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PRIMAQUINE-INDUCED HEMOLYTIC ANEMIA: SUSCEPTIBILITY OF NORMAL VS. GLUTATHIONE-DEPLETED RAT ERYTHROCYTES TO 5-HYDROXYPRIMAQUINE

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D) Abbreviations:

5-HPQ, 5-hydroxyprimaquine
G6PD, glucose-6-phosphate dehydrogenase
GSH, reduced glutathione
GSSG, oxidized glutathione (glutathione disulfide)
PSSG, protein-glutathione mixed disulfides
MAQ-NOH, 6-methoxy-8-hydroxylaminoquinoline
DEM, diethyl maleate
PBSG, isotonic phosphate-buffered saline with glucose

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ABSTRACT

Primaquine is an important antimalarial agent because of its activity against exoerythrocytic forms of *Plasmodium sp*. Methemoglobinemia and hemolytic anemia, however, are dose-limiting side effects of primaquine therapy. These hemotoxic effects are believed to be mediated by metabolites, though 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-hydroxylaminoquinoline, 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 ^{51}Cr-labeled erythrocytes to 5-HPQ induced a concentration-dependent decrease in erythrocyte survival (TC_{50} 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 vs. phenolic metabolites to the overall hemotoxicity of primaquine remains to be assessed.

Malaria is a widespread, life-threatening parasitic disease that is responsible for 300-500 million acute illnesses and an estimated 1.5-2.7 million deaths worldwide each year (Kain and Keystone, 1998). Primaquine, an 8-aminoquinoline anti-malarial 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 *P. ovale* (Tracy and Webster, 2001). Primaquine is also used in combination with chloroquine to combat the problem of multiple drug resistance in *P. 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). Since 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-hydroxylaminoquinoline (MAQ-NOH), 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 (Bolchoz 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 primaquine hemotoxicity. In particular, hydroxylation of primaquine at the 5-position of the quinoline ring

(Fig. 1) is known to yield redox active derivatives that are capable of inducing oxidative stress within normal and G6PD-deficient human erythrocytes. Several of these compounds, including 5-hydroxyprimaquine (5-HPQ), 5-hydroxy-6-desmethylprimaquine and their N-dealkylated derivatives, were synthesized in the 1960s and made available to investigators by the World Health Organization. Studies with these compounds in isolated suspensions of red cells have shown that they can induce methemoglobin formation, glutathione (GSH) depletion and stimulation of hexose monophosphate shunt activity (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). However, there is a notable lack of evidence for their hemolytic activity *in vivo*.

Progress towards 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 re-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 in order 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 metal, stannous chloride, potassium trifluoroacetate and GSH were obtained from Sigma-Aldrich (St. Louis, MO). $Na_2{}^{51}CrO_4$ in sterile saline (1 mCi/ml, pH 8) was obtained from New England Nuclear (Billerica, MA). All other chemicals and reagents were of the best grade commercially available.

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-methoxy-8-[4-amino-1-methylbutylamino]quinoline) 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 LC-MS. The reaction mixture contained four major components: 5-HPQ (m/z 275.5-276.5; 4.60 min, 21.0%), 5-HPQ quinoneimine (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 dithionite 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). ^{1}H NMR (in D_2O): δ 8.62 (dd, J=1.4, 4.4 Hz, 1, H-2), 8.43 (dd, J=8.5, 1.4 Hz, 1, H-4), 7.46 (s, 1, H-7), 4.40 (dd, 4.3, 8.5 Hz, 1, H-3), 3.76 (m, 2, H-1'), 3.82 (s, 3, OCH_3), 2.77 (m, 2, H-4'), 1.65 (m, 1, H-2'), 1.65 (m, 1, H=3'), 1.56 (m, 1, H-2'), 1.52 (m, 1, H-3'), 1.19 (d, J=6.6 Hz, 3, H-5'). ^{13}C NMR (in D_2O): δ 148.6 (C-2), 142.7 (C-6), 139.7 (C-5), 134.9 (C-9), 132.4 (C-4), 122.4 (C-8), 121.8 (C-3), 120.4 (C-10), 112.8 (C-7), 57.9 (C-1'), 57.6 (OCH_3), 38.9(C-4'), 29.7 (C-2'), 23.1 (C-3'), 15.9 (C-5'). Because 5-HPQ is unstable, even when stored in the dark under argon at –80˚C, it was routinely prepared for immediate use (i.e., within 24-48 hr) from its more stable precursor, 5-methoxyprimaquine, as described above.

