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# INHIBITORS OF GLUTATHIONE REDUCTASE AS POTENTIAL ANTIMALARIAL DRUGS. KINETIC COOPERATIVITY AND EFFECT OF DIMETHYL SULPHOXIDE ON INHIBITION KINETICS

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We have developed inhibitors of glutathione reductase that improve on the inhibition of literature lead compounds by up to three orders of magnitude. Thus, analogues of Safranine O and menadione were found to be strong, reversible inhibitors of yeast glutathione reductase. Safranine O exhibited partial, uncompetitive inhibition with $K_i$ and $\alpha$ values of 0.5 mM and 0.15, respectively. Thionine O was a partial (hyperbolic) uncompetitive inhibitor with $K_i$ and $\alpha$ values of 0.4 μM and 0.15, respectively. LY83583 and 2-anilino-1,4-naphthoquinone also showed (hyperbolic) partial, uncompetitive inhibition with micromolar $K_i$ values. For Nile Blue A a model for two-site binding with (parabolic) uncompetitive inhibition fitted the data with a $K_i$ value of 11 μM and a kinetic cooperativity between the sites of 0.12, increased to 0.46 by preincubation of the enzyme and Nile Blue A in the presence of glutathione disulphide. Analysis of the effects of preincubation on the kinetics and cooperativity indicated the possibility of a slow conformational change in the homodimeric enzyme, the first such indication of kinetic cooperativity in the native enzyme to our knowledge. Further evidence of conformational changes for this enzyme came from studies of the effects of dimethyl sulphoxide which indicated that this co-solvent, which at low concentrations has no apparent effect on initial velocities under normal assay conditions, induced a slow conformational change in the enzyme. Thionine O, Nile Blue A and LY83583 were redox-cycling substrates producing superoxide ion, detectable by means of cytochrome c reduction, but leading to no loss of glutathione reductase activity, under aerobic or anaerobic conditions. The water-soluble Safranine analogues Methylene Blue, Methylene Green, Nile Blue A and Thionine O (5 mg/kg i.p. × 5) were effective antimalarial agents *in vivo* against *P. berghei*, but their effect was small and a higher dose (50 mg/kg i.p. × 1) was toxic in mice. Comparison was made with human glutathione reductase and its literature-reported interactions with several tricyclic inhibitors as studied by X-ray diffraction. It is possible that the conformational changes detected in the present study from alterations

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in detailed kinetic inhibition mechanisms may shed light on information transfer through the glutathione reductase molecule from the dimer interface ligand pocket to the active-site.

*Keywords:* Glutathione reductase; Antimalarial drugs; Dimethyl sulphoxide; Inhibition

*Abbreviations:* DMSO, dimethyl sulphoxide; GSSG, oxidized glutathione; GR, glutathione reductase



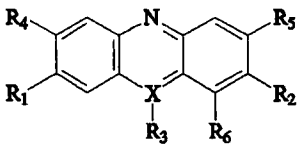
## INTRODUCTION

In many of its numerous physiological roles reduced glutathione (GSH)[1] becomes oxidised to glutathione disulphide (GSSG), which is then recycled to GSH by reaction (1), catalysed by glutathione reductase (GR):

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ \quad (1)$$

In view of the physiological importance of GR and of the known 3-dimensional structures of GR and of complexes with both of its substrates,[2] this enzyme is of appreciable medical interest[3] and has been proposed as a potential drug target, especially in the field of antimalarials.[4–6] During malarial infection by *Plasmodium vivax* GR activity has been reported to decrease steadily during increasing parasitaemia, normalising again on successful chloroquine therapy and cure.[7] Analogues (10-aryl-substituted 3-methylflavins) of the tricyclic flavin nucleus of FAD, the cofactor of GR, have been reported to have antimalarial activity *in vitro* and *in vivo*[8,9] and such molecules have been shown to be strong inhibitors of GR ($K_i$ values of the order of 1 μM).[10] However, it has been argued that inhibition of human erythrocyte GR may not be their primary mode of antimalarial action.[11] In a detailed X-ray structural analysis of enzyme/ligand complexes it was shown that Safranine O (**1**) and menadione (**2**)[12] bind preferentially in a cavity in the interface region between the two subunits[13] of human erythrocyte GR. X-ray diffraction analysis of two complexes of human GR with 10-substituted isoalloxazines showed that the ligands bind (one per dimer) in a cavity at the dimer interface, bounded by H75, F78, Y407 and their symmetrical equivalents on the other subunit.[14] This is similar in general location to the site occupied by menadione and safranine.[12]

During our studies of glutathione reductase and trypanothione reductase[15] we became interested in finding molecules capable of binding to such a region of the interface as potential protein–protein interaction inhibitors. Consequently we studied the inhibition of yeast glutathione reductase by several analogues of Safranine O (Figure **1**–**13**) to provide insight into the



| No. | Name | X | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Safranine O | $N^+$ | $NH_2$ | $NH_2$ | Ph | $CH_3$ | $CH_3$ | H |
| 2 | Thionine O | $S^+$ | $NH_2$ | $NH_2$ | — | H | H | H |
| 3 | Azure A | $S^+$ | $NMe_2$ | $NH_2$ | — | H | H | H |
| 4 | Azure B | $S^+$ | $NMe_2$ | NHMe | — | H | H | H |
| 5 | Methylene Green | $S^+$ | $NMe_2$ | $NMe_2$ | — | $CH_3$ | $CH_3$ | $NO_2$ |
| 6 | New Methylene Blue N | $S^+$ | NHEt | NHEt | — | $CH_3$ | $CH_3$ | H |
| 7 | Acridine Yellow G | C | $NH_2$ | $NH_2$ | H | $CH_3$ | $CH_3$ | H |
| 8 | 9-Aminoacridine | C | H | H | $NH_2$ | H | H | H |
| 9 | Janus Green | $N^+$ | $NEt_2$ | $-N=NC_6H_4pNMe_2$ | Ph | H | H | H |

