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Apicomplexan Parasites

Molecular Approaches toward Targeted Drug Development

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7
The Reducing Milieu of Parasitized Cells as a Target of Antimalarial Agents: Methylene Blue as an Ethical Drug

*Peter Meissner, Heike Adler, Denis Kasozi, Karin Fritz-Wolf, and R. Heiner Schirmer**

Abstract

There are five species of the apicomplexan genus *Plasmodium* which cause human malaria. The diseases are distinct entities, and may require different drugs for their treatment. For the different intervention goals, specific parasitized cells and tissues must be considered as targets of the antimalarial agents. As suggested by large-scale *experimenta naturae*, such as the effects of pro-oxidant glucose-6-phosphate dehydrogenase (G6PD) alleles and pro-oxidant foodstuffs, the compromised antioxidant capacity of erythrocytes protects against severe malaria. One mechanism underlying this protection is the IgG-mediated, early phagocytosis of parasitized erythrocytes. As illustrated here for glutathione reductase (GR) deficiency, this mechanism cannot be observed in cultures of parasitized erythrocytes. Pro-oxidant agents such as the GR inhibitor and subversive substrate methylene blue (MB), dapsone, or primaquine most likely result in pharmacological phenocopies of natural protection mechanisms.

Introduction

Drugs used to treat malaria – a disease of the poor – should be also developed for ethical reasons. By using methylene blue (MB) as an example, it is possible to illustrate Sir James Black's statement that, [The] "...most fruitful basis for the discovery of a new drug is to start with an old drug." Recently, MB has been shown to be an analog of the bacterial redox pigment and quorum sensor, pyocyanin, which might explain why it behaves like a natural compound shaped for biological effects and biological compatibility in evolution. MB is active against both schizonts and gametocytes, and should be included in combination therapies against malaria. In this chapter, the details are reported of recent efforts to develop an MB-based drug combination to treat pediatric malaria, not only via clinical trials but also through anthropological and pharmacological studies.

*Corresponding author

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The Redox Milieu of Parasite-Bearing Cells as a Natural Target

Human malaria is caused by a handful of different *Plasmodium* species; simultaneous infections by two or more *Plasmodium* strains, or even species, are not uncommon in tropical countries [1]. Likewise, two other apicomplexan protozoal parasites, *Babesia microti* and *Babesia divergens* [2], also cause a disease in humans that resembles malaria.

Cells and tissues carrying malarial parasites are most sensitive to oxidative stress, a situation confirmed by *experimenta naturae*, which show that persons in whom the erythrocytes are exposed to pro-oxidative conditions are protected from severe forms of malaria. The basis of this pro-oxidative situation may be a genetic disposition, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, the intake of foodstuffs containing pro-oxidant redox-cycling compounds (e.g., divicine in broad beans, or certain food additives in red suya dishes), or the administration of oxidizing drugs such as primaquine, dapsone, or MB [3, 4]. As in most other cells, the redox milieu of parasitized erythrocytes is a fragile, delicate biological entity. Typically, the overall redox potential of cytosolic spaces is less negative than −250 mV, and thus far below that of the extracellular spaces of aerobically living organisms, where the redox milieu is governed by atmospheric oxygen. One explanation of the need for a reducing intracellular milieu is that living cells originated in an environment where thiol groups are stable, and where such thiol groups are essential for numerous central life processes, such as enzyme catalysis, deoxynucleotide reduction, and the detoxification of xenobiotics. However, following the intrusion of oxygen into the biosphere, the thiophilic environment was – and still is – challenged enormously by oxidative processes. Consequently, in order to survive, all living cells had to protect themselves by employing redoxin-based networks fueled by the reducing equivalents of NADPH and NADH (Figure 7.1). The network of proteins which maintains redox homeostasis *in situ* is the target of natural antimalarial mechanisms. It should be noted that in the parasitized cells a number of pro-oxidant foodstuff ingredients and synthetic drugs act not only as enzyme inhibitors but also as redox-cycling compounds (Figure 7.2). In this way, the enzyme–drug complexes mimic the action of the antimicrobial enzyme NADPH oxidase [3].

The development of drugs targeting the redox-metabolism of *Plasmodium falciparum*, as a representative of the apicomplexan parasites, has been comprehensively described in reviews which have lost neither their timeliness nor relevance [1, 3, 5, 6]. Hagai Ginsburg's schemes and contribution represent another source of continuous inspiration (see Malaria parasite metabolic pathways; http://sites.huji.ac.il/malaria/). In addition, the discovery and development of naphthoquinone drugs directed against the antioxidative homodimeric flavoenzymes of the glutathione reductase (GR) family has been detailed by Elisabeth Davioud-Charvet and Don Antoine Lanfranchi (see Chapter 20 and Ref. [4]). The biosynthesis of glutathione and dihydrolipoic acid, the major low-molecular-weight thiols of parasites and host cells, is described by Sylke Müller and colleagues in Chapter 10.

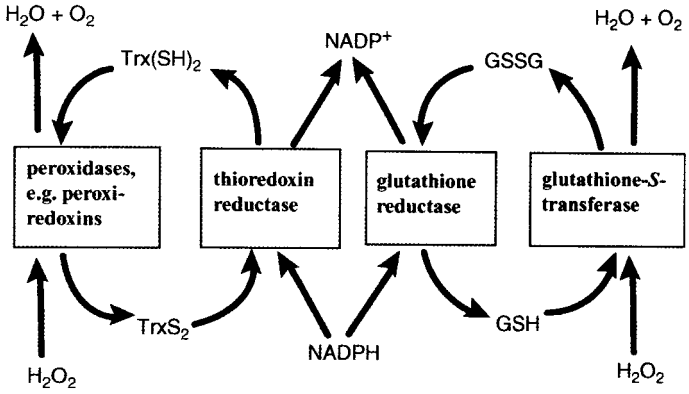

Figure 7.1 Network of antioxidative thiol- and dithiol proteins in *P. falciparum*. The functions of the system include the detoxification of reactive oxygen species and the production of deoxynucleotides from nucleotides. Trx, thioredoxin; GSH, glutathione; GSSG, glutathione disulfide. For *P. falciparum*, five peroxiredoxins and ten Trx- and glutaredoxin-like proteins are known. Individual thioredoxins and glutaredoxins can undergo redox reactions with each other, and with GSH (not shown here). The rate constants of these thiol-disulfide exchanges are in the order of 10 to 100 $M^{-1} s^{-1}$. Glutathione-*S*-transferase catalyzes the formation of glutathione conjugates, can complex heme, and – because of its high intracellular concentration – can act as an efficient glutathione peroxidase. *P. falciparum* does not contain Se-dependent glutathione peroxidase and catalase [1, 3, 5].

