UNIVERSITÀ DEGLI STUDI DI TORINO

New chemotherapeutic strategies against malaria, leishmaniasis, and trypanomiases

Mario Zucca[1], Sara Scutera[2], Dianella Savoia[1*]

[1] *Department of Clinical and Biological Sciences,*
[2] *Department of Public Health & Microbiology,*

University of Torino, Italy

Running title: Antiprotozoal strategies

*Corresponding author: Dianella Savoia, Dept. of Clinical and Biological Sciences, at S. Luigi Gonzaga Hospital, University of Torino, Regione Gonzole 10, 10043 Orbassano (To), Italy; Tel: +39 0116705427; Fax +39 0112365427. E-mail: dianella.savoia@unito.it

Abstract: Due to the persistent lack of suitable vaccines, chemotherapy remains the only option for the treatment of patients infected by protozoan parasites. However, most available antiparasitic drugs have serious disadvantages, ranging from high cost and poor compliance to high toxicity and rapid induction of resistance. In recent decades basic research laboratories identified a considerable number of promising new molecules, but their development has not been pursued in depth by pharmaceutical firms because of poor prospects of economic return. The establishment of adequately funded public-private partnerships is currently reversing the trend. This review deals with new drugs against *Plasmodium, Leishmania* and *Trypanosoma* parasites, focusing on the molecules that are in the most advanced stage of development. The purpose of this article is to provide the reader with a panoramic view of the updated literature on the challenges and strategies of contemporary antiprotozoal drug research, paying the due attention to the already published reviews.

Keywords: Antibiotics, antiprotozoal chemotherapy, *Leishmania*, natural products, *Plasmodium*, *Trypanosoma*.

1. INTRODUCTION

Infections caused by parasitic protozoa take an enormous toll on human health. Their prevalence is higher in tropical and equatorial countries, where the major number of deaths is due to malaria, leishmaniasis, and African and American trypanosomiasis. High mortality rates are associated to poor sanitary conditions, high percentages of untreated HIV-infected individuals, and lack of efficient prophylactic measures [1]. In the developed countries the situation is not so tragic, but nonetheless it has its drawbacks. If it is true that in these countries most HIV-infected patients are treated, the number of chronically immuno-deficient carriers of transplanted organs is set to grow. In these patients latent parasitic infections, such as leishmaniasis and toxoplasmosis, can reappear as opportunistic infections. Moreover, the climate changes linked to global warming in temperate countries are extending the insect vector habitats, as is the case of the northward spread of leishmania-transmitting sandflies, assessed by epidemiological surveys of canine leishmaniasis in Italy [2-4]. Some additional cases of parasitic infections have also been registered due to "transplant tourism", i.e. travel with the intent of receiving or donating an organ, a practice that is rapidly increasing [5]. A further danger for industrialized countries comes from massive migration of infected subjects from endemic countries: a typical example is the presence of thousands of *Trypanosoma cruzi*-infected people in North America and Spain [6].

In the absence of prophylactic or curative vaccines the only available choice is chemotherapy. However, this relies on drugs which are tens of years old, afflicted with serious disadvantages, such as heavy side-effects, selection of resistant strains, or high production costs. Unfortunately, this situation is not likely to improve in the short term because, as many authors have emphasized, market forces are insufficient to drive the discovery and development of new drugs [1]. In a profit-driven context in which pharmaceutical firms, like any private industry, respond to economic rather than social or human interests, the development of drugs for parasitic diseases by private companies has no future, even in the presence of major technical breakthroughs such as the recent insights in parasite biology and the increasing use of computer-aided virtual screening [7]. To counteract the disengagement of pharmaceutical companies from R&D of drugs for tropical diseases that took place in the 1970s, some public-private partnerships (PPPs) were established, such as the Medicines for Malaria Venture (MMV), the Drugs for Neglected Diseases initiative (DNDi) [8], the Institute for One World Health, and the Italian Malaria Network. These PPPs, generously funded by, among others, the Bill and Melinda Gates Foundation and the Rockefeller Foundation, exploit the synergy resulting from the coordination of the basic research undertaken by universities and public healthcare organizations with the pharmaceutical industry expertise in medicinal chemistry and technology necessary for the development of leads and drug candidates. This topic is thoroughly discussed by Nwaka and Ridley [9]. The production cost remains a fundamental issue, as the new drugs should be available on the markets of

developing countries at an affordable price. This factor must be taken into account to evaluate the real value of any R&D program aimed at producing new effective and safe antiparasitic drugs to be used in endemic countries [10].

The strategies to optimize the identification of new active compounds rely on the integration of medicinal chemistry, pharmacokinetics, and project planning. The publicly-founded sequencing of parasitic genomes and the recent breakthroughs in the fields of genomics, proteomics, bioinformatics, high-throughput screening (HTS), high content screenings (HCS), structure-based design, chemioinformatics and pharmacogenomics, already in use to identify molecular targets in major diseases like cancer and neurodegenerative or inflammatory disorders, can be used to identify new parasite-specific targets [11-13]. Computer-assisted search for drug targets is making the process of drug finding less costly and time consuming, and *in silico* approaches such as virtual screening by molecular docking are becoming more and more efficient and cost-effective [14, 15]. Today the choice is among single-target, multi-target or phenotypic screening. The large-scale screening against well identified singular targets is more easy to perform, and can take advantage of the availability of the full genome sequence of *Plasmodium*, *Leishmania*, *Trypanosoma* and *Toxoplasma* species, that provides the possibility to develop drugs targeting biochemical processes common to different parasites [16]. This strategy could produce huge savings in the development, toxicity testing and marketing procedures. However, single target drugs frequently show reduced *in vivo* efficacy and/or undesired safety and resistance profiles, due to target redundancy, crosstalk, compensatory and neutralizing actions, and on-target and off-target toxicities [17]. Multi-target drugs would be more safe, more active and with a lower capacity of selecting resistant strains. Nowadays multi-target drugs are obtained by the combination of two different molecules, as in antimalarial combination treatments, but it can be envisaged that the availability of *in silico* models of cellular systems will make the identification of new single molecules active on more than one target possible. A stimulating discussion on the advantages of the multi-target approach has been written by Keith *et al*. [18].

The alternative/complementary approach to *in silico* strategies is phenotypic screening, in which compounds are screened *in vitro* against the whole organism. This most traditional technique offers increased chances of efficacy, potentially slow development of resistance, and the possibility to be used for the screening of natural products such as plant derivatives [19, 20]. A serious drawback of this approach is that *in vitro* screening can be performed, by definition, only on the parasite stages that can be grown *in vitro*. Consequently, for example, relatively few drugs are available to inhibit liver stage malaria parasites [21]. Three groups, from academia (St. Jude Children's Research Hospital) [22], industry (GlaxoSmithKline, GSK) [23], and academic/industry consortia (Novartis) [24] have reported the results of phenotypic HTS of compounds from chemical libraries, and the structures of thousands of active compounds have been deposited in public databases [25]. A number of other screenings are still ongoing, whose results have not been published yet [26].

This review discusses the problems and the up-to-date perspectives regarding new drugs against *Plasmodium, Leishmania* and *Trypanosoma* parasites, focusing on the molecules that are in the most advanced stage of development.
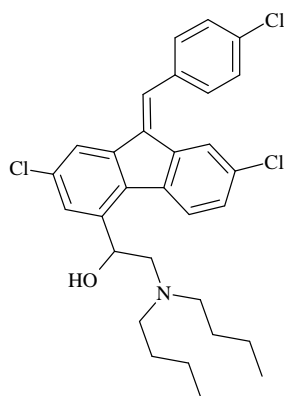
2. *PLASMODIUM*

Malaria parasites were discovered in 1880 by Alphonse Laveran (1907 Nobel Prize Winner), a French military doctor who identified motile forms in the blood of a febrile patient [27]. About 20 years later, Ronald Ross (1902 Nobel Prize Winner) clarified the transmission role of *Anopheles* mosquitoes. Among the human-infecting *Plasmodium* species, *P. falciparum* (*malaria tropica*) is responsible for the majority of malaria-linked deaths in sub-Saharan Africa, where it has been around for more than 6000 years, as demonstrated by the finding of its DNA in ancient Egyptian mummy tissues dating back to ~4,000 years ago [28]. *P. vivax* (*malaria tertiana*), endemic in tropical and subtropical areas of Asia, North and South America, in the Middle East, North Africa, and in the South Pacific, is traditionally considered more benign but it causes as much as 25-40% of the global malaria cases, and PCR detection methods show that *P. falciparum* and *P. vivax* co-infections may be as high as 50-65% in Thailand [29]. The low diffusion of *P. vivax* in sub-Saharan Western Africa and in Papua New Guinea is related to the lack of the Duffy glycoprotein antigen on the surface of red blood cells of the indigenous populations [30]. For a comprehensive review of the mechanisms of genetic resistance to malaria, correlated with hemoglobinopathies, erythrocyte polymorphisms, enzymopathies, and immunogenetic variants, the reader is referred to Lopez *et al.* [31]. The other two main human-infecting species are *P. ovale* and *P. malariae*, which represent a small percentage of infections. In addition, some cases of human malaria are due to the primate parasite *P. knowlesi*, common in Southeast Asia, which has a replication asexual erythrocytic cycle of about 24 hours [32].

Malaria is the fifth cause of death from infectious diseases worldwide (after respiratory infections, HIV/AIDS, diarrheal diseases, and tuberculosis), and it is the second leading cause of death from infectious diseases in Africa, after HIV/AIDS [33]. In the absence of an effective licensed vaccine, the global antimalarial strategy relies on a multi-faceted approach based on prevention (i.e. vector control and pharmacological prophylaxis), quick and reliable diagnostic procedures, and treatment with effective antimalarial drugs. The Malaria Eradication Research Agenda (malERA) initiative [34], created in 2007, re-established the long-term goal of malaria eradication, that, at least for now, is destined to remain elusive, since currently available drugs and protocols are mostly directed to treat patients by killing the parasite asexual stages rather than to prevent transmission by gametocyte elimination. Eradication attempts based on the blockage of malaria transmission should rely on gametocydal drugs. However, to test potential gametocydal drugs, both an improved gametocyte production method and a reliable assay to assess the compound gametocydal activity are required. In this

regard, new affordable and reliable techniques to grow malaria gametocytes *in vitro* to be used for drug activity assessment are currently being developed at GSK Tres Cantos laboratories [35].

In order to identify the optimal treatment for each malaria case, some key differences among *Plasmodium* species must be taken into account. Following the bite of an infected mosquito, the sporozoites injected into the skin (on average one hundred) travel from the dermis to the liver and penetrate into the hepatocytes, where by schizogony thousands of merozoites originate and are successively released into the bloodstream [36]. In the case of *P. vivax* and *P. ovale,* following hepatocyte infection some of the sporozoites do not replicate, but become dormant hypnozoites that may reactivate and cause relapses after latency periods of weeks, months or even several years [32]. Therefore, in these infections the availability of hypnozoite-killing drugs is crucial to eradicate the parasite from the host. A second difference among *Plasmodium* species concerns the replication cycle duration, which is reflected in the timing of the fever peaks (~48 hours for *P. falciparum, P. vivax* and *P. ovale,* ~ 60-72 hours for *P. malariae*). The third difference is the timing of the appearance of circulating gametocytes and their persistence into the bloodstream, especially relevant for transmission control. Whereas *P. falciparum* gametocytes appear several days after the initial parasitaemia, *P. vivax* gametocytes appear concurrently or even before asexual parasites. Therefore, the ideal drug for *P. vivax* eradication should kill all blood stages of the parasite, gametocytes included. For a detailed review of available treatments for *P. vivax*, see Price *et al.* [37]. Currently, malaria treatment relies on four families of drugs: quinolines and their derivatives, peroxides (artemisinin and its semisynthetic derivatives), antifolates, and the hydroxynaphthoquinone atovaquone [38]. In order to limit the emergence of resistance the WHO recommends 7 days treatments based on peroxides and antibiotics such as tetracycline, doxycycline, or clindamycin, or 3 days treatments based on peroxides and amodiaquine, mefloquine, sulfadoxine-pyrimethamine, or lumefantrine (Fig. **1**), a racemic fluorene derivative that conforms structurally, physicochemically and functionally to the quinolines [39].

lumifantrine

Fig. (**1**)

The treatment protocols should take into account the *Plasmodium* species and drug-resistance profile, which vary from place to place, and should be optimized in order to address severe malaria cases or uncomplicated malaria, including disease in special risk groups (young children, pregnant women, HIV-positive patients, travelers from non-endemic regions).

2.1. Quinine and quinine analogs

Quinine (Fig. **2**) is an alkaloid found in the bark of the cinchona tree native to the tropical Andes forests of western South America, introduced into Europe in the early 1600s. It rapidly kills intra-erythrocytic malaria schizonts of all species and gametocytes of *P. vivax* and *P. malariae*, but not those of *P. falciparum*. It also has analgesic, but not antipyretic properties. Quinine was the best antimalarial drug available until the 1920s, when more effective synthetic derivates became available, but its anti-malarial mechanism of action has not yet been fully resolved [40, 41].
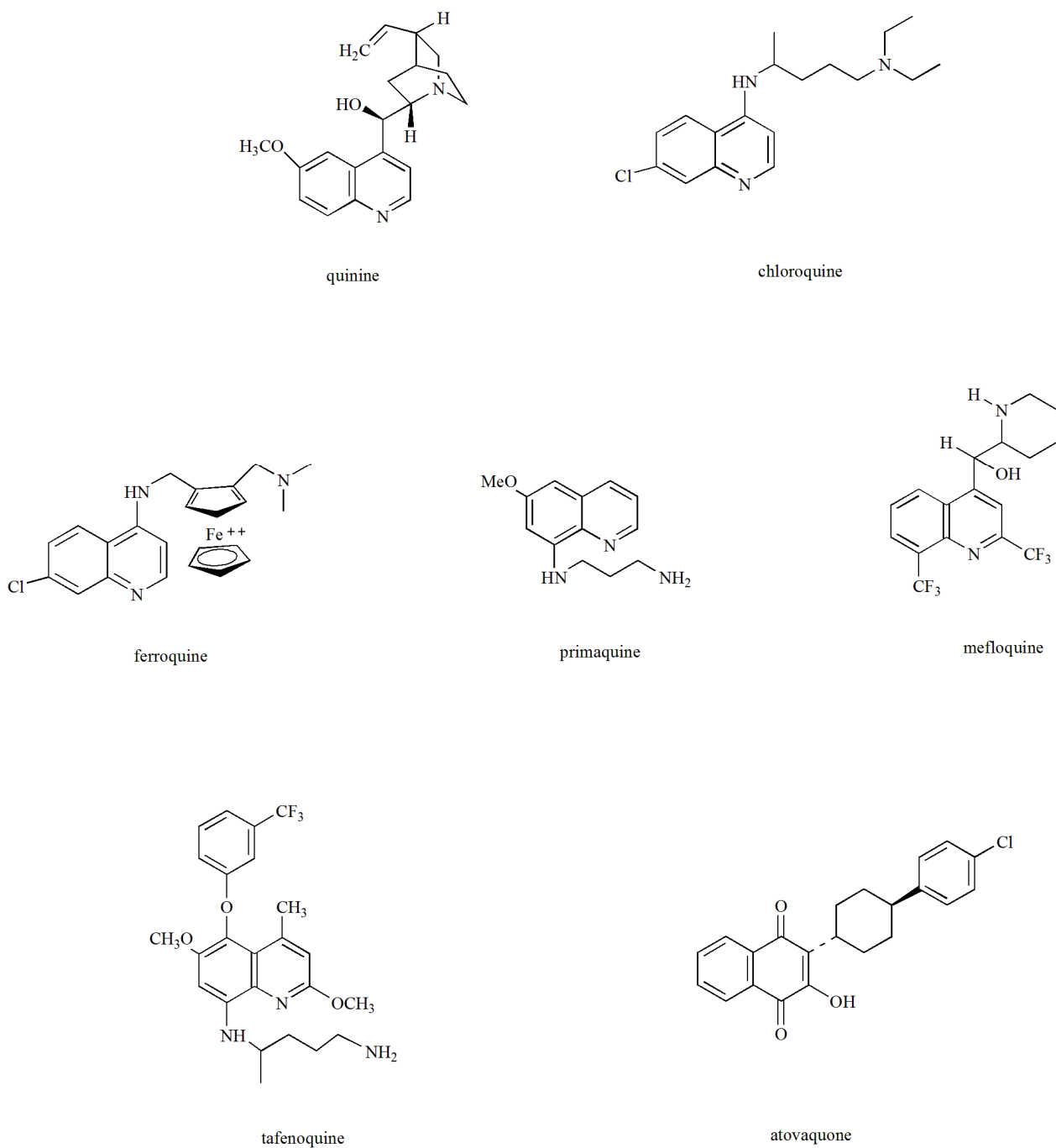
quinine

chloroquine

ferroquine

primaquine

mefloquine

tafenoquine

atovaquone

Fig. (**2**). Chemical structures of antimalarial drugs currently in use or in advanced stages of development.

The most successful quinine analog is chloroquine (Fig. **2**), a 7-chloroquinoline which was first introduced in the 1940s and quickly became the antimalarial drug of choice [42], thanks to its good efficacy and long half-life, coupled with low cost and low toxicity that makes it safe for children and pregnant women. Unfortunately, within about a decade the development and rapid spread of resistant strains caused the collapse of the chloroquine-dependent dream of global malaria

eradication, and today the WHO recommends that chloroquine for the treatment of *P. falciparum* malaria be avoided, except in specific areas where susceptible strains are still present [42]. Nonetheless, the initial chloroquine success prompted the feeling that, as Andrews *et al.* [43] put it, "chloroquine's safety and economic advantages are simply too strong to abandon", encouraging the development of two lines of research, both aimed to overcome parasite resistance. The first line pursues the production of analogs able to bypass resistance. The second exploits the linkage of chloroquine with molecules known as reversal agents or chemosensitizers, which, by inhibiting the enhanced chloroquine export from the digestive vacuole, thwart the basic process involved in chloroquine resistance [42]. Two generations of reversed chloroquine drugs have so far been developed, the first one based on existing molecules known for their ability to produce a chloroquine build-up inside parasites, such as verapamil and imipramine (Fig. **3**), the second and more recent one based on new modified molecules.
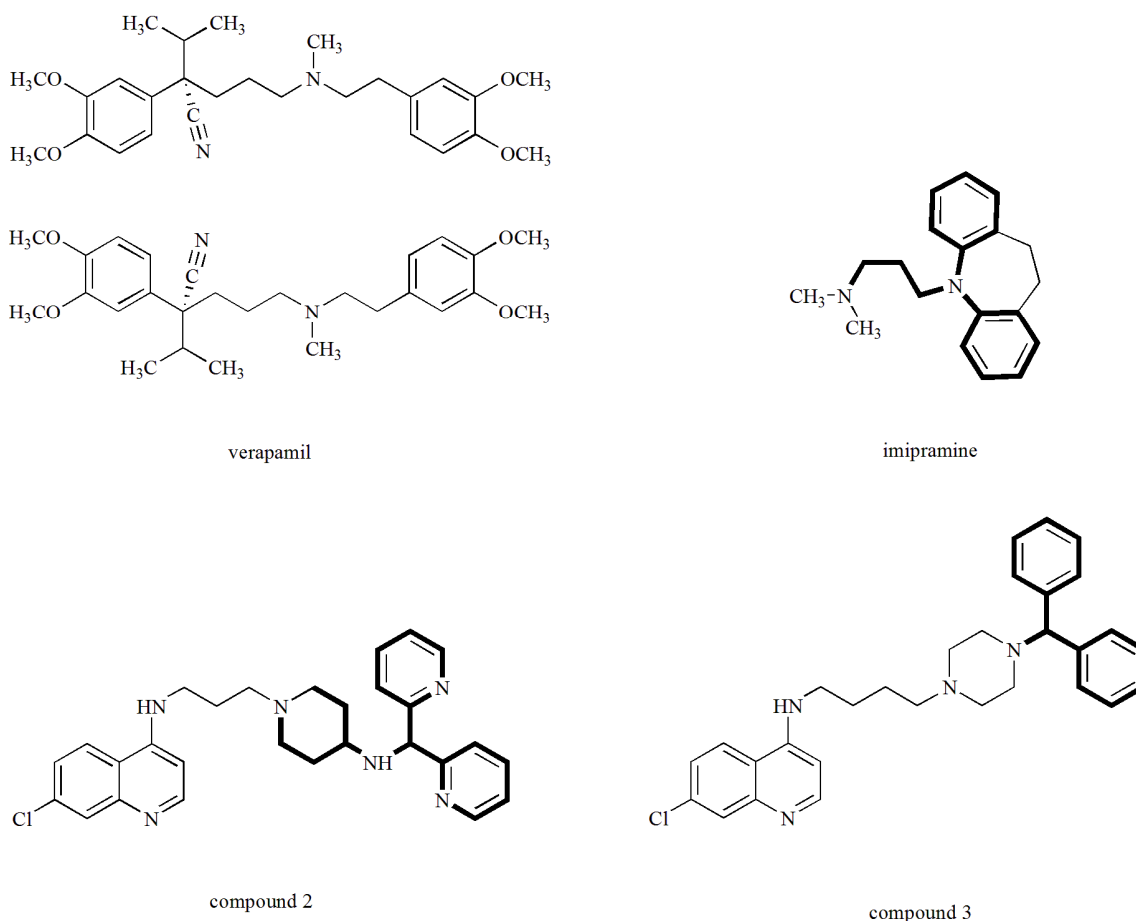

verapamil

imipramine

compound 2

compound 3

Fig. (**3**). Chemical structures of chloroquine chemosensitizer drugs.

Among these, as reported by Peyton [44], compounds 2 and 3 (Fig. **3**) showed good activity both *in vitro* and in murine experimental models. The search for modified and more active chloroquine derivatives led to the development of

ferroquine (Fig. **2**) [45], a second generation 4-amino-quinoline, which is currently the most advanced organo-metallic drug candidate that just completed phase I clinical trials as a treatment for uncomplicated malaria, being active against both chloroquine-susceptible and chloroquine-resistant strains of *P. falciparum* and *P. vivax*. Due to its long half life and activity, ferroquine, together with an appropriate partner drug, might qualify for the development of a single-dose antimalarial combination treatment. These promising properties need, however, further evaluation in clinical phase II and III studies [46].

Two other quinine analogs are currently in use: primaquine and mefloquine (Fig. **2**). Primaquine, an 8-aminoquinoline, is the only approved drug against *P. vivax* and *P. ovale* hepatic stages, including both hypnozoites and parasites acutely infecting the liver. It can be used to achieve radical cure of *P. vivax* infections thanks to its ability to kill both the gametocyte and hypnozoite stages. It is thought to be converted in the liver to an active quinone metabolite acting on the parasite mitochondria. This mechanism of action may be related to that of napthoquinones such as atovaquone (Fig. **2**), which was shown to inhibit the cytochrome bc1 complex of the mitochondrial respiratory chain and collapse the mitochondrial membrane potential [40]. Patients with an attack of *vivax* malaria and parasitized red blood cells should receive a course of chloroquine, which rapidly destroys the erythrocytic parasites and terminates the paroxysm, and a concurrent primaquine administration in order to eradicate liver hypnozoites and circulating gametocytes. Primaquine would be a very good agent for causal prophylaxis of *vivax* and *falciparum* malaria, but some of its properties, such as the short elimination half-life and the ability to induce severe haemolysis in glucose-6-phosphate dehydrogenase(G6PD)-deficient subjects, diminish its therapeutic utility and hamper its use for prophylaxis [47].

Tafenoquine, also known as WR 238605 (Fig.**2**), is a new 8-aminoquinoline that was developed by the Walter Reed Army Institute of Research and GSK in the search for a safer, more effective and longer acting replacement for primaquine. Tafenoquine possesses greater activity against all stages of malaria parasites, and thanks to its long elimination half-life (2-3 weeks), it can be used both for *P. falciparum* and *P. vivax* chemoprophylaxis, and for *P. vivax* residual liver parasites eradication [47]. It is currently in phase IIb/III clinical trials. A clinical study initiated in 2009 and continuing in 2012 has been undertaken by MMV and GSK to assess the dose safety in G6PD heterozygous subjects, aimed at identifying the maximum safe dose. The efficacy and safety of tafenoquine is also currently being assessed in a Phase IIb clinical study commenced in Peru and Thailand by using a loose combination of tafenoquine and chloroquine [48]. Researchers at the Walter Reed Army Institute of Research are testing a series of novel 5-aryl-8-aminoquinoline derivatives, aimed at separating the antimalarial activity of tafenoquine from its haemolytic side effects in G6PD-deficient patients [49].

Mefloquine, belonging like quinine to the quinolinemethanol family, is indicated for the treatment of mild to moderate acute malaria caused by mefloquine-susceptible strains of *P. falciparum* (both chloroquine-susceptible and -resistant strains) or by *P. vivax*. Mefloquine is also indicated for the prophylaxis of *P. falciparum* and *P. vivax* infections, including prophylaxis of *P. falciparum* chloroquine-resistant strains.

