1.1- Presenting Malaria

1.1.1-What is Malaria?

Malaria is a serious disease, with staggering human dimensions: it is by far the most devastating and deadly parasitic disease in the world. It has been a deadly enemy of humankind for thousands of years. Although an ancient disease, the failure of drugs once used to control it has made death by malaria more frequent now than at any point in history. It remains a major public health problem in more than 90 countries, inhabited by more than 2.4 billion people- 40% of the world's population[1]. The disease is estimated to kill a child every 30 seconds and to cause up to half a billion episodes of clinical *Plasmodium falciparum* malaria[2] and 2.7 million deaths worldwide annually[3]!

Malaria is a parasitic disease and is transmitted when a person is bitten by an infected mosquito. Only *Anopheles* female infected mosquitoes transmit malaria and to do so the mosquito must have been infected by having drawn blood from a person infected with malaria. There are four malaria parasite species that produce human disease: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malaria* and *Plasmodium ovale*. Of these, *P. falciparum* is the most lethal strain, if not prompt and adequately diagnosed and treated.

The key factor contributing to the continued inability to control this disease is the increasing resistance of malaria parasites to available drugs [4].

1.1.2- Where do we find Malaria? The Actual Situation Scale

Malaria occurs in many locations of the poor tropical world and also in some locations of the subtropics: Sub-Saharan Africa, Asia and Oceania and Latin America.

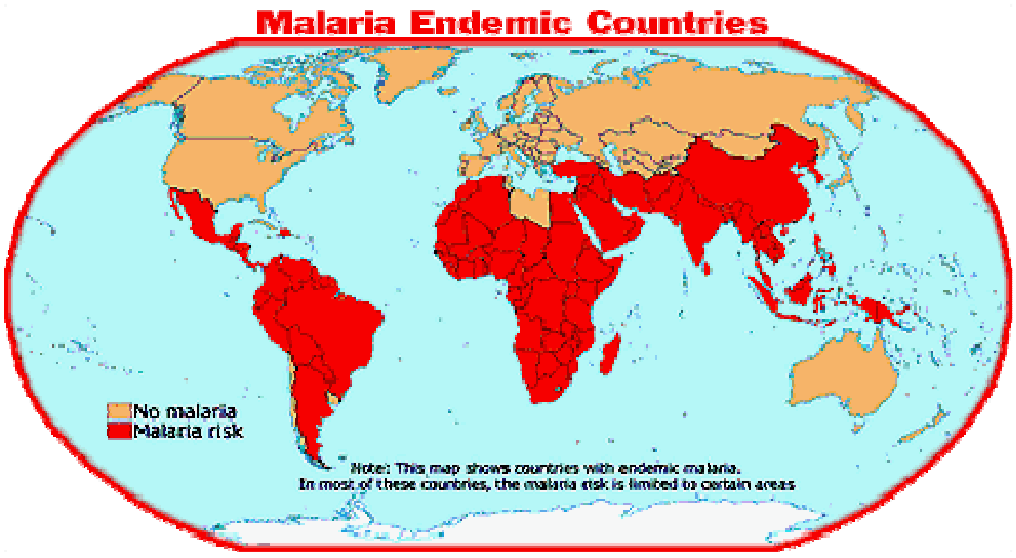


Figure 1. Actual Global Distribution of Malaria

Ninety per cent of the world's malaria cases occur in Africa, where *P. falciparum*, the most dangerous of the four species of human malaria, is predominant. It is also widespread in many countries of Asia, like India, Sri Lanka, Thailand, Indonesia, where *P. falciparum* infections have increased due to resistance of the parasite to used drugs, like chloroquine. In Central and South America, the most common species is *P. vivax*, although there is also an increase in *P. falciparum* cases. Even in Europe, there is malaria in Turkey, Armenia and Azerbaijan, caused by *Plasmodia vivax*, *malarie* and *ovale,* who have worldwide patchy distributions. North America, Europe, Australia and Mexico have controlled and overcome the disease in the past fifty years.

1.1.3- How? Burden of Disease: the Symptoms

- The misery starts with a seemingly innocent mosquito bite…

The parasites are injected into the human along with the mosquito saliva. Once in the human bloodstream, the parasites arrive in the liver and penetrate hepatocytes, where they remain for 9-16 days, multiplying within the cells. On release, they return to the blood and penetrate red blood cells and the clinical symptoms begin.

Malaria produces fever (the temperature may rise above 41ºC, over several days) of different frequency, depending on the causing parasite. Headache, fever relapses, aches, pain all over the body, diarrhoea and abdominal pain are often present. Spleen and liver are easily palpable on clinical examination. In case of severe and complicated malaria (usually caused by delay in treating an uncomplicated attack of *P. falciparum*), impaired consciousness, weakness, anaemia and jaundice (because of the loss of red blood cells) will be present. Other complications are cerebral malaria (unrousable coma), generalised convulsions, renal failure, hypoglycaemia, fluid disturbances, pulmonary oedema, circulatory collapse, shock, disseminated intravascular coagulation and others. These symptoms may occur singly or in combinations.

- Host response to infection with malaria parasites

Initial infections with *P. falciparum* are commonly fatal, but patients who survive develop some immunity. However this response is incomplete, strain-specific, short-lived and only maintained by frequent reinfection. Responses to infection are also affected by genetic factors. Overall, the process is extremely complex[5].

1.1.4- Since when?

- Going back in time…

From the Italian for "bad air," mal'aria has probably influenced to a great extent human population and human history. Malaria parasites have been with us since the dawn of time. They probably originated in Africa where fossils of mosquitoes up to 30 million years have been found. Malaria or a disease resembling malaria has been noted for more than 4,000 years. First records of the symptoms of malaria date back to 2700 BC, described by ancient Chinese medical writings, edited by Emperor Huang Ti. Malaria became widely recognized in Greece by the 4th century BC, being responsible for the decline of many city-state populations. Hippocrates was the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived - before this, the supernatural was blamed. The association with stagnant waters (breeding grounds for Anopheles) led the Romans to begin drainage programs, the first intervention against malaria.

In China, during the 2nd century BC, the Qinghao plant (*Artemisia annua L*), known in the West as the annual or sweet wormwood tree, was described in a medical treatise, due to its anti-fever properties. The active ingredient of Qinghao was isolated by Chinese scientists in 1971. Known as artemisinin, it is today a very potent and effective antimalarial drug, especially in combination with other medicines. On the opposite side of the world, in the early 17th century[6], following their arrival in the New World, the Spanish learned of a medicine used for the treatment of fevers. Spanish Jesuit missionaries in South America learned of a medicinal bark from indigenous Indian tribes. With this bark, the Countess of Chinchón, the wife of the Viceroy of Peru, was

cured of her fever. The tree was then named Cinchona after the countess. The medicine from the bark is now known as the antimalarial, quinine. Quinine remains an important and effective treatment today, although some quinine resistance has been observed.

Not until 1889 was the protozoal cause of malaria elicited by Laveran, a French army surgeon working in Algeria, and only in 1897 was the Anopheles mosquito demonstrated to be the vector for the disease. At this point the major features of the epidemiology of malaria seemed clear, and control measures started to be implemented. Eradication of the vector, the mosquito, by dichloro-diphenyl-trichloroethane (DDT), in the forties, was one of the attempts, but despite success, there was a complete failure to eradicate malaria from Sub-Saharian Africa. Also, strong environmental issues were raised and this strategy abandoned[7].

History tells us that many of our past breakthroughs in malaria control were driven by the needs of the military and of the colonial powers; lets have hope that the present urgency in finding efficient ways to control malaria doesn't have to rely on wars or colonialism…

- At present times

A number of antimalarial drugs are available, but their use is limited by high cost, toxicity and increasing parasite resistance. Most likely, the successful control of the disease will require advances in many areas, in a joint effort, including the control of the mosquito vectors, the development of effective vaccines together with the optimal use of available drugs and the development of new approaches to antimalarial chemotherapy. Also, the recently sequenced genomes of *P. falciparum* and anopheles mosquito bring a new hope into the fight against malaria[8].

1.1.5- The Malaria Cycle Trap: Man, Mosquito and Parasite

The *Plasmodium* genuses of protozoal parasites (mainly *Plasmodia falciparum*, *vivax*, *ovale*, and *malariae*) have a life cycle which is split between a vertebrate host and an insect vector. The *Plasmodium* species, with the exception of *P. malariae* (which may affect the higher primates) are exclusively parasites of man. The mosquito is always the vector, and is always an Anopheline female mosquito, although, out of the 380 species of Anopheline mosquito, only 60 can transmit malaria.