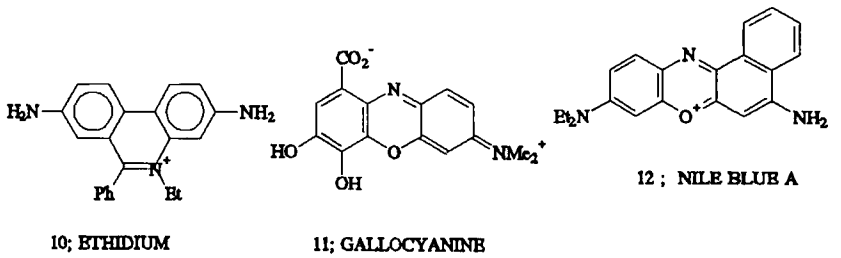



FIGURE 1 Structures of the inhibitors of glutathione reductase used.

interactions and a kinetic basis for the inhibition. We also provide details of studies on menadione analogues related to LY83583, the selective repressor of cyclic GMP formation, which we recently showed to be a strong, redox-cycling inhibitor of glutathione reductase.[16] The effects of members of the Safranine class of GR inhibitors on *Plasmodium berghei* infection of mice are also reported in the light of the potential antimalarial activity described above for GR inhibitors.

## MATERIALS AND METHODS

### Materials

Thionine O (certified), Safranine O (certified), 1,8-dihydroxyanthraquinone, 2-anilino-1,4-naphthoquinone, 9-aminoacridine, acridine yellow, 2-acetamidofluorene, 2-aminoanthraquinone, chrysin, 2-aminofluorenone,

2-hydroxy-1,4-naphthoquinone, apigenin and anthraquinone-2-carboxylic acid were from Aldrich Chemical Co., Nile Blue A, Azure A, Azure B, New Methylene Blue, Methylene green, ethidium bromide, Janus Green from Fluka and lapachol was from Sigma. *O*-Methyl-lapachol and norlapachol were from a previous study.[17]

## Methods

GR (yeast) was purchased from Sigma for this study, and from Boehringer and Calbiochem. In some experiments GR from bovine intestinal mucosa (Sigma) was used and homogeneity to SDS PAGE determined as well as the value of $k_{cat}$ as a measure of specific activity. GR was assayed as previously described[16] except that a total volume of 3 ml was used. The concentrations in the assay were: GR 4–6 nM; GSSG (0.5 $K_m$, $K_m$, 1.5 $K_m$, 2 $K_m$); inhibitor (Thionine O: 0.1, 0.25, 0.5 μM; Nile Blue A: 2, 4, 6 μM; 2-anilino-1,4-naphthoquinone: 5, 10, 15 μM; Safranine O: 0.2, 0.5, 0.8 mM). Reversibility of inhibition was assessed for thionine by equilibrating a mixture of GR (10 μM) and thionine (83 μM) for 20 min at 25°C in assay buffer to a total volume of 240 μl, followed by separating protein and inhibitor on a PD-10 column (Pharmacia). Protein-containing fractions were assayed for GR activity and the results compared with those for a control run identically, but in the absence of inhibitor. For a typical kinetic run to study inhibition, GSSG (at the appropriate concentration), NADPH (0.1 mM) and inhibitor were mixed in buffer at ambient temperature, equilibrated at 25°C in the thermostatted cuvette compartment of the spectrophotometer and the assay initiated by adding a small aliquot of GR. Inhibition type was diagnosed by the use of three different plots (the Lineweaver–Burk plot, $1/V_0$ versus $1/[S_0]$ at various values of [I], the Dixon plot, $1/V_0$ versus [I] at various values of $[S_0]$ and the Cornish–Bowden plot, $[S_0]/V_0$ versus [I] at various values of $[S_0]$).[18] Values of $I_{50}$ (the concentration of inhibitor which gives 50% inhibition) were determined by varying the inhibitor concentrations using GR (4 nM) and GSSG and NADPH at saturating concentrations of 1 and 0.1 mM, respectively.

Preincubation-based experiments were carried out as follows. Enzyme and inhibitor were preincubated for a fixed time (80 min) in the presence of GSSG substrate at ambient temperature in the dark. The assay in such cases was started by adding a small aliquot of NADPH (to give a final concentration of 0.1 mM). When the assay was performed in the presence of DMSO, the DMSO content (200 μl, 7% v/v) was kept constant in all measurements.

When Thionine O (5 μM) and Nile Blue A (20 μM) were tested as substrates for GR (0.7 μM), oxidation of NADPH (0.1 mM) was measured

at 340 nm. In addition, enzyme activity was monitored by coupling superoxide ion radical formation to reduction of cytochrome c (20 μM) and measuring absorbance changes ($\epsilon_{550} = 21{,}000\ M^{-1}\ cm^{-1}$). Anaerobic measurements were performed in rubber-stoppered cuvettes, flushed repeatedly with argon. All buffers and solutions used in such assays were treated in this way. Kinetic parameters were obtained from the data by means of the Enzfitter© programme of R.J. Leatherbarrow, distributed by Elsevier Biosoft.

Male MF1 mice weighing 18–26 g were used, experiments being conducted at an environmental temperature of 23 ± 2°C. Mice were housed in groups of 12 on a 12 h dark (20:00 to 08:00)/light (08:00 to 20:00) cycle with free access to food and water. *P. berghei* (N/13/1A/4/203), maintained by serial blood passage in MF1 mice, was injected i.v. into experimental animals ($2 \times 10^7$ parasitised red blood cells/mouse), which were then treated topically with a 2.5% solution of monosulfiram to prevent infection by *Eperythrozoon coccoides*. Azure A, Azure B, Methylene Blue, Methylene Green, Nile Blue A and Thionine O were the water soluble compounds assessed for antimalarial activity *in vivo* in this study. Compounds were dissolved in Water for Injection BP and injected i.p. 4 h after inoculation with *P. berghei* (ca. 15:30) and subsequently twice daily (ca. 09:30 and 15:30) on each of the next two days. Three days after inoculation, following 5 doses i.p., malarial parasitaemia was determined using light microscopy as the percentage of erythrocytes containing Leishman positive bodies (%).

