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PharmGKB summary: methylene blue pathway

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Introduction

The many uses of methylene blue

Methylene blue (methylthioninium chloride) is a 'jack of all trades' with a litany of clinical uses. *In vivo*, it is indicated for use as a therapy for drug-induced methemoglobinemia [1-3], can be used for the treatment of infections, pathologies, or poisoning, and as a dye for diagnostics. It is also commonly used as a dye *in vitro* – for example, as a component in staining of cells, tissues, DNA, parasites, and bacteria [4-6]. In the 1890s, Ehrlich demonstrated its use to target the malarial parasite, and more recently it has been reinvestigated for inclusion in antimalarial regimens in the wake of parasite drug resistance [7-9]. Further examples of its clinical use include treatment of ifosfamide-induced neurotoxicity (although treatment has been reported ineffective), an antidote for cyanide poisoning, visualization of fallopian tubes or ruptured membranes, a marker of tumors, and even as a potential therapy for septic shock and ischemic brain injury [5,8,10-18]. In phase II clinical trials, a modified version of methylene blue is reported to slow cognitive decline in mild–moderate Alzheimer's disease patients compared to placebo, and phase III trials are planned, although these results remain unpublished [19]. The mechanism of action is unclear – possibly by preventing tau protein aggregation or increasing amyloid-β clearance by

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Conflicts of interest

There are no conflicts of interest.

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enhancing proteasome activities [19,20]. As a photodynamic therapy, methylene blue could be used to treat psoriasis, West Nile virus infection, AIDS-related Kaposi's sarcoma, antibiotic-resistant bacterial strains and decontaminate blood before transfusion [5,21-25]. Methylene blue has also contributed to drug development, forming the structural chemical basis of other therapeutic drugs, including the antimalarial drug, chloroquine; the antihistamine, promethazine; and the antipsychotic, chlorpromazine [5]. Acknowledging the many uses of methylene blue, this article focuses on the pharmacodynamics of methylene blue in the context of methemoglobinemia treatment and the pathways surrounding this, due to known pharmacogenetic associations that relate to these pathways.

Methemoglobinemia

Methemoglobinemia is an increase in the methemoglobin (MetHb) content of red blood cells (RBCs) [26,27]. MetHb is formed when heme iron atoms within hemoglobin are oxidized and can no longer bind oxygen or carbon dioxide [26-30]. Normal levels of MetHb in circulating RBCs are around 1%. Methemoglobinemia occurs when these levels increase and can be due to inherited factors (hereditary) or induced by exogenous oxidizing agents such as therapeutic drugs (acquired) [26,27]. Cyanosis occurs at around 15% MetHb, and tissue hypoxia can occur as levels rise further–MetHb levels of 70% or higher can be fatal [27].

G6PD deficiency

Methylene blue is an effective treatment for reducing MetHb however, it is associated with adverse reactions in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals (Table 1). Drug labels for methylene blue contraindicate or advise precaution for use in G6PD-deficient individuals due to a risk of hemolytic anemia and/or methemoglobinemia [1-3]. Three hundred and thirty million individuals are estimated to have a deficiency in the G6PD enzyme, with the highest prevalence found in Africa, the Middle East, and Asia [48]. G6PD-deficient individuals are more susceptible to RBC oxidative stress induced by exogenous agents such as therapeutic drugs because reduced nicotinamide adenine dinucleotide phosphate (NADPH) production cannot meet the demand required by regulatory mechanisms (described below in further detail) [46,49,50]. The variants within the *G6PD* gene that have been identified (currently > 180) are categorized into WHO classes (I–V) depending on the extent of enzyme deficiency and clinical manifestations they confer (see the PharmGKB G6PD very important pharmacogene summary http://www.pharmgkb.org/vip/PA28469) [50-54].

Here, we use three pathway diagrams to explain the pharmacogenetics underlying the mechanism of action of methylene blue in the treatment of methemoglobinemia. We introduce the essential role of G6PD in the production of NADPH via the pentose phosphate pathway (PPP) in RBCs (Fig. 1) and detail some of the mechanisms that are dependent on NADPH to regulate oxidative stress in RBCs (Fig. 2), including methylene blue treatment (Fig. 3). Many of the steps within these pathways involve cycling redox reactions – oxidation is the loss of electrons and reduction is the accompanied gain of electrons [29]. These pathways help to explain why individuals with genetic variants that confer G6PD deficiency may be more susceptible to methemoglobinemia triggered by exogenous agents

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and why methylene blue treatment is ineffective in these individuals, possibly even exacerbating RBC oxidative stress. Interactive versions of these pathways, with links to gene and drug pages can be found on the PharmGKB website at: http://www.pharmgkb.org/search/browse/pathways.action.

