Glucose-6-Phosphate Dehydrogenase Deficiency and Antimalarial Drug Development

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Abstract. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is relatively common in populations exposed to malaria. This deficiency appears to provide some protection from this infection, but it can also cause hemolysis after administration of some antimalarial drugs, especially primaquine. The risk of drug-induced G6PD deficiency-related hemolysis depends on a number of factors including the G6PD variant, the drug and drug dosage schedule, patient status, and disease factors. Although a great deal is known about the molecular biology of G6PD, determining the potential for drug-induced hemolysis in the clinical setting is still challenging. This report discusses the potential strategies for assessing drug-induced G6PD deficiency-related hemolytic risk preclinically and in early clinical trials. Additionally, the issues important for conducting larger clinical trials in populations in which G6PD deficiency is prevalent are examined, with a particular focus on antimalarial drug development.

INTRODUCTION

Hemolytic anemia after administration of the antimalarial drug pamaquine (also known as plasmoquine and plasmoquin) was reported as early as 1926.1 However, it was not until the 1950s that a series of investigations by United States Army researchers identified glucose-6-phosphate dehydrogenase (G6PD) deficiency as the cause of hemolysis after administration of the related antimalarial primaquine.2 These studies first showed that erythrocytes from subjects who were sensitive to the antimalarial drug primaquine had lower glutathione levels than those who were non-sensitive to primaquine,3 and subsequently that erythrocytes from primaquine-sensitive individuals were unable to maintain glutathione levels after challenge with acetylphenylhydrazine.4 These findings focused attention on the pathway that maintains glutathione in the reduced state, and eventuated in the discovery that primaquine-sensitive cells were G6PD deficient.5

Glutathione metabolism consists, in large part, of its oxidation to the disulfide and recycling to the reduced form by the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH) by mediation of glutathione reductase (Figure 1). Reduced glutathione is important in the detoxification of free radicals. In normal cells, NADPH is regenerated by G6PD during oxidative stress. Impairment of this step prevents reduced glutathione recycling, exposing the cell to oxidative damage. Alternative pathways to G6PD-dependent NADPH production exist in most human cells but not in erythrocytes, and the lack of protein synthetic machinery deprives the erythrocyte of the opportunity to replace enzyme that has been lost. For these reasons, these cells are uniquely vulnerable to oxidative stress in G6PD deficiency.

Many different drugs, as well as fava beans (favism), can cause G6PD deficiency-related hemolysis.6 The most probable cause of drug-induced G6PD deficiency-related hemolysis is a reaction with oxyhemoglobin, which forms superoxide that decomposes to hydrogen peroxide (Figure 1). If G6PD activity is low, peroxides accumulate, resulting in hemoglobin denaturation and binding to the cell membrane, seen morphologically as Heinz bodies.7 Hemolysis occurs when erythrocytes pass through the spleen and the Heinz bodies are removed along with a portion of cell membrane. After several passes through the spleen, the cell membrane loses competency and the erythrocyte is destroyed.

Over the past 50 years, our knowledge of the genetics, molecular biology, and epidemiology of G6PD deficiency, (reviewed extensively elsewhere†), has expanded enormously. However, the application of these findings to drug development and the conduct of clinical trials remains challenging. This report, based on a Working Group meeting among researchers and clinicians in G6PD and malaria, examines these issues, with a particular focus on the development of new antimalarial drugs.

MOLECULAR EPIDEMIOLOGY OF G6PD DEFICIENCY

Located at the q28 locus on the X-chromosome,9 the G6PD gene is 18 kb long and contains 13 exons.10,11 Polymorphisms of the G6PD gene are numerous, with G6PD deficiency due to single point mutations, deletions, insertions and, rarely, splicing variants; approximately 200 variant alleles have been described and 140 variants were characterized at the DNA level by 2002.12 Phenotypically, G6PD deficiency is most often seen in hemizygous males. Heterozygous females do display partial G6PD deficiency, showing mosaicism consisting of normal G6PD and G6PD-deficient erythrocyte populations13; homozygous females are less common. G6PD deficiency is thought to have been maintained in populations exposed to Plasmodium spp. due to a protective effect against the parasite.14–17 For example, in two large case-control studies of more than 2,000 African children, the common African form of G6PD deficiency (G6PD A−) was associated with a 46–58% reduction in the risk of severe ma-
laria for both female heterozygotes and male hemizygotes. The mechanism for this protective effect may be the higher sensitivity of G6PD-deficient erythrocytes to hydrogen peroxide produced by the parasite. This causes erythrocyte damage that impairs parasite growth and/or leads to early erythrocyte phagocytosis.14,18,19

