Primaquine-Induced Hemolytic Anemia: Formation and Hemotoxicity of the Arylhydroxylamine Metabolite 6-Methoxy-8-hydroxylaminooquinoline

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ABSTRACT

Primaquine is an important antimalarial agent because of its activity against exoerythrocytic forms of Plasmodium spp. However, methemoglobinemia and hemolytic anemia are dose-limiting side effects of primaquine therapy that limit its efficacy. These hemotoxicities are thought to be mediated by metabolites; however, the identity of the toxic species has remained unclear. Since N-hydroxy metabolites are known to mediate the hemotoxicity of several arylamines, the present studies were undertaken to determine whether 6-methoxy-8-aminoquinoline (6-MAQ), a known human metabolite of primaquine, could undergo N-hydroxylation to form a hemotoxic metabolite. When 6-MAQ was incubated with rat and human liver microsomes, a single metabolite was detected by high performance liquid chromatography (HPLC) with electrochemical detection. This metabolite was identified as 6-methoxy-8-hydroxylaminooquinoline (MAQ-NOH) by HPLC and mass spectral analyses. As measured by decreased survival of 51Cr-labeled erythrocytes in rats, MAQ-NOH was hemolytic in vivo. Furthermore, in vitro exposure of 51Cr-labeled erythrocytes to MAQ-NOH caused a concentration-dependent decrease in erythrocyte survival (EC50 of 350 μM) when the exposed cells were returned to the circulation of isologous rats. MAQ-NOH also induced the formation of methemoglobin when incubated with suspensions of rat erythrocytes. These data indicate that 6-MAQ can be metabolized to MAQ-NOH by both rat and human liver microsomes and that MAQ-NOH has the requisite properties to be a hemotoxic metabolite of primaquine. The contribution of MAQ-NOH to the hemotoxicity of primaquine in vivo remains to be assessed.

Malaria is considered to be the most widespread parasitic infection in the world, with nearly one-third of the world population threatened by Plasmodium spp. infections (Miller, 1997). Primaquine, an 8-aminoquinoline drug, has been an important antimalarial agent for over 40 years because of its unique effectiveness against exoerythrocytic forms of the parasite. Primaquine is currently the only drug capable of treating the persistent liver stages of P. vivax and P. ovale responsible for relapsing malaria (Tracy and Webster, 1996). Furthermore, the importance of primaquine has grown recently due to development of resistance against other antimalarial drugs, and because of its potential for use against opportunistic Pneumocystis carinii pneumonia in acquired immunodeficiency syndrome patients (Kantor, 1992).

Despite its broad antiparasitic activity, primaquine therapy is limited by its capacity to induce a Heinz-body anemia, particularly in individuals with deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity (Dern et al., 1955; Degowin et al., 1966). Deficiency in erythrocytic G6PD has been shown to confer resistance to infection by the malarial parasite, which presumably accounts for the high incidence of G6PD deficiency in areas endemic for malaria (Beutler, 1978). Early mechanistic studies established that 1) primaquine hemotoxicity was due to metabolite(s) of the drug, rather than the parent compound; 2) glutathione became oxidized to glutathione disulfide and was lost from the red cell; 3) hemoglobin became denatured and formed large insoluble aggregates that were attached to the inner surface of the red cell membrane (i.e., Heinz bodies); and 4) the hemolytic activity was associated with an accelerated removal of the damaged (but intact) red cells from the circulation by the spleen and liver (for review, see Beutler, 1969). The identity of the hemotoxic metabolite(s), however, was not determined, and the mechanism underlying the hemolytic response to primaquine remains unknown.

Phenolic metabolites of primaquine have long been sug-

ABBREVIATIONS: G6PD, glucose-6-phosphate dehydrogenase; 6-MAQ, 6-methoxy-8-aminoquinoline; MAQ-NOH, 6-methoxy-8-hydroxylaminooquinoline; DMSO, dimethyl sulfoxide; PBSG, phosphate-buffered saline supplemented with glucose; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; HPLC-EC, high performance liquid chromatography with electrochemical detection.
gested as candidate hemotoxic species (Tarlov et al., 1962). These oxidation products have been identified as metabolites of primaquine in the biological fluids of experimental animals (Strother et al., 1981, 1984; Idowu et al., 1995), and they have been observed to cause oxidative damage to normal and G6PD-deficient erythrocytes (Strother et al., 1984; Agarwal et al., 1988; Fletcher et al., 1988). However, phenolic metabolites have not been detected in humans after administration of primaquine (Baty et al., 1975; Mihaly et al., 1984) or in primaquine-treated human liver microsomes (Bangchang et al., 1992).

