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Review

Activation of pyruvate kinase as therapeutic option for rare hemolytic anemias: Shedding new light on an old enzyme

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ABSTRACT

Novel developments in therapies for various hereditary hemolytic anemias reflect the pivotal role of pyruvate kinase (PK), a key enzyme of glycolysis, in red blood cell (RBC) health. Without PK catalyzing one of the final steps of the Embden-Meyerhof pathway, there is no net yield of adenosine triphosphate (ATP) during glycolysis, the sole source of energy production required for proper RBC function and survival. In hereditary hemolytic anemias, RBC health is compromised and therefore lifespan is shortened. Although our knowledge on glycolysis in general and PK function in particular is solid, recent advances in genetic, molecular, biochemical, and metabolic aspects of hereditary anemias have improved our understanding of these diseases. These advances provide a rationale for targeting PK as therapeutic option in hereditary hemolytic anemias other than PK deficiency. This review summarizes the knowledge, rationale, (pre)clinical trials, and future advances of PK activators for this important group of rare diseases.

1. Introduction

Pyruvate kinase (PK) is a glycolytic enzyme, which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, resulting in generation of adenosine triphosphate (ATP). Since red blood cells (RBCs) are fully dependent on anaerobic glycolysis for ATP production, PK plays a pivotal role in regulating RBC metabolism. Activation of PK and subsequently increasing the RBC's energy content is currently considered a new therapeutic option for various hereditary hemolytic anemias and is the subject of several clinical trials. This review will highlight the role of PK in hereditary hemolytic anemias, thereby providing a rationale for PK activators in the treatment of these diseases.

2. Pyruvate and PK in RBC metabolism

2.1. RBC function

During their normal 120-day lifespan, RBCs require energy to maintain vital cellular functions and fulfill their important task of oxygen delivery to tissues. These intrinsic functions include the maintenance of: (1) cellular metabolism and anaerobic glycolysis, (2) cellular hydration and homeostasis through functional activity of ion pumps in the RBC membrane, (3) antioxidant capacity to protect oxygen-carrying and iron-containing hemoglobin (Hb) molecules and other proteins from continuous oxidative stress and damage, (4) membrane phospholipid asymmetry to prevent phagocytosis, and (5) RBC shape and deformability of the cytoskeleton to facilitate the flow in (micro)vessels [1]. Mature RBCs lack a nucleus and cytoplasmic organelles including mitochondria, and, consequently, the ability to synthesize proteins. This makes RBCs entirely dependent on anaerobic glycolysis, and key regulatory enzymes including PK to generate sufficient amounts of the energy carrier molecule, ATP. Insufficient levels of ATP inevitably lead to a shortened RBC lifespan [2].

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2.2. Discovery of pyruvate and its role in RBCs

Glycolysis was the first catabolic pathway to be discovered, dating back to the second half of the 19th century (Fig. 1) [3,4]. Already in 1834, Pelouze extracted an unidentified organic acid apart from glutaric acid from tartaric acid, a distillate of grape extract. It was characterized by Berzelius in the following years and named "pyruvic acid", because it was distilled using heat ("pyro-" from Ancient Greek πῦρ (pûr, "fire")) [5,6]. The molecular structure of pyruvic acid was discovered in the 1870s, with the conjugate base pyruvate, $C_3H_3O_3^-$ [4]. In the following decades, the glycolytic [Embden-Meyerhof(-Parnas)] pathway was elucidated by biochemists Embden, Meyerhof and Parnas [7]. This discovery of this first metabolic reaction sequence was a breakthrough, acknowledging pyruvate as an important product of glycolysis [8].

Pyruvate is crucial for several energy-generating metabolic pathways and can be transformed in humans into different biochemical end products (Fig. 2). The six known metabolic fates of pyruvate are: (1) The reversible reduction to lactate, catalyzed by lactate dehydrogenase (LDH) with concomitant oxidization of nicotinamide adenine dinucleotide (NADH) to NAD^+ in the last step of anaerobic glycolysis, (2) The oxidation to acetyl coenzyme A, mediated by pyruvate dehydrogenase complex in cells with mitochondria including RBC precursors, to serve as main input of the tricarboxylic acid (TCA) cycle (*i.e.*, Krebs or citric acid cycle), (3) The anaplerotic conversion to oxaloacetate by pyruvate carboxylase, which replenishes TCA intermediates and is used for gluconeogenesis, (4) The transamination to alanine, especially in muscle tissue, which can be recycled in the liver and is also used for gluconeogenesis (Cahill cycle), (5) The malic enzyme-mediated conversion to malate, and (6) Acetate biosynthesis *via* hydrogen peroxide, pyruvate dehydrogenase complex or α-ketoglutarate dehydrogenase [9]. In addition, pyruvate has been acclaimed as an active scavenger of reactive oxygen species (ROS), namely hydrogen peroxide [10].

Regarding the role of pyruvate in RBCs, Wilbrandt reported in 1937 that RBCs exposed to fluoride shrank as a result of potassium (K^+) loss, but that this could be prevented by the addition of pyruvate to the medium [11]. Later, this water loss along with K^+ efflux was attributed to the Gardos channel, a calcium (Ca^{2+})-sensitive K^+ channel present in several tissues including RBCs, where it is involved in cell volume regulation [12]. It has been suggested that pyruvate would favor the synthesis of ATP by the RBC's NADH/NAD^+ redox system [13]. NAD^+, formed by oxidation of NADH in the conversion of pyruvate to lactate, could stimulate glycolysis and thereby ATP synthesis since it is used upstream in glycolysis by the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (Fig. 3) [14,15]. RBC's ATP stimulates the plasma membrane Ca^{2+} ATPase (PMCA), which maintains low intracellular Ca^{2+} concentration and thus prevents the Gardos channel from becoming active [13,16]. Maintenance of cellular hydration is critical for RBC survival, and disturbed cellular hydration may lead to premature RBC destruction (hemolysis). In dehydrated hereditary stomatocytosis (DHS type 1, also known as hereditary xerocytosis), for example, gain-of-function variants in *PIEZO1* encoding the nonselective mechanosensitive cation channel PIEZO1, lead to increased Ca^{2+} influx in RBCs, in turn activating the Gardos channel with subsequent dehydration and premature RBC destruction [17]. This historical example demonstrates that sufficient "pyruvic acid" is required to maintain RBC function, as it is essential for RBC energy metabolism.

2.3. PK and its role in glycolysis

PK has been highly conserved throughout evolution (Fig. 4). Its name is actually a misnomer, as it does not directly catalyze phosphorylation of pyruvate, but rather transfers a phosphate group from PEP to adenosine diphosphate (ADP) through substrate-level phosphorylation at the end of the Embden-Meyerhof pathway (Fig. 3) [18,19]. Thus, anaerobic glycolysis, favored under low-oxygen conditions, has a net production of two molecules of ATP per molecule glucose. In addition, unique and intriguing aspects of RBC glycolysis are regulation of nicotinamide adenine dinucleotide (NADH) levels and 2,3-diphosphoglycerate (2,3-DPG, also known as 2,3-bisphosphoglyceric acid or 2,3-BPG) levels through the Rapoport-Luebering shunt. NADH is required for the enzymatic reduction of methemoglobin to Hb, thereby reducing heme iron from the ferric (Fe^{3+}) to the ferrous form (Fe^{2+}), which can bind oxygen. 2,3-DPG is required for modulation of Hb-oxygen affinity, thereby oxygen release to tissues [20]. High-oxygen conditions on the other hand favor glucose metabolism through the hexose monophosphate pathway, also called pentose phosphate pathway (Fig. 3) [1,21]. This pathway recycles nicotinamide adenine dinucleotide phosphate (NADPH), powering the thiol-based antioxidant system to provide the cell with sufficient amounts of reduced glutathione (GSH) as scavenger of hydrogen peroxide [21]. Both pathways are linked as the first glycolytic intermediate, glucose-6-phosphate, enters the hexose monophosphate pathway (Fig. 3).

