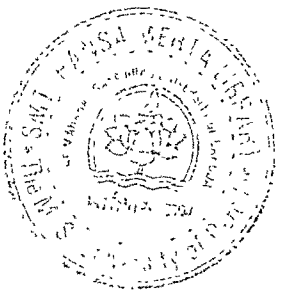


Chapter 1.

Introduction

1 INTRODUCTION

BACKGROUND

Malaria, African sleeping sickness, leishmeniasis, Chagas' disease, and other protozoan diseases of humans and their livestock continue to have a devastating impact worldwide. However, none of these protozoan diseases has had as enormous effect upon civilization, either historically or in modern times, as malaria. The hard statistics about malaria is far from comforting. Killing over one million victims every year and infecting another 500 million, the disease continues to have a catastrophic effect in many developing countries, striking the most vulnerable members of society: pregnant women and children under the age of five[1]. The World Health Organization (WHO) estimates[2] that malaria costs many afflicted states 1.3% of their annual gross domestic product, cutting their economies by over 30% since 1960. Exacerbated by poverty, malaria exerts a high toll on populations that can least afford a cure. These protozoan diseases are once again exerting an enormous toll in terms of mortality and morbidity on the populations of developing countries. Although there are reasons for this degenerating situation, including administrative difficulties, complex political and socioeconomic factors (such as military conflicts, collapsing health programmes and population displacement) it is clear that the parasites themselves are also fighting back. In the 1950s, many a people were optimistic that the disease could be eradicated with the development of powerful mosquitocides, environmental control and drug treatments. As a result there was a marked drop in the incidence of the disease in some regions, even complete eradication of the malaria carrier, *Anopheles gambiae.* But, by the end of the 20th century, concerns about the environmental safety of some mosquitocides and the parasite's increasing drug resistance saw rise in the disease's incidence.

Since the market for the antiparasitic drugs is in poor third world countries, innovative and cost effective approaches to antiparasite drug discovery and development are urgently needed. Much of the world's malaria occurs in countries with an annual per capita expenditure on health of less than $ 10. There is little interest shown by the pharmaceutical industry in developing new

antimalarial drugs, as the risks are great due to low returns on investment made in this direction. If drug resistance in *Plasmodium falciparum* (*P. falciparum*) continues to increase at the current rate, malaria may become untreatable in parts of Southeast Asia.

Furthermore, as international travel becomes more common, malaria is not confined to the tropical zones of the world, and imported malaria is an increasingly serious problem. The main reason for the dramatic increase in deaths from malaria in Africa is attributed to the spread of resistance to the mainstay antimalarial drug chloroquine; and the influence of drug resistance on malaria mortality is almost certainly underestimated[3]. Actually, morbidity and mortality are rising, not because of ineffective treatment of severe malaria, but because of ineffective first-line oral treatment that causes an increase in proportion of patients who develop severe disease. In addition, the transmission of resistant strains is facilitated by unsuccessful treatment[4]. Given the inexorable spread of drug resistance, and until the development of an effective antimalarial vaccine, the search for effective, safe, and affordable drugs for falciparum malaria is one of the most pressing health priorities worldwide. The widespread and rapid development of drug resistance requires that measures should be taken to prevent this evolution with the remaining effective compounds and with the new compounds that might be produced in the future. Ideally, drugs should be used only when needed (i.e., only for treatment, not for prophylaxis) and should be given to patients who are compliant with respect to the treatment regimen, in order to avoid treatments at subtherapeutic doses. The choice of drug combinations is indeed necessary.

The WHO has started a new program (called Roll - Back Malaria) in an effort to combine knowledge gained from new scientific efforts with education of the potential victims of the malaria. Malaria control efforts include attempts to develop an effective vaccine, eradicate mosquito vectors and develop new drugs[5]. 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 remains an economically and environmentally viable alternative.

1.1 REPLICATIVE CYCLE OF MALARIA[6]

In 1897, Ronald Ross reported that the parasite *Plasmodium* could infect a female mosquito, thus showing the complete parasite cycle. The malaria parasite exhibits a complex life cycle (Figure 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.

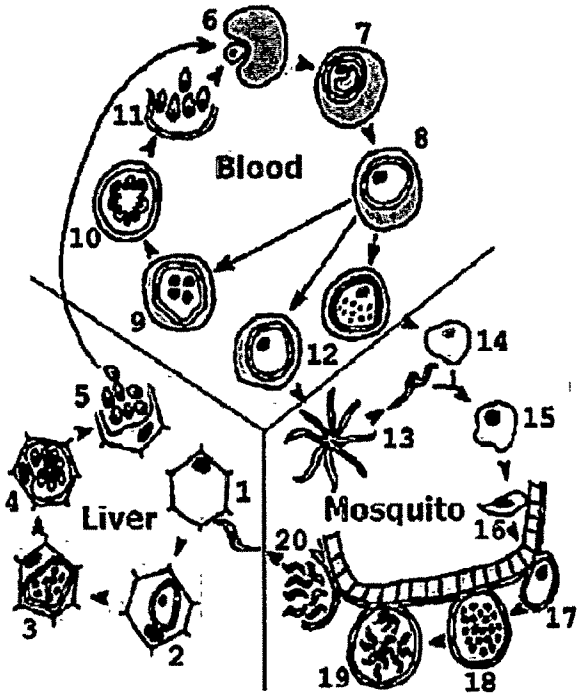


Figure 1: The life cycle of malaria parasite.

Infection is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver and invade hepatocytes (1). 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). A proportion of the liver-stage parasites from *P. vivax* and *P. ovale* go through a dormant period (not shown in Figure 1)

instead of immediately going through the asexual replication (i.e., temporarily stay at step 2). These hypnozoites 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 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 intermittent fevers often associated with malaria are due to the synchronous rupture of infected erythrocytes and release of merozoites. 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 escape from the host erythrocyte. Factors, which participate in the induction of gametogenesis, include: a drop in temperature, an increase in carbon dioxide 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 Figure 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). A 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 regards to the types of cells or tissues they invade and their motility.

1.2 CURRENTLY AVAILABLE ANTIMALARIAL DRUGS

In most cases, antimalarial drugs are targeted against the asexual erythrocytic stage of the parasite. The parasite degrades hemoglobin in its acidic food vacuole[7], producing free heme able to react with molecular oxygen and thus to generate reactive oxygen species as toxic by-products. A major pathway of detoxification of heme moieties is polymerization as malarial pigment[8]. 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[9]. The main classes of active schizontocides are 8-aminoquinolines, 4-aminoquinolines, aryl-alcohols 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 and its semisynthetic derivatives and synthetic analogs. Some antibiotics are also used, generally in association with quinoline-alcohols[10]. A 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. 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[11] 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[12] with a longer elimination half-life (14 days compared to 4 hours for primaquine). It also has a larger therapeutic index than primaquine. This molecule may be useful for chemoprophylaxis of *P. falciparum* and for prevention of relapses of vivax malaria[13].

