Chloroquine resistance of *Plasmodium berghei*: biochemical basis and countermeasures

R. I. Salganik, T. G. Pankova, T. V. Chekhonadskikh, & T. M. Igonina

Microsomal monooxygenases, enzymes that metabolize xenobiotics, may be responsible for the chloroquine resistance of malarial parasites. Plasmodium cells contain cytochrome P-450 and exhibit aryl hydrocarbon hydroxylase and aminopyrine N-demethylase activity; two monooxygenases that inactivate chloroquine. The activities of these monooxygenases are considerably higher in chloroquine-resistant strains of *Plasmodium berghei* than in the chloroquine-sensitive strain of the parasite. Inhibitors of microsomal monooxygenases have the potential to overcome the chloroquine resistance of *Plasmodium spp.*, and, of those inhibitors tested, the copper-lysine complex, copper(lysine)₄, was the most effective.

The spread of strains of *Plasmodium falciparum* that are resistant to chloroquine and other antimalarial drugs is one of the main reasons for the resurgence of malaria and for the problems in treating the disease (1). Also the basis for the drug resistance is unclear in most cases, and this makes it difficult to develop countermeasures.

The selection and wide distribution of *Plasmodium* spp. mutants with elevated drug metabolic rates has been proposed as a possible cause of the observed chloroquine resistance of the malarial parasite. Elimination of hydrophobic xenobiotics from eukaryotic cells involves microsomal monooxygenases, of which cytochrome P-450 plays a central role (2). In this enzymic oxidation the xenobiotics are converted into more polar metabolites, ensuring their ultimate elimination. Chloroquine is also inactivated by N-demethylation and hydroxylation by monooxygenases such as aminopyrine N-demethylase (AND) and aryl hydrocarbon hydroxylase (AHH).

Mutations that increase the activity of plasmodium microsomal monooxygenases might therefore accelerate the inactivation and elimination of chloroquine. Thus monooxygenase inhibitors potentially could be used to restore the parasite's sensitivity to chloroquine and other antimalarials. Furthermore, inhibition of liver and other monooxygenases may prolong the effect of chloroquine by suppressing its metabolism and thus maintaining a high blood level of the drug.

**MATERIALS AND METHODS**

**Strains of *P. berghei* and their laboratory passage**

The following laboratory strains of *Plasmodium berghei* were used in the study: the chloroquine-sensitive S-strain; the LNK-65 strain that exhibits a two- to three-fold spontaneous reduction of chloroquine sensitivity; and the CR strain that has a 7- to 10-fold induced resistance to chloroquine. These strains were maintained by weekly syringe passage through mice aged 1.5 months. The mice were inoculated intraperitoneally with 10⁷ parasitized erythrocytes isolated from the blood of mice infected with *P. berghei*. In order to retain induced resistance to the CR strain, donor mice infected with the parasite were treated with chloroquine phosphate (500 mg per kg single oral dose) 24 hours before inoculation. Blood was drawn from mice 8 days after inoculation with the chloroquine-sensitive strain of *P. berghei* and 14-15 days after inoculation with the chloroquine-resistant strains.

* Strains were provided by the Martzinsky Institute of Medical Parasitology and Tropical Medicine, USSR Ministry of Health, Moscow.
Isolation of P. berghei from the infected blood

Blood was collected in tubes containing a 3.8% solution of sodium citrate. The tubes were then centrifuged at 600 g for 10 minutes and the precipitate suspended and washed twice in five volumes of a sodium chloride/glucose phosphate buffer (pH 7.4). The erythrocytes were lysed in a solution of saponin (0.14 g/l) to release the parasite. The pellet was resuspended in a 0.25 mol/l solution of sucrose to remove haemoglobin and centrifuged at 10 000 g for 15 minutes. The resultant pellet was homogenized and used for subsequent assays.

