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FLAVIN ANALOGS WITH ANTIMALARIAL ACTIVITY AS GLUTATHIONE REDUCTASE INHIBITORS

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Abstract—10-(4′-Chlorophenyl)-3-methylflavin has antimalarial activity *in vitro* and *in vivo* (Cowden *et al.*, *J Med Chem* **31**: 799, 1988). This flavin analog and two of its derivatives were found to inhibit the antioxidant flavoenzyme glutathione reductase from human erythrocytes in its isolated form as well as in hemolysates. The mixed-type inhibition was completely reversible, the K_i-values being of the order of 1 μM. Surprisingly, the drugs were not competitive with FAD, but with GSSG, one of the enzyme's substrates. Malaria parasite glutathione reductase, extracted from *Plasmodium falciparum*, could also be inhibited by the compounds. Studies on the effects of the substances on *P. falciparum in vitro*, which were demonstrated morphologically and by growth inhibition, confirmed previous observations with 10-(4′-chlorophenyl)-3-methylflavin and showed similar parasiticidal characteristics for the two new derivatives. The activities of five other erythrocytic enzymes tested were not impaired by the drugs, nor was the nucleotide metabolism of erythrocytes and/or parasites significantly changed. Permeation into red blood cells was demonstrated for one compound by ^{19}F-NMR-spectroscopy. Inhibition of glutathione reductase might contribute to, or account for, the antimalarial activity of this group of flavin analogs.

The production of reactive oxygen species (ROS)¶ by activated phagocytes is known to contribute to the host response to intraerythrocytic malaria parasites [1–5]. Agents which generate ROS kill malaria parasites *in vitro* and *in vivo* [1–4]. Thus, oxidative stress has become a concept of potential malaria therapy. The oxidative stress on a cell can be raised by either directly increasing the input of ROS or by suppressing its antioxidant capacity [5], and both of these manoeuvres damage malaria parasites.

Recently, the riboflavin analog 10-(4′-chlorophenyl)-3-methylflavin (**1**) was shown to have antimalarial activity *in vitro* and *in vivo* [6, 7]. Riboflavin deficiency is known to reduce the activity of the antioxidant flavoenzyme glutathione reductase in erythrocytes [8, 9] and to have antimalarial effects [10, 11]. Preliminary experiments showed that compound **1** inhibited the activity of glutathione reductase in lysates of mouse erythrocytes [6]. Therefore, compound **1** and two of its derivatives (**2** and **3** in Fig. 1) now have been tested as inhibitors of this enzyme from human erythrocytes and malaria parasites.

In the present work we also report on further characteristics of these promising antimalarial compounds, such as the lack of interactions with other erythrocytic enzymes, their effects on the nucleotide metabolism of red blood cells and parasites, their *in vitro* actions on parasite growth and morphology, and their membrane permeation.

‡ To whom correspondence should be addressed.
¶ Abbreviation: ROS, reactive oxygen species.

MATERIALS AND METHODS

Reagents and medium

All reagents (from Merck, Darmstadt, F.R.G. and Serva, Heidelberg, F.R.G.) were of the highest purity available. RPMI-1640 medium was obtained from Biochrom, Heidelberg, F.R.G.

Drugs

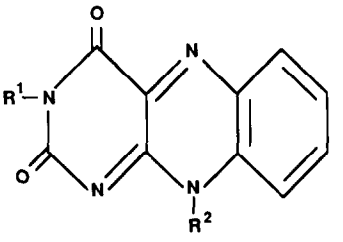
The three flavin analogs, 10-(4′-chlorophenyl)-3-methylflavin (**1**), 10-(3′-trifluoromethylphenyl)-3-methylflavin (**2**) and 10-(4′-chlorophenyl)-flavin (**3**), were prepared by the action of nitrosobenzene on 6-anilinouracils in the presence of acetic anhydride [7] according to the method of Yoneda *et al.* [12]. The drugs were dissolved in dimethyl sulphoxide.

