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CORRESPONDENCE

Glycolytic activity and in vitro effect of the pyruvate kinase activator AG-946 in red blood cells from low-risk myelodysplastic syndromes patients: A proof-of-concept study

To the Editor,

Lower-risk myelodysplastic neoplasms (LR-MDS) are characterized by anemia due to ineffective erythropoiesis. The latter is sustained by increased erythron iron turnover along with impaired red blood cell (RBC) utilization, but also by a disrupted metabolism.[1–3] Glycolysis is the prominent metabolic cascade in RBCs and is currently targeted with pyruvate kinase (PK) activators in clinical trials in rare anemias. AG-946 is an investigational, small-molecule, potent allosteric activator of PK with the potential to enhance RBC functionality and survival by stimulating glycolysis and to improve differentiation of erythroid cells in bone marrow.[4] The drug may thus improve anemia caused by ineffective erythropoiesis even in MDS, and a clinical trial is ongoing.[5]

To characterize RBC metabolism in anemic patients with LR-MDS and the in vitro effect of AG-946, we enrolled 66 adult LR-MDS subjects (LR-MDS as per IPSS/IPSS-R and Hb < 11 g/dL), within the observational and biologic study CYTOPAN [NCT05931718] (Supplementary methods). Briefly, enzymatic activities (hexokinase [HK], glucose phosphate isomerase [GPI], phosphofructokinase [PFK], aldolase [ALD], triose phosphate isomerase [TPI], glyceraldehyde phosphate dehydrogenase [GAPD], phosphoglycerate kinase [PGK], and PK) and ATP levels were evaluated in peripheral blood by the standard spectrophotometric method according to Beutler et al (PK and HK in all 66 patients, the other enzymes in 5, ATP levels in 56). RBC deformability by Osmoscan analysis was performed in six patients. In the in vitro study, samples from further 10 patients and five healthy controls were incubated for 6 and 24 h at 37°C in the presence or absence of AG-946 (1, 5 and 50 μM). Thereafter, enzymatic activities (HK, PK) and ATP levels, Osmoscan analysis, and metabolomics were evaluated. A patient with hereditary PK deficiency was also tested as positive control. All samplings were performed after at least 4 weeks from the last RBC transfusion to avoid contamination.

As shown in Table S1, LR-MDS patients were mainly elderly males and mostly belonged to IPSS low (68%) and IPSS-R very low/low groups (76%). The majority had MDS with low blast (MDS-LB, 54%) according to WHO 2022, followed by MDS-SF3B1 (35%), and del5q syndrome (11%). At sampling, 56% were transfusion dependent, two of three were receiving recombinant erythropoietin, and one of three iron chelation with deferasirox due to iron overload. PK activity levels were reduced in 23 cases (35%; normal reference range 11.9–16.7 IU/gHb), with median levels being 13.7 IU/gHb, (range 7.5–35.9). These 23 patients had significantly lower levels of Hb and RBC compared with those with normal PK activity ($p = .01$ for both, Figure 1A). HK activity values were increased in 60 patients (91%; normal reference range 0.78–1.32 IU/gHb), median levels being 2.3 (0.8–8.1) IU/gHb. This resulted in an abnormally reduced PK/HK ratio (median 5.45, range 2–15) in 63 subjects (95%; normal reference range: 11.55–17.65). ATP levels were reduced in 33/56 tested patients (59%; normal reference range 3.6–4.9 mmol/gHb), median values being 3.4 mmol/gHb (range 1.3–6.5), and 32 of them had a simultaneous reduction of PK/HK activity ratio (Figure 1A), and 11 decreased PK activity. No differences were found between patients with iron overload or on chelation. Osmoscan, performed in six patients, did not show altered RBC deformability (Figure S1). Finally, in five patients, randomly selected among the original cohort of 66 subjects, PFK, GAPD, PGK, ALD, GPI, and TPI activity were tested, with median values falling almost within the normal range except GAPD and ALD, which were slightly above normal (Table S2).

Further 10 LR-MDS patients (9 males and 1 female, median age of 82 years, range: 58–87) were selected with the same inclusion criteria of the initial cohort and were tested in the in vitro study. Six had MDS RS/SF3B1, three MDS LB, and one MDS with 5q deletion. Baseline PK activity, PK/HK ratio, and ATP levels were confirmed reduced versus healthy controls (Figure 1B). After 24 h in vitro incubation with AG-946, five of 10 patients (3 MDS-RS/SF3B1, 1 MDS-LB, 1 MDS-del5q) had a significant increase in mean PK activity (Figure 1C) across all AG-946 concentrations compared to DMSO-treated controls ($p < .01$). Patients who demonstrated an increase in PK activity also had higher HK levels (Figure 1D), and reduced PK/HK ratio (Figure 1E). Four of five patients who demonstrated an increase in PK activity (1 excluded for experiment failure) maintained stable ATP levels across all evaluated concentrations of AG-946 (Figure 1F). No differences were found between patients with iron overload or on chelation. Osmoscan curve was similar among patients and controls in all experimental conditions; after 24 h incubation with or without AG-946 an expected overhydration was observed (Table S3).