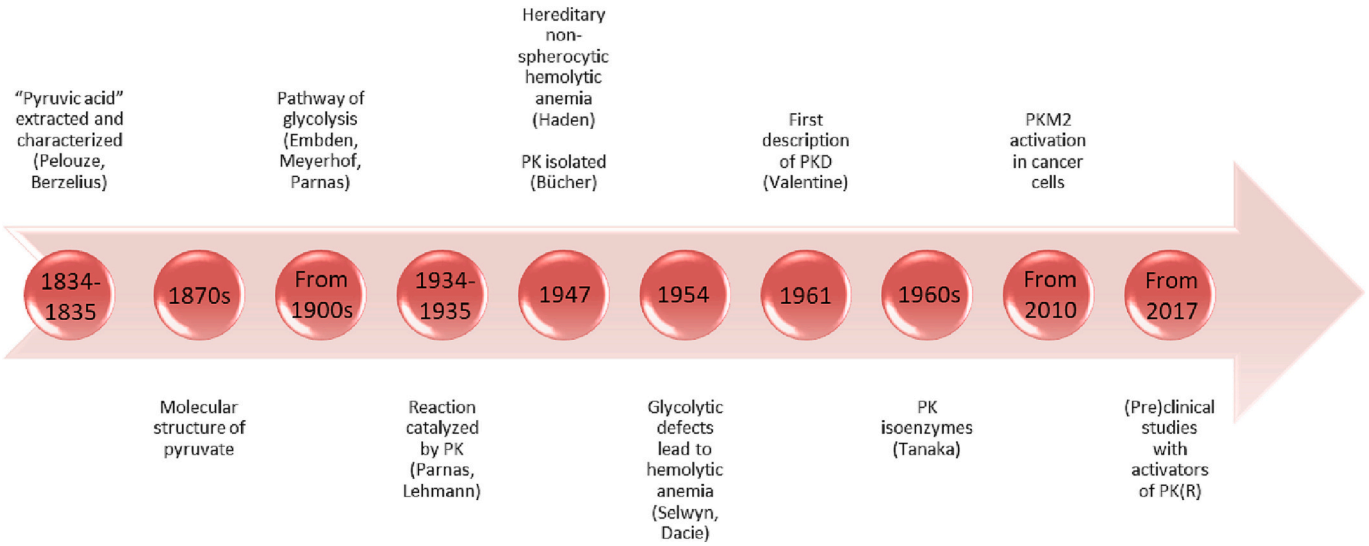

Fig. 1. Timeline of some key discoveries in studies of PK [3–7,22,32,40,53,54,56,136,137].
PK, pyruvate kinase; PKD, pyruvate kinase deficiency; PKM2, PK muscle isoenzyme 2; PKR, the PK isoenzyme unique to RBCs.

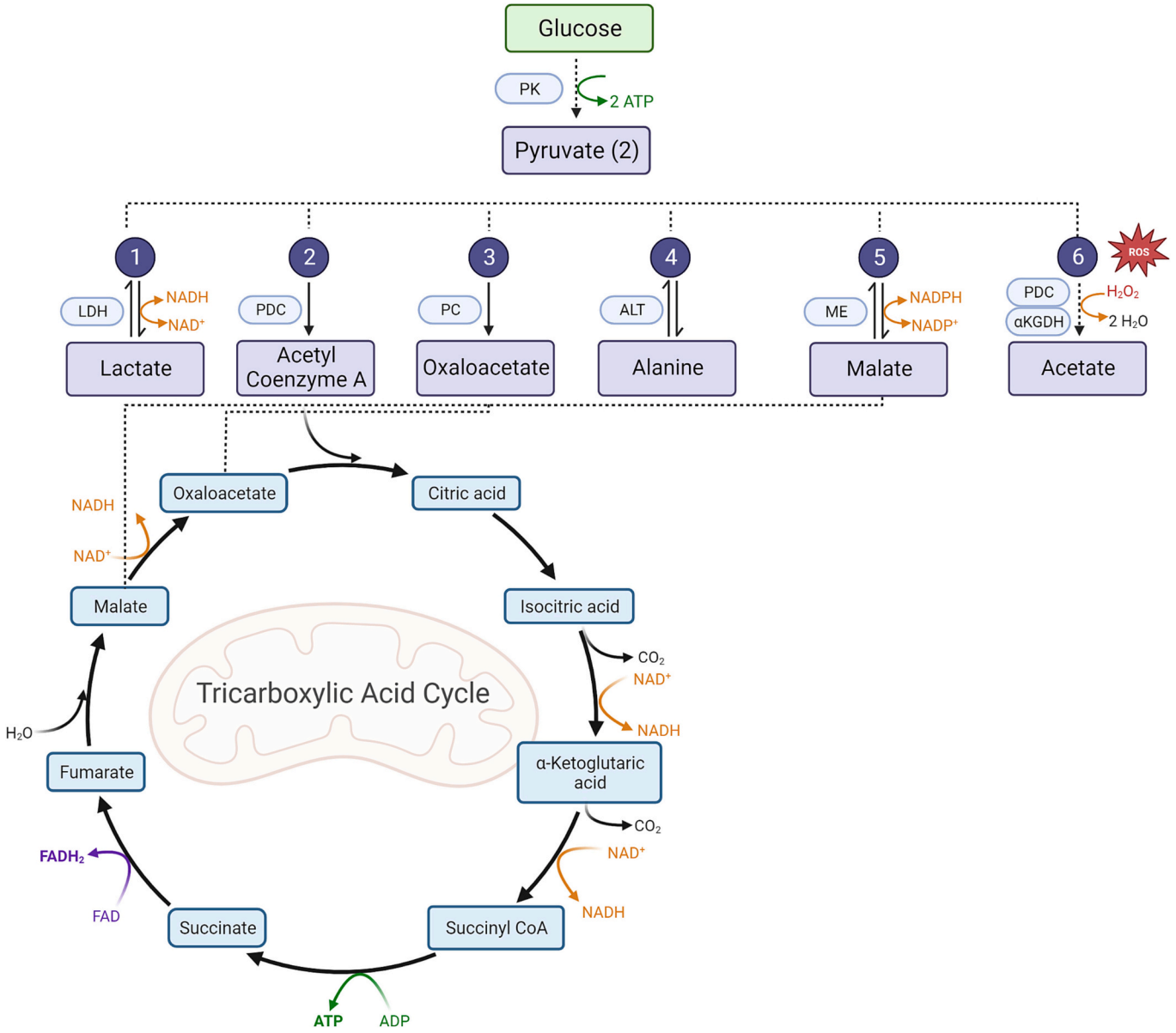

Fig. 2. Fate of pyruvate.
PK, pyruvate kinase; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; NADH/NAD^+, oxidized/reduced forms of nicotinamide adenine dinucleotide; PDC, pyruvate dehydrogenase complex; PC, pyruvate carboxylase; ALT, alanine aminotransferase; ME, malic enzyme; NADPH/$NADP^+$, nicotinamide adenine dinucleotide phosphate; αKGDH, α-ketoglutarate dehydrogenase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; H_2O, water; CO_2, carbon dioxide; ADP, adenosine diphosphate; FAD/$FADH_2$, oxidized/reduced flavin adenine dinucleotide. Created with BioRender.com

