1. Introduction

Parasite-borne diseases are having devastating impact on human civilizations. However, none of the parasitic diseases has had as enormous an impact on humans as that of malaria, which is one of the major causes of morbidity and mortality of human civilizations in the past, and even today it remains one of the deadliest diseases on the planet. It displays full explosive power of vector-borne infections, erupting suddenly and with intensity that can overwhelm vulnerable communities.[1] Even today malaria, particularly the one caused by *Plasmodium falciparum*, remains a serious health problem in Africa, South America and many parts of Asia.[2] As per the recent WHO report malaria is having devastating impact on the lives of more than 3 billion people of the world, about half of the humanity.[3]

An estimated 600 million people are at risk of contracting malaria infection and the disease kills more than 1 million children and a large number of pregnant women every year. About 90% of these casualties occur in tropical Africa and majority of them are children under the age of five. Exacerbated by poverty, malaria exerts a high toll on populations that can least afford a cure.[4] Despite a heavy toll of tropical parasitic diseases on human life in the developing world, present antimalarial therapy still rely on drugs developed decades ago. There are several reasons for re-emergence and spread of the infection across the globe as a major public health burden; the most common ones are complex life cycle of the parasite, resistance developed by parasite against common antimalarial drugs, inadequate control of the mosquito vector, and despite tremendous impetus and thrust instituted legitimately, the dream of developing an effective, safe and economical vaccine is a big illusion till date. The other major contributors to the re-emergence of the disease and its spread in the new locations and populations are increased population density, global warming, continuing poverty and political instability. The business travels to malaria endemic areas further added the spread of the disease to the developed countries where it has been thought to be totally eradicated a long time ago. The prophylaxis of malaria is also complex because of resistance, safety and tolerability issues of the existing drugs, so there are very limited antimalarial drug options available.[5]

WHO aims for the global roll back of malaria with intensified efforts in providing access to safe, affordable and effective antimalarial combination treatments worldwide along with preventive measures.[6] Science still has no magic

bullet for malaria and doubt remains whether such a single solution will ever exist. In many cases, the clinical usefulness of new compounds is limited due to poor efficacy, toxicity and constant attrition rate of drugs due to development of resistance. The absence of profit incentives has resulted in the lack of investment by the pharmaceutical industry in developing new chemotherapies[7] because the disease is more prevalent among the people of the developing countries who hardly have any purchasing power. Efforts in the direction of vaccine development and control/eradication of the mosquito vectors have either failed or met with limited success. Development of new antimalarial drugs only remains an economically and environmentally viable alternative.

Overall, there is an alarming situation as the parasite has developed resistance to all of the available drugs except artemisinins, and to prevent resistance development against artemisinins, these are generally used in combination with other antimalarials. But some recent reports have challenged the effectiveness and use of artemisinins alone or in combination due to reports on their reduced efficacy on Thai-Combodian border, historically a site of emerging antimalarial drug resistance and also because vaccine development has not yet yielded any fruitful results.[6] Hence there is an urgent need for identification and characterization of new targets for antimalarial chemotherapy. Fortunately the *P. falciparum* genome sequencing has revealed a number of new targets for drug and vaccine development. But only the development of newer antimalarial drugs remains an economically and environmentally viable alternative to fight out the menace of the disease.[8]

1.1 Replicative life cycle of malaria

Malaria is caused by four species of protozoan parasites of the Plasmodium genus. These are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, each of which presents slightly different clinical symptoms. Geographically *P. falciparum* is the most widespread and the most pernicious of the four species causing the major malaria related morbidity and mortality.[9]

In 1897, Ronald Ross first reported that the parasite *Plasmodium* could infect the female anopheles mosquito, parasites are transmitted from one person to another by this insect vector, thus completing parasite cycle. The malaria parasite exhibits a complex life cycle (Fig. 1) involving an insect vector (mosquito) and a

vertebrate host (human). The major phases of the life cycle are: liver stage, blood stage, sexual stage and sporogony.[9]

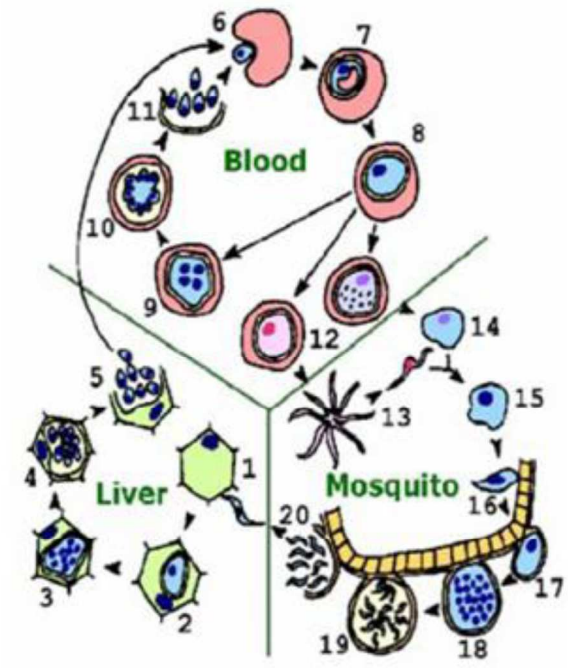


Figure 1: The life cycle of malaria parasite

Malaria infection is initiated when sporozoites are injected into the blood of human host along with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver where they invade parenchymal cells of the hepatocytes (1), a process involving receptor ligand mediated adhesion. The intracellular parasite undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocyte (2-4). Exoerythrocytic schizogony culminates in the production of merozoites, which are released into the bloodstream (5). During development and multiplication in the liver the host is asymptomatic. In nonrelapsing malaria (falciparum, malariae) no parasites are left in the liver as merozoites infection moves entirely in the blood. In relapsing malaria, a proportion of the liver-stage parasites from *P. vivax* and *P. ovale* go through a non dividing dormant stage known as hypnozoite (not shown in Fig. 1) instead of immediately going through the asexual replication (i.e., temporarily stay at step 2). These hypnozoites are reservoir of parasites which will reactivate after several weeks to months (or years) after the primary infection and are responsible for relapses. Merozoites invade erythrocytes (6) and undergo a trophic period in

which the parasite enlarges (7-8). The early trophozoite is often referred to as 'ring form' because of its morphology. Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into constituent amino acids. Multiple rounds of nuclear division manifest the end of the trophic period without cytokinesis resulting in a schizont (9). Merozoites bud from the mature schizont, also called a segmenter (10) and the merozoites are released following rupture of the infected erythrocyte (11). Invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle (6-11). The time intervals between the intermittent fever and chills often associated with malaria is due to the synchronous rupture of infected erythrocytes and release of merozoites, parasitic waste and the cell debris. The debris is the cause of the episodes of fever and chills. Trophozoite and schizont infected erythrocytes are rarely found in the peripheral circulation during *P. falciparum* infections. Erythrocytes infected with these stages adhere to endothelial cells and sequester in the microvasculature of vital organs, especially brain, heart and lungs. Sequestration in the brain is a contributing factor in cerebral malaria. As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro or microgametocytes (12). The gametocytes are large parasites, which fill up the erythrocyte, but only contain one nucleus. Ingestion of gametocytes by the mosquito vector induces gametogenesis (i.e., the production of gametes) and their escape from the host erythrocyte. Factors which participate in the induction of gametogenesis include a drop in temperature, an increase in carbon dioxide concentration and mosquito metabolites. Microgametes formed by a process known as exflagellation (13) are flagellated forms, which will fertilize the macrogamete (14) leading to a zygote (15). The zygote develops into a motile ookinete (16), which penetrates the gut epithelial cells and develops into an oocyst (17). The oocyst undergoes multiple rounds of asexual replication (18) resulting in the production of sporozoites (19). Rupture of the mature oocyst releases the sporozoites into the hemocoel (body cavity) of the mosquito (20). The sporozoites migrate to and invade the salivary glands (not shown in Fig. 1), thus completing the life cycle. In summary, the malaria parasite undergoes three distinct asexual replicative stages exoerythrocytic schizogony, blood stage schizogony and sporogony, resulting in the production of invasive forms (merozoites and sporozoites). Sexual reproduction occurs with the switch from

vertebrate to invertebrate host and leads to the invasive ookinete. All invasive stages are characterized by the apical organelles typical of apicomplexan species. The invasive stages differ in regard to the types of cells or tissues they invade and their motility.

1.2. Currently available antimalarial drugs

A systemic malaria control program began after discovery of the malaria parasite by Laveran (Nobel Prize for medicine, 1907) in 1880 and the demonstration by Ross in 1897 that the female anopheles mosquito was the vector of malaria. The mosquito control combined with therapeutic and chemoprophylatic approaches in parallel are used in the successful disease control. The efforts of complete eradication of the disease were limited by the resistance developed by the parasite against common antimalarial drugs and also by the mosquito vector.[9] Mostly, antimalarial drugs are targeted against the asexual erythrocytic stage of the parasite. The parasite degrades hemoglobin in its acidic food vacuole,[10] producing free heme, capable of reacting with molecular oxygen and thus generating reactive oxygen species as toxic by-products. A major pathway of detoxification of heme moieties is polymerization as malarial pigment.[11] Majority of antimalarial drugs act by disturbing the polymerization (and/or the detoxification by any other way) of heme, thus killing the parasite with its own metabolic waste.[12] The main classes of active schizonticides are 8-aminoquinolines, 4-aminoquinolines, arylalcohols including quinoline alcohols and antifolate compounds which inhibit the synthesis of parasitic pyrimidines. A newer class of antimalarials is based on the natural endoperoxide artemisinin, its semisynthetic derivatives and synthetic analogs. Some antibiotics are also used, generally in association with quinoline-alcohols.[13] Very few compounds are active against gametocytes and also against the intra-hepatic stages of the parasite.

1.2.1 8-Aminoquinolines

This is the only class of gametocytocides available to the therapeutic purposes. Primaquine has been widely used for the treatment of the hypnozoites (liver reservoirs) responsible for the relapsing forms of *P. vivax* and *P. ovale*. However, primaquine was recently reconsidered for malaria chemoprophylaxis to eliminate *P. falciparum* at the early stage of infection, when parasite develops in

the liver, thus preventing the clinical disease. Despite its good oral absorption, this molecule has a short half life and needs to be administered daily. Serious toxicity can be a major problem in patients with glucose-6-phosphate dehydrogenase deficiency. Primaquine is interfering with the mitochondrial function of *Plasmodium*. Tafenoquine (WR 238605) is a primaquine analog with a longer elimination half-life (14 days compared to 4 hours for primaquine). It also has a higher therapeutic index than primaquine. This molecule may be useful for chemoprophylaxis of *P. falciparum* and for prevention of relapses of vivax malaria.

1.2.2 4-Aminoquinolines

The main antimalarials are the 4-aminoquinolines because they have proven to be a highly successful class of compounds for the treatment and prophylaxis of malaria. They are easy to synthesize, cheap and generally well tolerated. These compounds, as well as the quinoline alcohols, are active against the intra-erythrocytic stages of the parasite. The 4-aminoquinolines are able to accumulate in high concentrations within the acid food vacuole of *Plasmodium*, to kill the parasite.[14] Chloroquine (CQ) was introduced in 1944-1945 and soon became the mainstay of therapy and prevention, as this drug was cheap, nontoxic and active against all strains of malaria parasites. In 1994, CQ was the third most widely consumed drug in the world after aspirin and paracetamol.[15] The precise mode of action of the quinoline antimalarials and the mechanism of parasite resistance are still not completely understood. On the mode of action of CQ, one can cite [14] many hypotheses like (i) direct-heme binding, (ii) inhibition of an unidentified heme ferriprotoporphyrin- IX “polymerase”, (iii) inhibition of vacuolar phospholipase, (iv) inhibition of protein synthesis and (v) interaction with DNA. However, the main mode of action of CQ seems to be related to the accumulation of this weak base in the acidic lysosome and binding to ferriprotoporphyrin-IX polymerase, thereby preventing the detoxification of ferriprotoporphyrin-IX by polymerization and thus killing the parasite.[16] CQ resistance was observed in Southeast Asia and South America at the end of the 1950s and in Africa in the late 1970s. Resistant parasites accumulate CQ less avidly than do sensitive ones. Resistance can be reversed *in vitro* using drugs known to reverse drug resistance in tumor cells, such as verapamil.[17] The observation that CQ resistance appeared

rather late, about 10 years after its widespread use, has been considered as an argument to support the hypothesis that CQ resistance has genetic basis.[18]

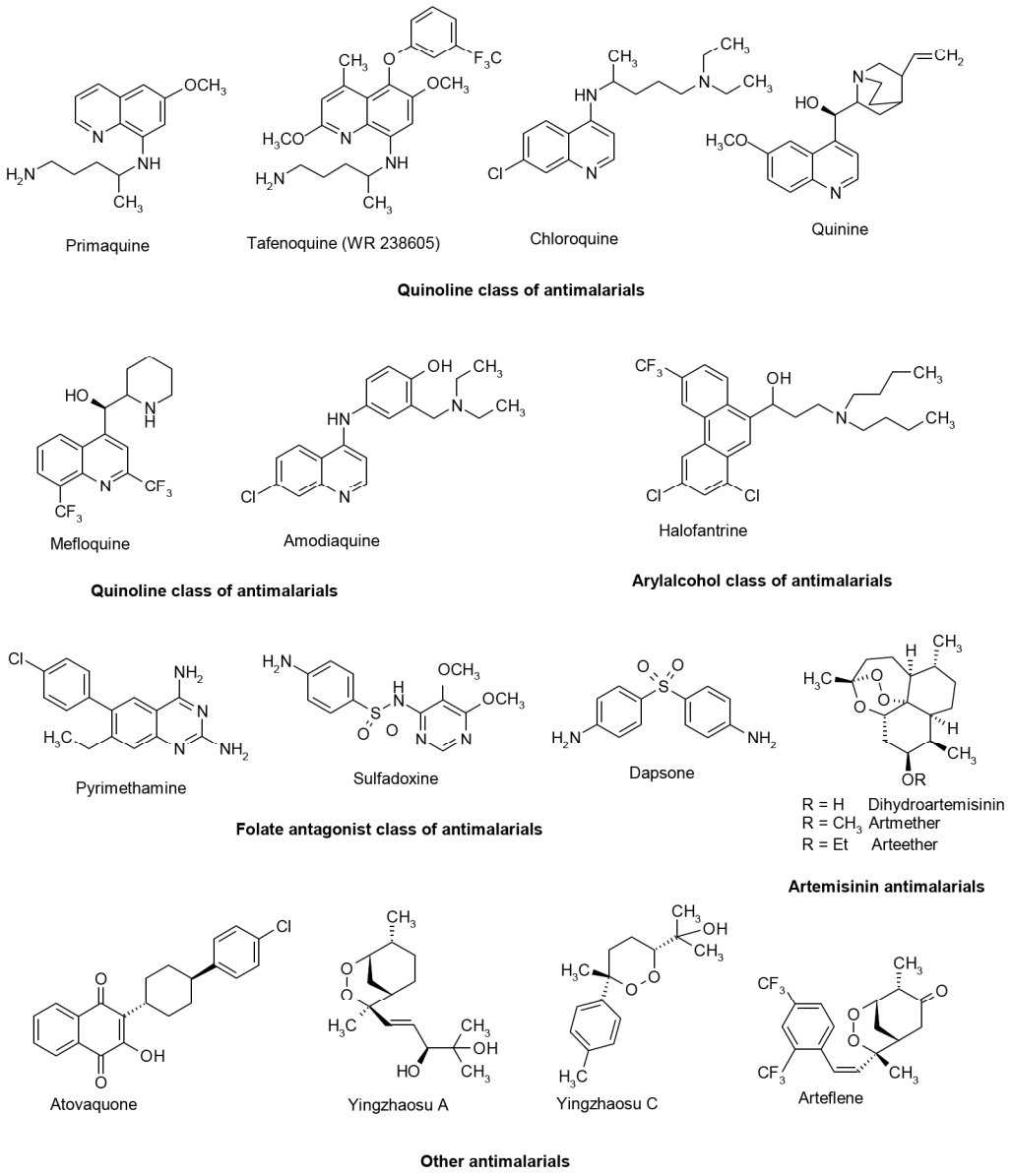


Figure 2: Examples of some common antimalarial drugs

In spite of its reduced efficacy, CQ is still the most widely used antimalarial drug in Africa, both for the reason of cost and because of the widespread prevalence of resistance among people infected with the parasite towards a large number of other antimalarial drugs. Moreover, tumor necrosis factor (TNF), a cytokine responsible for some cerebral damages, which is

produced by immune system during the malaria crisis, has been proven to have a synergistic effect with chloroquine, thus enhancing the effect of the drug.[19] Amodiaquine is chemically related to CQ, but is more effective than CQ for clearing parasitemia in cases of uncomplicated malaria, even against some chloroquine-resistant strains.[20] However, drug resistance and potential hepatic toxicity limit its use. Amodiaquine has been shown to bind to heme and to inhibit heme polymerization *in vitro*, with a similar efficiency as CQ.[21] Furthermore, amodiaquine exhibits cross-resistance with CQ suggesting that it exerts its activity by a similar mechanism.[22]

1.2.3 Quinolinemethanols

Quinine, the active ingredient of cinchona bark, introduced into Europe from South America in the 17th century, had the longest period of effective use and relieved more human suffering than any other drug for almost 3 centuries. But now there is an overall decline in its clinical response for *P. falciparum* in some areas. As on today quinine is not a first line treatment drug because of its small therapeutic index, longer period of treatment and the toxicity associated with it. Nevertheless, it remains an essential antimalarial drug for severe falciparum malaria and intravenous infusion is, in this case, the preferred route of its administration. The addition of a single dose of artemisinin enhances the parasite elimination rate and thus increases the cure rate.[23] Quinine interacts weakly with heme, but has been shown to inhibit heme polymerization *in vitro*. The mechanism of resistance to quinine is unknown, but a similar one as that of mefloquine has been suggested.[21] Combination of quinine and clindamycin[24] or quinine and allopurinol[25] significantly shorten the duration of treatment with respect to quinine used alone.