Enzymes

Glutathione reductase ($NADPH + GSSG + H^+ \rightarrow NADP^+ + 2\,GSH$; EC 1.6.4.2) from human red blood cells was prepared and assayed according to Krohne-Ehrich *et al.* [13]. The FAD-free apoenzyme was prepared by the method of Fritsch *et al.* [14].

The activities of other erythrocytic enzymes were measured according to Berghäuser [15] for adenylate kinase ($2\,ADP \rightarrow ATP + AMP$), lactate dehydrogenase and pyruvate kinase, and according to Kuby *et al.* [16] for glucose-6-phosphate dehydrogenase, hexokinase and adenylate kinase ($ATP + AMP \rightarrow 2\,ADP$).

For inhibition tests on parasite glutathione reductase a crude extract from *Plasmodium falciparum* was prepared. The erythrocytes from 100 ml of a parasitized red blood cell culture (hematocrit 5%, parasitemia 20%, in RPMI-1640 medium



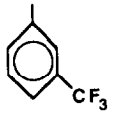
	R^1	R^2	M_r
1	$-CH_3$		338
2	$-CH_3$		372
3	$-H$		324

Fig. 1. Structures of the flavin analogs **1**, **2** and **3**.

according to Moore *et al.* [17]) were lysed in a 10-fold volume of saponin solution (15 mg/100 ml in Ringer solution [18]). After centrifugation (7 min, 300 *g*, 4°) the parasites were washed twice and then kept in 200 μl Ringer solution. Their membranes were lysed by freezing and thawing (three times). The supernate of the following ultracentrifugation (40 min, 110,000 *g*, 4°) was used for the enzymatic assays. To prove the quality of this extract, the activity of glutamate dehydrogenase [19], the marker enzyme for the parasites [20, 21], and the activity of glucose-6-phosphate dehydrogenase [22, 23], were determined. According to these measurements the contamination of the extract with erythrocytic components was less than 10%. The parasite glutathione reductase was assayed under the same conditions as the erythrocytic enzyme [13].

Parasites

The parasites (FCB-strain of *Plasmodium falciparum*) were cultured [24, 25] using a 5% suspension of ARh^+ erythrocytes in RPMI-1640 medium [17]. The medium was supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, 50 μg/ml hypoxanthine, 100 μg/ml gentamycin and 10% ARh^+ human serum. The cultures were kept at 37° in an incubation chamber which contained a gas mixture of 75% N_2, 6% CO_2 and 19% O_2.

Four hundred μl volumes of the cultures (hematocrit 5%, initial parasitemia 1–2% at early ring stage of sorbitol-synchronized [26] parasites) were exposed to the three drugs at concentrations between 1 μM and 20 μM. Control samples contained either no additions or DMSO (0.01%–0.2% v/v). The medium was changed twice a day, maintaining constant inhibitor concentrations.

Giemsa stained smears of cells were prepared every 12 hr. Percentage parasitemia was determined microscopically by examining 2000 erythrocytes. Morphological alterations of the parasites were noted. All experiments were performed three times.

Purine/pyrimidine metabolism

Extraction of parasitized erythrocytes. All manipulations were done at 4°. The cells were separated from incubation medium by centrifugation (10 min, 500 *g*), washed by resuspension in isotonic PBS and again pelletted. The weight of the pellet was determined. Metabolites were extracted from cells by addition of 0.6 M ice-cold $HClO_4$ to a final concentration of 0.4 M. After standing in ice for 30 min, cellular debris were removed by centrifugation and an aliquot of the supernate was titrated to pH 6.5 by addition of 1.1 molar equivalents of KOH (0.6 M) and $KHCO_3$ (0.2 M). The precipitated $KClO_4$ was removed after 20 min by centrifugation and the neutralized cell extract was stored at −20° for subsequent analysis.