The main defects of the quinine-family molecules are the ability to select resistant parasite strains, documented for *P. falciparum, P. malariae* and *P. vivax*, and a plethora of negative side-effects mostly involving the gastrointestinal, nervous, and respiratory systems. Nevertheless, in the near future quinine will continue to play a significant role, particularly in limited-resource settings, where less toxic but more costly drugs (i.e. peroxides) are not available, and in the management of malaria in the first trimester of pregnancy [41]. For comprehensive reviews on the currently used antimalarial drugs the reader is referred to Kappagoda *et al.* [50] and Wells *et al.* [32].

2.2. Artemisinin and its derivates

Artemisinin (Fig. **4**) is a sesquiterpene lactone containing an unusual peroxide bridge that is believed to be responsible for the drug's mechanism of action. The natural molecule, isolated from the plant *Artemisia annua* (sweet wormwood), and its semi-synthetic derivatives artemether, artesunate and dihydroartemisinin (Fig. **4**) are potent antimalarial drugs. However, a decline of the efficacy of artemisinin and artesunate monotherapy due to the emergence of resistant strains was detected in Western Cambodia [51], in the Thai-Cambodian border [52], and in the Thailand-Myanmar border [53].
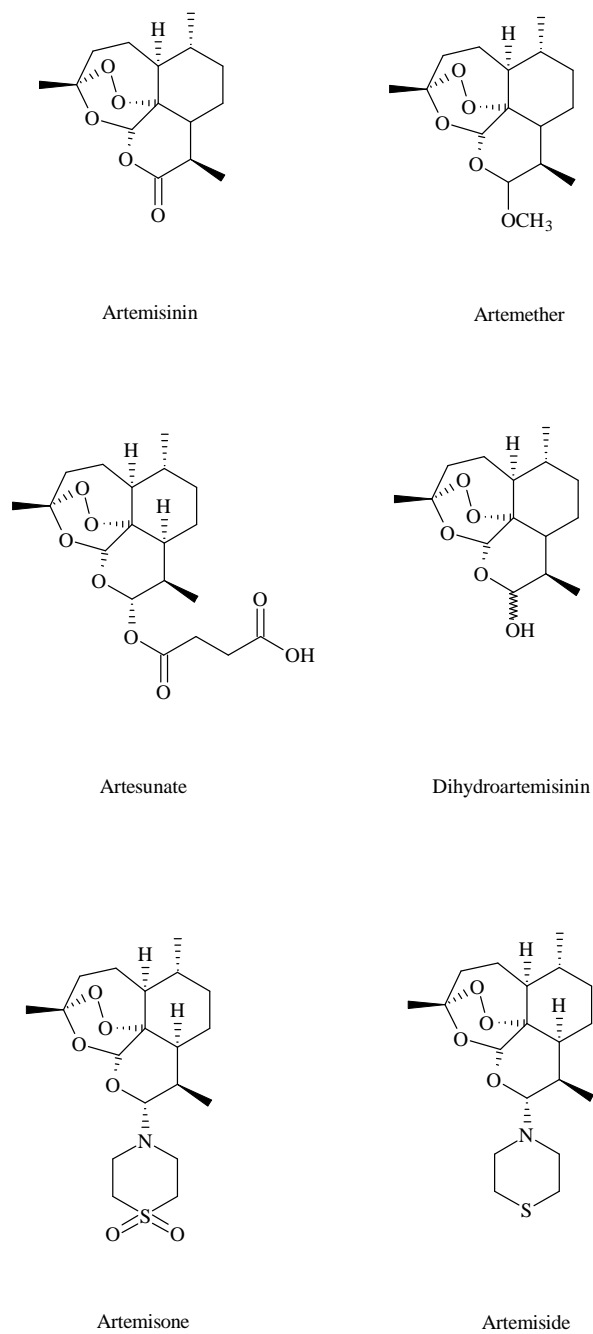
Fig. (**4**). Chemical structures of antimalarial peroxides.

Since the spread of artemisinin resistance would be disastrous for global malaria control, the WHO strongly

recommends the association of artemisinin with other antimalarials [54, 55]. The Artemisinin-based Combination Therapies

(ACT), active on the asexual blood stages of *P. falciparum*, are assumed to also be fully active against the other

Plasmodium species and are currently considered the treatment of choice for malaria tropica, being rapidly effective and

well tolerated by patients. However, in practice artemisinin is in short supply and remains unaffordable for most people living in malaria-endemic zones, because the artemisinin content of the plant is low and the chemical synthesis of the compound is complex and uneconomic. In 2005 the estimated demand for the fixed combination drug artemether-lumifantrine, containing the dose of artemether required to cure a malaria patient (i.e. 500 mg) was for 120 million adult treatment courses, corresponding to approximately 114 tons of artemisinin, given that the overall chemical yield of artemether from artemisinin is 53% [56]. Following WHO recommendations in 2001, within 8 years 88 countries, including all African countries, had officially adopted ACTs as first-line treatment for uncomplicated malaria. However, only 14 countries report distributing enough ACTs to treat at least 50% of reported malaria cases in the public system, and only 5 countries reported distributing enough ACTs to treat all cases in 2008 [57]. For a review of cost-effectiveness of malaria treatment and prophylaxis, see van Vugt *et al.* [58].

Many attempts are underway to enhance the yield of artemisinin through traditional breeding and *in vitro* plant tissue cultures or heterologous expression systems involving the use of genetically-modified microbes [59]. So far, financial considerations have limited the development of artemisinin derivatives and no second generation artemisinin derivatives are yet in use [60]. The most promising molecules, artemisone (BAY 44-9585) and artemiside (Fig. **4**), are semisynthetic 10 alkylaminoartemisinins that can be synthesized from dihydroartemisinin [61]. Both molecules showed very promising activity in the treatment of cerebral malaria in a murine model [62]. Artemisone, that possesses a longer half-life, lower curative dose, and superior bioavailability compared with first generation artemisinin derivatives, was shown to be devoid of neurotoxicity in human preclinical testing [63].

2.3. Antifolates

The first-generation antifolates, pyrimethamine and cycloguanil (Fig. **5**), were developed in the 1940s as antimalarial drugs that targeted the parasite's dihydrofolate reductase (DHFR) blocking the parasite DNA and protein synthesis, thereby inhibiting parasite growth. The sulfadoxine-pyrimethamine combination was widely used in antimalarial therapy because it is inexpensive, relatively safe and effective, as it requires single-dose treatments [64]. However, single-point mutations of DHFR can cause parasite resistance, and since the use of the chloroquine + sulfadoxine/pyrimethamine combination for the treatment of uncomplicated malaria has not been discontinued despite the introduction of ACT, antifolate-resistant *P. falciparum* strains are common in Bangladesh, Thailand and Cambodia [65]. The use of probenecid (Fig. **5**) to increase the efficacy of the sulphadoxine/pyrimethamine association for intermittent preventive treatment in pregnancy was proposed, however additional information is needed on its safety and pharmacokinetics in pregnancy [66].

Empirical drug screenings by the Walter Reed Army Institute of Research led to the discovery in the mid 1960s of second-generation antifolates, namely WR99210 (Fig. **5**), which possess marked activity against both pyrimethamine- and chloroquine-resistant isolates of *P. falciparum* [67].

pyrimethamine

cycloguanil

probenecid

WR99210

PS-15

JPC-2056

JPC-2067

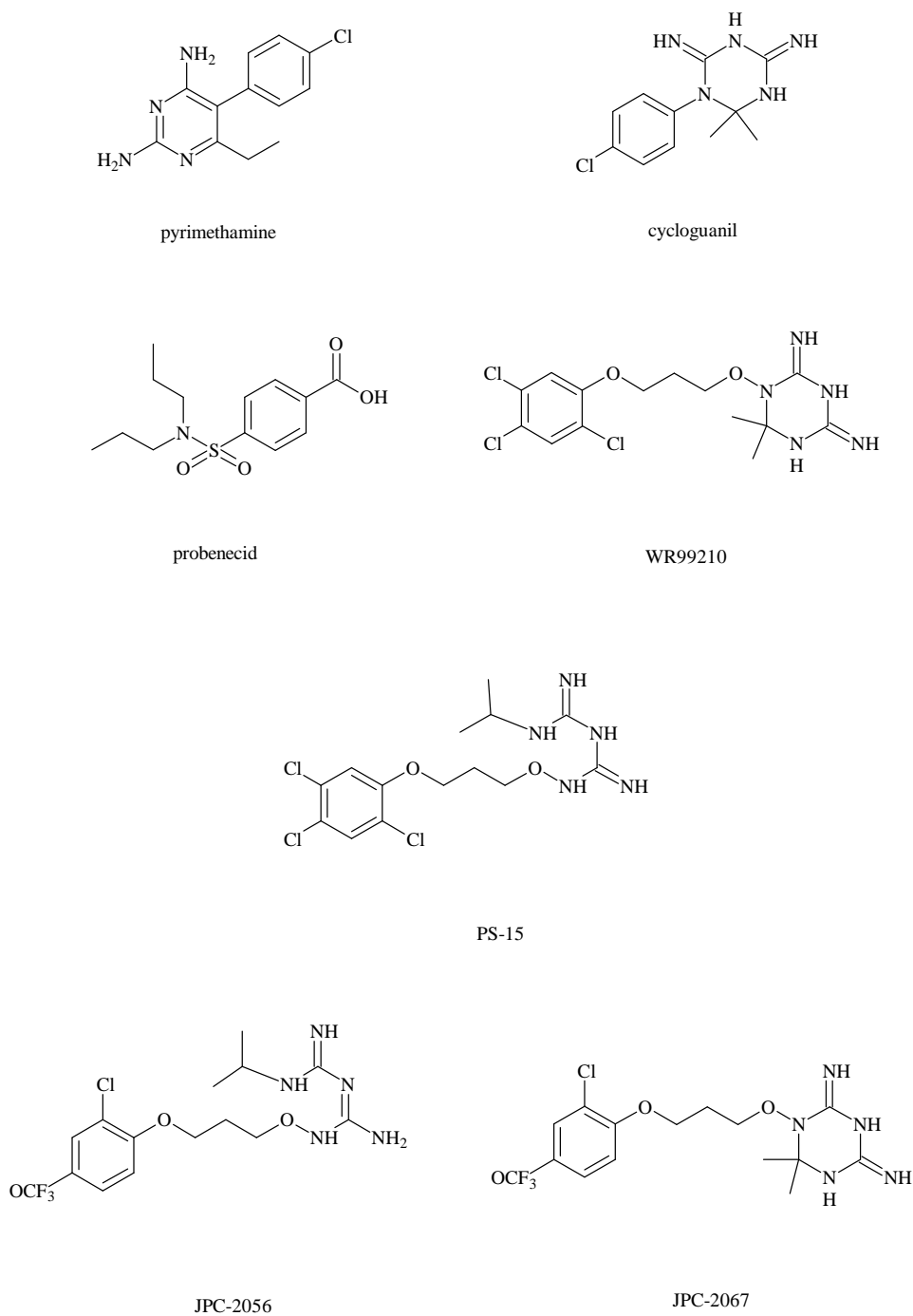Fig. (**5**). Chemical structures of antifolates.

However, the development of the drug was abandoned, because it showed poor bioavailability and during clinical trials induced severe gastrointestinal symptoms in volunteers [47]. In an effort to circumvent these problems PS-15 (Fig. **5**), the phenoxypropoxybiguanide precursor for WR99210, was synthesized [68]. However, further assessment of PS-15 was suspended because of regulatory restrictions in the use of the starting material, i.e. 2,4,5-trichlorophenol, used to produce PS-15. To overcome the safety and regulatory restrictions associated with PS-15 production, third-generation phenoxypropoxybiguanide prodrugs such as PS-26, JPC2005 and JPC2056 (Fig. **5**), with their corresponding active dihydrotriazine metabolites, were synthesized. Of these compounds JPC2056, which is metabolized to JPC2067 (Fig. **5**), was selected as the lead candidate for preclinical development [69]. JPC2067 is currently being developed for the treatment of infections from *P. falciparum*, *T. gondii* and other non-protozoal pathogens, such as *Mycobacterium* and *Nocardia* spp [70]. Research aimed at the identification of new antifolates is in the stage of lead identification, mostly performed by preliminary assessment of the most promising molecules by molecular docking, followed by *in vitro* antimalarial evaluation. Recently structural and mutagenic studies on the *P. falciparum* bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) led to the definition of the features essential for achieving effective inhibition, and to the identification of hybrid phenyl thiazolyl-1,3,5-triazine analogues as promising molecules [71].

2.4. Atovaquone

Known at first for its activity against *Pneumocystis carinii* (now *P. jiroveci*) and *T. gondii*, atovaquone (Fig. **6**) was further developed as an antimalarial drug and finally introduced on the market in 1997 as a fixed-dose combination with proguanil (Fig. **6**), for the treatment and prophylaxis of multidrug-resistant *P. falciparum* malaria in areas endemic with chloroquine resistant strains.
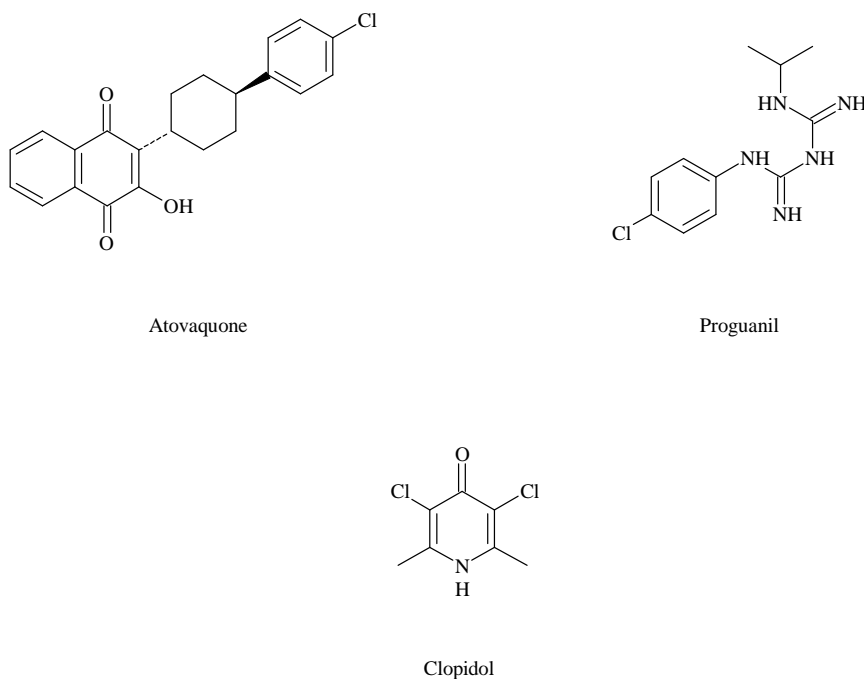
Atovaquone

Proguanil

Clopidol

Fig. (**6**). Chemical structures of drugs targeting cytochrome *bc*1

The need for the combination was due to the rapid development of resistance when atovaquone was used alone [72]. Proguanil, a biguanide, enhances atovaquone activity, thereby reducing the likelihood of resistance emergence, by a mechanism that needs to be clarified. Both atovaquone and proguanil are active against the hepatic and the erythrocytic stages of the parasite, but despite its effectiveness the high cost of the combination prevented its widespread use in malaria-endemic areas, especially sub-Saharan Africa [73]. Atovaquone is a hydroxynapthoquinone which targets the parasite respiratory chain by inhibiting mitochondrial electron transport through the cytochrome *bc*1 complex. Because of its poor pharmaceutical properties, such as low bioavailability and high plasma protein binding, a series of attempts, so far unsuccessful, aimed at improving its bioavailability were undertaken (for review, see [74]). The discovery of the action mechanism of atovaquone prompted the search for other molecules targeting the *P. falciparum* mitochondrial respiratory chain. Along this line, a GSK team is developing a series of 4-pyridones related to the anti-coccidial drug clopidol (Fig. **6**). This family of cytochrome *bc*1 inhibitors seems the most promising, but a number of these molecules showed high toxicity in clinical trials. However the very fact that they reached clinical trials indicates that their development is worthwhile [74].

2.5. Antibiotics

The antimalarial activity of antibiotics such as chloramphenicol and tetracyclines was serendipitously discovered in the late 1940s, but because of their slowness of action, this class of molecules was not considered clinically useful

(reviewed in [75]). However, if antibiotics were no match for chloroquine in the early1950s, the worldwide spread of chloroquine-resistant *P. falciparum* in the 1960s led to the re-evaluation of their use in the 1970s. In 1983 Geary and Jensen [76] by *in vitro* studies demonstrated that the antimalarial activity was a prerogative of antibiotics that interfere with protein or nucleic acid synthesis, but not of those that disrupt cell wall synthesis. They also observed that the killing effect became evident after 96 h of treatment, a period corresponding to two full erythrocytic cycles (delayed-death effect). More recent studies suggest that these antibiotics act by disrupting essential functions of the apicoplast, a vestigial, nonphotosynthetic remnant of a chloroplast, derived from a red alga that resided symbiotically within a protozoan ancestor of the *Apicomplexa* [77]. This intracellular organelle features a 35-kb cyanobacterial genome (reviewed in [77, 78]). Parasites treated *in vitro* for few hours with clinically relevant concentrations of tetracyclines or clindamycin were able to fulfill a first erythrocytic cycle with release of merozoites that invaded new erythrocytes and completed most of a second cycle of development, forming multinucleated schizonts. However, these schizonts appeared grossly abnormal and were unable to form functional merozoites capable of rupturing the host cell [79]. It seems that these antibiotics block some apicoplast housekeeping functions, such as the synthesis of type II fatty acids, iron-sulfur clusters, heme and isoprenoids needed for mitochondrial functions, resulting in non-functional apicoplasts in developing progeny [80].
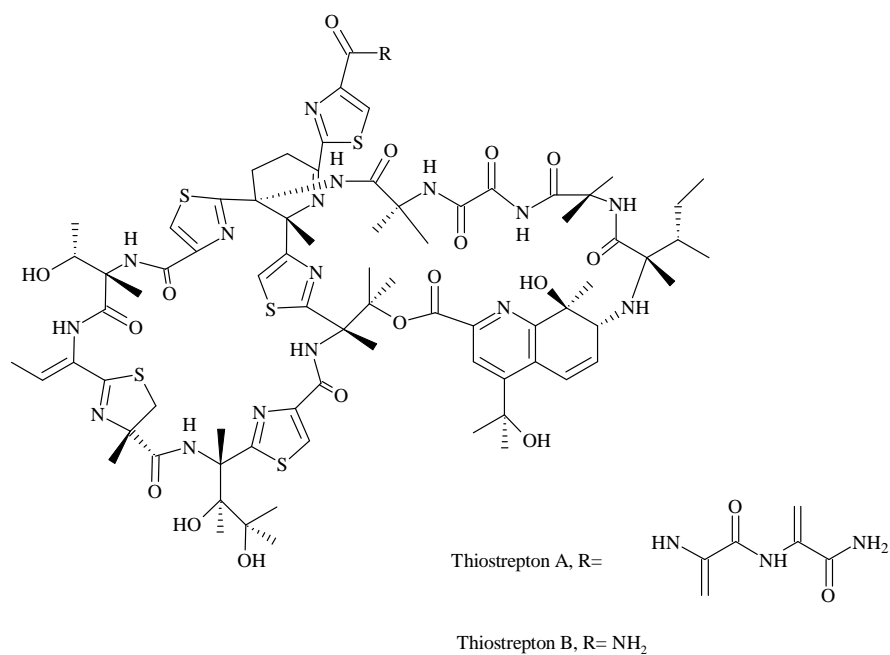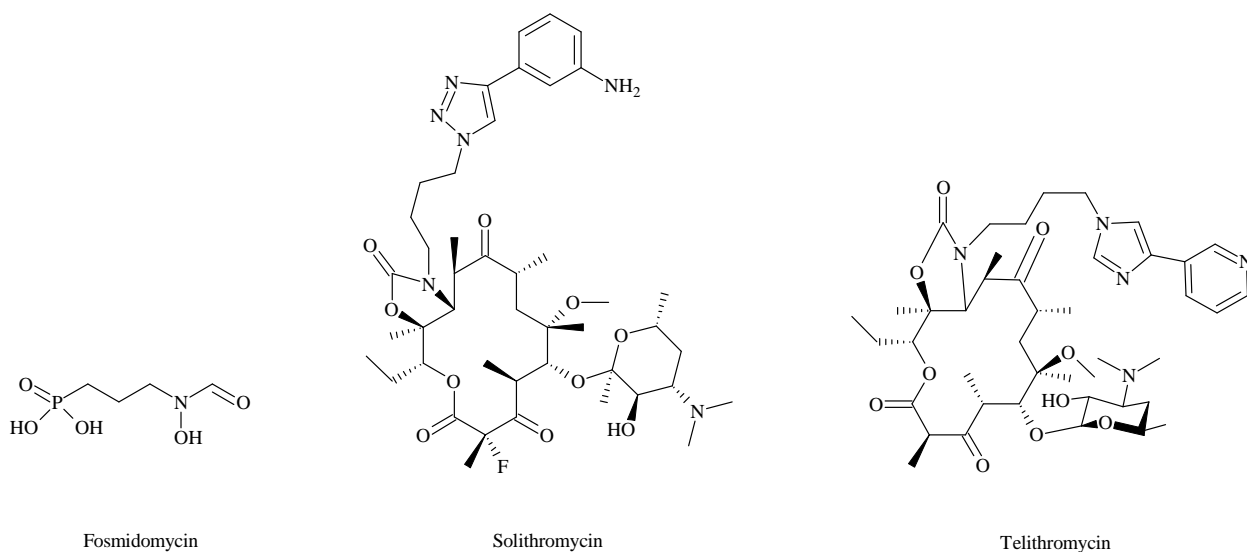
Fosmidomycin

Solithromycin

Telithromycin

Thiostrepton A, R=

Thiostrepton B, R= NH$_2$

Fig. (**7**). Chemical structures of antibiotics with antimalarial activity.

Fosmidomycin (Fig. **6**), isolated in the 1970s as a natural antibiotic from S*treptomyces lavendulae* and currently produced by chemical synthesis, was considered a promising antimalarial agent and a possible alternative to artemisinins since the discovery of its inhibitory action on 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme of the non-mevalonate pathway of isoprenoid biosynthesis that is absent in humans [81]. Fosmidomycin does not display the delayed-

death effect, showing a rapid onset of action, but it is not gametocydal. Its relatively weak activity can be improved by combining it with clindamycin. *In vitro* synergistic activity of the fosmidomycin/clindamycin combination against various strains of *P. falciparum* was demonstrated, and clinical trials confirmed the safety and efficacy of fosmidomycin alone or combined with clindamycin for the treatment of uncomplicated *P. falciparum* in adults. However, recent clinical trials of the fosmidomycin/clindamycin combination on children <3 years of age gave disappointing results that warrant further investigation [82].

Recently, it was shown that some thiopeptide antibiotics which affect malaria apicoplast causing the delayed-death effect can also rapidly kill the parasite by targeting the cell proteasome. Rapid parasite killing was already observed by Goodman *et al.*, who in 2007 demonstrated that thiostrepton (Fig. **7**) shared the immediate killing effect with ciprofloxacin and rifampicin, but the mechanism involved was not fully investigated [83]. Protein regulation is important for the parasite because of its high replication rate in the human liver and blood stages. Moreover, the temperature shift to which the parasite must rapidly adapt when it moves back and forth between humans and mosquitoes induces a stress response that is managed by the ubiquitin-proteasome system. *In silico* predictions indicate that over half of the parasite proteins represent targets for ubiquitination [83, 84]. Therefore, proteasome inhibitors are considered promising antimalarial agents. An essential role of the *Plasmodium* proteasome has been demonstrated for the liver, blood and gametocyte stages [83, 85-91]. Thiostrepton ($C_{72}H_{85}N_{19}O_{18}S_5$), first isolated from *Streptomyces azureus* in 1954 and later also from *Streptomyces laurentii*, is often referred to as the parent compound of the thiopeptide antibiotics (for review, see [92]). It is effective against Gram-positive bacteria with activity comparable to that of the penicillins, but it was not developed for clinical use as antibacterial because of its low aqueous solubility and consequent early induction of resistance [92]. Thiostrepton, as well as other antibiotics such as azithromycin, telithromycin (Fig. **7**), and tetracyclines, inhibits the prokaryotic-like translation machinery of the malaria apicoplast, resulting in delayed death. However, thiostrepton and some of its more active newly generated derivatives also target the *P. falciparum* 20S proteasome, resulting in rapid parasite elimination prior to DNA replication. Semisynthetic thiostrepton derivatives seem highly promising, being fast-acting molecules which are not subject to rapid development of resistance, because their gametocytocidal activity prevents the transmission of resistance-conferring genotypes [83].