**Prevalence of G6PD deficiency.** Table 1 summarizes the main deficient variants found in different regions, although information on the molecular epidemiology of G6PD deficiency is patchy globally.20–37 In Africa, the prevalence of G6PD deficiency has been reported as high as 28.1% in southwest Nigeria,22 22.5% in Congo (Brazzaville),38 15.7% in Mali (Bamako),39 13.0% in Uganda40 and 9.0–15.5% in Gabon.41,42 Molecular characterization data have been mostly lacking for Africa, although more are becoming available, most recently, from Mali.21 The available data consistently indicate that G6PD A− is by far the most common deficient variant (Table 1).21,22,43,44 This variant is the result of three different combinations of mutations: 202A,376G, which is the predominant combination; 376G,968C, which has been found in the extreme western region of Africa (Senegal)23; and 376G,680T, which is rare.43 The variant G6PD Santamaria (376G,542T) has also been found in west Africa.23

In southeast Asia, the prevalence of G6PD deficiency differs greatly by region and ethnic group and variants are similarly diverse (Table 1).24–30 For example, in Myanmar, Iwai et al. found prevalences of G6PD deficiency as high as 10.8% for the Shan people, 7.3% in the dominant ethnic group the Burma, and absent in the Akha, despite a regional proximity to the Shan.27 In some populations, there may be a predominant variant, such Viangchan in Cambodia and Laos, but in other populations several variants may be present with subtle differences from region to region even within the same (broad) ethnic group (Table 1).24–30 For example, in southern Thailand, G6PD variants Viangchan, Kaiping and Mahidol comprised only 68.6% of 134 G6PD-deficient subjects, with G6PD Canton (9.7%), G6PD Union (2.2%), G6PD Gaohe (1.5%), G6PD Quing Yuan (0.7%), G6PD Mediterranean (0.7%), and G6PD Songklanagarind (0.7%) also present; 15.7% remained uncharacterized.30

In India, the population is diverse and the government has tried to map G6PD deficiency more accurately. A recent review summarized G6PD prevalence across the country.45 Prevalence of G6PD deficiency is generally 0–10%, although some communities may have higher prevalences; 27.5% for the Vatalia Prajapati community in Western India46 and 27.1% for the Angami Nagas, a tribal group in northeastern
Among 15 tribes in Orissa region, the prevalence of G6PD deficiency varied. Overall, the prevalence of G6PD Mediterranean appears to be the most common deficient variant in the tribal populations in southern India. In Latin America, the prevalence of G6PD deficiency is generally lower (≤ 2%), Table 1, and G6PD A– appears to be the most common deficient variant. In Brazil, 98.6% of G6PD-deficient blood bank samples were G6PD A–, with only 1 of 4,621 samples G6PD Mediterranean. Spanish and African ancestry contribute to most cases of G6PD deficiency; G6PD A– (202A,376G), formerly known as Betica, is a common variant in Spain. There appears to be considerable regional variation in the prevalence of G6PD deficiency and this pattern continues to change because of migration. G6PD deficiency is rare in the native American population.

### Table 1: Prevalence of most common glucose-6-phosphate dehydrogenase (G6PD) variants in regions where antimalarials are used