Pathway descriptions

The pentose phosphate pathway and production of NADPH in RBCs (Fig. 1)

Glucose is converted to glucose-6-phosphate by hexokinase (*HK1*), and then enters either the glycolysis pathway via conversion to the isomer fructose-6-phosphate or the PPP (also known as hexose monophosphate shunt) via oxidation into 6-phosphogluconolactone [29,55-61]. Two steps within the PPP produce NADPH (Fig. 1); the conversion of glucose-6-phosphate to 6-phosphogluconolactone by G6PD and the conversion of 6-phosphogluconate to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (*PGD, 6PGD*) [55-58,62]. The end product of the pathway is ribose-5-phosphate, utilized for the production of nucleotides, polysaccharides, and coenzymes, and used in RBCs for phosphoribosylpyrophosphate (PRPP) production to generate ADP for use in the Embden–Meyerhof glycolysis pathway [57,63,64]. Within RBCs, NADPH is required for the regulation of oxidative stress and is utilized for methylene blue function [27,57,65]. The only source of NADPH in RBCs is the via the PPP, in which G6PDis the rate-limiting step [29,49,50,55-57]. As RBCs age, enzyme activities involved in glucose metabolism diminish, including that of G6PD, reducing energy production and the ability to protect cell membrane integrity and hemoglobin from oxidation [60,66].

The role of NADPH in neutralization of ROS in RBCs

Release of reactive oxygen species (ROS) such as superoxide (O_2^-) and/or peroxide (e.g. hydrogen peroxide, H_2O_2) can be triggered by exogenous oxidizing agents such as therapeutics or their metabolites [30,50]. For example, H_2O_2 is produced as a byproduct of the conversion of uric acid to allantoin by the drug rasburicase [67,68]. RBCs are constantly subjected to oxidative stress, from their role as an oxygen transporter to their exposure to xenobiotics in circulation [29,30]. NADPH is utilized as reducing power in many of the processes that protect RBCs from these oxidative stresses (Figs 2 and 3). Inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by H_2O_2 is thought to reroute glucose from glycolysis to the PPP to increase NADPH production [58,69].

The oxidative stress regulatory pathway (erythrocytes)

The oxidative stress regulatory pathway focuses on some of the mechanisms requiring NADPH from the PPP to neutralize ROS such as O_2^- and H_2O_2 (Fig. 2). O_2^- can be converted to H_2O_2 by superoxide dismutase (*SOD1*) [50,62]. Glutathione reductase (*GSR*) utilizes NADPH to convert oxidized glutathione (GSSG) to reduced glutathione (GSH) [55-57,70,71]. GSH is then oxidized back to GSSG by glutathione peroxidase (*GPX1*) in a cyclical reaction to neutralize $2H_2O_2$ into $2H^2O$ and O_2 [50,55-57,70]. GSH also protects hemoglobin by preventing and reversing oxidation that causes the formation of disulfide crosslinks between globin chains, which distorts hemoglobin structure, potentially resulting in the precipitation of 'Heinz bodies' [29]. RBCs with normal G6PD enzyme activity have

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higher PPP activity and levels of GSH compared to G6PD-deficient RBCs, however, deficient cells can cope with low levels of available NADPH under normal conditions [49,50]. When oxidative stress occurs, G6PD ‘normal’ RBCs maintain GSH levels by enhancing PPP activity, whereas in G6PD-deficient cells the PPP remains at minimum capacity and GSH levels decrease [49]. Because the PPP in G6PD-deficient RBCs is already close to the maximum activity rate obtainable under normal conditions, these cells cannot cope with oxidative stresses and are more susceptible to lysis triggered by oxidative stress, which can lead to hemolytic anemia [49,50].

Another key system requiring NADPH is the conversion of oxidized thioredoxin (*TXN*) to a reduced form by thioredoxin reductase (*TXNRD1*), reduced thioredoxin is then utilized as an electron donor by the enzyme peroxiredoxin to neutralize H_2O_2 [70-72]. In RBCs, peroxiredoxin 2 (Prx 2, *PRDX2*) is the most abundant isoform, and also plays a protective role by binding to and stabilizing hemoglobin [72,73]. The importance of *PRDX2* is demonstrated in knockout mice who display morphological RBC defects such as Heinz bodies, and upon exposure to H_2O_2 blood samples from knockout mice have enhanced MetHb formation compared to wildtype samples [73].

Both GSH and thioredoxin reductase can convert fully oxidized vitamin C (dehydroascorbic acid) to its reduced form (ascorbate, ascorbic acid), which can in turn donate electrons and hydrogens to O_2^-, H_2O_2, and oxygen free radicals [71,74-76]. Because humans cannot synthesize vitamin C, recycling it from an oxidized state is important in maintaining RBC and plasma levels of the antioxidant form [71,74,75].