Finally, metabolomic analysis showed that most metabolites were significantly higher in MDS versus healthy controls at baseline, including those ascribed to Warburg effect, pentose phosphate shunt, glycolysis, carnitine, and pyruvate metabolism confirming an altered RBC-metabolic status of MDS versus HC at baseline (Figure S2A,B). In addition, we observed an increased level of amino acids and

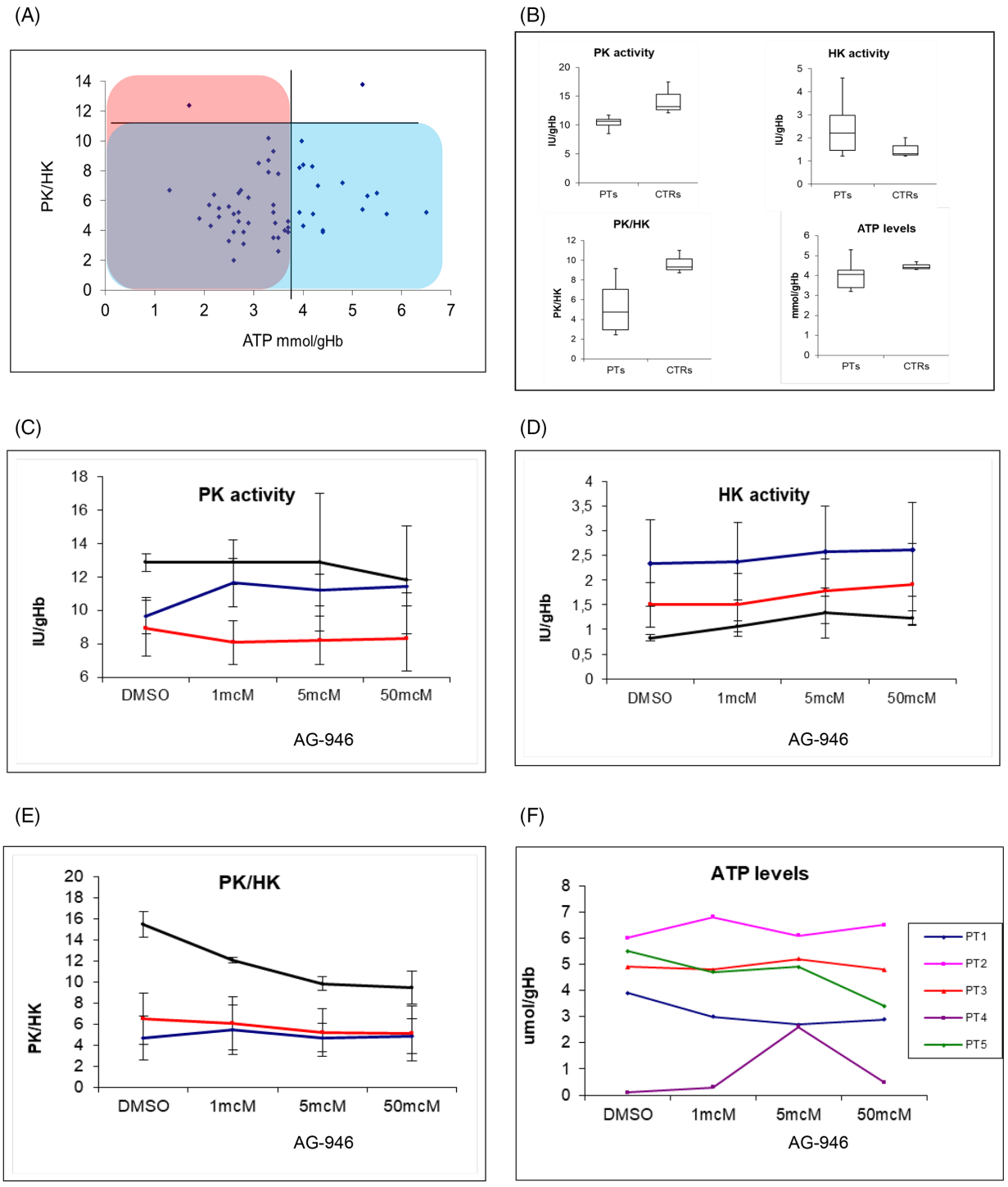

FIGURE 1 Hematologic features and glycolytic activity in myelodysplastic neoplasms (MDS) patients in the observational (panel A) and in the in vitro part of the study (panel B–F). Panel (A). Scatter plot of PK/HK activity ratio and ATP levels in 66 patients with LR-MDS. PK/HK ratio was reduced in nearly all patients (blue box) and ATP levels were decreased in two of three subjects (pink box). Lower limit of normality (LLN) for PK/HK 11.5; LLN for ATP 3.6 µmol/gHb. Panel (B). Baseline pyruvate kinase (PK), hexokinase activity (HK), PK/HK ratio, and ATP levels in 10 patients with low-risk myelodysplastic syndromes (PTs) and five healthy controls (CTRs) included in the in vitro part of the study. Values are shown as box plots of 10 PTs and 5 CTRs studied in the second part of the study evaluating AG-946 effect in vitro. Normal ranges for PK activity were 11.9–16.7 IU per g of Hb (IU/gHb); for HK 0.78–1.32 IU/gHb; for ATP 3.6–4.9 µmol/gHb. Panels (C–E, and F). Effect of AG-946 on glycolytic activity in vitro. (C–E). PK and HK activity and ratio after 24 h of incubation with dimethyl sulfoxide (DMSO, control) and 1, 5, and 50 µM AG-946 in 10 LR-MDS patients and five healthy controls. Values are shown as mean ± SD. PK activity increased in five patients (blue line) after incubation with all AG-946 concentrations (DMSO: mean 9.68 IU/gHb [SD 1.4], 1 µM: 11.68 IU/gHb [SD 0.9], 5 µM: 11.2 IU/gHb [SD 0.89], 50 µM: 11.54 IU/gHb [SD 0.8]) and was unaffected in five patients (red line) and healthy controls (black line). (F). Individual ATP levels in the five patients (PT) displaying increased PK activity after incubation with AG-946.

nucleotide metabolism with an intriguing increased level of pseudouridine, a post-transcriptional RNA modification of uridine. Moreover, in seven of 10 patients, incubation with AG-946 led to an increase of pyruvate with 50 μM at 6 h and with all concentrations (1 to 50 μM) at 24 h. Finally, AG-946 at concentrations from 1 to 50 μM induced an increase of lactate at 6 and 24 h (Figure S3A,B) further supporting possible modulation of glycolysis by AG946.

