The bacterial redox signalling molecule pyocyanin as an antiparasitic agent: comparisons with its thioanalog methylene blue

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The quorum sensor and signalling molecule pyocyanin (PYO) contributes significantly to the pathophysiology of *Pseudomonas aeruginosa* infections. Comparison to phenothiazine drugs suggests that the antimalarial compound methylene blue (MB) can be regarded as a sulfur analog of PYO. This working hypothesis would explain why the synthetic drug MB behaves as a compound shaped in biological evolution. Here we report on redox-associated biological and biochemical properties of PYO in direct comparison to its synthetic analog MB. We quantitatively describe the reactivity of both compounds toward cellular reductants, the reactivity of their reduced leuco-forms towards O₂, and their interactions with FAD-containing disulfide reductases. Furthermore, the interaction of PYO with human glutathione reductase was studied in structural detail by x-ray crystallography, showing that a single PYO molecule binds to the intersubunit cavity of the enzyme. Like MB, also PYO was also found to be active against blood schizonts of the malaria parasite *P. falciparum* *in vitro*. Furthermore, both compounds were active against the disease transmitting gametocyte forms of the parasites, which was systematically studied *in vitro*. As shown for mice, PYO is too toxic to be used as a drug. It may, however, have antimalarial activity in numerous human patients with concomitant *Pseudomonas* infections. MB, in contrast to PYO, is well tolerated and represents a promising agent for MB-based combination therapies against malaria. Current and future clinical studies can be guided by the comparisons between MB and PYO reported here. Additionally, it is of interest to study if and to what extent the protection from malaria in patients with cystic fibrosis or with severe wound infections is based on PYO produced by *Pseudomonas* species.

Keywords: Diaphorase, Enzyme inhibition, Gametocytocidal drugs, *Plasmodium falciparum*, Redox-cycling drugs, Stage-specific drug effects, Toxicology, Wound infections

Introduction

Phenazines, nitrogen-containing heterocyclic compounds synthesized by *Pseudomonas* species and a number of other bacteria, are secondary metabolites with important biological activities. Pyocyanin (5-methyl-1-hydroxyphenazine, PYO, Fig. 1A) is a blue, redox-active phenazine produced and secreted by *Pseudomonas aeruginosa* where it serves as a respiratory factor, a quorum sensor, and an anti-microbial molecule.^{1–4} *P. aeruginosa* as a leading bacterial pathogen in hospital settings is the primary cause of sepsis and death in patients immunocompromised as a result of

neutropenia, burns, or cystic fibrosis. Concentrations of up to 100 µM PYO have been detected in the sputa of *P. aeruginosa*-infected cystic fibrosis patients.⁵

PYO is known to exhibit pro-oxidant activity and, as a zwitterion with both hydrophobic and hydrophilic regions; it can easily interact with and penetrate cell-biological membranes at pH 7.0. PYO can directly accept electrons from reducing agents such as NADH, NADPH, and reduced glutathione (GSH). Electrons are then transferred to oxygen in order to generate reactive oxygen species (ROS) such as hydrogen peroxide and singlet oxygen⁶ at the expense of host antioxidant capacity.³ GSH levels of several cell lines exposed to PYO have been shown to be depleted by up to 50%^{6,7} and H₂O₂ production has been demonstrated to increase dramatically.⁸ The concentration-,

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Figure 1 (A) Structures of PYO and MB in oxidized and reduced forms. (B) PYO and MB as redox cycling catalysts *in vivo*: NAD(P)H-dependent reduction of the blue oxidized forms and rapid re-oxidation of the leuco forms by auto-oxidation. (C) PYO and MB as diaphorase substrates of *P. falciparum* mitochondrial lipoamide dehydrogenase. In the Lineweaver–Burk diagram, the ordinate axis intercepts represent the reciprocal values of the specific activity for PYO (squares) and MB (circles). The enzyme preparation described in the experimental section was used. The OH group of PYO_{ox} has a pK_a of 4.9.

time-, and O₂-dependent reactions of GSH with PYO were recently analyzed in detail by Cheluvappa *et al.*⁹

Studies on the role of PYO in *P. aeruginosa* virulence *in vivo* using alternative model hosts revealed that PYO participates in the rapid killing of the nematode *Caenorhabditis elegans* by producing ROS¹⁰ and severely affects numerous other organisms including bacteria,¹¹ yeast, fungi,² insects,¹² plants,¹³ and mice.^{3,14} *P. aeruginosa* mutants lacking PYO are greatly attenuated in both acute and chronic infection as shown in mouse models.³

In order to find physiological targets of PYO, Ran *et al.*⁸ screened a *Saccharomyces cerevisiae* deletion library. Sixty percent of the hits have orthologs in humans and indicate that cell cycle, electron transport and respiration, epidermal cell growth, protein sorting, vesicle transport, and the vacuolar ATPase may be targeted. Beside these properties, which are mainly

attributed to increased ROS levels, PYO shows physical effects affecting ciliary function and immunological effects interfering with interleukins, neutrophil apoptosis, and T cell response, for example. These effects might result in part from increased ROS levels (for a review see Liu and Nizet¹⁵ and references therein). However, the interaction of PYO with central cellular redox enzymes, including disulfide reductases like glutathione reductase (GR) and thioredoxin reductase (TrxR), has not yet been studied in sufficient detail.

The phenothiazine compound methylene blue (MB; methylthionine hydrochloride or 3,7-bis(dimethylamino)phenothiazin-5-ium chloride, Fig. 1A), the first synthetic compound to be used as a drug, was described by Guttman and Ehrlich in 1891 as effective against malaria.¹⁶ MB is registered in most countries as the treatment of choice for acute and

chronic methemoglobinemia. It has been intensely studied and its numerous favorable properties have been described, including the differential staining of cell biological structures and protein crystals, its medicinal applications, and its unique physicochemical and photochemical characteristics.^{17–21} Like PYO, MB exhibits a remarkable reactivity toward cellular reductants and interacts with signaling proteins, flavoenzymes, haem proteins, or transmembrane transporters.^{19,20} Thus, MB behaves almost as a molecule shaped in biological evolution and indeed it has striking structural and functional similarities with PYO (Fig. 1).

