SUPPLEMENTARY APPENDIX

AG-348 (mitapivat), an allosteric activator of red blood cell pyruvate kinase, increases enzymatic activity, protein stability, and adenosine triphosphate levels over a broad range of *PKLR* genotypes

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 doi:10.3324/haematol.2019.238865

Received: September 25, 2019.

Accepted: January 23, 2020.

Pre-published: January 23, 2020.

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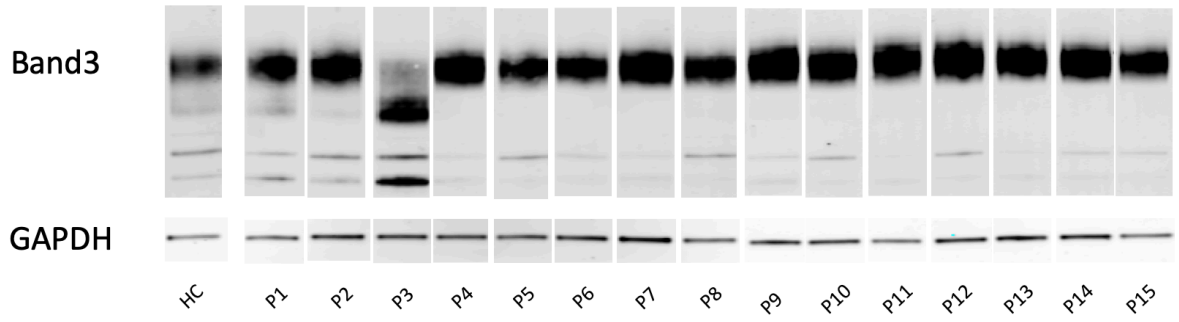
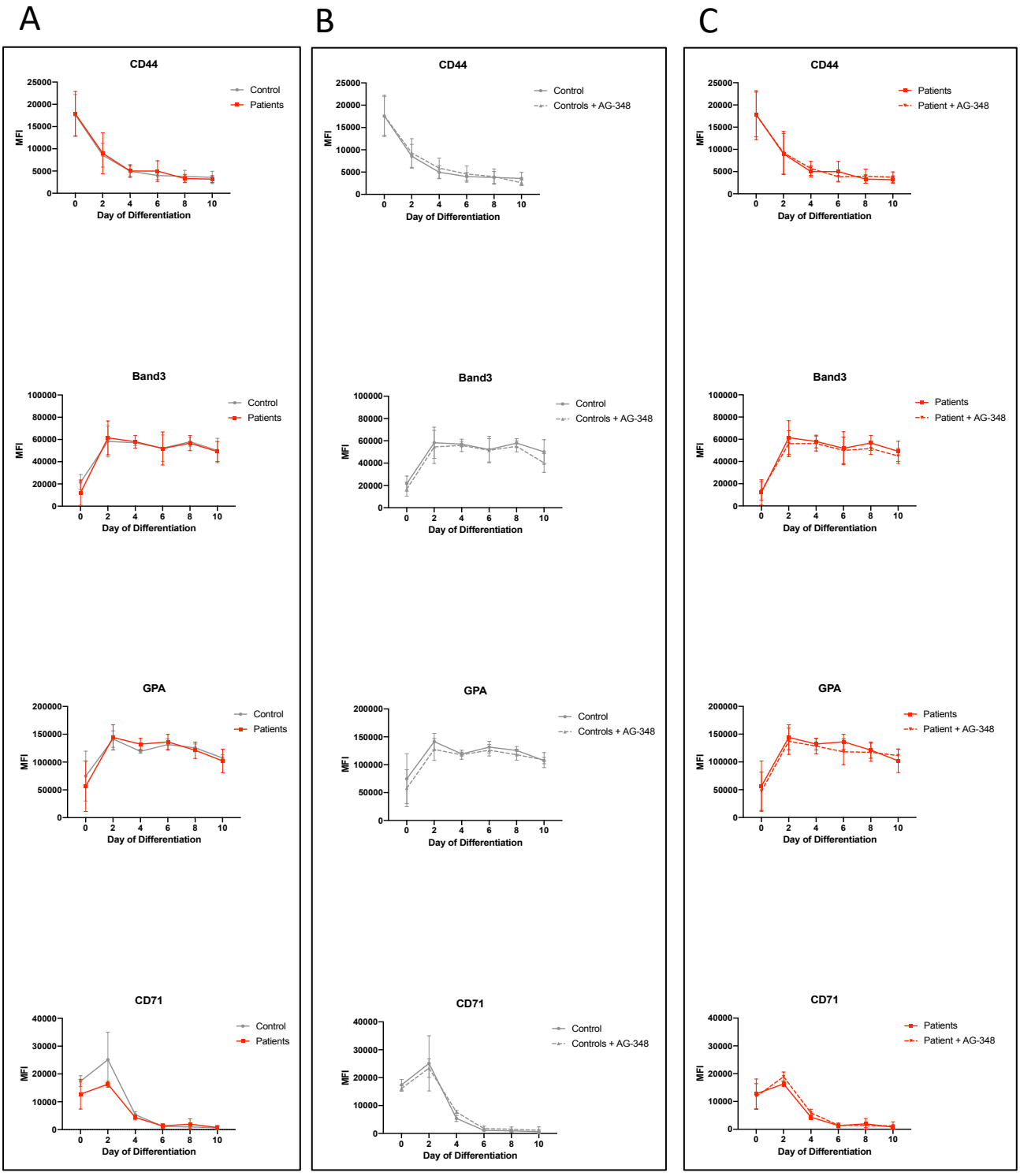
SUPPLEMENTAL DATA

