were the only plasmepsins demonstrated to be involved in the hemoglobin catabolism of \textit{P. falciparum}.\textsuperscript{32,106} Recently, Plm IV and HAP have also been localized in the food vacuole and shown to be capable of hemoglobin digestion.\textsuperscript{35}

It appears as the proform of all food vacuole plasmepsins are processed into mature Plm I, II, IV, and HAP by an atypical calpain-like protease, which has also been suggested as a potential target for drug development.\textsuperscript{107} The temporal expression of the four hemoglobin-degrading plasmepsins differs during the erythrocytic stage. Plm I is transcribed in the early ring stage followed by Plm II, which is optimally expressed in the trophozoite stage.\textsuperscript{106} HAP and Plm IV are only detectable from the trophozoite stage and all four persist to schizogony.\textsuperscript{35} Furthermore, differences in cleavage site specificity and pH optimum have been demonstrated.\textsuperscript{35} In addition to hemoglobin degradation, Plm II and IV might also be involved in cleavage of the host erythrocyte membrane skeleton.\textsuperscript{35,109,110}

As the name implies, the histo-aspartic protease (HAP) has a histidine at the position of one of the two catalytic aspartates.\textsuperscript{111} Whether this results in an aspartic protease-like mechanism or not has been the subject of discussion.\textsuperscript{112,113}

High levels of sequence homology (~60-70\%)\textsuperscript{105} are observed between Plm I, II, IV and HAP, which also lies in a cluster on the same gene.\textsuperscript{42} Compared to Plm II, the binding site region of Plm I, IV, and HAP show 84\%, 68\%, and 39\% identity, respectively.\textsuperscript{114} Plm V-X are not clustered and show much lower sequence similarity.\textsuperscript{42}

Crystal structures of Plm II, IV, and \textit{P. vivax} food vacuole Plm are presently available in the protein data bank (PDB).\textsuperscript{115}

1.6.1 Cathepsin D

Selectivity versus human aspartic proteases is important when developing inhibitors of pathogenic enzymes. The two major concerns are toxicity and/or diminution of effective concentrations reaching the pathogen.\textsuperscript{42} The plasmepsins have a varied degree of sequence homology with the human aspartic proteases, the most similar being cathepsin D (Cat D).\textsuperscript{116} Compa-
son between Plm II and Cat D shows a 35% sequence identity and even higher identity at the active site. \(^{117}\)

Cat D is a lysosomal enzyme present in most cell types. Its essentiality for survival has been demonstrated with knockout mice lacking Cat D.\(^{42}\)

### 1.7 Plasmepsin Inhibitors

The use of plasmepsin inhibitors has killed malaria parasites both in culture and in animal models, establishing proof of concept that these proteases are viable as drug targets.\(^{31,32,100,118}\) Analyses of substrate preferences and active site mutations have provided insight into the binding specificities of the different plasmepsins.\(^{119-127}\) Additionally, library screening and similarities to other aspartic proteases have been utilized in the search for active compounds. Both peptidomimetic substrate-based inhibitors and non-peptide inhibitors have been identified, and the advances have been continuously reviewed.\(^{63,100,128-131}\)

At the beginning of the 1990s Plm I and II were the only malarial plasmepsins characterized. Difficulties in obtaining active recombinant Plm I and an early available crystal structure of Plm II in complex with pepstatin A meant that the design efforts were primarily directed towards Plm II.\(^{117,118,132}\) Over the years characterization of additional plasmepsins has increased the number of targets for inhibitor design.\(^{42}\) Besides Plm I and II the food vacuolar Plm IV and HAP have been the subjects of attention.\(^{133}\) Despite the fact that aspartic protease inhibitors kill *P. falciparum* in culture, it is not clear which plasmepsin(s) is(are) essential for survival.\(^{42}\) Recent experiments with *P. falciparum* plasmepsin knockout clones indicate the importance of inhibiting several of these aspartic proteases in order to combat the parasite.\(^{134,135}\) Design of one compound that is able to inhibit several plasmepsins could be favorable not only for efficient killing of the parasite, but also to impede the emergence of parasite resistance. Major obstacles have been low selectivity over the human Cat D and insufficient activity in parasite-infected erythrocytes.

#### 1.7.1 Peptidomimetic Inhibitors

Most of the plasmepsin inhibitors identified so far have been peptidomimetic in structure and some examples are shown in Figures 10 (A-E) and 11 (F-I). Early inhibitors with greater activity against Plm I than Plm II were discovered by library screening (e.g. the aldehyde A, Fig. 10).\(^{32,118}\)

The peptidomimetic inhibitors can be divided according to their transition state isostere.
• **Statines**

The general aspartic protease inhibitor pepstatin A (B, Fig. 10), with high affinity for all four food vacuolar plasmepsins (Plm I, II, IV and HAP),\(^{31,35,136}\) has been used as a starting structure for optimization.\(^{117,137}\) Additionally, Cat D homology and statine-based libraries have been exploited in the search for active inhibitors.\(^{117,138-140}\) In this way several potent inhibitors have been identified (e.g. inhibitors C-E, Fig. 10). Evaluation of the stereogenic center at the statine hydroxyl indicated a preference for the \((S)\)-configuration, which is in agreement with the hydroxyl configuration in pepstatine A.\(^{138}\)

Cyclization strategies have been employed in inhibitor design (C and D, Fig. 10).\(^{117}\) A connection between the P1 and P3 positions, as in C, appears favorable for Plm II inhibition, while a P2-P3’ bridge, as in D, results in much lower affinity. The difference in potency is suggested to be attributed to the flap Val78 in Plm II interfering with the cyclic portion of the P2-P3’ bridge.\(^{117}\)

![Chemical structures](image)

**Figure 10.** An early identified peptidomimetic Plm I selective inhibitor (A) and examples of statine-based plasmepsin inhibitors (B-E). P. falciparum IC\(_{50}\) denotes the effect in cell culture, i.e. the inhibitory effect on P. falciparum growth in infected erythrocytes.

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**Figure 10.** An early identified peptidomimetic Plm I selective inhibitor (A) and examples of statine-based plasmepsin inhibitors (B-E). P. falciparum IC\(_{50}\) denotes the effect in cell culture, i.e. the inhibitory effect on P. falciparum growth in infected erythrocytes.
Selectivity versus Cat D has been demonstrated to be imparted by the P2 and P3 side chains, with a preference for \( \beta \)-branched groups in the P2 position towards Plm II.\textsuperscript{138,141} Moreover, biological screening of a combinatorial statine library reveals that selectivity of statine-based inhibitors can also be obtained by manipulation on the prime side.\textsuperscript{140}

Recently, the structure of Plm II in complex with inhibitor E has been presented by X-ray crystallography (1W6H). This inhibitor was also shown to be effective in reducing parasite growth in red blood cells.\textsuperscript{141}

Some modestly potent inhibitors of Plm I and II, referred to as reversed-statines (where a retro-amide replaces the amide bond on the prime side of the statine), have also been described.\textsuperscript{142,143}

- **Allophenylnorstatines**
  By investigating allophenylnorstatine-based compounds, initially designed against the HIV-1 protease, potent and selective plasmepsin inhibitors have been identified (e.g. F, Fig. 11).\textsuperscript{144} The (S)-configuration of the transition state mimicking hydroxyl was found to be superior over the (R)-isomer and a t-butylamide in P2' induced selectivity over Cat D.\textsuperscript{144}

  Allophenylnorstatines have also been employed in designing adaptive inhibitors.\textsuperscript{133} Adaptive inhibitors bind with high affinity to a primary target and maintain significant potency against the remaining enzyme family members. Plm II was selected as the primary target and retained activity was measured against Plm I, IV and HAP. The highest overall affinity was obtained with compound F (Fig. 11).\textsuperscript{133,145}

- **Hydroxyethylamine**
  Screening of a large hydroxyethylamine-based library designed against Cat D resulted in the identification of low molecular weight inhibitors of Plm II (inhibitor G, Fig. 11).\textsuperscript{146} Piperidine-based side chains in the P2' position appeared to be most important for plasmepsin selectivity. It was hypothesized that the basic side chains were favorable for trapping the inhibitors in the acidic food vacuole. Inhibitor G in this series has been crystallized in complex with Plm II (1LF3), revealing a significant flexibility of the S1' subsite.\textsuperscript{147}

  Based on what was known in the literature at the beginning of 1999 a series of Plm I and II inhibitors encompassing a basic hydroxyethylamine transition state isostere were designed (e.g. inhibitor H, Fig. 11).\textsuperscript{148,149} In general, large P1' side chains were preferred for high potency against the plasmepsins.\textsuperscript{148,149}

- **1,2-dihydroxyethylene**
  \( C_2 \)-symmetric N-terminal duplicated compounds originally developed as HIV-1 protease inhibitors have been redesigned against Plm I and II (e.g. inhibitor I, Fig. 11).\textsuperscript{150}
1.7.2 Non-Peptide Inhibitors

The structural relationship between the two aspartic proteases Plm II and renin, involved in blood pressure regulation, has been utilized in the search for non-peptide inhibitors. An X-ray structure of a 3,4-disubstituted piperidine in complex with renin revealed an unexpected flexibility. Scientists at Roche hypothesized that the flexible Plm II could probably accommodate this type of conformationally demanding ligand in a similar way. This was also confirmed by an unpublished crystal structure discussed in a recent review. Screening of the 3,4-disubstituted renin inhibitors led to the identification of Plm II inhibitors with high activity against *P. falciparum* in infected erythrocytes (e.g. inhibitor J, Fig. 12).

The X-ray structure of the piperidine-based inhibitor from Roche in complex with renin has also been used as a starting point for the rational design of Plm II and IV inhibitors incorporating an azabicyclic core structure. However, only inhibitors in the micromolar range were identified.

High throughput screening of a large commercial library and further optimization resulted in non-peptidic 4-amino-piperidine inhibitors of Plm II with significant selectivity versus Cat D. Some of the inhibitors were also active in mice infected with *P. berghei* (e.g. inhibitor K, Fig. 12).

