G6PD deficiency and the need for a novel treatment to prevent kernicterus

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Introduction

Approximately 80% of newborns worldwide have some degree of hyperbilirubinemia and jaundice.[1] Severe cases of hyperbilirubinemia can progress to kernicterus, leading to permanent developmental disorders. Several risk factors contribute to hyperbilirubinemia and kernicterus, including G6PD deficiency, which is one of the most common human enzymopathies. In the developing world, lack of access to common treatments for hyperbilirubinemia, along with a high rate of G6PD deficiency, leads to high incidence of kernicterus. Below, we highlight the need for a novel therapy to prevent kernicterus, and discuss the pharmacological activation of G6PD as a promising therapeutic strategy.

Hyberbilirubinemia and kernicterus

Hyperbilirubinemia results from increased bilirubin production coupled with inefficient bilirubin excretion. Hemolysis and subsequent heme breakdown produces bilirubin, which can be conjugated and excreted in the liver. However, lower liver function in newborns leads to reduced bilirubin removal and increased levels of serum bilirubin.

Low to moderate levels of serum bilirubin (<20 mg/dL) are non-toxic and can be reduced by non-invasive treatment such as phototherapy, which uses light in a narrow wavelength band with a peak around 460-490 nm to break down bilirubin into excretable byproducts. In cases of severe hyperbilirubinemia, exchange transfusion is used to reduce bilirubin concentration in the blood. Other therapies, less commonly used, include intravenous immune globulin (mechanism of action unknown) and pharmacologic therapies to reduce bilirubin production or increase bilirubin conjugation.[2]

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If severe hyperbilirubinemia is left untreated, bilirubin that crosses the blood-brain barrier can rise to toxic levels in the brain, leading to acute bilirubin encephalopathy (kernicterus). Kernicterus is characterized by lethargy, decreased feeding, high-pitched cry, fever, seizures, and even death. Up to 84% of infants with kernicterus will develop chronic bilirubin encephalopathy, characterized by permanent movement disorders, mental retardation, and hearing loss.[3]

The incidence of kernicterus is difficult to estimate due to delayed diagnosis, an error in the diagnosis code, under-reporting, or lack of reporting in third-world countries.[4] The prevalence of severe hyperbilirubinemia is estimated to be 2-45 per 100,000 births, and of kernicterus 0.4-2.7 per 100,000 births in developed countries.[5] The incidence of kernicterus in developing countries is higher due to a variety of factors, such as genetic differences, prematurity, low birth weight, underfeeding, and sepsis.[5,6]

Glucose-6-phosphate dehydrogenase (G6PD) deficiency

G6PD deficiency is one of the most common human enzymopathies, estimated to affect 400 million people worldwide.[7] The deficiency is caused by single nucleotide polymorphisms (SNPs) leading to single amino acid changes in the protein glucose-6-phosphate dehydrogenase (G6PD). Over 400 SNPs, responsible for 160 different amino acid changes, have been observed in G6PD deficiency.[8,9] These mutations can cause deficiency of varying severity, and are classified into four clinical categories (Table 1). The majority of G6PD deficiency can be accounted for by a few common and relatively mild (Class II or III) mutations, while severe (Class I) mutations are more rare.[10] As G6PD is X-linked, heterozygous females can carry severe mutations and remain symptomless, whereas hemizygous males with Class I mutations suffer from chronic nonspherocytic hemolytic anemia (CNSHA).[11,12] Class II and III mutations lead to episodes of hemolytic anemia after stressors such as ingestion of fava beans (favism), ingestion of certain prescription drugs, or infection.

Recent completion of the 1000 Genomes Project[13] has allowed us to update the estimation of the prevalence of G6PD deficiency (Fig 1). The 1000 Genomes Project sequenced the entire genomes of ~3,500 individuals from five main human populations, revealing the presence of 22 single mutations and 5 double mutations in the G6PD protein. The majority of mutations were Class III, with few Class II and no known Class I mutations observed. Extrapolation of these numbers using data from the 2015 Population Reference Bureau[14] estimates that 7.6% of the world population, or 560 million people, carries a mutated G6PD allele. On average, weighted by the world population distribution, 5.3% of males have G6PD deficiency.

