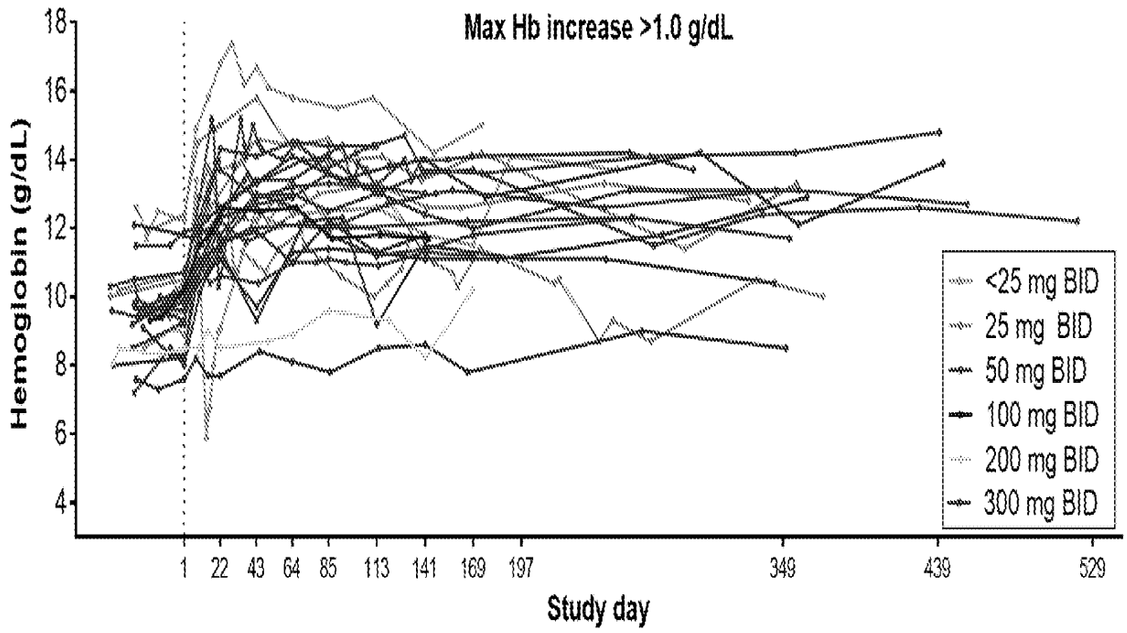
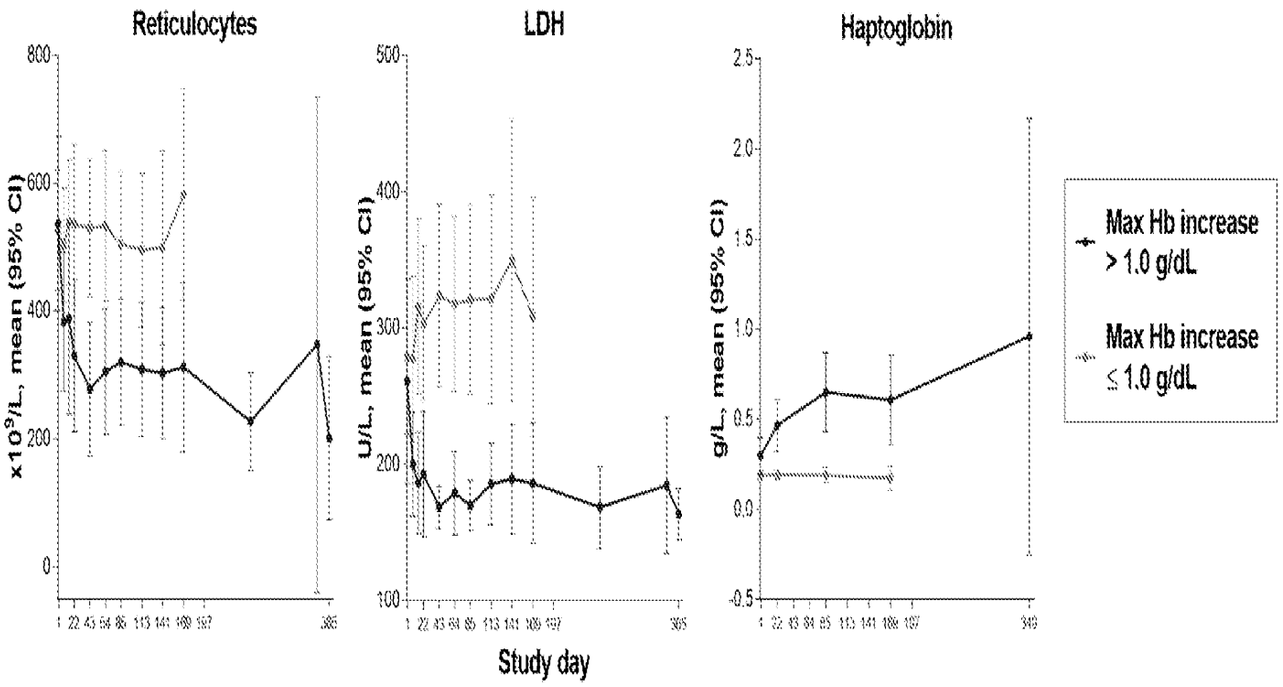
# Majority of Hb increases are rapid and sustained

- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range 7–141 days)
  - Median baseline Hb in subjects who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.5–12.3 g/dL) vs. 8.0 g/dL (range 6.5–10.1) in subjects who did not
- In 8 patients, the dose had to be held or reduced due to rapid rise in Hb



# Haemolysis markers improved in patients with Hb increase of >1.0 g/dL

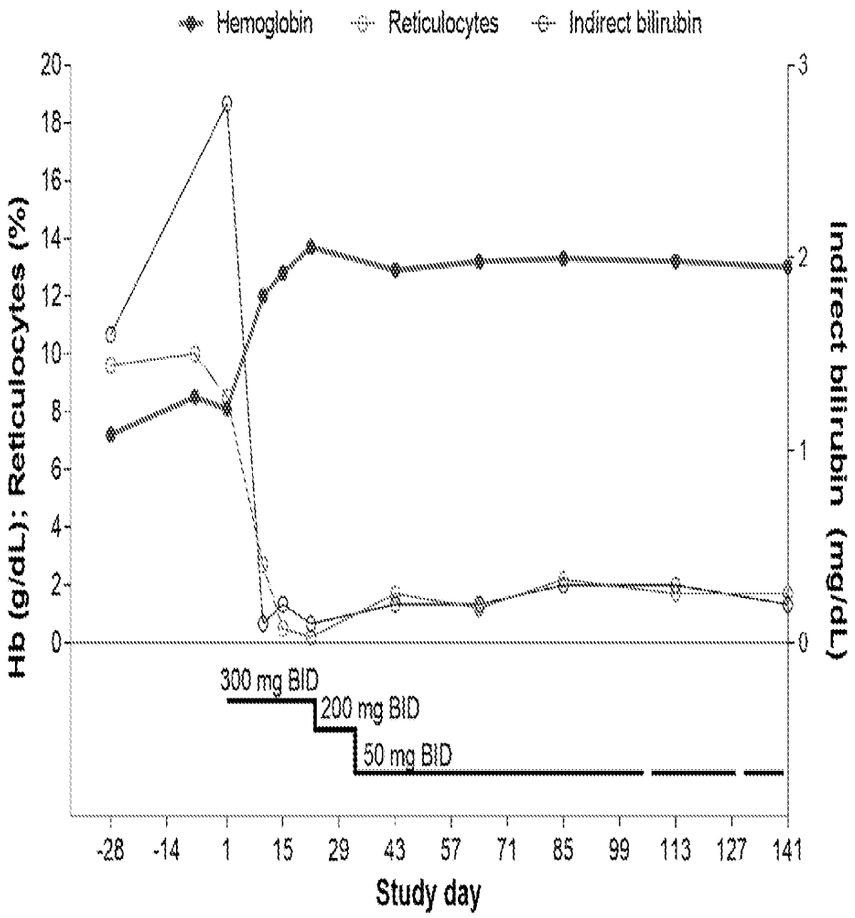
- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin




Only visits with ≥5 subjects included. Figure shows central laboratory results. LDH = lactate dehydrogenase

# Hb response with improvements in haemolysis parameters: single subject experience with AG-348

- Female 35 years of age, white, missense/missense genotype (T384M/R479H)



# DRIVE PK conclusions

- AG-348 is a novel, first-in-class PK-R activator in clinical testing as a potential disease-altering therapy for patients with PK deficiency
- Chronic daily dosing with AG-348 is well tolerated
  - Clinical significance of AG-348 aromatase inhibition is unclear
  - One grade 4 serious AE of elevated triglycerides was observed
- 25 of 52 (48%) subjects had a maximum Hb increase of >1.0 g/dL
  - Responses are rapid in onset and durable
  - Other parameters (reticulocytes, LDH, and haptoglobin) indicate decreased haemolysis in responders
  - Some genotype–Hb response correlations were observed
- These data highlight the potential of AG-348 to be the first disease-altering treatment for patients with PK deficiency, providing a rationale for pivotal studies



Ex. 2001-0553

# Acknowledgments


- We would like to thank the patients who agreed to participate in this study
- We would also like to thank all the clinical research sites and study investigators:
  - Boston Children's Hospital; Rachael F Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet L Kwiatkowski
  - Central Pennsylvania Clinic; D Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin HM Kuo
  - Hammersmith Hospital; D Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Hôpital Henri Mondor; Frédéric Galactéros
  - Hôpital Saint Vincent de Paul; Christian Rose
  - Hôpital de la Timone; Emmanuelle Bernit
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
- We would like to thank Drs Ellis Neufeld and David Nathan for helpful discussions
- This clinical study was funded by Agios Pharmaceuticals

Editorial support was provided by Christine Ingleby, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios




Ex. 2001-0554

# Abstract Submission

***26. Red blood cells and iron - Clinical***

EHA-1830

**EFFECTS OF AG-348, A PYRUVATE KINASE ACTIVATOR, ON ANEMIA AND HEMOLYSIS IN PATIENTS WITH PYRUVATE KINASE DEFICIENCY: EARLY DATA FROM THE DRIVE PK STUDY**

Rachael F Grace*[1], Christian Rose[2], D Mark Layton[3], Wilma Barcellini[4], Janet L Kwiatkowski[5], Bruce Silver[6], Elizabeth Merica[7], Charles Kung[7], Marvin Cohen[8], Hua Yang[7], Jeffrey Hixon[7], Penelope A Kosinski[7], Lee Silverman[7], Lenny Dang[7], Zheng (Jason) Yuan[7], Ann J Barbier[7], Samuel Agresta[7], Bertil Glader[9]

[1]Dana-Farber Boston Children's Cancer and Blood Disorder Center, Boston, United States, [2]Hôpital Saint Vincent de Paul, Lille, France, [3]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom, [4] Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, [5]The Children's Hospital of Philadelphia and Perelman School of Medicine, Philadelphia, [6]Bruce A Silver Clinical Science and Development, Dunkirk, [7]Agios Pharmaceuticals, Inc., Cambridge, [8]MBC Pharma Solutions, Newtown, [9]Stanford School of Medicine, Palo Alto, United States

**Background:** Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by deficiency of the glycolytic enzyme red cell PK (PK-R), required for maintenance of adenosine triphosphate (ATP) in red blood cells. AG-348 is an orally-available, small molecule, allosteric activator of PK-R that activates wild-type and a range of mutated PK-R enzymes *in vitro* and increases PK-R activity and restores ATP levels in red blood cells from patients with PK deficiency *ex vivo*.

**Aims:** To describe early data from the ongoing DRIVE PK study (NCT02476916), an open-label, dose-ranging trial of AG-348 in transfusion-independent adults with PK deficiency.

**Methods:** After providing informed consent, patients are randomized to AG-348 50 mg or 300 mg orally twice daily (BID) for 6 months. Transfusion independence is defined as no more than 3 units of red blood cells transfused in the 12 months preceding first dose and no transfusions in the 4 months preceding first dose. Patients are followed weekly for Weeks 1–3, then every 3 weeks until Week 12, and then monthly until Week 24. Hormone and iron status are evaluated at Baseline, Week 12 and End of Study.

**Results:** As of 15 Jan 2016, 10 patients have received treatment for between 1 and 24 weeks. Adverse events (AEs) have been mild-to-moderate, with nausea (CTCAE Grade 1–2) the most commonly reported AE: 2 out of 5 (40%) patients in the 50 mg BID arm and 3 out of 5 (60%) patients in the 300 mg BID arm. No serious AEs have been reported. There have been no dose modifications for AEs. A single patient has had dose modifications for an increase in hemoglobin (Hb) above the protocol mandated maximum. Dosing was temporarily interrupted and reduced twice (100 mg BID, then 50 mg BID) and the patient has since been maintained on 50 mg BID for several weeks. Pharmacokinetic data showed exposure in line with expectations from earlier healthy volunteer studies with AG-348 for both doses. At data cutoff, pharmacodynamic analysis of 2,3-diphosphoglycerate (2,3-DPG) and ATP was available for 5 patients. Baseline 2,3-DPG levels were elevated in 4 of 5 patients, as expected. All patients with a Hb response of ≥1.5 g/dL also showed a substantial decrease in 2,3-DPG levels. Further data are being collected to evaluate relationships between hematological parameters and pharmacodynamic markers (2,3-DPG and ATP). Genotype–response relationships are also being assessed.

**Summary/Conclusion:** Early clinical data from DRIVE-PK indicate that AG-348 is well tolerated. Data on all patients (n~15–20), including safety, hematological responses, pharmacodynamic effects and genotype–response relationships will be presented.