The use of broad spectrum antibiotics with antimalarial activity, such as azithromycin, clindamycin and doxycycline, in combination with quinine could be recommended for the parenteral treatment of severe malaria cases in hospitalized patients [93]. A clindamycin-quinine combination is recommended by the WHO as the drug of choice for the oral treatment of *P. falciparum* infection in the first trimester of pregnancy, as a second-line drug following first-choice

treatment failures, for malaria in the second and third trimesters of pregnancy, for travelers and for the oral phase of severe malaria treatment [94]. The efficacy of this combination versus other anti-malarial drugs in the treatment of uncomplicated *falciparum* malaria is thoroughly reviewed in [95]. Pfizer and MMV are working together to develop a fixed-dose combination tablet of azithromycin and chloroquine (AZCQ) for Intermittent Preventative Treatment in Pregnant Women. The combination is designed to be synergistic against chloroquine-resistant strains of *P. falciparum* and showed efficacy in the treatment of symptomatic malaria in two multi-center clinical trials performed on adult subjects in sub-Saharan Africa. Both azithromycin and chloroquine were shown to be safe in children and pregnant women over a number of years. Furthermore, the activity of azithromycin against common sexually transmitted infections may provide additional benefit in improving pregnancy outcomes [48]. In this regard, the effect of antibiotics on the host bacterial flora must be considered: if in some cases antibiotics may be useful for their activity on bacterial co-infections or on malaria-unrelated bacterial fevers that in endemic areas lacking proper diagnostic facilities could be misdiagnosed as severe malaria, in all other cases, especially when widely used as oral formulations for therapy and prophylaxis, they may select unwelcome resistant bacterial strains [96].

Among new antibiotics solithromycin (Fig. **7**), a next-generation oral and intravenous fluoroketolide macrolide effective against malarial parasites, can be considered a promising partner drug in a combination therapy. Unlike telithromycin, solithromycin does not contain a pyridine moiety, which was shown to inhibit nicotinic acetylcholine receptors and is a potential cause of the severe adverse events associated with telithromycin (Ketek effects). In a murine model of *P. berghei* infection solithromycin monotherapy had cure rates similar to that of artesunate. Several phase I studies showed that solithromycin is well tolerated, and this compound recently completed a successful phase II trial for the treatment of community-acquired bacterial pneumonia [97].

2.6. New approaches

Plasmodium genome sequencing brought into light a vast amount of information regarding protein hypothetical functions in both the human host and mosquito vector [98]. Since the 1980s and until the mid-2000s the development of new antimalarials was carried out trying to modify existing chemotypes or to assess the validity of new targets identified through genetic and/or *in silico* insights [26]. However, these strategies did not lead to the deployment of new antimalarials, and have been generally recognized as unsatisfactory because of the small number of available scaffolds, for the lack of whole-cell activity, and/or for the rapid *in vitro* development of resistance [23, 26]. Therefore, to identify novel antimalarial leads major pharmaceutical firms and academic institutions chose to screen large chemical libraries using high throughput,

whole cell screening assays based on *P. falciparum* proliferation. In recent years, thousands of new molecules with potent

activity against *Plasmodium falciparum* were identified and information about them publicly released. The St. Jude

Children's Research Hospital group performed the screening of more than 300,000 compounds against intra-erythrocytic *P.*

falciparum identifying 172 representative molecules, whose structure and biological activity were disclosed to provide the

scientific community with new starting points for further investigation [22]. Screening a GSK proprietary 2,000,000

compound chemical library, Gamo *et al*. identified about 11,500 validated hits, referred to as the Tres Cantos Antimalarial

Set [23]. Among these, a new chemical class of inhibitors, designated cyclopropyl carboxamides, which possess potent

inhibitory activity against *P. falciparum* strains and are effective by oral administration in malaria mouse models, were

considered for lead optimization. However, they showed an unexpected propensity to select for resistant parasites, and this

problem, if not solved, will hamper further development [99]. By using the same type of whole cell screening assay,

Novartis scientists identified spiroindolones as effective antimalarials which act by rapidly suppressing the parasite protein

synthesis [100]. Spiroindolones represent a family of molecules that being based on a different molecular scaffold are active

on parasite strains resistant to currently used antimalarials. The compound NITD609 (Fig. **8**), a spirotetrahydro-β-carboline,

has good drug-like attributes and displays physicochemical properties compatible with conventional tablet formulation; it is

as effective as artesunate, with potency in the low nanomolar range against all *P. vivax* and *P. falciparum* isolates.
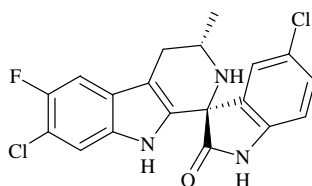

NITD$_{609}$

Fig. (**8**).

NITD609 is also similar to artesunate in its ability to kill both mature trophozoite and immature *P. vivax* ring stages

[101]. The ability to inhibit gametocytogenesis makes NITD609 a good candidate for malaria prophylaxis and, thanks to its

potential for transmission reduction, also for a global malaria-eradication program. Further safety and pharmacological

preclinical evaluations are currently ongoing to support the initiation of human clinical trials [102]. A second class of

molecules, i.e. imidazolopiperazines, still in the stage of hit to lead optimization, are being developed at Novartis

laboratories [103]. At Merck Research Laboratories, the screening of the proprietary compound collection through the *P.*

falciparum 3H-hypoxanthine assay and the application of semi-automated technology, resulted in the discovery of MK-

4815 (Fig. **9**), a compound with potent *in vitro* activity against multidrug-resistant *P. falciparum* and able to cure *P. berghei* infected mice by oral administration [104].
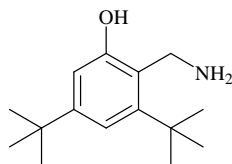
MK-4815

Fig. (**9**).

The compound, a 2-aminomethyl-3,5-di-*tert*-butylphenol, belongs to the Mannich base family of compounds, previously reported to have various degrees of antimalarial activity [105]. The mechanism of action of MK-4815 has not been elucidated yet, but the available data show that short-term exposure (4 to 7 h) can kill mature *P. falciparum* parasites *in vitro,* probably due to MK-4815 selective accumulation in late stage parasites. MK-4815 is undergoing further evaluation and was accepted as a preclinical candidate by the MMV [104]. High priority molecules in the lead optimization, preclinical or phase I development stage are thoroughly reviewed in [26].

2.7. Natural products

Traditional plants are still widely used as a first-line treatment especially in rural areas of malaria endemic countries [106-108]. However, clinical research in this field is scanty and underfunded, probably due to practical and theoretical concerns such as biodiversity, sustainable use and conservation of the plant extracts, and to legal concerns regarding property rights and the equitable distribution of economical benefits [109]. A partnership between the Global Initiative For Traditional Systems (GIFTS) of Health at the Oxford University (UK) and international researchers working on traditional medicine for malaria gave origin in 1999 to the Research Initiative on Traditional Antimalarial Methods (RITAM), funded by the Rockefeller Foundation, the Multilateral Initiative on Malaria (MIM) and the Tropical Disease Research (TDR) Division of the WHO. Currently RITAM has a network of over 300 scientists, organised into four specialist groups, covering pre-clinical and clinical assessment of traditional medicinal plants used for malaria treatment, insect repellents and vector control, and its objectives include the review of current knowledge on traditional anti-malarial

methods, determination of research priorities, the design ofoptimal research methodologies, and the avoidance of research replication [110].

The study of traditional plant remedies is complicated by the need to collect a series of ethnobotanical informations (location and abundance of the plant, parts used, form of use, duration of treatment), and by the problems in dosage definition due to variations in the concentrations of active ingredients linked to harvest time, storage and preparation modalities [111]. So far, the remarkable amount of published ethnobotanical and pharmacological studies on the more than 1200 known anti-malarial plants would need a systematic review, but a standard procedure is lacking [112, 113]. The development of a score system to select and prioritize plants for further research as anti-malarial drug candidates is in progress [112], and the reader interested in detailed lists of plant-derived antimalarial agents identified in the last decade is referred to a series of existing exhaustive reviews [109, 111, 114, 115]. Given the abundance of natural molecules with antimalarial properties, what is needed is the willingness of private firms or public institutions to bear the costs of further investigation. Most natural bioactive molecules are susceptible to chemical modification to give origin to antimalarial drugs based on new molecular scaffolds, possibly active against *Plasmodium* multidrug resistant strains. This approach is more attractive to the pharmaceutical industry than the use of crude extracts, offering more patenting opportunities. Nowadays the chemistry of natural products is considered a highly promising provider of new leads for drug discovery [116], following a standby period that lasted from the end of the 1980s until recent years [117]. A great number of natural molecules with antimalarial activity have been and are being identified, and all of them, in the words of their discoverers, are worthy of "further investigation" as antiplasmodial agents. By way of example here we mention β-lactams, which besides their antibacterial activity acquired a prominent place in organic chemistry as synthetic building blocks (synthons) for further elaboration [118]. Following the initial observation that 1-aminopropan-2-ols are active against *P. falciparum* in the micromolar range [119], a library of gamma-aminoalchools was produced through the ring opening of *cis*-3-alkoxy-4-aryl-β-lactams [120]. These compounds also showed antimalarial activity *in vitro*. By means of a different approach, i.e. the direct *in vitro* screening of a number of marine sponge extracts, monamphilectine A (Fig. **10**), a new diterpenoid β-lactam alkaloid with potent antimalarial activity, was isolated from *Hymeniacidon* spp. Puerto Rican marine sponges [121].
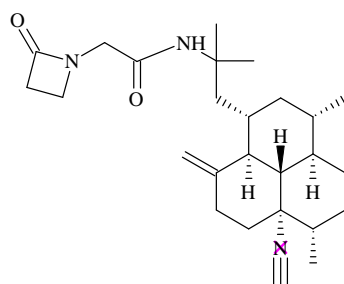
Monamphilectine A

Fig. (**10**).

The identification of active constituents in plant extracts can be performed by a two-step miniaturized procedure: the screening of extract libraries for antiplasmodial activity is followed by the active compound identification, performed by means of HPLC-based activity profiling, a technique introduced in 2001 [122] and successively optimized for antiprotozoal drug discovery [123]. This procedure was recently used to screen a library of 254 extracts from European plants [124].

3. *TRYPANOSOMATIDAE*

The family *Trypanosomatida* includes flagellated parasitic protozoa belonging to the genera *Trypanosoma* and *Leishmania*, responsible for sleeping sickness, Chagas disease and leishmaniasis. The clinical management of these infections is based on chemotherapeutics that are far from the ideal because of cost, toxicity and resistance problems. Unfortunately, the urgent need to identify and develop new therapeutic alternatives comes up against the lack of funds for R&D. To facilitate target-focused analyses for pathogens prioritized by the WHO Special Programme for Research and Training in Tropical Diseases (TDR), the database TDRtargets.org was created as a central repository of target-related data designed to facilitate multiple approaches to target prioritization [125].

3.1. Leishmaniae

Leishmaniae constitute a genus of dixenic *Kinetoplastida* protozoa, which are present as flagellated promastigotes in the sandfly vector and as aflagellated sferoidal amastigotes within the mammalian host macrophages. The term leishmaniasis denotes a spectrum of clinical manifestations including the visceral form (VL), in which the parasite colonization of bone marrow, liver and spleen results in host immunosuppression and death in the absence of treatment, and the cutaneous (CL) and mucocutaneous (MCL) forms, in which parasites remain localized in the epithelial tissues. Further distinctions can be made according to the species of the parasite, of the insect vector and of the reservoirs. Anthroponotic VL is caused by

Leishmania donovani, whereas zoonotic VL is caused by *L. infantum*; antroponotic CL is caused by *L. tropica*, and zoonotic CL is caused by *L. major*. The vector species belong to the genera *Phlebotomus* and *Lutzomyia*, whereas the reservoir species vary with the geographic region and include wild rodents, wild and domestic carnivores, and humans. Leishmaniasis is endemic in Southern Europe, Middle East and Asia, Africa and Central and South America. According to the 2012 WHO update on leishmaniasis [126], approximately 200,000 to 400,000 VL cases and 700,000 to 1.2 million CL cases occur globally each year, with 12 million humans infected and 350 million at risk of infection. It is estimated that 90% of all VL cases occur in Bangladesh, Brazil, India (Bihar), Nepal and Sudan, 90% of MCL occurs in Bolivia, Brazil and Peru, and 90% of CL cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. VL infection does not necessarily lead to clinical disease: most infections remain asymptomatic, but malnutrition and immune suppression, notably HIV [127, 128], predispose to clinical disease. A tentative estimate of mortality suggests the occurrence of 20,000 to 40,000 VL deaths per year. Despite the above numbers, leishmaniasis fully belongs to the group of Neglected Tropical Diseases. In 2007, the World Health Assembly approved a resolution on the control of leishmaniasis that enabled WHO to take a leading role in providing technical assistance for the initiation, maintenance and expansion of leishmaniasis control programmes. An Expert Committee convened in Geneva in 2010 reached the conclusion that adequate worldwide leishmaniasis control is feasible with the medicines and diagnostic tools currently available [129]. However, the same Committee recognized that there is a crucial lack of funding, political commitment and national and international cooperation, and leishmaniasis remains a major neglected tropical disease with limited therapeutic options.

3.1.1 Current therapy of leishmaniasis

Most currently available antileishmanial drugs present problems related to toxicity and/or resistance, or require hospitalization, being therefore inadequate in the field. Despite the improvements recently achieved by combination therapies, which reduce the time and cost of treatments, new drugs are still urgently needed [130]. The conventional therapy of leishmaniasis traditionally relies on three basic drugs: pentavalent antimonials, pentamidine, and amphotericine B (Fig. **11**). Following the pioneering work of Gaspar Vianna, who discovered the effect of trivalent antimony (Sb^{III}) for the treatment of *L. braziliensis* infections [131], less toxic compounds based on pentavalent antimony (Sb^V), i.e. meglumine antimoniate and sodium stibogluconate, (Fig. **11**) were introduced in the 1940s. Despite their severe cardiac, hepatic, pancreatic and renal toxicity, they are still in use for the treatment of any type of leishmaniasis, mostly due to their low cost and high cure rates (85-95%). Antimonials must be administered daily for at least three weeks, and the intramuscular injections are often associated with local pain systemic side effects such as nausea, vomiting, weakness and myalgia,

abdominal colic, diarrhea, and skin rashes [132]. In addition, their widespread use in areas of high endemicity led to the

increasing emergence of resistant strains in Bihar (India), Iran and Peru [133, 134].

Pentamidine was also discovered in the 1940s and it is currently used in the treatment of *Pneumocystis jiroveci*

pneumonia, first-stage African trypanosomiasis and CL in South America. Pentamidine was originally available in two

galenic formulations, i.e. the pentamidine isethionate and the pentamidine methanesulfonate lyophilized salts. In the early

1990s the production of pentamidine methanesulfonate was stopped and now only pentamidine isethionate is on the market

[135]. The major side effects of pentamidine are hypotension, diabetes mellitus and renal impairment. Its use in India has

been abandoned, but presently it is under evaluation for the prophylaxis of VL relapses in HIV co-infected patients [136].

Amphotericin B deoxycholate is the main treatment option where resistance to antimonials is reported. It has a high

cure rate (~100%) at a dose of 0.75–1 mg/kg for 15–20 intravenous infusions either daily or on alternate days. However, it

has many adverse effects, which necessitate close monitoring and hospitalization for 4–5 weeks, which ultimately increases

the cost of therapy. [137]. The drug has renal toxicity, but it has the advantage that primary resistance is not reported in

immunocompetent individuals. The replacement of deoxycholate with other lipids was a major breakthrough in

amphotericin B therapy. Due to targeted delivery to macrophages in the liver, spleen and bone marrow, the lower organ

exposure to the free drug results in a net decrease of side effects. The possibility to deliver larger doses of the drug per

single dose makes VL treatment much shorter, with huge savings in hospitalization costs [138]. Of the three available

formulations, i.e. liposomal amphotericin B (AmBisome®; Gileas Sciences, Inc.), amphotericin B lipid complex (ABLC;

Abelcet®, Enzon Pharmaceuticals), and amphotericin B cholesterol dispersion (ABCD; Amphotec™, InterMune Corp.),

AmBisome has the best safety profile. It has been extensively tested and has been approved by FDA for the treatment of

VL. Based on a study performed in Bihar, a single dose of 10mg/kg body weight was as effective as the conventional

amphotericin B deoxycholate administered in 15 infusions of 1 mg/kg, given every other day during a 29-day

hospitalization [139]. This finding, coupled with the preferential price of US$ 18 per 50-mg vial negotiated by WHO [140],

makes the liposomal amphotericin B single infusion therapy an excellent option for VL in India [137].

The drugs that have been brought into the market more recently are paromomycin and miltefosine (Fig. **11**).

Paromomycin is an aminoglycoside antibiotic, which was granted orphan drug status in 2005 and is used for the treatment

of both VL and CL in parenteral and topic formulations. It was shown to be non-inferior to amphotericin B, its main

advantages being safety and low cost, approximately US$10 per patient. The disadvantages include the need for the

administering of intramuscular injections, and the monitoring of serum transaminases [138, 141]. Miltefosine, also known

as hexadecylphosphocholine, was registered in India as the first oral antileishmanil agent in 2002. It can achieve long-term cure rates of 94% following treatment courses of 28 days. Its major limitations are the high cost, the need for monitoring of gastrointestinal side-effects and occasional hepatic and renal toxicity. In addition, miltefosine is teratogenic; therefore, contraception for the duration of treatment and for the subsequent 3 months is mandatory for women of childbearing age. It is recommended that drug assumption by patients be monitored, because the drug's high cost and rapid efficacy may induce patients to prematurely discontinue the treatment, which favours the development of parasite resistance [137].For updated and comprehensive overviews that cover the subject of leishmaniasis and its treatment the reader is referred to: Kobets *et al.*, Croft and Olliaro, Murray, Antinori *et al.* [142-145]

3.1.2 Novel short-term therapeutic options

The bulk of research on the therapy of leishmaniasis is currently focused on the comparative evaluation of in use antileishmanial drugs and of their combinations with drugs originally developed for other purposes [146]. Accordingly, the introduction of short-term therapeutic novelties relies on formulation improvements or combinations of existing drugs. A new and very interesting perspective is offered by the finding that the combination of paromomycin and miltefosine, by interacting with the Toll-like receptor 4, induces the release of nitric oxide and tumour necrosis factor by human macrophages, so enhancing the killing of *L. donovani* promastigotes *in vitro* [147]. These results indicate that the activity of some drug combinations can go beyond the direct killing of the parasite, with implications for the modulation of the host immune response and the overcoming of parasitic resistance, and support the value of further investigations in this area.

The efficacy of the combination of classic antileishmanials with drugs not yet on the market, such as sitamaquine (see below) are being investigated [148]. New perspectives are offered by the proven activity of orally administered meglumine antimoniate-β-cyclodestrin conjugates [149], and by the development of a liposome formulation of meglumine antimoniate, which in a dog infection model was effective at a 20-fold-lower cumulative dose of Sb than is used for conventional antimonial treatment [150]. For a comprehensive review on the perspectives of the new delivery strategies of antimonials see [151]. The main problem in the identification of new antileishmanial drugs is that they should be tested against the intra-macrophagic amastigote, which is the clinically relevant stage of the parasite in the mammalian host. For a thorough review of *in vitro* and *in vivo* experimental models, see [152]. The assessment of the activity of amphotericin B, miltefosine, sodium stibogluconate and paromomycin on *L. donovani* intracellular amastigotes infecting different cell types, such as mouse peritoneal macrophages, mouse bone marrow-derived macrophages, human peripheral blood monocyte-derived macrophages, or differentiated THP-1 cells, performed by Seifert et al. indicates that the activity of the drugs varies

according to the host cell type [153]. These important results should be taken into account because of their heavy

implications on the *in vitro* evaluation of new drugs and on the assessment of drug susceptibility of clinical isolates.
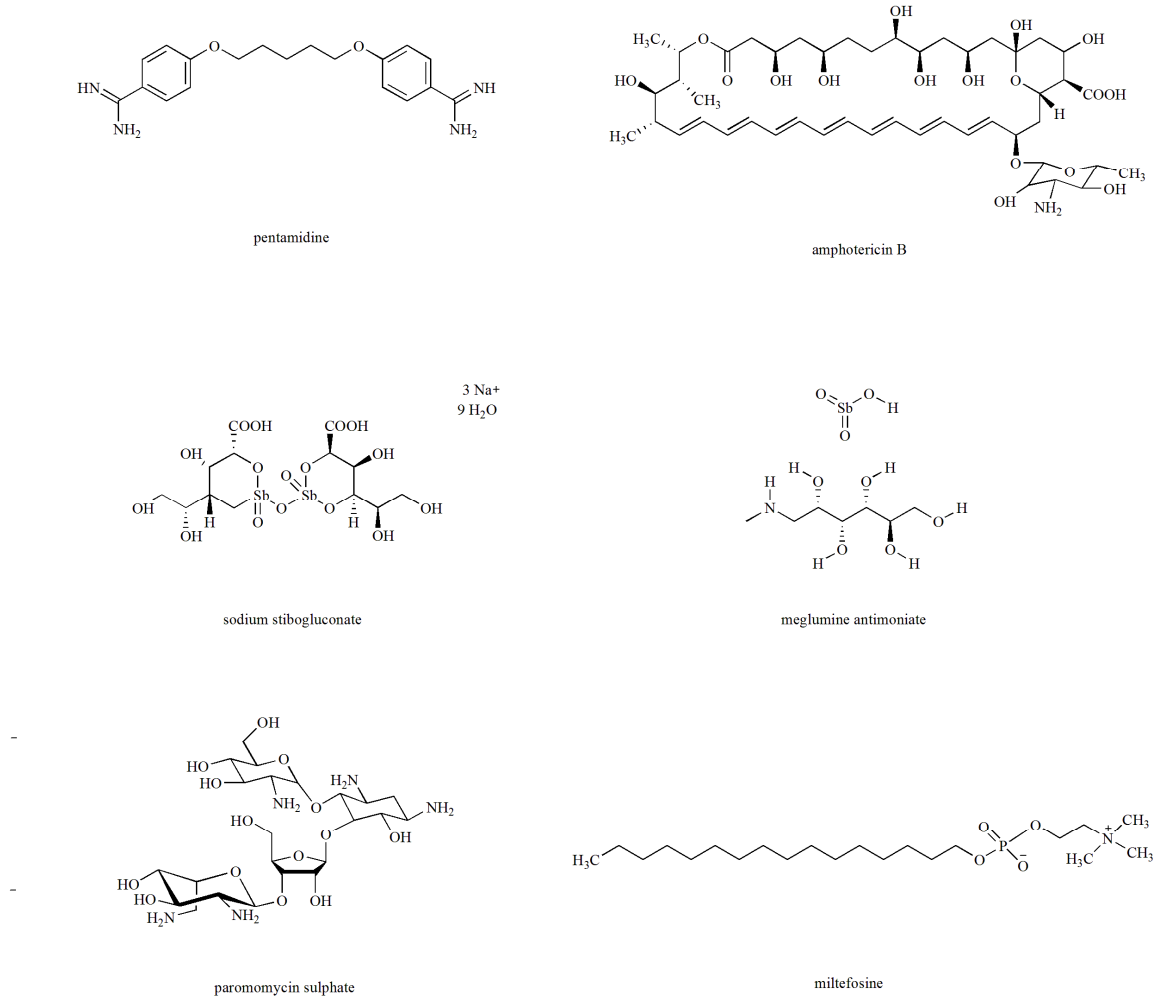

pentamidine

amphotericin B

sodium stibogluconate

meglumine antimoniate

paromomycin sulphate

miltefosine

Fig. (**11**). Chemical structures of currently used antileishmanial drugs.

A high content assay that allows the contemporary assessment of parasite growth inhibition and of compound

cytotoxicity by simultaneous visualization of both host cell nuclei and parasite kinetoplasts was developed by De Muylder

et al. [154]. According to these authors, in this kind of assay all steps are amenable to automation and could be reduced to

384-well format resulting in a high-throughput screening methodology. This technique was used to screen a set of 909

compounds against both *L. donovani* promastigotes and intracellular amastigotes. As a result, all but one of the hits

identified with the intracellular amastigote screen were also found in the promastigote screen, whereas fifty-six percent of

the hits from the promastigote screen were not found in the intracellular amastigote screen, indicating that the promastigote

screen failed to identify all active compounds and led to 56% of compounds being likely false positives. The compound that specifically inhibited intracellular amastigotes, i.e. naloxonazine, was completely inactive against both promastigotes and axenic amastigotes. This behaviour indicates that its activity must be dependent on some macrophage function, which according to available data could involve opioid receptors. The discovery of parasite growth inhibitors that interact with the host cells opens innovative and quite attractive possibilities, because in principle these inhibitors should not select resistant strains, and underscores the importance of evaluating compound activity against intracellular amastigotes [154].

