Supplemental materials for Kung et al. 'AG-348 enhances pyruvate kinase activity in red blood cells from patients with pyruvate kinase deficiency'

Supplemental Methods.

Biochemistry:

Protein expression and purification:

PK-R wild-type and mutant proteins were expressed in the Escherichia coli strain BL21 (Invitrogen) as an N-terminal His6-fusion in the pET28a vector (Novagen). Overnight cultures were diluted 1:100 and grown in LB media plus 5% (w/v) sucrose to an optical density at 600 nm (OD600) of 0.4–0.5 at 37°C then induced with 0.5 mM isopropylthiogalactoside (IPTG) for 3 hours at 27°C before harvest; cell pellets were stored at -80°C until use. Thawed cell pellets were resuspended in Lysis Buffer (50 mM Tris pH 7.5, 100 mM potassium chloride (KCl), 20% glycerol 25 mM magnesium chloride (MgCl2), 25 µg/mL deoxyribonuclease I (DNase I), stirred on ice for 30 minutes and disrupted by three passes through a microfluidizer at 15,000 pounds per square inch (psi). Inclusion bodies were collected by centrifugation for 30 minutes at 20,000g and washed three times in Wash Buffer (50 mM Tris pH 7.5, 1% v/v octyl-fl-D-thioglucopyranoside [OTG]) before resuspension in Nickel (Ni)-Column Buffer A (50 mM Tris pH 8.0, 6 M guanidine-HCl, 10 mM imidazole). After clarification for 30 minutes at 20,000g, the resuspended inclusion bodies were applied to a 5 mL Ni-sepharose high performance (HP) column (GE Life Sciences) equilibrated with Ni-Column Buffer A. The column was washed to OD280 baseline with Ni-Column Buffer A, then eluted with a 20 column volume (CV) linear gradient of 0-100% Ni-Column Buffer B (50 mM Tris pH 8.0, 6 M guanidine-HCl, 500 mM imidazole). Fractions containing PK-R were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and brought to a final concentration of 25 mM dithiothreitol (DTT). Refolding of PK-R was accomplished by 1:10 dropwise dilution into Refolding Buffer (50 mM Tris pH 8.0, 100 mM KCl, 20% glycerol, 100 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 100 mM non-

detergent sulfobetaine [NDSB-201]) and for 12 hours at 4°C. The refolded protein solution was centrifuged at 20,000g for 20 minutes, concentrated to 10 mL by a 10,000 molecular weight cut-off (MWCO) Centricon (Millipore), then centrifuged again at 20,000g for 20 minutes. The sample was then applied to a 320 mL Sephacryl S200 gel filtration column (GE Life Sciences) equilibrated in Sizing Column Buffer (50 mM Tris pH 7.5, 100 mM KCl, 20% glycerol, 1 mM DTT) and eluted with 10 column volumes of the same. PK-R active fractions were identified by SDS-PAGE and enzymatic activity, then snap frozen in liquid nitrogen and stored at -80°C. A similar procedure was used to express and purify PKM2 and PKL enzymes.

Pyruvate kinase activity assay:

PK-R activity was measured by a coupled enzyme system with lactate dehydrogenase (LDH) in which the pyruvate produced by PK-R was reduced to lactate with the concomitant oxidation of NADH to NAD. Reaction progress was followed by a change in the oxidation state of the cofactor spectrophotometrically at 340 nm, using an extinction coefficient of 6300/M·cm. Reactions were performed in 200 µL of 1x Buffer (100 mM KCl, 50 mM Tris 7.5, 5 mM MgCl2, 1 mM DTT, 0.03% bovine serum albumin (BSA), 180 µM NADH) containing 10–100 ng PK-R enzyme and 0.5 units of LDH at room temperature. The supplemental table lists enzyme, PEP, and ADP concentrations used for reaction conditions for each mutant isoform. These concentrations were selected for each mutant enzyme based on those which gave the maximal signal difference between the inactivated state and the activated state with the natural ligand, fructose-1,6-bisphosphate (FBP). For determination of kinetic parameters, ADP was held constant at 5 mM, PEP was varied (0.2–20 mM), and 5 µM AG-348 was added.

