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The necessity to develop drugs against parasitic diseases

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This review focuses on the most significant trends in the development of drugs for the treatment of malaria, African sleeping sickness and toxoplasmosis. In the case of malaria, those include new fixed-dose artemisinin combinations, antifolates and new targets in the apicoplast of *Plasmodium falciparum*. Targets in the treatment of trypanosomiasis are the biosynthesis of glycosylphosphatidylinositol and enzymes incolved in the biosynthesis of trypanothione. Efforts to develop a vaccine against toxoplasmosis are discussed as well.

1. Introduction

The research and development process (R & D) of a drug is of high risk. Research and development usually requires 3 to 7 years before a compound or a target gene reaches the clinical trial (Fig. 1). Most of the industrial projects are discontinued after a short period of time because of the high costs of investment. However, the immense costs from a serious R & D process are more or less the costs of failed projects (Fig. 2).

For the past few years a lack of interest from the pharmaceutical companies and their economical situation caused a delay in development of new drugs against parasitic diseases. Recently it has been shown that there are alternatives for the development of drugs in collaboration with the pharmaceutical industry. One of those alternatives e.g. is "Medicines for Malaria Venture" (MMV) [1] which is a collaboration with pharmaceutical companies, non governmental organizations and academic institutions. The mission of MMV is to renew and sustain the supply of affordable antimalarials. MMV is also being supported and supervised by the WHO in connection with the "Roll Back Malaria Programme". A key advantage offered by MMV is the development of a portfolio of projects under competitive criteria and the combination of the pharmaceutical industry with its expertise in drug discovery and the public sector with its expertise in basic biology and clinical medicine.

This review strictly focuses on new strategies in therapy for the treatment of malaria, African sleeping sickness and toxoplasmosis. We primarily review the most significant trends in R & D and drug therapy.

2. Benefits and limitations in current antimalarial therapy and new directions for the future

Malaria today is still a disease of poverty since it mostly appears in Africa. 700000 children die per year mainly in the Sahara since they have not developed immunity [2]. According to WHO reports the number of clinical cases is estimated to be between 300 to 500 million people.

Recent hypotheses for a better understanding of malaria were based on the theory that gene polymorphisms such as sickle cell trait, thalassemia and a defect in glucose-6-phosphate dehydrogenase were selected under evolutionary pressure to combat malaria [3]. One of the main driving forces to develop new antimalarials is the increase in resistance against currently used antimalarials or to produce a more cheaper chloroquine analogue.

Resistance against the 4-aminoquinoline chloroquine [4] and the fixe combination of pyrimethamine/sulphadoxine [5] inhibiting folate biosynthesis developed stepwise but on a global scale. In the Table we summarize the benefits and limitations of the currently used antimalarials focusing on resistance, side effects, costs and compliance.

The fact that quinine, an alkaloid of the cinchona bark (*Cinchona succirubra*, Rubiaceae), has a neurotoxic potential such as tinnitus and its application thrice a day for seven days, led to the development of fully synthetic 4-aminoquinolines with chloroquine as the main precursor of this group (Table). However, the costs for the new aminoquinolines are high and resistance occurred as soon as they were on the market. Even mefloquine should only be taken in endemic areas and thus the number for 4-aminoquinoline antimalarials is limited.

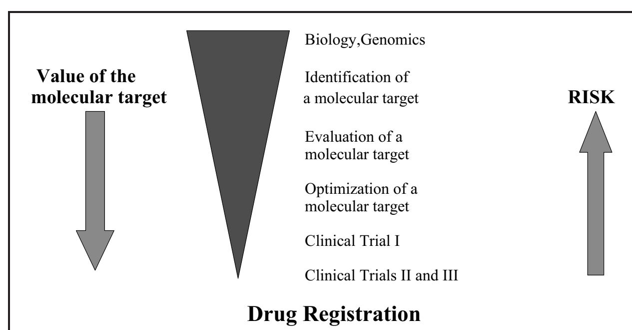


Fig. 1: The development of a new drug. Many potential targets obtained from the databases of biology/genomics exist, but only a few are valuable targets. It is shown that the value of a component increases before it finally requires medicinal chemistry. However, the risk of a project decreases with the evaluation of the target structure