Enzyme assays

Aryl hydrocarbon hydroxylase. The activity of aryl hydrocarbon hydroxylase (AHH) was determined as described previously (3). The incubation mixture contained NADPH (1 mol/l), magnesium chloride (5 mmol/l), tri(hydroxymethyl)aminomethane hydrochloride (TRIS-hydrochloride) (50 mmol/l), 600 µg heptic microsome protein or plasmodial homogenate, and 80 µmol/l ^3H-benz[a]pyrene (46.25 × 10⁶ Bq/µmol).

The radioassay for benz[a]pyrene monoxygenase relies on a single extraction to separate the metabolites, mainly ^3H-3-hydroxybenz[a]pyrene, from the residual substrate. Separations were performed in dimethyl sulfoxide, while for scintillation counting the fluid described by Bray was used (4).

Aminopyrine N-demethylase. The activity of aminopyrine N-demethylase (AND) was determined as described by Anchakov et al. (5). The incubation mixture contained 3 mmol/l NADPH, 100 mmol/l TRIS-hydrochloride (pH 7.5), 1.5 mmol/l aminopyrine, 5 mmol/l magnesium chloride, and 0.2-1.0 mg of pellet. The reaction was carried out at 37 °C and was stopped after 20 minutes by adding a 5% solution of trichloroacetic acid and the amount of formaldehyde in the supernatant was determined (6).

The activity of AH2 was expressed as the number of pmoles of 3-hydroxybenz[a]pyrene formed per mg of protein and that of AND as the number of pmoles of formaldehyde per mg of protein. The significance of the differences was evaluated using Student’s t test (7).

Removal of leukocytes. Samples of infected cells were passed through a Whatman CC-31 cellulose column, as previously described (8), to remove leukocytes.

Induction of reticulocytosis

Reticulocytosis was induced in mice by intraperitoneal injection of 20 mg/kg phenylhydrazine each day for 8 days (9). Mice were killed 48 hours after the last phenylhydrazine inoculation, and microscopic examination showed that by this time nearly 90% of the erythrocytes were reticulocytes. The lysate of the reticulocytes was assayed for AHH activity.

Isolation of microsomes from P. berghei

Fifty to sixty mice aged 3–4 weeks (weight range 12–15 g) were infected with a high dose of P. berghei. Each mouse was injected intraperitoneally with undiluted blood (0.1 ml per mouse) from mice with 60–80% parasitaemia. Three to four days after infection with the parasite, before the number of leukocytes and reticulocytes had increased, and when the level of parasitaemia did not exceed 50–80%, samples of blood were taken from the mice. P. berghei was freed from the infected blood by addition of 0.01 g saponin in 100 ml of a 0.01 mol/l phosphate buffer containing 0.01% magnesium chloride and 0.9% sodium chloride. The pellet of freed parasites obtained after centrifugation (600 g) was washed with buffer and homogenized mechanically in a 0.25 mol/l solution of sucrose for 1 minute. The homogenate was then centrifuged twice at 15 000 g for 25 minutes, and the microsomes were sedimented at 105 000 g for 1 hour. Finally, the microsomes obtained were resuspended in a 0.25 mol/l solution of sucrose containing 25% (v/v) glycerine and stored at −20 °C till used.

Spectroscopic studies

The absorption spectra of the microsomes were recorded between λ = 400 nm and λ = 500 nm at room temperature. The procedure used has been described previously (10); the absorption of cytochrome b₅ at these two wavelengths was obtained by addition of the reducing agent sodium hydrosulfite to the microsomes in the sample cuvette; the presence of oxyhaemoglobin was detected by gassing the suspension with carbon monoxide for 1–2 minutes; and the absorption spectrum of cytochrome P-450 was obtained by addition of sodium hydrosulfite to both the sample and reference cuvettes, and by gassing only the sample cuvette with carbon monoxide. The concentration of protein was determined as described by Lowry et al (11) and that of DNA as described by Burton (12).