Fold-change analysis showed that incubation with 50 μM AG-946 increased ATP and decreased 2,3 DPG levels in healthy controls at 6 h, as well as in patient with inherited PK deficiency. In MDS, an increase of glyceraldehyde 3 phosphate was noted with 50 μM AG-946 at 6 and 24 h, whilst no clear changes in ATP and 2,3 DPG levels were observed (Figure S4).

This is the first comprehensive analysis demonstrating impacted RBC glycolytic metabolism in a large cohort of LR-MDS patients, including biochemical and metabolomic assays. We found that RBC metabolism differs substantially from that of healthy controls in that MDS-RBCs show reduced PK and increased HK activity, and consequently, an altered PK/HK ratio in almost all cases. Additionally, two of three patients displayed reduced RBC-ATP levels indicating a general "lack of energy" in MDS. Possible explanations of metabolic differences between MDS and healthy controls have been linked to the different maturation/proliferation status of MDS stem cells as well as to iron overload and proinflammatory microenvironment in the bone marrow.[1] Erythropoiesis is a highly demanding process, requiring high-energy input obtained through a shift from glycolysis to mitochondrial-based metabolism in normal cells.[6] In MDS, a deficiency of mitochondrial subunits or enzymes has been described.[1] Consistently, an increase in extramitochondrial glucose catabolism despite adequate oxygen availability (aerobic glycolysis), namely the Warburg effect, has been observed. This results in the hypoxia-independent activation of hypoxia-inducible factor 1α (HIF1α) through different intermediate metabolites of the tricarboxylic acid cycle, such as the oncometabolite 2-hydroxyglutarate.[1,6] It is worth reminding that iron overload is also associated with a reduction of mitochondrial activity, decreased ATP generation, and increased production of reactive oxygen species (ROS) affecting lipids, DNA, and proteins such as glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase and PK.[6] The above illustrated mechanisms, observed in nucleated bone marrow precursors, may be similar in peripheral MDS-RBCs that mainly rely on glycolysis for ATP production.[6] In this study, metabolomic analysis confirmed an altered RBC-metabolic status of MDS versus controls, with a significant increase of several metabolites indicating a general upregulation of various pathways such as Warburg effect, glycolysis, pentose phosphate shunt, and pyruvate metabolism. The apparent discrepancy between increased glycolysis and reduced PK activity and ATP may reside in the regulation of glycolysis by oxidant stress. In hypoxic state, rate-limiting enzymes, including GAPD and PK,[1,6] are inhibited to redirect glucose oxidation to the pentose phosphate pathway to produce reducing equivalents that counteract oxidative stress.