**Keywords**: Clinical trial, Hemolytic anemia, Treatment

# Disclosures

S466

- This study was funded by Agios Pharmaceuticals Inc.
- Rachael F Grace: Agios – study co-principal investigator and scientific advisor
- Christian Rose: Agios – study investigator
- D Mark Layton: Agios – study investigator
- Wilma Barcellini: Agios – study investigator, no other disclosures
- Janet L Kwiatkowski: Agios – study investigator; Shire, Sideris, and Ionis Pharmaceuticals – consultant
- Bruce Silver, Marvin Cohen: Agios – consultants
- Bertil Glader: Agios – study co-principal investigator and scientific advisor
- Elizabeth Merica, Charles Kung, Hua Yang, Jeffrey Hixon, Penelope A Kosinski, Lee Silverman, Lenny Dang, Zheng (Jason) Yuan, Ann J Barbier, Sam Agresta: Agios – employees and stockholders
- Editorial assistance was provided by Christine Tomlins, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios



Ex. 2001-0556

# Effects of AG-348, a pyruvate kinase activator, on anemia and hemolysis in patients with pyruvate kinase deficiency: early data from the DRIVE-PK study

**Rachael F Grace[1], Christian Rose[2], D Mark Layton[3], Wilma Barcellini[4], Janet L Kwiatkowski[5], Bruce Silver[6], Elizabeth Merica[7], Charles Kung[7], Marvin Cohen[8], Hua Yang[7], Jeffrey Hixon[7], Penelope A Kosinski[7], Lee Silverman[7], Lenny Dang[7], Zheng (Jason) Yuan[7], Ann J Barbier[7], Samuel Agresta[7], Bertil Glader[9]**

*[1]Dana-Farber Boston Children's Cancer and Blood Disorder Center, Boston, USA;*
*[2]Hôpital Saint Vincent de Paul, Lille, France;*
*[3]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, UK;*
*[4]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy;*
*[5]The Children's Hospital of Philadelphia and Perelman School of Medicine, Philadelphia, USA;*
*[6]Bruce A Silver Clinical Science and Development, Dunkirk, USA;*
*[7]Agios Pharmaceuticals Inc., Cambridge, USA;*
*[8]MBC Pharma Solutions, Newtown, USA;*
*[9]Stanford University School of Medicine, Palo Alto, USA*

*Presented at the 21st Congress of the European Hematology Association, 11 June 2016, Copenhagen, Denmark*

Ex. 2001-0557

# Pyruvate kinase (PK) deficiency: a severe congenital anemia

**Description**

- Presents in childhood with severe hemolytic anemia
- Estimated 2,400 patients diagnosed in the US and EU

**Etiology**

- Caused by mutations in the *PK-LR* gene coding for erythrocyte pyruvate kinase (PK-R)

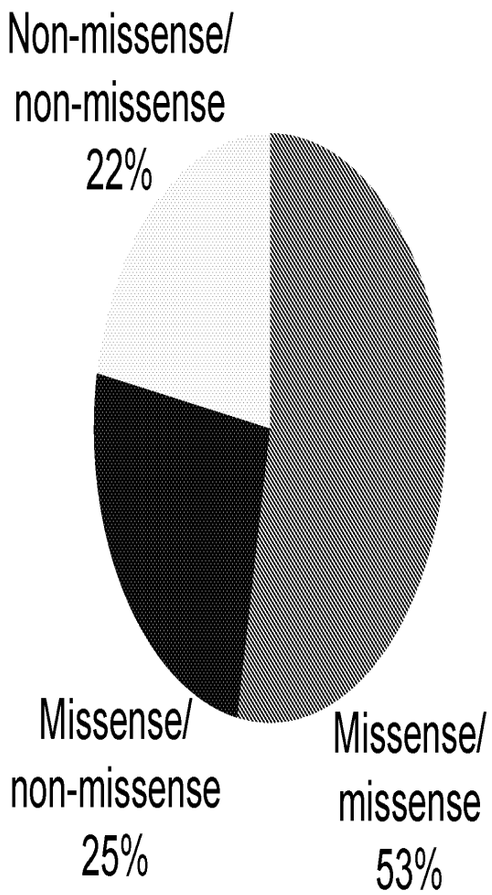
**Disease Burden**

- Lifelong hemolytic anemia
- Iron overload and jaundice
- Infection risk post-splenectomy

**Diagnosis/ Treatment**

- PK-R enzyme activity and/or genetic testing
- Supportive treatment: transfusions, splenectomy, iron chelation

**Type of *PK-LR* mutations found in 74 unrelated cases enrolled in the PK deficiency natural history study**



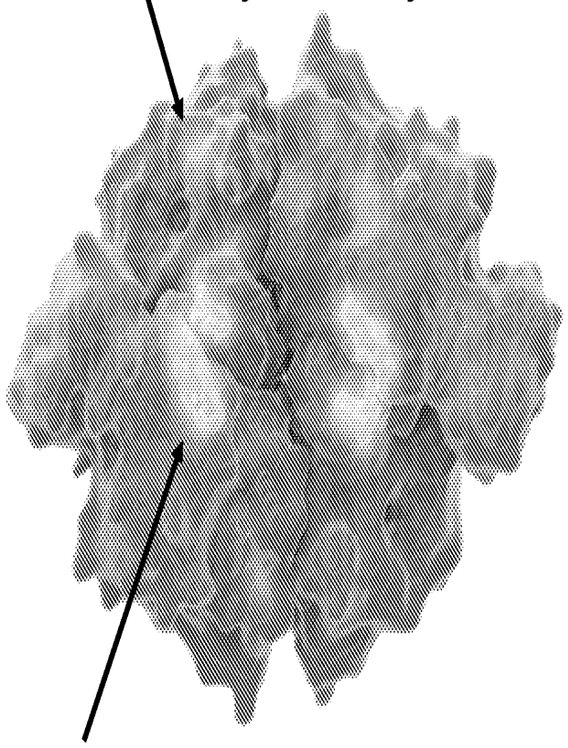

Source: Grace R et al. *Am J Hematol* 2015;90(9):825-30; Bianchi P et al. 57th ASH Annual Meeting 2015, Abstract 3337



Ex. 2001-0558

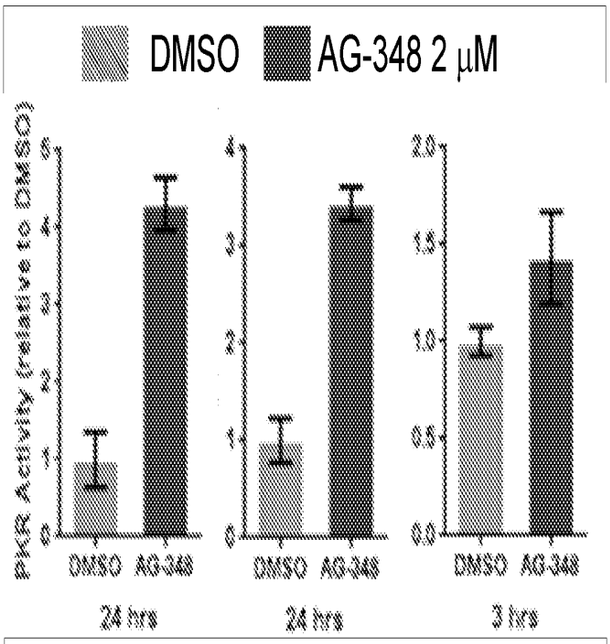
# AG-348: allosteric activator of wild-type and mutant PK-R

Active PK-R is a tetramer; mutations (green) decrease the catalytic activity



AG-348 (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

AG-348 activates mutant PK-R in blood from PK deficient patients




| Patient A (R510Q/ G511R) | Patient B (R486W/ D390N) | Patient C (A495V/ E241stop) |
|---|---|---|

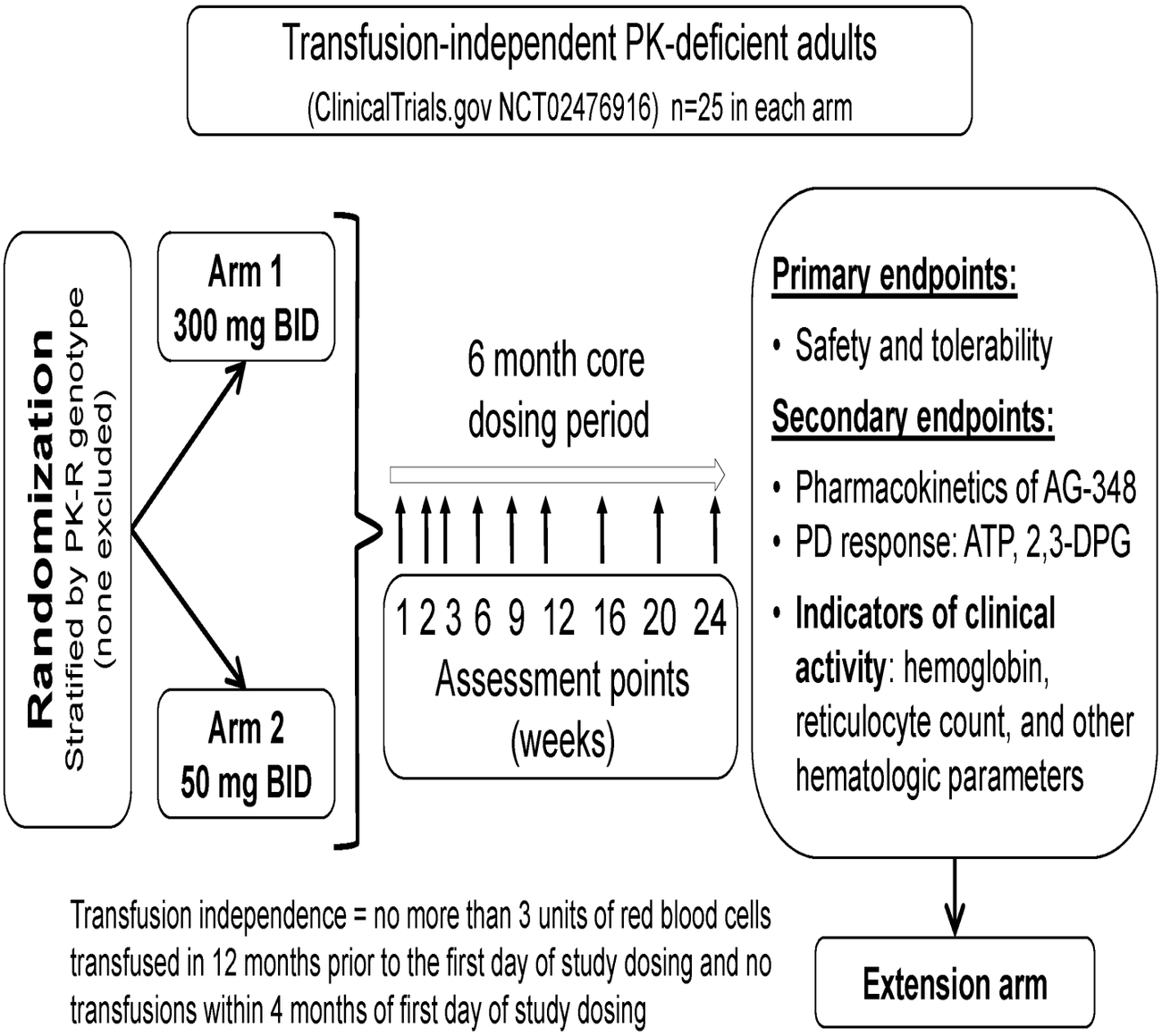
Kung C et al. 55th ASH Annual Meeting 2013, Abstract 2180; Kung C et al. 56th ASH Annual Meeting 2014, Abstract 4010



Ex. 2001-0559

# Study design

## Open-label, global phase 2 study: 14 centers in the US, Canada, and EU




Transfusion independence = no more than 3 units of red blood cells transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

All patients provided written informed consent.

BID = twice daily; PD = pharmacodynamic



Ex. 2001-0560

# Demographics and disposition

- The study was initiated in June 2015; data cut-off 27 March 2016
- 18 patients have been treated, with no early discontinuations
- Three patients have completed the 24-week core period
  - Two remain on treatment in the extension arm

| Characteristic | 50 mg BID, n=9 | 300 mg BID, n=9 | Total, N=18 |
|---|---|---|---|
| Men/Women, n | 7/2 | 5/4 | 12/6 |
| Age in years, mean (range) | 25.9 (19–41) | 35.3 (22–61) | 30.6 (19–61) |
| Race[a] white, n | 8 | 9 | 17 |
| Hemoglobin baseline, g/dL, mean (SD) | 10.0 (1.5) | 8.5 (1.6) | 9.3 (1.7) |
| Duration of treatment, weeks, median (range) | 10.7 (3.0–24.4) | 10.9 (3.0–24.0) | 10.8 (3.0–24.4) |
| Splenectomized, n | 6 | 7 | 13 |

[a]Not reported in 1 subject



Ex. 2001-0561

# Safety and adverse event (AE) summary

- AG-348 was generally well tolerated; the majority of AEs were grade 1–2
  - One subject received a dose reduction due to rapidly increasing hemoglobin
  - One grade 2 'allergic reaction', successfully re-challenged with lower dose
  - One grade 2 AE of osteoporosis has been reported since the cut-off date
- No significant changes in ECGs and clinical safety laboratory parameters

| | 50 mg BID n=9 | 300 mg BID n=9 | Total N=18 |
|---|---|---|---|
| Subjects experiencing at least 1 AE, n | 7 | 8 | 15 |
| Most frequent AEs (≥3 subjects), n | | | |
| Nausea | 4 | 5 | 9 |
| Headache | 2 | 2 | 4 |
| Hot flush | 1 | 2 | 3 |
| Insomnia | 0 | 3 | 3 |
| Subjects experiencing at least 1 drug-related AE, n | 6 | 7 | 13 |
| Subjects experiencing at least 1 serious AE | 0 | 0 | 0 |
| Subjects experiencing at least 1 grade ≥3 AE[a] | 0 | 2 | 2 |
| Subjects experiencing at least 1 grade ≥3 drug-related AE | 0 | 1 | 1 |

AEs were graded using National Cancer Institute Common Terminology Criteria, version 4.03

[a]Two grade 3 events (unrelated hypertension, related hypertriglyceridemia)

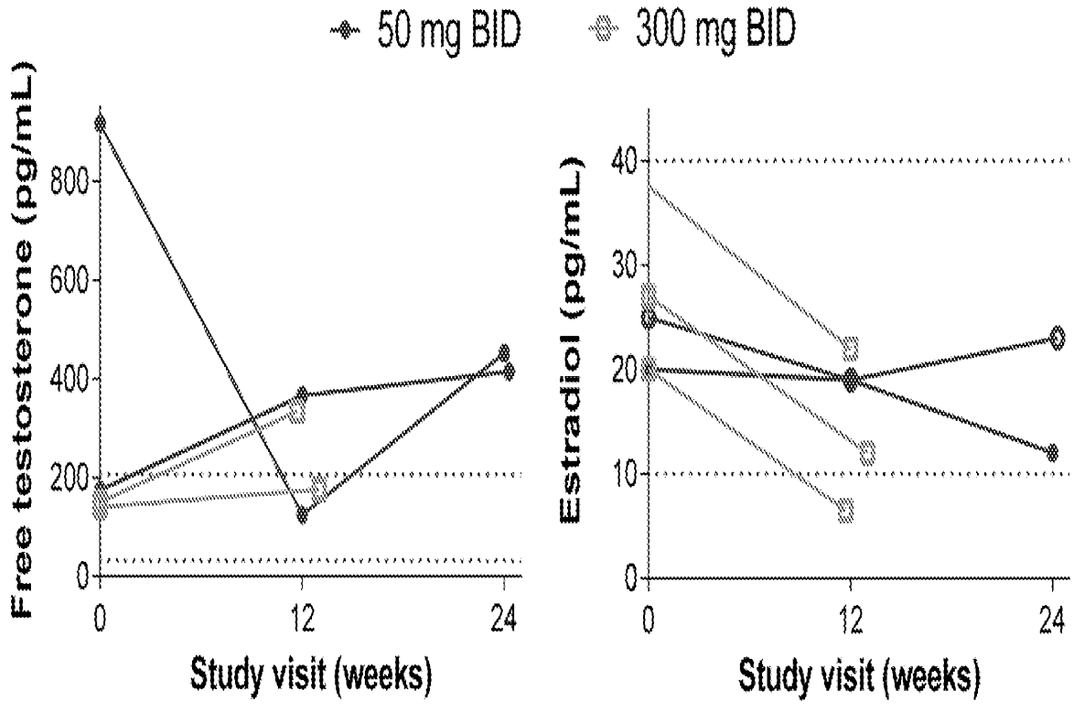


Ex. 2001-0562

# Effect of AG-348 on sex steroids

- Sex steroids were assessed at baseline, week 12 and week 24
- An upward trend in free testosterone and a downward trend in estradiol were observed in male patients