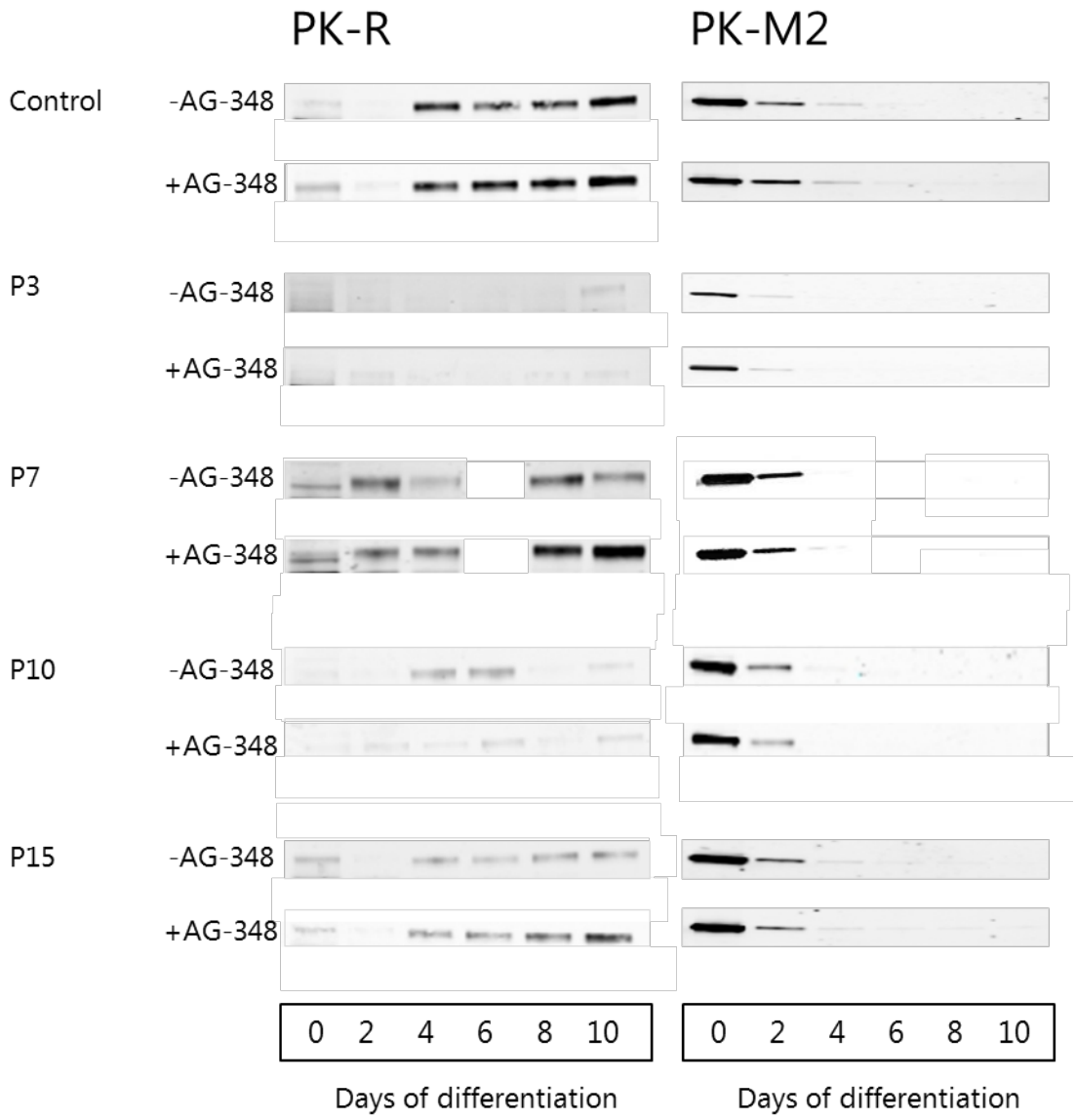
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Band 3 expression levels are comparable between PK-deficient patients and healthy controls. Erythroid surface marker Band 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were determined by Western Blot analysis and the Band 3/GAPDH ratio was used to evaluate band 3 expression levels. Band 3 expression levels of PK-deficient patients (median band3/GAPDH ratio: 2.7, range 1.0-4.8, n=14) were comparable to healthy controls (median ratio: 2.3, range 1.9-2.7, n=3). Patient 3 was excluded from this analysis because this sample may be degraded. A mutated form of band 3 is unlikely because of an essentially normal osmoscan profile as measured on this patient (not shown).