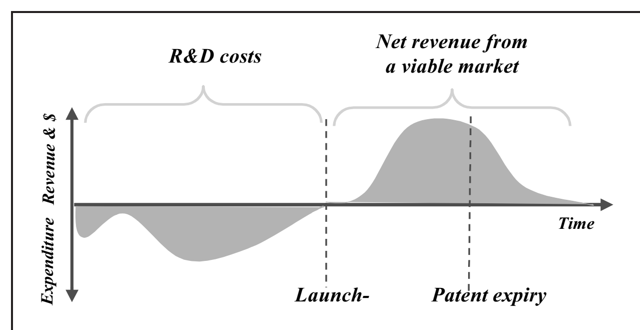


Fig. 2: The economical aspect of research and development (RD). The research and development process is of high economical risk. Thus, a company investing in drug R & D has to calculate the costs before its patent expires. The costs for any product are limited by its patent life

Table: Drugs that are currently used as antimalarials with their main indications and limitations

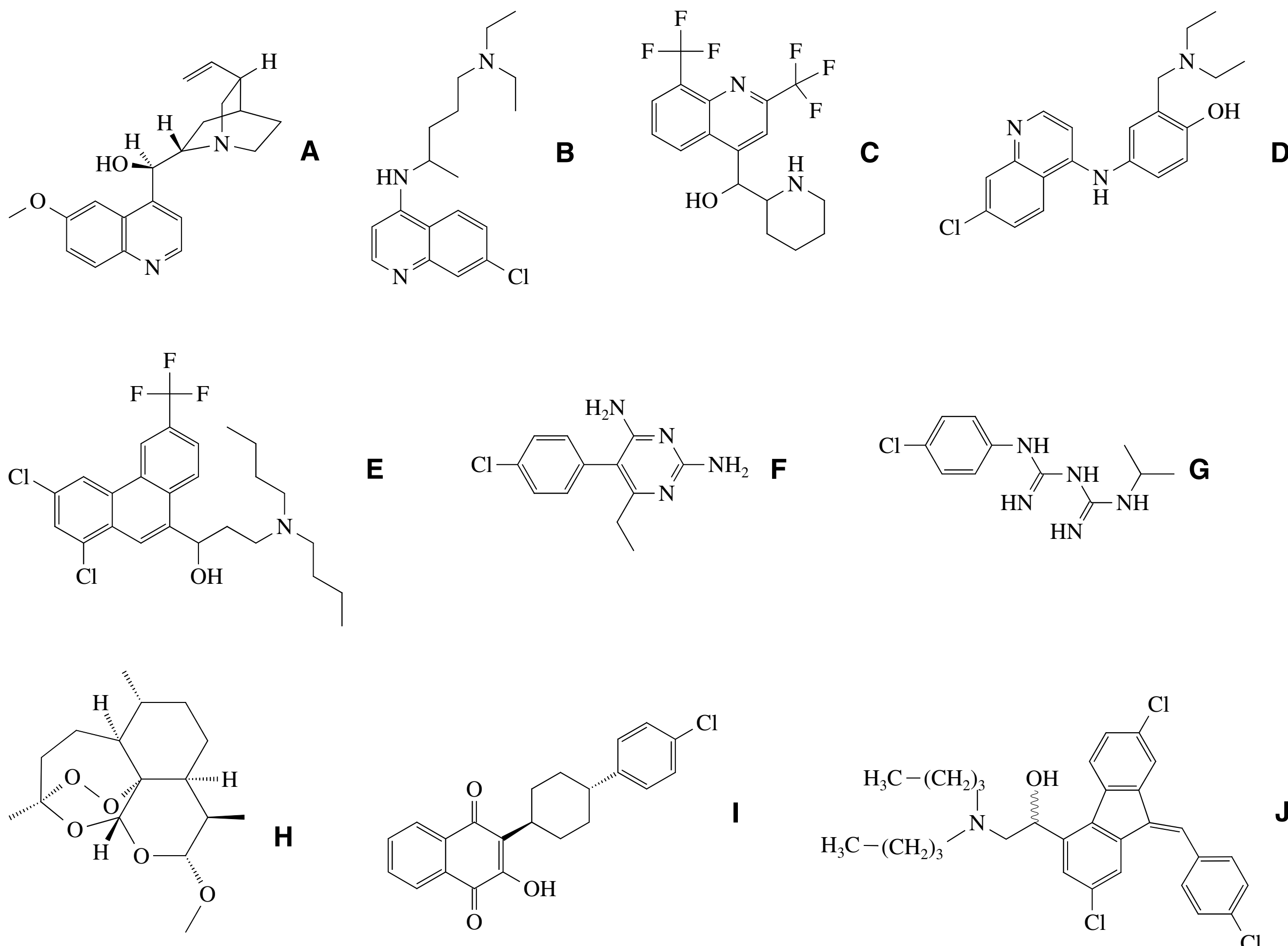
Drug	Limitations
Chloroquine	Resistance
Quinine	Compliance, side effects, resistance
Amodiaquine	Side effects, resistance
Mefloquine	Side effects, resistance, costs
Halofantrine	Side effects, resistance, costs
Artemisinin	Compliance, costs
Artemether,	Compliance, costs
Arteether,	Compliance, costs
Artesunate	Compliance, costs
Sulphadoxine/ Pyrimethamine	Resistance
Atovaquon/ Proguanil	Resistance, potential costs
Lumefantrin/Artemether	Compliance Resistance Potential costs