Hormone levels in male patients with available data




Normal reference low and high limits for men shown as horizontal dotted lines

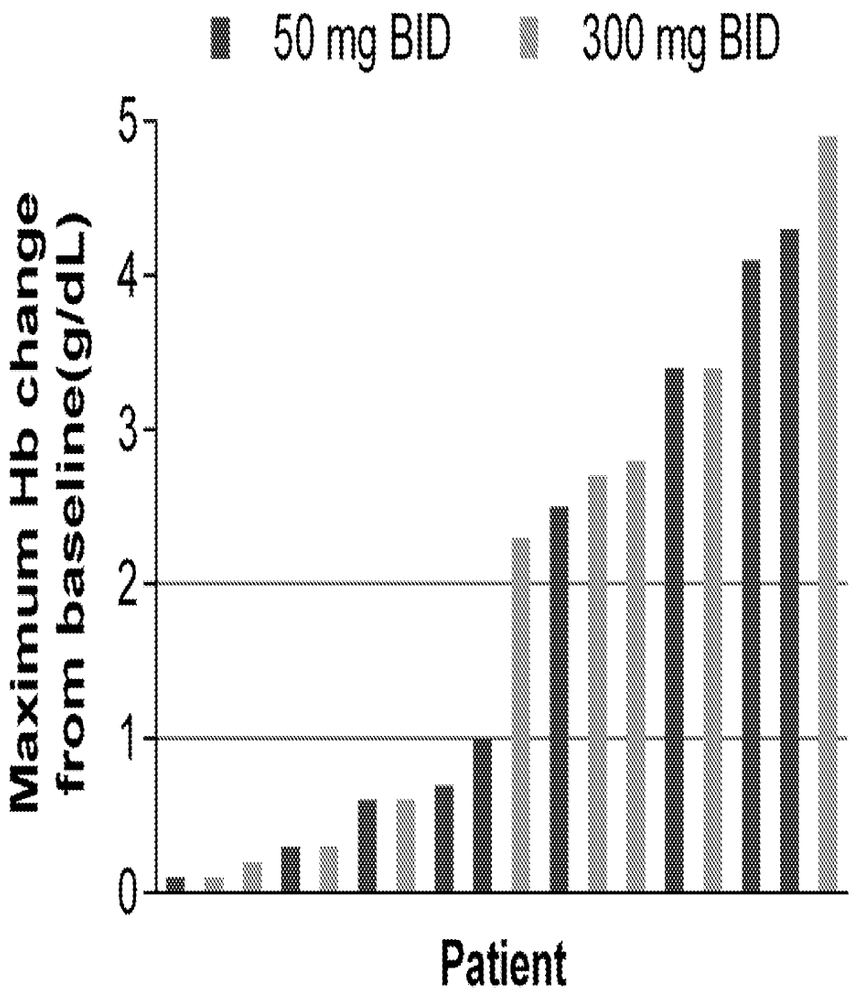


Ex. 2001-0563

# Clinical Activity Results

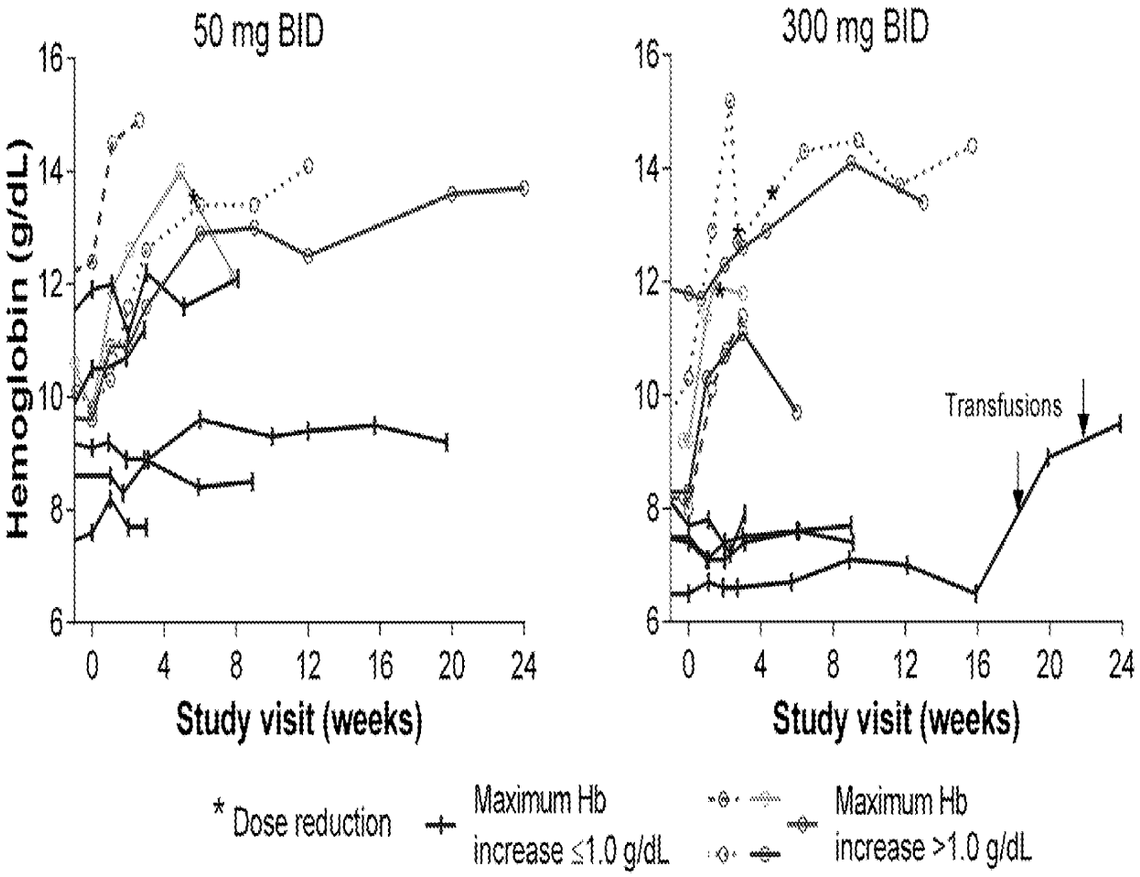
# Maximum change in hemoglobin (Hb) in the 50 and 300 mg BID dose groups

- 9 of 18 patients had an increase in Hb >1.0 g/dL



# Hb increases are rapid

- Median time to a Hb increase >1.0 g/dL was 1.9 weeks (range, 1.1–9.1 weeks)
- In patients who had Hb increases >1.0 g/dL:
  - The mean maximum increase was 3.4 g/dL (range, 2.3–4.9 g/dL)



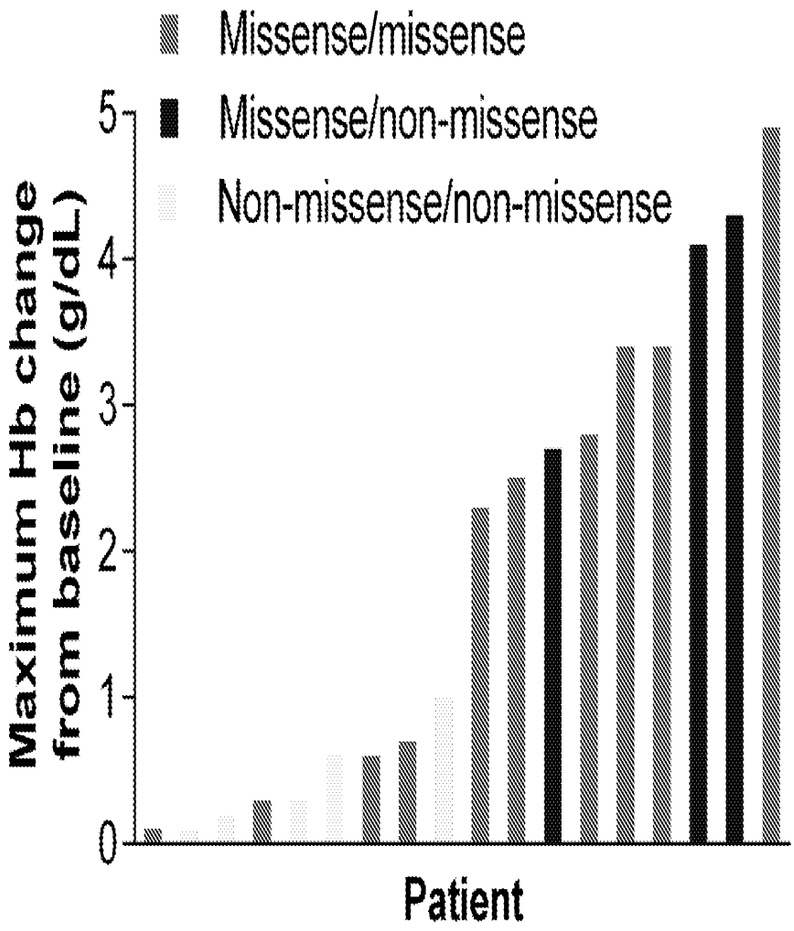

N=18, each line represents an individual patient



Ex. 2001-0566

# Genotype profile and Hb change

- Across all genotypes, 9 of 18 patients had an increase in Hb >1.0 g/dL
- Of 13 patients with at least one missense mutation, 9 had an increase in Hb >1.0 g/dL






Ex. 2001-0567

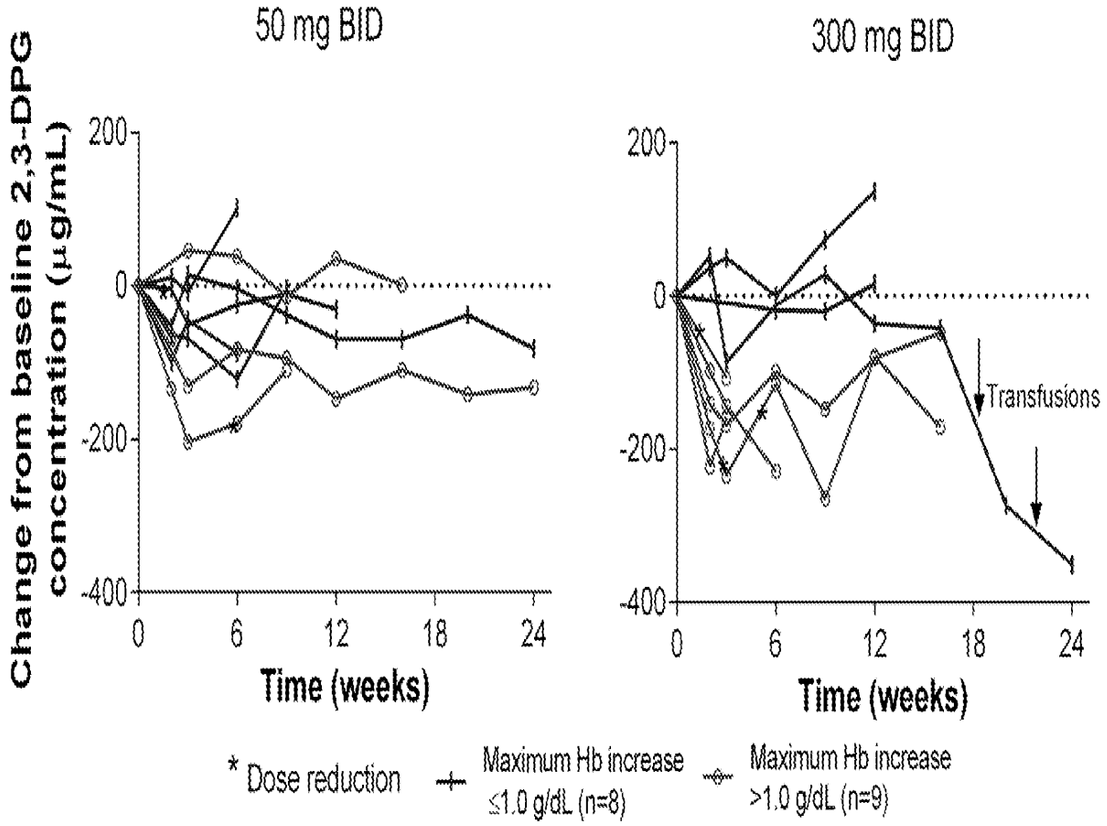
# Pharmacodynamic Results



Ex. 2001-0568

# Effect of AG-348 on pharmacodynamic marker 2,3-DPG

- Levels of 2,3-DPG and ATP in whole blood assessed at baseline and weeks 2, 3, 6, 9, 12, 16, 20 and 24
- No discernible effect on ATP observed
- More data are needed to clarify if any correlation exists between 2,3-DPG decreases and Hb increases >1.0 g/dL






Ex. 2001-0569

# DRIVE-PK conclusions

- AG-348 is a novel, first-in-class, PK-R activator in clinical testing as a disease-altering therapy to restore metabolic function in patients with PK deficiency
- Daily dosing with AG-348 for up to 6 months is well tolerated
- AG-348 demonstrates proof-of-concept with rapid and sustained Hb increases in patients with PK deficiency
- Preliminary genotype correlations were observed:
  - Missense/missense and non-missense/missense genotypes are associated with Hb increases
  - Non-missense/non-missense genotypes have not shown increases in Hb >1.0 g/dL



Ex. 2001-0570

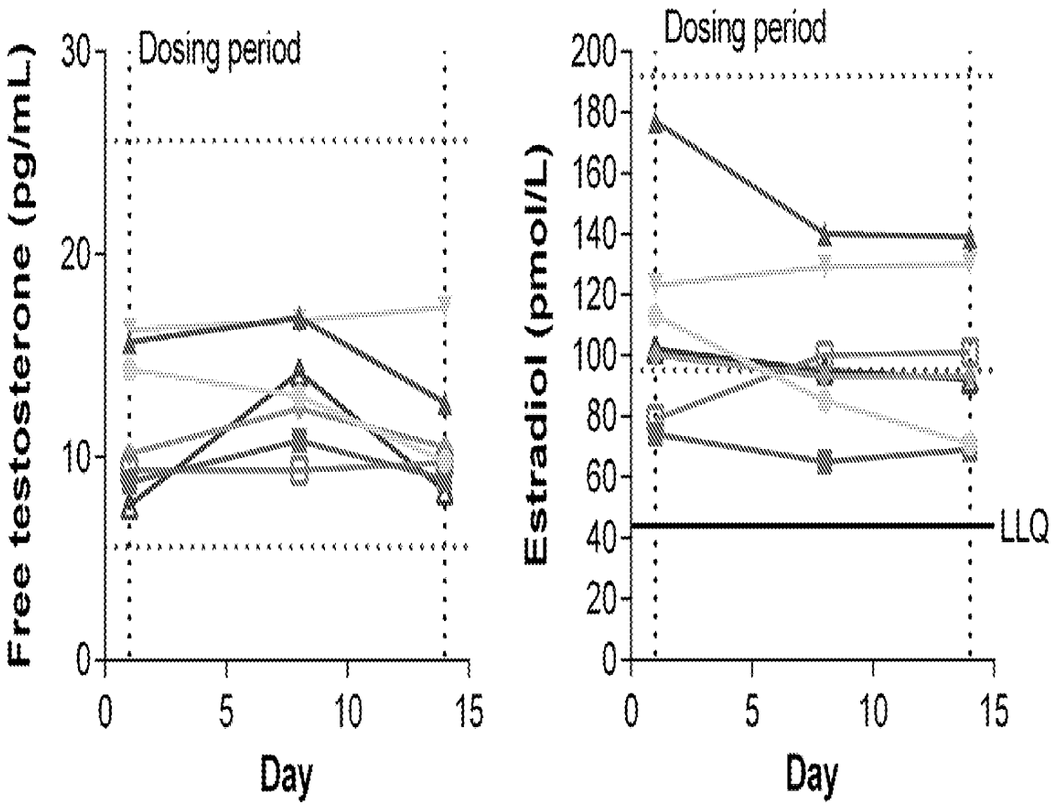
# Acknowledgments


We would like to thank the patients who agreed to participate in this study

- We would like to thank Drs. Ellis Neufeld and David Nathan for helpful discussions
- We would also like to thank all the clinical research sites and study investigators:
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  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet Kwiatkowski
  - Central Pennsylvania Clinic; Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin Kuo
  - Hammersmith Hospital; Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Hôpital Henri Mondor; Frederic Galacteros
  - Hôpital Saint Vincent de Paul; Christian Rose
  - Hôpital de la Timone; Emmanuelle Bernit
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini

