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Targeting the red cell enzyme pyruvate kinase with a small allosteric molecule AG-348 may correct underlying pathology of a glycolytic enzymopathy

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Mature red blood cells (RBC) lack a nucleus and mitochondria, relying almost entirely on their own glycolytic degradative processes to generate energy. One of the major reaction pathways in RBC is the Embden-Meyerhof in which a series of enzymes convert glucose anaerobically into usable energy: adenosine triphosphate (ATP).[1] The pathway utilizes two ATP to initiate the reaction, with ultimately two more ATP being produced. This pathway is essential to meeting the energy demands of RBC, including maintenance of red cell membrane flexibility and, therefore, impacting RBC shape.[1]

In the Embden-Meyerhof pathway, a pyruvate kinase (PK) isoform unique to RBC, PK-R, is a rate-limiting enzyme that plays a critical role in the formation of pyruvate from phosphoenolpyruvate (PEP) with the simultaneous generation of ATP from adenosine diphosphate (ADP)[2] (Figure 1). Among the most common enzyme defects related to the Embden-Meyerhof pathway is an inherited disorder in which homozygote individuals display signs and symptoms of hemolytic anemia due to the deficiency of the PK-R enzyme. PK defects have been documented worldwide, although most cases have been identified in people of Northern European ancestry.[3]

This rare hereditary disorder is characterized by changes in RBC metabolism including manifestation of anemia and a compromised energetic profile (ATP production). RBC deficient in PK cannot produce enough energy to maintain normal membrane function. Potassium and water leak from the cell, while calcium concentrations increase. As a result, these cells become rigid, lose flexibility, and are more susceptible to premature hemolysis.[4]

In mammals, two PK genes are expressed depending on anatomical region and cell type: PK muscle (PKM) and PK liver and red blood cell (PKLR). PKLR controls the expression of the red blood cell (PK-R) or liver (PK-L) isoforms from tissue-specific promoters. Mutations in the PKLR gene cause PK deficiency with clinical symptoms apparently confined to RBC. PK-R of RBC is a tetrameric enzyme that exists in equilibrium between a less active T-state and a more active R-state that can be induced by binding to the glycolytic intermediate fructose bisphosphate (FBP). Therefore, intervention strategies designed to counter this condition included the stabilization of the active R state of PK-R, directly restoring PK activity above and beyond the endogenous activation by FBP.[5]

An early investigation that screened for drugs targeting the PK enzyme resulted in the discovery of a small molecule, AG-348, which allosterically activates wild type PK as well as the mutant form of the enzymes. The activities of this molecule were demonstrated *in vitro*, in mice, and *ex vivo* in human RBC.[6] A partially resolved crystal structure of AG-348 bound to PK-R (2.75 A° resolution) showed that AG-348 is bound in the PK enzyme pocket at the dimer–dimer interface away from the FBP-binding domain and is buried in a cluster of apolar amino acids inducing post binding conformational change to the final R-state reminiscent of the classic R to T transition in another red cell protein, hemoglobin.[7]

