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ANTIMALARIAL ACTION OF FLAVIN ANALOGUES SEEMS NOT TO BE DUE TO INHIBITION OF GLUTATHIONE REDUCTASE OF HOST ERYTHROCYTES

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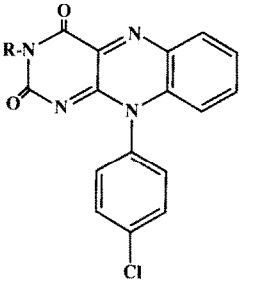
Abstract—A series of 10-(4′-chlorophenyl)-3-substituted flavins (**1a–f**) were examined with respect to their antimalarial properties. They were tested against *Plasmodium falciparum in vitro* and *Plasmodium vinckei vinckei in vivo*. The propostion that they might act through glutathione reductase (GR) (EC 1.6.4.2) inhibition has been studied. Inhibition of *P. falciparum in vitro* by these compounds shows only slight variation between analogues; in contrast, inhibition of human erythrocyte GR by members of the same series is highly variable, indicating that this is probably not their primary mode of antimalarial action. Results of the *P. vinckei vinckei* screen showed that 10-(4′-chlorophenyl)-3-methyl, 3-ethyl and 3-propyl substituted flavins are active *in vivo* over the dose range screened (10–70 mg/kg).

Thurnham and co-workers found that riboflavin deficiency suppressed *Plasmodium falciparum* infection in humans [1] and *Plasmodium berghei* infections in rats [2]. With this in mind, we synthesized a number of potential riboflavin antagonists, including 10-(4′-chlorophenyl)-3-methylflavin (**1b**) which proved to be a potent antimalarial agent *in vivo* and *in vitro* [3] and a good inhibitor of glutathione reductase (GR§) [3, 4].

Reduced glutathione (GSH) plays an essential role in the anti-oxidant defence system of the red blood cell. GSH is maintained in its reduced form via the flavoenzyme GR [5]. Zhang *et al.* [6, 7] have shown that depleting intraerythrocytic levels of GSH via GR inhibition is an effective way of preventing the growth of malaria parasites both *in vitro* and *in vivo*, most probably as a consequence of the susceptibility of *Plasmodium* species to oxidant stress [5, 8]. These findings suggested that the antimalarial action of **1b** could be due to inhibition of the host's erythrocytic GR.

To investigate this possibility, the structure-activity relationship of a series of 10-(4′-chlorophenyl)-3-substituted flavins (**1a–f**; Fig. 1) was examined. These analogues exhibited considerable variation in their ability to inhibit human GR, which did not correlate with their inhibition of *P. falciparum* growth *in vitro*. These findings suggest that inhibition of human erythrocyte GR is probably not the primary mode of antimalarial action of this class of compounds.

We have also tested the new analogues, **1a** and **1c–f**, against *P. vinckei vinckei* in mice in order to determine their effectiveness as antimalarials *in vivo*.



1a, R = H **1d**, R = n-C_3H_7
1b, R = CH_3 **1e**, R = C_6H_5
1c, R = C_2H_5 **1f**, R = $CH_2C_6H_5$

Fig. 1. Structure of the 10-(4′-chlorophenyl)-3-substituted flavins, **1a–f**.

METHODS AND MATERIALS

10-(4′-Chlorophenyl)-3-substituted flavins. The flavins, **1a–f**, were formed by the action of three equivalents of nitrosobenzene on 6-(4′-chloroanilino)-3-substituted uracil in the presence of acetic anhydride. The synthesis of **1b** has previously been described using this method [9]. The compounds **1c** (m.p. 330°), **1d** (m.p. > 360°), **1e** (m.p. > 360°) and **1f** (m.p. 351°) were synthesized in the same manner. Flavin **1a** was synthesized as in Cowden *et al.* [10]. Melting points were determined in open capillaries and are uncorrected. All flavins analysed correctly

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§ Abbreviations and chemical names: GR, glutathione reductase; GSH and GSSG, reduced and oxidized glutathione; NADPH and $NADP^+$, reduced and oxidized nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; IC_{50}, concentration at which 50% of activity/growth is inhibited.