Supplemental Figure 2. Erythroid surface marker expression during erythroid differentiation is comparable between PK-deficient patients and healthy controls and is not influenced by AG-348. (**A**) Erythroid surface markers CD44, Band 3, glycophorin A (GPA) and CD71 were measured on day 0, 2, 4, 6, 8 and 10 of differentiation in cultured erythroid cells of PK-deficient patients and healthy controls. (**B**) Erythroid surface markers measured during differentiation in erythroid cells from healthy controls cultured in absence (solid grey line) and presence of AG-348 (dashed grey line). (**C**) Erythroid surface markers measured during differentiation in erythroid cells of PK-deficient patients cultured in absence (solid red line) and presence of AG-348 (dashed red line).



Supplemental Figure 3. PK-R and PK-M2 expression during ex vivo erythropoiesis is not influenced by AG-348. PK-R and PK-M2 levels were determined by Western Blot analysis on consecutive days during erythroid differentiation. Levels of PK-M2 gradually decreased during differentiation whereas PK-R levels increased, although not evidently recapitulated in all patients. Erythroid cells from healthy controls, and patient samples 7 and 15 showed an increase in PK-R expression when grown in presence of AG-348.

SUPPLEMENTAL METHODS

Whole blood from patients with PKD was collected according to protocol that was approved by the ethical committee of the University Medical Center Utrecht (Protocol 14-571/M). Whole blood of healthy controls was collected through the Mini Donor Service, which is approved by the ethical committee of the University Medical Center Utrecht and in accordance to the declaration of Helsinki.

Hematological laboratory parameters.

Routine hematological parameters were measured using the Abbott Cell-Dyne Sapphire (Abbott Diagnostics, Santa Clara, CA, USA).

Metabolic Profiling

Relative abundance of central carbon metabolites was performed by UPLC-MS. Frozen whole blood samples were thawed on ice and 10 µL aliquots were extracted with 120 µL of hot 70% ethanol containing 100 ng/mL L-glutamate (13C5, D5, 15N) as an internal standard. Samples were vortexed and centrifuged at 14000 rpm for 10 minutes at 4º C; supernatants were dried down and resuspended in water with 2-ketobutyrate (13C4, D2) internal standard. Metabolites were analyzed by high resolution accurate mass detection (HRAM) on a QExactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific) as described previously.[1] Peak identification and integration was done with Maven software.[2] Quantitative analysis of ATP and 2,3-DPG was performed as described previously.[3]

PK activity measurements, protein levels, and thermostability test.

RBCs were purified from whole blood using a microcrystalline cellulose-α-cellulose column according to standardized methods.[4,5] Columns were centrifuged and washed twice with NaCl 0.9% before further experiments. PK and hexokinase (HK) activity was measured in lysates of purified RBCs as described.[4,5] PK thermostability was measured essentially as described[6] on the same hemolysates, after 0, 5, 10, 20, 40 and 60 minutes of incubation at 53°C. Aliquots were chilled for 5 minutes and centrifuged for 3 minutes at 600 g.

PK activity measurements after *ex vivo* treatment with AG-348 were performed at low substrate conditions (final concentration phosphoenol pyruvate (PEP) 0.5 mM). Protein levels of PK-R were determined by Mesoscale Assay (MesoScale Discovery) as described[7] using Goat anti-PKLR antibody (Aviva) and Mouse anti-PKLR antibody (Abcam) both in Tris buffered saline + 0.5% Tween 20 (TBS-T) as sandwich antibodies in a multi-array 96 well plate. SULFO-TAG goat anti-mouse (Mesoscale discovery) was used as the detection antibody and the signal was measured using a MesoScale Discovery instrument. The PKR protein level was determined by normalizing the light intensity of the SULFO-TAG electrochemiluminescence (ECL) to the protein concentration of the lysate (final units: ECL/g of protein).