# BACKUP

- AG-519 does not demonstrate off-target aromatase inhibition or effect steroid hormones




Male subjects receiving AG-519 375 mg BID. LLQ, lower limit of quantification; dotted red lines indicate reference range upper and lower limits



Ex. 2001-0572

# Abstract Submission

***27. Enzymopathies, membranopathies and other anemias***

EHA-2605

## EFFECTS OF AG-348, A PYRUVATE KINASE ACTIVATOR, IN PATIENTS WITH PYRUVATE KINASE DEFICIENCY: UPDATED RESULTS FROM THE DRIVE PK STUDY

Rachael F Grace*[1], D Mark Layton[2], Frédéric Galactéros[3], Christian Rose[4], Wilma Barcellini[5], D Holmes Morton[6], Eduard van Beers[7], Hassan Yaish[8], Yaddanapudi Ravindranath[9], Kevin Kuo[10], Sujit Sheth[11], Janet L Kwiatkowski[12], Bruce Silver[13], Charles Kung[14], Marvin Cohen[15], Hua Yang[14], Penelope A Kosinski[14], Lei Hua[14], Ann Barbier[14], Bertil Glader[16]

[1]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, United States, [2]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom, [3]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, [4]Hôpital Saint Vincent de Paul, Lille, France, [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, [6]Central Pennsylvania Clinic, Belleville, United States, [7]Universitair Medisch Centrum Utrecht, Utrecht, Netherlands, [8]University of Utah, Salt Lake City, [9]Wayne State University School of Medicine, Children's Hospital of Michigan, Detroit, United States, [10]University of Toronto, Toronto, Canada, [11]Weill Cornell Medical College, New York, [12]Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia, [13]Bruce A Silver Clinical Science and Development, Dunkirk, [14]Agios Pharmaceuticals, Inc., Cambridge, [15] MBC Pharma Solutions, Newtown, [16]Stanford University School of Medicine, Palo Alto, United States

**Please indicate all of which apply to the presenting author:** MD

**Background:** Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by deficiency of the glycolytic enzyme, red cell PK (PK-R). AG-348 is an orally available, small molecule, allosteric activator of PK-R that activates the wild-type and a range of mutated PK-R enzymes *in vitro*, and increases PK-R activity and restores adenosine triphosphate levels in red blood cells from patients with PK deficiency *ex vivo*.

**Aims:** To report on preliminary efficacy and safety data from the use of AG-348 in the ongoing DRIVE PK study (NCT02476916), an open-label dose-ranging trial of AG-348 in transfusion-independent adults with PK deficiency.

**Methods:** After providing informed consent, patients were randomized to AG-348 50 mg or 300 mg orally twice daily (BID) for 6 months (Core Period). At the end of the Core Period, patients can continue on treatment for another 2 years in the Extension Period. Transfusion independence is defined as ≤3 units of red blood cells transfused in the 12 months preceding the first dose of AG-348 and no transfusions in the 4 months preceding the first dose. Patients are followed weekly for Weeks 1–3, every 3 weeks for Weeks 4–12, monthly for Weeks 13–24 and then every 3 months until the end of the study. Hormone and iron status are evaluated at Baseline, Week 12 and End of Core Period, and then every 6 months in the Extension Period.

**Results:** As of 18 Jan 2017, goal enrolment has been met and all 52 patients are evaluable for safety and efficacy; 24 have completed the Core Period and 23 are ongoing in the Core Period. Five patients discontinued from the Core Period, owing to adverse events (AEs) (n=2) or consent withdrawal (n=3). Of the 24 subjects who completed the Core Period, 21 entered the Extension Period and 20 are still on treatment; 1 was discontinued by the investigator. Patients are currently receiving doses ranging between <25 mg BID and 300 mg BID. As of the previous data cutoff date of 23 Sep 2016 (where N=34), AG-348 had an acceptable AE profile, with headache (44%), nausea (41%) and insomnia (38%) being the most frequently reported events. Serum levels of sex steroids measured at Baseline, Week 12 and Week 24 in the Core Period demonstrated increases in free and total testosterone, and decreases in estradiol and estrone, consistent with the known effect of aromatase inhibition by AG-348. Of the 32 patients evaluable for efficacy at 23 Sep 2016, 15 (47%) had a maximal increase in hemoglobin (Hb) >1 g/dL. Hb responses were seen across a range of four doses, and were rapid and sustained. For a subset of patients (n=8), the rate of glycolytic metabolism in peripheral blood samples was assessed before and after treatment, and a positive correlation was observed between increases in glycolytic flux through the PK-R pathway and increases in Hb. Updates on safety, clinical efficacy measures (including Hb levels) and genotype–response correlations will be provided.

**Summary/Conclusion:** AG-348 is a novel, first-in-class PK-R activator undergoing clinical testing in patients with PK deficiency. The ongoing DRIVE PK study has now met goal enrolment of 52 patients, and data from these patients will be available at the time of presentation. Chronic daily dosing with AG-348 is well tolerated and has demonstrated clinically relevant, durable increases in Hb across a range of doses from <25 mg BID to 300 mg BID. These data highlight the potential of AG-348 to be the first disease-altering treatment for patients with PK deficiency.

**Keywords**: Clinical trial, Hemolytic anemia, Treatment

# Effects of AG-348, a pyruvate kinase activator, in patients with pyruvate kinase deficiency: Updated results from the DRIVE PK study

Rachael F Grace[1], D Mark Layton[2], Frédéric Galactéros[3], Christian Rose[4], Wilma Barcellini[5], D Holmes Morton[6], Eduard van Beers[7], Hassan Yaish[8], Yaddanapudi Ravindranath[9], Kevin HM Kuo[10], Sujit Sheth[11], Janet L Kwiatkowski[12], Bruce Silver[13], Charles Kung[14], Marvin Cohen[15], Hua Yang[14], Penelope A Kosinski[14], Lei Hua[14], Ann Barbier[14], Bertil Glader [16]

*[1]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, MA USA; [2]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, UK; [3]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, France; [4]Hôpital Saint Vincent de Paul, Lille, France; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [6]Central Pennsylvania Clinic, Belleville, PA, USA; [7]Universitair Medisch Centrum Utrecht, Utrecht, Netherlands; [8]University of Utah, Salt Lake City, UT, USA; [9]Wayne State University School of Medicine, Children's Hospital of Michigan, Detroit, MI, USA; [10]University of Toronto, Toronto, Canada; [11]Weill Cornell Medical College, New York, NY, USA; [12]Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA, USA; [13]Bruce A Silver Clinical Science and Development, Dunkirk, MD, USA; [14]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [15] MBC Pharma Solutions, Newtown, PA, USA; [16]Stanford University School of Medicine, Palo Alto, CA, USA*

*Presented at the 22nd Congress of the European Hematology Association, 22–25 June 2017, Madrid, Spain*

# Disclosures

- This study was funded by Agios Pharmaceuticals Inc.
- Rachael F Grace: Agios – study co-principal investigator and scientific advisor
- D Mark Layton: Agios – study investigator
- Frédéric Galactéros – study investigator
- Christian Rose: Agios – study investigator
- Wilma Barcellini: Agios – study investigator, no other disclosures
- D Holmes Morton – study investigator
- Eduard van Beers – study investigator
- Hassan Yaish – study investigator
- Yaddanapudi Ravindranath – study investigator
- Kevin HM Kuo – study investigator
- Sujit Sheth – study investigator
- Janet L Kwiatkowski: Agios – study investigator; Agios, Shire, Sideris, and Ionis Pharmaceuticals – consultant
- Bruce Silver, Marvin Cohen: Agios – consultants
- Bertil Glader: Agios – study co-principal investigator and scientific advisor
- Charles Kung, Hua Yang, Penelope A Kosinski, Lei Hua, Ann J Barbier: Agios – employees and stockholders
- Editorial assistance was provided by Christine Ingleby, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios

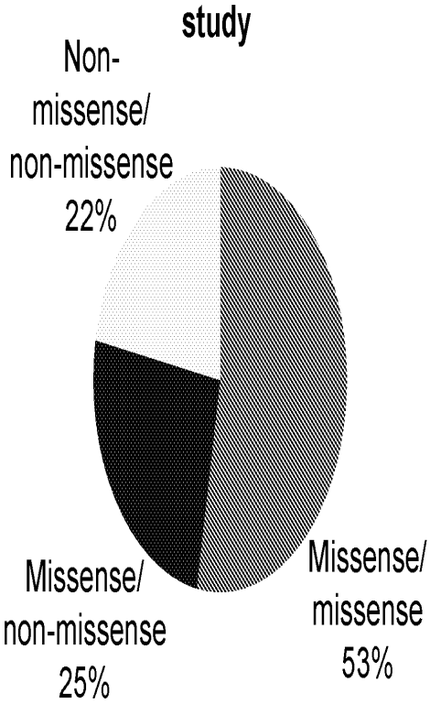


Ex. 2001-0575

# Pyruvate kinase (PK) deficiency: a severe congenital anemia

| | |
|---|---|
| **Description** | • Presents in childhood with severe hemolytic anemia |
| **Etiology** | • Caused by mutations in the *PK-LR* gene coding for erythrocyte pyruvate kinase (PK-R) |
| **Disease burden** | • Lifelong hemolytic anemia<br>• Iron overload and jaundice<br>• Infection risk post splenectomy |
| **Diagnosis/ treatment** | • PK-R enzyme activity and/or genetic testing<br>• Supportive treatment: transfusions, splenectomy, iron chelation |

**Type of *PK-LR* mutations found in 74 unrelated cases enrolled in the PK deficiency natural history study**



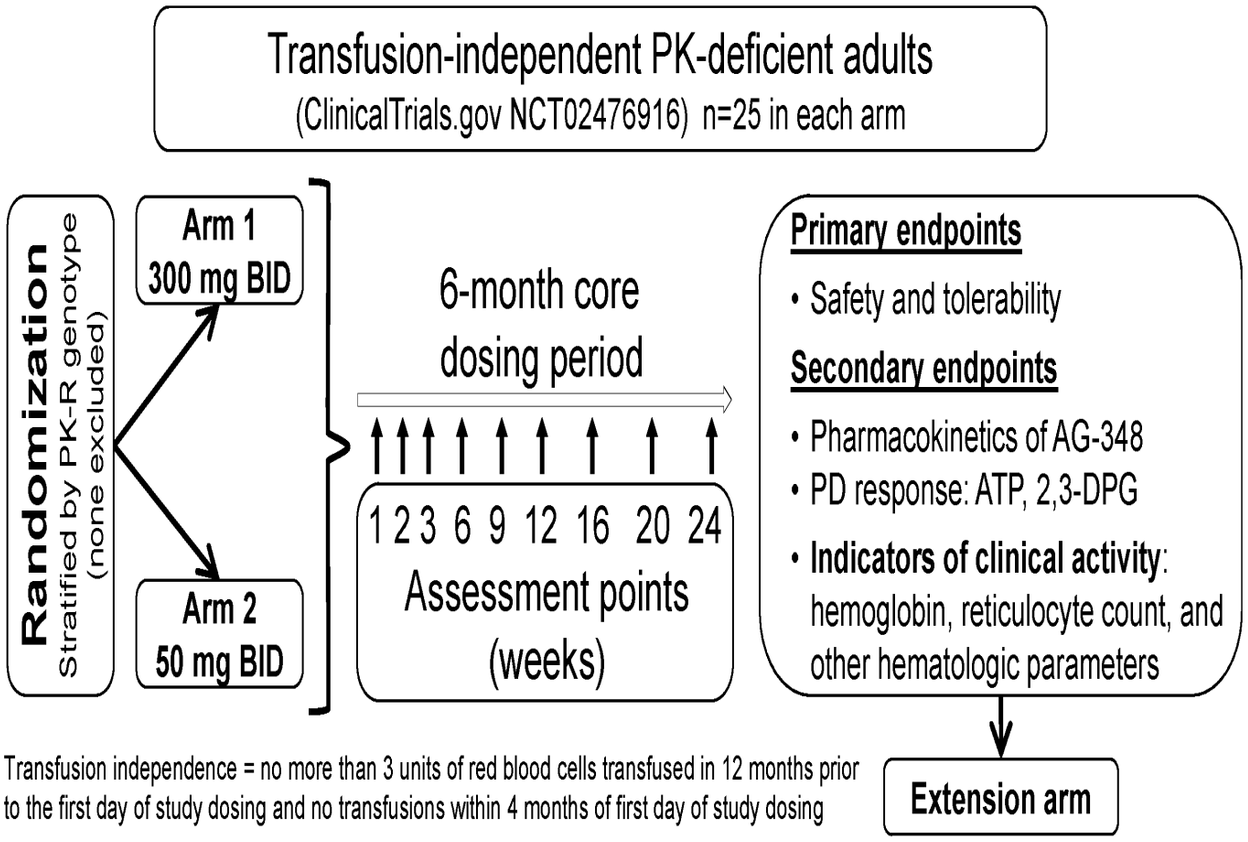

Source: Grace R et al. *Am J Hematol* 2015;90(9):825-30; Bianchi P et al. 57th ASH Annual Meeting 2015, Abstract 3337



Ex. 2001-0576

# Study design

## Open-label, global phase 2 study: 14 centers in the US, Canada, and EU




Transfusion independence = no more than 3 units of red blood cells transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

Fully enrolled as of November 2016

All patients provided written informed consent. 2,3-DPG = 2,3 diphoshphoglycerate; BID = twice daily; PD = pharmacodynamic



Ex. 2001-0577

# Demographics

| Characteristics[a] | 50 mg BID n=27 | 300 mg BID n=25 | Total N=52 |
|---|---|---|---|
| Men, n (%) | 18 (66.7) | 14 (56.0) | 32 (61.5) |
| Age at randomization in years, median (range) | 29 (18–58) | 40 (21–62) | 34 (18–62) |
| Race white[b], n (%) | 22 (81.5) | 20 (80.0) | 42 (80.8) |
| Hemoglobin (Hb) baseline, median (range) | 9.6 (6.9–12.3) | 8.6 (6.5–12.0) | 8.9 (6.5–12.3) |
| Duration of treatment, weeks, median (range) | 23.0 (13.0–76.9) | 26.3 (12.9–70.9) | 24.9 (12.9–76.9) |
| Splenectomized, n (%) | 23 (85.2) | 20 (80.0) | 43 (82.7) |
| Iron chelation prior to enrolment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (76.0) | 38 (73.1) |

[a]Data cut-off March 27, 2017; [b]Not reported in 3 patients, 3 patients were Asian, and 4 were "other"



