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Primaquine-Induced Hemolytic Anemia: Hemotoxicity of 5-Hydroxyprimaquine (5-HPQ)

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PRIMAQUINE-INDUCED HEMOLYTIC ANEMIA:

HEMOTOXICITY OF 5-HYDROXYPRIMAQUINE (5-HPQ)

by

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Cell and Molecular Pharmacology

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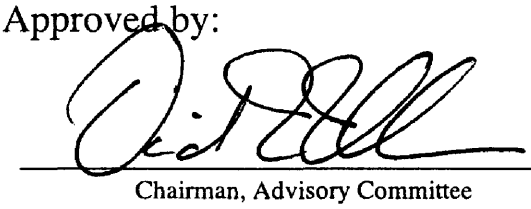
Approved by:



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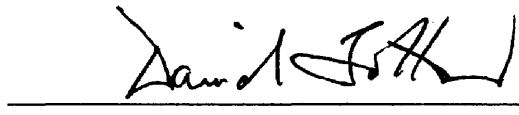

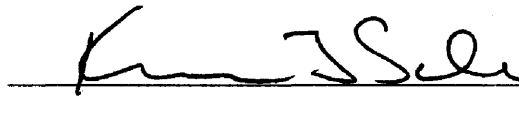

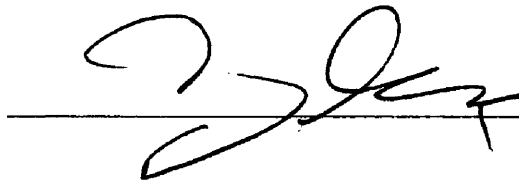

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KEY TO ABBREVIATIONS

RES	reticuloendothelial system
Hb	hemoglobin
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
PS	phosphatidylserine
GSH	reduced glutathione
G6PD	glucose-6-phosphate dehydrogenase
PQ-CX	carboxyprimaquine
MAQ	6-methoxy-8-aminoquinoline
MAQ-NOH	6-methoxy-8-hydroxylaminoquinoline
5-HPQ	5-hydroxyprimaquine
HPLC-EC	high-performance liquid chromatography with electrochemical detection
PBSG	isotonic phosphate-buffered saline with glucose
GSSG	oxidized glutathione (glutathione disulfide)
PSSG	protein-glutathione mixed disulfides
DEM	diethyl maleate
DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
NEM	N-ethyl maleimide
HBSS	Hank's buffered saline solution
CH	cumene hydroperoxide
DTT	dithiothreitol

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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 mechanism 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} ~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.

To investigate the fate of erythrocytes *in vivo* after *in vitro* exposure to 5-HPQ, rat ^{51}Cr-labeled erythrocytes were incubated with hemolytic concentrations of 5-HPQ and then re-administered intravenously to rats. The time-course of loss of radioactivity from blood and uptake into the spleen and liver was measured. In rats given 5-HPQ-treated erythrocytes, an increased rate of removal of radioactivity from the circulation was observed as compared to the vehicle control. The loss of blood radioactivity was accompanied by a corresponding increase in radioactivity appearing in the spleen but not in the liver. When rats were pretreated with clodronate-loaded liposomes to deplete splenic macrophages, there was a decreased rate of removal of radioactivity from the circulation and a markedly diminished uptake into the spleen. A role for phagocytic removal of 5-HPQ-treated red cells was confirmed *in vitro* using the J774A.1 macrophage cell line. Furthermore, depletion of red cell GSH with diethyl maleate significantly enhanced *in vitro* phagocytosis of 5-HPQ-treated red cells. These data indicate that splenic macrophages are responsible for removing 5-HPQ-treated red cells and support a role for depletion of GSH as a key event in the process leading to macrophage recognition and phagocytosis of 5-HPQ-damaged erythrocytes.

To investigate the mechanism underlying the hemolytic activity of 5-HPQ, we have examined the effect of hemolytic concentrations of 5-HPQ on ROS formation within rat erythrocytes using the cellular ROS probe, 2',7'-dichlorodihydrofluoresein diacetate (DCFDA). In addition, we examined the effect of 5-HPQ on membrane lipids and cytoskeletal proteins. The data indicate that 5-HPQ causes a prolonged, concentration-dependent generation of ROS within erythrocytes. Interestingly, 5-HPQ-generated ROS was not associated with the onset of lipid peroxidation or an alteration in

phosphatidylserine asymmetry. Instead, 5-HPQ induced oxidative injury to the erythrocyte cytoskeleton, as evidenced by changes in the normal electrophoretic pattern of membrane ghost proteins. Immunoblotting with an anti-hemoglobin antibody revealed that these changes were due primarily to the formation of disulfide-linked hemoglobin-skeletal protein adducts. These data suggest that cytoskeletal protein damage, rather than membrane lipid peroxidation or loss of phosphatidylserine asymmetry, underlies the process of removal of erythrocytes exposed to 5-HPQ.

Cummulatively, the data presented in this dissertation describe a mechanism by which exposure to 5-hydroxyprimaquine leads to the hemolytic removal of red cells; i.e. the generation of intracellular oxidative stress causes protein oxidation and hemoglobin binding to the membrane and subsequent phagocytosis by macrophages. A relationship between drug-induced hemolytic anemia and mechanisms of red cell senescence is discussed, as are possible implications for the antimalarial therapeutic effect of primaquine. The relative contribution of N-hydroxy vs. phenolic metabolites to the overall hemotoxicity of primaquine remains to be assessed.

CHAPTER 1

Background and Introduction

Normal Red Cell Senescence

Physiology

The mature red cell is a "marvel of functional design" that has been stripped of all organelles and nuclear material, leaving only a "wafer-shaped membranous bag of protein," 8μm in diameter (Jandl, 1996). Human red cells are formed from progenitor cells in the bone marrow prior to being released into the circulation, where they begin an arduous journey of 100 to 200 total miles, which lasts approximately 120 days (~60 days in the rat). During this journey, each "membranous bag of protein" travels at an incredible rate, such that it passes through the heart once every 1 to 3 minutes, while squeezing through capillary channels as small as 3μm across and managing constant exposure to oxidative stress (Jandl, 1996).

The red cell is perfectly engineered and well equipped to handle these normal stresses. However, certain changes are thought to accumulate on the red cell surface over its lifespan, which define the cell as "senescent" and lead to its uptake and degradation by resident macrophages of the reticuloendothelial system (RES) by 120 days (Rifkind, 1966). Given the massive number of red cells our circulation (~43 trillion), the removal of 1/120th of the total number circulating erythrocytes every day represents a truly remarkable feat. Approximately 360 billion senescent red cells are removed from the circulation every day, amounting to almost 5 million red cells every second (Bratosin et al., 1998). However, despite representing a very significant physiological event, the

specific changes that occur to the red cell and the mechanism(s) by which it is recognized and removed from the circulation are not well defined.

Oxidative Stress in the Red Cell

Red cells are ideally designed to deliver oxygen to the tissues. The hemoglobin (Hb) content of the red cell is approximately 5mM (~34 g/dl) with a heme-iron content of ~20 mM, representing ~95% of total cellular protein, (Jandl, 1996). Such high hemoglobin concentrations allow the red cell to be highly specialized for the transport of oxygen and carbon dioxide between the lungs and tissues. However, it is this specialized capacity that also renders the red cell susceptible to chronic oxidative stress.

Oxidative stress occurs in the red cell, because the exposure of its iron-rich contents to oxygen-rich environments leads to auto-oxidation of hemoglobin and subsequent formation of methemoglobin and hydrogen peroxide (Fig. 1.1). Thanks to the activities of several anti-oxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, the red cell is adequately equipped to handle this stress under normal conditions. Methemoglobin is readily reduced to hemoglobin by the NADH-dependent methemoglobin reductase (Passon and Hultquist, 1972), and superoxide is converted to hydrogen peroxide by superoxide dismutase (Fridovich, 1975), which is then acted on by the catalase and glutathione peroxidase/reductase systems (Eaton, 1991) as follows:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

$$2GSH + 2H_2O_2 \xrightarrow{GSH\ Px} 2H_2O + GSSG$$

$$GSSG \xrightarrow{GSH\ Red} 2GSH$$

Importantly, the actions of each of these enzymes (with the exception of superoxide dismutase) require the presence of NADPH as a cofactor. The only source of NADPH in the red cell is by generation from the pentose phosphate pathway, also called the hexose monophosphate (HMP) shunt (Fig. 1.2). The shunt acts by shuttling glucose-6-phosphate through several steps, the first two of which are oxidations that generate NADPH from NADP+. Normally the HMP shunt operates at only 3-5% of its maximum capacity, but when confronted with excess ROS generation through the pathway shown above or otherwise, the red cell responds by stimulating shunt activity (discussed below) and producing sufficient NADPH to deal with the stress. Any deficiency in this system or with the production of NADPH has devastating consequences for the ability of red cells to handle oxidative stress.

Proposed Mechanisms of Red Cell Senescence.

The mechanisms by which aged red cells are recognized and removed from the circulation are unclear. However, it is currently thought that oxidative damage in the red cell accumulates over time and leads to specific cell-surface changes, which mark the red cell for removal from the circulation, defining it as "senescent" (Bartosz, 1991). Many

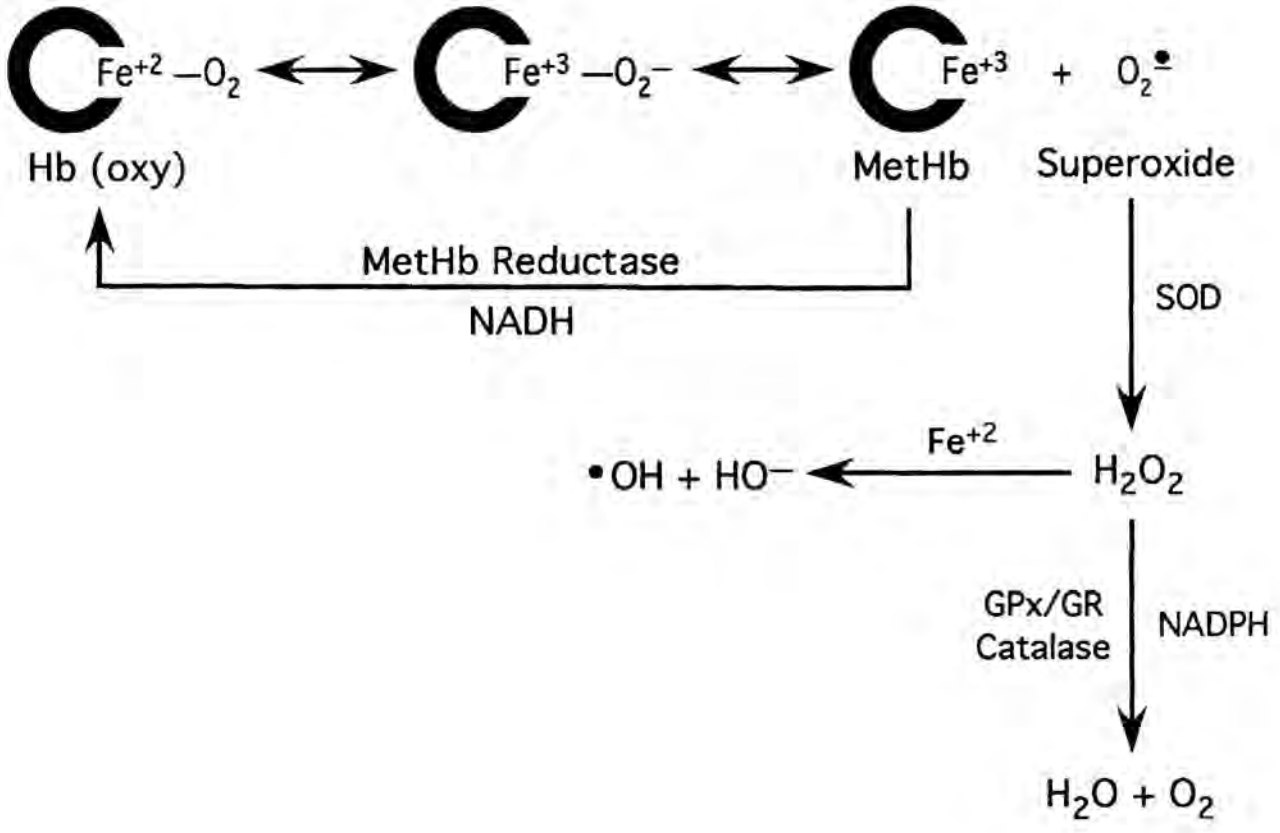


Fig. 1.1. Normal production of active oxygen species within the red cell as a consequence of ferric iron-superoxide anion dissociation from oxyhemoglobin. Hb, oxyhemoglobin; MetHb, methemoglobin; SOD, superoxide dismutase; Gpx/GR, glutathione peroxidase/glutathione reductase.

(Jollow and McMillan, 2001)

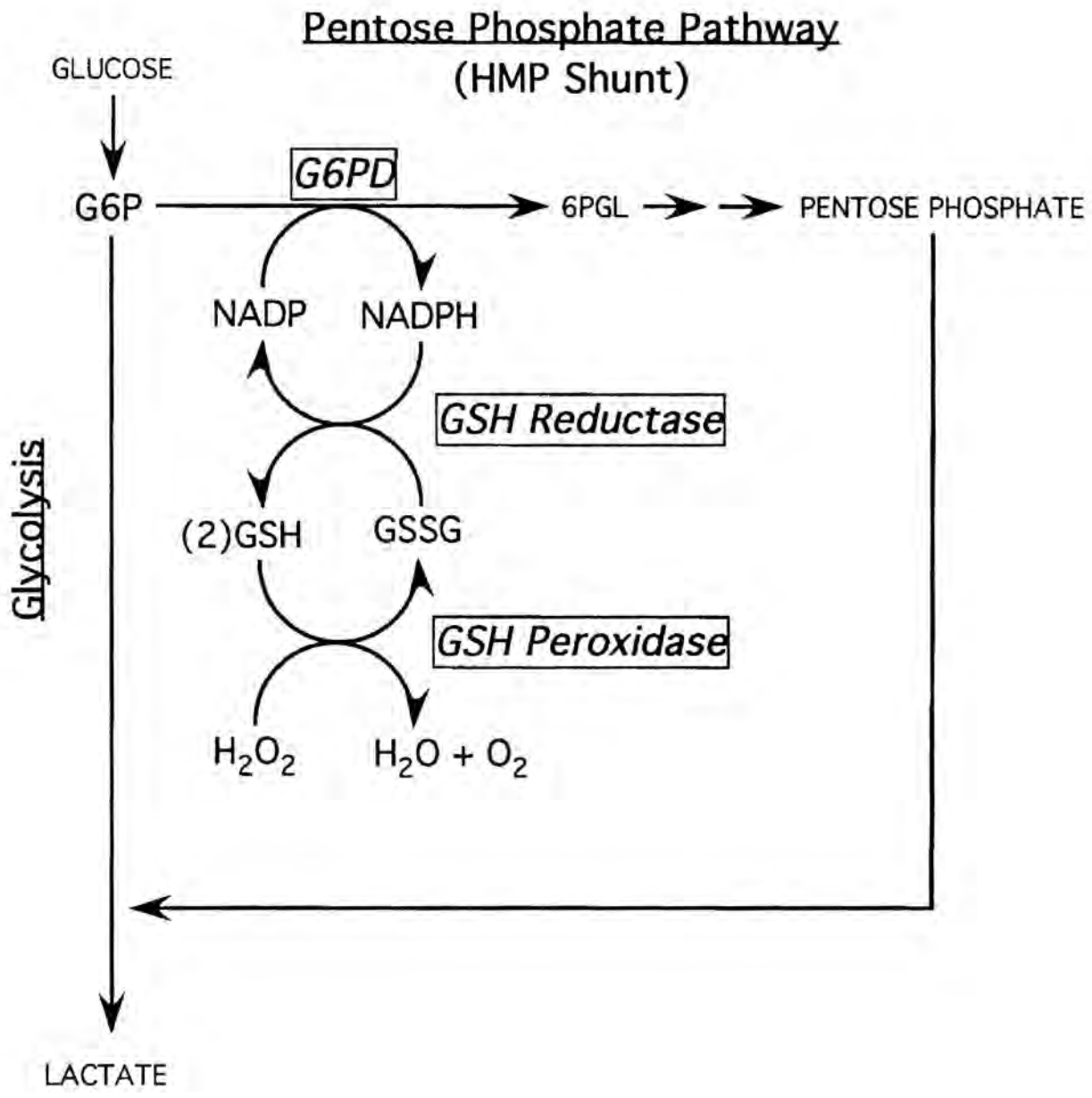


Fig. 1.2. Pentose phosphate pathway, or hexose monophosphate (HMP) shunt, of red cells. This cyclic pathway is activated by changes to cellular NADP/NADPH ratios that favor NADP and accelerate the regeneration of NADPH, which is necessary for detoxification of ROS and the maintenance of GSH and cellular enzymes in a reduced state. The first, rate-limiting enzyme of the HMP shunt is G6PD.

studies have attempted to identify the precise target(s) of oxidative damage and to determine the mechanism(s) by which they lead to erythrophagocytosis by macrophages. The major hypotheses are: 1) that specific modifications to the red cell membrane result in the exposure of "senescence antigens" (protein, lipid, or carbohydrate) on the red cell surface that are recognized by circulating "senescence antibodies," which opsonize senescent red cells for phagocytosis (Kay, 1994); 2) that enzymatic removal of sialic acid residues from red cell glycolipids or glycoproteins results in recognition by a macrophage receptor (Bratosin et al., 1998); 3) that red cell aging is analogous to the apoptotic response observed in other (nucleated) cell types, i.e. oxidative stress results in caspase activation and alterations to the normal asymmetric distribution of phospholipids in the membrane (Mandal et al., 2002; Kuypers and de Jong, 2004); or 4) that protein-protein interactions are necessary for recognition of "self," and oxidative damage alters these interactions among the cytoskeletal, integral membrane, and cell surface proteins (Oldenborg, 2004).

While each of these hypotheses is appealing, there is no consensus at present as to which represents the final erythrocyte death signal. It has been suggested that the mechanism of red cell senescence might not be able to be reduced to a single hypothesis, but that "when a metabolism or a cellular phenomenon is of vital importance it is always protected by multiple pathway systems" (Bratosin et al., 1998). Much work remains to solve the molecular and cellular mechanism of erythrophagocytosis, but two hypotheses have arisen very recently that appear to be particularly relevant.

Phospholipid Asymmetry

Phospholipid asymmetry was first established in the red cell, and most of what is known about its regulatory process was also discovered in erythrocytes. In all cell types, including normal circulating blood cells, an asymmetric conformation of membrane phospholipids is tightly regulated. Phosphatidylserine (PS) is actively and exclusively maintained within the inner leaflet of the plasma membrane and only becomes exposed to the outer leaflet in situations that require recognition and removal of damaged cells, i.e. apoptosis, or initiation of the coagulation cascade (Kuypers and de Jong, 2004). Phosphatidylethanolamine (PE) is also found within the inner leaflet, but it is not as tightly regulated as PS (Fig. 1.3). On the other hand, the choline-containing phospholipids, phosphatidylcholine and sphingomyelin, are found mainly in the outer leaflet of the plasma membrane. Three classes of integral membrane enzymes have been shown to be responsible for regulation of phospholipid asymmetry. Specifically, an ATP-driven aminophospholipid translocase, also called "flippase," very efficiently transports PS and PE from the outer leaflet to the inner leaflet of the plasma membrane (Fig. 1.4). Additionally, a calcium-activated "phospholipid scramblase" causes non-specific bidirectional movement of multiple phospholipids, and an ATP-dependent "floppase," outwardly directs PC and cholesterol.

It has been shown that PS exposure is one of the earliest detectable features in all apoptotic cells and facilitates recognition and removal of apoptotic bodies by macrophages expressing a PS receptor (Kuypers and de Jong, 2004). Additionally, transport of PS to the outer leaflet of cell membranes has been shown to occur as a result

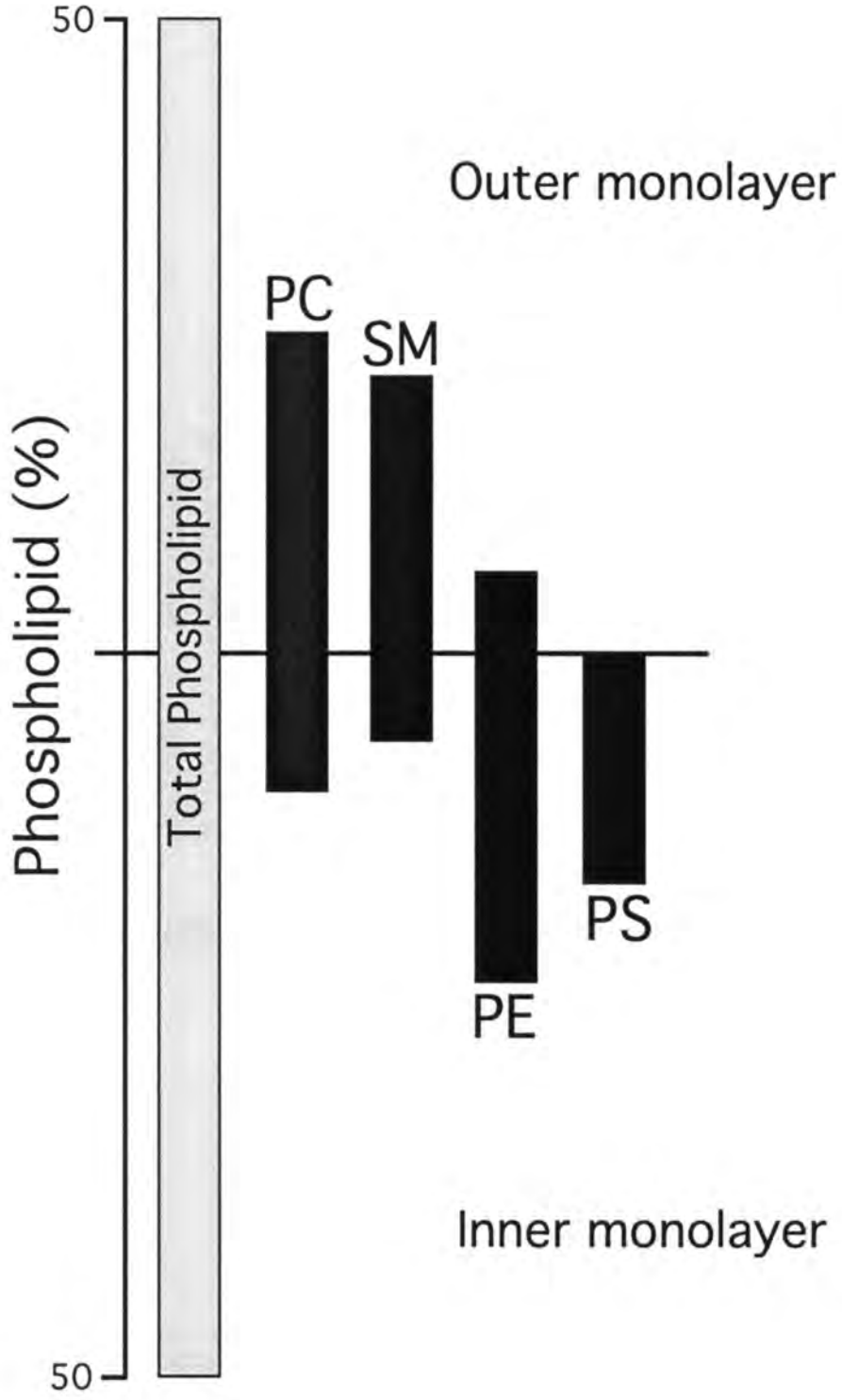


Fig. 1.3. Asymmetrical distribution of phospholipids in RBC membranes, shown as a percentage of total membrane phospholipid. PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

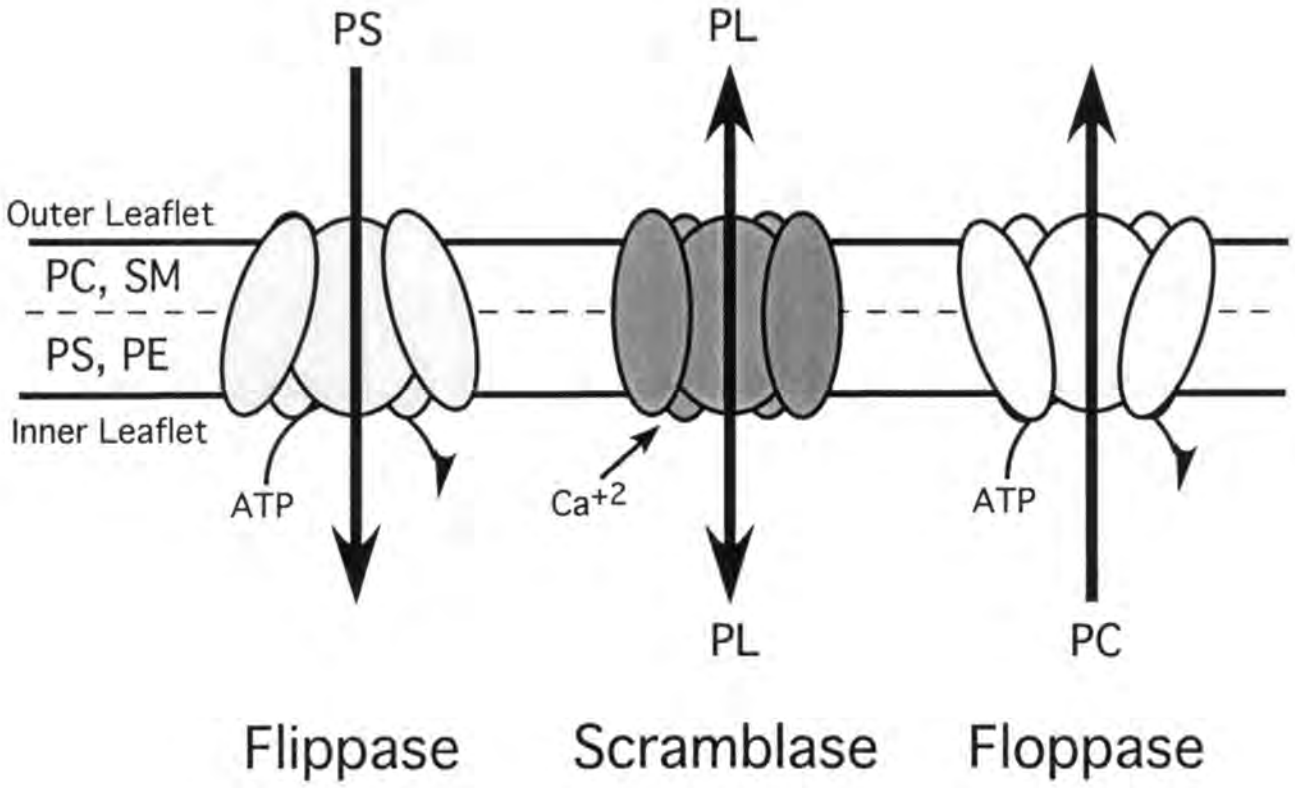


Fig. 1.4. Schematic representation of the action of lipid transporters in the plasma membrane. PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine. "Flippase" (aminophospholipid translocase) is an ATP-dependent transporter, which inwardly directs PS and PE. "Floppase" represents a class of ATP-dependent transporter, which transports PC and SM outwardly. "Scramblase" is an ATP-independent, Ca^{+2}-activated transporter, which non-specifically disrupts phospholipids asymmetry via bidirectional transport.

of accumulation of malonyldialdehyde (MDA) following lipid peroxidation or damage to spectrin (Jain, 1984), as well as from non-specific Ca^{++}-induced cell membrane scramblase activation (Bevers et al., 1998). Interestingly, senescent red cells appear to retain portions of the apoptotic machinery and to also share the common apoptotic endpoint of PS exposure. Mandal et al. demonstrated that procaspase 3 was converted to caspase 3 in red cells oxidatively damage by *t*-butyl hydroperoxide, which resulted in the exposure of PS and subsequent phagocytosis by macrophages (Mandal et al., 2002). Although red cells lack mitochondria, it has been hypothesized that intracellular oxidative stress in the erythrocyte mimics the apoptotic response observed in nucleated cells, such that activation of caspase 3 negatively regulates flippase and causes extracellular exposure of PS (Bratosin et al., 1997; Mandal et al., 2002). These studies led to the suggestion that there may be a significant correlation between red cell senescence, oxidative stress and loss of phospholipid asymmetry.

Protein-Protein Interactions and Recognition of "self"

It has been proposed that normal protein-protein interactions among the cytoskeletal and integral membrane proteins of erythrocytes are necessary to confer the recognition of "self" to macrophages (Oldenborg et al., 2000). CD47, *i.e.* integrin-associated protein (IAP), is a ubiquitously expressed transmembrane glycoprotein with five hydrophobic transmembrane-spanning domains and a short, alternatively spliced, hydrophobic tail. It was first identified in the placenta and neutrophil granulocytes as a protein that associates with $\alpha_v\beta_3$ integrins, regulates integrin function and signals leukocyte responses to RGD-containing extracellular matrices (Oldenborg, 2004). Like other cell types, red cells

express CD47; however, unlike other cell types, red cells do not express integrins, suggesting that there may be a unique role for this protein in erythrocytes.

CD47 was demonstrated to be a marker of "self" in red cells, and this was hypothesized to represent a potential pathway for the expression of hemolytic anemia. In their initial study, Oldenborg et al. showed that red cells from CD47 knockout mice were rapidly cleared when administered to the circulation of wild type mice (Oldenborg et al., 2000). Furthermore, they showed that CD47 on normal blood cells could prevent elimination by binding to the macrophage receptor, signal regulatory protein alpha (SIRPα), which is thought to send an inhibitory signal to the macrophages (Fig. 1.5). SIRPα contains intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which become tyrosine-phosphorylated in response to CD47. ITIM phosphorylation causes the recruitment of the SH2 containing phosphatase (SHP-1) to the membrane, which subsequently inhibits tyrosine kinase-dependent signaling pathways thereby preventing phagocytosis (Oldenborg, 2004). Based on the finding that CD47 functions as a "marker of self" on erythrocytes, a role for CD47 in senescent red cells was explored. Several studies showed that aged murine red cells and stored or banked erythrocytes, which have been associated with increased clearance *in vivo*, both had reduced CD47 expression, supporting the hypothesis that phagocytosis of senescent red cells may be regulated by CD47-SIRPα interaction (Anniss and Sparrow, 2002; Oldenborg, 2004).

CD47 is a member of the band 3 protein-based macrocomplex of integral and peripheral proteins in the red cell membrane (Bruce et al., 2003). In its tetrameric form, band 3 (anion exchanger 1 or AE1) binds to protein 2.1 (ankyrin), protein 4.2, and CD47,

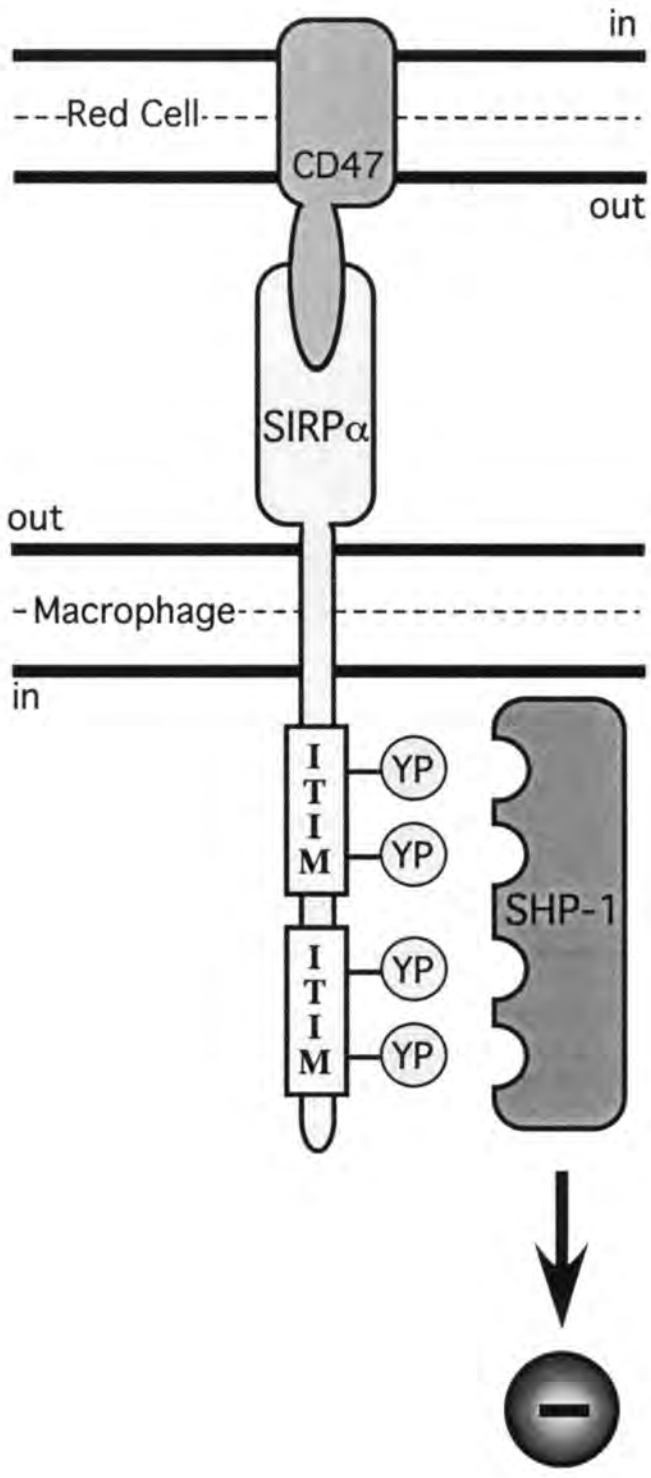


Fig. 1.5. Model for the regulation of erythrophagocytosis by the CD47/SIRPα system. Interaction of normal red cell CD47 with the macrophage SIRPα receptor results in tyrosine phosphorylation and subsequent recruitment of SHP-1 to the membrane, which negatively regulates phagocytosis by inhibiting downstream signaling.

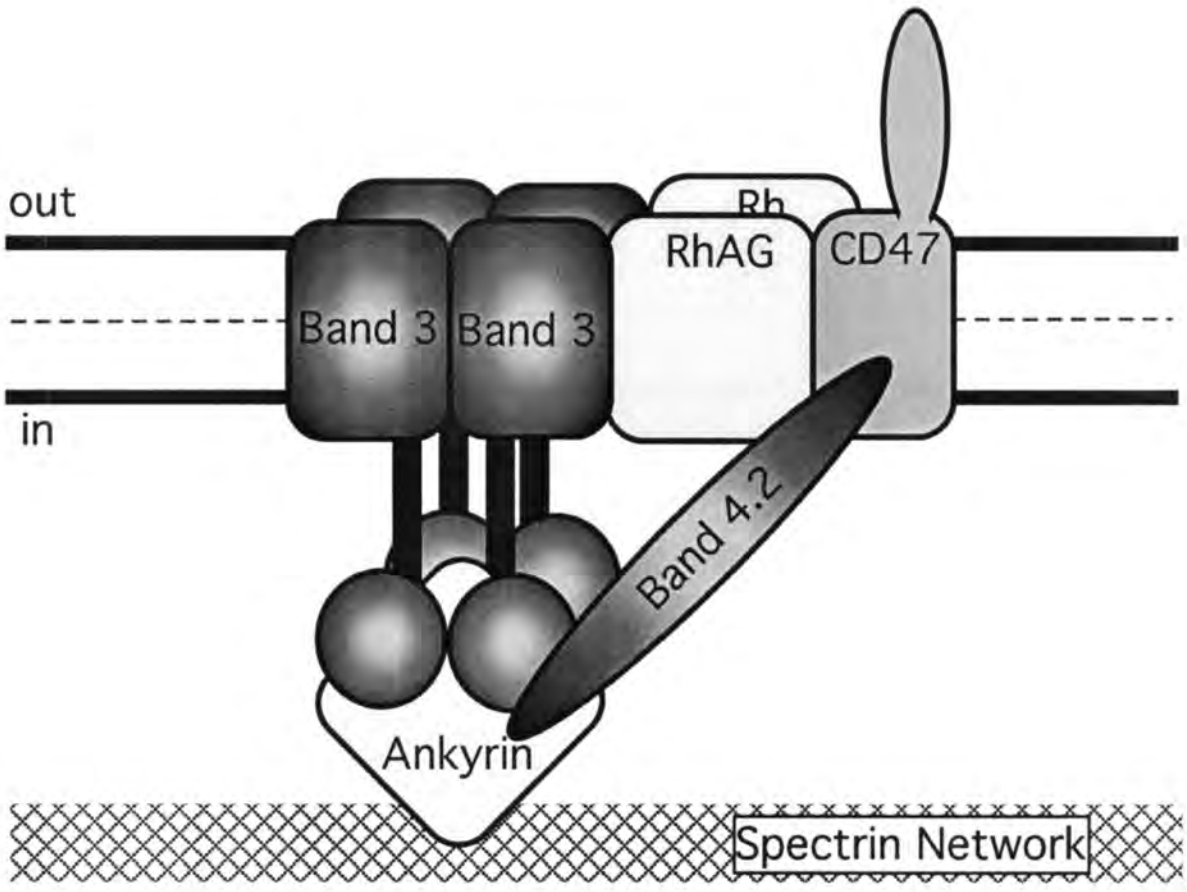


Fig. 1.6. Schematic diagram of the band 3 macrocomplex. Tetramers of band 3 are attached to the spectrin cytoskeleton through ankyrin (band 2.1). Protein 4.2 binds ankyrin, band 3, and CD47, providing vertical attachment of CD47 to the cytoskeleton. CD47 is also horizontally associated with band 3 through the Rh proteins.