Small non-peptidyl inhibitors of Plm II have also been discovered by screening of compounds in the Walter Reed chemical database and by natu-
ral product screening. However, most of these inhibitors exhibit low Plm potencies.

\[\text{Figure 12. Examples of non-peptide inhibitors (J and K) and the generic structure of a bifunctional potential plasmepsin and falcipain inhibitor (L). P. falciparum IC}_{50}\text{ denotes the effect in cell culture, i.e. the inhibitory effect on P. falciparum growth in infected erythrocytes.}\]

1.7.3 Bifunctional Inhibitors

A few examples of bifunctional plasmepsin inhibitors have been reported in the literature. In addition to plasmepsin inhibition these molecules also demonstrate other mechanisms of action against the parasite. Molecules with the ability to inhibit both the plasmepsins and the parasite falcipains have been claimed in a patent from 2001 (L, Fig. 12).

Several plasmepsin inhibitors have demonstrated higher potency in cell culture than in in vitro plasmepsin assays implying additional parasite targets. Detailed investigations of some of these inhibitors indicate additional inhibition of the parasite heme polymerization.

Statine-based Plm II inhibitors have been linked to primaquine (Sec. 1.4) in an effort to design antimalarials using a “double-drug” approach.
2 Aims of the Present Study

At the initiation of this project in the beginning of 2000, the only characterized *P. falciparum* plasmepsins were the food vacuolar Plm I and Plm II. The essential importance of these aspartic proteases for parasite survival had been demonstrated in cell culture.\textsuperscript{31,32} Today, at least eight additional plasmepsins have been identified in the *P. falciparum* genome. Two of these, Plm IV and HAP, have also been found to be involved in the hemoglobin catabolism in the parasite food vacuole.\textsuperscript{35}

The overall aim of this study was:

- to design and synthesize inhibitors of the *P. falciparum* plasmepsins.

The Specific objectives were:

- to identify a scaffold active against Plm II with potential for further optimization, (During the course of this project inhibition of other plasmepsins, such as Plm I and Plm IV, have emerged as important objectives.)

- to investigate the impact of scaffold manipulation on the affinity for Plm I, II, and IV aiming at selective as well as adaptive inhibitors,

- to establish structure-activity relationships guided by computational methods,

- to obtain activity against the *P. falciparum* parasites in infected erythrocytes based on the concept of plasmepsin inhibition, and

- to attain selectivity over the most homologous human aspartic protease Cat D.
3 Design of Plasmepsin Inhibitors

As described previously, the rational design of aspartic protease inhibitors relies on the stabilization of the tetrahedral intermediate of the enzymatic catalysis. By employing a chemical functionality mimicking the tetrahedral intermediate in combination with optimal design of the remaining parts of the molecule, high-affinity inhibitors can be achieved.

In this project the above strategy was applied to identify inhibitors active against the *P. falciparum* plasmepsins. When this project started only a few mechanism-based inhibitors of Plm I and II had been identified. However, previous efforts at discovering renin and HIV-1 protease inhibitors provided a pool of possible transition state isosteres that could be utilized in the search for potent inhibitors.

Rough computational modeling in Plm II together with a short proposed synthetic route from commercially available mannitol led to the emergence of 1,2-dihydroxyethylene as an attractive transition state isostere for the design of plasmepsin inhibitors. This type of scaffold had previously been developed in an effort to explore C-terminal duplicated inhibitors of the HIV-1 protease. In contrast to the malarial plasmepsins the HIV-1 protease is C2-symmetric in nature, which has also been exploited in the design of these inhibitors. Therefore, the first compounds assessed for plasmepsin inhibitory activity were those with a C2-symmetric structure.

Carbohydrates are usually convenient starting materials since they are often commercially available and encompass a number of defined stereocenters. The C2-symmetric inhibitors were obtained in three steps from mannitol, as outlined in Figure 13. Unsymmetrical inhibitors relying on the same scaffold were later developed specifically aiming at the plasmepsins.

We wanted to guide our design by computational tools. The X-ray structures of Plm II available at the beginning of 2000 revealed considerable flexibility of the enzyme. Especially the S1' subsite was found to be of varying size depending on the accommodated inhibitor. This Plm II flexibility was confirmed by thermodynamic experiments. Hence, a method that could take enzyme flexibility into account was important for reliable results. Molecular dynamics (MD) in combination with the linear interaction energy (LIE) method appeared to be a promising computational tool. During the MD simulation the enzyme-ligand complex is allowed to relax in order to attain the optimum fit. Since no other *P. falciparum* plasmepsin crystal
structure was available in the PDB at the initiation of this project only Plm II was used in the computational predictions.

Before starting a more thorough design process we wanted to investigate which stereochemistry, that originating from D- or L-mannitol, was most propitious for the establishment of favorable interactions between the scaffold and the active site, thus yielding the most active inhibitors (Paper I). Thereafter, based on the mannitol enantiomer with the optimal stereochemical configuration these $C_2$-symmetric diamide inhibitors were optimized by elongation of the $P_1$ and $P_1'$ side chains (Paper II). In the subsequent study we explored unsymmetrical inhibitors and different replacements of the inhibitor amide bonds (Paper III).

Cyclization of peptidic inhibitors reduces the number of flexible bonds and also the loss of entropy upon ligand binding. In the last study we adopted this concept to our plasmepsin inhibitors (Paper IV).

**Figure 13. Retrosynthetic analysis of symmetrical and unsymmetrical inhibitors derived from mannitol.**
4 Synthesis of Potential Plasmepsin Inhibitors

4.1 Synthesis of $C_2$-Symmetric Diamides (Papers I and II)

The $C_2$-symmetric diamides 8-18 (Scheme 1) were synthesized essentially according to a previously developed procedure for preparation of HIV-1 protease inhibitors.$^{161}$ Depending on the desired stereochemistry of the four central carbon atoms of the final diamide, either L-mannonic $\gamma$-lactone or D-mannitol was used as the starting material. The synthetic route is based on a procedure reported by Linstead et al.,$^{163}$ where nitric acid and heating were used to accomplish direct oxidation of D-mannitol to the bicyclic D-mannaro-1,4:3,6-dilactone 2. The unnatural L-mannitol was commercially available in the semi-oxidized form, L-mannonic $\gamma$-lactone. Starting from the semi-oxidized lactone facilitated the oxidation of the second lactone ring resulting in higher yields (60% from L-mannonic $\gamma$-lactone compared to 20% from D-mannitol).$^{161,163}$

Since the bislactone 1 was known to be unstable under basic conditions the subsequent alkylation yielding products 3-7 was performed under mildly acidic conditions using the appropriate trichloroacetimidate in dry dioxane. Initially, trifluoromethanesulphonic acid was employed as a catalyst in accordance with the previously published procedure.$^{161,164}$ However, a change to boron trifluoride etherate resulted in less by-products and higher yields. The phenyl bislactones 3 and 6 as well as the vinyl bislactone 7 were prepared using trifluoromethanesulphonic acid in 51%, 68%, and 51% yields, respectively, while the vinyl bislactone 4 and the (E)-bromovinyl bislactone 5 were synthesized using boron trifluoride etherate in 85% and 91% yields, respectively.

Of the three different trichloroacetimidates used only the benzyl trichloroacetimidate was commercially available. The allyl trichloroacetimidate and the (E)-bromoallyl trichloroacetimidate (see Paper II) were obtained from trichloroacetanitride and the corresponding alcohols.$^{165}$

In an attempt to produce the dipropynyl bislactone the propargyl trichloroacetimidate was prepared. However, the subsequent propargylation of bislactone 1 was not successful. A complex mixture of by-products was
formed and purification proved to be difficult. An effort was made to stabilize a plausible propargylic cation by converting the triple bond to a (ethynyl)dicobalt hexacarbonyl complex. Both the triple bond of the propargyl trichloroacetimidate and that of propargyl alcohol were converted to cobalt complexes and evaluated in the alkylation of bislactone 1, but without any success. However, the propargyl alcohol in complex with dico
bolt hexacarbonyl was successful in alkylationing cyclopentanol in a test reaction.

Nucleophilic ring opening of the bislactones 3-7 using excess amine (D- or L-valine methylamide or (1S,2R)-1-amino-2-indanol) at reflux resulted in the target diamides 8-18. Slightly increased yields of the diamides 9 and 14-16 were obtained when adding the nucleophile at 0 °C and slowly allowing the reaction to attain room temperature before reflux.

Scheme 1
4.1.1 Amide Reduction
In order to obtain a potential diamine inhibitor the diamide 13 was refluxed in THF with an excess of lithium aluminum hydride (Scheme 2). Surprisingly, the monoamine 20 was formed to a larger extent than the diamine 19, according to the analysis of the reaction mixture by LC-MS. Attempts to optimize the reaction conditions were made by increasing the molar equivalents of LiAlH₄. Unfortunately, this did not seem to affect the results significantly. Only the monoamine 20 was formed in a sufficient quantity to allow isolation, although in a low final yield.

Scheme 2

4.2 Synthesis of Inhibitors Comprising Amide Bond Replacement (Paper III)
Symmetrical and unsymmetrical plasmepsin inhibitors incorporating a replacement of one or both of the two amide bonds in the backbone were synthesized. A diacylhydrazine element, a 1,3,4-oxadiazole and a 1,2,4-triazole were chosen as diverse amide bond replacements. Only symmetrical inhibitors comprising the diacylhydrazine and 1,3,4-oxadiazole were prepared (Scheme 3). Vinyl bromides were consistently used in the P1 and P1’ positions since they were intended to serve as handles in future palladium-catalyzed transformations (Sec. 4.3.1).

4.2.1 Symmetrical Diacylhydrazines and 1,3,4-Oxadiazoles
The synthetic route to the symmetrical diacylhydrazines (21 and 22) and the symmetrical 1,3,4-oxadiazoles (23 and 24) is outlined in Scheme 3. The oxadiazoles were prepared via the diacylhydrazines. Nucleophilic ring open-
ing of the (E)-bromobislactone 5 using excess hydrazide at reflux furnished the diacylhydrazines 21 and 22.

As the inhibitors incorporate several functionalities mild synthetic conditions were preferred. Thus, commercially available Burgess reagent (Fig. 14) was used in the cyclodehydration reactions to produce the 1,3,4-oxadiazoles instead of the more harsh reagents usually employed, e.g. SOCl₂, POCl₃, polyphosphoric acid, or sulfuric acid.