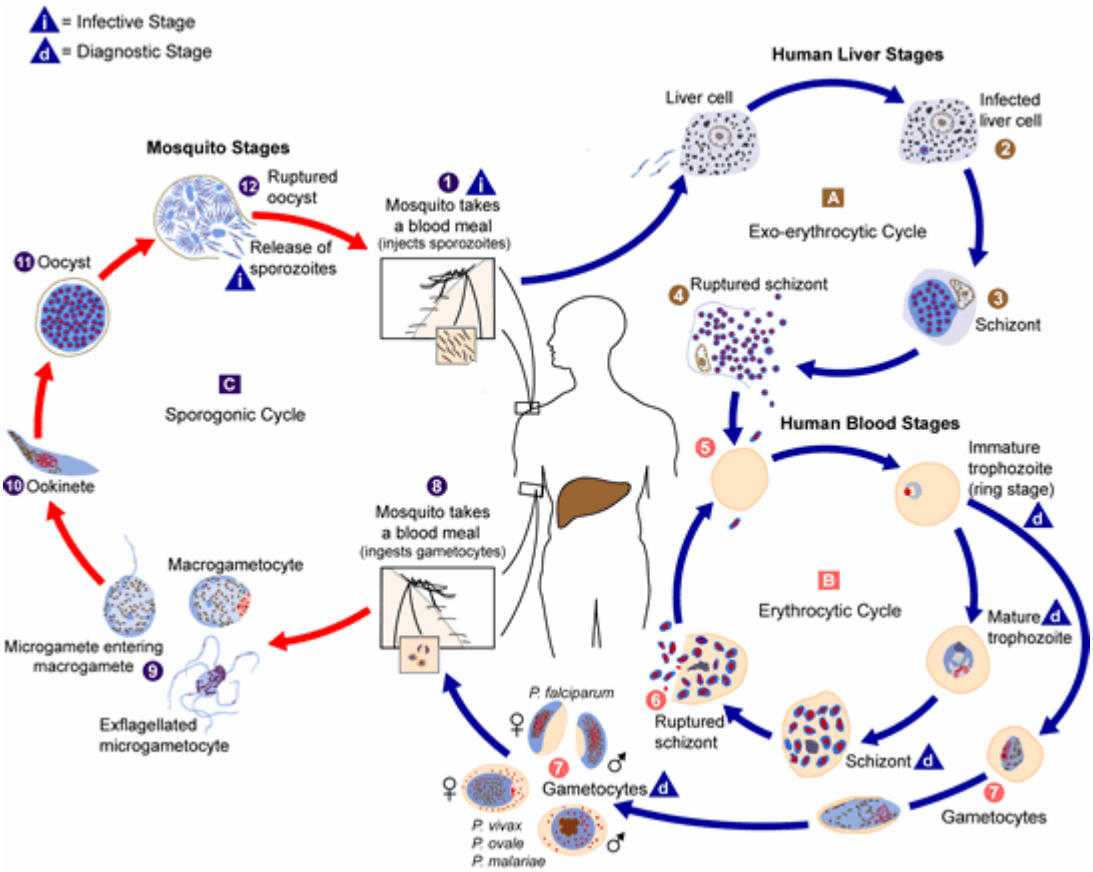


Figure 2. The basic life cycle of the parasite[9]

During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host(1). Sporozoites infect liver cells (2)and mature into schizonts(3), which rupture and release merozoites(4). (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony(A)), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony(B)). Merozoites infect red blood cells(5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites(6). Some parasites differentiate into sexual erythrocytic stages (gametocytes)(7). Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an Anopheles mosquito during a blood meal(8). The parasites' multiplication in the mosquito is known as the sporogonic cycle(C). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes(9). The zygotes in turn become motile and elongated (ookinetes) (10)which invade the midgut wall of the mosquito where they develop into oocysts(11). The oocysts grow, rupture, and release sporozoites(12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites (1)into a new human host perpetuates the malaria life cycle.

1.2- Fighting Malaria

1.2.1- Concerns and Hopes…

It is a fact that the resistance of malaria parasites to available drugs continues to grow, increasingly limiting the ability to control the disease. Chloroquine and sulfadoxine-pyrimethamine, which for several decades were the foundation for malaria therapy, have now their clinical usefulness eroded, due to the emergence and consolidation of resistance to these drugs. Quinine and mefloquine also show a few focus of resistance. It is worth noting that in addition to inadequate drug efficacy, new therapies may fail because of other factors, such as inappropriate use, inadequate absorption, poor adherence, intolerability, the use of counterfeit drugs or improper manufacture of drugs, or prohibitive cost[10].

On the positive side, it is reassuring that many new approaches to antimalarial drug discovery are under development or evaluation, which suggests that, given the adequate support, there will be valuable new strategies to fight malaria in the near future! And also, the parasite's entire genome has been released, which can dramatically accelerate the early steps of drug discovery by identifying new plasmodial targets. However, this doesn't change the fact that high-quality biological studies are needed firstly in order to validate such drug targets.

1.2.2 – The Present and the Future Within the Parasite

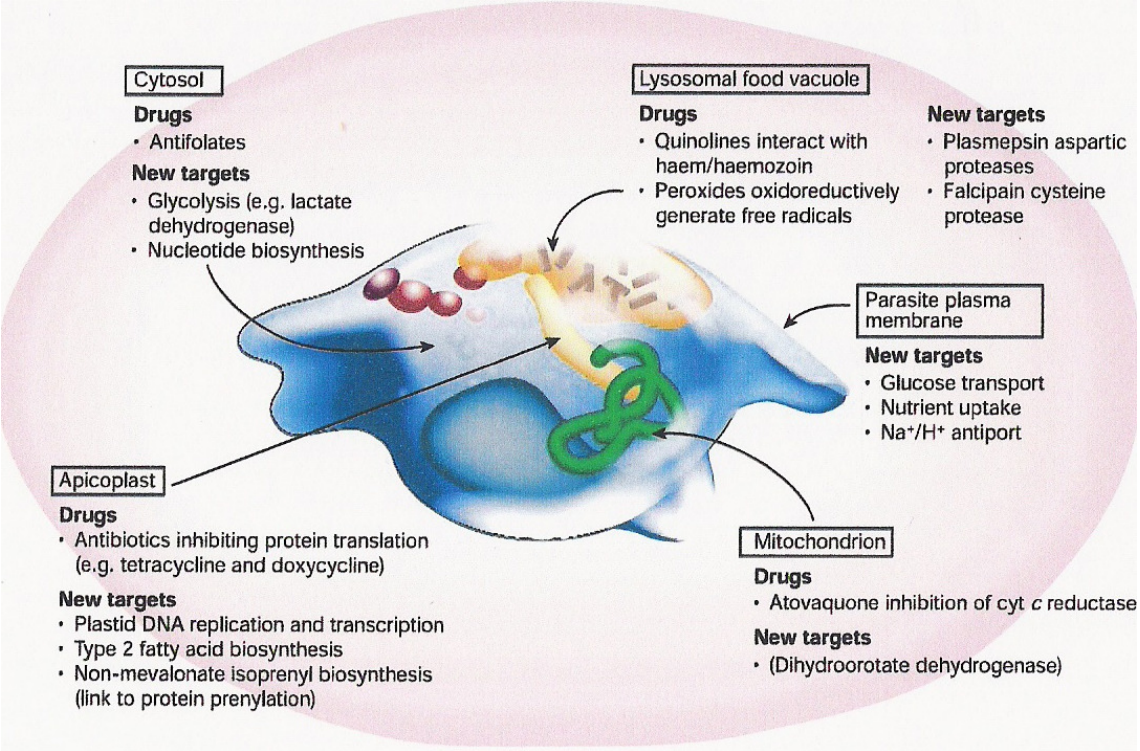


Figure 11. A *P. falciparum* trophozoite residing in an erythrocyte (drug targets are highlighted[11]).

The malaria parasite offers a variety of possible drug targets associated with its various organelles: cytosol, apicoplast, mitochondrian, parasite plasma membrane and lysosomal food vacuole. Traditionally-seen essential surviving processes to the parasite have been targeted as well as new ones, which have been spotted from more recent studies.

1.2.3- Established Antimalarial Drugs and Parasite Resistance

Some antimalarials have been traditionally used since centuries ago, proved its efficacy and also failures (in Figure 3, the structures of some well-known antimalarials are presented).

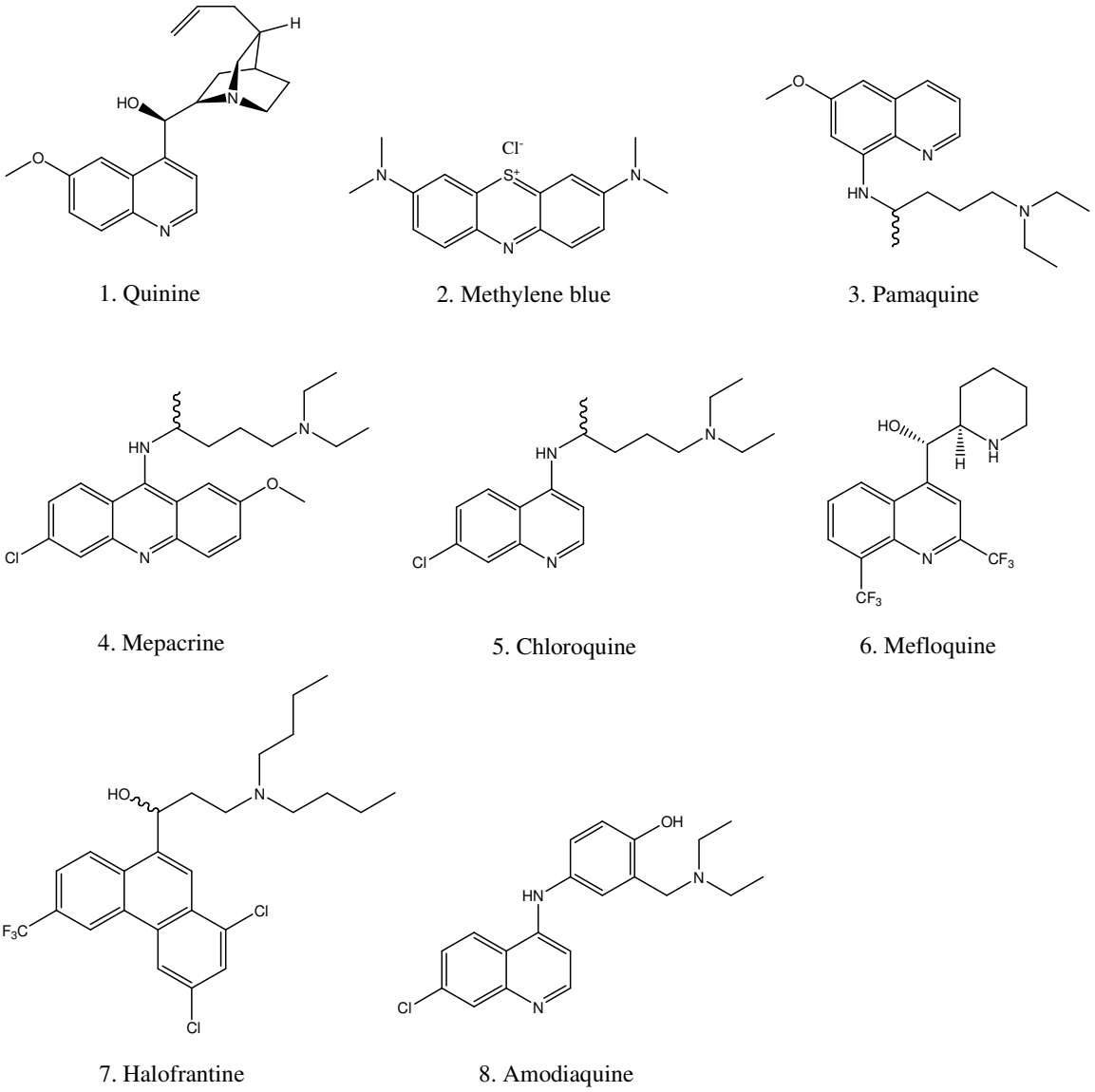
In 1820, **quinine** (1, Figure 3) was isolated as the active ingredient and successively replaced the crude bark for the treatment of malaria. Thus, malaria was among the first diseases to be treated with a pure chemical compound. Quinine is still used in the treatment of multiresistent malaria, despite its low efficacy and tolerability.