An alternate hypothesis is that the hemotoxicity of primaquine is associated, in part or in whole, with an arylhydroxylamino metabolite. Primaquine is known to undergo N-dealkylation in humans to yield 6-methoxy-8-aminquinoline (6-MAQ) (Baty et al., 1975). In previous studies with other arylamines, such as aniline and dapsone, we have observed that their N-hydroxy metabolites are direct-acting hemotoxicants and are responsible for the hemolytic activity of the parent compounds (Harrison and Jollow, 1986; Grossman and Jollow, 1988). It is thus plausible that 6-MAQ could be converted to its N-hydroxy analog (Fig. 1), and that this metabolite could be capable of inducing methemoglobinemia and hemolytic damage.

To investigate whether an arylhydroxylamine metabolite could be involved in primaquine hemotoxicity, we have synthesized the putative N-hydroxy metabolite 6-methoxy-8-arylhydroxylaminquinoline (MAQ-NOH), and have examined the capacity of rat and human liver microsomes to form this metabolite. In addition, we have assessed the ability of MAQ-NOH to induce methemoglobin formation and hemolytic damage in rat erythrocytes. We report that MAQ-NOH was formed in both rat and human liver microsomes, and that MAQ-NOH is a direct-acting methemoglobinemic and hemolytic agent in rats. These results suggest that N-dealkylation of primaquine followed by N-oxidation of 6-MAQ may be a contributing pathway for the expression of primaquine hemotoxicity.

**Experimental Procedures**

**Chemicals and Materials.** 6-Methoxy-8-nitroquinoline, platinum oxide, stannous chloride, and granular tin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Glutathione, NADP⁺, and DMSO-d₆ were obtained from Sigma Chemical Co. (St. Louis, MO). Na₂⁵¹CrO₄ in sterile saline (1 mCi/ml, pH 8) was purchased from New England Nuclear (Billerica, MA). Male Sprague-Dawley rat and pooled human liver microsomes were purchased from In Vitro Technologies (Baltimore, MD). All other chemicals were of the best commercially available grade.

**Animals.** Male Sprague-Dawley rats (75–100 g) were obtained from Harlan Laboratories (Indianapolis, IN), and were 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 isotonic phosphate-buffered saline supplemented with glucose (PBSG) (110 mM NaCl, 20 mM Na₂HPO₄, 4 mM KH₂PO₄, 10 mM d-glucose, pH 7.4) to remove the plasma anduffy coat. The cells were resuspended in PBSG and used the same day they were collected.

**Synthesis of 6-MAQ and MAQ-NOH.** To prepare 6-MAQ, 6-methoxy-8-nitroquinoline was reduced to the amine using tin/stannous chloride as described previously (Furniss et al., 1989). The crude product was recrystallized from methanolic HCl as the hydrochloride salt. HPLC analysis of the crystallized product revealed a single UV absorbance peak (280 nm). Mass spectral analysis revealed a molecular ion at m/z 175. The NMR spectrum of 6-MAQ showed chemical shifts (in ppm referenced to the solvent) at 3.86 (s, 3H, 6-OCH₃), 6.92 (d, J = 2.5 Hz, 1H, H₃), 6.96 (d, J = 2.5 Hz, 1H, H₄), 7.74 (dd, J = 4.8 Hz, J = 8.4 Hz, 1H, H₅), 8.59 (d, J = 8.4 Hz, 1H, H₆), and 8.77 (dd, J = 1.5 Hz, J = 4.7 Hz, 1H, H₇): the 8-amino protons were not observed.