(Bruce et al., 2003)

and is the major attachment site of the red cell membrane to the cytoskeleton (Fig. 1.6). Given its importance for self-recognition, it is conceivable that oxidative damage to members of the band 3 macrocomplex, or CD47 itself, perhaps in the form of free sulfhydryl modification, could alter normal CD47-presentation to macrophages, resulting in a loss of self-recognition and phagocytosis.

Drug-Induced Hemolytic Anemia

Anemia, defined as a reduction in the concentration of circulating red cells, can occur from either a decrease in red cell production or from an increase in red cell destruction. Hemolytic anemia is the generic term used to describe the disease process that results from an increased destruction of red cells. Despite what the term "hemolytic anemia" might imply, frank red cell lysis in the circulation, *i.e.* "intravascular hemolysis," is relatively rare. Rather, the more common mechanism of destruction of red cells is due the recognition and phagocytosis of intact red cells by splenic macrophages, *i.e.* "extravascular hemolysis" (Jandl, 1996). Clinically, a decreased hematocrit causes hemolytic anemia patients to present acutely with fatigue, pallor, shortness of breath, and a rapid heart rate. As a result of hemoglobin catabolism, jaundice and dark urine also occur. Additional signs may include an increased spleen size, an increased reticulocyte count, and/or hemoglobinuria.

A variety of drugs and environmental chemicals are known to cause hemolytic anemia, some of the more common of which are listed in Table 1-1. The "drug-induced hemolytic anemias" are collectively referred to as the Heinz body hemolytic anemias because of the "spheroidal inclusions of denatured hemoglobin that are a late sign of prior

Table 1-1. Drugs and chemicals that cause hemolytic anemia

Categories	**Representative compounds**
Antimalarials	Primaquine Pamaquine Pentaquine
Sulfonamides	Sulfanilamide Sulfapyradine Sulfacetamide Sulfamethoxazole
Sulfones	Dapsone Thiazolesulfone Diaphenylsulfone
Analgesics	Acetanilid
Antipyretics	Phenylhydrazine
Nitrofurans	Nitrofurantoin
Miscellaneous	Naphthalene Toluidine blue Methylene Blue Trinitrotoluene Nalidixic Acid Niridazole Pyridium Phosphine
Naturally-occuring compounds	Vicine/Convicine (fava beans) Lawsone (henna)

(Jandl, 1996)

oxidation" (Borges and Desforges, 1967). While the exact mechanism by which the drugs listed in Table 1-1 cause the premature removal of damaged, but intact, red cells is unclear, the Heinz body hemolytic anemias tend to share some common characteristics: 1) they are thought to occur following an initial oxidative stress, which results from free radical metabolites; 2) this oxidative stress is handled by the normal defense mechanisms of the cell, *i.e.* GSH peroxidase and catalase, which protect free sulfhydryl groups on Hb, SH-containing enzymes, membrane proteins, and membrane lipids; 3) GSH peroxidase and catalase, which are both dependent on NADPH, are eventually overwhelmed by this oxidative stress; 4) free radicals then attack various cellular targets, including hemoglobin, SH-containing enzymes, membrane protein, and/or membrane lipids; and 5) intracellular oxidative damage leads to cellular changes that are recognized by splenic macrophages and result in erythrocyte phagocytosis. While the precise mechanism by which intracellular oxidative damage is translated into an external cell-surface signal that marks the cell for removal is not clear, the common findings of oxidative stress and splenic sequestration have led to the hypothesis that drug-induced hemolytic anemia is due to an acceleration of the normal physiologic aging processes, whatever those mechanisms might be (Azen and Schilling, 1963; Tizianello et al., 1968).

Interestingly, there is some evidence available to suggest that there may be more than one mechanism by which hemolytic agents impose oxidative damage. Clinical studies by Degowin et al. (Degowin et al., 1966) made an important distinction between the hemolytic effect of primaquine and another hemolytic drug, dapsone (Fig. 1.7). In their studies, equivalent doses of primaquine or dapsone were administered to normal and

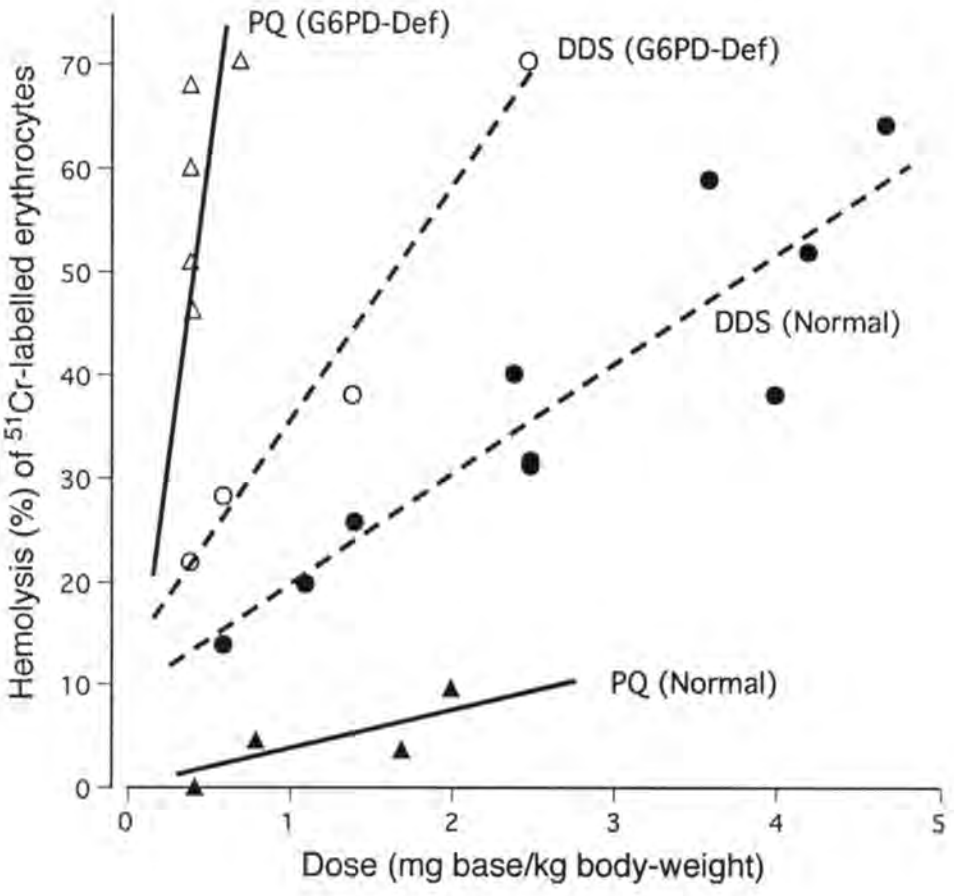


Fig. 1.7. Effect of G6PD (A-) deficiency on the hemolytic activity of dapsone and primaquine in volunteers. PQ (Normal), primaquine in normal individuals; PQ (G6PD-Def), primaquine in A- G6PD-deficient individuals; DDS (Normal), dapsone in normal individuals; DDS (G6PD-Def), Dapsone in A- G6PD-deficient individuals.

(Degowin, 1966)

African (A^-) variant G6PD deficient volunteers. In normal volunteers, dapsone was shown to be the more potent hemolytic agent. However, in the G6PD-deficient individuals (see below), primaquine was the much more potent hemolytic agent. Dapsone showed only a two-fold increase in toxicity in G6PD-deficient individuals, but the toxicity of primaquine was multiplied 30-fold. While the reason for the difference is unclear, it may be related to the fact that dapsone is metabolized to a single hydroxylamine-type metabolite that is hemolytic, whereas primaquine metabolism is complex with multiple non-toxic and toxic pathways (see below). Thus the hemolytic activity of primaquine may be mediated by the synergistic action of multiple toxic metabolites, including N-hydroxy-, quinoneimine- and quinone-types (see below). At a minimum though, the data show that there is marked variation in the hemolytic response to different agents, which suggests that more than one mechanism may be involved in drug-induced hemolytic anemia.

Glucose-6-Phosphate Dehydrogenase Deficiency

Historical

The potential risk of hemolytic anemia from use of the 8-aminoquinoline antimalarial-drug, pamaquine, was first reported in 1926. Observations over the next 30 to 40 years, showed that pamaquine, and then later primaquine, were not universally toxic, but only caused hemolytic anemia in a subset of uniquely susceptible individuals, who were termed "primaquine sensitive." For normal individuals, pamaquine and primaquine (as well as many of the other drugs listed in Table 1-1) would cause hemolytic anemia only when given in very large doses, well above the therapeutic

window. However, "primaquine sensitive" individuals presented with hemolytic anemia even when receiving conventional therapeutic doses of primaquine. In response to these initial observations, extensive work was done in the 1950s and 1960s to elaborate the mechanism of "primaquine sensitivity," and those studies showed that the sensitivity was associated with an intrinsic abnormality in the red cell, which was later identified as an enzymatic defect of glucose-6-phosphate dehydrogenase, i.e. G6PD (Beutler et al., 1955).

Glucose-6-Phosphate Dehydrogenase, the enzyme

G6PD is a cytosolic "housekeeping" enzyme that catalyzes the first and rate-limiting step of the pentose phosphate pathway (i.e. the hexose monophosphate shunt or HMP shunt), and in erythrocytes, this pathway is the sole source for NADPH. NADPH, in turn, is an important reducing cofactor for a variety of antioxidant enzymes, including catalase and GSH peroxidase/reductase (Fig. 1.2), which are necessary for the detoxification of ROS in the cell.

The human G6PD monomer consists of 515 amino acids with a molecular weight of 59.2 kDa (Beutler, 1994), but the active enzyme exists as a dimer that has binding sites for both glucose-6-phosphate and NADP. While the precise mechanisms of control for this important pathway are not yet completely understood, there appear to be at least two distinct roles for the cellular NADP/NADPH ratio (Jollow and McMillan, 2001). In addition to being a cofactor for the enzyme, NADP is necessary for conversion of the inactive G6PD monomer to the active dimer. Consequently, in the presence of high NADP concentrations, the conversion of monomer to dimer promotes G6PD, and hence HMP shunt activity (Jollow and McMillan, 2001). Conversely, NADPH provides a

potent inhibitory feedback control that is dependent on substrate (glucose-6-phosphate) concentration, such that the inhibition is greatest when the concentration of G6P is lowest (Yoshida and Lin, 1973). Under quiescent conditions, the NADPH/NADP ratio favors NADPH, hence these controls are estimated to limit the activity of normal G6PD to less than 1% of the V_{max} value of the enzyme (Yoshida and Lin, 1973). However, under enhanced oxidative stress conditions, NADPH is rapidly consumed and converted to NADP by the activities of catalase and GSH peroxidase/reductase. As the NADPH/NADP ratio drops, the inhibitory control of NADPH is diminished, and the activity of G6PD increases sufficiently to generate enough NADPH to handle the stress.

Genetics

G6PD-deficiency is an X-linked disorder, which, with several classes of variants, is considered to be the most common genetic deficiency in the world, affecting over 400 million individuals worldwide (Beutler, 1994). Most of these variants are point mutations that result in a single amino acid change, which leads to loss of activity as the red blood cell ages (Beutler, 1990; Jollow and McMillan, 1998). Thus aged cells are thought to have decreased levels of active G6PD and are hence more susceptible to oxidative damage. G6PD deficient individuals include approximately 10% of African-American males (A^- variant) who have a residual G6PD activity that is approximately 10% of normal. A^- red cells that are older than 55 days are considered to have lost all activity. About 15% of Caucasians of Mediterranean descent (Med^- variant), and 70% of Kurdish Jews, have a much more severe deficiency and red cells older than only 20 days are considered to have lost all activity. There are also population groups in Asia, India

and the Middle East that may express the same or other variants (Jollow and McMillan, 1998).

Forty-five years ago, several studies suggested that G6PD deficiency actually confers a resistance to falciparum malaria infection (Beutler, 1994), which has been postulated to have created a selective advantage for the G6PD deficiency in areas where malaria is endemic. The geographical correlation between a high prevalence of G6PD deficiency and areas where malaria is historically endemic is striking (Ruwende et al., 1995). That G6PD deficiency is so common in those countries most affected by malaria complicates and significantly limits the use of drugs like primaquine in those countries where it is most needed.

Malaria

Malaria is a life threatening parasitic disease of epidemic proportions that is a major public health concern for developing countries. Every year, infection is responsible for 300-500 million acute illnesses and an estimated 1.5-2.7 million deaths worldwide (Kain and Keystone, 1998). Tropical and subtropical regions with dense populations, typically the poorest in the world, are most affected but tend to be the least economically equipped to handle the severe disease burden or to contribute to the development of malarial treatment and prevention. Areas where malaria is considered to be endemic include: Africa, parts of Asia, Central and South America, Oceania and certain Caribbean Islands (Kain and Keystone, 1998).

Malaria is a protozoan disease that is caused in humans by four different species of the genus Plasmodium: P. falciparum (falciparum malaria), P. vivax (vivax malaria), P.

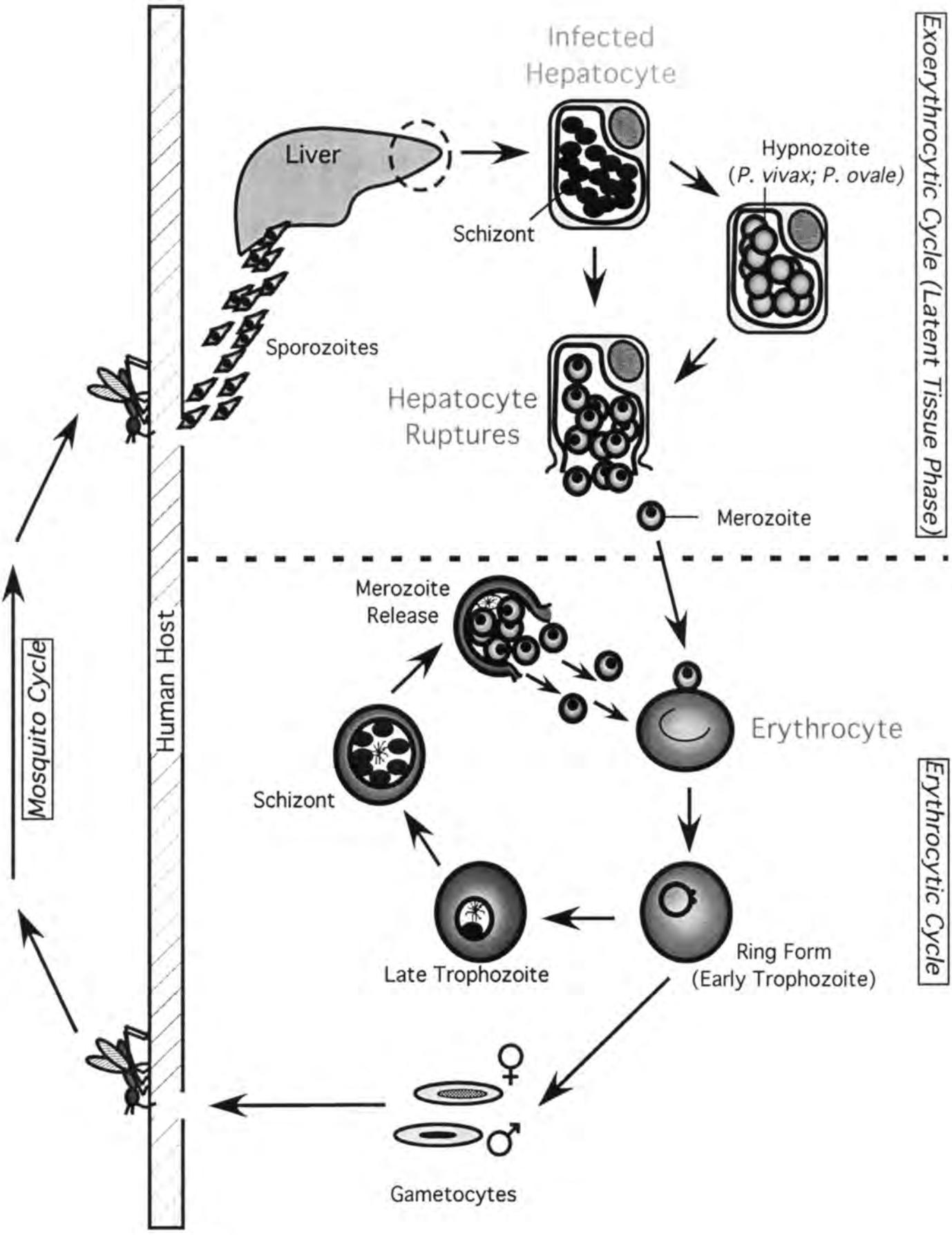


Fig. 1.8. The life cycle of the malaria parasite (see text for details).

ovale, and P. malariae. The disease is characterized by extreme exhaustion associated with paroxysms of high fever, sweating, shaking chills and anemia. Malarial transmission occurs when an infected mosquito injects the malarial sporozoites into the bloodstream of a human host while taking a blood meal (Fig. 1.8). After a brief period of about 30 minutes, the sporozoites disappear from the bloodstream as they either migrate to the liver, where they infect hepatocytes, or are destroyed by phagocytes. Those sporozoites that infect hepatocytes form cyst-like structures and continue to develop and multiply during what is referred to as the "exoerythrocytic phase" of the parasite's development. This period can vary significantly depending on the malarial species involved. In particular, P. falciparum and P. malariae schizonts will develop directly into merozoites, but P. vivax and P. ovale schizonts are able to develop into hypnozoites as well as merozoites. The hypnozoite forms can remain dormant in the liver for months or even years before being reactivated into merozoites to continue the cycle, causing malarial fever spikes and chills long after the initial infection (i.e. vivax malaria or "relapsing malaria"). The mechanism responsible for the reactivation of hypnozoites is unknown; regardless, the sporozoites eventually develop into merozoites and multiply until they overload the cell and force it to rupture. When rupture occurs, the merozoites are released into the bloodstream, where they infect red blood cells and initiate the "erythrocytic cycle." It is this cycle that is responsible for the severe fever spikes and chills typically associated with malaria infection. Similar to the exoerythrocytic cycle, the erythrocytic schizonts will eventually overload and rupture the red blood cell, releasing malarial forms that either reinfect other red cells to continue the cycle of fever spikes or develop into gametocytes, which are taken up by a new mosquito taking a blood

meal. The sexual phase of the malarial life cycle occurs in the mosquito and is completed when the mosquito injects sporozoites into a new host during another blood meal.

Antimalarial Therapy.

Historical

Until the middle of the 20th century, malaria was endemic in parts of Europe and North America. Prior to the 18th century, the most accepted treatment for malaria was the so-called Gaelen method, which aimed to "expulse" the disease through "bleeding and purging". The first attempts at specifically treating malaria date back to the early 17th century when the bark of Cinchona trees from Peru was used. By the early 18th century, its use had become widespread, and in 1820, two French pharmacists isolated quinine as the active Cinchona bark ingredient. Quinine quickly replaced the crude bark for treatment of malaria and is credited with destroying the traditional expulsion methods of Gaelen. Over the next 100 years, demand for quinine increased, but the drug still had to be extracted from the Cinchona bark. As the world's overwhelming need for natural sources of quinine expanded, so did the search for more readily available synthetic drugs (Wernsdorfer and McGregor, 1988; Wiesner et al., 2003).

In 1891, Paul Ehrlich cured two malaria patients with methylene blue, based on the observation that the dye was taken up by malarial parasites. By 1925, modification of the basic structure of methylene blue resulted in the synthesis of the first 8-aminoquinoline antimalarial compound, pamaquine. Unfortunately, pamaquine proved to be much too toxic and largely ineffective against P. falciparum. However, it did prove to be the first drug that was capable of preventing malarial relapse due to P. vivax. In 1932, mepacrine

(atebrine, quinacrine) was developed by attaching the basic side chain of pamaquine to acridine instead of quinoline. Mepacrine proved to be active against the erythrocytic stages of P. falciparum and was extensively used in combination with pamaquine during World War II. Studies by German scientists in the 1930s led to the synthesis of chloroquine, a 4-aminoquinoline blood schizontocide structurally derived from quinine that is effective for the treatment of both P. falciparum and P. vivax. Chloroquine quickly became the most effective and important antimalarial drug and was even used in several programs aimed at global eradication of malaria. Extensive studies in the 1940s then resulted in the development of most of the antimalarial therapies used today (Wernsdorfer and McGregor, 1988; Wiesner et al., 2003).

Malaria Chemotherapy and Drug Resistance

Therapeutic regimens against P. falciparum, which typically consist of various combinations of blood schizontocides, have been very successful in the past. However extensive use these drugs, in particular the 4-aminoquinoline, chloroquine, has lead to increasing resistance throughout the world to almost all blood schizontocidal antimalarial agents currently used (Bjorkman and Phillips-Howard, 1990; White, 1998). Plasmodium falciparum resistance to chloroquine was first encountered within 15 years of its introduction and has steadily expanded ever since (Peters, 1987). Today, chloroquine is used primarily to treat malaria only in Central America, Haiti, the Dominican Republic and most of the Middle East; while malarial infection in every other endemic region of the world (Africa, Asia, and parts of the Middle East) is considered to be chloroquine-resistant. Additionally, chloroquine-resistant parasites have become resistant to other,

sometime chemically unrelated drugs, such as quinine, mefloquine, proguanil, and pyrimethamine, among others. Because of this increased resistance, quinine has actually re-emerged as a drug of choice, but only in combination with other drugs. The latest search for an effective drug has lead to artemisinin and its derivatives, compounds derived from the ancient Chinese herbal qinghao and its active ingredient qinghaosu. No resistance has yet developed against the artemisinins, but the potential is thought to exist (Krishna et al., 2004). Importantly, none of these drugs have been shown to be effective against relapsing P. vivax and P. ovale malaria.

As is shown in Table 1-2, the development of antimalarial therapies has mainly concentrated against the erythrocytic cycle, while primaquine remains the *only* tissue schizontocide approved for the radical cure of latent tissue stages of malarial parasites. Additionally, no significant resistance has developed against primaquine; it continues to be effective against the latent tissue stage of all four human malarial species and for the treatment and prophylaxis of the most lethal species, P. falciparum (Shanks et al., 2001). Primaquine thus has the potential to provide an important tool to combat malarial transmission and resistance, if something can be done to control its side-effects.

Primaquine

Primaquine (6-methoxy-8-[4-amino-1-methylbutylamino]quinoline; Fig. 1.9) is the prototype 8-aminoquinoline antimalarial drug. It was one of three drugs (pentaquine, isopentaquine and primaquine) that were developed during the Second World War by the antimalarial research initiative of the US Army that were shown to be more effective against *vivax* malaria than pamaquine but less toxic. Of these three drugs, primaquine

Table 1-2. Current Drugs for Antimalarial Treatment and Prevention

Target	**Drug**
Erythrocytic Cycle *(Blood Schizontocides)*	Chloroquine Hydroxychloroquine Quinine Quinidine Mefloquine Proguanil Atovaquone Sulfadoxine-pyrimethamine Cycloguanil Clindamycin Tetracyclines Sulfonamides Dapsone Promin Artemether Artemisinin
Exo-erythrocytic Cycle ***(Tissue Schizontocides)***	**Primaquine**
Mosquito *(Vector Control)*	Insecticides Netting

remains the most effective available compound for elimination of the hypnozoite stages of P. vivax and P. ovale (Wernsdorfer and McGregor, 1988). However, despite its unique importance for antimalarial therapy for over fifty years, the mechanism of therapeutic action of primaquine remains unknown. Limited data suggests that possible targets might include the parasitic inner mitochondrial membrane, dihydroorotate synthase, or DNA (Howells et al., 1970; Boulard et al., 1983; Peters, 1984). It has also been suggested that primaquine is converted to redox active metabolites in the liver, which contribute to the antimalarial activity of primaquine by generating reactive oxygen species (Bates et al., 1990), although direct evidence for this postulate is lacking.

Primaquine is effective against the latent tissue forms of all four human malarial species and has been gaining use for both the treatment and prophylaxis of *P. falciparum* in addition to the relapsing species (Shanks et al., 2001). Interestingly, primaquine is also useful when combined with clindamycin for the treatment of *Pneumocystis carinii* pneumonia (PCP), an infection that is common in immunosuppressed individuals and definitive for the conversion of HIV to AIDS, another worldwide epidemic (Toma et al., 1998). However, despite its importance and unique therapeutic effectiveness, use of primaquine is limited by its toxicity in G6PD deficient individuals, which, along with multi-drug resistance to most of the blood schizontocides, highlights the need for more effective antimalarial drugs with higher therapeutic ratios.

Primaquine Metabolism.

Previous studies have shown that redox-active metabolites rather than the parent drug are responsible for causing the major toxicities of primaquine, and possibly its

therapeutic effects as well. Primaquine is rapidly absorbed with a bioavailability of 96% in humans but is rapidly and extensively metabolized, resulting in removal of less than 2% of the administered dose by renal excretion (Mihaly et al., 1985). However, little is known about the metabolic fate of primaquine, since the extraction, identification and quantification of the major redox-active metabolites has been significantly complicated by their instability and poor organic solubility (Idowu et al., 1995).

Despite the difficulty in identifying metabolites, the metabolism of primaquine is thought to be complex and involve oxidation to both the aminoquinoline ring and the alkylamino side-chain (Fig. 1.9). Carboxyprimaquine (PQ-CX) is formed by oxidation of the terminal carbon of the side chain, and was found in the plasma of humans and rats in concentrations significantly higher than the parent compound (Mihaly et al., 1985). Although PQ-CX is the most abundant metabolite detected, it is neither redox active itself, which was shown by its inability to stimulate HMP shunt activity or form methemoglobin in red cells (Link et al., 1985; Baird et al., 1986); nor does it appear in the urine, which indicates further metabolism prior to excretion (Mihaly et al., 1984). The only other human metabolite of primaquine that has been detected is the N-dealkylated derivative, 6-methoxy-8-aminoquinoline or MAQ (Baty et al., 1975), which was also unable to stimulate HMP shunt activity or cause methemoglobin formation (Link et al., 1985; Baird et al., 1986). Recently, our laboratory used human and rat liver microsomes to show that MAQ could be metabolized *in vitro* to MAQ-NOH, which was subsequently shown to have significant hemolytic activity (Bolchoz et al., 2001). Other than these three metabolites (PQ-CX, MAQ, and MAQ-NOH), no other human metabolites have been observed to be formed either *in vivo* or *in vitro*.

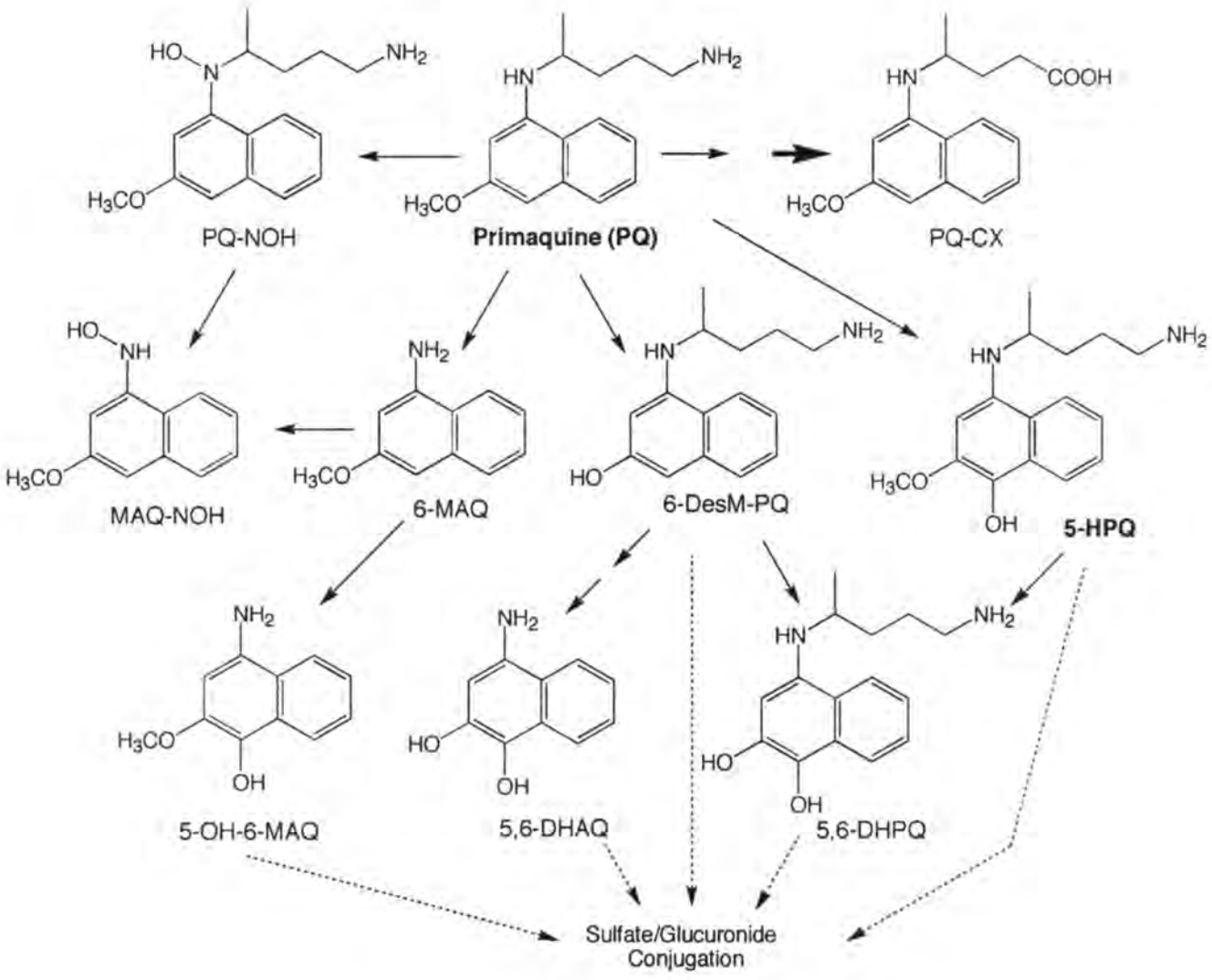


Fig. 1.9. Putative pathways of primaquine metabolism. PQ-NOH, *N*-hydroxy-primaquine; PQ-CX, 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline; MAQ-NOH, 6-methoxy-8-hydroxylaminoquinoline; 6-DesM-PQ, 6-desmethylprimaquine; 5-HPQ, 5-hydroxyprimaquine; 6-MAQ, 6-methoxy-8-aminoquinoline; 5-OH-6-MAQ, 5-hydroxy-6-methoxy-8-aminoquinoline; 5,6-DHAQ, 5,6-dihydroxy-8-aminoquinoline; 5,6-DHPQ, 5,6-dihydroxyprimaquine.

(Bolchoz et al., 2001)

5-Hydroxyprimaquine

Following the suggestions of Tarlov et al. (Tarlov et al., 1962), much attention has been given to the putative phenolic metabolites of primaquine (5-HPQ, 5,6-DHPQ, 5-OH-6-MAQ, and 5,6-DHAQ), which are thought to support quinone- or quinoneimine-type redox-cycling and generation of ROS as shown in Fig. 1.10 (Link et al., 1985). To examine the hemotoxic potential of these putative metabolites, several of the phenolic metabolites were synthesized in the 1980s and made available to investigators under a contract funded by the WHO. Studies with these derivatives in isolated erythrocytes showed that they were able to oxidize hemoglobin, stimulate hexose monophosphate shunt activity and deplete erythrocytic GSH (Allahyari et al., 1984; Link et al., 1985; Agarwal et al., 1988; Fletcher et al., 1988). Additionally, more recent studies from our laboratory assessed the hemotoxicity of MAQ-NOH, a hydroxylamine-type metabolite, which was shown to be directly hemotoxic to red cells (Bolchoz et al., 2001).

Collectively, these studies have led our laboratory to propose that the complex metabolism of primaquine can be simplified into three types of reactive metabolites: N-nitroso, *p*-quinoneimine-, and *o*-quinone-type metabolites (Fig. 1.10). While the quantitative importance of each of these metabolites is not yet known, each type is thought to have the ability to redox cycle and thereby generate reactive oxygen species according to the scheme shown in Fig. 1.11. While the initial experiments effectively demonstrated the oxidative activity of phenolic metabolites *in vitro,* they failed to establish a relevant model to assess whether their activities *in vitro* were relevant to the hemotoxicity observed *in vivo.* Thus, little is known about the capacity of phenolic metabolites to affect red cell survival *in vivo*, and efforts to detect these metabolites in

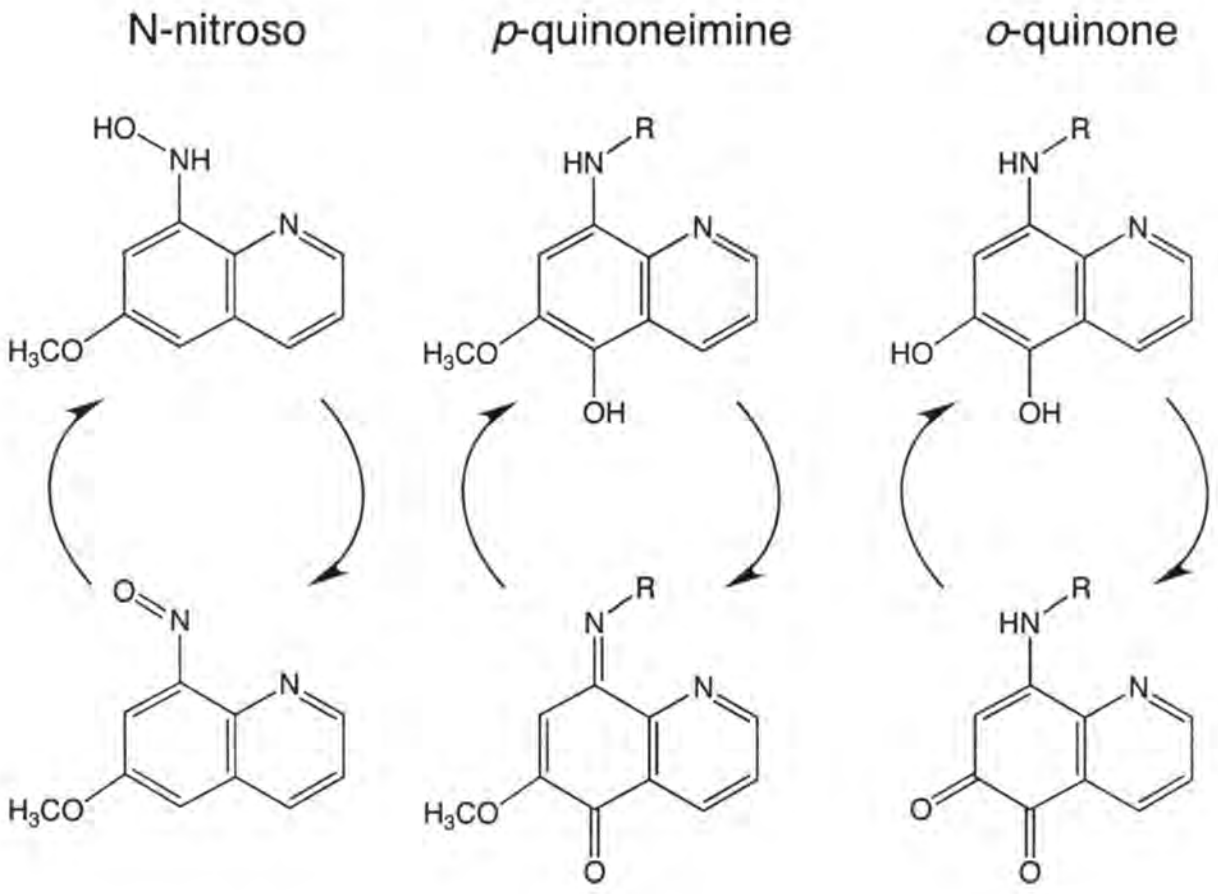


Fig. 1.10. Structures of types of primaquine reactive metabolite redox pairs. R=alkyl side-chain or H.