By the end of the 19th century, a synthetic drug, **methylene blue** (2, Figure 3), was for the first time used in humans, based on the fact that it was observed to be selectively taken up by the parasite in microscopic specimens[12]. Today it is known that this drug inhibits glutathione reductase thereby disturbing the redox homoeostasis of the parasite [13,14]. By modifying the methylene blue structure, **pamaquine** (3, Figure 3), an 8-amino-quinoline, and **mepacrine** (4, Figure 4) were developed. Both drugs were used extensively in World War II, especially in the Southwest Pacific.

Research to discover a substitute for quinine led to the synthesis in 1934 of **chloroquine** (CQ, 5, Figure 3), a synthetic 4-aminoquinoline, highly effective against parasites and well-tolerated by humans. Since then, chloroquine turned out to be the most effective and important antimalarial ever and became the drug of choice. Possibly due to its uncontrolled use, the first cases of chloroquine resistance appeared at the end of the 1950s. Today, chloroquine-resistant strains of *P. falciparum* (and to some extent of *P. vivax*) are common in all endemic areas throughout the world.

During the Vietnam War, the development of new antimalarial drugs was encouraged by the American military and resulted in the development of **mefloquine** (6, Figure 3) and **halofantrine** (7, Figure 3), aryl amino alcohols similar to quinine. In ten years time resistance also began to appear along with evidence of some strong side effects [15,16]. **Amodiaquine** (8, Figure 3), another 4-aminoquinoline, has been used since the 1980s, but with limitations due to adverse effects. Cost and side effects are then the main draw-backs with these effective drugs against chloroquine resistant parasites.

The parasite ingests haemoglobin from the host erythrocyte; from haemoglobin proteolysis, there is release of the heme moiety, which can be potentially toxic to the parasite. To avoid this, the parasite converts it to hemozoin or the so-called malaria pigment through oxidative processes, or destroys it through the existing reduced glutathione in the parasite's cytoplasm. There is convincing data that these 4-amino quinolines interfere with the detoxification of free heme[16,17].Whether this is also the case for the aryl amino alcohols is less clear[18].

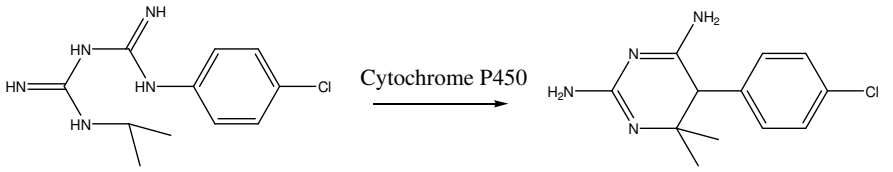


1. Quinine
2. Methylene blue
3. Pamaquine
4. Mepacrine
5. Chloroquine
6. Mefloquine
7. Halofrantine
8. Amodiaquine

Figure 3. Established 4-aminoquinolines and aryl amino alcohols of historical and current importance.

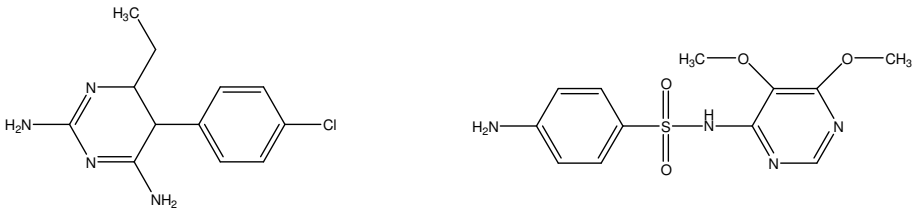
One hypothesis to explain CQ-resistance, and probably the resistance shown by other amino-quinolines is the observed small decrease in the vacuolar pH of resistant parasites, leaving significantly less free heme available for the formation of toxic complexes with CQ, thus reducing CQ accumulation[19].

During World War II, British researchers developed **proguanil** (9, Figure 4), a pyrimidine derivative, which in turn served as a lead compound for the development of **pyrimethamine** (11, Figure 4) in 1950. Pyrimethamine has been used in a synergistic combination with **sulfadoxine** (12, Figure 4), a sulphonamide. Proguanil, precursor of cycloguanil (10, Figure 4), the bioactive cyclic derivative, is more commonly applied in combination with chloroquine (5, Figure 3)[15]. These drugs are called antifolates because they interfere with the parasite's folate mechanism, an essential enzymatic pathway. From these enzymes, dihydropteroate synthase and dihydrofolate reductase are well studied and characterized as is the mechanism of action of antifolate inhibitors. Unfortunately parasite resistance arose extremely rapid in settings where these drugs were widely used.



9. Proguanil

10. Cycloguanil



11. Pyrimethamine

12. Sulfadoxine

Figure 4. Some Antifolate Inhibitors

A completely different group of drugs was found when the sesquiterpene **Artemisinin** (13, Figure 5) was isolated in 1972 from *Artemisia annua*, better known to Chinese herbalists for more than 2000 years as Qinghao. Artemisinin showed potent antimalarial

activity[20]. The most commonly used semisynthetic derivatives are water-soluble **artesunate** (17, Figure 5), the oil-soluble **artemether** (15, Figure 5) and **arteether** (16, Figure 5). These drugs are metabolized to **dihydroartemisinin** (14, Figure 5), which appears to be the main bioactive form of the drugs.

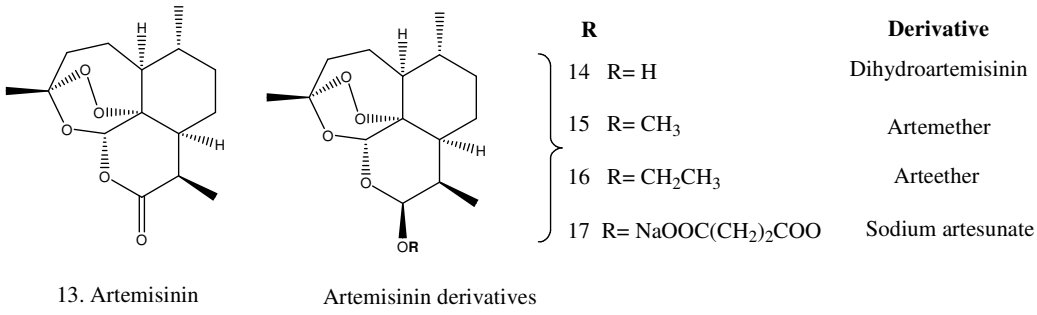
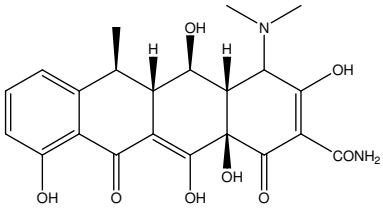


Figure 5. Structures of artemisinin and derivatives

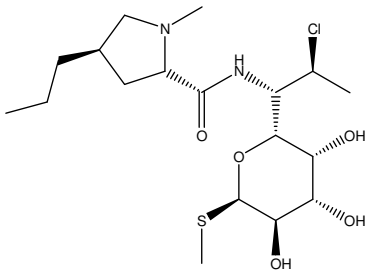
There are a number of pieces of evidence that the killing action of artemisinin depends on the cleavage of the endoperoxide bridge, after contact with the abundant heme-iron II in the parasite's food vacuole derived from the breaking down of host cell haemoglobin. The artemisinin free-derived radicals formed can then alkylate the heme molecule, thus inhibiting detoxification of free heme[21] and also alkylate essential parasite proteins[22].

At present, artemisinin derivatives are routinely administered for the treatment of malaria, in particular in Southeast Asia, with no reported cases of adverse reactions or parasite's resistance. Artesunate (17, Figure 5) is administrated in combination with mefloquine (6, Figure 3)[11], and artemether (15, Figure 5) with benflumetol (lumefantrine, 20, Figure 7); this happens because, though very effective, artemisinin derivatives have short half-lives (parasite clearance times are shorter than with any other antimalarial[23]), and coexistence of parasite multiple stages in the host could lead to recrudescence, unless there is a longer half-life drug being used simultaneously.

Acting on other sub cellular drug target of the parasite are some antibiotics. There is a range of antibiotics with antimalarial activity, whose effects possibly include specific inhibition of the parasite's plastid-like organelle, the apicoplast, where protein synthesis happens[24,25], or inhibition ofn mitochondrial functions, another symbiogenic compartment within the parasite[26]. Nowadays, combination treatment of acute infections can be made with antibiotics such as tetracyclines, clindamycin, doxycycline, azithromycin, and rifampicin. **Doxycycline** (18, Figure 6) is the most frequently used antibiotic in antimalarial therapy applied in combination with quinine (1, Figure 3) or artesunate (17, Figure 5) in cases of multiresistance[15]. **Clindamycin** (19, Figure 6) in combination with quinine (1, Figure 3) is effective to treat patients with falciparum malaria who could not take doxycycline, that is, children and pregnant woman [27]. The use of an antibacterial agent within a combination of drugs offers safety and good pharmacokinetics, so tells us experience with antibiotics in other areas. The recent malaria genome sequencing will certainly highlight the specific functional pathways for each parasite organelle and lead to focus again on the use of antibiotics as alternative therapeutic agents.