To circumvent reaction between the hydroxyl groups and Burgess reagent the hydroxyls were first protected using chlorotrimethylsilane. Cyclodehydration was then effected with Burgess reagent and deprotection using potassium fluoride provided the symmetrical 1,3,4-oxadiazoles (23 and 24). The yields were significantly higher in the reactions involving the 4-tert-butylphenyl side chain on the hydrazide (22 and 24) than those with a methyl in the same position (21 and 23).

Initially a different approach to the diacylhydrazines was tried starting with hydrazine as the nucleophile in the ring opening of bislactone 5 followed by acylation using acid chloride. The hydroxyls had to be protected in order not to react with the acid chloride. However, difficulties in discriminating between the hydroxyls and the hydrazides during protection and a longer synthetic pathway made this route less attractive.

Scheme 3

Figure 14. Burgess reagent.
4.2.2 Unsymmetrical Diacylhydrazines and Heterocycles

The unsymmetrical diacylhydrazines (27-36) and the unsymmetrical 1,3,4-oxadiazoles (37-42) demonstrated in Scheme 4 were synthesized following essentially the same route as outlined for the symmetrical compounds. The major difference was that the bislactones (4 and 5) were opened in two successive steps resulting in the unsymmetrical monolactone intermediates 25 and 26. The reaction conditions used in the mono-opening were optimized for bislactone 5.

Scheme 4

1. TMSCl, NEt₃, CH₂Cl₂
2. Burgess reagent, THF
3. KF/H₂O, MeOH

2-Hydroxypyridine, CH₂Cl₂

R₁ = H
R₁ = Br

O
O
O
O

R₁
R₁

4: R₁ = H
5: R₁ = Br

R₁
R₁

25: R₁ = H (42%)
26: R₁ = Br (44%)

27: R₁ = H, R₂ = -CH₃CH₂ (74%)
28: R₁ = Br, R₂ = -CH₃ (64%)
29: R₁ = Br, R₂ = -CH₂CH₂ (71%)
30: R₁ = Br, R₂ = -CH₂ (69%)
31: R₁ = Br, R₂ = -CH₂ (70%)
32: R₁ = Br, R₂ = -CH₂CH₂ (74%)
33: R₁ = Br, R₂ = -CH₂CH₂CH₂ (74%)
34: R₁ = Br, R₂ = -CH₂CH₂ (51%)
35: R₁ = Br, R₂ = -CH₂ (45%)
36: R₁ = Br, R₂ = -CH₂CH₂NCH₂H₂ clipped ×2HCl (22%)

37: R₁ = Br, R₂ = -CH₃ (20%)
38: R₁ = Br, R₂ = -CH₂ (70%)
39: R₁ = Br, R₂ = -CH₃CH₂ (65%)
40: R₁ = Br, R₂ = -CH₂ (38%)
41: R₁ = Br, R₂ = -CH₂CH₂ (41%)
42: R₁ = Br, R₂ = -CH₂CH₂CH₂ (42%)
To suppress unwanted $\beta$-elimination, known to be a side-reaction during ring opening of analogous bislactones, 2-hydroxypyridine was employed as a catalyst. 2-Hydroxypyridine has previously been demonstrated to be an efficient catalyst of the conversion of esters to amides. The highest yield (44%) was achieved with 1 equivalent 2-hydroxypyridine and 1 equivalent (1S,2R)-1-amino-2-indanol in CH$_2$Cl$_2$ (at 0 °C to room temperature). Optimization of the reaction was a balance between the formation of di-opened lactone and unreacted starting material. In a procedure not published at the time employing a benzyloxy analog, 0.1 equivalents of 2-hydroxypyridine were used at reflux with a similar yield.

Another route to similar unsymmetrical compounds using solid phase chemistry has previously been developed. However, since bislactone was found to be unreactive towards solid supported (1S,2R)-1-amino-2-indanol the bislactone had to be transformed into the more reactive bissuccinimidyl ester, resulting in additional synthetic steps and thus a lower final yield.

The second lactone ring was opened with excess hydrazide to produce the unsymmetrical hydrazides, generally in good yields. In accordance with the previous observations the reaction rate for opening of the second lactone ring was considerably faster than the opening of the first. Some of the hydrazides needed to generate the unsymmetrical diacylhydrazines were not commercially available and were prepared from the corresponding esters using hydrazine, following procedures described in the literature.

Trimethylsilyl protection of the hydroxyls followed by cyclodehydration and deprotection delivered the 1,3,4-oxadiazoles in overall acceptable yields over three steps. A test reaction using Burgess reagent and unprotected diacylhydrazine resulted in a complex mixture devoid of any detectable product (1,3,4-oxadiazole) according to LC-MS. Thus, it was necessary to protect the hydroxyls during the cyclodehydration.

The 1,2,4-triazole was employed as an alternative heterocycle incorporating a hydrogen bond donor. One test compound incorporating the triazole was prepared as depicted in Scheme 5. 3,5-Disubstituted triazoles can be prepared via acyl amidrazone by condensation of the corresponding hydrazide and amidine. Nucleophilic ring opening of monolactone with hydrazine provided the hydrazide in very good yield. The hydrazide was then reacted with acetamidine to the acyl amidrazone (not isolated) and thermal cyclization afforded the 1,2,4-triazole in modest yield.
4.3 Palladium-Catalyzed P1 and P1′ Extensions (Papers II and III)

In order to extend the P1 and P1′ side chains of the diamide inhibitor 14 and the unsymmetrical diacylhydrazine inhibitor 32 different palladium-catalyzed coupling reactions were utilized. These coupling reactions are very useful since carbon-carbon bonds can be formed under mild conditions in the presence of various functionalities. The ability to make carbon-carbon bonds is crucial in the processes of building complex molecules from simple precursors in organic synthesis, and numerous reactions of this kind based on palladium catalysis have been developed.178,179

For the P1/P1′ extensions three different types of palladium-catalyzed coupling reactions were employed: Suzuki, Sonogashira, and Heck reactions. All three reactions follow roughly the same mechanistic pathway, schematically outlined in Figure 15: starting with a) oxidative addition (of e.g. an aryl or vinyl halide/triflate) to Pd(0) resulting in a Pd(II) species, followed by b) transmetalation (in the Suzuki and Sonogashira reactions) or $S$-complex formation, insertion and $E$-elimination (in the Heck reaction), and finally c) reductive elimination which regenerates the catalyst Pd(0).

Vinyl bromides with (E)-stereochemistry of the olefin were employed as handles in all palladium-catalyzed reactions. Initially different aryl halides or aryl triflates were used as coupling partners to the vinyls of 10 (in the Heck reactions) and the corresponding alkyl boranes of 10 (in the Suzuki reactions). However, no product was formed in Heck couplings between 10 and phenyl triflate and hydroboration of 10 was unsuccessful when using
Early attempts revealed, however, that the \((E)\)-bromoallyl compound 14 could easily be synthesized and that Suzuki reactions with this compound and phenyl boronic acid were successful. This led us to discard the first strategy and continue using the new approach based on vinyl bromides as coupling partners in the palladium-catalyzed reactions.

**Figure 15.** General mechanism of Pd-catalyzed couplings. The Suzuki and Sonogashira reactions involve transmetalation, while the Heck reaction includes \(\pi\)-complex formation, insertion, and \(\beta\)-elimination.

Microwave heating, extensively explored at our laboratory,\textsuperscript{180-184} was employed in all palladium-catalyzed couplings and the reactions were carried out in septum-sealed reaction vessels using a single-mode synthesizer with temperature and pressure control.

### 4.3.1 Suzuki Couplings

The Suzuki coupling, or the Suzuki-Miyaura coupling, was developed by Akira Suzuki and Norio Miyaura in 1979.\textsuperscript{185} In this reaction organic groups attached to boron are transferred to palladium during transmetalation. It has been shown that the addition of suitable bases exerts a remarkable effect on the transmetalation rate. The nucleophilicity of organic groups on boron is enhanced by coordination of a negatively charged base to the boron atom, which is thought to facilitate transmetalation.\textsuperscript{186} In addition, the choice of palladium catalyst (ligands), organoboron species, solvent, and temperature influences the reactivity and outcome of the reaction.\textsuperscript{186-188}

The \((E)\)-bromo vinyl precursor 14 was reacted with eight different arylboronic acids, as demonstrated in Table 1, to afford the \((E)\)-extended diamide.
inhibitors 45-52. The following reaction conditions were employed; 6 equiv aryl boronic acid, a catalytic amount 13-20 mol% Pd(PPh₃)₄, sodium carbonate as base, and the solvent system DME/H₂O/EtOH (12:4:3)¹⁸⁰,¹⁸⁹. Microwave heating, at 90 °C for 30 min, resulted in complete consumption of the starting material. Generally, no mono-coupled or dehalogenated by-products were detected by LC-MS. The moderate yields could to some extent be attributed to chromatographic purification on a non-optimized preparative LC system.

Table 1. Palladium-Catalyzed Suzuki Couplings Between the (E)-Bromo Vinyl Precursor 14 and Different Boronic Acids

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Boronic Acid</th>
<th>R-group</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>(HO)₂B</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>46</td>
<td>(HO)₂BF</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>47</td>
<td>(HO)₂B</td>
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<td>42</td>
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<td>48</td>
<td>(HO)₂B</td>
<td></td>
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</tr>
<tr>
<td>49</td>
<td>(HO)₂BO</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>50</td>
<td>(HO)₂B</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>51</td>
<td>(HO)₂B</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>52</td>
<td>(HO)₂B</td>
<td></td>
<td>61</td>
</tr>
</tbody>
</table>

The two extended unsymmetrical diacylhydrazines 53 and 54 were prepared from the diacylhydrazine 32 (Scheme 6). The unsymmetrical diacylhydrazine 32 was found to react much more sluggishly in the Suzuki couplings than the symmetrical diamide 14. The reaction conditions above were unsuccessful in attaining complete consumption of starting material and significant dehalogenation was detected by analysis of the crude reaction mixture using LC-MS. One possible explanation of the unreactive nature of compound 32 is trapping of the palladium catalyst by coordination to the diacylhydrazine element. Several different palladium catalysts (Pd(PPh₃)₄, Herrmann’s cata-
lyst\textsuperscript{190}/[(t-Bu)_3PH]BF_4, Pd_2(dba)_3/[(t-Bu)_3PH]BF_4, Pd(OAc)_2/2-
(dimethylamino)-2′-dicyclohexylphosphinobiphenyl), bases (Na_2CO_3, KF, Ba(OH)_2·8H_2O), solvents (DME/H_2O/EtOH 12:4:3, DME/EtOH 3:1, DME/H_2O 9:1, THF), and temperatures (90-150 °C) were investigated to optimize the reaction.