### 3.1.3 Mid- and long-term therapeutic options

The discovery and licensing of drugs based on novel molecular scaffolds for the treatment of leishmaniasis can be considered a mid- or long-term objective [129], but there are some molecules that have been around for a certain period and are now in advanced phase clinical trial. One of these is sitamaquine (Fig. **12**), formerly known as WR6026, an 8-aminoquinoline that was initially developed by the Walter Reed Army Institute of Research. The elucidation of its mechanism of action that results in parasite apoptosis is under way. The strong points of sitamaquine are its short elimination half-life that prevents the rapid emergence of resistance, and the oral route administration. The results of phase IIb clinical trials against VL in India and Kenya by GSK were encouraging [155], but the selection of a sitamaquine-resistant *L. donovani* clone in the laboratory and some adverse effects, such as methemoglobinemia and nephrotoxicity evidenced in clinical trials, are being considered for a further development decision [156].

sitamaquine

fexinidazole
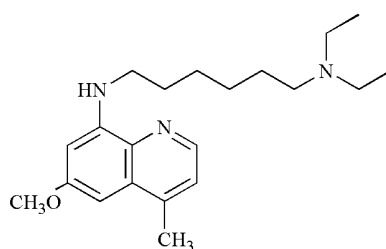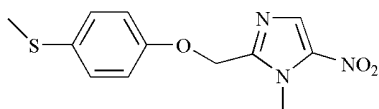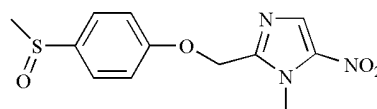
fexinidazole sulfoxide
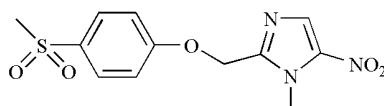
fexinidazole sulfone
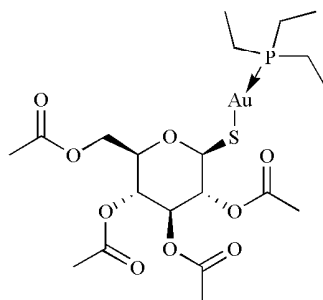
auranofin

Fig. (**12**). Chemical structures of antileishmanial drugs under development.

A second promising antileishmanial molecule in advanced stage of development is tafenoquine (Fig. **2**), already discussed in the *Plasmodium* section [47, 48]. This 8-aminoquinoline, that can be administered by oral route and is currently in clinical trials for the treatment of *P. vivax* malaria, when tested *in vitro* against intracellular amastigotes and in BALB/c

mouse models of *Leishmania* infection showed similar potency to sitamaquine against both pentavalent antimony-sensitive and -resistant *L. donovani* strains [157]. As with sitamaquine, the major limit of tafenoquine, i.e. the toxicity for G6PD-deficient subjects, suggests the need for the investigation of appropriate treatment protocols and the development of suitable combination therapies.

Fexinidazole (formerly Hoe 239) (Fig. **12**), is a new oral nitroimidazole that was rediscovered by the Drugs for Neglected Disease initiative (DNDi) and is now undergoing phase I clinical trials for treatment of African sleeping sickness [158]. Following the observation that a bacteria-like nitroreductase is implicated in the mode of action of nitro drugs in *Trypanosoma brucei* and *T. cruzi*, and considering that the genomes of leishmania parasites contain a homologous nitroreductase gene, the Fairlamb group at Dundee University (UK) decided to investigate whether fexinidazole could be an effective treatment for VL. Their results suggest that fexinidazole or its metabolites, fexinidazole sulfoxide and fexinidazole sulfone (Fig.**12**), have the potential to become safe and effective oral antileishmanial drugs [159].

3.1.4 Target-based development of new drugs

In the field of leishmaniasis the de novo target-based drug discovery is still in the stage of elucidating the best strategies to prioritize pathogen proteins based on whether their properties meet criteria such as sequence-derived information (e.g., molecular mass) and functional data on expression, essentiality, phenotypes, metabolic pathways, assayability and druggability, that are considered desirable in a drug target. The search for potential new targets is focused on enzymes involved in metabolic pathways, which are essential for the parasite but differ significantly from their mammalian counterparts in order to achieve selective toxicity [146].

The synthesis of polyamines and of their precursors is essential for leishmania parasites. Polyamines in their protonated forms behave as natural polycations, which affect both DNA and RNA functions. Consequently, the enzymes involved in spermidine synthesis and utilization, i.e. arginase, ornithine decarboxylase, S-adenosylmethionine decarboxylase, spermidine synthase, tryparedoxins, tryparedoxin-dependent peroxidases and, in particular, trypanothione synthetase and trypanothione reductase, are attractive targets for drug development [160]. Auranofin (Fig. **12**), a gold(I)-containing drug already in clinical use as an antiarthritic agent (aggiungere formula a fig 11) kills leishmania parasites by inhibiting the trypanothione reductase [161]. Inhibitors of the same enzyme are being identified by *in silico* structure-based virtual screenings [162], whereas the tertiary structure of spermidine synthase, another important enzyme of the polyamine synthetic pathway, is being assessed with the aim to identifying suitable inhibitors [163].

Peptidases (proteases) are critical for the survival and pathogenicity of parasites. Leishmaniae possess a family of peptidases that includes aspartic, cysteine, metallo, serine and threonine peptidases., Among these, the best known are the cysteine peptidases designated as CPA, CPB and CPC. These enzymes are important virulence factors that modulate the immune response of the mammalian host and facilitate tissue host invasion, and as such are considered attractive potential targets for chemotherapy [164]. A cysteine protease database has recently been made available with the aim of providing a summary of data on these enzymes and on their inhibitors [165]. The only aspartic protease present in leishmaniae belongs to the family of the A2 retroviral-like aspartic proteases, and is inhibited by drugs originally developed as inhibitors of HIV protease [166]. Nelfinavir was shown to be highly active against strains of *L. braziliensis, L. donovani* and *L. chagasi,* with inhibition rates superior to 90% [167]. These findings are of particular interest, given the suboptimal outcome of liposomal amphotericin B therapy in immunodeficient HIV-infected patients [137], and constitute a further example of the fortuitous possibility to treat leishmaniasis with a drug originally developed for other less neglected pathogens. The metallo-protease gp63 present on the surface of leishmania parasites is a key virulence factor which may be delivered within the host cells [168]. This protein protects the parasite from complement-mediated lysis, promotes the intracellular survival of amastigotes and modulates the host macrophage signaling [169] by cleaving macrophagic protein tyrosine phosphatases [170]. At present the research on gp63 is not focused on the development of inhibitors, but mainly deals with the role of this molecule as a possible vaccine [171].

To survive inside macrophages, leishmania amastigotes must counteract the parasiticidal effect of reactive oxygen and reactive nitrogen species generated by the host cell. All trypanosomes and leishmaniae possess a unique thiol metabolism in which the glutathione/glutathione reductase system is replaced by a trypanothione/trypanothione reductase system. The dithiol trypanothione [bis(glutathionyl)spermidine] is the donor of reducing equivalents and the reduction of trypanothione disulfide is crucial for the maintenance of a reducing intracellular milieu. Since the early 1990s, a large number of trypanothione reductase inhibitors that do not affect human glutathione reductase has been identified (for review, see [172]). The search for new scaffold trypanothione reductase inhibitors is progressing by means of traditional whole cell screenings and *in silico* structure-based virtual screenings [162, 173], but so far there are no new molecules in this category are under development.

The complex life cycle of leishmania parasites involves tightly regulated morphologic and metabolic changes of the parasite to cope with the greatly differing environments found in the sandfly vector and in the mammalian host: beyond the temperature difference, in the sandfly the parasite multiplies outside cells, whereas in the mammalian host it lives and multiplies inside macrophages. Protein kinases regulate cellular processes including cell cycle progression and

differentiation, and have drawn a lot of attention as potential drug targets for a wide range of diseases and syndromes, such as cancer, cardiovascular disease and Alzheimer's disease [174]. Among the various families of protein kinases so far identified, the most studied are the cyclin-dependent and the mitogen-activated protein kinases. Members of the former family have been characterized as potential targets for cancer therapy, and the screening of a library of inhibitors of human cyclin-dependent kinases against axenic amastigotes of *L. donovani* showed that a series of disubstituted pyrazol[4-3*d*]pyrimidines is active in a 1.5-12.4 μM range [175]. In addition, several small chemical inhibitors of cyclin-dependent kinases are undergoing clinical trials to assess their effectiveness in cancer treatment. By means of high throughput screening techniques, molecules based on azapurine and thiazole cores, inactive against human cyclin-dependent kinases but active against *L. major* promastigotes and amastigotes were identified. These hits are meant to be used for future hit-to-lead synthesis programs [176]. However, there are evidences that molecules highly active against *L. mexicana* cyclin-dependent cdc2-related serine/threonine protein kinase CRK3 do not show any leishmanicidal activity *in vitro*, suggesting that the activity of this specific enzyme is not indispensable for parasite survival contrary to indications provided by genetic manipulation studies [177]. This finding underscores the necessity of performing biological validation as early as possible in the drug development process.

The predominant sterols found in *Leishmania* species are ergosterol and stigmasterol, whose synthetic pathways differ from that of their mammalian counterpart cholesterol. The enzyme 24-methyltransferase, which is a vital enzyme in ergosterol biosynthesis, represents a good target. Some azasterols able to inhibit 24-methyltransferase showed antileishmanial activity. However, other compounds of the same family that did not affect the enzyme showed nonetheless antileishmanial activity by a mechanism that still needs to be elucidated [178, 179]. Sterols synthesis may also be affected by plant extracts: sterols isolated from the roots of the plant *Pentalion andrieuxii* showed marked antileishmanial activity [180], as well as the steroidal glycoalkaloid α-tomatine, derived from Lycopersicon aesculentum [181].

Dihydrofolate reductase (DHFR) is a key enzyme in folate metabolism, whose inhibition prevents the biosynthesis of thymidine. Following crystallization of the enzyme from *L. major* and *T. cruzi*, structural data are available to design selective inhibitors [146]. However, it has been shown that leishmaniae can overcome DHFR inhibition by overexpressing pteridine reductase 1 (PTR1), an enzyme mainly involved in the reduction of biopterin but also able to reduce other pterins and folates [182]. Based on this evidence, the successful treatment of leishmaniasis with antifolates would require the simultaneous inhibition of both DHFR and PTR1 by drugs which do not affect the activity of human DHFR. Research is being carried out on both fronts. Pteridine reductase inhibitors optimization is currently being pursued [183]. In leishmania

DHFR forms a functional complex with thymidylate synthase (TS). By means of an *in silico* virtual screening technique, the initial screening of 126,923 compounds yielded 21 compounds able to dock to the binding site of the leishmania DHFR-TS. Among these, the molecule 571633 was identified as a promising lead worthy of further development [184].

Unlike their mammalian and insect hosts, leishmaniae cannot synthesize the purine ring de novo and utilize the salvage pathway to produce purine bases. The purine salvage pathway is therefore considered an attractive target, and as such has been thoroughly investigated during the last 30 years. These studies identified the enzyme GMP synthase as a potential target within the pathway, because its activity is required in order to avoid the depletion of guanylate nucleotides [185].

Other families of enzymes that have potential as prospective parasite drug targets are topoisomerases, metacaspases and myristoyltranferases [186; 146]. Metabolic network analysis and high-throughput computationally enhanced screens are powerful tools to identify new chemotypes or already approved drugs that target leishmania parasites [187-189]. The whole field of target-based new drugs for leishmaniasis looks very promising, but it is still at a very basic developmental stage.

3.1.5 Phenotypic screening and natural products

Phenotypic screening is the most direct way to identify new antiparasitic drugs, and constitutes a valid alternative or complement to computational methods. It has the advantage that the waste of time and resources which may be spent to develop a drug that in the end may show no activity is avoided. This technique is the most suitable for screening natural products, which are abundantly offered by all nature kingdoms. The renewed interest in plant secondary metabolites is well described in the updated and comprehensive reviews by Schmidt *et al.*, to which the interested reader is referred [190, 191]. Natural products such as cyclic peptides, various flavonoids, chalcones, lignans, coumarins, iridoids, monoterpenes, saponins, toxoids, curcumin, quinoline alkaloids, and polyketides exhibit interesting anti-leishmanial activity [192; 193]. A series of plant derivatives, including acriflavine, saponins, plumbagin and the herbicide trifluralin, were tested alone or in combination in a mouse-*L. major* model of infection by Malwali *et al.* [194]. These authors observed that single drugs were effective in reducing pad lesions and liver and spleen parasite burden, with the following order of potency: saponin > plumbagin > trifluralin > acriflavine. However, the parasites reappeared at the infection site 90-150 days after the end of the treatment. Combination treatments were performed with the six possible combinations of drug pairs, and after 7 days of treatment the parasites disappeared from the lesions, which completely healed in a period of 30 days in all the infected animals. Combination treatments were more effective and allowed the use of lower doses. The treatment did not achieve the eradication of the parasites from the liver and spleen of the infected mice, but these results underscore the importance of

35

plant derivative investigations, suggesting that these compounds could be used with traditional drugs to obtain synergic effects and to limit the emergence of resistance.

HTS are also being used to identify molecules based on new molecular scaffolds. Two new antileishmanial compounds were identified as promising leads and validated against both promastigotes and intracellular *L. donovani* amastigotes by Siqueira-Neto et al. starting from a chemical library of 26,500 chemicals [195]. The two molecules, CA272 and CH 872, are based on the hydrazine and 4-hydroxyquinoline scaffolds, respectively, both of which are new as antileishmanials. An *ex vivo* splenic explant system was recently perfected and successfully used to screen chemical libraries containing 4,035 compounds, yielding 202 hits [196]. Eighty-four of these hits were classified as lead compounds, of which sixty-nine were previously unknown to have antileishmanial activity.

The target-free phenotypic screening of antileishmanial compounds was chosen by scientists at the Genomic Institute of the Novartis Research Foundation, who performed a HTS of 700,000 compounds against axenic amastigotes in a 1536 well plates format [197]. The Institut Pasteur Korea (IPK), together with the Drugs for Neglected Diseases initiative (DNDi), developed a High-throughput screening/High content screening (HTS/HCS) visual screen in a 384 well plate format using the *L. donovani*-infected human macrophage cell line THP-1 as the host cell. This assay, which can be performed by image-based analysis without the need for a reporter gene, was used for screening 26,500 compounds, generating 123 hits, 62 of which were also active against promastigotes. In addition to the intrinsic value of the generated hits, in the authors' words the development and validation of this HTS protocol for *Leishmania* infection of human macrophages is a major breakthrough in the field of antileishmanial drug discovery [198]. An analysis of patents filed before 2009 for antileishmanial drugs indicates that they are in the range of 500-600 [199]. If to this we add the flourishing identification of extracts with antileishmanial activity derived from Central and South American plants [200], we can say that the near future holds some promises.

The issues and problems of natural product development were addressed in the malaria section of this paper. To avoid repetitions, here we only add that leishmaniasis, as well as trypanosomiases, fully belongs to the family of neglected diseases. With an estimated 12 million people infected, compared to about 245 million people infected with malaria, leishmaniasis drug market is far less attractive than that of drugs for malaria. In this situation, the development of any compound is unlikely, irrespective of its natural or chemical origin. Whereas the identification of new targets and hits is highly rewarding for individual researchers, unfortunately the further development of the molecules is not rewarding for anyone. In this situation, the problem of funding drug development involves the political will of States and international health organizations.

3.2. Trypanosomes

3.2.1. Human African Trypanosomiasis (HAT)

African trypanosomes, the etiological agents of sleeping sickness, are extracellular flagellates transmitted by infected tsetse flies belonging to the genus *Glossina*. The prevention of HAT is based essentially on vector control and active surveillance of the population. This strategy, implemented by colonial powers, brought to the almost complete eradication of the disease in the early 1960s. However, after the advent of independence most endemic countries faced a collapse of surveillance and control activities, chiefly due to civil conflicts, with a progressive re-emergence of the disease, which reached a peak in the late 1990s in the Democratic Republic of the Congo (DRC), Angola, Central African Republic, southern Sudan, and Uganda [201]. It is currently estimated that 60 million people in 36 sub-Saharan African countries are at risk, and 50,000 to 70,000 people are infected.

In west and central Africa, the anthroponotic parasite *Trypanosoma brucei gambiense* causes a chronic disease that can last many years, whereas in Eastern and Southern Africa the zoonotic *T. b. rhodesiense*, which accounts for less than 10% of cases, is responsible for an acute form of the disease that may last weeks to months. Both infections, that invariably lead to coma and death if left untreated, occur in two stages: in stage 1, characterized by non-specific clinical symptoms, trypanosomes are restricted to the lymph and blood systems, whereas in stage 2, characterized by severe neurological symptoms, parasites can be found in the brain and in cerebrospinal fluid [201].

Trypanosomes are protected from lytic factors present in human plasma by a variant surface glycoprotein (VSG) codified by about 2000 genes that are expressed in succession. The consequent high degree of antigenic variation makes the development of a vaccine unlikely. In this situation the prevention of the disease mainly relies on the vector control and treatment of infected patients. Currently available treatment is based on four licensed drugs and one licensed drug combination. All of them are hindered by high toxicity, complexity of administration procedures and progressive loss of efficacy in some geographical regions [202]. Treatment is parasite- and stage-specific, depending on the compound ability to cross the blood-brain barrier. Early stage *T. b. gambiense*, but not *T. b. rhodesiense* infections are treated with pentamidine (Fig. **13**), introduced in 1941. This drug, whose mechanism of action is unknown, has broad-spectrum anti-parasitic activity, being also used for the treatment of antimony-resistant leishmaniasis and *Pneumocystis jiroveci* pneumonia, but it must be administered by i.m. injection for 7 consecutive days and has many adverse side effects. Suramin (Fig. **13**), introduced in 1922, is the only available drug for first-stage *T. b. rhodesiense* infection. Its mechanism of action is

37

also unknown, and its use against *T. b. gambiense* infection is generally avoided, especially where *Onchocerca* spp are also present, because its high activity against these parasites can expose patients to the risk of severe allergic reactions. The recommended dose regimens for suramin are complex and last up to 30 days. Both second-stage *T. b. gambiense* and *rhodesiense* infections can be treated with melarsoprol (Fig. **13**), which was introduced in 1949 and, being the less expensive drug, is still the most widely used in areas where eflornithine is not available or affordable. Moreover, it is the only choice for second-stage *T. b. rhodesiense* cases. Melarsoprol is considered one of the most toxic compounds ever used in therapy, being responsible for frequent adverse reactions ranging from skin reactions to a severe or even fatal encephalopathic syndrome. Moreover, treatment failures due to melarsoprol resistance are a current and increasing problem [203]. Eflornithine (Fig. **13**) is the only new molecule for the treatment of *T. b. gambiense,* registered in 1990. Being less toxic than melarsoprol, it is recommended as the first-line treatment for second-stage *T. b. gambiense* infection, whereas it is not advised against *T. b. rhodesiense*, due to poor susceptibility [201]. However, the drug is difficult to administer, requiring one slow infusion every 6 h for 14 days because of its short half-life. The association of eflornithine with nifurtimox (Fig. **13**), a drug used for the treatment of Chagas disease and administrable by the oral route, showed advantages in terms of administration regimen, cost and convenience, with less adverse side-effects and minor probability of resistance induction, thanks to the different mechanism of action of the two drugs: eflornithine inhibits ornithine decarboxylase, whereas nifurtimox leads to the production of intracellular free radicals [204].
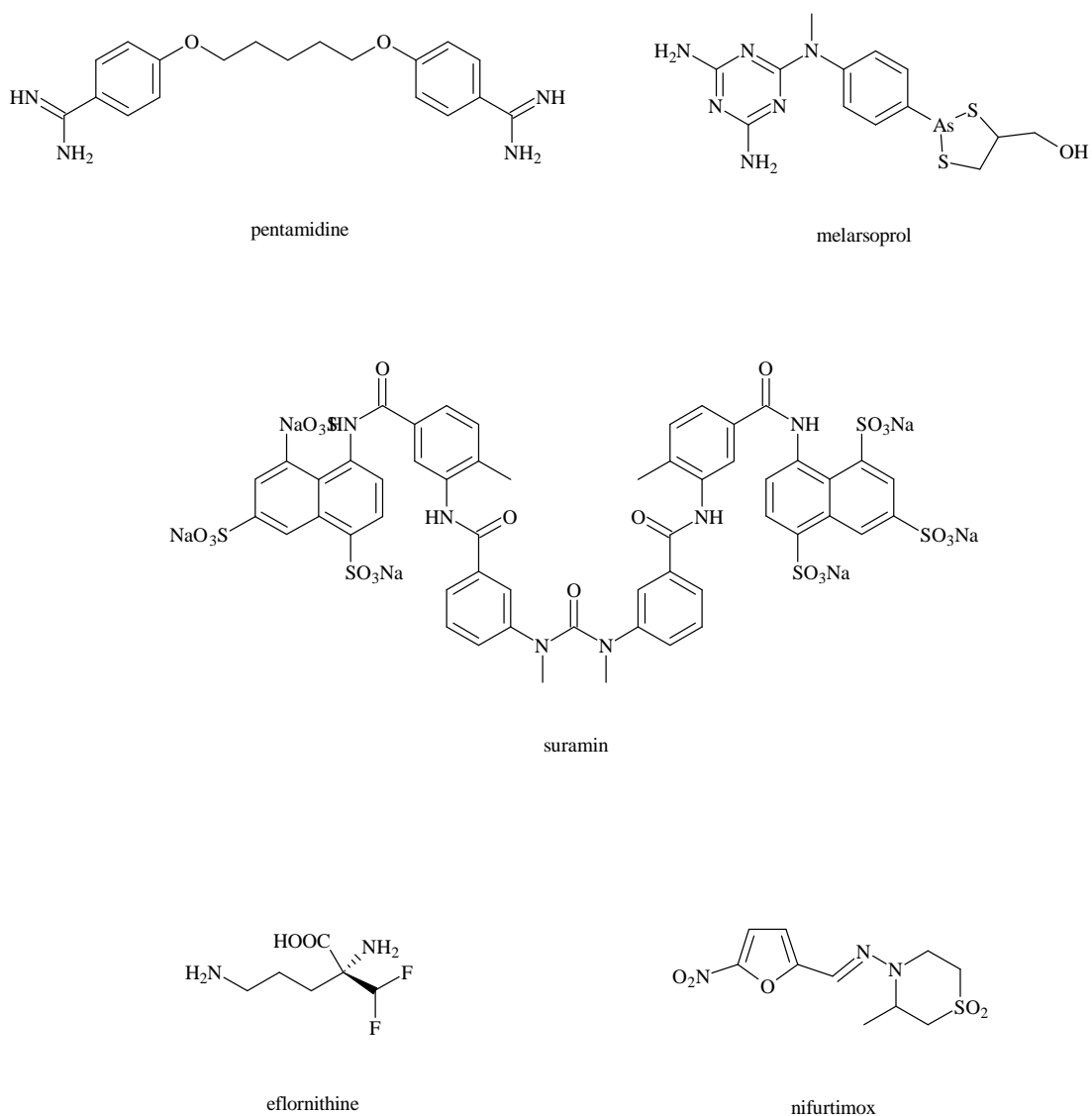
pentamidine

melarsoprol

suramin

eflornithine

nifurtimox

Fig. (**13**). Chemical structures of drugs currently used against HAT.

The nifurtimox–eflornithine combination (NECT) was added to the WHO's Essential Medicines list in 2009 [205]. Despite being a clear improvement with reduced toxicity and treatment duration, the eflornithine requirement for i.v. administration is still a limitation. Moreover, the lack of a single agent effective against both parasite species and both disease stages requires accurate species and stage diagnosis, a difficult task to perform in rural community hospitals that lack modern medical equipment [206]. It is estimated that less than 20% of currently infected people have access to treatment or are under any HAT surveillance, due to the difficulty of performing diagnosis and treatment in remote or conflict zones [202]. The regained awareness that by the late 1990s HAT had again reached epidemic levels triggered the

joint funding of new research programs and a WHO donation agreement with the HAT drug manufacturers, resulting in eflornithine availability to non-governmental organizations. The screening of over 2000 new diamidines against *T. brucei* performed by the Consortium for Parasitic Drug Development [207] led to the identification of an initial lead, furamidine (Fig. **14**), with excellent efficacy but poor oral bioavailability and blood–brain barrier penetration. These limitations were overcome by the corresponding methoxime prodrug DB289 (parafuramidine) (Fig. **14**), which became the first oral trypanocide for early stage HAT to enter phase III clinical trials that were successfully completed. But when an additional phase I trial was conducted testing a 14 days schedule that would cover the treatment of pneumocystosis, cases of renal toxicity observed about 8 weeks post treatment stopped further development of parafuramidine [208].
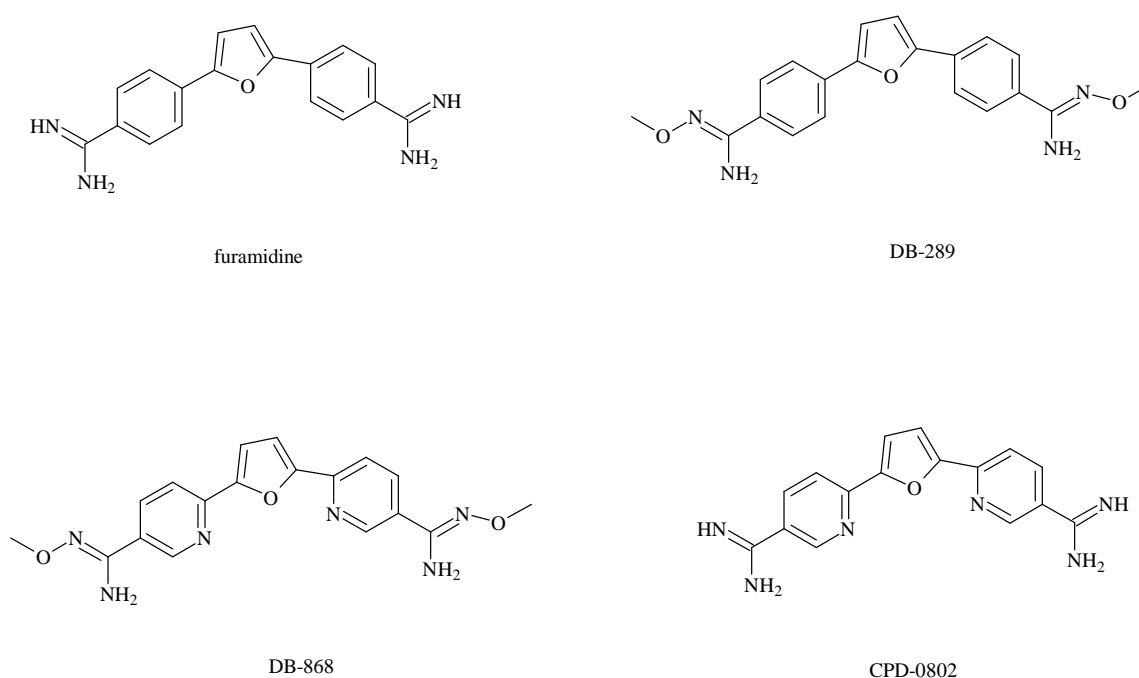

furamidine

DB-289

DB-868

CPD-0802

Fig. (**14**). Chemical structures of anti-HAT drugs under development.

