Hemolysis and Metabolic Lesion of G6PD Deficient RBCs in Response to Dapsone Hydroxylamine in a Humanized Mouse Model. *

Karolina H. Dziewulska, Julie A. Reisz, Ariel M. Hay, Angelo D’Alessandro and James C. Zimring

University of Virginia School of Medicine, Department of Pathology, Charlottesville, VA USA (K.H.D., A.M.H, and J.C.Z)

University of Colorado Anschutz Medical Campus, Department of Biochemistry and Molecular Genetics, Aurora, CO, USA (J.A.R. and A.D.)
Running Title: Dapsone Mediated Hemolysis of G6PD-Deficient RBCs

Corresponding Author

James C. Zimring MD, PhD
University of Virginia School of Medicine
P.O. Box 801386
Charlottesville, VA 22908
email: jcz2k@virginia.edu

Number of text pages = 13
Number of tables = 0
Number of figures = 4
Number of references = 36
Words in abstract = 247
Words in introduction = 553
Words in discussion = 1435

Nonstandard Abbreviations. Glucose 6-phosphate dehydrogenase (G6PD), G6PD deficiency (G6PDd), pacific blue (PB), green fluorescent protein (GFP), methanol (MeOH), liquid chromatography-mass spectrometry (LC-MS), 6-phosphogluconate (6P-gluconate), monoacetyldapsone hydroxylamine (MADDS-NOH), hemoglobin (Hb), methemoglobin (MetHb), methemoglobin reductase (MR), pentose phosphate pathway (PPP), citrate phosphate dextrose adenine buffer (CPDA-1), dapsone hydroxylamine (DDS-NOH), Mediterranean variant of G6PD (Med-), humanized G6PD-deficient mouse (hG6PDMed-), pacific blue (PB)

Recommended Section Assignment = Metabolism, Transport, Pharmacogenomics
Abstract:

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in humans (~5% of all individuals). G6PD deficiency (G6PDd) is caused by an unstable enzyme and manifests most strongly in red blood cells (RBCs) that cannot synthesize new protein. G6PDd RBCs have decreased ability to mitigate oxidative stress due to lower levels of NADPH, as a result of a defective pentose phosphate pathway. Accordingly, oxidative drugs can result in hemolysis and potentially life-threatening anemia in G6PDd patients. Dapsone is a highly useful drug for treating a variety of pathologies but oral dapsone is contraindicated in patients with G6PDd due to oxidative stress-induced anemia. Dapsone must be metabolized to become hemolytic. Dapsone hydroxylamine (DDS-NOH) has been implicated as the major hemolytic dapsone metabolite, but this has never been tested on G6PDd RBCs with in vivo circulation as a metric. Moreover, the metabolic lesion caused by DDS-NOH is unknown. We report that RBCs from a novel humanized mouse expressing the human Mediterranean G6PD-deficient variant have increased sensitivity to DDS-NOH. In addition, we show that DDS-NOH damaged RBCs can either undergo sequestration (with subsequent return to circulation) or permanent removal in a dose-dependent manner, with G6PD-sufficient RBCs mostly being sequestered, and G6PDd RBCs mostly being permanently removed. Finally, we characterize the metabolic lesion caused by DDS-NOH in G6PDd RBCs and report a blockage in terminal glycolysis resulting in a cellular accumulation of pyruvate. These findings confirm DDS-NOH as a hemolytic metabolite and elucidate metabolic effects of DDS-NOH on G6PDd RBCs.
Significance Statement

These findings confirm that dapsone hydroxylamine, an active metabolite of dapsone, causes \textit{in vivo} clearance of murine RBCs expressing a human variant of deficient G6PD, an enzymopathy that affects half a billion individuals (G6PD deficiency). Both cellular mechanisms of clearance (sequestration \textit{vs.} destruction) and specific metabolic disturbances caused by dapsone hydroxylamine are elucidated, providing novel mechanistic understanding.
**Introduction:**