Mefloquine is structurally related to quinine and its long half-life (14-21 days) has probably contributed to the rapid development of resistance towards this drug. For this reason, mefloquine should be used in combination with other antimalarial agents. It binds with high affinity to membranes, causes morphological changes in the food vacuole of *Plasmodium* and interacts relatively weakly with free heme. The plasmodial P-glycoprotein 1 (Pgh 1) plays a role in mefloquine resistance and it may also be the target of this drug.[26,27] Lumefantrine was synthesized by the Academy of Military Medical Sciences in Beijing. It

belongs to the quinoline alcohol group that includes quinine and mefloquine. Its mechanism of action involves the interaction of the drug with heme and it is active against CQ resistant *P. falciparum* infection. The antibiotics like tetracycline are used in combination with quinine for multidrug resistant *P. falciparum* infection,[28] and doxycycline and azithromycin[29] are used for the purpose of prophylaxis. Fluoroquinolone antibiotics showed activity only in the *in vitro* tests.

1.2.4 Other arylalcohols

Halofantrine is effective against chloroquine-resistant malaria.[30] Despite this, cardiotoxicity has limited its use as a therapeutic agent.[31] Mefloquine usage appears to lead to selection of parasites resistant to halofantrine.[32] Furthermore, it is an expensive drug without availability of parenteral formulations. Pyronaridine, an acridine derivative, is a synthetic drug widely used in China that may have utility for multiresistant falciparum malaria.[33] Its current Chinese oral formulation is reported to be effective and well tolerated, but its oral bioavailability is low and this contributes to an unacceptably high cost of the treatment. It seems likely that drug resistance would emerge rapidly if pyronaridine was used in monotherapy. As reported above, resistance to a lot of antimalarial drugs has been observed in clinical isolates, but resistance to mefloquine, quinine and halofantrine appears to be inversely correlated with resistance to chloroquine and amodiaquine, suggesting that the development of a high level of resistance to chloroquine makes the parasite more sensitive to the arylmethanols.[34]

1.2.5 Folate antagonists

These compounds inhibit the synthesis of parasitic pyrimidines and thus of parasitic DNA. There are two groups of antifolates (i) the dihydrofolate reductase (DHFR) inhibitors like the antimetabolite antimalarial drugs, pyrimethamine and proguanil and (ii) the dihydropteroate synthase (DHPS) inhibitors having sulfones and sulphonamides like sulfadoxine and dapsone.

Due to a marked synergistic effect, a drug of the first group is usually used in combination with a drug of the second one. Unfortunately, resistance is widespread in Asia and now in Africa.[35] Pyrimethamine-sulfadoxine (SP, Fansidar) is the most widely used combination. It is cheap, practicable (only one dose is needed because of the slow elimination of the drugs from the body) and

currently efficient in many parts of Africa. However, it is poorly active against highly chloroquine-resistant strains. SP is also particularly prone to rapid emergence of resistance. The mechanism of resistance for this combination has been shown to be due to mutations in the genes of DHFR and DHPS.[36] Proguanil has also been combined with CQ in some oral formulations. It should be mentioned that proguanil is a prodrug, its P-450 metabolite cycloguanil is the active compound. Chlorproguanil is a chlorinated analog of proguanil that is also metabolized as an active triazine compound. It is more efficient and has a larger therapeutic index than proguanil and its combination with dapsone is eliminated more rapidly than SP, offering the possibility of lowering the selection pressure for resistance.[37] Finally, the development of a combination of proguanil and atovaquone (Malarone) would provide another useful way for the treatment of malaria.[38] Atovaquone, a hydroxynaphthoquinone derivative, is an analog of ubiquinone, a parasite mitochondrial electron-carrier which is a cofactor of the dihydroorotate dehydrogenase enzyme. Atovaquone acts by inhibiting parasite mitochondrial electron transport. However, the mechanism of synergy of proguanil with atovaquone is complex. This combination is well tolerated and more effective than CQ alone, CQ-SP [39] or mefloquine,[40] against acute uncomplicated multidrug resistant *P. falciparum*. It is also effective in regions where proguanil alone is ineffective due to resistance. Unfortunately, atovaquone is expensive and not easily affordable in most of the African countries.

1.2.6 Artemisinin derivatives

To date, resistance has emerged to all classes of antimalarial drugs except artemisinins[41,42] and therefore, most of the African countries have adopted artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria but some recent reports have challenged credibility and use of ACTs due to reduced efficacy of certain artemisinin-based combination therapies.[6] Artemisinin derivatives are the fastest acting antimalarial drugs.[43] Four compounds that have been used are the parent artemisinin extracted from Chinese herb ‘qinghao’ (*Artemisia annua.*)[44] and three derivatives that are actually more active than artemisinin itself.[43] One of them is a water-soluble hemisuccinate artesunate and the other two are oil-soluble ethers, artemether and arteether. All of them are readily metabolized to the biologically active metabolite,

dihydroartemisinin. Artemisinin is active in nanomolar concentrations *in vitro* on both CQ-sensitive and resistant *P. falciparum* strains. These drugs are fast acting and act against gametocytes, the sexual forms of the parasite that infect mosquitoes. The treatment of several million patients with artemisinin derivatives for acute malaria failed to detect any significant toxicity,[45] even for pregnant women,[46] despite the fact that neurotoxicity was observed in animals with higher doses than used clinically. Artemisinin and its derivatives appear to be the best alternative for the treatment of severe malaria[47] and arteether has been included in the WHO List of Essential Drugs for the treatment of severe multidrug resistant *P. falciparum* infection. Arteether is having advantage over artemether as it is more lipophilic and gives less toxic metabolite (ethanol versus methanol). In this family, The Walter Reed Institute of Research has patented a stable, water-soluble derivative called artelinic acid that is now being tested in animals. When compared with other artemisinin derivatives, artelinic acid had the highest plasma concentration, the highest oral bioavailability, the longest half-life, the lowest metabolism rate and the lowest toxicity at equivalent doses.[48] A key advantage of these endoperoxide containing antimalarial agents, which have been used for nearly two decades is the absence of any drug resistance. When several strains of *P. berghei* or *P. yoelii* were exposed to a selection pressure by artemisinin or synthetic analogs within infected mice, resistance proved very hard to induce. A low level of resistance has been observed which disappeared as soon as the drug selection pressure was withdrawn. Furthermore, with a synthetic analog of artemisinin, BO7, resistance to the drug was lost when drug pressure was removed; it was not regained once drug pressure was re-applied.[49] Remarkably, the introduction of artemisinin derivatives in routine treatment in some areas of Southeast Asia has been associated with a significant reduction of incidences of falciparum malaria. In fact, artemisinin derivatives prevent gametocyte development and therefore reduce the transmission.

The major drawback of artemisinin derivatives is their short half-life (3-5 hours). When used in monotherapy, a treatment as long as 5 days is required for complete elimination of the parasites. They are then preferentially used in combination with other antimalarial agents such as sulfadoxine-pyrimethamine,[50] benflumetol,[45] mefloquine[51] or chlorproguanil-dapsone to increase cure rates and to shorten the duration of therapy in order to minimize the emergence of resistant

parasites. Combination of artemether or artesunate and mefloquine has been used in areas of multidrug resistance in Southeast Asia. When associated with lumefantrine (benflumetol, a slow eliminated oral drug,) artemether is as effective as the artesunate-mefloquine combination and better tolerated. Artemether clears most of the infection and the lumefantrine concentration that remains at the end of the 3 to 5-day treatment course is responsible for eliminating the residual parasites. This combination is safe in patients with uncomplicated falciparum malaria and even in children. It clears parasites rapidly and results in fewer gametocytes carriers. The only limiting factor of artemisinins in comparison to common antimalarials is their treatment cost and duration of course in monotherapy, which make them least affordable in case of the low income countries sharing world's major malaria episodes and its associated deaths.[52] The relatively high cost and erratic supply of the natural parent compound artemisinin make it necessary to develop new synthetic and cheap endoperoxide-based antimalarials.[53,54]

1.2.7 Other antimalarials

Yingzhaosu A and Yingzhaosu C, two naturally occurring peroxides with bisabolene skeleton have been isolated from the roots of the plant yingzhao (Artabotrys uncinatus), both showing significant antimalarial activity *in vitro*.[54-56] Arteflene a synthetic analog of Yingzhaosu A is under clinical developement. The compound has low human toxicity as it interacts with specific parasite proteins, fast acting and equipotent to artemisinin and has better chemical stability. Its single dose can lasts for longer period of time.[57-59]

1.3 New antimalarial drug development approaches

Due to complex life cycle and different causative strains of the parasite and high mutability of the genome of *Plasmodium falciparum*, development of malaria vaccine has met with failures time and again hence, identification of new targets have been explored for the development of new drugs acting via different mechanisms in order to avoid fast development of resistance.[60] The *P. falciparum* genome sequencing has revealed a number of new targets for drug and vaccine development.[61,62] But discovery of new targets, the developmental time and costs are also likely to be too high for such an approach. Inhibition of the proteases that

specifically degrade hemoglobin in the parasite food vacuole, the plasmepsins, falcipains and falcilysins, is an interesting area of research.[8] Also very promising are targets associated with pathways specific to the apicoplast, such as the shikimate pathway and the 2-deoxy-*D*-xylulose-5-phosphate (DOXP) pathway.[63] Cellular and biochemical targets in the mosquito vector are worthy of consideration as well.[64] One group of workers has demonstrated the viability of using a molecular connectivity QSAR approach to identify new active antimalarial agents.[65]

1.3.1 Old targets new compounds

The main challenges associated with antimalarial drug development program are high costs for developing new targets, development of resistance to the available drugs by the parasite and general lack of interest among the major pharma players, as the disease belongs to the poor third world countries. Other challenges associated with it are the cost, efficacy, target specificity, oral-bioavailability and safety. A new antimalarial drug must meet all standard drug development criteria of a new molecule so, an alternative strategy is the exploitation of the existing targets.[66] As the major target population is from Africa and other developing countries, any new drug development program must address the challenge of cost and drug resistance along with all standard drug development criteria. The free heme liberation in the parasite food vacuole is also an "old" but always an attractive pharmacological target. It could be the most specific target that can be exploited since it comes from the hemoglobin digestion by the parasite that occurs only in infected erythrocytes.

Many chemical entities are directed toward this well known target, such as CQ and artemisinin derivatives. CQ is a cheap and easy-to-prepare molecule that has proved its credibility as it is highly effective, safe and well-tolerated drug for treatment and prophylaxis. However, the spread of resistance has resulted in a huge reduction in the utility of CQ. Many chemical modifications have been attempted to obtain a molecule as affordable as CQ and equally active even on resistant strains. These modifications carried out are substitutions in the quinoline nucleus, variations in the side chain, synthesis of bisquinolines and more recently, the introduction of a ferrocenyl moiety.[67] A little modification could provide a

new compound that would be active on resistant strains; but the wait for a safe and effective chloroquine alternative still continues.

Artemisinin and its derivatives (artemether, arteether and artesunate) are increasingly used in Asia and Africa where multidrug-resistant *P. falciparum* is prevalent. They are rapidly effective and well-tolerated treatments, but the total synthesis is too complex to be exploited and the yield of extraction from the plant is too low, despite research efforts toward the enhancement of artemisinin or one of its precursor's productions in *A. annua*. As a result, they remain expensive treatments that are hardly accessible to people in endemic areas. The cost will also be limitative for sophisticated artemisinin derivatives.[68] Synthetic trioxanes, simplified analogs of artemisinin retaining the crucial endoperoxide bridge have been developed, but none of them could enter into clinical trials successfully.[69]

1.3.2 Proteases-general introduction

Proteases or proteolytic enzymes found in plants, bacteria, virus, protozoa, fungi and mammals are essential for replication and are involved in a series of essential metabolic and catabolic processes, such as protein turnover,

Table I: Proteases in disease propagation and their functions[70]

Protease	**Function**	**Involved in disease**
HIV-1 Protease	HIV replication	AIDS
Renin	Generation of angiotensin I	Hypertension
Thrombin	Blood coagulation	Stroke, vascular clots
Tryptase	Phagocytosis	Asthma
Cathepsin K	Bone resorption	Osteoporosis
ACE	Generation of angiotensin II	Hypertension
Neutral endoprotease	Release of ANP	Hypertension
Rhinovirus 3C protease	Viral replication	Common cold
Falcipain	Proteolysis	Malaria
Plasmepsin I & II	Proteolysis	Malaria
Cruzain	Proteolysis	Chagas' disease

digestion, blood coagulation, apoptosis, fertilization, cell differentiation and the immune response system.[71] Proteases have enormous commercial importance in industrial enzyme applications, mainly in detergent, food and leather industries.[72] Proteases selectively catalyze hydrolysis of large polypeptides into smaller peptides and further into amino acids thus facilitating their absorption by the cells.[73] Their virtual control over protein synthesis in human beings and other microorganisms enable them to regulate physiological processes.[74] They have been implicated in many health problems (Table-I) involving the circulatory system, immune system, allergies, sports injuries and infectious diseases. They catalyze important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms and viruses. This makes proteases a valuable target for the development of newer drugs.

1.3.3 Protease inhibitors as drugs

Proteases are involved in a number of diseased states and thus have gained tremendous attention as therapeutic targets with regard to viral and parasitic infections, stroke, cancer, Alzheimer's disease, neuronal cell death and arthritis. They are designated either as endo or exopeptidases that cleave peptide bonds within a protein or peptide and remove amino acids from the N or the C-terminus. Depending on the catalytic residue responsible for peptide hydrolysis, the proteases have been divided into classes like serine, cysteine, aspartic and metalloproteases.[71,75]

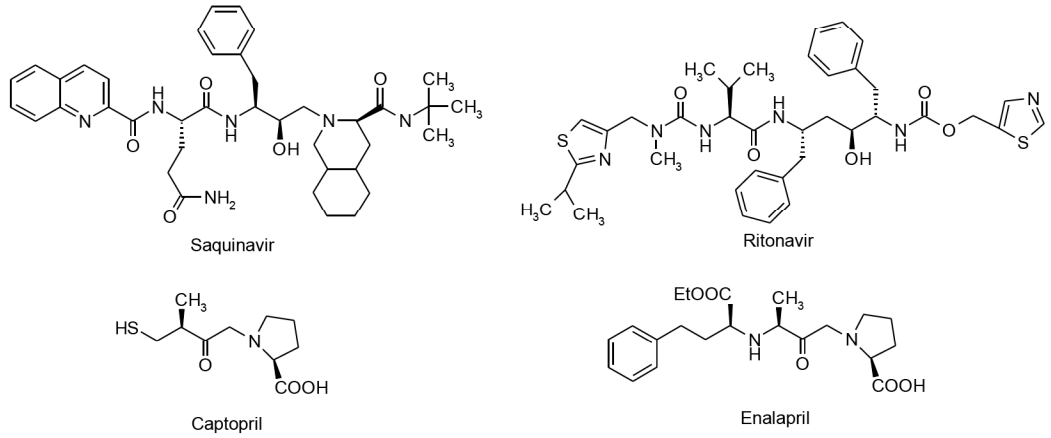


Figure 3: Protease inhibitor drugs available in market

Proteases are crucial for propagation of certain diseases and are emerging as promising therapeutic targets to be used in the treatment of these

diseases. These diseases include inflammation, tumor progression, parasitic, fungal and viral infections (e.g. malaria, schistosomiasis, *C. albicans*, HIV, herpes and hepatitis), immunological, respiratory, neurodegenerative and cardiovascular disorders. Protease inhibitors thus, have considerable potential utility for therapeutic intervention in a variety of disease states.[73,76] There are many examples of protease inhibitors as highly successful drugs.