Separation of nucleotides by HPLC. A 100 μl sample of the cell extract was loaded onto a Partisil 10 SAX column (0.42 × 22 cm, Whatman) equilibrated with Solvent A (7.0 mM KH_2PO_4, pH 3.0). The sample was eluted at a flow rate of 2.0 ml/min with a concave gradient (Waters curve 7) from Solvent A to Solvent B (250 mM KH_2PO_4, 500 mM KCl, pH 3.8) for 45 min followed by isocratic elution with

Table 1. Inhibition of glutathione reductase from human erythrocytes and from *P. falciparum* by flavin analogs

Enzyme preparation	IC_{90} [μM] of the flavin analogs 1	2	3
Purified enzyme (1 U/ml)	9.0	31	10
Hemolysate (1 U/ml)	8.1	30	10
Plasmodium falciparum extract (0.2 U/ml)	21.0	23	17

One ml standard assay mixture containing 1 mM GSSG and 0.1 mM NADPH was used [13], the sample size ranging from 2 μl (hemolysate) to 10 μl (*Plasmodium* extract). Shown are IC_{90}-values, i.e. the inhibitor concentrations required to reach 90% enzyme inhibition. Owing to the high GSSG-concentration, the type of inhibition is predominantly non-competitive. Accordingly, the inhibition constants K_i were assessed using the equation $v_i/v = K_i/([I] + K_i)$, [29]. In the case of human glutathione reductase the K_i-values (1.0 μM for compound **1**, 3.4 μM for compound **2** and 1.1 μM for compound **3**) agree with the data of Fig. 2.

Solvent B for a further 45 min [27]. Separated metabolites were monitored by acquisition of whole UV-spectra in the range 190 nm to 370 nm. For further details concerning data processing and assignment of peaks see Ref. 27.

NMR measurements

To examine whether the flavin analogs permeated into non-parasitized erythrocytes, 5 ml samples of red blood cells (1:1 diluted in PBS containing 4 mg/ml glucose) were incubated for 12 hr at 25° with 50 μM compound **2**, a drug which contains a trifluoromethyl-group. After centrifugation (7 min, 300 *g*, 4°) the cells were washed twice in PBS with glucose. To identify quantitatively intracellular **2**, ^{19}F-NMR-spectroscopy (470 MHz, 300°K) was carried out on pelletted erythrocytes (*ca.* 2.5 ml). The control sample contained 50 μM **2** in PBS.

RESULTS

Enzymatic tests

Isolated glutathione reductase was inhibited by all three compounds in a dose-dependent manner. Adding 10 μM flavin analog to the enzyme assay decreased glutathione reductase activity by 91% (**1**), 74% (**2**) or 90% (**3**). Similar results were obtained for inhibition of enzyme activity in hemolysates (Table 1). The observed inhibition did not show any time dependence and was completely reversible by dilution. Preincubation with 100 μM NADPH did not change the results of the inhibition, which indicates that the reaction is not dependent on the redox state of the enzyme. Adding FAD (0.1 μM–10 μM) to the assays did not lead to significant changes in the inhibitory effects of the compounds, nor could the reconstitution of the apoenzyme with FAD [14] be disturbed by the flavin analogs. These observations suggest that the inhibition is not competitive with the flavin moiety of FAD.

The K_m of NADPH towards glutathione reductase (8.5 μM [28]) did not change in the presence of the flavin analogs (shown for 1 μM **1** and 1 μM **3** in Fig. 2), whereas they were found to compete with the other substrate, GSSG. Figure 3 shows the Dixon-plot [29] for compound **1** at GSSG concentrations of $\geq$100 μM. According to this diagram, the substance acts as an inhibitor competitive with GSSG, the K_i being 0.4 μM. A similar result was observed for drug **3**. As discussed below, this competitive inhibition cannot account for the effects described in Table 1.

None of the other tested erythrocytic enzymes (adenylate kinase, pyruvate kinase, lactate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase) was found to be signficantly inhibited (>20%) by 10 μM of any of the three flavin analogs.