Western blot analysis of PK-R and PK-M2 was performed as previously described[8] using polyclonal antibodies directed against PK-L[9] and monoclonal antibodies raised against PK-M2 (PodiCeps, Utrecht, The Netherlands). Blots were visualized using Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit antibodies (Li-Cor, Invitrogen) and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

***Ex vivo* treatment of wild type and PK-deficient RBCs with AG-348 .**

Purified RBCs or whole blood of patients and controls were incubated for 3, 6 and 24 hours at 37^{o}C in presence or absence of AG-348. Purified RBCs or whole blood was added to phosphate-buffered saline containing 1% glucose, 170 mg/L adenine, and 5.25 g/L mannitol (pH 7.40) (AGAM buffer) prior to incubation. Purified RBCs from patients and healthy control subjects were incubated with different dosages of AG-348 (up to 10 μM) up to 24 hours at 37°C. After 6 and 24 hours PK-R activity and ATP levels (CellTiterGlo; Promega, Madison, WI) were measured. For determination of PK-R thermostability, RBC lysates were incubated for 2 hours with AG-348 (37°C) prior to testing. Because in the latter case AG-348 does not need to cross the membrane, a lower concentration of AG-348 was used (2 μM).

Band 3 expression levels

Western blot analysis of band 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on purified RBCs using monoclonal antibodies directed against human band 3 (Sigma) and polyclonal anti-human GAPDH antibodies (Sigma). Blots were visualized using Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit antibodies (Li-Cor, Invitrogen) and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Band 3/GAPDH ratio was calculated to evaluate band 3 expression levels.

Osmotic gradient ektacytometry (osmoscan) and deformability measurements

Osmotic gradient ektacytometry (osmoscan) and deformability measurements were performed using the Laser Optical Rotational Red Cell Analyzer (Lorrca, RR Mechatronics, Zwaag, The Netherlands) as described.[10,11] Elongation index (EI) was calculated from height and width of the diffraction pattern and reflects RBC deformability. To study the effect of AG-348 AGAM buffer was added to whole blood (1:10 ratio), and supplemented with either AG-348 (final concentration 20 µM) or DMSO (untreated samples). Samples were incubated for 24 hours at 37°C, after which deformability was measured.

Ex vivo production of red blood cells and treatment with AG-348

Erythroid cells from PK-deficient patients and healthy controls were produced from peripheral blood mononuclear cells (PBMC) as described.[12] Briefly, PBMCs were isolated from peripheral blood by density purification using Percoll (GE Healthcare, Little Chalfont, UK). For proliferation, cells were cultured for 10 days in CellQuin medium (kind gift from Emile van den Akker, Sanquin, Amsterdam, The Netherlands), supplemented with Stem Cell Factor (100ng/mL, Amgen), Epo (2U/mL, EPREX), dexamethasone (1µM, Sigma), and 1ng/mL IL-3 (R&D systems). After 10 days, cells were washed three times with PBS and reseeded at $2E^6$ cells/mL in CellQuin medium supplemented with EPO (10 U/mL, Eprex), heparine (5U/mL, Leo Pharmacy), human blood group AB plasma (3%, Sigma). At day 5 of differentiation CellQuin medium was replaced by RetQuin medium (Emile van den Akker, Sanquin, Amsterdam, The Netherlands). AG-348 was added at a final concentration of 2 µmol/L at days 0, 1, 3-10 during proliferation and day 0, 2, and 4 during differentiation. Under these conditions mean AG-348 levels in medium are 2.4 µmol/L (range 1.4 - 3.8 µmol/L) as determined by a validated liquid chromatography with tandem mass spectrometry (LC-MS/MS) method.[13]

Statistical Analysis

Statistical analysis was performed using Graphpad Prism (v7.04). T-test, Mann-Whitney test or Wilcoxon test was used when appropriate. One-way ANOVA (Post-hoc Tukey's test or Dunnett's test) was carried out or a Kruskal-Wallis test, followed by Dunn's tests for post-hoc analysis was used when appropriate. Pearson's correlation was used to determine correlations of laboratory parameters with enzyme activity measurements. A p-value of <0.05 was considered statistical significant.

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