At frequent intervals throughout the experimental period, mice were assessed visually for possible behavioural and autonomic effects including the adoption of a huddled posture, changes in cutaneous vasomotor tone, the development of piloerection and motor disturbances. Body weight and colonic temperature were measured before inoculation and at the end of the experiment 72 h after *P. berghei*. Colonic temperature was measured using a Yellow Springs Instrument Series 400 small animal rectal thermistor, inserted under minimal manual restraint, and displayed on a YSI model 47TA Tele-Thermometer. At the end of the experiment, exploratory locomotor activity of mice was measured over a 5 min period following transfer of animals to an unfamiliar plastic cage (41 cm length × 25 cm width × 12.5 cm depth) positioned on a LKB Animex Activity Meter.

## Statistics

Results for observations *in vivo* are expressed as mean ± s.e.m. for groups of 4 mice. The probabilty ($P$) of the difference between means was evaluated by the non-parametric Mann–Whitney $U$-test.

## RESULTS

GR from yeast was found to be inhibited by several analogues of Safranine O and menadione. Both families of analogues eventually provided potent inhibitors of GR (see Table I) based on their $I_{50}$ values. As a representative example, the inhibition was shown to be reversible for Thionine O. When GR was equilibrated with thionine and the protein reisolated, free of Thionine O, on a PD-10 column GR activity to a level of $91 \pm 1\%$ of control could be restored. The most potent inhibitors (Figure 1), namely Thionine O, Nile Blue A and 2-anilino-1,4-naphthoquinone were studied in greater detail. Safranine O was chosen as the reference compound in view of the X-ray diffraction data available for its complex with human erythrocyte GR.[2]

### Safranine O

Steady-state inhibition studies of Safranine O with yeast GR were carried out using GSSG concentrations varying from 0.5–2 $K_m$ at fixed Safranine O concentrations (0.2, 0.5, 0.8 mM). The Lineweaver–Burk plot (Figure 2A) of the kinetic data was found to consist of a series of parallel lines, indicating an uncompetitive type of inhibition. The replot of the Lineweaver–Burk ($1/V_0$) intercept versus inhibitor concentration of the data of Figure 2A was convex downward, indicating partial inhibition (Figure 2B). Consistent with

TABLE I $I_{50}$ values of Safranine O and Menadione analogues as inhibitors of yeast glutathione reductase

| *No.* | *Safranine analogues* | $I_{50}$ (μM) | *Menadione analogues* | $I_{50}$ (μM) |
|---|---|---|---|---|
| **1** | Safranine O | 700 | 2-Acetamidofluorene | — |
| **8** | 9-Aminoacridine | 5% at 100 | 2-Aminoanthraquinone | — |
| **7** | Acridine Yellow G | 10% at 100 | Chrysin | — |
| **9** | Janus Green | 20% at 50 | Lapachol | 4000 |
| **10** | Ethidium Bromide | 200 | Norlapachol | 2000 |
| **11** | Gallocyanine | 200 | 2-Aminofluorenone | 15% at 1000 |
| **5** | Methylene Green | 80 | 2-Hydroxy-1,4-naphtho-quinone | 8% at 350 |
| **6** | New Methylene Blue N | 20 | Apigenin | 10% at 100 |
| **4** | Azure B | 7 | Menadione | 30 |
| **3** | Azure A | 6 | Anthraquinone-2-carboxylate | 70 |
| **12** | Nile Blue A | 4 | 1,8-Dihydroxyanthraquinone | 30 |
| **2** | Thionine O | 1 | O-Methyllapachol 2-Anilino-1,4-naphthoquinone | 3 |

10% DMSO was present; assay conditions at 25°C: GR (4 nM), GSSG (1 mM), NADPH (0.1 mM), 0.2 M potassium phosphate buffer pH 7.0 containing 2 mM EDTA and appropriate inhibitor concentrations. NADPH oxidation was followed at 340 nm.