Compounds **1**, **2** or **3** in the range 5 to 30 μM inhibited the activity of *Plasmodium falciparum* glutathione reductase [30] in a dose-dependent manner. To achieve >90% enzyme inhibition drug concentrations of *ca.* 20 μM were required (Table 1). This is approximately twice as much as is needed for comparable inhibition of the human enzyme.

Growth inhibition of Plasmodium falciparum *in vitro*

Incubation of *P. falciparum* with the flavin analogs (1–20 μM) reduced the viability of the cultures drastically. After 48 hr, which corresponds to the duration of an asexual life cycle of the protozoa under the chosen conditions, the parasites of the control samples were in the early ring stage again, whereas the parasites of the drug-containing cultures had been arrested, dose-dependently, at preceding stages of their development: as trophozoites, at the late ring stage or, at the highest drug concentrations, at the early ring stage [31]. Also the degree of parasite deformation increased in an inhibitor-dependent way. At no drug concentration did we observe inhibition of merozoite invasion into erythrocytes.

The morphologically intact parasites were counted to determine the parasitemia of each sample. Figure 4 shows the dose-dependent decrease of parasitemia compared to controls (=100%) after 48 hr and 65 hr incubation with the flavin analogs. DMSO was not found to have a significant influence on parasite growth (<15% inhibition) in the relevant range from 0.01% to 0.2% (v/v).

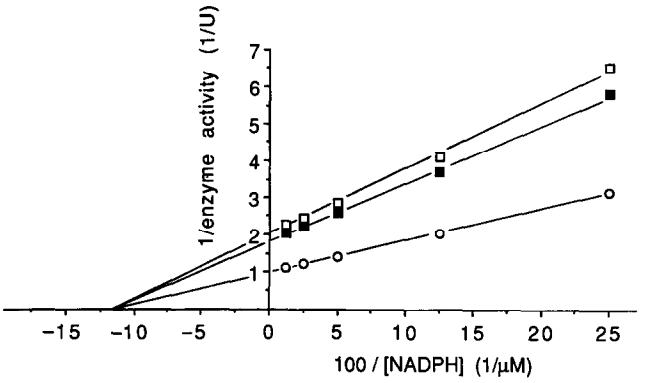


Fig. 2. Effects of 1 μM compound **1** (□) or **3** (■) on glutathione reductase activity at different NADPH concentrations (4–100 μM); [GSSG] = 1 mM. The control contained 1% DMSO (○). All lines in the Lineweaver–Burk plot cut the abscissa at -0.117 ($1/\mu M$) = $-1/K_m$. Consequently the K_m-value is 8.5 μM under all three experimental conditions, which indicates that neither inhibitor competes with NADPH. The K_i-values as determined from the slopes are 1.0 for inhibitor **1** and 1.1 for inhibitor **3**.

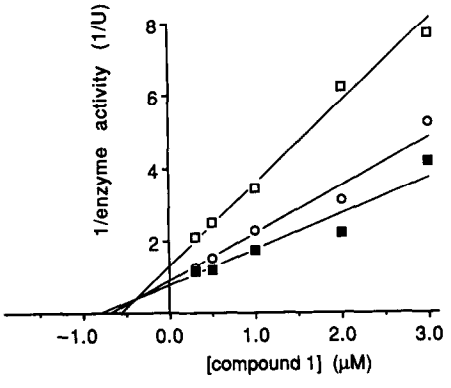


Fig. 3. Dixon plot [29] for compound **1** measured at GSSG concentrations of 100 μM (□), 300 μM (○) and 1000 μM (■). The lines intersect in the upper left quadrant above [S] = -0.4 μM. This is consistent with compound **1** competing with GSSG, the inhibition constant being 0.4 μM. An identical K_i-value was found for compound **3**. In the presence of these competitive inhibitors the Michaelis constant for GSSG ($K_m = 61$ μM) rises to $K'_m = 61\ (1 + [I]/0.4)$ μM [28, 29]. As GSSG at 1 mM does not disturb the absorption spectrum of 20 μM flavin analog **1** in the range 200 to 600 nm, it is unlikely that the two molecules interact with each other directly.