PK was first isolated in 1947; however, it was not until the 1960s that different PK isoenzymes were identified (Fig. 1) [22]. There are four PK isoenzymes expressed in human tissues, from two separate genes, PKLR and PKM. PKLR produces two isoenzymes: PKR, the PK isoenzyme unique to RBCs, and the liver-specific isoenzyme of PK (PKL). Both these proteins are produced from the *PKLR* gene by using alternative promotors. PK muscle isoenzyme 1 (PKM1), expressed in skeletal muscle, brain and heart, and PKM2, expressed in embryonic cells, but also widely distributed in adult tissues and proliferating cells including leukocytes and platelets, are alternative splice isoenzyme of the *PKM* gene [23]. During erythroid differentiation, erythroblasts gradually switch expression from the M2 to the R form. In healthy mature RBCs PKM2 is absent [24,25]. PKR consists of two distinct species: PK-R1 and PK-R2. The homotetramer PK-R1 (also called L′4) predominates in reticulocytes and young RBCs, whereas the heterotetramer PK-R2 (L2L′2) predominates in mature RBCs [26]. The enzymatic activity of PKR decreases with increasing RBC age [27]. The enzyme's active, tetrameric, high-affinity relaxed (R) state is stimulated by PEP and the allosteric activator fructose-1,6-bisphosphate (FBP), and requires cations magnesium (Mg^{2+}) and K^+. The inactive, low-affinity tight (T) state is favored by its product ATP [18,28]. The allosteric transition from the T state to the R state involves a combination of domain and subunit rotations coupled to a defined conformational change. This triggers distortion of the PEP binding site and results in active site architecture and the catalytic mechanism. Regulation mechanisms of PK activity are not yet fully elucidated, but one of the proposed models involves altered intracellular redox state and subsequent inhibition of the enzyme through oxidation of cysteine residues [29,30]. So, even though PK is one of the most conserved and abundant proteins of the evolution tree and its role in glycolysis is well understood, allosteric regulation mechanisms differ between isoenzymes and are still under investigation.

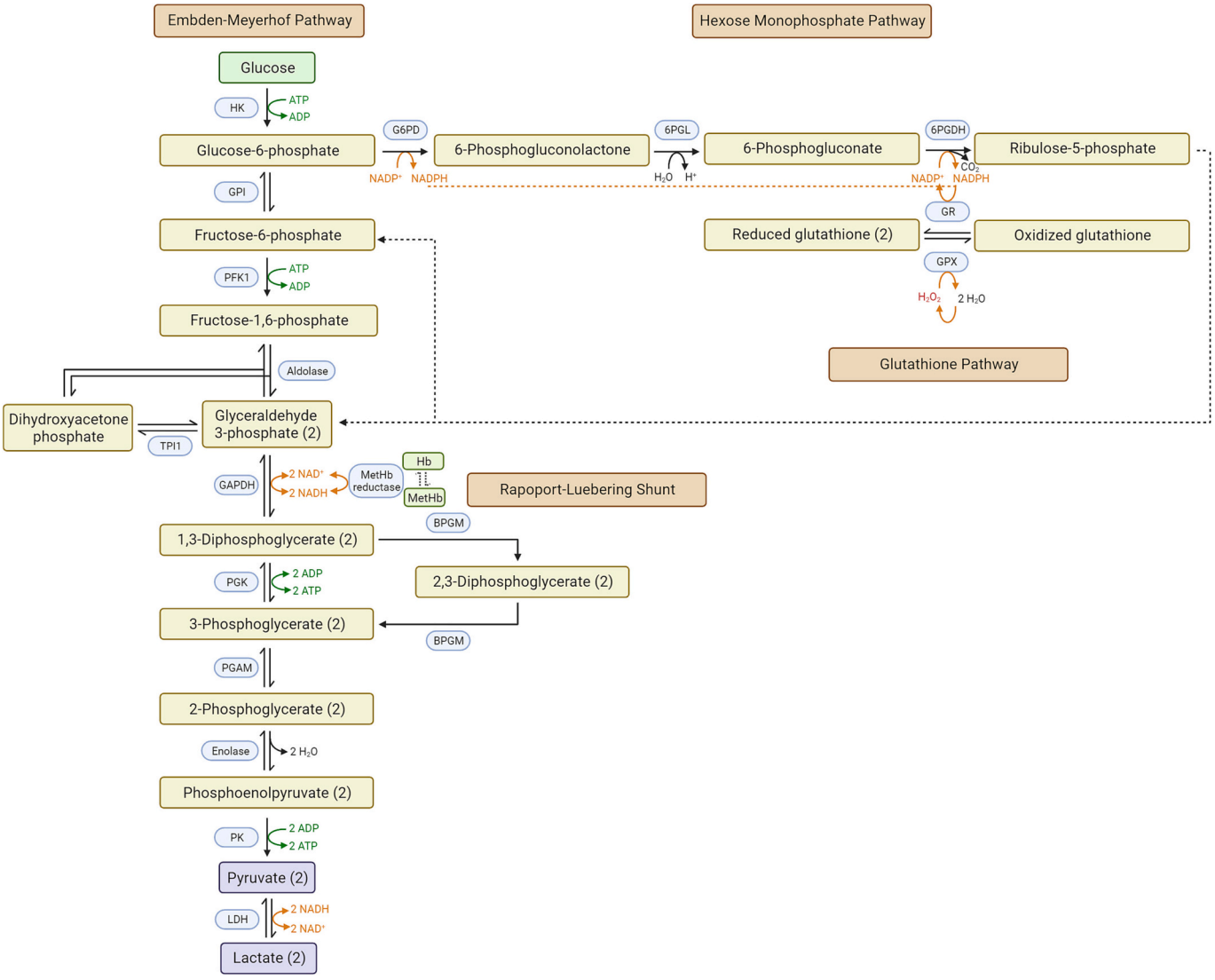

Fig. 3. Schematic glycolytic pathway of RBCs essential for energy metabolism.
HK, hexokinase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GPI, glucose-6-phosphate isomerase; PFK1, phosphofructokinase-1; TPI1, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NADH/NAD^+, oxidized/reduced forms of nicotinamide adenine dinucleotide; (Met) Hb, (met)hemoglobin; BPGM, bisphosphoglycerate mutase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; H_2O, water; PK, pyruvate kinase; LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; NADPH/$NADP^+$, nicotinamide adenine dinucleotide phosphate; 6PGL, 6-phosphogluconolactonase; H^+, hydrogen ion; 6PGDH, 6-phosphogluconate dehydrogenase; CO_2, carbon dioxide; GR, glutathione reductase; GPX, Glutathione peroxidase; H_2O_2, hydrogen peroxide. Created with BioRender.com