Meanwhile amodiaquine, a 4-aminoquinoline which has been shown to cause agranulocytosis in patients who took the drug prophylactically is used in therapy again because of its efficiency against chloroquine-resistant *Plasmodium falciparum* strains. A contraindication for the phenantrene halofantrine were heartrhythm problems. The effect of 4-aminoquinolines is based on their interference with the haem catabolite pathway in the food vacuole of the parasite. Within the 48 h cycle of infection there is invasion, growth and release of the parasite from the infected erythrocyte. In the first step of invasion aspartic proteases (plasmepsine) [6] and the cysteine protease falcipain 2 [7] are involved. As shown later in this review (Fig. 5) both enzymes will possibly be new drug targets in the future. Haem is finally sequestered to the non toxic haemozoin that comprises aggregated haem dimers. The planar aromatic structure of the 4-aminoquinolines interacts with haem and thus prevents the formation of haemozoin. Since there is no single target enzyme which is inhibited by the 4-aminoquinolines, resistance increased stepwise against these drugs.

A lot of work in the past partly contributed to the understanding of resistance against chloroquine in *P. falciparum*. Resistance against chloroquine is linked so far to two identified genes, i.e. the *P. falciparum* chloroquine transporter *PfCRT* gene [8] and secondly a P-glycoprotein [9] *mdr* that leads to an altered susceptibility of mefloquine, halofantrine and artemisinin.

2.1. New fixed – dose combinations of artemisinin as an alternative strategy in therapy for the future

Some synthetic derivatives of artemisinin the main constituent of the chinese herb *Artemisia annua* (Asteraceae) which was used in China traditionally against fever are meanwhile used in therapy. The synthetic derivatives con-



Drugs that are currently in use as antimalarials. A) quinine B) chloroquine C) mefloquine D) amodiaquine E) halofantrine F) pyrimethamine G) proguanil H) artemisinin I) atovaquone J) lumefantrine

sist of artemether, arteether and artesunate which are all metabolized to dihydroartemisinin and have to be taken for 5–7 days because of the short half lives of the compounds. However, the main advantages of artemisinins are their rapid clearance of parasites and their effect against gametocytes, the sexual stages of the parasite which infect the mosquito. The WHO has started a large scale rectal artesunate development programme for malaria patients who currently die because they lack immediate access to injectable treatment [10].