The best results were obtained with barium hydroxide octahydrate as the base and Pd_2(dba)_3/[(t-Bu)_3PH]BF_4 as the catalytic system. Microwave irradiation at 110 °C for 15 min generated the (E)-methylenedioxyphenyl 54, while a longer reaction time (40 min) was necessary with the less reactive electron-deficient 4-acetylphenylboronic acid to furnish the (E)-acetylphenyl 53. Despite optimization the extended products 53 and 54 were both isolated in low yields.

4.3.2 Sonogashira Couplings

A mild method for palladium-catalyzed cross-coupling of terminal alkynes with aryl or vinyl halides was developed by Sonogashira in 1975.\textsuperscript{191} The Sonogashira coupling is believed to proceed via transmetalation between a copper acetylide and Pd(II) with subsequent reductive elimination to yield the new C-C bond.\textsuperscript{192}

By reacting diamide 14 with two different acetylenes, phenylacetylene and trimethylsilylacetylene, the enyne extended products, 55 and 56, were obtained (Scheme 7). The reaction conditions employed were based on previously optimized conditions for trimethylsilylacetylene and 4-bromo anisole.\textsuperscript{193} Catalytic amounts of Pd(PPh_3)_4 and CuI were used in the presence of a base (diethylamine) in DMF. Microwave heating at 120 °C for 10 min with phenylacetylene furnished (E)-phenylacetylene 55 in high yield. The reaction with the less acidic and thus less reactive trimethylsilylacetylene to the (E)-trimethylsilyl-acetylene 56 was optimally performed at a lower temperature (90 °C) in combination with a longer reaction time (30 min).
The trimethylsilyl group of compound 56 was cleaved off using potassium fluoride to generate enyne 57 in excellent yield (Scheme 7).

Scheme 7

4.3.3 Heck Couplings

Moritani-Fujiwara194 and Heck195 independently discovered palladium-mediated vinylic substitution reactions in the late 1960s. Catalytic versions of these reactions were later reported by Heck196 and Mizoroki197 and further developed by Heck into the general method now termed Heck coupling.198 After oxidative addition, the organo Pd(II) complex coordinates to the π bond of the olefin and subsequent syn-insertion leads to the formation of a new C-C σ-bond.

Scheme 8

The regioselectivity of the insertion is influenced by electronic and steric factors. Several attempts have been made to control the regioselectivity.199-202
In the present work, acyclic, mono-substituted electron-deficient olefins were used which in the presence of monodentate ligands usually provide predominantly terminal insertion.\textsuperscript{200} The olefin product is released by β-hydride elimination, most often resulting in the thermodynamically stable (E)-isomer.\textsuperscript{201} Palladium(0) is finally regenerated by base-induced reductive elimination of the palladium(II) hydride.

Diamide 14 was reacted with styrene or methylacrylate to generate the dienes 58 and 59, as outlined in Scheme 8. The reactions were performed using Herrmann’s catalyst\textsuperscript{190}, diisopropylethylamine as base, and aqueous DMF. Microwave irradiation at 170 °C for 25 min and 150 °C for 30 min furnished the dienes 58 and 59 in 46% and 84% yields, respectively. As higher temperatures were needed to complete the Heck reactions, the thermally stable Herrmann’s catalyst was employed instead of the more labile Pd(PPh\textsubscript{3})\textsubscript{4}.

### 4.4 Synthesis of Macrocyclic Inhibitors (Paper IV)

To rigidify the mannitol-derived 1,2-dihydroxyethylene inhibitors ring-closing olefin metathesis was employed. Cyclization was performed between the side chains, resulting in a 13-membered and a 16-membered macrocycle.

Olefin metathesis (Fig. 16), which is yet another transition-metal-catalyzed C-C bond-forming reaction, was discovered during a study of Ziegler polymerizations.\textsuperscript{203} The initial “Ziegler type” catalysts were only compatible with unfunctionalized olefins. Together with a better understanding of the mechanism, new and more tolerant catalysts were developed.\textsuperscript{203} Among the most recently developed are the second generation ruthenium-based Grubbs catalysts, which combine high reactivity and robustness with a broad tolerance of functional groups.\textsuperscript{204} This new family of catalysts includes an $N$-heterocyclic carbene ligand instead of the previous phosphine ligands.\textsuperscript{205} In the present work, the commercially available second generation Grubbs catalyst incorporating a saturated 1,3-dimesitylimidazol-2-ylidene ligand was used (Scheme 9).\textsuperscript{206} The generally accepted mechanism of ruthenium-catalyzed ring-closing olefin metathesis, schematically outlined in Figure 16, consists of a sequence of reversible [2+2] cycloadditions/cycloreversions.\textsuperscript{203,205,207} The precise details of the individual steps are still under discussion.\textsuperscript{203,205,207}
Figure 16. A schematic description of the generally accepted mechanism of ruthenium-catalyzed ring-closing metathesis (RCM).

The synthetic route to the macrocycles 65 and 66 is illustrated in Scheme 9. The unsymmetrical diamides 63 and 64 were synthesized following the previously described synthesis (Scheme 4) of the unsymmetrical diacylhydrazines.

In order to obtain the unsymmetrically alkylated bislactone 60, 1.5 equiv. allyl trichloroacetimidate and 1.5 equiv. benzyl trichloroacetimidate were simultaneously added to bislactone 1, using boron trifluoride etherate as catalyst. This yielded a mixture of the dibenzylated and diallylated bislactones together with the asymmetrically alkylated 60. (1S,2R)-1-Amino-2-indanol was used in the next step to mono-open bislactone 60. No significant preference for either of the two lactones of 60 could be detected from the UV absorbance on LC-MS. An approximately 1:1 inseparable mixture of 61 and 62 was formed. The second lactone ring was opened using excess 7-octenamine to furnish the unsymmetrical diamides 63 and 64 in a total yield of 73%. Initially, the commercially available 10-undecenamine (incorporating three extra carbons in the alkyl chain) was employed to open the second lactone ring. However, the macrocycles formed with this chain length could not be separated.

Since the 7-octenamine was not commercially available it was prepared from the corresponding bromo octene via azide substitution followed by LiAlH₄ reduction to the amine (72% yield over two steps).

As the two diamides 63 and 64 could not be separated the product mixture was used in the ring-closing metathesis. A catalytic amount of second generation Grubbs catalyst was used in 1,2-dichloroethane at reflux to provide the macrocycles 65 and 66 in a total yield of 48%. The two macrocycles, 65 and 66, as well as the (E)- and (Z)-isomers of each cycle were separated by LC and the E/Z ratios were determined by NMR. The double-bond configurations were assigned from 3J couplings between the olefinic protons and from NOE experiments. Both the 13-membered (65) and the 16-membered...
ring (66) were formed with preference for the (E)-isomer, with E/Z ratios of ~19:1 and ~5:3, respectively.

Scheme 9

Structural assignments of compounds 65 and 66 were made using C-H connectivity information from HMBC NMR experiments starting from proton 1.
(Scheme 9). This proton has a long-range coupling to the carbonyl carbon 2, which in turn has a coupling to proton 3. Proton 3 has long-range couplings to three carbons, one of which (H-4) has couplings to either aromatic (in 65) or olefinic (in 66) protons, depending on the macrocycle.

Hydrogenation on Pd/C reduced the ring C-C double bond to give the saturated macrocycles 67 and 68 (Scheme 10). The only by-product detected by LC-MS was a small amount of debenzylated product.

Scheme 10
5 Biological and Computational Results

To investigate the ability of the synthesized target compounds, described in Section 4, to inhibit the \textit{P. falciparum} plasmepsins estimates of inhibition constants ($K_i$) were made from measurements in Plm I, II, and IV enzyme assays. In order to examine selectivity versus human Cat D, the target compounds were also assessed in a Cat D assay. All target compounds (8-18, 20-24, 27-42, 44-59, 65-68) were evaluated in the Plm II assay and most of them (12-14, 20-24, 27-42, 44-59, 65-68) were also assessed in the Plm I and Cat D assays. Since the Plm IV assay was not available at the beginning of this project, only some of the compounds described in Papers I-III (13, 20, 48-50, and 59) were selected for examination in this assay. However, all of the macrocyclic target compounds described in Paper IV (65-68) were investigated in the Plm IV assay.

In addition to high potency in enzyme assays the inhibitor must also be able to reach its site of action. The target organelle for the plasmepsin inhibitors is the food vacuole of the parasite, which is in turn situated inside the erythrocyte. This means that several membranes have to be crossed in order to reach the plasmepsins. To examine the ability of our compounds to reach the plasmepsin targets and kill the parasite in cell culture a set of compounds was selected among those described in Papers II and III (13, 20, 22, 24, 28, 30-32, 39, 40, 48-50) to be evaluated in \textit{P. falciparum}-infected erythrocytes. The ability to inhibit parasite growth was estimated as inhibition at a specific inhibitor concentration, expressed as a percentage.

Pharmacokinetic properties, including metabolism and membrane permeability, were investigated for a few inhibitors (8, 12, 45, and 47). The results are presented in Section 5.4 but have not been presented in any of the Papers I-IV.

All attempts to crystallize Plm II with different inhibitors in the series studied (30, 32, 37, 38, 41, 45, 47-51, 52-55, 59) were unsuccessful. In the absence of a crystal structure and to guide the design process the LIE method in combination with MD was employed. In contrast to several other computational methods, this approach allows enzyme flexibility. All inhibitors described in Paper I (8-11, 13 and 15-18) and selected inhibitors presented in Paper II (14, 20, 45, 48, 50, and 52) and Paper III (27, 28, 32, 37, 41, 53, and 54) were investigated using this method.
5.1 The Linear Interaction Energy Method

A number of theoretical approaches to predicting ligand-enzyme binding, ranging from rigid docking to more flexible methodologies, have been described in the literature. A detailed description of all these methods is beyond the scope of this presentation, but for a recent review, see ref 208.

In 1994, Åqvist et al. reported a new semi-empirical method for calculating free energies of binding from MD simulations. This method, which is referred to as the LIE method, is based on the interaction energies of the ligand with its surroundings in the “bound” and “free” states (Fig. 17).