3. Development of PK activators

3.1. PKM2 activation in cancer therapy

Since the discovery of PK, research has focused on the PKM2 isoenzyme, whose dimeric, low-active form is overexpressed in many human cancer cells [31,32]. Cancer cells have an altered metabolism in which nutrients are divided between energy production and macromolecular biosynthesis to support cell growth and proliferation [33]. Unlike normal cells that catabolize glucose by the TCA cycle and oxidative phosphorylation in mitochondria, cancer cells produce energy predominantly and faster by converting glucose into lactate even in conditions of sufficient oxygen. This phenomenon is called aerobic glycolysis, also known as the Warburg effect, and used to support high metabolic demands [34]. It has been demonstrated that altered expression and activity of PKM2 has a key role in the Warburg effect [35]. The low-active, dimeric form of PKM2, whose expression is increased by oncoproteins and oxidative stress, supports the synthesis of glycolytic intermediates to fulfill biosynthetic requirements of cell growth and proliferation. In addition, dimeric PKM2 enhances flux through the hexose monophosphate pathway, and, thereby, the generation of NADPH. NADPH is involved in nucleotide synthesis and is used as reducing equivalent by glutathione reductase to cope with high levels of ROS [30]. Based on this, PKM2 is considered a new target for cancer treatment by using both inhibitors that block the catalytic activity of PKM2 (*e.g.*, shikonin and alkannin) or activators that enhance tetramerization of PKM2, thus suppressing the Warburg effect and tumorigenesis (*e.g.*, TEPP-46, DASA-58; as monotherapy or in combination with an inhibitory glucose analogue 2-deoxy-D-glucose) [36]. The first described PKM2 activators were discovered in a high-throughput screen of approximately 300,000 compounds [32,37]. Although many preclinical studies have reported that targeting the dual regulation of PKM2 could decrease growth and proliferation of cancer cells [32,38,39], none have advanced to clinical trials to date.

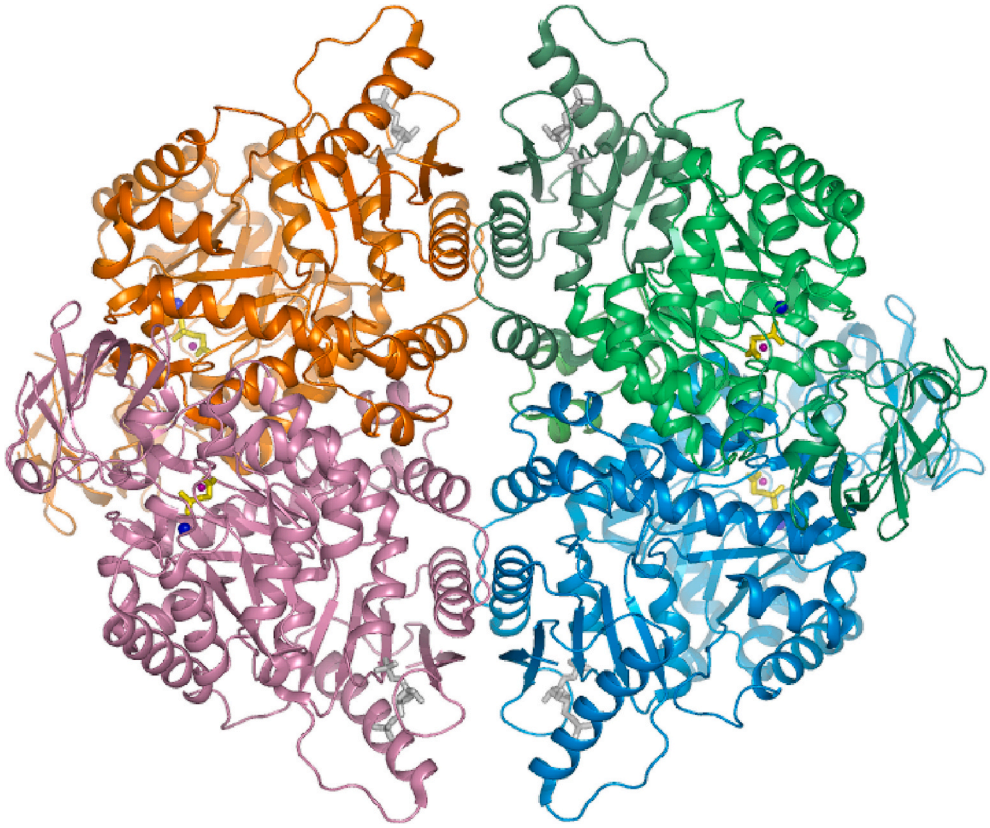

Fig. 4. Representative 3D-structure of tetrameric PK.
Figure adapted from Chapter 48 Erythrocyte Enzyme Disorders, Kaushansky K, Prchal JT, Burns LJ, Lichtman MA, Levi M, Linch DC. *Williams Hematology, 10e;* 2021.
Available at: https://accessmedicine.mhmedical.com/content.aspx?bookid=2962&sectionid=252528620
Accessed: March 23, 2023.

Table 1
Main preclinical study results of subjects treated with PK activators in hereditary hemolytic anemia.

Authors	Study population and design	Efficacy outcomes	Safety outcomes
Kung et al. [40]	Mitapivat		NR
	- *In vivo*: C57/BL6 mice, 7d (gavage)	- ↑PK activity, ↓2,3-DPG, ↑ATP	
	- *Ex vivo*: RBCs of HCs	- ↑PK activity, ↑ATP	
	- - *Ex vivo*: RBCs of patients with PKD	- ↑PK activity, ↓2,3-DPG, ↑ATP	
Rab et al. [45]	Mitapivat		NR
	- *Ex vivo*: RBCs of HCs ($n = 15$)	- ↑PK activity, ↑ATP	
	- *Ex vivo*: RBCs of patients with PKD ($n = 15$)	- ↑PK activity, ↑ATP, ↑PK thermostability	
Schroeder et al. [50]	Etavopivat		NR
	- *Ex vivo*: *SCD*, single dose ($n = 17$)	- ↓p50, ↓RBC sickling	
	- *In vivo*: non-human primates, 5d ($n = 4$)	- ↓2,3-DPG, ↑ATP	
Mattè et al. [47]	Mitapivat	- ↑markers of erythroid maturation, ↓ apoptosis	NR
	- *Ex vivo*: RBC precursors ($CD34^+$ cells) of patients with β-thalassemia	- ↑Hb, ↓circulating erythroblasts, ↓EPO, ↓ROS, ↑GSH/glutathione disulfide ratio, ↓markers of mitochondrial dysfunction, ↓liver/duodenal iron overload	
	- *In vivo*: $Hbb^{th3/+}$ mouse model of β-thalassemia, 21d (gavage) or 56d (diet)		
Rab et al. [46]	Mitapivat *Ex vivo*: RBCs or buffy coat-depleted blood of patients with SCD ($n = 7$-11)	↑PK activity, ↑PK thermostability, ↑ATP/2,3-DPG ratio, ↓RBC sickling	NR
Rab et al. [51]	AG-946 *Ex vivo*: buffy coat-depleted blood of patients with SCD ($n = 5$)	↑PK activity, ↑PK thermostability, ↑ATP/2,3-DPG ratio, ↓p50, ↓RBC sickling	
Quezado et al. [109]	Mitapivat *In vivo*: Townes SCD mouse model, 4w (diet)	↑ATP, ↓leukocytes, ↓ROS, ↓mitochondria retention	NR
Mattè et al. [123,138]	Mitapivat *In vivo*: band $4.2^{-/-}$ mouse model of HS	↑Hb, ↓reticulocyte count, ↓markers of hemolysis, ↓EPO, ↓spleen/mouse weight ratio, ↓PS-positive RBCs, ↓osmotic fragility	NR

d, days; RBC, red blood cell; HC, healthy control; PK(D), pyruvate kinase (deficiency); 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; NR, not reported; *n*, number of subjects treated with PK activator (excluding placebo); SCD, sickle cell disease; Hb, hemoglobin; EPO, erythropoietin; ROS, reactive oxygen species; GSH, glutathione; p50, partial oxygen pressure at which hemoglobin is 50% saturated; w, weeks; HS, hereditary spherocytosis; PS, phosphatidylserine.