Glucose 6-phosphate dehydrogenase (G6PD) is the first and rate limiting enzyme in the pentose phosphate pathway (PPP), which generates NADPH that fuels antioxidant pathways (Luzzatto et al., 2020). However, approximately half a billion individuals (1 in every 20) has a G6PD deficiency (G6PDd), presumably as it confers resistance to malaria (Luzzatto et al., 2020). Due to decreased NADPH generated by the PPP, G6PDd individuals have less ability to mitigate oxidative stress through the multiple pathways that utilize NADPH as an electron donor, either directly or through other redox reactions (e.g., glutathione reductase (Reed, 1990), peroxiredoxins (Rhee and Kil, 2017), glutaredoxins (Meyer et al., 2009), thioredoxin (Meyer et al., 2009), ascorbate/tocopherol (Gegotek and Skrzydlewska, 2023), etc.). The absence of G6PD is embryonic lethal in mice (Longo et al., 2002) and has not been described in humans; rather, G6PDd in humans is due to enzyme variants (mostly missense mutations), many of which have decreased enzyme stability (Gomez-Manzo et al., 2016). As such, G6PDd manifests particularly in red blood cells (RBCs) as they have neither the ability to synthesize new protein nor alternate sources of NADPH (i.e., mitochondria). Thus, G6PDd individuals are prone to potentially life-threatening anemia due to RBC destruction (hemolysis) in response to oxidative stress from illness, certain drugs, or certain foods.

In most cases, drugs known to induce hemolysis in G6PDd patients can be avoided if the G6PDd status of a person is known. However, some drugs that are hemolytic in G6PDd patients do not have good alternatives. Dapsone is the primary treatment for a number of dermatologic diseases for which alternative treatments are less effective (Rybak-d'Obyrn and Placek, 2022; 2023). Dapsone-containing regimens for treating chloroquine-resistant malaria have also been developed, and while efficacious in eliminating plasmodial infection, their use had to be discontinued due to life-threatening anemia in G6PDd patients.(Luzzatto, 2010) Thus, G6PDd is a serious barrier to dapsone-containing therapy for multiple illnesses and in a large number of people.
Although the hemolytic effect of dapsone in G6PDd individuals is well known, its mechanisms of hemolysis remain only partially understood. Importantly, dapsone is not itself hemolytic, but must first be metabolized into a hemolytic form. It has been reported that dapsone hydroxylamine (DDS-NOH) is a hemolytic dapsone metabolite, causing redox cycling and extravascular hemolysis of G6PD-sufficient rat RBCs, which is increased if a G6PD inhibitor (epiandrosterone) is included (Grossman and Jollow, 1988; Grossman et al., 1992; Grossman et al., 1995; McMillan et al., 1995; Bradshaw et al., 1997). However, effects of DDS-NOH have not been tested in a model of G6PDd that recapitulates the human biology of an unstable enzyme.

RBCs from humans with the Mediterranean variant of G6PD (Med-) have approximately 5% of normal G6PD activity due to an unstable enzyme (Piomelli et al., 1968; Kahn et al., 1974; Matte et al., 2020) and can undergo brisk hemolysis in response to dapsone. We have recently described a humanized mouse model of G6PDd where the mouse G6PD gene was replaced with human genomic DNA encoding the Med- variant (D’Alessandro et al., 2021b). The resulting animal (hG6PD_{Med-}) has 5% of normal G6PD activity in RBCs consistent with enzyme levels reported in humans with Med- G6PD (Gomez-Manzo et al., 2016). In the current report, we demonstrate that DDS-NOH selectively causes in vivo clearance of hG6PD_{Med-} RBCs and characterize the metabolic lesion caused by DDS-NOH.
Materials and Methods:

Mice:

hG6PDMed- and HOD mice were generated as previously described. (Desmaret s et al., 2009; D’Alessandro et al., 2021b) The data shown in this manuscript were generated with the hG6PDMed- mouse that has previously been described in detail (D’Alessandro et al., 2021b); however, as the studies reported herein were being carried out, a transition was made to a new version of the hG6PDMed- mouse that was generated with an identical approach as described (D’Alessandro et al., 2021b), but engineered to not have the 13 residual base pairs from CRE excision of mouse G6PD. This new mouse has the same characteristics as the previously described animal, the same sensitivity of its RBCs to DDS-NOH and is being described in a separate report (manuscript in preparation). C57BL/6J mice (cat # 000664) and B6-GFP mice (cat# 004353) were purchased from Jackson labs. All experiments were carried out under an approved IACUC protocol at the University of Virginia.