However, recent data on the efficacy of aza analogs of parafuramidine, such as CPD-0802 and its prodrug DB-868 (Fig. **13**) in murine and monkey models of CNS infection, offer new hope for dicationic molecules to treat stage 2 HAT [209].

Oxaborole carboxamides, developed by Anacor Pharmaceuticals (Palo Alto, CA), constitute a second promising class of new compounds with potent and selective trypanocidal activity. Due to the unique properties of boron which facilitates reversible interactions with biochemical targets, the incorporation of this element into drug candidate molecules provided interesting lead molecules active against malaria [210], HAT and Chagas disease parasites [211]. The screening of

an oxaborole library in a whole cell *T. brucei* viability assay revealed that these compounds are effective inhibitors of parasite growth at concentrations as low as 0.02 mg/mL [212]. The compound SCYX-6759 (Fig. **15**) was the most potent, showing good *in vitro* and *in vivo* activity. Further optimization was focused on the improvement of brain permeability and pharmacokinetics, yielding SCYX-7158 (Fig. **15**) as a clinical drug candidate that got the clearance for a phase I clinical trial started in March 2012 [212].
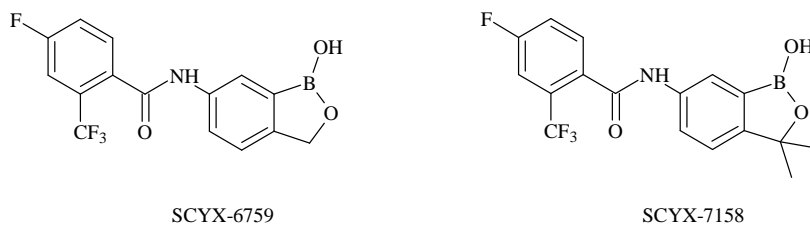

Fig. (**15**). Chemical structures of new oxaboroles.

As part of a DNDi project aimed to the systematic review and profiling of nitroheterocyclic compounds, in 2006 more than 700 already existing but long forgotten molecules were screened against *T. brucei*. This work brought about the rediscovering of fexinidazole (Fig. **12**), a 2-substituted 5-nitroimidazole, as a drug candidate [213]. Fexinidazole was in preclinical development in the 1970s and early 1980s as a broad-spectrum antimicrobial agent by Hoechst AG (now Sanofi-Aventis). In 1983, its *in vivo* activity against African trypanosomes was further substantiated, but its development was not pursued at the time. Recent experiments showed that fexinidazole is effective in both the acute mouse model and, more importantly, in the chronic mouse model with established brain infection. Fexinidazole ended phase I clinical trials and will undergo a phase II/III efficacy and safety study in mid 2012 [214]. Fexinidazole is the first new oral drug candidate in 30 years that is in clinical development for both stage 1 and stage 2 HAT, and considering the recent discovery of its antileishmanial activity [158], it could become the first much needed breakthrough for both HAT and VL therapy.

The investigation of the mechanisms by which pathogenic African trypanosomes escape innate human immunity open opportunities for the identification of new targets. Humans are immune to infections by highly prevalent African trypanosomes such as *T. brucei brucei, T. congolense* and *T. vivax* thanks to the activity of two potent immune molecules called trypanosome lytic factors (TLF-1 and TLF-2) [215]. TLF-1 is a minor subclass of human high density lipoprotein of approximately 500 kDa which interacts with a *T. b. brucei* surface receptor and is transported to the parasite lysosome. Following activation by the acidic pH, TLF-1 destabilizes the lysosomal membrane causing parasite lysis. TLF-2 is a lipid-poor protein complex of approximately 2 mDa containing significant amounts of IgM, and its mechanism of action is still

unclear. *T. b. rhodesiense* survives the action of TLF-1 because it possesses a serum resistance associated (SRA) protein that is an atypical VSG of shorter than average length (410 amino acids instead of approximately 490) [216]. SRA protein inhibits TLF-1 efficient transport to lysosome by interacting with the carboxy terminal of the apolipoprotein L-I moiety of TLF-1. SRA is not expressed by *T. b. brucei*, which can be made resistant to TLF-1 by transfection of the SRA gene. The two main approaches to disrupt SRA/TLF-1 interaction involve either the mutation of apoL-I or the use of small molecules/peptides to inhibit the binding. The resistance of T. b. gambiense to TLF-1 is not due to the SRA protein, but to the functional loss of the receptor-mediated uptake of TLF-1. In this case, the conjugation of TLF-1 to targeting molecules or alternative ligands could restore parasite sensibility. It can be envisaged that in the future this it will be possible to introduce a population of wild and domestic transgenic animals resistant to *T. b. rhodesiense*, so decreasing the reservoir and the spread of infection [215].

The research aimed at evaluating plant extracts based on ethnobotanical remedies active against HAT is considered a valid alternative to the investigation of huge chemical libraries, which usually yield a very low number of hits. African plants are being investigated with *in vitro* and *in vivo* experiments [217-219, 190, 191. Active extracts are a potential, and so far untapped, source of new antitrypanosomal agents (for review, see Alviano et al [193]).

3.2.2. Chagas' disease

Chagas' disease is caused by the Kinetoplastid hemoflagellate *Trypanosoma cruzi,* transmitted by haematophagous domestic and sylvatic mites belonging to the *Triatominae* subfamily (of the *Reduviidae* family), present in Central and South America. Infection may also occur by blood transfusion or organ transplantation, and by transplacental, mucosal (conjunctiva) and oral route (breastfeeding and food contaminated by mite faeces). Although the vectorial and transfusional transmission cases have been progressively reduced, Chagas' disease is still a major public health problem and one of the leading causes of morbidity, disability and mortality in Latin America. It is estimated that nearly 90 million people are at risk of infection and more than 8 million are infected, with an annual death toll of about 50,000 people in 18 endemic countries [220]. The yearly incidence rate due to vectorial transmission is estimated to be around 42,000, whereas that of congenital cases is estimated to be nearly 15,000 [221]. Due to migration from Chagas-endemic Latin American countries to developed countries, the infection is becoming a global health problem. Nearly 300,000 infected people live in the United States, which induced most U.S. blood banks to begin the screening for Chagas' disease in 2007, and more than 80,000 cases are reported in Spain. Cases of *T. cruzi* infection are no longer rare in any country with high numbers of Latin American immigrants, which poses serious public health problems concerning blood transfusions and organ transplants, not

to mention that the disease must be considered for differential diagnosis as possible aetiology of cardiomyopathy cases [220, 222].

The infection can occur at any age, but it is most frequent between 1 and 5 year olds, with a fatality rate under 10% in untreated cases. Chagas' disease is characterized by a first acute toxemic phase, during which the parasite can be easily detected in peripheral blood. Usually a local reaction at the entry site is followed by a general malaise lasting 6-8 weeks, during which a wide array of symptoms, such as fever, hepatosplenomegaly, meningoencephalitis, and myocarditis may be manifested. Patients that overcome the acute phase undergo the indeterminate form of the chronic phase, in which the infection is asymptomatic and can be detected only by means of serological or parasitological tests. This condition may last indefinitely, but 10-20 years after infection 10-40% of individuals (the percentage varies according to the geographic region) will develop the symptomatic, highly invalidating chronic form, that may severely affect the gastrointestinal tract, the central and peripheral nervous system, and myocardium, with eventually lethal outcome due to cardiac failure, severe arrhythmia, venous thromboembolism, and sigmoid volvulus [220, 223].

Chagas' disease cannot be eradicated because it is a zoonosis with a large variety of reservoirs including more than 100 species of marsupial and placental mammals autochthonous to the American continent. No drugs are available for prophylaxis, and there is no vaccine to protect susceptible individuals. Consequently, the control of vectorial transmission by using insecticides, and the improvement of houses to avoid mite colonization are the only feasible ways to reduce the possibility of human infection [224]. Beyond the implementation of vector control and epidemiologic surveillance, the main challenge is to provide effective medical treatment to already infected people and to support the development of efficient and non toxic drugs with adequate funding. Currently available drugs, i.e. nifurtimox (Fig. **11**) and benznidazole (Fig. **15**), introduced in the 1970s, must be administered by the oral route for 60-90 days.
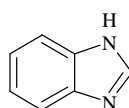

benzimidazole

Fig. (**16**).

Both drugs cause common side effects such as anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy, that lead to poor patient compliance. However, their major limitation resides in their uselessness for the treatment of the chronic phase of the disease. Both drugs are indicated for the treatment of the acute phase, congenital

infection, early chronic disease (mostly children under 15), and infection reactivation in AIDS patients. The discovery of effective treatments for chronic adult cases is the present challenge [222]. The review of the literature on the subject highlights the lack of coordination between basic research laboratories and the structures that should carry out drug development. Progress is hindered at both stages by the difficulty to obtain suitable animal models of the chronic form of the disease, and to monitor chronic phase patients in clinical trials to obtain proof of cure [224]. Despite these problems, thanks to the new insights into *T. cruzi* biology and metabolism, during the last decade new targets were identified, and some promising compounds are in different stages of development. The most promising targets are: cruzipain; the ergosterol biosynthesis pathway; trypanothione synthesis and thiol-dependant redox metabolism.

Cruzipain (cruzain) is a cysteine protease closely related to the cathepsin family, whose activity is critical for the parasite viability. The cruzipain irreversible inhibitor K-777 (Fig. **17**) was thoroughly studied at the Sandler Center for Drug Discovery, and reached the state of clinical candidate for phase I safety trials [225]. Irreversible cruzipain inhibitors contain an electrophilic functional group "warhead" (i.e., vinyl sulfone or 2,3,5,6-tetrafluorophenoxymethyl ketone) that can covalently bind to cruzipain via nucleophilic attack of the active site cysteine. The current challenge is to identify and develop a small molecule acting as a reversible covalent inhibitor of cruzipain, with potentially fewer off-target side-effects, often associated with irreversible enzyme inhibitors [226]. Novel small molecule inhibitors of cruzipain were recently identified by Rogers *et al.* [227], and a new line of cruzipain inhibitors is being developed by a Merck team [228].
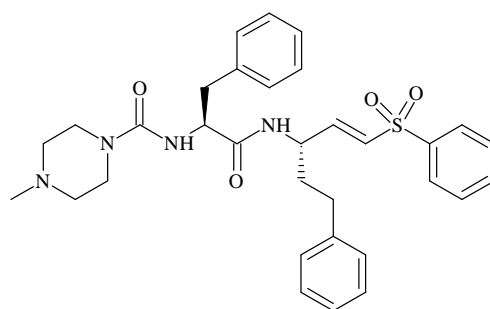
K$_{777}$

Fig. (**17**). A cruzipain irreversible inhibitor.

Following the observation that, similarly to fungi and yeasts, *T. cruzi* is strictly dependent on endogenously produced sterols, the antiparasitic characterization of antifungal azoles inhibiting sterol C-14α-demetylase (CYP51), an enzyme that catalyzes the conversion of lanosterol to ergosterol, led to the identification of posaconazole (Fig. **18**), developed by Schering-Plough, as an active trypanosomicidal [229].
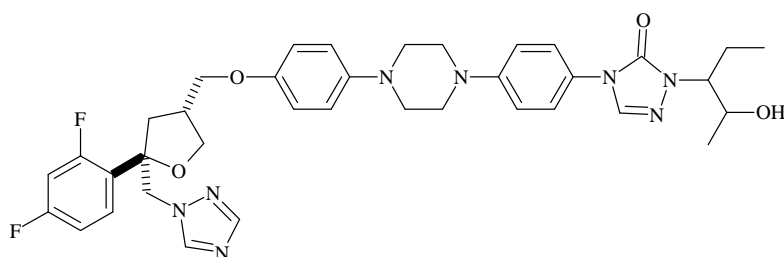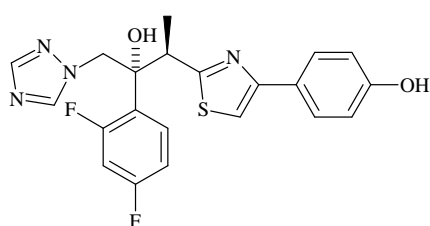
Fig. (**18**). Chemical structures of drugs being developed against Chagas' disease.

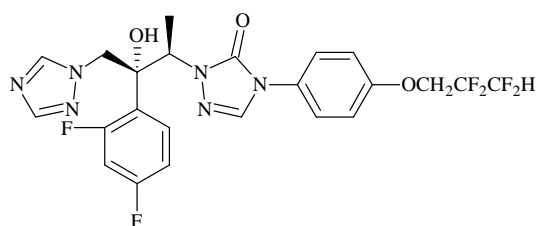Posaconazole is a potent broad spectrum antifungal, approved by the U.S. Food and Drug Administration in 2006 as salvage therapy for invasive fungal infections in immunocompromised patients. Posaconazole treatment achieved radical cure in both acute and chronic mouse models of Chagas' disease, probably thanks to its potent and selective intrinsic anti-*T. cruzi* activity (the minimal growth-inhibitory concentrations against the intracellular amastigote form is in the nanomolar to sub-nanomolar range) and special pharmacokinetic properties (long terminal half-life and large distribution volumes) [230]. A successful case, in which an off-label treatment with posaconazole was performed on a compassionate basis on a patient with chronic Chagas' disease and systemic lupus erythematosus, was reported in Spain. In this case posaconazole, administered at the dosage of 400 mg every 12 hour for 90 days, was well-tolerated, and *T. cruzi* blood PCR was consistently negative throughout the follow-up interval of 13 months after the start of treatment (nine consecutive negative PCR tests) [231]. Other molecules that inhibit CYP51 are ravuconazole (Fig. **18**) and its water-soluble prodrug E1224, a mono-lysine derivative, both developed by Eisai, (Japan) in collaboration with DNDi, and Tak-187 (Fig. **18**) [232], which completed phase I trials [224]. Concerns regarding the potential development of resistance to azole-based compounds suggest looking for new agents acting via different mechanisms. As mentioned above in the HAT section, a Scynexis team is developing new compounds based on the benzoxaborole scaffold [162]. Benzoxaboroles demonstrated activity against *P. falciparum* and *T. brucei*, and the screening against *T. cruzi* led to the identification of SCYX-6759 (Fig. **15**), a molecule

45

with interesting antiparasitic and pharmacokinetic properties. In mice infected with a sub-lethal dose of parasites, the compound achieved 100% inhibition of bloodstream parasitaemia following oral administration of 10 mg/kg once daily for 5 days, whereas it did not eradicate the parasite in chronically infected and cyclophosphamide-immunosuppressed mice, a model in which other compounds, including posaconazole, have proved curative. The development of oxaborole-containing derivatives is proceeding on an empiric basis, given the present ignorance about their mechanism of action and the incomplete knowledge of *T. cruzi* biology [211].

A recent paper by Gunatilleke *et al.* reports the identification of novel chemotypes targeting *T. cruzi* CYP51. Starting from a library of 104,000 chemical compounds, by means of a target-based high-throughput screening, these authors identified 11 hits. These highly different compounds were able to inhibit 50% of parasite endocytosis and intracellular growth at sub-μM concentration ranges (for comparison, posaconazole, used as positive control, worked at 1 nM concentration). The discovery of new structurally different leads has favourable implications on chemical development, resistance avoidance, possibility of therapeutic combinations, and, last but not least, patenting procedures [233].

The trypanosomicidal activity of series of 23 compounds, selected by means of high throughput screening technologies starting from the 303,286 molecule NIH collection, was characterized by Andriani and co-workers. The IC50 of the compounds was assessed in vitro on T. cruzi amastigotes infecting NHI/3T3 cells. Firefly luciferase-expressing *T. cruzi* trypomastigotes grown on monolayers of LLcMK2 monkey kidney epithelial cells were used to assess the activity of compounds in an in vivo inhibition assay based on the imaging of infected mice following luciferine injection. The *in vitro* assay demonstrated that 11 compounds had no activity, and only five compounds were active at concentration ranges ≤ 1 μM. The twelve active compounds were then assayed in vivo, with the following results: some of the compounds had no activity, others increased parasite loads possibly by interfering with the immune response of the mice, whereas two compounds resulted in severe and significant decreases of the mice parasite burden, compared to the control group. These two compounds are closely related, belonging to the 1-(4- Halogeno-benzyl)-2,4,6-triphenyl-pyridinium series and differ only in the nature of halogen on the para position of the benzyl (Fluorine or Chlorine) [234]. The fact that the two compounds are closely related suggests the efficacy of the basic chemical structure, which is worthy of further development.

Two compounds, amiodarone and dronedarone (Fig. **19**), already on the market as antiarrhythmic, are potent and selective anti-*T. cruzi* agents. Their mechanism of action is based on the lethal disruption of intracellular Ca^{2+} homeostasis, due to the mobilization of Ca^{2+} contained in the *T. cruzi* intracellular storage sites, i.e. mitochondrion and acidocalcisomes [235]. Dronedarone is an amiodarone derivative in which the 2,5-diiodophenyl moiety of the parental drug was replaced with an unsubstituted phenyl group, and a methyl sulfonyl group, aimed at reducing the thyroid toxicity and lipophilicity of

the parental drug, was incorporated. This new drug was already approved by FDA for use as an antiarrhythmic in humans and is replacing amiodarone as the drug of choice due to its improved safety profile and apparent absence of associated thyroid or pulmonary toxicity. The characterization of dronedarone trypanocidal activity supports its possible repurposing as a single or combination drug for the chronic phase of Chagas' disease [235].
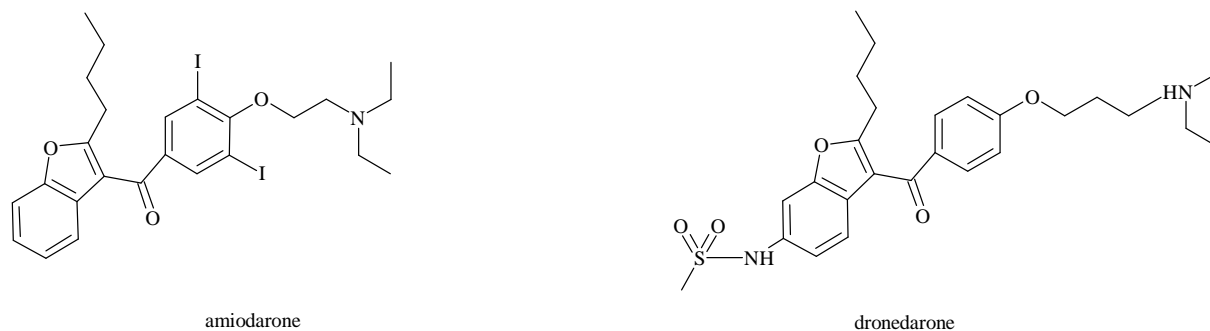

amiodarone   dronedarone

Fig. (**19**). Chemical structures of antiarrhythmic drugs with potent and selective activity against *T. cruzi.*

4. CONCLUSIONS

Many compounds identified by means of both the target-based approach and the whole cell screens are currently awaiting further development. At the state of the art, both approaches are useful and their integration may be the key of success [236]. For a detailed review on the strategies that can be used to develop new drugs against malaria parasites, including targeting *Plasmodium* apoptosis and using hybrid molecules, the interested reader is referred to [237, 238].

Besides the already discussed drug-based prophylaxis and treatment options, a promising new strategy is the modulation of the immune system of infected subjects. It has been shown that in humans *P. falciparum* infection is associated with a CD4^{+} T cell dysfunction, which negatively affects the induction of protective *Plasmodium*-specific antibody responses, and is a major underlying factor for prolonged blood-stage *Plasmodium* infection. In addition, it is known that the immune system of chronically ill VL patients is anergic. In patients co-infected with HIV and malaria or leishmaniasis, the damage to the immune system is cumulative. In this regard, it is interesting to note that a number of protease inhibitors currently in use in the therapy of AIDS showed activity *in vitro* against *Plasmodium*, *Leishmania* and other parasitic protozoa (for a thorough review, see [239]). Affecting both viral and protozoal proteases, in HIV/parasite co-infected patients these drugs achieve the double goal of contributing to the efficiency of the host immune system and of interfering with parasite virulence and survival. Even though their interest is mostly academic, because in malaria and VL

endemic areas most HIV-infected subjects do not receive specific antiretroviral treatment, these findings deserve further studies, and support the approach aimed to the identification of molecules with an interspecific range of pleiotropic effects.

Recent experiments performed on a mouse model of lethal *P. yoelii* infection indicate that mice pretreatment with polysaccharides derived from *Achyranthes bidentata*, a traditional Chinese medicinal herb which possesses immuno-modulatory and anti-inflammatory functions, significantly extended the mice survival time and decreased the parasitaemia [240]. Again, in a mouse model of *P. yoelii* infection, the administration of anti-programmed death ligand 1 (Anti-PD-L1)- and anti-lymphocyte activation gene 3 (Anti-LAG-3)-specific blocking antibodies markedly improved the effector and CD4$^+$ T cell responses, as well as the antibody-producing B cell response, which resulted in a rapid decrease of parasitemia and in accelerated parasite clearance [241]. It is worth noting that biological agents targeting these inhibitory pathways, designed to improve T cell responses to neoplastic diseases, are already in clinical trials [242-244]. This strategy has two major advantages over the classic drug-based parasite-killing approach: first, it does not induce parasite resistance; second, by re-establishing the normal functionality of the immune system, it improves the resistance of the subject to intercurrent infections by other viral, bacterial and protozoal intracellular pathogens. It can be envisaged that the chronic forms of leishmania and trypanosoma infection could also take advantage of this immunomodulatory approach, although knowledge concerning the response of the human immune system to these infections is still incomplete and somewhat controversial [245, 246]. The validity of targeting host mechanisms instead of using direct antiparasitic drugs is supported at the conceptual and practical level by the problem of resistance, which is due to be developed, sooner or later, against virtually all drugs targeting a definite metabolic step of the parasite [247]. The long-term advantages of host resistance improvement are attested by the field observation that all of the malaria-related haemoglobin variants found in Africa result in a physiologically significant reduction in parasite adhesion to endothelial cells [248], and in the ultimate better outcome of the immune response [249]. From an evolutionary standpoint, the long-term persistence of haemoglobinopathies indicates that they do not exert parasite selection. Accordingly, drugs targeting cytoadherence do not impose a strong selective pressure on the parasite. Plant components such as cocoa flavonoids could be promising candidates for malaria therapy, not so much as to lead to healing, but as agents able to reduce the severity of cytoadherence-mediated complications (cerebral malaria and acute respiratory distress, which are not rapidly affected even by fast-acting parasiticidal drugs) and to facilitate immunity-mediated parasite clearance [247]. New anti-cytoadherence compounds were also identified by using the crystal structure of human ICAM 1 as a basis to screen *in silico* for inhibitors of cytoadherence. One compound, (+)-epi-2-galloylcatechin-3-gallate (Fig. **20**), at micromolar concentrations inhibited the binding of two variant ICAM 1-binding malaria parasites in a highly specific, dose-dependent manner [250].
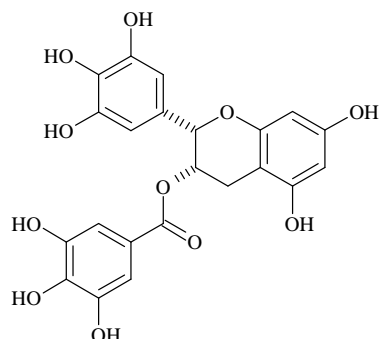
(+)-EGCG

Fig. (**20**).