For pyruvate kinase activity measurements from red cells, activity was assessed as above, except with substitution of 10 µL red cell lysate for the recombinantly expressed protein as the source of pyruvate kinase in the assay, omission of DTT from the master mix, and standardization of the PEP concentration at 0.5 mM for mutant RBC and 0.1 mM for WT RBC.[1]

For activity samples from patient B (Figure 4D), an alternative method of detecting PK-R activity was used which relied on direct detection of the pyruvate product using liquid chromatography/mass spectrometry (LC/MS). The reaction was run as above (except for omission of LDH coupling enzyme and NADH from the master mix) for a fixed time of 30 minutes at 37°C and then quenched using 300 µL 95% acetonitrile (ACN)/5% H_2O containing a pyruvate-$13C_2$ extraction standard (Isotec) at 10 µM. After thoroughly mixing the samples by pipette, the plate was spun in a 4°C centrifuge at 4000 rpm for 10 minutes to pellet excess protein. 50 µL of the supernatant was transferred to a shallow 96-well plate which was then heat-sealed to prevent evaporation. The plate was put into an autosampler kept at 4°C and 10 µL of each sample was injected onto a tandem Waters UPLC/ABSciEx 4000 QTrap Triple-quadrupole Mass Spectrometer. The mass spectrometer was operated in multiple-reaction monitoring (MRM) mode, detecting newly produced pyruvate. The extraction standard, pyruvate-$13C_2$ was also monitored to account for sample-to-sample extraction differences.

The protein concentration used to normalize both spectrophotometric and direct pyruvate detection methods was determined using a bicinchoninic acid (BCA) kit (Pierce). LC/MS peaks were integrated using MultiQuant software. Data was exported as area under the pyruvate peak, normalized to the area under the pyruvate-$13C_2$ extraction standard peak.

Thermostability assay:

For determination of thermostability, R510Q mutant protein was diluted to 3 µg/mL in Assay Buffer (100 mM KCl, 50 mM Tris 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.03% BSA, 180 µM NADH). AG-348 or DMSO was added and the mixture was incubated at room temperature for 30 minutes and subsequently at 53°C for the specified amount of time when activity was measured, as above, except at saturating PEP concentration (2 mM).

Crystallography:

The PK-R protein was purified by Ni column chromatography. Protein concentration was 5.4 mg/ml in buffer containing 20 mM Na+/K+ P_i, pH 6.0, 50 mM KCl. The protein sample was supplemented with 5mM FBP, 1 mM AG-348, then mixed with equal

volume of the well solution 50 mM Mes/KOH, pH 6.0, 10 mM $MnSO_4$, and 10.5% w/v PEG8000. The crystallization tray was incubated at 21°C. Crystals were harvested after 5 days. The cryoprotectant was the well solution supplemented with 30% PEG 400.

Mouse studies:

C57BL/6 mice received seven days twice daily oral dosing (12 hours interval) of AG-348 at 1 mg/kg/dose, 10 mg/kg/dose, 50 mg/kg/dose or 150 mg/kg/dose (10 mL/kg) via oral gavage. 32 mice were treated in each dose level. During the course of this study, no abnormal clinical symptoms or behavior of the animals were noted. AG-348 was prepared as a suspension in 0.5% methylcellulose in water. Approximately 400 uL of blood was collected via cardiac puncture into K_2EDTA tubes at 0, 3, 6, 12, 24, 32, 48, and 72 hours (individual mice in groups of 4 were used per time point) following the last dose on Day 7 (the 13th dose) from all animals. Two sets of blood samples (25 µL each) were frozen on dry ice immediately after collection for 2,3-DPG and ATP assays. The remaining samples were centrifuged within 15 minutes at 2000*g* for 5 minutes to collect plasma samples. Red blood cell pellets were saved for PK-R activity assay. The concentration of AG-348 in plasma samples and the concentration of ATP and 2,3-DPG in whole blood samples were determined using tandem mass spectrometry (LC-MS/MS).