The human immunodeficiency virus protease (HIV-1 protease) has proved to be an attractive target due to its essential role in the replicative cycle of HIV. Several low molecular weight HIV-1 protease inhibitors (MW < 1000 Da) are now used as drugs, including saquinavir, ritonavir, indinavir, nelfinavir and amprenavir. These are among the first successful examples of receptor/structure based designer drugs. Keeping in mind the prior knowledge of inhibition of other aspartic proteases (eg. renin) these compounds were designed and developed by docking these structures on the active site of HIV-1 protease.[70] Captopril and enalapril are the examples of inhibitors of a metalloprotease, angiotensin-converting enzyme (ACE) used successfully for the control of hypertension.[77]

1.3.4 Cysteine proteases and related aspects

Cysteine (thiol) proteases exist in three structurally distinct classes,[75] which are papain-like (e.g. cathepsins), ICE-like (caspases) and picorna-viral type. Several members of the cysteine protease family of enzymes have been implicated as possible causative agents in a variety of diseases. More notable examples include cathepsin K in degradation of bone matrix, cathepsin-L and S for MHC-II antigen presentation, the caspases in programmed cell death, rhinovirus 3C protease for viral processing, falcipain and cruzain in parasitic infections and the possible role played by the gingipains in periodontal diseases. The biggest problem in designing inhibitors for cysteine proteases is the similarity in their substrate affinities and proteolytic mechanisms with the serine proteases. Although, the spatial configurations of the catalytic triads of serine and cysteine proteases are quite similar, it appears that the oxyanion hole and the negatively charged tetrahedral intermediate are the central features of the catalytic mechanisms of serine proteases, while cysteine proteases stabilize the later more neutral acyl intermediate with the help of imidazole ring of histidine. This

mechanistic difference could be exploited in the development of potent, reversible and selective transition state analogs as potential drugs.

Like serine proteases, cysteine proteases tend to have relatively shallow solvent exposed active sites that can accommodate short substrate/inhibitor segments of protein loops or strands. Most of the inhibitors developed to date tend to be 2-4 amino acids or their equivalents in chain length, interacting with the nonprime subsites of the enzymes and terminating with various electrophilic isosteres. Recently it has been convincingly demonstrated, for a wide range of proteases including cysteine protease, that they universally bind their inhibitors/substrates in extended or β-strand conformations.

1.4 Malarial cysteine proteases: new target for chemotherapy

1.4.1 Malarial cysteine proteases' role in hemoglobin degradation

Erythrocytic stage of the malaria parasite *P. falciparum* has developed complex proteolytic machinery for haemoglobin digestion.[78] Malaria parasite ingests and degrades most of the host cell hemoglobin during the morphologically separate phases inside the erythrocyte (ring, trophozoite and schizont stages).[79] Hemoglobin degradation is essential to the survival of the parasite, as interruption of this process with a variety of protease inhibitors leads to death.[80] The amino acids derived from hemoglobin degradation are incorporated into parasite proteins or utilized for energy metabolism.[81,82] It has also been suggested that removal of hemoglobin through degradation provides space in the red blood cell for the growing parasite and prevents premature erythrocyte lysis.[83]

Massive degradation of hemoglobin generates a large amount of toxic heme, which causes membrane damage due to its peroxidative properties.[84] The released hematin is detoxified to a cyclic dimer, β-hematin. These dimers crystallize to give hemozoin (insoluble malaria pigment).[11] This massive catabolic feat is carried out in a specialized organelle, the food vacuole and is most pronounced during the trophozoite stage of development.[10] More recent studies indicate that the crystallization process from β-hematin to hemozoin takes place in or closely associated with, neutral lipid nanospheres in the aqueous content of the vacuole.[85]

A major function of the food vacuole is to degrade the host red cell hemoglobin sequestered through the cytostome machinery and provide amino

acids to the parasite. This is brought about by a variety of proteases and the inhibition of this process is well investigated as a drug target.[86,87] These enzymes act at an optimum *p*H in the range of 4.5-5.0, i.e. *p*H of the digestive vacuole.[78] A low digestive vacuolar *p*H further promotes biomineralization of heme to hemozoin.[88]

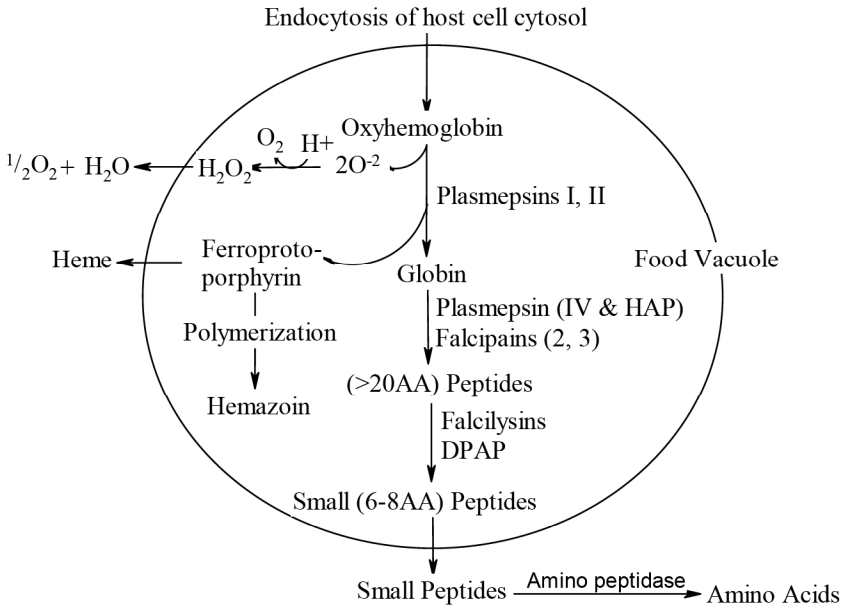


Figure 4: Role of cysteine proteases in hemoglobin digestion[89,90]

In *P. falciparum*, proteases involved in hemoglobin degradation have been proposed to act in a semi-ordered fashion as outlined in Fig. 4. Ten aspartic proteases named plasmepsins (PMs) were identified in the genome of *P. falciparum* and four of them, namely palsmepsin I, II and IV and histo-aspartic protease (HAP) participate in hemoglobin degradation.[89,90] Plasmepsin I and II have been postulated to initiate hemoglobin degradation, by hydrolyzing the peptide bond between residues Phe33 and Leu34 of native hemoglobin. [90,91] This initial cleavage is thought to promote unfolding and release of the heme moiety followed by further degradation by the plasmepsins,[87] the cysteine proteases (named falcipain-2 and falcipain-3),[92-94] a metalloprotease (named falcilysin)[95] and the lately discovered dipeptidyl aminopeptidase (DPAP).[96] However, the precise sequence of events particularly whether a plasmepsin or a falcipain catalyzes the initial degradation step is still under debate.[80]

Disruption of each plasmepsin gene was achieved and these studies confirmed that all these four vacuolar enzymes play important roles for parasite

development though not an essential one, as PMs knockouts possess the ability to compensate for the loss of individual plasmepsin function.[97] On the other hand, it is known that inhibition of cysteine proteases turns out to be lethal for parasites. Cysteine protease inhibitors irreversibly block the rupture of host cell membrane, which prevent parasites to invade fresh erythrocytes.[98] The metalloprotease, falcilysin is only able to cleave small polypeptides, up to 20 amino acids, producing even shorter oligopeptides.[95] DPAP1 cleaves off dipeptides from hemoglobin-derived oligopeptides in the food vacuole.[96] Finally, small peptides may be pumped out of the food vacuole into the cytoplasm and an amino peptidase activity provides amino acids essential for parasite's survival.[99,100]

1.4.2 Falcipains' role in proteolysis

The best characterized *P. falciparum* cysteine proteases initially known as trophozoite cysteine protease (TCP) and later termed as falcipains share sequence identity and other features with papain family cysteine proteases. Falcipain-1 (FP-1),[92] nearly identical copies of two falcipain-2 (FP-2) (falcipain-2 and falcipain-2', also known as falcipain-2A and falcipain-2B, respectively)[93,101] and falcipain-3 (FP-3)[94] are all expressed during the erythrocytic stage of the Plasmodium life cycle.[102] Falcipain-2' is not fully understood and the knock out studies have revealed that there is no significant change in its absence in parasite's function in erythrocytic stage.[101]

FP-1, located on chromosome 14, shares approximately 38-40% sequence identity with FP-2 and FP-3. The function of FP-1 remains uncertain because of its low abundance and inadequate systems for the production of recombinant enzyme. FP-1 has been proposed to play a role in parasite invasion as a relatively specific inhibitor of FP-1 blocked parasite invasion of host erythrocytes independent of hemoglobin degradation.[103] However, a subsequent study showed that FP-1 knockout parasites developed normally in erythrocytes, suggesting that this protease alone is neither required for parasite invasion nor during the intracellular development within erythrocytes.[92] The gene disruption studies clearly demonstrated an important role for FP-1 in oocyst production during parasite development in the mosquito midgut, but not an essential role in asexual or gametocyte/gamete development. FP-1 could be directly involved in the actual transition from gamete to oocyst by activating proteins by proteolytic

processing or, if secreted, it could degrade the peritrophic matrix or midgut endothelium facilitating the migration of the ookinete.[104]

In contrast, the well-characterized function of FP-2 and FP-3 includes degradation of host hemoglobin required for sustaining the metabolic needs of rapidly growing parasite.[93] They show a high level of amino acid sequence homology (65%) but a different substrate specificity,[105] although both enzymes favor substrates with Leu at P_2.[93,94] FP-2 and FP-3 are primarily localized in the food vacuole, consistent with their roles in hemoglobin digestion. Both falcipains are synthesized over fairly long interval during the erythrocytic cycle as membrane-bound pro-forms that are processed to soluble mature forms. However, FP-2 is synthesized earlier, in the beginning of early trophozoites and is processed more quickly than FP-3 which showed peak expression in late trophozoites/early schizonts stage. Cysteine protease inhibitors and the lactone antibiotic brefeldin-A, blocked the processing of both enzymes, suggesting that FP-2 and FP-3 are trafficked to the food vacuole via the endoplasmic reticulum/Golgi network and that they are processed by autohydrolysis (which occurs at neutral *p*H) before arrival at the food vacuole.[106]

Genetic ablation of FP-2 gene, located on chromosome 11 underscored the critical importance of this protease in hemoglobin degradation,[107] which is actually considered the prime target for discovery of novel antimalarial drugs. During the late trophozoite and schizont stages, FP-2 is involved in the degradation of erythrocyte membrane skeletal proteins, including ankyrin and the band 4.1 protein.[108] This activity displays a *p*H optimum in the range of 7.0-7.5 and is thought to contribute to destabilization of the erythrocyte membrane, leading to host cell rupture and release of the mature merozoites, hence FP-2 shows stage specific activity i. e. in early trophozoite stage it degrades hemoglobin in acidic *p*H and in neutral *p*H it cleaves the host erythrocytic skeletal membrane proteins ankirin and protein 4.1, vital for the stability of RBC membrane.[93,109]

The disruption of FP-2 gene revealed that there is transient block in hemoglobin hydrolysis but the loss of this enzyme alone is not sufficient to cause parasite lethality, the parasite recovered from the defects later presumably due to expression of FP-3 in the later stage of life cycle of the parasite, thus suggesting the participation of additional cysteine proteases for parasite invasion and growth

in human erythrocytes. Alternatively, the development of parasite lethality might require concomitant deletion of multiple cysteine proteases. Unlike other falcipains, the FP-3 gene could not be disrupted, but its replacement with a tagged functional copy has been recently achieved.[110] These studies revealed that FP-3 is essential to erythrocytic parasites, suggesting that efforts to develop cysteine protease inhibitors as antimalarial drugs should probably be focused on FP-2 and FP-3.

FP-2' was the last cysteine protease to be identified through analysis of genetic variability among isolates of *P. falciparum*. These studies revealed that *P. falciparum* contains a nearly identical copy of the FP-2 gene, also located on chromosome 11, encoding a distinct enzyme. These two genes are actually paralogs, which share 96% identity at the nucleotide level and 93% identity at the amino acid level. The products of these paralogs were designated as FP-2 and FP-2'.[111] Their amino acid sequences differ at seven positions within their mature forms, including three amino acid replacements localized close to residues that are predicted to interact with substrate and consequently may affect its affinity or specificity.[112]

Recombinant FP-2' protein expressed in bacteria exhibits protease activity as FP-2 in erythrocytic parasites but importantly, the recombinant FP-2' cleaves host ankyrin but not protein 4.1. Functional analysis revealed similar biochemical properties to those of FP-2, including *p*H optima (*p*H 5.5-7.0), reducing requirements and substrate preference. Notwithstanding its predicted hemoglobinase function, the *P. falciparum* FP-2' may contribute and orchestrate selective proteolytic events during the exit of malaria parasite from human red blood cells.[113] However, gene disruption studies demonstred that FP-2' knockout parasites showed no differences in morphology neither in sensitivities to cysteine or aspartic proteases compared to the wild-type parasites.[110] These results indicate a subordinate role for FP-2' in parasite development.

All falcipains except FP-1 are involved in the conversion of proplasmepsins into their active forms. FP-2' and FP-3 are expressed later in the erythrocytic cycle of the parasite. This would suggest that FP-2 might serve as the dominant proplasmepsin maturase, although the contribution of FP-2' and FP-3 cannot be ruled out.[114]

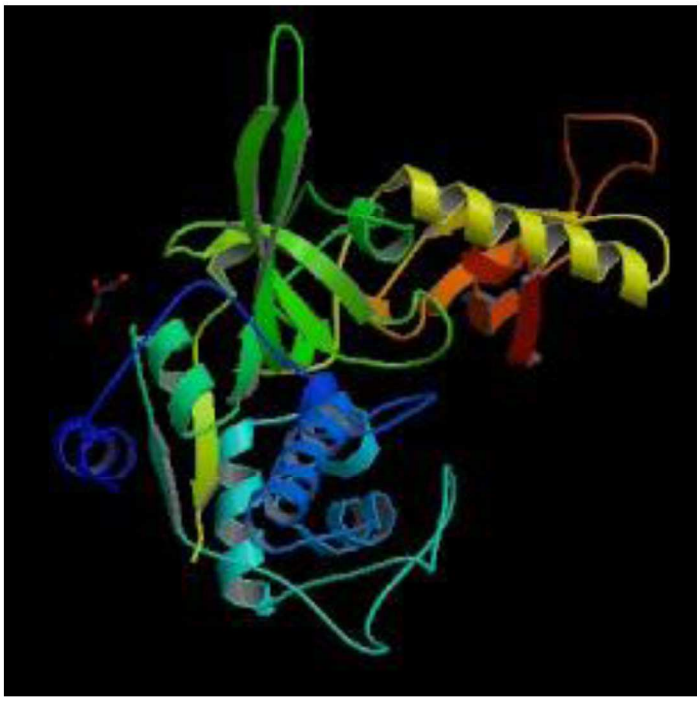
1.4.3 General structural features of falcipain-2

The mature FP-2 is a single polypeptide chain of 241 amino acids, synthesized during the trophozoite stage as a membrane-bound proenzyme comprising 484 amino-acid residues.[93] The proenzyme is transported to the food vacuole through the endoplasmatic reticulum/Golgi system and during this process the N-terminal 243 residues containing the membrane anchor are proteolytically removed. An autoproteolytic processing mechanism was suggested on the basis of inhibitor studies[110] and from the observation that the recombinant proenzyme undergoes spontaneous processing during *in vitro* refolding.[93]

The crystal structure of the free FP-2 (inactivated by iodoacetamide, pdb code 2GHU)[115] and in complexation with cystatin (1YVB pdb code)[116] (Fig. 5) has been deposited in the Protein Data Bank. More recently, the crystal structures of FP-2 in complexation with epoxysuccinate E-64 (3BPF) and FP-3 with aldehyde leupeptin have also been reported.[117]

FP-2 generally adopts a classic papain-like fold in which the protease is divided into L (left) and R (right) domains. The catalytic residues Cys42, His174 and Asn204 (Cys25, His159 and Asn175 following papain convention) are located in a cleft at the junction between the two domains (Fig. 6). Like papain and the majority of C1A proteases, FP-2 also has the disulfide bond Cys168-Cys229 to fix the upper loops defining the S_2 and S_1' substrate-binding sites. An additional Cys99-Cys119 disulfide bond, predicted on the basis of homology modeling studies,[118] appears to play an important role together with the Cys73-Cys114 disulfide in stabilizing the long $\alpha 4/\beta 2$ loop (~30 residues) that blankets the lateral surface of the L-domain.