<table>
<thead>
<tr>
<th>Country, region or population</th>
<th>% G6PD deficiency/total sample (n/N)</th>
<th>Three most prevalent G6PD variants</th>
<th>Total, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td></td>
<td></td>
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<tr>
<td>Comoros Islands</td>
<td>3.6% (17/467)</td>
<td>82.4% A– (376G, 202A)</td>
<td>17.6% Mediterranean</td>
</tr>
<tr>
<td>Mali, Dogon of Bandiagara</td>
<td>16.5% (81/488)</td>
<td>100% A– (376G, 202A)</td>
<td>100</td>
</tr>
<tr>
<td>Mali, Malinké of Kangaba</td>
<td>14.9% (340/2,277)†</td>
<td>100% A– (376G, 202A)</td>
<td>100</td>
</tr>
<tr>
<td>Nigeria</td>
<td>28.0% (170/606)</td>
<td>100% A– (376G, 202A)</td>
<td>100</td>
</tr>
<tr>
<td>Senegal, Niakhar region</td>
<td>16.3% (70/430)</td>
<td>81.4% A– (376G, 968C)</td>
<td>10.0% A– (376G/202A)</td>
</tr>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cambodia, Chanthaburi</td>
<td>15.8% (34/215)</td>
<td>82.4% Viangchan</td>
<td>3.0% Union</td>
</tr>
<tr>
<td>Cambodia, remote areas</td>
<td>7.9% (47/670)</td>
<td>97.9% Viangchan</td>
<td>2.1% Union</td>
</tr>
<tr>
<td>China, southern region</td>
<td>8.1% (382/4,704)</td>
<td>32.2% Anant</td>
<td>24.3% Canton</td>
</tr>
<tr>
<td>Indonesia, Ambonese</td>
<td>6.0% (42/654)</td>
<td>100% Vanua Lava (11/11)</td>
<td>100</td>
</tr>
<tr>
<td>Laos, Lao group</td>
<td>7.2% (21/270)‡</td>
<td>100% Viangchan (9/9)</td>
<td>100</td>
</tr>
<tr>
<td>Malaysia, Malay population</td>
<td>10.8% (4/33)‡</td>
<td>37.2% Viangchan</td>
<td>26.7% Mediterranean</td>
</tr>
<tr>
<td>Myanmar, Shan†</td>
<td>6.6% (62/933)‡</td>
<td>92.8% Mahidol (26/28)</td>
<td>3.6% Union (1/28)</td>
</tr>
<tr>
<td>Thailand, Bangkok</td>
<td>9.4% (49/522)‡</td>
<td>54.0% Viangchan</td>
<td>100.0% Canton</td>
</tr>
<tr>
<td>Thailand, southern region</td>
<td>10.8% (134/-)‡</td>
<td>31.3% Viangchan</td>
<td>20.1% Kaiping</td>
</tr>
<tr>
<td>India</td>
<td>10.5% (332/3,166)</td>
<td>60.4% Mediterranean</td>
<td>24.5% Kerala-Kalyan</td>
</tr>
<tr>
<td>Latin America</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil, Amazonia, Belem</td>
<td>100% (196/-)</td>
<td>82.1% A– (376G, 202A)</td>
<td>7.1% A– (376G/968C)</td>
</tr>
<tr>
<td>Brazil, Bragança Paulista, Sáo Paulo State</td>
<td>1.7% (804/621)§</td>
<td>98.6% A– (376G, 202A)</td>
<td>100</td>
</tr>
<tr>
<td>Mexico, Pacific coast</td>
<td>1.1% (21/1,985)¶</td>
<td>54.3% A– (376G, 202A)</td>
<td>25.7% A– (376G/668C)</td>
</tr>
<tr>
<td>Middle East</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iran, Golestanian province</td>
<td>25% (71/-)</td>
<td>69.0% Mediterranean</td>
<td>26.7% Chatham</td>
</tr>
<tr>
<td>Oman</td>
<td>23% (23/-)</td>
<td>73.9% Mediterranean</td>
<td>8.7% Chatham</td>
</tr>
<tr>
<td>Saudi Arabia, Eastern province</td>
<td>154/-</td>
<td>84.0% Mediterranean</td>
<td>5.8% A–</td>
</tr>
</tbody>
</table>

* – Analysis performed on G6PD-deficient subjects, N population sample unknown.
† Prevalence determined by phenotype in males, genotyping performed on 63 samples including 11 female heterozygotes and two homozygotes; n/N G6PD-deficient samples are shown in parentheses after variant.
§ 70/80 G6PD-deficient subjects were genotyped.
‡ G6PD deficiency sample included an additional 14 subjects from other areas, total = 35.

India. Among 15 tribes in Orissa region, the prevalence of G6PD deficiency ranged between 5.1% and 15.9%. In general, the prevalence of G6PD deficiency in India is not related closely to malaria endemicity; in central India, where 50% of P. falciparum malaria cases occur, the prevalence of G6PD deficiency is 6.1%. Overall, G6PD Mediterranean appears to be the most common deficient variant (60.4%), followed by Kerala-Kalyan (24.5%) and Orissa (13.3%) (Table 1). However, different variants may dominate in different areas (Table 1).

In Latin America, the prevalence of G6PD deficiency is generally low (≤ 2%), Table 1, and G6PD A– appears to be the most common deficient variant. In Brazil, 98.6% of G6PD-deficient blood bank samples were G6PD A–, with only 1 of 4,621 samples G6PD Mediterranean. Spanish and African ancestry contribute to most cases of G6PD deficiency; G6PD A– (202A,376G), formerly known as Betica, is a common variant in Spain. There appears to be considerable regional variation in the prevalence of G6PD deficiency and this pattern continues to change because of migration. G6PD deficiency is rare in the native American population.
DRUG-INDUCED G6PD DEFICIENCY-RELATED HEMOLYSIS

Hemolytic anemia is the prototype of the clinical manifestation of G6PD deficiency. This is probably true partly because of the history of how G6PD deficiency was discovered, i.e., through investigation of primaquine-induced hemolysis, and it may be in part because this type of hemolytic reaction is most easily defined and most easily studied. This review deals with this specific manifestation of this enzyme deficiency, but it is important to bear in mind that hemolysis in G6PD deficiency is often the result of infections of various types. Thus, that a patient received drug and developed hemolysis is by no means absolute proof that the drug caused the hemolysis. The most catastrophic consequence of G6PD deficiency is neonatal jaundice, leading in severe cases to kernicterus. Here, the problem is not one primarily of hemolysis, but rather the inability of the G6PD-deficient liver of the newborn to adequately conjugate bilirubin.

However, here we focus on drug-induced hemolysis. A decrease in the hemoglobin level is the most readily ascertained sign of drug-induced G6PD deficiency-related hemolysis and is easy to monitor. In addition, patients may experience weakness, breathlessness, back pain, aches, and occasionally renal failure. The clinical course of drug-induced G6PD deficiency-related hemolysis is variable and may be influenced by the G6PD variant, the drug, the dose of that drug, patient status, and disease factors.