PK activation has been shown to improve glycolytic activity in normal RBCs, and also in RBCs from patients with PK deficiency, thalassemia, and sickle cell disease.[4] These in vitro evidence fostered the clinical trials of PK activation allowing anemia improvement and reducing transfusion needs in these patients. Here we reported the beneficial effect of AG-946 in improving RBC glycolytic activity in MDS. In vitro incubation with AG-946 led to an increase in PK activity and PK/HK ratio and stable ATP levels in RBCs of half of LR-MDS patients across all WHO classifications. Increased PK and PK/HK activity occurred with 1 μM of AG-946 and were maintained at concentrations up to 50 μM. RBC samples of MDS patients with improved PK activity showed stable ATP levels across all AG-946 concentrations. This was accompanied by increased levels of pyruvate and lactate by metabolomic analysis in RBCs of most patients with a maximal effect at 50 μM, as well as by an increase of products of early glycolysis. These findings, although preliminary and in a limited number of patients, support the possible activity of AG-946 in boosting glycolysis of MDS-RBCs, with variable effect likely linked to the high biological and metabolic heterogeneity of these patients versus healthy controls.

In conclusion, our study demonstrates dysregulated glycolytic activity in red blood cells from a large cohort of anemic patients with LR-MDS, with a possible beneficial effect of AG-946 in vitro. A clinical trial of AG-946 in anemic patients with LR-MDS is ongoing.[5]

AUTHOR CONTRIBUTIONS

BF and WB designed the study and experimental protocol, followed patients, collected and analyzed data, wrote the manuscript, and revised it for important intellectual content. CV, AM, EF, PB, and AZ designed the experimental protocol, collected data, performed the biochemical tests, wrote the manuscript, and revised it for important intellectual content. PP and MWR revised the study design and the article for important intellectual content. LP, MB, and CL followed patients, collected data, and revised the article for important intellectual content. MB and DG performed the metabolomic analysis and revised the article for important intellectual content. FP collaborated to results conceptualization and revised the article for important intellectual content. All authors approved the final version of the article.

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CONFLICT OF INTEREST STATEMENT

WB received consultancy honoraria from Agios, Alexion, Sanofi, and Novartis, and research funding from Alexion; BF received consultancy honoraria from Alexion, Janssen, and Sobi, and research funds from Agios and Zenas Biopharma; PB received consultancy honoraria from Agios Pharmaceutics; PP and MWR are current Agios employees and Agios stockholders. The other authors have no conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

All data have been included in the article and supplementary files. Additional information may be obtained upon reasonable request to the corresponding author.

DATA SHARING STATEMENT

All data have been included in the manuscript, for additional data please contact bruno.fattizzo@unimi.it.

Bruno Fattizzo[1,2], Cristina Vercellati[1], Anna Marcello[1], Parija Patel[3], Megan Wind-Rotolo[3], Loredana Pettine[1], Marcella Bonanomi[4,5], Daniela Gaglio[4,5], Marta Bortolotti[1,2], Claudia Leoni[1,2], Elisa Fermo[1], Paola Bianchi[1], Anna Zaninoni[1], Francesco Passamonti[1,2], Wilma Barcellini[1]

[1]*SC Ematologia, SS Fisiopatologia delle Anemie, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*

[2]*Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy*

[3]*Agios Pharmaceuticals, Inc., Cambridge, Massachusetts, USA*

[4]*Institute of Molecular Bioimaging and Physiology of the Italian National Research Council (IBFM-CNR), Segrate (MI), Italy*

[5]*ISBE Elixir European Infrastructure for Systems Biology, Milan, Italy*

Correspondence

Bruno Fattizzo, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 35, 20100, Milan, Italy.

Email: brunofattizzo@gmail.com

ORCID

Bruno Fattizzo https://orcid.org/0000-0003-0857-8379

Loredana Pettine https://orcid.org/0000-0001-9553-2082

Marta Bortolotti https://orcid.org/0000-0001-5147-970X

Paola Bianchi https://orcid.org/0000-0001-5976-5233

Anna Zaninoni https://orcid.org/0000-0002-8614-3904

Wilma Barcellini https://orcid.org/0000-0003-1428-9944

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.