In the absence of suitable specific vaccines, the eradication of vector-borne protozoal diseases can be envisioned for anthroponotic infections only, i.e. malaria and VL due to *L. donovani*. Malaria elimination was achieved in European countries, such as France, Italy, Greece, Spain, and Portugal, essentially thanks to mosquito control by abundant DDT spraying [251].The use of DDT, now banned for toxicological and ecological concerns, achieved malaria transmission interruption but not anopheles elimination. The ongoing presence of vectors and the ever increasing presence of infected travelers or immigrants from endemic countries may result in the reappearance of malaria in these countries. Indeed, the recent finding of several autochthonous malaria cases in European countries of the Mediterranean basin supports the need for a careful monitoring of the mosquito population and for an increased vigilance by health professionals [252]. In Europe, climatic changes favoring sandfly diffusion are widening the areas of VL endemicity, whereas the international migratory flows from endemic countries are making *T. cruzi* infection a global problem. This situation could at last reverse the lack of sense of urgency that characterizes the field of the struggle against tropical parasites, compared, for example, to the struggle against HIV. It can be envisaged that when a critical threshold in industrialized countries is exceeded, perhaps the market will be more rewarding and malaria and neglected diseases will be less neglected.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1]   Pink, R.; Hudson, A.; Mouries, A.M.; Bendig, M. Opportunities and challenges in antiparasitic drug discovery. *Nat. Rev. Drug Discov.,* **2005,** *4*, 727-740.

[2]   Maroli, M.; Rossi, L.; Baldelli, R.; Capelli, G.; Ferroglio, E.; Genchi, C.; Gramiccia, M.; Mortarino, M.; Pietrobelli, M.; Gradoni, L. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. *Trop. Med. Int. Health,* **2008**, *13*, 256-264.

[3]   Morosetti, G.; Bongiorno, G.; Beran, B.; Scalone, A.; Moser, J.; Gramiccia, M.; Gradoni, L.; Maroli, M. Risk assessment for canine leishmaniasis spreading in the north of Italy. *Geospat. Health*, **2009**, *4*, 115-127.

[4]   Otranto, D.; Paradies, P.; de Caprariis, D.; Stanneck, D.; Testini, G.; Grimm, F.; Deplazes, P.; Capelli, G. Toward diagnosing *Leishmania infantum* infection in asymptomatic dogs in an area where leishmaniasis is endemic. *Clin. Vaccine Immunol.*, **2009**, *16*, 337-343.

[5]   Kotton, C.N. Transplant tourism and donor-derived parasitic infections. *Transplant Proc.*, **2011,** *43*, 2448-2449.

[6]   Schmunis, G.A.; Yadon, Z.E. Chagas disease: a latin american health problem becoming a world health problem. *Acta Tropica*, **2010**, *115*, 14–21.

[7]   Trouiller, P. Drug development and registration for parasitic diseases: what are the barriers? In: *Drugs against parasitic diseases: R&D methodologies and issues*, Fairlamb, Ridley, Vial, Ed.; WHO, Geneva, **2003**, pp. 23-30.

[8]   Chatelain, E.; Ioset, J.R. Drug discovery and development for neglected diseases: the DNDi model. *Drug Des. Devel. Ther.*, **2011**, *5*, 175-181.

[9]   Nwaka, S.; Ridley, R.G. Virtual drug discovery and development for neglected diseases through public-private partnerships. *Nat. Rev. Drug Discov.,* **2003**, *2*, 919-928.

[10]   Traorè, M. Role of endemic countries in research and development. In: *Drugs against parasitic diseases: R&D methodologies and issues*, Fairlamb, Ridley, Vial, Ed.; WHO, Geneva, **2003**, pp.31-34.

[11] Birkholtz, L.; van Brummelen, A.C.; Clark, K.; Niemand, J.; Marechal, E.; Llinas, M.; Louw, A.I. Exploring functional genomics for drug target and therapeutics discovery in Plasmodia. *Acta Tropica*, **2008**, *105*, 113–123.

[12] Leroy, D.; Doerig, C. Drugging the *Plasmodium kinome*: the benefits of academia-industry synergy. *Trends Pharmacol. Sci.,* **2008**, *29*, 241-249.

[13] Wang, Y.; Wu, Y. Computer-assisted searches for drug targets with emphasis on malarial proteases and their inhibitors. *Curr. Drug Targets Infect. Disord.*, **2004**, *4,* 25-40.

[14] Wolf, A.; Shahid, M.; Kasam, V.; Ziegler, W.; Hofmann-Apitius, M. *In silico* drug discovery approaches on grid computing infrastructures. *Curr. Clin. Pharmacol.,* **2010**, *5*, 37-46.

[15] Kasam, V.; Salzemann, J.; Botha, M.; Dacosta, A.; Degliesposti, G.; Isea, R.; Kim, D.; Maass, A.; Kenyon, C.; Rastelli, G.; Hofmann-Apitius, M.; Breton, V. WISDOM-II: screening against multiple targets implicated in malaria using computational grid infrastructures. *Malar. J.,* **2009**, *8*, 88.

[16] Prado-Prado, F.J.; García-Mera, X.; González-Díaz, H. Multi-target spectral moment QSAR versus ANN for antiparasitic drugs against different parasite species. *Bioorg. Med. Chem.*, **2010,** *18*, 2225–2231.

[17] Ma, X.H.; Wang, R.; Tan, C.Y.; Jiang, Y.Y.; Lu, T.; Rao, H.B.; Li, X.Y.; Go, M.L.; Low, B.C.; Chen, Y.Z. Virtual screening of selective multitarget kinase inhibitors by combinatorial support vector machines. *Mol. Pharm.*, **2010**, *7*, 1545-1560.

[18] Keith, C.T.; Borisy, A.A.; Stockwell, B.R. Multicomponent therapeutics for networked systems. *Nature Rev. Drug Discov.*, **2005**, *4*, 1-8.

[19] Gilbert, I.H.; Leroy, D; Frearson, J.A. Finding new hits in neglected disease projects: target or phenotypic based screening? *Curr. Top. Med. Chem.*, **2011**, *11*, 1284-1291.

[20] Kamaraj, C.; Kaushik, N.K.; Rahuman, A.A.; Mohanakrishnan, D.; Bagavan, A.; Elango, G.; Zahir, A.A.; Santhoshkumar, T.; Marimuthu, S.; Jayaseelan, C.; Kirthi, A.V.; Rajakumar, G.; Velayutham, K.; Sahal D. Antimalarial activities of medicinal plants traditionally used in the villages of Dharmapuri regions of South India. *J. Ethnopharmacol*., **2012**, *141*, 796-802.

[21] Derbyshire, E.R.; Prudêncio, M.; Mota, M.M.; Clardy, J. Liver-stage malaria parasites vulnerable to diverse chemical scaffolds. *Proc. Natl. Acad. Sci. USA*, **2012**, *109*, 8511-8516.

[22] Guiguemde, A.; Shelat, A.A.; Bouck, D.; Duffy, S.; Crowther, G.J.; Davis, P.H.; Smithson, D.C.; Connelly, M.; Clark, J.; Zhu, F.; Jiménez-Díaz, M.B.; Martinez, M.S.; Wilson, E.B.; Tripathi, A.K.; Gut, J.; Sharlow, E.R.; Bathurst, I.; El Mazouni, F.; Fowble, J.W.; Forquer, I.; McGinley, P.L.; Castro, S.; Angulo-Barturen, I.; Ferrer, S.; Rosenthal, P.J.;

Derisi, J.L.; Sullivan, D.J.; Lazo, J.S.; Roos, D.S.; Riscoe, M.K.; Phillips, M.A.; Rathod, P.K.; Van Voorhis, W.C.; Avery, V.M.; Guy, R.K. Chemical genetics of *Plasmodium falciparum*. *Nature*, **2010**, *465*, 311-315.

[23] Gamo, F.J.; Sanz, L.M.; Vidal, J.; de Cozar, C.; Alvarez, E.; Lavandera, J-L.; Vanderwall, D.E.; Green, D.V.S.; Kumar, V.; Hasan, S.; Brown, J.R.; Peishoff, C.E.; Cardon, L.R.; Garcia-Bustos, J.F. Thousands of chemical starting points for antimalarial lead identification. *Nature*, **2010**, *465*, 305-310.

[24] Plouffe, D.; Brinker, A.; McNamara, C.; Henson, K.; Kato, N.; Kuhen, K.; Nagle, A.; Adrián, F.; Matzen, J.T.; Anderson, P.; Nam, T.G.; Gray, N.S.; Chatterjee, A.; Janes, J.; Yan, S.F.; Trager, R.; Caldwell, J.S.; Schultz, P.G.; Zhou, Y.; Winzeler, E.A. *In silico* activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. *Proc. Natl. Acad. Sci. U S A*, **2008**, *105*, 9059-9064.

[25] https:// www.ebi.ac.uk/chemblntd/, **2012**, June 18.

[26] Guiguemde, W.A.; Shelat, A.A.; Garcia-Bustos, J.F.; Diagana, T.T.; Gamo, F.J.; Guy, R.K. Global phenotypic screening for antimalarials. *Chem Biol.*, **2012**;*19*,116-29.

[27] http://www.cdc.gov/malaria/about /history/laveran.html, **2012**, May 11.

[28] Nerlich, A.G.; Schraut, B.; Dittrich, S.; Jelinek, T.; Zink, A.R. *Plasmodium falciparum* in ancient Egypt. *Emerg. Infect. Dis.*, **2008,** *14*, 1317-1319.

[29] Mayxay, M.; Pukrittayakamee, S.; Newton, P.N.; White, N.J. Mixed-species malaria infections in humans. *Trends Parasitol.*, **2004,** *20*, 233-240.

[30] Mueller, I.; Galinski, M.R.; Baird, J.K.; Carlton, J.M.; Kochar, D.K.; Alonso, P.L.; del Portillo, H.A. Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect. Dis.*, **2009,** *9*, 555-566.

[31] López, C.; Saravia, C.; Gomez, A.; Hoebeke, J.; Patarroyo, M.A. Mechanisms of genetically-based resistance to malaria. *Gene*, **2010**, *467*, 1-12.

[32] Wells, T.N.C.; Alonso, P.L.; Gutteridge, W.E. New medicines to improve control and contribute to the eradication of malaria. *Nature Rev. Drug Discov.*, **2009**, *8*, 879-891.

[33] World Health Organization. *World malaria report*: **2011**, http://www.who.int.

[34] http://malera.tropika.net, **2012**, May 25.

[35] Lelievre, J.; Almela, M.J.; Lozano, S.; Miguel, C.; Franco, V.; Leroy, D.; Herreros, E. Activity of clinically relevant antimalarial drugs on *Plasmodium falciparum* mature gametocytes in an ATP bioluminescence ''transmission blocking'' assay. *PLoS ONE,* **2012**, *7*, e35019.

[36] Silvie, O.; Mota, M.M.; Matuschewski, K.; Prudencio,M. Interactions of the malaria parasite and its mammalian host. *Curr. Opin. Microbiol.*, **2008**, *11*, 352–359.

[37] Price, R.N.; Douglas, N.M.; Anstey, N.M.; von Seidlein, L. *Plasmodium vivax* treatments: what are we looking for? *Curr. Opin. Infect. Dis.*, **2011**, *24*, 578-585.

[38] Gemma, S.; Travagli, V.; Savini, L.; Novellino, E.; Campiani, G.; Butini, S. Malaria chemotherapy: recent advances in drug development. *Recent Pat. Antiinfect. Drug Discov.,* **2010**, *5*, 195-225.

[39] Ezze, F.; Van Vugt, M.; Nosten, F.; Looareesuwan, S.; Whire, N.J. Pharmacokinetics and pharmacodynamics of lumefantrine (Benflumetol) in acute Falciparum Malaria. *Antimicrob. Agents Chemother.*, **2000**, *44*, 697–704.

[40] Foley, M.; Tilley, L. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacol. Ther.*, **1998***,79*, 55–87.

[41] Achan, J.; Talisuna, A.O.; Erhart, A.; Yeka, A.; Tibenderana, J.K.; Baliraine, F.N.; Rosenthal, P.J.; D'Alessandro, U. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malar. J.*, **2011**, *10*, 144-156.

[42] Burgess, S.J.; Kelly, J.X.; Shomloo, S.; Wittlin, S.; Brun, R.; Liebmann,K.; Peyton, D.H. Synthesis, structure-activity relationship, and mode-of-action studies of antimalarial reversed chloroquine compounds. *J. Med. Chem.*, **2010**, *53*, 6477–6489.

[43] Andrews, S.; Burgess, S.J.; Skaalrud, D.; Kelly, J.X.; Peyton, D.H. Reversal agent and linker variants of reversed chloroquines: activities against *Plasmodium falciparum. J. Med. Chem.*, **2010**, *53*, 916–919.

[44] Peyton, D.H. Reversed chloroquine molecules as a strategy to overcome resistance in malaria. *Curr. Top. Med. Chem.*, **2012**, *12*, 400–407.

[45] Biot, C.; Glorian, G.; Maciejewski, L.A.; Brocard, J.S. Synthesis and antimalarial activity in vitro and in vivo of a new ferrocene-chloroquine analogue. *J. Med. Chem.*, **1997**, *40,* 3715-3718.

[46] Supan, C.; Mombo-Ngoma, G.; Dal-Bianco, M.P.; Ospina Salazar, C.L.; Issifou, S.; Mazuir, F.; Filali-Ansary, A.; Biot, C.; Ter-Minassian, D.; Ramharter, M.; Kremsner, P.G.; Bertrand Lell, B. Pharmacokinetics of ferroquine - a second generation 4 amino-quinoline - in asymptomatic carriers of *Plasmodium falciparum* infections. *Antimicrob. Agents Chemother.*, **2012**, *56*, 3165-3173.

[47] Shanks, G.D.; Edstein, M.D. Modern malaria chemoprophylaxis. *Drugs*, **2005**, *65*, 2091-2110.

[48] http://mmv.org. **2012**, April 27.

[49] Shiraki, H.; Kozar, M.P.; Melendez, V.; Hudson, T.H.; Ohrt, C.; Magill, A.J.; Lin, Ai J. Antimalarial activity of novel 5-aryl-8-aminoquinoline derivatives. *J. Med. Chem.*, **2011**, *54*, 131-142.

[50] Kappagoda, S.; Singh, U.; Blackburn, B.G. Antiparasitic therapy. *Mayo Clin. Proc.*, **2011**, *86*, 561-583.

[51] Noedl, H.; Se, Y.; Schaecher, K.; Smith, B.L.; Socheat, D.; Fukuda, M.M. Evidence of artemisinin-resistant malaria in Western Cambodia. *N. Engl. J. Med.*, **2008**, *359*, 2619-20.

[52] O'Brien, C.; Henrich, P.P.; Passi, N.; Fidock, D.A. Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum. Curr. Opin. Infect. Dis.*, **2011**, *24*, 570-577.

[53] Phyo, A.P.; Nkhoma, S.; Stepniewska, K.; Ashley, E.A.; Nair, S.; McGready, R.; Ier Moo, C.; Al-Saai, S.; Dondorp, A.M.; Lwin, K.M.; Singhasivanon, P.; Day, N.P.; White, N.J.; Anderson, T.J.; Nosten, F. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet*, **2012**, *379*, 1960-1966.

[54] Rehwagen, C. WHO ultimatum on artemisinin monotherapy is showing results. *B.M.J.*, **2006,** *332*, 1176.

[55] Dondorp, A.M.; Nosten, F.; Yi, P.; Das, D.; Phyo, A.P.; Tarning, J.; Lwin, K.M.; Ariey, F.; Hanpithakpong, W.; Lee, S.J.; Ringwald, P.; Silamut, K.; Imwong, M.; Chotivanich, K.; Lim, P.; Herdman, T.; An, S.S.; Yeung, S.; Singhasivanon, P.; Day, N.P.; Lindegardh, N.; Socheat, D.; White, N.J. Artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.*, **2009**, *361*, 455-467.

[56] Haynes, R. K.; Fugmann, B.; Stetter, J.; Rieckmann, K.; Heilmann, H-D.; Chan, H-W.; Cheung, M-K.; Lam, W-L.; Wong, H-N.; Croft, S.L.; Vivas, L.; Rattray, L.; Stewart, L.; Peters, W.; Robinson, B.L.; Edstein, M.D.; Kotecka, B.; Kyle, D.E.; Beckermann, B.; Gerisch, M.; Radtke, M.; Schmuck, G.; Steinke, W.; Wollborn, U.; Schmeer, K.; Römer, A. Artemisone - a highly active antimalarial drug of the artemisinin class. *Angew. Chem. Int. Ed.*, **2006**, *45*, 2082-2088.

[57] Roederer, M.W.; McLeod, H.; Juliano, J.J. Can pharmacogenomics improve malaria drug policy? *Bull. World Health Organ.*, **2011**, *89*, 838-845.

[58] van Vugt, M.; van Beest, A.; Sicuri, E.; van Tulder, M.; Grobusch, M.P. Malaria treatment and prophylaxis in endemic and nonendemic countries: evidence on strategies and their cost-effectiveness. *Future Microbiol.*, **2011**, *6*, 1485-500.

[59] Barbacka, K.; Baer-Dubowska, W. Searching for artemisinin production improvement in plants and microorganisms. *Curr. Pharm. Biotechnol.*, **2011**, *12*, 1743-1751.

[60] Nagelschmitz, J.; Voith, B.; Wensing, G.; Roemer, A.; Fugman, B.; Haynes, R.K.; Kotecka, B.M.; Rieckmann, K.H.; Edstein, M.D. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamic antimalarial activity of the new artemisinin derivative artemisone. *Antimicrob. Agents Chemother.*, **2008**, *52*, 3085-3091.

[61] Guo, J.; Guiguemde, A.W.; Bentura-Marciano, A.; Clark, J.; Haynes, R.K.; Chan, W-C.; Wong, H-N.; Hunt, N.H.; Guy, R.K.; Golensera, J. Synthesis of artemiside and its effects in combination with conventional drugs against severe murine malaria. *Antimicrob. Agents Chemother.*, **2012**, *56*, 163-173.

[62] Waknine-Grinberg, J.H.; Hunt, N.; Bentura-Marciano, A.; McQuillan, J.A.; Chan, H-W.; Chan, W-C.; Barenholz, Y.; Haynes, R.K.; Golenser, J. Artemisone effective against murine cerebral malaria. *Malaria Journal*, **2010**, *9*, 227-242.

[63] Pooley, S.; Fatih, F.A.; Krishna, S.; Gerisch, M.; Haynes, R.K.; Wong, H-N.; Staines, H.M. Artemisone Uptake in *Plasmodium falciparum*-infected erythrocytes. *Antimicrob. Agents Chemother.*, **2011**, *55,* 550-556.

[64] Hawkins, V.N.; Joshi, H.; Rungsihirunrat, K.; Na-Bangchang, K.; Hopkins Sibley, C. Antifolates can have a role in the treatment of *Plasmodium vivax. Trends Parasitol.*, **2007**, *23*, 213-222.

[65] Marma, A.S.; Mita, T.; Eto, H.; Tsukahara, T.; Sarker, S.; Endo, H. High prevalence of sulfadoxine/pyrimethamine resistance alleles in *Plasmodium falciparum* parasites from Bangladesh. *Parasitol. Intern.,* **2010**, *59*, 178–182.

[66] Gutman, J.; Kachur, S.P.; Slutsker, L.; Nzila, A.; Mutabingwa, T. Combination of probenecid-sulphadoxine-pyrimethamine for intermittent preventive treatment in pregnancy. *Malaria J.*, **2012**, *11*, 39-48.

[67] Diaz, D.S.; Kozar, M.P.; Smith, K,S.; Asher, C,O.; Sousa, J.C.; Schiehser, G.A.; Jacobus, D.P.; Milhous, W.K.; Skillman, D.R.; Shearer, T.W. Role of Specific cytochrome P450 isoforms in the conversion of phenoxypropoxybiguanide analogs in human liver microsomes to potent antimalarial dihydrotriazines. *Drug Metab. Dispos.*, **2008**, *36*, 380–385.

[68] Jensen, N.P.; Ager, A.L.; Bliss, R.A.; Canfield, C.J.; Kotecka, B.M.; Rieckmann, K.H.; Terpinski, J.; Jacobus, D.P. Phenoxypropoxybiguanides, prodrugs of DHFR-inhibiting diaminotriazine antimalarials. *J. Med. Chem.,* **2001**, *44*, 3925-3931.

[69] Edstein, M.D.; Kotecka, B.M.; Ager, A.L.; Smith, K.S.; DiTusa, C.A.; Diaz, D.S.; Kyle, D.E.; Schiehser, G.A.; Jacobus, D.P.; Rieckmann, K.H.; O'Neil, M.T. Antimalarial pharmacodynamics and pharmacokinetics of a third-generation antifolate-JPC2056-in cynomolgus monkeys using an in vivo-in vitro model. *J. Antimicrob. Chemother.*, **2007,** *60*, 811–818.

[70] Mookherjee, S.; Shoen, C.; Cynamon, M. *In vitro* activity of JPC 2067 alone and in combination with sulfamethoxazole against *Nocardia* species. *Antimicrob. Agents Chemother.*, **2012**, *56*, 1133–1134.

[71] Gahtori, B.; Ghosh, S.K.; Parida, P.; Prakash, A.; Gogoi, K.; Bhat, H.R.; Singh, U.P. Antimalarial evaluation and docking studies of hybrid phenylthiazolyl-1,3,5-triazine derivatives: a novel and potential antifolate lead for Pf-DHFR-TS inhibition. *Exp. Parasitol.*, **2012**, *130*, 292–299.

[72] McKeage, K.; Scott, L.J. Atovaquone/Proguanil. A review of its use for the prophylaxis of *Plasmodium falciparum* malaria. *Drugs*, **2003**, *63*, 597-623.

[73] Yeates, C.L.; Batchelor, J.F.; Capon, E.C.; Cheesman, N.J.; Fry, M.; Hudson, A.T.; Pudney, M.; Trimming, H.; Woolven, J.; Bueno, J.M.; Chicharro, J.; Fernández, E.; Fiandor, J.M.; Gargallo-Viola, D.; Gómez de las Heras, F.; Herreros, E., León, M.L. Synthesis and structure-activity relationships of 4-Pyridones as potential antimalarials. *J. Med. Chem.*, **2008,** *51,* 2845–2852.

[74] Barton, V.; Fisher, N.; Biagini, G.A.; Ward, S.A.; O'Neill, P.M. Inhibiting Plasmodium cytochrome bc1: a complex issue. *Curr. Opin. Chem. Biol.*, **2010**, *14*, 440-446.

[75] Coatney, G.R.; Greenberg, J. The use of antibiotics in the treatment of malaria. *Ann. N. Y. Acad. Sci.*, **1952,** *55*, 1075-1081.

[76] Geary, T.G.; Jensen, J.B. Effects of antibiotics on *Plasmodium falciparum* in vitro. *Am. J. Trop. Med. Hyg.*, **1983**, *32*, 221-225.

[77] McFadden, G.I. The apicoplast. *Protoplasma,* **2011**, *248*, 641-650.

[78] Ekland, E.H.; Schneider, J.; Fidock, D.A. Identifying apicoplast-targeting antimalarials using high-throughput compatible approaches. *FASEB J.*, **2011**, *25*, 3583-3593.

[79] Goodman, C.D.; Su, V.; McFadden, G.I. The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.*, **2007**, *152,* 181–191.

[80] Dahl, E.L.; Rosenthal, P.J. Apicoplast translation, transcription and genome replication: targets for antimalarial antibiotics. *Trends Parasitol.*, **2008**, *24*, 279-284.

[81] Lell, B.; Ruangweerayut, R.; Wiesner, J.; Missinou, M.A.; Schindler, A.; Baranek, T.; Hintz, M.; Hutchinson, D.; Jomaa, H.; Kremsner, P.G. Fosmidomycin, a novel chemotherapeutic agent for malaria. *Antimicrob. Agents Chemother.*, **2003**, *47*, 735–738.

[82] Lanaspa, M.; Moraleda, C.; Machevo, S.; González, R.; Serrano, B.; Macete, E.; Cisteró, P.; Mayor, A.; Hutchinson, D.; Kremsner, P.G.; Alonso, P.; Menéndez, C.; Bassat, Q. Inadequate efficacy of a new formulation of fosmidomycin-clindamycin combination in Mozambican children less than three years old with uncomplicated *Plasmodium falciparum* malaria. *Antimicrob. Agents Chemother.*, **2012**, *56*, 2923–2928.

[83] Aminake, M.N.; Schoof, S.; Sologub, L.; Leubner, M.; Kirschner, M.; Arndt, H-D.; Pradel, G. Thiostrepton and derivatives exhibit antimalarial and gametocytocidal activity by dually targeting parasite proteasome and apicoplast. *Antimicrob. Agents Chemother.*, **2011**, 1338-1348.

[84] Ponts, N.; Saraf, A.; Chung, D-W.; Harris, A.; Prudhomme, J.; Washburn, M.P.; Florens, L.; Le Roch, K.G. Unraveling the ubiquitome of the human malaria parasite. *J. Biol. Chem.*, **2011**, *286*, 40320-40330.

[85] Gantt, S.M.; Myung, J.M.; Briones, M.R.; Li, W.D.; Corey, E.J.; Omura, S.; Nussenzweig, V.; Sinnis, P. Proteasome inhibitors block development of Plasmodium spp. *Antimicrob. Agents Chemother.*, **1998,** *42*, 2731-2738.

[86] Lindenthal, C.; Weich, N.; Chia, Y.S.; Heussler, V.; Klinkert, M.Q. The proteasome inhibitor MLN-273 blocks exoerythrocytic and erythrocytic development of Plasmodium parasites. *Parasitology*, **2005**, *131*, 37-44.