Here we report – in the context of intracellular redox reactions – on further important properties of PYO and MB. This includes comparative studies on their redox cycling behavior, the quantitative reduction of PYO and MB by diaphorases, and the inhibition of disulfide reductases by PYO and MB. In addition to these kinetic data, we provide the x-ray structure of PYO bound to human GR. Furthermore, we show that both compounds are active against the human malaria parasite *Plasmodium falciparum*, including stage-specific effects and a remarkable activity against the disease-transmitting gametocytic forms.

Results

Redox cycling of PYO in the presence of intracellular reductants and triplet oxygen

Many pharmacological effects of PYO and MB are due to spontaneous or enzyme-catalyzed redox-cycling reactions (see Tables 1 and 2 and ¹⁹). The reducing equivalents required for these reactions are supplied by major intracellular reductants such as NADPH, NADH, or glutathione; the final electron receptor is often O₂ (Fig. 1B). The rate constant for the spontaneous second-order reaction PYO + NADPH + H⁺ → LeucoPYO + NADP⁺ (Fig. 2) was determined as previously described for MB and found to be 80 M^{−1} second^{−1} at pH 7.3 and 37°C; values for 25°C are given in Table 1; assuming cytosolic concentrations of 40 μM NADPH and 25 μM PYO, the oxidation of NADPH would correspond to approx. 5 μM/minute. In order to slow down the spontaneous reaction between PYO and NAD(P)H in pharmacological experiments we recommend choosing a pH of 8.0.

PYO reacts much more slowly than MB with intracellular thiol compounds such as thioredoxin or glutathione, both in the absence and presence of daylight.¹⁹

Table 1 Properties of PYO in comparison to MB

Property	Pyocyanin	MB
Chemical class	Phenazine	Phenothiazine
Color	Blue	Dark blue
Colorless reduction product	LeucoPYO	LeucoMB (solubility only 25 μM)
λ _{vis,max}	690 ± 100 nm	663 nm
M _r of the heterocycle	210 Da	284 Da
Milestones	Forbes 1860, Wrede 1924, 1929, Friedman and Michaels 1931, Newman 2006	Caro 1884, Bernthsen 1887, Ehrlich 1891, Wieland 1922, Clark 1925
Functions <i>in vivo</i>	In <i>P. aeruginosa</i> respiratory pigment, quorum sensor, transcription factor, antimicrobial agent ¹	Numerous technical, industrial, scientific and medical applications
Clinical dosage for various diseases	Not applicable	3 mg per kg per day ²²
Concentrations <i>in vivo</i>	<100 μM in infected bronchial mucus (see Ref 5)	5–30 μM in whole blood ²²
Midpoint potential at pH 7	−34 mV ²⁴ (see Acknowledgements)	+10 mV
Intracellular reductants (<i>k</i> values in M ^{−1} s ^{−1} at pH 7 and 25°C)	NADPH (46) > NADH (26) > dihydrolipoamide (3.2) > GSH (<0.5)	Dihydrolipoamide (53) > thioredoxin (27) > NADPH (6.6) > NADH (3.5) > GSH (<0.5) ¹⁹
Redox cycling catalyst <i>in vivo</i>	Yes, reduction by NADPH, reoxidation by O ₂	Enzyme-catalyzed reduction by NADPH, reoxidation by O ₂
Reduction by flavoenzymes	LipDH and TrxR	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 ²⁵
IC ₅₀ against <i>P. falciparum</i> blood stages <i>in vitro</i>	86 ± 4.5 nM [3D7 strain] 59 ± 3.3 nM [K1 strain]	3.1 ± 0.4 nM [3D7 strain] 6.6 ± 1.1 nM [K1]
IC ₅₀ against early (and mature) <i>P. falciparum</i> gametocytes <i>in vitro</i>	180 nM (560 nM)	34 nM (60 nM)
Tested for biological conduct <i>in vivo</i>	In >100 Ma of evolution in bacteria-containing biotopes	In an unprecedented variety of uses in medicine and biotechnology

Data on PYO listed from line 'Redox-cycling catalyst' to the bottom line are from this paper. This applies also to the IC₅₀ values of MB; most other data on MB were taken from Ref 19. IC₅₀ is the concentration of an inhibitor where biological and/or biochemical activity is reduced to 50%.

Table 2 Enzyme-kinetic data for interactions of PYO and MB with disulfide reductases

Enzyme	K_M for PYO (μM)	k_{cat} for PYO (second^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{second}^{-1}$)	IC_{50} for PYO (μM)	K_M of MB (μM)	k_{cat} for MB (second^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{second}^{-1}$)	IC_{50} for MB (μM)
Human GR	43	0.17	3950	7	6.3	0.03	4760	16.4
Human GR C63A	600	3.9	6500	n.a.	56	0.62	11 100	n.a.
<i>Pf</i> GR	38	0.03	790	15	50	2.50	50 000	5.4
Porcine LipDH	25	1.2	48 000	>500	28	3.20	114 000	>1000
<i>Pf</i> mtLipDH	60	0.9	15 000	>500	31	4.00	129 000	>1000
Human TrxR	170	>0.9	>5300	n.d.	95	10.00	105 000	30
<i>Pf</i> TrxR	31	0.7	22 600	7	68	6.00	88 200	59

P. falciparum GR has a catalytic efficiency of only $790 \text{ M}^{-1} \text{second}^{-1}$ at pH 8.0 and even lower values at physiological pH values. The abbreviations are *Pf*, *P. falciparum*; GR, GR; LipDH, dihydrolipoamide dehydrogenase; TrxR, thioredoxin reductase; n.a., not applicable, because the C63A mutant of GR, lacking one catalytic-site cysteine, has no disulfide reductase activity. Most data for MB are from.¹⁹ Not listed is the enzyme-catalyzed auto-oxidation rate which refers to the reaction between NAD(P)H and atmospheric O_2 . It ranges from $0.003 \text{ second}^{-1}$ for human GR to $0.260 \text{ second}^{-1}$ for *P. falciparum* mitochondrial LipDH.