Table 2. Drug concentrations required for 50% or >95% inhibition of *P. falciparum* viability after 48 hr or 65 hr incubation

	After 48 hr		After 65 hr	
Inhibitor	IC_{50} (μM)	$IC_{>95}$ (μM)	IC_{50} (μM)	$IC_{>95}$ (μM)
1	2.8	12	0.5	10
2	1.9	8	0.2	5
3	1.2	15	0.4	7

After 48 hr incubation with the compounds, followed by a 70 hr interval of drug-free incubation, the parasitemia of the cultures which had contained less than 10 μM inhibitor started increasing again, whereas none of the parasites treated with ≥10 μM were found to be viable. This observation shows that the parasites considered alive after 48 hr with ≥10 μM inhibitor (Fig. 4) were in fact already irreversibly damaged. Addition of 20 mM GSH to the cultures [25, 32] did not influence the growth inhibition.

Effects of the flavin analogs on nucleotide metabolism

Comparisons of elution profiles for extracts from parasitized erythrocytes untreated or exposed to compound **1** (15 μM) for 27 hr suggest that this flavin analog did not affect pyrimidine or purine nucleotide biosynthesis and/or metabolism (Fig. 5).

The pyrimidine nucleotides apparent in these profiles (UTP, CTP, UDP, CDP and UDP-sugars) can be attributed to *P. falciparum*; they do not occur in uninfected erythrocytes. Two unknown malarial metabolites (indicated by "X" in Fig. 6) were detected in both sample and control cultures. These compounds were not observed in uninfected red blood cells.

Membrane permeation

The flavin analog **2** was detected quantitatively (*ca.* 50 μM) in non-parasitized red blood cells by ^{19}F-NMR-spectroscopy (Fig. 6). The position of the peak is identical for control and sample and the calculated drug concentrations seem to correspond. (Because of the different numbers of accumulations the control spectrum has to be multiplied by 6.12 to be compared directly with the sample spectrum [33].) This result indicates that compound **2** and, based on the structural similarities, probably also the other two flavin analogs, can permeate into non-parasitized erythrocytes.

DISCUSSION

The three tested flavin analogs (**1**, **2** and **3**; Fig.

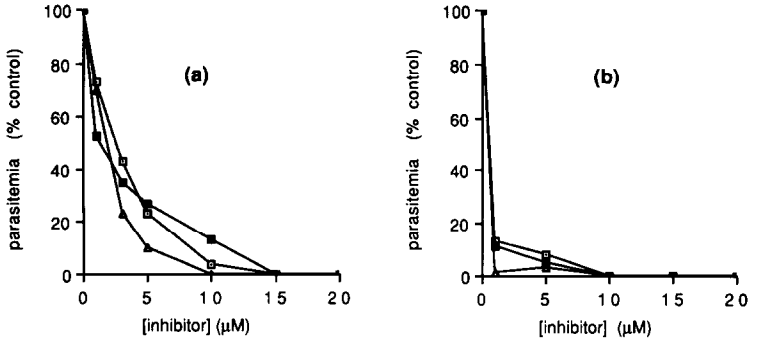


Fig. 4. Growth inhibition of *Plasmodium falciparum in vitro* after incubation with flavin analog **1** (⊡), **2** (△) or **3** (■): (a) after 48 hr, (b) after 65 hr.

