Primaquine-Induced Hemolytic Anemia: Susceptibility of Normal versus Glutathione-Depleted Rat Erythrocytes to 5-Hydroxyprimaquine

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ABSTRACT

Primaquine is an important antimalarial agent because of its activity against exoerythrocytic forms of Plasmodium spp. Methemoglobinemia and hemolytic anemia, however, are dose-limiting side effects of primaquine therapy. These hemotoxic effects are believed to be mediated by metabolites, although the identity of the toxic specie(s) and the mechanism underlying hemotoxicity have remained unclear. Previous studies showed that an N-hydroxylated metabolite of primaquine, 6-methoxy-8-hydroxy-laminoquinoline, was capable of mediating primaquine-induced hemotoxicity. The present studies were undertaken to investigate the hemolytic potential of 5-hydroxyprimaquine (5-HPQ), a phenolic metabolite that has been detected in experimental animals. 5-HPQ was synthesized, isolated by flash chromatography, and characterized by NMR spectroscopy and mass spectrometry. In vitro exposure of 51Cr-labeled erythrocytes to 5-HPQ induced a concentration-dependent decrease in erythrocyte survival (TC50 of ca. 40 μM) when the exposed cells were returned to the circulation of isologous rats. 5-HPQ also induced methemoglobin formation and depletion of glutathione (GSH) when incubated with suspensions of rat erythrocytes. Furthermore, when red cell GSH was depleted (>95%) by titration with diethyl maleate to mimic GSH instability in human glucose-6-phosphate dehydrogenase deficiency, a 5-fold enhancement of hemolytic activity was observed. These data indicate that 5-HPQ also has the requisite properties to contribute to the hemotoxicity of primaquine. The relative contribution of N-hydroxy versus phenolic metabolites to the overall hemotoxicity of primaquine remains to be assessed.

Malaria is a widespread, life-threatening parasitic disease that is responsible for 300 to 500 million acute illnesses and an estimated 1.5 to 2.7 million deaths worldwide each year (Kain and Keystone, 1998). Primaquine, an 8-aminoquinoline antimalarial drug, is effective against the exoerythrocytic forms of all four of the malarial species that infect humans and is the only radically curative drug for the latent tissue forms of Plasmodium vivax and Plasmodium ovale (Tracy and Webster, 2001). Primaquine is also used in combination with chloroquine to combat the problem of multiple drug resistance in Plasmodium falciparum (Shanks et al., 2001). Despite its clinical importance and effectiveness, use of primaquine has long been known to be limited by its capacity to induce hemolytic anemia, particularly in individuals with a hereditary deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity (Dern et al., 1955; Degowin et al., 1966). Because G6PD deficiency is prevalent in malarial areas, this dose-limiting toxicity can have a major impact on the usefulness of this drug in these populations.