The catalase (*CAT*) enzyme also neutralizes H_2O_2, involving reaction steps that form different states of the enzyme – NADPH is not required for the functional activity of catalase, rather the prevention of forming an inactive state of the enzyme [77]. The first reaction between resting catalase (ferricatalase) and H_2O_2 forms compound I and H_2O, subsequent reaction with a further H_2O_2 molecule returns catalase to resting state and releases H_2O and O_2 [77,78]. Reduction of compound I can also form the inactive compound II state, that slowly spontaneously reverts back to catalase [77,78]. Formation of compound II can be prevented by the production of NADPH by G6PD from glucose-6-phosphate [77,79,80]. Evidence suggests that NADPH may also function to reduce compound I back to catalase under conditions that prolong this state of catalase, a strong oxidant (e.g. low H_2O_2) [77].

Methylene blue pathway, PD

The methylene blue pathway focuses on the mechanisms involved in oxidation of hemoglobin to MetHb and protection from this process, or reduction of MetHb back to hemoglobin (Fig. 3). Hemoglobin in RBCs has interchangeable structural forms that enable the uptake of oxygen and release of carbon dioxide in the lungs and the release of oxygen and uptake of carbon dioxide in the tissues (reviewed extensively in [81]). Deoxyhemoglobin is the tense conformation – as oxygen binds to one heme group, the structure relaxes and affinity for oxygen increases, enabling oxygen molecules to rapidly bind the remaining three heme groups (oxyhemoglobin) [28,81]. MetHb is formed through

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the oxidation of one or more heme iron atoms within deoxyhemoglobin from a ferrous to the ferric state (Fe^{2+} to Fe^{3+}) by compounds such as H_2O_2 and O_2^- [26-30].

To prevent the formation of MetHb, RBCs have several different mechanisms that work either by reducing ROS in the cell, to prevent MetHb formation (including mechanisms described in the oxidative stress regulatory pathway; Fig. 2), or by reverting the ferric iron back to a ferrous state by reduction [26,27,29]. The main RBC enzyme that reduces MetHb *in vivo* is the soluble form of cytochrome b5 reductase (*CYB5R3*, also known as NADH-dependent MetHb reductase or diaphorase-1), utilizing the electron donor NADH to reduce cytochrome b5 (*CYB5A*) which can then in turn reduce MetHb [26-28,30,82]. More than 40 genetic variants within the *CYB5R3* gene have been associated with recessive congenital methemoglobinemia [82]. Extracellular NADH in the presence of lactose dehydrogenase can enhance the rate of MetHb reduction in human erythrocytes [83].

A second enzyme, flavin reductase (NADPH) [biliverdin reductase B (*BLVRB*), NADPH-MetHb reductase, NADPH-MetHb-diaphorase] undertakes around 5% of this activity in RBCs under normal conditions, requiring NADPH and an electron acceptor cofactor [26,27,30,84]. Riboflavin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) can all act as electron acceptors to then reduce MetHb [85-87].

Pharmacodynamics of methylene blue

Another such cofactor for BLVRB that greatly accelerates the reduction of MetHb is methylene blue [26,27,30]. Methylene blue is reduced to leukomethylene blue by BLVRB, accepting electrons from NADPH [27]. Leukomethylene blue acts as an electron donor to reduce MetHb to hemoglobin, converting back to methylene blue in a cyclical redox reaction [27,30]. Conversely, because methylene blue is an oxidizing agent, at high concentrations it can cause methemoglobinemia by oxidizing hemoglobin [27,30]. The efficacy of methylene blue treatment can be affected by numerous factors, for example an aniline intermediate may block RBC uptake of methylene blue [88].

Pharmacogenetics – G6PD deficiency

Early *in-vitro* studies demonstrate that the rate of MetHb reduction in G6PD-deficient RBCs incubated with methylene blue and glucose is severely reduced compared to normal RBCs [89]. However, this can be increased by incubating G6PD-deficient and ‘normal’ RBCs together. However, this can be increased by incubating G6PD deficient and ‘normal’ RBCs together-possibly by the diffusion of leukomethylene blue into G6PD-deficient RBCs [89]. Due to dependency on NADPH, methylene blue treatment is often ineffective at ameliorating methemoglobinemia in G6PD-deficient patients [33-35,37,39,40], and may exacerbate the condition and/or induce hemolysis in individuals with G6PD deficiency (Table 1) [14,26,31-33,35-38,40,41]. In fact, failure of methylene blue to reduce MetHb was developed as a diagnostic test for G6PD deficiency [90,91].

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Is methylene blue safe in G6PD-deficient individuals?

A recent evidence-based review concluded that methylene blue treatment should be avoided in patients with G6PD deficiency due to a risk of hemolysis [47], backed by other reviews [46,62,92], the Italian G6PD Deficiency Association [93], and drug labels [1-3]. Alternative treatments for methemoglobinemia include vitamin C, an electron donor that can reduce ROS and therefore inhibit the production of MetHb (Fig. 2) [13,26,33,94]. However, methylene blue is a more potent and rapid reducer of MetHb than other options, such as riboflavin [95], vitamin C treatment is not always effective [35,37,40], and several NADPH-dependent mechanisms are required for the recycling of oxidized vitamin C [71,74,75].