DNA isolation and sequencing:

Genomic DNA was isolated from 0.5 mL whole blood utilizing the QIAamp DNA Blood Midi Kit as stated in the protocol handbook. DNA sequencing to identify the mutations in the PK-R gene was conducted by SeqWright Inc (now part of GE Healthcare).

Red cell studies:

For WT red cell studies, fresh blood drawn from healthy volunteers into K_2EDTA tubes was received from a commercial vendor (Research Blood Components). Patient samples were obtained from hospitals following each institution's IRB protocol (Boston Children's Hospital, Tufts Medical Center, St. Jude Children's Research Hospital). Patient samples E and F were received ~24 hrs post-blood draw via overnight courier (stored at 4°C during shipment). All other patient samples were received on the same

day of blood draw (time to receipt was variable, up to 8 hrs). Whole blood was pelleted by spinning at 500g for 10 minutes. The plasma layer was removed and the pellet was resuspended in 5x volume of phosphate buffered saline (PBS). Cells were spun at 500g for 10 minutes. The supernatant was removed and the cell pellet was resuspended at 50% hematocrit in PBS. Red blood cells were purified by density centrifugation over Percoll. The RBC pellet was washed by adding 5x volumes of 1x PBS and spinning at 500g for 10 minutes. Supernatant was removed and the RBC pellet was resuspended in AGAM media (1x PBS, 1% glucose, 170 mg/L adenine, 5.25 g/L mannitol) at a density of $4x10^9$ cells/mL.

For PK-R activity assays, AG-348 was prepared in 100% DMSO as a 10 mM stock. Serial dilutions were performed in 96-well V-bottom storage plate and then added to 96-well V-bottom plates. RBCs were diluted in AGAM media containing 10% fetal bovine serum (FBS) to a density of $1x10^8$ cells/mL before added to assay plates (final compound concentration at 0.1% DMSO concentration). Assay plates were sealed using aluminum foil seals and incubated overnight at 37°C in a humidified chamber. For ATP assays, cells were prepared as described except at a density of $1x10^7$ cells/mL in black assay plates and ATP levels were read out using Cell-Titer-Glo (Promega).

PK-R protein assay:

The PK-R protein in the diluted lysates was evaluated as follows: 40 µL of a 1 µg/ml solution of goat anti-PKLR antibody (Aviva, OAGA00912) in phosphate buffered saline (PBS) was added to each well of a multi-array 96 well plate (MesoScale Discovery) and incubated overnight at 4°C. The plate was decanted and 150 µL blocking buffer (PBS + 0.5% Tween 20 [PBS-T] + 3% BSA) was added and the plate shaken at room temperature for 2 hours. The plate was washed 3x with PBS-T with 5 minutes shaking in between each wash. The plate was decanted and 25 µl (3-6 µg protein/well) of diluted lysate was added to the appropriate wells and shaken at room temperature for 2 hours. The plate was washed as above. 25 µL of a 1 µg/ml solution of mouse anti-PKLR antibody (Abcam, ab89071) in antibody buffer (PBS-T + 1% BSA) was added to each well and shaken at room temp for 1 hours. The plate was washed as above in PBS-T. 25 µL of a 1 µg/ml solution of SULFO-TAG goat anti-mouse (MSD) in antibody buffer

was added to each well and shaken at room temp for 1 hour followed by washing with PBS-T as above. 25 µL of 1X read buffer (MSD) was added to each well and the plate was read on a MesoScale Discovery instrument. The PK-R 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/µg of protein).

Metabolite measurements:

300 µL aliquot of RBCs was frozen on dry ice to lyse cells and quench metabolism. Samples were then stored at -80°C until all samples were harvested and ready for metabolite extraction. For extraction, samples were removed from the -80C° freezer and thawed on a water bath kept at 37°C for 4 minutes. 450 µL of ACN was added to each sample to precipitate protein. Samples were then spun at 14,000 rpm for 10 minutes in a centrifuge cooled to 4°C. 50 µL of supernatant was transferred to a 96-well plate and the samples were dried under a nitrogen stream. Extracts were then reconstituted in 50 µL of H_2O containing 1 µg/mL 2-ketobutyric acid-$13C_4$-d_2 internal standard (Isotec). The plate was put into an autosampler kept at 4°C and 10 µL of each sample was injected onto a tandem Waters UPLC/ABSciEx 4000 QTrap Triple-quadrupole Mass Spectrometer as described previously.[2] Whole blood samples were extracted in 12 volumes of 70% ethanol at 70°C. Samples were then spun at 14,000 rpm for 10 minutes in a centrifuge cooled to 4°C. 60 µL of supernatant was transferred to a 96-well plate and the samples were dried. Extracts were reconstituted in 60 µL of H_2O containing 1 µg/mL 2-ketobutyric acid-$13C_4$-d_2 internal standard (Isotec) and 10 µl were injected onto a Thermo Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. For RBC analysis the mass spectrometer was operated in MRM mode using negative electrospray ionization and for whole blood analysis the mass spectrometer was operated in Full Scan mode using negative electrospray ionization.

For the RBC metabolite analysis LC/MS peaks were integrated using MultiQuant software. Data was exported as area under the peak for each metabolite. Fold-change values were calculated at a given time point by taking the peak areas for the drug-treated sample and dividing by the peak areas of the DMSO control. Propagation of

error was used to determine error bars. For absolute quantitation in whole blood (supplemental Figure 3), we used a 2,3-$^{13}C_2$ PEP label (Cambridge Isotope Laboratories) to generate a calibration curve in blood matrix. LC/MS peaks were integrated using Quan Browser software and concentrations were calculated from the linear fit of the calibration curve.

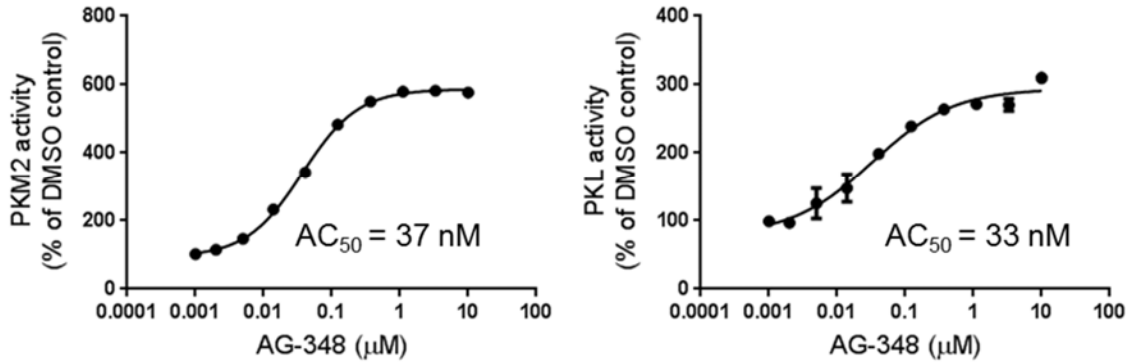
1. Beutler E, *Red Cell Metabolism: A Manual of Biochemical Methods*. 1975, New York: Grune & Stratton.
2. Kung C, Hixon J, Choe S, et al. Small molecule activation of PKM2 in cancer cells induces serine auxotrophy. *Chem Biol*. 2012;19(9):1187-1198.