Importantly, primaquine is not directly toxic to erythrocytes at clinically relevant concentrations. Although the hemotoxicity of primaquine has long been considered to be dependent on metabolism, the metabolite(s) responsible and the underlying mechanism(s) have remained unclear. We have reported recently that 6-methoxy-8-hydroxy-laminoquinoline, an N-hydroxylated metabolite of primaquine, is a direct-acting hemolytic and methemoglobinemic agent in rats, and therefore may be a contributor to the hemotoxicity observed in primaquine-treated humans (Bolezho et al., 2001). Metabolism of primaquine, however, is relatively complex, and a variety of known and putative phenolic metabolites have also been considered to be capable of mediating prima-
5-Methoxyprimaquine (5,6-dimethoxy-8-[4-amino-1-methylbutylamino]quinoline) was prepared from 6-methoxy-8-nitroquinoline as described previously (Elderfield et al., 1955). 5-HPQ (5-hydroxy-6-desmethylprimaquine) was synthesized from 5-methoxyprimaquine by HBr-catalyzed hydrolysis using a modification of an established method (Allahyari et al., 1984). The composition of the reaction mixture was monitored as a function of time via liquid chromatography-mass spectrometry. The reaction mixture contained four major components: 5-HPQ (m/z 275.5–276.5; 4.60 min, 21.0%), 5-HPQ quinoneimine (m/z 273.5–274.5; 4.41 min, 42.8%), 5-methoxyprimaquine (m/z 289.5–290.5; 9.58 min, 21.1%), and 5-hydroxy-6-desmethylprimaquine (m/z 259.5–260.5; 4.09 min, 15.1%). The yield of 5-HPQ was optimized by adjusting the reaction temperature to 120°C and the reaction time to 20 min. The yield was increased further by reducing the quinoneimine to the hydroquinone using sodium dithionate and maintaining the reaction mixture under argon to minimize oxidation of the hydroquinone. The reaction mixture was then purified in two steps using SepPak Plus C18 cartridges (Waters Corporation, Milford, MA). 5-HPQ was eluted from the first cartridge with 5% acetonitrile/0.05% aqueous trifluoroacetic acid, lyophilized, and then applied to a second cartridge. 5-HPQ was eluted from the second cartridge with 5% acetonitrile in water containing 5 mM HBr. After removal of the solvent by lyophilization, elemental analysis confirmed the presence of the trihydrobromide salt of 5-HPQ (purity >99% as judged by HPLC and NMR analysis).

Progress toward understanding the role of phenolic metabolites in primaquine-induced hemolytic anemia has been hampered because they are no longer available, the synthetic methods to prepare them are relatively difficult, and the products are highly unstable. As a first step in our investigation of the potential contribution of phenolic metabolites to primaquine-induced hemolytic anemia, we have synthesized 5-HPQ and examined its stability and redox behavior. In addition, we have assessed the hemolytic potential of 5-HPQ in GSH-normal and GSH-depleted rat red cells. In view of the critical role proposed for oxidative stress in the mechanism underlying primaquine-induced hemolytic anemia, we measured the formation of methemoglobin and monitored red cell sulfhydryl status under hemolytic conditions to correlate the hemolytic response with these indicators of intracellular oxidative damage. We report that 5-HPQ is an extremely potent direct-acting hemolytic agent in rats and that hemolytic activity is associated with methemoglobin formation and a marked depletion of erythrocytic GSH. When GSH was depleted from rat red cells to mimic GSH instability of human G6PD-deficient red cells (Gaetani et al., 1979), the hemolytic activity of 5-HPQ was markedly enhanced. The significance of the data with regard to the overall contribution of metabolites to primaquine-induced hemolytic anemia is discussed.

**Materials and Methods**

**Chemicals and Materials.** 6-Methoxy-8-nitroquinoline, ferrous bromide, sodium stannous metal, potassium trifluoroacetate, and GSH were obtained from Sigma-Aldrich (St. Louis, MO). Na$_{51}$CrO$_4$ in sterile saline (1 mM/mL, pH 8) was obtained from New England Nuclear (Billerica, MA). All other chemicals and reagents were of the best grade commercially available.

**Fig. 1.** Putative metabolism of primaquine to 5-HPQ.
hauser effect spectroscopy (NOESY) experiment was acquired in the phase sensitive mode by collecting 2 × 256 fids. Digital resolution in F1 was increased by linear prediction to 1024 points, processed using the GERT transform function, and then Fourier transformed. Presence of a methoxy group in the 6-position of 5-HPQ was verified by the NOESY experiment.

Mass spectra were obtained using a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). A 150-mm Alltech Platinum EFS C18 reverse phase column was used. The sample was eluted with 10% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min. The column effluent was split and 10% was directed to the ESI source. Instrument parameters were as follows: ESI needle voltage, 4.5 kV; ESI capillary temperature, 200°C; ion energy, 45%; isolation window, 1 amu; and scan range, 150.0 to 1000.0 amu. Mass spectrometry and tandem mass spectrometry data were acquired automatically using Xcalibur software (version 1.2).