18. Doxycycline

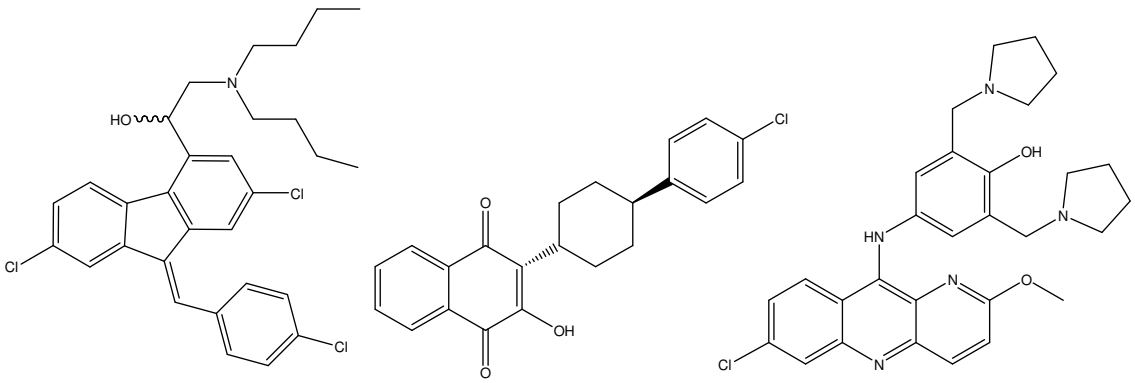


19. Clindamycin

Figure 6. Some antibiotics used in malaria chemotherapy

1.2.4 Newer Antimalarials in Clinical Use or in Clinical Development

A combination of an aryl amino alcohol similar to halofrantine, **lumefantrine** (20, Figure 7), and artemether (15, Figure 5), has recently been approved for the treatment of uncomplicated *P. falciparum* malaria. But poor and variable bioavailability and ease of resistance development to quinoline methanols may limit future interest with these drugs[28]. **Malorone**, an atovaquone- proguanil combination, is another recent approved drug. **Atovaquone** (21, Figure 7), a naphtoquinone antimicrobial agent, affects parasite mitochondrial functions selectively[29]. It couldn't be used as a monotherapy because the parasite developed a mutation of a cytochrome *b* gene localized in the mitochondrial genome [30]; addition of proguanil (9, Figure 4) solved the resistance problem and this synergistic interaction has overcome the rate of treatment failure. Malorone kills the parasites in their early stage of host development, in the liver, before infection of the erythrocytes takes place, which is advantageous for the prophylactic application[31].

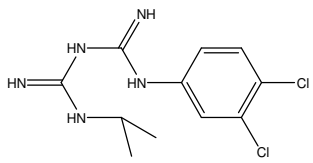


20. Lumefantrine 21. Atovaquone 22. Pyronaridine

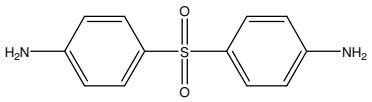
Figure 7. New components of combination therapy

The most active of the 4-amino quinolines is **Pyronaridine** (22, Figure 7), a very active Mannich-base antimalarial that possibly acts via a common mechanism with other 4-aminoquinolines, by blocking hemozoin formation. It has been used as a monotherapy, but there is ongoing work towards the development of a synergistic combination with artesunate (17, Figure 5) or artemisinin (13, Figure 5).

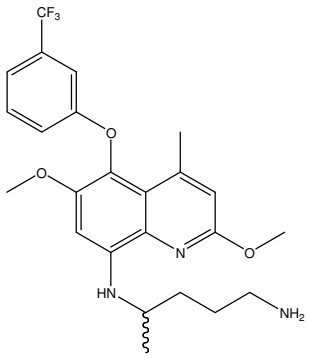
An interesting new approach is the combination **chlorproguanil** (23, Figure 8) with **dapsone** (24, Figure 8), commonly referred as **Lapdap,** now under development. Chlorproguanil is a close analogue of proguanil (9, figure 4) and dapsone is a dihydropteroate synthase inhibitor that has been widely used to treat leprosy [32]. Lapdap is a rapidly eliminated combination, so it is expected to be less vulnerable to antifolate resistance, though, for the same reason, cannot be expected to have much of a chemoprophylatic effect. Its optimal use may be as a three-drug combination, with artesunate (17, Figure 5). **Tafenoquine** (25, Figure 8) is an improved derivative of pamaquine (3, Figure 3). This drug is active against the liver stages of the parasite, but also against erythrocytic stages[33]. Its mode of action, as with other 8-aminoquinolines, is largely unknown and also has the major disadvantage of inducing haemolytic anaemia in individuals with a glucose 6-phosphate dehydrogenase deficiency.



23. Chlorproguanil



24. Dapsone



25. Tafenoquine

Figure 8. Lapdap combination and Tafenoquine

Fosmidomycin (26, Figure 10), a compound originally isolated as an antibiotic, is another antimalarial whose molecular target and activity has only been known recently[34]. It inhibits a pathway in isopropenoid biosynthesis within the parasite, which is distinctive from the human one (Figure 9).

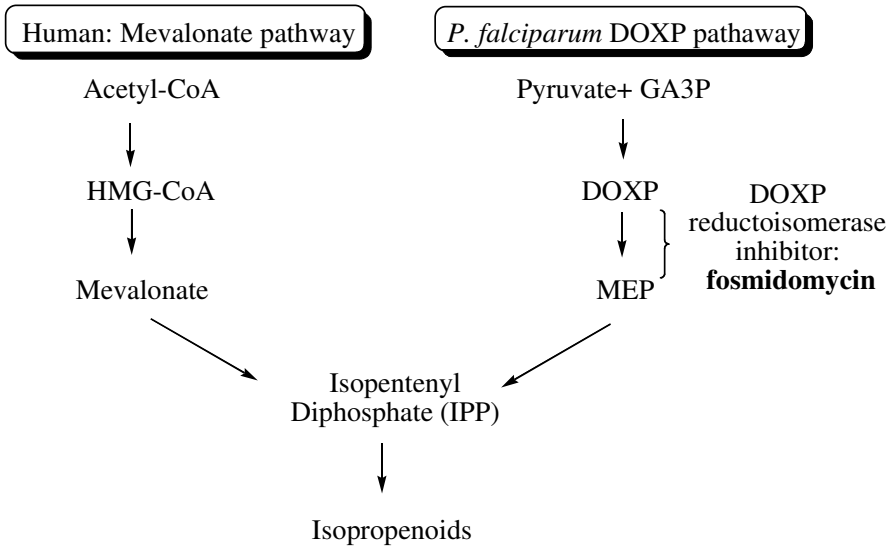
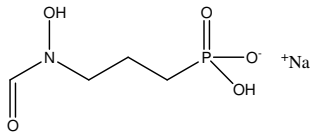
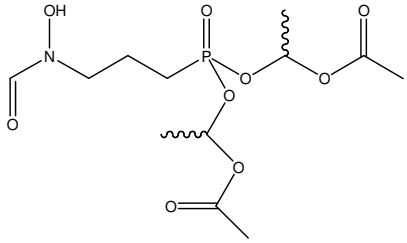


Figure 9. Isopropenoid biosynthesis in humans and in *P. falciparum*[33]

The quasi-irreversible binding in the enzyme induces conformational alterations that result in very high affinity[35]. Fosmidomycin prodrug derivatives, such as **FR900098** (27, Figure 10) have been prepared in order to increase oral bioavailability[36,37].



26. Fosmidomycin



27. FR900098

Figure 9. DOXP inhibitors

1.2.5 New Approaches: Attacking the Parasite

1.2.5.1- New Heme-Polymerization Inhibitors

The emergence of parasite resistance against chloroquine (5, Figure 3) has prompted the need to investigate other 4-aminoquinolines, and also 8-aminoquinolines, bisquinolines and quinolinemethanols[28], all of these with action directed towards the food vacuole target. Although the mechanism of resistance is still unclear, it is suggested to be linked to multiple factors, including changes in transport proteins[38]. It is worth noting that it has taken much longer for the parasite to develop resistance to this class of compounds than to many others, and, truth to be said, the fact that there was an excessive and uncontrolled use of chloroquine didn't help. In this context, there is still high interest in this class of compounds.

Various mechanisms have been proposed to rationalize the mode of action of chloroquine and aminoquinolines in general: chloroquine accumulates to high concentrations within the parasite's food vacuole[39] by a mechanism commonly referred as the weak base effect[40] or, as more recently suggested, by a mechanism of intraparasitic receptor binding with heme[41,42]. Chloroquine and analogues are believed to exert its activity by interfering with the heme detoxification processes the parasite uses to eliminate heme following haemoglobin digestion. This interaction with heme leads to increased levels of heme or heme/ drug complex which get to be unbearably toxic to the parasite, killing it.

Amodiaquine (7, Figure 3) is a 4-aminoquinoline effective against many chloroquine falciparum resistant strains, which has had severely restricted clinical uses because of

associated side effects: hepatotoxicity and agranulocytosis[43,44]. So, one possible research strategy would be to try to improve amodiaquine. This drug contains a *p-hydroxyanilino* moiety which is believed to undergo P-450- catalysed oxidation to a chemically reactive metabolite which can bind to host cellular macromolecules and initiate hypersensitivity reactions[45]. A new series of amodiaquine analogues was synthesised bearing a 3´hydroxyl and a 4´Mannich side-chain function, in order to avoid formation of toxic metabolites via P-450 metabolism (28, Figure 12). **Isoquine**, (29, Figure 12), is one of the new analogues bearing this structural change, showing superior activity against CQ resistant strains and also having minimal cross-resistance with chloroquine and cheap production price[45].