G6PD deficiency increases the risk of neonatal hyperbilirubinemia and kernicterus

Infants with G6PD deficiency are significantly more likely to develop hyperbilirubinemia;[15] a recent meta-analysis of five studies including over 20,000 subjects found that G6PD-deficient infants are almost four times more likely to develop hyperbilirubinemia and three times more likely to receive phototherapy compared to G6PD-normal infants.[16]

Moreover, G6PD-deficient infants are more likely to develop kernicterus. In the United States, 20% of infants who develop kernicterus have G6PD deficiency, compared to an estimated 4-7% prevalence of G6PD deficiency in the average population.[17,18] Of infants with kernicterus, 15% of those with G6PD-deficiency died, compared to 1% mortality rate in the G6PD-normal infants.[18] A summary of kernicterus and G6PD deficiency in other countries is summarized in Table 2. Enrichment of G6PD deficiency in kernicterus is seen in almost all cases.

Interestingly, African-American neonates exhibit lower peak levels of serum bilirubin compared to Caucasian neonates, yet are more likely to develop kernicterus after discharge from the hospital, accounting for 25% of all the kernicterus cases in the USA.[19] As G6PD deficiency is highly common in African-Americans (up to 21% of males in certain regions), this suggests that G6PD-deficient neonates develop signs of kernicterus at lower levels of serum bilirubin than G6PD-normal neonates.

In many Asian, African, Mediterranean, and Middle Eastern countries, where G6PD deficiency is common, all newborns are screened for G6PD deficiency. This has been associated with reduction of the instance of severe hyperbilirubinemia and kernicterus in several countries. In regions where G6PD deficiency is historically less common, an increase in global population movement has raised the question of whether G6PD deficiency screening should be implemented everywhere.[17]

How does G6PD deficiency contribute to kernicterus?

G6PD is the rate-limiting enzyme in the pentose phosphate pathway, catalyzing the oxidation of glucose-6-phosphate to 6-phospho-gluconate while reducing $NADP^+$ to NADPH. NADPH then fuels the regeneration of reduced glutathione (GSH), which in turn neutralizes reactive oxygen species (ROS) (Fig. 2). G6PD is a major source of NADPH, along with mitochondrial enzymes isocitrate dehydrogenase and malic enzyme.[20] However, in erythrocytes, which lack mitochondria, G6PD is the sole source of NADPH and therefore plays a critical role in protection against ROS. Indeed, Class I G6PD mutations cause CNSHA due to lack of protection against ROS in red blood cells. Similarly, G6PD deficiency in infants leads to an increase in hemolysis and subsequent rise in serum bilirubin levels from heme breakdown, and partially explains the higher prevalence of hyperbilirubinema in G6PD-deficient babies.

The mechanism by which G6PD deficiency contributes to kernicterus is less well understood.[21] Unconjugated bilirubin accumulates in the brain, specifically in neurons, neuronal processes, and microglia.[22] A high rate of hemolysis may cause serum bilirubin to be produced more quickly than it can be conjugated or diffuse into skin and other body tissues; during this spike in which serum bilirubin concentrations are higher than the diffusion threshold, bilirubin penetrates the blood-brain barrier and enters brain cells.[23] Furthermore, because bilirubin is lipophilic, it preferentially accumulates in fatty tissue and therefore does not diffuse out of the brain even at high concentrations.

Bilirubin toxicity begins at the cell membranes, as bilirubin is lipophilic and is highly concentrated in membrane compartments.[24] This bridges membrane permeability, leads to

lipid peroxidation, and inhibits the functions of membrane-bound proteins, such as ATPases. [25] Similarly, bilirubin also targets mitochondrial membranes, leading to disruption of the electron transport chain and membrane-bound proteins, which in turn results in mitochondrial swelling, membrane permeability, depolarization, cytochrome *c* release and cell death by apoptosis and necrosis. [26–30]

Bilirubin itself has antioxidant properties, yet generation of ROS is a hallmark of bilirubin toxicity in the brain. [30–32] At high bilirubin concentrations, increased production of superoxide radical anion, [33] depletion of reduced glutathione (GSH), and increase in the oxidized disulfide form of glutathione (GSSG) are observed. [34] High concentrations of ROS lead to protein oxidation, lipid peroxidation, and DNA damage, which activates pathways signaling for neuroinflammation, cell cycle arrest and apoptosis. [21,35] In G6PD-deficient infants, kernicterus and increased bilirubin-induced neurotoxicity can be partially attributed to the lack of GSH regeneration due to decreased G6PD activity.

In general, the brain is sensitive to damage from increased ROS because of its high utilization of oxygen and high concentration of oxidizable polyunsaturated fatty acids. [36] Additionally, infants (especially pre-term) have lower levels of antioxidant enzymes and scavengers such as vitamin E. [37–39] Furthermore, the developing brain exhibits very low mitochondrial antioxidant activity and relies primarily on cytoplasmic enzymes (such as G6PD) to maintain redox homeostasis. [36]

Although the mechanism of bilirubin-induced neurotoxicity is not completely understood, it is clear that G6PD deficiency contributes to kernicterus via at least two mechanisms: initially, through increased hemolysis-induced spike in serum bilirubin levels and subsequent accumulation of bilirubin in the brain, and secondly, by reduced buffering capacity against bilirubin-induced ROS (Fig. 3). This second mechanism may explain why G6PD-deficient infants develop kernicterus even at lower levels of serum bilirubin.