Figure 2 Spectra of PYO in oxidized and NADPH-reduced form in 50 mM potassium phosphate buffer of pH 7. The peak at 340 nm in the reduced spectrum (dotted line) is largely due to excess NADPH.

Enzymatic reduction of PYO by diaphorases

In Table 2, PYO and MB are compared as diaphorase substrates (Figs 1B and C) of disulfide reductases. Diaphorases or NAD(P)H:acceptor oxidoreductases [EC 1.6.5.2.] are enzymes that can catalyze the transfer of electrons from NAD(P)H to reducible aromatic compounds. A typical flavoprotein with strong diaphorase activity is dihydrolipoamide dehydrogenase (LipDH).²¹ Both PYO and MB are reduced enzymatically and subsequently reoxidized in spontaneous reactions with oxygen. Each redox cycle results in the loss of NADPH and the production of toxic H_2O_2 (Fig. 1B). This pro-oxidant process contrasts with the physiological functions of the disulfide reductases which normally produce thiol groups as antioxidants. The flavoenzyme GR for instance catalyzes the reaction $\text{NADPH} + \text{GSSG} + \text{H}^+ \rightarrow \text{NADP}^+ + 2 \text{ GSH}$. GSSG and GSH stand for glutathione disulfide and reduced glutathione, respectively. TrxRs ($\text{NADPH} + \text{TrxS}_2 + \text{H}^+ \rightarrow \text{NADP}^+ + \text{Trx}(\text{SH})_2$) produce reduced thioredoxin, and the back reaction of LipDH ($\text{NADH} + \text{lipoamide} + \text{H}^+ \rightarrow \text{NAD}^+ + \text{dihydrolipoamide}$) can contribute reducing equivalents in the form of dihydrolipoamide. These thiol-producing systems work in parallel but, via redoxins, they can closely interact and support each other in a number of pathophysiological situations.²⁸

Figure 3 Human GR homodimer with bound PYO. PYO (blue) and FAD (yellow) are represented as ball-and-stick models. Additionally, the surfaces of the catalytic cysteines Cys58/Cys63 and Cys58'/Cys63' (yellow) and of PYO (blue) are shown. The atomic coordinates and their structure factors determined by x-ray diffraction analysis have been deposited at the PDB (Protein Data Bank) under the accession code (PDB ID) 3SQP. Molecular graphics images were produced using the UCSF Chimera package.²⁶

Figure 4 Binding of PYO to human GR. For clarity only one of the two observed orientations of PYO (blue carbon atoms) is shown. Green C_α -atoms indicate amino acids at distances of less than 5 Å from the ligand. O atoms are shown in red, N atoms in blue, and sulfur atoms in yellow.

PYO as an inhibitor of disulfide reductases

Like MB, PYO can interact with enzymes not only as a substrate but also as a dissociable inhibitor (Table 2). The binding site for PYO as an inhibitor of human GR, shown in Figs 3 and 4, is described in detail

below. In contrast to MB, PYO is a better inhibitor of human erythrocytic GR (see x-ray structure) than e.g. of *P. falciparum* GR (see IC₅₀ values in Table 2). The IC₅₀ values referring to the inhibition of the GR-catalyzed reaction can be interpreted as K_i values if the inhibition type is non-competitive.^{27,28} This means that the inhibitor binds only to the free enzyme, not to enzyme–substrate complexes. While the enzyme kinetics clearly indicated non-competitive inhibition under standard conditions, a change from pH 7.3 to pH 8.0 led to an uncompetitive inhibition type as shown by Cornish-Bowden and Dixon-diagrams²⁹ (data not shown). In these studies, both the concentration of the substrate GSSG (in the range between 58 and 390 μ M) and the PYO concentration (between 0 and 10 μ M) were varied. The K_i determined for this uncompetitive inhibition was 7.5 μ M for human GR. This means that at pH 8.0 the inhibitor PYO does not bind to the free enzyme but rather to the enzyme–substrate complex ES. Like MB, PYO does not inhibit the LipDHs of *P. falciparum* and man (Table 2).

The GR inhibition observed *in vitro* can be of pathological significance since rather high (up to 100 μ M) extracellular PYO concentrations have been reported for infections caused by the PYO-producing bacterium *P. aeruginosa*.³ Furthermore, in certain cell compartments such as the cytosol of erythrocytes and in lysosomes of other cells PYO is known to accumulate.

Structural analysis of human GR complexed with PYO

Human GR is structurally known in atomic detail.³⁰ As with other disulfide reductases, it contains a so-called drug-binding site in a solvent-filled cavity between the two subunits of the homodimeric enzyme. It is here where heteroaromatic inhibitors such as safranin and xanthene and a number of isoalloxazines and napthoquinones are bound,²⁸ some of them being sandwiched in an aromate-binding subsite (see below). The function of this site *in vivo* is unknown. Using PYO as an inhibitor of crystalline human GR we studied the interactions between protein and ligand in detail.