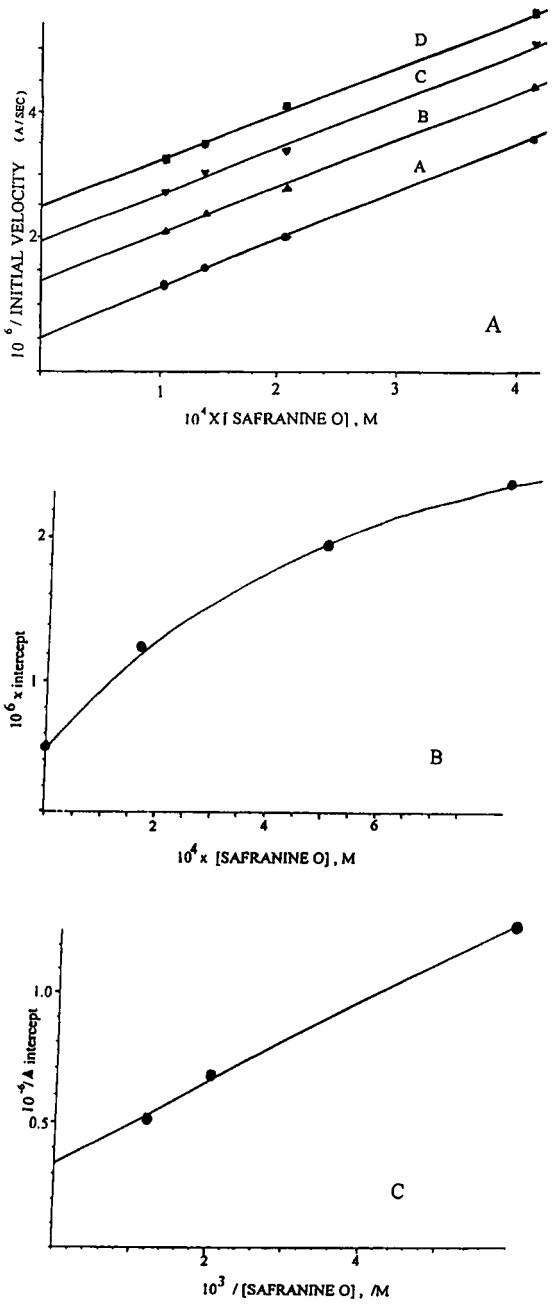



FIGURE 2 Inhibition of yeast glutathione reductase by Safranine O. (A) Lineweaver–Burk plot. Inhibitor concentrations: A, ● (0 mM); B, ▲ (0.2 mM); C, ▼ (0.5 mM); D, ■ (0.8 mM). (B) Secondary plot: intercept versus [inhibitor]. (C) $1/\Delta_{\text{intercept}}$ versus 1/inhibitor replot.

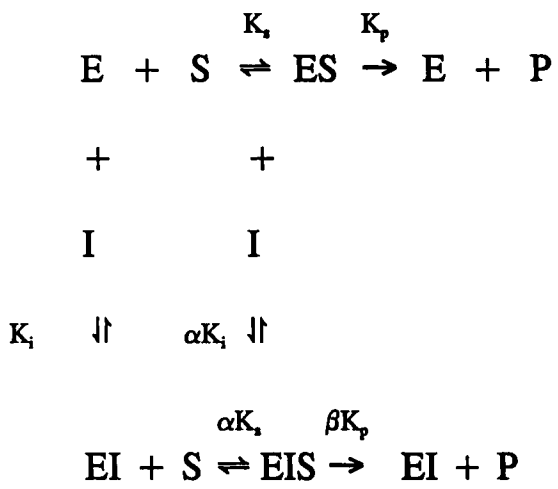



FIGURE 3 General scheme for hyperbolic mixed-type inhibition: $\alpha = \beta$ for partial uncompetitive inhibition.[19]

this, the Dixon and Cornish–Bowden plots were also curved. The kinetic scheme which describes partial, uncompetitive inhibition is shown in Figure 3.

To determine the values of $K_i$ and $\alpha$ (in this case $\alpha = \beta$), $1/\Delta_{intercept}$ was plotted versus 1/[inhibitor], where $\Delta_{intercept}$ is the value of the Lineweaver–Burk intercept (on the $1/V_0$ axis) minus the value of this intercept at $1/[S_0] = 0$. A straight line was obtained with a slope of $V_{max} \cdot \alpha \cdot K_i/(1-\alpha)$ and an intercept of $V_{max}/(1-\alpha)$ (Figure 2C).[18,19] From these data, the values of $K_i$ and $\alpha$ were determined to be 0.5 mM and 0.15, respectively (Table II). For the kinetic scheme of Figure 3, an $\alpha$ value of 0.15 indicates that however high the inhibitor concentration, the enzyme will still be active to a level of 0.15 $V_{max}$. Therefore Safranine O with a $K_i$ value in the millimolar range is only ever a weak inhibitor of yeast GR.

### Thionine O

Steady-state inhibition kinetics of Thionine O with yeast GR were performed in a similar way to the studies of Safranine O, but in view of the lower $I_{50}$, the fixed Thionine O concentrations used were lower, viz. 0.1, 0.25, 0.5 μM. The Lineweaver–Burk plot of the kinetic data indicated an uncompetitive type of inhibition, and, as for Safranine O, the replot of the intercept versus inhibitor for Thionine O was convex downward, indicating

TABLE II Kinetic parameters for yeast glutathione reductase inhibitors. Under the sites of action column, the number of binding sites is given by $n^0$ and the cooperativity constant as $c$. Values of $c$ and $\alpha$ were determined as described in the text

| *Entry* | *Inhibitor* | *Inhibition* | *Sites of action* ($n^0$, *cooperativity*, $c$) | $K_i$ (μM) | $\alpha$ | *DMSO* (%) | *Preincubation* (min) |
|---|---|---|---|---|---|---|---|
| 1 | Safranine O | Hyperbolic-Uncompetitive | 1 | 500 | 0.15 | 0 | 0 |
| 2 | Thionine O | Hyperbolic-Uncompetitive | 1 | 0.4 | 0.15 | 0 | 0 |
| 3 | Thionine O | Hyperbolic-Uncompetitive | 1 | 1.2 | 0.08 | 7 | 80 |
| 4 | Nile Blue A | Parabolic-Uncompetitive | 2, $c = 0.12$ | 11 | — | 0 | 0 |
| 5 | Nile Blue A | Parabolic-Uncompetitive | 2, $c = 0.46$ | 7 | — | 0 | 80 |
| 6 | Nile Blue A | Parabolic-Uncompetitive | 2, $c = 0.31$ | 9 | — | 0 | 80 |
| 7 | Nile Blue A | Parabolic-Uncompetitive | 2, $c = 0.22$ | 10 | — | 0 | 80 |
| 8 | Nile Blue A | Hyperbolic Uncompetitive | 1 | 1.3 | 0.13 | 7 | 80 |
| 9 | Anilino-1,4-naphtho-quinone | Hyperbolic Uncompetitive | 1 | 21 | 0.19 | 0 | 0 |
| 10 | Anilino-1,4-naphtho-quinone | Hyperbolic Uncompetitive | 1 | 5 | 0.06 | 7 | 80 |
| 11 | LY-83583 | Hyperbolic Uncompetitive | 1 | 14 | 0.14 | 0 | 0 |

partial inhibition. From the $1/\Delta_{intercept}$ versus 1/[inhibitor] replot the values of $K_i$ and $\alpha$ were determined to be 0.4 μM and 0.15, respectively.