To synthesize MAQ-NOH, controlled reduction of 6-methoxy-8-nitroquinoline to the hydroxylamine was carried out by hydrogenation using platinum oxide as the catalyst (Allahyari et al., 1984). The crude product was recrystallized once from acetone. HPLC analysis of the product showed <1% residual 6-methoxy-8-nitroquinoline. Mass spectral analysis revealed a molecular ion (M⁺) at m/z 191, and a predominant fragment ion at m/z 175. MS/MS analysis of the M⁺ revealed fragment ions at m/z 176 (M⁺ − 15), 173 (M⁺ − 18), and 162 (M⁺ − 29). The NMR spectrum of MAQ-NOH showed chemical shifts at 3.84 (s, 3H, 6-OCH₃), 6.69 (d, J = 2.7 Hz, 1H, H₃), 6.76 (d, J = 2.7 Hz, 1H, H₄), 7.43 (dd, J = 4.2 Hz, J = 8.3 Hz, 1H, H₅), 8.13 (dd, J = 1.6 Hz, J = 8.3 Hz, 1H, H₆), 8.56 (dd, J = 1.7 Hz, J = 4.2 Hz, 1H, H₇), 8.67 (s, 1H, -NH₂), and 8.79 (s, 1H, -NOH). Analysis of the double quantum-filtered correlation spectrum confirmed the assignment of the aromatic ring protons. The synthetic compounds were stable under argon at −80°C.

**HPLC Analysis.** Chromatography was performed on a Waters HPLC system (Milford, MA) consisting of a model 600A pump, a Rheodyne injector (20-μl loop), and a 250-mm Alltech Platinum EPS C18 reverse phase column. 6-MAQ was eluted with 35% acetonitrile in water at a flow rate of 1.2 ml/min, and was detected on a Waters model 481 UV-Vis variable wavelength detector set at 280 nm. MAQ-NOH was eluted with 40% methanol in water containing 0.1% tri-fluoroacetic acid, and 10 mM ammonium acetate at a flow rate of 1.5 ml/min, and was detected using either 1) a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy carbon working electrode (oxidation mode, +0.35 V) and a Ag/AgCl reference electrode, or 2) a Waters Millennium model 996 photodiode array detector.

**NMR Spectroscopy and Mass Spectrometry.** Mass spectral analysis was performed on a Finnigan MAT Quadrapole/EISI tandem mass spectrometer. The instrument was operated in positive-ion mode with electrospray ionization (ESI) needle voltage, 4.2 kV; ESI capillary temperature, 200°C; isolation width, 2 amu; and scan range, 100–500 amu. MS/MS data were simultaneously acquired for the selected parent ion (m/z 191). Helium was used to fragment the ion with a collision energy of 35 eV. Proton NMR spectra were acquired on a Varian VXR 400 spectrometer operating at 400 MHz with conventional quadrature detection.

**Microsomal Metabolism of 6-MAQ.** 6-MAQ (1 mM) was incubated with male Sprague-Dawley rat and pooled human liver microsomes (1.0 mg of protein/ml) in 100 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of an NADPH-generating system (163 μM β-NAD⁺, 1.7 mM glucose 6-phosphate, and 0.375 U of glucose-6-phosphate dehydrogenase). The incubation was conducted in a shaking water bath at 37°C. Aliquots (100 μl) were removed at 1, 3, 5, 10, 20, and 30 min, added to 100 μl of cold methanol, and centrifuged at 13,500g for 1 min. An aliquot of the supernatant (20 μl) was then injected immediately into the HPLC for analysis. The concentration of MAQ-NOH in the microsomal incub-
tions was quantified by measuring the peak height against a standard curve obtained by addition of synthetic MAQ-NOH to microsomal protein.

To obtain a mass spectrum of the microsomal metabolite, three microsomal suspensions (1 ml) were concentrated using Oasis HLB 1-ml C18 extraction cartridges (Waters). The cartridges were washed with 1 ml of methanol and equilibrated with 1 ml of water. After loading the microsomal incubation on the cartridge, it was washed with 5% methanol/water. The metabolite was eluted with 1 ml of 100% methanol and dried in a Speed Vac concentrator. The three samples were then pooled and dissolved in 75% methanol/water, and injected onto the HPLC. The peak corresponding to the metabolite was collected, and an aliquot was injected directly into the ion source of the mass spectrometer for analysis.