The structure as determined by x-ray crystallography (Table 3) shows one PYO molecule bound in the cavity of human GR (Figs 3 and 4). The aromate-binding pocket is formed by seven amino acids (Asn71, Val74, His75, Phe78, Met79, His82, Tyr407) of one subunit and the corresponding residues of the other subunit. PYO is directly sandwiched by residues Val74, Phe78, and their symmetry mates Phe78' and Val74'.

Superpositions of 461 C α atoms per subunit of this model with a high resolution structure of human GR³⁰ and with the human GR–xanthene complex³¹

Table 3 Summary of data collection and structure refinement of the crystalline GR–PYO complex

Protein	Human GR
Space group	P1
Unit cell dimensions (Å)	$a = 62.08$, $b = 67.37$, $c = 78.86$ $\alpha = 68.98^\circ$, $\beta = 66.8^\circ$, $\gamma = 52.54^\circ$
Resolution range (Å)*	37.71–2.21 (2.27–2.21)
Completeness (%)	93.9 (82.4)
Mean redundancy	3.3 (3.0)
R_{sym}^{**} (%)	2.9 (6.2)
(I/σ)	29.0 (17.4)
Reflections used in refinement	47 819
Protein atoms	7116
Solvent molecules	858
rmsd bonds (Å)/rmsd angles	0.01/0.9
R_{cryst}^{***} (%) / R_{free}^{****} (%)	14.3/18.1

*Values within parentheses represent data in the last resolution shell.

** $R_{\text{sym}} = 100 \sum |I_h - \bar{I}| / \sum I_h$.

*** $R_{\text{cryst}} = 100 \sum \|F_{\text{obs}}| - |F_{\text{calc}}|\| / \sum |F_{\text{obs}}|$ calculated for all observed data. No sigma cut-off was used.

**** R_{free} calculated for 3% of randomly chosen unique reflections that were excluded from the refinement.

revealed rms deviations of 0.29 and 0.31 Å, respectively. Differences to the ligand-free structure occur mainly at the positions of the amino acids His75, Phe78, Met79, and His82 and their symmetric counterparts. In the ligand-free structure, these amino acid residues adopt multiple conformations while in the structures with bound PYO or with bound xanthene³¹ they are rigid and form a defined binding pocket. No remarkable differences were found between the two structures containing an inhibitor. Preliminary crystallographic studies on partially demethylated MB derivatives such as azure B indicate that phenothiazine drugs are also sandwiched between the aromatic rings of phenylalanine 78 and phenylalanine 78' (Fritz-Wolf, unpublished data). However, the width of the aromate-binding site (about 10 Å) limits the size of tricyclic compounds that can bind in the same orientation as PYO or the aromatic moiety of xanthene. Larger compounds would clash with Asn71 and Tyr407 and their symmetry mates (Fig. 4). These four amino acid residues do not appear to be flexible, because they are involved in multiple interactions with other amino acid residues even in ligand-free GR. PYO is only 7.3 Å long, whereas MB has a length of 12.4 Å and is thus too large to bind in the same way as PYO.

The kinetic finding that PYO can act as a non-competitive inhibitor of human GR suggested that the binding of PYO might lead to major structural changes at the catalytic sites. This, however, did not become evident through the structural analyses. On the other hand, PYO is bound to the basis of the rigid helices in both subunits which carry the catalytic residues Cys58 and Cys63. Very subtle changes at the

base of the helices could interfere with the catalytic reduction of GSSG. Another explanation for the lack of observable structural changes is that the ligand exerts its effects only when the substrate glutathione is bound to the crystalline enzyme. This interpretation is consistent with the finding that PYO, as other bi- and tricyclic compounds, can also exhibit uncompetitive inhibition³² (see above). For testing this, work is in progress to prepare suitable crystals of GR complexed with both the substrate GSSG and the inhibitor PYO.

Effects of PYO on the malaria-causing asexual forms of *P. falciparum*

The biochemical and crystallographic results strongly suggested systematically studying the antiparasitic activity of PYO in direct comparison with MB. The activity of PYO against chloroquine (CQ)-sensitive and -resistant laboratory strains of *P. falciparum* was tested as described for MB by Vennerstrom *et al.*³³ and Akoachere *et al.*¹⁷ The IC₅₀ values for PYO were determined to be 86 ± 4.5 nM on the CQ-sensitive strain 3D7 (IC₅₀ for CQ: 8.8 ± 0.9 nM) and 59 ± 3.3 nM on the CQ-resistant K1 strain (IC₅₀ for CQ: 158 ± 12 nM). Thus both parasite strains were about 10–30 times more susceptible to MB than to PYO (IC₅₀ values for of MB: 3.1 ± 0.4 nM for 3D7; 6.6 ± 1.1 nM for K1).

In addition to the IC₅₀ values, we assessed the specificity of PYO action on different developmental stages of intraerythrocytic parasites. For this purpose, seven different PYO concentrations ranging from $\sim 1 \times$ IC₅₀ to $\sim 100 \times$ IC₅₀ were added to ring, trophozoite, and schizont stages of 3D7 parasites. After exposure for 1, 6, 12, or 24 hours, PYO was removed and the parasites were incubated for another 24 hours.³⁴ As shown in Fig. 5, PYO showed marked parasitocidal effects already after 1–6 hours of treatment, with schizont stages of the parasites exhibiting the highest susceptibility. For detailed comparison with data on MB and CQ, please consult the papers of Akoachere *et al.*¹⁷ and Sturm *et al.*³⁴

Drug combination assays demonstrated that *in vitro* PYO acts additively with MB (FIC₅₀ value = 1.0) and synergistically with artemisinin (FIC₅₀ value = 0.73). A synergy with artemisinin and related compounds has also previously been described for MB.¹⁷ This supports the notion that the mechanism of antiparasitic action is similar for MB and PYO.