### Nile Blue A

Steady-state inhibition kinetics with yeast GR were carried out at fixed Nile Blue A concentrations of 2, 4 and 6 μM. The Lineweaver–Burk plot gave a series of parallel lines indicating uncompetitive inhibition. The replot of intercept versus [inhibitor] was non-linear, but the curvature was now concave upwards (Figure 4) and parabolic indicating more than one site of interaction of Nile Blue A with yeast GR. A multiple-site analysis was carried out according to Segel.[19] The derivation of the appropriate equation was based on the comparable equation[19] for uncompetitive inhibition, Eq. (2):

$$1/V = K_m/V_{max}[S] + (1/V_{max}) \cdot (1 + [I]/K_i), \quad (2)$$

$$1/V = K_m/V_{max}[S] + (1/V_{max}) \cdot (1 + [I]/K_i)^2. \quad (3)$$

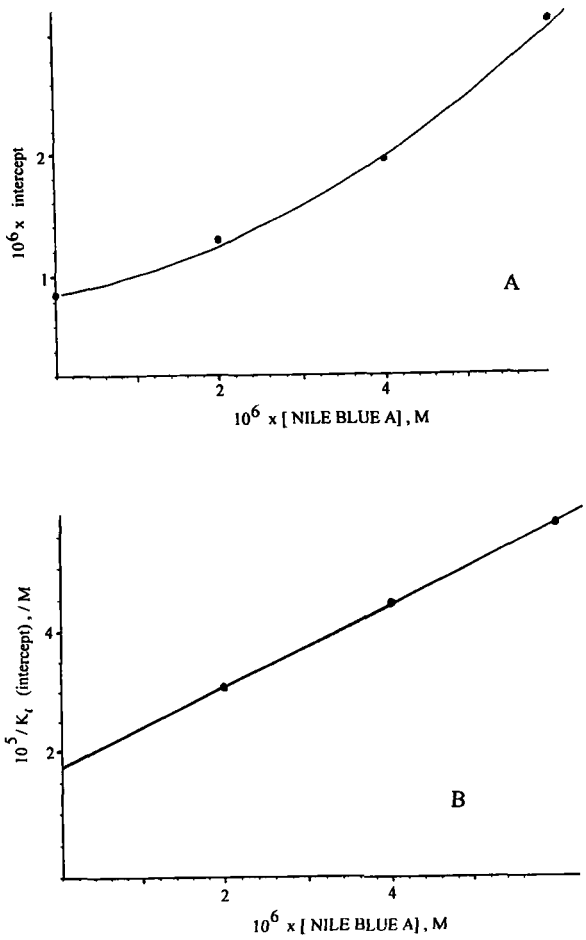



FIGURE 4 Inhibition of yeast glutathione reductase by Nile Blue A. (A) Secondary plot: intercept versus inhibitor concentration. (B) Plot of $1/K_i$ (intercept) versus inhibitor concentration.

For a two-site system Eq. (3) is appropriate, but the calculated $K_i$ value is no longer a true dissociation constant for an EI complex, but rather is a complex function, which varies with [I]. This can be understood in terms of Eq. (4), which describes the intercept of the $1/V_0$ versus $1/[S_0]$ plot (Eq. 4) when $1/[S_0] = 0$:

$$\text{intercept} = (1/V_{max}) \cdot (1 + [I]/K_i)^2 = (1/V_{max})\ (1 + [I]/K_{i,\text{intercept}}), \quad (4)$$

where

$$K_{i,\,intercept} = [I]/(intercept \cdot V_{max} - 1). \quad (5)$$

Equation (4) can be rearranged as

$$1/K_{i,\,intercept} = (1/K_i)^2 \cdot [I] + 2/K_i. \quad (6)$$

Thus, if the reciprocals of the $K_{i,\,intercept}$ values determined from the $1/V_0$ versus $1/[S_0]$ plot, are replotted versus the corresponding values of [I], the replot will be a straight line with a slope of $1/K_i^2$ and an intercept of $2/K_i$ (Eq. 4). If there is cooperative binding, the slope of this replot is $1/cK_i^2$, and the intercept is still $2/K_i$.

The above procedure for the analysis of a two-site system was applied to the kinetic data for Nile Blue A. A plot of $1/K_{i,\,intercept}$ versus [I] yielded a straight line (Figure 4), indicating the applicability of the analysis and hence the interaction of Nile Blue A at two different sites. The slope and the intercept of the line revealed a cooperativity of $c = 0.12$ and a $K_i$ value of 11 μM. When Nile Blue A was preincubated with GR for 80 min in the presence of substrate and the reaction initiated by adding NADPH, the type of inhibition was unchanged (Table II, entry 5). However, the cooperativity factor ($c$) was increased approximately four-fold. Two more experiments were carried out to elucidate the origin of this interesting increase in the cooperativity constant, $c$. Firstly, GR was preincubated with the GSSG substrate. After 80 min, Nile Blue A was added, followed by NADPH to initiate the reaction (Table II, entry 6). A drop in cooperativity to $c = 0.31$ was found. In a second experiment, GR was preincubated with Nile Blue A. After 80 min substrate (GSSG) was added, followed by NADPH with a delay of about 30 s (Table II, entry 7). Under these conditions, the cooperativity factor was increased only two-fold. Thus, the outcome of the experiment with no preincubation (Table II, entry 4) could be closely mimicked by one with a preincubation of 80 min, but in the absence of disulphide substrate (Table II, entry 7). This is readily explicable because in classical, uncompetitive inhibition the inhibitor is thought to bind mainly to the ES-complex[19] and during preincubation no such complex can be formed. However, this cannot explain the increased cooperativity for entry 5 in Table II. The only reasonable factor left is the time of preincubation. It was observed that after a preincubation of 80 min (Table III, entry 2) the enzymatic activity dropped by about 18% and the $K_m$ value was increased by about 13%. This fact likewise is very unlikely to be responsible for the increase in cooperativity. With the