The most extraordinary features of FP-2 are two unique structural elements: a ''noselike'' projection (at the N-terminus) connecting the L and R domains and an ''arm-like'' structure (near the C-terminus of the mature protein) extending away from the protease surface.[116] FP-2 nose consists of 17 amino acids and deletional analysis of this sequence[119] confirms that FP-2 nose is fundamental for proper folding; generally it possesses a short significant element of secondary structure and the amino acid Glu138 forms a buried hydrogen bond with Tyr4 and a salt bridge with Arg5. This clearly indicates that FP-2 nose, even if shorter than the standard papain family prodomain, may play a critical role as chaperone for protease folding. Furthermore, in a different way from the other members of the



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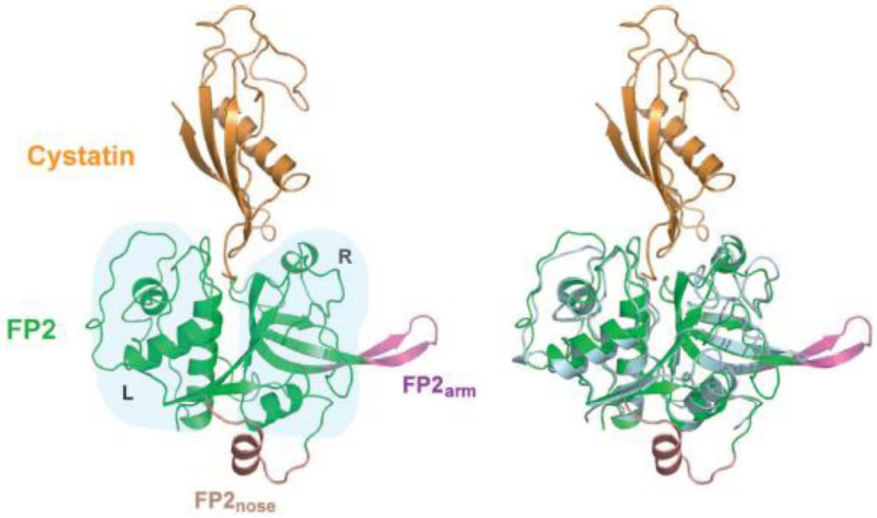


b

Figure 5: a) Crystal structure of FP-2 and cystatin complex. b) Overall structure of FP-2-cystatin is depicted in stereo in a conversational orientation for papain-like cysteine proteases: with the L domain (Left), the R domain (Right) and the active site on the top facing forward.[116]

papain family, interactions of a residue in the protein core (Glu138) with the FP-2 nose may provide the additional binding energy essential to stabilize initial interactions for folding.

FP-2 arm is composed of 14 amino acids located at the distal end of the R domain (residues 183-196). It is a protruding arm-like structure of two extended β– strands connected by an abrupt turn and generally it does not establish any contact with the protease core. Mutagenesis studies suggest that FP-2 arm may interact with the natural substrate of FP-2, hemoglobin. However, because FP-2 arm is $\geq$ 25A° away from the protease active site, FP-2 arm probably functions in hemoglobin hydrolysis through distal-site binding rather than direct participation in substrate cleavage.

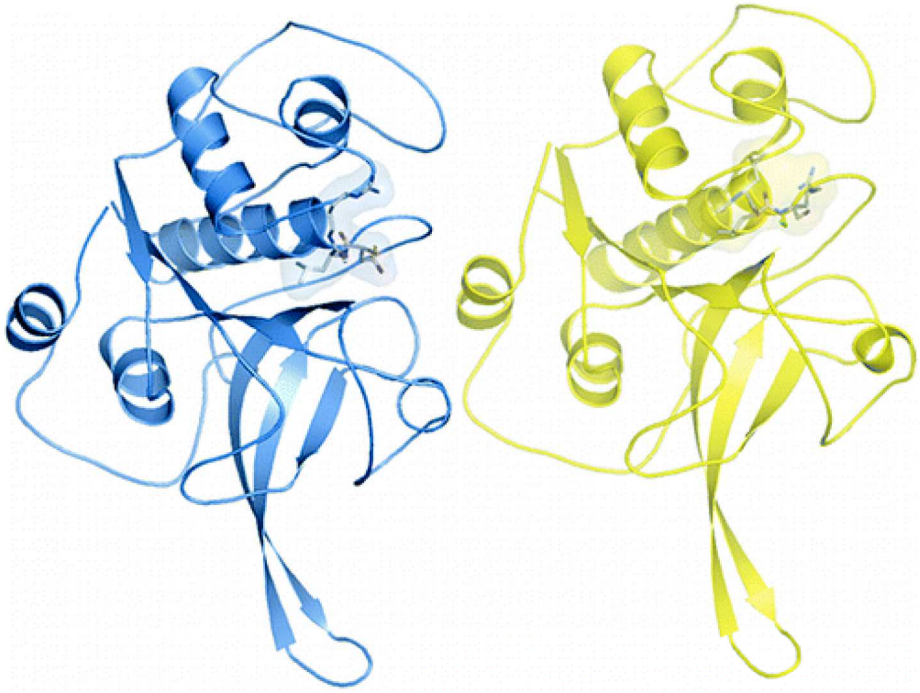


Figure 6: Crystal structure of FP-2 and FP-3 bound to small molecule inhibitors; implications for substrate specificity [117]

The 14-residue hemoglobin-binding β-hairpin exhibits a high degree of conformational flexibility when freely exposed to solvent bulk. The large positional deviations exhibited by the β-hairpin, while comparing the structure of the free FP-2 and that of the FP-2-cystatin complex further underscores an inherent flexibility and plasticity of this element that may have functional implications in hemoglobin binding.

Recently, the co-crystallized FP-3-leupeptin structure (3BPM) has been reported; FP-3 also possesses the two unique insertions discussed above, that distinguishes plasmodial cysteine proteases from all other structurally characterized papain family enzymes: the noselike domain located at the N-

terminus (AA 1-25) and the flexible 14-residue β-hairpin at the C-terminus outside of the two unique motifs discussed above. The rest of FP-2/3 is structurally similar to homologous proteases in the C1A family. The active site is located in a cleft between the structurally distinct domains of the papain-like fold. The S_2 pocket is the major determinant of specificity for most cysteine proteases and its predominantly hydrophobic nature in FP-2 is confirmed by the fact that FP-2 has a strong preference for substrates with a hydrophobic residue, particularly Leu, at the P_2 position.[93] The crystal structure reveals that although the S_2 subsite is generally hydrophobic, the electronegative side chain of Asp234 is positioned very deep in the S_2 pocket. Therefore, the presence of basic side chain in the P_2 position could form a salt-bridge interaction with Asp234, adding further insights useful to explore the structural determinants of FP-2 specificity.

1.5 Falcipain-2 inhibitors

To date, many chemotypes have been identified as inhibitors of FP-2 such as peptides, peptidomimetics, isoquinolines, thiosemicarbazones, nonpeptidic chalcones, pyrimidinenitriles and others which are able to inactivate the enzyme in a reversible or irreversible manner. The majority of malarial cysteine protease inhibitors are peptidic or peptidomimetic compounds in which the hydrolysable amide bond is replaced by an electrophilic functionality. In this way, the catalytic thiol of the enzyme reacts with the inhibitor to form a covalent complex. Until recently, most potent cysteine protease inhibitors were irreversible inhibitors, in which the electrophilic "warhead" would alkylate the enzyme, through nucleophilic displacement or conjugate addition. Examples of this approach include fluoromethyl ketones, acyloxymethyl ketones, vinyl sulfones, or epoxysuccinates.

Alternatively, potent reversible inhibition can be achieved through highly electrophilic warheads like aldehydes, ketoamides and nitriles, which form reversible, covalent bonds to the thiol active site. As synthetic peptide inhibitor of the *P. falciparum* schizont cysteine protease, Pf 68 inhibited erythrocyte invasion by cultured parasites[120]. The most effective peptide, GlcA-Val-Leu-Gly-Lys-NHC_2H_5 inhibited the protease and blocked parasite development at high micromolar concentrations (IC_{50} = 900 μM)[120]. The natural triterpene betulinic acid and its analogs (betulinic aldehyde, lupeol, betulin, methyl betulinate and

betulinic acid amide) caused concentration dependent alterations of erythrocyte membrane shape towards stomatocytes or echinocytes according to their hydrogen bonding properties.[121] Thus, analogs with a functional group having a capacity of donating a hydrogen bond (COOH, CH_2OH and $CONH_2$) caused formation of echinocytes, whereas those lacking this ability (CH_3, CHO, $COOCH_3$) induced formation of stomatocytes. Both kinds of erythrocyte alterations were prohibitive with respect to *P. falciparum* invasion and growth; all compounds were inhibitory with IC_{50} values in the range of 7-28 μM and the growth inhibition correlated well with the extent of membrane curvature changes assessed by transmission electron microscopy. Although these results do not demonstrate the levels of inhibition expected to be therapeutically relevant, they suggest that a specific protease activity is required for erythrocyte invasion by malaria parasites and thus is a potential target for antimalarial drugs.

The active site of papain and related proteases is large and can be divided into pockets at each side of the catalytic site, S_1-S_4 and S_1'-S_3', with each pocket accommodating one amino acid residue of the peptide substrate.[122] The general structure of a cysteine protease inhibitor (Fig. 8) should comprise an electrophilic moiety for interaction with the cysteine residue of the enzyme and one or two series of substituents, P_1-P_4 and/or P_1'-P_3', responsible for interactions with the different binding pockets of the protease.

The irreversible inhibitors are better characterized by means of kinetic constants: the dissociation constant K_i measures the affinity of inhibitor for the enzyme, the k_{inac} describes the velocity of formation of covalent enzyme inhibitor complex and the second-order rate constant of inhibition k_{2nd} that determines their potency.

1.5.1 Peptide-based falcipain-2 inhibitors

1.5.1.1 Peptidyl fluoromethyl ketones

Fluoromethyl ketones are in general, highly reactive and selective irreversible inhibitors of cysteine proteases.[123] A number of peptidyl fluoromethyl ketones were tested against FP-2,[124] previously named as *P. falciparum* trophozoite cysteine proteinase (TCP). In this context CBZ-Phe-Arg-CH_2F (**1**) (Fig. 7) proved to be the most potent inhibitor of FP-2 at picomolar concentration (IC_{50} = 0.36 nM), to block the hemoglobin degradation (IC_{50} = 0.10 mM) and to

inhibit the development of cultured malaria parasites in the nanomolar range (IC_{50} = 64 nM).[125] CBZ-Phe-Arg-CH_2F (**1**) was effective at nanomolar concentrations on four different strains of *P. falciparum* (Itg2, FCR3, W2, D6) and proved to be nontoxic to four human cell lines.

A number of fluoromethyl ketones characterized by the presence of a morpholineurea (Mu) group at P_3 position was tested against FP-2; the most effective inhibitors were Mu-Phe-HomoPhe-CH_2F (**2**) (IC_{50} = 3 nM) and Mu-Leu-HomoPhe-CH_2F (**3**) (IC_{50} = 0.42 nM). Mu-Phe-HomoPhe-CH_2F (**2**), which proved to be the most effective inhibitor against *P. vinckei* cysteine protease (IC_{50} = 5.1 nM against *P. vinckei* protease) is biochemically similar in terms of molecular mass, *p*H optimum, substrate specificity and inhibitor sensitivity to FP-2. It was also tested *in vivo* in a murine malaria model.[126] When administered parenterally

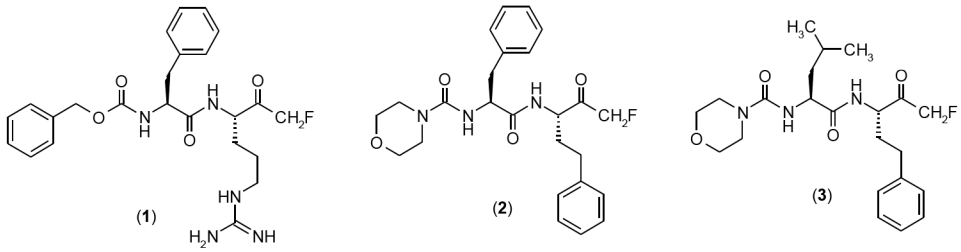


Figure 7: Structures of peptidyl fluoromethyl ketones against FP-2

four times a day for 4 days to *P. vinckei* infected mice, the inhibitor (**2**) cured 80% of the treated mice. However, the use of peptidyl fluromethyl ketones in therapy had an important limitation due to their proteolysis by serum, tissue and gut proteinases. These *in vivo* assays clearly evidenced that the serum half-life of **2** was short and that the efficacy of the inhibitor could be improved only by its frequent administrations

1.5.1.2 Peptidyl vinyl sulfones

Vinyl sulfones (VS) well known covalent inhibitors of FP-2 are able to inactivate the enzyme by irreversible addition of the thiol group of active site Cys42 to the electrophilic vinyl sulfone moiety, which behaves as a Michael acceptor.[127] It is proposed that a hydrogen bond from the protonated active site His174 to one of the sulfone oxygens might polarize the vinyl group, thus further activating the β-carbon proposed that a hydrogen bond from the protonated active site His174 to one of the sulfone oxygens might polarize the vinyl group, thus

further activating the β-carbon proposed that a hydrogen bond from the protonated active site His174 to one of the sulfone oxygens might polarize the vinyl group, thus further activating the β-carbon toward nucleophilic attack (Fig. 9).[123]

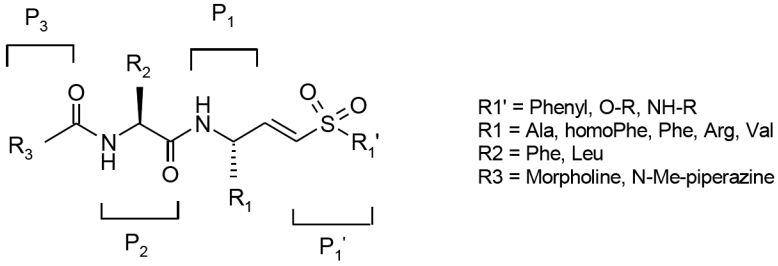


Figure 8: General structure of vinyl sulfones, vinyl sulfonate esters and vinyl sulfonamides

The obtained negative charge at the α-carbon is eliminated via protonation by the histidinium residue.[128]

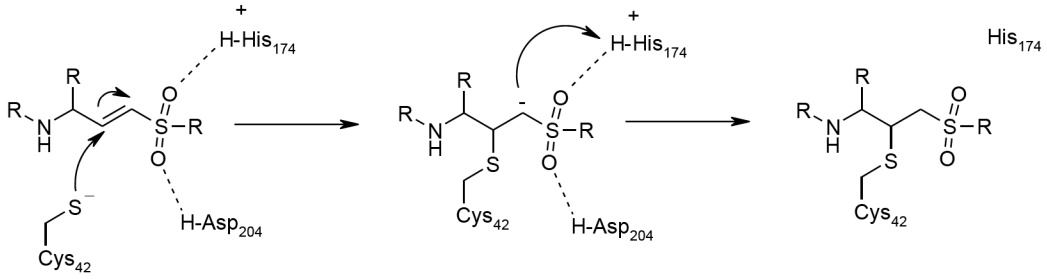


Figure 9: Mechanism of FP-2 inhibition by vinyl sulfones

Furthermore vinyl sulfones proved to be unreactive toward serine proteases, metalloproteases, aspartyl proteases, nonactive-site cysteines and circulating thiols such as glutathione; moreover, substituted vinyl sulfones proved to be less reactive toward nucleophiles than the analogous vinyl ketones or esters and sufficiently inert without the target.[127]

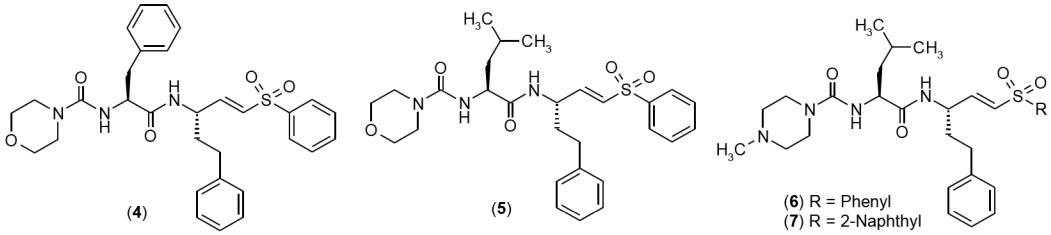


Figure 10: Structures of vinyl sulfones containing morpholineurea

A series of vinyl sulfones as novel peptide-based FP-2 cysteine proteinase inhibitors were reported in *P. vinckei*.[129] For most of the compounds, inhibitory effects against FP-2 and *P. vinckei* proteases were similar; however, in some cases efficacy against the two related enzymes proved to be different and this clearly indicated a critical role of the peptide portion of the inhibitors in their

specificity toward similar enzymes. In this context, Mu-Phe-HomoPhe-VSPh (**4**) (Fig. 10) has been proven to be a mid-nanomolar inhibitor of both proteinases (IC_{50} = 0.08 μM against FP-2 and IC_{50} = 0.1 μM against *P. vinckei* protease); on the contrary, the structurally similar peptide Mu-Leu-HomoPhe-VSPh (**5**) containing a Leu residue at P_2 site improved effectiveness against FP-2, while activity against the *P. vinckei* enzyme decreased (IC_{50} = 0.003 μM against FP-2 and IC_{50} = 0.2 μM against *P. vinckei* protease).

An improved *in vivo* effectiveness resulted when the morpholineurea group was replaced with the N-methylpiperazineurea (N-Pipu) group in compound (**6**) due to increased aqueous solubility and bioavailability. The phenyl vinyl moiety was also replaced with a 2-naphthalene group.[130] Both of the compounds (**6** and **7**) (Fig. 10) showed antimalarial effects correlating with potency against FP-2. Both of them strongly inhibited hemoglobin degradation, development and metabolic activity in cultured *P. falciparum* parasites in the nanomolar range. The compounds (**6** and **7**) displayed better activity than the parent molecule (**5**) in the inhibition of FP-2 and this clearly showed positive effect of the replacement of morpholine nucleus with the *N*-methylpiperazine. Also the extension of the aromatic area at the P_1' site (i.e. **7**) resulted more fruitful. When the most active compound (**7**) was administered orally to mice, it markedly delayed the progression of murine malaria and cured about 40% of the treated animals. However, many problems common to peptide-based inhibitors still remained, like: (i) poor pharmacological profile, (ii) reduced absorption through cell membranes, (iii) susceptibility to protease degradation and (iv) limited bioavailability.