1.2.2 4-Aminoquinolines

The main antimalarials are the 4-aminoquinolines because they have proven to be the most highly successful class of compounds for the treatment and prophylaxis of malaria. They are easily synthesized, 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, since this drug was cheap, non-toxic 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. Among the proposed hypotheses on the mode of action of CQ, one can cite[14]: (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 bases[18]. In spite of its reduced efficacy, CQ is still the most widely used antimalarial drug in most parts of Africa, both for reason of cost and because of the widespread prevalence of resistance among people infected with the parasite towards a large number of 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 Quinoline-methanols

Quinine, the active ingredient of cinchona bark, introduced into Europe from South America in the 17th century, had the longest period of effective use, but there is now a decrease of the clinical response on *P. falciparum* in some areas[23]. Nevertheless, it remains an essential antimalarial drug for severe falciparum malaria and intravenous infusion is, in this case, the preferred route. 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 for mefloquine has been suggested[21]. Combination of quinine and clindamycin significantly shorten the duration of treatment with respect to quinine, used alone[24]. 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 (Pgh 1) plays a role in mefloquine resistance and Pgh 1 may also be the target of this drug[21, 25].

1.2.4 Other aryl-alcohols

Halofantrine is effective against chloroquine-resistant malaria[26]. Despite this, cardiotoxicity has limited its use as a therapeutic agent[27]. Mefloquine usage appears to lead to selection of parasites resistant to halofantrine[28] also. 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[29]. The 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 aryl-methanols[30].

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[31]. Pyrimethamine-sulfadoxine (SP) 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[32]. 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 being 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[33]. Finally, the development of the combination of proguanil and atovaquone (Malarone) would provide another useful way for the treatment of malaria[34]. 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[35], or mefloquine[36], 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 African countries.

1.2.6 Artemisinin derivatives

Artemisinin derivatives are the fastest acting antimalarial drugs[37]. Four compounds have been used, the parent one artemisinin, extracted from Chinese herb 'qinghao' (*Artemisia annua*)[38] and three derivatives that are actually more active than artemisinin itself[37]. One of them is a water-soluble hemisuccinate, artesunate; two others 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 stages of the parasite that infect mosquitoes. The treatment of several million patients with artemisinin derivatives for acute malaria failed to detect any significant toxicity[39], even for pregnant women[40], 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[41] and artemether has been included in the WHO List of Essential Drugs for the treatment of severe multiresistant malaria. 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[42]. 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 has been 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[43]. 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 h). 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[44], benflumetol [45], or mefloquine[46] 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

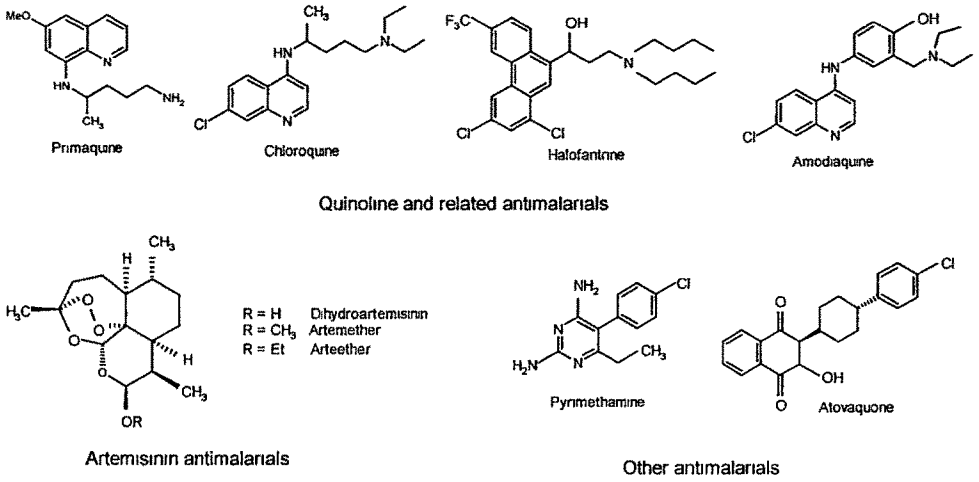


Figure 2: Examples of some drugs used to treat malaria

rapidly and results in fewer gametocytes carriers. However, the relatively high cost and erratic supply of the natural parent compound artemisinin make necessary the development of new synthetic and cheap endoperoxide-based antimalarials[47, 48].

1.3 NEW ANTIMALARIAL DRUGS

A lot of efforts have been made for the development of malaria vaccines without success till date[49]. The near availability of the complete sequencing of *P. falciparum* genome should improve the chances of existence of a vaccine. However, a positive result is not likely to be next door. The genome sequencing would allow the identification of new parasite proteins to be inhibited. But, time requirement is long, from discovering targets to developing new therapeutic means and costs are also likely to be high for such an approach.

1.3.1 Old targets, new compounds

An alternative strategy is the exploitation of known targets such as the phospholipid (PL) metabolism of infected erythrocytes[50]. The free heme liberated in the parasite food vacuole is also an "old" but always 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, among them are CQ and artemisinin derivatives. CQ is a cheap and easy-to-prepare molecule that has proved to be a 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 are substitutions in the quinoline nucleus, variations in the side chain, synthesis of bisquinolines, and more recently, the introduction of a ferrocenyl moiety[51]. A little modification seems to be enough to make a compound that would be active on resistant strains, but the wait for a safe and effective chloroquine alternative 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 still low, despite the investigations 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[52]. Synthetic trioxanes, simplified analogs of artemisinin retaining the crucial endoperoxide bridge, have been developed, but none of them could enter into clinical trials successfully[53].

1.4 PROTEASES - GENERAL INTRODUCTION

Proteases or proteolytic enzymes form one of the largest and more important groups of enzymes. They selectively catalyze the hydrolysis of

polypeptide bond. Now a days, proteases command enormous commercial importance. Proteases are one of the three largest classes of industrial enzymes accounting for about 60 % of the total sales of enzymes, worldwide. They have a variety of applications, mainly in detergent, food and leather industries[54].

Proteases assist the hydrolysis of large polypeptides into smaller peptides and further into amino acids thus, facilitating their absorption by the cells[55]. Their virtual control over protein synthesis in human beings and other microorganisms enables them to regulate physiological processes such as digestion, fertilization, growth, cell differentiation/signaling/migration, immunological defense, wound healing, and apoptosis[56]. They have been implicated in many health problems (**Table-1**) involving the circulatory system, immune system, allergies, sports

Table 1: Proteases in Disease Propagation and Their Functions

Protease	**Function**	**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	Amino acid synthesis	Malaria
Plasmepsin I & II	Amino acid synthesis	Malaria
Cruzain	Amino acid synthesis	Chagas' disease

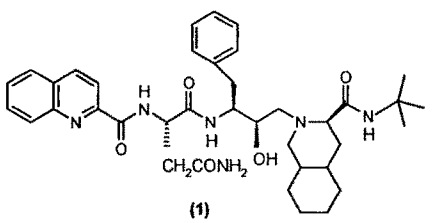
injuries and infectious diseases. They catalyze important proteolytic steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms and viruses. This makes proteases a valuable target for the development of newer drugs.