TABLE III Kinetic constants for GSSG as a substrate of yeast GR, under various conditions of incubation. GR (4 nM) was assayed with NADPH (0.1 mM) and various GSSG (0.3 to 3 $K_m$) concentrations in 0.1 M K-phosphate buffer pH 7.0, 1 mM EDTA at 25°C. In entry 1 the assay was initiated with GR and the decay of NADPH was followed at 340 nm. In entries 2 and 3 the reaction was initiated by adding NADPH

| *Entry* | *Preincubation* (mm) | *DMSO* (%) | $K_m$ (M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
|---|---|---|---|---|---|
| 1 | 0 | 0 | $5.2 \times 10^{-5} \pm 0.3 \times 10^{-5}$ | $236 \pm 5$ | $4.6 \times 10^6 \pm 0.3 \times 10^6$ |
| 2 | 80 | 0 | $5.9 \times 10^{-5} \pm 0.1 \times 10^{-5}$ | $194 \pm 8$ | $3.3 \times 10^6 \pm 0.3 \times 10^6$ |
| 3 | 60 | 7 | $10.2 \times 10^{-5} \pm 0.4 \times 10^{-5}$ | $219 \pm 5$ | $2.1 \times 10^6 \pm 0.2 \times 10^6$ |

currently available data we are unable to explain the observed increase of cooperativity during the preincubation. One possibility is that the homodimeric form of the enzyme can undergo a very slow conformational change leaving the value of $K_i$ almost unchanged, but significantly affecting the cooperativity. In summary, Nile Blue A is a good inhibitor of yeast GR which provides the first indication of kinetic cooperativity for this native homodimeric enzyme.

### 2-Anilino-1,4-naphthoquinone

Steady-state inhibition kinetics of anilino-1,4-naphthoquinone, an analogue of menadione, with yeast GR were carried out at fixed inhibitor concentrations of 5, 10 and 15 μM and showed partial, uncompetitive inhibition of yeast GR according to the Lineweaver–Burk and secondary plot criteria described. Values of $K_i$ and $\alpha$, calculated as for Safranine O, are recorded in Table II.

### Effect of Dimethyl Sulphoxide

In our preliminary studies DMSO was used as a co-solvent to levels of up to 10% in the assay, and under these circumstances all compounds (see Table I) had one novel, common feature with respect to GR inhibition. They appeared to show a slow-onset of inhibition on monitoring the progress curve at 340 nm. This apparent slow-onset of inhibition, however, was not observed when the DMSO was omitted. In addition, the apparent slow-onset of inhibition in the presence of DMSO was no longer observed when the assay was carried out under equilibrated conditions with a preincubation period of 60–80 min. This observation led us to investigate the effect of DMSO on yeast GR. Under normal assay conditions ([GSSG] $\gg K_m$ and [NADPH] $\gg K_m$), the presence of up to 10% v/v DMSO in the assay

cuvette had no detectable rate effect on the initial velocity of the reaction. Under such conditions, DMSO does not appear to affect the velocity of the GR-catalysed reaction. When the enzyme was preincubated with GSSG in the presence of 7% DMSO for 60 min the assay activity was only slightly reduced (Table III, entry 3). However, the $K_m$ value was doubled and the value of $k_{cat}/K_m$ dropped two-fold in consequence. The apparent slow-onset of inhibition could now be partially explained by simple substrate depletion since the $K_m$ value increased in the presence of DMSO. However, this does not explain the elimination of the curved response after a preincubation period. It is likely that DMSO also induces a slow conformational change in the enzyme. This hypothesis is strongly supported by the fact that DMSO can both increase the $K_i$ value of one inhibitor (Table II, entry 3) and decrease those of others (entry 8 and 10) as well as altering the type of inhibition from parabolic to hyperbolic (entry 8). One should, therefore, cautiously interpret enzymic kinetic data of protein modification data, which have been acquired in the presence of DMSO with GR.

## Redox-Cycling

When Thionine O (5 μM) and Nile Blue A (20 μM) were tested as substrates for GR (0.7 μM) (replacing GSSG as substrate) under aerobic conditions, NADPH (0.1 mM) oxidation was linear with time and several molar equivalents of NADPH were consumed per mole of dye. Thus, these compounds probably undergo enzyme-catalysed reduction with subsequent reoxidation by $O_2$ and concomitant production of superoxide ion (redox-cycling), as observed for some quinones with trypanothione reductase[20] and for LY83583 with GR.[16] This was confirmed for Thionine O and Nile Blue A by monitoring cytochrome c reduction by the superoxide ion produced in the coupled assay described above. The most efficient of these redox-cyclers was Thionine O. This compound had an apparent $K_m$ of 18 μM and a $k_{cat}/K_m$ ratio of $2.33 \times 10^6\ M^{-1}\ min^{-1}$(see Table IV). When Thionine O (10 μM) was incubated with GR (8 nM) and NADPH (0.1 mM) for 30 min under aerobic and anaerobic conditions, respectively, and then thoroughly dialysed, no loss of enzyme activity was observed compared to a control without dyes.