Several studies show no association between risk of methylene blue-induced hemolysis and G6PD deficiency. No cases of severe hemolysis were observed in 24 children with G6PD deficiency in a study to treat uncomplicated malarial infection with methylene blue and chloroquine, a drug combination also found to be safe in 74 healthy G6PD-deficient men [96,97]. Both studies were carried out in an area of West Africa where *G6PD* class III variants are common [96-98].

It has therefore been debated whether methylene blue is safe to use in G6PD-deficient individuals or not. The answer may depend on the type of deficiency, as WHO class III *G6PD* variants are considered to confer less severe deficiency compared to class I or II [50,53,54,96,97,99]. However, one argument against using methylene blue in this patient population is that testing for G6PD deficiency is still based on enzyme activity in the majority of cases around the world, rather than genotyping or characterization of the underlying variant, and so methylene blue should be administered with extreme caution to those with known G6PD deficiency [48,99 (Author reply)]. Of the case studies identified in this extensive literature review (Table 1), only two publications describe the characterization of the G6PD electrophoretic variant (A –), and genotyping of the specific underlying genetic variants is not always reported (see G6PD A – haplotypes http://www.pharmgkb.org/gene/PA28469?tabType=tabHaplotypes) [33,43]. Another argument is that despite previous perceptions, *G6PD* A– should not clinically be regarded as 'mild' as patients are still at risk of life-threatening acute hemolytic anemia when challenged with a potent agent [100], and the distinction between WHO class II and III *G6PD* that variants is no longer clinically useful [101]. Illustrating this, a recent combined analysis of four randomized controlled trials of methylene blue treatment observed significant but small reductions in hemoglobin levels in hemi/homozygous children with class III *G6PD* A– compared to wildtype or heterozygous children (Table 1) [43]. This effect was described as being of limited clinical consequence (with only two children of 844 displaying severe anemia: one heterozygous and one hemizygous), though monitoring of adverse hematological events in G6PD-deficient patients was advised [43].

As it is possible that leukomethylene blue diffuses into G6PD-deficient RBCs [89], heterozygous females may be less at risk for methylene blue-induced hemolysis, though to our knowledge this mechanism has yet to be examined.

Other genetic factors may contribute to risk – for example, individuals with cytochrome b5 reductase, catalase, or GSH synthetase deficiency may be at a higher risk of drug-induced

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methemoglobinemia and hemolytic anemia [40,102-107]. Newborns are particularly susceptible to methemoglobinemia due to low cytochrome b5 reductase levels, reduced catalase and glutathione peroxidase activity [27,28,108]. Similar to G6PD, hexokinase is able to enhance the rate of the PPP under conditions of oxidative stress (e.g. treatment with methylene blue), as demonstrated by a lack of enhanced PPP rate in cells when hexokinase is inhibited [109], and patients with hexokinase deficiency exhibit nonspherocytic hemolytic anemia [110]. BLVRB deficiency, a rare or potentially asymptomatic condition as indicated by a lack of literature, was described in an individual who had sufficient G6PD activity but an abnormal methylene blue screen test [111]. Due to role of BLVRB in the pharmacodynamics of methylene blue, methylene blue treatment may be unsuccessful in BLVRB-deficient patients, as reflected in European Union drug labeling, which contraindicates the use of the drug in these individuals [2,3,112,113].

Disease context may also play a role – patients with methemoglobinemia are already displaying clinical signs of oxidative stress within their RBCs and therefore may be at a higher risk for hemolysis. As an oxidizing agent, the dosage of methylene blue administered should also be considered [27,30]. Other factors, for example, baseline hemoglobin levels in patients with malarial that correlate with parasitemia levels, may also affect risk [43].

Does methylene blue cause hemolysis in G6PD-deficient individuals, or is it the agent that initiated methemoglobinemia?

Several of the same agents listed that can trigger acquired methemoglobinemia are also known to cause hemolytic anemia in G6PD-deficient individuals, for example, primaquine, acetanilide, and toluidine [27,46]. It is therefore difficult to pinpoint whether methylene blue is the cause of hemolysis in G6PD-deficient individuals being treated for acquired methemoglobinemia, rather than the precursor agent, though methylene blue is listed both as an agent that can cause hemolytic anemia in G6PD-deficient individuals and as an agent that can cause acquired methemoglobinemia [27,46]. In one study from our literature review, the development of methemoglobinemia in a G6PD-deficient patient was associated with methylene blue (Table 1) [13].

Are G6PD-deficient individuals also more susceptible to methemoglobinemia risk?