Depending on the active moiety in the proteases they have been categorized into four major classes[57]:

- Aspartic proteases
- Cysteine proteases
- Serine proteases
- Metalloproteases

1.4.1 Protease inhibitors as drugs

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) and immunological, respiratory, neurodegenerative, and cardiovascular disorders. Protease inhibitors thus, have considerable potential utility for therapeutic intervention in a variety of disease states[55, 58]. There are many glaring examples of development of highly successful drugs for the treatm-



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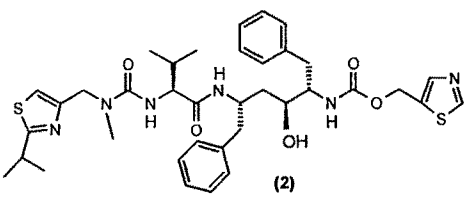

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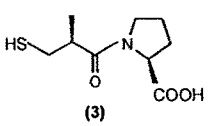

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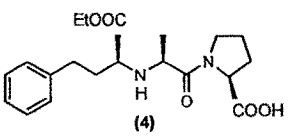


(4)

-ent of diseased conditions based on inhibition of certain proteases. The protease of the human immunodeficiency virus (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 inhibitors of HIV-1 protease (MW < 1000 Da) are now used as drugs, including saquinavir **(1)**, ritonavir **(2)**, 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 (e. g. renin) these compounds were designed and developed by docking these structures on the active site of HIV-1 protease[59]. Captopril **(3)** and enalapril **(4)** are some examples, of inhibitors of a metalloprotease, angiotensin-converting enzyme (ACE), used successfully for the control of hypertension[60].

1.4.2 Cysteine proteases and related aspects

Cysteine (thiol) proteases[57] exist in three structurally distinct classes, which are papain-like (e.g. cathepsins), ICE-like (caspases), or 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 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 inhibitors developed to date tend to be 2-4 amino acids or their equivalent in 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, that they universally bind their inhibitors/substrates in extended or β-strand conformations; that is, the peptide backbone or equivalent

is drawn out in a linear arrangement[61]. This common conformational requirement for recognition by proteases suggests *new efforts to develop conformationally restricted inhibitors that adopt receptor-binding conformations and thus are entropically advantaged for binding to a protease.* On the other hand, most of the many thousands of protease inhibitors that have been developed to date are relatively flexible molecules that have to use energy to rearrange into a protease-binding conformation. A possible trend in the development of more selective and potent protease inhibitors may be the use of more conformationally restricted molecules that are fixed in the protease-binding conformation.

Most proteases are sequence-specific, the size and hydrophobicity/hydrophilicity of enzyme sites defining possible binding amino acid side chains of polypeptide substrates. The standard nomenclature[62] used to

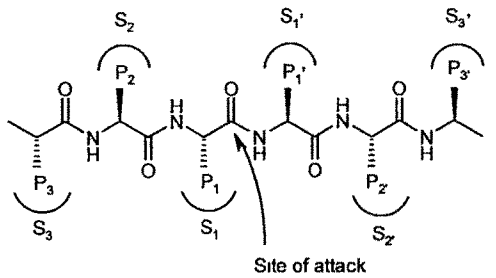


Figure 3: Standard nomenclature for substrate residues and their corresponding binding sites in the enzyme, protease.

designate protein substrate/inhibitor residues (e.g. P_3, P_2, P_1, $P_{1'}$, $P_{2'}$, $P_{3'}$) that bind to corresponding enzyme subsites (S_3, S_2, S_1, $S_{1'}$, $S_{2'}$, $S_{3'}$) is shown in Figure 3. Some common examples of proteases are described below:

1. Cathepsin K
2. Cathepsin B
3. Cathepsin L
4. Cathepsin S
5. Caspases
6. Calpains
7. Falcipain
8. Rhinovirus 3C protease
9. Cruzain

1.4.3 Cysteine protease inhibitors

Most of the inhibitors presented in the coming sections have evolved following the usual structural scheme for development of protease inhibitors. This comprises a peptide segment, for recognition by the enzyme, corresponding to the sequence of a good substrate i.e. it must contain two or more amino acids to achieve good affinity, at least for endopeptidases. The peptide segment is then bound to a nucleophilic attackable/substitutable group which can react with the cysteine residue of the active site.

1.4.3.1 Aldehydes

Development of peptidyl aldehydes as inhibitors of cysteine proteases is based on two independent research strategies: (1) The assumption that a tetrahedral intermediate is involved in enzymatic hydrolysis has led to investigation of the effect of carbonyl compounds on this protease[63] and (2) During screening of culture filtrates of different *Streptomyces* strains, a number of peptidyl aldehydes like leupeptin **(5)** (IC_{50} (papain) = 0.5 μM, IC_{50} (Cathepsin B) = 0.4 μM) and elastinal were isolated with inhibiting activity towards cysteine proteases. Peptidyl aldehydes are not selective inhibitors *per se* and are reversible inhibitors despite binding covalently to the enzyme.

A tetrahedral hemithioacetal (Figure 4), formation has been shown by NMR[64] studies, through nucleophilic attack of the thiolate anion. A leupeptin analogue Cbz-Leu-Leu-Leu-CHO **(6)** is a potent inhibitor of cathepsin K (Ki_{app}=1.4 nM), inhibits parathyroid hormone (PTH) -stimulated resorption, and,

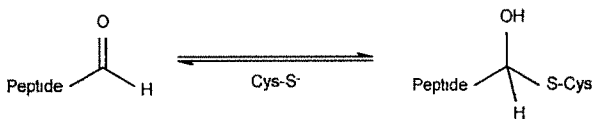
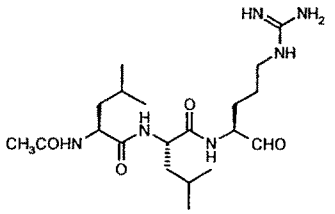


Figure 4: Inhibition of cysteine proteases by peptidyl aldehydes.

when administered at 30 mg/kg i.p., significantly reduces bone loss and hind paw edema in an adjuvant arthritic rat model[65].

Many dipeptidyl and tripeptidyl aldehydes have been synthesized. The selectivity can be achieved by varying the amino acid sequence. MDL 28170 (Z-Val-Phe-CHO) is a well known inhibitor of calpain. Optimization of this structure



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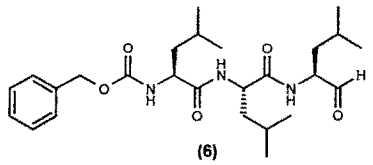

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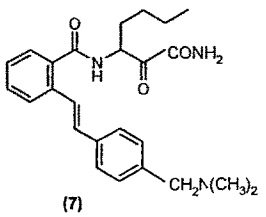

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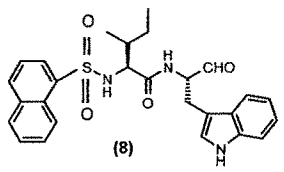
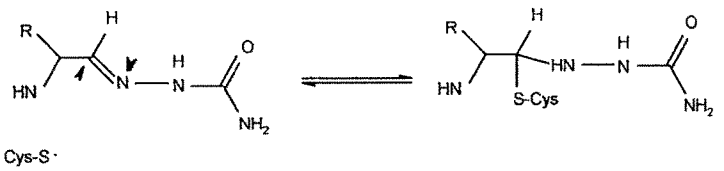


(8)

led to the development of potent calpain inhibitor (7) which exhibited substantially improved pharmacokinetic profiles, in particular water solubility and metabolic stability. It showed significant neuroprotective efficacy even when administered after inducing the injury in rats[66]. A series of peptidic α-acylamino aldehydes have been reported[67] as potent, selective, and reversible inhibitor of cathepsin L [compound (8) IC_{50} = 1.9 nM]. These compounds inhibited the release of Ca^{+2} and hydroxyproline from bone in *in vitro* bone culture system and also prevented bone loss in ovariectomized mice at an oral dose of 50 mg/kg.