HPLC Analysis. Chromatography was performed on a Waters HPLC system (Milford, MA) consisting of a model 510 pump, a Rheodyne injector (5-ml loop), and a 250-mm Alltech Platinum EPS C18 reverse phase column. 5-HPQ was eluted with 10% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 1.1 ml/min, and was detected on a Waters model 481 UV-Vis variable wavelength detector set at 254 nm. For stability studies, a Bioanalytical Systems HPLC system (West Lafayette, IN) consisting of a model PM-80 pump, a Rheodyne 7125 injector (20 μl loop), and a 150-mm Alltech Platinum EPS C18 reverse phase column was used. 5-HPQ was eluted with 7% acetonitrile in water containing 0.05% trifluoroacetic acid and 50 mM potassium trifluoroacetate at a flow rate of 1.0 ml/min, and was detected using a Bioanalytical Systems Epsilon electrochemical detector equipped with a glassy carbon working electrode (oxidation mode, +0.35 V) and a Ag/AgCl reference electrode.

NMR Spectroscopy and Mass Spectrometry. Proton and carbon NMR spectra were obtained on a Varian Inova spectrometer operating at 400 and 100 MHz, respectively. Proton assignments were made by employing the double quantum filtered COSY experiment acquired in the phase sensitive mode. 2x256 fids were acquired. Digital resolution in F1 was increased by linear prediction to 1024 points, processed using the Gaussian weighting function, then Fourier transformed. The chemical shifts of unresolved multiplets were based on the chemical shifts of the cross peaks. Carbon resonances were assigned using gradient versions of the heteronuclear single quantum coherence (HSQC) and heteronuclear multi-bond correlation (gHMBC) experiments. In the HSQC 128 fids were acquired. Linear prediction increased the points in F1 to 512, Gaussian weighted, then Fourier transformed. In the HMBC 400 fids were acquired, linear prediction increased the points in F1 to 1200, sinebell weighted, then Fourier transformed. The nuclear Overhauser effect spectroscopy (NOESY) experiment was acquired in the phase sensitive mode by collecting 2x256 fids. Digital resolution in F1 was increased by linear prediction to 1024 points, processed using the Gaussian weighting function, 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 Instrument Systems Inc., San Jose, CA). A 150-mm Alltech Platinum EPS 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; scan range, 150.0-1000.0 amu. MS and MS/MS data were acquired automatically using Xcalibur software (version 1.2).

Electrochemical Activity of 5-HPQ. Cyclic voltammetry was performed using a Bioanalytical Systems (West Lafayette, IN) CV-27 voltammograph, 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 an Ag/AgCl reference electrode.

Animals and Erythrocyte Incubation Conditions. Male Sprague-Dawley rats (75-100 g) were purchased from Harlan Laboratories (Indianapolis, IN), and maintained on food and water *ad libitum*. Animals were acclimated for 1 week to a 12-h light-dark cycle prior to 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 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 ^{51}Cr-labeled red cells was determined *in vivo* after *in vitro* incubation with various concentrations of 5-HPQ (25-300 μM). After incubation for 2 hr at 37˚C, the erythrocytes were washed once and resuspended in PBSG (40% hematocrit). Aliquots (0.5 ml) were administered intravenously to isologous rats. T_0 blood samples were taken from the orbital sinus 30 min after administration of labeled red cells. Additional blood samples were taken every 48 hr 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 T_0 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 hr 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 Sulfhydryl 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 (Evelyn and Malloy, 1938) as described previously (Harrison and Jollow, 1987).

For determination of sulfhydryl 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 as described previously (Grossman et al., 1992). The amount of sulfhydryl 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 previously described (Bolchoz et al., 2002). Briefly, DEM (750 μM) dissolved in acetone was added to packed red cells. Following a 15 minute incubation at 37°C, the red cells were analyzed for GSH content by HPLC with electrochemical detection as described above. Under these conditions, GSH was reduced to about 5% 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 hr 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 to 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 sec in the absence of red cells and about 30 sec in their presence. Since 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 Augusto, 1992), which suggests that this compound has the ability to redox cycle. To determine directly whether oxidation of 5-HPQ to its quinoneimine form is reversible, we examined its electrochemical activity in argon-purged PBSG (pH 7.4) using cyclic voltammetry. As shown in fig. 3, when an excitation potential scan was initiated in the positive direction, two oxidation products (peaks A and B) were observed. When the potential scan was reversed (in the negative direction), a reduction product (peak C) was 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 ^{51}Cr-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 T_0 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 hr 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 as compared with controls. Fig. 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 TC_{50} 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 peak methemoglobin level of only about 20%, which nevertheless remained constant over the 2 hr incubation period. Fig. 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 (TC_{50} ca. 100 μM).