G6PD-deficient individuals may be more susceptible to acquired methemoglobinemia, though this direct association is unclear in the published literature. The development of methemoglobinemia has been linked to G6PD deficiency [114]. Rasburicase (contraindicated in G6PD-deficient patients) has been associated with both methemoglobinemia and hemolytic anemia in several reported cases of patients with G6PD deficiency [38,39,56,115-118]. Deficiency in G6PD may contribute to exacerbation of acquired methemoglobinemia in several ways via a decrease in available NADPH in RBCs, as described above, though the NADH-dependent cytochrome b5 reductase pathway dominates reduction of MetHb [26,27].

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Conclusion

Methylene blue can be used for the treatment of methemoglobinemia, a condition triggered by oxidative stress in RBCs. However, the mechanism of action of methylene blue is dependent on the intracellular capacity for NADP/NADPH recycling, and as the PPP is the only source of NADPH in RBCs, methylene blue treatment to reduce MetHb relies on G6PD enzyme activity. G6PD deficiency is found in around 5% of the world's population, with more than 180 genetic variants described that confer varying degrees of enzyme deficiency [48,52]. Methylene blue is therefore an unsuitable treatment option for methemoglobinemia in G6PD-deficient individuals, and has been associated with hemolysis likely caused by the exacerbation of oxidative stress in RBCs deficient in the G6PD enzyme.

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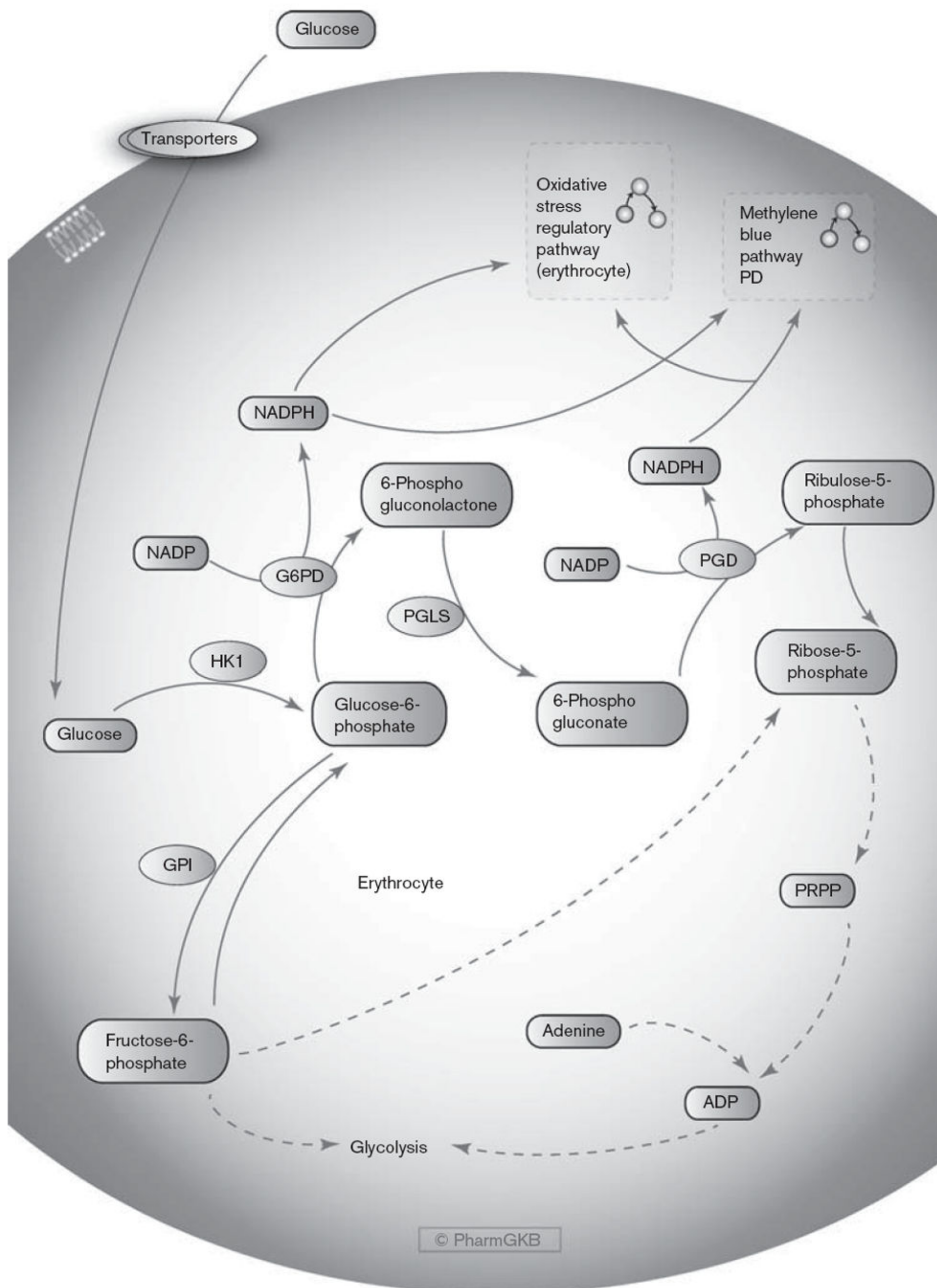

Fig. 1.