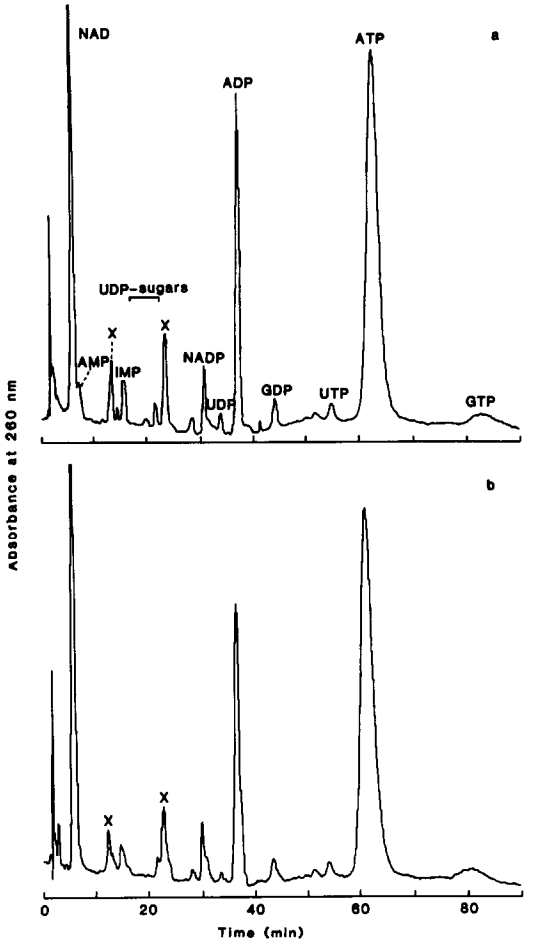


Fig. 5. Effect of flavin analog **1** on the nucleotide metabolism of *P. falciparum*-infected erythrocytes: (a) control (b) cells exposed to 15 μM compound **1** for 27 hr. Purine and pyrimidine metabolites were separated by anion-exchange HPLC. Peaks marked "X" remain to be identified.

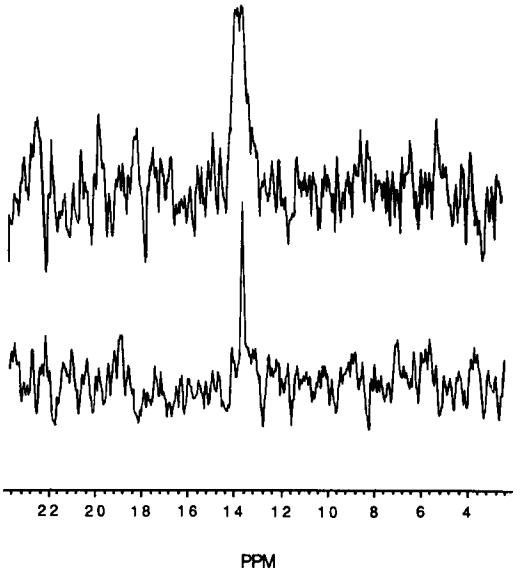


Fig. 6. Quantitation of compound **2** inside non-parasitized erythrocytes using ^{19}F-NMR-spectroscopy at 470 MHz. T = 300°K; sample volume = 2.5 ml. Upper spectrum: Sample, number of accumulations: 75000. Lower spectrum: Control; number of accumulations: 2000.

1) represent effective inhibitors of erythrocytic and parasitic (*P. falciparum*) glutathione reductase. These results are consistent with previous, preliminary observations on the mouse enzyme [6]. Because the inhibition was found to be reversible, not time-dependent and not dependent on the redox-state of the enzyme, the three drugs are unlikely to bind covalently. Another important negative finding is that the flavin analogs do not compete with the flavin moiety of the cofactor FAD.

The mixed-type inhibition indicated by the data in Table 1 and in Figs 2 and 3 suggests that the inhibitor might be sandwiched between the aromatic side chains of Phe78 and Phe78s in the large intersubunit cavity of glutathione reductase. The situation would be analogous to that with the structurally-similar dye safranin [34], where one inhibitor molecule at this position can influence both catalytic sites and both GSSG-binding sites of the dimeric enzyme. X-ray diffraction analyses of crystalline glutathione reductase/flavin analog-complexes are expected to

elucidate the interactions in atomic detail (Ermler, Becker and Schulz, work in progress).