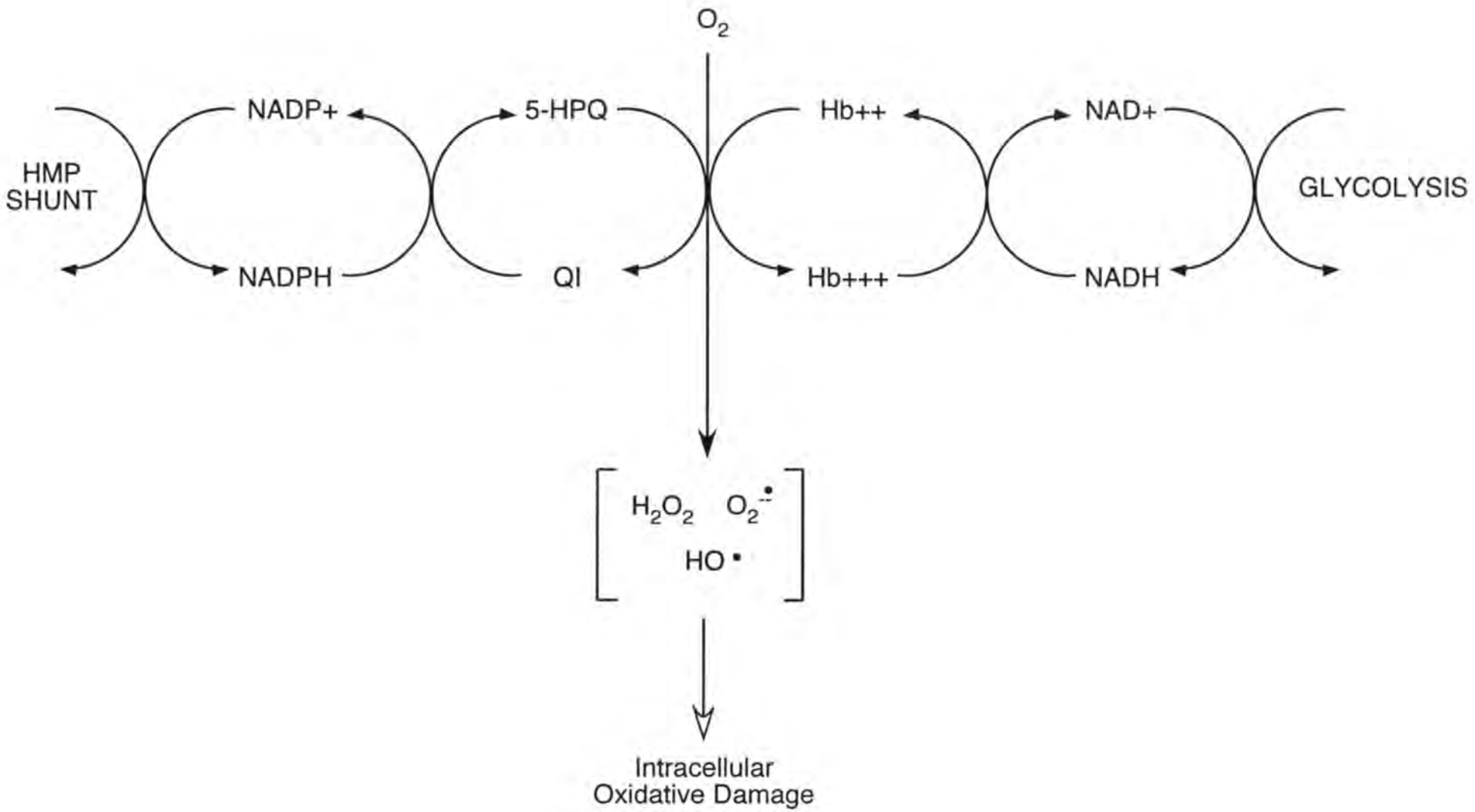


Fig. 1.11. Working hypothesis for quinoneimine-induced oxidative stress in red cells. 5-HPQ = 5-hydroxyprimaquine;QI = 5-hydroxyprimaquine-quinoneimine; Hb++ = ferrohemoglobin; Hb+++ = ferrihemoglobin; HMP SHUNT = hexose monophosphate shunt

primaquine-treated humans have not been successful. Furthermore, the compounds initially offered by the WHO are no longer available, are difficult to synthesize, and are extremely unstable.

5-HPQ, which is formed by hydroxylation in the 5-position of the quinoline ring, is the most simple of the putative phenolic metabolites. Although it has not been found in humans, formation of 5-HPQ from primaquine is expected, since it would arise by a single metabolic step and has been shown to be formed in several experimental animals, including rats, dogs and monkeys (Strother et al., 1981; Fletcher et al., 1984; Ni et al., 1992). Additional studies have shown that 5-HPQ can generate H_2O_2 and hydroxyl radicals within the red cell. It was suggested that this formation of ROS was the result of an iron-catalyzed reaction between the semiquinone intermediate and H_2O_2 (Vasquez-Vivar and Augusto, 1992). It is conceivable that this generation of intracellular ROS could mediate the hemolytic damage of primaquine in G6PD-deficient individuals, who are unable to sufficiently replenish GSH.

Similar to the other phenolic metabolites mentioned above, 5-HPQ has been shown to deplete glutathione, form methemoglobin, and stimulate HMP-shunt activity. However, there is no evidence that 5-HPQ has a direct hemolytic effect, and it is not known whether the conditions under which 5-HPQ was shown to cause oxidative damage *in vitro* are relevant to those that cause hemolytic damage *in vivo*.

Rationale and Specific Aims.

The mechanism underlying primaquine-induced hemolytic injury is unknown. Current thinking is that primaquine itself is not responsible, but rather cellular injury

results from an oxidative stress generated within the red cell by redox-active metabolites. A classical approach to identify the toxic metabolites (i.e. administration of primaquine *in vivo* in order to match toxicity to the formation of a metabolite) cannot be used, because rats (and humans with normal G6PD activity) do not respond to primaquine with toxicity, as noted above. In addition, the reactivity of certain metabolites in blood, i.e. 5-HPQ, does not allow for the correlation of blood levels with toxicity. Thus an alternative approach must be used.

Three types of redox-active metabolites are known or postulated to arise during the hepatic metabolism of primaquine: an N-hydroxy derivative (MAQ-NOH); and two phenolic derivatives, 5-HPQ and 5-hydroxy-6-desmethyl primaquine. Previous studies in our lab focused on MAQ-NOH, a hydroxylamine-type metabolite. MAQ-NOH was shown to be a direct-acting hemolytic agent in rats and to have the requisite properties to contribute to the overall hemotoxicity of primaquine (Bolchoz et al., 2001). The following studies are focused on the more simple of the two putative phenolic metabolites, 5-HPQ, since it would arise by a single metabolic step and hence may be formed in larger amounts. Previous studies showed that 5-HPQ could redox cycle to its quinoneimine analog, generate methemoglobin and cause loss of GSH when added to red cells *in vitro* (Allahyari et al., 1984; Link et al., 1985; Agarwal et al., 1988; Fletcher et al., 1988). Thus, 5-HPQ appears to be an redox-active metabolite that might be able to mediate the overall hemotoxicity of primaquine.

The long-term objective of these studies is to design a "better primaquine"; that is, a derivative that retains antimalarial activity but has little or no hemolytic capacity. Our fundamental contention is that an improved drug can only be developed if the mechanism

of toxicity is understood. To this end, the present studies will seek to establish: 1) the direct hemolytic activity of 5-HPQ; 2) the nature of injury induced within the red cell by 5-HPQ, and 3) the relationship between these internal oxidative changes with the premature sequestration of 5-HPQ-damaged red cells. The information obtained should be useful in modulating metabolism and/or RBC in the rat to confer "primaquine sensitivity" and to examine the pattern of oxidative damage in order to determine which type(s) of metabolites contribute. These studies will also significantly advance our understanding of the general mechanism of drug-induced hemolytic anemia, i.e. examination of the nature of the cellular oxidative damage will allow us to assess the relevance of specific lesions to the premature removal of damage cells, which could also provide insight into the normal, physiologic removal of senescent erythrocytes.

Hypothesis: 5-HPQ causes the hemolytic removal of damaged erythrocytes in rats by initiating an intracellular oxidative stress that specifically alters membrane components and leads to recognition and uptake of intact cells by splenic macrophages.

Specific Aim 1. To characterize the chemical properties, hemolytic response and pattern of oxidative injury induced in rat erythrocytes by 5-HPQ.

Specific Aim 2. To determine the role of splenic macrophages in the fate of 5-HPQ-damaged rat erythrocytes.

Specific Aim 3. To examine the role of membrane lipid peroxidation and cytoskeletal protein alterations in the hemotoxicity of 5-hydroxyprimaquine.

CHAPTER 2

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

Introduction

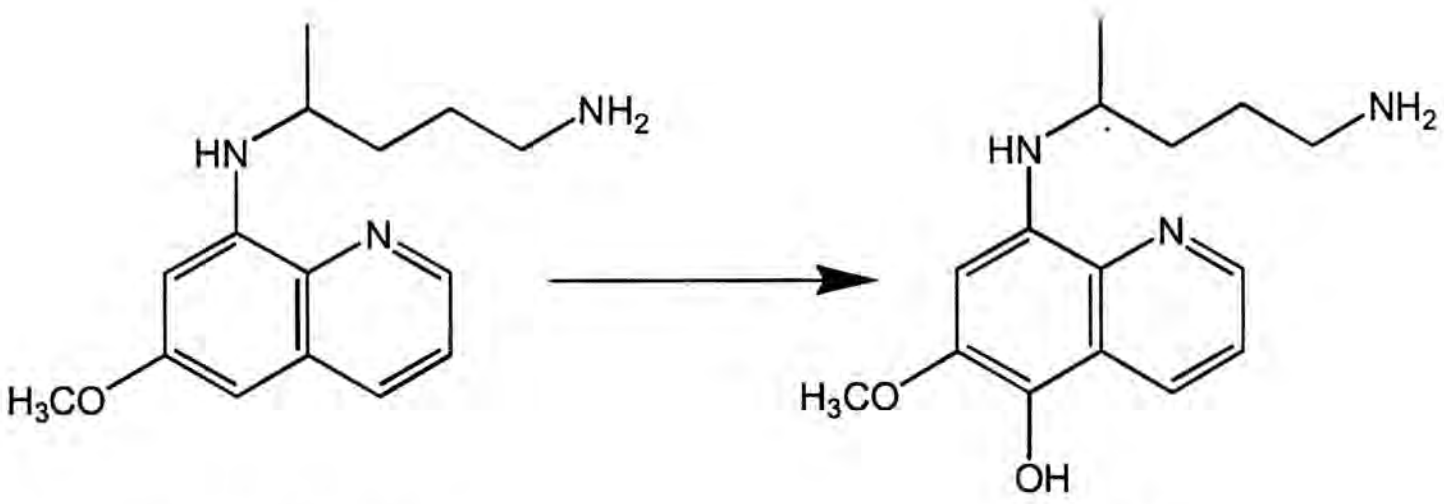
Malaria is a widespread, life-threatening parasitic disease that is responsible for 300-500 million acute illnesses and an estimated 1.5-2.7 million deaths worldwide each year (Kain and Keystone, 1998). Primaquine, an 8-aminoquinoline anti-malarial drug, is effective against the exoerythrocytic forms 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. 2.1) 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 1980s 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



Primaquine (PQ) 5-HPQ

Fig. 2.1. Putative metabolism of primaquine to 5-HPQ.

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 pre-pared from 6-methoxy-8-nitroquinoline as described previously and shown in Fig. 2.2 (Elderfield et al., 1955). 5-HPQ (5-hydroxy-6-methoxy-8-[4-amino-1-methyl-butylamino]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

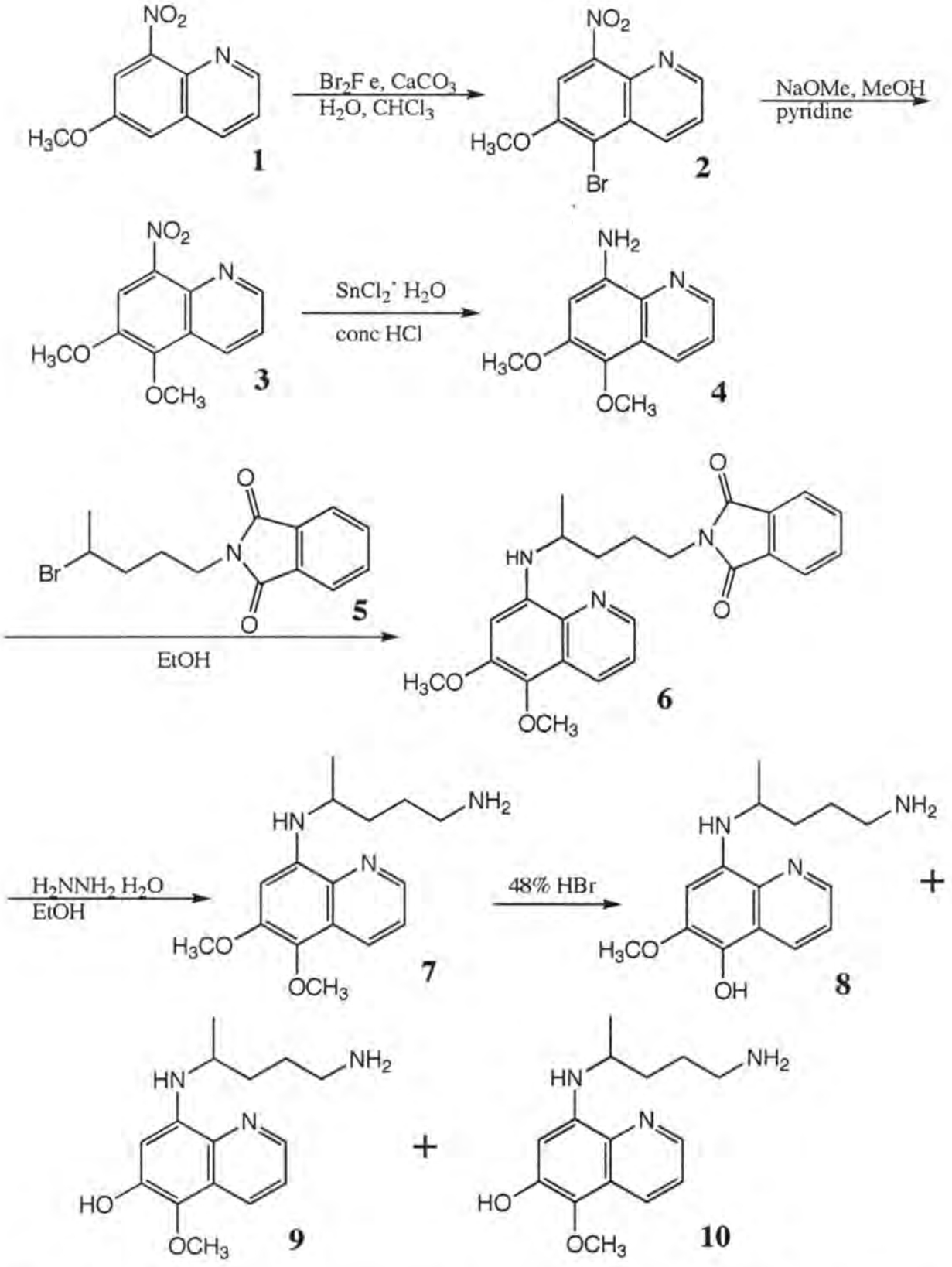


Fig. 2.2. Schematic representation of the synthesis of 5-HPQ. 6-methoxy-8-nitroquinoline (**1**) was brominated by electrophilic substitution to form 5-bromo-6-methoxy-8-nitroquinoline (**2**). Nucleophilic aromatic substitution of **2** with sodium methoxide produced 5,6-dimethoxy-8-nitroquinoline (**3**), which was then reduced with tin(II) chloride to form 5,6-dimethoxy-8-aminoquinoline (**4**). 4-bromo-1-phthalimido-pentane (**5**) was used to alkylate 4 to form 5,6-dimethoxy-8-(4-phthalimido-1-methyl-butylamino)-quinoline (**6**). Hydrazinolysis of 6 formed 5-methoxyprimaquine (**7**), which yielded 5-HPQ (**8**) and a mixture of products (**9,10**) after selective hydrolysis with HBr.

reaction mixture contained four major components (Fig. 2.3): 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; Fig. 2.4): 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.

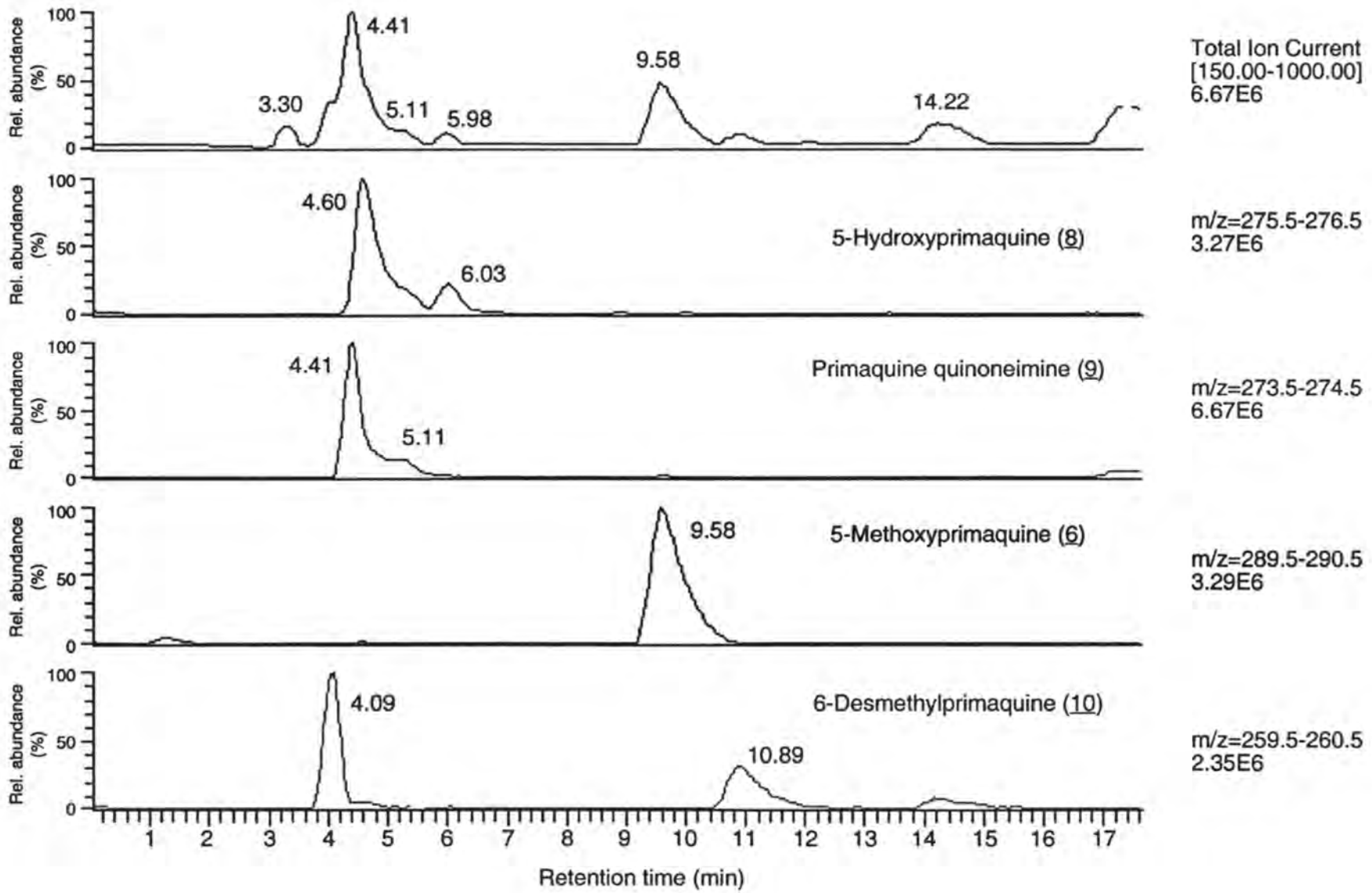


Fig. 2.3. LC-MS analysis of key reaction products from the selective hydrolysis of 5-methoxyprimaquine.

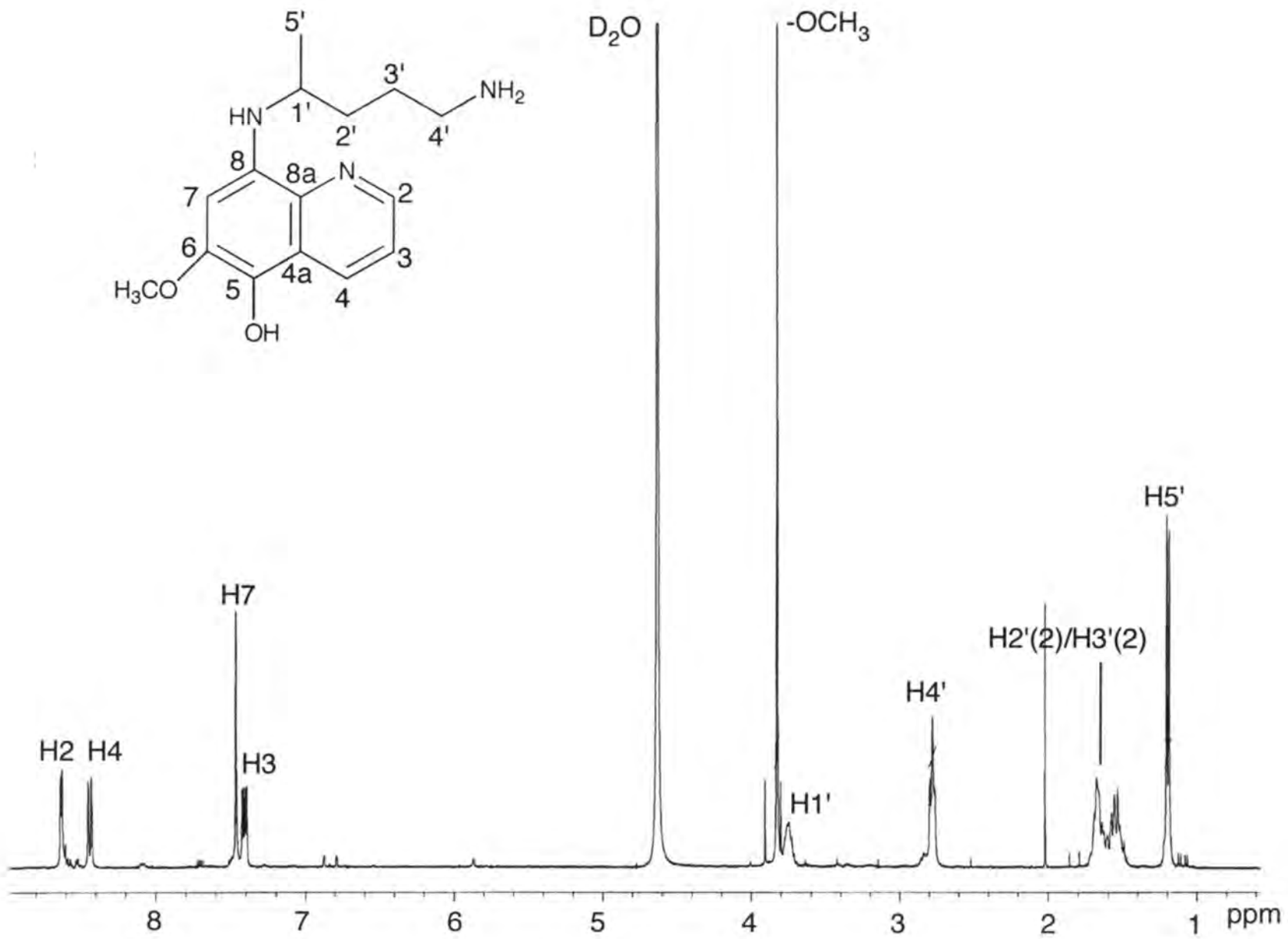


Fig. 2.4. ^{1}H NMR spectrum of 5-HPQ

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., 2002b). 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. 2.5). 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

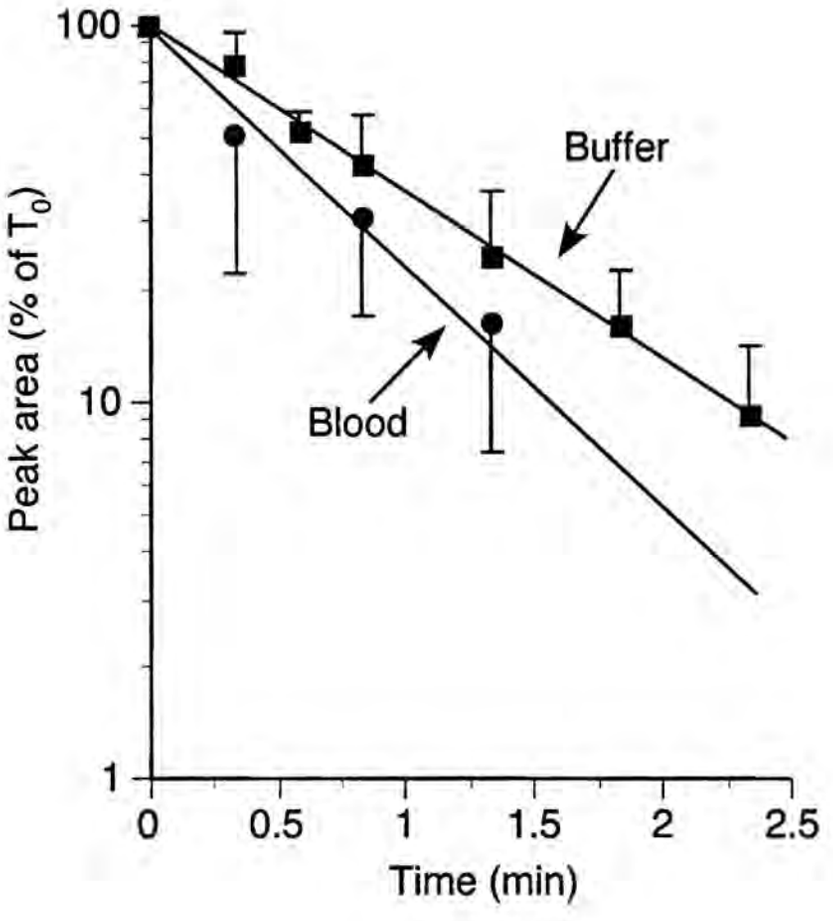


Fig. 2.5. Stability of 5-HPQ in blood versus buffer. 5-HPQ (500μM) was added to buffered saline in the absence and presence of erythrocytes (40% hematocrit) and allowed to incubate aerobically at 37°C. Aliquots were removed at designated intervals, treated with dithionite, and assayed for 5-HPQ concentration using HPLC with electrochemical detection. Values are expressed as a percentage of the concentration at T_0 and are means ± S.D. ($n = 3$).

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. 2.6, 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. 2.7A, 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. 2.7B shows the concentration

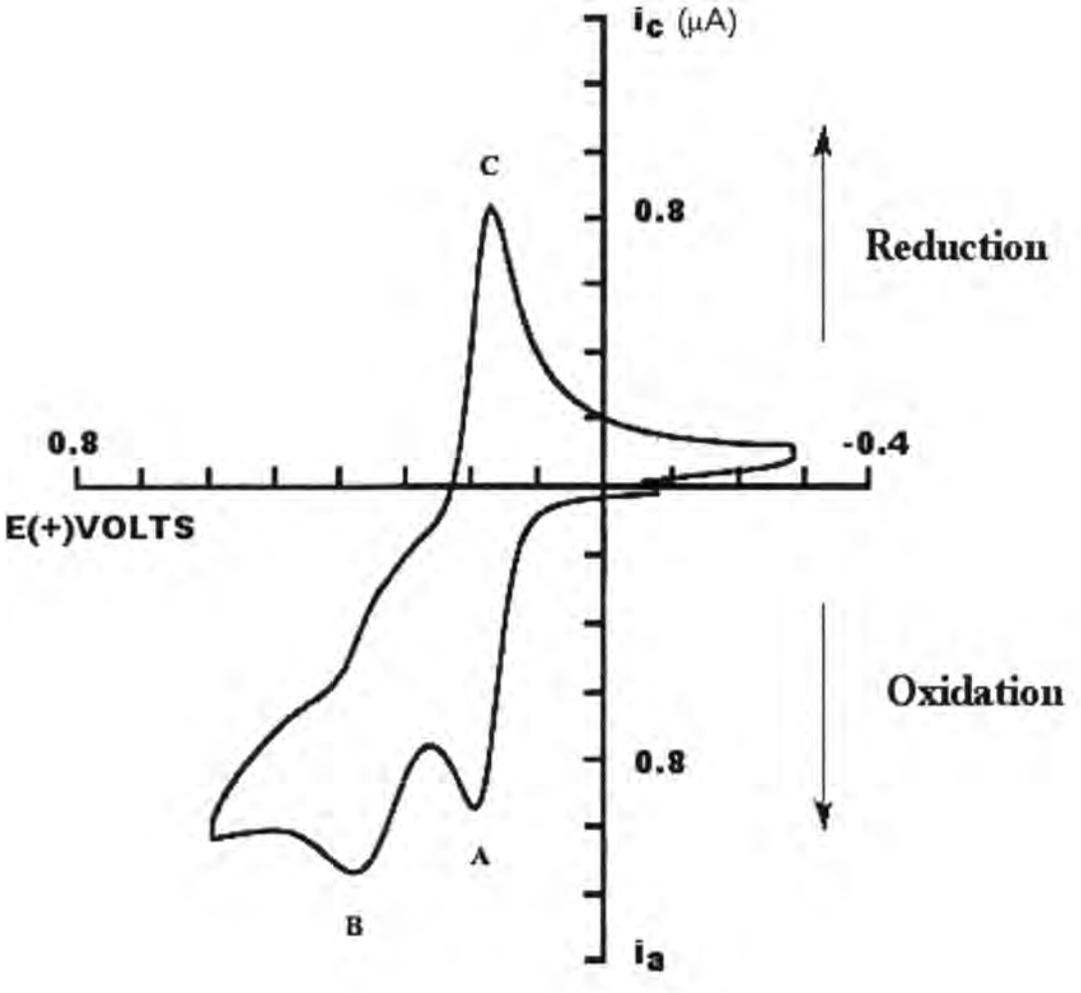


Fig. 2.6. Cyclic voltammogram of 5-HPQ (245 μM) in argon-purged PBSG (pH 7.4) at room temperature. Working electrode, carbon paste; reference electrode, Ag/AgCl; auxiliary electrode, platinum. Scan rate, 150 mV/sec. A and B, anodic (oxidation) peaks; C, cathodic (reduction) peak.

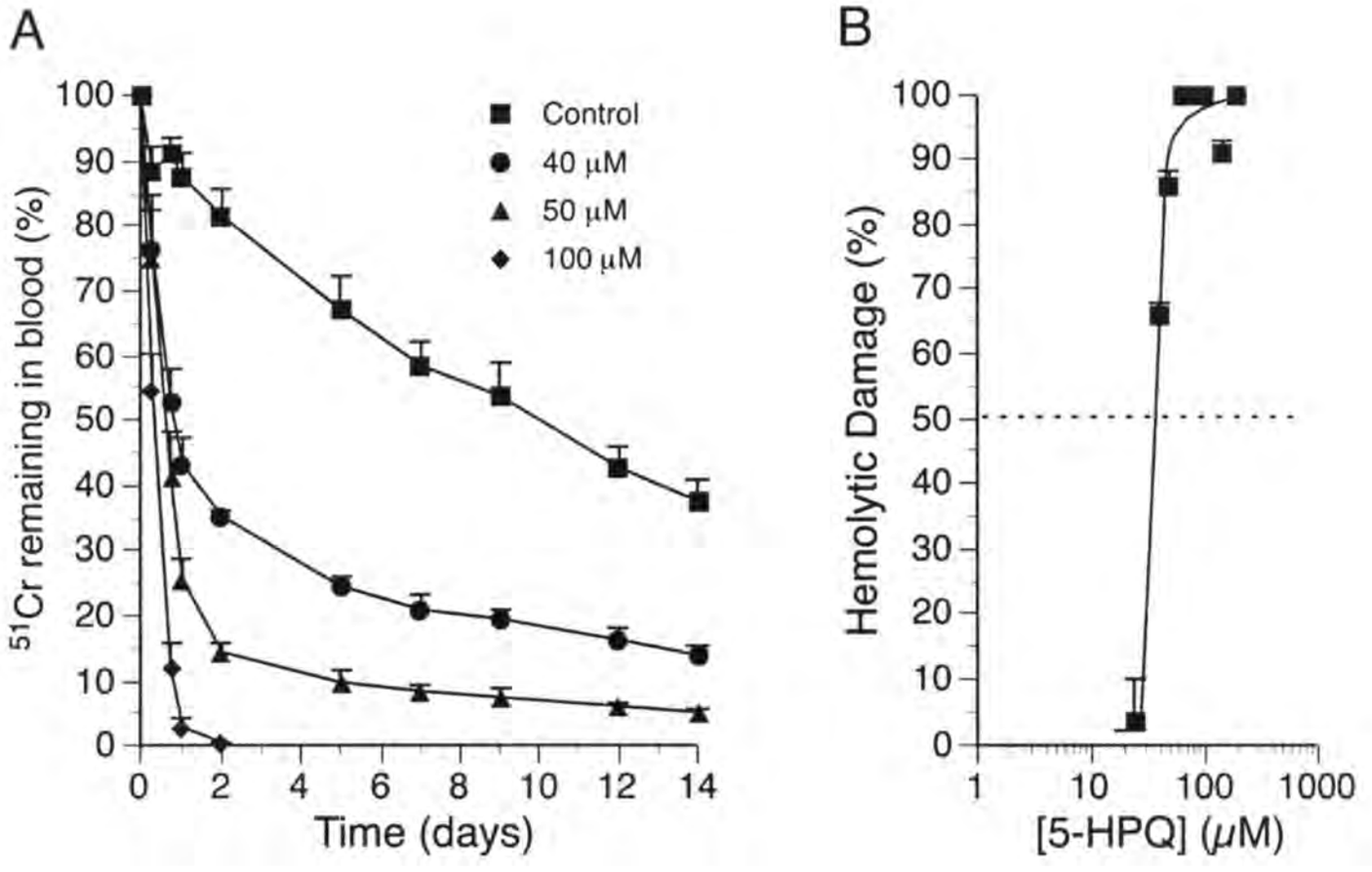


Fig. 2.7. A, survival of rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to 5-HPQ. Radiolabeled erythrocytes were incubated for 2 hr at 37°C with the indicated concentrations of 5-HPQ; control cells were incubated with vehicle (10 μl H_2O). The erythrocytes were then washed and re-administered 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).