Searching for Ethical Drugs Against Malaria

Drugs used to treat malaria, as a disease of the poor, should be also developed as *ethical drugs*, characterized by the acronym *bonaria*, where *bon* means safe and effective, *a* affordable for the patients who need it, *r* already registered for other medical indications, and *ia* for internationally accessible (K. Becker and R.H. Schirmer, personal communication; this aspect will be further dealt with in the Discussion).

In this chapter, attention is focused on what antimalarial drug discovery, especially in the field of pro-oxidant agents, can learn from clinical malaria and from natural phenomena related to this disease. Furthermore, the redox metabolism offers convincing examples of parasite and host coevolution, as well as evolution of pathogen–host cell interactions [7].

Different Goals Require Different Antimalarial Agents

In all forms of human malaria, the actual disease (characterized *inter alia* by fever bouts) is caused by the rapid multiplication of so-called "schizonts" in erythrocytes. There are, however, other host cells and host tissues essential for maintenance of the parasite lifestyles in humans and in the *Anopheles* mosquito [7, 8]. Consequently, a distinction can be made between three major indications for antimalarial drugs: (i) the prevention and cure of the actual disease caused by blood schizonts; (ii) the

prevention of disease transmission by sporozoites, blood schizonts (via transfusion–transmission), or gametocytes; and (iii) the prevention of relapses caused, for example, by liver hypnozoites of *Plasmodium vivax* [9].

Target Cells and Tissues

When an *Anopheles* mosquito attacks the human body, it injects approximately 20 *P. falciparum* sporozoites; the sporozoites subsequently enter 20 hepatocytes, in which they then multiply 30 000-fold within 10–14 days. The liver schizonts are then released and enter the erythrocytes, where they multiply asexually by a factor of up to 16 within 48 h. When the merozoites are subsequently released, it is essential that they find fresh erythrocytes within 1 min; otherwise they will not survive.

Some erythrocytic parasites develop within 14 days into so-called female and male gametocytes which, after being taken up by a female mosquito, can mate to form zygotes that, in the midgut of the mosquito, develop into ookinetes and then oocysts. The latter mature and release sporozoites, which become concentrated in the mosquito's salivary glands from where they are injected into the human skin. The oocysts, which impose as huge tumors in the mosquito, are combated by the insect host via pro-oxidant processes such as encapsulation in melanin-rich cocoons, or destruction with reactive oxygen species (ROS) produced by phagocytes of the hemolymph. Although about 90% of the population of most mosquito species are refractory to *Plasmodium* transmission (i.e., they are successful in killing all of the parasites), the remaining 10% can quite easily support the propagation of *Plasmodia* [7, 10]. Challenging the oocysts, for example with inhibitors of plasmodial glutathione synthesis or GR (K. Buchholz, personal communication), would cause the parasites to be killed in the mosquito, relieve the vector insect from an enormous disease burden, and this would interrupt the transmission pathway from one human host to another. A more practical, and less mosquitophilic, approach is to use insecticides.

Typically, the targeting of *extracellular* forms of the parasite in the human host as a prophylactic measure against malaria is a domain of vaccine research rather than of drug research. Sporozoite motility, however, can also be inhibited effectively by drugs. Recent *in vitro* studies conducted by Frischknecht and coworkers at Heidelberg University have revealed that MB, at submicromolar concentrations, blocks the gliding motility that is characteristic of sporozoites during their long journey through their insect and human hosts, before they settle in their final hepatocyte destination (J. Hellmann and F. Frischknecht, personal communication).

In addition, several mechanisms exist whereby the blood schizonts – and thus malaria as a clinical disease – are transferred directly from person to person by blood transfusion, by organ transplantation, and by therapeutic inoculation (a procedure which was used to cure antibiotic-resistant neurosyphilis by injecting malarial parasites, for which Wagner-Jauregg was awarded the Nobel Prize in 1927). In addition, accidental inoculation can occur among drug addicts who share needles, syringes, or – even worse – contaminated drug solutions for injections. With regards to transfusion–transmission [11], special agents are required for blood sterilization;

typically, the recipient of parasitized blood can be treated with schizonticides and gametocytocidal agents and, in the case of *P. vivax*, later with hypnozoite-eliminating agents such as primaquine.

Another upcoming possibility is pathogen inactivation in suspicious blood units, on the basis of photoactivatable MB or dimethylene blue [11].

Congenital malaria, caused by the transmission of blood schizonts and gametocytes from mother to baby before or during childbirth, is not uncommon in so-called *pregnancy malaria*, where the maternal side of the placenta carries a pool of parasitized erythrocytes adhering to the chorionic villi.

To date, no cases have been reported of malaria as a sexually transmitted disease (STD). It is important to recognize this point as a negative aspect, since STDs attract relatively high public attention and concern on a worldwide basis.

Primaquine and its Redox-Cycling Metabolite, 5-Hydroxyprimaquine, Target Hypnozoites, Gametocytes, and Blood Schizonts

To date, very few clinical studies have been conducted with drugs aimed at combating gametocytes, because it was an accepted principle of ethics commissions that therapeutic measures must exclusively serve the patient, while "altruistic" drugs or vaccines that would prevent a patient from spreading the disease to other persons were considered "unethical." Among the few drugs used against gametocytes the most prominent are primaquine and the artemisinins.

Treatment with the 8-aminoquinoline tissue schizontocide, *primaquine*, has been regarded as a radical cure for relapsing malaria caused by *P. vivax* and other *Plasmodia* that form liver hypnozoites [9, 12]. In addition, primaquine has gametocytocidal activity, thus preventing the transmission of viable parasites from man to mosquito. Primaquine has also been used as one partner of drug combinations used against blood schizonts, the disease-causing form of malarial parasites. Unfortunately, under certain circumstances the clinical utility of primaquine is restricted by its hemolyzing effect in patients with certain types of genetic erythrocytic G6PD deficiency [13]. However, the statement that primaquine should not be administered to any patient with G6PD deficiency is too general, as this would prevent its use in more than 500 million persons worldwide.