Electrochemical Activity of 5-HPQ. Cyclic voltammetry was performed using a CV-27 voltammograph (BAS Bioanalytical Systems), C-1A/B cell stand, and a model RXY recorder. Stock solutions of 5-HPQ (245 μM) were prepared in argon-purged isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM d-glucose (PBSG). Samples were scanned at a rate of 150 mV/s at room temperature under an argon atmosphere using a carbon-paste working electrode, a platinum auxiliary electrode and a Ag/AgCl reference electrode.

Animals and Erythrocyte Incubation Conditions. Male Sprague-Dawley rats (75–100 g) were purchased from Harlan (Indianapolis, IN) and maintained on food and water ad libitum. Animals were acclimated for 1 week to a 12-h light/dark cycle before their use. Blood from the descending aorta of anesthetized rats was collected into heparinized tubes and washed three times with PBSG to remove the plasma and buffy coat. The cells were resuspended to a 40% hematocrit in PBSG and used on the same day they were collected. Stock solutions of 5-HPQ in argon-purged water were prepared to deliver the appropriate concentration of 5-HPQ in 10 μl to erythrocyte suspensions (1–3 ml).

Measurement of Hemolytic Activity. The survival of rat 51Cr-labeled red cells was determined in vivo after in vitro incubation with various concentrations of 5-HPQ (25–300 μM). After incubation for 2 h at 37°C, the erythrocytes were washed once and resuspended in PBSG (40% hematocrit). Aliquots (0.5 ml) were administered intravenously to isologous rats. T0 blood samples were taken from the orbital sinus 30 min after administration of labeled red cells. Additional blood samples were taken every 48 h for 14 days. At the end of the experiment, the samples were counted in a well-type gamma counter, and the data were expressed as a percentage of the T0 blood sample. The hemolytic response was quantified by calculating the fraction of radiolabeled red cells that were removed from the circulation within the first 48 h for each animal by linear regression as described previously (McMillan et al., 2001). Statistical significance was determined with the use of Student’s t test.

Determination of Methemoglobin Formation and Sulphydryl Status. Methemoglobin levels in erythrocyte suspensions treated with 5-HPQ (25–1000 μM) were measured using a modification of the spectrophotometric technique of Evelyn and Malloy (1938) as described previously (Harrison and Jollow, 1987).

For determination of sulphydryl status, aliquots (200 μl) of the erythrocyte suspensions were removed at various intervals after addition of 5-HPQ and assayed for GSH, oxidized glutathione (GSSG), and glutathione-protein mixed disulfides (PSSG) by HPLC with electrochemical detection (HPLC-EC) as described previously (Grossman et al., 1992). The amount of sulphydryl present in the samples was determined by comparison of peak areas to prepared standards.

GSH Depletion of Erythrocyte Suspensions. Diethyl maleate (DEM) was used to deplete GSH in red cell suspensions as described previously (Bolchoz et al., 2002). Briefly, DEM (750 μM) dissolved in acetone was added to packed red cells. After a 15-min incubation at 37°C, the red cells were analyzed for GSH content by HPLC-EC as described above. Under these conditions, GSH was reduced to about 40% of initial levels. The cells were resuspended to a 40% suspension in PBSG and used on the same day that they were collected.

Results

Stability and Electrochemistry of 5-HPQ. NMR studies undertaken as part of the characterization of the newly synthesized 5-HPQ indicated that it was stable for over 24 h when maintained at low pH under strictly anaerobic conditions. This indicated that it could be prepared and kept as a solution without significant degradation before its experimental use in erythrocyte suspensions. On the other hand, previous work had shown 5-HPQ to be unstable in the presence of oxygen (at pH 8.5) due to its facile conversion into its quinoneimine form (Vasquez-Vivar and Augusto, 1990).