1.4.3.2 Peptidyl semicarbazones

Peptidyl semicarbazones have been used as intermediates in the synthesis of aldehydes. It has therefore been shown that they are inhibitors of cysteine

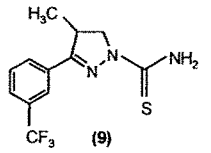


Cys-S·

Figure 5: Inhibition of cysteine proteases by peptidyl semicarbazones.

proteases themselves although, much weaker than the corresponding aldehydes. Inhibition takes place due to formation of a tetrahedral adduct[68] by attack of

thiolate on the protected carbonyl carbon (Figure 5) rather than the regeneration of aldehyde by the hydrolysis of the semicarbazone. This reaction is also revervsible that is why semicarbazones, like aldehydes, are suitable as ligands for purification of cysteine protease by affinity chromatography. Simple tripeptidyl semicarbazone, Z-Arg-Ile-Phe-Sc, inhibit papain (Ki = 23 nM) and cathepsin B (K_I = 500 nM) [68]. A novel series[69] of potent cyclized pyrazoline thiosemicarbazones



(9)

have been reported as inhibitors of *Trypanosoma cruzi* cysteine protease cruzain out of which compound **(9)** is noteworthy (IC_{50}= 40 nM).

1.4.3.3 Methyl Ketones and Trifluoromethyl Ketones

Peptidyl aldehydes inhibitors are rapidly oxidized *in vivo* to a carboxylic acid. For this reason, the aldehyde group was replaced by the metabolically more stable[70] trifluoromethyl ketone function (TFMK). Here also, it has been shown that a tetrahedral hemiketal is formed as covalent enzyme-inhibitor adduct[68]. Methyl ketone and TFMK derivatives, like the aldehydes, are reversible inhibitors of cysteine proteases so that here too, the formation of a tetrahedral hemithioketal is assumed. The low inhibitory activity of these compounds towards cysteine proteases has been suggested to be due to steric hindrance[71]. Simple di- and tripeptide TFMK have been reported as inhibitors of cysteine protease enzyme. Z-Phe-Ala-CF_3 inhibits Cathepsin B (K_I = 300-470 μM) and Z-Val-Phe-CF_3 inhibits α-chymotrypsin[72] (K_I = 2.4 μM).

1.4.3.4 α-Keto Acids, α-Keto Esters, α-Keto Amides, and Diketones

Since the synthesis of TFMK and methyl ketone peptides did not bring about any improvement in inhibitory activity towards cysteine proteases, the TFMK group was replaced by other electron-attracting groups. Many derivatives have been synthesized and tested for their activity towards calpains, cathepsin B, and papain[73] including Z-Phe-Gly-COOH, which inhibits Cathepsin B (K_I = 2 μM), Ac-Phe-Gly-$COOCH_3$ which inhibits papain (K_I = 2 μM) and Z-Phe-Gly-

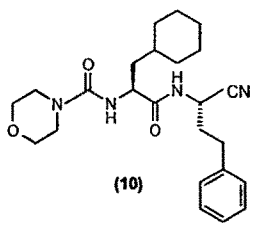
$CO_2CH_2CO_2Et$ which inhibits both papain (K_I = 1 μM) and cathepsin B (K_I = 0.2 μM).

1.4.3.5 Nitriles

Peptidyl nitriles are known to be inhibitors of cysteine proteases and were first reported by Hanzlik as inhibitors of the plant protease, papain[74]. These

Figure 6: Inhibition by peptidyl nitriles.

peptide nitriles were shown by NMR to form covalent bonds[75] with the active site cysteine, and this bond formation was shown to be reversible (Figure 6). Recently a series of dipeptidyl nitriles[76] have been reported as inhibitors of Cathepsin S. Out of them compound **(10)** is worth mentioning with IC_{50} = 5 nM. Starting with the previously reported nitrile, Z-Phe-NH-CH_2CN (IC_{50} = 62 μM) a series of dipeptidyl nitriles have been reported as Cathepsin B inhibitors with



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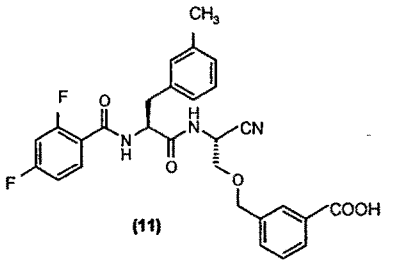


(11)

excellent selectivity[77] over other cysteine cathepsins. Compound **(11)** has shown high selectivity for Cathepsin B (IC_{50} = 6.8 nM) over Cathepsin L (IC_{50} = 554 nM) and Cathepsin S (IC_{50} = 937 nM).

1.4.3.6 Halomethyl Ketones

TPCK [1-(Tosylamino)-2-phenylethyl chloromethyl ketone] has been known as a reagent for affinity labeling of the histidine residue of the chymotrypsin active site for a long time[78]. Cysteine proteases also react with this reagent. X-ray structural analysis of papain-inhibitor adducts show that instead of the histidine, it is the cysteine residue of the active site that is irreversibly

alkylated. There are two possible pathways: (A) the thiolate anion can react directly with the carbon of the chloromethyl group in a nucleophilic substitution;

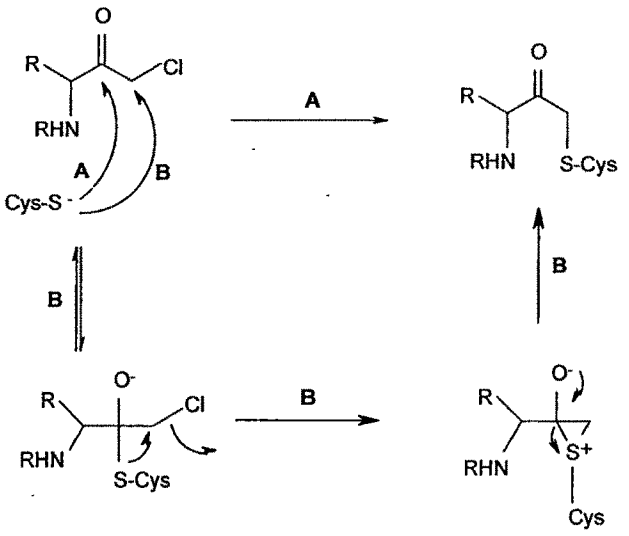


Figure 7: Inhibition by chloromethyl ketones

(B) the sulfur attacks the carbonyl carbon and the product is formed via a tetrahedral intermediate and a sulfenium ion in a 3-step reaction (Figure 7). This leads to the development of halomethyl ketones as cysteine protease inhibitors. Many peptidyl choromethanes have been synthesized and a certain amount of selectivity can be achieved within the group of cysteine proteases by variation of the P_1 and P_2 amino acids. For example Z-Leu-Tyr-CH_2F is selective for cathepsin B[78] (second order rate constant = 640 M^{-1} s^{-1}) over Cathepsin L (second order rate constant = 12000 M^{-1} s^{-1}). A disadvantage of the chloromethyl ketones is their high reactivity and therefore lack of selectivity. Not only do they react with serine proteases, but also with other SH-containing molecules, such as glutathione or nonproteolytic enzymes, which means that they are not suitable for *in vivo* applications as they would be too toxic[79]. This led to the development of monofluoromethyl ketone derivatives in which replacement of chlorine with fluorine could have the potential for lower reactivity during alkylation.