In vivo efficacy and toxicity

In addition to the *in vitro* studies, PYO and MB were tested in a murine *Plasmodium berghei* model as described by Peters.³⁵ Preceding *in vitro* experiments had indicated that *P. berghei* proliferation is susceptible to PYO with an IC₅₀ of about 115 nM (16 hours incubation with the compound followed by 8 hours incubation with ³H-hypoxanthine).

In the first experimental setup for the *in vivo* testing, the compounds were applied subcutaneously for 3 days after infection. Doses of 3×100 mg/kg ($n = 4$) and 3×30 mg/kg ($n = 4$) MB s.c. were found to be toxic, already leading to death on day 1 (after the first dose). A dosage of 3×100 mg/kg PYO s.c. ($n = 3$) led to rapid death of two animals and to markedly enhanced survival (15 days in comparison with 4–6–7 days for the parasitized non-treated controls) of one animal.

Oral application of 3×100 mg/kg MB ($n = 4$) was found to be very efficient with 0% parasitemia on day 4 (corresponding to 100% activity) and an average mouse survival of 13 days (11, 13, 14, 14 days) in comparison with 4–6–7 days for the parasitized non-treated controls, which were euthanized on day 4. Oral application of 3×30 mg/kg MB ($n = 4$) showed $76.7 \pm 5.4\%$ activity on day 4 (parasitemia reduced to 23% of controls) and survival of 14 days for all mice. In direct comparison 3×100 mg/kg PYO given orally ($n = 2$) showed less than 40% activity with respect to parasitemia and no prolonged survival of the animals who additionally suffered from PYO toxicity. In this standard procedure for *in vivo* testing of antimalarials, treated control animals which are severely ill and about to die 4 days after infection are

Figure 5 Stage-specific effects of various concentrations of PYO (x-axis, given in nM) on different developmental stages of the asexual forms of *P. falciparum* in cultured erythrocytes. The data indicate a rapid antiparasitic effect, with the schizont stage exhibiting the highest susceptibility.

euthanized on day 4. The efficiency of a drug is expressed by two parameters, the parasitemia and the survival time, which allows one to compare different drugs, concentrations, and combinations with standard clinically employed drugs such as CQ.

In conclusion, MB proved to be a highly efficient and non-toxic antimalarial agent in the *P. berghei*/mouse model when applied orally in lower doses. In contrast, PYO showed intolerable *in vivo* toxicity in relation to its antiplasmodial effects in mice.

In vitro activity of MB and PYO against *P. falciparum* gametocytes

Elimination of blood gametocytes, the sexual form of the parasite, prevents the transmission of disease parasites from man to mosquito and thus interrupts the malaria cycle.^{36,37} Gametocytes are resistant to most antimalarial drugs except artemisinins and 8-aminoquinolines such as primaquine^{37,38} and – as indicated by a recent clinical study – to MB.³⁷ In order to complement the clinical study, we systematically investigated the efficacy of MB and PYO on gametocytes *in vitro*. CQ – still the most used schizonticidal antimalarial drug – was included in these experiments as a reference drug.

MB, PYO and CQ were found to be active against gametocytes of the *P. falciparum* strain 3D7 on day 9 (stages II and III) and day 13 (stages IV and V). The IC₅₀-values are given in Table 4. The day 9 gametocytes were 2, 3, and 50 times more sensitive to MB, PYO, and CQ, respectively, than the day 13 gametocytes.

Discussion

PYO as an important redox-active signaling and antimicrobial molecule is synthesized from chorismate through a series of complex steps.^{4,39,40} This synthesis is regulated by quorum sensing (for a review see Winstanley *et al.*⁴¹), a process involving a cell-density-dependent accumulation of low molecular weight signal molecules that enable bacteria to modulate the expression of virulence genes and to form biofilms.^{1–3,42} With its antibiotic function, PYO provides *Pseudomonas* with comparative growth advantages, which is supported by the fact that, at high cell

densities, phenazine biosynthesis was found to be upregulated (up to 300 μM of PYO⁴³). Using a wild-type and a mutant strain defective in phenazine production, Price-Whelan *et al.*⁴³ studied the physiological role of PYO in *Pseudomonas* with respect to redox and energy metabolism in more detail. The authors found that the mutant accumulated more NADH in the stationary phase than the wild type which correlated with a decrease in oxygen availability. PYO addition to the phenazine-null mutant decreased intracellular NADH levels, suggesting that PYO reduction facilitates redox balancing. So beside oxygen and nitrate, PYO could be used as an alternative (terminal) electron acceptor. In addition, an inhibition of the pyruvate dehydrogenase complex by PYO through the generation of superoxide has been postulated.⁴³ As a consequence, less pyruvate is converted to acetylCoA for feeding the citric acid cycle and as a result less NADH is produced keeping the NADH/NAD⁺ ratio in acceptable regions. Additionally to these observations and as delineated in our introduction, a large number of further targets of PYO have been described in *Pseudomonas* and other organisms – many of them in the context of their redox sensitivity.^{8,15} In this work we studied the reactivity of PYO and its synthetic analog MB toward important cellular reductants such as NADH, NADPH, or dihydrolipoamide, the reactivity of their reduced leuco-forms toward triplet O₂, and their interactions with FAD-containing disulfide reductases in detail.

As shown in Table 1, PYO reacts rapidly with NADPH and NADH but is >10 times less reactive than MB toward intracellular dithiol compounds such as thioredoxin or dihydrolipoamide. GSH is a very poor reducing agent for both PYO and MB but the intracellular concentrations of GSH (>1 mM) are one to two orders of magnitude higher than those of other physiological reductants.