Effect of 5-HPQ on Rat Erythrocyte Sulfhydryl Status. To examine the fate of red cell GSH following 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. 6A, 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 TC_{50} 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, ^{51}Cr-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 hr 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 sub-hemolytic concentration of 5-HPQ (10 μM) was also not significantly different from the controls (fig. 7A). In contrast, exposure of GSH-depleted red cells to a 10 μM 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 μM.

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 which 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 prior to 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.

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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-hr 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 μM) was about 3.5-fold lower than that of dapsone hydroxylamine (TC_{50} ca. 150 μM), 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 MAQ-NOH (TC_{50} ca. 350 μM), 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} ca. 7.5 μM).

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

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concentration response data suggest that the mechanisms underlying methemoglobin formation and hemolytic activity of 5-HPQ may be unrelated.

Taken together, these data strongly support a role for 5-HPQ in primaquine-induced hemolytic anemia, and furthermore, 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 vs. 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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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Putative metabolism of primaquine to 5-hydroxyprimaquine (5-HPQ).

Fig. 2. Stability of 5-HPQ in blood vs. buffer. 5-HPQ (500 μM) was added to buffered saline in the absence and presence of erythrocytes (40% hematocrit), and allowed to incubate aerobically at 37°C. Aliquots were removed at designated intervals, treated with dithionite and assayed for 5-HPQ concentration using HPLC with electrochemical detection. Values are expressed as a percentage of the concentration at T_0 and are means ± S.D. (n=3).

Fig. 3. Cyclic voltammogram of 5-HPQ (245 μM) in argon-purged PBSG (pH 7.4) at room temperature. Working electrode, carbon paste; reference electrode, Ag/AgCl; auxiliary electrode, platinum. Scan rate, 150 mV/sec. A and B, anodic (oxidation) peaks; C, cathodic (reduction) peak.

Fig. 4. A, survival of rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to 5-HPQ. Radiolabeled erythrocytes were incubated for 2 hr at 37°C with the indicated concentrations of 5-HPQ; control cells were incubated with vehicle (10 μl H_2O). The erythrocytes were then washed and re-administered intravenously to isologous rats. T_0 blood samples were taken 30 min after administration of labeled cells. Data points are means ± S.D. (n=4). B, concentration dependence for the hemolytic response following 5-HPQ exposure. Values are means ± S.D. (n=4).

Fig. 5. Effect of 5-HPQ on methemoglobin formation in rat erythrocytes. A, rat erythrocytes were treated with 5-HPQ (100 μM) and assayed for methemoglobin levels over time; control cells were incubated with vehicle (10 μl H_2O). Data points are means ± S.D. (n=3). B, concentration-dependence for methemoglobin formation. Aliquots of the incubation mixture

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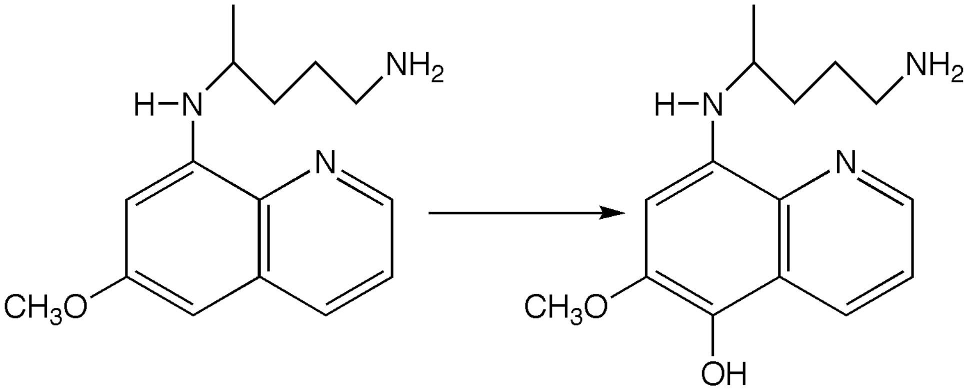
were assayed for methemoglobin 30 min after exposure to 5-HPQ (25-1000 μM). Data points are means ± S.D. (n=3).