## Effect on *P. berghei* Infection of Mice

*P. berghei* parasitaemia in mice injected i.p. with vehicle (Water for Injection BP, 10 ml/kg) was 62.3 ± 1.6% at the end of the experimental period 72 h

TABLE IV Apparent kinetic constants of cytochrome reduction by the superoxide ion produced in the reaction of yeast glutathione reductase and NADPH with Thionine O, Nile Blue A and LY-83583. Cytochrome c reduction was monitored at 550 nm. The experiments were done at 25°C under aerobic conditions in 0.2 M potassium phosphate buffer, pH 7.0, containing 2 mM EDTA, 20 μM cytochrome c, 120 μM NADPH, 0.7 μM GR and various dye concentrations

| *Compound* | $K_m$ [μM] | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($M^{-1} min^{-1}$) |
|---|---|---|---|
| Thionine O | $18 \pm 2$ | $42 \pm 1.7$ | $2.33 \times 10^6 \pm 0.2$ |
| Nile Blue A | $82 \pm 7$ | $21.8 \pm 1$ | $2.65 \times 10^5 \pm 0.3$ |
| LY-83583 | $158 \pm 11$ | $5.0 \pm 0.3$ | $3.2 \times 10^4 \pm 0.3$ |

after inoculation. Parasitaemia in these mice was associated with a colonic temperature of $37.5 \pm 0.1$°C, a loss in body weight of $-3.4 \pm 1.0$ g and decreased exploratory locomotor activity. Parasitaemia was significantly reduced by treatment with Methylene Blue ($46.9 \pm 5.6\%$, $P = 0.0382$), Methylene Green ($51.5 \pm 3.8\%$, $P = 0.0401$) and Nile Blue A ($52.3 \pm 1.9\%$, $P = 0.0089$) but not ($P < 0.05$) by Thionine O ($57.8 \pm 2.5\%$, $P = 0.1816$), Azure A ($54.8 \pm 2.9\%$, $P = 0.5518$) or Azure B ($55.3 \pm 10.9\%$, $P = 0.0615$) at a dose level of 5 mg/kg. The antimalarial activity of a higher dose was not assessed in this study because a single injection i.p. of 50 mg/kg of each of these compounds caused symptoms of distress, which prompted humane killing, within minutes of administration. In contrast, 5 mg/kg was, from behavioural assessments, well tolerated on repeated administration. Colonic temperature at the end of the experimental period was lower in all experimental groups ($P < 0.05$) when compared with the value of $37.5 \pm 0.1$°C for vehicle-treated malarial mice: Azure A ($36.7 \pm 0.2$°C), Azure B ($36.8 \pm 0.3$°C), Methylene Blue ($36.8 \pm 0.2$°C), Methylene Green ($36.6 \pm 0.1$°C), Nile Blue A ($35.7 \pm 0.2$°C) and Thionine O ($36.7 \pm 0.2$°C). The mean loss in body weight caused by *P. berghei* infection was less in all six experimental groups than that in control mice ($-3.4 \pm 1.0$ g), but the difference was significant ($P < 0.05$) for only Methylene Green ($-0.7 \pm 0.2$ g) and Thionine O ($-0.6 \pm 0.3$ g). Exploratory locomotor activity in all experimental groups was higher than that for control malarial animals (data not shown).

## DISCUSSION

Many parasites including the causative agents of malaria appear to be more sensitive to oxidative stress than their mammalian hosts.[5] Therefore, glutathione reductase as a thiol-generating antioxidant enzyme has been

proposed as a potential target for chemotherapy, especially in the case of malaria. Some GR inhibitors are known (e.g. some quinones,[21] BCNU-derivatives,[22,23] 2,4-dihydroxybenzylamine,[24] valproic acid,[25] oltipraz[26]) with antischistosomal action and some GR inhibitory flavin derivatives[10] have antimalarial activity. In this study, based on the observation that Safranine O and menadione bind to GR,[12] we have been able to find inhibitors for which inhibition strength has been increased by 3 and 2 orders of magnitude, respectively. Thionine O was shown to be a hyperbolic uncompetitive inhibitor ($K_i = 0.4\,\mu M$) of GR. Nile Blue A and 2-anilino-1,4-naphthoquinone are an order of magnitude less potent than Thionine O.

From our kinetic studies we cannot deduce the exact binding site(s) of these inhibitors on GR. However, we strongly believe that they interact with GR in the interface region of the homodimer, as established for Safranine O and menadione[12] and 10-substituted isoalloxazines[14] for the following reasons: (1) structural analogy, (2) related chemical reactivity, (3) identical kinetic behaviour with GR, (4) identical type of inhibition.

The flavin analogues (**13**) were mixed inhibitors of GR, competing with GSSG based on the Dixon plot, but not competing with FAD.[10] Safranine O (**1**)[12] and the flavin-based inhibitors (**13**)[10] were proposed to bind to human GR, sandwiched between the phenyl rings of F78 and F78′ in the intersubunit cavity of GR. In view of their overall structural similarity to safranine and family (**13**), it is reasonable to propose that the new inhibitors, **2–12**, bind in a similar general region. This cavity is quite large and the present study indicates that it is quite promiscuous in its selection of a binding partner. Comparison of the Safranine O (**1**) and 10-aryl-3-substituted flavins (**13**), with Thionine O (**2**), Azure A (**3**) Azure B (**4**), Methylene Green (**5**), New Methylene Blue (**6**), Acridine Yellow, (**7**), 9-aminoacridine (**8**) and gallocyanine (**11**) shows that the phenyl substituent on the central ring of the tricyclic is unnecessary for good inhibition. Previous workers found that the N10-position of isoalloxazines was tolerant of substitution for the human GR system also.[14] The ability of GR to accept a fused ring either in a tricyclic (**10**) or tetracyclic (**12**) arrangement indicates that the binding site is quite extensive and able to interact with a variety of structures, so that it should be possible to improve even on the strong inhibitions reported here.

There were no major structural changes detectable at 3 Å resolution on binding the 10-substituted isoalloxazines[14] and it was proposed that enzyme activity could be altered by such ligands by transmission of effects through a long helix to the active site.[14] The inhibition kinetics observed with some of the ligands in the present study in the presence of DMSO may shed some light on this area of potential configurational/conformational change for GR.