However, the reduction of PYO as part of redox cycles can also be brought about by so-called diaphorases (Fig. 1B). These are haem- or flavin-containing enzymes that have narrowly defined physiological functions – e.g. the reduction of disulfides – but as a moonlighter activity they are capable of reducing

Table 4 *In vitro* activities of PYO, MB, and CQ against immature (day 9) and mature (day 13) *P. falciparum* gametocytes

Compound	IC ₅₀ (95% CI) [nM]	
	Day 9 gametocytes (stages II and III)	Day 13 gametocytes (stages IV and V)
PYO	178 (147–215)*	557 (262–1190)
MB	33.8 (32.1–35.7)	59.5 (37.3–94.8)
CQ	8.60 (3.02–24.7)	487 (278–854)

*95% Confidence interval. The IC₅₀s between early (day 9, stages II and III) and late (day 13, stages IV and V) gametocyte stages were significantly different for all compounds, the *P*-values being 0.0021 for PYO, 0.0013 for MB, and 0.0146 for CQ.

numerous heteroaromatic compounds at the expense of NADPH or NADH. Taking into account the data from Table 2 as well as intracellular enzyme concentrations, TrxR and LipDH are likely to be the pharmacologically most relevant PYO-reducing enzymes, since the intracellular concentrations of these enzymes (0.1–1.0 μM) are as high as or even higher than those used for the diaphorase assays.²⁸ Thus, in an example calculated for malaria parasite infected red blood cells, an intracellular concentration of 20 nmol/ml GR is estimated to induce the diaphorase-mediated oxidation of more than 300 μmol NAD(P)H per ml of parasitized cells per day. The corresponding rate for the spontaneous intracellular oxidation of NADPH by PYO is assumed to be about 1% of this value.

The mechanism of the diaphorase activity of flavoenzymes is important for interpreting the action of PYO and MB. Taking human GR as an example, the reducing equivalents are likely to follow a pathway from NADPH via flavin/reduced flavin and PYO/leucoPYO to O_2 .¹⁹ The strongest evidence against an involvement of the active site redox pair Cys58/Cys63 in the diaphorase activity of the disulfide reductases is the finding that mutant proteins which lack one or both active site cysteines have a higher turnover number k_{cat} and a higher catalytic efficiency $k_{\text{cat}}/K_{\text{M}}$ with PYO as a substrate than the wild-type enzyme; a case in point is the human GR mutant C63A (Table 2). In contrast to the diaphorase activity of GR, reduction of the cell-physiological substrate GSSG totally depends on both cysteine residues.

Protein-bound flavin-adenine dinucleotide is known to reduce heteroaromatics even when the reaction partners are not precisely oriented to each other.^{23,44} Regarding the diaphorase activity of GR, a possible binding site of PYO close to reduced flavin is the pocket which accommodates the nicotinamide moiety of NADPH.^{28,30} In contrast, the binding of PYO in the intersubunit cavity of GR (Fig. 3) is unlikely to allow electron transfer from FAD to PYO. The mechanism of the enzymic diaphorase activity will be studied further by structural analyses of crystalline LipDH–PYO complexes. LipDH is preferable to GR as it has a closely related structure but is not inhibited by heteroaromatics. The notorious instability of recombinant *P. falciparum* LipDH⁴⁵ was overcome by modifications of protein isolation and storage (see Experimental procedures).

The studied disulfide reductases – with the exception of LipDH – are inhibited by heteroaromatics like PYO and MB. PYO was found to be a stronger non-competitive inhibitor of human GR than of *P. falciparum* GR; in contrast MB is a better inhibitor for

the parasite enzyme. So far we have not been successful in obtaining crystals of the GR–MB complex that diffract to high resolution. However, in the corresponding GR–PYO complex we were able to identify the binding site of the inhibitor (Fig. 4). This has given us new clues for the binding mode of MB and its demethylated derivatives to disulfide reductases. One hypothesis deduced from our results is that not MB itself but demethylation products of MB might be bound – sandwiched between two symmetrical phenylalanine residues – at the so-called aromate-binding site of GR *in vivo*.

PYO has been shown to be active against multi-drug-resistant *Staphylococcus aureus* and against *Candida albicans* in culture.² As reported here PYO at nanomolar concentrations is also active against the protozoal parasite *P. falciparum* *in vitro*. PYO showed marked parasitocidal effects even after 1–6 hours, with schizont stages of the parasites exhibiting the highest susceptibility. *P. falciparum* causes more than 300 million cases of clinical malaria per year in tropical regions; groups at risk to develop severe malaria are children under five, pregnant women, and tourists.³⁶ The actual disease is due to growth and multiplication of the parasite in human erythrocytes. The sexual blood forms, so-called gametocytes, do not contribute to clinical disease but are capable of developing in the insect vector thus maintaining the malaria cycle between man and mosquito.^{37,38} Both PYO and MB show activity against *P. falciparum* gametocytes in the nanomolar range (Table 4). Our systematic *in vitro* studies furthermore confirm that, in contrast to CQ, MB is indeed highly active against all stages of gametocytes. For the action of CQ on gametocytes, our results were found to be consistent with previous reports.⁴⁶ The enhanced expression of proteins involved in protection against oxidative stress in mature gametocytes – including glyoxalases, glutaredoxin, and 2-Cys peroxiredoxin^{47,48} – may contribute to their reduced susceptibility to the tested compounds when compared with asexual red blood cell stages. The fact that MB nevertheless retains great potency against mature gametocytes supports the antimalarial potential of MB – both as a gametocytocidal drug and a drug suitable for schizonticidal drug combinations.³⁷

Because of its toxic effects PYO is not suited as a candidate for antimalarial therapy. Nevertheless it may play a role *in vivo*. In many wars, *Pseudomonas* has been responsible for wound infections that give rise to greenish secretions with a typical sweetish odor. Infections in patients with mild and severe forms of cystic fibrosis are also often caused by *Pseudomonas* species. It remains to be studied to what extent these patients are protected from *P. falciparum* and *P. vivax* malaria.⁴⁹ Further studies on