Oxidant stress challenge (see Figure 1 for graphic of experimental design):

RBCs were collected in standard citrate phosphate dextrose adenine buffer (CPDA-1), washed in PBS, and incubated with DDS-NOH at the indicated concentrations in 5 mM [1,2,3-\textsuperscript{13}C\textsubscript{3}]glucose -PBS at a hematocrit of 40%. DDS-NOH was purchased from Toronto Research Chemicals, Toronto, ON (Cat# D193260), dissolved in 100% methanol (76 mM) and stored in single use aliquots under argon gas at -80°C. After dilution, the final concentration of MeOH in the treatment mixture was a maximum of 1.3% for the highest concentration of DDS-NOH. Control cells were incubated with 1.3% MeOH for a vehicle control. After 1 hour incubation at 37°C, RBCs were washed 3 times in PBS. Aliquots of all treated samples were snap frozen and stored at -80°C for subsequent metabolomics analysis. Treated RBCs were then mixed at a 2:1 ratio with untreated RBCs from HOD transgenic mice that express a detectable tracer gene on...
RBCs. The mixture was transfused into B6-GFP recipients. All transfusions were carried out by lateral tail vein injection at a 20% hematocrit in a final volume of 250 microliters.

Quantifying in vivo circulatory lifespan of DDS-NOH treated RBCs:
Peripheral blood was obtained at the indicated time points. RBCs were stained with anti-HOD followed by a secondary antibody conjugated to Pacific Blue (PB) as described. (Howie et al., 2019) Flow cytometry was used to separately enumerate recipient RBCs (GFP+), tracer RBCs (PB+) and experimental RBCs (no fluorescence). The channels used were confirmed to not inadvertently exclude any DDS-NOH treated RBCs due to autofluorescence as a result of methemoglobin formation. This approach allows the ability to measure RBC circulation without any need for genetic or biochemical labeling that may alter RBC biology. Because RBC survival was calculated as a function of an untreated tracer population that was co-injected with test RBCs, any variation due to small differences in transfusion or phlebotomy volume was controlled. Post-transfusion ratios of test:tracer were normalized to pre-transfusion ratios using the formula \[ \frac{\text{post-transfusion (test RBCs/tracer RBCs)}}{\text{pre-transfusion (test RBCs/tracer RBCs)}} \].

Mass spectrometry-based metabolomics:
Frozen RBC samples were thawed on ice and then metabolites from a 10 μL aliquot were extracted using 90 μL of cold 5:3:2 methanol:acetonitrile:water (v/v/v).

After sample randomization, 10 μL of extracts were injected into a Thermo Vanquish UHPLC system (San Jose, CA, USA) and resolved on a Kinetex C18 column (150 × 2.1 mm, 1.7 μm, Phenomenex, Torrance, CA, USA) at 450 μL/min through a 5 min gradient. The negative mode gradient utilized mobile phases: A = 95% water, 5% acetonitrile, 1 mM ammonium acetate; B = 95% acetonitrile, 5% water, 1 mM ammonium acetate) in negative ion mode. Solvent gradient:
0-0.5 min 0% B, 0.5-1.1 min 0-100% B, 1.1-2.75 min hold at 100% B, 2.75-3 min 100-0% B, 3-5 min hold at 0% B. The positive mode gradient utilized mobile phases: A = water, 0.1% formic acid; B = acetonitrile, 0.1% formic acid. Solvent gradient: 0-0.5 min 5% B, 0.5-1.1 min 5-95% B, 1.1-2.75 min hold at 95% B, 2.75-3 min 95-5% B, 3-5 min hold at 5% B. Eluant was introduced to the mass spectrometer via electrospray ionization. The Q Exactive mass spectrometer was operated in MS$^1$ mode with the following settings: resolution 70,000, scan range 65-950 m/z, maximum injection time 200 ms, microscans 2, automatic gain control (AGC) 3 x 10$^6$ ions, source voltage 4.0 kV, capillary temperature 320 °C, and sheath gas 45, auxiliary gas 15, and sweep gas 0 (all nitrogen).