When treating malaria patients with primaquine, the parasite-bearing erythrocytes are thought to be recognized by splenic macrophages as the equivalent of senescent red cells. This leads to a selective removal of the parasitized erythrocytes from the circulation [14]. The mechanism of this process is interesting for the present authors' program, which is aimed at developing pro-oxidant agents as antiparasitic drugs (see Figure 7.2 and Table 7.2). 5-Hydroxyprimaquine (5-HPQ) is a human metabolite of primaquine that forms a redox pair with its quinoneimine form; continuous cycling of this redox pair is thought to generate ROS within the erythrocytes. For example, when rat erythrocytes were incubated with 5-HPQ *in vitro*, and subsequently injected into the veins of isologous rats, they were removed rapidly from the circulation as compared to untreated, control erythrocytes [14]. Thus, the ROS attack on the *cytosolic* surface of the membrane generates a signal to remove

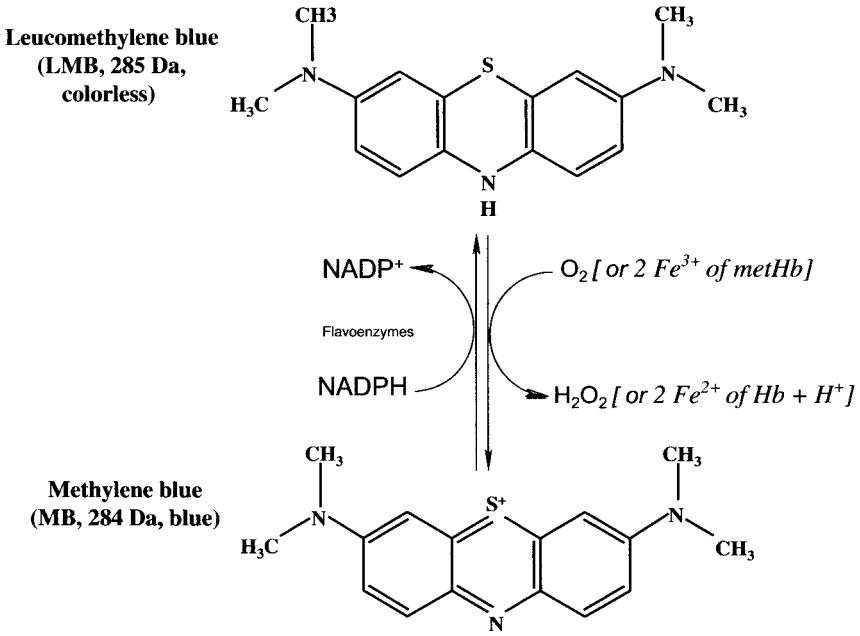

Figure 7.2 Methylene blue (MB) as a redox-cycling catalyst *in vivo*. Shown are the NAD(P)H-dependent reduction of the blue oxidized form and the rapid reoxidation of the color-free leuco form by auto-oxidation. Pyocyanin undergoes the same redox cycle. In contrast, both divicine and isouramil are reduced by glutathione (GSH) and reoxidized by O_2. The other product, glutathione disulfide, is reduced by NADPH in a glutathione reductase-catalyzed reaction. In all cases, the balance equation for one redox cycle is $NADPH + H^+ + O_2 \rightarrow NADP + H_2O_2$ (see Ref. [15]).

damaged erythrocytes from the circulation. The nature of the crucial target(s), and the mechanism of transfer of this signal across the membrane, are discussed below in the context of MB as a drug.

Pro-Oxidant Mechanisms for Preventing and Curing Malaria as an Acute Disease

Malaria as a clinical disease is caused by blood schizogony – that is, the asexual propagation of the parasite within the erythrocytes.

A number of *experimenta naturae* have suggested that malarial parasites require a highly reducing intra-erythrocytic environment for schizogony [3, 15, 16]. Notably, *P. vivax* may require an even higher antioxidative capacity of the erythrocyte metabolism than other plasmodia, as this parasite is able to propagate only in reticulocytes. Such young red blood cells are biochemically characterized *inter alia* by a very stable redox milieu.

Erythrocytes are normally well equipped with antioxidant cell-biochemical tools, including high activities of catalase, superoxide dismutase, peroxiredoxins and other

peroxidases, as well as enzymes that guarantee high concentrations of NADPH and thiol groups in the form of glutathione (2 mM) and protein-bound cysteine residues of hemoglobin (e.g., 10 mM Cys-93). One challenge for the reducing metabolism is that, during maturation of the precursor cells, enormous amounts of free iron ions and heme groups must be handled for hemoglobin production; both, the free iron ions and heme can readily catalyze the formation of ROS, including the OH-radical [17]. Another cause of continuous pro-oxidative challenges is the absence of mitochondria from mature erythrocytes; these organelles maintain physically dissolved O_2 at levels below 10 µM (http://sites.huji.ac.il/malaria/).

In addition, the binding mode of oxygen includes features of O_2^--binding to ferrihemoglobin, which suggests that superoxide or another ROS can be released from the O_2-transporting hemoglobin [1].

In mature erythrocytes, the tetrameric hemoglobin molecules are tightly packed in quasi-crystalline arrays. However, when pro-oxidant conditions prevail in the erythrocytic subregions, disturbances of the hemoglobin structure lead to the formation of hemichrome aggregates that contain intermolecular disulfide bridges and loosened heme groups as products of hemoglobin destabilization and degradation. The hemichromes possess a strong affinity for cytoskeletal proteins [14] and/or the cytoplasmic domain of the plasma membrane anion channel [18]. Following this binding, channel protein oxidation and oligomerization ensue.

The clustering of oxidized anion channels sets off a series of events that lead to changes at the erythrocyte surface which are recognized by specific immunoglobulin G (IgG) molecules. Typically, these normally recognize the band 3 protein clusters of a senescent cell, which accumulate at the end of its lifespan.