Ex. 2001-0578

# Safety summary

- AG-348 was generally well tolerated
- The majority of adverse events (AEs) were grade 1–2
- Treatment-related AEs leading to discontinuation, n=3
  - Chest discomfort/pleural effusion, pharyngitis/nausea, anemia
- 13 serious AEs in 10 patients
  - Six drug-related SAEs in 5 patients: Withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia; pharyngitis
    - Grade 4 hypertriglyceridemia at Week 24, resolved upon AG-348 discontinuation (Grade 1 at baseline)

| Patient disposition by study period | Ongoing, N | Completed, N | Discontinued[a], N |
|---|---|---|---|
| Core | 15 | 29[b] | 8 |
| Extension | 21 | 0 | 3 |

[a]Reasons for discontinuation: AEs (3), investigator decision (4), and withdrew consent (4)

[b]Five subjects completing the Core period did not enter the Extension



Ex. 2001-0579

# Safety summary: most common AEs

| AEs, regardless of causality (occurring in >5 patients) | 50 mg BID n=27 | | 300 mg BID n=25 | | Total N=52 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n (%) | 25 (92.6) | 7 (25.9) | 25 (100.0) | 5 (20.0) | 50 (96.2) | 12 (23.1) |
| Headache | 9 (33.3) | 0 | 14 (56.0) | 0 | 23 (44.2) | 0 |
| Insomnia | 5 (18.5) | 1 (3.7) | 15 (60.0) | 1 (4.0) | 20 (38.5) | 2 (3.8) |
| Nausea | 10 (37.0) | 0 | 9 (36.0) | 0 | 19 (36.5) | 0 |
| Viral upper respiratory tract infection | 7 (25.9) | 0 | 2 (8.0) | 0 | 9 (17.3) | 0 |
| Arthralgia | 5 (18.5) | 0 | 3 (12.0) | 0 | 8 (15.4) | 0 |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Back pain | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Cough | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Dizziness | 4 (14.8) | 0 | 3 (12.0) | 1 (4.0) | 7 (13.5) | 1 (1.9) |
| Vomiting | 3 (11.1) | 0 | 4 (16.0) | 0 | 7 (13.5) | 0 |
| Hot flush | 1 (3.7) | 0 | 6 (24.0) | 0 | 7 (13.5) | 0 |
| Diarrhea | 3 (11.1) | 0 | 3 (12.0) | 0 | 6 (11.5) | 0 |
| Influenza | 5 (18.5) | 1 (3.7) | 1 (4.0) | 0 | 6 (11.5) | 1 (1.9) |

Grade 3 AEs not reported in previous slide or table above: colitis (n=1), thrombosis (n=1), pharyngitis (n=1), post-procedural hemorrhage (n=1), hypertension (n=1)

AEs graded using National Cancer Institute Common Terminology Criteria, version 4.03



Ex. 2001-0580

# Dose titration in DRIVE PK

- Patients randomized to starting dose of 50 mg or 300 mg BID AG-348
  - Dose decreased:
    - AEs (e.g. insomnia)
    - Hb exceeding midpoint of normal range (Male: >15.0 g/dL; Female: >13.5 g/dL)
  - Dose increased:
    - Lack of Hb response
- Range of doses (5 mg QD–300 mg BID) used
- Efficacy and steroid hormone levels analyzed by the dose received for the longest duration in the Core period

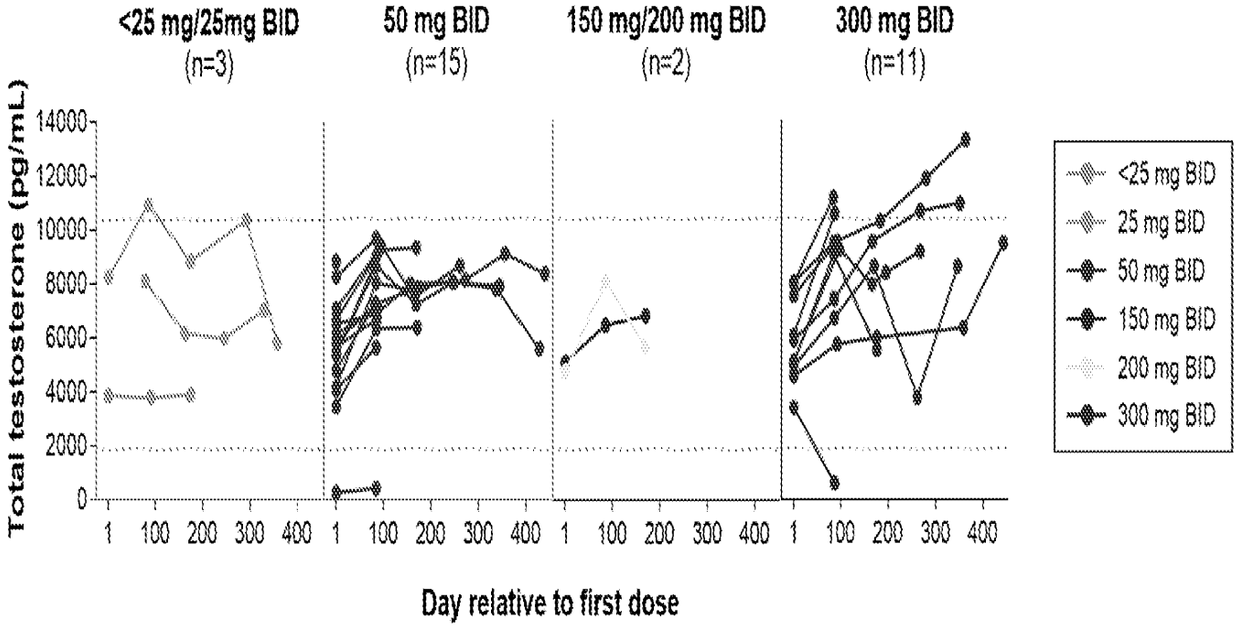
QD = once daily



Ex. 2001-0581

# Effect of AG-348 on hormones: total testosterone in men

- Preliminary findings are consistent with aromatase inhibition by AG-348 across multiple dose levels in men
  - Most testosterone values remained within the normal range
  - DEXA scan data inconclusive
  - Longer follow-up required to assess clinical impact




Normal reference low and high limits shown as horizontal dotted lines; DEXA = dual energy X-ray absorptiometry

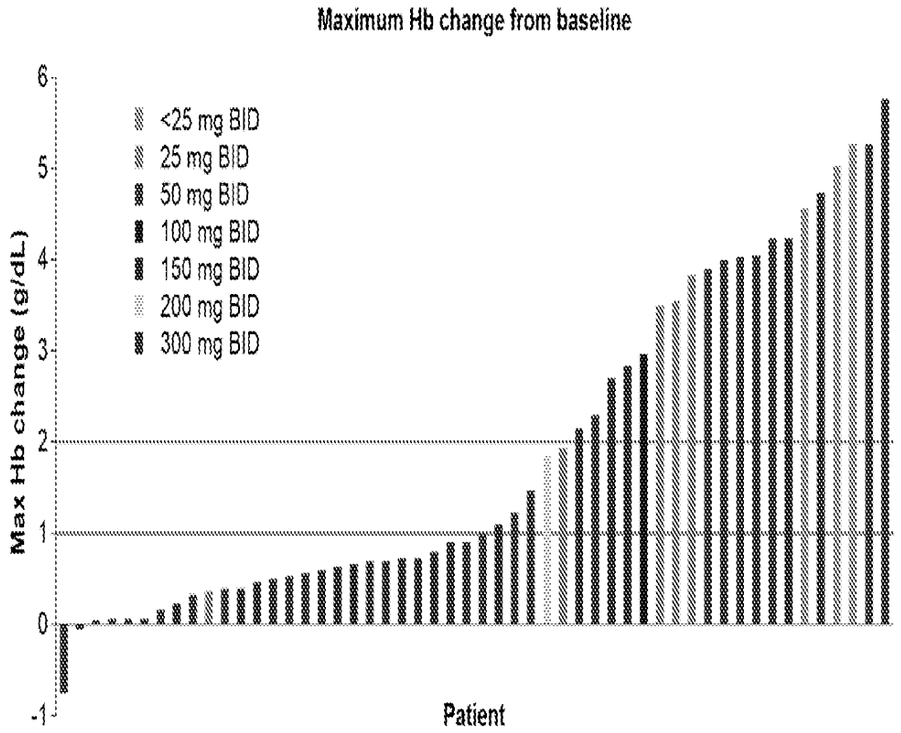


Ex. 2001-0582

# Clinical activity results

# Maximum Hb increase observed during the Core period

- 25/52 (48%) patients had a maximum Hb increase of >1.0 g/dL
  - The mean maximum increase was 3.5 g/dL (range 1.1–5.8 g/dL)



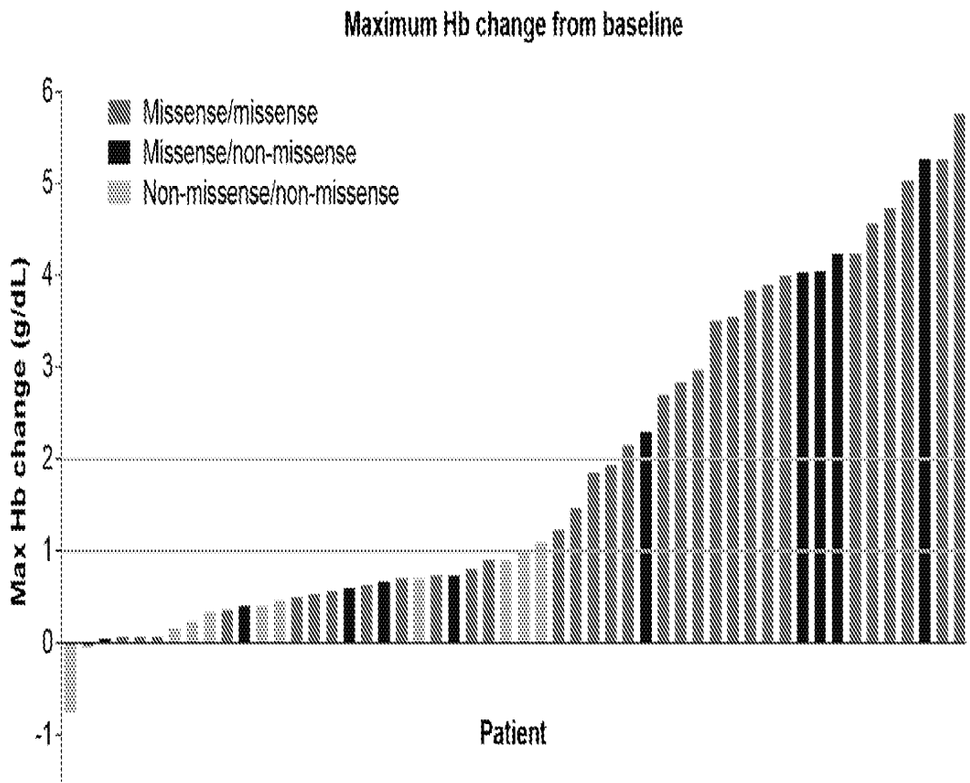

The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)



Ex. 2001-0584

# Maximum Hb increase observed by genotype

- 25/52 (48%) patients had a maximum increase in Hb >1.0 g/dL
  - 24/42 (57%) patients who had ≥1 missense mutation had Hb increase >1.0 g/dL



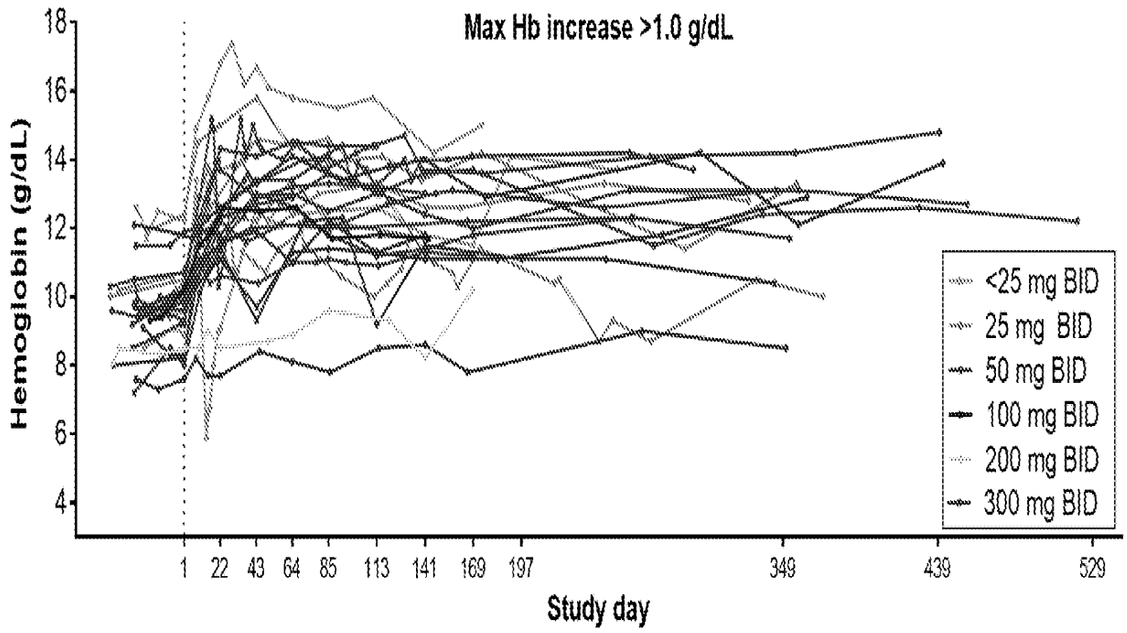

The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)



Ex. 2001-0585

# Majority of Hb increases are rapid and sustained

- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range 7–141 days)
  - Median baseline Hb in subjects who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.5–12.3 g/dL) vs. 8.0 g/dL (range 6.5–10.1) in subjects who did not
- In 8 patients, the dose had to be held or reduced due to rapid rise in Hb