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. 2.8A, 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. 2.8B 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. 2.9A, addition of 100 μM 5-

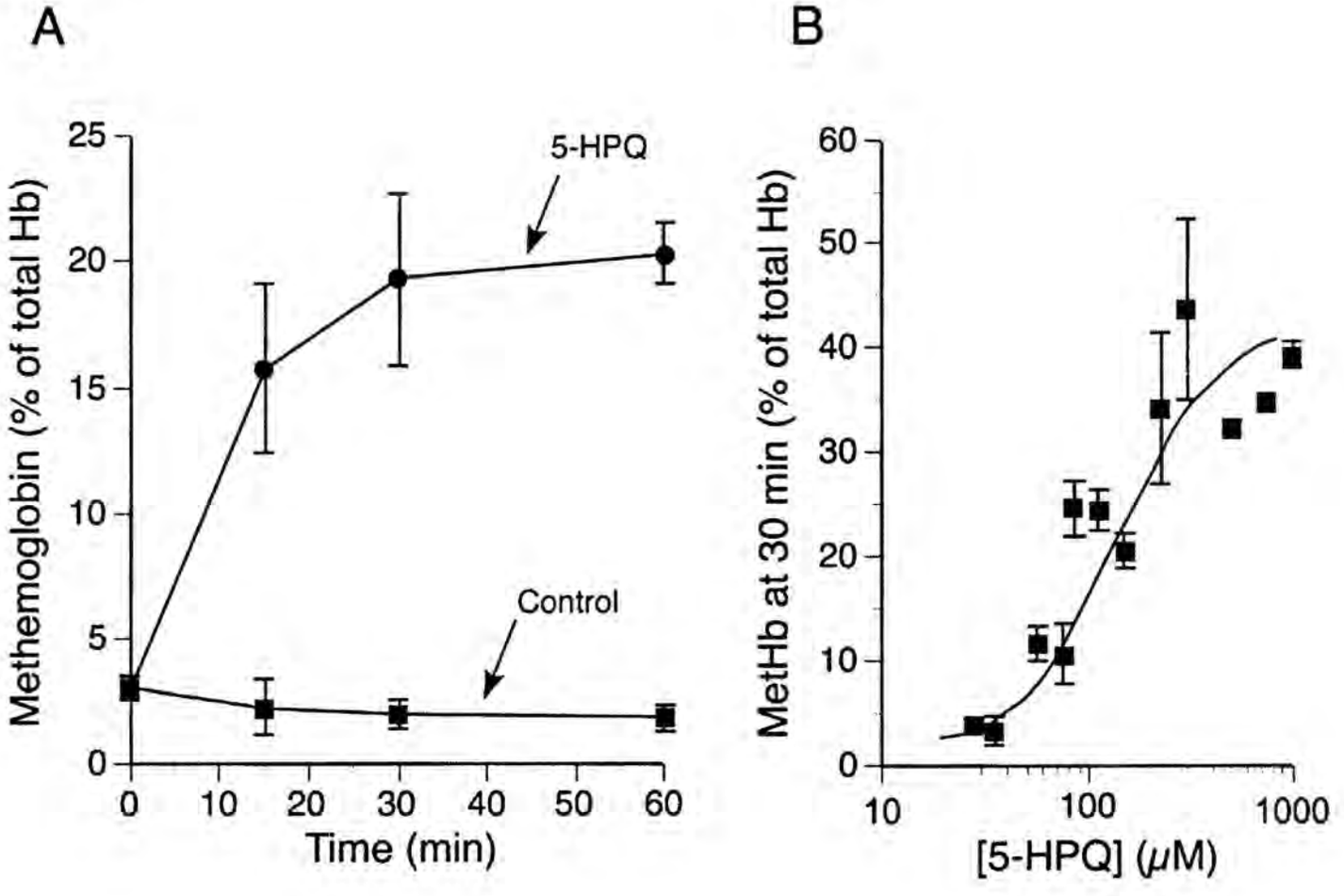


Fig. 2.8. Effect of 5-HPQ on methemoglobin formation in rat erythrocytes. A, rat erythrocytes were treated with 5-HPQ (100 μM) and assayed for methemoglobin levels over time; control cells were incubated with vehicle (10 μl H_2O). Data points are means ± S.D. (n=3). B, concentration-dependence for methemoglobin formation. Aliquots of the incubation mixture were assayed for methemoglobin 30 min after exposure to 5-HPQ (25-1000 μM). Data points are means ± S.D. (n=3).

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. 2.9B. As with the hemolytic response (Fig. 2.7B), 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. 2.10A, 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. 2.10A). 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. 2.10B) revealed the concentration-response curve to be shifted significantly to the

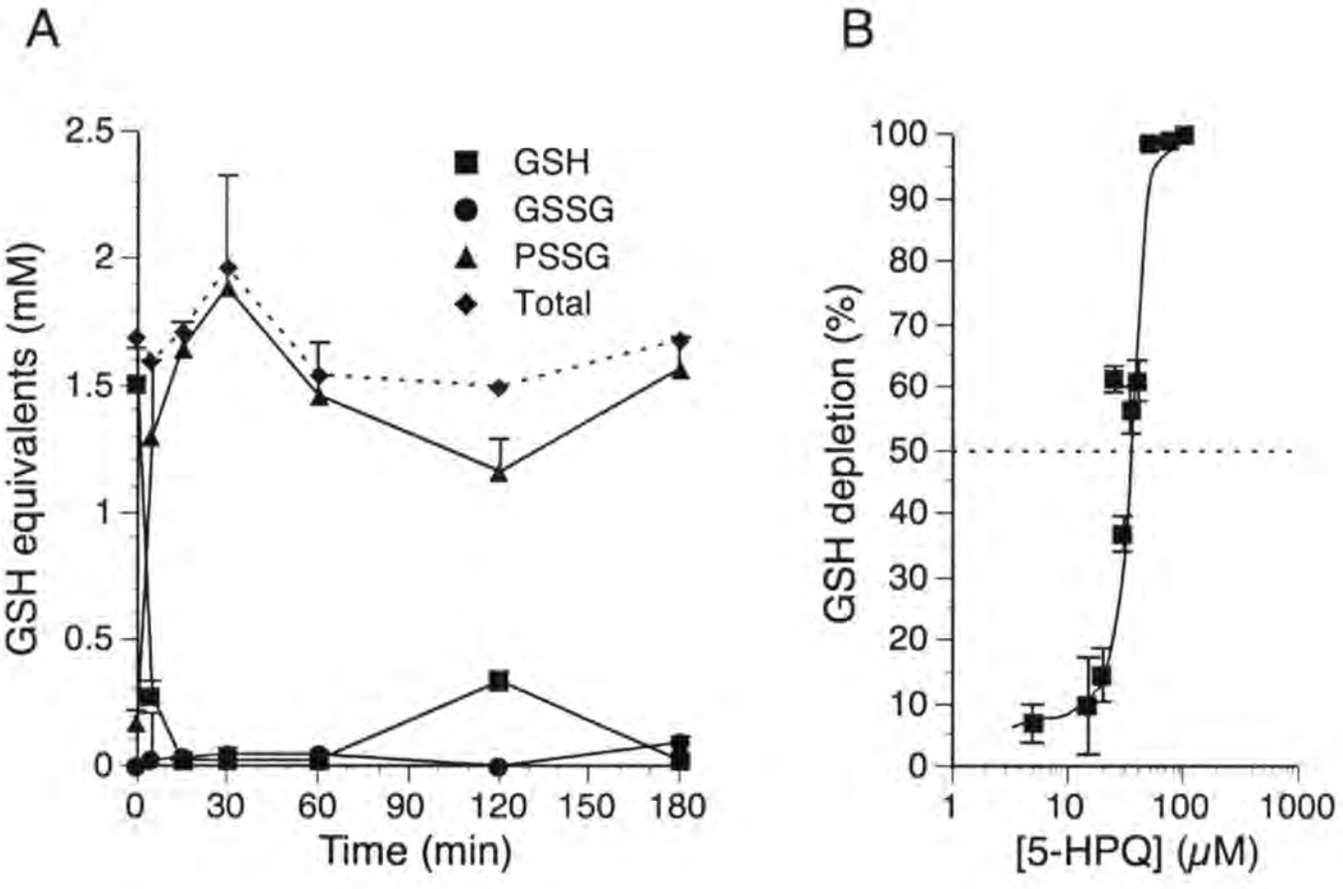


Fig. 2. 9. Effect of 5-HPQ on rat erythrocyte sulfhydryl status. A, rat red cells were incubated at 37°C in PBSG containing 5-HPQ (100 μM). At the indicated time points, aliquots were withdrawn and assayed for GSH, GSSG, and glutathione-protein mixed disulfides (PSSG). Data points are means ± S.D. (n=3). 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).

left of the response curve for GSH-normal cells (Fig. 2.7B), 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. 2.7). This loss of erythrocyte viability *in vivo* was correlated with a rapid and extensive depletion of GSH (Fig. 2.9A), which exhibited a concentration dependence that coincided with that of the hemolytic response (Fig. 2.9B). The disappearance of GSH was matched by the

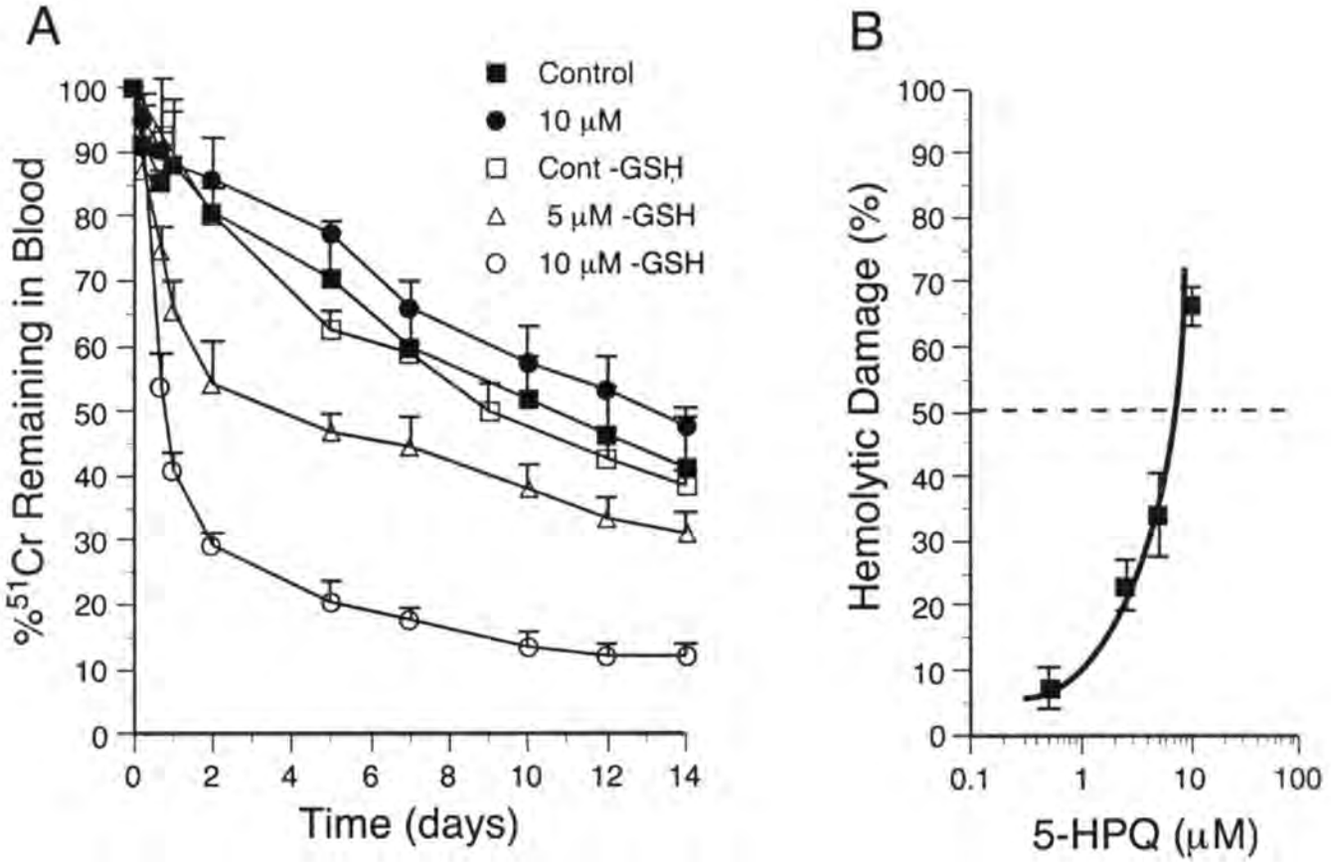


Fig. 2.10. A, survival of normal vs. GSH-depleted rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to 5-HPQ. Radiolabeled red cells were treated with DEM to deplete intracellular GSH (>95%). The cells were incubated for 2 hr at 37°C with the indicated concentrations of 5-HPQ; GSH-depleted 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).

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. 2.10A, 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.

Although the *in vitro* exposure/*in vivo* survival data presented in Fig. 2.7 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. 2.8A, hemolytic concentrations of 5-HPQ were associated with the formation of methemoglobin, however, the concentration response curve for methemoglobin formation (Fig. 2.8B) 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. 2.5). 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.

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.

CHAPTER 3

Primaquine-Induced Hemolytic Anemia: Role of Splenic Macrophages in the Fate of 5-Hydroxyprimaquine-Treated Rat Erythrocytes

Introduction

The hemolytic anemia induced by the antimalarial drug, primaquine, has long been known to be due to an accelerated uptake of oxidatively damaged erythrocytes into the spleen (Beutler, 1969). Individuals who have a deficiency in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity have an enhanced susceptibility to primaquine because they are unable to generate sufficient NADPH, and hence reduced GSH, to detoxify reactive oxygen species (ROS) generated by the drug. It is also well accepted that primaquine itself is not biologically active in erythrocytes at toxicologically relevant concentrations, but that redox active metabolites, produced by CYP-catalyzed ring- and N-hydroxylation, are responsible for the hemolytic injury that occurs after administration of the parent compound.

One of the ring-hydroxylated metabolites, 5-hydroxyprimaquine (5-HPQ), has long been postulated to be capable of mediating primaquine hemotoxicity *in vivo*. In support of this postulate, we recently demonstrated that 5-HPQ is directly hemolytic in rat red cells; that is, when rat ^{51}Cr-labeled-red cells are incubated with 5-HPQ *in vitro* for 2 hrs at 37°C and then given intravenously to isologous rats, the tagged red cells are removed rapidly from the circulation (Bowman et al., 2004). Loss of erythrocyte viability *in vivo* was associated with oxidative stress responses *in vitro*, including methemoglobin formation, depletion of erythrocytic GSH and formation of glutathione-protein mixed disulfides. Furthermore, when erythrocytic GSH was partially depleted (by 95%) by

titration with diethyl maleate (DEM) to mimic human G6PD deficiency, the hemolytic activity of 5-HPQ was enhanced in the rat erythrocytes by more than 5-fold.

The potency and pro-oxidant nature of the hemolytic activity of 5-HPQ in rats supports its role as a major contributor to primaquine-induced hemolytic anemia; however, little is known about the fate of these erythrocytes *in vivo*; i.e., do they undergo intravascular lysis and uptake of the cell fragments into the liver, or do the cells remain intact and undergo selective uptake into the spleen? In addition, the role of macrophages in the process of removal of damaged erythrocytes has been presumed, yet no data exist that demonstrate a requirement for phagocytic activity in order to provoke a hemolytic response.

The present studies were undertaken to determine the fate of 5-HPQ-treated erythrocytes in rats, and to clarify the role of macrophages in this process. Experimentally, we have compared uptake of 5-HPQ-treated erythrocytes into the spleens and livers of normal rats with that of rats pretreated with clodronate-loaded liposomes. This pretreatment is known to deplete splenic macrophages and hepatic Kupffer cells (Van Rooijen and Sanders, 1994). We report that 5-HPQ-treated ^{51}Cr-labeled erythrocytes are rapidly taken up into the spleen in preference to the liver when re-administered intravenously to untreated rats. In clodronate-loaded liposome pretreated rats, the survival of the 5-HPQ damaged red cells in the circulation was markedly prolonged, coinciding with a virtual abolition of splenic uptake and a much delayed hepatic uptake. Parallel studies using J774A.1 macrophage cultures demonstrated the uptake of 5-HPQ-damaged, but visually intact, rat red cells into "jackpot" cells in the culture (Cocco and Ucker, 2001). The uptake was concentration-dependent in regard to 5-HPQ, and was enhanced markedly by prior depletion of erythrocytic GSH by DEM titration. The data indicate that the premature removal of 5-HPQ-damaged red cells in

the rat results from their selective recognition and uptake by splenic macrophages. Of importance, the reproduction of the *in vivo* macrophage-dependent toxicity *in vitro* indicates that the J774A.1 macrophage culture can be used as a model to look for external cell surface markers that initiate splenic sequestration of erythrocytes inflicted with hemolytic injury.

Materials and Methods.

Chemicals and materials.

5-HPQ was synthesized by HBr-catalyzed hydrolysis of 5-methoxyprimaquine as described previously (Bowman et al., 2004). $Na_2{}^{51}CrO_4$ in sterile saline (1mCi/ml, pH 8) was obtained from New England Nuclear (Billerica, MA). Phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphocholine), cholesterol and clodronate (dichloromethylene bisphosphonate) were purchased from Sigma-Aldrich (St. Louis, MO). J774A.1 cells are a monocyte-derived murine macrophage cell line that was obtained from the ATCC (Manassas, VA). All other reagents were of the best grade commercially available.

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 HBSS (with Ca^{2+} and Mg^{2+}) to remove the plasma and buffy coat. The cells were resuspended to a 40% hematocrit in HBSS and used the same day they were collected. Stock solutions of 5-HPQ in argon-purged water were prepared to deliver the appropriate concentrations of 5-HPQ in 10 μl to erythrocyte suspensions (1-3 ml).

In some experiments, diethyl maleate (DEM) was used to deplete GSH (>95%) in erythrocyte suspensions as described previously (Bolchoz et al., 2002b). Briefly, DEM (750 μM) dissolved in acetone was added to packed red cells. After 15 min of incubation at 37°C, the content of erythrocytic GSH was determined from an aliquot by HPLC-EC.

Preparation and administration of clodronate-loaded liposomes.

Clodronate-loaded liposomes were prepared as described previously (Van Rooijen and Sanders, 1994). The liposomes were washed once and centrifuged to remove free clodronate, and the liposomes were administered intravenously (0.1 ml/10 g body wt) via the tail vein to rats 24 hr before administration of the radiolabeled erythrocytes. Intravenous administration of saline served as a control to ensure that these animals had normal, non-blocked, non-suppressed and non-activated macrophages.

Measurement of erythrocyte disposition in vivo.

The survival and fate of erythrocytes was determined in saline-treated or clodronate-loaded liposome-treated rats after *in vitro* exposure to 5-HPQ as described previously (Bowman et al., 2004). Briefly, ^{51}Cr-labeled erythrocytes were incubated with 5-HPQ (40 μM) for 2 h at 37°C. After incubation the erythrocytes were washed once and resuspended in HBSS (40% hematocrit). Aliquots (0.5 ml) of the labeled cells were administered intravenously to isologous rats. T_0 blood samples (75 μl) were taken from the orbital sinus 1 hr after administration of labeled red cells. Additional blood samples were taken at 48 hr intervals for 7 days. At the end of the experiment, the samples were

counted in a well-type gamma counter and the data are expressed as a percentage of the T_0 blood sample.

To determine the disposition of the ^{51}Cr label, livers and spleens were excised at designated intervals from groups of rats that had received the labeled erythrocytes as described above. The tissues were weighed and then counted concurrently in a well-type gamma counter, and the values were corrected for ^{51}Cr activity of residual erythrocytes within the tissue vasculature. In experiments to determine the disposition of broken red cells, untreated ^{51}Cr-labeled erythrocytes (40% hematocrit) were subjected to three freeze-thaw cycles using a dry ice-acetone bath to induce complete cell lysis. Aliquots of the (unwashed) broken cell preparation were administered intravenously to rats in the same manner as described above for the intact erythrocytes.

Measurement of in vitro *erythrophagocytosis.*

J774A.1 macrophages were plated in 12-well culture plates at 2 x 10^5 cells/well to prepare semiconfluent monolayers. Twenty-four hours after plating the macrophages, aliquots (50 μl) of control and 5-HPQ-treated ^{51}Cr-labeled packed erythrocytes (treated as described above) were added to the macrophage cultures and allowed to incubate for up to 24 hr at 37°C. After incubation, non-ingested erythrocytes were removed by washing once with NH_4Cl (0.8% w/v) followed by washing three times with HBSS. The adherent macrophages were released from the plates with 0.5 ml NaOH (0.5 N), and the extent of phagocytosis was assessed by counting the radioactivity in the macrophages using a well-type gamma counter.

Results

Disposition of ^{51}Cr-labeled erythrocytes after in vitro *exposure to 5-HPQ.*

Although the direct hemolytic activity of 5-HPQ in rats has been established (Bowman et al., 2004), the fate of 5-HPQ-damaged red cells *in vivo*; i.e., intravascular lysis *vs.* splenic sequestration, is unknown. To determine the anatomic site(s) of sequestration of 5-HPQ-damaged red cells and the role of macrophages in this process, rat ^{51}Cr-labeled erythrocytes were incubated with 5-HPQ (40 μM) for 2 hr at 37°C. After the incubation, the erythrocytes were washed and returned to the circulation of saline-pretreated or clodronate-loaded liposome-pretreated rats. At designated intervals a blood sample was taken from each rat followed by excision of the spleen and liver for determination of uptake of the radiolabel.

Upon readministration to saline-pretreated rats, 5-HPQ-treated erythrocytes showed an initial rapid disappearance from the circulation (Fig. 3.1A). This loss of blood radioactivity coincided with a marked increase in splenic radioactivity (Fig. 3.1B), which was not observed in rats that received saline-treated control cells. The splenic radioactivity reached an apparent steady state by day 1 corresponding to processing of the tagged red cells and subsequent release of the radioactivity for urinary excretion (data not shown). The levels of radioactivity in the livers of rats given 5-HPQ-treated erythrocytes were slightly higher than those in the controls (Fig. 3.1B), and these levels remained constant over the course of the experiment. On a whole organ basis, splenic radioactivity in saline-pretreated rats was 4 to 5 times that of the liver and 50 to 60 times higher when expressed per gram of tissue.

In contrast to the rapid disappearance of blood radioactivity in saline-pretreated rats, the rate of removal of 5-HPQ-treated erythrocytes in rats pretreated with clodronate-loaded liposomes was markedly reduced (Fig. 3.2A), and was not accompanied by significant uptake of radioactivity into the spleen (Fig. 3.2B). As shown in Fig. 3.3A, the decreased rate of removal of blood radioactivity was associated with a time-dependent decrease in the spleen/body weight ratio (40% decrease at day 7), which presumably reflects extensive depletion of macrophages from this tissue. Administration of clodronate-loaded liposomes had no effect on liver/body weight ratio (Fig. 3.3B). The amount of ^{51}Cr detected in the livers of rats pretreated with saline or clodronate-loaded liposomes was relatively low, suggesting that under normal conditions, hepatic Kupffer cells do not have an active role in removing 5-HPQ-damaged erythrocytes. Of note, the clodronate-loaded liposome-pretreated rats showed a significant increase in liver radioactivity on day 7 (Fig. 3.2B), which suggests that Kupffer cells may have begun to repopulate the liver and remove damaged erythrocytes in the absence of a functional splenic uptake mechanism. This observation is in agreement with previous studies which showed that Kupffer cells repopulate the liver after liposome treatment more rapidly than macrophages repopulate the spleen (Van Rooijen et al., 1990). Although uptake into the liver may be considered to reflect intravascular lysis of erythrocytes, it has been shown that Kupffer cells will take on the role of removing senescent and damaged erythrocytes in splenectomized animals (Ganick et al., 1977).

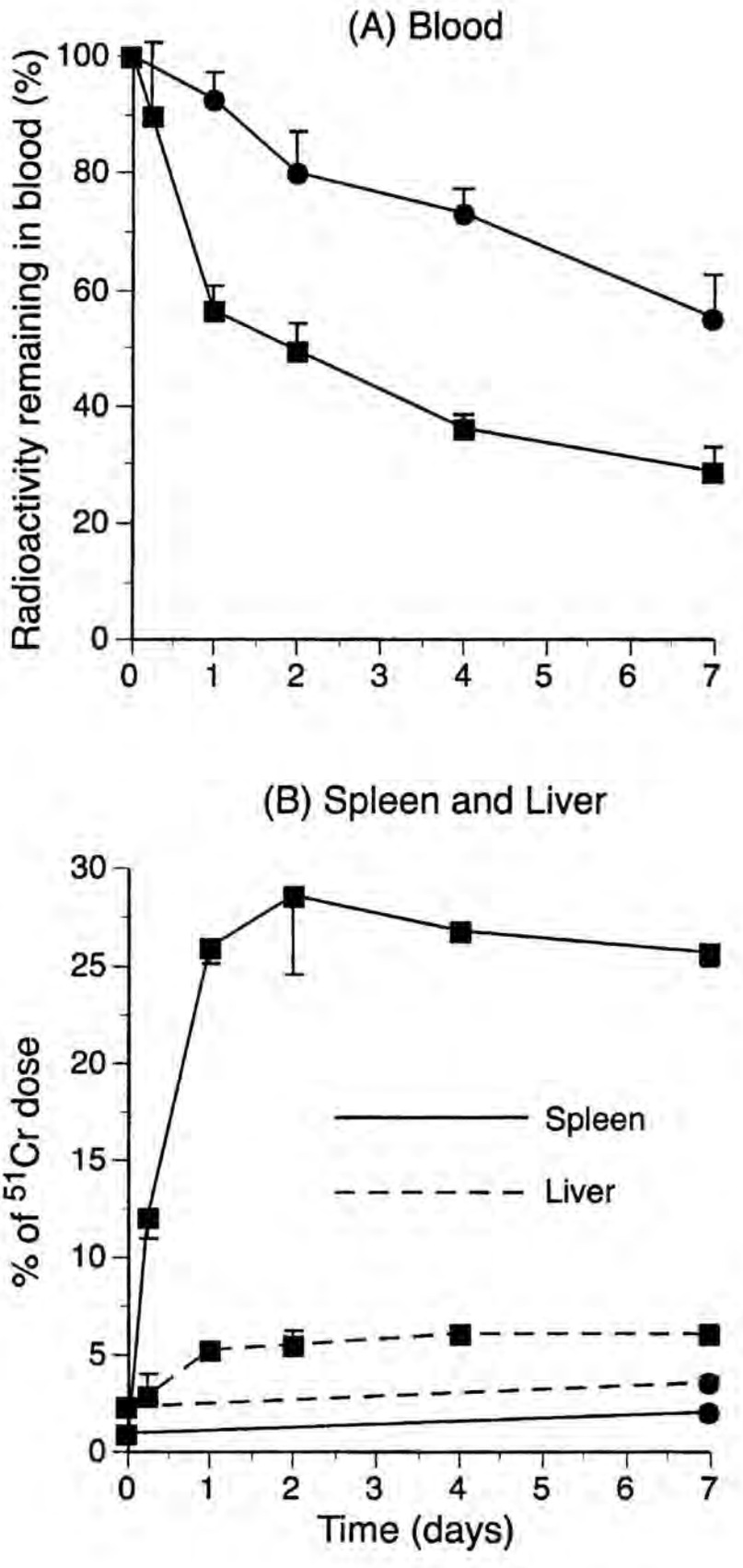


Fig. 3.1. Disposition of the ^{51}Cr label in (A) blood and (B) spleens and livers of rats infused with ^{51}Cr-labeled erythrocytes exposed *in vitro* to the vehicle (●) or 40 μM 5-HPQ (■) for 2 hr at 37°C. Data points are means ± S.D. (n = 3).

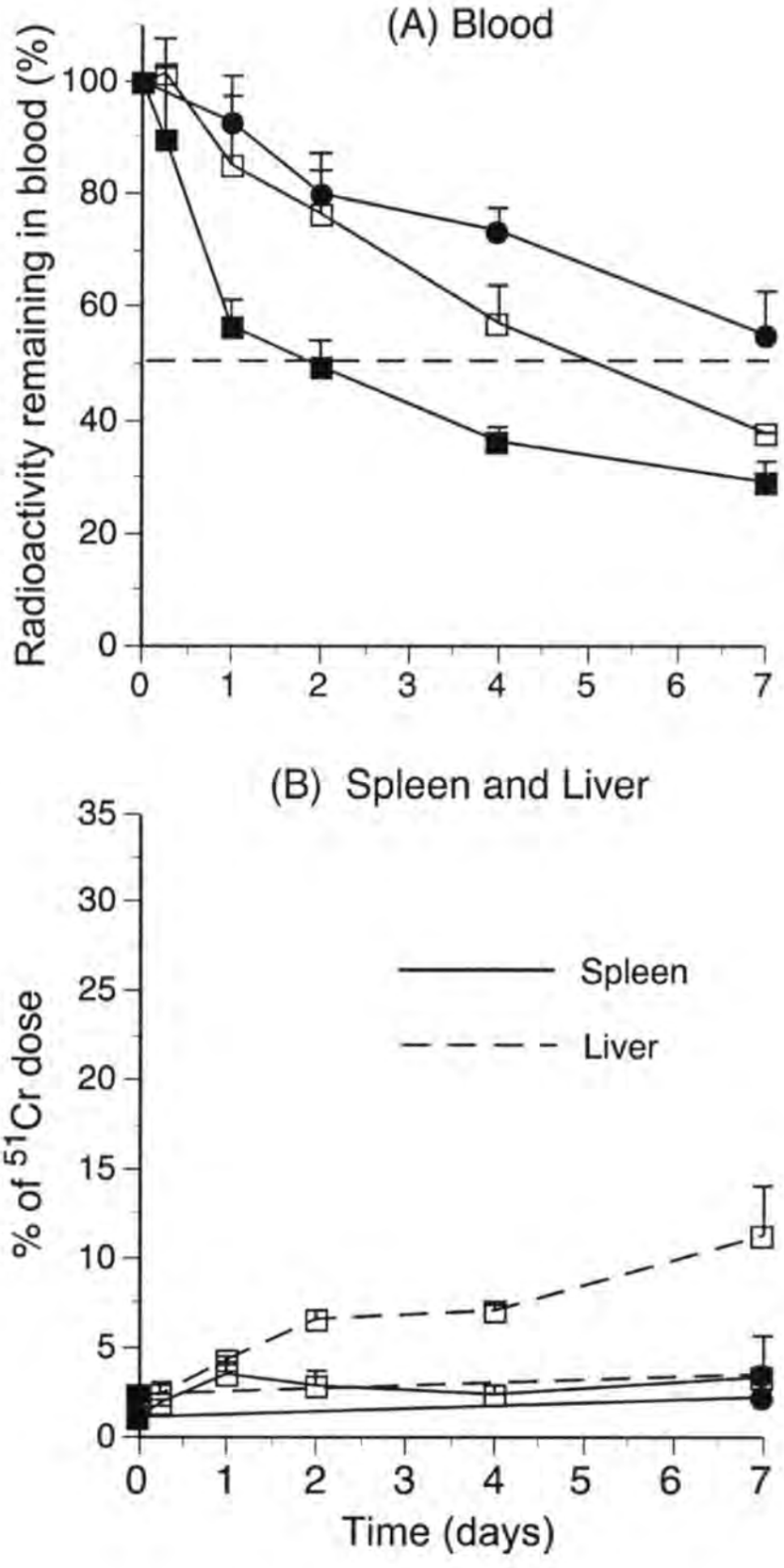


Fig. 3.2. Effect of pretreatment of rats with saline (*closed symbols*) or clodronate-loaded liposomes (open symbols) on the disposition of the ^{51}Cr label in (A) blood and (B) spleens and livers of rats infused with ^{51}Cr-labeled erythrocytes exposed *in vitro* to the vehicle (circles) or 40 μM 5-HPQ (squares) for 2 hr at 37°C. Data points are means ± S.D. ($n = 3$).

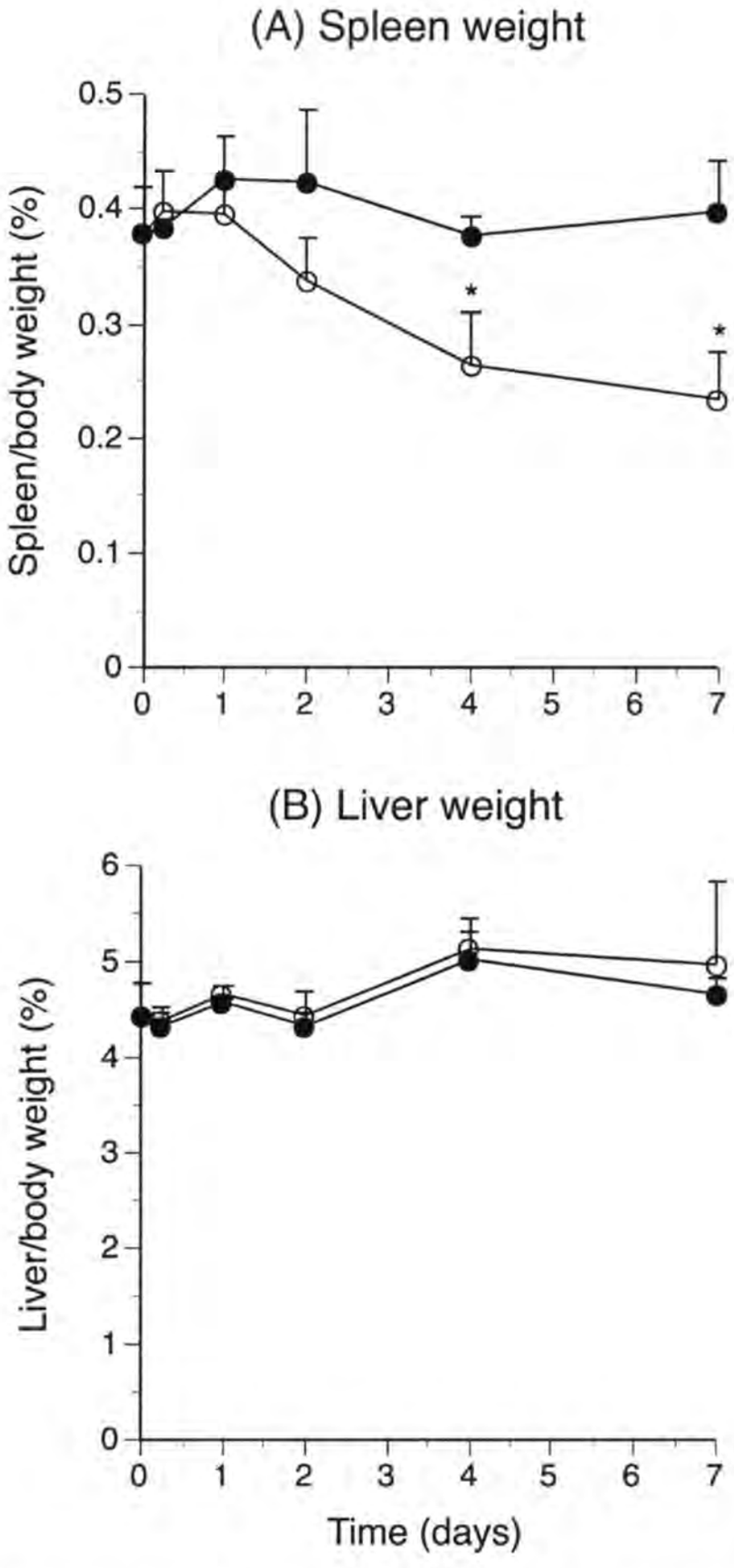


Fig. 3.3. Effect of pre-treatment of rats with clodronate-loaded liposomes on (A) spleen and (B) liver weight/body weight ratios. Rats were given saline (●) or clodronate-loaded liposomes (O) intravenously. At the indicated time points, the spleens and livers were excised and weighed. Data points are means ± S.D ($n = 3$). *Significantly different from PBS-pretreated rats ($p < 0.05$).