3.2. Development of PKR activators

The therapeutic concept of PKR activation was based on the previously described small-molecule allosteric activators of PKM2 that bind in a pocket distinct from the endogenous FBP binding site [32,39]. The first-in-class, oral, twice daily, small-molecule agonist mitapivat (AG-348) that targets the pocket in PKR, but also PKL and PKM2, was identified by Agios Pharmaceuticals Inc. [40,41] Initially this therapy was developed to specifically target the underlying pathophysiology of patients with PK deficiency (PKD; OMIM 266200), a genetically defined RBC disorder with unmet needs. In preclinical and phase 1 trials, mitapivat has been shown to significantly increase activity of both wild type and numerous mutant forms of PKR, increasing ATP production and reducing 2,3-DPG levels by inducing enhanced glycolytic flux (ClinicalTrials.gov: NCT02108106 and NCT02149966) (Tables 1 and 2) [40,42–47]. In 2021 and 2022, Forma Therapeutics (now acquired by Novo Nordisk) published the first preclinical and phase 1 study results of etavopivat (FT-4202), a once-daily, oral, small-molecule allosteric activator of PK (NCT03815695) (Tables 1 and 2) [48–50]. Proof of concept without significant adverse events was demonstrated in controls treated up to 14 days with 7-day follow-up. Currently, novel highly potent activators of PK, such as AG-946, are being developed and evaluated in preclinical and early-phase clinical studies (NCT04536792) [51,52].

4. PK activation therapy in PKD

4.1. History of PKD

It was not until 1947 that Haden published the first case of hereditary nonspherocytic hemolytic anemia (Fig. 1) [53]. By then, most studies on RBC metabolism were performed to search for optimal RBC storage conditions for transfusion therapy. In 1954, the first association between impaired RBC glycolysis and hereditary hemolytic anemia was observed by Selwyn and Dacie in "atypical" hereditary nonspherocytic hemolytic anemias (Fig. 1) [54,55]. An increased rate of *in vitro* "autohemolysis" was found in some cases, that was not restored in the presence of excess glucose. RBCs of these cases were thus unable to utilize glucose at a normal rate. Furthermore, decreased ATP and increased 2,3-DPG levels of RBCs were found, and the addition of ATP reduced the rate of *in vitro* autohemolysis. These results prompted Valentine and coworkers to assess RBC enzyme activity of different glycolytic enzymes in families with hereditary nonspherocytic hemolytic anemias [56]. Eventually, this led to the discovery of PKD (Fig. 1). Nowadays, PKD is the canonical example of disturbed energy metabolism and the dependance on ATP of RBCs.

4.2. Pathophysiology of PKD

PKD is one of the most common causes of chronic hereditary nonspherocytic hemolytic anemia and is characterized by molecular and clinical heterogeneity; some patients with PKD only suffer from mild hemolysis, whilst others show signs of severe organ damage [57]. Complications of PKD include iron overload due to regular RBC transfusions and increased erythropoietic drive resulting in erythroferrone-mediated suppression of hepcidin and thus increased iron absorption [58,59]. The disease is inherited in an autosomal recessive manner and caused by variants in the *PKLR* gene, predominantly missense variants. Characterization of mutant proteins showed that amino acid substitutions can affect catalytic efficiency, thermostability, and response to allosteric effectors [60].

Mutant PKR decreases PK activity and stability, which leads to defective glycolysis and an inadequate production of ATP. Thus, RBC energy demands are not met. This affects RBC homeostasis and ultimately promotes premature clearance of RBCs in the spleen [61]. Whereas the exact mechanisms are unknown, these likely involve: (1) Increased RBC dehydration as a result of an increase in intracellular Ca^{2+} by the less active ATP-dependent PMCA which, in turn, activates the Gardos channel (as described above), (2) Disturbance of the entwined balance between ATP production and antioxidant systems

Table 2
Main clinical study results of subjects treated with PK activators in hereditary hemolytic anemia.

Authors	Study population and design	Efficacy outcomes	Safety outcomes
Yang et al. [43] NCT04000165	Mitapivat - Phase 1: HC, SAD ($n = 36$) - Phase 1: HC, MAD ($n = 36$)	↓2,3-DPG, ↑ATP	TEAEs mainly at doses ≥700 mg 1 TEAE grade ≥ 3 (abnormal liver function tests)
Schroeder et al. [50] NCT03815695	Etavopivat Phase 1: HC, single dose ($n = 6$)	↓p50, ↓2,3-DPG	NR
Forsyth et al. [49] NCT03815695	Etavopivat - Phase 1: HC, SAD ($n = 24$) - Phase 1: HC, 14d MAD ($n = 36$)	- ↓2,3-DPG, ↓p50 - ↓2,3-DPG, ↑ATP, ↓p50	- 21% ≥1 TEAE - 42% ≥1 TEAE (grade 1; mostly headache), no SAEs
Iyer et al. [52] NCT04536792	AG-946 Phase 1: HC, SAD (4 cohorts of $n = 6$) Phase 1: HC, 14d MAD (2 cohorts of $n = 6$)	- ↓2,3-DPG, ↑ATP - ↓2,3-DPG, ↑ATP	- 10% ≥1 TEAE (grade 1) - 24% ≥1 TEAE (mostly grade 1) 1 non-treatment related SAE
Grace et al. [84] NCT02476916	Mitapivat (DRIVE-PK) Phase 2: adults with *PKD* not regularly transfused, 50 mg or 300 mg twice daily, 24w ($n = 52$)	50% Hb increase ≥1 g/dL (all without two non-missense or homozygous R479H variants)	100% ≥1 AE (mainly low-grade, transient, incl. headache, insomnia, nausea) 18 SAEs (pharyngitis and hemolytic anemia in $n \geq 2$)
Al-Samkari et al. [86] NCT03548220	Mitapivat (ACTIVATE) Phase 3: adults with *PKD* not regularly transfused with ≥1 non-R479H variant, MAD 5-20-50 mg twice daily, 24w ($n = 40$)	40% Hb increase ≥1.5 g/dL ↓markers of hemolysis Improved patient-reported outcomes	58% ≥1 TEAE 3 TEAEs grade ≥ 3 1 treatment-related SAE (musculoskeletal pain)
Glenthøj et al. [87] NCT03559699	Mitapivat (ACTIVATE-T) Phase 3: adults with *PKD* regularly transfused with ≥1 non-R479H variant, 16w dose-optimization and 24w fixed-dose period ($n = 27$)	37% had a reduction in transfusion burden by ≥33%	100% ≥1 AE (mainly grade 1-2) 2 TEAEs grade ≥ 3 were treatment-related (joint swelling, AST increase) 3 non-treatment related SAEs