Meanwhile artemisinins are combined with different partner drugs with longer half-life to improve the compliance for the patients. One of these partner drugs is lumefantrine an aryl alcohol component in the fixed-dose combination Riamet® which has been registered in Germany a year ago. It is still a matter of debate whether a 2-days (4-dose) or 4-days (6-dose) regimen is the most appropriate treatment in Africa [11].

Interestingly in some parts of Thailand [12] artemisinin is used as a fixed-dose combination with mefloquin. Meanwhile different combinations of artemisinin with various drugs are currently studied in certain epidemological situations [13].

There are recent reports from Bayer Vital and MMV [14] about the production of artemisone, a derivative of artemisinin that kills malaria parasites even more rapidly than other structural analogues. By the year 2003 the drug should be able to pass clinical trials and should be accessible for all people in developing countries.

Fixed-dose combinations with artemisinin and a drug with a long half life are considered very critical in particular for the situation in Africa: i) The long half life of the partner drug of artemisinin may be unsuitable in case of intense transmission. ii) The chance of resistance development against the partner drug is high. iii) The costs for the artemisinin fixed-dose combinations are high. iiii) Complex drug regimens have to be supervised in regions with poor healthcare infrastructure.

The mechanism of action of the artemisinins parallels that of the 4-aminoquinolines. The oxidative cleavage of the sesquiterpenoid structure and interaction with haem in the food vacuole of the parasite leads to an interruption of the haem catabolite pathway.

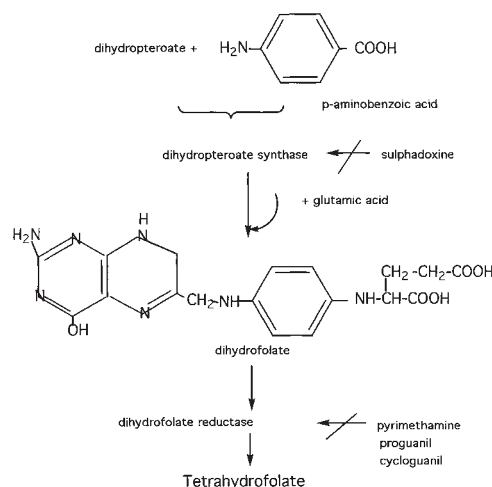
2.2. *The status of antifolates in malaria therapy and a view into the "industrial pipeline"*

Reduced folates are essential for the key one-carbon transfer needed for the biosynthesis of nucleic acids which are essential for proliferation of the parasite. The most important folate inhibitor used as an antimalarial drug is a fixed-dose combination between pyrimethamine and sulphadoxine, Maloprim® which can be used for intermittant treatment in pregnancy. While pyrimethamine inhibits dihydrofolate reductase (DHFR), sulphadoxine interferes with dihydropteroate synthase (DHPS) (Scheme 1). Thus inhibition is based on two targets of the folate pathway.

There is strong development of resistance in Africa against the fixed-dose combination pyrimethamine/sulphadoxine. A second concern is the appearance of Lyell- or Steven-Johnson syndrome which is an allergic reaction of the skin.

Meanwhile biguanides like proguanil which inhibits DHFR are used to overcome the resistance to pyrimethamine. Proguanil functions as a prodrug which is metabolized to cycloguanil by cytochrom P450 [15]. A derivative of proguanil, i.e. chlorproguanil will probably achieve re-

Scheme 1



The folate pathway starting with dihyropteroate, p-aminobenzoic acid and glutamic acid. Under catalysis of dihydropteroate synthase dihydrofolate is formed. Sulphadoxine is a specific inhibitor of dihydropteroate synthase. Dihydrofolate reductase catalyses the reduction of dihydrofolate to tetrahydrofolate. Dihydrofolate reductase is inhibited by pyrimethamine, proguanil and cycloguanil.

gistration as a fixed dose combination with artemisinin, dapsone and artesunate [16] this year.