**Hemotoxicity Studies.** The level of methemoglobin in erythrocyte suspensions treated with MAQ-NOH (100–2500 μM) was measured as described previously (Harrison and Jollow, 1987). The survival of 51Cr-labeled red cells was determined in vivo after in vitro exposure to various concentrations of MAQ-NOH (0–750 μM) as described previously (Harrison and Jollow, 1986). Briefly, MAQ-NOH dissolved in DMSO (10 μl) was added to erythrocyte suspensions (40% red cells, 2.0 ml) and allowed to incubate aerobically for 2 h at 37°C. After the incubation, the red cells were washed once, resuspended in PBSG, and 0.5-ml aliquots were administered intravenously to isologous rats. Thirty minutes after administration of the labeled red cells a T₀ blood sample was taken from the orbital sinus, and then serial samples were taken at 48-h intervals for 14 days. At the end of the experiment, the radioactivity in the blood samples was counted concurrently in a well type gamma counter, and the counts above background were expressed as percentage of the T₀ sample. The time for blood radioactivity to decrease to 50% of initial levels (51Cr-T₅₀) was determined for each animal by regression analysis. Statistical significance was determined with the use of Student’s t test.

To determine the hemolytic activity of MAQ-NOH in vivo, untreated 51Cr-labeled red cells were administered intravenously to isologous rats. A T₀ blood sample was taken via the orbital sinus 48 h after administration of the labeled cells. MAQ-NOH (150 and 250 mg/kg i.p.) dissolved in DMSO (0.5 ml/kg i.p.) was administered immediately after the T₀ blood sample was taken. Serial blood samples were taken at the designated intervals and assayed as described above for the in vitro incubation/in vivo survival protocol.

**Results**

**Microsomal Metabolism of 6-MAQ.** To determine whether an N-hydroxy metabolite of 6-MAQ could be formed by rat and human liver microsomes, 6-MAQ (1 mM) was incubated with liver microsomes (1 mg/ml) containing an NADPH-generating system for 3 min at 37°C. At the end of the incubation period, an aliquot (100 μl) was removed, added to ice-cold methanol (100 μl), and centrifuged. An aliquot of the supernatant (20 μl) was then injected immediately onto the HPLC-EC. As shown in Fig. 2C, a single chromatographic peak was observed from the rat microsomal sample. This peak was not observed in the absence of an NADPH-generating system (Fig. 2B). The retention time of the metabolite peak was identical to that of synthetic MAQ-NOH (Fig. 2A), and its UV-visible absorbance spectrum also was identical (data not shown). Formation of the metabolite was time dependent, being linear for about 3 to 5 min (Fig. 3A).

To confirm the identity of the microsomal metabolite, rat microsomal incubations were analyzed by ESI mass spectrometry. To generate a sufficient amount of the metabolite for MS analysis, three separate microsomal incubations were carried out with 6-MAQ (0.1 mM) for 10 min, and then the reaction mixtures were pooled and subjected to solid-phase extraction. The extract was concentrated in a Speed Vac, injected onto the HPLC, and the metabolite peak was collected and injected into the mass spectrometer. The mass spectrum of the metabolite (Fig. 4A) showed a molecular ion at m/z 191, consistent with the addition of a hydroxyl group on 6-MAQ. A fragment ion at m/z 175 (M⁺ − 16; −O) was also observed, indicating the loss of elemental oxygen from the
molecular ion. This loss of 16 m/z units has been observed previously with other N-hydroxyarylamine compounds (Lay et al., 1986). The molecular ion (m/z 191) was then selected and subjected to ESI MS/MS. The MS/MS spectrum of the microsomal metabolite (Fig. 4B) showed three fragment ions at m/z 176 (215; –CH₃), 173 (218; –H₂O), and 162 (229; –CHO) (Fig. 3B), and was identical to the MS/MS of synthetic MAQ-NOH.

Incubation of 6-MAQ with human microsomes in the presence of NADPH (Fig. 2D) resulted in the formation of a metabolite with the same retention time and UV spectrum as that of the rat (Fig. 2C). The formation of MAQ-NOH by human microsomes was linear for about 5 min (Fig. 3A). Of interest, the extent of formation of MAQ-NOH by human microsomes was about twice that of rat microsomes.