In this issue of *Haematologica*, Rab *et al.*[8] report on the effects of AG-348 on RBC from a small number of patients with PK deficiency. In this investigation, the group carried out *ex vivo* experiments on RBC from a broad range of patient phenotypes, including measuring several parameters such as activities of the intermediates in the glycolytic pathway and ATP levels. They showed that AG-348 affects thermostability of the PK-R, protein levels and the shape of RBC. They also reported a modest increase in ATP and improvement in PK thermostability.
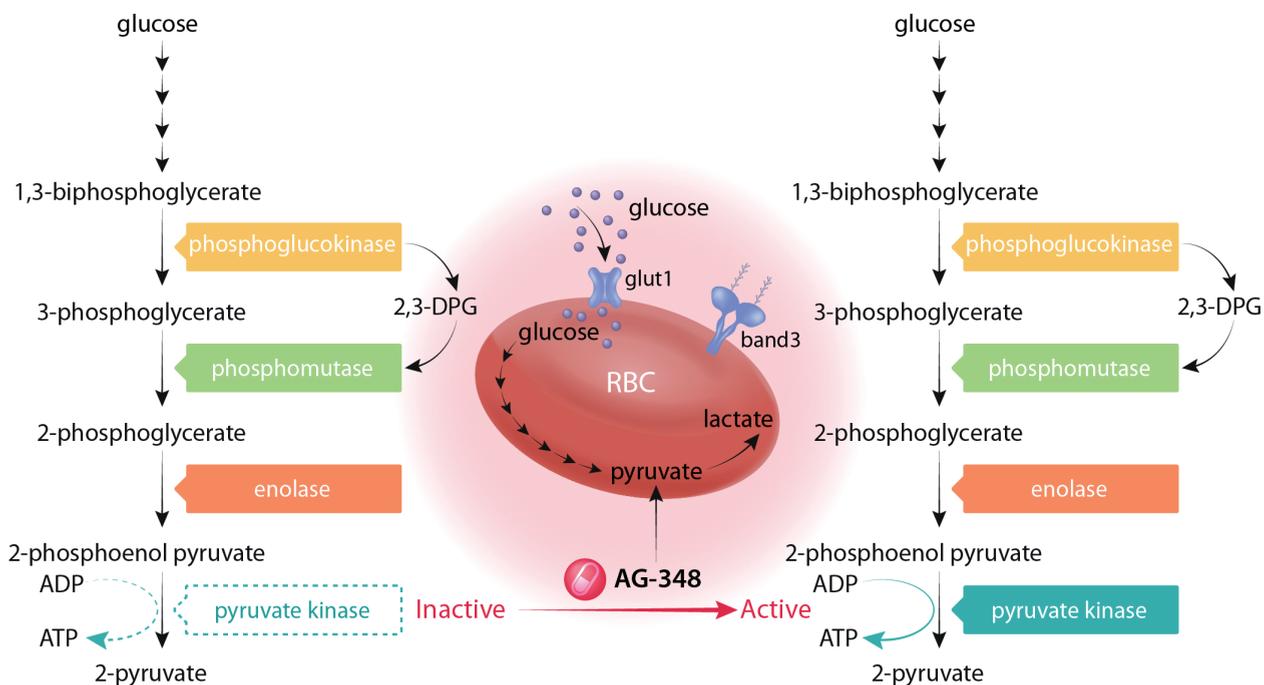
Figure 1. Schematic representation of the action of AG-348 on the enzyme pyruvate kinase in the final step of the glycolytic degradation of glucose in red blood cells (RBC). Glycolysis, the first step in cellular respiration begins with one glucose molecule (6 carbons) which is subsequently broken down in a series of enzymatic steps *via* ten enzymes into two pyruvic acid molecules (2 carbons) as well as releasing adenosine triphosphate and reduced nicotinamide adenine dinucleotide. The schemes on both sides (normal RBC metabolism and in pyruvate kinase deficiency) begin with the formation of 1,3 biphosphoglycerate after several enzymatic steps of phosphorylation and molecular arrangements. 2,3-bisphosphoglyceric acid (2,3-BPG), also known as 2,3-diphosphoglyceric acid (2,3-DPG) (allosteric affecter of hemoglobin), is a three-carbon isomer of the glycolytic intermediate 1,3-BPG. The final critical step in this reaction pathway involves the formation of pyruvate from phosphoenolpyruvate, a reaction catalyzed by the enzyme pyruvate kinase. The red cell in the center of the figure where these reactions occur is shown with two important receptors, Glut 1 and band 3, which play key roles in glucose transport and metabolism. The small molecule AG-348 penetrates the red cell and activates the enzyme pyruvate kinase.

Additionally, protein analyses using mass spectrometry suggest improvement in key glycolytic intermediates as a result of this treatment.[8]

Glycolytic pathways are also known to be regulated by band 3 in concert with hemoglobin-confirmation dependent transition from R to T states.[9] However, western blot analysis in this manuscript surprisingly shows no differences in band 3 concentrations in samples from PK-deficient patients compared to healthy controls. These findings suggest that the effects of AG-348 may occur in a band 3-independent manner, although a more comprehensive proteomic analysis in the presence and absence of AG-348 may still reveal changes in band 3 and its associated proteins at different time points following treatment.