PYO will contribute to our understanding of the biological and cell-biochemical properties of this versatile compound and of its pharmacological analog MB.^{25,50}

Experimental procedures

Enzymes

With the exception of recombinant mitochondrial LipDH of *P. falciparum*, all enzymes were produced and assayed as described by Buchholz *et al.*¹⁹ The *P. falciparum* LipDH was cloned, heterologously over-expressed, and purified according to McMillan *et al.*⁴⁵ but with the following modifications. To obtain higher yields, we cloned the PCR fragment into pET28 (Novagen, Darmstadt). After induction with IPTG, the cultures were grown over night at 18°C. A one-step purification procedure using Ni-NTA His-Bind resin (Novagen) resulted in more than 10 mg/l cell culture which is 100-fold higher than the previously reported yield.⁴⁵ For storing and stabilizing the protein according to Blumenstiel *et al.*,¹⁸ *P. falciparum* LipDH was precipitated with ammonium sulfate and dissolved in a minimum of 100 mM potassium phosphate of pH 7.5. Subsequently, glycerol was added to a final concentration of 30%, and this suspension was stored at -20°C. The enzyme suspension kept its reducing activity versus lipoamide, PYO, and MB for more than 2 months.

Enzyme inhibitors

The concentration of PYO (from Cayman) was determined using an $\epsilon^{520\text{ nm}}$ of $2.46\text{ mM}^{-1}\text{ cm}^{-1}$ in 0.1 M HCl⁵¹ or $\epsilon^{690\text{ nm}} = 4.3\text{ mM}^{-1}\text{ cm}^{-1}$ in 50 mM MOPS buffer of pH 7.2. 1 mM stock solutions of PYO (in DMSO or in a 50 mM phosphate buffer of pH 8.0) and of MB (in H₂O; $\epsilon^{613\text{ nm}} = 40.0\text{ mM}^{-1}\text{ cm}^{-1}$) were freshly prepared at least once a week and kept in dark bottles at room temperature. For biochemical and pharmacological studies the standard buffer of physiological ionic strength (0.3 M) was 200 mM KCl, 46.5 mM potassium phosphate, 1 mM EDTA, pH 6.9 at 25°C.^{28,52} The reactions of the various enzymes were performed as described previously¹⁹ with or without added PYO. In experiments where PYO was tested as a substrate, the spontaneous reaction between NADPH and PYO had to be slowed down. This was achieved by increasing the pH to 8.0. As an alternative one might stabilize the NADPH concentration at less than 10 μM using an NADPH-generating biochemical system.¹⁹

Crystallization of human GR

Recombinant human GR was purified as described.⁵² Crystals were grown at 22°C using the hanging-drop vapor diffusion technique: 10 mg/ml human GR in 120 mM ammonium sulfate, 100 mM potassium phosphate, pH 7.5 was equilibrated by dialysis with

660 mM ammonium sulfate in 100 mM potassium phosphate, pH 7.5. The crystals (>250 μm in each dimension) grew within 1 week. They were washed with 50 mM potassium phosphate of pH 7.3 and soaked in 1 mM PYO in GR assay buffer of pH 7.5 for more than 7 days. Prior to the measurements, the crystals were soaked in mother liquor containing 35% glycerol. Human GR crystals prepared in this way are very stable even in the absence of mother liquor and can be equilibrated with a broad variety of aqueous solutions. This renders human GR crystals uniquely suitable for soaking experiments with pharmacological agents or for using them as indicators of redox potential in living cells.⁵³

X-ray crystallographic studies

X-ray diffraction data were collected at the Max Planck Institute for Medical Research with the rotation method and processed by the program XDS.⁵⁴ Crystal data were recorded at 100 K by an image plate detector (Mar345 image plate, xrayresearch, Norderstadt, Germany) using CuK α radiation from a rotating anode (Rigaku, MicroMax-007HF, operating at 40 kV and 30 mA). Reflection phasing and refinements were conducted with the PHENIX program suite,⁵⁵ and model correction was carried out using the graphics program O.⁵⁶ The structure was solved by molecular replacement, using a high-resolution structure of human GR³⁰ as a search model.

The crystals obey C2 space group symmetry and contain one monomer in the asymmetric unit, with the enzymatically active homodimer generated by a crystallographic two-fold axis. The initial calculated difference map ($|F_o| - |F_c|$) revealed the presence of electron density resembling PYO. The molecule was found to be bound in the cavity located at the interface of the two GR subunits. The two-fold crystallographic symmetry axis overlaps with PYO, which is not a completely symmetric molecule (Figs 1A and 4); consequently we did all calculations in space group P1, using non-crystallographic symmetry constraints for both subunits.

The inhibitor was placed manually in the additional density. PYO is bound in two orientations, related by a two-fold non-crystallographic axis. Refinement of the full model was completed after a few rounds of model correction (resolution: 2.2 Å, $R_{\text{free}} = 18\%$ with a coordinate error of 0.22 Å and an overall temperature factor of 41.7 Å²). The model displays good stereochemistry (Table 3) as verified by Procheck.⁵⁷

Cultivation of *P. falciparum*

The CQ-sensitive *P. falciparum* strain 3D7-Netherlands and the CQ-resistant strain K1 were grown in continuous culture as described by Trager and Jensen⁵⁸ with slight modifications. Unless

otherwise stated, parasites were maintained at 1–10% parasitemia and 3.3% hematocrit in an RPMI 1640 culture medium supplemented with A+ erythrocytes, 0.5% lipid-rich bovine serum albumin (Albumax), 9 mM (0.16%) glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, and 22 µg/ml gentamicin. All incubations were carried out at 37°C in 3% O₂, 3% CO₂, and 94% N₂. Synchronization of parasites in culture to ring stages (<70% for drug testing and >90% for stage specificity studies) as starting population was carried out by repetitive treatment with 5% (w/v) sorbitol.⁵⁹ The parasites were used for the experiments delineated below.