1.4.3.7 Diazomethanes

The development of diazomethanes is based on the observation that the antibiotic azaserine[80] inhibits cellular growth by alkylation of a thiol group on the

amidotransferase involved in purine synthesis. At the same time it was known that diazomethyl ketones used in synthesis of chloromethyl ketones have no activity toward serine proteases. Thus, diazomethyl ketones could be suitable as potential selective inhibitors of cysteine proteases. Z-Phe-CHN_2 was the first compound discovered[81] as active and irreversible inhibitor of papain. The fact that serine proteases are not inhibited by these substances and they do not react with simple thiols like mercaptoethanol or dithiothreitol led to synthesis of a multitude of derivatives, some of which have noteworthy selectivity within the

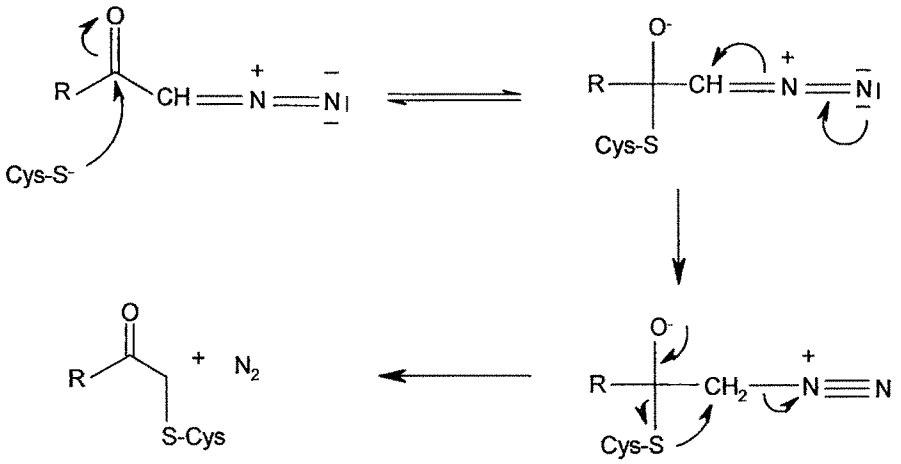


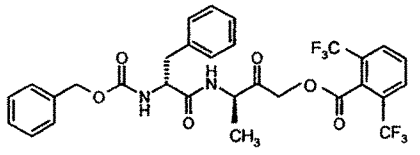
Figure 8: Inhibition by diazomethyl ketones.

cysteine protease group, for example Z-Phe-Ala-CHN_2 is selective[82] for Cathepsin H (second order rate constant = 0.6 M^{-1} s^{-1}) over Cathepsin L (second order rate constant =620 000 M^{-1} s^{-1})and Cathepsin B (second order rate constant = 500-630 M^{-1} s^{-1}) . The exact mechanism of their inhibition is not known at present. It is thought that the carbonyl carbon undergoes nucleophilic attack by the thiolate to form a hemithioketal. The diazomethyl carbon is subsequently protonated by the imidazolium ion of the histidine, the rate-determining step of the reaction, and the thioether end product is formed via a three-membered transition state with concomitant cleavage of nitrogen[79] (Figure 8).

1.4.3.8 Acyloxymethyl Ketones

The peptidyl acyloxymethyl ketones are another group of inhibitors whose development was based on a concept similar to that of the peptidyl

monofluoro ketones[83]. Reduced chemical activity is achieved by a space-filling leaving group which is only weakly active in SN_2 reactions. Wide variation of the structure is possible via both the peptide sequence and the structure of the leaving group. Thus, both selectivity for cysteine proteases, corresponding to the S1'subsites, and reactivity can be controlled. On the basis of NMR spectroscopic comparisons with peptidyl chloromethyl ketones, it is likely that the same type



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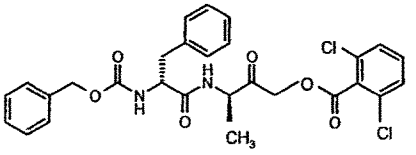

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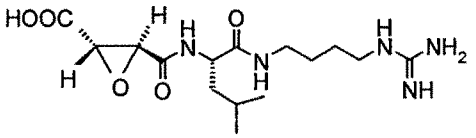
of enzyme-inhibitor adduct is formed[83]. Among the reported inhibitors[84] includes compound **(12)** (Cathepsin B k_{inact}/K_{inact} = 1,600,000 M^{-1} s^{-1}) and **(13)** (Cathepsin S k_{inact}/K_{inact} = 686,000 M^{-1} s^{-1}).

1.4.3.9 Epoxysuccinyl Derivatives

In 1978, Hanada *et al* succeeded in isolating a highly active, irreversible inhibitor of papain from culture extract[85] of *Aspergillus japonicus*. The substance



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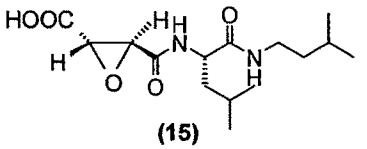

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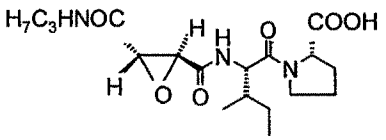

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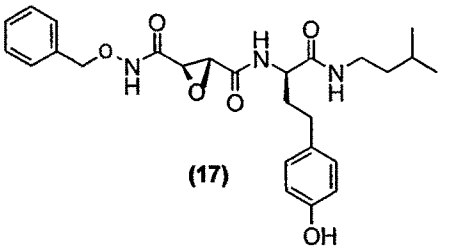

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was identified as 1-[(*N*-(*L*-3-*trans*-carboxyoxiran-2-carbonyl)-*L*-leucyl)amino]-4-guanidinobutane, E-64 **(14)**. Systematic studies were carried out to investigate the role of the different structural components of the inhibitor in enzyme inhibition and the *trans*-*L*-(*S*, *S*)-epoxysuccinic acid was discovered to be the reactive group essential for inhibition[86]. A change of configuration of the epoxide residue or the

neighbouring amino acids reduces the activity by a factor of 10-100. In contrast to other microbial inhibitors, epoxysuccinyl peptides inhibit only cysteine proteases[85]. Similarly, simple thiols do not react with these substances, at least, in physiological conditions.

NMR spectroscopic investigations[87] show that the active-site thiolate attacks at C-3 of the oxirane ring (Figure 9) and the epoxide ring is opened with

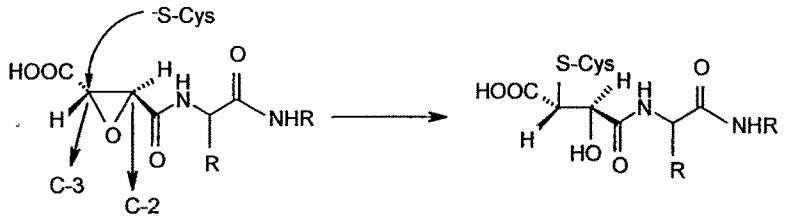


Figure 9: Inhibition by epoxysuccinyl derivatives.