The structure-activity relationships of a new series of peptidyl vinyl sulfones, vinyl sulfonate esters and vinyl sulfonamides (Table-II, III) for inhibition of the protease FP-2 and of *P. falciparum* development has been reported.[131] Compounds containing the core sequence Phe-homoPhe showed modest activity against FP-2 and substitutions at position P_3 (Fig. 11) in these compounds had relatively little impact on activity. Compounds with the core sequence Phe-O-(phenyl)Ser had similar activities, with IC_{50} for the inhibition of FP-2 in the mid-to-high nanomolar range. The Leu-homoPhe compounds revealed to be very active against FP-2, with IC_{50} values generally in the high picomolar to low nanomolar range. Identical compounds, except for the P_1' substituent

displayed the general rank order of activity: vinyl sulfonate esters > vinyl sulfonamides > phenyl vinyl sulfones (Tables-II, III). The vinyl sulfones (**8**) were potent inhibitors with IC_{50} in the low nanomolar range.

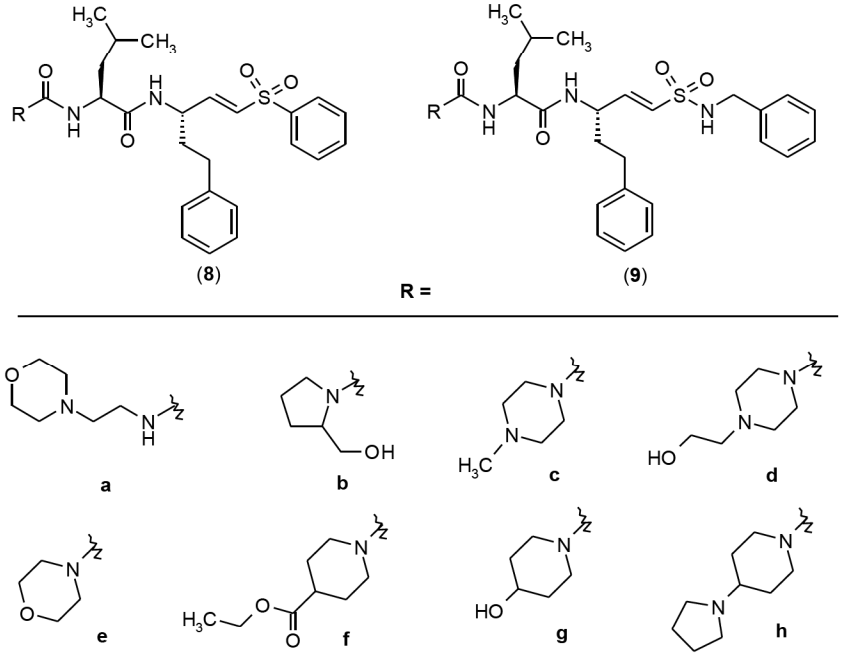


Figure 11: P_3 (R) substituents of peptidyl vinyl sulfones (**8**) and vinyl sulfonamides (**9**)

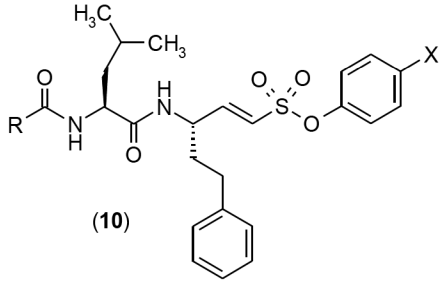
Sulfonates (**10**) and sulfonamides (**9**), despite their greater activity against FP-2, were generally less active against the parasite. In this case, for compounds with identical structures and with variable P_1' portion, the antiparasitic activity order was generally (**8**) > (**9**) > (**10**); an order which was opposite with respect to the activity against FP-2 (Table-I, II). In the vinyl sulfonates (**10**) antiparasitic order was observed with 'X' group as OCH_3 > F > H, opposite to that reported for the enzymatic inhibition (Table-III).

It is noteworthy that P_3 substituents (Fig. 11) showed a marked impact on the activity against parasite cultures. Three of the six vinyl sulfonate esters (**10**) with the highest antiparasitic activities contained an isonipecotic ester moiety (**8f**, **9f**, **10f**), while the other ones contained a tertiary amine (**8c**, **8h** and **9c**). It is interesting to observe that compounds containing the isonipecotic ester moiety proved to be active both against the enzymes as well as the cultured parasites. On the contrary, those compounds containing a prolinol substituent at P_3 (**8b**, **9b** and **10b**) were typically potent against the enzymes but showed relatively poor antiplasmodial activity. The measured second-order rate constants ($k_{ass}M^{-1}$ sec^{-1})

Table II: Effects of R substituents (P_3 Unit) on the activity of peptidyl vinyl sulfones (**8**) and vinyl sulfonamides (**9**) toward FP-2 development of *P. falciparum*

R	Compound 8		Compound 9	
	FP-2 IC_{50} (nM)	***P. falciparum* IC_{50} (nM)**	**FP-2 IC_{50} (nM)**	***P. falciparum* IC_{50} (nM)**
a	35	200	14	1500
b	27	1800	3.6	220
c	8.7	4.5	2.3	4.4
d	9.2	15	2.5	22
e	3	22	2.2	46
f	6.9	3.9	2.2	1.6
g	9.9	21	-	-
h	6.7	1.6	-	-

Note : Compounds (**8e**) and (**8c**) correspond to compounds (**5**) and (**6**), respectively.



(**10**)

Table III: Effects of R substituents (P_3 Unit) on the activity of vinyl sulfonate esters (**10**) toward FP-2 and development of *P. falciparum*

R	X	FP-2 IC_{50} (nM)	*P. falciparum* IC_{50} (nM)
b	H	0.8	390
b	F	0.8	394
b	CH_3O	0.9	220
e	H	0.7	49
e	F	0.7	84
e	CH_3O	0.7	14
f	H	0.7	42
f	F	0.7	120
f	CH_3O	0.9	9.7

for the inhibition of FP-2 and FP-3 of the most potent vinyl sulfone (**8**), sulfonamide (**9**) and sulfonate (**10**) inhibitors were generally similar against both

of the recombinant enzymes. As seen with IC_{50} determinations, the vinyl sulfonate esters were the most potent inhibitors, with k_{ass} values against both the proteases being $>10^5 M^{-1}$ sec^{-1} for all of the tested compounds. The second-order rate constants for the phenyl vinyl sulfones and vinyl sulfonamides were lower but they were consistently $>10^4$ M^{-1} sec^{-1}.

1.5.1.3 Peptidyl aldehyde and α-ketoamide derivatives

It was found that peptidyl aldehydes and α-ketoamide derivatives were effective to inhibit the enzymatic activity of FP-2 at very low nanomolar range. The best two inhibitors were revealed to be the aldehyde (**11**) and the ketoamide (**12**) with an IC_{50} value of 1 nM. The other representative inhibitor was Z-Leu-homoPhe-al (**13**) exhibiting an IC_{50} value of 2 nM.[132] Compounds (**11-13**) (Table-IV) proved to be active against multiple strains of *P. falciparum* with the best activity shown by compound (**11**) (Table-IV).[132]

In agreement with older studies of vinyl sulfone inhibitors,[129] compounds containing Leu at the P_2 position were about an order of magnitude more potent than those with Phe at this position. The key difference between the plasmodial cysteine proteases and many other papain family cysteine proteases, including the abundantly available host proteases cathepsin B and cathepsin L suggests the potential for specific inhibition of plasmodial enzymes.[133]

The lead inhibitors (**11-12**) (Table-IV) when evaluated against recombinant FP-2 and FP-3 proved to be similarly active against both of the enzymes. Cysteine proteases of rodent malaria parasites that appear to be orthologs of FP-2 and FP-3 were also characterized. A single ortholog of the two *P. falciparum* proteases appeared to be present in *P. vinckei* and in three other rodent malaria parasites.[134] Interestingly, all the rodent parasite cysteine proteases contain an unusual active site substitution that, in the case of the *P. vinckei* protease vinckepain-2, mediates unusual kinetics.[135] Taking into consideration that assays in mouse models are generally a key step of malaria drug discovery, it was of interest to check the activity of peptidyl aldehydes against *P. falciparum* and *P. vinckei* cysteine proteases. The obtained results revealed that vinckepain-2 was substantially less inhibited by the best inhibitors by about an order of magnitude; inhibition was particularly poor with a compound containing Phe at P_2 consistent with literature data.

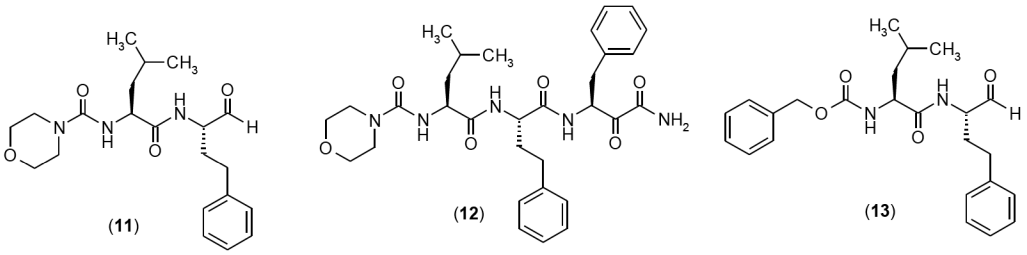


Table IV: Falcipain-2 inhibition and activity against different strains of *P. falciparum* of peptidyl aldehyde and a-ketoamide derivatives. (**11**-**13**)

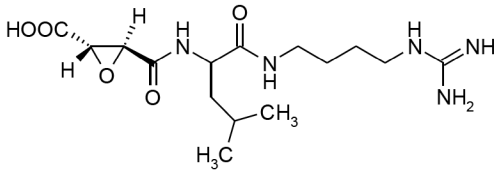
Compound	FP-2 IC_{50} (nM)	IC_{50} (nM)				
		W2	ItG	D6	Dd2	HB3
11	1	1.1	0.96	1.4	1.9	2.6
12	1	2.9	5.3	2.5	2.4	3.1
13	2	8.2	19	12	19	16

Although activity against vinckepain-2 was weak, the *in vivo* antimalarial activity of the lead compound Mu-Leu-homoPhe-al (**11**) was checked against *P. vinckei* malaria and these studies demonstrated for this inhibitor: (i) very poor pharmacokinetic profile, (ii) problems with its formulation and (iii) need of subcutaneous infusion pumps for administration. This clearly emphasizes that differences between *P. falciparum* and *P. vinckei* targets may contribute to the limited *in vivo* efficacies of some cysteine protease inhibitors, if it is considered that antimalarial drug discovery is based on validation with rodent models before advancement to a full scale development.

1.5.1.4 E-64

The *N*-(*L*-3-*trans* carboxyoxiran-2-carbonyl)-*L*-leucyl)-amido(4-guanido) butane (E-64, Fig. 12), was the first epoxysuccinyl peptide isolated from a culture of *Asperigillus japonicus*,[136] and its structure was determinated by Hanada in 1978.[137] E-64 is an irreversible inhibitor of papain-like cysteine proteases (clan CA, family C1), including papain, bromelain, ficin,[136] cathepsin B,[138] cathepsin L,[139] calpain II [140,141] and cruzain,[142] but it does not inhibit clan CD proteases such as caspases,[143] legumains,[144] and gingipains,[145] and inhibits clostripain very slowly.[146] It shows a second-order rate constant (k_{2nd}) of FP-2 inhibition of $1.16x10^3$ M^{-1} sec^{-1} and IC_{50} values of 0.015 μM and of 0.075 μM

toward FP-2 and FP-3, respectively. Additionally, when tested against *P. falciparum* strain FCBR, it revealed an IC_{50} value of 5.3 μM.[147]



E-64

Figure 12: Structure of E-64

E-64 contains a *trans* (2*S*,3*S*) configured epoxide ring, whereas the amino acid residues of the peptidyl part of the inhibitor have the *L*-configuration. On the epoxide ring the substituents at C-2 and C-3 are in *trans* position to one another.[148,149] *Cis* configuration leads to total loss of inhibition activity. E-64 inhibits cysteine proteases by S-alkylation of the active site cysteine, which results in the opening of the epoxide ring.[150,151] Its leucine side chain mimics the P_2 amino acid of the substrate, occupying the target's S_2 binding pocket, while the agmatine moiety binds in the S_3 position.[152]

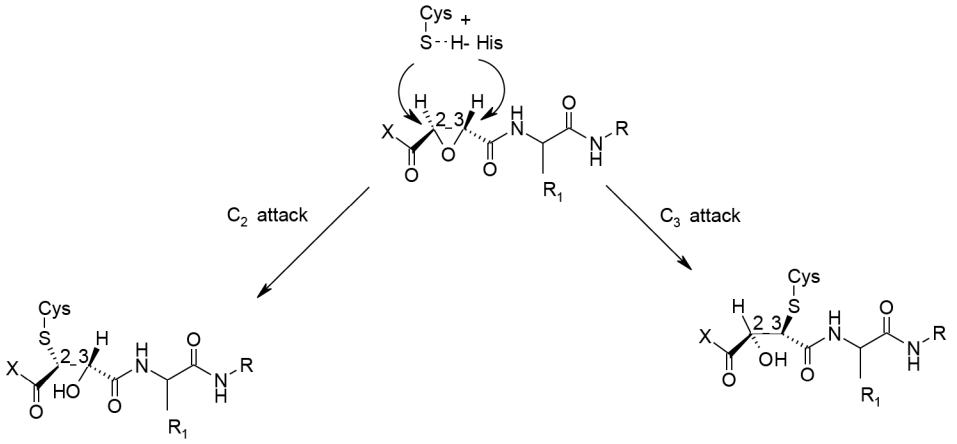


Figure 13: Mechanism of inhibition of cysteine protease by Epoxysuccinates

Peptidyl epoxysuccinates can inhibit the enzyme by forming a thioether bond via a nucleophilic attack at C-2 or C-3 of the epoxide ring by the active site cysteine residue.[153] Attack occurs at either C-2 or C-3 depending on the orientation of the epoxysuccinate in the active site (Fig. 13). In case of E-64, the site of attack was identified only at C-2 from NMR experiments.[150] The stereochemistry of the enzyme- inhibitor adduct has undergone an inversion of configuration at the reaction site due to a nucleophilic attack by the active site

thiolate in an SN_2 reaction. For instance, E-64, which has the 2*S*,3*S* configuration before the nucleophilic attack, will become 2*R*,3*R* after the covalent bond between the cysteine residue and C-2 of the oxirane ring is formed.

Initially, it was postulated that when E-64 inhibited papain, the oxirane ring would be protonated by His159. This suggestion was rejected by Varughese and co-workers,[154,155] from the crystal structure of papain inhibited by E-64. They argued that the epoxide was more likely protonated by water (Fig. 14) because of the distance (5.5A°) of the resulting hydroxyl group from His159.[156]

1.5.1.5 Peptidyl aziridines

Aziridines are aza-analogs of epoxides, that are equally susceptible to ring opening by nucleophiles.[123,157] This class of inhibitors has been tested against several types of proteases, including serine proteases, aspartyl proteases and metalloproteases, but aziridines are irreversible and selective inhibitors of cysteine proteases.[158] Further, aziridines such as aziridine-2-carboxylates and aziridine-2,3-dicarboxylates, are hydrolyzed by serine proteases.[159,160] There are three types of aziridinyl peptides (Table-V) that are classified according to their structural differences. [123] Type I are N-acylated aziridines with the aziridine moiety located on the C-terminus of the peptides or amino acids. Type II are aza-analogs of epoxysuccinyl peptides that have the aziridine-2,3-dicarboxylic acid moiety at the N-terminus of the amino acid. Type III are N-acylated aziridines such as type I, but the aziridine moiety is located in the middle of the peptide chain. Besides the common inhibition mechanism, several differences can be found between the epoxide and the aziridine heterocycles. These include reactivity, the influence of the *p*H of medium, selectivity and stereospecificity of inhibition.[161]

The stereospecificity of the inhibition is one major difference between aziridines and epoxides. Aziridines have been shown to be more reactive at low *p*H,[162] with maximum activity at *p*H 4, while the epoxides are more reactive at *p*H 6-7 due to protonation of aziridine nitrogen atom, which translates into the increased reactivity of the inhibitor toward papain. Another difference between aziridines and epoxides is the hydrogen-bonding abilities.[163] Aziridines (Type-II) are H-bond donors, whereas the epoxides are H-bond acceptors. These differences suggest that the two classes of inhibitors may have different binding modes and possibly show variable interactions with cysteine proteases. The aziridine ring can

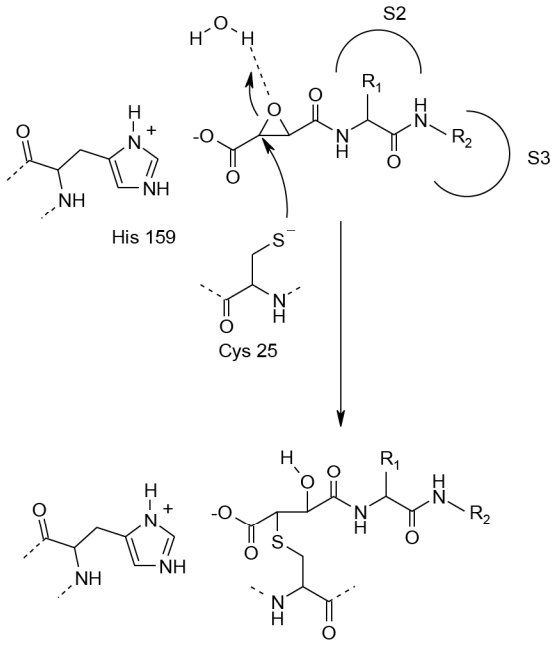


Figure 14: Proposed inhibition mechanism of papain-like cysteine protease inhibitors containing three-membered heterocycles

be opened by nucleophiles in two different ways-besides C-N bond cleavage, C-C bond cleavage is also possible.[164] Unlike epoxides, the *R*,*R* configuration of the

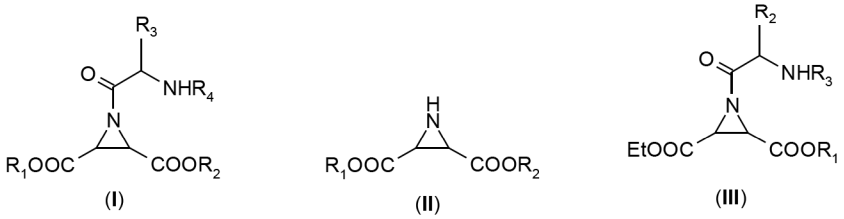


Table V: Aziridinyl peptide compounds.