Determination of IC₅₀ values of drugs and fractional inhibitory concentration (FIC) values of drug combinations

Isotopic drug sensitivity assays by means of the semi-automated microdilution technique⁶⁰ were employed to investigate the susceptibility of *P. falciparum* to various compounds and their effects in combination with other clinically used antimalarials. The procedure depends on the incorporation of radioactive ³H-hypoxanthine which is taken up by the parasite as a precursor of purine deoxynucleotides for DNA synthesis and was performed according to the modifications of Fivelman *et al.*⁶¹ In 96-well microtitre plates (Nunc^R), a two-fold serial dilution of the starting concentration of each pharmacologically active compound to be tested was carried out. Parasites were incubated at a parasitemia of 0.25% (>70% ring forms for the drug testing) and a hematocrit of 1.25% in hypoxanthine-free medium. After 48 hours, 0.5 µCi ³H-hypoxanthine was added to each well, and the plates were incubated for another 24 hours. The cells of each well were then harvested on a glass fiber filter (Perkin-Elmer, Rodgau-Jügesheim, Germany), washed and dried. Their radioactivity in counts per minute was assumed to be proportional to the number of parasites in the well. All IC₅₀ values were determined in quadruple.

In order to assess the effects of drug combinations, the two compounds were applied alone and in fixed concentration ratios of 1:1, 1:3, and 3:1 as described by Fivelman *et al.*⁶¹ IC₅₀ and IC₉₀ values were calculated and the FICs of the respective drugs were determined.

Stage-dependent effects of PYO were tested *in vitro* on synchronous (>90% obtained by repetitive sorbitol synchronization steps) cultures of the *P. falciparum* strain 3D7 according to Maerki *et al.*⁶² For this purpose, seven different PYO concentrations ranging from ~1 × IC₅₀ to ~100 × IC₅₀ were added to ring, trophozoite, and schizont stage parasites at a parasitemia of 0.15% and a hematocrit of 5%. After exposure for 1, 6, 12, or 24 hours, the plates were washed four times

with compound-free medium in order to dilute the free compound to less than 1% of its starting concentration before adding ³H-hypoxanthine and continuing as described above. Compound effects are expressed as a percentage of the untreated controls. Suspensions of uninfected erythrocytes were used for background subtraction. Samples were measured in 96 well plates with 250 µl total volume per well. Typically, the positive controls (untreated) had about 3000 counts per minute (cpm), the negative controls (uninfected erythrocytes) had below 30 cpm. The parasite multiplication rate per red blood cell cycle was in the controls 5.4 ± 0.5. For most conditions tested, an initial enhancement of ³H-hypoxanthine incorporation was observed before the growth inhibition became apparent. This phenomenon is observed for many drugs and likely to be based on enhanced metabolic activity under pharmacological stress.

In vitro assay for *P. falciparum* gametocyte sensitivity toward PYO, MB, and CQ

P. falciparum (strain 3D7) gametocytes were cultured according to Ifediba and Vanderberg⁶³ with modifications. Briefly, following synchronization with 5% sorbitol,⁶⁰ a ring stage culture was set up (day 1) with 1.5–2% parasitemia at 6% hematocrit with fresh A+ erythrocytes; no further erythrocytes were used during the experiment. Gametocytes were cultured in RPMI 1640 medium (Gibco) supplemented with A+ human serum (10%), 1.96 g/l glucose, 50 mg/l hypoxanthine, and 22 µg/ml gentamycin. Cultures were kept under 3% O₂, 3% CO₂, and 94% N₂ at 37°C. The medium was changed daily and Giemsa-stained slides were prepared for monitoring gametocyte development. On day 3 the cultures were diluted from 6 to 3% hematocrit. To inhibit further asexual parasite growth,⁶⁴ cultures were supplemented with 50 mM N-acetylglucosamine (Sigma) from day 5 onwards and then treated with 5% sorbitol on day 8 as described.⁶⁵ In order to remove hemozoin, the cultures were washed and centrifuged (at 750 g for 10 minutes) four times. For eliminating ring stages and to concentrate gametocytes, the cultures were passed through a magnetically mounted MACS[®] LS separation column fitted with a 21 G flow resistor (Miltenyi Biotech, Germany) as described.⁶⁶ Cultures were continued at a 2% gametocytemia and 3% hematocrit until day 9 (stages II and III) or day 13 (stages IV and V) when drug treatment started.

Activity of PYO, MB, and CQ against gametocytes was assessed as described by Chutmongkonkul *et al.*,⁶⁷ with modifications. Briefly, stock solutions of CQ diphosphate (Sigma) and MB (Sigma) were freshly dissolved in bidistilled water, PYO was dissolved in DMSO. Twenty microliters of 50% hematocrit (final hematocrit ~2.0%) with 2% gametocytemia and

500 µl of drug-containing medium or 500 µl solvent-containing medium (for controls) were added to each well in 24-well plates. (Solvent – DMSO or water – refers to the volume of the stock solution used in the respective experiment). After 24 hours, solvent-containing media (controls) and drug-containing media were replaced. After 48 hours all wells received fresh medium without drugs for a further 2 days. On day 14 (for immature gametocytes) and day 18 (for mature gametocytes) thin blood films were prepared, then Giemsa-stained, and gametocytes were counted per 10 000 erythrocytes. The 50% inhibitory concentrations (IC₅₀) were determined by curve fitting the percentage gametocyte inhibition (in relation to control) against log drug concentration with a variable-slope sigmoidal function and compared using the F-test (Prism 4.0 GraphPad Software, San Diego, CA).

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