Effect of G6PD genotype. G6PD variants are classified according to the phenotypic effect: class 1, enzyme deficiency with chronic nonspherocytic hemolytic anemia; class 2, severe enzyme deficiency (< 10% activity); class 3, moderate/mild enzyme deficiency (10–60% activity); class 4, very mild or no enzyme deficiency (≥ 60–100% activity); class 5, increased enzyme activity. For example, the common African variant G6PD A− is a usually a mild/moderate deficiency (10–15% of normal activity, hemizygous males). In contrast, the G6PD Mediterranean variant is more severe (< 1% of normal activity).

The difference in G6PD deficiency due to the different variants appears to be related to their proximity and/or importance of the mutation to the glucose-6-phosphate and NADP+ binding and subunit contact sites, although data are conflicting for different variants. Some variants with mutations distant from the NADP+ binding site, such as the African A− variant, have normal affinity for glucose-6-phosphate and NADP+, with G6PD deficiency being caused by a reduced number of catalytically active molecules per erythrocyte. In this case, abnormal polypeptide folding appears to make the enzyme less stable, possibly making it more susceptible to proteases and reducing survival time within the cell. For variants that cause more severe G6PD deficiency, such as G6PD Mediterranean, there may also be a reduced catalytic efficiency of molecules in addition to greatly decreased stability versus normal and G6PD A−. For patients with G6PD A−, this has important clinical consequences. In this case, G6PD activity is greatest in younger cells, so as the erythrocyte population becomes replenished, G6PD activity increases and hemolysis slows. Thus, drug-induced G6PD deficiency-related hemolytic anemia in G6PD A− subjects is usually self-limiting. For example, in the case of G6PD A− and primaquine, 30 mg/day, hemoglobin remains stable for approximately 48–72 hours before decreasing steeply from approximately 14 g/dL to anywhere between 6 g/dL and 10 g/dL, sometimes with black urine and general weakness, before recovering. In comparison, for the G6PD Mediterranean variant hemolysis continues with no compensatory effect. Thus, from a public health perspective, the clinical impact of drug-induced G6PD deficiency-related hemolysis depends not only on the prevalence of G6PD deficiency in the population, but also on its molecular genetic basis.

Effect of different drugs and dose-response. Table 2 lists drugs that are associated with clinically important hemolysis in G6PD deficiency. Some additional drugs may be hemolytic in cases of class 1 G6PD deficiency. The time course of hemolysis is different for different drugs and/or drug doses. Hemolysis caused by primaquine is detectable between 1 and 3 days after drug administration. In contrast, favism may take only hours to affect a significant hemolysis.

Primaquine was the index drug for studies in drug-induced hemolysis in G6PD deficiency. This agent is still widely used, mainly against P. vivax and usually without G6PD screening, because of a lack of suitable alternatives. However, despite extensive experience with primaquine, the dose-response for G6PD deficiency-related hemolysis for different phenotypes/genotypes is complex, depending not only on the drug dose and G6PD variant, but also on the time span over which the total dose of drug is administered. For example, in a study conducted in Myanmar, hemolysis did not occur in two patients hemizygous for severe deficiency (G6PD Myanmar) after treatment with quinine, 600 mg three times a day for seven days, followed by primaquine, 45 mg in a single dose for gametocytes and 45 mg a week for eight weeks for P. vivax malaria. In contrast, treatment of 22 G6PD-deficient patients in Thailand (where G6PD Mahidol is predominant) with a three-day course of chloroquine (total dose = 1,500 mg) followed by primaquine, 15 mg a day for 14 days, resulted in a significant decrease in hematocrit, although blood transfusion was not required. Thus, there does appear to be a dose-response effect, and characterizing this in the clinical setting where alternatives to primaquine are being evaluated would be useful for determining the risk–benefit of new agents.

Patient status. The time course of G6PD deficiency-related hemolysis may not be the same for well subjects versus ill patients, although the effect of different patient factors is not well understood. For example, although patients who present with low hemoglobin levels may appear to be at greater risk

<table>
<thead>
<tr>
<th>Drug</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>Dimercaptosuccinic acid†</td>
<td>Phenazopyridine</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>Phenylhydrazine</td>
</tr>
<tr>
<td>Gilbenclamide†</td>
<td>Primaquine†</td>
</tr>
<tr>
<td>Isobutyl nitrite</td>
<td>Sulfacetamide‡</td>
</tr>
<tr>
<td>Lawsone (henna)</td>
<td>Sulfanilamide†</td>
</tr>
<tr>
<td>Methylene blue‡</td>
<td>Sulfapyridine‡</td>
</tr>
<tr>
<td>Nalidixic acid†‡</td>
<td>Thiolasulone‡</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Toluidine blue†</td>
</tr>
<tr>
<td>Nitrazole</td>
<td>Trimitrotoluene</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>Urate oxidase‡</td>
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</tbody>
</table>

* Adapted from Beutler.† Single case report: cause and effect not certain.‡ Agents used for the treatment of malaria.
of clinically important hemolysis, in the case of G6PD A−, anemic subjects are likely to have a relatively young erythrocyte population and greater G6PD activity and may, therefore, be better able to tolerate an antimalarial therapy associated with drug-induced G6PD-related hemolysis.