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9 Hitachi-556 spectrophotometer.
Table 1. Activity of aryl hydrocarbon hydroxylase (AHH) in the haemolysates of P. berghei-infected and non-infected mice

<table>
<thead>
<tr>
<th>Subjects</th>
<th>AHH activity[^a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected mice</td>
<td>26 ± 26</td>
</tr>
<tr>
<td>Infected mice</td>
<td></td>
</tr>
<tr>
<td>5 days after infection</td>
<td>26 ± 19</td>
</tr>
<tr>
<td>8 days after infection</td>
<td>170 ± 30[^b]</td>
</tr>
</tbody>
</table>

[^a] Activities are expressed in pmol 3-H-3-hydroxybenz(a)pyrene per mg protein.
[^b] Statistically different from the values for non-infected mice and infected mice 5 days after infection at the P<0.05 level (n = 5).

Reagents

The following reagents were used in the study: compound S (17α-hydroxydeoxyxycorticosterone), compound S acetate (17α-hydroxydeoxyxycorticosterone 21-acetate)[^c] and 4-bromomethyl-2,2,5,5-tetramethyl-3-imidazoline-3-oxide-N-oxyl[^d] (RBr). The copper-lysine complex, copper(lysine)₂, was synthesized as described previously [13]. Chloramphenicol was used as the standard drug. Phenylhydrazine was obtained commercially as were benzo[a]pyrene and saponin.

RESULTS

The activity of AHH was assayed in the homogenate of saponin-lysed erythrocytes obtained from the blood of mice infected with the chloroquine-sensitive strain of P. berghei (strain S). There was no measurable AHH activity in the erythrocytes of the control and infected mice on day 5 after the infection, when parasitaemia was only poorly developed; in contrast, high AHH activity was measured on day 8, when parasitaemia reached maximum values (Table 1). The number of leukocytes in the blood of infected mice increased; however, there was no relation between the number of leukocytes in the infected blood and AHH activity. Thus, the leukocyte count on day 5 after infection was 10- to 20-fold higher than in the infected mice than in the controls, although the two groups exhibited the same AHH activity. Leukocytes were removed by passing blood through a Whatman CC-31 cellulose column [8]. This procedure ensured their complete removal, as indicated by microscopy, without affecting the AHH activity.

The development of P. berghei infection in mice is associated also with reticulocytosis [14]. To determine whether the measured AHH activity in P. berghei-infected blood was due to reticulocyte debris in the lysates, we induced reticulocytosis by administering phenylhydrazine to the mice. Blood from these mice exhibited no AHH activity after it had been treated with saponin. Radioassay of protein (600 mg) for AHH activity gave 2151 ± 667 cpm for the background controls, 2190 ± 240 cpm for intact mice, and 1975 ± 14 cpm for mice with reticulocytosis.

Hydroxylation of benzo[a]pyrene in the plasmodium homogenate was inhibited by metyrapone, a highly specific inhibitor of monoxygenases [15]. Addition of metyrapone to the plasmodium homogenate at the same concentration as the substrate (10⁻⁴ mol/l) significantly inhibited AHH activity, while an increase in the concentration of metyrapone to 10⁻³ mol/l further decreased the AHH activity (Fig. 1).

Fig. 1. Activity of aryl hydrocarbon hydroxylase (AHH) in haemolysates from P. berghei-infected mice in the presence of metyrapone. (a) Control; (b) 10⁻⁴ mol/l metyrapone added to the incubation mixture; and (c) 10⁻³ mol/l metyrapone added to the incubation mixture.

[^c] A gift from Dr. A. V. Samerovskaya, Institute of Organic Chemistry, USSR Academy of Sciences, Moscow, USSR.
[^d] Provided by Institute of Organic Chemistry, USSR Academy of Sciences, Novosibirsk, USSR.
Fig. 2. Absorption spectra between \( \lambda = 400 \) nm and \( \lambda = 500 \) nm of microsomes isolated from *Plasmodium berghei*. (a) After addition of sodium hydrosulphite to the sample cuvette; (b) after addition of sodium hydrosulphite and carbon monoxide to the sample cuvette and of sodium hydrosulphite to the reference cuvette; and (c) peak at \( \lambda = 459 \) nm shown on an expanded scale.