Parallel stability studies, in which a known amount of MAQ-NOH was added to rat microsomal suspensions, indicated that MAQ-NOH disappeared in an apparent first order manner with a t₁/₂ of about 6 min (Fig. 3B). Thus, it is likely that the observed production of MAQ-NOH in rat and human microsomal suspensions is an underestimation of the enzymatic capacity of the microsomes to N-oxidize 6-MAQ.

In Vivo Hemolytic Activity of MAQ-NOH. To examine the hemolytic potential of MAQ-NOH in vivo, groups of rats were infused with ⁵¹Cr-labeled erythrocytes 48 h before i.p. administration of various doses of MAQ-NOH (dissolved in DMSO) or the vehicle alone. After an initial blood sample (T₀), serial blood samples were obtained to allow determination of the time necessary for the radioactivity to decline to 50% of the T₀ value (⁵¹Cr-T₅₀) for each animal. As shown in Fig. 5, vehicle-treated controls exhibited a gradual decline in blood radioactivity (⁵¹Cr-T₅₀ of 9.27 ± 0.34 days) that is considered to reflect primarily the normal removal of senescent erythrocytes. Erythrocyte survival after administration of a 150-mg/kg (0.94-mmol/kg) dose of MAQ-NOH was not significantly different from controls. However, the 250-mg/kg (1.57-mmol/kg) dose produced a statistically significant increase in the rate of removal of the radiolabeled erythrocytes (⁵¹Cr-T₅₀ of 6.36 ± 1.1 days). Unfortunately, administration of higher doses of MAQ-NOH (>300 mg/kg) were lethal due to acute respiratory depression, which precluded the construction of a complete dose-response curve for MAQ-NOH hemolytic activity in vivo.

Direct Hemotoxicity of MAQ-NOH. To determine whether MAQ-NOH acts directly upon red cells to produce hemolytic damage, rat ⁵¹Cr-labeled erythrocytes were resuspended in PBSG (40% hematocrit) and incubated with various concentrations of MAQ-NOH in vitro for 2 h at 37°C. No evidence for hemolysis was observed under these incubation conditions. The cells were then washed once, administered intravenously to isologous rats, and serial blood samples were taken for 15 days. As shown in Fig. 6A, exposure of the labeled cells to MAQ-NOH induced a concentration-depen-
maximal level of methemoglobin achieved for each incubation. Data were incubated for 2 h at 37°C with the indicated concentrations of MAQ-NOH; control cells were incubated with vehicle (10 μM DMSO) alone. The erythrocytes were then washed and administered intravenously to isologous rats. T₀, blood samples were taken 30 min after administration of the labeled cells. Data points are means ± S.D. (n = 4).

B, concentration-response relationship for reduction in the T₅₀ of 5¹Cr-labeled erythrocytes after MAQ-NOH exposure. The values are means ± S.D. (n = 4).

Fig. 6. A, survival of ⁵¹Cr-labeled erythrocytes in vivo after in vitro exposure of the labeled cells with MAQ-NOH. Radiolabeled erythrocytes were incubated for 2 h at 37°C with the indicated concentrations of MAQ-NOH; control cells were incubated with vehicle (10 μM DMSO) alone. The erythrocytes were then washed and administered intravenously to isologous rats. T₀, blood samples were taken 30 min after administration of the labeled cells. Data points are means ± S.D. (n = 4). B, concentration-response relationship for reduction in the T₅₀ of ⁵¹Cr-labeled erythrocytes after MAQ-NOH exposure. The values are means ± S.D. (n = 4).

To examine the capacity of MAQ-NOH to induce methemoglobinemia in vitro, the time and concentration dependence of methemoglobin formation was examined in rat erythrocyte suspensions exposed to MAQ-NOH. As shown in Fig. 7A, incubation of erythrocyte suspensions with MAQ-NOH (750 μM) resulted in the rapid formation of methemoglobin. Methemoglobin levels reached a peak of about 25% within an hour of MAQ-NOH exposure, and then declined gradually over the next 2 h. In contrast, the level of methemoglobin in control incubates remained low and constant during the course of the experiment. The MAQ-NOH concentration dependence of this response after 60 min of incubation is shown in Fig. 7B. Methemoglobin levels under these conditions ranged from about 10% methemoglobin at 100 μM MAQ-NOH to about 65% methemoglobin at 2.5 mM MAQ-NOH.