High fever (37–40°C) appears to exacerbate the clinical severity of G6PD deficiency-related hemolysis.79 This does not appear to be related to parasitemia or severity of malaria. However, parasitemia in peripheral blood may not reflect true parasite load overall because parasites are also sequestered in the visceral circulation, and this may require further investigation.

Tumor necrosis factor-α (TNF-α) is one of the key cytokines that influence the pathology of microbial infections. Polymorphisms in the TNF-α gene promoter have been claimed to confer an increased susceptibility to severe forms of *P. falciparum* malaria and may affect the clinical course of the disease, such as increased fever.80

**Disease factors.** Erythrocyte lysis occurs during the *Plasmodium* life cycle to enable the release of daughter merozoites. Acute hemolysis can occur in *P. falciparum* malaria independent of drug therapy, and appears to be related linearly to the level of parasitemia.81 It is unknown if there is an interaction between disease-related and G6PD deficiency-related hemolysis. In *P. vivax* infection, a decrease in the hemoglobin level is almost certainly due to drug-induced hemolysis owing to the low parasite count. Uncommonly, hemolysis may continue after malaria is cured, possibly due to persisting antibodies against erythrocytes,82,83 although their role must be considered controversial.84

**DETERMINING THE G6PD DEFICIENCY-RELATED HEMOLYTIC POTENTIAL OF ANTIMALARIAL DRUGS**

There is no reliable, validated method of testing antimalarial hemolytic potential in G6PD deficiency either preclinically or clinically. At present, initial testing of antimalarial candidates in industry is performed using an *in vitro* erythrocyte lysis assay. Briefly, serial dilutions of the experimental compound are prepared and added to a 2% erythrocyte–phosphate-buffered saline suspension. The percentage of hemolysis is estimated from the hemoglobin absorbance at approximately 412 nm. Controls include erythrocytes without solvents/compound, erythrocytes with solvent only, and erythrocytes with 0.5% sodium dodecyl sulfate (100% lysis). These *in vitro* tests are then compared with the results of animal toxicity testing looking at standard hematologic parameters in case of a metabolite effect. Further studies are performed only if a problem is identified. However, these standard tests do not accurately identify hemolytic drugs and the potential for G6PD-related hemolysis is not specifically investigated. In clinical studies, G6PD deficiency-related hemolysis has been evaluated in standard phase 2 and 3 clinical trials as a safety issue, i.e., to determine risk–benefit, rather than as a research issue to determine hemolytic potential *per se*.

Developing a classification system of antimalarial drugs for hemolytic risk in G6PD deficiency based on preclinical tests, possibly using primaquine as a reference point, would be valuable. Such a system would help to reduce the risk to patients and resources as new agents were progressed to clinical trials. A number of possibilities for achieving this aim require further investigation.

**Pre-clinical testing of hemolytic potential.** Currently, there is no simple, validated method to allow one to predict the hemolytic potential of a drug. Primaquine is the prototype of such drugs, and it was apparent in early *in vitro* studies that when challenged with primaquine, normal and G6PD-deficient cells were indistinguishable from one another by means such as hemolysis, osmotic fragility, antigenicity, mechanical fragility, and a variety of other parameters.85 However, *in vitro* differences between the reaction of G6PD-deficient and normal cells to drugs could be demonstrated with respect to Heinz body formation,7 maintenance of reduced glutathione levels,86 and the oxidation of the 1-carbon of glucose.87

Unfortunately, the response of erythrocytes to various drugs in such *in vitro* systems does not reliably parallel their hemolytic potency *in vivo*. Notably, ascorbic acid, cysteine, and several other essentially benign compounds have the same effect as substances such as primaquine or naphthol, which are capable of causing hemolysis. The underlying problem would seem to be the metabolic fates of drugs. An attempt has been made to address this difficulty by using plasma from patients ingesting drug and determining the capacity of this plasma to stimulate the hexose monophosphate shunt of normal erythrocytes.88 This method was validated by showing that plasma from subjects ingesting two sulfonamides that produced hemolysis *in vivo*, sulphormethoxine and sulphalene, stimulated glucose oxidation in normal cells, but the non-hemolytic drug chloroquine did not.89 Although first described some 35 years ago, this technique has not been used with additional drugs.

Another subsequently described approach consisted of incubating the test drug with mouse liver microsomes, challenging G6PD-deficient erythrocytes with the products of this process, and measuring reduced glutathione levels.90 A considerable number of drugs were tested in this system, and it proved to have good predictive power. However, 3,4-dihydroxy-L-phenylalanine (L-DOPA), which the authors considered to be of unknown hemolytic effect, was classified by this approach as hemolytic, although previous 51Cr-labeling studies had shown that this compound was devoid of *in vivo* hemolytic effect.91 There are obviously going to be some differences between drug metabolism in mouse microsomes and the intact human body and this may explain the discrepancy in these findings.