[87] Reynolds, J.M.; El Bissati, K.; Brandenburg, J.; Günzl, A.; Mamoun, C.B. Antimalarial activity of the anticancer and proteasome inhibitor bortezomib and its analog ZL3B. *BMC Clin. Pharmacol.*, **2007**, *7*, 13-18.

[88] Kreidenweiss, A.; Kremsner, P.G.; Mordmüller, B. Comprehensive study of proteasome inhibitors against *Plasmodium falciparum* laboratory strains and field isolates from Gabon. *Malaria J.*, **2008**, *7*, 187-194.

[89] Prudhomme, J.; McDaniel, E.; Ponts, N.; Bertani, S.; Fenical, W.; Jensen, P.; Le Roch, K. Marine actinomycetes: a new source of compounds against the human malaria parasite. *PLoS One*, **2008**, *3*, e2335.

[90] Czesny, B.; Goshu, S.; Cook, J.L.; Williamson, K.C. The proteasome inhibitor epoxomicin has potent *Plasmodium falciparum* gametocytocidal activity. *Antimicrob. Agents Chemother.*, **2009**, *53*, 4080-4085.

[91] Schoof, S.; Pradel, G.; Aminake, M.N.; Ellinger, B.; Baumann, S.; Potowski, M.; Najajreh, Y.; Kirschner, M.; Arndt, H.D. Antiplasmodial thiostrepton derivatives: proteasome inhibitors with a dual mode of action. *Angew. Chem. Int. Ed. Engl.*, **2010**, *49*, 3317-3321.

[92] Bagley, M.C.; Dale, J.W.; Merritt, E.A.; Xiong, X. Thiopeptide antibiotics. *Chem. Rev.*, **2005**, *105*, 685-714.

[93] Noedl, H. ABC-antibiotics-based combinations for the treatment of severe malaria? *Trends Parasitol.*, **2009**, *25*, 540-544.

[94] WHO, Guidelines for the treatment of malaria. 2nd ed., **2010**, WHO, Geneva, Switzerland.

[95] Obonyo, C.O.; Juma, E.A. Clindamycin plus quinine for treating uncomplicated falciparum malaria: a systematic review and meta-analysis. *Malaria J.*, **2012**, *11*, 2-12.

[96] Chung, M.C.; Ferreira, E.I.; Santos, J.L.; Giarolla, J.; Rando, D.G.; Almeida, A.E.; Bosquesi, P.L.; Menegon, R.F.; Blau, L. Prodrugs for the treatment of neglected diseases. *Molecules*, **2007**, *13*, 616-677.

[97] Wittlin, S.; Ekland, E.; Craft, J.C.; Lotharius, J.; Bathurst, I.; Fidock, D.A.; Fernandes, P. *In vitro* and *in vivo* activity of solithromycin (CEM-101) against *Plasmodium* species. *Antimicrob. Agents Chemother.*, **2012**, *56*, 703–707.

[98] Chung, D-W.; Ponts, N.; Cervantes, S.; Le Roch, K.G. Post-translational modifications in Plasmodium: More than you think! *Mol. Biochem. Parasitol.*, **2009**,*168*, 123-134.

[99] Sanz, L.M.; Jimenez-Díaz, M.B.; Crespo, B.; De-Cozar, C.; Almela, M.J.; Angulo-Barturen, I.; Castaneda, P.; Ibanez, J.; Fernandez, E.P.; Ferrer, S.; Herreros, E.; Lozano, S.; Martínez, M.S.; Rueda, L.; Burrows, J.N.; García-Bustos, J.F.; Gamo, F.J. Cyclopropyl carboxamides, a chemically novel class of antimalarial agents identified in a phenotypic screen. *Antimicrob. Agents Chemother.*, **2011**, *55*, 5740–5745.

[100] Yeung, B.K.S.; Zou, B.; Rottmann, M.; Lakshminarayana, S.B.; Ang, S.H.; Leong, S.Y.; Tan, J.; Wong, J.; Keller-Maerki, S.; Fischli, C.; Goh, A.; Schmitt, E.K.; Krastel, P.; Francotte, E.; Kuhen, K..; Plouffe, D.; Henson, K.; Wagner, T.; Winzeler, E.A.; Petersen, F.; Brun, R.; Dartois, V.; Diagana, T.T.; Keller, T.H. Spirotetrahydro β-carbolines (spiroindolones): a new class of potent and orally efficacious compounds for the treatment of malaria. *J. Med. Chem.*, **2010**, *53*, 5155–5164.

[101] Rottmann, M.; McNamara, C.; Yeung, B.K.S.; Lee, M.C.S.; Zou, B.; Russell, B.; Seitz, P.; Plouffe, D.M.; Dharia, N.V.; Tan, J.; Cohen, S.B.; Spencer, K.R.; González-Páez, G.E.; Lakshminarayana, S.B.; Goh, A.; Suwanarusk, R.; Jegla, T.; Schmitt, E.K.; Beck, H.P.; Brun, R.; Nosten, F.; Renia, L.; Dartois, V.; Keller, T.H.; Fidock, D.A.; Winzeler, E.A.; Diagana, T.T. Spiroindolones, a potent compound class for the treatment of malaria. *Science*, **2010**, *329*, 1175-1180.

[102] v Pelt-Koops, J.C.; Pett, H.E.; Graumans, W.; vd Vegte-Bolmer, M.; v Gemert, G.J.; Rottmann, M.; Yeung, B.K.S.; Diagana, T.T.; Sauerwein, R.W. The spiroindolone drug candidate NITD609 potently inhibits gametocytogenesis and blocks *Plasmodium falciparum* transmission to Anopheles mosquito vector. *Antimicrob. Agents Chemother.*, **2012**, *56*, 3544-3548.

[103] Wu, T.; Nagle, A.; Kuhen, K.; Gagaring, K.; Borboa, R.; Francek, C.; Chen, Z.; Plouffe, D.; Goh, A.; Lakshminarayana, S.B.; Wu, J.; Ang, H.Q.; Zeng, P.; Kang, M.L.; Tan, W.; Tan, M.; Ye, N.; Lin, X.; Caldwell, C.; Ek, J.; Skolnik, S.; Liu, F.; Wang, J.; Chang, J.; Li, C.; Hollenbeck, T.; Tuntland, T.; Isbell, J.; Fischli, C.; Brun, R.; Rottmann, M.; Dartois, V.; Keller, T.; Diagana, T.; Winzeler, E.; Glynne, R.; Tully, D.C.; Chatterjee, A.K. Imidazolopiperazines: hit to lead optimization of new antimalarial agents. *J. Med. Chem.*, **2011**, *54*, 5116–5130.

[104] Powles, M.A.; Allocco, J.; Yeung, L.; Nare, B.; Liberator, P.; Schmatz, D. MK-4815, a potential new oral agent for treatment of malaria. *Antimicrob. Agents Chemother.*, **2012**, *56*, 2414–2419.

[105] Shanks, G. D. Treatment of falciparum malaria in the age of drug resistance. *J. Postgrad. Med.*, **2006,** *52*, 277-280.

[106] Krettli, A.U.; Andrade-Neto, V.F.; Brandão, M.G.; Ferrari, W.M. The search for new antimalarial drugs from plants used to treat fever and malaria or plants randomly selected: a review. *Mem. Inst. Oswaldo Cruz*, **2001**, *96*, 1033-1042.

[107] Vigneron, M.; Deparis, X.; Deharo, E.; Bourdy, G. Antimalarial remedies in French Guiana: a knowledge attitudes and practices study. *J. Ethnopharmacol.*, **2005**, *98*, 351-360.

[108] Soh, P.N.; Benoit-Vical, F. Are West African plants a source of future antimalarial drugs? *J. Ethnopharmacol.,* **2007**, *114*, 130-140.

[109] Wright, C.W. Recent developments in research on terrestrial plants used for the treatment of malaria. *Nat. Prod. Rep.*, **2010**, *27*, 961-968.

[110] Willcox, M.L.; Burton, S.; Oyweka, R.; Namyalo, R.; Challand, S.; Lindsey, K. Evaluation and pharmacovigilance of projects promoting cultivation and local use of *Artemisia annua* for malaria. *Malaria J.*, **2011**, *10*, 84-90.

[111] Oliveira, A.B.; Dolabela, M.F.; Braga, F.C.; Jácome, R.L.; Varotti, F.P.; Póvoa, M.M. Plant-derived antimalarial agents: new leads and efficient phythomedicines. Part I. Alkaloids. *An. Acad. Bras. Cienc.*, **2009,** *81*, 715-740.

[112] Graz, B.; Falquet, J.; Diallo, D.; Willcox, M.; Giani, S. Screening of traditional herbal medicine: First, do a retrospective study, with correlation between diverse treatments used and reported patient outcome. *J. Ethnopharmacol.*, **2005**, *101*, 338–339.

[113] Graz, B.; Falquet, J.; Elisabetsky, E. Ethnopharmacology, sustainable development and cooperation: the importance of gathering clinical data during field surveys. *J. Ethnopharmacol.*, **2010**, *130*, 635–638.

[114] Batista, R.; Silva Ade, J. Jr.; de Oliveira, A.B. Plant-derived antimalarial agents: new leads and efficient phytomedicines. Part II. Non-alkaloidal natural products. *Molecules*, **2009**, *14*, 3037-3072.

[115] Kaur, K.; Jain, M.; Kaur, T.; Jain, R. Antimalarials from nature. *Bioorg. Med. Chem.*, **2009**, *17*, 3229-3256.

[116] Ojima, I. Modern natural products chemistry and drug discovery. *J. Med. Chem.*, **2008**, *51*, 2587-2588.

[117] Newman, D. J. Natural products as leads to potential drugs: an old process or the new hope for drug discovery? *J. Med. Chem.*, **2008**, *51*, 2589–2599.

[118] Alcaide, B.; Almendros, P. Allenyl-b-lactams: versatile scaffolds for the synthesis of heterocycles. *Chem. Rec.*, **2011,** *11*, 311–330.

[119] Robin, A.; Brown, F.; Bahamontes-Rosa, N.; Wu, B.; Beitz, E.; Kun, J.F.J.; Flitsch, S.L. Microwave-assisted ring opening of epoxides: a general route to the synthesis of 1-aminopropan-2-ols with anti malaria parasite activities. *J. Med. Chem.*, **2007**, *50,* 4243-4249.

[120] D'hooghe, M.; Dekeukeleire, S.; Mollet, K.; Lategan, C.; Smith, P.J.; Chibale, K.; De Kimpe, N. Synthesis of novel 2-alkoxy-3-amino-3-arylpropan-1-ols and 5-alkoxy-4-aryl-1,3-oxazinanes with antimalarial activity. *J. Med. Chem.,* **2009,** *52,* 4058–4062.

[121] Avilés, E.; Rodrìguez, A.D. Monamphilectine A, a potent antimalarial-β-lactam from marine sponge *Hymeniacidon* sp: isolation, structure, semisynthesis, and bioactivity. *Org. Lett.,* **2010**, *12*, 5290-5293.

[122] Schobel, U.; Frenay, M.; Van Elswijk, D.A.; McAndrews, J.M.; Long, K.R.; Olson, L.M.; Bobzin, S.C.; Irth, H. High resolution screening of plant natural product extracts for estrogen receptor α and β binding activity using an online HPLC-MS biochemical detection system. *J. Biomol. Screen.,* **2001**, *6*, 291-303.

[123] Adams, M.; Zimmermann, S.; Kaiser, M.; Brun, R.; Hamburger, M. A protocol for HPLC-based activity profiling for natural products with activities against tropical parasites. *Nat. Prod. Comm.*, **2009**, *4*, 1377-1381.

[124] Zimmermann, S.; Thomi, S.; Kaiser, M.; Hamburger, M.; Adams, M. Screening and HPLC-based activity profiling for new antiprotozoal leads from European plants. *Sci Pharm.*, **2012**, *80*, 205–213.

[125] Crowther, G.J.; Shanmugam, D.; Carmona, S.J.; Doyle, M.A.; Hertz-Fowler, C.; Berriman, M.; Nwaka, S.; Ralph, S.A.; Roos, D.S.; Van Voorhis, W.C.; Agüero, F. Identification of attractive drug targets in neglected-disease pathogens using an in silico approach. *PLOS Negl. Trop. Dis.*, **2010**, *4*, (8) e804.

[126] http://www.who.int/leishmaniasis/resources/Leishmaniasis_worldwide_epidemiological_ and_drug_access_update.pdf**. 2012**, June 29.

[127] Alvar, J.; Canavate, C.; Gutierrez-Solar, B.; Jimenez, M.; Laguna, F.; Lopez-Velez, R.; Molina, R.; Moreno, J. *Leishmania* and Human Immunodeficiency Virus coinfection: the first 10 years. *Clin. Microbiol. Rev.*, **1997**, *10*, 298-319.

[128] Cruz, I.; Nieto, J.; Moreno, J.; Canavate, C.; Desjeux, P.; Alvar, J. Leishmania/HIV co-infections in the second decade. *Indian J. Med. Res.*, **2006**, *123*, 357-388.

[129] WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March **2010**. (WHO technical report series; no. 949).

[130] Freitas, L.H.; Chatelain, E.; Kim, H.A.; Siqueira-Neto, J.L. Visceral leishmaniasis treatment: What do we have, what do we need and how to deliver it? *Intern. J. Parasitol.: Drugs and Drug Resist.*, **2012***, 2*, 11–19.

[131] Vianna, G. Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. 7 Congresso Brasileiro de Medicina Tropical de São Paulo, São Paulo, Brasil, **1912**, 4, 426-428.

[132] Frézard, F.; Demicheli, C; Ribeiro. R.R. Pentavalent antimonials: new perspectives for old drugs. *Molecules*, **2009**, 14, 2317-2336

[133] Sundar, S.; More, D.K.; Singh, M.K.; Singh, V.P.; Sharma, S.; Makharia, A.; Kumar, P.C.; Murray, H,W. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin. Infect. Dis.*, **2000**, 31, 1104-1107.

[134] Le Pape, P. Development of new antileishmanial drugs--current knowledge and future prospects. *J. Enzyme Inhib. Med. Chem.*, **2008**, 23, 708-718.

[135] Dorlo, T.P.; Kager, P.A. Pentamidine dosage: a base/salt confusion. *PLoS Negl. Trop. Dis.*, **2008**, 2, e225.

[136] ClinicalTrials.gov. Prophylaxis of Visceral Leishmaniasis Relapses in HIV Co-infected Patients with Pentamidine: A Cohort Study (NCT01360762). **2011**. http://clinicaltrials.gov/ct2/show/NCT01360762 (November 12 2012, date last accessed).

[137] Sundar, S.; Singh, A.; Rai, M.; Prajapati, V.K.; Singh, A.K.; Ostyn, B.; Boelaert, M.; Dujardin, J-C.; Chakravarty, J. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin. Infect. Dis.*, **2012**, May 31, Epub ahead of print.

[138] Sundar, S.; Rai, M. Treatment of visceral leishmaniasis. *Expert Opin. Pharmacother.*, **2005**, 6,2821-2829.

[139] Sundar, S.; Chakravarty, J.; Agarwal, D.; Rai, M.; Murray, H.W. Single-Dose Liposomal Amphotericin B for Visceral Leishmaniasis in India. *N. Engl. J. Med.*, **2010,** 362, 504-512.

[140] http://www.who.int/leishmaniasis/research/978_92_4_12_949_6_Annex6.pdf, last accessed 11/12/2012.

[141] Sinha, P.K.; Jha, T.K.; Thakur, C.P.; Nath, D.; Mukherjee, S.; Aditya, A.K.; Sundar, S. Phase 4 Pharmacovigilance Trial of Paromomycin Injection for the Treatment of Visceral Leishmaniasis in India. *J. Trop. Med.*, **2011**, 2011, 645203.

[142] Kobets, T.; Grekov, I.; Lipoldová, M. Leishmaniasis: Prevention, Parasite Detection and Treatment. *Curr Med Chem.*, **2012**, 19,1443-74.

[143] Croft, S.L.; Olliaro, P. Leishmaniasis chemotherapy-challenges and opportunities. *Clin. Microbiol. Infect.*, **2011**, *17*, 1478-1483.

[144] Murray, H.W. Review: leishmaniasis in the United States: treatment in 2012. *Am. J. Trop. Med. Hyg.*, **2012**, *86*, 434-440.

[145] Antinori, S.; Schifanella, L.; Corbellino, M. Leishmaniasis: new insights from an old and neglected disease. *Eur. J. Clin. Microbiol. Infect. Dis.*, **2012**, 31, 109-118.

[146] Singh, N.; Kumar, M.; Singh, R.K. Leishmaniasis: current status of available drugs and new potential drug targets. *Asian Pac. J. Trop. Med.*, **2012**, 5, 485-497.

[147] Das, S.; Rani, M.; Pandey, K.; Sahoo, G.C.; Rabidas, V.N.; Singh, D.; Das, P. Combination of paromomycin and miltefosine promotes TLR4-dependent induction of antileishmanial immune response in vitro. *J. Antimicrob. Chemother.*, **2012**, 67, 2373-2378.

[148] Seifert, K.; Munday, J.; Syeda, T.; Croft, S.L. In vitro interactions between sitamaquine and amphotericin B, sodium stibogluconate, miltefosine, paromomycin and pentamidine against *Leishmania donovani*. *J. Antimicrob. Chemother.*, **2011**, *66*, 850-854.

[149] Ribeiro, R.R.; Ferreira, A.W.; Martins, S.P.; Neto, L.R.; Rocha, G.O.; Le Moyec, L.; Demicheli, C.; Frezard, F. Prolonged absorption of antimony(V) by the oral route from non-inclusion meglumine antimoniate-beta-cyclodextrin conjugates. *Biopharm. Drug Dispos.,* **2010**, *31*, 109-119.

[150] Ribeiro, R.R.; Moura, P.E.; Pimentel, M.V.; Sampaio, M.W.; Silva, M.S.; Schettini, A.D.; Alves, F.C.; Melo, A.F.; Tafuri, L.W.; Demicheli, C.; Melo, N.M.; Frezard, F.; Michalick, S.M. Reduced tissue parasitic load and infectivity to sand flies in dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi* following treatment with a liposome formulation of meglumine antimoniate. *Antimicrob. Agents Chemother.,* **2008**, *52*, 2564-72.

[151] Frézard, F.; Demicheli, C. New delivery strategies for the old pentavalent antimonial drugs. *Expert Opin. Drug Deliv.*, **2010**, 7, 1343-1358.

[152] Gupta, S.; Nishi. Visceral leishmaniasis: experimental models for drug discovery. *Indian J. Med. Res.*, **2011**, *133*, 27-39.

[153] Seifert, K.; Escobar, P.; Croft, S.L. In vitro activity of anti-leishmanial drugs against *Leishmania donovani* is host cell dependent. *J. Antimicrob. Chemother.*, **2010,** *65*, 508-511.

[154] De Muylder, G.; Ang, K.K.N.; Chen, S.; Arkin, M.R.; Engel1, J.C.; McKerrow, J.K. A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Negl. Trop. Dis.*, **2011**, *5*, e1253.

[155] Carvalho, L.; Luque-Ortega, J.R.; López-Martín, C.; Castanys, S.; Rivas, L.; Gamarro ,F. The 8-aminoquinoline analogue sitamaquine causes oxidative stress in *Leishmania donovani* promastigotes by targeting succinate dehydrogenase. *Antimicrob. Agents Chemother.*, **2011**, *55(9)*, 4204-4210.

[156] Loiseau,P.M.; Cojean, S.; Schrével, J. Sitamaquine as a putative antileishmanial drug candidate: from the mechanism of action to the risk of drug resistance. *Parasite*, **2011**, *18*, 115-119.

[157] Yardley, V.; Gamarro, F.; Croft, S.L. Antileishmanial and antitrypanosomal activities of the 8-Aminoquinoline tafenoquine. *Antimicrob. Agents Chemother.*, **2010**, *54*, 5356-5358.

[158] Torreele, E.; Bourdin Trunz, B.; Tweats, D.; Kaiser, M.; Brun, R.; Mazué, G.; Bray, M.A.; Pécoul, B. Fexinidazole - a new oral nitroimidazole drug candidate entering clinical development for the treatment of sleeping sickness. *PLoS Negl. Trop. Dis.***, 2010,** *4*, e923.

[159] Wyllie, S.; Patterson, S,.; Stojanovski, L:, Simeons, F.R.; Norval, S.; Kime, R.; Read, K.D.; Fairlamb, A.H.. The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. *Sci. Transl. Med.*, **2012**, *4*, 119re1.

[160] Colotti, G.; Ilari, A. Polyamine metabolism in Leishmania: from arginine to trypanothione. *Amino Acids*, **2011**, 40, 269-285.

[161] Ilari, A.; Baiocco, P.; Messori, L.; Fiorillo, A.; Boffi, A.; Gramiccia, M.; Di Muccio, T.; Colotti, G. A gold-containing drug against parasitic polyamine metabolism: the X-ray structure of trypanothione reductase from *Leishmania infantum* in complex with auranofin reveals a dual mechanism of enzyme inhibition. *Amino Acids*, **2012**, 42, 803-811.

[162] Venkatesan, S.K.; Saudagar, P.; Shukla, A.K.; Dubey, V.K. Screening natural products database for identification of potential antileishmanial chemotherapeutic agents. *Interdiscip. Sci*., **2011**, 3, 217-231.

[163] Grover, A.; Katiyar, S.P.; Singh, S.K.; Dubey, V.K.; Sundar, D. A leishmaniasis study: Structure-based screening and molecular dynamics mechanistic analysis for discovering potent inhibitors of spermidine synthase. *Biochim. Biophys. Acta*, **2012**, 1824, 1476-1483.

[164] Caroselli, E.E.; Assis, D.M.; Barbiéri, C.L.; Júdice, W.A.; Juliano, M.A.; Gazarini, M.L.; Juliano, L. *Leishmania (L.) amazonensis* peptidase activities inside the living cells and in their lysates. *Mol. Biochem. Parasitol*., **2012**, 184, 82-89.

[165] Rana, S.; Dikhit, M.R.; Rani, M.; Moharana, K.C.; Sahoo, G.C.; Das, P. CPDB: cysteine protease annotation database in *Leishmania* species. *Integr. Biol. (Camb)*., **2012,** 4, 1351-1357.

[166] White, R.E.; Powell, D.J.; Berry C. HIV proteinase inhibitors target the Ddi1-like protein of Leishmania parasites. *FASEB J*., **2011**, 25, 1729-1736.

[167] Santos, L.O.; Vitório, B.S.; Branquinha, M.H.; Silva, C.M.; Santos, A.L.; d'Avila-Levy, C.M. Nelfinavir is effective in inhibiting the multiplication and aspartic peptidase activity of Leishmania species, including strains obtained from HIV-positive patients. *J. Antimicrob. Chemother*., **2012**, Oct 28 [Epub ahead of print].

[168] Gómez, M.A.; Olivier, M. Proteases and phosphatases during Leishmania-macrophage interaction: paving the road for pathogenesis. *Virulence,* **2010**, 1, 314-318.

[169] Isnard,A.; Shio, M.T.; Olivier, M. Impact of Leishmania metalloprotease GP63 on macrophage signaling. *Front. Cell. Infect. Microbiol.*, **2012**, 2, 72.

[170] Heneberg, P. Finding the smoking gun: protein tyrosine phosphatases as tools and targets of unicellular microorganisms and viruses. *Curr. Med. Chem.*, **2012**, 19, 1530-1566.

[171] Mazumder, S.; Maji, M.; Ali, N. Potentiating effects of MPL on DSPC bearing cationic liposomes promote recombinant GP63vaccine efficacy: high immunogenicity and protection. *PLoS Negl. Trop. Dis.*, **2011**, 5, e1429.

[172] Krauth-Siegel, R.L.; Inhoff, O. Parasite-specific trypanothione reductase as a drug target molecule. *Parasitol. Res.*, **2003**, 90, Suppl 2, S77-85.

[173] Rodrigues, R.F.; Castro-Pinto, D.; Echevarria, A.; dos Reis, C.M.; Del Cistia, C.N.; Sant'Anna, C.M.; Teixeira, F.; Castro, H.; Canto-Cavalheiro, M.; Leon, L.L.; Tomás, A. Investigation of trypanothione reductase inhibitory activity by 1,3,4-thiadiazolium-2-aminide derivatives and molecular docking studies. *Bioorg. Med. Chem.*, **2012**, 20, 1760-1766.

[174] Naula, C.; Parsons, M.; Mottram, J.C. Protein kinases as drug targets in trypanosomes and Leishmania. *Biochim. Biophys. Acta*, **2005**, 1754, 151-159.

[175] Jorda, R.; Sacerdoti-Sierra, N.; Voller, J.; Havlíček, L.; Kráčalíková, K.; Nowicki, M.W.; Nasereddin, A.; Kryštof, V.; Strnad, M.; Walkinshaw, M.D.; Jaffe, C.L. Anti-leishmanial activity of disubstituted purines and related pyrazolo[4,3-d]pyrimidines. *Bioorg. Med. Chem.*, Lett., **2011**, 21, 4233-4237.

[176] Walker, R.G.; Thomson, G.; Malone, K.; Nowicki, M.W.; Brown, E.; Blake, D.G.; Turner, N.J.; Walkinshaw, M.D.; Grant, K.M.; Mottram, J.C. High throughput screens yield small molecule inhibitors of Leishmania CRK3:CYC6 cyclin-dependent kinase. *PLoS Negl. Trop. Dis.*, **2011**, *5*, e1033.