Type (I)	Type (II)	Type (III)
R_1= Et, H	R_1= Et, H	R_1= amino acid, peptide
R_2= Et, Bn	R_2= amino acid	R_2= amino acid, side chain
R_3= amino acid side chain	-	-
R_4= protecting group, amino acid, dipeptide	-	R_3= protecting group, amino acid

aziridine ring is preferred for inhibition in both types II and III aziridine inhibitors, whereas the type I aziridine inhibitors with the *S*,*S* configuration are better inhibitors.[165] More recently,[166] several aziridines were screened against FP-2, FP-3 and cultured *P. falciparum*. They can be divided in three groups (i) aziridine-2-carboxylic acid derivatives, (ii) N-acylated aziridine-2,3-dicarboxylic acid

derivatives and (iii) aziridine-2-carboxylates containing a lysine residue. Within the series of aziridine-2-carboxylic acid derivatives with free 3-position, inhibitor (**14**) (Fig. 15) is the most active one with IC_{50} value of 2.2 μM against FP-2. Among the N-acylated aziridine-2,3-dicarboxylic acid derivatives, **15** and **16**

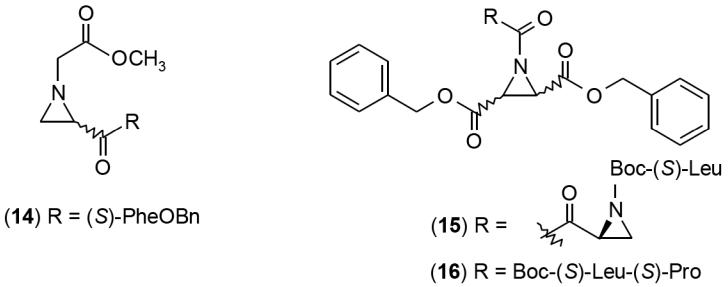


Figure 15: Aziridine-2-carboxylic and 2,3-dicarboxylic acid derivatives.

offered the most potent inhibitors of FP-2 (IC_{50} = 0.079 and 5.4 μM, respectively) and FP-3 (IC_{50} = 0.25 and 39.8 μM, respectively).

1.5.1.6 The 1,2,4-trioxolane compounds

In a prodrug approach, 1,2,4-trioxolane carbonyl protected compounds were reported to act by their cleavage/decomposition by iron chelation. The peptidic carbonyl protected (aldehyde) morpholineurea (**17**) (Fig. 17) was reported

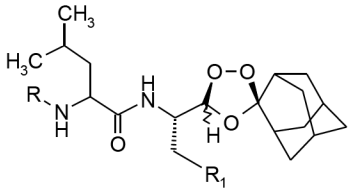


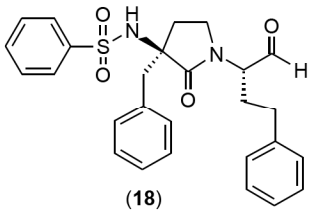
Figure 16: 1,2,4-Trioxolane compounds

to be weak falcipain inhibitor than parent aldehyde but it showed good antimalarial activity with IC_{50} value of 9.3 nM in *P. falciparum* 3D7 strain.[167]

1.5.1.7 The γ-lactam or pyrrolidinone isosteres

The γ-lactam or pyrrolidinone isostere has proven to be an effective conformational constraint for inhibitors of other classes of proteases.[168] A series of conformationally constrained pyrrolidinone inhibitors attached with electrophilic

aldehyde[169] were reported as inhibitors of falcipain. Compound (**18**) (Fig. 17) exhibited excellent binding affinity (50 nM) for falcipain.



(**18**)

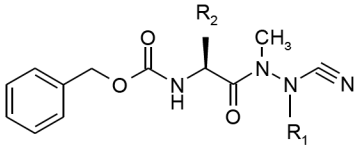
Figure 17: The γ-lactam or pyrrolidinone derivative

1.5.1.8 Peptidic fumaric acids and other peptidic analogs

Fumaric acid derived oligopeptide compounds library was successfully identified from a high-throughput screening of a solid phase-bound combinatorial library with the glycine, asparagine or phenylalanine in P_1 and phenylglycine and cyclohexylglycine in P_2 site. These fumaric acid derivatives from the library were found to be the most potent.[170] This information was used for synthesis of more potent vinylogous Michael-type peptidic cysteine protease inhibitors, displaying different inhibition mechanisms depending on the configuration of the double bond and the peptide sequence. It has been reported that the *E* configured vinylogous Gln-*tert*-esters are time-dependent inhibitors and on the other hand, *Z* or *E* configured maleic and fumaric acid derivaites containing two phenyl residues are reversible inhibitors. Some of the compounds show preferential selectivity to FP-2 over FP-3.[171]

1.5.1.9 Azapeptide nitriles derivatives

Azadipeptide nitrile cysteine protease inhibitors display structure dependent antimalarial activity against both chloroquine sensitive and chloroquine



(**19a**) R_1 = -*i*.Pr, R_2 = -Methyl
(**19b**) R_1 = -CH_2Ph, R_2 = -Pentyl
(**19c**) R1 = -CH_2Ph, R_2 = -$(CH_2)_2$Ph

Figure 18: Azapeptide nitrile derivatives

resistant *P. falciparum* malaria parasites. Inhibition of parasite's hemoglobin degrading cysteine proteases was also investigated, revealing the azadipeptide

nitriles as potent inhibitors of FP-2 and 3. A correlation between the cysteine protease inhibiting activity and the antimalarial potential of the compounds was observed. The FP-2, IC_{50} for the compound derivatives are more potent than phenylalanine peptides suggesting that the S_2 pocket of the enzyme is deep rather than shallow.[172]

1.5.2 Peptidomimetic falcipain-2 inhibitors

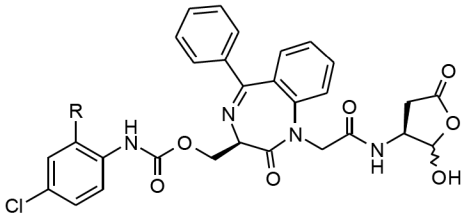
1.5.2.1 Peptidomimetics based on a 1,4-benzodiazepine scaffold

Even though several peptide-based FP-2 inhibitors have been identified, their utility as therapeutic agents is limited due to their susceptibility to protease degradation and poor absorption through cell membranes; a common strategy to improve pharmacokinetic and pharmacodynamic parameters is to lock a defined conformation of the peptide into a rigid scaffold, which could mimic protein's secondary structure. In this context the β-turn is a structural motif that has been postulated in many cases for the biologically active form of linear peptides and in recent years several scaffolds mimicking β-turns have been reported.[173-176]

The benzodiazepine (BZD) nucleus with its similar molecular dimensions has been proven to be a good β-turn mimetic.[177,178] Coupled with the fact that BZDs possess good oral bioavailability as a drug class that are certainly well tolerated; some novel peptidomimetic FP-2 inhibitors have been designed. They are based on a 1,4-BZD scaffold [179] as β-turn mimetics introduced internally to a peptide sequence which mimics the dipeptide *D*-Ser-Gly, and on a C-terminal aspartyl aldehyde building block, which inhibits the enzyme by forming a reversible covalent bond with the active site cysteine. The serine hydroxyl group was functionalized with various aryl isocyanates.

All new peptidomimetics displayed a significant inhibition of both FP-2A and FP-2B with IC_{50} values in the range of 8-26 μM. In this context compound (**20**) (Fig. 19) proved to be the most active inhibitor with an IC_{50} = 8.2 μM for the FP-2A and of 11.2 μM for FP-2B. Trying to define a structure-activity relationship, in this test series the phenyl derivative proved to be the least potent inhibitor (IC_{50} about 20 μM in both the enzymes); the introduction of an electron-donating or an electron-withdrawing group at position 4 of the phenyl ring of the carbamoyl moiety increased the inhibitory activity in both the cases. The best

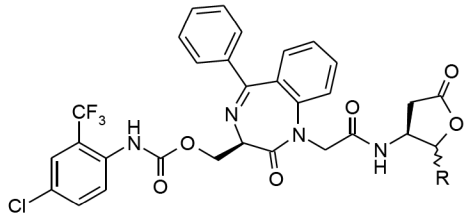
results were obtained when a methyl (**20**) or a trifluoromethyl (**21**) group was introduced at position 2 of 4-chloro derivative. Also the extension of the arom-



	FP-2A	FP-2B
	(IC_{50} in mM)	
(**20**) R = CH_3	8.2	11.2
(**21**) R = CF_3	8.7	25.9

Figure 19: Inhibition of FP-2A and FP-2B by peptidomimetic 1,4-benzodiazepine compounds.

atic area seemed to be fruitful. It is interesting to see that all new peptidomimetics inhibit both FP-2A and FP-2B at a similar level and this is a key step in the development of a FP-2 inhibitors, as it is well known that the disruption of FP-2A gene and in this case a decrease of the expression of this paralog seems to be compensated by the increased expression of FP-2B.[86] Furthermore, all compounds proved to possess good selectivity when tested against other cysteine proteases; such a panel of active recombinant human caspases (i.e., caspases 1-9) did not show any inhibitory activity up to 50 μM. The peptidomimetics bearing a protected aspartyl aldehyde warhead led to the thioacylals (**22**) (Fig. 20) and the acylals (**23**) with increased antiplasmodial activity in comparison to the parent molecule (**21**).[180]

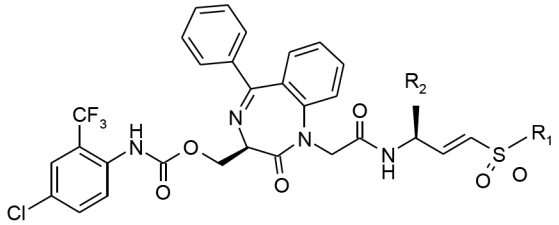


	FP-2	FP-2
	Ki (μM)	IC_{50} in μM
(**22**) R = *S*-*t*-Bu	8.2	28.5
(**23**) R = OBn	8.9	49.5

Figure 20: Thioacylal and acylal compounds

In order to further investigate the structural requirements of this class of inhibitors, the peptidomimetics were modified by replacing the P_1 aspartyl

aldehyde in the lead compound (**20**) with a vinyl sulfone moiety which was able to interact as classical Michael acceptor with the active site cysteine leading to an irreversible inactivation of the target enzyme as described above. In particular, two different series of vinyl sulfones were synthesized,[181] first one containing, at



(**24**) R_1 = H
(**25**) R_1 = CH_2CH_2Ph

Table VI: Inhibition of FP-2 and antiplasmodial activity of compounds (**25**)

Compound	R_1	k_{2nd} (M^{-1} min^{-1})	$k_{inac}M^{-1}$ /k_{inac} μM	*P. falciparum* IC_{50} (nM)
25a	Methyl	432,000	0.16/0.39	55.4
25b	Ethyl	307000	0.1/0.32	9.1
25c	Phenyl	175000	0.11/0.60	9.2
25d	4-OCH_3-C_6H_4	634000	0.034/0.053	59.6

the P_1 site, a homoPhe residue (e.g. **25**, Table-VI), an amino acid known to strongly increase the potency of FP-2 inhibitors and second one containing in the same position, a glycine (e.g. **24**, Table-VI), useful to evaluate the relevance of the amino acid side chain in the process of recognition of the ligand by the enzyme.[130] It was observed that all of the compounds (**24-25**) exhibited high second-order rate constants in the range of 161,000-634,000M^{-1} min^{-1}, in particular compounds (**25**) (Table-VI) generally showed higher second order rate constants with respect to analogs (**24**). Furthermore, it was possible to observe that the introduction, at the P_1' site, of a methoxy group in position 4 of the phenyl ring, strongly stabilized the enzyme-inhibitor interaction, probably acting as hydrogen bond acceptor, as shown in the most active compound (**25d**) with the highest value of the second order rate constants of inhibition (K_{2nd} = 634,000M^{-1} min^{-1}) and the lowest dissociation constant value (K_{inac} = 53 nM). Although there was no clear correlation between the antiplasmodial activity and the inhibitory potency, compounds (**25**) proved to be more active than their glycine analogs (**24**). It is interesting to point out that compound (**25c**), one of the lesser potent inhibitors against the enzyme, displayed the highest antiplasmodial activity, while the

presence of the methoxy group para to the phenyl ring (i.e. **25d**), basic for the enzyme inhibitor interaction, decreased the activity against the parasite, probably reducing the penetration through cell membranes.

Selectivity assays were also performed against papain family human cysteine proteases cathepsins B and L by demonstrating good selectivity of all vinyl sulfones toward FP-2. Other efforts were made to further optimize the pharmacophore portion by replacing the vinyl sulfone moiety with a vinyl phosphonate one, generating unsatisfactory results; a decrease of potency and selectivity was obtained.[182]

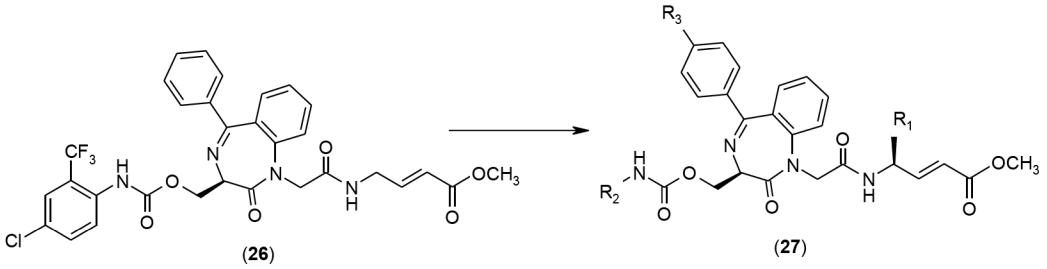


Table VII: Inhibition of falcipain-2 and antiplasmodial activity of compounds (**27**).

Comp.	R_1	R_2	R_3	k_{2nd} (M^{-1} min^{-1})	$k_{inac}M^{-1}$ /k_{inac} μM	*P. falciparum* IC_{50} (nM)
27a	H	4-Cl, 2-CF_3-C_6H_4	Br	24,500	0.038/1.56	6.33
27b	H	4-Cl, 2-CF_3-C_6H_4	H	3570000	0.06/0.017	12
27c	H	1-Adamantyl	H	1160000	0.013/0.014	10.7
27d	H	1-Naphthyl	H	*	*	17.6

* Time independent inhibition.

Based on the constrained peptidomimetic vinyl ester (**26**) (IC_{50} 12 μM)[183] an irreversible inhibitor of FP-2, the constrained vinyl ester peptidomimetics were evaluated; compound (**27a**) (Table-VII) was found to be the most potent one having an IC_{50} of 10.7 nM. Any further modification in the parent compound leads to decrease in FP-2 inhibition.[184]

1.5.2.2 Peptidomimetics based on a pyridine/pyrimidone ring scaffold

Recently, another nonpeptidic scaffold was incorporated on a peptide sequence suitable for FP-2 recognition, in a way to lock the amino acid backbone. The pyridone ring core was selected as a replacement for the peptidic leucine residue, at the P_2 position, within the inhibitor's framework. Aldehyde, vinyl sulfone or vinyl ester moieties were the chosen pharmacophores.[185] The new

synthesized peptidomimetics showed *in vitro* antimalarial activity against the 3D7 parasite strain in the range of 5-40 μM, with the vinyl sulfone (**28**) (Fig. 21) as the best inhibitor in the series (IC_{50} = 5.7 μM).