Fig. 6. Effect of 5-HPQ on rat erythrocyte sulfhydryl status. A, rat red cells were incubated at 37°C in PBSG containing 5-HPQ (100 μM). At the indicated time points, aliquots were withdrawn and assayed for GSH, GSSG, and glutathione-protein mixed disulfides (PSSG). Data points are means ± S.D. (n=3). B, concentration dependence for GSH depletion by 5-HPQ (5-100 μM) in rat erythrocytes. GSH concentration was determined before addition of 5-HPQ and again at 15 min post-exposure. Values are expressed as a percentage of the initial level and are means ± S.D. (n=3).

Fig. 7. A, survival of normal vs. GSH-depleted rat ^{51}Cr-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 hr at 37°C with the indicated concentrations of 5-HPQ; GSH-depleted control cells were incubated with vehicle (10 μl H_2O). 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)



Primaquine

5-Hydroxyprimaquine (5-HPQ)

FIGURE 1

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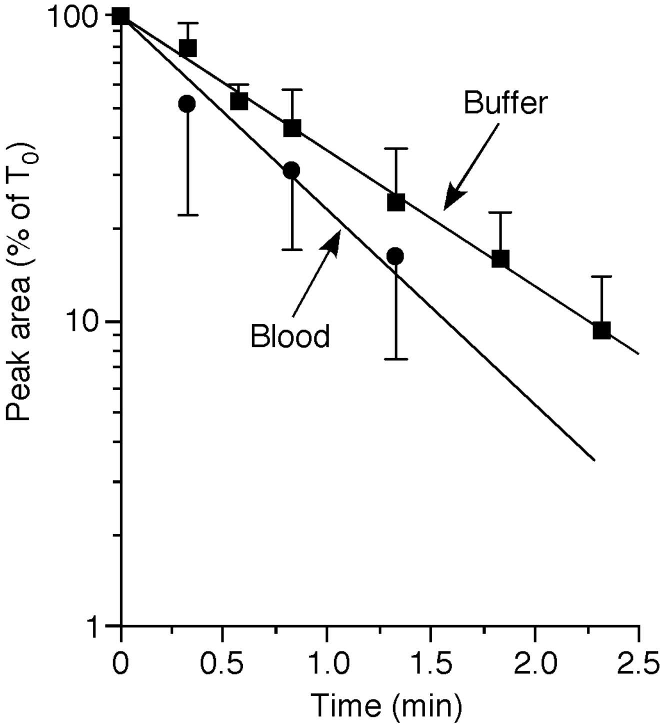