The mechanisms which lead to resistance against antifolates have been intensively studied. The cause for resistance to pyrimethamine are mutations of the DHFR at amino acid positions Serine 108 → Asp, Cys 59 → Arginine, Asparagine 51 → Isoleucine and Isoleucine 164 → Leucine [5]. Resistance against DHPS resulting from mutations at codons 436, 437, 581 and 613 correlate with sulphadoxine resistance [17].

2.3. *Registered, new fixed-dose combinations*

One of the most recent fixed-dose combination that has been registered is Malarone® with atovaquone and proguanil. Atovaquone structurally belongs to hydroxynaphtoquinones and inhibits cytochrome c reductase in the respiratory chain. The spontanous development of resistance results from a point mutation in the cytochrome reductase gene [18]. Recent results from a randomized, double blind-study of atovaquone-proguanil versus mefloquine showed that atovaquone-proguanil was better tolerated and had less neuropsychiatric adverse events. Since malaria parasites live partly under anaerobic conditions they strictly depend on ATP from glycolysis and from ATP which they gain from the phosphorylation reactions in the respiratory chain. Malaria parasites have an incomplete redox electron transport chain in comparison to other eukaryotes. However, there are significant elements which might be new potential targets in drug therapy for the future. A new class of cytochrome c reductase inhibitors are β-methoxyacrylates [19]. The main problem, however, is that resistance against β-methoxyacrylates developed rapidly in rodent malaria.

Even antibiotics, which inhibit bacterial protein biosynthesis like tetracyclines, doxycyclines and clindamycin will be combined with other antimalarials because they are thought to inhibit "protein like bacterial" synthesis in the apicoplast, an organelle that is unique to *Plasmodium* species. In Germany doxycycline is not allowed to be used for antimalarial chemotherapy. Nevertheless in parts

of South-east Asia quinine and doxycycline is a commonly used combination. In Africa this fixed-dose combination is limited to children younger than 8 years [20] and doxycycline is substituted by clindamycin.

2.4. *New targets located in the apicoplast of P. falciparum*

The fact that *P. falciparum* has an apicoplast, which does not exist in *human*, raises the hope to find new targets in the future (Fig. 3).
One of the so far identified targets is the *fabI* (fatty acid biosynthesis) gene, which codes for the parasitic enoyl-ACP-reductase gene and is inhibited by triclosan [21]. The enoyl-ACP-reductase catalyses the final step in parasitic fatty acid biosynthesis which is the hydration of crotonyl-CoA to butyryl-CoA.
Another target is the inhibition of the isoprenoid pathway in *P. falciparum*. In *P. falciparum* the formation of isoprenoids starts from pyruvate and glyceraldehyde to form 1-deoxy-D-xylulose-5-phosphate (DOXP) under DOXP synthase catalysis. This first step is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DOXP Synthase). In the second step catalyzed by DOXP-reductoisomerase, 2-C-methyl-D-erythritol-4-phosphate is formed. DOXP-reductoisomerase is inhibited by the antibiotic fosmidomycin [22].
A new target is the phosphatidylcholine biosynthesis from *P. falciparum* [23]. This pathway does not exist in *human*. The prototype of inhibitors of phosphatidylcholine biosynthesis is the substance G25 [1,16 Hexadecylmethylen-bis(*N*-methyl-pyrrolidiniumbromid]. This substance inhibits growth of *P. falciparum* and *P. vivax* *in vivo* and *in vitro*.