How do we prevent kernicterus?

The incidence of kernicterus has risen in recent years due to a variety of factors. [5,40,41] Infants are often discharged from the hospital within 24-48 hours of birth, whereas bilirubin levels often peak four to five days after birth; lack of proper monitoring at home allows the development of kernicterus, which might have otherwise been prevented if the infant were to remain at the hospital. [42] Increasing popularity of breastfeeding has also contributed to hyperbilirubinemia and kernicterus. [40,43]

In developing regions, access to phototherapy and exchange transfusions is often limited. [44] Even in developed nations, despite these simple and accessible treatments, up to 6.6% of G6PD-deficient babies will develop kernicterus [45] and 12-50% of G6PD-deficient infants with kernicterus will die. [46] Especially in cases where an acute hemolytic event in G6PD-deficient infants triggers rapid rise of bilirubin concentration in the brain, kernicterus may be impossible to prevent by conventional treatments. [47] Furthermore, exchange transfusion leads to adverse events in 5% of infants, and death in 0.4% of infants. [48]

There is a clear need for a novel therapy to prevent kernicterus and its complications, especially in G6PD-deficient newborns. Below, we propose possible approaches to treat or prevent kernicterus.

Strategies to reduce serum bilirubin levels—One approach is to reduce the amount of circulating bilirubin by increasing bilirubin conjugation, increasing the binding capacity of albumin for serum bilirubin, or by binding bilirubin with an exogenous therapeutic agent before it can cross the blood-brain barrier. Phenobarbital increases expression of UGT1A1, which enhances bilirubin conjugation in the liver, and has been shown to reduce peak serum bilirubin levels. However, the use of phenobarbital is no longer recommended due to its slow onset of effect, long duration of effect, and ineffectiveness when given in the first 12 hours of life.[49]

Previously, therapeutic attempts to increase binding of bilirubin by albumin via a small molecule have been unsuccessful.[50,51] The normal range of bilirubin concentration in the blood is 6 mg/dL (102 uM), and at least 25 mg/dL (427 uM) in cases of severe hyperbilirubinemia.[42] With this extremely high concentration of circulating bilirubin, a therapeutic bilirubin-binding agent would need to reach an unreasonably high concentration in the blood in order to have a physiologically significant effect.

Treatment with antioxidants—Major bilirubin toxicity stems from GSH depletion and imbalanced redox equilibrium. Although bilirubin induces upregulation of genes involved in GSH homeostasis, NADPH homeostasis, and antioxidant defense,[52,53] the temporal delay in upregulation still allows ample time for spikes in bilirubin-related ROS to exert neuronal damage. Studies have shown that levels of antioxidant vitamins A, C, and E are reduced in hyperbilirubinemic infants;[54] however, a clinical study showed that treating infants with hyperbilirubinemia with the antioxidant vitamin E was unsuccessful.[55]

A more recent study showed that total antioxidant capacity remains unchanged or may increase in hyperbilirubinemia, possibly due to the antioxidant activity of bilirubin.[56] This interplay between antioxidant activity and ROS is characteristic of other ROS-related diseases, and although bilirubin-induced ROS is a main driver of kernicterus, the use of antioxidants to prevent kernicterus is likely to be ineffective. Antioxidant treatment has proven ineffective in many other ROS-related diseases,[57,58] possibly because these antioxidants may be scavenging both 'good' and 'bad' ROS.[59] Further, the transient, spike-like nature of bilirubin-induced ROS is difficult to pinpoint, and administration of antioxidants too early or too late will not be effective.

Therefore, restoring redox equilibrium by improving endogenous antioxidant defense represents a more promising treatment for kernicterus and ROS-related diseases in general.

Restoring endogenous GSH: activating G6PD—Specifically, GSH depletion and increase in GSSG are hallmarks of bilirubin toxicity; a therapy directed at restoring the GSH/GSSG balance may be more helpful than general treatment with antioxidants. For example, pre-treatment with N-acetylcysteine (NAC), a GSH precursor, reduced bilirubin toxicity in rat neuronal cells in culture.[34,52] NAC has been administered to preterm infants

with no ill effects, but its effect on development of hyperbilirubinemia and/or kernicterus has not been reported.[60] However, supplementing with GSH or GSH precursors does not bypass the issue of timing, and sudden increase in GSH may have unwanted impacts on glucose and/or iron metabolism.[58,61] Moreover, GSH does not efficiently cross the blood-brain barrier,[62] and it us unknown whether NAC can cross the blood-brain barrier.[63] Rather, a therapy directed at maintaining endogenous control of the GSH/GSSG balance is desirable.