FIGURE 2

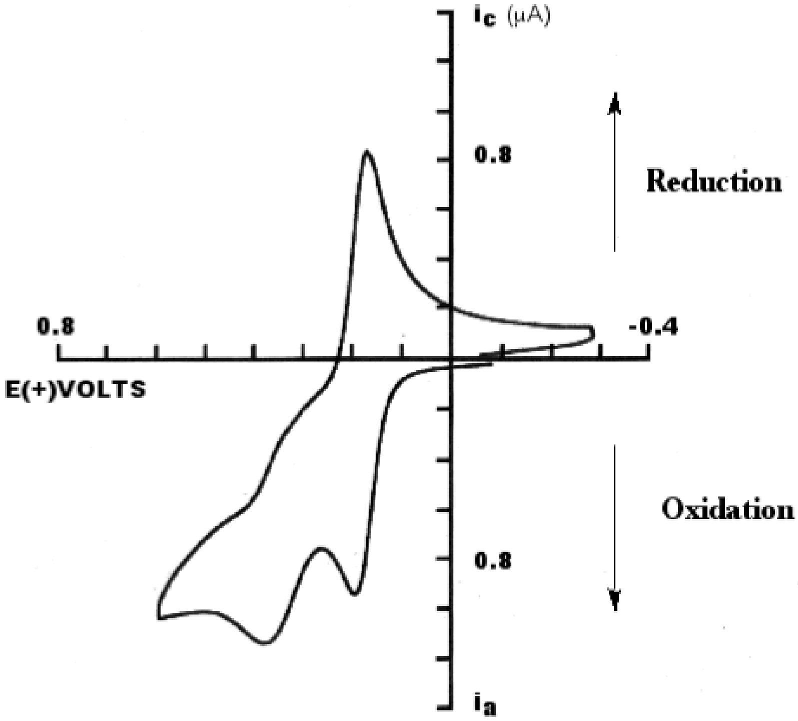

FIGURE 3

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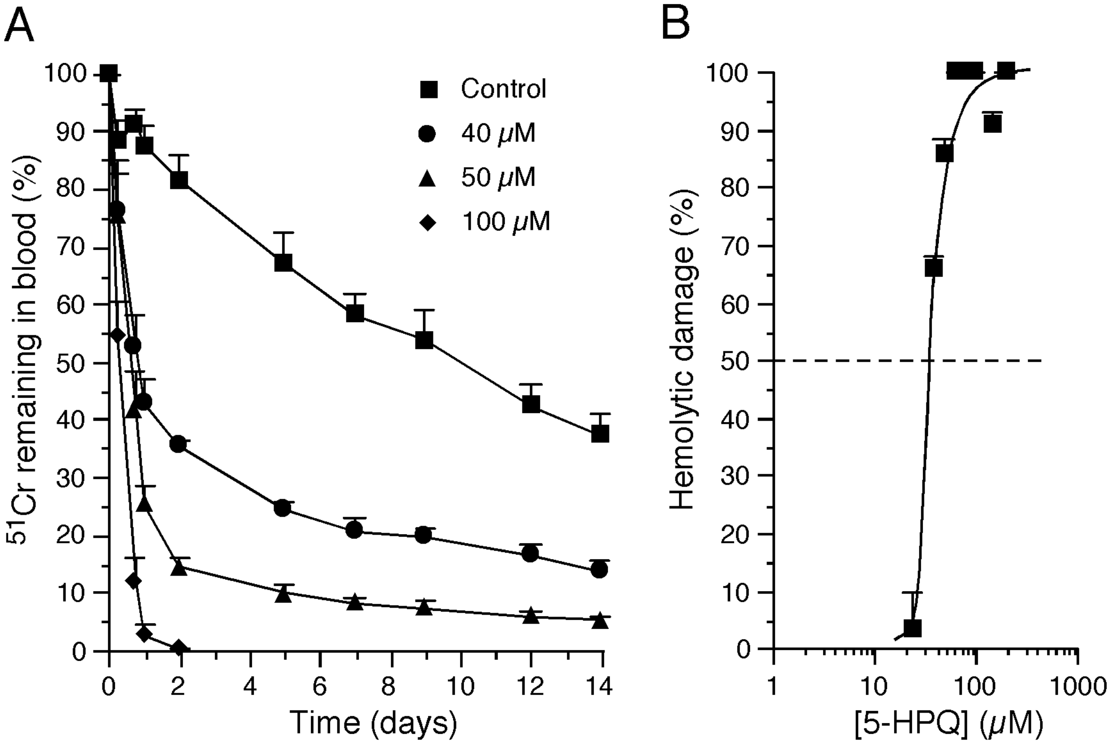