![Figure 17. The LIE method calculates the absolute binding free energy (ΔG_{bind}) based on ligand-surrounding interactions in the “bound” and “free” states.](image)

According to the LIE method the absolute binding free energy (ΔG_{bind}) of a ligand-enzyme complex can be calculated from Equation 1:

\[
\Delta G_{\text{bind}} = \alpha\langle V_{l-s}^{vdW}\rangle_p - \langle V_{l-s}^{vdW}\rangle_w + \beta\langle V_{l-s}^{el}\rangle_p - \langle V_{l-s}^{el}\rangle_w + \gamma
\]  

where the subscript \(l-s\) denotes ligand-surrounding interactions, and the superscripts \(vdW\) and \(el\) denote nonpolar (van der Waals) and polar (electrostatic) components of the interaction energies, respectively. Thus, two MD simulations are needed; one with the ligand free in water (w), and one with the ligand in complex with the solvated protein/enzyme (p). By calculating the time averages from the MD trajectories the average interaction energies between the ligand and its surroundings can be obtained. The constant \(\beta\) originates from the linear response approximation for electrostatic forces, and \(\alpha\) is an empirical constant calibrated against experimental data. For some complexes introduction of a nonzero constant \(\gamma\) is needed in order to reproduce absolute binding free energies. Here the following values were used: \(\beta=0.33\), \(\alpha=0.181\), and \(\gamma=0.210\).

The inhibition constant, \(K_i\), can then be calculated from the absolute free energy, using \(\Delta G_{\text{bind}} = RT\ln K_i\).

Prior to this project the LIE method has been used successfully to predict the affinities of inhibitors of other enzymes e.g. the HIV-1 protease, DHFR, and human thrombin. At the initiation of this project only one crystal structure, that of Plm II in complex with pepstatine A (1SME), was available in the PDB. Consequently, this structure was employed as a starting point for all simulations described in Paper I. Although new X-ray structures became available we
decided to continue using 1SME in the LIE predictions for two reasons: 1) more reliable comparisons could be made between different ligands if the same starting structure was employed, and 2) it was found that the active sites of the more recently available X-ray structures were much more open than that of 1SME. If a small ligand is used this results in no initial interactions with some parts of the active site, rendering this kind of structure more difficult to simulate.

In order to obtain reliable results from the LIE method it is important to start from well-docked conformations of the enzyme-ligand complex. Since the S1’ subsite is collapsed in 1SME, automated docking with our inhibitors and a rigid enzyme was unsuccessful. Instead, manual docking, based on the similarities between our inhibitors and pepstatin A, was employed to generate the starting structures, as described in Papers I-III.

5.2 Inhibitor Stereochemistry and P2/P2’ Side Chains (Paper I)

The 3D orientation of the inhibitor functionalities is of essential importance for the establishment of optimal interactions with the enzyme active site. Thus, stereochemistry plays a crucial role in molecular recognition. For instance the capability of hydrogen bond formation is usually a key determinant for specificity.211,214 We decided at an early stage to examine which stereochemical configuration of the four central carbon atoms of the inhibitors, originating from the natural D or the unnatural L-mannitol, was preferred for optimal affinity for Plm II. Since it was known from the literature that $\beta$-branched side chains were allowed both as P2 and P2’ substituents, valine methylamide was selected as a starting point at these positions.120,138 The stereochemistry of the P2/P2’ side chains was investigated simultaneously with the stereochemistry of the central backbone. In addition, the size of the P1/P1’ substituents was studied by using allyloxy or benzyloxy groups. This resulted in the two series of stereoisomers shown in Table 2, benzyloxy (8, 9, 15, and 16) and allyloxy (10, 11, 17, and 18). The results obtained from a standardized Plm II assay and from MD in combination with the LIE method are summarized in Table 2.

As anticipated, the stereochemical configuration was of vital importance in attaining inhibitory potency. Only the \((SRRRRS)\) isomers 8 and 10, originating from L-mannitol and L-valine, demonstrated activity in the Plm II assay at concentrations up to 10 $\mu$M. The same stereochemical ranking was obtained from the MD simulations with the LIE method ($K_i$ Calc, Table 2). The difference in affinity between the \((SRRRRS)\) inhibitors with benzyloxy (8) or allyloxy (10) P1/P1’ side chains was negligible in the Plm II assay,
while the computational results indicated a more pronounced preference for benzyloxy substituents.

Table 2. Experimental and Calculated Plm II Inhibition Constants and Interaction Energies of the D- and L-Mannitol Derived 8-11 and 15-18

<table>
<thead>
<tr>
<th>Cpmd</th>
<th>R-group</th>
<th>Config</th>
<th>( K_i ) (( \mu )M)</th>
<th>( \Delta G_{\text{bind}} ) (kcal/mol)</th>
<th>Ligand-surrounding interactions (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Exp</td>
<td>Calc</td>
<td>Exp</td>
</tr>
<tr>
<td>8</td>
<td>phenyl</td>
<td>SRRRSS</td>
<td>3.8</td>
<td>0.52</td>
<td>-7.4</td>
</tr>
<tr>
<td>9</td>
<td>phenyl</td>
<td>RRBBBB</td>
<td>&gt;10</td>
<td>160</td>
<td>nd</td>
</tr>
<tr>
<td>15</td>
<td>phenyl</td>
<td>SSSSSS</td>
<td>&gt;10</td>
<td>7.8 (-10^3)</td>
<td>nd</td>
</tr>
<tr>
<td>16</td>
<td>phenyl</td>
<td>RSSSSR</td>
<td>&gt;10</td>
<td>1.5 (-10^4)</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>vinyl</td>
<td>SRRRSS</td>
<td>4.1</td>
<td>11</td>
<td>-7.4</td>
</tr>
<tr>
<td>11</td>
<td>vinyl</td>
<td>RRBBBB</td>
<td>&gt;10</td>
<td>330</td>
<td>nd</td>
</tr>
<tr>
<td>17</td>
<td>vinyl</td>
<td>SSSSSS</td>
<td>&gt;10</td>
<td>1.3 (-10^4)</td>
<td>nd</td>
</tr>
<tr>
<td>18</td>
<td>vinyl</td>
<td>RSSSSR</td>
<td>&gt;10</td>
<td>9.4 (-10^3)</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd, not determined. * See Paper I for standard deviations.

An investigation of the nonpolar and polar energies in Table 2 reveals that all stereoisomers of each series had favorable nonpolar interactions with the Plm II active site. Actually, the (SRRRSS) isomers displayed the least favorable hydrophobic interactions. On the other hand, only the two (SRRRSS) isomers 8 and 10 had favorable polar interactions with Plm II. All of the other stereoisomers in both series exhibited repulsive polar interactions to Plm II. Thus, it is evident that the stereoselectivity mainly depends on electrostatic interactions, such as H-bonds, while hydrophobic interactions are responsible for nonspecific affinities.

In the simulations the two (SRRRSS) isomers 8 and 10 were found to adopt the same conformations and interaction patterns with the Plm II active site. The predicted binding conformation of inhibitor 8 is shown in Figure 18 superimposed on the crystal structure 1SME. A schematic picture of the same complex is shown in Figure 19.

The hydrogen bonding pattern between the inhibitors and Plm II on the prime side was essentially the same as that of pepstatine A in the initial crystal structure. The prime side hydroxyl was positioned in a similar fashion to the transition state mimicking hydroxyl of pepstatin A, i.e. between the two
carboxylic acids of Asp34 and Asp214. Due to a small rotation of the prime side methylamide a hydrogen bond to the carbonyl of Asp76 was lost. The P1’ side chain, which was modeled into the collapsed S1’ subsite, exhibited nonpolar interactions with Val78, Tyr192, Ile212, Thr298, and Ile300. The only movement observed in our MD simulations when accommodating the P1’ group was rotation of the Phe294 side chain. This is in contrast to the recent X-ray 1LF3,147 where the backbones of the two loops Met75-Val82 and Leu292-Pro197 are displaced to fit a bulky P1’ side chain. The P2’ valine was found to be oriented into the S2’ subsite in a similar fashion as the P2’ Ala of pepstatine A.

As a consequence of the C₂-symmetry of our inhibitors the amide bonds on the nonprime side are reversed compared to pepstatine A. Hence, the hydrogen bonding pattern on this side, described in Figures 18 and 19, differs significantly compared to pepstatine A. Notably, a water molecule, not found in 1SME, entered during the MD simulation and formed a bridge of hydrogen bonds between the nonprime hydroxyl and the S2 Thr217. Moreover, the P1 group was situated in S1 turning towards S3, and the P2 valine, though somewhat displaced compared to the P2 valine of peptatin A, was found in the S2 pocket.

Figure 18. Stereo view (MD average) of the predicted Plm II (yellow) binding of inhibitor 8 (green), superimposed on the X-ray structure 1SME117 of pepstatine A (purple) in complex with Plm II (light blue). The water molecule found to bridge between the nonprime hydroxyl and Thr217 is denoted “Wat”.

The compounds in the initial series of isomers were only weak inhibitors of Plm II. In order to increase affinity and at the same time reduce the peptide-like properties a replacement of the P2/P2’ valines was considered. 2-Indanol which has successfully been used in these positions in HIV-1 protease inhibitors,161,215 appeared to be an interesting alternative. Hence, the optimal L-
mannitol derived (RRRR) stereochemistry was applied to the central scaffold and the P2/P2’ valines were replaced by (1S,2R)-1-amino-2-indanol. In order to computationally investigate the effect of the indanols alone, the smaller allyloxy P1/P1’ side chains were employed. The resulting inhibitor 13 was biologically and computationally assessed. The results are demonstrated in Table 3.

Table 3. Experimental and Calculated Plm II Inhibition of Inhibitor 13

<table>
<thead>
<tr>
<th>Plm II ( K_i ) (( \mu \text{M} ))</th>
<th>( \Delta G_{\text{bind}} ) (kcal/mol)</th>
<th>Ligand-surrounding interactions (kcal/mol)¹</th>
</tr>
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<tbody>
<tr>
<td>Exp</td>
<td>Calc</td>
<td>Exp</td>
</tr>
<tr>
<td>0.096</td>
<td>0.24</td>
<td>-9.6</td>
</tr>
</tbody>
</table>

¹See Paper I for standard deviations.