Therefore, to determine the stability of the 5-HPQ hydroquinone/quinoneimine redox pair under our experimental conditions, 5-HPQ (500 μM) was added to aerobic PBSG (pH 7.4) in the absence and presence of red cells. Aliquots were withdrawn at intervals, treated with an excess of sodium dithionite, and then assayed for 5-HPQ by HPLC-EC (Fig. 2). Rapid loss of 5-HPQ occurred in both situations with a half-life of about 45 s in the absence of red cells and about 30 s in their presence. Because the hydroquinone and quinoneimine forms of 5-HPQ were not well separated on the HPLC-EC column, LC-MS analysis (in which both halves of the redox pair could be detected independently by selected ion monitoring) confirmed that the disappearance of 5-HPQ was not due simply to its oxidation to the quinoneimine during chromatographic analysis, but instead was due to the complete degradation of the redox pair (data not shown).

Previous studies have shown that in the presence of an excess of NADPH and a catalyst (ferredoxin:NADP+ oxidoreductase), 5-HPQ can generate greater than stoichiometric amounts of hydrogen peroxide (Vasquez-Vivar and Au-
Various concentrations of 5-HPQ for that 37 s response curve for 5-HPQ was extremely sharp, with an apparent concentration dependence of hemolytic activity of 5-HPQ. The concentration-response curve observed. These data are consistent with a concerted oxidation of 5-HPQ hydroquinone to 5-HPQ quinoneimine (peak B) via a semiquinone radical intermediate (peak A), as suggested by previous work (Vasquez-Vivar and Augusto, 1992). Peak C corresponds to the reduction of the oxidized products formed on the forward scan back to 5-HPQ hydroquinone. These electrochemical data support the concept that 5-HPQ can undergo cycling as a fully reversible redox couple at physiological pH.

Direct Hemolytic Activity of 5-HPQ. Although a variety of studies on the oxidative activity of 5-HPQ in red cells have been published, its direct hemolytic activity has not been established. To investigate the hemolytic potential of 5-HPQ, rat 51Cr-labeled erythrocytes were incubated with various concentrations of 5-HPQ for 2 h at 37°C. The cells were then washed and returned to the circulation of isologous rats. A T0 blood sample was taken from the orbital sinus 30 min after administration of the labeled red cells and then serial blood samples were taken at 48-h intervals for 14 days. As shown in Fig. 4A, exposure of the labeled cells to 5-HPQ caused a concentration-dependent increase in the rate of removal of radioactivity from the circulation compared with controls. Figure 4B shows the concentration response curve for the hemolytic activity of 5-HPQ. The concentration-response curve for 5-HPQ was extremely sharp, with an apparent threshold concentration of about 25 μM, a TC50 of approximately 40 μM, and a maximal response at about 100 μM.

Methemoglobin Formation by 5-HPQ. 5-HPQ has been previously shown to deplete red cell GSH and induce methemoglobin formation (Allahyari et al., 1984; Link et al., 1985; Baird et al., 1986; Agarwal et al., 1988; Fletcher et al., 1988; Vasquez-Vivar and Augusto, 1994). To determine the relationship between these endpoints and the hemolytic response, we examined the time and concentration dependence of methemoglobin formation in rat erythrocyte suspensions exposed to 5-HPQ. As shown in Fig. 5A, incubation of a rat red cell suspension with a maximal hemolytic concentration of 5-HPQ (100 μM) resulted in the rapid formation of methemoglobin. This concentration produced a peak methemoglobin level of only about 20%, which nevertheless remained constant over the 2-h incubation period. Figure 5B depicts the concentration dependence of the methemoglobinemic response to 5-HPQ at 30 min post-exposure. Methemoglobin levels ranged from approximately 3.5% at 25 μM 5-HPQ to a maximum of about 40% at 300 μM (TC50 of ca. 100 μM).