The bound IgGs activate complement, and finally trigger the phagocytosis of altered red blood cells. A number of hereditary and acquired pro-oxidant disorders, including the intracellular development of malarial parasites, exacerbates this phenomenon, leading to a more precocious and effective elimination of the diseased erythrocytes [19, 20]. Examples of pro-oxidant protein polymorphisms in persons exposed to malaria, or whose ancestors have been exposed to malaria for numerous generations, include G6PD deficiency, sickle-cell trait, HbSC (a double Hb mutant that contains HbS and HbC in each erythrocyte), beta-thalassemia and, as discussed below, GR deficiency and pyruvate kinase deficiency [20–25]. Pyruvate kinase, as a glycolytic enzyme, contributes to antioxidative defense [24], most likely because it produces the ATP required for the synthesis of glutathione. Some of the above mutant proteins reach polymorphism frequencies; that is, the corresponding alleles occur in more than 1% of the population. In the case of pro-oxidant G6PD mutants, between 10% and 20% of the male population is affected in many countries [13], notably in India, tropical Africa, and Polynesia.

Pro-oxidative food ingredients, such as isouramil and divicine of fava beans, or food additives in certain red suya meals [15], and pro-oxidative drugs such as primaquine, MB, dapsone, and 1,4-naphthoquinones (see Chapter 20) appear to protect the individual from severe malaria in a similar way as do pro-oxidant gene alleles.

Inherited Erythrocyte GR Deficiency as a Model for Malaria Treatment with Human GR Inhibitors

The above discussion on the protective effects of pro-oxidative G6PD alleles led to the following question: Do GR deficiency or drug-induced GR inhibition also lead to the selective removal of red blood cells containing ring stages of *Plasmodium*, by the process of phagocytosis?

In *P. falciparum*-infected red blood cells, the homodimeric flavoenzyme GR regenerates reduced glutathione, which *inter alia* is essential for antioxidant defense. Notably, GR utilizes NADPH which is produced in the pentose phosphate shunt by G6PD and another enzyme. It should be stressed here that NADPH-binding is also essential to maintain the stable, functional form of catalase; thus, low levels of NADPH will lead, in turn, to low levels of this antioxidant enzyme.

A deficiency in GR resulting from an insufficient saturation of the enzyme with its prosthetic group flavin adenine dinucleotide (FAD; a derivative of the vitamin riboflavin) is common. This situation has been studied in the Maremma region of Italy, that has always been notorious for its high incidence of malaria [25, 26]. In some cases, the so-called "nutritional" GR insufficiency may have a hereditary component, with a low affinity of the apoenzyme for the prosthetic group, FAD. In contrast, hereditary apoGR deficiency is rare. In 1976, the group of Dirk Roos described the case of a young woman who presented with favism after a meal of broad beans. She and two of her siblings were found to have normal, stable G6PD levels, but no activity of GR in the red and white blood cells. Subsequent Western blotting of the cell extracts yielded a negative result for GR as a protein. More recently, an investigation was conducted as to whether the GR-deficient erythrocytes would be suitable as host cells for *P. falciparum*, and whether the infected cells were sensitive to IgG-mediated ring-stage phagocytosis [20]. In these studies, the parasite multiplication rate was found to be equal in both GR-deficient and GR-sufficient erythrocytes, while practically no differences were apparent in terms of drug sensitivity (Table 7.1). Hence, GR deficiency may induce changes in the parasite–host unit similar to those described for G6PD deficiency and other pro-oxidant genetic dispositions, and phagocytosis of the ring-stage-infected red blood cells is more pronounced in antioxidation-compromised erythrocytes than in normal red blood cells. In this context, it should be noted that the ring stages do not yet produce hemozoin, a paralyzing poison for phagocytes [19].

Consequently, GR deficiency adds to the paradigm of malaria-protective genetic variations which are based on enhanced IgG-mediated ring-stage phagocytosis, rather than on impaired parasite growth [19].

Erythrocytes pretreated with the disulfide reductase inhibitor bis-chloronitrosourea (BCNU) either *in vivo* or *in vitro*, have been shown suitable for corroborating and/or extending the findings of the present report. In analogy to the animal model of primaquine treatment [14], the plan is to conduct the following study: to take blood samples from BCNU-treated patients [27], to use the erythrocytes as host cells of *P. falciparum* and, subsequently, to test if the ring stages are more susceptible to phagocytosis than parasitized matched control cells from patients not treated with

Table 7.1 Comparison of the growth and biochemical properties of *P. falciparum* grown in glutathione reductase (GR)-deficient and in control erythrocytes.[a].

	GR-deficient host cells	Normal host cells
Total glutathione in the parasite (nmol mg^{-1} protein)	68 ± 2.1	73 ± 1.4
GR activity in parasites (mU mg^{-1})	160 ± 6	270 ± 14
Parasite multiplication rate per red blood cell cycle	4.9 ± 0.3	5.4 ± 0.5
IC_{50} of chloroquine (nM)	8.2 ± 0.2 (4.9 ± 0.2)	8.0 ± 0.2 (5.9 ± 0.6)
IC_{50} of chloroquine for K1 (nM)[b]	160 ± 10	190 ± 11
IC_{50} of methylene blue (nM)	3.9 ± 0.2 (4.2 ± 0.3)	3.8 ± 0.2 (4.2 ± 0.3)
IC_{50} of methylene blue for K1 (nM)[b]	8.8 ± 0.5	8.1 ± 0.4
IC_{50} of artemisinin (nM)	14 ± 0.5	16 ± 0.8

a) Before determining the biochemical parameters in the parasites, *P. falciparum* 3D7 was grown over four to five cycles (corresponding to 8–10 days) in the respective red blood cells. Control cells had the same blood group as the patient's. All values given represent mean values of two to three parallel determinations.

b) The chloroquine-resistant strain K1 is expectedly less sensitive to chloroquine than 3D7, but it is similarly sensitive to MB. All data were taken from Ref. [20].

BCNU. In this context, it would also be of interest to study whether patients under BCNU-treatment have ever contracted malaria [3]. A previous observation that BCNU-pretreated erythrocytes did not serve as host cells of *P. falciparum in vitro* is explained by the additional damage caused by very high BCNU concentrations, for instance, on the glutathione-synthesizing enzymes [20, 28].

Furthermore, the data acquired had an impact on antimalarial drug development strategies, as they indicated that the antimalarial effects of compounds capable of manipulating or inhibiting the activities of human GR are difficult to assess in cell cultures not containing IgG and phagocytes. Thus, the inhibitors of human GR would be expected to be efficient *in vivo*, but not *in vitro*.