Discussion

The present results demonstrate that 6-MAQ, a known human metabolite of primaquine, can be N-hydroxylated to form MAQ-NOH by both rat and human liver microsomes (Fig. 2, C and D). Incubation of rat red cells with this metabolite caused a concentration-dependent formation of methemoglobin (Fig. 7B). When ⁵¹Cr-tagged red cells were incubated with MAQ-NOH, washed, and then administered to isologous rats, survival of the tagged red cells in the circulation was reduced in a concentration-dependent manner. Under the incubation conditions chosen (aerobic for 2 h at 37°C in buffer containing glucose), no evidence for frank hemolysis was observed, and the EC₅₀ for the hemolytic response was about 350 μM MAQ-NOH (Fig. 6B). Furthermore, MAQ-NOH was able to provoke a hemolytic response when administered directly to rats (Fig. 5).

Although the crucial role of metabolism in primaquine hemotoxicity has been accepted for over 40 years (Fraser and Vesell, 1968), the hemotoxic metabolite(s) has not been identified. Lack of progress is due in large part to the multiplicity of the known and proposed pathways of primaquine metabolism (Fig. 8), all of which can give rise to metabolites with redox potential. Moreover, poor organic solubility and instability of these putative metabolites have complicated their extraction and quantification from biological media (Idowu et al., 1995). These obstacles, combined with the difficulty in provoking a hemolytic response in laboratory animals with primaquine (Lee et al., 1981; Jollow et al., unpublished data), have hampered efforts to determine the contribution each metabolite makes toward methemoglobin formation and hemolytic damage in vivo.

To overcome these problems, investigators have synthesized phenolic derivatives of primaquine and have examined their effects in erythrocyte suspensions. Following the suggestion of Tarlov et al. (1962), much attention has been given to the 5-hydroxy- and 5,6-dihydroxy metabolites of prima-
primaquine (Fig. 8), which (via 5,6-quinone formation) could support redox cycling and the generation of active oxygen species (Link et al., 1985). In support of this postulate, these compounds were shown to induce a variety of oxidative effects in both normal and G6PD-deficient red cells, including stimulation of hexose monophosphate shunt activity (Baird et al., 1986), hemoglobin oxidation and glutathione depletion (Strother et al., 1981; Agarwal et al., 1988; Fletcher et al., 1988). More recently, Vasquez-Vivar and Augusto (1994) showed that a 500 μM concentration of the quinoneimine derivatives of these metabolites (prepared by treatment of the phenols with hydrogen peroxide) could induce oxidative damage and increase the osmotic fragility of rat erythrocytes. When examined under the same experimental conditions, 6-MAQ was found by these investigators to be inactive. Thus, they concluded that phenolic metabolites were the toxic species and that an N-hydroxylated metabolite was unlikely to contribute to the hemotoxicity of primaquine. However, MAQ-NOH was not synthesized in these studies and tested directly, and it is not clear that sufficient levels of MAQ-NOH could have been generated from 6-MAQ under their experimental conditions to allow adequate test of the postulated role of this metabolite. Moreover, since hemolytic anemia in humans is associated with sequestration of intact red cells rather than with intravascular hemolysis (Rifkind, 1966), the relevance of their observed increases in osmotic fragility is uncertain.

Although the in vivo survival data in Fig. 6 clearly indicate that MAQ-NOH is directly hemolytic to G6PD-normal rat red cells, these data do not allow for direct assessment of the role of MAQ-NOH in primaquine hemotoxicity. It is noteworthy, however, that the pattern of the response produced by MAQ-NOH exposure, i.e., the rate of 51Cr uptake with no evidence of frank lysis, was similar to that observed with arylhydroxylamine metabolites of aniline and dapsone. The type of damage inflicted by these direct-acting hemolytic agents has been shown to be consistent with an acceleration of the normal removal of “aged” but intact red cells from the circulation by the spleen (Jollow and McMillan, 1998). The hemolytic potency of MAQ-NOH (EC50 of 350 μM) toward G6PD-normal rat red cells, as measured in the in vitro/in vivo assay, was about 2-fold lower than that of dapsone hydroxylamine (EC50 of ca. 150 μM), which mediates the hemolytic activity of dapsone (Grossman and Jollow, 1988), and about 3-fold more potent than N-hydroxyphenetidine (EC50 of ca. 900 μM), which mediates the hemotoxicity of phenacetin (Jensen and Jollow, 1991).