HC, healthy control; SAD, single ascending dose; MAD, multiple ascending dose; *n*, number of subjects treated with PK activator (excluding placebo); 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; TEAE, treatment emergent adverse events; NR, not reported; d, days; PK(D), pyruvate kinase (deficiency); SCD, sickle cell disease; p50, partial oxygen pressure at which hemoglobin is 50% saturated; (S)AE, (serious) adverse events; Hb, hemoglobin; w, weeks; AST, aspartate aminotransferase.

including the NADH/NAD^+ redox system to respond to (patho)physiological stresses (Fig. 3), (3) Tagging of RBC surfaces with an 'eat me' signal for macrophages by exposing the phospholipid phosphatidylserine (PS). Phospholipids are asymmetrically distributed in the lipid bilayer of cell membranes, and this asymmetry is maintained by enzymes. As a net result of less ATP-dependent flippase activity and more Ca^{2+}-activated scramblase activity, PS can be 'scrambled' to the outer layer, but not 'flipped' back to the inner layer, so that PS exposed on RBC surfaces can trigger macrophages to remove them from the blood stream, and (4) ATP-depletion-mediated changes in RBC membrane integrity and deformability. ATP is essential in regulatory cascades involving phosphorylation and ion transport mechanisms in RBC membranes, such as the membrane-bound ATPases exchanging sodium (Na^+) for potassium (K^+) and PMCA [21,45,62–66]. RBCs deform temporarily when they flow in narrow vessels. Shear stress triggers ATP release through Pannexin1 channels upon Ca^{2+} influx evoked by the mechanosensitive Piezo1 channel. The normal biconcave shape of RBCs is maintained by multiprotein complexes including Band 3-ankyrin linkage, which bind the membrane to the skeleton [67,68]. With increasing periodic squeezes, intracellular ATP is reduced. Consequently, there is decreased phosphorylation of protein 4.1R by the ATP-dependent Protein kinase C, which increases the affinity of the interaction with Band 3 in the RBC membrane, preventing ankyrin from binding to Band 3. In the end, ATP depletion likely will lead to progressive changes in morphology and gradual loss of deformability and, eventually, hemolysis [69,70].

In addition to insufficient ATP levels, PK deficiency in RBCs leads to a buildup of glycolytic intermediates, including 2,3-DPG [71,72]. Increased 2,3-DPG levels shift the oxygen-dissociation curve to the right, lowering Hb-oxygen affinity. This facilitates a better release of oxygen to tissues, ameliorating symptoms of reduced Hb levels [73].

Ineffective erythropoiesis, *i.e.*, the imbalance between erythroid proliferation and differentiation, has been described as an additional pathophysiological consequence of PKD [74,75]. The exact underlying mechanism is unclear. However, decreased antioxidant response in a PK deficient cell line has been associated with increased apoptosis of RBC precursors [76]. It has been hypothesized that less available pyruvate, required for the TCA cycle in RBC precursors, leads to increased consumption of other sources of anaplerotic carbon including glutamine. As glutamine is necessary for the generation of GSH, and GSH is a cofactor for GSH peroxidase 4 (GPX4), an antioxidant that plays a major role in mitophagy during reticulocyte maturation, this could hamper the final steps of erythropoiesis in PKD [77]. This emphasizes the need of functional glycolysis, also in the context of anti-oxidative response. Possibly related to ineffective erythropoiesis, a striking difference between PKD and most other hereditary hemolytic anemias is the drastic reticulocytosis post-splenectomy. Reticulocytes require much more ATP compared to mature RBCs but can rely on the mitochondrial TCA cycle and oxidative phosphorylation. However, in the hypoxic splenic environment, reticulocytes of patients with PKD must rely on glycolysis, which does not meet its ATP demand, leading to hemolysis. After splenectomy, and in contrast to other hereditary hemolytic anemias such as hereditary spherocytosis, reticulocyte counts increase in PKD [78]. Overall, PKD is the canonical example what happens with ATP-depleted RBCs.

4.3. Rationale for PK activation therapy in PKD

Whilst the underlying molecular cause of PKD has been identified more than half a century ago, the progress in translating this knowledge into improved patient care has hampered since [57,79,80]. Treatment options are mostly supportive and include splenectomy, iron chelation and RBC transfusions. Experience with hematopoietic allogeneic stem cell transplantation and experimental gene therapy in PKD is limited [81–83]. The development of PK activators, which specifically target the underlying pathophysiology of PKD, demonstrated an increase in PK activity, ATP levels and RBC deformability *ex vivo* (Table 1) [45]. In a phase 2 clinical trial in non-transfused patients with PKD (NCT02476916), 50% of the patients treated with mitapivat had an increase in Hb level of greater than 1.0 g/dL (Table 2) [84]. This was accompanied by a decrease in markers of hemolysis. Notably, Hb responses (defined as an increase of >1.0 g/dL from baseline at more than 50% of the assessments in the core period) were only observed in patients who had at least one *PKLR* missense variant whereas patients with two non-missense variants or homozygous p.(Arg479His) splicing variants (most common in the Amish community) did not show this response in Hb levels. Moreover, a relationship between PK protein level and Hb response was observed in this study, suggesting that some level of PK protein is required for allosteric activation by mitapivat. Generally, twice daily mitapivat was well tolerated with the majority of adverse events of Grade 1 or 2, most commonly being headaches, insomnia and nausea. Several serious adverse events were reported, including hemolysis related to abrupt drug withdrawal, hypertriglyceridemia and osteoporosis, the latter being potentially related to the reversible mild aromatase inhibitor effect of mitapivat [84]. However, a large-pooled analysis showed that bone mineral density remained stable over time in adult patients with PKD on long-term treatment with mitapivat [85]. Recently, with the completion of two successful phase 3 clinical trials demonstrating the safety and efficacy of mitapivat therapy in PKD (NCT03548220, NCT03559699) (Table 2) [86,87], the FDA and EMA approved mitapivat as first-disease modifying therapy for hemolytic anemia in adults with PKD (Pyrukynd®, 2022). Main results of these phase 3 trials were the significant increases in Hb level, the decrease in markers of hemolysis, and improved patient-reported outcomes. In patients with PKD who regularly received RBC transfusions, a reduction in transfusion burden was found. In addition, no new safety signals were identified. The most common treatment-emergent adverse events included transaminase increase, headache and nausea [86,87]. Extension studies are ongoing (NCT03853798), demonstrating improvements in patient-reported outcomes and iron overload [88,89]. Studies of mitapivat in pediatric participants are initiated (NCT05144256, NCT05175105). In general, the past few years the development of PK activators has led to a more rapid change in the therapeutic landscape of patients with PKD than in the many decades before.