The pentose phosphate pathway in erythrocytes. A graphical representation of the genes involved in the generation of NADPH in red blood cells. The NADPH produced can be utilized in mechanisms that regulate oxidative stress and by methylene blue to reduce methemoglobin. An interactive version of this pathway with links to genes and the interconnecting pathways can be found on the PharmGKB website: http://www.pharmgkb.org/pathway/PA165971634. G6PD, glucose-6-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; HK1, hexokinase 1; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate;

PGD, 6-phosphogluconate dehydrogenase; PGLS, 6-phosphogluconolactonase; PRPP, phosphoribosylpyrophosphate.

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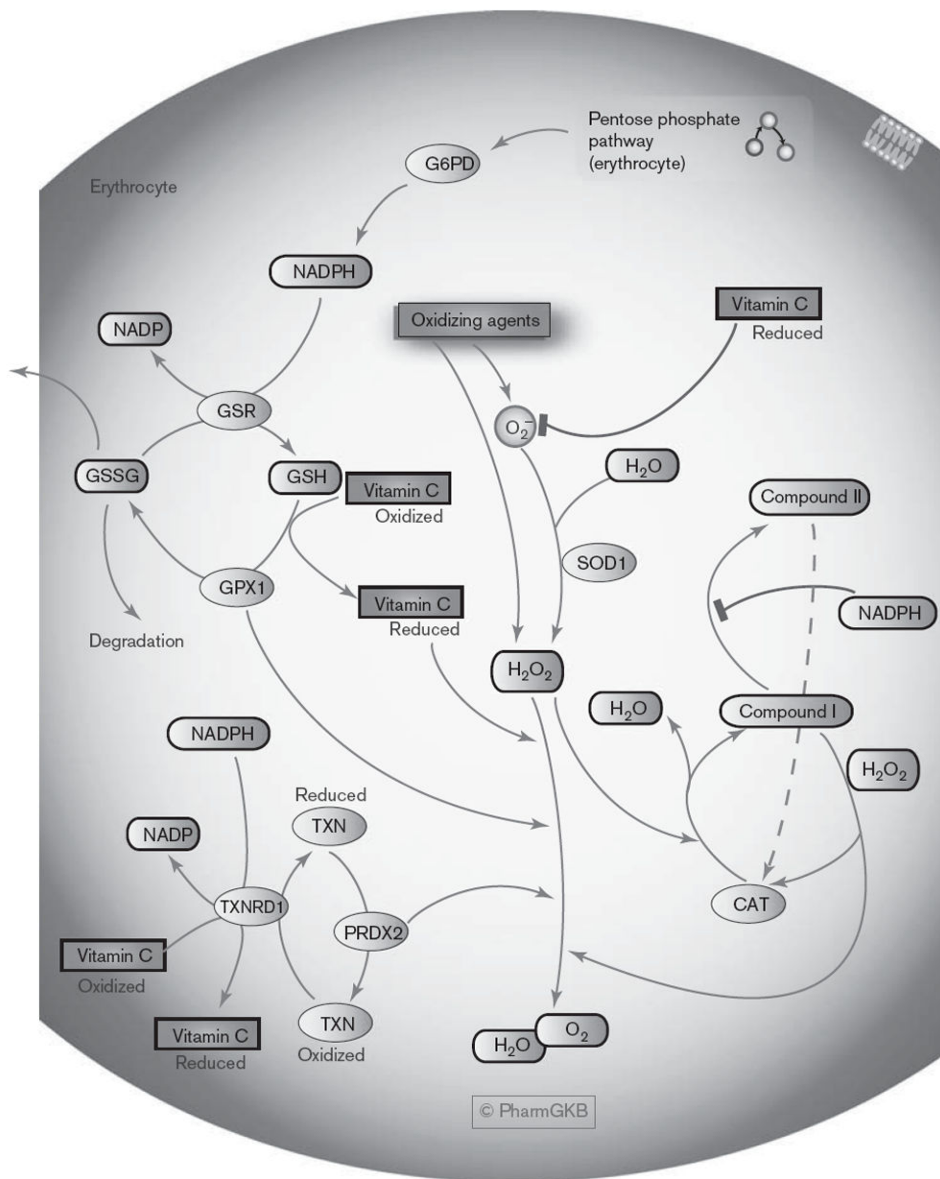

Fig. 2.

The oxidative stress regulatory pathway in erythrocytes. A graphical representation showing several of the regulatory mechanisms that prevent oxidative stress in red blood cells, many of which require NADPH from the pentose phosphate pathway. An interactive full color version of this pathway with links to genes and the interconnecting pathways can be found on the PharmGKB website: http://www.pharmgkb.org/pathway/PA165980399. CAT, catalase; G6PD, glucose-6-phosphate dehydrogenase; GPX1, glutathione peroxidase; GSH, reduced glutathione; GSR, glutathione reductase; GSSG, oxidized glutathione; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PRDX2, peroxiredoxin 2; SOD1, superoxide dismutase 1; TXN, thioredoxin; TXNRD1, thioredoxin reductase.