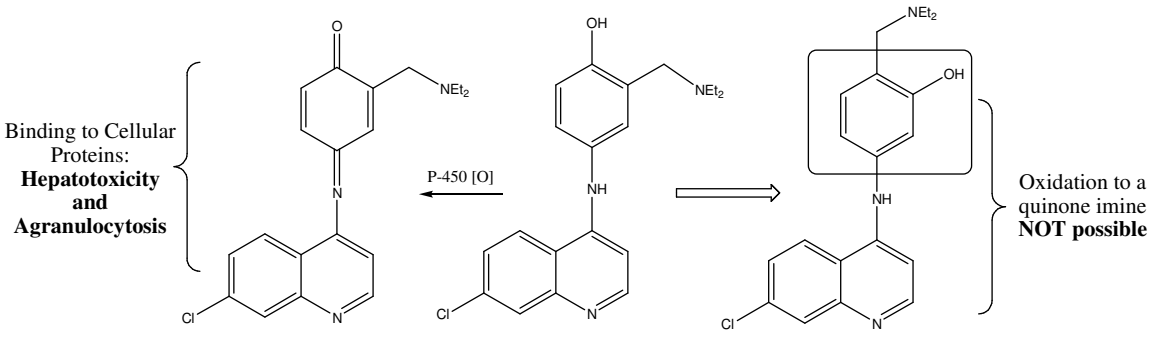
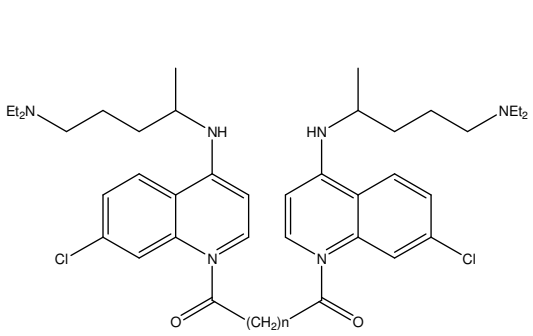


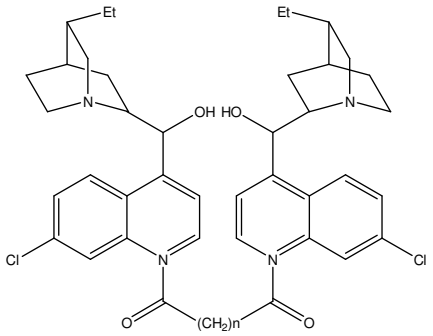
Figure 12. Bio activation of amodiaquine to toxic quinoneimine and amodiaquine redesign to prevent enzyme oxidation[45]

Current work is also being focused on the development of chloroquine derivatives with two quinoline cores, connected by various linkers (**bisquinolines**, Figure 13). Several modifications have been tried, most of them successful, suggesting that the place of attachment of the linker is irrelevant, retaining activity both if anchored onto the quinoline nucleus or the 4-, 6-, or 8- positions[28]. Alkyl ether and piperazine bridges

were shown to be more effective in-vivo, due to increased water solubility and drug absorption[46].



30. Bis-4-aminoquinolines



31. Bis-4-quinolinemethanols

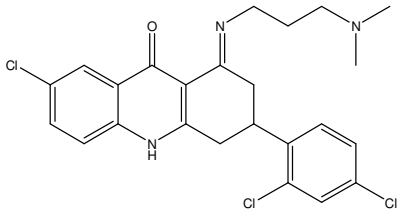
Figure 13. Some bisquinolines developed

From mepacrine (4, Figure 3), a new highly effective derivative has been synthesised, **dihydroacridinedione** WR243251 (32, Figure 14). This drug has a mixed mechanism of activity, inhibiting heme polymerization[47] and, similarly to atovaquone (21, Figure 7), also inhibiting the cellular respiration of the parasite[48].

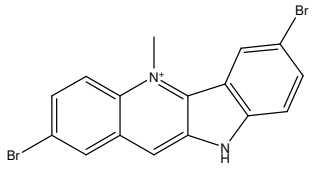
Plant derived compounds, such as cryptolepine, an indoloquinoline alkaloid used in traditional medicine, also offer an approach to antimalarial chemotherapy. But, as a result of its DNA-intercalating properties, this natural compound is cytotoxic[49]. However, one of its synthetic derivatives, **2,7-dibromocryptolepine** (33, Figure 14) has shown high antimalarial activity and lower toxicity in mice[50].

Aiming at the development of completely new inhibitors of heme polymerization, an *in-vitro* microassay was established in which ^{14}C haematin was incorporated into insoluble β-haematin, which is chemically indistinguishable from hemozoin. Over 100 000 compounds were screened and two of them identified as such inhibitors, **Ro 06-9075** (34, Figure 14) and **Ro 22-8014** (35, Figure 14). Also, compounds originally developed

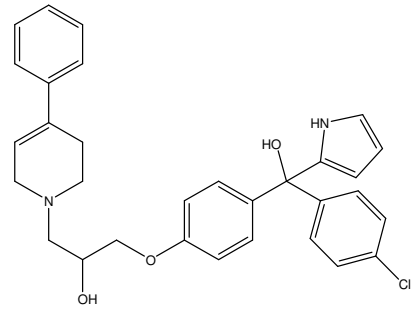
as **protease inhibitors**, like compound 40 (36, Figure 15), turned out to be potent inhibitors of heme polymerization[51,52].



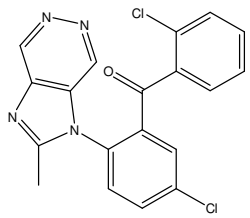
32. WR243251



33. 2,7-dibromocryptolepine



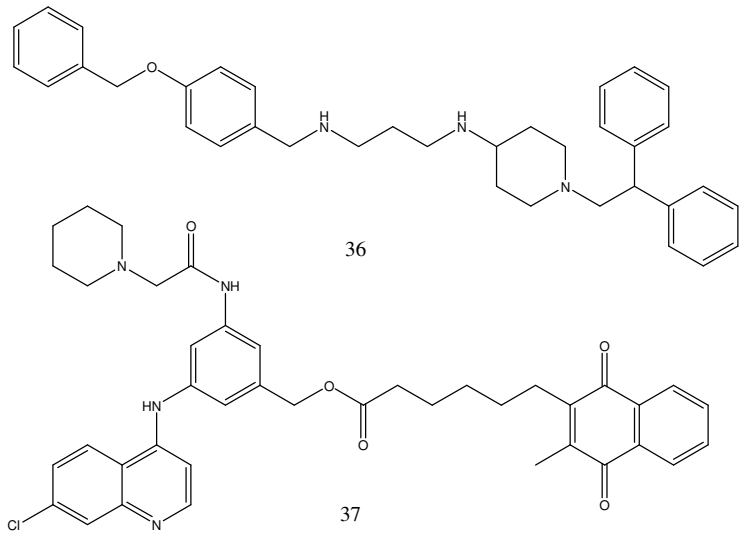
34. Ro 06-9075



35. Ro 22-8014

Figure 14. New inhibitors of heme polymerization

Chloroquine-resistant parasites have a decreased accumulation in food vacuole. Alterations in the vacuolar pH[53] and competition with FP-binding proteins are hypothesis to explain this fact. Another possibility is the fact that CQ-resistant parasites have increased levels of glutathione reductase, possibly used to detoxify heme; so it seems that the parasite has developed an alternative mechanism for hemozoin disposal, decreasing parasite susceptibility to CQ[54,55]. Inhibitors of glutathione reductase partially reverse QC resistance[56]. The synthesis of prodrugs, like compound (37, Figure 15) incorporating a 4-aminoquinoline with proved antimalarial activity and a glutathione reductase inhibitor, could be the answer to overcome CQ resistance.



36

37

Figure 15. A protease inhibitor and a chimeric prodrug

1.2.5.2- Artemisinin Derivatives and Other Peroxides

Artemisinin (13, Figure 5) is a sesquiterpene containing a 1,2,4-trioxane, and the dialkylperoxide function is the key of its biological activity[20,57]. Existing evidence suggests that artemisinin and related peroxidic antimalarial drugs exert their parasiticidal activity subsequent to reductive activation by haem, released as a result of haemoglobin digestion by the parasite[58,59]. This irreversible redox reaction produces carbon-centred free radicals, leading to alkylation of haem[60] and proteins[61], one of which- the sarcoplasmic-endoplasmic reticulum ATPase PfATP6- may be critical for parasite survival. The apparent mode of action of this class of compounds makes them very rapid onset of action. Currently, artemisinin derivatives are the most effective antimalarials when taken in drug combination therapy to avoid recrudescence due to their short half-lives. The erratic supply of the parent compound artemisinin makes it necessary to design new, cheap and accessible synthetic drugs based in the same

mechanism of action. Based on the structure of artemisinin, numerous semisynthetic derivatives and totally synthetic peroxides have been prepared and tested for their antimalarial properties. Manipulation of the lactone function of artemisinin led to a number of semisynthetic derivatives, such as **artelinic acid** (39, Figure 16), considerably more stable and soluble than artesunate (17, Figure 5) [62] and others, like compounds **40-42**[63] (Figure 16). Compound **42** was rationalized to incorporate the piperazine ring as means of enriching the drug inside the parasite's food vacuole, through the weak base effect[33]. Artemisinin derived trioxane dimmers (43, Figure 16) have also been investigated and have demonstrated excellent antimalarial response[64].