Effect of 5-HPQ on Rat Erythrocyte Sulphhydryl Status. To examine the fate of red cell GSH after treatment with hemolytic concentrations of 5-HPQ, aliquots were taken at various intervals and analyzed for GSH, GSSG, and PSSG levels by HPLC-EC. As shown in Fig. 6Aa, addition of 100 μM 5-HPQ to rat red cells resulted in a complete depletion of GSH within 15 min. The loss of GSH was matched by an increase in PSSG; GSSG remained low throughout the incubation period. The concentration dependence of the 5-HPQ-induced depletion of GSH is shown in Fig. 6B. As with the hemolytic response (Fig. 4B), a sharp concentration-response curve was observed, with a TC50 of approximately 40 μM.

Hemolytic Activity of 5-HPQ in GSH-Depleted Erythrocytes. The enhanced susceptibility displayed by G6PD-deficient individuals to primaquine-induced hemolytic anemia is thought to be due to an inability to maintain sufficient levels of NADPH, and thus reduced glutathione, in response to the oxidative stress. To reproduce in rat erythrocytes the instability of GSH known to occur in human G6PD-deficient erythrocytes, 51Cr-labeled red cells were titrated with DEM to deplete GSH by >95%. The GSH-depleted red cells were then exposed to...
various concentrations of 5-HPQ in vitro for 2 h at 37°C, and their survival was determined in vivo. As shown in Fig. 7A, the survival of untreated GSH-depleted red cells (T_{50} = 11.0 ± 1.9 days) was not significantly different from the survival of GSH-normal red cells (Fig. 4A; T_{50} = 9.8 ± 0.8 days). As expected from the previous experiment, the rate of removal of GSH-normal red cells exposed to a subhemolytic concentration of 5-HPQ (10 \mu M) was also not significantly different from the controls (Fig. 7A). In contrast, exposure of GSH-depleted red cells to a 10 \mu M concentration of 5-HPQ provoked a dramatic increase in their rate of removal. Quantitation of the hemolytic response for GSH-depleted red cells (Fig. 7B) revealed the concentration-response curve to be shifted significantly to the left of the response curve for GSH-normal cells (Fig. 4B), with a TC_{50} under these conditions of about 7.5 \mu M.

**Discussion**

Oxidative metabolism has long been known to play a critical role in the onset of primaquine-induced hemotoxicity, and phenolic metabolites have been considered the most likely candidates for mediating both the hemolytic and methemoglobinemic responses that have been observed during the course of therapy with this antimalarial drug. Considerable attention has been given to the 5-hydroxy- and 5,6-dihydroxy metabolites of primaquine because they have the potential to redox cycle (via quinoneimine and 5,6-quinone formation, respectively) and generate reactive oxygen species. Support for the importance of phenolic metabolites has come from a variety of in vitro studies that showed that these compounds were able to induce oxidative changes within red cells, such as stimulation of hexose monophosphate shunt activity, GSH depletion, and hemoglobin oxidation. What has been missing from these efforts, however, is evidence that links these biochemical changes observed in vitro to loss of erythrocyte viability in vivo.

The present results demonstrate that a redox active phenolic metabolite of primaquine, 5-HPQ, is a direct-acting hemolytic agent in the rat (Fig. 4). This loss of erythrocyte viability in vivo was correlated with a rapid and extensive depletion of GSH (Fig. 6A), which exhibited a concentration...
dependence that coincided with that of the hemolytic response (Fig. 6B). The disappearance of GSH was matched by the formation of mixed disulfides between GSH and the soluble protein of the red cell. The importance of GSH status in determining the sensitivity of rat red cells to this hemolytic agent is illustrated by the data in Fig. 7A, which shows that depletion of GSH with DEM before 5-HPQ exposure caused a marked enhancement of the hemolytic response. These data strongly support the concept that the hemolytic response has a discrete dose threshold and that this threshold is dependent on the presence GSH in the red cell.