5. PK activation therapy in thalassemias

5.1. Pathophysiology of thalassemias

Thalassemias are a group of recessively inherited disorders of Hb, first described in 1925 by Cooley [90]. Thalassemias are characterized by an imbalance in the quantity and/or quality of α-chains in α-thalassemia (OMIM 604131), or β-chains in β-thalassemia (OMIM 613985). This imbalance in the α- to β-globin ratio leads to precipitation and aggregation of the excess globin chains, causing oxidative damage to the RBC [91]. This subsequently results in a phenotype marked by ineffective erythropoiesis, hemolytic anemia and iron overload [91]. Treatments are mainly supportive and include RBC transfusions, iron chelation therapy and in some cases splenectomy [92]. Despite significant advances in treatments (*e.g.*, luspatercept to reduce transfusion need, hematopoietic stem cell transplantation for pediatric patients, and clinical trials in gene therapy) [93,94], continued challenges remain in the management of transfusion-dependent and non-transfusion-dependent thalassemias due to limited access, costs and side effects [92,95].

5.2. Rationale for PK activation therapy in thalassemia

One novel treatment option in development for thalassemia is activation of PK in RBCs, thereby enhancing glycolysis and increasing the availability of ATP. In a preclinical study, RBCs from thalassemia patients were found to have a decreased PK/hexokinase (HK) ratio,

indicating a relative shortage of PK activity when corrected for RBC age including the presence of reticulocytosis, even though the protein itself was not mutated. *Ex vivo* treatment with mitapivat increased PK activity and ATP levels [44]. Since excess globin chains are cleared by ATP-dependent proteolytic mechanisms, it was hypothesized that PK activators could improve the ability of thalassemic RBCs to survive in the setting of ongoing oxidative damage [96]. In the $Hbb^{th3/+}$ mouse model of β-thalassemia, a significant decrease in the level of ROS associated with an increased GSH/glutathione disulfide ratio was observed (Table 1). However, it remains to be established how PK activation exactly results in increased RBC antioxidant capacity *via* the intertwined metabolic pathways (Fig. 3). In addition, probably in the same way as previously suggested for PKD, treatment with mitapivat led to amelioration of hemolytic anemia in the β-thalassemia mouse model. Also, markers of ineffective erythropoiesis (*e.g.*, serum erythropoietin, circulating RBC precursor count) and iron homeostasis (*e.g.*, erythroferrone, hepcidin) improved [47]. Since studies have shown conflicting results on the intracellular 2,3-DPG level in thalassemic RBCs, the effect of modulating intracellular 2,3-DPG levels by PK activators should be evaluated [97–100]. In a phase 2, open-label trial of mitapivat in adult patients with α- or β-thalassemia who were non-transfusion-dependent (NCT03692052), sustained improvements in Hb level, markers of hemolysis and erythropoiesis were observed with a safety profile consistent with that of previously published studies concerning mitapivat (Table 2) [91,101]. Two phase 3 randomized controlled trials with mitapivat treatment in patients with transfusion- and non-transfusion-dependent α-thalassemia or β-thalassemia are currently ongoing (NCT04770753, NCT04770779) (Table 2) [102]. As for etavopivat, currently a trial on patients with thalassemia or sickle cell disease (SCD) is ongoing (NCT04987489) [103]. Although no results on the effect of etavopivat in patients with thalassemia have yet been published, the data generated to date provide a strong rationale for PK activation therapy in patients with thalassemia.

6. PK activation therapy in SCD

6.1. Pathophysiology of SCD

SCD (OMIM 603903) is a debilitating hereditary RBC disorder, with the first identification of sickle shaped RBCs by Irons and Herrick in 1910 [104]. SCD is characterized by the presence of sickle hemoglobin (HbS). HbS is encoded by a single point variant of the β-globin gene changing glutamic acid to valine in codon 6. In homozygotes and compound heterozygotes, deoxygenation of the mutant HbS leads to polymerization, resulting in sickled RBCs. HbS polymerization causes RBC membrane injury resulting in chronic hemolysis and vasculopathy, and initiates a complex pathophysiological cascade including roles of cellular adhesion, inflammation and oxidative stress [105]. Eventually, vascular occlusion leads to acute and painful episodes, the hallmark of SCD and responsible for the major clinical disease burden, particularly in young patients. Due to the complexity and heterogeneity of SCD, a wide variety of therapeutic targets are evaluated, such as HbS polymerization, RBC adhesion and amino acid disturbances in the RBC. Moreover, allogenic stem cell transplantation has increasingly become an important curative treatment option, and trials with gene therapy and gene editing are ongoing [106]. To this date, next to hydroxyurea which increases fetal Hb and reduces HbS polymerization, only recently a few new therapies were approved by the FDA and EMA.

6.2. Rationale for PK activation therapy in SCD

While absolute levels of PK activity are elevated in RBCs of patients with SCD, PK/HK ratio and PK thermostability are decreased compared to RBCs of controls, indicating a relative deficiency in PK activity when corrected for RBC age [46]. Although ATP levels appear to be normal in the total fraction of SCD RBCs, decreased ATP levels were found in reticulocyte-poor RBC fractions [107]. Moreover, increased ATP consumption has been demonstrated in Berkeley SCD mouse models, although whole blood levels of ATP were higher in Townes SCD mice compared to control mice [108,109]. Decreased ATP has been associated with increased ROS formation, PS exposure and an increased number of irreversibly sickled RBCs [107,108]. The higher levels of intracellular 2,3-DPG also play a pivotal role in the pathophysiology of SCD, since it lowers Hb-oxygen affinity and is thus directly associated with RBC sickling due to promoting HbS polymerization and decreasing HbS solubility upon deoxygenation [46,110]. These sickled RBCs are more prone to hemolysis. Indeed, the antisickling effect of 2,3-DPG depletion *ex vivo* was shown as therapeutic potential decades ago [111]. Less sickled RBCs were observed by microscopy in 2,3-DPG depleted SCD RBCs treated with glycolate, thereby generating glycolate-2-phosphate as activator of 2,3-DPG phosphatase. However, obstacles to inducing 2,3-DPG depletion *in vivo* were reported, including the nonpenetrating nature of glycolate-2-phosphate and toxic concentration of glycolate used. Taken together, the rationale for activation of wild type PK as a treatment option in SCD is based on restoring PK-activity and -stability, causing a beneficial increase in ATP production, as well as decreasing the level of intracellular 2,3-DPG [46]. Indeed, treatment of Townes SCD mice with mitapivat increased ATP levels, decreased extramedullary hematopoiesis, leukocytosis, mitochondrial retention and oxidative stress in RBCs (Table 1) [109]. In clinical trials, mitapivat has been evaluated in two phase 1-2 trials of patients with SCD [NCT04000165, EudraCT 2019-003438-18 (the ESTIMATE study)] (Table 2) [112,113]. Findings suggest safety and efficacy on the short term. Effects include the proposed increase in ATP level and decrease in 2,3-DPG level, accompanied by improvements in markers of hemolysis and sickling. The 6- to 8-week treatment duration of these studies does not allow yet for an extensive evaluation of the effect of mitapivat on clinical complications including vaso-occlusive episodes. Extension studies are ongoing, and a phase 2/3 trial in SCD is currently enrolling (NCT05031780) [41]. In addition, *ex vivo* treatment of RBCs from patients with SCD with another activator, AG-946, shows promising first results and a phase 1 trial with AG-946 in patients with SCD is ongoing (NCT04536792) [51]. Similar potential benefit of PK activator therapy in SCD was reported for etavopivat in a phase 1 study [114]. Phase 2/3 studies of etavopivat treatment in patients with SCD are ongoing (NCT04624659, NCT04987489). Preliminary results show beneficial effects such as a rapid increase in Hb level alongside a decrease in absolute reticulocyte count and indirect bilirubin levels [114,115]. Even though absolute PK activity is increased in patients with SCD due to reticulocytosis, PK activation therapy is promising to restore pathophysiological effects of relative PK deficiency.