**Clinical testing of hemolytic potential.** The difficulty with all pre-clinical models is obtaining human-relevant drug metabolites. The best approach to address this is to use plasma from normal subjects after drug dosing as suggested by Welt and others.88 If possible, G6PD deficiency-related hemolytic potential should be determined in early clinical studies. This would be of great benefit for the design of later trials and allow more rigorous investigations that would not be practical in the setting of a large clinical field trial.

A conservative approach is first to give a new antimalarial agent to G6PD normal individuals to obtain plasma levels of the drug metabolites and then assess hemolytic potential *in vitro*. Because G6PD deficiency-related hemolysis is due to erythrocyte clearing by the spleen, these semi *in vitro* methods require the use of surrogate markers of hemolytic poten-
tial. Markers that could be used include increased carbon dioxide production due to stimulation of the ribose-5-phosphate pathway in G6PD normal erythrocytes \(^8\) and reduction in glutathione levels in cross-matched G6PD-deficient erythrocytes \(^\text{9,92}\) (Figure 1). Erythrocyte deformability may also be influenced by oxidative stress and, although technically more difficult, could conceivably be used as a marker. \(^\text{93}\) However, whether these experiments can be extrapolated to the relative clinical risk of hemolysis for the different drug doses and different variants is unknown.

As a further step, hemolytic potential could be evaluated in G6PD-deficient disease-free individuals; there is no evidence that hemolysis would be more severe in patients with malaria unless patients have concurrently taken other antimarial drugs. In fact, the younger erythrocyte population in G6PD A− individuals with malaria may mitigate hemolysis because G6PD activity will be higher. Studying G6PD-deficient subjects has the advantage that erythrocyte survival can be determined using isotope labeling without the risk of allogeneic transfusion. \(^\text{94}\) However, recruiting G6PD-deficient volunteers may be difficult because of the potential risk of adverse events.

G6PD A− heterozygous women may be a good compromise because approximately half of their erythrocytes will be resistant to hemolysis, which provides a large safety factor should the drug being tested be very potently hemolytic. Subjects could, therefore, be included providing their fraction of G6PD normal cells exceeds 50% of their total based on biochemical assays or cytochemical staining. \(^\text{95}\) This type of trial has been given ethical approval in the past but a sufficient number of volunteers could not be found. Also, mild drug-induced G6PD deficiency-related hemolysis may be difficult to detect or to differentiate from non-G6PD deficiency-related hemolysis in controls. However, if there was hemolysis for <25% of G6PD-deficient cells in this group, then studies could be progressed to class 3 G6PD A− hemizygous males or homozygous females, with and without malaria, and then to class 2 variants, such as G6PD Mediterranean, to discover any effects in severe G6PD deficiency.

**ISSUES FOR THE CONDUCT OF ANTIMALARIAL CLINICAL TRIALS IN G6PD DEFICIENCY POPULATIONS**

**Patient population.** In administering antimalarial drugs, it would be impractical to have G6PD deficiency as an exclusion criterion on the label because G6PD deficiency screening is not usually available in the areas where these drugs are used. Thus, G6PD-deficient patients need to be included in clinical trials. It is important that clinical trial data are generated for the areas in which the disease is most important, the drugs are most needed, and the prevalence of G6PD deficiency is highest, i.e., sub-Saharan Africa and southeast Asia. If the hemolytic effect of the test drug in G6PD-deficient patients is essentially unknown, then risk should be assessed preferably first in phase I trials in heterozygous G6PD A− females (as described above). If no significant problem arises, then new agents should be tested in the target population to determine the relative risk versus other available agents. In some cases, drugs are already registered for use without G6PD deficiency screening and this provides an opportunity to enroll G6PD-deficient patients in the trial to investigate the possible hemolytic risk.

If a given antimalarial drug, or one agent in a combination therapy, has known hemolytic potential then a risk–benefit can be determined. Such antimalarial drugs may still have useful applications in some regions and situations depending on the hemolytic risk for the most common variants and/or if they have certain clinical benefits, such as activity against hypnozoites and gametocytes. Where primaquine is currently the only available drug for the treatment of *P. vivax*, it would be especially useful to know the relative risk of hemolysis with new agents versus that of primaquine.

When studies are conducted in partnership with the World Health Organization Special Program for Research and Training in Tropical Diseases, the first step is to obtain ethical approval from the World Health Organization ethical research committee, followed by local ethical committee approvals. Other international partnerships have similar systems. Additionally, the sponsor’s country institutional review boards should approve the clinical trial design. \(^\text{96}\) This country will normally be a developed country to ensure that the standards expected in the developed world from a pharmaceutical company are maintained globally. Severe adverse events management facilities should be built into the study site if not already available. An independent data monitoring committee should be included in the study protocol to further protect the study volunteers. Importantly, data including G6PD-deficient patients are necessary for registration in some countries, such as India. If serious hemolysis due to G6PD deficiency is a known adverse event, then it may be difficult to obtain ethical approval unless it is the only drug available and then on a compassionate basis under supervision.