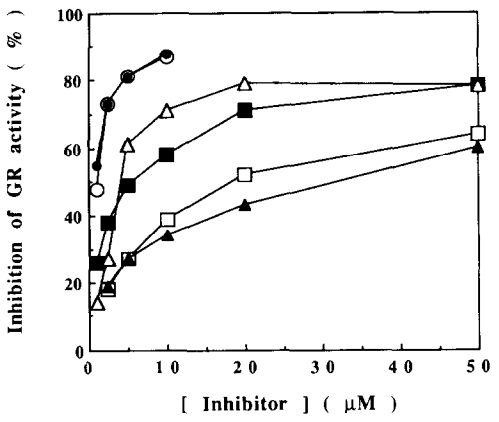

Fig. 2. Inhibition of human glutathione reductase by flavin analogues **1a–f**, **1a** (○), **1b** (●), **1c** (■), **1d** (□), **1e** (▲) and **1f** (△).

for carbon, hydrogen and nitrogen. The mass spectra of the new flavins were determined with a V.G. Micromass 7070F mass spectrometer using an Incos data system and were consistent with their assigned structures.

GR assay. Enzyme activity was measured essentially by the method of Krohne-Ehrich *et al.* [11]. The GR reaction was monitored with a Varian DMS 100 UV/visible spectrophotometer at 25° using the decrease in absorbance at 340 nm that occurs when NADPH is converted to $NADP^+$. The assay mixture had a volume of 1 mL and a pH of 7.0. It contained 50 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, 1 mM GSSG, 0.1 mM NADPH and 3 nM GR purified from human erythrocytes (a generous gift from Dr Heiner Schirmer, Heidelberg, F.R.G.). After the assay mixture had been incubated with the inhibitor for 2 min the reaction was initiated by addition of GSSG. Stock solutions (1 mM) of the flavin compounds in dimethyl sulphoxide were used. Reaction rates were obtained at various inhibitor concentrations; control samples contained dimethyl sulphoxide without inhibitor. The values presented are the means of three experiments; the experimental values deviated from the mean by less than 7%.

In vitro *inhibition of* P. falciparum *growth*. The inhibition of the growth of *P. falciparum* (FC27, a Papua New Guinea strain maintained *in vitro* over several years) by flavins was determined by [^{3}H]hypoxanthine incorporation over 48 hr incubation as in Cowden *et al.* [3]. The values presented at each concentration are the means of three separate experiments.

In vivo P. vinckei vinckei *screening*. *In vivo* antimalarial activity was screened by intraperitoneal injection of the flavins (**1a–f**) into mice infected with *P. vinckei vinckei* (V52) as described previously [10]. The percentage of animals cured, in groups of five mice at the doses 10, 30 and 70 mg/kg, was used to quantify activity.

RESULTS

Flavin inhibition of GR

Concentration–inhibition curves for flavins **1a–f** were determined (Fig. 2). Double reciprocal plots of GR inhibition against flavin concentration gave straight lines ($r^2 > 0.97$), which allowed the calculation of IC_{50} values (Table 1). Based on the IC_{50} the best inhibitor, 10-(4′-chlorophenyl)-3-methylflavin, **1b** ($IC_{50} = 0.8\ \mu M$), was 57 times more active than the worst inhibitor, 10-(4′-chlorophenyl)-3-phenylflavin, **1e** ($IC_{50} = 46.2\ \mu M$).

Table 1. Effects of 10-(4′-chlorophenyl)-3-substituted flavins on human erythrocyte GR

Flavin compound	IC_{50}* (μM)
1a	1.1
1b	0.8
1c	4.3
1d	19.0
1e	46.2
1f	5.7

* Concentration required to inhibit 50% of GR activity.

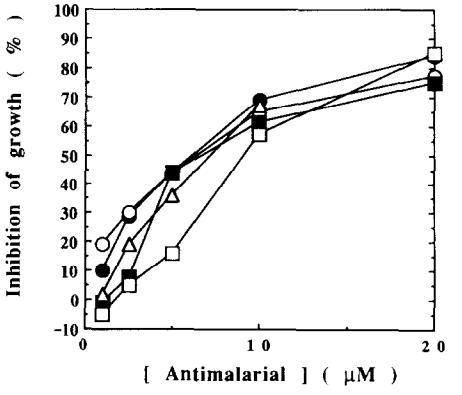

Fig. 3. Growth inhibition of *P. falciparum in vivo* after 48 hr incubation with flavin analogues, **1a** (●), **1b** (○), **1c** (■), **1d** (△) and **1e** (□).

Flavin inhibition of P. falciparum *growth* in vitro

Figure 3 shows inhibition of *P. falciparum* growth by compounds **1a–e**; it can be seen that all compounds inhibited to a similar extent. The IC_{50} of all the flavins lie in the narrow range of 6 to 9 μM. Results for compound **1f**, at all concentrations, and **1d** at 20 μM could not be obtained due to insolubility.