Consistently, with binding features previously reported, compounds bearing a homoPhe residue at P_2 site proved to be more active than those bearing a phenylalanine in the same position. Some of the synthesized peptidomimetics were also evaluated on recombinant FP-2 and FP-3. In this evaluation they proved to be weak inhibitors of FP-2 (IC_{50} 10-20 μM) and inactive toward FP-3 (IC_{50} > 25 μM). The best inhibitory profile against FP-2 was shown by compound (**29**) (IC_{50} = 10.9 μM), whereas it was not possible to test the most active compound (**28**) because of its low solubility.

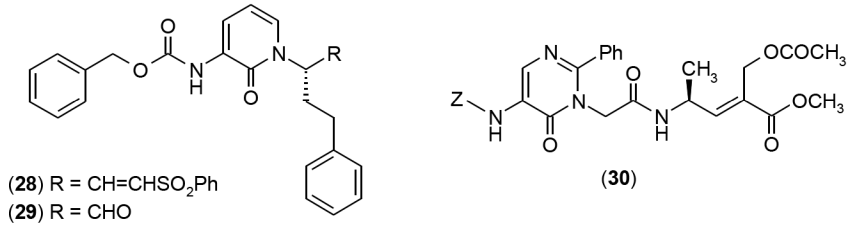


Figure 21: Pyridone and pyrimidone compounds

A new class of pyrimidinyl peptidomimetics (**30**) was reported as malarial cysteine protease inhibitors. The core structure of the new agents consists of a substituted 5-aminopyrimidone ring and a Michael acceptor side chain methyl 2-hydroxymethyl-but-2-enoate. The most effective compound (**30**) of the series (IC_{50} = 9 ng/ml) showed comparable efficacy to that of CQ (IC_{50} = 6 ng/ml). In general, this class of compounds exhibited weak to moderate *in vitro* cytotoxicity against neuronal and macrophage cells and less toxicity in colon cell line. Preliminary results indicated that compound (**30**) is active against *P. bergehi* prolonging the life span of the parasite-bearing mice from 6 days for untreated control to 16-24 days for drug treated animals.[186]

1.5.3 Nonpeptidic falcipain-2 inhibitors

1.5.3.1 Chalcones

Modeling studies on acylhydrazones have been made on a model structure of FP-2, designed on the basis of the X-ray structures of papain and actinidin. This investigation on the most active compound of this class, the oxalic

bis[(2-hydroxy-1-naphthylmethylene)hydrazide], clearly revealed that one naphthyl group filled the hydrophobic S_2 site while the other one interacted with the Trp177 of the S_1' site. On the basis of these results, the length of the backbone was shortened; the aromatic rings being replaced with heteroaromatic nucleus in order to improve the water solubility and to enhance interaction with His67 of the S_2 pocket. Finally, to improve the metabolic stability, the hydrazide linker was replaced with an α,β-unsaturated ketone backbone leading to calchones.[162] Some chalcones were thought to inhibit malarial cysteine proteases and today their ability to inhibit the protease of *P. falciparum* FP-2 is well known. Chalcones are biosynthetic precursors of flavonoids.

Licochalcone A (Fig. 22), a natural product isolated from Chinese liquorice roots, was the first compound of this family to exhibit *in vivo* and *in vitro* antimalarial activity.[187,188] Chalcones-artemisinins combination therapy have been reported to have synergistic or additive effect in the treatment of malaria.[189]

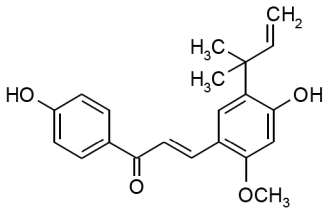


Figure 22: Licochalcone

First study on synthetic chalcones was performed by Li and co-workers in 1995.[190] The most active chalcone derivative, 1-(2,5-dichlorophenyl)-3(4-quinolinyl)-2-propen-1-one (**31**) (Fig. 23) showed an IC_{50} value of 200 nM against both CQ-resistant (W2) and CQ-sensitive (D6) strain of *P. falciparum*. The main conclusions of this work were: (i) the C_2-C_3 double bond was revealed to be essential for the inhibitory properties, because it kept the molecular structure in extended conformation leading to a better interaction with the active site, (ii) substitutions on the bridge portion caused a pronounced decrease in the inhibitory activity, probably due to steric interactions, (iii) introduction of halogens on ring A (fluorine and chlorine at 2,3 or 2,4-positions e.g. **33**) and electron-donating groups on the B ring increased the antimalarial activity and (iv) the presence of quinolinyl group in ring A resulted in increased activity. Under acidic conditions, nitrogen-containing heterocycles can be protonated within the acidic food vacuole of the parasite facilitating the interaction with His67 of FP-2. Additionally, this

chemical entity which is common in established antimalarials may interfere with heme detoxification affecting the whole process.

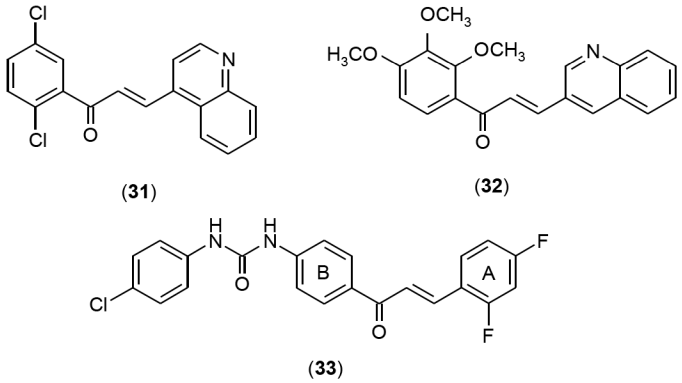


Figure 23: Chalcone compounds.

Later, a broad series of alkoxylated and hydroxylated chalcones have been synthesized by Liu and co-workers in 2001,[191] with the most active compound 1-(2',3',4'-trimethoxyphenyl)-3-(3-quinolinyl)-2-propan-1-one (**32**) (Fig. 23) showing an *in vitro* IC_{50} value of 2 μM against a strain of CQ-resistant human malaria parasite, *P. falciparum* (K1). The whole series was not tested directly against FP-2. Its FP-2 inhibitory activity was presumed. The results indicated that (i) the substitution pattern on the B ring could influence the antimalarial activity, (ii) hydrophobicity and size factors, respectively, for the A and B rings were important parameters for activity, (iii) presence of halogens on A ring did not necessarily increase the antimalarial activity while the influence of the latter groups on A ring seemed to be influenced by substitution pattern on B ring and (iv) an interesting observation was the association of good antimalarial activity with the presence of a 3-quinolinyl A ring. This was observed with 3-quinolinyl A ring and several B ring series of compounds except 4'-hydroxylchalcone. The 4-quinolinyl A ring derivatives were significantly less active than their 3-quinolinyl counterparts indicating that a steric element is also involved in the interaction with the target.

Dominguez et al. reported phenylurenyl chalcones to possess good inhibitory properties against FP-2.[192] The best inhibitor of this series, was 1-[4'-*N*-(*N'*-*p*-chlorophenylurenyl)phenyl]-3-(3,4,5-trimethoxyphenyl)-2-propen-1-one disp-laying an IC_{50} value of 1.76 μM toward the protease, coupled with a satisfactory inhibition of cultured malaria parasite development (IC_{50} = 3 μM).

Quantum chemical QSAR study on the chalcone derivatives confirmed that their activity against W2 and D6 *P. falciparum* strains depended upon steric and hydrophobic factors. Generally, a width limiting chemical substituent on A ring is optimal for activity on W2 and D6 strains. Molecular weight, which is related to molecular volume, appears to influence only the activity of D6 strain. This study also indicates that chalcones are capable of taking resonance structures along unsaturated chain, thus they can be good Michael acceptors. This would result in irreversible inhibition. The oxygen of the carbonyl is subject to the protonation in the internal acidic environment of the digestive vacuole of plasmodium, intensifying the positive partial charges on carbons C-1 and C-3 and favoring the nucleophilic addition at these carbons. However, these studies were performed in the absence of crystal structure of the parasitic cysteine protease, giving a rough evaluation for enzyme-inhibitor interaction mechanism.[193]

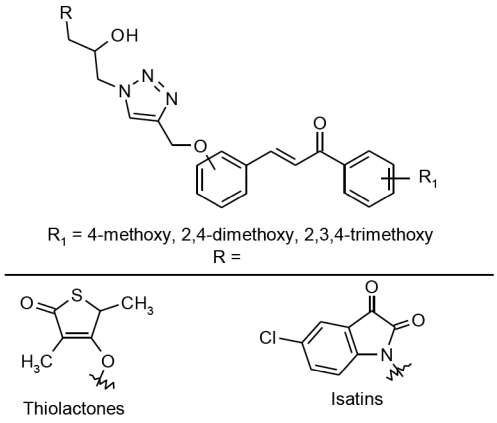


Figure 24: β-Amino alcohol thiolactone-chalcone and isatin-chalcone derivatives

Hans et al. reported two types of chalcone, thiolactone and isatin scaffold- containing compounds (Fig. 24). A 36-member β-aminoalcohol triazole library showed that the thiolactone-chalcones, with IC_{50} ranging from 0.68 to 6.08 μM, were more active against W2 strain of *P. falciparum* than the isatin-chalcones with IC_{50} values of 14.9 μM or less. Interestingly, isatin-chalcones diaplayed FP-2 inhibitory activity whereas the thiolactone-chalcones lacked enzyme inhibitory activity. No correlation was observed between the antiplasmodial activity and the FP-2 enzyme inhibition suggesting that it may not be the primary mode of action of the chalcones.[194]

1.5.3.2 The α-pyranochalcones and pyrazoline derivatives

Wanare G et al. reported the α-pyranochalcones and pyrazoline derivatives as falcipain inhibitors. Docking studies (GLIDE) on (*E*)-3-(3-(2,3,4-

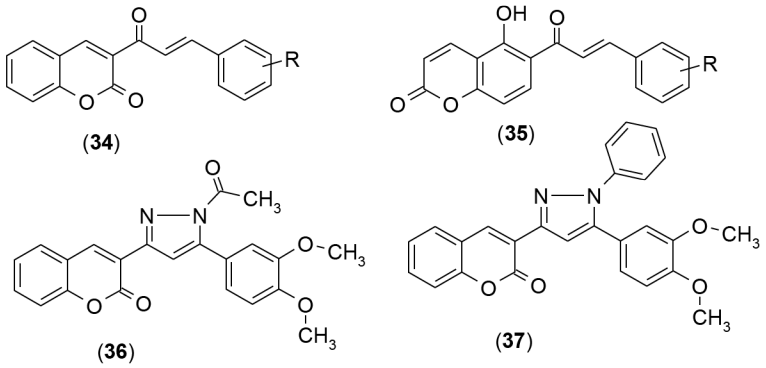


Figure 25: Chalcone compounds

Table VIII: α-Pyranochalcones and pyrazoline falcipain inhibitors

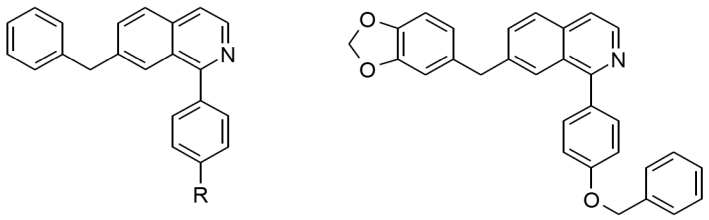
Compound	R	Antimalarial activity *P. falciparum* (mg/ml)			
		3D7 IC_{50}	**3D7 IC_{80}**	**RKL9 IC_{50}**	**Resistance index (IC_{50} D7/IC_{50} RKL9)**
34a	3-OCH_3-	11	24	4.9	2.2
34b	3,4-di OCH_3-	26.7	100	-	-
34c	2,3,4-tri OCH_3-	3.1	6.2	1.1	2.8
35a	2-Cl	20	25	13.5	1.5
35b	2,5-di OCH_3-	>100	-	-	-
36	-	10	10	9	1.1
37	-	10	44	7.6	1.3

trimethoxyphenyl)-acryloyl)-2*H*-chromen-2-one with active site residue Cys42 of falcipain enzyme revealed the requirement of hydrogen bond acceptor in ring B. The compound (**34c**) with trimethoxy substituent (Table-VIII) was found to be the most potent compound.[195]

1.5.3.3 Isoquinoline derivatives

Isoquinolines derivatives (Fig. 26) were designed as FP-2 inhibitors according to homology modeling studies and subsequent validation by docking. It was found that isoquinoline framework was not essential for the activity against FP-2.[196] The planarity of the isoquinolines was unimportant as both the dihydroisoquinoline and isoquinoline cores were found to have IC_{50} values in the

same range. Moreover, all of the compounds bearing a *p*-methoxyphenyl substituent at position 1 of the isoquinoline core were almost equipotent to the



(**38**) R= OMe, IC_{50} 3 μM
(**39**) R= OBu, IC_{50} 3 μM

(**40**) IC_{50} 3 μM

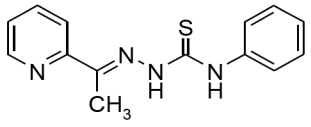
Figure 26: Isoquinoline derivatives

earlier reported ligands.[197] On the other hand, compounds with *p*-benzyloxyphenyl group (**38-40**, Fig. 26) docked well into the active pocket of the homology-modeled FP-2 and were found to be more potent than the corresponding hydroxyl analogs suggesting that S_2 pocket of the enzyme prefered to accommodate the hydrophobic groups.[196]

1.5.3.4 Thiosemicarbazones

1.5.3.4.1 2-Acetylpyridine-4-phenyl-3-thiosemicarbazone

The first thiosemicarbazone synthesized as potential antimalarial agent was 2-acetylpyridine-4-phenyl-3-thiosemicarbazone (**41**, Fig. 27). Klayman and co-workers[198] prepared a series of thiosemicarbazone derivatives from the parent compound (**41**) and tested them against *P. berghei* in mice. The main conclusions of this work were (i) the 2-pyridylethylidene group was important for antimalarial



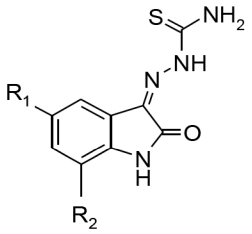
(**41**)

Figure 27: 2-Acetylpyridine-4-phenyl-3-thiosemicarbazone

activity and (ii) at N-4 position the presence of unsubstituted phenyl, benzyl, phenethyl or cycloalkyl groups such as adamantyl and cyclohexyl could also contribute to antimalarial activity.

1.5.3.4.2 Isatin thiosemicarbazones and other thiosemicarbazone compounds

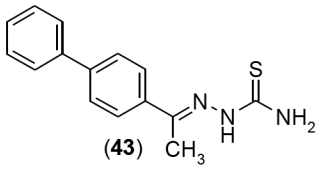
Chiyanzu et al. designed a new class of thiosemicarbazones as FP-2 inhibitors bearing the isatin scaffold (Fig. 28).[199] It was evident that the commercially available isatin was inactive, while the corresponding thiosemicarbazones showed certain degree of activity. The most promising FP-2 inhibitor of this series was revealed to be compound (**42**) with an IC_{50} value of 4.4 μM.