Disposition of ^{51}Cr-labeled broken cells in vivo.

To determine if intravascular lysis could have contributed to the hemolytic response to 5-HPQ, the disposition of untreated ^{51}Cr-labeled broken red cells was examined. Broken erythrocytes were prepared by subjecting ^{51}Cr-tagged cells to multiple freeze-thaw cycles. As shown in Fig. 3.4A, rapid and complete disappearance of the ^{51}Cr-label from the blood occurred within 2 days, and the loss of blood radioactivity was accompanied by a modest increase in liver radioactivity that probably reflects uptake of red cell fragments by Kupffer cells (Fig. 3.4B). In contrast to 5-HPQ-treated erythrocytes, only a minor amount of radiolabel from the broken cells was taken up into the spleen. About 30% of the administered radioactivity appeared in the urine during the seven-day observation period; the remainder was unaccounted for and was presumed to be occluded within the microcirculation.

Role of macrophages on the hemotoxicity of 5-HPQ in vitro.

To determine whether splenic macrophage-dependent uptake of 5-HPQ-treated erythrocytes could be reproduced *in vitro*, ingestion of ^{51}Cr-labeled rat erythrocytes was assessed using J774A.1 cells, a mouse monocyte-derived macrophage cell line that has been shown previously to bind and ingest both necrotic and apoptotic cells (Cocco and Ucker, 2001). Radiolabeled erythrocytes were incubated with hemolytic concentrations of 5-HPQ for 2 hr at 37°C. The erythrocytes were then washed and incubated with macrophages for various time periods. At the end of each incubation period, the macrophages were rinsed with HBSS to remove non-adherent erythrocytes, and then treated with ammonium chloride to remove adherent (but non-ingested) erythrocytes.

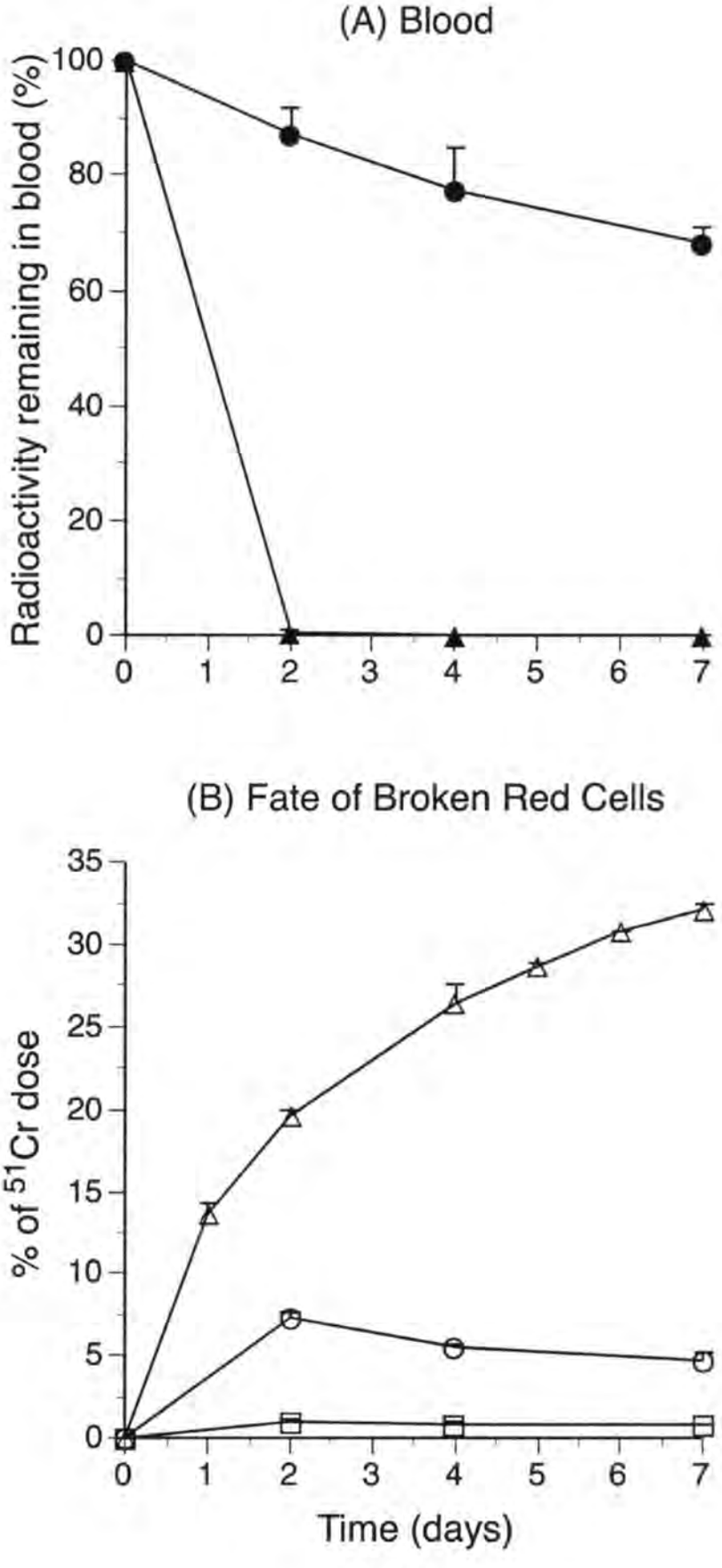


Fig. 3.4. Disposition of the ^{51}Cr label in (A) blood of rats infused with ^{51}Cr-labeled normal (●) and broken (▲) erythrocytes and in (B) the spleens (□), livers (○) and urine (△) of rats infused with ^{51}Cr-labeled broken erythrocytes. Data points are means ± S.D. (n = 3).

As shown in Fig. 3.5, there was a concentration-dependent increase in radioactivity associated with the macrophages after 18 hr of incubation. The level of radioactivity associated with the macrophages at earlier time points (6 and 12 hr) was not significantly different from the controls (data not shown). Of importance, the concentration dependence for this response coincided with the concentration dependence for hemolytic activity *in vivo*.

Visual confirmation of the phagocytic response was carried out by microscopic examination of the macrophages after 18 hr of incubation and prior to red cell lysis with ammonium chloride. In cultures containing control erythrocytes, only an occasional red cell could be observed within some of the macrophages (Fig. 3.6A). In contrast, large numbers of intact erythrocytes could be observed within many of the macrophages that received 5-HPQ-treated erythrocytes (Fig. 3.6B). Of note, a subset of the J774A.1 macrophages appeared to account for a majority of the uptake of the damaged red cells. This phenomenon has been observed previously and suggests that within the clonal population, some macrophages, designated "jackpot cells," are more competent at erythrocyte recognition than are others. The mean values for macrophage radioactivity incorporate macrophages that have ingested no erythrocytes and others that contain several ingested erythrocytes.

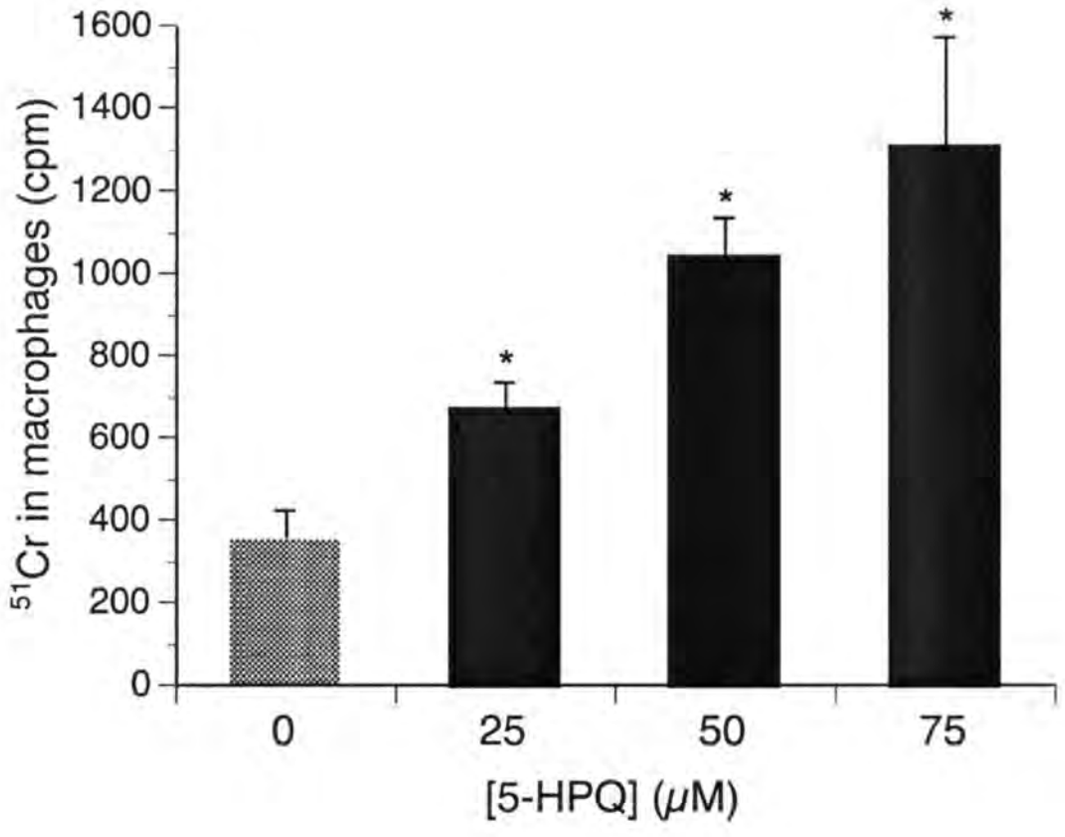


Fig. 3.5. Effect of 5-HPQ on the uptake of rat ^{51}Cr-labeled red cells by J774A.1 macrophages. ^{51}Cr-labeled erythrocytes were incubated with the indicated concentrations of 5-HPQ for 2 hr at 37°C. The erythrocytes were then washed and added to J774A.1 macrophage cultures for 24 hr. The cultures were washed with saline and ammonium chloride to remove unbound and bound (but non-ingested) erythrocytes, respectively. The macrophages were then scraped from the plates and the radioactivity was counted. Data points are means ± S.D. (n=3). *Significantly different from controls ($p < 0.01$).

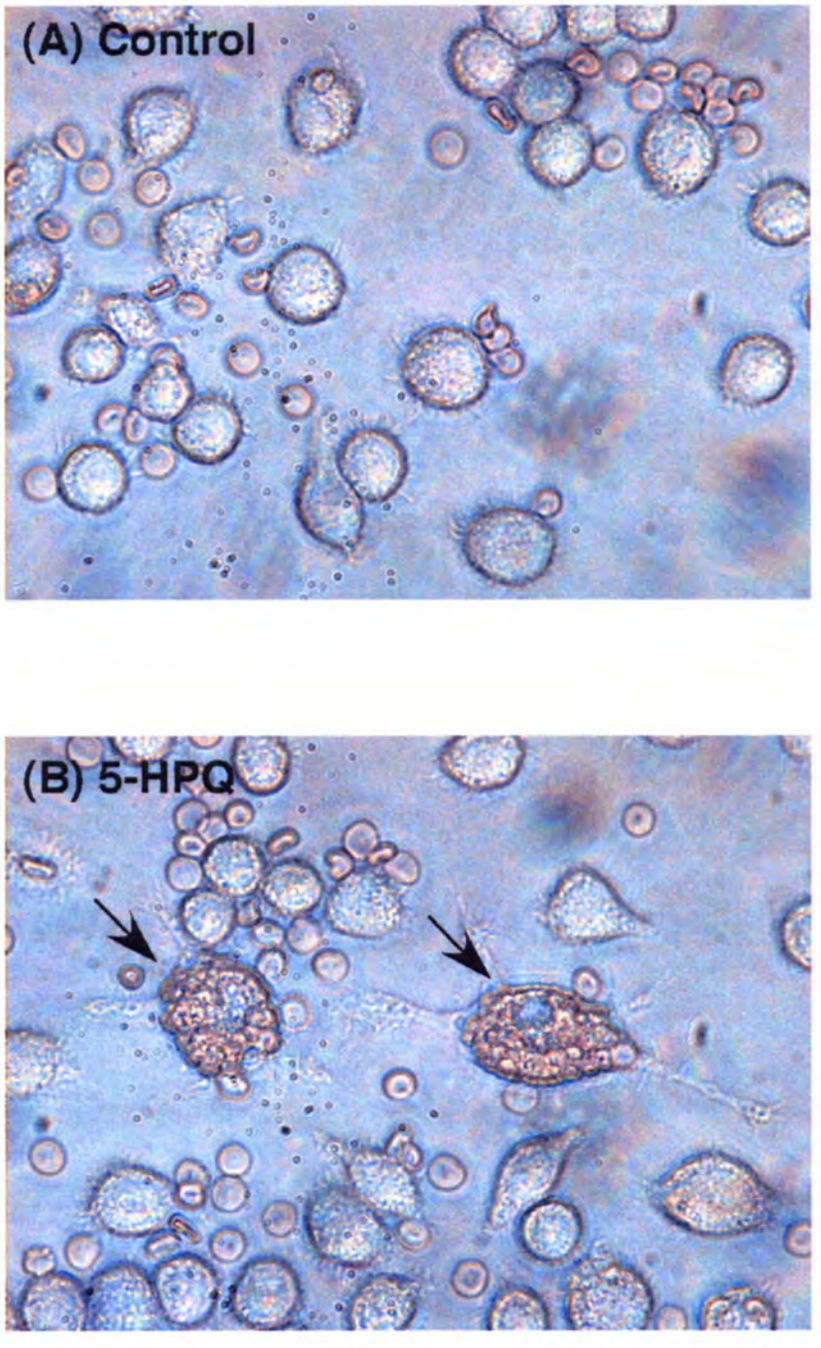


Fig. 3.6. Light micrographs of J774A.1 macrophage cultures after incubation with (A) control and (B) 5-HPQ-treated rat erythrocytes. Rat erythrocytes were treated with the vehicle or 5-HPQ (50 μM) for 2 hr at 37°C before being incubated with the macrophages for 18 hr. The arrows indicate the presence of intact 5-HPQ-treated erythrocytes within "jackpot cells." Magnification 63x.

Effect of GSH depletion on phagocytic uptake.

G6PD-deficient individuals have long been known to have an enhanced susceptibility to primaquine-induced hemolytic anemia. We have shown previously that this enhanced susceptibility can be reproduced by prior depletion of red cell GSH (Bolchoz et al., 2002; Bowman et al., 2004). In the case of 5-HPQ, the hemolytic susceptibility of GSH-depleted rat erythrocytes is about 5-fold greater than GSH-normal red cells in response to treatment with 5-HPQ (Bowman et al., 2004). To determine the effect of GSH depletion on phagocytic removal *in vitro*, ^{51}Cr-labeled erythrocytes were titrated with diethyl maleate to deplete GSH (*ca.* 95%) prior to exposure to a low concentration of 5-HPQ. After 2 hr of incubation at 37°C, control and GSH-depleted erythrocytes were incubated with J774A.1 macrophages for 18 hr. As shown in Fig. 3.7, treatment of GSH-normal erythrocytes with 25 μM 5-HPQ did not induce a significant increase in phagocytic uptake by macrophages as compared with the control. However, the GSH-depleted erythrocytes showed a significant increase in their phagocytic uptake as compared with the control. This observation is in agreement with the previous *in vitro* exposure/ *in vivo* erythrocyte survival study (Fig. 2.10), which showed that 25 μM 5-HPQ was sub-hemolytic in GSH-normal erythrocytes but was hemolytic in GSH-depleted erythrocytes (Bowman et al., 2004).

Discussion

Human erythrocytes have a predetermined lifespan in the circulation of approximately 120 days. During this time, damage is thought to accumulate within erythrocytes to the point that these cells are recognized as senescent and are removed from the circulation by macrophages of the reticuloendothelial system. Although 360

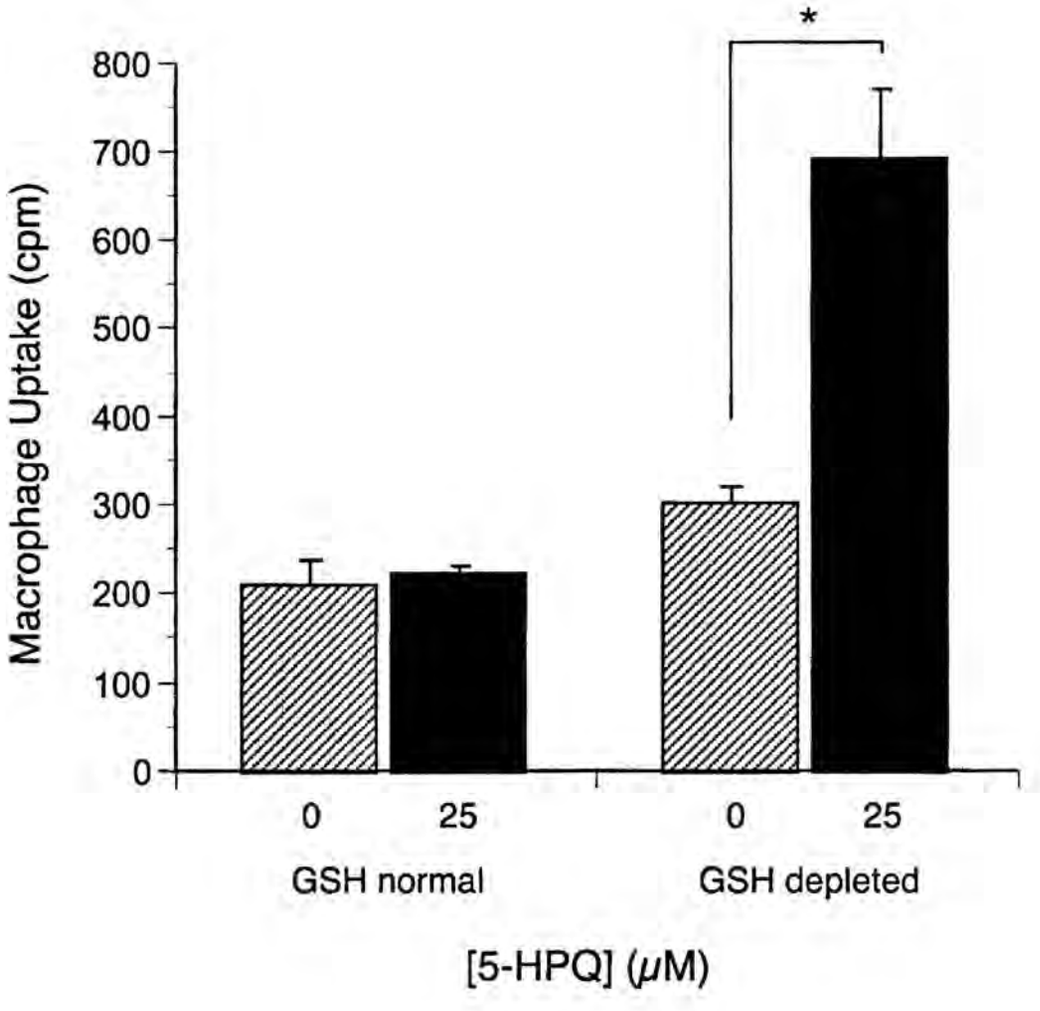


Fig. 3.7. Effect of depletion of erythrocytic GSH on uptake of 5-HPQ-treated erythrocytes by J774A.1 macrophages. ^{51}Cr-Labeled erythrocytes were titrated with DEM to deplete GSH by 95%. GSH-normal and GSH-depleted erythrocytes were then incubated with the vehicle or 5-HPQ (25 μM) for 2 hr at 37°C. The erythrocytes were then washed and incubated with J774A.1 macrophages for 18 hr. Data points are means ± S.D. (n=4). *Significantly different from controls ($p < 0.05$).

billion senescent erythrocytes are removed from the circulation per day by this system, surprisingly little is known about the changes red cells undergo as they age or the mechanism by which these cells are recognized and removed from the circulation (Jandl, 1996). Studies supporting specific mechanisms are numerous and often conflicting, but most have in common the idea that an accumulation of damage to the membrane (in some form or another) occurs as a consequence of normal chronic, low-level oxidative stress (Clark et al., 1984). How intracellular oxidative stress might provide a signal for phagocytic uptake is not understood, however, a number of theories have been proposed.

On the one hand, oxidative stress may stimulate erythrocyte phagocytosis through a non-specific mechanism referred to as "culling". In this process, oxidative stress is thought to alter the membrane cytoskeleton leading to a decrease in erythrocyte deformability, thereby restricting progress of erythrocytes through the splenic red pulp and increasing their exposure to resident macrophages (Chadburn, 2000). On the other hand, oxidative stress may induce discrete changes to the external cell surface that stimulate specific phagocytic receptors on macrophages. Although what constitutes a recognition ligand on the erythrocyte surface is not yet known, extensive studies have supported several competing viewpoints, including: 1) unmasking of a cryptic antigenic site leading to opsonization by circulating autologous IgG (Kay, 1994); 2) unmasking of carbohydrate epitopes by desialization of glycoproteins, such as glycophorin A (Kay, 1993); 3) externalization of the membrane phospholipid, phosphatidylserine (Mandal et al., 2002); and 4) loss of self recognition caused by alterations in the cell surface expression or lateral distribution of the macrophage inhibitory ligand, CD47 (Oldenborg et al., 2000).

As part of ongoing studies in our laboratory to identify redox-active metabolites of primaquine that contribute to its hemotoxicity, we have demonstrated that a phenolic metabolite, 5-HPQ, is a direct-acting hemolytic agent in the rat (Bowman et al., 2004). More specifically, we have shown that treatment of erythrocytes with 5-HPQ *in vitro* induces a GSH-dependent acceleration in the removal of the treated red cells *in vivo*. The present studies were undertaken to determine the fate of these erythrocytes and to ascertain whether splenic macrophages play a significant role in this process.

To determine the fate of 5-HPQ-treated erythrocytes *in vivo*, we compared the extent of uptake of control, broken and 5-HPQ-treated erythrocytes into the spleen and liver, which are the two major organs for erythrocyte clearance. Broken red cells and erythrocytes exposed to 40 μM 5-HPQ *in vitro* were both removed rapidly from the circulation as compared to the control. However, radioactivity from the broken cells was distributed to the liver and was ultimately recovered in the urine (Fig. 3.4B), whereas the radiolabel from 5-HPQ-treated erythrocytes was distributed primarily to the spleen (Fig. 3.1B). These data indicate that the spleen is the primary anatomic site of sequestration for erythrocytes exposed to 5-HPQ, and suggest that intravascular lysis does not contribute significantly to the loss of erythrocytes from the circulation. A similar observation was reported in G6PD-deficient individuals receiving primaquine (Degowin et al., 1966). Those studies demonstrated that the radiolabel was taken up into both the spleen and the liver; however, the role of the spleen was significantly greater when uptake was expressed relative to organ weight. Importantly, these observations lend further support to the hypothesis that 5-HPQ has the requisite properties to mediate primaquine toxicity *in vivo*.

The selective depletion of macrophages from specific tissues has been shown to be beneficial for the determining their role in defined biological processes (Van Rooijen and Sanders, 1994). Macrophages have long been thought to be responsible for the splenic sequestration that is observed following administration of hemolytic agents. If macrophages are the key players and erythrocyte removal is not due simply to non-specific trapping in the splenic cords, then substances that cause a loss of macrophage viability or otherwise interfere with the recognition mechanism should have an inhibitory effect on splenic sequestration. To test the dependence of the hemolytic response on the presence of functional macrophages, rats were given clodronate-loaded liposomes 24 hr before administration of the radioalabeled erythrocytes. After being ingested the liposomes are degraded within macrophages by lysosomal phospholipase, releasing clodronate into the cytosol where it induces macrophage cell death by apoptosis (Van Rooijen and Sanders, 1994). A dose of 0.1 ml of clodronate-liposome suspension per 10 g body weight has been reported to be sufficient for depletion of splenic macrophages and hepatic Kupffer cells, and the decline in spleen/body weight ratio observed over the course of the experiment (Fig. 3.3) is consistent with selective loss of macrophages from the spleen.

The time it takes to remove 50% of the ^{51}Cr-labeled erythrocytes for 5-HPQ-treated erythrocytes in clodronate-loaded liposome-pretreated rats was significantly delayed (by ~3 days) in relation to that of the saline-pretreated rats (Fig. 3.2A). In addition, the accumulation of radioactivity that was observed in the spleens of saline-pretreated rats was markedly reduced in clodronate liposome-pretreated rats (Fig. 3.2B). Interestingly, uptake of the label into the livers of clodronate liposome-pretreated animals increased

significantly 4 days after administration of the labeled cells (Fig. 3.2B). This observation is in agreement with previous studies which showed that Kupffer cells repopulate the liver more rapidly than macrophages repopulate the spleen following treatment with the liposomes (Van Rooijen and Van Kesteren-Hendrikx, 2003), and it reflects the ability of Kupffer cells to increase their phagocytic activity towards erythrocytes in the absence of a functional splenic uptake mechanism (Ganick et al., 1977).

An *in vitro* assay for hemolytic anemia has long been desired. Although the rat ^{51}Cr-labeled erythrocyte *in vitro* exposure/*in vivo* survival assay (e.g., Fig. 3.1A) is a useful experimental tool for identifying direct-acting hemolytic agents and for studying the mechanism underlying the hemolytic response, this assay is limited by the need for large numbers of animals, it does not easily permit erythrocyte-macrophage interactions to be examined in detail, and it offers no way to investigate the hemolytic response in human erythrocytes. Measurement of drug-induced erythrocyte lysis in the test-tube (i.e., osmotic fragility) is not a valid substitute for the hemolytic response *in vivo* because of the recognition that the toxic endpoint is splenic sequestration. Efforts to develop cell culture assays based on isolated splenic macrophages or Kuppfer cells are hindered by the low numbers of cells that can be harvested from these tissues.

In an effort to reproduce *in vitro* the uptake of 5-HPQ-treated erythrocytes that was observed *in vivo*, the phagocytic activity of murine monocyte-derived macrophage cell lines towards control and 5-HPQ-treated erythrocytes was examined. In initial experiments we utilized RAW 264.7 macrophages, but they did not respond to 5-HPQ-treated erythrocytes (data not shown), though these cells did ingest erythrocytes that had been treated with neuraminidase (which cleaves sialic acid residues). In contrast, J774A.1

macrophages were found to respond selectively to 5-HPQ-treated erythrocytes. Uptake of the ^{51}Cr label by these macrophages was dependent on the concentration of 5-HPQ (Fig. 3.5), and this concentration-dependence correlated closely with that of the hemolytic response (Bowman et al., 2004). Importantly, uptake of the ^{51}Cr label was due to phagocytosis of intact erythrocytes (Fig. 3.6), which supports the conclusion that erythrocyte removal *in vivo* is due to uptake of intact erythrocytes rather than to intravascular lysis. Moreover, the data suggest that restrictive passage of erythrocytes through the splenic architecture may not be necessary for removal of the damaged cells from the circulation.

To test the validity of this cell culture system as an experimental model for 5-HPQ-induced hemolytic anemia, the effect of depletion of erythrocyte GSH on phagocytic uptake was examined. In agreement with previous studies (Bowman et al., 2004), GSH depletion induced the uptake of erythrocytes that were treated with a sub-hemolytic concentration of 5-HPQ in GSH-normal erythrocytes (Fig. 3.7). Taken together, these data indicate that J774A.1 macrophages can be used as a valid model to identify cell surface changes that signal macrophage recognition and phagocytosis.

For many years, "pitting" and "culling" have been commonly accepted mechanisms for the functional removal of oxidatively damaged red cells by the spleen. "Culling" is thought to occur as damage to the red cell results in increased transit time through the tight meshwork of the splenic red pulp, which increases the likelihood of exposure to and recognition by cordal macrophages (Chadburn, 2000). Possible mechanisms of trapping that have been suggested include: 1) that the hemoconcentration that occurs in the splenic red pulp creates an acidic, hypotonic environment that results in cell swelling and

trapping within the splenic meshwork; or 2) that removal is directly related to profound morphological alterations and/or damage to the red cell membrane that renders them less flexible and thus more susceptible to becoming trapped (Chadburn, 2000). "Pitting" is thought to lead to the removal of damaged cells as cell inclusions or appendages, such as Heinz bodies, cause parts of the red cell to be trapped in the sinus wall and subsequently amputated by macrophages, allowing the pliant part of the cell to slip through. Although the cell survives, it does so with a reduction of membrane surface, which can ultimately lead to spherocytosis and trapping in the spleen. Erythrophagocytosis by J774A.1 macrophages *in vitro* provides evidence that "pitting" and "culling" are not necessary for the recognition of 5-HPQ-damaged red cells. Although a role for culling cannot be ruled out, since it could still work by increasing the time of exposure of damaged red cell to macrophages, it is likely that the macrophages recognize a specific signal on the red cell surface rather than a general structural alterations.

In summary, we have demonstrated that splenic macrophages play a crucial role in the removal of 5-HPQ-treated rat red cells from the circulation, and that this response can be reproduced in vitro using J774A.1 macrophages. This observation further supports the notion that 5-HPQ is an important contributor to the hemolytic response induced by primaquine. Future studies are warranted to determine the nature of the signal that commits the damaged erythrocytes to premature splenic sequestration.

CHAPTER 4

Primaquine-Induced Hemolytic Anemia: Role of Membrane Lipid Peroxidation and Cytoskeletal Protein Alterations on the Hemotoxicity of 5-Hydroxyprimaquine

Introduction

Malaria is a life-threatening parasitic disease of epidemic proportions, and it is a major public health concern for developing countries. Each year malarial infection is responsible for an estimated 300-500 million acute illnesses and 2 million deaths in tropical and subtropical regions worldwide (Kain and Keystone, 1998). Primaquine, the prototype 8-aminoquinoline tissue schizontocide, is effective against all four malarial species that infect humans and is the only drug approved for the radical cure of relapsing malaria. As resistance has developed from extensive use of blood schizontocides, such as chloroquine, the importance of primaquine in combating multiple drug resistance has increased, and it is now used in combination with several blood schizontocides for the treatment and prophylaxis of all forms of malaria, including the most lethal malarial species, *Plasmodium falciparum* (Shanks et al., 2001). Primaquine is also effective in the treatment of *Pneumocystis carinii* pneumonia, a common infection in AIDS patients (Toma et al., 1998). The clinical utility of primaquine, however, is restricted by its hemotoxicity, particularly in patients who are genetically deficient in erythrocytic glucose-6-phosphate dehydrogenase (G6PD) activity (Beutler, 1990).

The hemolytic activity of primaquine has long been known to be due to an intra-erythrocytic oxidative stress that is mediated by redox-active metabolites rather than by the parent drug itself. The oxidatively damaged erythrocytes are thought to be recognized by splenic macrophages as the equivalent of senescent red cells, resulting in their selective removal from the circulation (Rifkind, 1966). 5-Hydroxyprimaquine (5-HPQ) is

a putative human metabolite of primaquine that forms a redox pair with its quinoneimine form; continuous cycling of this redox pair is thought to generate reactive oxygen species (ROS) within erythrocytes (Vasquez-Vivar and Augusto, 1992). We have shown that when 5-HPQ is incubated with rat red cells *in vitro*, damage occurs within the cells such that they are removed rapidly from the circulation of isologous rats as compared to untreated controls (Bowman et al., 2004). While it is clear that ROS attack on the cytosolic surface of the membrane leads to a signal for removal of damaged erythrocytes from the circulation, neither the nature of the crucial target(s), whether lipid or protein, nor the mechanism of transfer of the signal across the membrane is known.

The mechanism(s) by which senescent or damaged (but intact) erythrocytes are selected for splenic sequestration are not known. Two current hypotheses are: 1) that red cell damage is analogous to the apoptotic response observed in other (nucleated) cell types, resulting in changes to the normal asymmetric distribution of phospholipids (Mandal et al., 2002); and 2) that protein oxidation interferes with normal protein-protein interactions among the cytoskeletal, integral membrane and cell surface proteins that confer the recognition of "self" (Oldenborg et al., 2000; Bruce et al., 2003). The present study examines the role of the postulated truncated apoptotic pathway in the hemolytic activity of 5-HPQ, specifically in regard to potential roles for membrane lipid peroxidation and loss of phospholipid (i.e., phosphatidylserine) asymmetry. We also address the alternate hypothesis, that alterations to skeletal membrane proteins in 5-HPQ-damaged red cells provide a crucial link between internal oxidative stress and external recognition and removal of erythrocytes from the circulation.

The data indicate that under *in vitro* incubation conditions that provoke premature splenic sequestration of red cells *in vivo*, 5-HPQ generates intracellular ROS but does not induce lipid peroxidation or cause loss of phosphatidylserine asymmetry in the plasma membrane of the red cell. In contrast, profound alterations occur to certain proteins of the cytoskeleton in 5-HPQ-treated erythrocytes, and these alterations are due primarily to the formation of disulfide-linked hemoglobin-skeletal protein adducts. Further studies are warranted to determine the specific signal for erythrocyte phagocytosis, however, the present data are consistent with the concept that cytoskeletal protein damage, rather than alterations in the lipid bilayer, underlies the process of splenic uptake of red cells damaged during the course of primaquine therapy.

Materials and Methods

Chemicals and materials.

5-HPQ was synthesized by HBr-catalyzed hydrolysis of 5-methoxyprimaquine as described previously (Bowman et al., 2004). Bovine factor V, bovine factor Xa and bovine prothombin were obtained from Enzyme Research Laboratories (South Bend, IN). Chromogenic substrate S-2238 was purchased from Chromogenix (DiaPharma Group, Inc., Westchester, OH). Alexafluor 647-conjugated annexin V and 2',7'-dichloro-dihydrofluorescein diacetate (DCFDA) were obtained from Molecular Probes, Inc. (Eugene, OR). Calcium ionophore A23187, N-ethylmaleimide (NEM), cumene hydroperoxide (CH) and rabbit anti-rat hemoglobin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest grade commercially available.

Erythrocytes and incubation conditions.

Male Sprague-Dawley rats (75-100 g) were obtained 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 before their use. Blood was collected from the descending aorta of anesthetized rats into heparinized tubes and washed 3 times with Hanks' Balanced Salt Solution with calcium (HBSS, Invitrogen, Carlsbad, CA) to remove the plasma and buffy coat. The cells were resuspended to a 40% hematocrit in HBSS and used on the same day they were collected. Erythrocytes were washed 3 times with HBSS and resuspended to a 40% hematocrit.

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 (1-2 ml). The erythrocyte suspensions (40% hematocrit) were allowed to incubate for up to 2 hr at 37°C, and then washed once with HBSS prior to biochemical analyses. The effects of DTT on intact red cells were determined as follows: 1.) red cells in PBSG were incubated for the indicated times up to forty minutes in the presence or absence of 50μM 5-HPQ; 2.) Cells were washed once with PBSG and reincubated for 1 additional hour in the presence or absence of 5 mM DTT; 3.) at the end of the incubation with DTT, the red cells were washed once again with PBSG and prepared for the appropriate assay as described below.

Measurement of ROS formation in erythrocytes.

Rat erythrocytes were suspended in isotonic phosphate-buffered saline (pH 7.4) supplemented with 10 mM D-glucose (PBSG) to a 10% hematocrit. DCFDA (600 μM),

dissolved in DMSO, was added to the erythrocyte suspension and allowed to incubate for 15 min at 37°C. Immediately after the addition of various concentrations of 5-HPQ, fluorescence was measured at 2 min intervals for 20 min (excitation 488 nm, emission 529 nm) on a Molecular Devices SprectraMAX Gemeni XS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA).

Preparation of erythrocyte membrane ghosts.