Flavin inhibition of P. vinckei vinckei in vivo

Table 2 summarizes the antimalarial activity of **1a–f** against *P. vinckei vinckei*. Clearly, in this screen, compounds **1b** and **1c** were the most effective, with **1d** becoming active only at the highest dose. Compounds **1a**, **1e** and **1f** were not active in the dose range screened. These results clearly demonstrate the influence of substituents in position 3 on activity *in vivo*.

DISCUSSION

This study was undertaken to delineate the role that inhibition of host erythrocytic GR plays in the antimalarial activity of 10-(4′-chlorophenyl)-3-substituted flavins, a novel class of antiprotozoal agents

Table 2. Parenteral antimalarial activity of 10-(4'-chlorophenyl)-3-substituted flavins against *P. vinckei vinckei* in mice

Flavin	Percentage cured* at dose		
	10 mg/kg	30 mg/kg	70 mg/kg
1a	0	0	0
1b	20	100	0†
1c	100	100	100
1d	0	0	100
1e	0	0	0
1f	0	0	0

* Animals were considered cured if still living 60 days post-treatment with a single intraperitoneal injection in olive oil (100 μL).

† Toxic.

[3]. A series of flavin analogues was prepared with the following substituents at the 3-N position: hydrogen, methyl, ethyl, propyl, phenyl and benzyl. This series of compounds, with a wide range of lipophilic, electronic and steric properties, was tested for its ability to inhibit human GR *in vitro*. This inhibition was compared with the antimalarial activity of these compounds against cultured *P. falciparum*. A lack of correlation, throughout the series, between the two test systems suggests that inhibition of host erythrocytic GR is probably not the principal mode of antimalarial action of this class of drugs. We cannot, of course, exclude the possibility that these compounds are metabolized within the erythrocyte or parasite to a common active metabolite which inhibits GR, though we have no evidence to support this proposition.

When tested against human GR a substantial change in effectiveness was observed across the series. On the other hand, in the *P. falciparum* assay, the compounds proved to be essentially equipotent throughout the series. These results indicate the importance of the substituent in the 3-N position of 10-phenylflavins in terms of host red cell GR inhibition, a factor apparently not crucial in their antimalarial action against *P. falciparum* in culture.

In an effort to explain the antimalarial activity of **1b**, a number of erythrocytic flavoenzymes have previously been investigated as possible targets by Becker *et al.* [4]. In that report, GR presented itself as the most likely possibility. However, it was found that addition of GSH *in vitro* did not block the antimalarial action of 10-(4'-chlorophenyl)-3-methylflavin against *P. falciparum*, an observation consistent with the present conclusion that inhibition of host erythrocytic GR is probably not the main site of antimalarial action for these drugs. It should be noted that **1b** has also been shown to be an inhibitor of parasite GR and the flavins conceivably could be exerting their antimalarial activity via this route [4].

When the series was screened in mice against *P. vinckei vinckei* the 3-methyl, 3-ethyl and 3-propyl substituted compounds were effective antimalarials. 10-(4'-Chlorophenyl)-3-ethylflavin, **1c**, was the most potent and did not show any signs of toxicity at the doses administered. The 3-unsubstituted, 3-phenyl and 3-benzyl flavins failed to show any activity in the dose range tested. The differences in the antimalarial action of the flavins against *P. falciparum* and *P. vinckei vinckei* can probably be attributed to pharmacokinetic effects. The changes in the pharmacokinetic properties of the flavins, especially lipophilicity, caused by the different 3-N substituents, may have a large effect on physiological distribution and elimination. This could also explain the considerable variation in potency seen among the flavins in the *P. vinckei vinckei* screen.

In conclusion, the present work suggests that inhibition of human erythrocyte GR is probably not the primary mode of action of the 10-(4'-chlorophenyl)-3-substituted flavin antimalarials. Additionally, substituents in the 3-N position of these compounds have a direct effect on their enzyme inhibitory activity. The flavins tested had approximately equal activity against *P. falciparum* in culture but not in the *P. vinckei vinckei* screen where the order of activity, **1c** > **1b** > **1d** > **1a** = **1e** = **1f**, illustrates the influence that 3-N-substituents have on activity *in vivo*. Work is continuing to determine how this new class of antimalarials may function.

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