**G6PD deficiency screening.** The NADPH fluorescence test is a qualitative test and is the gold standard for G6PD deficiency screening. \(^\text{97,98}\) This test is rapid, reliable, easily performed, and is almost equivalent to a point of care test, taking approximately 15 minutes to perform. It requires only a blood spot on filter paper. For example, a study conducted in rural Burkino Faso found good agreement between the NADPH fluorescence test (adjusted for hemoglobin) and polymerase chain reaction (PCR)−based G6PD genotyping with an estimated (relative) sensitivity of 98.2% (95% confidence interval [CI] = 95.8−99.6%) and specificity of 97.1% (95% CI = 94.2−99.2%). \(^\text{99}\) There is no advantage in buying commercial tests because the reagents can be made by experienced investigators cheaply and in bulk and are also robust in the field.

For patient screening purposes, qualitative tests are enough. However, these tests will only reliably detect G6PD deficiency in males and homozygous females. Among heterozygous females, some subjects will always overlap in enzyme activity with normal subjects and even quantitative tests will not necessarily resolve this problem. \(^\text{100,101}\) In this case, screening for G6PD deficiency could, for practical purposes, differentiate phenotypic results into positive, negative, and +/− and accept that in some cases the phenotype will not reflect the genotype. This would identify the severely deficient patients at most risk of hemolysis.

In clinical trials of a new antimalarial combination therapy combining chlorproguanil, dapsone, and artesunate being conducted in Africa (Burkina Faso, Ghana, Kenya, Mali, Nigeria and Tanzania) G6PD-deficient patients are not excluded. Blood to determine the G6PD activity is collected on
a filter paper on day 1 before the first dose of antimalarial drug is given and tested within 15 days in a South African central laboratory. In these trials, patients are under supervision on days 1–3 and hemolysis should be observed within this time frame. After day 3, patients are followed-up at home by a field worker on days 4–6, then at hospital on day 7, 14, 28, and so on as required.

**G6PD deficiency genotyping.** Although phenotyping would be sufficient for G6PD deficiency screening, in terms of determining hemolytic potential in a clinical research setting the genotype should be determined for all patients. Not only does including all patients simplify the protocol, but it provides data that could be valuable in interpreting the results, for example, G6PD-deficient patients that had no hemolytic event. Also, with DNA available, other mutations could be examined if necessary (e.g., unstable hemoglobins).

In addition, genotyping is required to provide a clear epidemiologic picture for determining the risk of drug-induced hemolysis across a population. This may be particularly important in areas where few epidemiologic data are available. However, this must be done with caution because it may not be possible to extrapolate epidemiology from clinical trials too widely. For example, the common African form of G6PD deficiency, G6PD A–, is associated with a reduction in risk of severe malaria for both female heterozygotes and male hemizygotes. Thus, the number of G6PD-deficient patients enrolled into a trial may be smaller than the background epidemiology would suggest. Also, the prevalence of G6PD deficiency and the genotype may vary considerably between regions and even between different ethnic communities within the same region. However, in Africa, where most individuals will be A–, multicenter clinical trial samples could provide a reasonable estimation of the frequency of G6PD deficiency in the malaria-endemic population.

In the first instance, genotyping needs to cover the most common G6PD deficiency mutation in the population. Approximately 3–4% of subjects will not have a mutation on genotyping for common mutations because they have a polymorphic mutation derived from a different ethnic group, or they have a rare, sporadic mutation. There may be some false-positive results if blood samples are not properly handled or if samples are mixed up. If necessary, the entire coding region of the G6PD gene can be sequenced.

Chip resequencing is a developing technology and can be automated. It can be expensive, but may be cost-effective for a large number of samples. This technology is in development as a commercial test for analyzing patient genomes for the cytochrome P450 genes CYP2D6 and CYP2C19, which are linked to a number of serious adverse drug reactions. Similar technology has been used to scan for common Chinese G6PD mutations in the G6PD gene, although this has yet to be applied in the clinical trial setting.

**Consent, sampling, and quality control.** For genomic epidemiology studies in malaria-endemic countries, practical issues should be considered in achieving valid informed consent in relation to comprehension, the ability to volunteer and competence of the study subject. Consent for DNA testing needs to be obtained before enrolment into a clinical trial. Genetic material needs to be collected at screening because patients may not return for follow-up visits. The consent should be sufficiently broad so that genes other than G6PD can be studied. A 7-mL blood sample should be obtained if possible. Blood samples in EDTA solution can be refrigerated and stored for at least a month. Heparin should be avoided because it interferes with the PCR. Anticoagulant-citrate-dextrose solution can also be used and the extra dextrose may be useful, although this anticoagulant is less readily available than EDTA. At ambient temperature, a sample will last for several days and using a cool bag will extend this time. Freezing will destroy erythrocytes, but frozen blood is a good source of DNA. Finger blood drops on filter paper could be used if necessary. Purifying DNA from blood spots can be somewhat cumbersome but is possible in field conditions where equipment and training are provided and has the advantage that samples are robust. For example, a study in Thailand found that the quality of genomic DNA in dried blood on filter paper was stable enough for DNA amplification when stored at ambient tropical conditions for up to 10 years.