Nile Blue A showed a very distinct behaviour with GR from yeast. In the presence of DMSO (7% v/v) the type of inhibition was hyperbolic-uncompetitive with one site of action (Table II, entry 8), whereas without DMSO parabolic-uncompetitive inhibition at two sites was observed (Table II, entries 4–7). DMSO not only changes the inhibition from parabolic to hyperbolic, reduces the sites of action from two to one but also increases the strength of inhibition by about one order of magnitude.

DMSO, used as a co-solvent, also influenced the inhibition of GR with Thionine O as well as with 2-anilino-1,4-naphthoquinone. In the former case the $K_i$ value was raised (Table II, entries 2, 3), in the latter the $K_i$ value was lowered (Table II, entries 9, 10). Since the time of preincubation (Table III) had but little influence on the kinetic parameters, it is likely that DMSO induces a slow conformational change in the enzyme. In addition, Nile Blue A represents a very interesting inhibitor of GR from yeast, since we have observed kinetic cooperativity for it (Table II, entries 4–7) when the assay was performed without DMSO. Apart from the order of addition of substrate, co-substrate, enzyme and inhibitor in the assay, the factor that affected the cooperativity most (Table II, entry 5) was time. Although we are unable to explain the observed increase of cooperativity during preincubation with currently available data, it is likely that the homodimeric form of the enzyme can undergo a slow conformational change leaving $K_i$ almost unchanged, but significantly affecting the cooperativity. It is interesting to note that the cooperative parabolic-uncompetitive inhibition with Nile Blue A is specific for GR from yeast and does not depend on different suppliers (Sigma, Calbiochem, Boehringer). For GR from bovine intestinal mucosa the type of inhibition of Nile Blue A was clearly linear-uncompetitive ($K_i = 3\,\mu M$, data not shown).

In addition to inhibiting GR powerfully, these families of compounds are also effective redox-cyclers. The superoxide anion formed by GR and these molecules in the presence of NADPH would increase oxidative stress to potential invaders (such as *Plasmodium* sp.). Hence Thionine O as well as 2-anilino-1,4-naphthoquinone are possible lead compounds as potential antimalarials. The redox-cycling inhibition of yeast GR by LY83583 can be compared with the published data for bovine intestinal GR,[16] for which the $K_m$ for NADPH oxidation was 79.9 μM (cf. 158 μM, Table IV). The efficiency of the redox-cycling reaction (Table IV and the literature[16]) for these quinones with glutathione reductase may explain some of the toxic side-effects of quinones[16] and contribute to the overall cellular process of redox-cycling, which is dominated by the actions of NADPH-cytochrome P450 reductase and DT diaphorase.[27] The broad distribution of glutathione

reductase further augments the potential of this mechanism of toxification. Methylene Blue is known to compete 100–600 times more effectively with paraquat for reduction by flavoenzymes (xanthine oxidase, NADH cytochrome c reductase and NADPH cytochrome c reductase), leading to decreased superoxide ion formation in the presence of haem proteins.[28] This led to the proposal that Methylene Blue may serve as an antidote to paraquat poisoning although this would be limited by its rapid clearance (< 12–24 h).[28] It is possible that some of the materials in Table I would act similarly to Methylene Blue with respect to paraquat detoxification, but measurements of their toxicity and clearance are required to assess their usefulness.

There are two genes[29] now cloned from *Plasmodium*-encoding GR-like enzymes. The PfGR1 gene encodes an enzyme with thioredoxin reductase properties[30,31] The PfGR2 gene appears to encode a more GR-like enzyme.[32] It would be instructive to compare inhibition details for the materials here reported between host and parasite GR.

The water-soluble Safranine analogues Methylene Blue, Methylene Green and Nile Blue A (5 mg/kg) had a small but significant antimalarial effect *in vivo* against *P. berghei*; Thionine O, Azure A and Azure B also lowered the group mean parasitaemic count, but their effect was not significant in this study. This dose was well tolerated on repeated (×5) injection although mice developed a slight hypothermia (about −0.8°C). However, the antimalarial activity of a higher dose of these compounds was not assessed due to toxicity evident at 50 mg/kg. No positive correlation between the antimalarial activity *in vivo* and the $I_{50}$ for yeast GR activity *in vitro* (Table I) was apparent for the compounds tested. The menadione analogues 1, 8-dihydroxyanthraquinone, anthraquinone-2-carboxylate and 2-anilino-1,4-naphthoquinone were not sufficiently water-soluble to be injected in a similar formulation as the Safranine analogues for comparison. We routinely use an oily formulation containing DMSO to administer s.c. novel antimalarial drugs in this mouse model of malaria. However, it is a consistent observation in our laboratory that DMSO (4% v/v) reduces *P. berghei* parasitaemia when compared with animals receiving either no vehicle or vehicle without DMSO (10 ml/kg). Interestingly, the reduction in parasitaemia produced by DMSO is similar to that produced by the Safranine analogues, raising the possibility that the antimalarial effect of DMSO may also involve GR.

In summary, Thionine O and 2-anilino-1,4-naphthoquinone are very potent reversible inhibitors of GR. Since these compounds are also found to produce superoxide anion radicals (redox-cycling) they represented potential lead compounds as antimalarials, but our studies on *P. berghei* infection

of mice indicate that the solubility and toxicity problems associated with such compounds are too great to warrant further development. The cooperative inhibition of Nile Blue A also represents a prototype for a novel form of regulation of the activity of this enzyme that has not been reported previously in that it indicates that an appropriate natural effector ligand could make use of this kinetic cooperativity. It is possible that the DMSO-induced conformational changes found for yeast GR, if also present in human GR, may provide a probe for structural changes on binding these ligands that may be detectable by X-ray diffraction methods.

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