Red cell ghosts were prepared from vehicle- and 5-HPQ-treated red cells as described previously with modification (Grossman et al., 1992). Briefly, washed red cells were centrifuged and the packed cells were lysed in 30 ml of ice-cold phosphate buffer (5 mM, pH 8.0). The membrane ghosts were pelleted by centrifugation at 20,000 x *g* for 10 min. The supernatant was removed by aspiration and the ghosts were repeatedly washed with phosphate buffer until the control cells yielded white ghosts, typically 3-4 washes.

Determination of lipid peroxidation in erythrocytes.

Lipid peroxidation was assessed by measuring the content of F_2-isoprostanes in red cell ghosts prepared from vehicle- and 5-HPQ-treated red cells as described previously (Bolchoz et al., 2002b). Briefly, the lipids were extracted from membrane ghost suspensions (250 μl), prepared with argon-purged phosphate buffer, with chloroform/methanol (2:1, v/v). The extracted lipids were then subjected to alkaline hydrolysis to release the esterified F_2-isoprostanes. The work-up procedure for quantifying F_2-isoprostanes by GC/MS is described in detail elsewhere (Morrow and Roberts, 1999). Treatment of red cell suspensions for 1 hr at 37°C with the lipid-soluble

peroxide, cumene hydroperoxide (1 mM), was used as a positive control for lipid peroxidation (van den Berg et al., 1992).

NEM and calcium ionophore treatment of erythrocytes.

As a positive control for translocation of phosphatidylserine from the inner to the outer leaflet of the lipid bilayer, rat erythrocytes were treated with NEM to inhibit aminophospholipid translocase, and calcium ionophore A23187 was added to induce membrane lipid scrambling as described previously (Kuypers et al., 1996). Briefly, rat erythrocytes were washed three times with HBSS (with calcium) and reconstituted to 40% hematocrit with HBSS. The red cell suspension (1 ml) was allowed to incubate with 10 mM NEM for 30 min at 37°C, and then washed twice with HBSS. Calcium ionophore A23187 (4 μM) in DMSO was then added and allowed to incubate for 1 hour at 37°C. After incubation, the red cells were washed with 2.5 mM EDTA (1 ml) to remove the calcium, and then washed twice more with *calcium-free* HBSS containing 1% bovine serum albumin (BSA) to remove the ionophore. The cells were then re-suspended in 1 ml of annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), and analyzed for annexin V labeling and prothrombinase activity as described below.

Determination of phosphatidylserine translocation in erythrocytes.

Erythrocyte labeling with annexin V was performed according to a modification of a flow cytometric technique (Kuypers et al., 1996). Following incubation with the vehicle, the positive control, or 5-HPQ (2 hr at 37°C), aliquots (50 μl) of the suspensions were removed and washed twice with annexin binding buffer (1 ml). After the last wash,

aliquots of the packed red cells (2 μl) were combined with annexin binding buffer (997 μl), and AlexaFluor 647-conjugated annexin-V solution (1 μl) was added to the erythrocyte suspensions to bring the total volume to 1 ml. After 30 min incubation in the dark at room temperature, the samples were washed and re-suspended in annexin binding buffer (1 ml), and analyzed on a Becton Dickinson FACSCalibur analytical flow cytometer (BD Biosciences, San Jose, CA).

Phosphatidylserine translocation was also assessed by measuring the conversion of prothrombin to thrombin using a modification of a method described previously (Kuypers et al., 1996). Briefly, after incubation with the vehicle or 5-HPQ, erythrocyte suspensions were washed once with HBSS, and 1 μl of packed cells was added to 1 ml of Tris buffer (pH 7.4) at 37°C. Bovine factor V (0.33 U/ml) and bovine factor Xa (0.33 U/ml) were added, followed by 0.13 mg of prothrombinase. After 4 min, the reaction was quenched with 15 mM EDTA. The erythrocytes were pelleted by centrifugation and 75 μl of the supernatant was added to 1 ml of chromogenic substrate S-2238 working solution. The increase in absorbance at 405 nm was determined over 1 min at 37°C in a Shimadzu UV-160 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with software for enzyme kinetics. The amount of thrombin formed per unit time was determined by comparison with thrombin standards.

Electrophoretic analysis of membrane cytoskeletal proteins.

Red cell ghosts from control and 5-HPQ-treated erythrocytes were washed exhaustively to remove unbound hemoglobin (4 washes). The ghost protein was then solubilized in NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA), with or without

40 mM dithiothreitol (DTT), and heated at 70°C for 10 min. Solubilized proteins (15 μg) were loaded and resolved on 4-12% NuPAGE Bis-Tris gels with MOPS Running Buffer (Invitrogen) at 200 V (constant) for 50 min. Resolved proteins were transferred to PVDF membranes for immunoblot analysis according to the Invitrogen protocol. For SDS-PAGE analysis, solubilized membrane ghosts were resolved on continuous gels under non-reducing conditions as described previously (McMillan et al., 1995), then stained with Gel Code Blue Stain Reagent (Pierce, Rockford, IL) and destained with water.

Rat red cell membrane proteins were identified according to molecular weight (Fairbanks et al., 1971) with the use of molecular weight protein standards (Invitrogen). Blotted proteins were blocked in TBST (pH 7.5) containing 5% non-fat dry milk and incubated in TBST containing 1% BSA and primary antibody (rabbit anti-rat hemoglobin, 1:10,000 vol/vol). After washing and incubation with the peroxidase-conjugated secondary antibody (anti-rat IgG), the immunoblots were developed using ECL detection (Amersham Biosciences, Piscataway, NJ).

GSH Depletion of Erythrocyte Suspensions.

Diethyl maleate (DEM) was used to deplete GSH (>95%) in red cell suspensions as described previously (Bolchoz et al., 2002b). Briefly, DEM (750μM) 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. The cells were re-suspended to a 40% hematocrit in HBSS and used on the same day that they were collected.

Measurement of Hemolytic Activity.

The survival of rat ^{51}Cr-labeled red cells was determined *in vivo* after *in vitro* incubation with 50μM 5-HPQ with or without 5mM DTT as described above. After incubation at the specified times at 37°C, the erythrocytes were washed once and resuspended in PBSG (40% hematocrit). Aliquots (0.5ml) were administered intravenously to isologous rats. T_0 blood samples were taken from the orbital sinus xx min after administration of labeled red cells. Additional blood samples were at sequential times over 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 use of Student's *t* test.

Erythrophagocytosis by J774 macrophages.

J774A.1 macrophages (ATCC) were plated at 200,000 cells/well in 12 well culture plates and allowed to adhere. After 24 hr, 50μl Cr-labeled, vehicle- or 5-HPQ-treated packed red cells were added to the macrophage monolayer and incubated for 24 hr at 37°C. Non-ingested RBCs were removed by lysis with NH_4Cl and washed three times with buffer. Adherent macrophages were released with NaOH and phagocytosis was assessed by measuring radioactivity in a well-type gamma counter. Data were expressed as either counts per minute (CPM) or as a percent of control.

Results

Effect of 5-HPQ on ROS generation in rat erythrocytes.

DCFDA is a lipid-soluble probe that can detect the intracellular formation of several types of ROS, including hydroxyl, peroxyl, alkoxyl, and nitroxyl free radicals, as well as peroxynitrite (Halliwell and Whiteman, 2004). To assess the formation of ROS in 5-HPQ-treated rat erythrocytes, erythrocyte suspensions were pre-incubated with DCFDA for 15 min followed by addition of 5-HPQ. Fluorescence was then measured at 2 min intervals for 20 min using a fluorescence microplate reader. Preliminary experiments indicated that the concentration of red cells in the suspensions had to be reduced from 40% to a 10% hematocrit to avoid significant quenching of the fluorescence signal by the cells. 5-HPQ concentrations were reduced accordingly (by 4-fold) to maintain the same 5-HPQ-to-red cell ratio that was used in the other experiments.

As shown in Fig. 4.1, incubation of erythrocytes with DCFDA in the absence of 5-HPQ resulted in a slight but measurable increase in fluorescence over a 20 min time period, consistent with the known low-level, steady production of ROS in normal red cells (Jandl et al., 1960). Inclusion of 5-HPQ (5-30 μM) in the erythrocyte suspension caused a marked and concentration-dependent increase in the generation of fluorescence, indicating enhanced production of ROS by 5-HPQ. This observation is consistent with previous observations which showed that 5-HPQ has the ability to redox cycle and to cause oxidative stress in rat erythrocytes, as evidenced by GSH oxidation and methemoglobin formation (Bowman et al., 2004). No fluorescence signal was detected in the absence of red cells or in the absence of DCFDA (data not shown).

Effect of 5-HPQ on erythrocyte membrane lipid peroxidation.

To determine whether ROS production by 5-HPQ resulted in lipid peroxidation, rat red cells were incubated for 2 hr at 37°C with concentrations of 5-HPQ corresponding to EC_{10} (10 μM), EC_{50} (40 μM) and EC_{90} (70 μM) concentrations for hemolytic activity (Bowman et al., 2004). The red cells were then washed and lysed in argon-purged hypotonic buffer for the preparation of red cell ghosts, as described previously (Grossman et al., 1992). Esterified isoprostanes were extracted from the ghosts and hydrolyzed, and the total F_2-isoprostane content in the samples was quantified by GC-MS (Morrow and Roberts, 1999). As shown in Fig. 4.2, F_2-isoprostane content in 5-HPQ-treated incubates was not different from that of control incubates, regardless of the concentration of 5-HPQ. In contrast, treatment of red cells with the positive control, cumene hydroperoxide (CH, 1 mM), resulted in a 3-fold increase in F_2-isoprostane formation. Treatment with this concentration of CH was the lowest that produced a significant elevation in F_2-isprostane formation that was not associated with significant frank lysis of the erythrocytes; concentrations of CH greater than 1 mM caused progressive and complete red cell lysis (data not shown).

Effect of 5-HPQ on red cell membrane phospholipid asymmetry.

To determine whether exposure to 5-HPQ resulted in a loss of phosphatidylserine asymmetry, the transmembrane location of phosphatidylserine was assessed by two methods: enhancement of prothrombinase activity, and annexin-V labeling of intact erythrocytes.

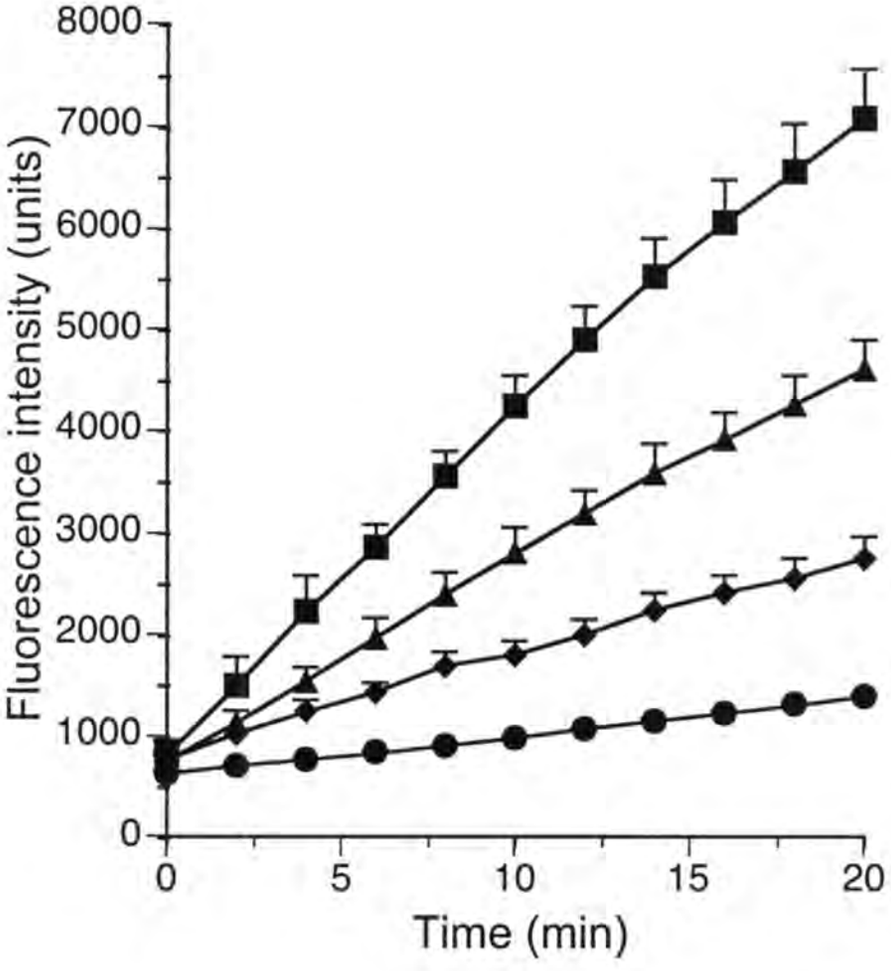


Fig. 4.1. Effect of 5-HPQ on ROS generation in rat erythrocytes. Erythrocyte suspensions (10% hematocrit) were incubated with DCFDA (600 μM) for 15 min. The red cells were then treated with the vehicle (●), 5μM (◆), 15 μM (▲), and 30 μM (■) 5-HPQ. Fluorescence emission intensity was monitored on a fluorescence microplate reader (excitation, 488; emission, 529 nm) at 2 min intervals. The values are means ± S.D. (n = 3).

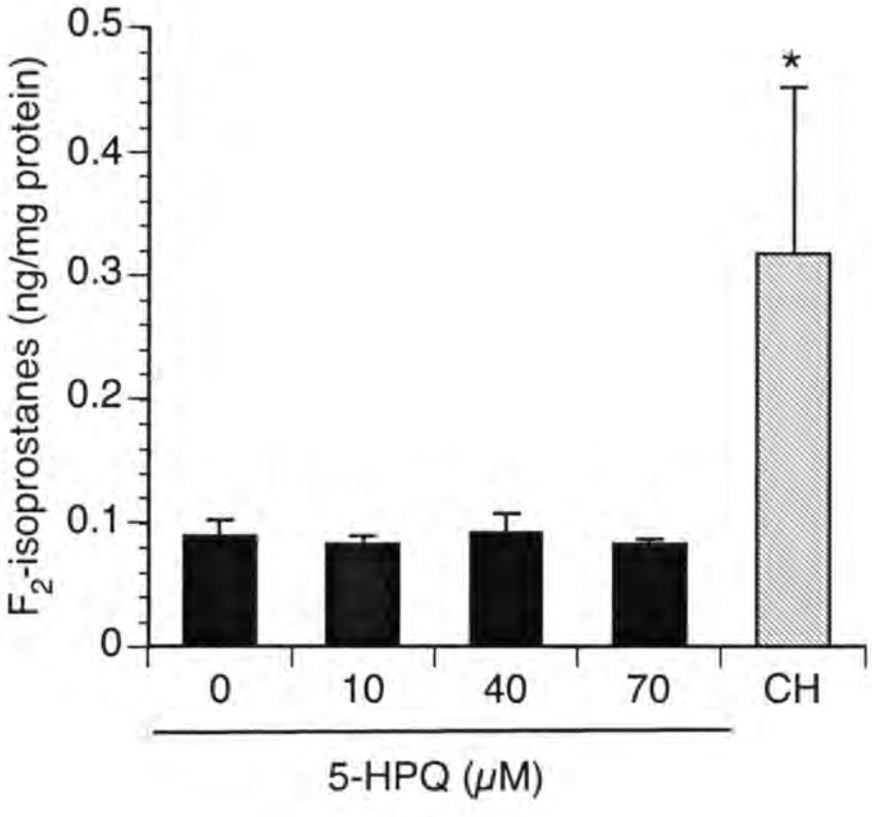


Fig. 4.2. Effect of 5-HPQ on F_2-isoprostane formation in rat erythrocytes. Erythrocytes were incubated for 2 hr at 37°C in HBSS containing the indicated concentrations of 5-HPQ. After incubation erythrocyte ghosts were prepared and analyzed for F_2-isoprostane content by GC-MS as described in the *Materials and Methods*. The values are means ± S.D. (n = 3). * Significantly different from control ($p < 0.05$).

Erythrocyte suspensions (40%) were incubated with hemolytic concentrations of 5-HPQ (10 to 70 μM) for 2 hr. At the end of the incubation, the red cells were washed, re-suspended in HBSS and analyzed for prothrombinase activity, which is dependent on cell surface exposure to phosphatidylserine (Bevers et al., 1982). For a positive control, erythrocytes were treated with 10 mM NEM for 30 min, followed by treatment with 4 μM calcium ionophore A23187 in the presence of calcium for 1 hr. As shown in Fig. 4.3A, thrombin formation in 5-HPQ-treated erythrocytes was not significantly different from the control. In contrast, the positive control, NEM plus ionophore and calcium, induced an 8-fold increase in prothrombinase activity as compared to the control.

The presence of phosphatidylserine in the outer leaflet of the lipid bilayer was confirmed by direct labeling of erythrocytes with fluorescently conjugated annexin V, which binds to acidic phospholipids, particularly phosphatidylserine (Kuypers et al., 1996). As shown in Fig. 4.3B, annexin-positive erythrocytes from 5-HPQ-suspensions constituted less than 2% of the red cells, which was similar to the vehicle-treated control. In contrast, treatment of red cells with NEM plus ionophore and calcium resulted in labeling of about 45% of the erythrocytes.

Effect of 5-HPQ on rat erythrocyte membrane skeletal proteins.

To determine whether 5-HPQ-induced hemolytic activity was associated with alterations to the membrane cytoskeleton, rat erythrocyte ghost proteins from control and 5-HPQ-treated erythrocytes were separated by SDS-PAGE and either stained with Gel Code Blue, or transferred to PVDF membranes and immunostained with antibodies to rat hemoglobin. As shown in Fig. 4.4A, 5-HPQ caused concentration-dependent changes to

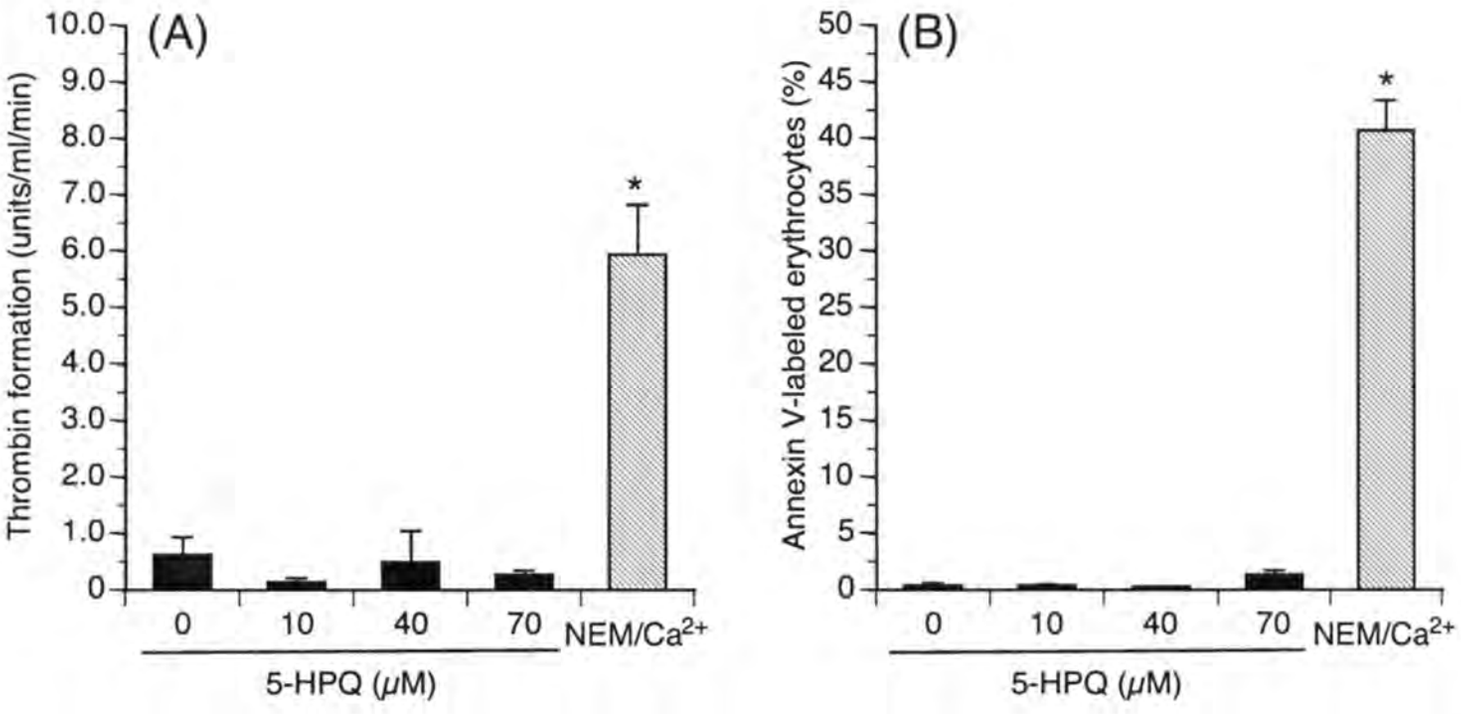


Fig. 4.3. Effect of 5-HPQ on phosphatidylserine asymmetry in rat erythrocytes. Erythrocyte suspensions (40% hematocrit) were incubated with the indicated concentrations of 5-HPQ for 2 hr at 37°C. NEM/Ca^{2+} was used as a positive control. (A) Prothrombinase activity and (B) AlexaFluor 647-conjugated annexin V labeling were determined as described in *Materials and Methods*. The values are means ± S.D. (n = 3). * Significantly different from control ($p < 0.05$).

the normal electrophoretic pattern of erythrocyte skeletal proteins as compared with that of the vehicle-treated control. 5-HPQ treatment induced the appearance of new protein bands at 16, 32 and 64 kDa, which is consistent with formation of membrane-bound hemoglobin monomers, dimers and trimers, respectively (Grossman et al., 1992). In addition, there was a concentration-dependent loss of resolution of protein bands 1 and 2 (α and ß spectrins), splitting of band 2.1 (ankyrin) and loss of band 4.2. On the other hand, band 4.1 and band 5 (actin) appeared not to be affected significantly by treatment with 5-HPQ.

Immunoblot analysis of the skeletal proteins from 5-HPQ-treated red cells showed a dramatic increase in the content of membrane-bound hemoglobin (Fig. 4.4B). Only insignificant amounts of hemoglobin were bound to skeletal proteins at sub-hemolytic concentrations of 5-HPQ (<40 μM), whereas increasing amounts of hemoglobin were bound to a wide variety of proteins at hemolytic concentrations. When 5-HPQ-treated ghosts were treated with DTT (40 mM at 70°C for 1 hr) prior to electrophoresis, binding of hemoglobin to skeletal proteins was reversed (Fig. 4.5). This observation indicated that hemoglobin was bound covalently to skeletal protein sulfhydryl groups through intermolecular disulfide bonds.

Effect of 5-HPQ on the formation of Hb-membrane adducts in GSH-depleted RBCs.

In order to further assess that the formation of Hb-adducts plays a direct role in the removal 5-HPQ-damaged red cells, we examined the formation of Hb-cytoskeletal protein adducts in red cells that were treated with sub-hemolytic concentrations of 5-HPQ that have been shown to be hemolytic to GSH-depleted red cells (Bowman et al., 2004).

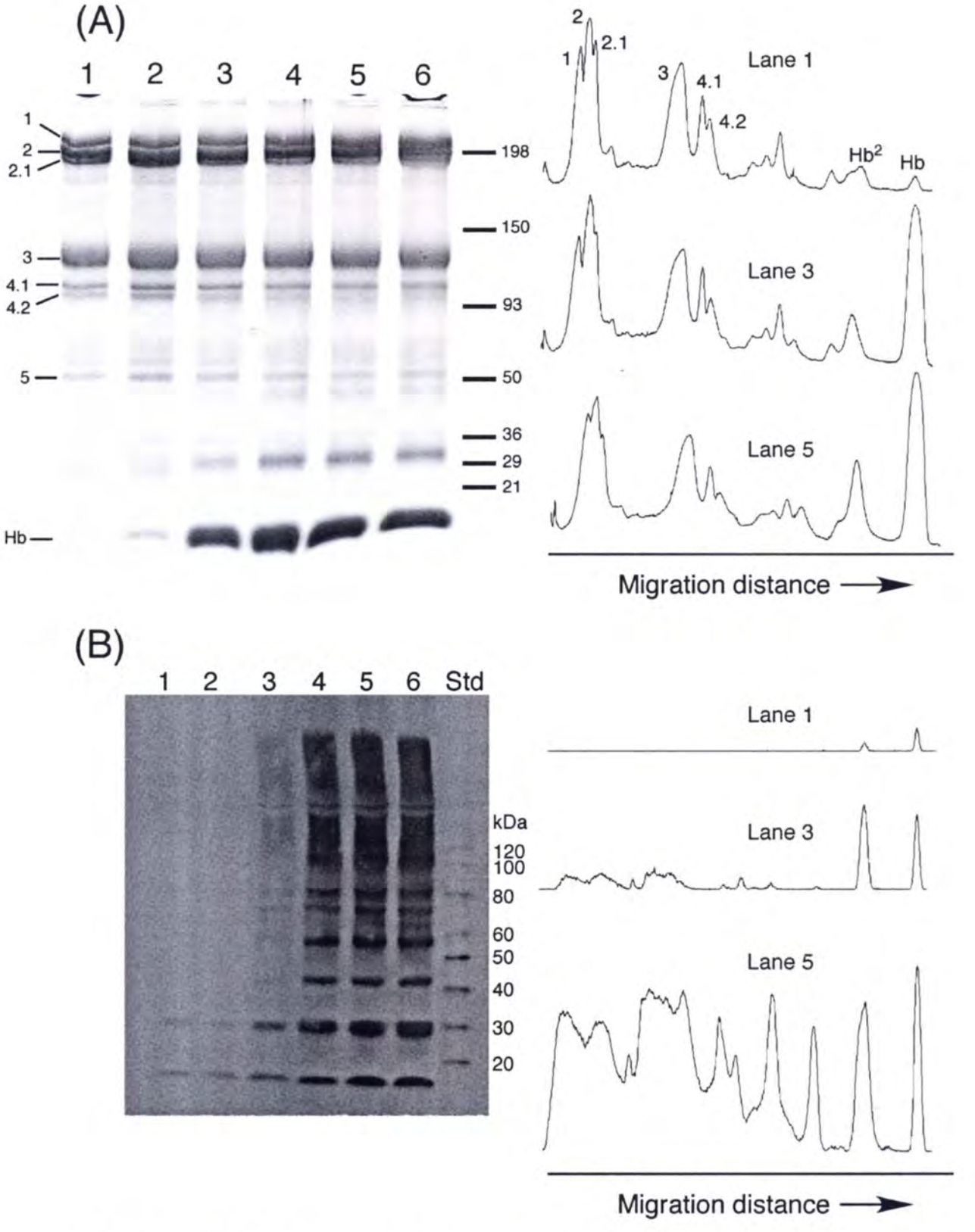


Fig. 4.4. Effect of 5-HPQ on rat erythrocyte membrane skeletal proteins. Red cells were incubated with vehicle (lane 1), 10 μM (lane 2), 25 μM (lane 3), 50 μM (lane 4), 75 μM (lane 5) and 100 μM (lane 6) 5-HPQ for 2 hr at 37°C. The cells were washed, and ghosts were prepared and washed exhaustively to remove unbound hemoglobin (Hb). The ghosts were then solubilized in SDS and subjected to PAGE. (A) Gel Code Blue-stained gel and densitometric scans; (B) immunoblot stained with polyclonal antibodies to rat hemoglobin. Molecular weight markers appear along the right side of the gel; the major cytoskeletal proteins are identified along the left side of the gel according to (Fairbanks et al., 1971).

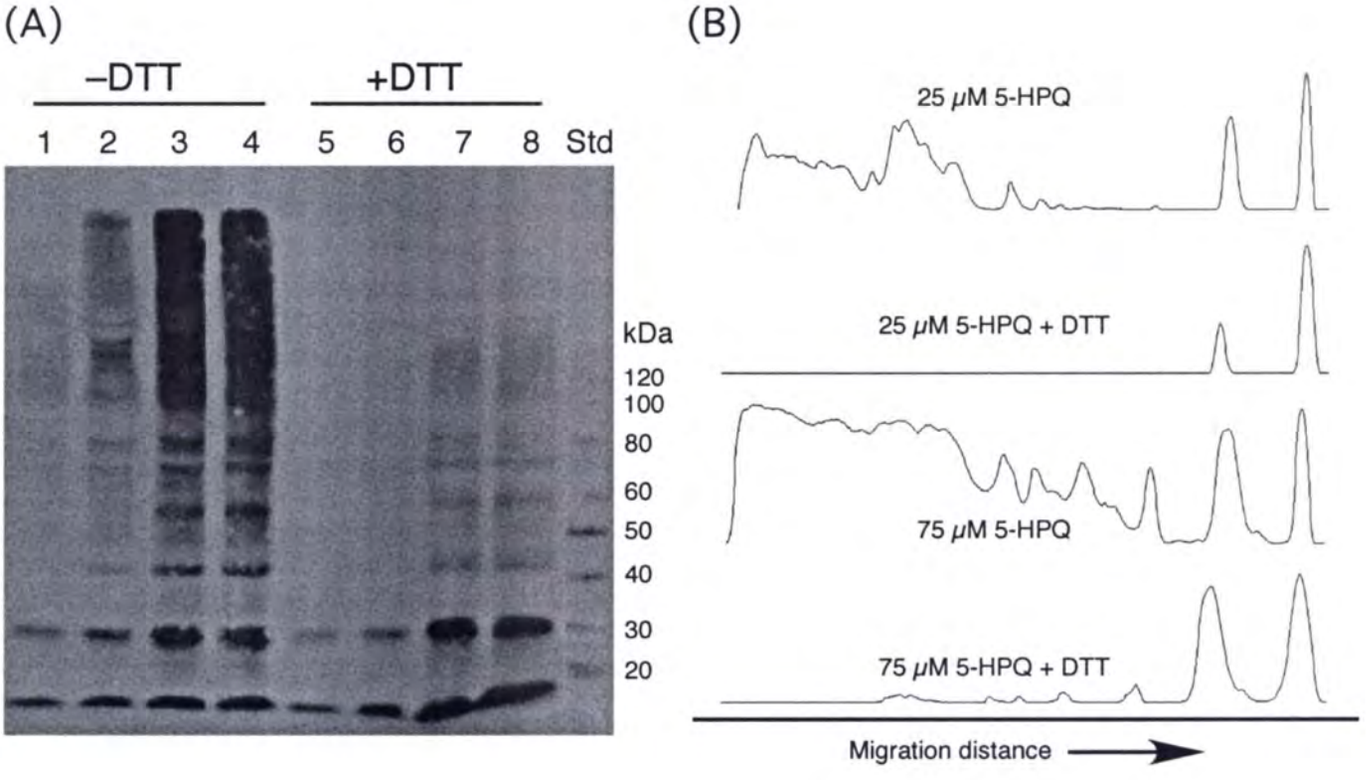


Fig. 4.5. Effect of DTT on 5-HPQ-induced hemoglobin binding to membrane skeletal proteins. Erythrocytes were incubated with 5-HPQ for 2 hr at 37°C. Ghosts were prepared, washed exhaustively to remove unbound hemoglobin, and each sample was divided into two aliquots. Prior to electrophoresis, one aliquot was incubated with water and the other with 40 mM DTT for 1 hr at 70°C. The samples were then subjected to SDS-PAGE and blotted onto PVDF membranes. (A) Immunoblot stained with polyclonal antibodies to rat hemoglobin. Vehicle control (lanes 1 and 5), 25 μM (lanes 2 and 6), 50 μM (lanes 3 and 7) and 75 μM (lanes 4 and 8) 5-HPQ. (B) Selected densitometric scans.

Red cell GSH was depleted by >95% with DEM prior to treatment with 5-HPQ for 2 h at 37°C. The range of 5-HPQ concentrations (2.5-25 μM) was chosen, since it was previously shown to span the red-cell survival concentration-response curve generated with GSH-depleted red cells treated *in vitro* were administered to rats *in vivo* (Bowman et al., 2004). After treatment with 5-HPQ, membrane ghosts were prepared with hypotonic phosphate buffer and washed extensively to remove unbound hemoglobin. Ghost protein (15μg) was then separated by SDS-PAGE, blotted to PVDF membranes, and immunostained with anti-rat hemoglobin antibodies. Immunoblot analysis showed enhanced binding of hemoglobin at concentrations as low as 2.5μM 5-HPQ (Fig. 4.6). Significantly, the sharp, threshold-like response seen in GSH-normal cells (Fig. 4.4B) was lost and the TC_{50} markedly reduced ~5-fold, from 25-50μM to approximately 5-10μM. This data demonstrate that there is a strong correlation between the formation of Hb-adducts with the hemolytic activity previously observed *in vivo* for both GSH-normal and GSH-depleted red cells, indicating that the formation of disulfide-linked Hb-adducts with skeletal membrane proteins likely underlies the mechanism for the recognition and removal of 5-HPQ-treated red cells by splenic macrophages.

Effect of DTT on 5-HPQ-induced Hb-adduct formation and hemolytic susceptibility.

While the correlation between the formation of Hb-cytoskeletal protein adducts and hemolytic activity is very strong, it was necessary to determine directly whether adduct formation is functionally significant for macrophage recognition and phagocytosis of damaged erythrocytes. We examined whether the reduction of Hb with DTT in intact cells could restore normal cytoskeletal protein sulfhydryl status to, in effect, "rescue" 5-

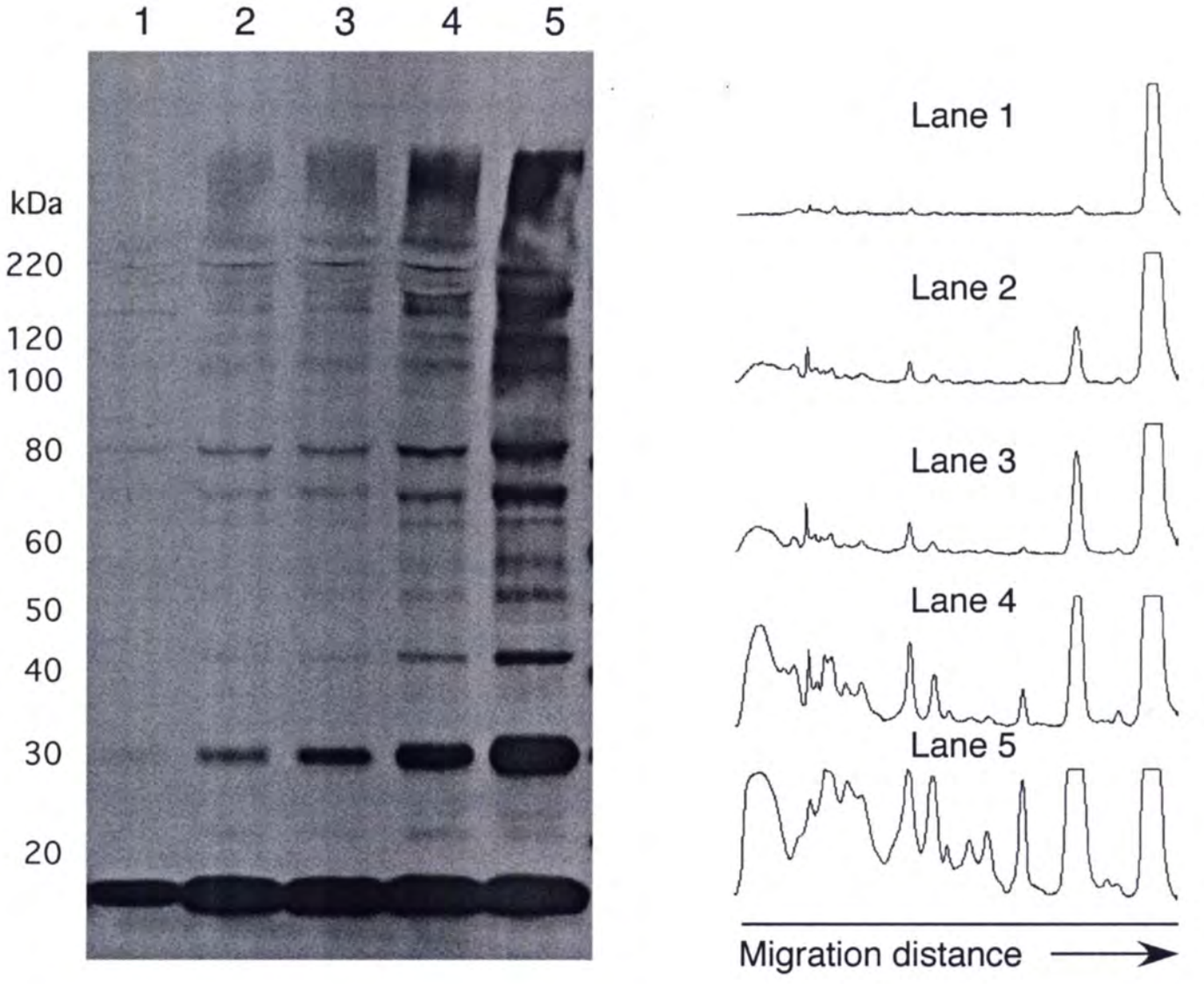


Fig. 4.6. Effect of 5-HPQ on Hb-cytoskeletal protein adduct formation in GSH-depleted rat erythrocytes. GSH depleted (>95%) rat erythrocytes were incubated in the presence of vehicle (lane 1), 2.5μM 5-HPQ (lane 2), 5μM 5-HPQ (lane 3), 10μM 5-HPQ (lane 4), or 25μM 5-HPQ (lane 5) for 2 hr at 37°C. Following exposure, the cells were washed and membrane ghosts were prepared and washed extensively to remove any unbound hemoglobin. The ghosts (15 μg of protein) were subjected to SDS-PAGE, blotted onto PVDF membrane, and stained with antibodies against rat hemoglobin. The densitometric tracings of the blot (left) are shown on the right.