Supplementary Table: Parameters for enzymatic assays

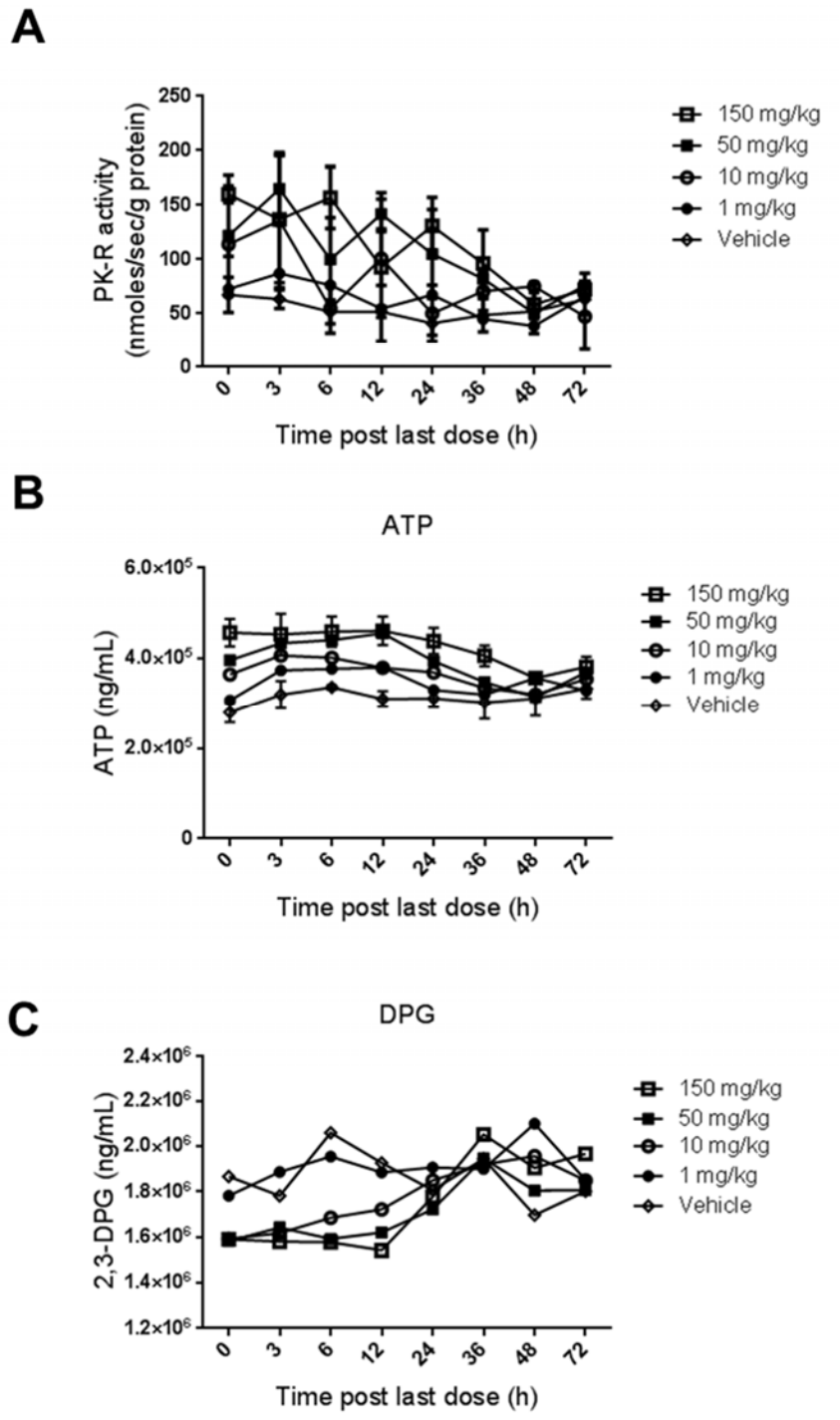
PK-R isoform	Enzyme (ng/assay well)	PEP concentration (mM)	ADP concentration (mM)
Wild-type	60	0.065	0.153
R532W	20	0.05	0.294
R510Q	40	0.11	0.2
R479H	100	0.23	0.6
R486W	250	0.27	1
R490W	200	0.16	0.36
G364D	200	0.2	0.6
G332S	80	0.95	0.3
T384M	300	0.19	0.05

Abbreviations: ADP = adenosine diphosphate; PEP = phosphoenolpyruvate; PK-R = red cell-specific form of pyruvate kinase