The relative contributions of competitive and non-competitive inhibition to the overall effect depend on the actual concentrations of GSSG. Non-competitive inhibition ($K_i = 1.0\ \mu M$) predominates in the standard enzyme assay characterized by an almost saturating GSSG concentration (1 mM = 16.4 × K_m; see Table 1). Under *in situ* conditions, however, with GSSG $\leqq K_m$, compounds **1** or **3** at 10 μM would be more effective as competitive than as non-competitive inhibitors (Fig. 3).

The flavin analogs have been reported to be but weak inhibitors ($K_i \geq 50\ \mu M$) of trypanothione reductase from *Trypanosoma cruzi* [35]. Since the major difference between trypanothione reductase and glutathione reductase is the mutually exclusive specificity for their disulfide substrates [36] the sensitivity of glutathione reductase towards the riboflavin analogs supports the hypothesis that these compounds influence the GSSG-binding site.

Except for glutathione reductase, none of the six (di)nucleotide-dependent erythrocytic enzymes tested was significantly influenced in its activity by the three flavin analogs. In addition, changes in the purine and/or pyrimidine metabolism of erythrocytes or parasites after exposure to 15 μM compound **1** could not be observed. This suggests that none of the enzymes involved in these metabolic reactions (some of which are flavoenzymes) was affected significantly and that the nucleotide biosynthesis remained intact. For these reasons it seems likely that the antimalarial actions of the drugs (*in vivo* and *in vitro*) are based, at least in part, on glutathione reductase inhibition, which leads to a decrease of the cells' antioxidant capacity and consequently to damage caused by ROS [4].

One observation arguing against the above hypothesis is the fact that the *in vitro* effects of the compounds on *P. falciparum* could not be prevented by the addition of 20 mM GSH, which was found to be the case when intracellular glutathione reductase had been inhibited by carmustine [25, 32]. These discrepancies might be explained by differences in the experimental conditions and/or by the different characteristics of the inhibiting substances. Carmustine modifies glutathione reductase irreversibly; consequently the erythrocytes were incubated with this compound and the excess reagent was washed off before the parasitizing inoculum was added to the cells [25]. In contrast, the flavin analogs act reversibly and had to be present in the cultures during the whole experiment. The possible advantages and disadvantages of reversible and irreversible glutathione reductase inhibition will be studied further.

All three flavin analogs were shown to have very effective antimalarial activity *in vitro*, their concentration required for ≥95% inhibition of parasite viability being *ca.* 10 μM. This suggests that the substances permeate into parasitized erythrocytes. Selective permeation into infected cells seemed possible, since merozoite invasion into erythrocytes was not inhibited by the drugs and because it is known that the membranes of parasitized and non-parasitized red blood cells show differences in their permeability [37]. However this hypothesis was shown to be unlikely by the ^{19}F-NMR-experiment, which demonstrated compound **2** in non-infected erythrocytes after incubation with the drug.

Whether the *in vitro* and/or *in vivo* effects of the compounds are related to inhibition of parasite glutathione reductase, or the erythrocyte enzyme, or both, remains to be established. However, in conclusion it should be emphasized that 10-(4′-chlorophenyl)-3-methylflavin and its two tested derivatives are very effective inhibitors of glutathione reductase *in vitro* and of parasite viability *in vitro* and *in vivo* [6, 7]. Since no significant side effects have been noticed *in vivo* (Hunt, unpublished observations) the compounds are of interest for exploiting the concept of oxidative stress in malaria therapy.

Note added in proof—In recent flow cytometric studies on human leukemia B-cells (Hedley D, Becker K and Hunt NH) incubation with compound **1** (BY-16) led to accumulation of cells arrested in the S-phase of the cell cycle which suggests that the substance interferes with DNA synthesis. This effect may also play a role in the inhibition of parasite growth by the riboflavin analog.

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