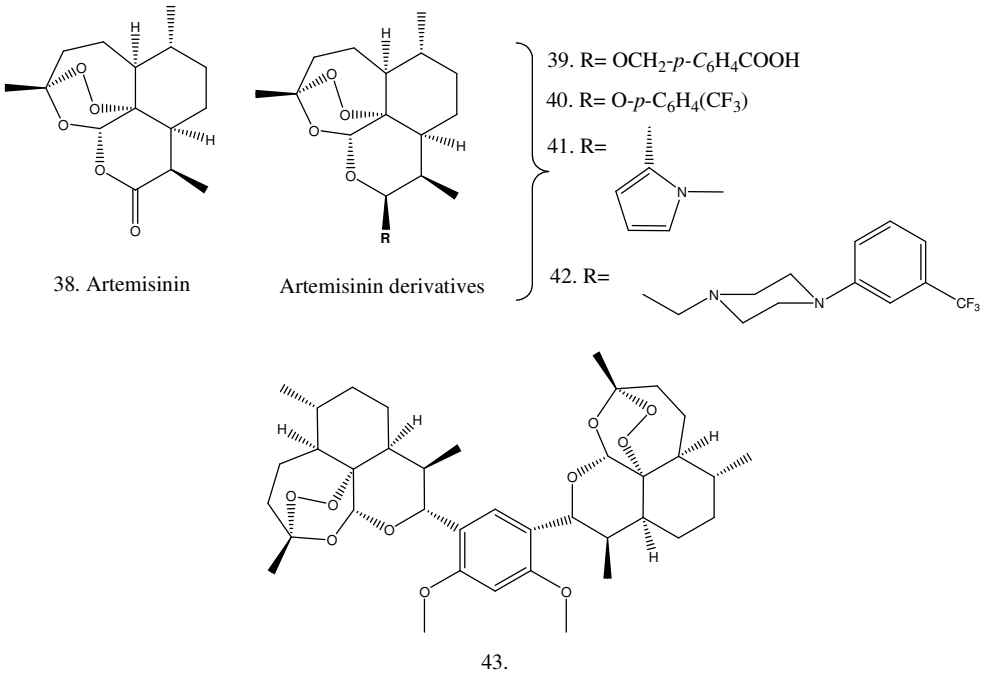
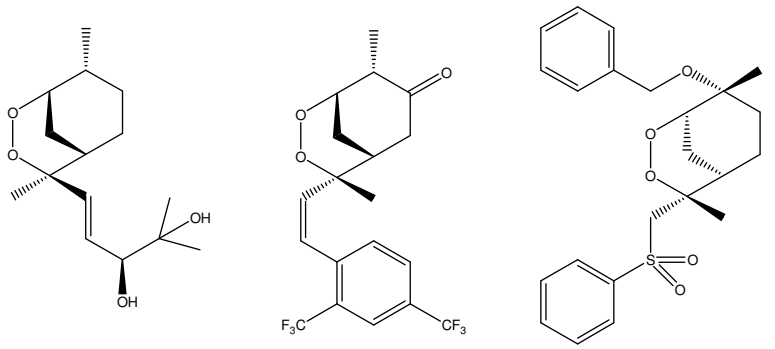


Figure 16. Some semisynthetic artemisinin derivatives

Though these artemisinin manipulations seem very effective, the short supply of natural artemisinin and its difficult and costly total synthesis still make it unaffordable to most. One recent approach to circumvent this question is to use a microbial source, like

engineered yeast, to obtain artemisinin or any derivative. This could be a cost effective, environmentally friendly, high quality and reliable answer to the problem[65]. An engineered strain of *Saccharomyces cerevisiae* has been designed to produce high titres (up to 100 mg/l) of artemisinic acid (artemisinin's immediate precursor) using an engineered melanovate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase from *A. annua* that performs a three step oxidation of amorpha-4,11-diene to artemisinic acid. The synthesized artemisinic acid is transported out and retained on the outside of the yeast and can be obtained from there in a simple and inexpensive purification process [66]. Yield optimization and industrial scale-up have to be considered.

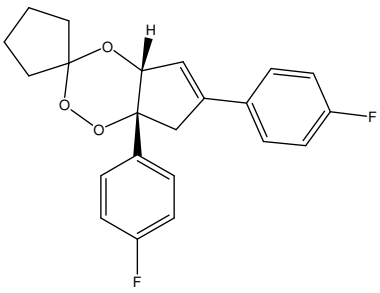
Yingzhaosu (44, Figure 17), isolated from the herb *Artabotorys uncinatus*, comes from traditional Chinese medicine. The structurally related synthetic derivative **arteflene** (45, Figure 17)), showed attractive properties[46], but also some inconsistencies in clinical trials that prevented further development of this compound [67]. A shorter and more efficient synthesis of this type of compounds afforded a new series of analogues such as endoperoxide sulfone (46, Figure 17)[68-70], which proved to have high antimalarial activity.



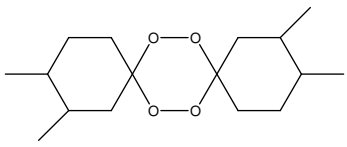
44. Yingzhaosu A 45. Arteflene 46. Sulfone endoperoxide

Figure 17. Yingzhaosu A and some analogues

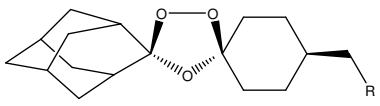
Unsaturated 1,2-dioxanes, simple bicyclic 1,2,4-trioxanes, tricyclic and tetracyclic 1,2,4-trioxanes, spiro ring-fused 1,2,4-trioxanes, spiro 1,2,4-trioxanes, 1,2,4,5-tetraoxanes, amine peroxides, acyclic peroxides and others are all peroxide chemical families, some of them with promising new compounds. From all of these, **Fenozan B07**[71] (47, Figure 18) is only marginally less effective than artemether (15, Figure 5) and compounds such as the 1,2,4,5-tetraoxane (48, Figure 18) showed activity comparable to artemisinin. A fully synthetic 1,2,7-trioxolane **OZ277** (49, Figure 18) is a drug candidate that exhibits structural simplicity and can be acceded via an economically feasible and scalable synthesis. It shows superior antimalarial activity and an improved biopharmaceutical profile[72]. It is now under a clinical development program[73]. Oxazines such as compound 50 (Figure 18) were also synthesised as potential antimalarial drugs, based on the fact that the N-O bond is also prone to undergo homolytic cleavage and generate radicals, having a standard bond dissociation energy similar to that of O-O bond. Parasite growth inhibition was achieved in the low micromolar range[74].



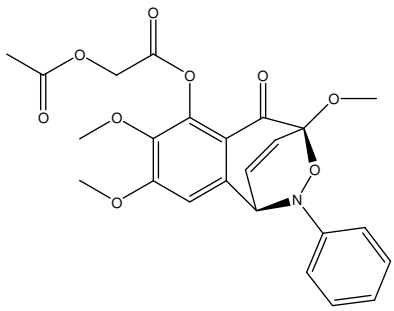
47. Fenozan B07



48.



49. OZ277 (R= $CONHCH_2C(CH_3)_2NH_2$



50.

Figure 18. Examples of new synthetic peroxides and oxazines

The development of compounds conjugating a 4-aminoquinoline entity with a trioxane motif, the so-called trioxaquines, has also proved to be a good approach. Compounds such as **DU-1301**[75,76](51, Figure 19) show very low IC50 values *in-vitro* and are active *in vivo*[77].

It is important to note that several peroxides also show antiproliferative and antitumor activity, and have therefore attracted interest for artemisinin-like compounds in anticancer chemotherapy.

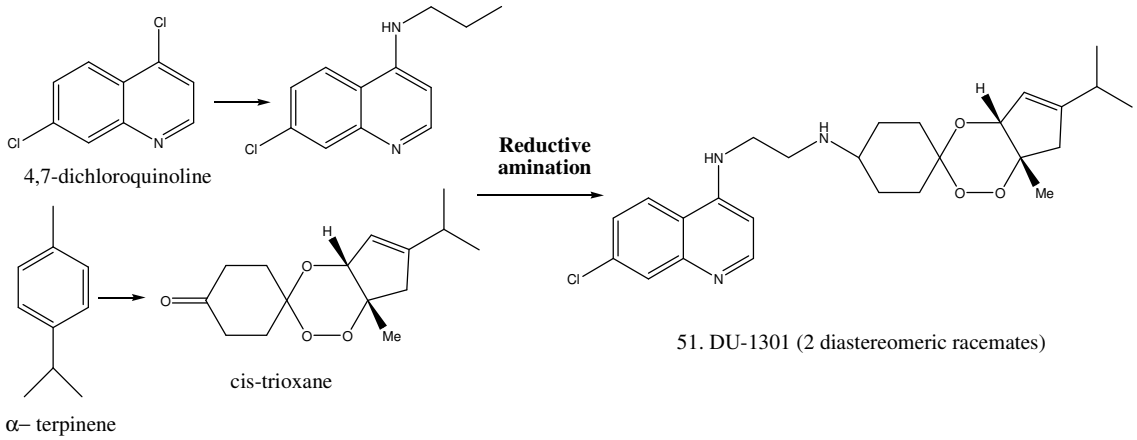


Figure 19. Convergent synthesis of trioxaquine DU1301[78]

1.2.5.3- Inhibiting the Parasite's Metabolic Pathways

- Protease inhibitors

Proteases have a significant role in the parasite survival. They appear to be required for several key parasite activities such as haemoglobin degradation, erythrocyte rupture and erythrocyte invasion.

Cysteine protease activity appears to be required for the erythrocyte rupture, at the completion of the parasite intraerythrocytic cycle[79]. Recent studies using E-64 showed that, in mature schizonts-stage parasites, this inhibitor blocked the lysis of the parasitophorous vacuole membrane [80] and also that the use of other cysteine protease inhibitors, leupeptin and antipain, blocked the lysis of the erythrocyte membrane [81].

During the process of erythrocyte invasion there is an irreversible junction between the apical end of the merozoites and the erythrocyte's receptors and secretory organelles, such as dense granules, are released from the apical end of merozoites[5]. PfSub1 and PfSub2 are well characterized proteases involved in this process belonging to the superfamily of subtilisin-like serine proteases. PfSub1 has shown to be expressed in merozoites and undergoes extensive post-translational processing during the transit through the secretory pathway of the parasite[82]. These proteases are, on their own right, potential chemotherapeutic targets.

It's during the intraerythrocytic growth, in their acidic food vacuole, that the malaria parasites degrade host haemoglobin for feeding purposes. The feeding process is catalyzed in a semi ordered manner, by a variety of proteases. It has been suggested that aspartic proteases plasmepsin I, II and IV, are responsible for initial cleavages of hemoglobin[83,84], that the cysteine protease falcipain-2 cleaves relatively large globin fragments, and that the metalloprotease falcilysin and HAP, an histoaspartic protease, cleaves smaller peptides [85,86]. Small globin peptides are then probably transported to the parasite cytosol where, from action of other proteases, the free amino acids are finally obtained. Specific inhibitors for plasmepsin II were obtained from modification of available inhibitors of cathepsin D, a lysossomal protease existing in mammalian cells. Selectivity of the new compounds, such as compound **52** (Figure 20) for plasmepsin over cathepsin D, is of particular importance, as cathepsin D is ubiquitous in mammals

and, therefore, inhibition of this enzyme would have severe side effects for the host[33]. Inhibitors of falcipain prevent the degradation of hemoglobin by P. falciparum, causing undegraded hemoglobin to accumulate in the parasite food vacuole and block parasite development[87]. Selected peptidyl fluoromethyl ketones[88-90], such as compound **53** (Figure 20) or vinyl sulfones[91,92], inhibited falcipain-2 and blocked *P. falciparum* development at low nanomolar concentrations .