For isotopic labeling experiments using [1,2,3-$^{13}$C$_3$]glucose, labeled metabolites were quantified as indicated in Figure 4 based upon their unique m/z ratios.

Raw data was converted to mzXML format with RawConverter then peaks annotated and integrated using Maven alongside the KEGG database, upon correction for natural $^{13}$C abundance. Quality control measures were performed as previously described. (Hay et al., 2022) Isotopologue data was graphed in GraphPad Prism 9.0.

**Instruments and statistics:**

All flow cytometry was carried out on an Attune cytometer and analyzed using FlowJo v10.8. Statistical analysis was carried out and graphs were generated using GraphPad Prism 9.
Results:

\textit{hG6PD}_{\text{Med}}. RBCs have decreased in vivo circulation in response to DDS-NOH

\textit{hG6PD}_{\text{Med}}. RBCs or wild-type G6PD RBCs were incubated with a titration of DDS-NOH for 1 hour (see Figure 1 for experimental design). Because most dapsone induced hemolysis in G6PDd patients is due to clearance of damaged RBCs by splenic or hepatic macrophages (i.e. extravascular hemolysis), an \textit{in vivo} circulation assay was utilized. After DDS-NOH treatment, RBCs were washed and then mixed 2:1 with HOD RBCs that express a transgene on the RBC surface that is easily stained with antibodies and visualized by flow cytometry. The 2:1 mixture was infused into GFP recipient mice and peripheral bleeds were taken at the indicated time points and stained with anti-HOD using antibodies that fluoresce on a different channel than GFP. This approach allows the gating out of all recipient RBCs (GFP+) and a determination of the ratio of test RBCs to control untreated HOD RBCs. This approach controls for differences in transfusion and/or phlebotomy volume as well as allowing a zero-time point based on the pre-transfusion ratio (input with no \textit{in vivo} clearance).

All measurements for DDS-NOH treated RBCs were normalized to vehicle only (MeOH) treatment from the same time point, as vehicle only RBCs were considered to represent a baseline 100% survival in the absence of DDS-NOH. No significant clearance was observed for MeOH only treated RBCs compared to untreated RBCs (data not shown). All doses of DDS-NOH caused increased clearance of both wild-type and \textit{hG6PD}_{\text{Med}}. RBCs in a dose dependent fashion but with greater clearance of \textit{hG6PD}_{\text{Med}}. RBCs compared to wild-type RBCs (Figure 2). At lower doses of DDS-NOH (250 and 500 \text{ \textmu M}), wild-type RBCs mostly reappeared in circulation by 20 hours and fully returned by 90 hours, indicating extravascular sequestration (likely in the spleen) with subsequent release. No return to circulation was seen with the highest dose of DDS-NOH (1000 \text{ \textmu M}) for wild-type RBCs. In contrast, only limited return to circulation was observed with any dose of DDS-NOH on \textit{hG6PD}_{\text{Med}}. RBCs. Together, these data
demonstrate that hG6PD<sub>Med</sub>- RBCs have increased sensitivity to DDS-NOH, as well as indicating that the injury is not an all or nothing effect, as a level of damage can occur that causes transient sequestration followed by release back into circulation.

\textit{Perturbations in glycolysis and PPP in hG6PD<sub>Med</sub>- and WT RBCs in response to DDS-NOH}

Aliquots of each RBC treatment condition were extracted and analyzed by liquid chromatography-mass spectrometry (LC-MS). Hierarchical clustering analysis of the top 50 metabolites by two-way ANOVA (genotype and dose) highlighted metabolic differences in hG6PD<sub>Med</sub>- RBCs (Figure 3A). hG6PD<sub>Med</sub>- RBCs had significantly lower levels of 6-phosphogluconate – the PPP product downstream to G6PD activity - and reduced glutathione, which requires PPP-derived NADPH for recycling of its oxidized form back to the reduced state by glutathione reductase (Figure 3B). Conversely, steady state levels of pyruvate, the penultimate metabolite of glycolysis, were significantly higher in hG6PD<sub>Med</sub>- RBCs compared to wild-type RBCs.