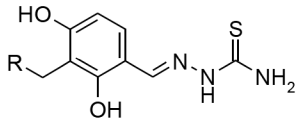
(**42**) $R_1 = NO_2$ $R_2 = H$

Figure 28: Isatin thiosemicarbazone derivatives

Recently, some thiosemicarbazones have been prepared and evaluated against FP-2, rhodesain and cruzain. Additionally all of the compounds were screened against the respective parasites. The obtained data revealed significant differences between the structural requirements essential for the inhibition of the

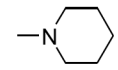


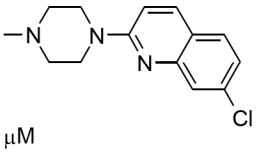
(**43**)



R =

(**44**) 

(**45**) 

(**46**) 

(**44**) FP-2 IC_{50} 5.8 μM: *P. falciparum* W2 ED_{50} >10 μM
(**45**) FP-2 IC_{50} 3.8 μM: *P. falciparum* W2 ED_{50} >10 μM
(**46**) FP-2 IC_{50} 2.25 μM: *P. falciparum* W2 ED_{50} >3.75 μM

Figure 29: Thiosemicarbazone compounds.

enzyme and the parasite. Compound (**43**) (Fig. 29) was the only one active against FP-2 (IC_{50} = 10 μM) but it was found to be almost inactive against the parasite

(ED_{50} >20 μM). On the contrary, all other compounds (**44-46**) (Fig. 29) showed good activity against FP-2. As the corresponding aldehyde precursors were practically inactive against FP-2, it was reasonable to assume that the (thio) semicarbazide moiety played an important role in the inhibition of FP-2; but compounds without mannich bases i. e. phenolic aldehydes and (thio)semicarbazides independently were found inactive against both FP-2 and the parasite. This clearly suggested that the activity of the discussed compounds was not due to the thiosemicarbazide side chain alone, but it was due to the combined effects of both, the mannich base of aldehyde and thiosemicarbazide components.[200] More recently, new mannich bases of phenolic benzaldehyde and 4-aminoquinoline-(thio)semicarbazide were synthesized. These small compounds showed good activity against CQ-resistant *P. falciparum* W2 strain but were very weak FP-2 inhibitors hence they primarily act some by some mechanism other than FP-2 inhibition.[201]

1.5.3.4.3 Gold thiosemicarbazones

The gold (I) thiosemicarbazone derivatives were evaluated for their FP-2 inhibiton. There was no correlation of antiplasmodial activity and FP-2 inhibition of the parent ligands hence these compounds were assumed to exhibit the antiplasmodial activity by inhibition of multiple targets.[202]

1.5.3.5 Acridinediones

Acridinediones like compound (**47**) (Fig. 30), have been shown to have antimalarial activity, but their mechanism of action remains unknown. From a

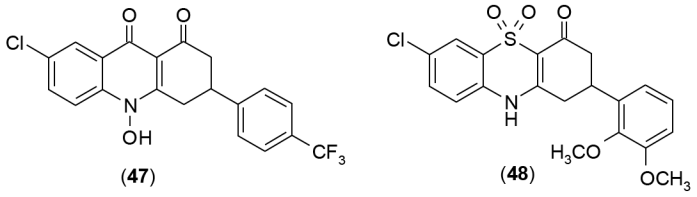


Figure 30: Acridione and isostere analog

series of chalcones, previously identified as inhibitors of falcipain, a conformationally constrained series was synthesized. This conformationally constrained series utilized a sulfur isostere of acridindione to afford the

phenothiazine (**48**) that blocked parasite metabolism and development at low micromolar concentrations.[203]

1.5.3.6 2-Cyanopyrimidines

Coteron et al. have reported a detailed study of optimization of the FP-2 and FP-3 activity on 5-chloro/bromo-2-cyanopyrimidine, (Fig. 31) an active scaffold. Based on initial studies on 4-chloro/bromo-2-cyanopyrimidines, it has been identified as an active scaffold at micromolar concentrations which was

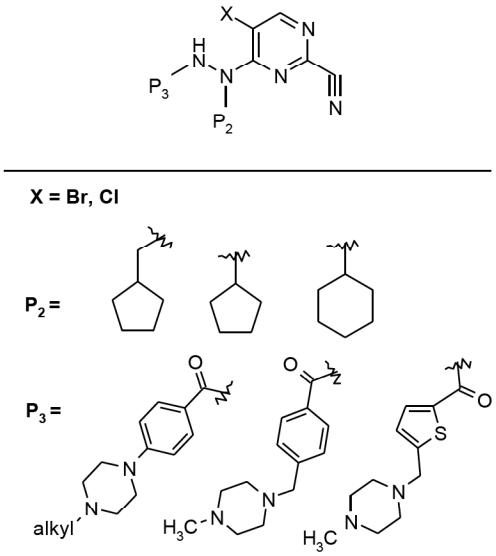
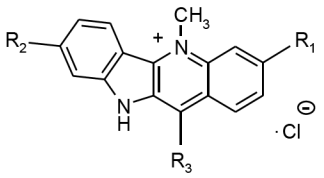


Figure 31: The 2-cyanopyrimidine derivatives

further subjected to optimization studies by varying the groups at X, P_2 and P_3 positions (Fig. 33) to obtain potent FP-2/FP-3 inhibitors. The FP-2/FP-3 inhibition concentrations were found to be in low nanomolar to picomolar range (IC_{50} 0.2 nM & 1nM in P*f* W2 strain).[204]

1.5.3.7 Cryptolepine derivatives

Cryptolepine derivatives (Fig. 32) containing basic side chains at the C-11 position containing propyl, butyl and cycloalkyl diamine groups significantly increased activity against chloroquine-resistant *P. falciparum* strains with less cytotoxicity, compared to chloroquine. Cryptolepine derivatives containing linear alkyl (eg. ethyl, propyl) or cyclic groups with terminal secondary or tertiary amines were found to be potent compounds with IC_{50} values of 20-90 nM. Any branching led to decrease in FP-2 inhibitory activity.[205]

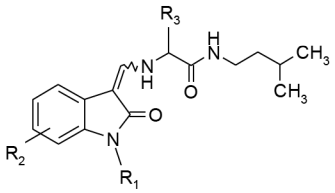


R_1, R_2 = H/Cl
R_3 = NH-Linker-NR_4R_5
R_4, R_5 = H, Alkyl, Cycloalkyl

Figure 32: Cryptolepine derivatives

1.5.3.8 Indolin-2-one derivatives

The 3-methyleneindolin-2-one derivatives (Fig. 33) showed moderate antimalarial activity (IC_{50} = 10 μM). The recognition moiety interaction of the Leu-*iso*-amyl sequence with the S_2 pocket plays important role in activity. Furthermore, the antiplasmodial activity could be significantly improved when a



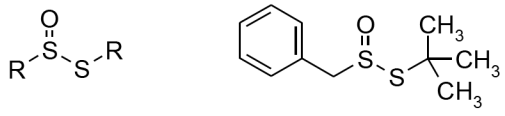
R_1 = H, methyl
R_2 = H, Cl
R_3 = H, isobutyl, phenyl ethyl/methyl

Figure 33: 3-Methyleneindolin-2-one derivatives

4-aminoquinoline moiety was coupled to the 3-methylene moiety of the indolin-2-one scaffold (IC_{50} = 140 nM).[206]

1.5.3.9 Allicin derivatives

Allicin (S-allyl-2-propenylthiosulfinate) and its derivatives were studied for FP-2 inhibition (Fig. 34). The mode of action is through the attack of active-site Cys residue on the primary carbon atom in vicinity to the thiosulfinate sulfur. Compounds **49** (allicin, K_i = 1.04 μM, IC_{50} = 5.21 μM), **55** (K_i = 1.8 μM, IC_{50} = 10.9 μM) and **51** (K_i = 4.5 μM, IC_{50} = 30.3 μM) showed good FP-2 inhibition.[207]



(**49**-**55**) (**56**)

Figure 34: Allicin derivatives

Table IX: Allicin derivatives and their antimalarial activity

Compound	R	FP-2 K_i (μM)	*P. falciparum* IC_{50} (μM)
49	allyl	1.04	5.21
50	*n*-propyl	26.4	78.3
51	benzyl	4.5	30.3
52	*tert*-butyl	>100	72.5
53	cyclohexyl	>100	34.4
54	*iso*-pentyl	3.04	54.7
55	*n*-hexyl	1.8	10.9
56	-	>100	52.9

1.5.4 Molecular modeling studies

1.5.4.1 Virtual screening of ChemBridge database

Virtual screening of ChemBridge database (consisting of approximately 2,41,000 compounds) was performed in an attempt to identify nonpeptide inhibitors of parasitic cysteine proteases as novel drugs.[208] The compounds were screened against homology models of falcipain-2 and falcipain-3 in three consecutive stages of docking. A total of 24 diverse inhibitors were identified, out

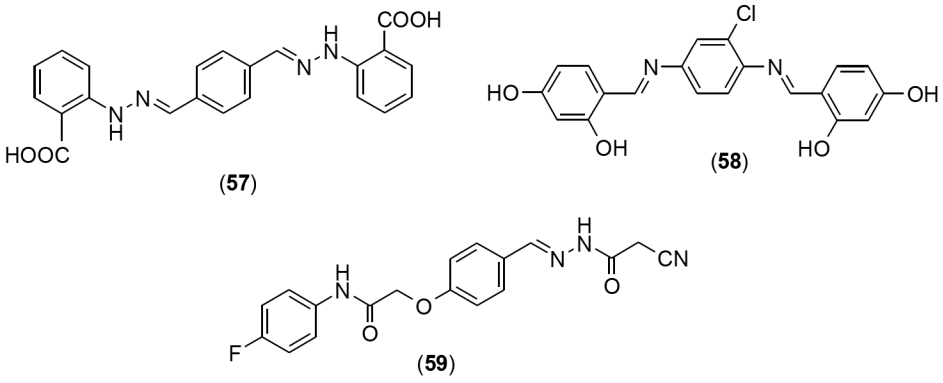


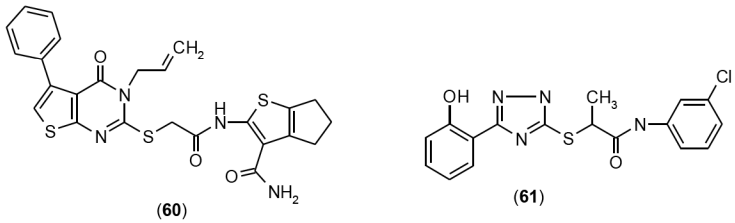
Figure 35: FP-2 and FP-3 dual inhibitors identified by virtual screening of ChemBridge database

of which 12 compounds appeared to be dual inhibitors of FP-2 and FP-3 (Fig. 35). Some of them showed preferential selectivity for FP-2 over FP-3. [203]

1.5.4.2 Docking studies on compounds of SPECS database

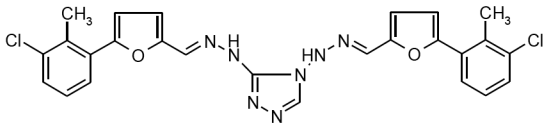
In another virtual screening study, the SPECS database (2,87,000) compounds were screened and reduced to about 80,000 using the drug likeness filter and subsequently docked by two methods Glide and GAs Dock, with different scoring

functions to identify leads for novel FP-2 inhibitors. The top 1000 compounds were selected using Glide with XP docking used for further accurate docking. The



(**60**)

(**61**)



(**62**)

	Inhibition rate(%)	IC_{50} μM
(**60**)	53.1	2.4
(**61**)	71.4	5.8
(**62**)	56.5	6.4

Figure 36: Potent compounds identified by docking studies on of SPECS database

[Note: Inhibition rate (%) calculated with respect to percent inhibition of control (E64)]

top 200 compounds were subjected to visual inspection of the docking geometry by (1) complementarity between the ligand and the hydrophobic S_2 pocket of the protein and (2) formation of hydrogen bonds between the ligand and residues near the catalytic cysteine (Cys42), resulting into identification of 53 compounds. In GAs Dock method top druglike 1000 compounds were selected by its energy score. The compounds were further evaluated and ranked using the CSCORE module of the Sybyl package and 154 compounds were then selected with the consensus score of 5. By visual analysis of the 154 docked poses by elimination criteria, 56 compounds were selected. Total 81 compounds were then screened for *in vitro* evaluation of FP-2 inhi bition. The compounds (**60-62**) (Fig. 36) were found to be the most potent ones.[209]

1.5.4.3 2-Amido-3-(1*H*-indol-3-yl)-*N*-substitued propanamides

2-Amido-3-(1*H*-indol-3-yl)-*N*-substitued-propanamides were designed and synthesized based on structure-based virtual screening in conjunction with

surface plasmon resonance (SPR) based binding assays. The compounds showed moderate FP-2 inhibition activity, with IC_{50} values ranging from 10.0-39.4 μM.[210]

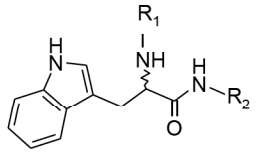


Figure 37: 2-Amido-3-(1*H*-indol-3-yl)-*N*-substitued propanamides

1.5.4.4 2-(3,4-Dihydro-4-oxothieno[2,3-*d*]pyrimidin-2-ylthio)acetamides

A series of novel small molecule FP-2 inhibitors have been designed and synthesized on the basis of structure-based virtual screening in conjunction with an enzyme inhibition assay. All of the 2-(3,4-dihydro-4-oxothieno[2,3-*d*]pyrimidin-2-ylthio)acetamide derivatives showed high inhibitory activity against FP-2 with IC_{50} values ranging from 1.46-11.38 μM.[211]

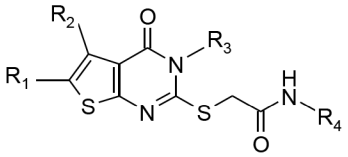


Figure 38: 2-(3,4-Dihydro-4-oxothieno[2,3-*d*]pyrimidin-2-ylthio)acetamides

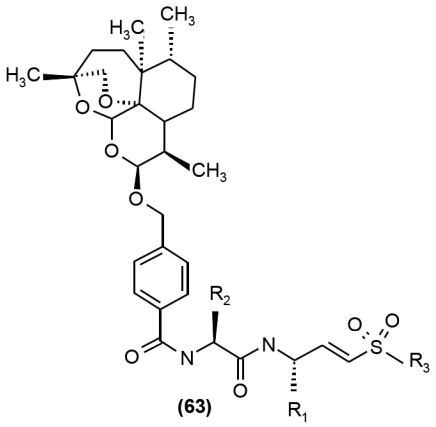
1.5.5 Combination or multi-target/hybrid structural approaches

Combination therapies are generally used to combat malaria that can minimize the chances of developing dug resistance. Certain combinations of falcipain inhibiting scaffolds and other antimalarial drugs or multi-target/hybrid therapy approaches have been studied in recent papers.

Aspartic and cysteine protease inhibitors act synergistically to degrade hemoglobin *in vitro*[212] therefore inhibitors of these enzymes would possess synergistic effects in inhibiting the growth of cultured parasites. So combination therapy of inhibitors of malarial cysteine and aspartic proteases may provide the most effective chemotherapeutic regimen and could possibly limit the development of parasite resistance to protease inhibitors in the best possible way.

Hans et al. reported two types of chalcones, thiolactone and isatin scaffolds containing hybrid compounds (Fig. 24) as antimalarial agents. [194] Chalcone-azole derivatives and artemisinins combination therapy have been reported to have synergistic or additive effect in the treatment of malaria. The

synergistic combinations decrease hemozoin formation in parasitized erythrocytes. These combinations do not affect new permeation pathways induced in the host cells. The combinations showed very potent FP-2 inhibition with IC_{50} values ranging from 0.29 to 10.63μM. The binding affinities and interaction modes were studied by molecular docking studies using AutoDock 3.05 for the potent compounds.[189]



R_1 = $(CH_2)_2Ph$, CH_2Ph
R_2 = CH_2CHMe_2, CH_2Ph, H
R_3 = Ph, Me

Figure 39: Artemisinin-dipeptidyl vinylsulfone hybrids

Peptidomimetic-thioacylals and acylals bearing protected aspartyl protease aldehyde warhead were studied for inhibiton of protozoal cysteine proteases falcipain-2 and rhodesain. Compounds showed potent anti-tripanosomal activity.[180] Artemisinin-dipeptidyl vinylsulfone hybrid molecules (**63**) were acting in the parasites food vacuole via endoperoxide activation and falcipain inhibition. Compounds showed FP-2 inhibition in micromolar range but almost all the compounds displayed IC_{50} values ranging from 2-6 nM in *P. falciparum* W2 strain.[213]

In another combination strategy through a single prodrug approach, 1,2,4-trioxolane carbonyl protected compounds were reported to act by their cleavage/decomposition by iron chelation. Peptidic aldehydes and esters were used to get hybrid compounds. The aldehyde endoperoxide hybrids showed activity in nanomolar concentration in 3D7 strain of *P. falciparum* but were observed to be not good falcipain inhibitors when compared with the parent peptidic aldehydes.[167]

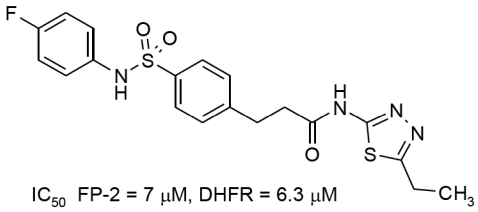


Figure 40: FP-2 and DHFR duel inhibitor

In a multitarget approach a series of small molecules were designed and synthesized. The compounds showed dual inhibition of falcipain-2 and dihydrofolate reductase as antimalarial agent and their potency increased 6 to 8 times in comparison to the individual inhibitors. The IC_{50} values for FP-2 ranged from 2.7–13.2 μM and for DHFR IC_{50} values ranged from 1.8–19.8 μM. The most potent compound (Fig. 40) exhibited moderate *in vivo* antimalarial activity in a dose dependent fashion. The molecular docking studies have been performed to identify the key structural information to attain dual inhibitory activity[214]

The existing armamentarium of antimalarial drugs is not sufficient to combat malaria, primarily because of resistance developed by the parasite. *P. falciparum* genome sequencing has revealed a number of drug targets. Although the enzymes of lethal malaria parasite species still has not drawn much attention of medicinal chemists, biologist and clinicians' worldwide to the extent it is required for the development of new drug. Ultimately, a better understanding of the biochemical and biological roles played by malarial proteases will foster the development of protease inhibitors that would specifically inhibit malaria parasite enzymes. There exist a great potential in the exploration of cysteine proteases falcipain inhibitor and thus might prove to be the most suitable drug target for the chemotherapy of malaria. This thesis work has been directed towards design and synthesis of FP-2 inhibitors. The details about design strategy, synthetic methodologies and biological screening results are described in the Chapter-2 Research Envisaged, Chapter-3 Result and discussion and Chapter-4 Experimental work sections.