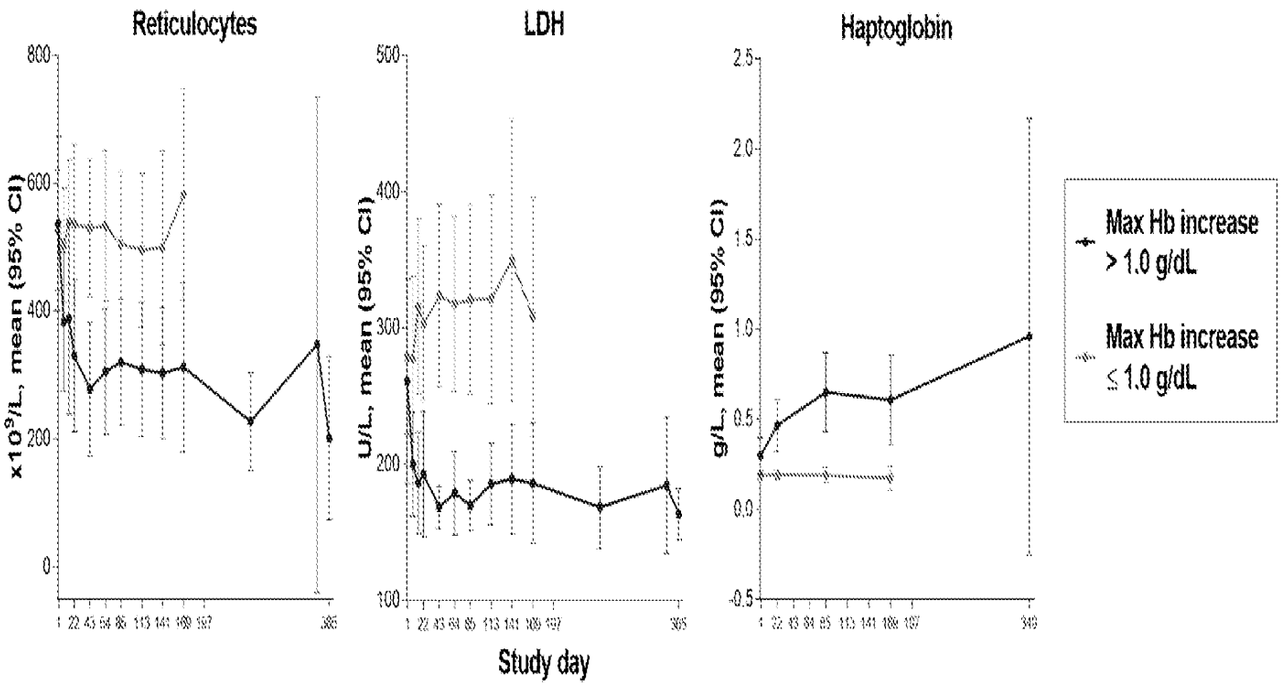





Ex. 2001-0586

# Hemolysis markers improved in patients with Hb increase of >1.0 g/dL

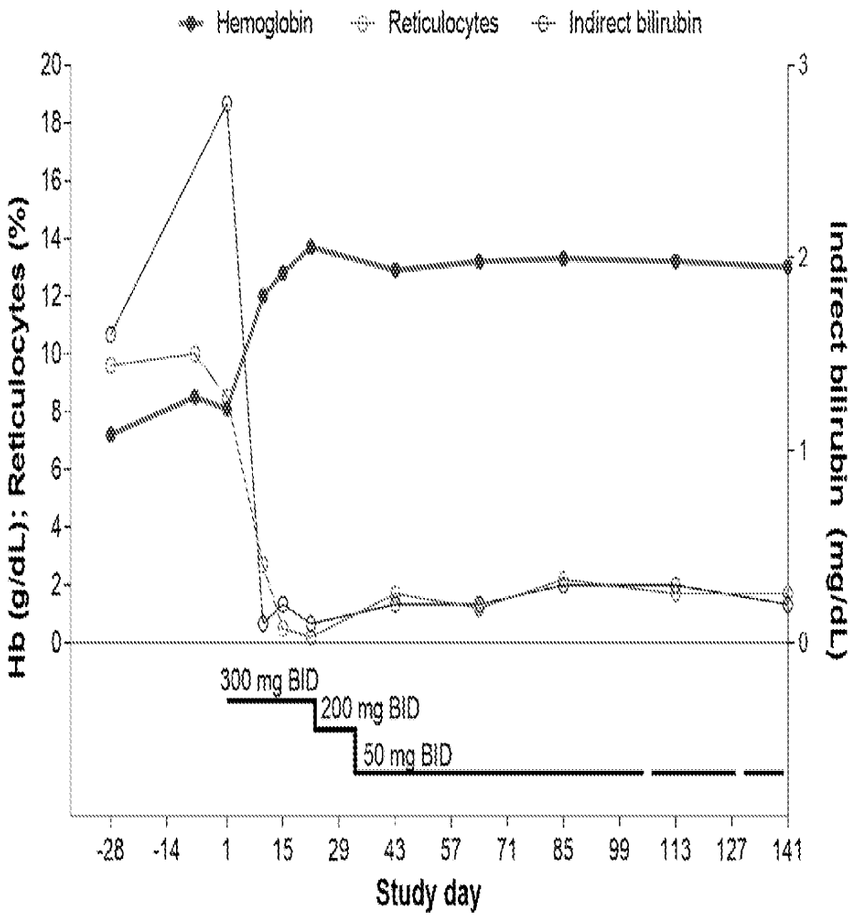
- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin




Only visits with ≥5 subjects included. Figure shows central laboratory results. LDH = lactate dehydrogenase

# Hb response with improvements in hemolysis parameters: single subject experience with AG-348

- Female 35 years of age, white, missense/missense genotype (T384M/R479H)






Ex. 2001-0588

# DRIVE PK conclusions

- AG-348 is a novel, first-in-class PK-R activator in clinical testing as a potential disease-altering therapy for patients with PK deficiency
- Chronic daily dosing with AG-348 is well tolerated
  - Clinical significance of AG-348 aromatase inhibition is unclear
  - One grade 4 serious AE of elevated triglycerides was observed
- 25 of 52 (48%) subjects had a maximum Hb increase of >1.0 g/dL
  - Responses are rapid in onset and durable
  - Other parameters (reticulocytes, LDH, and haptoglobin) indicate decreased hemolysis in responders
  - Some genotype–Hb response correlations were observed
- These data highlight the potential of AG-348 to be the first disease-altering treatment for patients with PK deficiency, providing a rationale for pivotal studies



Ex. 2001-0589

# Acknowledgments


- We would like to thank the patients who agreed to participate in this study
- We would also like to thank all the clinical research sites and study investigators:
  - Boston Children's Hospital; Rachael F Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet L Kwiatkowski
  - Central Pennsylvania Clinic; D Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin HM Kuo
  - Hammersmith Hospital; D Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Hôpital Henri Mondor; Frédéric Galactéros
  - Hôpital Saint Vincent de Paul; Christian Rose
  - Hôpital de la Timone; Emmanuelle Bernit
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
- We would like to thank Drs Ellis Neufeld and David Nathan for helpful discussions
- This clinical study was funded by Agios Pharmaceuticals

Editorial support was provided by Christine Ingleby, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios




Ex. 2001-0590

# Mitapivat (AG-348) long-term safety and efficacy in pyruvate kinase deficiency: 3-year results of the DRIVE-PK study

EP1461

Kathleen M. Glenn[1], Mark Connor[2], Antonio Piga[3], Wilma Barcellini[4], Rebecca Kuo[5], Ana Rothman[6], Ana Villegas[7], Ozan Beyan[8], Ana Kosmidis[9], Jonathan Kuo[10], Rose Ann Padilla[11], Sarah Ben-Dor[12], Kenneth I. Ataga[13]

## BACKGROUND

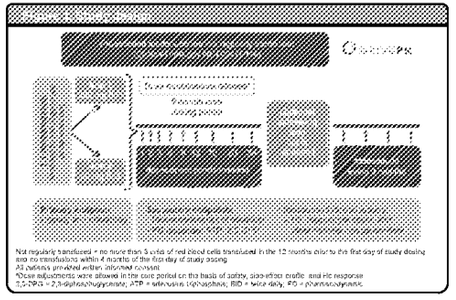
- Pyruvate kinase (PK) deficiency is an under-recognized autosomal recessive disease caused by mutations in the *PKLR* gene
- Pathogenic mutations lead to reduced activity of PKR, the red blood cell–specific PK enzyme, which results in lifelong hemolytic anemia[1,2]
- Acute and chronic complications of supportive care (eg, transfusions, splenectomy, or iron chelation) can additionally burden patients with PK deficiency
- Mitapivat (AG-348)—an oral, small-molecule, allosteric activator of both wild-type and mutant PKR[3,4]—is undergoing clinical evaluation in patients with PK deficiency
- DRIVE-PK is a phase 2, randomized, open-label, dose-ranging study of mitapivat in adults with PK deficiency who were not regularly receiving red cell transfusions. Results from the first 6 months of the study showed
  - A rapid increase from baseline of > 1.0 g/dL in hemoglobin (Hb) levels in 50% of patients
  - An acceptable safety profile[5]

## OBJECTIVE

- To report long-term safety and efficacy of mitapivat in patients with PK deficiency continuing in the extension period of DRIVE-PK (ClinicalTrials.gov NCT02476916)

## METHODS

- DRIVE-PK is a global study, with patients enrolled at 14 centers in the US, Canada, and Europe
- Patients who experienced clinical benefit without any concerning safety issues related to mitapivat (investigator discretion) could opt to enter the extension period (**Figure 1**)

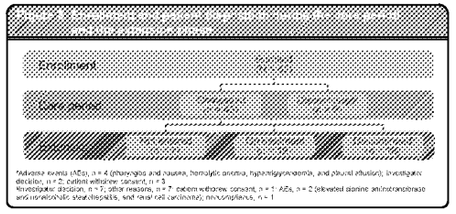



## METHODS (CONTINUED)

- Protocol amendments during the extension period required that:
  - Patients who did not have an increase from baseline Hb of ≥ 1.0 g/dL for ≥ 3 of the 4 prior measurements withdraw from the study
  - Patients treated with mitapivat doses > 25 mg BID undergo a dose taper and continue on a dose that maintained their Hb level at no lower than 1.0 g/dL below their pretaper Hb level

**Disposition**

- Of the 52 patients enrolled in the study, 43 completed the core period (24 weeks) and 18 remained in the study as of 27March2019 (**Figure 2**)




## RESULTS

**Treatment**

- Among the 18 patients continuing in the extension period, the last post-taper mitapivat doses were:
  - ≤ 25 mg BID (excluding missed doses; n = 12)
  - 50 mg BID (n = 5)
  - 200 mg BID (n = 1)
- Median (range) duration of treatment was 35.6 (28.7–41.9) months

**Table 1. Demographic characteristics of all patients and those who continued in the extension period**

| Characteristic | Total (N = 52) | Continued (N = 18) |
|---|---|---|
| Male, n (%) | 32 (61.5) | 10 (55.6) |
| Age at screening, median (range), years | 34 (18–61) | 33.5 (19–61) |
| White, n (%) | 43 (82.7) | 17 (94.4) |
| Hb baseline, median (range), g/dL | 8.9 (6.5–12.3) | 9.2 (7.9–12.0) |
| Splenectomy, n (%) | 43 (82.7) | 11 (61.1) |
| Cholecystectomy, n (%) | 38 (73.1) | 14 (77.8) |
| Iron chelation prior to enrollment, n (%) | 25 (48.1) | 5 (27.8) |
| Osteoporosis, n (%) | 8 (15.4) | 2 (11.1) |
| Mutation category, n (%) | | |
| Missense/missense | 32 (61.5) | 14 (77.8) |
| Missense/non-missense | 10 (19.2) | 4 (22.2) |
| Non-missense/non-missense | 10 (19.2) | 0 (0) |

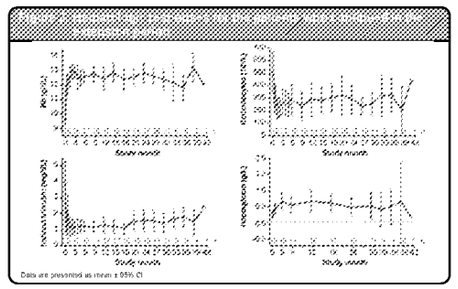
## RESULTS (CONTINUED)

**Baseline characteristics**

- No differences in age, race, or sex were observed between patients who continued in the extension period and those in the total cohort (**Table 1**)
- Patients continuing treatment had a lower splenectomy rate, and fewer had received prior iron chelation therapy
- The baseline Hb range of patients who continued in the extension period overlapped with the baseline range of the total cohort
- All continuing patients had at least one missense *PKLR* mutation, compared with 81% of patients originally enrolled

**Efficacy**

- Improvements in Hb and other markers of hemolysis, including reticulocytes, indirect bilirubin, and haptoglobin, achieved during the core period were sustained during the extension period (up to 42 months) (**Figure 3**)




**Safety**

- Mitapivat was generally well tolerated; the majority of AEs were grade 1–2
- AEs for patients who continued in the study (n = 18) were comparable in the core and extension periods (**Table 2**)
  - Headache, insomnia, fatigue, and nasopharyngitis were the most commonly reported AEs in the extension period
  - Fewer patients reported nausea and hot flush in the extension period than in the core period (2 vs 6 and 0 vs 5, respectively)
  - More patients reported nasopharyngitis in the extension period than in the core period (5 vs 1)
  - No new safety signals were identified in the extension period
- Ten patients had an increase from baseline of one grade or more in either aspartate transaminase or alanine aminotransferase on the basis of their worst grade
- Ten patients had an increase from baseline of one grade or more in triglycerides on the basis of their worst grade
- Male subjects experienced an increase in total and free testosterone and a decrease in estradiol and estrone consistent with previously reported mild off-target aromatase inhibition. Testosterone levels have been observed to remain in the normal range for the majority of subjects treated with mitapivat
- In a subset of patients where bone mineral density (femoral neck, total hip, and total lumbar spine) was analysed, no clinically meaningful trends were evident

**Table 2. Most common AEs in patients who continued in the extension period[a]**

| AE, n (%) | Core (N = 18) | Extension (N = 18) |
|---|---|---|
| Headache | 10 (55.6) | 7 (38.9) |
| Insomnia | 5 (27.8) | 5 (27.8) |
| Fatigue | 4 (22.2) | 5 (27.8) |
| Nasopharyngitis | 1 (5.6) | 5 (27.8) |
| Dizziness | 3 (16.7) | 3 (16.7) |
| Gastroenteritis | 2 (11.1) | 3 (16.7) |
| Pyrexia | 2 (11.1) | 3 (16.7) |
| Hypertriglyceridaemia | 1 (5.6) | 3 (16.7) |
| Nausea | 6 (33.3) | 2 (11.1) |
| Influenza | 4 (22.2) | 2 (11.1) |
| Arthralgia | 4 (22.2) | 2 (11.1) |
| Cough | 3 (16.7) | 2 (11.1) |
| Vomiting | 4 (22.2) | 1 (5.6) |
| Diarrhea | 4 (22.2) | 1 (5.6) |
| Dysmenorrhea | 4 (22.2) | 1 (5.6) |
| Hot flush | 5 (27.8) | 0 (0.0) |
| Chest discomfort | 3 (16.7) | 0 (0.0) |

[a]AEs coded using MedDRA, version 22.0.
AEs occurring in ≥ 15% of the 18 continuing patients in either the core or extension periods

## CONCLUSIONS

- Mitapivat is a novel, first-in-class, PKR activator in clinical testing as a potential disease-altering therapy for patients with PK deficiency
- Patients who responded to mitapivat had long-term durable responses:
  - Improvements in hemoglobin and other hemolysis markers were sustained at optimized individual doses during the extension period
- Chronic daily dosing with mitapivat for a median of 3 years and up to 42 months was well tolerated:
  - Consistent safety profile over the duration of treatment
  - No new safety signals observed
- Two phase 3 trials are underway to further study the effect of mitapivat in patients with PK deficiency

ACTIVATE ACTIVATE-T

**Acknowledgments** We would like to thank the patients taking part in the study. **Disclosures** This study was funded by Agios Pharmaceuticals, Inc. These data were previously presented in Glenn KF et al. *61st ASH Annual Meeting*, December 7–10, 2019, Orlando, FL, USA; Poster 3512. KFG: Agios – consultant, advisory committee member, research funding, Novartis – research funding. DML: Agios, Novartis – advisory committee member; Celgene – data safety monitoring board member. FC: Addmedica – advisory committee member. WB: Agios, Silvergate, Ionyx – consultant, advisory board member; Apellis – consultant; Alexion – consultant, research funding, speakers bureau member; Novartis – research funding, speakers bureau member. EJvS: Agios – advisory committee member, research funding. HMY: no conflict of interest to disclose. YR: Agios – study investigator, research funding. NHMK: Agios, Apellis, Bluebird Bio, Celgene, Pfizer – consultant; Alexion, Novartis – consultant, honoraria; Silvergate – data safety monitoring board member. SB: Celgene – consultant; Agonostherapeutics – data safety monitoring board member; CRISPR/Vertex – clinical trial steering committee member; JLK: Agios, Celgene, Ionyx – consultant; Apophanis, Novartis, Tenaya – research funding; Bluebird Bio – consultant, research funding. LH and CM: Agios – employment and stockholder; PPH: Agios – employment and stockholder; Bristol-Myers Squibb, Infinity Pharma, Jazz – stockholder. BO: Agios – consultant, advisory committee member, research funding. Editorial assistance was provided by Christine Ingleby, PhD, CMPP, Excel Medical Affairs, Horsham, UK, and supported by Agios. **References** 1. Grace RF et al. *Am J Hematol* 2018;93:525–30. 2. Perry AM et al. *Blood Cells Mol Dis* 2007;38:188–94. 3. Yang H et al. *Clin Pharmacol Drug Dev* 2019;8:266–78. 4. Kung C et al. *Blood* 2017;130:1347–56. 5. Grace RF et al. *N Engl J Med* 2018;381:933–44.