The results obtained suggested that human erythrocyte GR should not be neglected as a potential drug target [20, 28]. Another argument in favor of considering a host cell enzyme as a target is the *a priori* prevention of drug resistance.

P. falciparum GR as a Target of Inhibitors and Subversive Substrates

The inhibitors of *Plasmodium falciparum* glutathione reductase (PfGR), together with details of the natural defense mechanisms of the human host (e.g., peroxynitrite production and the fever reaction) that affect PfGR, are listed in Ref. [3].

As a word of warning for drug-design studies, the stable, intensely studied form of PfGR *in vitro*, namely E_{ox}, is but a minor form *in vivo*, whereas the reduced forms of the enzyme, which contain an active site dithiol, dominate in the cell. Only glutathionylated GR, NADPH-EH_2 and EH_4 are likely to occur in the parasite, and these molecular species should be considered as targets of redox state-specific inhibitors [1, 3, 4]. *P. falciparum* thioredoxin reductase (TrxR) is also

present *in vivo* in reduced forms. BCNU, for instance, does not affect the E_{ox} form of disulfide reductases, but is an irreversible inhibitor of reduced species, both *in vitro* and *in vivo*.

Fever Bouts Denature the Reduced Forms of PfGR

The conspicuous symptoms of malaria are the fever bouts (also known as "swamp fever," "Roman fever," *the* fever). Such bouts are dangerous for the patient, but much more so also for the parasites; indeed, evidence indicates that a large percentage of the parasites are killed during these fever phases. In culture, the blood schizonts multiply more than 10-fold in four days at 37 °C, but their numbers decrease to 20% when they are grown at 40 °C. These observations suggest that the inactivation of thermolabile enzyme species such as NADPH·GR contributes to the thermosensitivity of the malarial parasites [29]. Labile EH_2·NAD(P)H and EH_4, rather than stable E_{ox}, are the predominant forms of the disulfide reductases in active cells, and this should be accounted for in both pharmacological and pathophysiological studies of the enzymes. Additionally, redox-cycling agents and inhibitors targeting GR and TrxR at a physiological NADPH concentration of approximately 50 μM should be tested at 40 °C.

Elevated temperatures, even above 55 °C, do not affect human GR. Erythrocytic enzymes, which must remain functional for more than 100 days at 37 °C, have probably been selected for reasons of stability during the course of evolution.

Pharmaceutical Agents as Inhibitors and/or Substrates of Disulfide Reductases

Pharmaceutical agents that interact with GRs and other FAD-containing disulfide reductases can be grouped into two classes, namely enzyme inhibitors and diaphorase substrates. Diaphorase activity is an additional function of several oxidoreductases that are capable of catalyzing the reaction:

$$\text{NAD(P)H} + \text{oxidized heteroaromatic compound} \rightarrow \text{NAD(P)} + \text{reduced heteroaromatic compound.}$$

Many heteroaromatic, redox-active compounds (such as MB) can serve both as an inhibitor (of the disulfide reduction reaction) and as a diaphorase substrate. In this case, there are most likely two binding sites for the two modes of drug action on each enzyme.

The diaphorase substrates, which often are subdivided into turncoat inhibitors, subversive substrates and redox cyclers, are reduced by enzymes at the expense of NADPH, at a site which is not the binding site of the natural disulfide substrate. The reduction of the heteroaromatic compound is the actual diaphorase reaction. Subsequently, the reduced compound undergoes reoxidation or another cell-biochemical reaction. If the reoxidizing agent is O_2, the compound acts catalytically and serves as a redox-cycling agent. In each catalytic cycle NADPH and O_2 are consumed, while NADP and O_2^- or H_2O_2 are formed. Thus, in cooperation with the reductase and O_2, the drug catalyzes the same reaction as NADPH oxidase, a major enzymic

defense tool of higher organisms against pathogens. However, other intracellular components can also reoxidize leucoMB, an example being the heme group of methemoglobin. In this way, the effects of MB do not depend exclusively on O_2-driven redox-cycling.

At this point, dihydrolipoamide dehydrogenase (LipDH or DHLD) deserves special mention. This enzyme can serve as a highly efficient diaphorase using NADH and MB as the two substrates, although its physiological reactions are not inhibited by MB and/or other heteroaromatic compounds [3, 30]. In such a case, gene knockout experiments might lead to the conclusions that the enzyme is not essential for the parasite, and therefore not suitable as a target for drug development. However, the second conclusion is not correct because the enzyme – which serves as an NAD(P)H oxidase in the presence of a catalytic drug concentration – plays an essential drug-like role for the production of parasitotoxic compounds (K. Buchholz, personal communication).

The reaction sequence initiated by MB in the presence of molecular oxygen is:

$$\text{NADPH} + \text{MB} \rightarrow \text{NADP} + \text{leucoMB} \quad (\textit{diaphorase reaction of GR})$$
$$\text{leucoMB} + 2\,\text{O}_2 \rightarrow \text{MB} + \text{H}^+ + 2\,\text{O}_2^- \quad (\textit{auto-oxidation in vivo})$$
$$2\,\text{O}_2^- + 2\,\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (\textit{enzyme-catalyzed or spontaneous})$$
$$\text{NADPH} + \text{O}_2 + \text{H}^+ \rightarrow \text{NADP} + \text{H}_2\text{O}_2$$

Considering the first two lines, the balance equation is:

$$\text{NADPH} + 2\,\text{O}_2^- \rightarrow \text{NADP} + \text{H}^+ + 2\text{O}_2^-$$

This corresponds to the reaction catalyzed by the antimicrobial defense enzyme NADPH oxidase.

Is 100% Enzyme Inhibition Necessary?

In the lifestyles of the malarial parasite, where GR activity is essential, PfGR-targeting drugs do not have to lead to 100% inhibition in order to be effective. By using the *in vivo*-relevant form of the Michaelis–Menten equation with a K_M of 100 µM, $[S] = K_M\,(V_{max}/v - 1)^{-1}$, 93% inhibition is predicted to lead to an increase of the steady-state concentration in GSSG, from <4 µM to >800 µM, which is highly parasitotoxic [3]. The situation is similar for TrxR, where enzyme inhibition leads to a low steady-state concentration of reduced thioredoxin. Of course, these estimations are correct only if there is not more than one biological mechanism for the reduction of glutathione disulfide and oxidized thioredoxin, respectively.