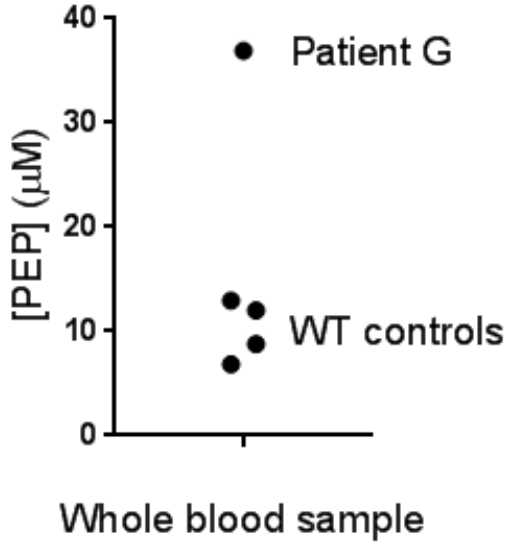
Supplemental Figures



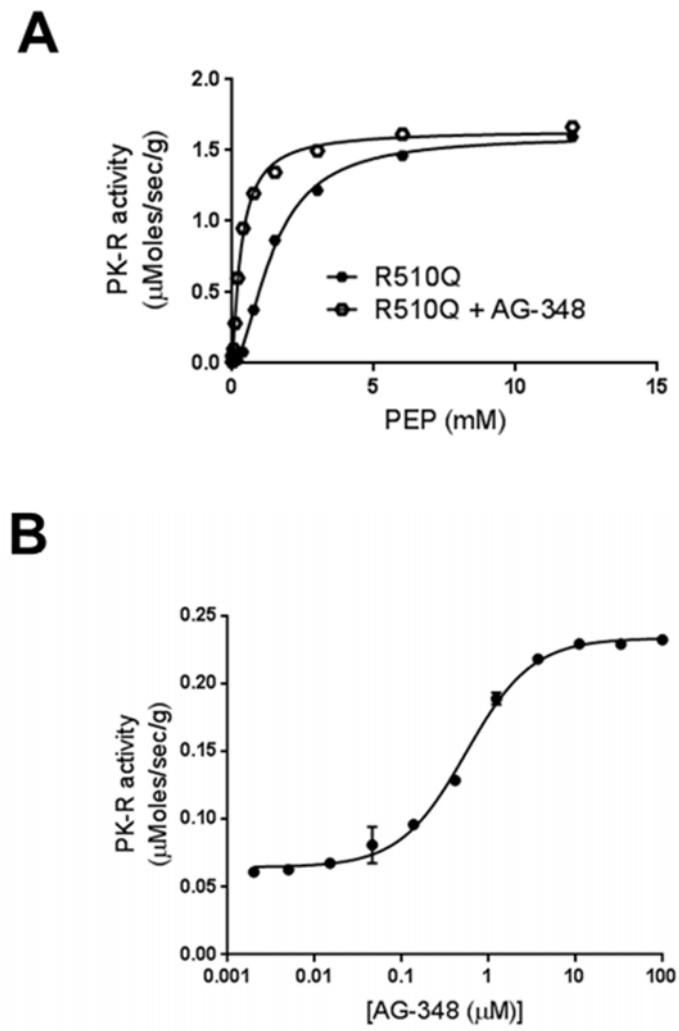
Supplementary Figure 1. Additional properties of AG-348. (A) Activity of recombinant PKM2 (left) or PKL (right) enzyme incubated with indicated concentrations of AG-348 ([PEP] = 0.1 mM).