G6PD is the rate-limiting producer of cytoplasmic NADPH, which in turn converts GSSG to GSH (Fig. 2). As discussed above, in the developing brain mitochondrial production of NADPH is low, and is made even lower by the toxic effect of bilirubin on the mitochondrial membrane. Therefore, G6PD is the main driver of GSH regeneration. The loss of this major source of GSH in G6PD deficiency explains why higher rates and worse outcomes of kernicterus are observed in G6PD-deficient babies. Therefore, we propose a small-molecule activator or chaperone of G6PD as a novel treatment for kernicterus. Discovery of molecular chaperones and activators is uncommon compared to the discovery of small-molecule enzyme inhibitors, but a few small-molecule chaperones have been used successfully.

One example is a pharmacological chaperone therapy for Gaucher disease, a lysosomal storage disease. The disease is caused by mutations in acid β-glucosidase leading to significant protein misfolding and subsequent degradation of the protein before it can be transported to the lysosome. Interestingly, binding of small-molecule inhibitors to the mutated protein rescues protein folding and allows the protein to be transported to the lysosome; these inhibitors have been termed "pharmacological chaperones". The pharmacological chaperone is competitively replaced by the highly concentrated substrate in the lysosome, leading to a net gain in acid β-glucosidase activity. Pharmacological chaperones have entered Phase II clinical trials to treat Gaucher disease in humans.[64]

Several common G6PD variants, such as G6PD A-, which is present in up to 45% of the population in some regions of Africa,[7] and G6PD Mediterranean, which is the most common G6PD mutation in Caucasians,[65] have been found to exhibit misfolding[66,67] and reduced half-life in erythrocytes.[68] Moreover, many Class I G6PD mutations exhibit highly decreased thermostability[69] and inefficient protein folding.[70] A pharmacological chaperone similar to the one found for Gaucher disease may be an effective strategy for correcting these types of G6PD mutations (Fig. 4a).

Another example of a small-molecule activator is Alda-1, an activator of aldehyde dehydrogenase 2 (ALDH2). A common ALDH2 variant, referred to as ALDH2*2, shows disordered folding in a small region of the protein caused by a single amino acid mutation. The ALDH2*2 variant remains viable, but exhibits <5% activity of ALDH2. Alda-1 increases the activity of ALDH2*2 by 11-fold and allosterically restores folding to the disordered region as demonstrated by a co-crystal structure.[71,72] Moreover, Alda-1 also increases the activity of wild-type ALDH2 by two-fold. Crystallography shows that Alda-1 binds near the active site, increasing productive encounters between the substrate and catalytic residues.[71]

Most G6PD mutants have not been crystallized, but if mutations induce disordered regions similar to ALDH2*2, they could potentially be corrected allosterically by a small molecule in a mechanism similar to Alda-1 (Fig. 4b). Canton G6PD, the most common mutation in East Asia, has been crystallized but shows no gross structural differences compared to wild-type G6PD.[73,74] In this case, a small molecule activator could be identified that binds in the catalytic site of G6PD and facilitates substrate and/or cofactor binding (Fig. 4c). Such a small molecule could also potentially activate wild-type G6PD in the same fashion; this activator could serve as a treatment to prevent kernicterus even in G6PD-normal infants with hyperbilirubinemia.

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Best Practices Box

What is the current practice?

- Jaundiced infants with serum bilirubin level above 20 mg/dL are subjected to phototherapy, and generally are given exchange transfusion at serum bilirubin level above 25 mg/dL.

- Phototherapy and exchange transfusion may not be available in developing nations where G6PD deficiency is most common.

What changes in current practice are likely to improve outcomes?

- We propose development of a novel treatment to prevent kernicterus in addition to current practice.

- A G6PD activator or pharmacological chaperone may reduce hemolysis and increase protection against bilirubin-induced ROS in the developing brain.

- Screening for G6PD deficiency even in regions where G6PD deficiency is uncommon may help identify infants at higher risk for developing kernicterus.

Summary Statement

Preventable kernicterus and the subsequent sequelae still occur with a regular frequency in both developed and developing nations. We propose activation of G6PD as a novel treatment for kernicterus in both G6PD-deficient and G6PD-normal infants.

KEY POINTS

- G6PD deficiency increases the risk of kernicterus in jaundiced newborns.