3. New strategies in the therapy of trypanosomiasis

3.1 *Biosynthesis of glycosylphosphatidylinositol*

Trypanosoma brucei is a protozoan parasite invading humans and other mammals by transmission via Tsetse flies. It causes "sleeping sickness" in humans and "nagana disease" in domestic animals in central Africa. *Trypanosoma brucei* has two distinct proliferative stages, a bloodstream stage living free in mammalian blood and a procyclic form in the midgut of the fly. The parasite circumvents the human immune system and also the digestion of coat proteins in the midgut of the insect by means of proteins tethered to the cell surface via glycosylphoshatidylinositol (GPI) anchors. Within the bloodstream stage 10% of these proteins are expressed while 1–3% of these proteins are expressed in the procyclic stage [24]. Disruption of the *TbGPI* 10 gene from *T. brucei* leads to a retarded growth of the procyclic forms. Infectivity of the tsetse flies is partially impaired particularly at the early stage.
The cloned *TbGPI* 10 gene from *T. brucei* encodes for a protein of 558 amino acids with 25% and 23% homology to the human gene and to that from *Saccharomyces cerevisiae* [25]. The gene is able to rescue the yeast mutant. From these results it is obvious that genes involved in the biosynthesis of glycosylphosphatidylinositol might be important targets against African trypanosomiasis.
Although there is a conserved core motif for the GPI anchor among all eukaryotes "EthN-P-6Manα-1,2Manα-1,6Manα-1,4GlucNα-1,6myoinositol-P-lipid" there are obviously significant differences between human GPI synthesis and GPI synthesis of parasitic protozoa [26]. Experiments with an inhibitor of terpenoid lactone structure YW 3548 showed no inhibition of GPI synthesis in an *in vitro* system of *T. brucei*, but inhibition in lymphoma and Hela cells. This inhibitor blocks the addition of the third mannose in GPI synthesis. However, synthetic structural GPI analoga, which are substituted with an acyl residue in position 2 of the inositol ring are better inhibitors of the GPI synthesis since in the GPI structure of Trypanosoma bloodstream form at position 2 of the inositol ring an acyl group naturally occurs [24, 27].

3.2. *Enzymes involved in the biosynthesis of trypanothione*

A well known and characterized target in trypanosomes is trypanothione and the enzymes involved in its biosynthesis and metabolism. Trypanothione is a (N^1, N^8, bisglutathionyl)spermidine which takes part in the detoxification of reactive oxygen species since trypanosomes lack glutathione reductase [28].
The biosynthesis of trypanothione involves two steps: In the first step spermidine is conjugated to glutathione under catalysis of glutathionylspermidine synthetase (Scheme 2). In the second step another glutathione molecule is added catalyzed by trypanothione reductase to form trypanothione.
The final steps of trypanothione dependent hydroperoxide metabolism comprise the concerted cascade of reduction equivalents from NADPH to trypanothione to a peroxidase with tryparedoxine and tryparedoxine reductase (Scheme 2, bottom part).
The design of inhibitors against glutathionylspermidine synthetase suffers from the absence of knowledge of the three dimensional-structure. However, phosphonates and phosphinates are non competitive inhibitors of the enzyme from *Crithidia fasciculata,* a trypanosomatid insect. The γ-glutamyl phosphapeptides combined with a spermidine residue have been shown to be more potent inhibitors. Unfortunately, these inhibitors were not effective on the growth of *Trypanosoma* and *Leishmania* [28].
Since trypanothione reductase has been characterized in its three dimensional structure a lot of specific inhibitors have