The use of [1,2,3-\textsuperscript{13}C\textsubscript{3}]glucose in the RBC treatments allows flux analysis through glycolysis and the PPP. Glucose that is metabolized through glycolysis generates metabolites that retain all 3 \textsuperscript{13}C atoms. In contrast, glucose that is metabolized through the PPP generates metabolites that shunt back into the glycolytic pathway labeled with only 2 \textsuperscript{13}C atoms, as the position 1 \textsuperscript{13}C anomeric carbon in glucose is lost as CO\textsubscript{2} in the PPP. (See figure 4, labeled carbon = red circles). This approach allows a targeted investigation of glycolytic vs. PPP flux in addition to total metabolite levels. (Nemkov et al., 2021; Rogers et al., 2021; Hay et al., 2022)

Analysis of the PPP demonstrated equivalent baseline 6-phosphogluconate (6P-gluconate) in wild-type and hG6PD<sub>Med</sub>- RBCs. However, upon DDS-NOH challenge, wild-type but not hG6PD<sub>Med</sub>- RBCs showed a substantial increase in 6P-gluconate, including increased
labeled metabolite. (Figure 4A). This demonstrates that RBCs have a compensatory increase in 
PPP flux in response to DDS-NOH and that hG6PD_{Med}- RBCs are defective in activating the 
PPP.

Both wild-type and hG6PD_{Med}- RBCs had a substantial increase in pyruvate upon DDS-
NOH treatment, but with a greater increase at lower doses of DDS-NOH in hG6PD_{Med}- RBCs. 
Conversely, lactate levels drop in both wild-type and hG6PD_{Med}- RBCs. Although lactate has a 
smaller fold decrease than the fold increase in pyruvate, lactate is present at a 20 fold molar 
excess over pyruvate in RBCs. (Sullivan and Stern, 1983) Thus, a block in conversion from 
pyruvate to lactate would show a much greater fold increase in pyruvate than decrease in 
lactate. Indeed, the isotopic tracing demonstrates that the increase in pyruvate and decrease in 
lactate are consistent with a blockage in the conversion of pyruvate to lactate by lactate 
dehydrogenase (LDH). This can be further visualized through the strong increase in pyruvate to 
lactate ratio. (Figure 4B) As predicted, the ratio of \(^{13}\text{C}_2 \text{pyruvate}/^{13}\text{C}_3 \text{pyruvate} \) is higher in wild-
type than hG6PD_{Med}- RBCs, suggestive of impaired PPP flux in hG6PD_{Med}- RBCs. (Figure 4B)
Discussion:

Dapsone is a drug mostly used in treating a variety of cutaneous pathologies and is hemolytic in G6PDd patients. In some cases, dapsone is used topically (e.g. the treatment of acne vulgaris) with limited systemic exposure (Jarratt et al., 2016) and no evidence of hemolysis in G6PDd patients. (Webster, 2010). However, dapsone is also taken orally in the treatment of numerous diseases, including (but not limited to) dermatitis herpetiformis, leprosy, pemphigus vulgaris, pyoderma gangrenosum, bullous systemic lupus erythematosus, and aphthous ulcers. (Kurien et al., 2023) Oral doses range from 25-300 mg daily and treatment can be transient or chronic, in some cases for years (i.e. in some leprosy patients), and good alternatives are limited. (Kurien et al., 2023) Although the exact frequency of G6PDd patients with the above conditions is difficult to determine, it is likely in the range of 3000 patients in the U.S. However, in addition to the above patients, the therapeutic barriers of dapsone-induced hemolysis in G6PDd patients are most pronounced in the treatment of malaria. Even in forms of G6PDd traditionally considered to be mild (i.e. the A- form), a study of 119 G6PD(A-) children reported greater than 90% of patients had hemolysis in response to anti-malarial regimens containing dapsone. (Pamba et al., 2012) One out of 5 patients had a drop in hemoglobin greater than 40% - one out of 10 patients required transfusion. (Pamba et al., 2012) Accordingly, regimens for treating malaria that contained dapsone have largely been abandoned, not because they are not useful, but rather due to hemolytic risk and inadequate G6PD point of care testing. (Luzzatto, 2010) Thus, risk of dapsone-mediated hemolysis in G6PDd patients represents a considerable pharmacogenomic barrier to treatment.