\[ \lambda = 420 \text{ nm and the other weak at } \lambda = 459 \text{ nm. The peak at } \lambda = 420 \text{ nm was not due to oxhaemoglobin impurities in the microsomal preparations, and passing the sample cuvette with carbon monoxide did not produce a signal at this wavelength.} \]

Comparison of the activities of monooxygenases from strains of *P. berghei* of different chloroquine sensitivity indicated that the activity of AHH and AND was much greater in the more chloroquine-resistant strains and that the degree of resistance was related to the monooxygenase activity (Table 2). In order to investigate whether inhibitors of plasmid monooxygenases could be used to overcome the chloroquine resistance of some strains of *P. berghei*, the following microsomal monooxygenase inhibitors that interact specifically with cytochrome P-450 were tested: compound S and its acetate (16), RBr (17), chloramphenicol (18), phenylhydrazine (19), and the copper complex copper(lysine) (20). The inhibitory effect of these compounds on AHH activity in mouse liver microsomes was determined in *vitro*. Because the structure of P-450 is similar in different eukaryotic cells, the data obtained could be helpful in selecting the appropriate inhibitors of monooxygenases for *Plasmodium* spp. The results in Table 3 indicate that the most effective inhibitors of mouse liver microsomal monooxygenases were the copper-lysine complex and phenylhydrazine; the others had a much weaker, yet appreciable, effect. The effects of these compounds on the activity of AHH in *P. berghei* was then investigated. In this case, compound S and its acetate had no inhibitory effect on the AHH activity of the parasite when present at the same concentration as the substrate; RBr exhibited a slight inhibitory effect; and chloramphenicol inhibited

<table>
<thead>
<tr>
<th>Strain</th>
<th>AHH activity ( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} )</th>
<th>AND activity ( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>125 ± 5.9</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>LNK-65</td>
<td>195.6 ± 12.5</td>
<td>–</td>
</tr>
<tr>
<td>CR</td>
<td>254.6 ± 29.7</td>
<td>7.9 ± 1.3</td>
</tr>
</tbody>
</table>

- \( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} \)
- \( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} \)
- Statistically different from the values for the N strain at the \( P > 0.95 \) level \( n = 5 \).

Table 3. Inhibition of activity, relative to that in controls, of aryl hydrocarbon hydroxylase (AHH) in mouse liver microsomes and in *Plasmodium berghei* cells by the substances tested in the study.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (mol/l)</th>
<th>Mouse liver microsomes ( % ) Activity</th>
<th>Plasmodium berghei ( % ) Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound S</td>
<td>( 10^{-4} )</td>
<td>86.5 ± 0.4 ( P &gt; 0.99 )</td>
<td>124.0 ± 25.0</td>
</tr>
<tr>
<td>Compound S acetate</td>
<td>( 10^{-4} )</td>
<td>79.0 ± 2.6 ( P &gt; 0.99 )</td>
<td>93.0 ± 22.0</td>
</tr>
<tr>
<td>RBr</td>
<td>( 10^{-4} )</td>
<td>73.4 ± 5.2 ( P &gt; 0.95 )</td>
<td>72.2 ± 2.2 ( P &gt; 0.95 )</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>( 4 \times 10^{-4} )</td>
<td>66.8 ± 4.0 ( P &gt; 0.999 )</td>
<td>68.2 ± 6.5 ( P &gt; 0.999 )</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>( 0.25 \times 10^{-4} )</td>
<td>3.0 ± 3.0 ( P &gt; 0.999 )</td>
<td>0.3 ± 0.3 ( P &gt; 0.999 )</td>
</tr>
<tr>
<td>Copper(lysine)</td>
<td>( 10^{-4} )</td>
<td>5.7 ± 4.1 ( P &gt; 0.999 )</td>
<td>0 ( P &gt; 0.999 )</td>
</tr>
</tbody>
</table>

- Activity of AHH in the controls (without inhibitor) was 888.1 ± 151.6 \( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} \).
- Activity of AHH in the controls (without inhibitor) was 130.9 ± 23.0 \( \mu \text{mol} \text{H}_2 \text{O}_2 \text{H-lysine per mg protein} \).
- Figures in parentheses indicate the level at which the result differed statistically from that of the control value.
- With *P. berghei*, copper(lysine) was used at a concentration of \( 10^{-5} \) mol/l.
AHH activity only by 30% when used at four times the concentration of the substrate (Table 3). In contrast, copper(lysine)$_2$ and phenylhydrazine completely suppressed the AHH activity of P. berghei, even when present at concentrations lower than that of the substrate (Table 3).