Attempts to develop nonpeptide inhibitors of falcipain are also underway. **Chalcones**[93](54, Figure 20) and **phenothiazines** [94](55, Figure 20) with in vitro antimalarial activity have already been identified.

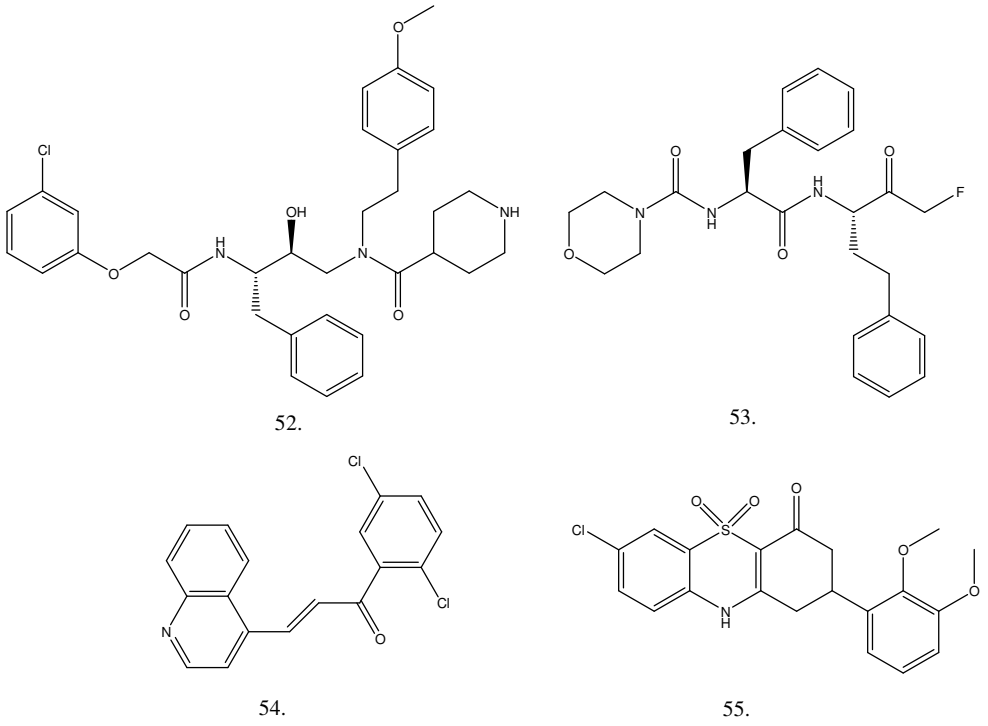


Figure 20. Protease Inhibitors

Recent studies showed that the mechanism of resistance to a vinyl sulfone falcipain inhibitor was complex and slow. The best way to avoid development of resistance is thought to be by the use of antimalarial combination therapy. It is also thought that

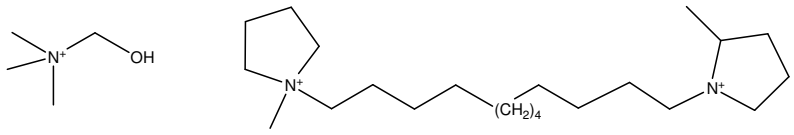
combining different classes of proteases that participate in the heme degradation process would probably have an overall synergistic effect in the fight to kill the parasite. The *P. falciparum* genome sequence brought prediction on the effects of more cysteine proteases[79], which, after being characterized, will certainly expedite drug discovery towards malaria eradication.

-Utilization of Parasite-Induced Transport

The membranes of normal mammalian cells, including erythrocytes, are endowed with many solute transporters that mediate the movement of ions and molecules between the plasma and the cell cytosol. Following malarial infection there appears to happen formation of new permeation pathways with functional characteristics quite different from those of the normal host transporters. It has been suggested that much of this malaria-induced transport is actually via one common pathway or transport system [95]. One of the approaches, considering this concept, is the simultaneous administration regime of a toxic nucleoside plus a transporter inhibitor. The nucleoside transporter inhibitor would protect normal host cells from the toxicity of the other compound, a citotoxic nucleoside that would enter the infected erythrocyte and destroy the parasite viability[96,97]. The other approach is based on the fact that this new permeation pathway allows transport of **L-purine** nucleosides, the non-physiological isomer, which is not taken by host cells; dimers consisting on a combination of this compounds, acting as carriers, with known antimalarials, would very likely provide greater antimalarial efficacy and specificity[98].

- Inhibitors of Phospholipid Metabolism

During the intraerythrocytic phase the malaria parasite needs to synthesize on its own considerable amounts of biological membrane in order to develop, since the host erythrocyte doesn't have this ability. Choline (56, Figure 21) is a precursor required for the synthesis of phospholipids, the predominant component of *plasmodium* membranes, and inhibition of the choline transporter with choline quaternarium ammonium analogues, such as **G25** (57. Figure 22)[99,100], has shown to be promising ($IC_{50} < 5$ nM), regardless of toxicity issues which will have to be assessed carefully.



56. Choline

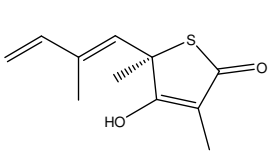
57. G25

Figure 21. Choline and a choline uptake inhibitor

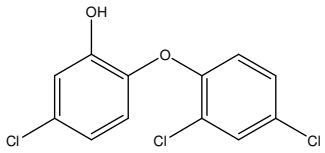
- Inhibitors of Type II Fatty Acid Synthesis

Fatty acids play an important role in providing precursors of biological membranes and represent an important form of metabolic energy. Mammals, fungi, and some mycobacteria synthesize fatty acids using a type I fatty acid synthase (FAS-I), a multifunctional polypeptide whose distinct regions catalyse each reaction. Differently, plant chloroplasts, most bacteria and the malaria parasite[101] use a type II system (FAS-II) where the same enzymatic steps are performed using separate, discrete enzymes, located inside the apicoplast. Since the human malaria parasite *P. falciparum* synthesizes fatty acids via a pathway that is absent in humans and well characterized,

this could be an important intervention focus for new antimalarials, namely antimicrobials. In fact, specific inhibitors of the FAS-2 pathway include **thiolactomycin** (58, Figure 22) and **triclosan** (59, Figure 23), which have proven to be valid targets. Triclosan inhibits ENR (FabI), an enoyl acyl carrier protein reductase and has shown significant activity against *P. falciparum* (IC50 in the lower micromolar range). PfENR, the translation product of the *pfnr* gene, has been crystallized as a binary complex with NADH and as a ternary complex bound to triclosan (and triclosan analogues) and with NADH [102], which will facilitate structure-based optimization of inhibitors.



58. Thiolactomycin



59. Triclosan

Figure 22. Inhibitors of fatty acid synthesis

- Protein Farnesylation Inhibitors

Protein prenylation refers to the post-translational modification of proteins by the covalent attachment of a 15 carbon farnesyl or a 20 carbon geranylgeranyl group. This allows creation of an hydrophobic tail that facilitates membrane association as well as protein interactions. Among known prenylated proteins are small GTPases, which play roles in cell signal transduction, vesicle trafficking and cell cycle progression [103]. Protein prenylation is mediated by three distinct enzymes, PFT, PGGT-I and PGGT-II. PFT, protein farnesyl transferase, transfers a farnesyl from farnesyl pyrophosphate to the thiol group of a cysteine existing in the CaaX motif of specific proteins [104] (Figure 23).

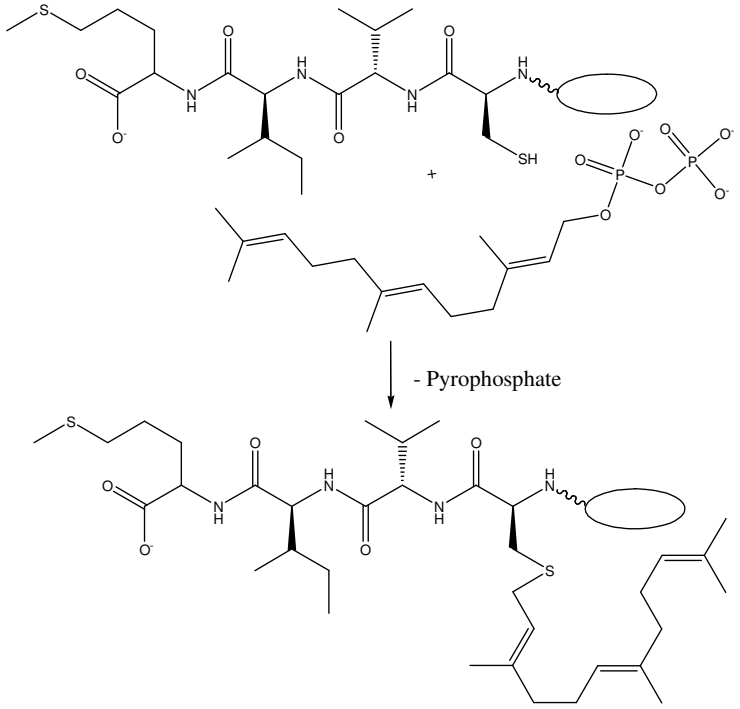
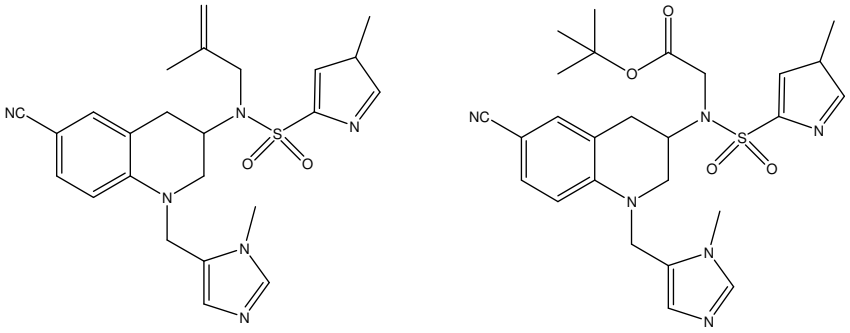


Figure 23. The prenylation reaction catalyzed by farnesyl transferase. The substrate protein shown has the recognition sequence cysteine-valine-isoleucine-methionine.