The in vitro exposure/in vivo erythrocyte survival assay allows the hemolytic damage observed in vivo to be reproduced in vitro under controlled conditions during a 2-h incubation period before the cells are returned to the circulation of isologous rats, and thus is a useful indicator of relative hemolytic potency among direct-acting hemolytic agents. However, this assay cannot be used to define the MAQ-NOH blood concentrations needed to cause a hemolytic response in vivo. Previous studies with dapsone hydroxylamine have demonstrated that hemolytic activity is proportional to the area under the curve of the metabolite and is not dependent on its concentration (Grossman and Jollow, 1988). Since the half-life of primaquine in humans is about 6 h (Fletcher et al., 1981), it is likely that 6-MAQ and the secondary metabolite MAQ-NOH will be produced at a low but steady rate during chronic primaquine therapy. Thus, although the concentration of MAQ-NOH in the circulation may never be very high, its area under the curve may be appreciable and reach toxicologically significant levels in G6PD-deficient patients.

Assessment of the contribution that MAQ-NOH makes to primaquine hemotoxicity is further complicated by the probability that the putative phenolic metabolites discussed above are formed in humans and hence also have the potential to induce hemolytic injury. In preliminary studies using the in vitro exposure/in vivo erythrocyte survival assay, we have observed that both 5-hydroxyprimaquine and 5-hydroxy-6-desmethylprimaquine are direct-acting hemolytic agents with hemolytic potencies similar to that of MAQ-NOH (D. C. McMillan and D. J. Jollow, unpublished results). Thus, primaquine could be metabolized to three types of hemotoxic species: phenolic, diphenolic, and arylhydroxylamine, each of which would be capable of undergoing redox cycling within the red cell. 5-Hydroxyprimaquine forms a redox pair with its p-quinoneimine analog, the 5,6-diphenolic metabolite with either an o-quinone or a p-quinonimine, and the 8-hydroxy-lamino metabolite (i.e., MAQ-NOH) with its nitroso analog. These considerations emphasize the need for more detailed studies on the metabolism of primaquine in rats and humans, and raise the possibility that more than one type of metabolite is responsible for primaquine hemotoxicity.

In the present studies on the metabolism of 6-MAQ, we did not detect a phenolic metabolite in either rat or human microsomal incubations. This does not indicate that these metabolites are not formed, but does raise the possibility that either they may not be major contributors to the hemotoxicity of 6-MAQ, or that their instability is much greater than that of MAQ-NOH under these experimental conditions. Further experiments will be necessary to determine whether phenolic metabolites of primaquine can be detected in humans. In addition, the effect of CYP inducers and inhibitors on the metabolism of primaquine and 6-MAQ will be necessary to identify CYP isoforms responsible for toxic versus nontoxic pathways, and will be crucial in the development of a primaquine-sensitive animal model.

In regard to the mechanism underlying primaquine-induced hemolytic anemia, data published by Degowin et al. (1966) first raised the possibility that oxidative damage to erythrocytes could arise by different mechanisms depending on the oxidant. These investigators reported that the doses of primaquine necessary to evoke signs of hemolysis in A- G6PD-deficient volunteers are about 20-fold lower than those required to elicit a similar response in G6PD-normal volunteers, whereas the doses of dapsone required to induce similar responses in G6PD-deficient versus normal differ only by a factor of 2. Although the underlying basis for these differences is not clear, it may be explained by the fact that dapsone hemotoxicity is mediated solely by N-hydroxy metabolites, whereas primaquine hemotoxicity could be mediated by multiple hemotoxic species, quinone, quinoneimine, and arylhydroxylamine (Fig. 8). Clearly, a quantitative assessment of the contribution each type of metabolite makes toward the hemotoxic response will be important in elucidating the mechanism underlying primaquine-induced hemolytic anemia.

In summary, we have demonstrated that a known human metabolite of primaquine, 6-MAQ, can be N-hydroxylated by both rat and human liver microsomes, and that this N-hy-
droxy metabolite is hemolytic in vivo in rats, and is directly hemotoxic to the rat red blood cell. The contribution of this metabolite to primaquine hemotoxicity remains to be assessed.

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