In this paper, we report several novel findings regarding the effects of DDS-NOH on hG6PD_Med. RBCs. Dapsone is not itself hemolytic but must first be metabolized into hemolytic forms (i.e. DDS-NOH and monoacetyldapsone hydroxylamine (MADDS-NOH)). The current findings demonstrate that the DDS-NOH-induced lesion is not binary (i.e., RBCs removed or
not); rather, there is a phenomenon of RBC sequestration and release back into circulation, especially for G6PD sufficient RBCs. The implication of this is that the rapid recovery from dapsone-induced anemia that can be observed when stopping treatment may not be entirely due to reticulocytosis, but release of sequestered RBCs may also contribute. This may also inform clinical timing of the decision to transfuse. The mechanisms of sequestration and release are unclear; however, it has been shown that damaged RBCs can be transiently sequestered in the spleen, where they adapt their membrane/volume ratio, before being released back into circulation (Safeukui et al., 2018). The precise cellular and molecular mechanisms of sequestration and release of DDS-NOH damaged RBCs will require further study.

We report in this paper the first characterization of the precise glycolytic/PPP metabolic lesion to G6PDd RBCs that are exposed to DDS-NOH. The increase in pyruvate and decrease in lactate, especially in hG6PDMed- RBCs exposed to DDS-NOH is a novel finding and suggests a decrease in LDH activity. It is well known that DDS-NOH induces methemoglobin (MetHb) formation in RBCs. (Vage et al., 1994) Consistent with this, although we did not formally quantify MetHb in the current report, DDS-NOH treatment of RBCs caused an obvious darkening of color that is a classic indication of MetHb formation (data not shown). MetHb is reduced back to hemoglobin (Hb) by methemoglobin reductase (MR), also called cytochrome b5 reductase. MR consumes NADH in the process. As DDS-NOH is a redox cycling compound it generates a continuous source of ROS with ongoing oxidation of Hb and consumption of NADH by MR. NADH is catalytic for glycolysis with no net change since it is generated by GAPDH and consumed by LDH. However, if NADH is consumed by MR then it is no longer available for LDH, which would inhibit the conversion of pyruvate to lactate resulting in the observed metabolic perturbation. Exacerbating this effect is that RBCs do not have mitochondria as an alternate source of NADH. In addition to inhibiting glycolysis, pyruvate is less easily excreted
from RBCs than is lactate, and its build up causes a drop in pH in the cytosol, further exacerbating the metabolic lesion. The proposed mechanism of LDH inhibition by NADH consumption by MR is a speculative part of this discussion but is consistent with known metabolism of RBCs. Although the current paper is the first metabolomics analysis of G6PDd RBCs treated with DDS-NOH, of which we are aware, the findings are in agreement with isolated metabolites measured in other studies (i.e. the decrease of GSH in G6PD sufficient rat RBCs treated with DDS-NOH). (McMillan et al., 1995)