[177] Cleghorn, L.A.; Woodland, A.; Collie, I.T.; Torrie, L.S.; Norcross, N.; Luksch, T.; Mpamhanga, C.; Walker, R.G.; Mottram, J.C.; Brenk, R.; Frearson, J.A.; Gilbert, I.H.; Wyatt, P.G. Identification of inhibitors of the Leishmania cdc2-related protein kinase CRK3. *Chem. Med. Chem.*, **2011**, 6, 2214-2224.

[178] Lorente, S.O.; Jimenez, C.J.; Gros, L.; Yardley, V.; de Luca-Fradley, K.; Croft, S.L.,; Urbina, J.A.; Ruiz-Perez, L.M.; Pacanowska, D.G.; Gilbert, I.H. Preparation of transition-state analogues of sterol 24-methyl transferase as potential anti-parasitics. *Bioorg. Med. Chem.*, **2005**, 13, 5435-5453.

[179] Gros, L.; Lorente, S.O.; Jimenez, C.J.; Yardley, V.; Rattray, L.; Wharton, H.; Little, S.; Croft, S.L.; Ruiz-Perez, L.M.; Gonzalez-Pacanowska, D.; Gilbert, I.H. Evaluation of azasterols as anti-parasitics. *J. Med. Chem.*, **2006,** 49, 6094-6103.

[180] Pan, L.; Lezama-Davila, C.M.; Isaac-Marquez, A.P.; Calomeni, E.P.; Fuchs, J.R.; Satoskar, A.R.; Kinghorn, A.D. Sterols with antileishmanial activity isolated from the roots of *Pentalinon andrieuxii. Phytochemistry*, **2012**, 82, 128-135.

[181] Medina, J.M.; Rodrigues, J.C.; De Souza, W.; Atella, G.C., Barrabin, H. Tomatidine promotes the inhibition of 24-alkylated sterol biosynthesis and mitochondrial dysfunction in Leishmania amazonensis promastigotes. *Parasitology*, **2012**, 139, 1253-1265.

[182] Cunningham, M.L.; Beverley, S.M. Pteridine salvage throughout the Leishmania infectious cycle: implications for antifolate chemotherapy. *Mol. Biochem. Parasitol.*, **2001**, 113, 199-213.

[183] Corona, P.; Gibellini, F.; Cavalli, A.; Saxena, P.; Carta, A.; Loriga, M.; Luciani, R.; Paglietti, G.; Guerrieri, D.; Nerini, E.; Gupta, S.; Hannaert, V.; Michels, P.A.; Ferrari, S.; Costi, P.M. Structure-based selectivity optimization of piperidine-pteridine derivatives as potent leishmania pteridinereductase inhibitors. *J. Med. Chem.*, **2012**, 55, 8318-8329.

[184] Rajasekaran, R.; Chen, Y.P. Probing the structure of Leishmania major DHFR TS and structure based virtual screening of peptide library for the identification of anti-leishmanial leads. *J. Mol. Model.*, **2012**, 18, 4089-4100.

[185] Boitz, J.M.; Ullman, B.; Jardim, A.; Carter, N.S. Purine salvage in Leishmania: complex or simple by design? *Trends Parasitol.*, **2012**, 28, 345-352.

[186] Bowyer, P.W.; Tate, E.W.; Leatherbarrow, R.J.; Holder, A.A.; Smith, D.F.; Brown, K.A. N-myristoyltransferase: a prospective drug target for protozoan parasites. *Chem. Med. Chem.*, **2008**, 3, 402-408.

[187] Sharlow, E.R.; Close, D.; Shun, T.; Leimgruber, S.; Reed, R.; Mustata, G.; Wipf, P.; Johnson, J.; O'Neil, M.; Grögl, M.; Magill, A.J.; Lazo, JS. Identification of potent chemotypes targeting *Leishmania major* using a high-throughput, low-stringency, computationally enhanced, small molecule screen. *PLoS Negl. Trop Dis.*, **2009**, 3, e540.

[188] Zhu, X.; Pandharkar, T.; Werbovetz, K. Identification of new antileishmanial leads from hits obtained by high-throughput screening. *Antimicrob. Agents Chemother.*, **2012,** 56, 1182-1189.

[189] Chavali, A.K.; D'Auria, K.M.; Hewlett, E.L.; Pearson, R.D.; Papin, J.A. A metabolic network approach for the identification and prioritization of antimicrobial drug targets. *Trends Microbiol.*, **2012**, 20, 113-123.

[190] Schmidt, T.J.; Khalid, S.A.; Romanha, A.J.; Alves, T.M.; Biavatti, M.W.; Brun, R.; Da Costa, F.B.; de Castro, S.L.; Ferreira, V.F.; de Lacerda, M.V.; Lago, J.H..; Leon, L.L.; Lopes, N.P.; das Neves Amorim, R.C.; Niehues, M.; Ogungbe, I.V.; Pohlit, A.M.; Scotti, M.T.; Setzer, W.N.; de N C Soeiro, M.; Steindel, M.; Tempone, A.G. The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases - part I. *Curr. Med. Chem.*, **2012**, 19, 2128-2175.

[191] Schmidt, T.J.; Khalid, S.A.; Romanha, A.J.; Alves, T.M.; Biavatti, M.W.; Brun, R.; Da Costa, F.B.; de Castro, S.L.; Ferreira, V.F.; de Lacerda, M.V.; Lago, J.H..; Leon, L.L.; Lopes, N.P.; das Neves Amorim, R.C.; Niehues, M.; Ogungbe, I.V.; Pohlit, A.M.; Scotti, M.T.; Setzer, W.N.; de N C Soeiro, M.; Steindel, M.; Tempone, A.G. The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases - part II. *Curr. Med. Chem.*, **2012**, 19, 2176-2228.

[192] Wink, M.; Ashour, M.L.; El-Readi, M.Z. Secondary Metabolites from Plants Inhibiting ABC Transporters and Reversing Resistance of Cancer Cells and Microbes to Cytotoxic and Antimicrobial Agents. *Front. Microbiol.*, **2012**, 3, Article 130.

[193] Alviano, D.S.; Barreto, A.L.; Dias Fde, A.; Rodrigues Ide, A.; Rosa Mdo, S.; Alviano, C.S.; Soares, R.M. Conventional therapy and promising plant-derived compounds against trypanosomatid parasites. *Front. Microbiol.*, **2012**, 3, Article 283.

[194] Makwali, J.A.; Wanjala, F.M.; Kaburi, J.C.; Ingonga, J.; Byrum, W.W.; Anjili, C.O. Combination and monotherapy of *Leishmania major* infection in BALB/c mice using plant extracts and herbicides. *J. Vector Borne Dis.*, **2012**, 49, 123-130.

[195] Siqueira-Neto, J.L.; Song, O.R.; Oh, H.; Sohn, J.H.; Yang, G.: Nam, J.; Jang, J.; Cechetto, J.; Lee, C.B.; Moon, S.; Genovesio, A.; Chatelain, E.; Christophe, T.; Freitas-Junior, L.H. Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. *PLoS Negl. Trop. Dis.*, **2010**, 4, e675.

[196] Osorio, Y.; Travi, B.L.; Renslo, A.R.; Peniche, A.G.; Melby, P.C. Identification of small molecule lead compounds for visceral leishmaniasis using a novel ex vivo splenic explant model system. *PLoS Negl. Trop. Dis.*, **2011**, 5, e962.

[197] Bustamante, J.M.; Park, H.J.; Tarleton, R.L. The Chagas Drug Discovery Consortium. Report of the 2nd Chagas Drug Discovery Consortium meeting, held on 3 November 2010; Atlanta GA, USA. *Expert Opin. Drug Discov.*, **2011**, *6(9)*, 965-73.

[198] Siqueira-Neto, J.L.; Moon, S.; Jang, J.; Yang, G.; Lee, C.; Moon, H.K.; Chatelain, E., Genovesio, A.; Cechetto, J.; Freitas-Junior, L.H. An image-based high-content screening assay for compounds targeting intracellular *Leishmania donovani* amastigotes in human macrophages. *PLoS Negl. Trop. Dis.*, **2012**, *6*, e1671.

[199] Monzote, L. Antileishmanial patents antileishmanial current drugs and relevant patents. *Recent Pat. Antiinfect. Drug Discov.*, **2011**, *6*, 1-26.

[200] García, M.; Monzote, L.; Scull, R.; Herrera, P. Activity of Cuban Plants Extracts against *Leishmania amazonensis.* ISRN Pharmacol., **2012**, *2012,* 104540, Epub 2012 Mar 15.

[201] Brun, R.; Blum, J.; Chappuis, F.; Burri, C. Human African trypanosomiasis. *Lancet*, **2010**, *375*, 148-159.

[202] Burri, C. Chemotherapy against human African trypanosomiasis: is there a road to success? *Parasitology*, **2010**, 137, 1987-1994.

[203] Alsford, S.; Eckert, S.; Baker, N.; Glover, L.; Sanchez-Flores, A.; Leung, K.F.; Turner, D.J.; Field, M.C.; Berriman, M.; Horn, D. High-throughput decoding of antitrypanosomal drug efficacy and resistance. *Nature*, **2012**, *482*, 232-236.

[204] Priotto, G.; Kasparian, S.; Mutombo, W.; Ngouama, D.; Ghorashian, S.; Arnold, U.; Ghabri, S.; Baudin, E., Buard, V.; Kazadi-Kyanza, S.; Ilunga, M.; Mutangala, W.; Pohlig, G.; Schmid, C.; Karunakara, U.; Torreele, E.; Kande, V. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet*, **2009**, *374*, 56–64.

[205] Barrett, M.P. Potential new drugs for human African trypanosomiasis: some progress at last. *Curr. Opin. Infect. Dis.*, **2010**, *23*, 603–608.

[206] Jacobs, R.T.; Nare, B.; Phillips, M.A. State of the art in African trypanosome drug discovery. *Curr. Top. Med. Chem.*, **2011**, 11, 1255–1274.

[207] www.thecpdd.org. **2012**, July 3

[208] Maser, P.; Wittlin, S.; Rottmann, M.; Wenzler, T.; Kaiser, M.; Brun, R. Antiparasitic agents: new drugs on the horizon. *Curr. Opin. Pharmacol.*, **2012**, *12*, 1-5.

[209] Brun, R.; Don, R.; Jacobs, R.T.; Wang, M.Z.; Barrett, M.P. Development of novel drugs for human African trypanosomiasis. *Future Microbiol.*, **2011**, *6*, 677–691.

[210] Zhang, Y-K.; Plattner, J.J.; Freund, Y.R.; Easom, E.E.; Zhou, Y.; Gut, J.; Rosenthal, P.J.; Waterson, D.; Gamo, F-J.; Angulo-Barturen, I.; Ge, M.; Li, Z.; Li, L.; Jian, Y.; Cui, H.; Wang, H.; Yang, J. Synthesis and structure–activity

relationships of novel benzoxaboroles as a new class of antimalarial agents. *Bioorg. Med. Chem. Lett.*, **2011**, *21*, 644–651.

[211] Jacobs, R.T.; Plattner, J.J.; Keenan, M. Boron-based drugs as antiprotozoals. *Curr. Opin. Infect. Dis.*, **2011**, *24*, 586–592.

[212] Jacobs, R.T.; Nare, B.; Wring, S.A.; Orr, M.D.; Chen, D.; Sligar, J.M.; Jenks, M.X.; Noe, R.A.; Bowling, T.S.; Mercer, L.T.; Rewerts, C.; Gaukel, E.; Owens, J.; Parham, R.; Randolph, R.; Beaudet, B.; Bacchi, C.J.; Yarlett, N.; Plattner, J.J.; Freund, Y.; Ding, C.; Akama, T.; Zhang, Y-K.; Brun, R.; Kaiser, M.; Scandale, I.; Don, R. SCYX-7158, an orally-active benzoxaborole for the treatment of stage 2 human African trypanosomiasis. *PLoS Negl. Trop. Dis.*, **2011**, *5*, e1151.

[213] Kaiser, M.; Bray, M.A.; Cal, M.; Bourdin Trunz, B.; Torreele, E.; Brun, R. Antitrypanosomal activity of fexinidazole, a new oral nitroimidazole drug candidate for treatment of sleeping sickness. *Antimicrob. Agents Chemother.*, **2011**, *55*, 5602-5608.

[214] www.dndi.org/portfolio/fexinidazole.html. **2012**, July 6.

[215] Stephens, N.A.; Kieft, R.; Macleod, A.; Hajduk, S.L. Trypanosome resistance to human innate immunity: targeting Achilles' heel. *Trends Parasitol.*, **2012**, 28, 539-545.

[216] Vanhamme, L.; Paturiaux-Hanocq, F.; Poelvoorde, P.; Nolan, D.P.; Lins, L.; Van Den Abbeele, J.; Pays, A.; Tebabi, P.; Van Xong, H.; Jacquet, A.; Moguilevsky, N.; Dieu, M.; Kane, J.P.; De Baetselier, P.; Brasseur, R.; Pays, E. Apolipoprotein L-I is the trypanosome lytic factor of human serum. *Nature*, **2003**, 422, 83-87.

[217] Nibret, E.; Wink, M. Trypanocidal and cytotoxic effects of 30 Ethiopian medicinal plants. *Z Naturforsch C.*, **2011**, 66, 541-546.

[218] Mann, A.; Ifarajimi, O.R.; Adewoye, A.T.; Ukam, C.; Udeme, E.E.; Okorie, I.I.; Sakpe, M.S.; Ibrahim, D.R.; Yahaya, Y.A.; Kabir, A.Y.; Ogbadoyi, E.O. In vivo antitrypanosomal effects of some ethnomedicinal plants from Nupeland of north central Nigeria. *Afr. J. Tradit. Complement. Altern. Med.*, **2011**, 8, 15-21.

[219] Abiodun, O.O.; Gbotosho, G.O.; Ajaiyeoba, E.O.; Brun, R.; Oduola, A.M. Antitrypanosomal activity of some medicinal plants from Nigerian ethnomedicine. *Parasitol. Res.*, **2012**, 110, 521-526.

[220] Salomon, C.J. First century of Chagas' disease: an overview on novel approaches to nifurtimox and benznidazole delivery systems. *J. Pharmac. Sci.*, **2011**, *101*, 888-894.

[221] Moncayo**, A.;** Silveira, **A.C.** Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. *Mem. Inst. Oswaldo Cruz*, **2009**, *104*, 17-30.

[222] Pinto Dias, J.C. The treatment of Chagas disease (South American Trypanosomiasis). *Ann. Intern. Med.*, **2006**, *144*, 772-774.

[223] World Health Organization. Control of Chagas' disease. Second Report of the WHO Expert Committee, **2002**, W.H.O. Tech. Rep. Ser. 905, Geneva, pp. 1–109.

[224] Clayton, J. Chagas disease: pushing through the pipeline. *Nature*, **2010**, *465*, S12-S15.

[225] McKerrow, J.H.; Doyle, P.S.; Engel, J.C.; Podust, L.M.; Robertson, S.A.; Ferreira, R.; Saxton, T.; Arkin, M.; Kerr, I.D.; Brinen, L.S.; Craik, C.S. Two approaches to discovering and developing new drugs for Chagas disease. *Mem. Inst. Oswaldo Cruz*, **2009**, *104 (Suppl. I)*, 263-269.

[226] Mott, B.T.; Ferreira, R.S.; Simeonov, A.; Jadhav, A.; Ang, K.K-H.; Leister, W.; Shen, M.; Silveira, J.T.; Doyle, P.S.; Arkin, M.R.; McKerrow, J.H.; Inglese, J.; Austin, C.P.; Thomas, C.J.; Shoichet, B.K.; Maloney, D.J. Identification and optimization of trypanosomal cysteine proteases: cruzain, rhodesain, and TbCatB. *J. Med. Chem.*, **2010**, *53*, 52–60.

[227] Rogers, K.E.; Keränen, H.; Durrant, J.D.; Ratnam, J.; Doak, A.; Arkin, M.R.; McCammon, J.A. Novel cruzain inhibitors for the treatment of Chagas' disease. *Chem. Biol. Drug Des.*, **2012**, 80, 398-405.

[228] Beaulieu, C.; Isabel, E.; Fortier, A.; Massé, F.; Mellon, C.; Méthot, N.; Ndao, M.; Nicoll-Griffith, D.; Lee, D.; Park, Y.; Black, W.C. Identification of potent and reversible cruzipain inhibitors for the treatment of Chagas disease. *Bioorg. Med. Chem. Lett.*, **2010**, *20*, 7444–7449.

[229] Lepesheva, G.I.; Hargrove, T.Y.; Anderson, S.; Kleshchenko, Y.; Furtak, V.; Wawrzak, Z.; Villalta, F.; Waterman, M.R. Structural insights into inhibition of sterol 14alpha-demethylase in the human pathogen *Trypanosoma cruzi*. *J Biol Chem.*, **2010**, *285*, 25582-25590.

[230] Urbina, J.A. Ergosterol biosynthesis and drug development for Chagas disease. *Mem. Inst. Oswaldo Cruz*, **2009**, *104*, *Suppl. 1,* 311-318.

[231] Pinazo, M.J.; Espinosa, G.; Gállego, M.; López-Chejade, P.L.; Urbina, J.A.; Gascón, J. Successful treatment with posaconazole of a patient with chronic Chagas disease and systemic lupus erythematosus. *Am. J. Trop. Med. Hyg.*, **2010,** *82*, 583-587.

[232] Corrales, M.; Cardozo, R.; Segura, M.A.; Urbina, J.A.; Basombrío, M.A. Comparative efficacies of TAK-187, a long-lasting ergosterol biosynthesis inhibitor, and benznidazole in preventing cardiac damage in a murine model of Chagas' disease. *Antimicrob. Agents Chemother.*, **2005,** *49*, 1556-1560.

[233] Gunatilleke, S.S.; Calvet, C.M.; Johnston, J.B.; Chen, C.K.; Erenburg, G.; Gut, J.; Engel, J.C.; Ang, K.K.; Mulvaney, J.; Chen, S.; Arkin, M.R.; McKerrow, J.H.; Podust, L.M. Diverse inhibitor chemotypes targeting *Trypanosoma cruzi* CYP51. *PLoS Negl. Trop. Dis.*, **2012**, 6, e1736.

[234] Andriani, G.; Chessler, A.D.; Courtemanche, G.; Burleigh, B.A.; Rodriguez, A. Activity *in vivo* of anti-*Trypanosoma cruzi* compounds selected from a high throughput screening. *PLoS Negl. Trop. Dis.*, **2011**, 5, e1298.

[235] Benaim, B.; Garcia, C.R. Targeting calcium homeostasis as the therapy of Chagas' disease and leishmaniasis - a review. *Trop. Biomed.,* **2011,** *28*, :471-481.

[236] Chatterjee, A.K.; Yeung, B.K.S. Back to the future: lessons learned in modern target-based and whole-cell lead optimization of antimalarials. *Curr. Top. Med. Chem.,* **2012***, 12,* 473-483.

[237] Muregi, F.W.; Kirira, P.G.; Ishih, A. Novel rational drug design strategies with potential to revolutionize malaria chemotherapy. *Curr. Med. Chem.*, **2011**, *18,* 113-143.

[238] Gemma, S.; Camodeca, C.; Sanna Coccone, S.; Joshi, B.P.; Bernetti, M.; Moretti, V.; Brogi, S.; Bonache, M.C.; Savini, L.; Taramelli, D.; Basilico, N.; Parapini, S.; Rottmann, M.; Brun, R.; Lamponi, S.; Caccia, S.; Guiso, G.; Summer, R.; Martin, R.; Saponara, S.; Gorelli, B.; Novellino, E.; Campiani, G.; Butini, S. Optimization of 4-Aminoquinoline/Clotrimazole-Based Hybrid Antimalarials: Further Structure-Activity Relationships, *in vivo* Studies, and Preliminary Toxicity Profiling. *J. Med. Chem.,* **2012,** Jul 12. [Epub ahead of print].

[239] Alfonso, Y.; Monzote, L. HIV Protease Inhibitors: Effect on the Opportunistic Protozoan Parasites. *Open Med. Chem. J.* **2011**, *5*, 40-50.

[240] Zhu, X.; Pan, Y.; Zheng, L.; Cui, L.; Cao, Y. Polysaccharides from the Chinese medicinal herb *Achyranthes bidentata* enhance anti-malarial immunity during *Plasmodium yoelii* 17XL infection in mice. *Malaria J.*, **2012**, *11*, 49-60.

[241] Butler, N.S.; Moebius, J.; Pewe, L.L.; Traore, B.; Doumbo, O.K.; Tygrett, L.T.; Waldschmidt, T.J.; Crompton, P.D.; Harty, J.T. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nature Immunol.*, **2012**, *13*, 188-195.

[242] Kline, J.; Gajewski, T.F. Clinical development of mAbs to block the PD1 pathway as an immunotherapy for cancer. *Curr. Opin. Investig. Drugs*, **2010**, *11*, 1354-1359.

[243] Sierro, S.; Romero, P.; Speiser, D.E. The CD4-like molecule LAG-3, biology and therapeutic applications. *Expert Opin. Ther. Targets*, **2011**, *15*, 91-101.

[244] Brahmer, J.R.; Tykodi, S.S.; Chow, L.Q.; Hwu, W.J.; Topalian, S.L.; Hwu, P.; Drake, C.G.; Camacho, L.H.; Kauh, J.; Odunsi, K.; Pitot, H.C.; Hamid, O.; Bhatia, S.; Martins, R.; Eaton, K.; Chen, S.; Salay, T.M.; Alaparthy, S.; Grosso, J.F.; Korman, A.J.; Parker, S.M.; Agrawal, S.; Goldberg, S.M.: Pardoll, D.M.; Gupta, A.; Wigginton, J.M. Safety and activity of anti–PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.*, **2012**, *366*, 2455-2465.

[245] Mansfield, J.M.; Paulnock, D.M. Regulation of innate and acquired immunity in African Trypanosomiasis. *Parasite Immunol.*, **2005**, *27*, 361-371.

[246] Stager, S.; Rafati, S. CD8+ T cells in Leishmania infections: friends or foes? *Front. Immunol.*, **2012**, *3*, 1-8.

[247] Maranz, S. An alternative paradigm for the role of antimalarial plants in Africa. *Scient.World J.*, **2012**, Article ID 978913, 1-9.

[248] Verra, F.; Bancone, G.; Avellino, P.; Blot, I.; Simporé, J.; Modiano, D. Haemoglobin C and S in natural selection against *Plasmodium falciparum* malaria: a plethora or a single shared adaptive mechanism? *Parassitologia*, **2007**, *49*, 209-213.

[249] Verra, F.; Simpore, J.; Warimwe, G.M.; Tetteh, K.K.; Howard, T.; Osier, F.H.A.; Bancone, G.; Avellino, P.; Blot, I.; Fegan, G.; Bull, P.C.; Williams, T.N.; Conway, D.J.; Marsh, K.; Modiano, D. Haemoglobin C and S role in acquired immunity against *Plasmodium falciparum* malaria. *PLoS ONE*, **2007**, e978.

[250] Dormeyer, M.; Adams, Y.; Kramer, B.; Chakravorty, S.; Tse, M.T.; Pegoraro, S.; Whittaker, L.; Lanzer, M.; Craig, A. Rational design of anticytoadherence inhibitors for *Plasmodium falciparum* based on the crystal structure of human intercellular adhesion molecule 1. *Antimicrob. Agents Chemother.*, **2006**, *50*, 724-730.

[251] Majori, G. Short history of malaria and its eradication in Italy with short notes on the fight against the infection in the Mediterranean basin. *Mediterr. J. Hematol. Infect. Dis.*, **2012**, *4*, e2012016.

[252] Odolini, S.; Gautret, P.; Parola, P. Epidemiology of imported malaria in the Mediterranean region. *Mediterr. J. Hematol. Infect. Dis.*, **2012**, *4*, e2012031.

FIGURE LEGENDS

Fig. (**1**)

Fig. (**2**). Chemical structures of antimalarial drugs currently in use or in advanced stages of development.

Fig. (**3**). Chemical structures of chloroquine chemosensitizer drugs.

Fig. (**4**). Chemical structures of antimalarial peroxides.

Fig. (**5**). Chemical structures of antifolates

Fig. (**6**). Chemical structures of drugs targeting cytochrome *bc*1

Fig. (**7**). Chemical structures of antibiotics with antimalarial activity.

Fig. (**8**).

Fig. (**9**).

Fig. (**10**).

Fig. (**11**). Chemical structures of currently used antileishmanial drugs.

Fig. (**12**). Chemical structures of antileishmanial drugs under development.

Fig. (**13**). Chemical structures of drugs currently used against HAT

Fig. (**14**). Chemical structures of anti-HAT drugs under development.

Fig. (**15**). Chemical structures of new oxaboroles.

Fig. (**16**).

Fig. (**17**). A cruzipain irreversible inhibitor.

Fig. (**18**). Chemical structures of drugs being developed against Chagas' disease.

Fig. (**19**). Chemical structures of antiarrhythmic drugs with potent and selective activity against *T. cruzi.*

Fig. (**20**).