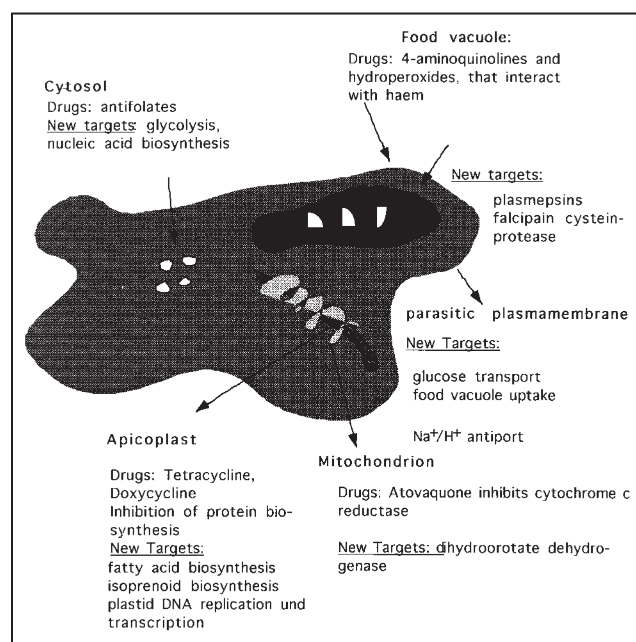
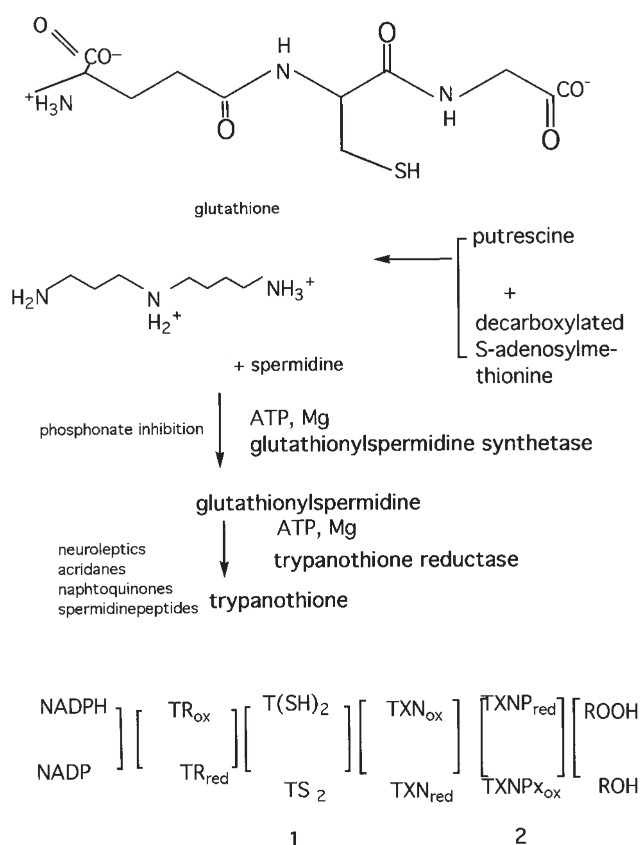


Fig. 3: Diagram of the most important organelles in *P. falciparum* and their associated drug targets. New targets are underlined and drugs applied in therapy are given

Scheme 2



Biosynthesis of trypanothione and trypanothione dependent hydroperoxide metabolism. The biosynthesis of trypanothione involves two steps (upper part): In the first reaction one glutathione molecule is conjugated to spermidine by glutathionylspermidine synthetase. In the second reaction trypanothione is formed by adddition of another glutathione moiety to glutathionylspermidine to form trypanothione. This reaction is catalyzed by trypanothione reductase.
The trypanothione antioxidant cascade (bottom part). The reduction equivalents are transported from NADP to a final peroxidase. The trypanothione antioxidant cascade consists of TR: trypanothione reductase, $T(SH)_2$ trypanothione, TS2: trypanothione disulphide, TXN: tryparedoxine; TXNP: tryparedoxine peroxidase

been developed. Most notably are the tricyclic antidepressants like clomipramine and neuroleptics with phenothiazine structure [29]. Acridines such as mepacrine, known for their antibacterial, antiparasitic and antitumor activity have also been shown to be potent inhibitors of trypanothione reductase.
Polyamine analogues which derive from spermidine and spermine turned out to be inhibitors of trypanothione reductase. Two natural compounds like lunarine and cadabacine consist of a cyclic spermidine structure [30]. Spermidine peptides are also inhibitors of trypanothione reductase. The naphtoquinones plumbagine and menadione are active inhibitors in the lower micromolar range [31].