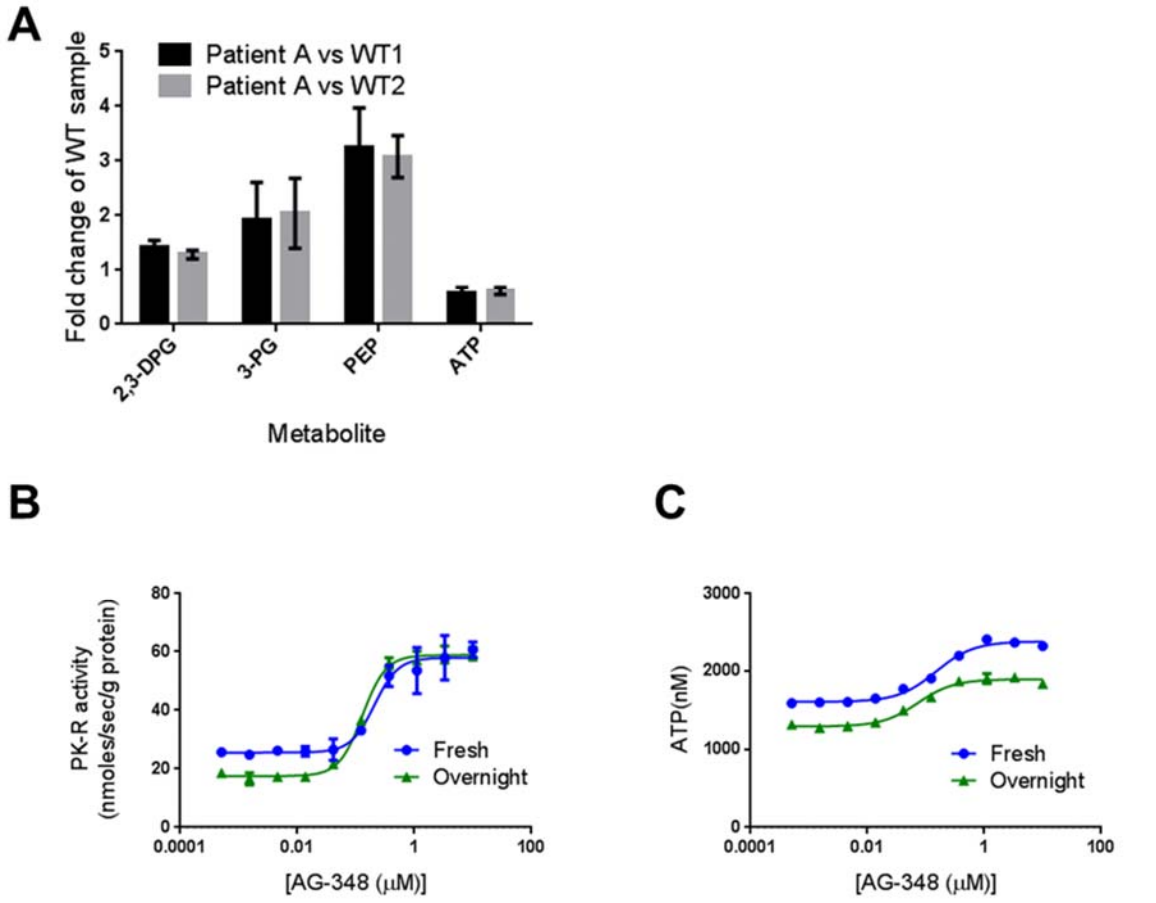
Supplementary Figure 2. AG-348 activates WT PK-R in mice. PK-R activity (A), ATP levels (B), and 2,3-DPG levels (C) in RBCs from mice dosed with AG-348 twice daily for 7 days as described in Figure 2. Time indicated is the time after the last dose.