HPQ-damaged red cells from their phagocytic fate. The ability of DTT (5mM) to reduce the disulfide-linked Hb-adducts, which formed during incubation with 5-HPQ was examined. Red cells were treated with a 50μM 5-HPQ in the absence of DTT for 15 min, washed once with PBSG, and then treated with 5 mM DTT for 1 hr. The cells were once again was with PBSG and red cell membrane ghosts were prepared and washed extensively. The ghost protein (15μg) was then subjected to SDS-PAGE and Western blot using anti-rat hemoglobin antibodies. As shown in Fig. 4.7, the amount of membrane bound Hb was markedly reduced in cells treated with DTT.

Next, we determined whether this reduction of Hb from cytoskeletal membrane proteins demonstrated any functional significance, i.e. whether DTT could "rescue" 5-HPQ-damaged erythrocytes. ^{51}Cr-labeld red cells were treated *in vitro* with 50μM 5-HPQ for either 20 or 40 min, washed once with PBSG, then treated with or without 5mM DTT for 1 hr. As shown in Fig. 4.8, administration of these erythrocytes to rats showed that DTT effectively "rescued" red cells treated with 5-HPQ for 20 min but not 40 min, suggesting that the red cells were committed to their fate by 40 min. This time-dependent commitment supports the concept that the appearance of an extracellular cell-surface signal is dependent on an inside-out signal transduction, from the intracellular membrane surface across the membrane to the extracellular cell surface.

Discussion

It is well recognized that primaquine administration can cause oxidative damage to erythrocytes, resulting in methemoglobinemia and hemolytic anemia. At pharmacologically relevant concentrations, primaquine is not directly toxic to red cells;

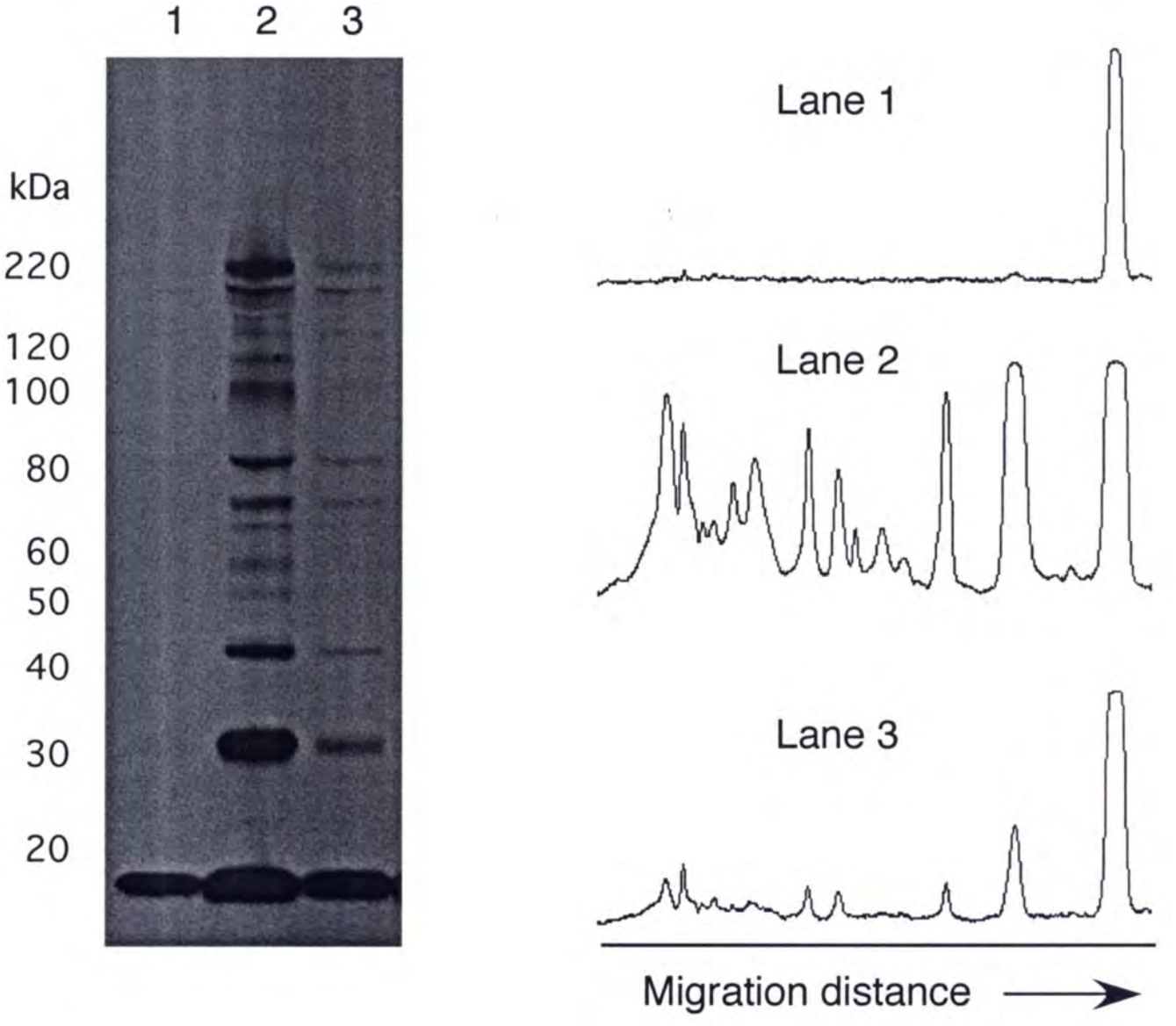


Fig. 4.7. Effect of DTT on 5-HPQ-induced Hb-cytoskeletal protein adducts in intact rat erythrocytes. Red cells were incubated with vehicle (lane 1) or 50μM 5-HPQ (lane 2 and lane 3) for 15 min at 37°C, then washed once with PBSG and treated with vehicle (lane 1 and lane 2) or 5 mM DTT (lane 3) for 1 hr at 37°C. After incubation, the cells were washed once more with PBSG, and ghosts were prepared and washed extensively to remove unbound Hb. The ghosts (15μg of protein) were then subjected to SDS-PAGE, blotted onto a PVDF membrane, and stained with antibodies to rat Hb. Densitometric tracings from the blot (left) are shown on the right.

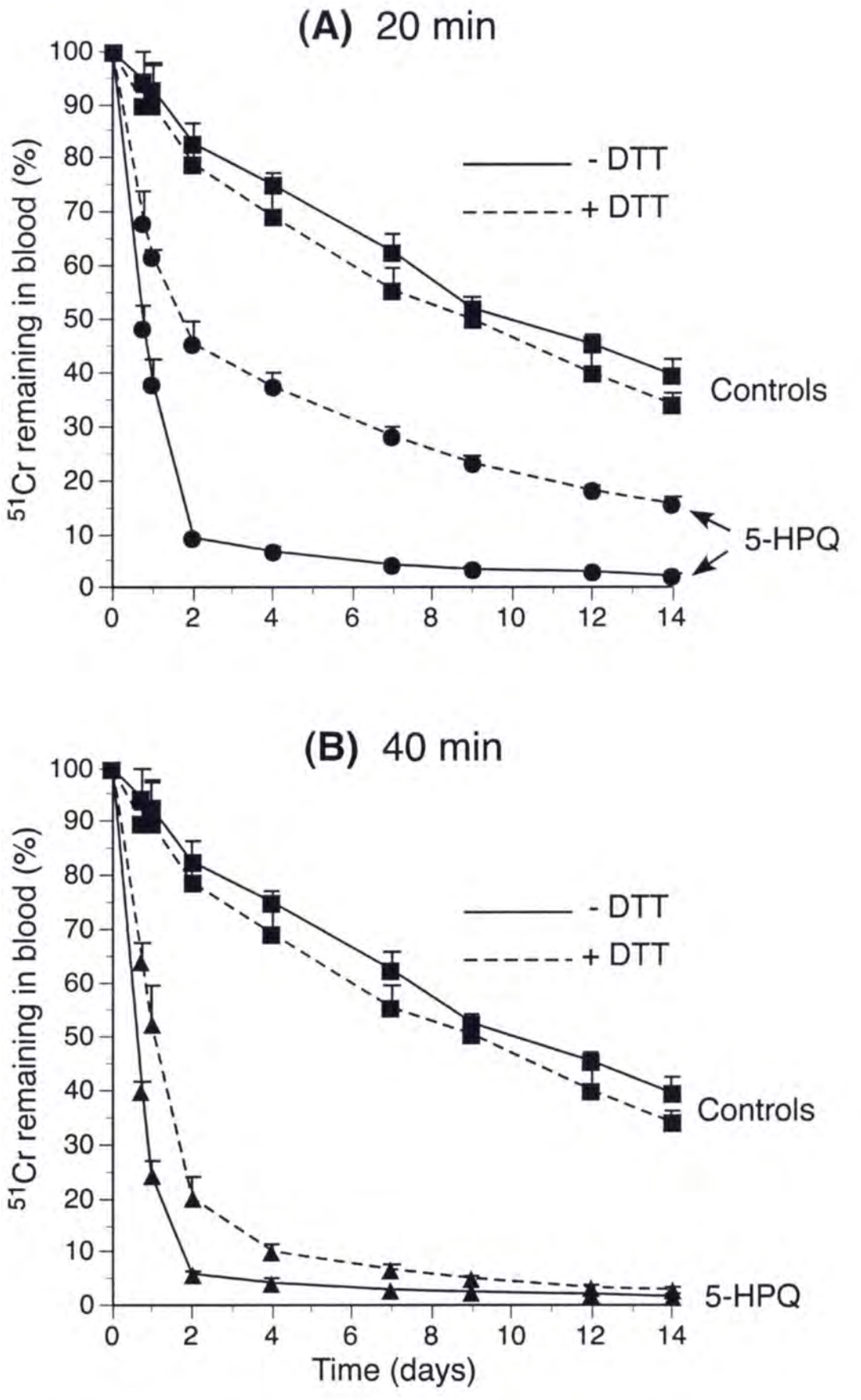


Fig. 4.8. Effect of DTT on the survival of rat ^{51}Cr-labeled erythrocytes *in vivo* after *in vitro* exposure to 5-HPQ (50μM) for (A) 20 min or (B) 40 min. The red cells were incubated with or without 5 mM DTT for 1 hr at 37°C after treatment with 5-HPQ, or with vehicle (H_2O) alone, for 20 or 40 min at 37°C. The erythrocytes were then washed and administered intravenously to isologous rats. T_0 blood samples were taken 35 min after administration of labeled cells. Data points are means ± SD (n = 4).

the toxicity is thought to be due to redox-active metabolites that generate ROS. When formed in sufficient quantity and rate, these ROS are considered to overwhelm cellular defenses and attack various protein and/or lipid components of the red cell. By identifying the toxic metabolite(s) and understanding the mechanism underlying the hemolytic response, it may be possible to undertake a rational redesign of the primaquine molecule to reduce toxicity while maintaining anti-malarial efficacy.

Previous studies have shown that the phenolic metabolite, 5-HPQ, has the capacity to redox cycle and generate ROS, and they suggested that it could contribute to the hemotoxicity of the parent drug (Vasquez-Vivar and Augusto, 1992). We have recently confirmed the hemolytic potential of 5-HPQ by demonstrating that it is a direct-acting hemolytic agent in the rat (Bowman et al., 2004). In these studies, ^{51}Cr-tagged erythrocytes were treated with 5-HPQ *in vitro* and then re-administered to isologous rats. The treated cells were rapidly removed from the circulation of the rats as compared with saline-treated controls. The concentration-dependence of this response was sharp, with a minimum response at 25 μM 5-HPQ, an EC_{50} at about 40 μM, and a maximal response at 75 μM. In addition, hemolytic activity was strongly correlated with depletion of GSH and formation of glutathione-protein mixed disulfides. These observations were similar to the pattern of oxidative stress-induced damage observed previously in red cells exposed to other direct-acting, pro-oxidant hemolytic agents, such as dapsone hydroxylamine (Bradshaw et al., 1997). However, the precise intracellular molecular targets of oxidative damage have not been identified, nor has the mechanism whereby internal oxidative damage is translated to an external cell-surface marker that enables splenic macrophages to recognize and remove the damaged red cells.

Since mature red cells lack nuclei and other organelles, the lipid and protein of the plasma membrane appear to be the most likely targets of intracellular ROS. In regard to lipids, it is well known that they are prime targets for ROS-initiated lipid peroxidation, which is thought to result in membrane blebbing and frank cell lysis. In addition, oxidative stress has been shown to alter the asymmetric distribution of phospholipids within the bilayer (Jain, 1984). A functional role for this asymmetry was demonstrated when it was shown that phosphatidylserine translocation to the outer leaflet resulted in an increased susceptibility of erythrocytes to phagocytosis by macrophages (Zwaal and Schroit, 1997).

More recently, studies on the normal, physiological removal of senescent erythrocytes have suggested that senescent erythrocytes undergo a process that is analogous to the apoptotic program observed in other cell types. For example, treatment of human red cells with *t*-butylhydroperoxide was shown to stimulate caspase 3 activation and phosphatidylserine externalization (Mandal et al., 2002). Additional studies have shown that peroxidation of phosphatidylserine in cells undergoing apoptosis causes a reduced affinity of aminophospholipid translocase for the oxidized phospholipid, resulting in the accumulation of phosphatidylserine in the outer leaflet (Tyurina et al., 2000). On the other hand, other investigators have found no association between oxidative stress and loss of phospholipid asymmetry in human erythrocytes (de Jong et al., 1997).

An alternate hypothesis is that erythrocyte proteins are critical targets of intracellular ROS. Previous studies have shown that hemoglobin and proteins of the membrane cytoskeleton are altered in response to pro-oxidant hemolytic agents, and this is reflected by the generation of reactive forms of hemoglobin, such as ferryl heme (Bolchoz et al.,

2002a) and hemoglobin thiyl free radicals (Bradshaw et al., 1995), and by changes in the normal electrophoretic pattern of skeletal proteins on non-reducing SDS-PAGE gels (Grossman et al., 1992; McMillan et al., 1995; McMillan et al., 2001). Evidence for the functional importance of normal protein-protein interactions among cytoskeletal, integral membrane and external cell surface proteins was illustrated by recent studies showing that these interactions are necessary to confer the recognition of "self" to circulating erythrocytes (Oldenborg et al., 2000; Bruce et al., 2002).

The studies presented in this chapter were undertaken to determine the effect of the redox-active primaquine metabolite, 5-HPQ, on generation of ROS in red cells and on the consequences of ROS generation to erythrocyte membrane lipids and proteins. Given the association between 5-HPQ-induced oxidative stress and its hemolytic activity, we analyzed rat red cell lipids for peroxidation and loss of phosphatidylserine asymmetry, and skeletal proteins for electrophoretic alterations and hemoglobin binding under experimental conditions associated with the generation of intracellular ROS.

DCFDA is a fluorescence-based probe that has been developed to detect intracellular production of ROS. DCFDA diffuses passively into cells where it is deacetylated to form 2',7'-dichlorodihydrofluorescein. 2',7'-Dichlorodihydrofluorescein is then converted to a fluorescent product, 2',7'-dichlorofluorescein, by ROS, thus providing a general assessment of intracellular oxidative stress (Hempel et al., 1999; Halliwell and Whiteman, 2004). The data presented in Fig. 4.1 show a concentration-dependent increase in DCFDA oxidation in rat erythrocytes treated with 5-HPQ, indicating that ROS formation is associated with the hemolytic response in both a concentration- and time-dependent manner. DCFDA oxidation continued for at least 20 min, which is of

interest because 5-HPQ has an extremely short half-life (<1 min) in erythrocyte suspensions (Bowman et al., 2004). Although the reason for this relatively long generation of ROS relative to 5-HPQ stability is not understood, it raises the intriguing possibility that some as yet unknown reactive intermediate with a longer half-life is generated in red cells under hemolytic conditions.

To assess whether lipid peroxidation was associated with 5-HPQ-induced hemolytic injury, the formation of F_2-isoprostanes was quantified in erythrocyte ghosts by GC-MS. Treatment with a range of concentrations of 5-HPQ, from sub-hemolytic (10 μM) to maximally hemolytic (70 μM), did not reveal any increase in the content of F_2-isoprostanes in 5-HPQ-treated erythrocytes as compared to the control (Fig. 4.2). In agreement with the lipid peroxidation data, analysis of phosphatidylserine externalization (Fig. 4.3) supported the concept that erythrocyte membrane lipids are not targets of 5-HPQ-generated ROS, and suggest that phosphatidylserine exposure on the external cell surface does not occur and thus is not a signal for uptake of 5-HPQ-treated red cells into the spleen.

In contrast to membrane lipids, exposure to 5-HPQ caused profound alterations to the red cell protein cytoskeleton (Fig. 4.4A). Of importance, the concentration dependence for this effect coincided with that of 5-HPQ-induced hemolytic activity. Gel Code Blue staining of ghost proteins resolved by SDS-PAGE showed alterations to several proteins, including the loss of resolution and/or splitting of protein bands in the spectrin/ankyrin region, loss of band 4.2, and the appearance of membrane-bound hemoglobin. Disappearance of band 4.2 was perhaps the most interesting effect. Band 4.2 has been shown to be a member of the band 3 complex, which includes band 3 (anion exchanger

1), band 2.1 (ankyrin) and CD47 (Bruce et al., 2003). Band 3 is an integral membrane protein that is anchored to the underlying cytoskeleton through its interaction with ankyrin. The extracellular domain of CD47 has been shown to act as a ligand for the inhibitory signal regulatory protein alpha (SIRPα) on splenic macrophages (Oldenborg et al., 2001). As noted above, this interaction is thought to confer self-recognition to normal red cells, preventing their phagocytic removal (Oldenborg et al., 2000). These findings are of relevance to the hemolytic response because other studies have shown that band 4.2 forms a vertical association between CD47 and ankyrin, thereby mediating cell-surface expression of CD47 and its membrane skeleton attachment in human erythrocytes (Dahl et al., 2004). While the fates of band 4.2 and ankyrin are not clear from these experiments, immunoblotting with anti-rat hemoglobin showed that significant amounts of hemoglobin are bound to multiple proteins of the band 3 complex (Fig. 4.4B). The pattern of Hb-binding correlated very strongly with the concentration-responses for the hemolytic activity of 5-HPQ in both GSH-normal (Fig. 4.4B) and GSH-depleted red cells (Fig. 4.6), indicating a direct role for the formation of Hb-cytoskeletal protein-adducts in the removal of 5-HPQ-damaged erythrocytes. Treatment of erythrocyte ghosts with the disulfide-reducing agent, DTT, showed that covalent binding of hemoglobin to these proteins occurred through the formation of intermolecular disulfide bonds (Fig. 4.5), and treatment of intact erythrocytes with DTT indicated that the covalent binding of hemoglobin could be reversed in the intact, viable cell (Fig. 4.7). In order to determine whether the reduction of 5-HPQ-induced Hb-adducts by DTT in whole cells showed any functional significance, we examined the hemolytic removal of ^{51}Cr-labeled, 5-HPQ-treated, DTT-"rescued" erythrocytes *in vivo* and found that these cells were "rescued"

after 20 min of treatment with 50μM 5-HPQ compared to controls not receiving DTT (Fig. 4.8A). Treatment with DTT reduced the fractional removal at 48 hrs of damaged red cells exposed to 5-HPQ for 20 min by ~35%. However, exposure to 5-HPQ for 40 min followed by treatment with DTT only reduced the fractional removal by ~5%, indicating that by 40 min virtually all of 5-HPQ-damaged red cells are committed to their fate. It is conceivable that intracellular damage by 40 min has been translated into an external cell surface signal that is no longer responsive to reduction by DTT.

When taken into consideration with previous studies, these results indicate that redox cycling of 5-HPQ causes enhanced ROS formation (Fig. 4.9), which overcomes antioxidant defenses. Hemoglobin, which is in close proximity to ROS generation, is a preferential target. Attack of ROS on hemoglobin may result in the formation of hemoglobin thiyl radicals (Bradshaw et al., 1995) and/or ferryl heme species (Bolchoz et al., 2002a), which are highly reactive but which lack the lipophilicity to penetrate into the membrane to initiate lipid peroxidation. Instead, these putative thiyl radicals could react with sulfhydryl-groups on a variety of structural proteins to form hemoglobin adducts (Jollow and McMillan, 2001). Given our previous observations of the formation of glutathione-protein mixed disulfides (Bowman et al., 2004) and our current observation of disulfide-linked hemoglobin-skeletal proteins (Figs. 4.5, 4.6, 4.7, and 4.8), it seems reasonable to hypothesize that 5-HPQ may be operating in a similar fashion. Additional studies will be necessary to determine whether thiyl radicals or other reactive species can be detected in response to treatment with 5-HPQ. Additional studies are also warranted to determine the precise protein targets of hemoglobin adduct formation that lead to splenic sequestration; however, it is not inconceivable to predict that binding of

hemoglobin monomers to proteins of the band 3 complex would lead to profound functional disturbances in cytoskeletal protein-protein interactions that are transmitted to the external surface of the erythrocyte, providing a signal for macrophage recognition.

In summary, we have shown that a hemolytic metabolite of primaquine, 5-HPQ, does not cause lipid peroxidation or loss of phosphatidylserine asymmetry despite the generation of ROS. Thus, lipids do not appear to be targets during the course of oxidative damage to 5-HPQ-treated rat erythrocytes. Rather, the data are consistent with the concept that cytoskeletal protein damage, in the form of disulfide-linked hemoglobin adducts, underlies the process of recognition and removal of 5-HPQ damaged erythrocytes. Additional studies are warranted to elucidate the process that underlies the loss of self-recognition.

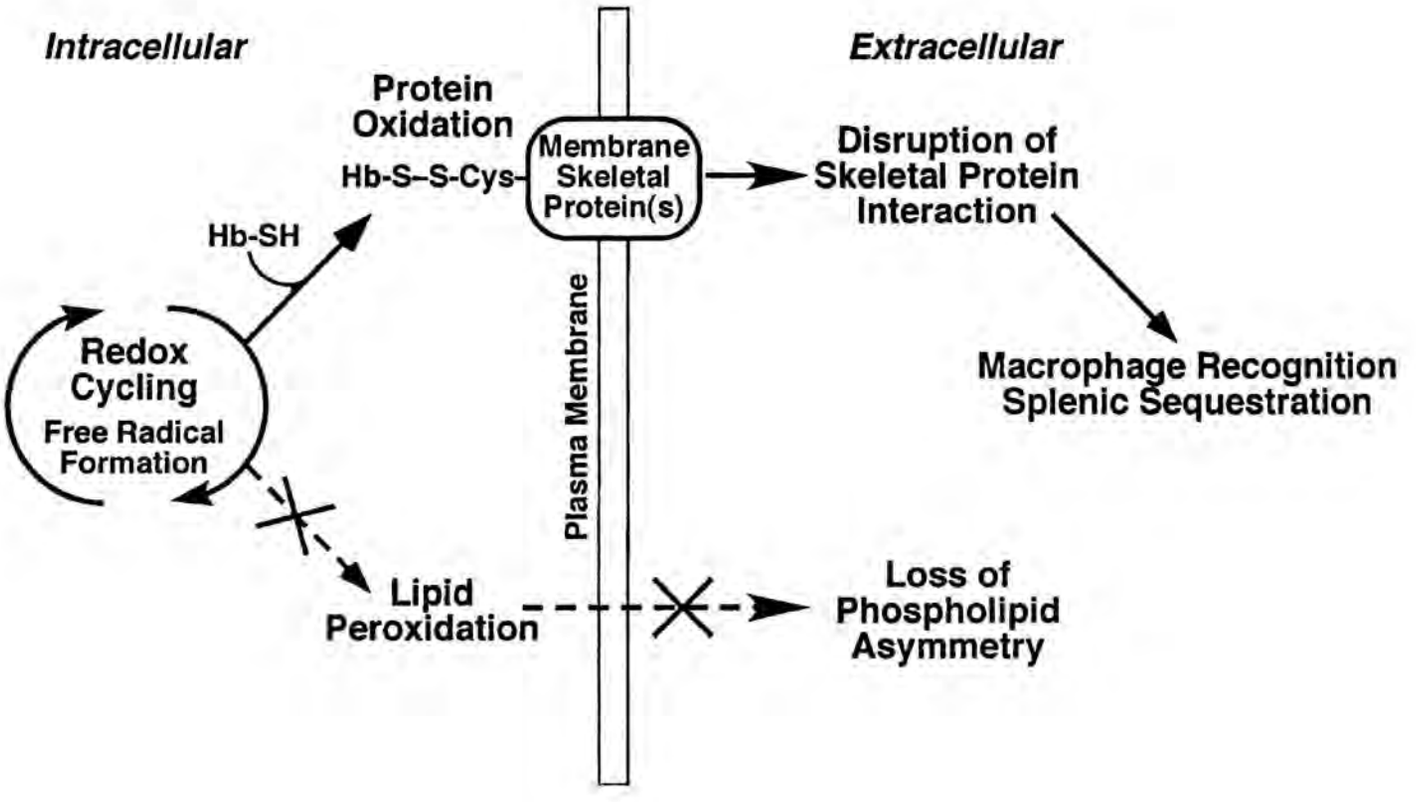


Fig. 4.9. Working hypothesis for the mechanism underlying the hemolytic activity of 5-HPQ in rat erythrocytes. See text for description.

CHAPTER 5

Summary, Discussion and Future Directions

Summary, Discussion and Future Directions

Hemolytic anemia was first recognized as a toxic side effect of pamaquine in 1926 (Cordes, 1926). Since that time, a wide variety of drugs and environmental chemicals have also been shown to elicit this toxic effect as well. As early as 1959, several observations from studies on the toxicity of primaquine supported the conclusion that hemolytic drugs cause the accelerated removal of red cells from the circulation by inflicting oxidative damage. Evidence for this included the following characteristic observations: 1) the depletion of cellular reduced glutathione, 2) oxidation of hemoglobin to methemoglobin, and 3) that "primaquine-sensitive" individuals also possessed an intrinsic red cell deficiency of glucose-6-phosphate dehydrogenase (Beutler, 1959).

The mechanisms of red cell sequestration for both hemolytic anemia and physiological erythrocyte senescence are thought to share the involvement of intracellular oxidative stress and uptake of damaged red cells by the spleen. This commonality has led to the hypothesis that both processes might operate through similar mechanisms, such that the mechanism of hemolytic anemia could result from an acceleration of the normal red cell senescence mechanism. Studies on the cellular and molecular mechanisms of senescent erythrophagocytosis have provided "numerous, and often conflicting, hypotheses" to address the question of "what is the critical signal that it, and it alone, will activate the resident macrophage to adhere to and engulf [the senescent red cell]" (Bratosin et al., 1998). A collection of studies has shown that the primary signal could either be protein, carbohydrate, or lipid in nature, and to potentially result from either the

unmasking of a "senescent cell antigen" (Kay, 1994) or from spatial remodeling of membrane proteins that are then either recognized by macrophages via opsonization by circulating "senescent antibodies" or by macrophage receptors directly. The only certainty is that a great deal of controversy remains with respect to the mechanisms of red cell senescence and hemolytic anemia.

Very early on, primaquine-induced oxidative damage was recognized to be mediated by redox active "metabolic products" rather than the parent drug itself (Earle et al., 1948). Studies have shown that the *N*-hydroxy metabolites of certain arylamine compounds, such as aniline and dapsone, are able to mediate the hemolytic activity of the parent compounds (Harrison and Jollow, 1986; Grossman and Jollow, 1988). Thus, in order to investigate whether an arylhydroxylamine metabolite contributed to the overall hemotoxicity of primaquine, the toxicity of the *N*-hydroxy type metabolite, MAQ-NOH, was recently examined in our laboratory. Laura Bolchoz demonstrated that MAQ-NOH could be formed by *N*-oxidation of 6-MAQ, an *N*-dealkylated primaquine metabolite that has been shown to be formed in humans (Bolchoz et al., 2001), and was a direct-acting methemoglobinemic and hemolytic agent in rats. She postulated that it induced its hemolytic injury via the generation of intracellular ROS, which oxidized lipids in GSH-normal rat erythrocytes and oxidized proteins GSH-depleted red cells. The results of her studies led to the conclusion that MAQ-NOH had the requisite properties to significantly contribute to the overall hemotoxicity of primaquine. However, neither the relative contribution of MAQ-NOH nor the potential contributions of other types of metabolites were assessed.

In 1962, Tarlov et al. suggested that the phenolic metabolites of primaquine were likely to play an important role in its hemotoxicity (Tarlov et al., 1962). However, experimental support for this hypothesis did not appear until the mid-1980s, when identification of phenolic metabolites in animals and synthesis of test compounds by the U.S Army Drug Development Program made research possible. Results from those studies demonstrated that *in vitro* incubation of red cells with the phenolic metabolites, 5-HPQ and 5,6-DHPQ, produced a number of oxidative effects, including: 1) the depletion of cellular reduced glutathione (Agarwal et al., 1988; Fletcher et al., 1988), 2) oxidation of hemoglobin to methemoglobin (Agarwal et al., 1988; Fletcher et al., 1988), and 3) stimulation of HMP shunt activity (Baird et al., 1986). Collectively, these data led to the postulate that quinoneimine and/or quinone metabolites of primaquine could redox cycle and thereby generate toxic levels of reactive oxygen species, which damage cellular components and lead to the accelerated removal of damaged cells from the circulation. However, direct evidence linking the biochemical changes observed *in vitro* to the hemolytic activity *in vivo* was lacking. Likewise data regarding the possible targets of oxidative damage, which would have illuminated the precise mechanism(s) by which metabolite-induced oxidative stress leads to the recognition and removal of damaged erythrocytes from the circulation, was also not available. Currently, any progress towards understanding the role of phenolic metabolites in primaquine-induced hemolytic anemia is hampered because the compounds are no longer available, the synthetic methods to prepare them are relatively difficult, and the products are very unstable.

The purpose of this dissertation was to examine the hemolytic potential of the quinoneimine-type phenolic metabolite, 5-hydroxyprimaquine, and our experimental

approach was as follows: 1) to synthesize 5-HPQ, characterize its chemical properties in order to learn how to properly handle and deliver the compound to experimental models; 2) to use an *in vitro* exposure/*in vivo* survival model to establish the direct hemolytic activity of 5-HPQ; 3) to characterize the pattern of 5-HPQ-induced oxidative stress within the red cell *in vitro* under toxicologically relevant conditions; 4) to examine possible membrane targets of oxidative damage; and 5) to determine a role for splenic macrophages in the hemolytic removal of 5-HPQ-damaged red cells. The data presented in this dissertation has provided evidence for the likely contribution of 5-HPQ to the overall hemotoxicity of primaquine, as well as significant insight into the mechanism of red cell removal in drug-induced hemolytic anemias and normal red cell senescence.

Synthesis and characterization of 5-HPQ, a quinoneimine-type metabolite

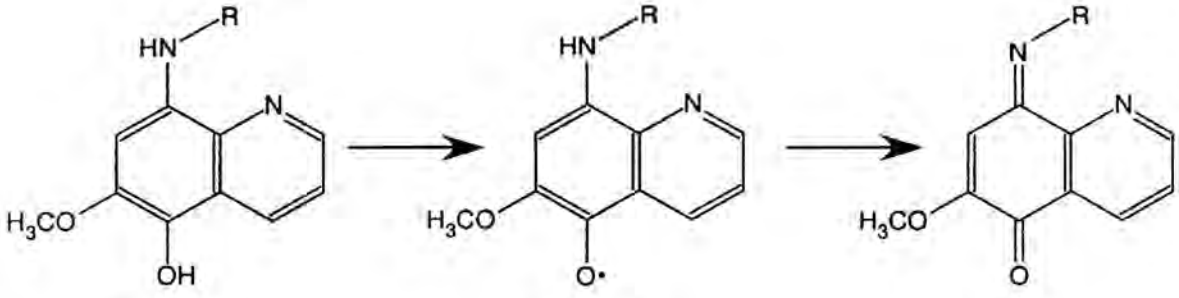
5-HPQ is no longer available from any source, commercial or otherwise. Thus, as a first step in our investigation, we re-synthesized 5-HPQ (Allahyari et al., 1984) and examined its chemical properties, including its stability and ability to redox cycle. The chemical instability of 5-HPQ in both aqueous solution and in the crystal state has been previously reported (Link et al., 1985), so in order to be confident that 5-HPQ was delivered to our experimental models intact, it was necessary to quantify this instability (Fig. 2.5). In agreement with the previous studies, we found that 5-HPQ was extremely unstable in blood and aerated buffer at physiological pH, and we determined that this instability was due to complete degradation of the redox pair and not merely a product of oxidation to the quinoneimine.

It was necessary then, given its instability, to develop a method that would allow us to reliably deliver 5-HPQ to red cell suspensions intact and in known concentrations. The observation that direct synthetic precursor of 5-HPQ, 5-methoxyprimaquine (Fig. 2.2) was stable and could be stored for extended periods of time allowed us to avoid significant degradation by freshly preparing 5-HPQ from its stable precursor as needed. Additionally, the NMR studies that were performed to initially characterize 5-HPQ showed that it would remain stable for over 24 hours when maintained at a low pH and under strictly anaerobic conditions, and thus provided us with a method to deliver 5-HPQ to experimental models. The high degree of reproducibility that was observed from one experiment to the next, e.g. the similar hemolytic response observed in three separate experiments, demonstrated our ability to reliably deliver 5-HPQ (Figs. 2.7, 3.1, and 4.8). Thus, despite the rapid degradation of 5-HPQ upon addition to red cell suspensions, we could be confident that we delivered 5-HPQ intact and in the appropriate concentrations.

Formation of 5-HPQ-induced ROS

5-HPQ caused a significant, concentration-dependent increase in ROS generation in red cells, which continued unabated for at least 20 min (Fig. 4.1), despite a very short half-life of 5-HPQ in the blood (Fig. 2.5). The observation that DCFDA oxidation in rat erythrocytes increases following treatment with concentrations of 5-HPQ that were shown to be hemolytic, indicates that the formation of ROS was associated with the hemolytic response. However, the finding that ROS formation was detected well after 5-HPQ was degraded raises the likelihood of some as yet unknown reactive intermediate with a longer half-life that mediates 5-HPQ-induced hemotoxicity.