Metabolic profiling of whole blood from some of the patients enrolled in this study from Rab *et al.* were carried out using liquid chromatography mass spectrometry (LC-MS/MS). The data confirmed the decrease in PK activity along with reduced levels of PEP, and the modest increase in 2,3-diphosphoglycerate (DPG). *Ex vivo* incubation of PK-deficient RBC however, increased PK activity and ATP production in a dose-dependent manner. Although 2,3-DPG levels were not systematically collected in this work, early *in vivo* experiments on RBC from mice treated with AG-348 showed modest (20%) decreases in 2,3-

DPG.[6] Because 2,3-DPG is quantitatively the most important organic phosphate in human RBC (being present in approximately four times the concentration of ATP), its measurement in those treated and untreated patients may better contribute to our understanding of the overall impact of this molecule on the metabolic pathways within RBC. Overall, *in vitro* assessment of AG-348 reported here in this manuscript by Rab *et al.* provides evidence that this molecule stimulates the activity of mutant PK-R enzymes, consistent with recently reported clinical responses to this drug in patients with PK deficiency.[10]

In an early investigation of a patient with a typical PK deficiency, elevated levels of 2,3-DPG in RBC with a corresponding right shifted oxygen equilibrium curve were found. This subject with a well-documented deficiency in PK had a marked increase in the concentration of 2,3-DPG (2.5-fold) and a 2.0-fold decrease in the oxygen affinity of whole blood.[11] In future studies it would be interesting to assess the role of AG-348 in another hemolytic condition, e.g., sickle cell anemia where both DPG and the RBC oxygen affinity are altered. Although no major glycolytic enzymatic abnormality is known to occur in this condition, several cytosolic and membrane alterations do occur, including changes in band 3 linked to glycolytic pathways which were recently reported in sickle cell disease mice.[12]

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The arrival of personalized genomics in bone marrow failure

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From the description of a first genetic bone marrow failure (BMF) syndrome (dyskeratosis congenita) in the literature[1] it took over 80 years to discover *FANCA* as the first gene causing BMF in humans.[2] Using a laborious process of positional cloning, which starts by finding patients with similar phenotype patterns, performing linkage analysis using polymorphic markers to finally localize the affected cDNA, a number of additional BMF genes have been identified in the following years. These include *DKC1* discovered in 1998,[3] *RPS19* in 1999[4] and *ELANE* in 1999,[5] to name just a few. Fueled by mapping the human genome and rapid advances in genome-wide technologies, a large proportion of genes predisposing to BMF and myelodysplastic syndromes (MDS) were identified using next-generation sequencing (NGS) technologies in the 21st century. Overall, roughly 100 genes have, thus, so far been associated with BMF/MDS syndromes. Classical inherited BMF syndromes can be categorized into Diamond-Blackfan anemia (DBA), Fanconi anemia (FA), severe congenital neutropenia (SCN), dyskeratosis congenita (DC), Shwachman-Diamond syndrome, and congenital thrombocytopenias (Figure 1A). From a biological perspective, all these entities are caused by loss of function of fundamental cellular pathways such as DNA repair, ribosome or telomere maintenance. In recent years we have witnessed a series of new discoveries on hereditary conditions predisposing to MDS.[6-8] The majority of these MDS predisposing genes are essential hematopoietic transcription factors and result in heterogeneous phenotypes that can bridge over to the classical BMF spectrum.

The landscape of germline genetic changes in BMF/MDS syndromes includes mutations in coding regions or non-coding alterations in promoters, regulatory elements, synonymous mutations, small deletions spanning single exons, and whole gene deletions (Figure 1B). Somatic alterations are mostly point mutations in leukemia driver genes and chromosomal gains or losses, but revertant uniparental disomies are also frequently found. Most BMF experts perform a phenotype-driven approach by first obtaining a family and medical history, followed by detailed physical examination and analysis of blood counts and bone marrow cellularity, morphology and cytogenetics. This approach most often guides the selection of an appropriate genetic testing platform to identify the causative gene (Figure 1C). For example, deletions (whole gene or intragenic) are a common cause of DBA and FA and for their detection, a well-established clinical copy number method is required (such as high resolution comparative genomic hybridization/single nucleotide polymorphism [CGH/SNP] array or multiplex ligation-dependent probe amplification [MLPA]). On the other hand, intronic non-coding mutations in *GATA2* might escape standard whole exome sequencing (WES) diagnostics because of coverage gaps, or simply due to missing expert knowledge about such lesions. The diagnostic genetic process for BMF and hereditary MDS is often challenging due to the continuous addition of novel genotypes and the evolving phenotype spectrum, but also the clinical need for a rapid turnaround