FIGURE 4

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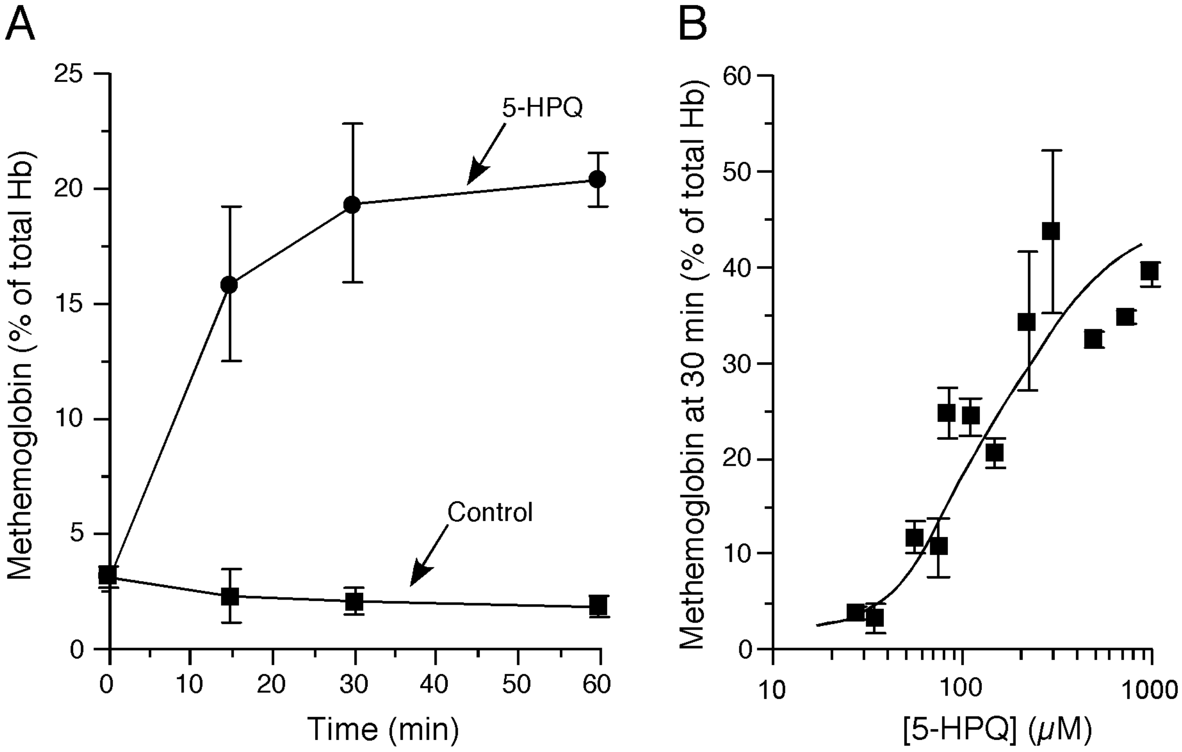

FIGURE 5

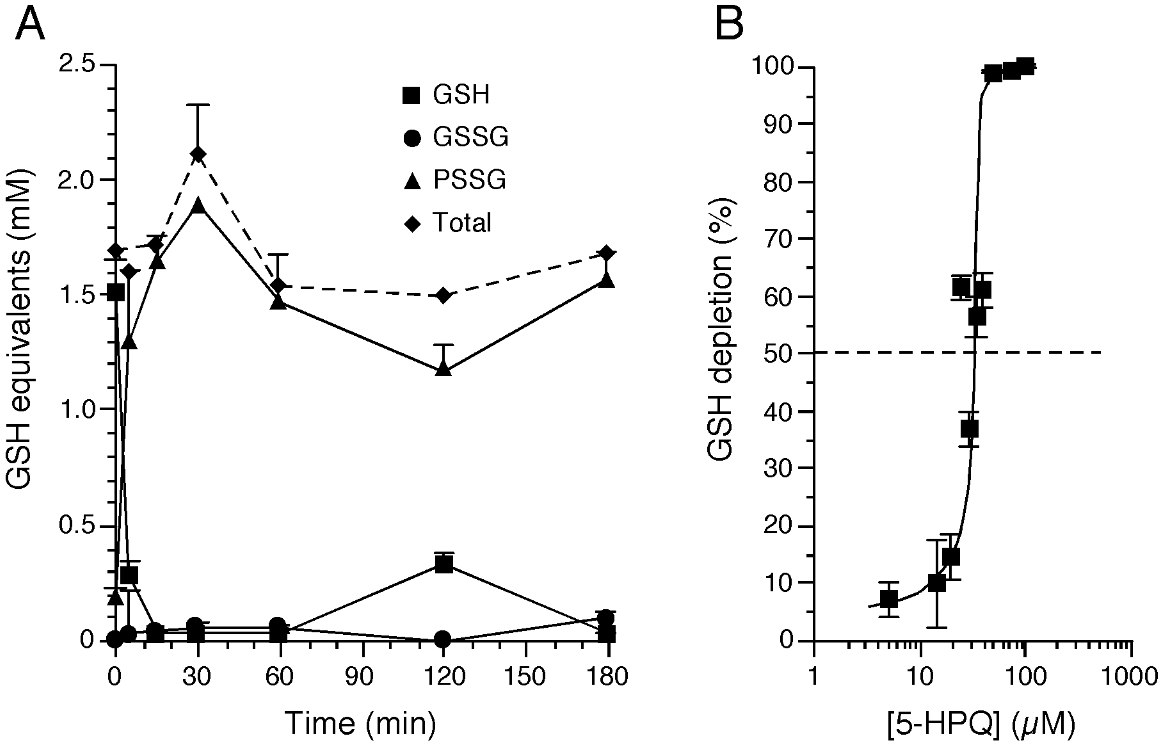
A
GSH equivalents (mM)
2.5
2.0
1.5
1.0
0.5
0
0
30
60
90
120
150
180
Time (min)
■ GSH
● GSSG
▲ PSSG
◆ Total
B
GSH depletion (%)
100
90
80
70
60
50
40
30
20
10
0
1
10
100
1000
[5-HPQ] (μM)