The current report has some limitations. The RBCs are treated with DDS-NOH at a high dose in vitro for a short period of time rather than the slower exposure at lower doses that would occur in a patient taking dapsone. Typical peak concentrations of DDS-NOH in humans taking a single dose of dapsone (100mg) are in the range of 2.2 μM, approximately 100-fold lower than the lowest dose used in the current study. (Mitra et al., 1995) However, the findings by Grossman et al. suggest that using a higher dose for a shorter period of time may not be problematic as the aggregate exposure to DDS-NOH (area under the concentration over time curve) determines clearance of RBCs. (Grossman and Jollow, 1988) Nevertheless, the concentrations used in the current study are clearly well above what would be achieved in vivo and we fully acknowledge that it is an artificial experimental maneuver. Importantly, it also has the advantage of allowing the metabolic study of RBCs that are damaged to the point that they would be cleared from circulation. This is not possible in vivo, because RBCs that are sufficiently damaged to be cleared cannot be sampled from circulation, resulting in a survivorship bias of blood samples that are drawn. Moreover, mice do not metabolize dapsone into dapsone hydroxylamine, preventing in vivo studies in the current system. (Tingle et al., 1997)

An additional limitation is that there are two active metabolites of dapsone known to generate ROS in RBCs (DDS-NOH and also monoacetyldapsone hydroxylamine (MADDS-
As the current paper only studies DDS-NOH, our observations are limited to this one metabolite. It seems a reasonable speculation that superoxide generated by redox cycling of MADDS-NOH will have similar biological effects to superoxide generated by redox cycling of DDS-NOH; however, we have no empirical data to test this speculation. Thus, the observations of the current manuscript are limited to only DDS-NOH.

A final limitation is that, of course, mice are not humans and while many biologies translate between species there are also many that do not. The humanization of the G6PD locus helps to mitigate translational risk, at least with regards to PPP dynamics. The precise differential sensitivity of RBCs to hemolytic effects of DDS-NOH between humans and rodents is difficult to determine, as studies treating human RBCs with DDS-NOH followed by infusion have not been reported. DDS-NOH causes the same general *in vitro* changes in human and rat RBCs with regards to morphology, GSH depletion, ROS generation, and hemoglobin adducts binding to the cellular membrane (McMillan et al., 1995; McMillan et al., 2005). However, human RBCs require higher doses of DDS-NOH (175-750 μM) to cause the same changes as 50-175 μM cause in rat RBCs. (McMillan et al., 1995) Conversely, RBCs from humans are more sensitive to DDS-NOH induced methemoglobin formation than RBCs from mice and rats. (Tingle et al., 1997) Some caution should be taken regarding the general category of “mice”, as C57BL/6J mice (as used herein) are a highly inbred strain and may differ from CD1 mice.

Of note, the metabolic observations reported herein are consistent with the accumulation of pyruvate in human RBCs from G6PDd subjects in response to oxidant stress-inducing iatrogenic interventions, such as storage in the blood bank – as we previously determined through metabolic quantitative trait loci analysis (Moore et al., 2022) and correlation of metabolic traits to hemolytic propensity in RBCs from human G6PDd blood donors (D'Alessandro et al., 2021a).
In aggregate, the current paper presents the first formal confirmation that DDS-NOH selectively causes *in vivo* clearance of G6PDd RBCs and also demonstrates the phenomenon of sublethal RBC damage that causes sequestration prior to reentry into circulation. Characterization of the RBC metabolic lesion in response to DDS-NOH demonstrates failure to induce PPP in response to ROS and decreased conversion of pyruvate into lactate. These findings advance our mechanistic understanding of the hemolytic toxicology of a highly useful drug (dapsone) in the context of G6PDd, for which variants are present in half a billion humans worldwide.
Acknowledgements: We would like to thank Dr. David Jollow and Dr. David McMillan for their scientific insight, helpful discussions, and kind encouragement.

Data Availability Statement: The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data. The raw data that are shown in the figures are freely available on request from the corresponding author.