Group training with a central reference laboratory is one approach to maintain quality control over different sites within a clinical trial. In this case, laboratory staff from the different study centers involved can attend technical training together and are sent reference samples before and throughout the study. In Africa, it would be difficult to ensure quality testing outside the district level and one would need at least one central laboratory in each country; further centralization would be impractical. However, building infrastructure in this way does aid in pharmacovigilance for post-registration safety studies.

**Clinical evaluations.** In addition to the above considerations, key information that should be collected at screening and at follow-up visits includes blood hemoglobin concentration, hematocrit, history of previous hemolysis, specific ethnicity (not merely a broad ethnic group), and history of other drugs used for this malaria episode. It is also useful to measure the reticulocyte count, but it should be borne in mind that there may be an effect of hemoglobin H disease for some automated reticulocyte-counting methods. There is no need to exclude patients with methemoglobinemia per se. An important consideration is that after transfusion of G6PD A– subjects with several units of blood it takes a considerable period of time before they will again be phenotypically G6PD deficient. In the context of clinical trials, a 28-day follow-up would be likely to capture this reconversion. Also, recent blood transfusions would also need to be taken into account during patient screening.

**Safety.** The decision for intervention after drug-induced G6PD deficiency-related hemolysis depends on the time course of the reduction in hemoglobin. A rapid decrease in hemoglobin requires that the drug be stopped immediately. Transfusion is clinically guided, and there is no cut-off for hemoglobin to define this decision. Importantly, transfusion may not be possible in remote areas. In general, a severe G6PD-related hemolytic crisis can be treated in a similar fashion to an incompatible blood transfusion.

Some approaches to reduce the severity of G6PD deficiency-related hemolysis have been investigated. Vitamin E has been shown to increase erythrocyte lifespan in G6PD Mediterranean subjects when given for a year. However, there is no evidence that there would be any benefit of administration for acute hemolytic crisis. Desferrioxamine has been used during acute hemolytic crisis in children and has been claimed...
to shorten the duration of the crisis and decrease the frequency of blood transfusion needed.\textsuperscript{111,112}

**Early detection of hemolysis.** Early indicators of G6PD deficiency-related hemolysis would be valuable for use in clinical trials both from a safety perspective and as outcomes investigating hematologic potential. Because the glutathione level decreases before hemolysis starts, this could possibly be used as an early indicator if facilities for detecting this are available. Mean hemoglobin concentration and mean hemoglobin concentration of reticulocytes could be used to detect early hemoglobin loss, but this has not been investigated for G6PD deficiency-related hemolysis. Plasma hemoglobin is present at only low concentrations and will not provide meaningful measurements. Heinz bodies (denatured hemoglobin) are present early during hemolysis, but can be difficult to see without considerable experience and this is usually impractical in field conditions. Similarly, carbon dioxide release would be accurate, but is impractical in the field, although it may be possible in a research setting.

**Pharmacovigilance.** Pharmacovigilance in developing countries is often a difficult issue. For example, drug quality may be poor due to inadequate storage or pharmaceutical counterfeiting and reporting networks for adverse events are often lacking. Planning for phase IV post-registration studies should, therefore, be conducted in concert with planning for phase III studies so that capacity and healthcare resources can be nurtured and scaled up where possible. Phase IV studies should also consider the possible effect of concomitant medications, including non-approved medicines, if the trial reflects a real-life setting. An important consideration is whether serious adverse events and deaths due to hemolytic anemia both before and after the introduction of a new drug can be compared.

**CONCLUSIONS**

Our understanding of G6PD polymorphism is impressive and the mechanism and consequences of drug-induced G6PD deficiency-related hemolysis are well described. Furthermore, although epidemiologic data are still patchy, screening programs in neonates and blood banks are increasing our knowledge of G6PD variant prevalence. In a clinical context, however, we still have much to learn when it comes to testing new antimalarial drugs and even in the case of agents that are already available. Pragmatically, for mild G6PD deficiency due to G6PD A–, drug-induced hemolysis is likely to be self-limiting and not life-threatening unless complicating factors that may accentuate the anemia or its consequences are present. In more severe forms of G6PD deficiency, greater caution needs to be used and risk–benefit calculated carefully, taking into account local facilities for detecting G6PD-deficient individuals and treating hemolytic crisis. Our objectives for the future should be not only to safeguard patients in clinical trials, but also to better characterize and quantify antimalarial drug hemolytic potential so that drugs can be used safely in a public health setting.

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