Although the in vitro exposure/in vivo survival data presented in Fig. 4 do not allow for a direct assessment of the role of 5-HPQ in primaquine hemotoxicity, this assay does permit the hemolytic damage observed in vivo to be reproduced in vitro under controlled conditions during a 2-h incubation period before the red cells are returned to the circulation of rats, and thus serves as a useful indicator of the relative potency among direct-acting hemolytic agents. Interestingly, 5-HPQ is the most potent hemolytic agent we have examined to date. The TC_{50} of 5-HPQ (ca. 40 \mu M) was about 3.5-fold lower than that of dapsone hydroxylamine (TC_{50} of ca. 150 \mu M), an N-hydroxy metabolite known to be the sole mediator of the hemolytic activity of dapsone, and about 8.5-fold lower than that of 6-methoxy-8-hydroxylaminoquinoline (TC_{50} of ca. 350 \mu M), an N-hydroxy metabolite shown recently by our laboratory to have the requisite properties to mediate primaquine hemotoxicity. Of interest, the potency of 5-HPQ was increased by more than 5-fold in GSH-depleted red cells (TC_{50} of ca. 7.5 \mu M).

As shown in Fig. 5A, hemolytic concentrations of 5-HPQ were associated with the formation of methemoglobin; however, the concentration-response curve for methemoglobin formation (Fig. 5B) was shifted well to the right of the hemolytic concentration-response curve. In addition, the methemoglobinemic efficacy of 5-HPQ was limited to about 40% of the maximum response, even when extremely high (1 mM) concentrations were used. Although the reason for this lack of efficacy and low relative potency is unknown and requires further investigation, it may be related to the marked instability of 5-HPQ in the presence of red cells (Fig. 2). Alternatively, 5-HPQ may interfere with the normal reduction of methemoglobin, either by depletion of reducing cofactors (NADH/NADPH) and/or inhibition of cellular reductases, or by generating more stable oxidants that continue to generate methemoglobin at a rate that exceeds its reduction. In any case, the concentration-response data suggest that the mechanisms underlying methemoglobin formation and hemolytic activity of 5-HPQ may be unrelated.

Together, these data strongly support a role for 5-HPQ in primaquine-induced hemolytic anemia, and furthermore, they may provide an explanation for the dramatic difference in primaquine sensitivity between G6PD-deficient and G6PD-normal individuals. Data published by Degowin et al. (1966) showed that doses of primaquine necessary to provoke a hemolytic response in G6PD-deficient humans are about 20-fold lower than those required to elicit a similar response in G6PD-normal individuals, whereas the doses of dapsone required to induce similar responses in G6PD-deficient versus normal differed by only a factor of 2. Although the reason for the difference in susceptibility between dapsone and primaquine is not yet understood, it may be related to the fact that dapsone hemotoxicity is mediated by a single hydroxylamine metabolite, whereas primaquine hemotoxicity may be mediated by the synergistic action of multiple toxic metabolites, including N-hydroxy, quinoneimine, and quinone.

In summary, we have demonstrated that a phenolic metabolite of primaquine, 5-HPQ, is directly hemotoxic to the rat red cell. We have also shown that the hemotoxicity is highly dependent on the level of GSH in the red cell, which suggests that GSH status may underlie the apparent threshold for primaquine hemotoxicity in G6PD deficiency. The actual contribution of this metabolite, however, to primaquine hemotoxicity remains to be assessed.

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Fig. 7. A, survival of normal versus GSH-depleted rat 51Cr-labeled erythrocytes in vivo after in vitro exposure to 5-HPQ. Radiolabeled red cells were treated with DEM to deplete intracellular GSH (>95%). The cells were incubated for 2 h at 37°C with the indicated concentrations of 5-HPQ. GSH-depleted control cells were incubated with vehicle (10 \mu l of H2O). Data points are means ± S.D. (n = 4). B, concentration dependence for the hemolytic response in GSH-depleted red cells. Values are means ± S.D. (n = 3).
Spectrometry Resource Facility for assistance in NMR and mass spectral analyses, respectively.

References


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