Both the Plm II assay and the LIE computations revealed a 40-fold improvement in binding affinity of inhibitor 13 compared to the corresponding divaline 10. The hydrophobic as well as the electrostatic energies from the MD simulations were found to be more favorable with 2-indanol as P2 and P2’ substituent than with valine residues in these positions (cf. 13 and 10).

The simulated binding mode of the central parts of inhibitor 13 (the two hydroxyls, P1/P1’ allyloxy substituents, and the mannitol-derived carbonyls) was virtually identical to the simulated binding mode of the corresponding parts in inhibitors 8 and 10. A schematic illustration of the predicted binding interactions of 13 is shown in Figure 19, and Figure 20 panel A (Sec. 5.3) shows the predicted binding conformation superimposed on the structure ISME. The aromatic ring of the P2’ indanol was aligned along the P2’ methylamide of inhibitor 8 and was stable in this position during the whole simulation. During most of the simulation the P2’ indan hydroxyl was involved in forming an intramolecular hydrogen bond with the amide carbonyl or with the carbonyl of Asn76. On the nonprime side the indanol was much more flexible. One side of the ring system was exposed to the solvent while the other had nonpolar interactions with Thr114, Ile290, Ser79 and the P1 allyloxy. Similar to the P2’ hydroxyl, the P2 hydroxyl also formed an intramolecular hydrogen bond with the nearby amide carbonyl.
5.3 P1 and P1’ Extensions of the Diamide Inhibitors
(Paper II)

Encouraged by the fact that the Plm II S1’ subsite exhibits a pronounced flexibility and by the notion that the S1 and S3 subsites constitute one large groove,117 we decided to investigate the impact of extending both the P1 and P1’ side chains simultaneously starting from inhibitor 13. In addition to Plm II, these inhibitors (12-14, 20, 45-52, and 55-59) were also examined against Plm I and the human Cat D. Six of the compounds (13, 20, 48-50, and 59) were evaluated for their ability to inhibit P. falciparum growth in cell culture. Recently, some of the inhibitors (13, 20, 48-50, and 59) were selected for further assessment in a Plm IV assay. All biological results as well as the computationally predicted inhibition constants for seven of the inhibitors (12-14, 20, 45, 48, 50, and 52) are presented in Table 4.

A comparison between the extended inhibitors 12, 14, 45-52, and 55-59 and the parent compound 13 demonstrates that elongation of the P1 and P1’ side chains generally results in higher affinity for both Plm I and II. However, the effect on Plm II was much less pronounced. In fact, some of the inhibitors with extended P1/P1’ substituents (47, 52, and 56) were less active against Plm II than the smaller P1/P1’ allyloxy inhibitor 13. One of the most potent inhibitors with a 4-acetylphenyl extension (48) demonstrated a >200-fold higher activity in the Plm I assay and a >10-fold higher activity in the Plm II assay than inhibitor 13.
Table 4. Inhibition Constants ($K_i$) and Inhibitory Activities Against *P. falciparum* in Infected Erythrocytes for P1/P1' Extended Compounds

<table>
<thead>
<tr>
<th>Structure</th>
<th>Enzyme $K_i$ (nM)</th>
<th>%inh @ 5 μM (Cell)</th>
</tr>
</thead>
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<td>Plm I Calc</td>
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</tr>
<tr>
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<td>&gt;2500</td>
</tr>
<tr>
<td><img src="image" alt="Structure 45" /></td>
<td>1.4</td>
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<tr>
<td><img src="image" alt="Structure 46" /></td>
<td>2.5</td>
<td>59</td>
</tr>
<tr>
<td>Structure</td>
<td>Enzyme $K_i$ (nM)</td>
<td>%inh @ 5 μM (Cell)$^b$</td>
</tr>
<tr>
<td>-----------</td>
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<td>-------------------------</td>
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<tr>
<td></td>
<td>Plm I</td>
<td>Plm II</td>
</tr>
<tr>
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<td>Calc</td>
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<tr>
<td><img src="image" alt="Structure 49" /></td>
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<td><img src="image" alt="Structure 51" /></td>
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</tbody>
</table>

$^a$ Calculated value

$^b$ Initial % inhibition at 5 μM concentration.

Table 4. (Continued)
<table>
<thead>
<tr>
<th>Structure</th>
<th>Enzyme $K_i$ (nM)</th>
<th>%inh ( \approx ) 5 μM (Cell)$^a$</th>
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</thead>
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<tr>
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<td>37</td>
</tr>
<tr>
<td>58</td>
<td>4.4</td>
<td>15</td>
</tr>
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</table>
The extended inhibitors were consistently more potent against Plm I than Plm II. Despite the lack of an X-ray structure of Plm I we reasoned from homology models that a possible explanation could be a more open active site, less sensitive to the small variations in the extended P1/P1’ arms.

Since poor solubility influences the inhibition constants the solubilities of the inhibitors (13, 14, 45-52, and 55-59) were measured in PBS buffer at pH 7.5. For all compounds the solubility was found to be safely above (at least 23 times) the inhibition constants.

Overall excellent agreement was obtained between the experimental Plm II $K_i$ values and the LIE-derived constants. The binding free energies and the contribution from nonpolar and polar interactions are given in Table 5.

The targeted plasmepsins are all active in the acidic food vacuole of the parasite. Thus, an inhibitor bearing a basic functionality, e.g. an amine, could be concentrated in the acidic organelle by protonation trapping. This strategy was utilized in the design of inhibitor 20 where one of the amides had been reduced to the corresponding amine. Unfortunately, this compound was completely inactive in all enzyme assays evaluated, including the Plm IV assay. The computational results from MD simulations of inhibitor 20, in both its charged and neutral states, with Plm II were consistent with this finding. The calculations indicate that the charge is better solvated in water than in the lipophilic binding site of the enzyme. Probably a positively charged compound must be favorably solvated by the enzyme in order to attain inhibitory potency. For example, basic transition state isosteres are thought to be stabilized by the catalytic aspartates, mimicking a late transition state.216 Although the neutral form was predicted to have some affinity for Plm II, correction for deprotonation at pH 4.5 resulted in a lower affinity than for the protonated form.

Notably, none of the selected Plm I and II inhibitors displayed any activity in the Plm IV assay at concentrations up to 5000 nM.

The most potent Plm II inhibitor 48, with the 4-acetylphenyl extensions, also exerted the greatest inhibition of parasite growth in infected erythrocytes (78% at 5 μM). Most striking of the cell assay results was that while
amine 20 was inactive in all of the three plasmepsin assays it displayed significant inhibition of parasite growth in infected erythrocytes. Possible explanations for this observation could be inhibition of other plasmepsins (than Plm I, II, and IV) or an entirely different mechanism of action.

Without exception, all of the inhibitors in this series were inactive in the Cat D assay. The reason for selectivity has been investigated using computational methods, but so far these methods have not been able to provide an explanation for the high Plm II/Cat D selectivity encountered.

Table 5. Experimental and Calculated Plm II Binding Free Energies

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>$\Delta G_{\text{bind}}$ (kcal/mol)</th>
<th>Ligand-surrounding interactions</th>
<th>(kcal/mol)$^a$</th>
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</thead>
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<td>Calc$^a$</td>
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<td>52</td>
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<td>-69.8</td>
</tr>
</tbody>
</table>

nd, not determined. $^a$ See Paper II for standard deviations. $^b$ The calculated binding free energy of the charged compound 20, corrected for the contribution of charged amino acids that were not taken into account in the simulation. $^c$ The calculated binding free energy of the neutral compound 20, corrected for the penalty of deprotonation at pH 4.5. $^d$ The binding free energy of compound 14 was calculated in Paper III.

As can be seen in Table 5, the largest contributions to Plm II binding of the P1/P1’ extended inhibitors were nonpolar interactions. Actually, the polar contribution was not even favorable for all of the investigated inhibitors. The proposed binding mode of the most potent Plm II inhibitor 48 is depicted in Figure 20, superimposed on the Plm II crystal structure in complex with pepstatine A (1SME, panel B) or in complex with G (1LF3, panel C). The central part of the inhibitor backbone (between the two amides) as well as the P2’ indanol was predicted to adopt essentially the same binding mode and hydrogen bonding pattern as inhibitor 13, shown in panel A (Fig. 21). According to the simulations the flexible S1’ pocket was able to accommodate large P1’ substituents. The most extended P1’ side chains, 4-acetylphenyl (48) and methylenedioxyphenyl (50), were actually reaching all the way out into solution. Besides a rotation of the Phe294 side chain, described above (Sec. 5.2) to accommodate bulky P1’ groups, a displacement of the Gly291-Pro295 loop backbone was also observed in the simulations with these two P1’ side chains.
Figure 20. Panels A and B: Stereo views (MD averages) of the predicted Plm II (yellow) binding of the inhibitors 13 (panel A, green) and 48 (panel B, green), both superimposed on the X-ray structure ISME\(^{117}\) of pepstatin A (purple) in complex with Plm II (light blue). Panel C: Stereo view (MD average) of the predicted Plm II (yellow) binding of inhibitor 48 (green), superimposed on the X-ray structure 1LF3\(^{147}\) of the hydroxyethylamine inhibitor G (purple, Sec. 1.7.1) in complex with Plm II (light blue). The water molecule found to bridge between the nonprime hydroxyl and Thr217 is shown in all three panels and denoted “Wat” in panel A. In panels B and C a second water molecule bridging the P2 indan hydroxyl and Thr221 is also shown.
The extended P1’ substituents were in fact predicted to be situated in a very similar position to the methylenedioxyphenethyl P1’ group of the crystallized G (Fig. 20, panel C). All the elongated P1 side chains were found to be oriented along the unobstructed S1-S3 cleft in the simulations of 12, 14, 45, 48, 50, and 52. This resulted in a relocation of the P2 indan from the S3 to the S2 subsite. Thus, the previous intramolecular hydrogen bond of the indan hydroxyl was broken and instead a hydrogen bond with the amide nitrogen of Ser218 and another to a water molecule bridging an interaction to Thr221 were observed. This conformational change could possibly account, to some extent, for the higher affinity of the P1/P1’ elongated inhibitors.

5.4 Caco-2 and Metabolism

In order to obtain information about the pharmacokinetic properties of this class of inhibitors the metabolism of two of the inhibitors (8 and 12 described in Paper I) and the cell permeability of four inhibitors (8 and 12 described in Paper I and 45 and 47 presented in Paper II) were examined.