In further studies, we examined the abilities of copper(lysine)$_2$, phenylhydrazine, and chloramphenicol to inhibit monoxygenases in the whole parasite and to prolong chloroquine activity in the host. For this purpose, we tested the effects of these three compounds on the AHH activity in vivo of mouse liver microsomes (Table 4). The results indicated that all three compounds markedly inhibited AHH activity 30 minutes after their injection. Interestingly, the inhibitory effect of phenylhydrazine and copper(lysine)$_2$ persisted up to 24 hours after injection, but that of chloramphenicol was shorter.

**DISCUSSION**

The selection of *Plasmodium* mutants with enhanced enzymic drug-metabolism may lead to the development of their resistance to chloroquine. *Plasmodium* spp. have a well-formed endoplasmic reticulum; however, to our knowledge, it has not previously been reported that, like other eukaryotes, the endoplasmic reticulum of *P. berghei* contains a system of monoxygenases involving cytochrome P-450 (21). In the experiments described, we determined the activity of the microsomal monoxygenases AHH and AND in the blood of mice infected with *P. berghei* that had developed parasitaemia. We also showed that AHH activity is not associated with the presence of leucocytes or reticulocytes but with the malarial parasite itself. The results obtained with metyrapone, a specific competitive inhibitor of microsomal monoxygenases, confirmed that benz[a]pyrene is hydroxylated by the microsomal mono-

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<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Dose (mg/kg body weight)</th>
<th>30 minutes</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>130</td>
<td>62.0 ± 11.1$^*$</td>
<td>110.7 ± 8.8</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>60</td>
<td>67.8 ± 6.2$^*$</td>
<td>74.9 ± 2.3$^*$</td>
</tr>
<tr>
<td>Copper(lysine)$_2$</td>
<td>20</td>
<td>48.7 ± 4.8$^*$</td>
<td>63.3 ± 7.0$^*$</td>
</tr>
</tbody>
</table>

$^*$ Different statistically from that of the control value at the P>0.999 level.

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$^a$ See footnote a, p. 381.
When injected in vivo, phenylhydrazine, the copper complex, and chloramphenicol inhibited the AHH activity of mouse liver microsomes.

The monoxygenase inhibitors described here decrease the metabolic degradation and excretion of chloroquine and thereby maintain high concentrations of the drug in the host. This novel approach appears to offer promise for the chemotherapy of malaria in patients infected with chloroquine-resistant strains of Plasmodium spp.

RÉSUMÉ

RÉSISTANCE À LA CHLOROQUINE DE PLASMODIUM BERGHEI:
BASES BIOCHIMIQUES ET MOYENS DE LA COMBATTRE

Les monoxygénases microsomales, enzymes qui métabolisent les composés xenobiotiques, pourraient être responsables de la résistance à la chloroquine chez la plasmodie, dont le cellule contient du cytochrome P-450 et présente une activité d’arylhydrocarbon-hydroxylase et d’aminopyrine N-diméthylase, deux monoxygénases qui activent la chloroquine. L’activité de ces monoxygénases est beaucoup plus intense chez les souches chloroquinorésistantes de Plasmodium berghei que chez la souche chloroquine-sensible du même parasite. Les inhibiteurs des monoxygénases microsomales ont donc la possibilité d’abolir la résistance à la chloroquine chez Plasmodium spp. et parmi ceux que nous avons expérimentés, c’est le complexe de cuivre et de lysine, CM(lysine), qui s’est révélé le plus efficace.

REFERENCES

12. BURTON, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric esti-