FIGURE 6

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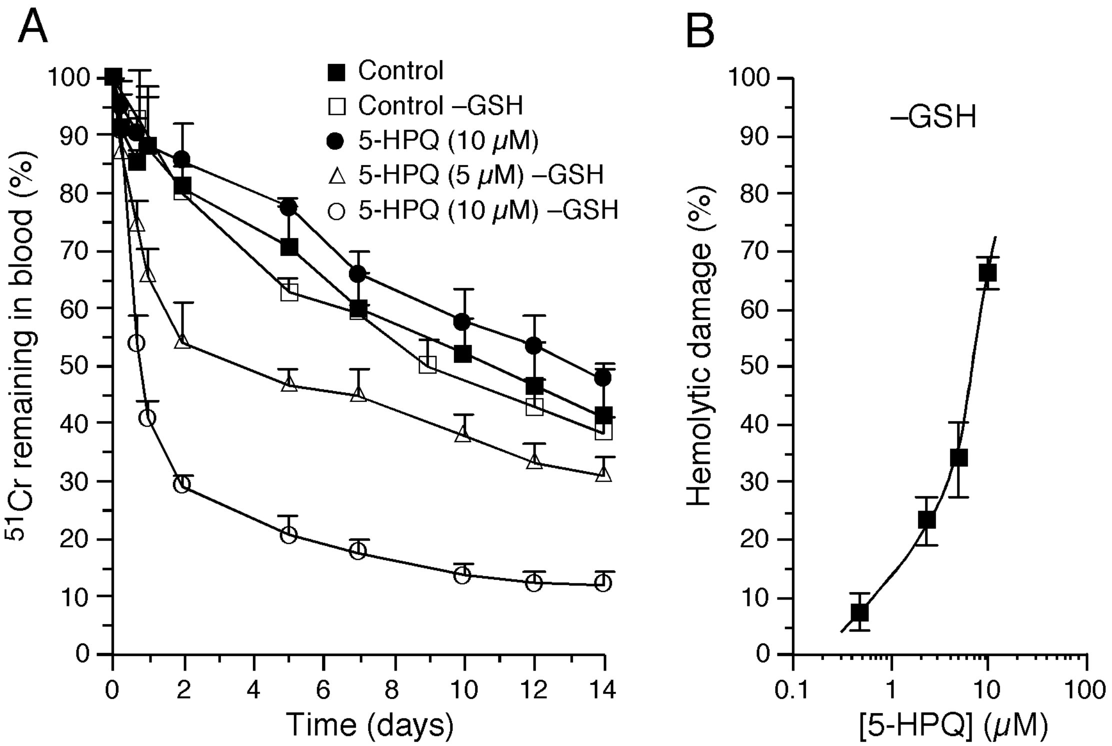
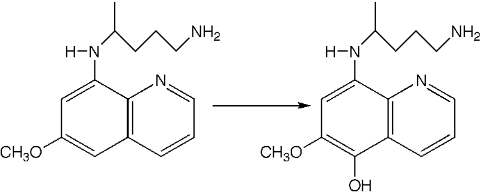
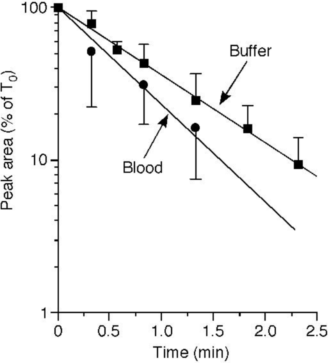
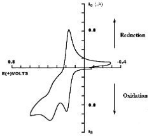