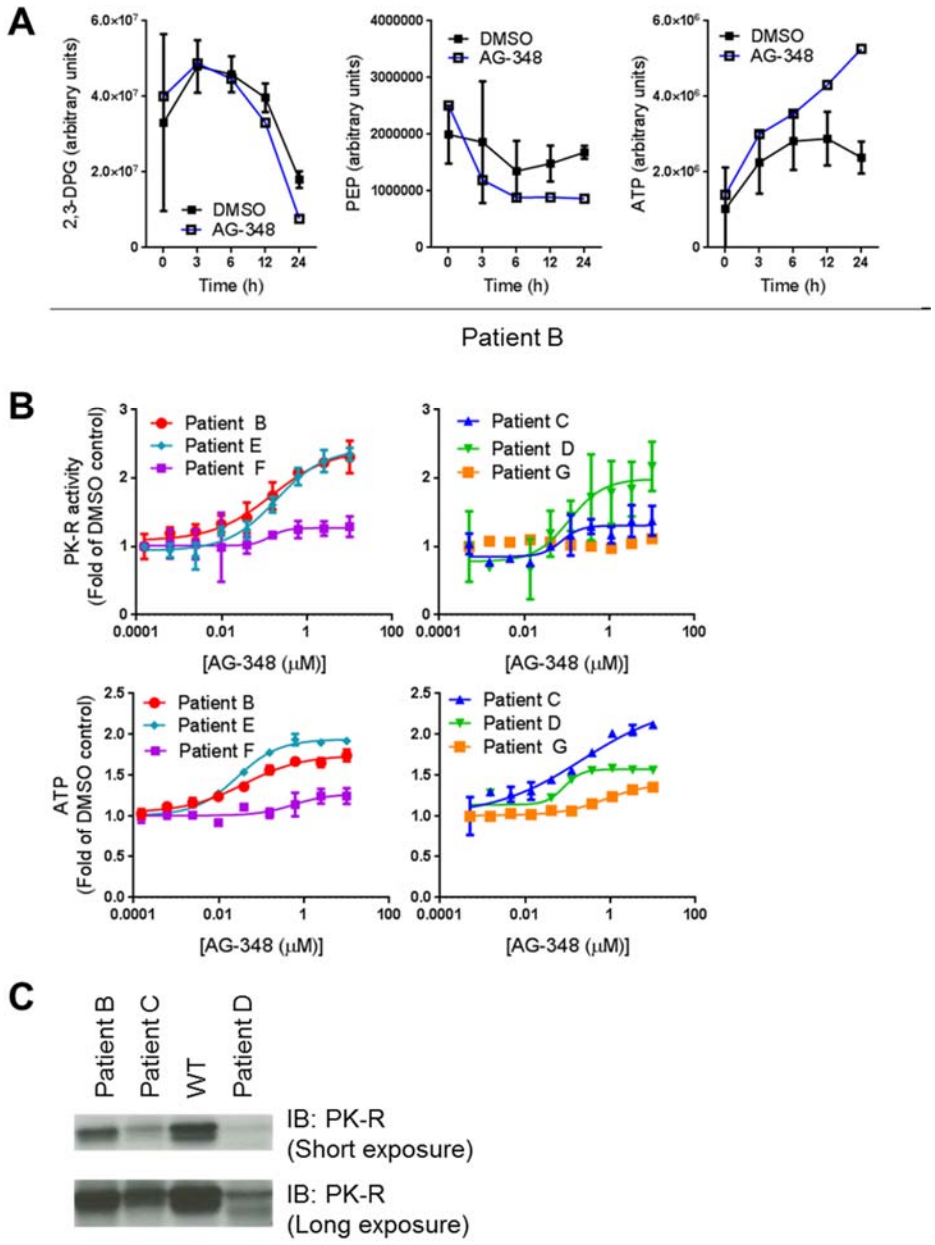
Supplementary Figure 3. Physiological PEP concentration. Absolute quantitation of levels of PEP in whole blood from patient G and 4 WT controls.



Supplementary Figure 4. AG-348 activates R510Q mtPK-R. (A) Plot of activity of recombinant R510Q mtPK-R enzyme stimulated with PEP with or without preincubation with AG-348 (5 μM). (B) PK-R activity of recombinant R510Q mtPK-R enzyme incubated with indicated concentration of AG-348 ([PEP] = 0.11 mM).



Supplementary Figure 5. Metabolite analysis in blood samples. (A) Relative metabolite concentrations in whole blood measured in patient A vs 2 WT donors. (B and C) WT RBCs were purified from whole blood received fresh after blood draw (blue) or from blood stored overnight at 4°C to simulate standard shipping conditions (green). RBCs were incubated with AG-348 for 24 hrs followed by assessment of (B) PK-R activity levels ([PEP] = 0.1 mM) and (C) ATP levels. There is a drop in ATP levels but not PK-R activity levels after overnight storage, and no overall change in response to AG-348.



Supplementary Figure 6. AG-348 activation of mtPK-R in RBCs from patients with PK deficiency. (A) Levels of 2,3-DPG, PEP, and ATP in RBCs from patient B at indicated times following treatment with AG-348. (B) Fold-change (relative to DMSO) in PK-R activity (top) and ATP levels (bottom) in PK-deficient RBCs incubated with AG-348 for 24 hours. (C) Western blot of PK-deficient RBCs from indicated patients shown at two different exposures. Patient C appears to have PK-R protein levels that fall roughly in between those of patients B and D. Antibody used was Abcam ab89071.