(A)



5-HPQ 5-HPQ• QI

(B)

(1) 5-HPQ + O_2 ⟶ 5-HPQ• + $O_2^{-\bullet}$

(2) 5-HPQ• + O_2 ⇌ QI + $O_2^{-\bullet}$

(3) $O_2^{-\bullet}$ + $O_2^{-\bullet}$ + $2H^+$ ⟶ H_2O_2 + O_2

(4) 5-HPQ + QI ⇌ 2 5-HPQ•

(5) 5-HPQ• + H_2O_2 ⟶ QI + •OH + H_2O

Fig. 5.1. Schematic representation of 5-HPQ autooxidation: (A) compound structures; (B) suggested reaction sequence (hydrogens and charges are not balanced to avoid changing the basic abbreviation used for 5-HPQ).

(Vasquez-Vivar and Augusto, 1990)

DCFDA is a non-fluorescent compound that is converted by reactive species into the fluorescent compound, DCF, and thus provides a valuable assay that can be utilized for the detection of free radical species. The DCFDA assay is able to detect the formation of peroxyl (RO_2•), alkoxyl (RO•), NO_2•, CO_3•$^-$, $ONOO^-$, and OH• radicals, but not H_2O_2 or superoxide directly (Halliwell and Whiteman, 2004). It should be noted that this list is not definitive for the specificity of the assay, and it could be that a one-electron oxidation of DCFH by a wide variety of free radicals and heme proteins (i.e. hemoglobin) might also produce a positive result (Halliwell and Whiteman, 2004). Thus, while it is reasonable to conclude that 5-HPQ generates the formation of reactive species in red cells in general, no definitive statement with respect to the specific species formed can be made based on this data (Fig. 4.1). Future experiments could be conducted using EPR spectroscopy and appropriate spin-trapping techniques to identify the specific free radicals present in red cell suspensions exposed to hemolytic concentrations of 5-HPQ.

Initial studies utilizing oxygen consumption and spin-trapping EPR led to the postulate that 5-HPQ has the capacity to redox-cycle and thereby generate H_2O_2, hydroxyl radicals, and a semiquinoneimine intermediate in aerated buffer according the schematic depicted in Fig. 5.1 (Vasquez-Vivar and Augusto, 1990; Vasquez-Vivar and Augusto, 1992). To confirm whether 5-HPQ could reversibly cycle, we examined its electrochemical activity in argon-purged PBSG (pH 7.4) using cyclic voltammetry (Fig. 2.6) and made observations that were consistent with the concept that 5-HPQ could undergo cycling as a fully reversible redox couple via a semiquinone radical intermediate at physiological pH.

It is well known that H_2O_2 reacts with hemoglobin to form ferryl-heme, and thus ferryl-heme levels in the red cell can reflect the formation of H_2O_2 in excess of its metabolic clearance (Harel and Kanner, 1988). Additionally, ferryl-heme itself is thought to be a strong cytotoxic oxidizing agent that is capable of peroxidation of unsaturated fatty acids (Kanner and Harel, 1985). To examine whether ferryl-hemoglobin, and by extension H_2O_2, were formed in red cells following exposure to hemolytic concentrations of 5-HPQ, we utilized a spectrophotometric scanning technique that can detect ferryl-hemoglobin formation in red cells (Bolchoz et al., 2002a). However, Fig. 5.2 shows that we could not detect any significant formation of H_2O_2 relative to controls. It should be noted that this assay does not allow us to specifically rule out the formation of H_2O_2, but it does suggest that, if formed, it does not react with hemoglobin. And at a minimum, ferrylhemoglobin does not appear to contribute significantly to 5-HPQ-induced hemotoxicity.

When discussing reactive species, it must be understood, that species such as H_2O_2 and hydroxyl radical are extremely reactive and short-lived, and are not able to account for the prolonged generation of ROS that we observed. Likewise, the rapid degradation of the 5-HPQ redox pair necessitates rapid degradation of any semiquinoneimine or other compound-centered free radical. Thus future studies are necessary to examine the identity or identities of longer-lasting intermediate reactive species. One attractive possibility is the potential involvemnt of reactive sulfur species (RSS), which have been recently been suggested to play a role in oxidative stress and to present an interesting possibility for longer-lasting reactive intermediate (Giles et al., 2001). Free thiol-groups are known to act as reducing agents that are easily oxidized to non-reactive disulfides.

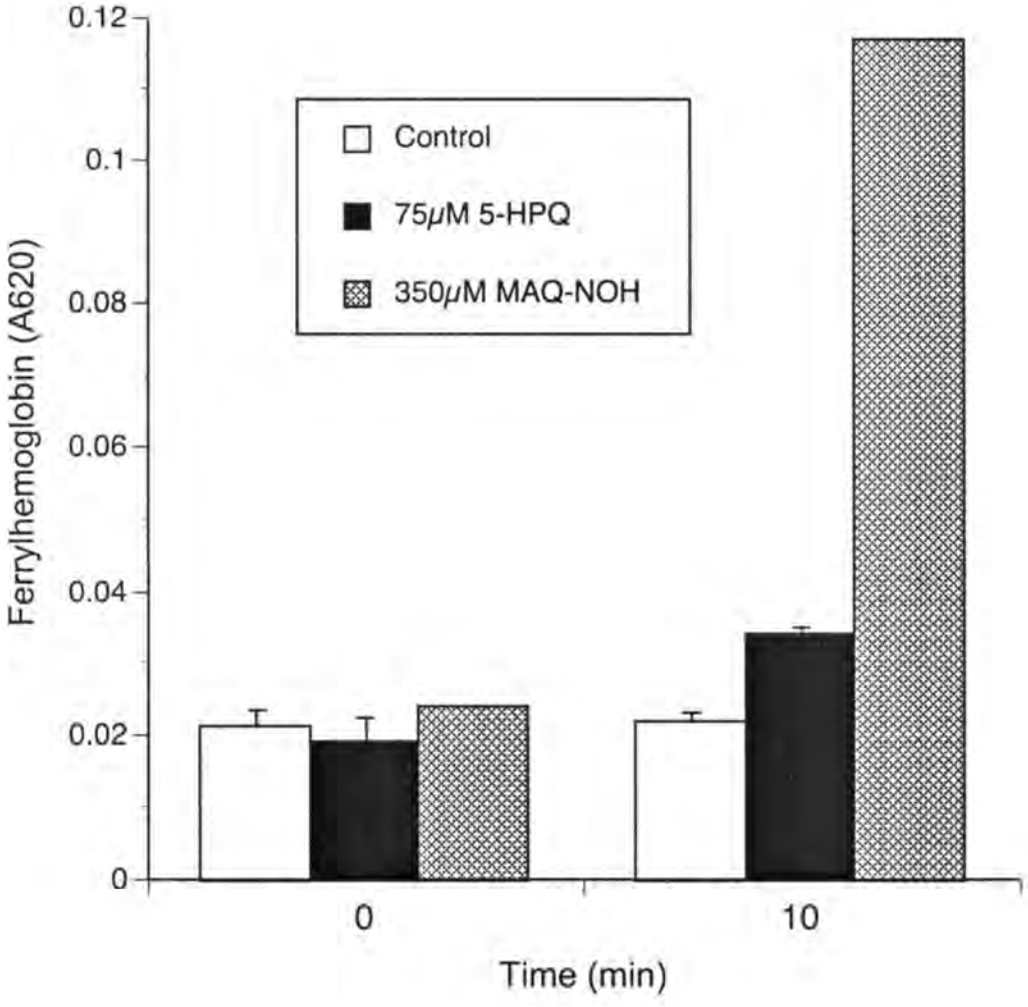


Fig. 5.2. The time-dependent, spectrophotometric detection of 5-HPQ-induced ferryl-hemoglobin formation. Rat erythrocytes (40% suspension) were pre-incubated with sodium sulfide (2 mM), which traps ferryl-heme species as sulfhemoglobin (λ_{max} 620 nm). Potassium cyanide (10%, 20 μl) was also added to remove any interference from methemoglobin (λ_{max} 620 nm). Values for control and 5-HPQ samples are means ± S.D. (n = 3). Values for MAQ-NOH samples represent an n of 1.

It has recently been shown that the disulfide can be further oxidized to either a disulfide-*S*-monoxide or a disulfide-*S*-dioxide under conditions of oxidative stress (Fig. 5.3). The sulfur atom in these molecules resides in a higher than normal oxidation state, which increases the reactivity of the disulfide toward other thiols via a sulfur-sulfur exchange reaction. As shown in Fig. 5.3, formation of protein mixed-disulfides by either the disulfide-*S*-monoxide or the disulfide-*S*-dioxide can also result in the production of sulfenic and sulfinic acid, which themselves are also strong oxidizing agents (Giles et al., 2001). Importantly, RSS are formed with relative ease under physiological conditions of oxidative stress, which underlies their potential to enhance oxidative stress *in vivo*. Although limited information is available regarding the formation of RSS, they do represent an intriguing possibility for an intermediate that might mediate a sustained oxidative stress long after the decay of 5-HPQ.

Hemolytic concentration-response relationship

Having synthesized 5-HPQ and developed a method to deliver it intact to red cell suspensions, our next step was to determine whether 5-HPQ could act as a direct acting hemolytic agent. To this end, we used an *in vitro* exposure/ *in vivo* survival model that has been previously developed and characterized by our laboratory (Harrison and Jollow, 1986). Specifically, ^{51}Cr-labeled rat erythrocyte suspensions were exposed to various concentrations of 5-HPQ *in vitro* for 2 hr at 37°C, and then cells were washed and returned to the circulation of isologous rats. Serial blood samples were taken at specific intervals, and loss of radioactivity from the circulation over time was measured as

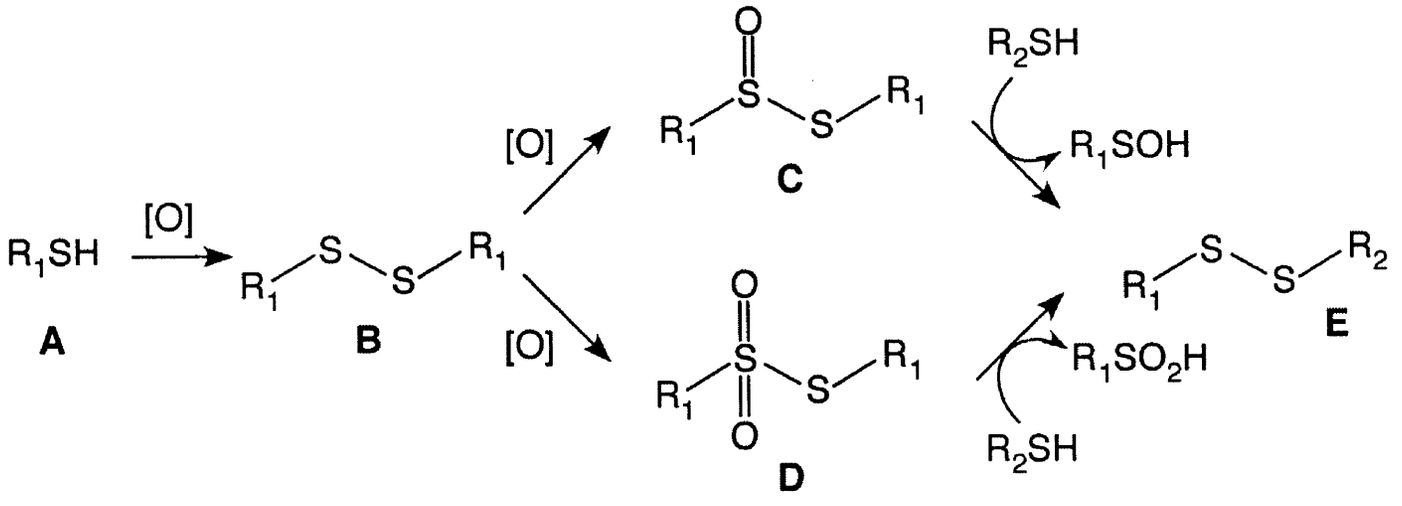


Fig. 5.3. Oxidative formation of disulfide-S-oxide RSS and subsequent sulfur-sulfur exchange to form the mixed disulfide. The disulfide B is formed from thiol A under oxidative conditions [O]. Further oxidation of the disulfide moiety yields either the disulfide-S-monoxide C or the disulfide-S-dioxide D. Activation of the disulfide via sulfur oxidation renders the bond more labile and promotes the reaction with a reduced thiol to form the mixed disulfide E and the sulfenic or sulfinic acid.

(Giles et al., 2001)

evidence of direct hemolytic activity. Importantly, it should be noted that the concentrations of 5-HPQ used in this model are not predictive of what would be required in vivo, but rather, this model permits the comparison of the relative potencies of a variety of direct-acting hemolytic agents. Additionally, specific cellular biochemical alterations can be directly correlated with the hemolytic removal from the circulation in vivo following the same in vitro treatment conditions. The data obtained from this model demonstrated that 5-HPQ is the most potent direct hemolytic agent that we have examined to date. Additionally, we were able to show that the direct hemolytic activity was dependent on intracellular levels of GSH and binding of Hb to membrane proteins.

It should also be noted that the cellular injury that results from drug-induced oxidative damage has been shown to be time-dependent (Grossman and Jollow, 1988; McMillan et al., 1991), which suggests that hemolytic injury is cumulative in nature rather than proportional to drug concentration. That is, the severity of hemolytic damage can be thought of as a series of "hits" on cellular targets that accumulate over time, rather than as a reversible drug-receptor association that is dependent on drug concentration. For example, 3,4-dichlorophenylhydroxylamine-treated red cells were shown to elicit a hemolytic response that was not found to correlate the peak plasma concentration, but with the area under the blood concentration versus time curve (McMillan et al., 1991).

Additionally, primaquine has a high bioavailability (>75%), a significant half-life (~7 hours), an extensive metabolism (renal elimination of <1% of the parent compound), and is chronically administered, thus it is conceivable that the AUC for 5-HPQ could be significant. Furthermore, if the toxicity of 5-HPQ is due to the accumulation of "hits" on red cells, the reactivity of 5-HPQ could cause damage to accumulate, despite a low or

undetectable peak plasma concentration at any given time. As a compromise between achieving a drug-AUC necessary to elicit a hemolytic response and the necessity to return cells to the circulation relatively quickly in order to maintain control-cell viability, we have chosen an incubation time of 2 hours for the *in vitro* exposure to hemolytic agents. In theory, a similar hemolytic response would be observed if lower concentrations of 5-HPQ were used and the treatment time increased, but the maintenance of control red cell viability decreases with longer incubation times. However, if a specific incubation time is chosen (i.e. 2 hours) and used consistently from one experiment to the other, the relative potency of a variety of toxic compounds can be assessed.

One additional point to be considered is that the hemolytic assay is generally performed using G6PD-normal rat erythrocytes. These cells have normal GSH levels and normal antioxidant enzyme activity when challenged by drug-induced oxidative stress, and thus the concentrations used must be able to overwhelm normal antioxidant defenses before eliciting a toxic response. Importantly, when GSH was depleted in red cells by >95% with diethyl maleate in order to mimic *in vitro* the GSH instability observed in G6PD-deficient red cells *in vivo*, the hemolytic response observed with 5-HPQ was markedly enhanced and an effect was observed with as little as 1-2μM following the relatively brief 2 hour incubation period.

Role of GSH in 5-HPQ-induced hemolytic anemia

Previous studies with 5-HPQ have shown that it causes an extensive depletion of GSH in isolated suspensions of rat and human erythrocytes (Allahyari et al., 1984; Agarwal et al., 1988; Fletcher et al., 1988). We demonstrated that the concentration-

dependence for 5-HPQ-induced GSH-depletion was sharp, exhibiting a threshold-like response (Fig. 2.9) that coincided with the concentration-dependence for the formation of mixed disulfides between GSH and soluble red cell protein (Fig. 2.9A). Furthermore, the concentration-response curve for GSH-depletion was found to closely coincide with the concentration-response curve for the hemolytic activity (Fig. 2.7). Additionally, depletion of GSH with DEM in red cells *in vitro* prior to 5-HPQ-exposure markedly enhanced the hemolytic response with a loss of the apparent threshold (Fig. 2.10). Collectively, these data strongly supported the concept that the hemolytic response induced by 5-HPQ had a discrete dose threshold and that this threshold was dependent on the presence of GSH in the red cell.

In view of the involvement of GSH in 5-HPQ-induced hemotoxicity, it is reasonable to speculate as to its specific role. Evidence exists that rat erythrocytes produce large quantities of hemoglobin-GSH mixed disulfides when exposed to oxidative stress (Grossman and Jollow, 1988; Lii and Hung, 1997; Rossi et al., 1998; Murakami and Mawatari, 2003). Given the significant production of these mixed disulfides, it is reasonable to conclude that 5-HPQ-induced depletion of GSH results primarily in the formation of Hb-GSH mixed disulfides (Fig. 2.9). Additionally, hemoglobin thiyl free radicals have been shown to be formed in red cells under oxidative-stress inducing conditions (Maples et al., 1988; Maples et al., 1990a; Maples et al., 1990b; Bradshaw et al., 1995), and although a thiyl radical has yet to be detected in red cells after exposure to 5-HPQ, our results agree with the hypothesis that hemoglobin is activated to a thiyl free radical by a 5-HPQ-induced reactive species. Under this hypothesis, cellular reduced GSH would react with the newly formed thiyl radical to form a mixed disulfide, initially

stifling the toxic species and protecting the red cell. However, under our treatment conditions, GSH was rapidly and completely depleted, which allowed the hemoglobin thiyl free radicals to attack other cellular components. Additional studies are necessary to determine whether the formation of thiyl radicals can be detected by EPR in response to treatment of red cells with 5-HPQ.

Cell membrane targets of 5-HPQ-induced oxidative damage

It is clear that 5-HPQ-induced ROS formation results in an attack on the cytosolic surface of the plasma membrane that leads to a signal for removal of damaged erythrocytes from the circulation. In order to determine the nature of the crucial targets of oxidative damage, we addressed two possibilities: 1) that red cell damage is analogous to the apoptotic response observed in other nucleated cell types, which results in loss of the normal asymmetric distribution of phosphatidylserine in the cell membrane (Mandal et al., 2002); and 2) that the oxidation of integral membrane proteins is involved in the link between internal oxidative stress and external recognition and removal of erythrocytes from the circulation.

With respect to the first hypothesis, lipid peroxidation and PS externalization have been previously shown to occur from peroxide-induced oxidative stress (Jain, 1984; Mandal et al., 2002). In order to determine whether these alterations occurred during the course of 5-HPQ-induced oxidative stress, we quantified lipid peroxidation by the direct measurement of free F_2-isoprostanes (Fig. 4.2), and PS externalization by both the annexin labeling of erythrocytes and the PS-dependent prothrombinase assays (Fig. 4.3).

None of these assays demonstrated any significant effect on red cell membrane lipids following exposure to hemolytic concentrations of 5-HPQ.

Given that we have shown the formation of reactive species that are capable of initiating lipid peroxidation, but that there was no lipid peroxidation, our data met some initial resistance with manuscript reviewers. However, we took special care to use the gold standard for the measurement of lipid peroxidation. Lipid peroxidation is a notoriously difficult response to measure, and the failure to detect it experimentally may not necessarily prove that it does not occur. Unfortunately, most of the commonly used methods that could be used to confirm its absence, such as the TBARS or conjugated-dienes assays, are well-known to be non-specific and unreliable (Moore and Roberts, 1998; Halliwell and Whiteman, 2004). Furthermore, it has been found that hydrogen peroxide-generating agents give rise to fluorescent pigments of hemoglobin, which have a maximum absorbance at 533 nm and thus interfere with the TBARS assay, which is read at 530 nm (i.e., give false positives). Other assays that have been used in our lab, including the fluorescent lipid probe, *cis*-parinaric acid, are also prone to artifacts and interference by the formation of methemoglobin (McMillan et al., 1998). For these reasons we utilized what is currently considered by most investigators in the field to be the "gold standard" for the quantification of lipid peroxidation, i.e. direct measurement of free F_2-isoprostanes by GC/MS. Furthermore, since the products of lipid peroxidation are thought to result in PS translocation, results from the two separate assays that assessed PS externalization, which were not influenced by fluorescent heme derivatives, confirmed the observed lack of lipid peroxidation.

The apparent contradiction between previously published results of oxidant induced lipid peroxidation and our own "controversial" data can be reconciled by considering the origin of oxidative stress. The high reactivity of hydroxyl radicals causes them to react in very close proximity to where they are formed. Previously, lipid alterations from oxidative stress were assessed following the addition of peroxides, such as H_2O_2, cumene hydroperoxide and *t*-butyl hydroperoxide to red cell suspensions. These reactive species encounter the membrane prior to any other cellular component and can be considered to cause an oxidative stress from the "outside-in". Alternatively, quinoneimine-, quinone-, or hydroxylamine-type compounds must first penetrate into the cytoplasm before they redox cycle and generate ROS from the "inside-out". Thus reactive species are more likely to react with cytoplasmic components in close proximity, i.e., hemoglobin, rather than membrane lipids.

In agreement with this hypothesis, exposure to 5-HPQ was found to cause profound alterations to the red cell hemoglobin and the protein cytoskeleton, principally in the form of disulfide-linked hemoglobin adducts (Figs. 4.4, 4.5, 4.6 and 4.7). The concentration-response for hemoglobin binding to skeletal membrane proteins correlated very strongly with the hemolytic response for GSH-normal cells, and when GSH was depleted with DEM prior to treatment with 5-HPQ, the pattern of hemoglobin binding was also remarkably similar to the hemolytic response for GSH-depleted erythrocytes. The observation that 5-HPQ treated red cells could be "rescued" from hemolysis by reduction with DTT, which was shown to release hemoglobin from skeletal membrane proteins, further supported a direct role for hemoglobin, or at least some redox sensitive protein, binding to skeletal membrane protein in the hemolytic response from 5-HPQ.

These findings are consistent with the hypothesis that 5-HPQ-exposure results in the formation of hemoglobin thiyl radicals, which first react with and deplete reduced GSH, and then react with a variety of sulfhydryl-containing structural proteins to form hemoglobin adducts to integral membrane and cytoskeletal proteins. Although, it could be that intracellular oxidative damage might directly alter reactive cysteines in Band 3 that leads to "Band 3 clustering" and subsequent with subsequent antibody recognition (Turrini et al., 2003). Regardless, additional studies are necessary to determine the precise internal protein targets that lead to an external signal for splenic sequestration. In view of the hypothesis that hemoglobin binding to numerous red cell skeletal membrane proteins is directly involved (Fig. 4.4B), it is likely the case that most of the binding of Hb would represent a bystander event. From Fig. 4.4A, it appears that members of the band 3 macrocomplex, specifically ankyrin and band 4.2, might be targets, and future experiments to identify specific inside-out protein alterations may involve the targeting hemoglobin for immunoprecipitation with subsequent mass spectral identification of bound proteins. Alternatively, biotinylation of cell surface amines, with subsequent avidin-column or gel isolation and identification of red cell surface proteins from 5-HPQ-treated red cells could be used to examine external cell-surface alterations.

Role for macrophages in the uptake of 5-HPQ treated erythrocytes

An examination of the literature reveals that the removal of damaged or senescent red cells from the circulation by macrophages in the spleen that is often taken for granted despite the sparcity of specific evidence. Thus, we examined the fate of 5-HPQ-treated red cells and demonstrated for the first time that they are taken up exclusively by the

spleen. Additionally, we demonstrated that it is the resident macrophages that are specifically responsible for the uptake of red. Furthermore, we developed an *in vitro* erythrophagocytosis assay using the J774A.1 macrophage cell line, which, in addition to suggesting that uptake by macrophages is signal-dependent rather than dependent on Heinz body trapping within the splenic cords, provides us with a very useful tool that can be used to identify specific cell-surface alterations that are responsible for macrophage recognition.

Studies to further validate and characterize our erythrophagocytosis assay are ongoing, and we are examining its ability to correlate with the specific effects that we previously observed *in vivo*. Specifically, depletion of GSH from red cells with DEM prior to treatment with 5-HPQ was shown to enhance red cell removal from the circulation of rats *in vivo* (Fig. 2.10), and we wanted to know whether depletion of GSH from red cells prior to treatment with 5-HPQ would enhance their erythrophagocytosis by J774A.1 macrophages *in vitro* as well. Figure 3.7 showed that phagocytosis of red cells treated with a subhemolytic concentration of 5-HPQ (25μM) was significantly enhanced in GSH-depleted versus GSH-normal rat red cells, and preliminary results suggest that the ability of DTT to "rescue" 5-HPQ-treated erythrocytes *in vivo* after *in vitro* exposure (Fig. 4.8) can also be reproduced using J774A.1 macrophages *in vitro* (Fig. 5.4).

One of several future directions that could be pursued using the erythrophagocytosis model *in vitro* involves the use of a fusion protein, which displays the extracellular domain of CD47 in order to restore proper recognition of "self" to the macrophages and specifically inhibit phagocytosis. Additionally, use of J774A.1 macrophages could become an important tool with which to study human hemotoxicity. Specifically, it

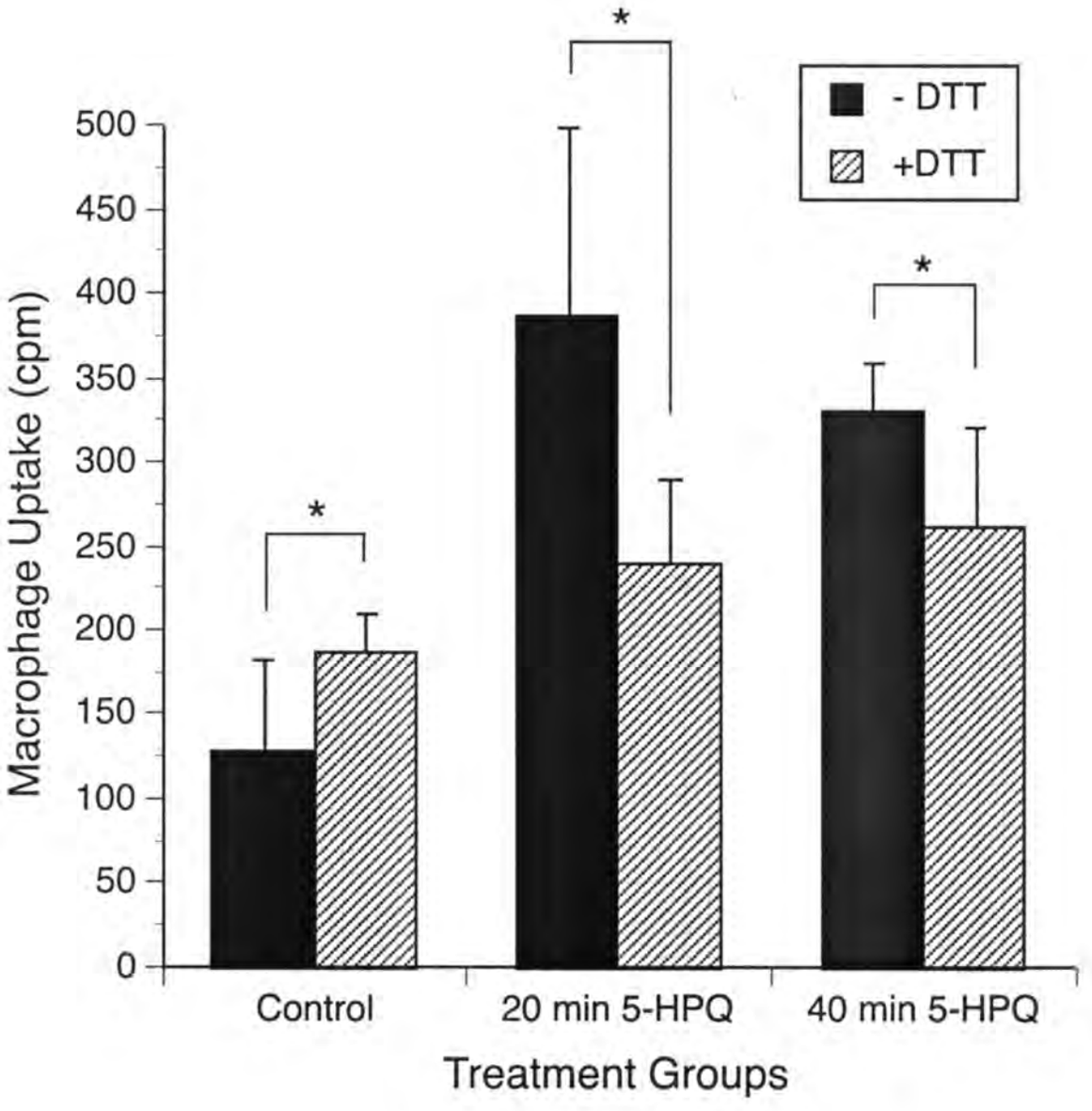


Fig. 5.4. Effect of DTT on the phagocytosis of rat ^{51}Cr-labeled erythrocytes after exposure to 5-HPQ (50μM) by J774A.1 macrophages *in vitro* (preliminary data). The red cells were incubated with or without 5mM DTT for 1 hr at 37°C after treatment with 5-HPQ (20 or 40 min), or with vehicle (H_2O) alone (40 min) at 37°C. The erythrocytes were then washed and allowed to incubate with plated macrophages for 18 hr. After the incubation period, non-ingested red cells were lysed with NH_4Cl and the macrophages extensively washed. Erythrophagocytosis was measured as radioactivity in macrophages (cpm). Data points are means ± S.D. (n=6) *, statistically significant, $p<0.05$.

would provide a model for examining concentration ranges of hemotoxic drugs, which result in phagocytosis by macrophages. Thus drug-induced biochemical alterations in human red cells could be studied within the context of drug concentrations that have been shown to be toxicologically relevant. Until now, one major problem with studying the effects of any hemolytic drug in human red cells is that there are no ethical or practical methods available that are equivalent to the in vitro exposure/ in vivo survival rat model to examine whether specific concentrations are toxicologically relevant, i.e. directly hemolytic, in humans.

Conclusion

Evidence has been presented in this dissertation to show that the phenolic primaquine metabolite, 5-HPQ, could play an important role in the overall hemotoxicity of the parent drug. While 5-HPQ is the most potent metabolite that has been examined in our experimental model, the determination of its relative contribution to the overall toxicity of primaquine will be dependent on its relative rate of formation, which has yet to be measured. However, in light of its potency, it is clear that in addition to being an important compound to study primaquine-induce hemolytic anemia, 5-HPQ can provide a useful tool that for studying the mechanism of hemolytic anemia and uptake of damaged cells in general.

Our working hypothesis is shown in Fig. 5.5. Specifically, (1) 5-HPQ is thought to passively diffuse into cells, where the coordinated iron in hemoglobin catalyzes a redox cycle between 5-HPQ and its quinoneimine. Reactive species are generated which non-specifically attack macromolecules in the vicinity of their formation. (2) Since red cells

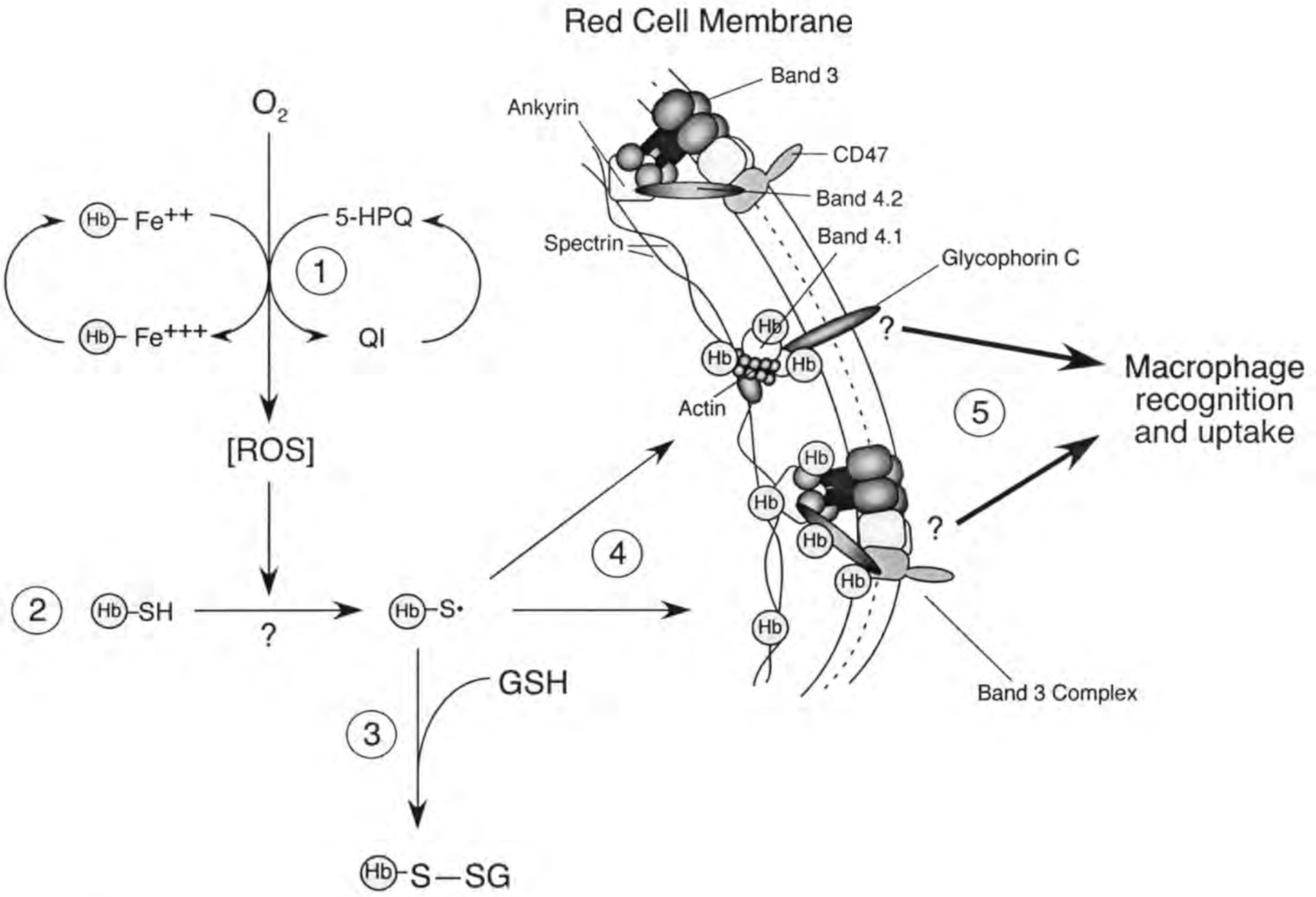


Fig. 5.5. Working hypothesis for 5-HPQ-induced hemolytic activiy (see text for detailed explanation).

are packed with hemoglobin, and ROS are formed intracellularly within the cytoplasm, hemoglobin is likely the primary target of oxidative damage. (3) It is thus thought that hemoglobin free thiols are activated to reactive thiyl radical intermediates, which are initially quenched by cellular reduced GSH, but then rapidly overwhelm this defense mechanism as radicals are formed faster than GSH is able to regenerate. (4) In the absence of sufficient GSH, hemoglobin thiyl radicals are free to attack other cellular targets, and our evidence suggests that hemoglobin binds non-specifically to free thiols on the cytoplasmic side of cytoskeletal and integral membrane proteins. (5) This addition of large protein adducts likely leads to specific cell surface alterations and/or loss of normal protein-protein interactions effecting a loss of recognition of "self" by splenic macrophages and subsequent phagocytosis.

In summary, the data presented in this dissertation has lead to the postulate of a mechanism by which quinoneimine-type metabolites of primaquine might lead to the hemolytic removal of red cells. While there are remarkable similarities to the mechanism of removal induced by hydroxylamine-type metabolites, the mechanism presented in this dissertation also differs significantly. It is believed that the novel evidence presented provides additional and important insight into the mechanism(s) of drug-induced hemolytic anemia normal red-cell senescence. Additionally, we hope that the additional information provided regarding of the mechanism by which primaquine might mediate its toxicity will eventually allow for the rational redesign of the primaquine molecule, in order to significantly lessen its toxicity while maintaining its therapeutic efficacy.

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