FIGURE 7

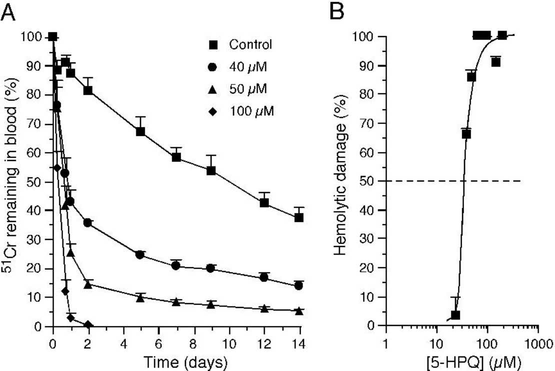


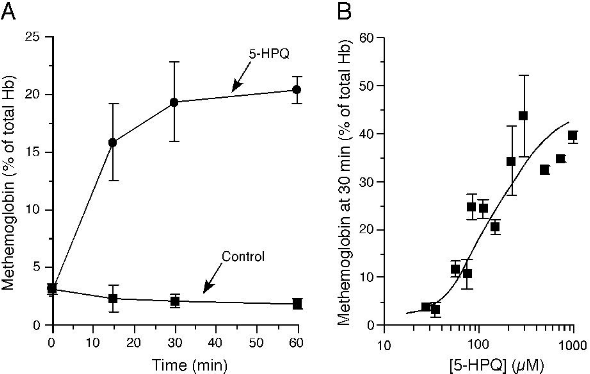
Primaquine 5-Hydroxyprimaquine (5-HPQ)

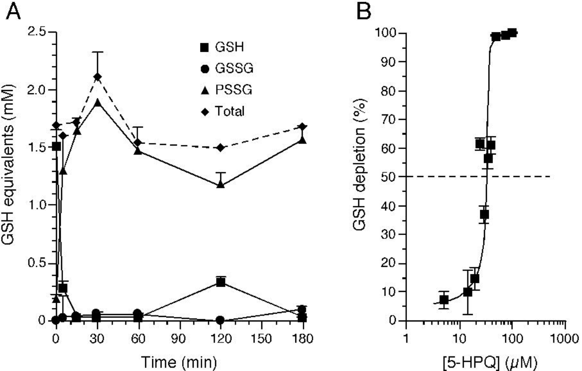

Buffer
Blood
Peak area (% of T_0)
Time (min)
100
10
1
0
0.5
1.0
1.5
2.0
2.5

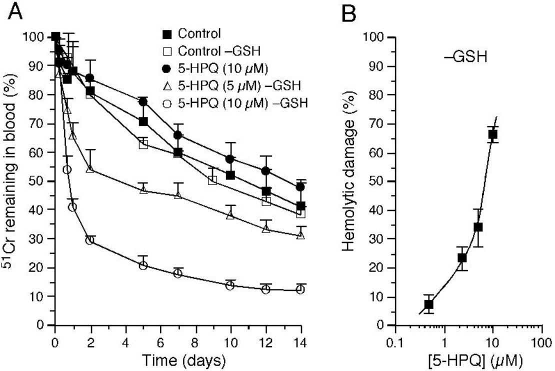


i_c (μA)
0.2
Reduction
0.8
-0.4
E(+)VOLTS
Oxidation
0.2
i_a


A
B
Control
40 μM
50 μM
100 μM
51Cr remaining in blood (%)
Time (days)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100
Hemolytic damage (%)
[5-HPQ] (μM)
1
10
100
1000


A
Methemoglobin (% of total Hb)
25
20
15
10
5
0
5-HPQ
Control
0
10
20
30
40
50
60
Time (min)
B
Methemoglobin at 30 min (% of total Hb)
60
50
40
30
20
10
0
10
100
1000
[5-HPQ] (μM)


A
GSH equivalents (mM)
0
0.5
1.0
1.5
2.0
2.5
Time (min)
0
30
60
90
120
150
180
■ GSH
● GSSG
▲ PSSG
◆ Total
B
GSH depletion (%)
0
10
20
30
40
50
60
70
80
90
100
[5-HPQ] (μM)
1
10
100
1000


A
B
Control
Control –GSH
5-HPQ (10 μM)
5-HPQ (5 μM) –GSH
5-HPQ (10 μM) –GSH
51Cr remaining in blood (%)
Time (days)
0
2
4
6
8
10
12
14
100
90
80
70
60
50
40
30
20
10
0
–GSH
Hemolytic damage (%)
[5-HPQ] (μM)
0.1
1
10
100