- G6PD is a major source of protection against bilirubin-induced oxidative stress in the developing brain.

- There is need both in developed and developing nations for a novel treatment for kernicterus, especially in regions with a high rate of G6PD deficiency.

- We propose a small-molecule activator or pharmacological chaperone for G6PD as a therapy for kernicterus in both G6PD-deficient and G6PD-normal infants with hyperbilirubinemia.

SYNOPSIS

Hyperbilirubinemia occurs frequently in newborns, and in severe cases can progress to kernicterus and permanent developmental disorders. G6PD deficiency, one of the most common human enzymopathies, is a major risk factor for hyperbilirubinemia and greatly increases the risk of kernicterus even in the developed world. Lack of access to common treatments for hyperbilirubinemia, coupled with a high rate of G6PD deficiency, leads to high incidence of kernicterus in the developing world. Therefore, a novel treatment for kernicterus is needed, especially for G6PD-deficient newborns. Oxidative stress is a hallmark of bilirubin toxicity in the brain. We propose activation of G6PD via a small-molecule chaperone as a strategy to increase endogenous defense against bilirubin-induced oxidative stress and prevent kernicterus.
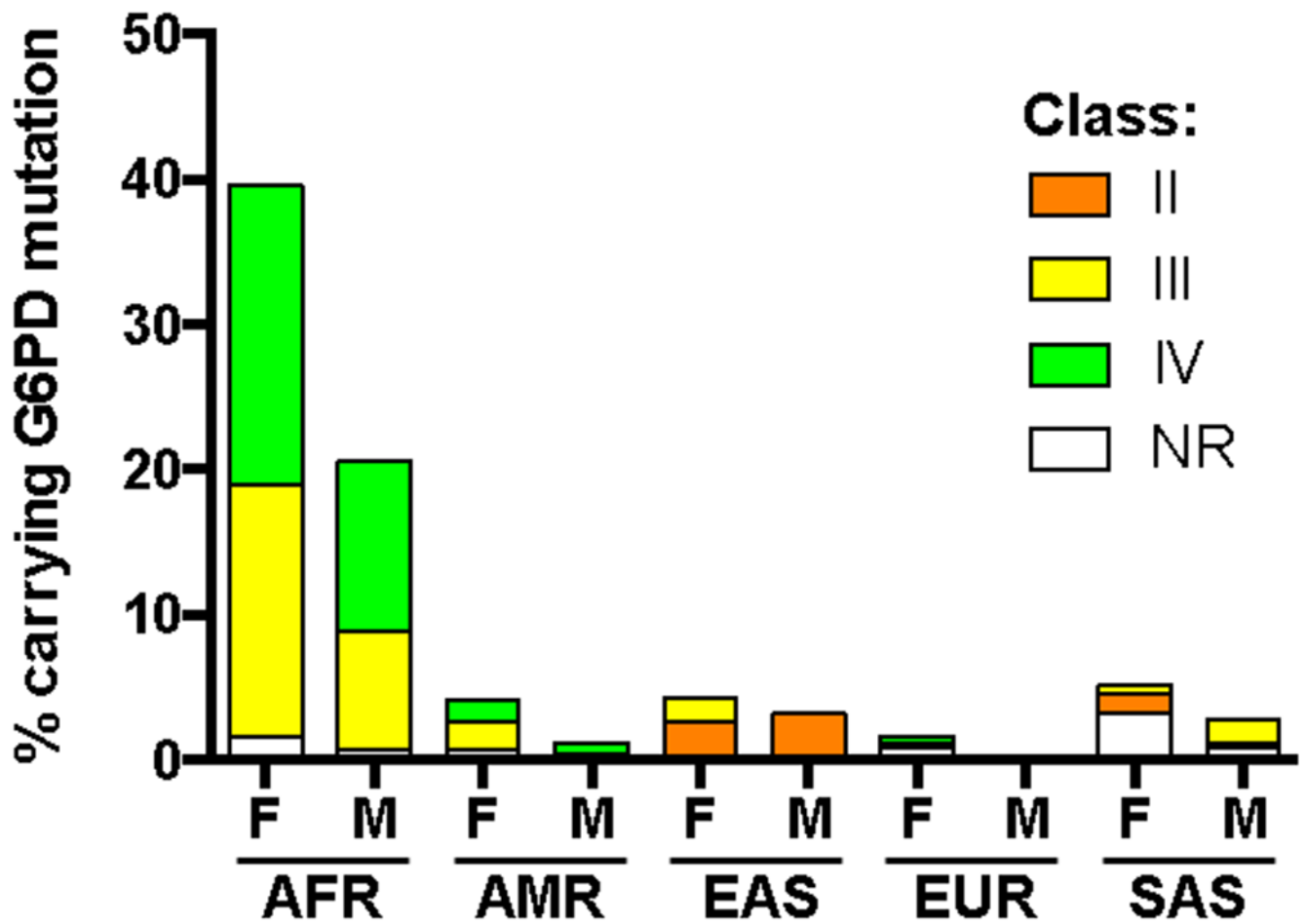
Figure 1:
Percent of females (F) and males (M) from the 1000 Genomes Project carrying a G6PD mutation. No Class I mutations were observed. AFR: African; AMR: Mixed American; EAS: East Asian; EUR: European; SAS: South Asian; NR: not reported.
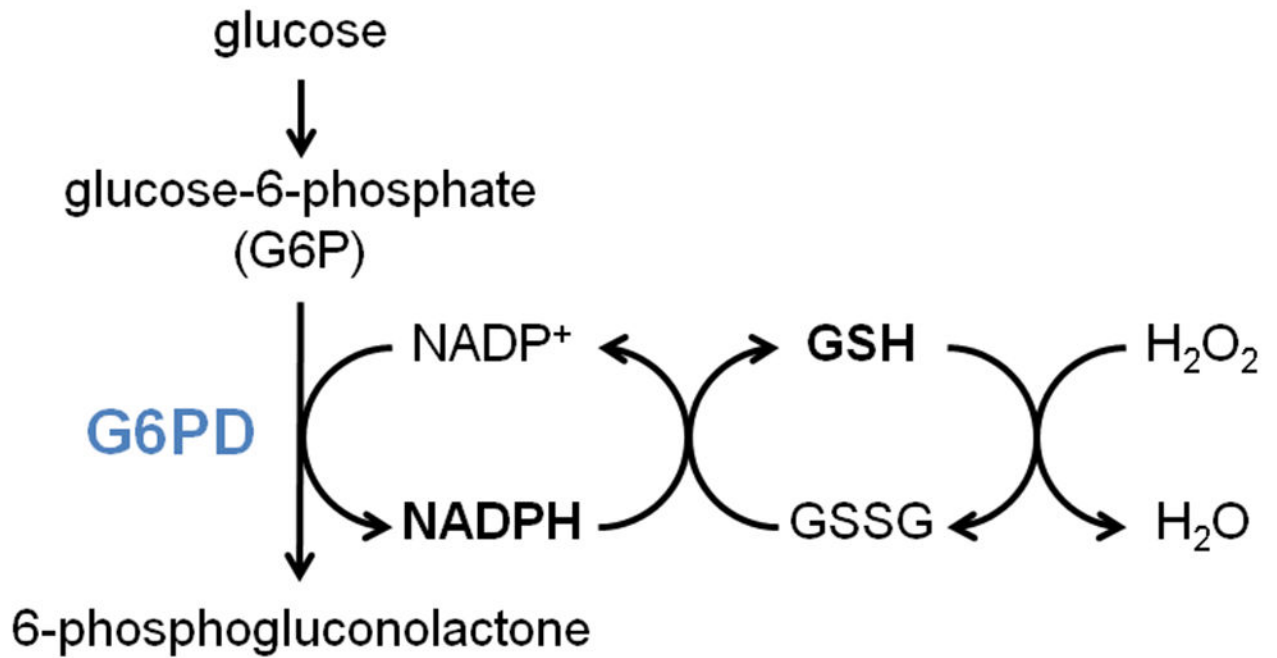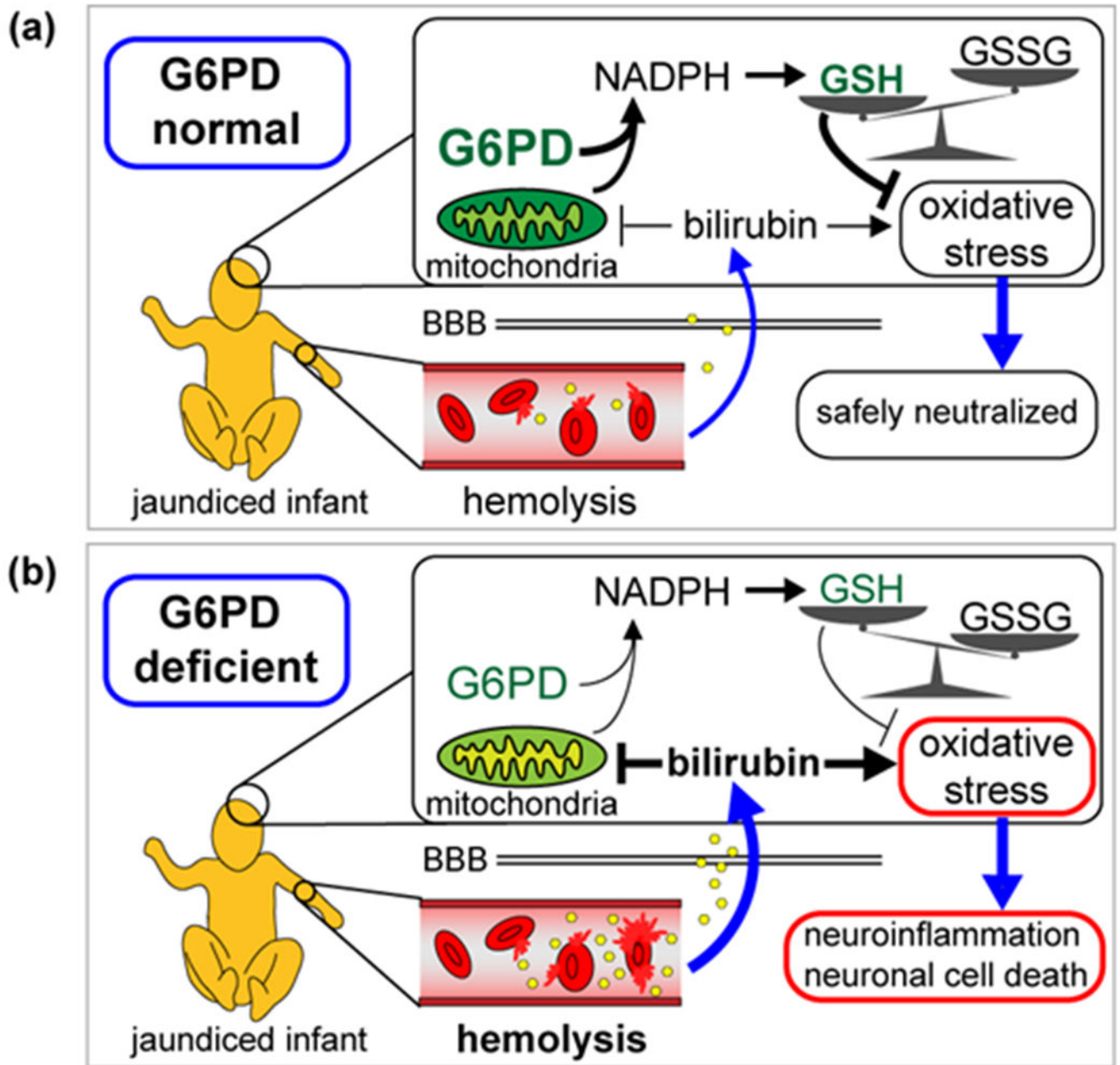
Figure 2:
Scheme of G6PD activity.