Presented at the Virtual Edition of the 25th European Hematology Association (EHA) Annual Congress, June 11–14, 2020

Ex. 2001-0591

conducted based on the pBABE-puro retrovirus vector (Addgene). To evaluate the mitochondrial respiration status, including the oxygen consumption rate (OCR), an XFe24 extracellular flux analyzer was used (Primetech).

**Results:** A microarray analysis based on control and FOG-1-overexpressed K562 cells demonstrated that 39 and 14 genes were upregulated and downregulated (> 2.5-fold), respectively, in the FOG-1-overexpressed cells. The upregulated gene ensemble included EPB41 (encoding Erythrocyte membrane protein band 4.1) and GLUT1. Quantitative RT-PCR confirmed significant upregulation of GLUT1 by FOG-1 overexpression. Because the GATA-1 ChIP sequencing analysis identified a GATA-1 peak in the GLUT1 promoter (Fujiwara et al, Mol Cell 2009), we performed a quantitative ChIP analysis and confirmed GATA-1 and GATA-2 chromatin occupancy at the GLUT1 locus. The chromatin occupancy of GATA-1 at the GLUT1 locus was significantly increased, whereas that of GATA-2 was significantly decreased by FOG-1 overexpression, indicating that FOG1 could have an important role in GLUT1 upregulation in erythroid cells.

During erythroid differentiation from human CD34-positive cells isolated from cord blood, GLUT1 was markedly upregulated in the later stages of erythroid differentiation, which reflects a high glucose demand in mature erythroblasts. When GLUT1 was overexpressed in K562 cells, the OCR rate initially significantly decreased and thereafter increased during maintenance culture. We are currently conducting detailed biological analyses to characterize the role of GLUT1 during erythroid differentiation.

**Summary/Conclusion:** Our findings describe an important link between the FOG-1-dependent transcriptional network and the glucose metabolic regulation of erythroid cells and provide a strong foundation for future studies that aim to elucidate information on glucose metabolic regulation during normal and aberrant erythropoiesis.

## EP1561 MITAPIVAT (AG-348) LONG-TERM SAFETY AND EFFICACY IN PYRUVATE KINASE DEFICIENCY: 3-YEAR RESULTS OF THE DRIVE PK STUDY

R. F. Grace[1*], D. M. Layton[2], F. Galactéros[3], W. Barcellini[4], E. J. van Beers[5], H. M. Yaish[6], Y. Ravindranath[7], K. H. Kuo[8], S. Sheth[9], J. L. Kwiatkowski[10], L. Hua[11], P. F. Hawkins[11], C. Mix[11], B. Glader[12]

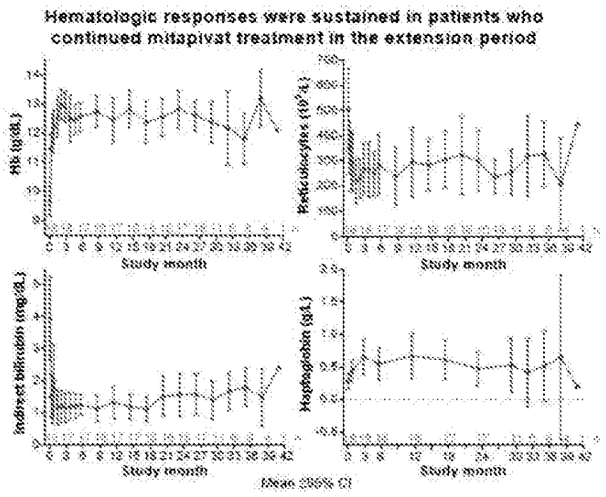
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**Background:** Pyruvate kinase (PK) deficiency is caused by PKLR gene mutations leading to reduced activity of PK-R, the red blood cell–specific PK enzyme. The resulting hemolytic anemia is lifelong, with treatment currently limited to supportive care. Mitapivat sulfate (AG-348) is a first-in-class, oral, small-molecule, allosteric activator of PK-R in clinical development for the treatment of PK deficiency. DRIVE PK is a phase 2, open-label, dose-ranging study of the safety and efficacy of mitapivat in adults with PK deficiency. In the 6-month core period, mitapivat rapidly increased hemoglobin (Hb) levels in 50% of patients, with acceptable safety (Grace et al. NEJM 2019;381:933).

**Aims:** To present the long-term safety and efficacy of mitapivat in patients with PK deficiency continuing treatment in the DRIVE PK study extension period (ClinicalTrials.gov NCT02476916).

**Methods:** Key inclusion criteria for the DRIVE PK study were age ≥18 years; diagnosis of PK deficiency confirmed by enzyme and molecular testing; baseline Hb levels ≤12.0 g/dL (men) or ≤11.0 g/dL (women); and ≤3 units of red blood cells transfused in the prior 12 months with no transfusions in the prior 4 months. Patients were randomized 1:1 to mitapivat 50 mg or 300 mg twice daily (BID), after which dose adjustment was permitted based on safety and efficacy. Patients could opt to enter the extension period if they had experienced clinical benefit with no concerning safety issues related to mitapivat, at investigator discretion. Extension period protocol amendments required: (1) withdrawal from the study for patients without an increase in Hb of ≥1.0 g/dL from baseline for ≥3 of the prior 4 measurements; and (2) dose tapering for patients who received mitapivat > 25 mg BID to the lowest dose maintaining Hb at ≥1.0 g/dL above their pretaper Hb level. Safety and efficacy were assessed every 3 months during the extension.

**Results:** Of 52 patients treated, 43 (83%) completed the core period and 36 (69%) entered the extension. At data cutoff, 18 patients had discontinued treatment due to investigator decision (n = 7), adverse events (AEs; n = 2), consent withdrawal (n = 1), noncompliance (n = 1), or other (n = 7). Eighteen patients were continuing mitapivat and had received ≥29 months' treatment (median duration 35.6 months [range 28.7–41.9]). Of these 18, 8 were female, 11 had a prior splenectomy, and mean baseline Hb was 9.7 g/dL (range 7.9–12.0). The posttaper BID doses of continued treatment were ≤25 mg (n = 12), 50 mg (n = 5), and 200 mg (n = 1). Improvements in Hb and hemolysis markers achieved in the core period were sustained in the extension period (Figure). Mitapivat was generally well tolerated in the 18 patients who continued treatment; the majority of AEs were grade 1–2. Similar AEs were reported in the extension vs core periods, with headache (38.9% vs 55.6%), insomnia (both 27.8%), and fatigue (27.8% vs 22.2%) reported most frequently. Fewer patients reported nausea (2 vs 6) and hot flush (0 vs 5) in the extension period. This AE profile in the extension is consistent with that seen in the overall patient population treated in the core period (N = 52). No new safety signals were observed.




**Summary/Conclusion:** These long-term safety and efficacy data support the potential for mitapivat to be the first disease-altering therapy for PK deficiency. Mitapivat is being further evaluated in patients with PK deficiency in two ongoing phase 3 trials.

## EP1562 MISSENSE OR NON MISSENSE: IS THAT THE QUESTION?

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**Background:** AG-348 is a drug that is currently being studied as a treatment of congenital hemolytic anemia due to pyruvate kinase (PK)



Ex. 2001-0592

ORIGINAL ARTICLE

# Safety and Efficacy of Mitapivat in Pyruvate Kinase Deficiency

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## ABSTRACT

### BACKGROUND

Pyruvate kinase deficiency is caused by mutations in *PKLR* and leads to congenital hemolytic anemia. Mitapivat is an oral, small-molecule allosteric activator of pyruvate kinase in red cells.

### METHODS

In this uncontrolled, phase 2 study, we evaluated the safety and efficacy of mitapivat in 52 adults with pyruvate kinase deficiency who were not receiving red-cell transfusions. The patients were randomly assigned to receive either 50 mg or 300 mg of mitapivat twice daily for a 24-week core period; eligible patients could continue treatment in an ongoing extension phase.

### RESULTS

Common adverse events, including headache and insomnia, occurred at the time of drug initiation and were transient; 92% of the episodes of headache and 47% of the episodes of insomnia resolved within 7 days. The most common serious adverse events, hemolytic anemia and pharyngitis, each occurred in 2 patients (4%). A total of 26 patients (50%) had an increase of more than 1.0 g per deciliter in the hemoglobin level. Among these patients, the mean maximum increase was 3.4 g per deciliter (range, 1.1 to 5.8), and the median time until the first increase of more than 1.0 g per deciliter was 10 days (range, 7 to 187); 20 patients (77%) had an increase of more than 1.0 g per deciliter in the hemoglobin level at more than 50% of visits during the core study period, with improvement in markers of hemolysis. The response was sustained in all 19 patients remaining in the extension phase, with a median follow-up of 29 months (range, 22 to 35). Hemoglobin responses were observed only in patients who had at least one missense *PKLR* mutation and were associated with the red-cell pyruvate kinase protein level at baseline.

### CONCLUSIONS

The administration of mitapivat was associated with a rapid increase in the hemoglobin level in 50% of adults with pyruvate kinase deficiency, with a sustained response during a median follow-up of 29 months during the extension phase. Adverse effects were mainly low-grade and transient. (Funded by Agios Pharmaceuticals; ClinicalTrials.gov number, NCT02476916.)

From the Dana–Farber/Boston Children's Cancer and Blood Disorders Center and Harvard Medical School, Boston (R.F.G.), and Agios Pharmaceuticals, Cambridge (A.J.B., S.B., L.H., C.K., P.H., M.-H.J., C.B.) — all in Massachusetts; Hôpital Saint Vincent de Paul, Lille (C.R.), and Unité des Maladies Génétiques du Globule Rouge, Centre Hospitalier Universitaire Henri Mondor, Créteil (F.G.) — both in France; Hammersmith Hospital, Imperial College Healthcare NHS Trust, London (D.M.L.); Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan (W.B.); Central Pennsylvania Clinic, Belleville (D.H.M.), and Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia (J.L.K.); Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands (E.J.B.); University of Utah, Salt Lake City (H.Y.); Wayne State University School of Medicine, Children's Hospital of Michigan, Detroit (Y.R.); University of Toronto, Toronto (K.H.M.K.); Weill Cornell Medical College, New York (S.S.); Bruce A. Silver Clinical Science and Development, Dunkirk, MD (B.S.); and Stanford University School of Medicine, Palo Alto, CA (B.G.). Address reprint requests to Dr. Grace at Boston Children's Hospital, 450 Brookline Ave., Dana 3-106, Boston, MA 02215, or at rachael.grace@childrens.harvard.edu.

*Deceased.

N Engl J Med 2019;381:933-44.
DOI: 10.1056/NEJMoa1902678

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Pyruvate kinase deficiency is an autosomal recessive enzymopathy in red cells that is caused by mutations in *PKLR*. These genetic alterations lead to a deficit of pyruvate kinase activity in red cells and to hemolytic anemia of variable severity.[1-8] In addition to anemia, pyruvate kinase deficiency is associated with serious complications that include gallstones, pulmonary hypertension, extramedullary hematopoiesis, and iron overload and its sequelae, which occur regardless of the degree of anemia or transfusion burden.[9,10] Patients most commonly have compound heterozygous mutations in the gene encoding the L and R isozymes of pyruvate kinase (*PKLR*), with more than 300 mutations described; most patients have at least one missense mutation.[9,11] Red-cell pyruvate kinase deficiency results in impaired glucose utilization and reduced ATP generation in red cells, which leads to compromised red-cell membrane homeostasis and hemolysis.[1,12-14]

Current management strategies, including blood transfusion and splenectomy, are supportive only and introduce both short- and long-term risks.[9,15-19] Hematopoietic stem-cell transplantation has been described in a small number of patients but has been associated with substantial risks of graft-versus-host disease and death.[20] No specific disease-modifying therapy exists.

Mitapivat (AG-348) is an oral, small-molecule allosteric activator of red-cell pyruvate kinase.[21] In vitro experiments have shown that mitapivat activates wild-type and a variety of mutant red-cell pyruvate kinase enzymes. Mitapivat increases pyruvate kinase activity ex vivo in red cells obtained from patients with pyruvate kinase deficiency.[21] In a dose-escalation study involving healthy volunteers,[22] investigators reported an acceptable safety profile and changes in glycolytic intermediates that were consistent with glycolytic pathway activation, findings that supported further investigation of mitapivat as a potential targeted treatment for pyruvate kinase deficiency. In this phase 2, multicenter study, we evaluated the safety and efficacy of mitapivat in adults with pyruvate kinase deficiency who were not receiving regular red-cell transfusions.

## METHODS

### STUDY DESIGN AND OVERSIGHT

We conducted the study at eight sites in North America and six sites in Europe. After a screening period of no more than 6 weeks, patients were randomly assigned in a 1:1 ratio to receive open-label mitapivat at a dose of 50 mg or 300 mg twice daily for a 24-week core period. The selection of the two doses was based on the results of a dose-escalation study involving healthy volunteers.[22] Randomization was stratified according to the *PKLR* mutation (R510Q vs. R486W vs. R479H vs. all other mutations) to maintain balance for the most frequently expected mutations. Eligible patients could opt to continue treatment in an extension phase, which is ongoing.

The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The protocol (available with the full text of this article at NEJM.org) was approved by the institutional review board or ethics committee at each study center. (Details are provided in the Supplementary Appendix, available at NEJM.org.) All the patients provided written informed consent before screening. The study was designed and analyzed by the sponsor (Agios Pharmaceuticals) in collaboration with the authors. A data and safety monitoring board consisting of the treating investigators and representatives of the sponsor reviewed the data on an ongoing basis. The manuscript was drafted by the first and last authors; all the authors contributed to reviews and revisions. A professional medical writer paid by the sponsor assisted the authors in the preparation of the manuscript. The authors vouch for the accuracy and completeness of the data and for the fidelity of the study to the protocol.

### PATIENTS

Adults (≥18 years of age) were eligible for participation if they had received a diagnosis of pyruvate kinase deficiency, as documented by lower activity of pyruvate kinase than that of other age-dependent enzymes in red cells and by the presence of at least two mutations in *PKLR* on genotyping. The hemoglobin level was 12.0 g per deciliter or less in men and 11.0 g per deciliter or less in women. None of the patients had received transfusions of more than 3 units of red cells in the previous 12 months or any transfusions in the previous 4 months.