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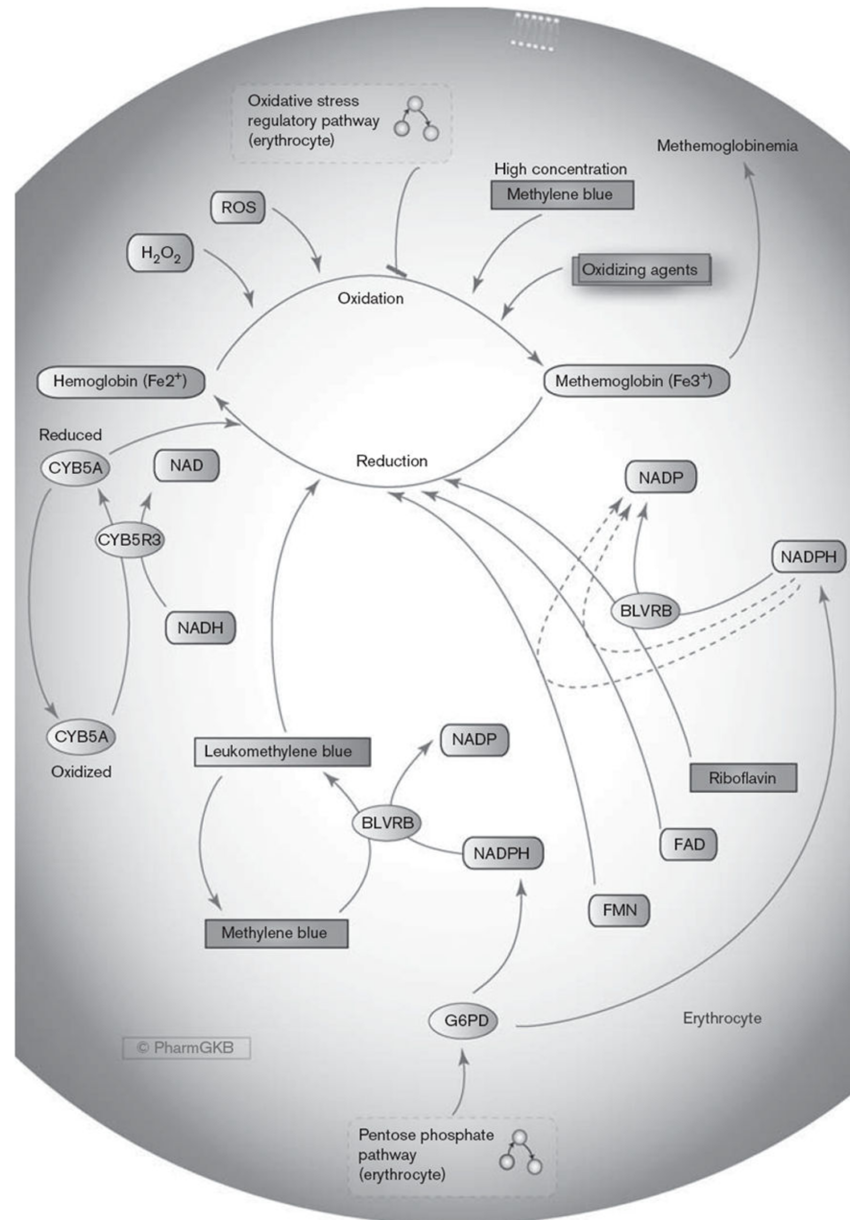

Fig. 3.

Methylene blue pathway, pharmacodynamics (PD). A graphical representation of some of the triggering agents that can cause methemoglobin production in red blood cells and the control mechanisms to prevent methemoglobinemia, including treatment with methylene blue which requires NADPH from the pentose phosphate pathway. An interactive full color version of this pathway with links to genes, drugs, and the interconnecting pathways can be found on the PharmGKB website:http://www.pharmgkb.org/pathway/PA165980834. BLVRB, biliverdin reductase B; CYB5A, cytochrome b5; CYB5R3, cytochrome b5 reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; G6PD, glucose-6-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide

phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

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Table 1

Cases of adverse reactions reported after methylene blue treatment in G6PD-deficient individuals