The metabolism was estimated as the percentage of metabolized compound using human microsomes. The divaline 8 was metabolized to 66% after ten minutes and the diindanol 12 was almost entirely metabolized (97%) after the same time. The rapid metabolism of inhibitor 12 was possibly due to the amino indanol group, which in indinavir is known to be readily oxidized in the benzylic position. Membrane permeability was assessed using a standardized Caco-2 assay as described earlier. The apparent permeability coefficients (Papp) of inhibitors 8, 12, 45, and 47 were found to be <0.9·10⁻⁶ cm/s, 9.2·10⁻⁶ cm/s, 5.0·10⁻⁶ cm/s, and 1.2·10⁻⁶ cm/s, respectively. Compounds with Papp values >1·10⁻⁶ cm/s are usually well absorbed across the human intestine. However, inhibitor 12, with the greatest Caco-2 permeability, exhibited extensive hepatic metabolism, which would probably result in negligible oral bioavailability.

5.5 Inhibitors Comprising Amide Bond Replacement (Paper III)

As described in Section 1.5.2, protease inhibitors usually originate from the preferred cleavage site of the natural peptide substrate, resulting in “peptide-like” structures. Peptidic compounds are known to be unsuitable as drugs due to their low metabolic stability, poor oral absorption, rapid excretion and low selectivity. Bioisosteric transformations of functionalities is a com-
mon approach to maintain the biological activity while improving the ADME properties.\textsuperscript{221,222} Especially bioisosteric replacements of the amide bonds have been the focus of attention when converting peptides to non-peptides.\textsuperscript{221}

In an effort to transform our inhibitors into less peptidic structures, replacement of one or both of the two amide bonds in the backbone of the scaffold was performed. We decided to use two diverse entities with respect to size, flexibility, and hydrogen bond donating/accepting capacities. Considering the direction of the P2 and P2’ side chains in the $C_2$-symmetric scaffold the 1,3,4-oxadiazole and diacylhydrazine appeared to be promising amide bond replacements. In addition, the diacylhydrazine unit has previously been employed in cysteine protease inhibitors,\textsuperscript{223} introducing a potential for developing bifunctional inhibitors, i.e. inhibitors with an intrinsic capacity to inhibit both the plasmepsins and the falcipains of the parasite. As described in Section 1.7.3 inhibitors based on such a concept have been patented.\textsuperscript{157} Moreover, synergistic effects of cysteine and aspartic protease inhibitors have been shown in both a $P. falciparum$ cell culture and in a murine malaria model.\textsuperscript{31,224} One test compound comprising a 1,2,4-triazole, instead of the 1,3,4-oxadiazole, was included to investigate the impact of a heterocycle with a hydrogen bond donating capacity.

As starting point the (E)-bromoallyloxy group was utilized in the P1 and P1’ positions and one of the indanol moieties was retained in the unsymmetrical inhibitors.

A series of diacylhydrazines (symmetrical 21 and 22, and unsymmetrical 27-36 and 53-54), 1,3,4-oxadiazoles (symmetrical 23, 24, and unsymmetrical 37-42), and one 1,2,4-triazole (43) were investigated in Plm I, Plm II, and Cat D assays. Ten of the inhibitors (diacylhydrazines 22, 30, 32, and 53 as well as 1,3,4-oxadiazoles 24 and 37-41) were selected for investigation in a Plm IV assay. The results of the enzyme assays are given as $K_i$ values in Table 6. Eight inhibitors including both diacylhydrazines and oxadiazoles (22, 24, 28, 30-32 and 39-40) were also examined with regard to their ability to inhibit parasite growth in cell culture (Table 6).

The binding modes of the unsymmetrical inhibitors (five diacylhydrazines 27, 28, 32, 53, and 54 and two 1,3,4-oxadiazoles 37 and 41) to Plm II were analyzed using MD simulations in combination with the LIE method. The predicted inhibition constants are presented in Table 6.

In general, the unsymmetrical compounds (27-43 and 53-54) were superior over the four symmetrical variants (21-24) as Plm I and II inhibitors. Only the symmetrical diacylhydrazine 22, with 4-t-butylphenyl P2/P2’ groups, exhibited a weak potency in the Plm I and II assays. It is possible that employment of P2/P2’ substituents other than methyl and 4-t-butylphenyl would result in more potent symmetrical Plm I and II inhibitors.
Table 6. Inhibition Constants ($K_i$) and Inhibitory Activities Against *P. falciparum* in Infected Erythrocytes for Compounds Comprising Amide Bond replacement

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<th>Structure</th>
<th>Enzyme $K_i$ (nM)</th>
<th>%inh @4 μM (Cell)</th>
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Table 6. (Continued)

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<tr>
<th>Structure</th>
<th>Enzyme $K_i$ (nM)</th>
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<tr>
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The diacylhydrazine was found to be a more suitable amide bond replacement than the 1,3,4-oxadiazole in Plm I and II inhibitors. The oxadiazoles (23, 24, and 37-42) demonstrated, at best, affinities in the micromolar range toward Plm I and II. In contrast, the corresponding diacylhydrazines (21, 22 and 28-33) exhibited up to hundreds of times higher potencies (cf. 32 and 41) in the same assays. Furthermore, the 1,2,4-triazole representing a heterocycle similar to the 1,3,4-oxadiazole but with a hydrogen bond donating NH was inactive in both the Plm I and II assays. Thus, properties associated with the diacylhydrazine unit such as length, flexibility, and hydrogen bond donating and accepting capability proved to be crucial for strong interactions with the active sites of Plm I and II. Several Plm I and II inhibitors with $K_i$ values in the low nanomolar range were identified in the diacylhydrazine series (e.g. 32-34). The distance between a phenyl and the diacylhydrazine fragment was found to be of importance, with an optimum of two carbons (cf. 29 with 31-33). However, saturated basic six-membered rings at the same distance gave inhibitors with significantly lower Plm I and II affinities (34 and 35).

Since extensions of the P1 and P1’ side chains previously led to a substantial increase in Plm I and II potency in the $C_2$-symmetric series (Sec. 5.3) we wanted to explore the same elongations of the unsymmetrical diacylhydrazines starting from the most potent phenethyl 32. The elongations of the previously most potent inhibitors, 4-acetylphenyl (48) and methylenedioxophenyl (50), were employed to generate the two inhibitors 53 and 54, respectively. Notably, these inhibitors were equally (54) or less (53) potent than the parent
compound 32. The origin of this difference in impact of P1/P1’ extensions on the affinity for Plm I and II between the C2-symmetrical inhibitors and the unsymmetrical diacylhydrazines could not be explained by MD and LIE calculations. Nevertheless, a shorter P1 and P1’ substituent as in the allyloxy 27 resulted in a decrease in Plm I and II activity compared to the parent 32, in accordance with the C2-symmetric series (cf. 13 and 14).

Of the compounds evaluated with regard to affinity for Plm IV the symmetrical oxadiazole 24 showed the highest affinity, with a $K_i$ value of 35 nM. Hence, the opposite relative activity between the two compounds with amide bond replacements, the diacylhydrazine and the 1,3,4-oxadiazoles, was demonstrated in the Plm IV assay. In addition, the symmetrical Plm IV inhibitors 22 and 24 were overall superior to the unsymmetrical Plm IV inhibitors (30, 32, 54, and 37-41). The only exception being the unsymmetrical P1/P1’ methylenedioxyphenyl 54, which was slightly more potent than the symmetrical 22. The reason for the affinity differences observed in the Plm IV assay compared to the Plm I and II assays is currently under computational analysis. Despite the fact that the most potent Plm IV inhibitor 24 was inactive in both the Plm I and II assays, this non-peptide inhibitor completely devoid of amide bonds, was also the most efficient of the inhibitors studied in killing P. falciparum parasites in erythrocytes (25% inhibition of parasite growth at 4 μM).

All of the previously investigated inhibitors were completely selective for the plasmepsins over the human Cat D at concentrations up to 2000 nM. In this series of inhibitors concentrations up to 6000 nM were employed in the Cat D assay. However, almost the entire series was still completely selective with no Cat D inhibitory activity. Only three of the inhibitors (the symmetrical diacylhydrazine 22 and the unsymmetrical P1/P1’ extended diacylhydrazines 53 and 54) demonstrated weak Cat D binding.

Table 7 presents the calculated binding free energies as well as the polar and nonpolar energy terms of the computationally analyzed inhibitors. Since these inhibitors are unsymmetrical, two possible binding orientations had to be investigated: one with the amide bond replacement (diacylhydrazine or 1,3,4-oxadiazole) oriented to the nonprime side, and one with these replacements located on the prime side. Overall the nonpolar (hydrophobic) term made the largest contribution to binding, while the polar (electrostatic) interactions provided the binding mode specificity. The favored binding mode of both the diacylhydrazine (27, 28, 32, 53, and 54) and the oxadiazole (37 and 41) inhibitors in the Plm II active site was that with the amide bond replacing group located on the prime side. Generally, the predicted binding free energies were in excellent agreement with the experimentally found energies. The ranking of the methyl diacylhydrazine and the methyl oxadiazole inhibitors (cf. 28 and 37) and the impact of the P1/P1’ bromine (cf. 32 and 27) were especially well-reproduced.
Table 7. Experimental and Calculated Plm II Binding Free Energies

<table>
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<tr>
<th>Cmpd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\Delta G_{\text{bind}}$ (kcal/mol)</th>
<th>Ligand-surrounding interactions (kcal/mol)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Calc&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Conf.1 and Conf.2 denotes the different orientations of the inhibitor in the active site. In conformation 1 the diacylhydrazine or oxadiazole linker is on the nonprime side, while in conformation 2 the amide bond replacements are on the prime side.

<sup>b</sup> See Paper III for standard deviations.

According to the MD simulations the central scaffold (the six carbons originating from mannitol as well as the P1 and P1’ side chains) and the indanol adopted the same binding conformation as in the previously investigated $C_2$-symmetric inhibitors.

In the binding mode with the diacylhydrazine located on the prime side a stable hydrogen bonding network was established between the diacylhydrazine and the active site residues Val78, Gly36, Asn 76, and Tyr192, as depicted in Figure 21 (panel B). This pattern of hydrogen bonds was very similar to that of pepstatin A in the X-ray 1SME (Figure 21, panel A).