7. PK activation therapy for other hereditary hemolytic anemias

7.1. Hereditary hemolytic anemias

More than 150 years ago, the first case descriptions of various abnormal RBCs detected by light microscopy were described in scientific literature [116–118]. We now know that hereditary hemolytic anemias encompass a heterogeneous group of disorders characterized by premature destruction of RBCs. Causes of hereditary hemolytic anemias include enzyme disorders [*e.g.*, PKD, glucose-6-phosphate dehydrogenase deficiency (OMIM 305900), glucose phosphate isomerase deficiency (OMIM 613470)], Hb disorders (*e.g.*, thalassemia, SCD) or RBC membrane disorders [*e.g.*, hereditary spherocytosis (HS) (OMIM 182900, 616649, 270970, 612653, 612690), hereditary elliptocytosis (OMIM 611804, 130600), or DHS (OMIM 194380, 616689)] [119]. Symptoms comprise variable degrees of acute or chronic hemolysis and ineffective erythropoiesis with acute and long-term complications including lifelong anemia, the formation of gall stones, increased risk for thrombotic complications, osteoporosis and iron overload [58]. Depending on disease severity, non-curative therapies include

splenectomy, associated with an increased risk of infectious and thrombotic complications, and RBC transfusion therapy, with the risk of iron overload. In spite of the diverse underlying causes, the group of hereditary hemolytic anemias have common aspects of hemolysis and thereby cellular energy demands [54].

7.2. Rationale for PK activation therapy in hereditary hemolytic anemias

Despite the fact that in some hereditary hemolytic anemias, the pathophysiological mechanisms are not completely understood yet, it is increasingly recognized that PK may play a key role because of generally increased cellular (oxidative) stress, glucose and energy utilization in these anemias [41,54]. In addition, the PK/HK ratio and/or PK thermostability have been shown to be compromised in patients with various forms of hereditary hemolytic anemia, including HS and DHS [44,120]. Impaired PK function could be related to impaired structural RBC membrane integrity, and consequently disrupted interactions between RBC membrane components and organized multi-enzyme complexes, including PK. This may result in local subcellular deficiency of PK [120,121]. Another factor explaining impaired PK function is oxidative stress; the catalytic domain of PK contains a number of cysteine residues that are easily oxidized, thereby reducing enzymatic activity of the enzyme [30,122]. Given the fact that PK activators increase the activity of wild type PK, the aforementioned beneficial effects of increasing ATP and reducing ineffective erythropoiesis can also apply to more types of hereditary hemolytic anemia.

Interestingly, a preclinical study with a Band $4.2^{-/-}$ mouse model of HS showed that mitapivat ameliorated hemolytic anemia, showing noninferiority of mitapivat compared with splenectomy [123]. Since HS is a genetically and phenotypically heterogeneous disorder, these interesting results in Band 4.2-/- mice should not be considered representative for all the various forms of HS, including more commonly occurring forms such as those due to ankyrin or β-spectrin defects. Next to HS, PK activation therapy might be considered in other RBC membrane disorders such as severe cases of hereditary elliptocytosis and hereditary pyropoikilocytosis [124].

In addition to PKD, we hypothesize that PK activation could be beneficial to compensate for impaired glycolysis due to other enzymes involved in the Embden-Meyerhof pathway, like glucose phosphate isomerase deficiency (Fig. 3). Enzyme deficiencies affecting the ATP-dependent synthesis of GSH, such as glutamate cysteine ligase deficiency, may also benefit from ATP generated by PK activation. Importantly, depending on the disorder, it remains to be established if and how stimulating the Embden-Meyerhof pathway and ATP generation through PK activation influences the intertwined energy, antioxidant, and other RBC metabolic pathways. Metabolomics and further analysis on the control of the balance between the Embden-Meyerhof and hexose monophosphate pathway could therefore be useful to unravel the (altered) glycolytic flux in RBCs, and to unravel the direct pathophysiological effects of ATP depletion and 2,3-DPG levels on RBC survival [125]. Moreover, concerns have been raised on the potential clinical effect of PK activators on Hb-oxygen affinity. One could argue whether any Hb increase obtained from reducing hemolysis is continuously able to (at least) compensate for reduced oxygen delivery in the microcirculation, or if in situations with fluctuating Hb levels, *e.g.* in the context of hemolytic or aplastic crises, such a treatment may be associated with an increased risk of tissue hypoxia and its sequelae [126]. So far, no such adverse events have been reported. However, this remains to be further elucidated, especially before PK activators are used in diseases that are known to show decreased 2,3-DPG levels such as DHS type 1 [127].

8. Future considerations

PK activation could be a therapeutic target for hypoplastic and hyporegenerative anemias in which decreased or ineffective erythropoiesis is present due to genetic alterations in hematopoietic stem cells. Anemias of interest in which decreased PK levels were found are for example Diamond-Blackfan anemia, congenital dyserythropoietic anemia type II and low-risk (LR) myelodysplastic syndrome (MDS) [128–131]. Phase 2 studies of treatment with AG-946 (NCT05490446) and etavopivat (NCT05568225) are currently recruiting patients to establish proof of concept and dose-selection in (LR-)MDS [132]. Relative deficiency of PK has also been described in other hematologic disorders, including acute leukemia, sideroblastic anemia and polycythemia vera [133]. In a broader sense, since PK activators also activate PKM2 its use may be investigated in non-hematological conditions that are associated with increased 2,3-DPG levels, as seen in some liver diseases, or (diabetic) nephropathy [134,135]. In conclusion, it seems reasonable to assume that the studies on PK activators for different disorders will be expanded further, prompted by more in-depth, later phase and follow-up studies.

9. Summary

Elucidation of metabolic mechanisms and pathophysiology of hereditary hemolytic anemias, especially of PKD, has provided tremendous insight into the essential role of PK for RBC energy metabolism and cellular homeostasis. The recent development of PK activators, mitapivat and etavopivat, has opened a new, encouraging potential scenario for treatment of an increasing number of hereditary hemolytic anemias. Patients with these disorders all show signs of lifelong hemolysis and/or ineffective erythropoiesis and are at risk for a diverse set of clinical symptoms and severe complications. PK activators, approved for adults with PKD and as investigational therapy for patients with various other hereditary hemolytic anemias, have thus the potential to substantially improve their disease course. In future, more studies on PK and PK activators in other diseases are needed to guide future advances.

Today, PK sheds new light on RBC metabolism in rare anemias.

Practice points

- Mitapivat is an approved targeted therapeutic option for adult patients with PKD.
- First results of clinical trials with PK activators in thalassemia and SCD demonstrate safety and efficacy.
- A preclinical study on a band $4.2^{-/-}$ HS mouse model showed that mitapivat ameliorated hemolytic anemia.

Research agenda

- Long-term safety and efficacy studies of PK activators in hereditary hemolytic anemias.
- The potential role of PK activators in hypoplastic and hyporegenerative anemias should be investigated.

Authors' contributions

M.v.D and R.v.W. conceptualized and wrote the manuscript; J.d.W., M.B., K.K., A.G., M.R. and E.v.B. revised the manuscript critically. All authors approved the final version submitted.

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