Study details	Methemoglobinemia reported to be triggered by	Consequence of subsequent methylene blue treatment	G6PD deficiency? Test carried out?	References
Case study, male adult (Filipino) with metastatic renal cell carcinoma	Triapine	Developed jaundice, hemoglobinuria, hemolysis	G6PD-deficient, enzyme activity assay	Foltz *et al*. [31] and Dalal *et al*. [32]
Case study, male adult (Mexican-American)	Cleaning liquid containing aniline and toluene	No change in MetHb levels, hemolysis on day 2 which may have been triggered by aniline, toluene, methylene blue (or the subsequent vitamin C[a] treatment)	G6PD deficiency A – variant, quantitative and qualitative enzyme activity assays	Rosen *et al*. [33]
Report of three premature neonates who were exposed to methylene blue prenatally (two male, one female)	Not applicable	Development of severe hemolysis resulting in hyperbilirubinemia, all required exchange transfusions	G6PD deficiency was confirmed in two of the three cases, enzyme activity assay	Gauthier [14]
Case study, 26-month-old boy	Nail removal fluid (containing nitroethane)	MetHb levels were only transiently reduced, and rose, but were resolved by RBC exchange	G6PD-deficient, enzyme activity, and 53% HbA, 41% HbS sickle trait	Golden *et al*. [34]
Case study, 23-year-old woman (India)	Aniline	Was also given vitamin C[a]. No improvement. Developed hemolysis and required transfusion	G6PD-deficient, methemoglobin reduction method	Mullick *et al*. [35]
A 3-month-old who had undergone cardiac surgery	Could not define, but the authors discuss that it may be due to the effects of cardiopulmonary bypass on enzyme activity, glyceryl trinitrate treatment, or other cardiac or respiratory causes	Low dose of methylene blue was given over 10 min and MetHb levels reduced. Developed jaundice and mild hematuria attributed to hemolysis	Prior knowledge of a partial G6PD deficiency	Maddali *et al*. [36]
A case study, 40-year-old man	Fungicide containing copper-8-hydroxyquinolate. He was initially treated with vitamin C[a]. Methemoglobinemia and hemolysis developed	Methylene blue was administered, along with continued treatment with vitamin C[a]. Treatment was not effective, and hemolysis became more severe	Underlying G6PD deficiency, as well as inhibition of G6PD activity by copper. Activity assay	Yang *et al*. [37]
A case study, adult, in a trial of 80 patients (patients with known G6PD deficiency were not enrolled)	Rasburicase	MetHb levels decreased but hemolysis worsened	Previously unknown G6PD deficiency, test not reported	Vadhan-Raj *et al*. [38]
A case study, 12-year-old boy (Laotian)	Rasburicase	Treatment was not effective	G6PD-deficient, enzyme activity test	Bhat *et al*. [39]
A case study, a male patient (Jordanian) with chronic renal failure	Metoclopramide (impaired renal function and cytochrome b5 reductase deficiency also contributed)	Also given vitamin C[a]. Treatment was not effective, his condition worsened, and he likely developed hemolysis. The patient died	Cytochrome b5 reductase-deficient, quantitative assay, and G6PD-deficient, enzyme activity assay	Karadsheh *et al*. [40]
A case study, 25-year-old man	Aniline	Symptoms diminished and MetHb levels reduced, however hemolytic anemia developed several days later	G6PD deficiency, assay	Liao *et al*. [41]

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Study details	Methemoglobinemia reported to be triggered by	Consequence of subsequent methylene blue treatment	G6PD deficiency? Test carried out?	References
A case study, 23-year-old woman undergoing laparoscopy	Methylene blue (administered through the cervix to visualize the fallopian tubes)	Cyanosis was treated with vitamin C[a] rather than with methylene blue	G6PD deficiency, enzyme levels	Bilgin *et al*. [13]
n = 409 children were genotyped, *n* = 88 were G6PD-deficient. Dose-finding study – treated with combination of chloroquine and different doses of methylene blue. Burkino Faso	Not applicable	In one child, hemoglobin dropped below 5 g/dl on day 5, and in seven children, hemoglobin value dropped by more than 3 g/dl	The child whose hemoglobin dropped below 5 g/dl was a G6PD-deficient hemizygous male. Three of the seven children with a greater than 3 g/dl drop in hemoglobin were G6PD-deficient. Genotyping method or variant not described	Meissner *et al*. [42]
Pooled analysis of four randomized control trials (*n* = 1005 children) (includes PMID: 16179085, 17026773 (above), 18286187, and unpublished data). A 21-month-old girl and a 28-month- old boy, both with malaria	Not applicable	*n* = 844 children were treated with methylene blue combined with other antimalarial drugs. In these two patients, hemoglobin levels fell to 5 g/dl or less (indicating severe anemia)	Female was heterozygous for G6PD A –, the male was hemizygous (likely that described above in Meissner *et al*. [42]). Specifics of genotyping were not provided	Muller *et al*. [43]

G6PD, glucose-6-phosphate dehydrogenase; MetHb, methemoglobin; PMID, PubMed identification number.

[a]It should be noted that vitamin C at high concentrations has also been associated with inducing hemolysis in G6PD-deficient individuals [44,45], although it is considered safe at therapeutic doses in patients who do not have WHO class I *G6PD* variants [46,47].