Pyocyanin, a Natural Analog of MB, is an Inhibitor and a Diaphoretic Substrate of GR

Recently, the blue pigment pyocyanin (PYO), which is known to act as a signal, quorum sensor, respiratory metabolite, and antimicrobial molecule in *Pseudomonas aeruginosa* and other bacteria [31], has been identified as having antimalarial activity

in vitro (K. Becker, personal communication). Comparisons of PYO with the phenothiazine derivative MB have suggested that the latter might be regarded as a sulfur analog of the natural phenazine compound PYO (Table 7.2). This working hypothesis explains, at least in part, why synthetic MB behaves like a compound that has been shaped by biological evolution. The similarities of MB and PYO include: (i) their characteristics as positively charged, heteroaromatic compounds; (ii) their reactivity toward cellular reductants such as NADPH or dihydrolipoamide; (iii) the reactivity of their reduced leuco-forms toward triplet O_2; and (iv) their interactions with signaling proteins, flavoenzymes, heme proteins, or transmembrane transporters. Both compounds were shown to have gametocytocidal and schizontocidal effects on malarial parasites in a submicromolar range. However, whereas PYO is too toxic, MB represents a promising agent for MB-based combination therapies against *P. falciparum* malaria in children (see below). Yet, pyocyanin may serve as a valuable tool for studying disease interference. Indeed, retrospective studies are currently under way to determine if patients suffering from *Pseudomonas aeruginosa* infections, which frequently occur in tropical countries, are protected from malaria by the secondary metabolite, PYO.

The binding of PYO to the flavoenzyme GR from human erythrocytes was studied using X-ray crystallography (Figure 7.3). As might be expected from the binding mode of other heterocyclic compounds [3], the structure shows one PYO molecule bound in the cavity located at the interface of the two subunits of the enzyme. The binding pocket is formed by seven amino acids of one subunit, and their counterparts of the other subunit, such that PYO is perfectly sandwiched between two phenylalanine residues (F78 and F78′). Azure B, a demethylated metabolite of MB with antimalarial activity [32], was also found to bind to the crystalline enzyme in a mode very similar to PYO (K. Fritz-Wolf, unpublished results).

Why Reintroduce MB as a Drug Against Pediatric Malaria?

In a famous paper, Sir James W. Black, M.D., the 1988 Nobel Laureate in Medicine [33], defined the basic philosophy of his approach as, "The most fruitful basis for the discovery of a new drug is to start with an old drug." The present authors greatly approve of this approach, and would like to add the term ". . .from a natural source" because, in that case, natural selection has shaped the compound for appropriate behavior in biological systems [7].

One very promising old drug (in fact, it was the very first synthetic drug) – MB – can serve as a noncompetitive inhibitor of PfGR, and much less of human GR, at therapeutically employed concentrations. This effect was identified by Petra Färber (of the present authors' group) when screening affordable drugs with antimalarial activities as PfGR inhibitors [34]. MB, which has long been used in the treatment of malaria [35], is today the standard medication against inherited and acquired methemoglobinemia, ifosfamid-induced neuropathy, and other pathological conditions (http://www.alzforum.org/new/Schirmer.asp; see also Ref. [36]). However, there is a contraindication for MB. A very recent discovery has been the functional

Table 7.2 Properties of pyocyanin in comparison to methylene blue.

Property	Pyocyanin	Methylene blue
Chemical class	Phenazine	Phenothiazine
Color	Blue	Dark blue
Colorless reduction product	LeucoPYO	LeucoMB (solubility only 25 μM)
$\lambda_{vis,max}$	690 ± 100 nm	663 nm
M_r of the heterocycle	210 Da	284 Da
Milestones	Forbes (1860), Wrede (1924, 1929), Newman (2006)	Caro (1884), Bernthsen (1887), Ehrlich (1891), Wieland (1922), Clark (1925)
Functions *in vivo*	In *P. aeruginosa* respiratory pigment, quorum sensor, transcription factor, antimicrobial agent	Numerous technical, industrial, scientific, and medical applications
Clinical dosage for various diseases	Not applicable, PYO is toxic	2–20 mg kg^{-1} per day
Concentrations *in vivo*	<100 μM in infected bronchial mucus	5–30 μM in whole blood
Midpoint potential at pH 7	−30 mV	+10 mV
Intracellular reductants (k-value at pH 7)	NADPH (86 $M^{-1} s^{-1}$) > NADH > dithiols	Dithiols > NADPH = NADH
Redox cycling catalyst *in vivo*	Reduction by NAD(P)H, reoxidation by O_2	Enzyme-catalyzed reduction by NAD(P)H, reoxidation by O_2
Reduction by flavoenzymes	LipDH and TrxR ≫ GR	LipDH > TrxR > GR
Inhibition of flavoenzymes	GR and TrxR, not LipDH	GR and TrxR, not LipDH
Crystal structure of enzyme-ligand complex	Human GR–PYO complex	Low-resolution data
IC_{50} against *P. falciparum* blood schizonts *in vitro*	86 ± 4.5 nM (3D7) 59 ± 3.3 nM (K1)	3.1 ± 0.4 nM (3D7) 6.6 ± 1.1 nM (K1)
IC_{50} against early (and mature) *P. falciparum* gametocytes *in vitro*	180 nM (560 nM)	34 nM (60 nM)
Tested for biological conduct *in vivo*	In >100 Ma of evolution in bacteria-containing biotopes [31]	In an unpresented variety of uses in medicine and biotechnology

Data on PYO listed from line "Redox-cycling catalyst" onwards were contributed by D. Kasozi, K. Fritz-Wolf and K. Becker (personal communication). This applies also to the IC_{50} values of MB. Most other data on MB were taken from Ref. [30].