Notably, it was found that the side chain of Met75 was rotated during the simulations with the phenethyl diacylhydrazine inhibitors 27, 32, 53, and 54 resulting in a large hydrophobic S2’ pocket able to easily accommodate the P2’ phenethyl (Fig. 22). Actually, this S2’ accommodation conferred a stabilization of the conformation forming the diacylhydrazine hydrogen bonds described above. With a smaller P2’ group, as the methyl of inhibitor 27, the hydrogen bonds between the diacylhydrazine and Plm II were weakened and the hydrogen bond to Tyr192 was completely lost.
Figure 21. Panel A shows the hydrogen bonding network between Plm II (green) and the two amides of the P2’ Ala in pepstatin A (yellow, ISME\textsuperscript{17}). Panel B shows the predicted Plm II (green) hydrogen bonding network of the diacylhydrazine (inhibitor 32, yellow, snapshot) and panel C shows the predicted Plm II (green) hydrogen bond of the 1,3,4-oxadiazole (inhibitor 41, yellow, snapshot).
The Met75 rotation was supported by published crystal structures of Plm II in complex with inhibitors encompassing bulky P2’ substituents. Thus, not only the Plm II S1’ pocket, but also the S2’ subsite seems to exhibit substantial flexibility.

The oxadiazole, with solely hydrogen bond accepting capacity, was more flexible during the simulations than the diacylhydrazine. No stable network of hydrogen bonds was formed with this moiety. Only one hydrogen bond between a ring nitrogen and Val78 was observed. Additionally, a repulsive interaction between the ring oxygen and the Gly36 carbonyl was noted. Since the five-membered ring constitutes a shorter linker than the diacylhydrazine, the P2’ phenethyl of the oxadiazole inhibitor 41 was not able to induce the Met75 rotation.

Figure 22. Surface representation of the Plm II S2’ subsite in complex with inhibitor 54: MD snapshot before Met75 rotation (panel A), and MD snapshot after Met75 rotation.
5.6 Macrocyclic Inhibitors (Paper IV)

Proteases (aspartic, cysteine, metallo-, and serine proteases) generally bind their substrates and inhibitors in an extended β-strand conformation (Sec. 1.5.1). Thus, preorganizing the inhibitor into the bioactive β-strand conformation, results in higher affinity due to a reduced loss of conformational entropy upon binding. Additionally, a decrease in flexibility might prevent interactions with other receptors, thus increasing the selectivity. One way of attaining conformational stability is by using macrocyclization. Several potent β-strand-mimicking macrocyclic inhibitors of aspartic proteases, as well as cysteine, metallo-, and serine proteases, have been reported in the literature. Especially macrocycles involving 3-4 amino acids and 13-17 ring atoms have been found to be suitable mimics of the extended β-strand conformation. Macrocyclic inhibitors have also shown higher proteolytic stability and improved cell permeability than their acyclic counterparts.

We wanted to explore the impact of constraining our 1,2-dihydroxyethylene-based inhibitors derived from L-mannitol by macrocyclization. Up to now, only two different macrocyclic plasmepsin inhibitors have been disclosed (the statine-based C and D, Fig. 10, Sec. 1.7.1). These inhibitors were both connected by amide bonds introducing additional hydrogen bond donors and acceptors. To avoid the addition of a hydrogen bond donating/accepting amide we decided to conduct cyclization via ring-closing metathesis, which was recently employed in the formation of macrocyclic β-secretase inhibitors.

In contrast to the previous inhibitors described in Papers I-III, the P1 and P1’ substituents had to be unequal in order to get selective metathesis reactions. An allyloxy group was chosen in one of the positions, to participate in the ring-closing metathesis, and on the other side a benzyloxy substituent was employed. Two different ring sizes, a 13-membered and a 16-membered ring encompassing either a double bond or a saturated ring system, were investigated. As previously, (with the P1/P1’ extended inhibitors, Sec. 5.3) the design utilized the fact that the S1 and S3 pockets form one continuous cleft. Hence, the 13-membered macrocycles (65 and 67) were connected between the P1 and P3 positions. The P2 side chain was omitted in the design. The 16-membered cycles (66 and 68) were designed to span over both the prime and nonprime sides incorporating the isosteric hydroxyls in the cycles.

All macrocycles were examined in Plm I, II, IV and Cat D assays and the results are presented as Ki values in Table 8. Only the pure (E)-isomers of the unsaturated cycles (65 and 66) could be isolated in quantities large enough for enzymatic measurements. To obtain information on the activity of the (Z)-isomers, mixtures of known isomeric ratios were also assessed.
Table 8. Inhibition Constants ($K_i$) for the Macrocyclic Compounds

<table>
<thead>
<tr>
<th>Structure</th>
<th>Enzyme $K_i$ (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Plm I</td>
</tr>
<tr>
<td>$65$</td>
<td></td>
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<tr>
<td>$66$</td>
<td></td>
</tr>
<tr>
<td>$67$</td>
<td></td>
</tr>
<tr>
<td>$68$</td>
<td></td>
</tr>
</tbody>
</table>

Overall the 13-membered inhibitors, $65$ and $67$, bridging the P1 and P3 positions, had significantly greater affinity for Plm I and II than the 16-membered prime-nonprime macrocycles $66$ and $68$. None of the 16-membered inhibitors demonstrated any affinity in the Plm I assay and only the unsaturated macrocycle $66$ was weakly active against Plm II. Val78, at the tip of the flap in Plm I and II, may constitute a steric hindrance to large cycles spanning over both the prime and nonprime sides. Plm IV has a
glycine residue instead of a valine in position 78 resulting in a more open active site. As can be seen in Table 8, all of the macrocyclic inhibitors except 67 demonstrated low affinities for Plm IV. Notably, the saturated 13-membered macrocyclic inhibitor 67 exhibited a 35-fold higher potency against Plm IV than the unsaturated counterpart (E)-65. Compound 67 is actually the second most potent Plm IV inhibitor obtained in the L-mannitol derived 1,2-dihydroxyethylene series.

A comparison between the plasmepsin $K_i$ values of the (E)-isomers and the E:Z mixtures revealed that the (E)-isomers were slightly superior to the (Z)-isomers in the 13-membered macrocycles against Plm I and II and in the 16-membered cycles against Plm II and IV. On the contrary, the (Z)-isomer of the 13-membered 65 was more potent than the (E)-isomer against Plm IV. The overall most potent plasmepsin inhibitor in the macrocyclic series was the saturated 13-membered inhibitor 67, with $K_i$ values of 179 nM, 121 nM, and 195 nM for Plm I, II, and IV, respectively.

To obtain a rough idea of the binding mode of the macrocyclic inhibitors in the active site of Plm II and IV, docking experiments keeping the enzyme rigid were carried out. Preliminary docking with Plm II (using the X-ray structures 1LF3147 and 1LF2225) indicate that the 13-membered macrocycles of (E)-65 and 67 are accommodated, as anticipated, in the S1-S3 groove, while the 16-membered cycles (E)-66 and 68 bridge the S1’ to the S2 pockets. Similar docking experiments with Plm IV (using both the PDB available 1LS5 and a homology model) provided solutions with the 13-membered cycles of (E)-65 and 67 accommodated on both the prime and nonprime sides. The larger 16-membered macrocycles, (E)-66 and 68, were accommodated as in Plm II along the S1’-S2 subsites. As expected, Val78 in the flap loop of Plm II seems to constitute a larger steric obstruction than Gly78 in Plm IV, resulting in a poorer fit in Plm II of the prime-nonprime macrocycles (E)-66 and 68, according to docking. As a consequence of the constraints imposed by the 16-membered cycles only one of the hydroxyls in (E)-66 and 68 was able to form hydrogen bonds with the catalytic aspartates in the active sites of Plm II and IV. The binding features of the macrocyclic inhibitors will be further investigated using MD and the LIE method.

All of the macrocyclic inhibitors were inactive in the Cat D assay at concentrations up to 5900 nM. Since the two earlier reported macrocyclic inhibitors (C and D) were also highly potent against Cat D, these new cyclic inhibitors constitute the first plasmepsin-selective macrocycles reported so far.
6 Concluding Remarks

During the course of this study significant progress has been made in the research on the malaria parasite plasmepsins. The difference between what is known today and what was known when this project started at the beginning of 2000 is considerable. Eight additional plasmepsins have been identified in the *P. falciparum* genome and knockout studies have demonstrated the relevance of inhibiting several of the food vacuole plasmepsins.

The present study has contributed by providing knowledge on structure-activity relationships of Plm I, II, and IV inhibitors, as summarized below.

- An L-mannitol derived scaffold with (RRRR)-configuration and incorporating a 1,2-dihydroxyethylene transition state mimicking unit with affinity for the *P. falciparum* Plm I, II, and IV has been identified.

- Scaffold manipulation with regard to a) P1/P1' and P2/P2' side chain modifications, b) amide bond replacement by diacylhydrazine, 1,3,4-oxadiazole, and 1,2,4-triazole, and c) macrocyclization resulted in both selective and adaptive Plm I, II, and IV inhibitors.

- Several inhibitors of Plm I and II with *K*<sub>i</sub> < 10 nM (e.g. inhibitor 48) were identified.

- One Plm IV selective inhibitor (24) comprising two oxadiazole rings with *K*<sub>i</sub> = 35 nM was identified. This compound represents the most potent non-peptide Plm IV inhibitor reported to date.

- One macrocyclic inhibitor (67) demonstrated high potency against all three Plm I, II, and IV but was lacking activity in the Cat D assay.

- Plasmepsin inhibitors with a considerable selectivity for the plasmepsins over the human aspartic protease Cat D have been discovered.

- The linear interaction energy (LIE) method in combination with molecular dynamics simulations predicted Plm II *K*<sub>i</sub> values in excellent agreement with assay results. We propose that this method constitutes a valuable design tool in future structure optimization.
• Plm I, II, and IV inhibitors with activity against the *P. falciparum* parasite in infected erythrocytes have been identified.

• One compound (20) containing a basic amine function inactive in Plm I, II, and IV assays demonstrated a significant activity against *P. falciparum* in erythrocytes, suggesting an effect on other parasite target(s).

• We believe that the identified plasmepsin inhibitors incorporating a diarylhydrazine element could provide suitable starting structures for the development of bifunctional plasmepsin and falcipain inhibitors in the future.
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