Author Contributions:

Participated in research design: Dziewulska, Reisz, D’Alessandro, and Zimring

Conducted experiments: Dziewulska, Reisz, and Hay

Contributed new reagents or analytic tools: Dziewulska, Reisz, D’Alessandro, and Zimring

Performed data analysis: Dziewulska, Reisz, D’Alessandro, and Zimring

Wrote or contributed to the writing of the manuscript: Dziewulska, Reisz, Hay, D’Alessandro, and Zimring

Conflict of Interests: JCZ is a co-founder and CSO of Svalinn Therapeutics. AD is a founder of Omix Technologies Inc, a consultant for Rubius Inc. and Forma Inc., and an advisory board member of Macopharma and Hemanext Inc. No funding for this study was received from any of the listed companies. This work received no external funding.
References:


Nemkov T, Stefanoni D, Bordbar A, Issaian A, Palsson BO, Dumont LJ, Hay A, Song A, Xia Y, Redzic JS, Eisenmesser EZ, Zimring JC, Kleinman S, Hansen KC, Busch MP,


Figure Legends:

**Figure 1:** Schematic of general experimental design. Test RBCs (hG6PD_{Med-} or wild-type) were incubated with DDS-NOH for 1 hour, washed, mixed 2:1 with tracer HOD RBCs, and infused into GFP recipient mice by tail vein injection. Flow cytometry was used to visualize test RBCs as non-fluorescent events and tracer RBCs by staining with PB-conjugated anti-HOD antibody – recipient RBCs (GFP+) were gated out. Post-transfusion RBC circulation was calculated by normalizing the ratio of test RBCs/tracer RBCs to the pre-transfusion ratio. This approach controls for differences in transfusion and/or phlebotomy volume as well as allowing a zero-time point based on the pre-transfusion (input) ratio. Figure was created with BioRender.com.

**Figure 2:** *In vivo* circulation of DDS-NOH treated RBCs. Wild-type (A) or hG6PD_{Med-} (B) RBCs were treated with the indicated concentrations of DDS-NOH and *in vivo* circulation was determined as described in main text and figure 1. Graphs show data from a representative experiment with test RBCs pooled from 3 donor animals. Error bars represent standard error of means and are biological replicates (n=3 recipient mice per sample tested). The same titration was performed twice with similar results (representative experiment shown). In additional studies, differential sensitivity of hG6PD_{Med-} vs. wild-type RBCs to 250 μM DDS-NOH was observed in 5 out of 5 experiments.

**Figure 3:** Metabolic phenotypes of hG6PD_{Med-} (G6PD) or wild-type (WT) RBCs in the presence (250, 500, or 1000 μM) or absence of dapsone hydroxylamine were characterized via untargeted metabolomics analysis. (A) Hierarchical clustering analysis of the top 50 metabolites by two-way ANOVA (genotype and dose). Color-coding indicates a high-level overview of pathway clustering. A version with full metabolite names appears in Supplementary
Figure 1; (B) substantial differences in 6-phosphogluconate, GSH, and pyruvate are noted between hG6PDMed- or wild-type RBCs. Data is presented as normalized peak areas versus DDS-NOH concentration.

Figure 4: Stable isotope tracing with [1,2,3-13C₃]glucose reveals alterations in glycolysis and the PPP in hG6PDMed- or wild-type RBCs exposed to DDS-NOH (0 – 1000 μM). Aliquots of hG6PD_{Med-} (G6PD) or wild-type (WT) RBCs were subjected to LC-MS for a targeted analysis of glycolysis and the PPP (n=3 for all samples analyzed). (A) Relative quantifications were determined for metabolites of glycolysis and the PPP, including labeled metabolites (see color coded legend). (B) Pyruvate to lactate ratios of labeled metabolites indicate decreased flux from pyruvate to lactate.
**FIGURE 1**

1. **TEST RBCs**
   - DDS-NOS 1 hour
   - Flow Cytometry (pre-trans ratio)
   - Recipient RBCs

2. **HOD Mouse**
   - hG6PD Med- or Wild-Type
   - GFP
   - anti-HOD

3. **GFP**
   - Recipient RBCs
   - Test RBCs
   - Tracer RBCs

4. **RBC Survival**
   \[
   \text{RBC Survival} = \frac{\text{test/HOD pre-trans}}{\text{test/HOD post-trans}}
   \]
FIGURE 3

A

B

6-P-Gluconate

Normalized levels

Glutathione (GSH)

Normalized levels

Pyruvate

Normalized levels

Dapsone hydroxylamine (uM)

Normalized levels

Amino acids

Fatty acids/carnitines

Energy metabolism

Redox/1-carbon metabolism