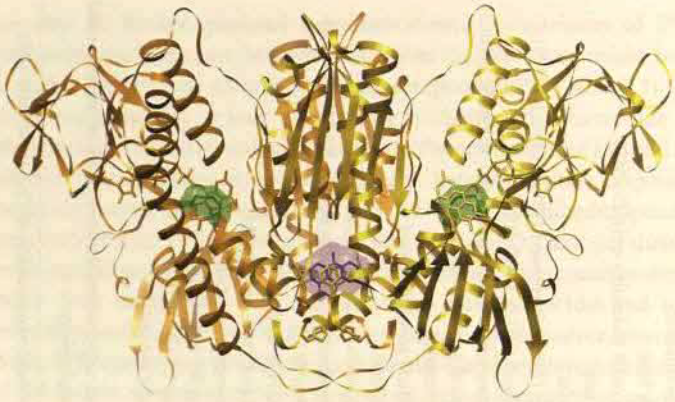

Figure 7.3 Human glutathione reductase homodimer with bound pyocyanin (PYO). The pyocyanin (blue) and FAD (yellow) are represented as ball-and-stick models. Additionally, the surfaces of the catalytic cysteines Cys 58/Cys63 and Cys58′/Cys63′ (green) and of PYO (blue) are shown.

interaction that occurs between MB and the serotonin-specific reuptake inhibitors (SSRIs) that are used as antidepressant agents. This undesirable synergy may lead to the potentially fatal "serotonin toxicity syndrome"; consequently, MB must not be administered to patients taking SSRIs [37].

Secondary Drug Design on the Basis of Phenothiazine Drugs?

There are two main reasons for the reluctance to design an optimized derivative of MB on the basis of its binding site in GR. The first reason is that, whilst demethylated MB species and selenoMB are interesting candidates, any derivative would result in major costs for testing the new compound as a drug candidate. The second reason is the fact that antimicrobial drugs acting as ideally fitting ligands of their targets are subject to rapid drug resistance development by the pathogen [38].

Drug resistance is in many cases due to drug tolerance at a molecular level. One reason for the occurrence of drug tolerance is that, in ligand–target interactions, the attractive forces are weak while the repulsive forces are strong. Thus, the introduction of an additional H-atom or a methyl group into the target protein, on the basis of a point mutation, can lead to the complete abolition of any interaction [3].

On a pharmacological level, drug optimization efforts by secondary design may lead to unexpected effects. For example, as shown by Eisenbrand, the carbamoylating agent BCNU was converted to the alkylating drug HeCNU by replacing a chlorine atom with an OH-group [3, 28].

Clinical Trials Using MB-Based Drug Combinations

The effective use of MB in two adult patients (probably with *P. vivax* malaria) was first reported by Guttmann and Ehrlich, in 1891 [39, 40]. Two years later, Ferreira

described in detail the first successful oral application of MB to 40 children with malaria in Rio de Janeiro [35]. In this case, oral daily doses of between 25 and 50 mg kg^{-1}, subdivided into several portions, were administered and well tolerated by infants and young children until the malaria symptoms subsided.

In 2003, MB was tested for the first time against uncomplicated *P. falciparum* malaria in Africa in clinical studies. Initially, the safety and pharmacokinetics of MB were studied in healthy and G6PD-deficient adult men [41, 42]. The G6PD-deficiency type G6PDA$^-$ that occurred in this and other study groups did not represent a contraindication for the use of MB, as no hemolysis was observed. It should be noted that about 150 types of G6PD mutation have been identified in populations with roots in malaria-endemic countries, that one in six Africans and one in five Indians carries a G6PD mutation, and that the hemolytic response to drugs is very different among the types and classes of G6PD deficiency [13, 43].

The safety studies were followed by randomized, controlled clinical trials conducted in young children (aged 6–59 months) with malaria [44]. Based on the intention to reverse parasite resistance against chloroquine, MB was combined with chloroquine; this drug combination was termed "Blue CQ." When administered at a dose of 2 mg kg^{-1} twice daily, MB proved to be ineffective, whereas clinical trials using higher doses of MB (6–12 mg kg^{-1} twice daily) showed it to be well tolerated and to have an efficacy of 66% ACPR (adequate clinical and parasitological response) [45]. Unfortunately, the positive effect of MB was overshadowed by parasites rapidly developing resistance to the partner drug, chloroquine. Moreover, the bitter taste of MB solutions necessitated the development of a taste-masked pediatric fluid formulation [35, 46] since, for children aged less than five years, the administration of tablets and capsules is not permitted.

Since 2006, MB has been administered in combination with amodiaquine in several clinical trials in young children in Burkina Faso. The combination proved to be highly effective (95% ACPR on day 28), and even superior to a combination of MB plus artesunate (62% ACPR on day 28), both of which are short-acting drugs [47]. When given as a monotherapy to semi-immune adults with uncomplicated malaria, MB proved to be effective (74% ACPR if given over three days), although several early treatment failures occurred due to the initial slower parasite clearance time of MB compared to the artemisinins [48]. Both experimental (Table 7.2) and clinical [49] evidence has been obtained showing MB to be effective not only against gametocytes (which would lead to MB becoming an asset for ongoing global malaria elimination efforts), but possibly also against hypnozoites (making it an interesting drug candidate for the treatment of *Plasmodium vivax*). Moreover, all of the pharmacokinetic studies with MB showed it to be better tolerated when administered orally than intravenously. Orally administered MB was also shown to provide a very good bioavailability of the drug [50]. In addition, MB demonstrated a much longer plasma half-life (ca. 20 h) than previously assumed [50], such that once-daily dosing should be considered [41, 50].

MB Leads to Blue Coloration of the Urine and, Consequently, of Napkins and Clothes

Although MB has been identified as a promising candidate for the treatment of malaria in the main risk group of young children, there is a major adverse side effect in all cases, in that it leads to a blue coloration of the child's urine and, consequently, also of the mother's clothes. Since the acceptability of such coloration effects was unknown, an anthropological study was undertaken in a malaria-endemic area of rural Burkina Faso to identify the community's perceptions regarding such blue coloration [51]. Perhaps not surprisingly, the results showed that people would accept the drug, even if its color was unusual and it stained the patient's clothing, as long as it is effective against malaria. Moreover, despite modern washing powders proving to be inferior for the purpose, the mothers quickly determined how MB-stained clothing could be cleaned, using traditional washing methods. In conclusion, these studies have not only shed some light on the relationship between color and drug preference in Africa, but also supported the importance of considering community attitudes before commencing public health interventions. An additional benefit was that the blue urine coloration not only improved patient compliance, but also helped to prevent the distribution of counterfeit drugs.

Notably, MB can be converted readily to color-free leucoMB [30]; indeed, REMBER™, a drug that is currently undergoing trials for the treatment of Alzheimer's disease, contains leucoMB [52] (see also http://www.alzforum.org/new/Schirmer.asp), which most likely has the same pharmacological properties as MB as it is readily auto-oxidized. Unfortunately, such instability of leucoMB in the presence of O_2 would render it impractical as an ingredient of antimalarial drug combinations.