4. Basic strategies for the development of a vaccine against toxoplasmosis

Toxoplasma gondii is a ubiquitous protozoan parasite that is pathogenic for humans and some animals. The final hosts are cats that develop oocysts. These oocysts contain sporocysts with four sporozoites each. The sporozoites become infectious when they are taken up by the final host or humans. After the infection *T. gondii* disseminates into bloodstream and lymph nodes before tachyzoites in the parasitophorus vacuole develop in brain, skeletal and heart muscle. The released tachyzoites are present in liquor and muscles where they persist as bradyzoites. The parasite is responsible for severe cogenital birth defects and fatal toxoplasmic encephalitis in immunocompromized people [32].
The problem of developing a vaccine against toxoplasmosis is the stage specific conversion of the parasite. A vaccine against specific tachyzoite surface proteins has been successfully used in sheep [33] to prevent abortion. The stage-specific expression of particular surface proteins from tachyzoites might be a new strategy to develop specific antibodies.
A new, fundamental strategy which is probably an approach for the development of a vaccine is the targeting of the “de novo pyrimidine pathway” (Fig. 4) [34]. *T. gondii* has a fragmented pathway for salvaging pyrimidine nucleobases from its host or the parasite itself. This pathway is funneled through uracil phosphoribosyltransferase. Disruption of the gene encoding uracil phosphoribosyltransferase does not affect the growth of *T. gondii* tachyzoites.
However, carbamoylsynthetase II (CSPII), a key enzyme of de novo pyrimidine biosynthesis shows distinct properties from the human host. In contrast to its human counterpart the CSPII domain of the parasite has a monofunctional domain with a glutamine amidotransferase domain which is not present in bacteria and fungi. Two knock out mutants were obtained with no CSPII activity. The mutants invaded normally but failed to replicate without uracil supplementation. A cloned 6.6 kb CSPII fragment rescued both mutants.
In a BALB/c mouse model for lethal toxoplasmosis the mutants do not cause an infection at inoculation doses of 10^3–10^6. An infection of an interferon deficient BALB/c mouse with the CSPII mutant resulted in no infection of the mouse. The cytokine interferon is responsible for host control of Toxoplasma infection. Balbc/mice with a cytokine IFN-γ knockout rapidly succumb to toxoplasmosis.

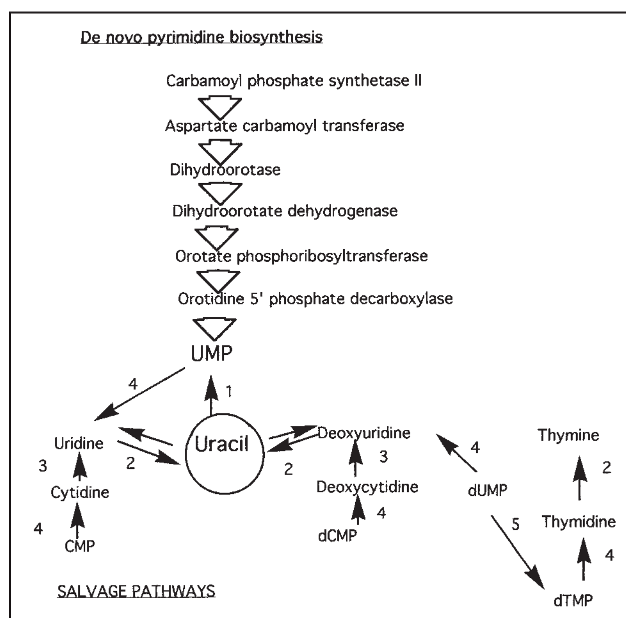


Fig. 4: Pyrimidine salvage pathway and *de novo* pyrimidine biosynthesis in *T. gondii*. Pathways that produce UMP, the precursor of pyrimidines are shown [34]. 1) nucleoside phosphorylase, 2) nucleoside deaminase 3) nucleoside 5′-monophosphate phosphohydrolase 4) thymidilate synthase 5) part of the bifunctional DHFR-Thymidilate synthase

The authors also showed that the *Toxoplasma* CSPII mutant strain protected from infection after challenge with an avirulent strain. Apparently the parasite's salvage pathway cannot salvage enough amounts of pyrimidines from its host cell. Thus, carbamoylsynthetase CSPII remains an interesting target enzyme.

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