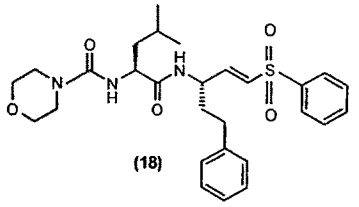
inversion of the configuration at C-3. More selective derivatives of E64 **(14)** have been developed such as E64c **(15)** (k_{second} = 8.7x10^3 M^{-1} s^{-1}) and CA-074 **(16)**, a selective inhibitor of cathepsin B.

CA-074 **(16)** is the most selective inhibitor of cathepsin B. It was reported to rescue 67 % of monkey hippocampal cornuammonis-1 (CA1) neurons from delayed neuronal death on day 5 after ischemia, when administered i.v. immediately after the ischemic insult[88]. Inhibitor **(15)** in the form of eye drops has also been shown to be effective in the prevention and treatment of cataracts probably by inhibition of calpain. The ethyl ester of E-64c (aloxistatin, loxistatin) was tested in clinical trials in Japan in 1986 as a prodrug for treatment of muscular dystrophy[89], but development was stopped in 1992 in phase III because the efficacy did not fulfill expectations. This inhibitor in the form of eye drops has also been shown to be effective in the prevention and treatment of cataracts probably by inhibition of calpain[90]. A D-homophenylalanine epoxysuccinate derivative **(17)** has been reported[91] as cruzain inhibitor (IC_{50} = 10 nM).

1.4.3.10 Peptidyl vinyl sulfones

The design of peptidyl vinyl sulfone template was based on the hydrogen bond acceptor capability and the polarizable nature of the vinyl

sulfone moiety. This group, tethered to an appropriate peptidyl recognition sequence, has served as an effective irreversible inhibitor of a variety of cysteine proteases. They are inert towards SH-containing molecules, such as glutathione or nonproteolytic enzymes[92]. The prototype vinyl sulfone **(18)** was reported to be



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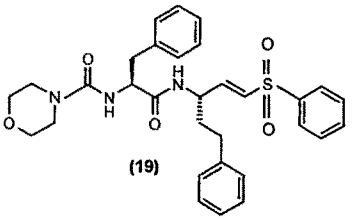

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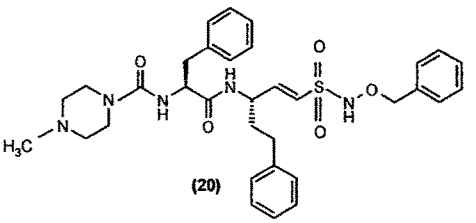


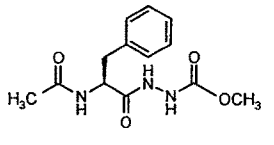
(20)

a potent inhibitor of Cathepsin S (K_i = 0.0059 μM) that is selective[92] over Cathepsins B (K_i = 39 μM) and L (K_i = 0.72 μM). Compound **(18)** is potent inhibitor of cruzain (K_i = 0.22 μM). Vinyl sulfone **(19)**, which incorporates a P_2 phenylalanine, is an effective inhibitor of cruzain (K_i = 0.36 μM) and cured *T.cruzi* infected macrophages at a concentration of 20 μM[93]. Evaluation of **(19)** in a mouse model of Chagas' disease showed that mice treated with **(19)** survived lethal infections with *T.cruzi* for the duration of the experiment (14-16 days after infection) while untreated mice died within 4-5 days. A second generation N-alkoxy vinylsulfonamides **(20)** has been reported[94] as effective inhibitor of cruzain, with significantly higher activity (second order inactivation constant = 6,480,000 M^{-1} s^{-1}) in tissue culture experiments over simple vinyl sulfonamide **(18)** (second order inactivation constant = 181,000 M^{-1} s^{-1}).

1.4.3.11 Aza-peptides

A series of aza-peptide amides, which utilize both primed and unprimed side binding elements, are irreversible inhibitors of papain. These pseudo-substrates were designed so that, upon attack of the active site cysteine, they would produce a thioacyl-enzyme intermediate which would hydrolyze slowly

relative to the parent peptide substrate. The incorporation of amino acid functionality, which acts as a leaving group and accesses the S1' leaving group binding pocket, was seen to increase the potency of these inhibitors. The rate of



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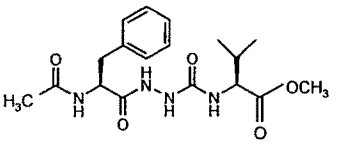

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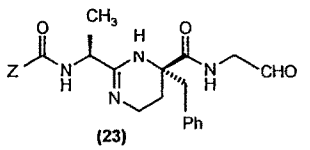
papain inactivation by the aza-peptide **(21)** was reported to have a k_{on} = 18 $M^{-1}s^{-1}$. Analog **(22)**, which incorporates a leaving group capable of interacting with the S1' binding pocket, inactivates papain at a rate 17 times that of **(21)**. The thioacyl enzyme intermediate formed between papain and **(22)** hydrolysed with a $t_{1/2}$ = 12 hours making these inhibitors essentially irreversible[95].

1.4.3.12 Inhibitors with other functionalities

In this section, some inhibitor structures will be discussed which have been developed just recently or for which only one single example has been



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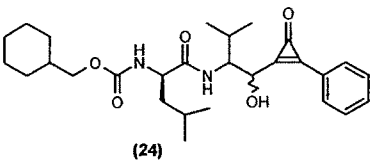

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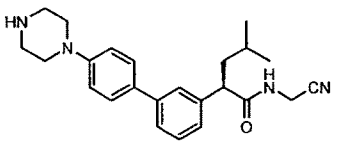


(25)

known. Conformationally constrained tetrahydropyrimidine[96] inhibitors **(23)** have been reported as competitive inhibitor of papain with K_I = 790 nM. The cyclopropenone derivative **(24)** bound to a peptide unit is a competitive inhibitor of papain[97]. The substance has no effect at concentrations of 100 μM on the serine proteases thrombin, cathepsin G and cathepsin D, and could be a representative of a new selective and potent class of cysteine protease inhibitors. A novel series of nonpeptidic biaryls[98] were reported as potent and reversible inhibitors of cathepsin K. Compound **(25)** is a potent inhibitor of cathepsin K (IC_{50} = 3 nM)

having selectivity over cathepsin B (IC_{50} = 3950 nM), cathepsin L (IC_{50} = 3725 nM) and cathepsin S (IC_{50} = 2010 nM). Unlike some peptidic nitrile inhibitors, the nitrile moiety of **(25)** is not converted to corresponding amide.

1.4.4 Malarial proteases: new targets for chemotherapy

The limitations of antimalarial chemotherapy as discussed in earlier sections underscore the need for new drugs, ideally directed against new targets. Among promising new targets for antimalarial chemotherapy are the malarial proteases. To date, eight different proteases have been identified in *P. falciparum* that are instrumental in the degradation process of hemoglobin, three cysteine proteases (Falcipain 1-3), three aspartyl proteases (Plasmepsin I, II and IV) one histo-aspartic protease (HAP), and one metalloprotease (falcilysin)[99]. The erythrocytic life cycle, which is responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites. Proteases appear to be required for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasites and for the degradation of hemoglobin by intraerythrocytic trophozoites. Plasmepsin I and II appear to perform the initial cleavage of hemoglobin. Plasmepsin IV and the HAP are only very weakly active on hemoglobin, but rapidly degrade denatured globin. The falcipains and falcilysin are believed to further degrade the peptides produced by the earlier cleavage events and are thus active in a late stage of the process. Although the specific roles of different classes of proteases are not completely clear, inhibitors of cysteine and serine proteases have consistently blocked erythrocyte rupture and invasion[100].