Prevention of Resistance Development to Key Antimalarials Using MB as an Additional Partner

Currently, artemisinin-based combination therapy (ACT) represents the first-line treatment of choice for malaria. Recently, however, Samarasekera described the development of artemisinin resistance at the Thai–Cambodia border that may also soon affect sub-Saharan Africa [53]. Hence, the question here is, "What can be done to avoid a repetition of the tragic history of chloroquine resistance?"

As discussed by Müller *et al.* [53], even if fixed-dose ACT were to be widely available, it would be only a question of time until resistance to the partner drug with the longer half-life would develop, leaving the artemisinin component unprotected. Thus, the addition of another antimalarial with a short half-life to existing ACTs might represent an innovative approach. MB, which has a broad activity against *P. falciparum* parasites and acts synergistically with artemisinins [54], might prove to be such a candidate. As noted above, MB has not only been demonstrated as both safe and effective in the malaria patients of Burkina Faso, but also has the potential to greatly reduce the number of *P. falciparum* gametocytes in clinical malaria cases [49].

Perhaps the most obvious question in this context would be whether any resistance of malaria parasites to MB has been observed. This is clearly not the case in animal

models, where only a very moderate increase in EC_{50} values can be provoked by administering MB for several months [3], nor in human malaria. The reason for the continuous sensitivity of *P. falciparum* appears to be that (leuco)MB and its metabolites have more than one target. In addition, some targets – such as the growing hemozoin double helix and the GR of the red blood cell – cannot be controlled by the genome of the parasite, which makes the development of rapid resistance against this drug very unlikely.

Discussion

Ethical Drugs: MB as an Example

Although the institutions of post-modern drug research are extremely efficient when developing and marketing drugs for the diseases of affluent societies, as well as "lifestyle" or "performance" drugs, they appear to have lost in part their competence to create drugs for diseases of the poor. Since 1975, less than 1% of all newly approved drugs have been registered for diseases that prevail in the developing countries. One such disease is *P. falciparum* malaria, which affects several hundred million people every year, with the high-risk groups among patients including unprotected tourists, pregnant women and, above all, children aged under five years. Whilst the tourists represent the "happy few" of all nations who have access to adequate prophylaxis or treatment, the main challenges are apparent in the other two high-risk groups. Clearly, the true burden of malaria – from personal, medical, and economical perspectives – is endured by the poor people of tropical countries [39, 55].

The present concept is to complement the modern procedures of drug development [38] by developing ethical drugs that fulfill the *bonaria* criteria. The term "ethical drugs" (*Ethische Präparate*, according to Robert Koch) implies that these drugs are necessary to prevent and cure disease, but that they are unlikely to generate profits. During the classical period of drug research – during the early decades of the twentieth century – physicians and medicinal chemists alike were highly successful at developing and distributing ethical drugs; indeed, at the time it was considered to be a crime, a sin of omission, to withhold treatment. Today, however, in periods of heightened safety concerns, and with the concept of an unlimited liability of both the physicians and the drug companies – the health services would prefer to leave hundreds of millions of people untreated rather than to risk a single case of real or putative toxic effects. This rigor with regards to ethical drugs should be contrasted with the relaxed attitude of today's Western society toward the often serious side effects of "lifestyle" and "performance" drugs.

A related problem to this situation is the "know–do" discrepancy. In other words, we know too much, and we do too little to alleviate the malarial burden [55]. Rather, the development, distribution, and administration of ethical drugs should be handed over to academic public institutions, anthropophilic foundations, and military services. Indeed, for more than 100 years military institutions have been highly

successful at developing drugs and vaccines to combat tropical epidemics and other diseases of the poor. Of course, help and support from pharmaceutical industries would be most welcome, but should their role become an essential part of the process?

While developing ethical drugs, it is in everybody's interest to ask challenging questions, including: How are the astronomic costs of developing new drugs calculated by drug providers [55]? Who determines the criteria for registering a drug? Who profits from the achievements of medical and scientific research? And, to whom are such drugs denied [56]?

Today, humankind lives in times of negative ethics, of avoiding mistakes, rather than in times of positive ethics where attempts are made to bring more justice, health, and progress to the world [56]. In his inaugural presidential address in 1937, Franklin D. Roosevelt characterized positive ethics in scientific and medical progress as follows: *"The test of our progress is not whether we add more to the abundance of those who have much; it is whether we provide enough for those who have too little."*

Conclusion

Both, MB and other pro-oxidant agents can be regarded as pharmacological phenocopies of inborn conditions, such as certain types of pro-oxidant G6PD deficiency or GR deficiency. The pro-oxidant genetic dispositions of red blood cells and pro-oxidant agents often do not affect parasite growth *in vitro*, but rather demonstrate their efficiency *in vivo* by inducing an IgG-mediated phagocytosis of erythrocytes carrying the ring stages of the parasites. The value of natural compounds, such as pyocyanin, as a natural analog of the pro-oxidant drug MB has been highlighted. Indeed, the absorption, distribution, metabolism and excretion (ADME) of administered MB can be better understood by making comparisons with pyocyanin released in *Pseudomonas* infections.

MB has several targets in *P. falciparum*-infected cells. *Inter alia*, it is a diaphorase substrate of *P. falciparum* disulfide reductases, in which case the parasite enzyme is an essential tool for mediating the drug effect. This must be accounted for when developing appropriate inhibitors of disulfide reductases, or when interpreting the results of gene knockout experiments.

The procedure applied to MB appears to show promise for drugs targeted against widespread diseases of the poor. Clearly, when a potential parasite-specific target such as PfGR becomes available for systematic testing, then all affordable registered drugs should be screened as inhibitors of this target. If an active compound were to be identified in this way, then the enormous costs of both preclinical and many clinical trials could be circumvented, and the forbiddingly narrow part of Ridley's "funnel of drug development" [38] could, accordingly, be avoided. Moreover, the desired drug should fulfill most, or all, of the *bonaria* criteria proposed for an ethical drug.

Ongoing studies continue to show that MB is active against not only schizonts but also gametocytes, which are responsible for malarial transmission and thus important in malarial elimination programs. This illustrates a further advantage of the

bonaria drugs, namely that there is a continuous dynamic interaction between the results of clinical trials and basic research.

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