Figure 3:

Contribution of G6PD deficiency to bilirubin-induced neurotoxicity. (a) Neonatal jaundice results from production of bilirubin (shown as yellow hexagons) following hemolysis. In G6PD-normal infants, GSH levels are properly maintained and low levels of bilirubin-induced oxidative stress are safely neutralized. (b) In G6PD-deficient infants, higher levels of hemolysis lead to higher concentration of bilirubin in the brain, which inhibits mitochondrial activity (indicated by yellowed mitochondria). Reduced G6PD activity leads to low NADPH levels, and GSH is depleted in favor of GSSG. Buildup of ROS leads to neuroinflammation, cell death, and kernicterus. BBB; blood-brain-barrier
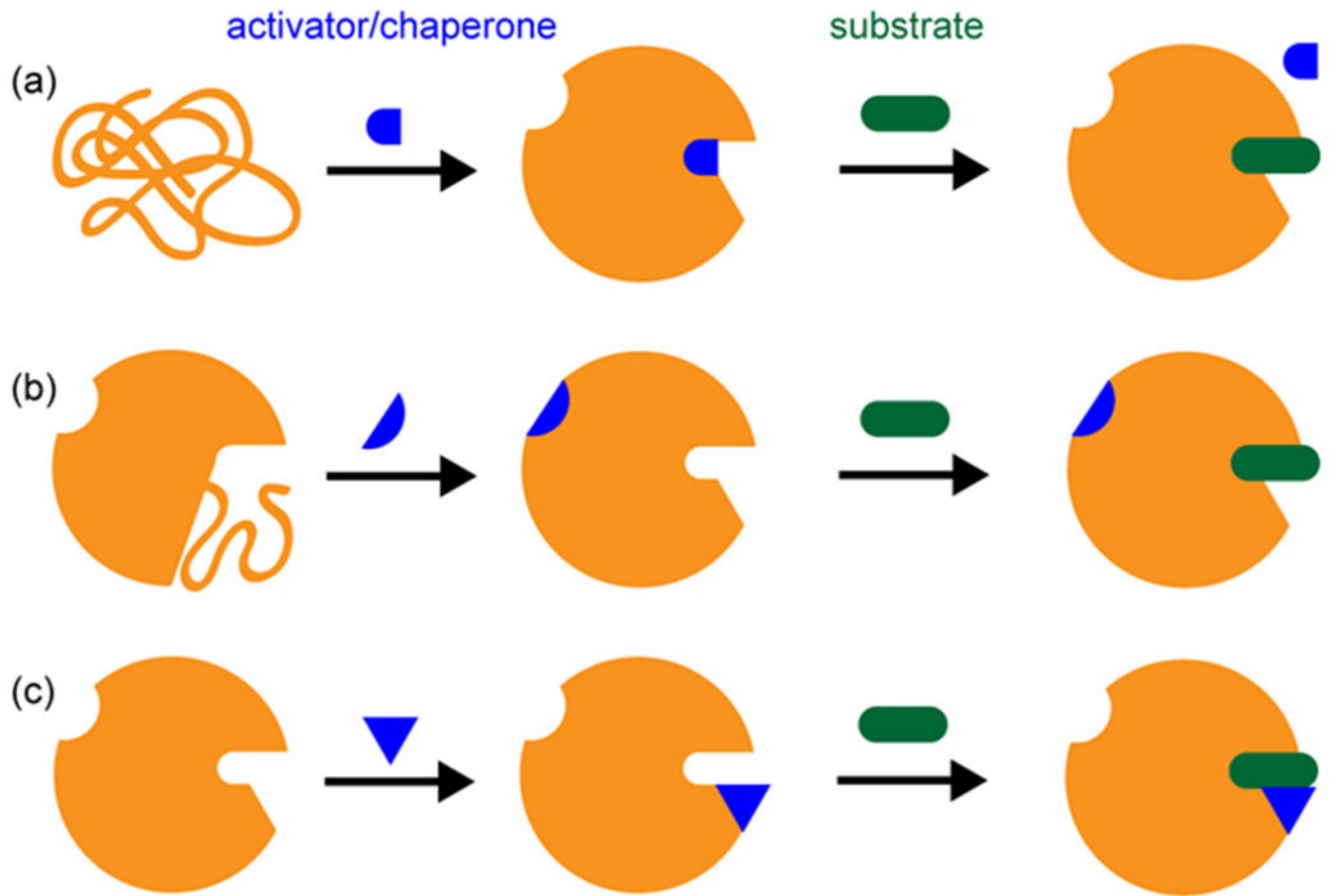
Figure 4.
Mechanisms of pharmacological chaperone or small-molecule activator. (a) A pharmacological chaperone rescues misfolding. In the case of pharmacological chaperones for acid β-glucosidase, the chaperone is competitively replaced by substrate. (b) A small-molecule activator correcting a disordered region allosterically. (c) A small-molecule activator binding in the active site, increasing productive interaction between substrate and catalytic residues.

Table 1:

Clinical classification of G6PD mutations. (adapted from [10])

Classification of G6PD mutations		Clinical outcome
Class I	< 10% activity	Severe; CNSHA
Class II	< 10% activity	Severe episodes of hemolytic anemia
Class III	10 - 60% activity	Mild episodes of hemolytic anemia
Class IV	60 - 150% activity	Asymptomatic

Table 2:

Kernicterus is over seven fold more likely to occur in G6PD deficient newborns worldwide.

Summary of known kernicterus and G6PD deficiency rates in various countries					
Country	Incidence of kernicterus (per 100,000)	% G6PD deficiency in infants with kernicterus	Expected % G6PD deficiency, based on general population	Fold over-representation of G6PD deficiency in infants with kernicterus	Ref
Canada	2.3 - 7.9	39 - 58	0 - 3	30 X	7,75–77
Cuba	4.6	5	2 - 4	1.6 X	78,79
Denmark	1.8 - 9	2	0 - 3	1 X	7,80,81
Hong Kong	269	55	3 - 6	12 X	82
Nigeria	800 - 1600	61 - 80	15	4.7 X	83–86
Oman	346	70	18	3.9 X	87
Singapore	N/A	43	2	22 X	88
Turkey	N/A	18	13	1.4 X	89
USA	1.5	20	1 - 7	5.6 X	7,18,90
UK/Ireland	0.9	21	0 - 3	13 X	7,91
Average				7.5 X	