1.4.4.1 Proteases and erythrocyte rupture

The rupture of erythrocytes by mature schizonts and the subsequent invasion of erythrocytes by free merozoites appear to require malarial protease activity, possibly to breach the erythrocyte cytoskeleton, a complex network of proteins. In addition, a number of malarial proteins are proteolytically processed

during the late schizont and merozoite life-cycle stages; for example, merozoite surface protein-1 is processed in such a manner that it is inhibited by serine protease inhibitors[101], presumably, to facilitate the complex series of events involved in erythrocyte rupture and invasion[102].

1.4.4.2 Proteases and Malarial Hemoglobin Degradation

Extensive evidence suggests that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasites, apparently to provide free amino acids for parasite protein synthesis[99]. In *P. falciparum*, hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most active metabolically. Trophozoites ingest erythrocyte cytoplasm and transport it to a large central food vacuole. In the food vacuole, hemoglobin is broken down into heme, a major component of malarial pigment[103], and globin, which is hydrolyzed to its constituent amino acids. The food vacuole is an acidic organelle analogous to lysosomes. Several lysosomal proteases are well characterized, including cysteine (cathepsins B, H, and L) and aspartic (cathepsin D) proteases[104], and malaria parasites contain analogous food vacuole proteases that degrade hemoglobin. At least two aspartic proteases and one cysteine protease have been isolated from purified *P. falciparum* food vacuoles[105].

Malarial aspartic protease activities have been identified[106]. Two aspartic proteases (plasmepsin I and plasmepsin II) are located in the food vacuole, have acid pH optima, and share sequence homology with other aspartic proteases. Furthermore, the aspartic proteases can cleave hemoglobin. One of the enzymes, plasmepsin I, cleaves native hemoglobin[105]. Plasmepsin II appears to prefer denatured globin as a substrate. On the basis of these data, plasmepsin I is thought to be responsible for initial cleavages of hemoglobin after the molecule is transported to the food vacuole[105]. Incubation of cultured *P. falciparum* parasites with the protease inhibitor leupeptin **(5)** caused trophozoite food vacuoles to fill with apparently undegraded erythrocyte cytoplasm[107]. Analysis of the leupeptin-treated parasites showed that they contained large quantities of undegraded globin, while minimal globin was detectable in control parasites. Leupeptin **(5)**

inhibits both cysteine and some serine proteases, but the highly specific cysteine protease inhibitor E-64 **(14)** also caused undegraded globin to accumulate. More recent studies that used nondenaturing electrophoretic methods demonstrated that cysteine protease inhibitors not only blocked malarial globin hydrolysis, but also inhibited earlier steps in hemoglobin degradation, including denaturation of the hemoglobin tetramer and the release of heme from globin[108]. Another study showed that E-64, inhibited the production of hemozoin (the malarial end product of heme) by cultured parasites[109]. These results suggest that a cysteine protease is required for initial steps in hemoglobin degradation by *P. falciparum*. A *P. falciparum* trophozoite cysteine protease with biochemical features expected for a food vacuole hemoglobinase has been identified[110] and biochemically and molecularly characterized[111]. This protease, called falcipain, degraded denatured and native hemoglobin *in vitro*; its acid pH selectivity for optimum activity, substrate specificity, and inhibitor sensitivity indicated that it was a papain family cysteine protease[111]. Specific inhibitors of falcipain blocked hemoglobin degradation and prevented parasite development. The degree of inhibition of falcipain by fluoromethyl ketones and vinyl sulfones correlated with their inhibition of hemoglobin degradation and parasite development, supporting the hypothesis that falcipain is the cysteine protease required for hemoglobin degradation.

The specific mechanism for hemoglobin degradation in the malarial food vacuole still remains unclear. As noted above, both, the aspartic protease plasmepsin I and the cysteine protease falcipain, have been identified in parasite food vacuoles and shown to cleave denatured and native hemoglobin *in vitro*[105]. Results showing that only cysteine protease inhibitors block hemoglobin processing and globin hydrolysis in cultured parasites suggest that falcipain is required for initial steps of hemoglobin degradation[112]. However, other studies have shown that native hemoglobin is cleaved by plasmepsin I, but not by falcipain, in nonreducing conditions that may be present in the food vacuole[107]. In any event, regardless of the exact sequence of hemoglobin processing, multiple enzymes, including at least the three proteases already identified, appear to

participate in the degradation of hemoglobin. These proteases are thus, logical targets for antimalarial drug development.

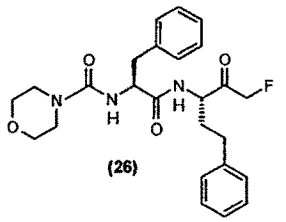
1.4.4.3 Drug Development Efforts for the Inhibition of Malarial Cysteine Proteases

The majority of malarial cysteine protease inhibitors are peptidic or peptidomimetic compounds in which the hydrolysable amide 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" alkylates 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 such as aldehydes, ketoamides and nitriles, which form reversible, covalent bonds to the thiol active site.

Synthetic peptide inhibitor of the *P. falciparum* schizont cysteine protease, Pf 68, inhibited erythrocyte invasion by cultured parasites[113]. 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) [113]. 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[114]. Thus, the 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 Plasmodium falciparum invasion and growth; all compounds were inhibitory with IC_{50} values in the range 7-28 mM, and the growth inhibition correlated well with the extent of membrane curvature changes assessed by transmission electron microscopy. Although these results do not demonstrate 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.

Numerous peptide-based irreversible cysteine protease inhibitors, including fluoromethyl ketones[115] and vinyl sulfones[116], inhibited falcipain at low nanomolar concentrations thereby inhibiting *P. falciparum* development and hemoglobin degradation at concentrations below 100 nanomoles. In a malaria animal model, fluoromethyl ketone **(26)** that inhibited falcipain at low nanomolar concentrations blocked *P. vinckei* protease activity *in vivo* after a single subcutaneous dose, and, when administered for 4 days, cured 80 % of murine



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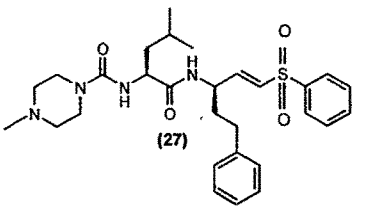

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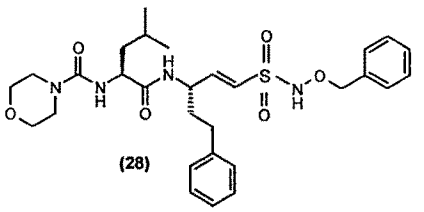

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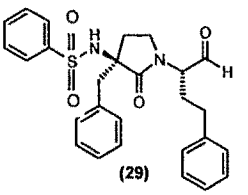

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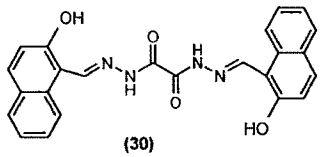
malaria infections. Thus, despite the theoretical limitations of potentially rapid degradation *in vivo* and inhibition of host proteases, peptide protease inhibitors show promise as candidate antimalarial drugs. Fluoromethyl ketones have subsequently shown toxicity in animal studies, but evaluations of related, apparently nontoxic inhibitors of falcipain as antimalarial drugs are under way.