Protein prenylation occurs not only in higher eukaryotic cells, where strong research and development arose regarding PFT inhibitors for the treatment of cancer, but also in a wide variety of pathogenic protozoa, including *Plasmodium falciparum*[105]. This similarity could bring questions regarding host toxicity, but existing studies have shown that PFT inhibitors are more toxic to cancer cells and parasites than normal mammalian cells, though the reason for this selectivity remains unclear yet. From the wide library of PFTI anticancer clinical candidates, several, like **BMS-388891** (60, Figure 24) and **BMS-386914** (61, Figure 24), inhibited *P. falciparum* enzymes at low nanomolar concentrations and had inhibitory effects on parasite growth. It was observed that compounds containing the tetrahydroquinoline (THQ) core possessed increased potency

[106]. Both the IC_{50} and ED_{50} results for the tested THQ's against *P. falciparum* strains from different geographic origin were extremely promising. The pharmacodynamic properties of these compounds have yet to be determined and assessed.



60. BMS-388891

61. BMS-386914

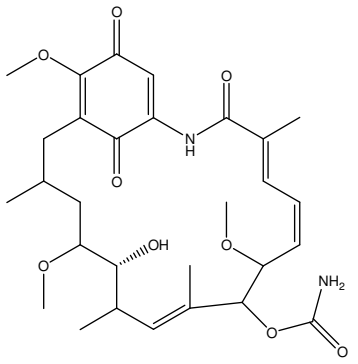
Figure 24: Potent protein farnesyl transferase inhibitors (PFTIs)

- Heat-Shock Protein Inhibitors

Heat-shock proteins are molecular chaperone proteins with an important role in stress tolerance, in the folding, activation and assembly of many proteins, such as steroid hormones, protein kinases and molecules regulating the cell cycle[107]. During host invasion, establishment and development, parasites are exposed to environmental stress. Parasitic heat-shock protein 90, known to be expressed by the parasite during the intraerythrocytic stages in the host[108], has an important role in the survival of these organisms. Although the gene coding for Hsp90 in *P. falciparum* has been cloned, there is still limited information available regarding its function in the parasite; nevertheless, Hsp90 has been shown to form complexes with *Plasmodium* proteins in the parasite's cytoplasm, and also participate in the parasite's growth in human erythrocytes. Addition of **geldanamycin** (62, Figure 25) to a *P. falciparum* culture resulted in inhibition of the parasite's Hsp90, affecting in particular the parasite's progression from ring to

trophozoite maximally[109]. This natural product binds with high affinity into the ATP binding pocket of Hsp90. The study of geldanamycin effects on the parasite's gene expression profiles during stage progression may bring to light details on this mechanism and possibly lead to identification of novel drug targets against malaria. Human host humoral response against Hsp90 of the parasite has been observed[110], making of this protein also an important antigen target[111].

Because Hsp90 "client" proteins play important roles in the regulation of the cell cycle, cell growth, cell survival, apoptosis, and oncogenesis, geldanamycin obstructs the proliferation of cancer cells and shows anti-cancer activity in experimental animals [112]. Although difficulties with solubility and toxicity should be overcome, Hsp90 inhibitors will also be potential and effective cancer chemotherapeutic drugs.



62. Geldanamycin

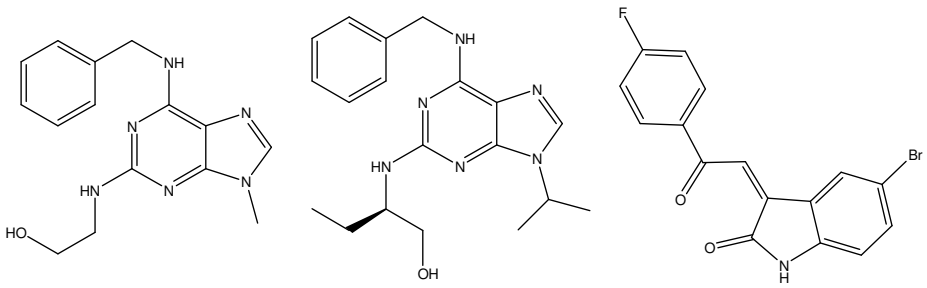
Figure 25. Hsp90 inhibitor

- Protein Kinase Inhibition

Protein kinases are key regulators of cell cycle control[113]. One family of kinases in particular, the cyclin-dependent protein kinases (CDKs), directly regulates the timely progression through the cell cycle and is activated by cyclin binding and phosphorilation. Loss of CDK regulation has been genetically linked to the

development of human cancers and there is considerable interest in the development of selective CDK inhibitors as novel therapeutics. Again, here is an area where research can intercross, through exchange of research information.

The regulatory mechanisms of the cell cycle remain largely unknown, but it is obvious that CDKs play an important part in the development of the parasite, since inhibition of PfPK5 and Pfmrk, two well characterized plasmodium CDKs, has been demonstrated. Purine derived CDK inhibitors like **olomoucine** (63, Figure 26) and **roscovitine** (64: Figure 26) effectively inhibited PfPK5 in the micromolar range, but had no effect on Pfmrk. However, **oxindole-based** compounds (65, Figure 26) were selective to Pfmrk, also in the micro molar range [114].



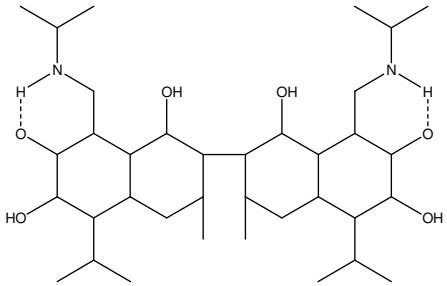
63. Olomoucine 64. Roscovitine 65. Oxindole-based compound

Figure 26. CDK inhibitors

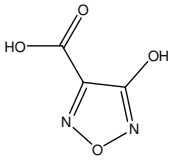
Comparison of plasmodial and mammalian CDK sequences reveals that there are structural differences within the ATP binding pocket that can be exploited to develop specific inhibitors [114].

- Glycolysis Inhibitors

Erythrocytic parasite life stages don't appear to have a functional citric acid cycle. Thus, the NADH required for glycolysis to proceed is regenerated from NAD+ via the conversion of pyruvate (the product of glycolysis) to lactate. This reaction is catalyzed by lactate dehydrogenase (LDH), the final enzyme of the glycolytic pathway in *Plasmodium falciparum.* Structural and kinetical discrepancies between these malarial enzymes and the human counterparts suggest that specific and potent malarial inhibitors can be designed or identified [115].



66. Isopropyl gossypol



67. OXD1

Figure 27. Lactate dehydrogenase inhibitors

Series of gossypol derivatives whose aldehyde groups have been modified, like **isopropyl gossypol** (66, Figure 27), have been tested against *P. falciparum* and shown to be inhibitors of LDH, with IC_{50} values of about 20 μM [116]. Heterocyclic azole-based compounds, like **OXD1** (67, Figure 27) selectively inhibit PfLDH at sub-micromolar concentrations[115]. OXD1 activity was more pronounced on trophozoite than on ring stages, probably because the trophozoite stage requires more energy[117]. PfLDH is among the most advanced targets of antimalarial structure-based drug design[118].

- Electron Transport Chain Inhibitors

Targeting the parasite's mitochondrial electron transport chain has been shown to be a successful chemotherapy strategy, due to existing differences between host and parasite in this process. This strategy has in fact led to the development of atovaquone (21, Figure 7), focused earlier, which has been a clinical success. The recently completed malaria genome project revealed that *P. falciparum* mitochondria lacked the conventional rotenone-sensitive complex I found in most mammalian mitochondria but instead had an alternative complex I[119] (or type II NADH:dehydrogenase), known as PfNDH2. Loss of PfNDH2 function, by its inhibition, provoked a collapse of mitochondrial transmembrane potential, leading to parasite death[120]. Targeting this enzyme and exploiting synergy with other targets may also be an effective antimalarial chemotherapy.

1.3. Summary

This general introduction presents malaria as a deadly parasitic disease with staggering human dimensions, and provides an overview of the significant advances and efforts made in malaria chemotherapy.

Identification of biological targets and mechanisms of drug action have been undeniable tools to the development of new strategies capable of delivering effective treatment of malaria in the close future, as well as the ability to prevent the development of resistance by the parasite to new drugs.

A promising and successful strategy to delay the development of resistance by the parasite is the Combination Theory Approach. The concept of combining two distinct chemical entities in the same molecule is currently under deep investigation, given the excellent preliminary results obtained from the combination of a 1,2,4-trioxane containing artemisinin derivative with another antimalarial pharmacophore such as an aminoquinoline or an aliphatic diamine, within the same molecule.

Chapter 2 describes the preparation of new Endoperoxide Cysteine Protease Inhibitor (ECPI) Pro-Drugs, in which an endoperoxide containing the 2,3-dioxabicyclo[3.3.1]nonane system incorporates a chalcone-type unit. Research was conducted towards the investigation of the mechanism of action of these hybrid drugs.

Chapter 3 describes the design and synthesis of novel Carbonyl Containing Cysteine Protease Inhibitors, following both a peptidic and a peptidomimetic approach, and the assessment of the inhibitors *in-vitro* activity. Optimisation of the peptidic/peptidomimetic moieties will lead to the synthesis of new pro-drugs, upon

combination with a trioxolane unit, affording a new approach in protease inhibitor design.

1.4 Bibliography

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