In an attempt to identify nontoxic peptide based cysteine protease inhibitors, a series of vinyl sulfones were synthesized and evaluated. These compounds exhibited strong antimalarial effects. Optimal activity was exhibited by compounds **(18)** and **(27)** in this series. They blocked hemoglobin degradation and development in cultured *P. falciparum* parasites and showed IC_{50} value of 10

nM and 5 nM, respectively. A second generation of vinyl sulfonamides **(28)**, which showed potent inhibition of falcipain, has been developed in order to improve *in vivo* activity and oral bioavailability. Experience with the peptidyl fluromethyl ketones and vinyl sulfones afforded important information about their structure activity relationships. Considering the amino acid sequence, Leu-Hph peptides gave excellent inhibition of falcipain[115,116]. Morpholineurea, N-methylpiperazine and Z groups are all acceptable at amino terminus of falcipain inhibitors.

The γ-lactam or pyrrolidinone isostere has proven to be an effective conformational constraint for inhibitors of other class of proteases[117]. A series of conformationally constrained pyrrolidinone inhibitors attached with electrophilic aldehyde[118] were reported as inhibitors of falcipain. Compound **(29)** exhibited excellent binding affinity (50 nM) for falcipain.

A structure-based design approach was used to identify nonpeptide inhibitors of falcipain[119]. Construction of homology-based structure of falcipain was performed using the known structure of papain and actinidin. Falcipain has 30 % overall homology to these cysteine proteases, but in the active site region is approximately 60% homologous. Screening of potential nonpeptide inhibitors



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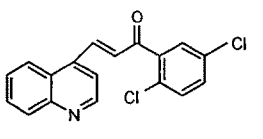

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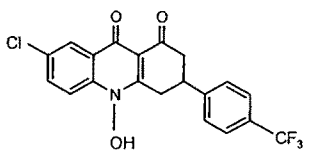
identified a lead compound **(30)** with activity at lower concentrations (IC_{50} = 7 μM). Subsequent synthesis and testing of small molecules based on the structure of the lead compound have identified biologically active falcipain inhibitors, including chalcone **(31)**, that block parasite metabolism at submicromolar concentrations[120] (IC_{50} = 200 nM).

Acridinediones, like compound **(32)**, have been shown to have antimalarial activity, but their mechanism of action remains unknown. From the



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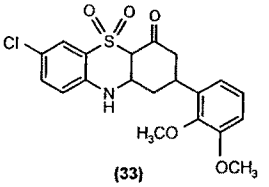

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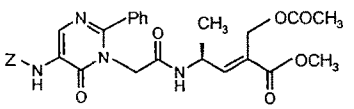
series of chalcones, previously identified as inhibitors of falcipain, a conformationally constrained series was synthesized. This conformationally constrained series utilized a sulfur isostere of the acridindiones to afford the phenothiazine **(33)** that block parasite metabolism and development at low micromolar concentrations [121].

A new class of pyrimidinyl peptidomimetic compounds was reported[122] 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-hyoroxymethyl-but-2-enoate. The most effective compound **(34)** 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*



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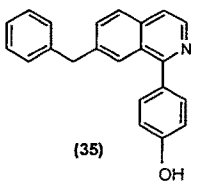
cytotoxicity against neuronal and macrophage cells and less toxicity in colon cell line. Preliminary results indicated that compound **(34)** is active against *P. bergehi*, prolonging the life span of parasite-bearing mice from 6 days for untreated control to 16-24 days for drug-treated animals.

Based on the homology model of falcipain with known vinyl sulfone inhibitors, a series of nonpeptidic isoquinoline[123] inhibitors were developed which exhibited *in vitro* enzyme inhibition at micromolar concentration [compound **(35)** IC_{50} = 8 μM]. The interaction of 1-(4-hydroxyphenyl) group with the Asp-234 in the S_2 pocket (Figure 3) was considered essential for activity.



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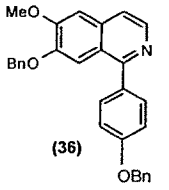

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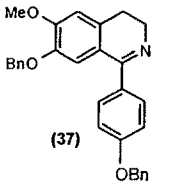

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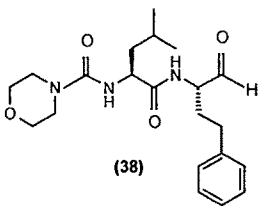
Based on this observation a new series of 1,6,7-trisubstituted isoquinolines [compound **(36)** IC_{50} = 3 μM] and dihydroisoquinoline [compound **(37)** IC_{50} = 4 μM] analogs were developed as falcipain inhibitors[124].

Recently a series of small size peptidyl aldehydes [compound **(38)** IC_{50} (falcipain-2) = 2 nM] and α-ketoamides [compound (**39**) IC_{50} (falcipain-2) = 1 nM] active against falcipain have been reported[125]. They are active against cultured *P. falciparum* parasites, and in murine malaria model. Optimized compounds inhibited falcipain-2 and falcipain-3, blocked hemoglobin hydrolysis, and prevented the development of *P. falciparum,* at nanomolar concentrations. The compounds were equally active against multiple strains of *P. falciparum* with varying sensitivities to standard antimalarial agents. The lead compound morpholinocarbonyl-leucine-homophenylalanine-aldehyde **(38)**, which blocked *P. falciparum* development at low nanomolar concentrations, was tested in a



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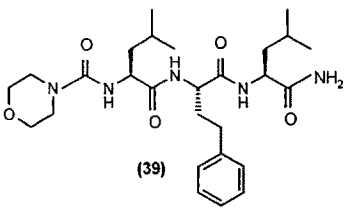

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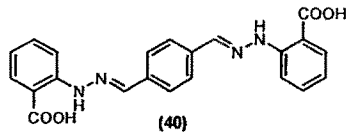
murine *P. vinckei* model. When infused continuously at a rate of 30 mg/kg of body weight/day, the compound delayed the progression of malaria but did not eradicate infections.

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[126] as novel drugs. 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



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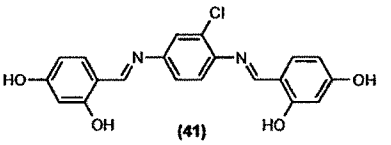

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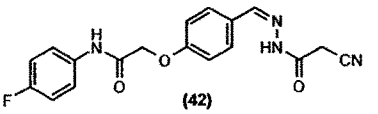

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of which 12 compounds appeared to be dual inhibitors of falcipain-2 and falcipain-3, e. g. compounds **(40)** and **(41)**. Some of them showed preferential selectivity **(42)** for falcipain-2 over falcipain-3.

Aspartic and cysteine proteases act synergistically to degrade hemoglobin *in vitro*[127] thereby inhibitors of these enzymes would possess synergistic effects in inhibiting the growth of cultured parasites. Therefore, the combination 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. Ultimately, a better understanding of the biochemical properties and biological roles played by malarial proteases, will foster the development of protease inhibitors that would specifically inhibit parasite enzymes and thus might prove to be the most suitable candidates for chemotherapy of malaria.