### DOSE SELECTION

Changes in dose were permitted during the core period on the basis of safety, side-effect profile, and hemoglobin response. Increases in dose were



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allowed if the hemoglobin level remained below the lower limit of the normal range (<13.0 g per deciliter in men and <11.6 g per deciliter in women) after at least 12 weeks of treatment. Dose decreases were allowed for adverse events thought to be related to mitapivat or if the hemoglobin level exceeded the midpoint of the normal range (>15.0 g per deciliter in men and >13.5 g per deciliter in women). Early in the trial, two patients had acute hemolysis after the abrupt discontinuation of the 300-mg dose of mitapivat following a rapid increase in the hemoglobin level. An additional seven patients for whom dose reduction was necessary because of a robust hemoglobin response underwent dose tapering, according to a protocol amendment, without acute hemolysis.

Patients initially were eligible for the extension phase for continued evaluation of safety if they did not have side effects that precluded the continued administration of mitapivat and were having clinical benefit, as determined by the investigator. Subsequently, after a protocol amendment, patients who did not have an increase from baseline of at least 1.0 g per deciliter in the hemoglobin level as evaluated in at least three of the last four measurements were withdrawn from the extension phase of the study. Patients who continued in the extension phase and were receiving doses of more than 25 mg of mitapivat twice daily underwent a tapering of the dose and continued to receive an individualized dose that maintained the hemoglobin level at a value that was no lower than 1.0 g per deciliter below the value that was reported before the taper began.

### END POINTS

The primary objective of this study was to assess the safety and side-effect profile of mitapivat administration in patients with pyruvate kinase deficiency. We assessed safety by monitoring the incidence of adverse events, laboratory measurements (hematologic and chemical analyses, urinalysis, coagulation, and endocrine analysis), and findings on physical examination, 12-lead electrocardiography, and dual-energy x-ray absorptiometry (DXA) scans. Serum levels of sex hormones (testosterone, estradiol, and estrone) and DXA scans were monitored because of reversible, mild, off-target aromatase inhibition that had been observed in preclinical studies (authors' unpublished data) and in healthy volunteers.[22] We also evaluated adverse events, treatment-related adverse events, serious adverse events, and specific events of interest (acute hemolysis after abrupt mitapivat discontinuation, osteoporosis, elevations in liver enzymes, hypertriglyceridemia, and insomnia).

Secondary objectives were characterizations of the pharmacokinetic and pharmacodynamic profiles of mitapivat and clinical efficacy, as measured by changes in hemoglobin and markers of hemolysis. Baseline levels of pyruvate kinase protein in red cells were measured as described previously.[21]

### STATISTICAL ANALYSIS

For the primary evaluation of the safety and side-effect profile of mitapivat, we determined that the enrollment of 25 patients in each of the two dose groups would provide a probability of 72% of observing a rate of adverse events of 5% in either group and a 93% probability of observing a rate of 10%. The safety analysis included all the enrolled patients who had received at least one dose of mitapivat. We summarized adverse events and other categorical safety measurements according to frequency distributions for the overall population and randomly assigned starting dose.

The efficacy analysis included all the patients who had received mitapivat for at least 3 weeks. Analyses of hemoglobin response included the number and proportion of patients who had an increase of more than 1.0 g per deciliter in the postbaseline hemoglobin level in more than 50% of assessments during the core period. In a post hoc analysis, these patients were defined as having had a hemoglobin response. For analyses of the hemoglobin response according to genotype, *PKLR* mutations were categorized into missense mutations and non-missense mutations, such as truncations or frameshift mutations. To account for dose modifications, we conducted some analyses according to the dose of mitapivat that the patients received for the longest duration in the core period (called the actual dose) rather than according to the randomly assigned dose.

## RESULTS

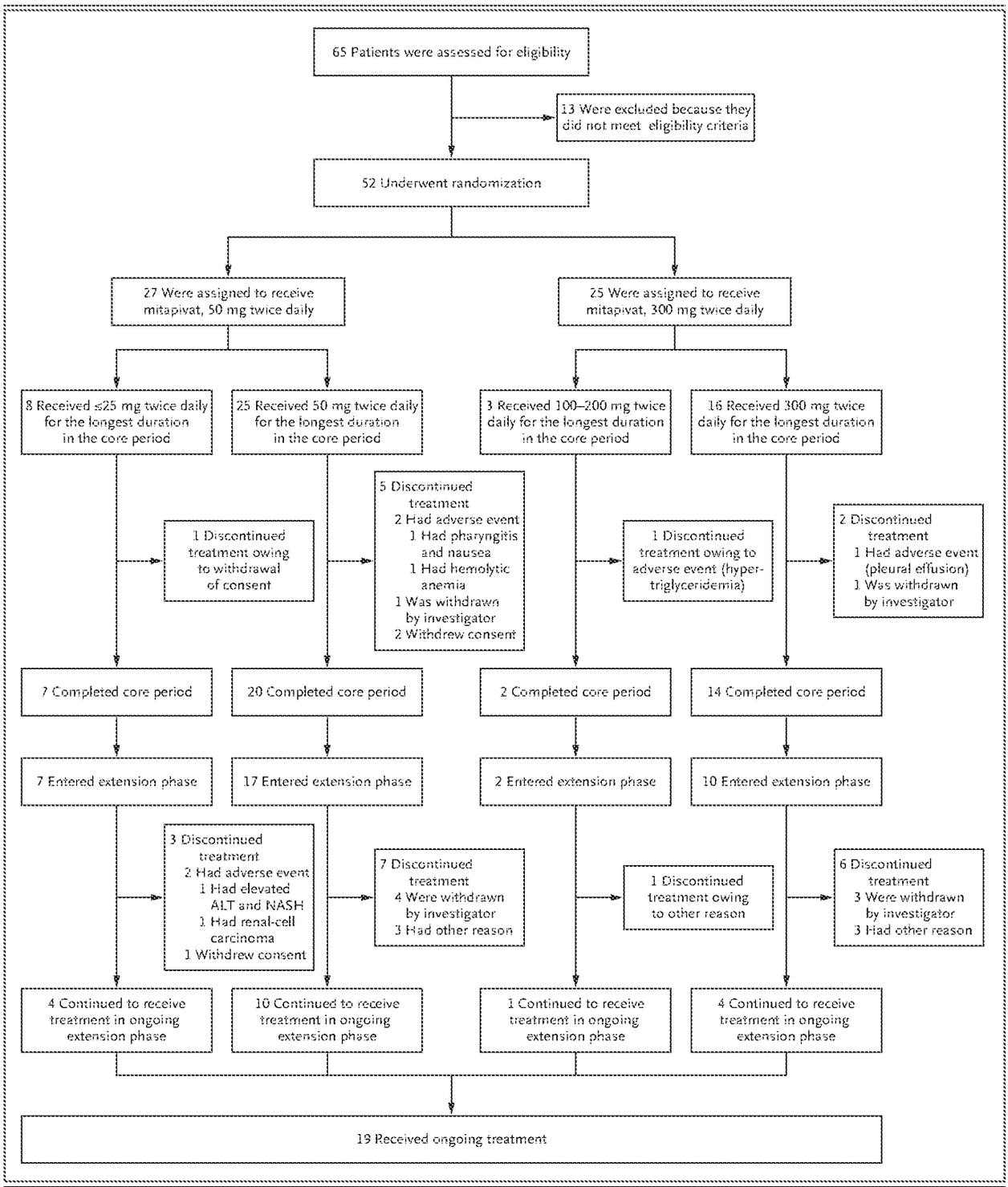
### PATIENTS

Of the 65 patients who had undergone screening, 52 were eligible for participation and were randomly assigned to receive mitapivat twice daily at a dose of 50 mg (27 patients) or 300 mg (25 patients)



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Figure 1 (facing page). **Enrollment during Core Period and Extension Phase, According to Actual Dose of Mitapivat.**

Shown are the numbers of patients who received the listed doses of mitapivat for the longest duration during the core period of the study, which was defined as the actual dose. According to the protocol, dose adjustments were allowed for reasons of safety, side-effect profile, and hemoglobin response. Provided in the Supplementary Appendix are a summary of dose adjustments (Table S1) and patients' assignments according to the randomized dose (Fig. S1). Of the 13 patients who were excluded during screening, 1 patient withdrew consent; 1 withdrew consent and had a concurrent medical condition; 3 had a concurrent medical condition, although 1 of these patients was successfully rescreened and enrolled; 1 was undergoing transfusion; 6 had a hemoglobin level that exceeded the entry criterion; and 1 did not complete screening assessments in the allotted time, although this patient was successfully rescreened and enrolled. ALT denotes alanine aminotransferase, and NASH nonalcoholic steatohepatitis.

(Fig. 1, and Fig. S1 in the Supplementary Appendix). Of the 52 patients, 43 (83%) completed the 24-week core period; 36 patients (69%) entered the extension phase of the study, and 19 (37%) continue to participate in the ongoing extension, with a median treatment duration of 29 months (range, 22 to 35 months) (Table 1). The doses of mitapivat that were administered and the rate of treatment adherence are summarized in Table S1 in the Supplementary Appendix.

The demographic and baseline clinical characteristics of the patients are shown in Table 1. The relative prevalence of *PKLR* mutation types was consistent with the literature, with 81% of the patients having at least one missense mutation. The median hemoglobin level at baseline was 8.9 g per deciliter (range, 6.5 to 12.3); 48% of the patients had a history of treatment with iron chelation despite the absence of regular red-cell transfusions. The majority of patients had undergone splenectomy (83%) and cholecystectomy (73%).

### SAFETY

All 52 of the patients who were included in the safety analysis had at least one adverse event, the majority of which were grade 1 or 2 in severity (Table 2). The most common adverse events were headache (in 24 patients), insomnia (in 22 patients), and nausea (in 21 patients). These events resolved within 7 days after the initiation of treatment in 60 of 65 episodes (92%) of headache, in 16 of 34 episodes (47%) of insomnia, and in 25 of 32 episodes (78%) of nausea. Headache and insomnia most often occurred within 2 weeks after drug initiation. Of the 52 patients, 19 (37%) had adverse events of grade 3 or higher, as reported by the investigator. Of these patients, 9 (17%) had 11 events that were deemed by the investigator to be possibly or probably related to mitapivat, including hypertriglyceridemia (in 4 patients [6%]), hemolytic anemia (in 2 [4%]), and hemolysis, dizziness, headache, renal-cell carcinoma in the left kidney, and insomnia (in 1 each [2%]).[23] The patient with renal-cell carcinoma had a kidney lesion that had been present at the time of enrollment and was identified retrospectively.

A total of 18 serious adverse events were reported in 15 patients, all as single events with the exception of pharyngitis and hemolytic anemia (in 2 patients each [4%]). With the exception of nasopharyngitis, the cumulative safety profile (during the core phase plus the extension phase) remained similar to that observed in the core period, which indicated no change in the safety profile to date with extended treatment.

Changes from baseline in sex hormone levels, the result of off-target aromatase inhibition, were observed in male patients, with most levels of testosterone and estradiol remaining within the normal range (Fig. S2 in the Supplementary Appendix).[22] Hormone levels returned to baseline in 4 patients who discontinued mitapivat for reasons unrelated to aromatase inhibition and for whom samples were available, a finding that was consistent with the reversibility that had been observed in healthy volunteers previously.[22] In the female patients, the interpretation of data with respect to sex hormones was confounded by variability in menopausal status and the use of hormonal contraception; these data are the subject of further investigation.

In 49 patients who were evaluated, there was no worsening of bone mineral density as determined with the use of DXA scans of the total



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Table 1. Demographic and Clinical Characteristics of the Patients at Baseline.*

| Characteristic | Mitapivat, 50 mg Twice Daily (N = 27) | Mitapivat, 300 mg Twice Daily (N = 25) | All Patients (N = 52) |
|---|---|---|---|
| Sex — no. (%) | | | |
| Female | 9 (33) | 11 (44) | 20 (38) |
| Male | 18 (67) | 14 (56) | 32 (62) |
| Median age (range) — yr | 28 (18–58) | 40 (20–61) | 34 (18–61) |
| Race — no. (%)† | | | |
| White | 22 (81) | 21 (84) | 43 (83) |
| Asian | 2 (7) | 1 (4) | 3 (6) |
| Not reported | 2 (7) | 1 (4) | 3 (6) |
| Other | 1 (4) | 2 (8) | 3 (6) |
| *PKLR* mutation type — no. (%) | | | |
| Missense/missense | 15 (56) | 17 (68) | 32 (62) |
| Missense/non-missense | 6 (22) | 4 (16) | 10 (19) |
| Non-missense/non-missense | 6 (22) | 4 (16) | 10 (19) |
| Median hemoglobin (range) — g/dl | 9.6 (6.9–12.3) | 8.6 (6.5–12.0) | 8.9 (6.5–12.3) |
| Splenectomy — no. (%)‡ | 23 (85) | 20 (80) | 43 (83) |
| Cholecystectomy — no. (%) | 19 (70) | 19 (76) | 38 (73) |
| Chelation therapy before enrollment — no. (%) | 14 (52) | 11 (44) | 25 (48) |
| Median ferritin (range) — ng/ml | 723 (41–3254) | 775 (346–2518) | 764 (41–3254) |
| Osteoporosis — no. (%) | 5 (19) | 3 (12) | 8 (15) |
| Completion of 24-wk core period — no. (%)§ | 21 (78) | 22 (88) | 43 (83) |

* *PKLR* encodes pyruvate kinase L and R isozymes. Percentages may not total 100 because of rounding.
† Race was reported by the patients. The 3 patients who selected "other" race were from North and West Africa.
‡ The type of splenectomy was not reported for 2 patients; all the others underwent a complete splenectomy.
§ The median treatment duration for the 52 patients who enrolled in the 24-wk core period was 16.4 months (range, 3.0 to 34.8). As of the data cutoff on August 31, 2018, the median treatment duration since randomization for the 19 patients who continued in the open-label extension phase was 28.9 months (range, 21.6 to 34.8).

hip, total lumbar spine, and femoral neck, which were obtained and interpreted locally during screening and during treatment over a median of 17 months (range, 4 to 30) (Table S2 in the Supplementary Appendix).

### EFFICACY

Of the 52 patients, 26 (50%) had an increase from baseline of more than 1.0 g per deciliter in the hemoglobin level (Table S3 and Fig. S3 in the Supplementary Appendix). Among these patients, the mean maximum increase in the hemoglobin level was 3.4 g per deciliter (range, 1.1 to 5.8). The median time until the first observed increase of more than 1.0 g per deciliter in the hemoglobin level was 10 days (range, 7 to 187). Of the 26 patients, 20 (77%) had an increase from baseline of more than 1.0 g per deciliter at more than 50% of the assessments in the core period, which met the definition of a hemoglobin response. The hemoglobin response was maintained in the 19 patients who were continuing to be treated in the extension phase, all of whom had at least 21.6 months of treatment (Fig. S4 in the Supplementary Appendix). Details regarding the hemoglobin response and the baseline characteristics of the patients who had a response are provided in Tables S4 and S5 in the Supplementary Appendix.

A relationship between genotype and hemoglobin response was observed (Fig. 2A; and Tables S5, S6, and S7 in the Supplementary Appen-



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dix). All the patients who had an increase from baseline of more than 1.0 g per deciliter had at least one missense mutation; none of the 10 patients who had two non-missense mutations and none of the 5 patients who were homozygous for R479H mutations (most common in the Amish population) had this level of hemoglobin response. Although the R479H mutation is a missense mutation, it leads to aberrant splicing owing to the location of the mutation at a splicing site.[24] A hemoglobin response occurred in 20 of 52 patients (38%; 95% confidence interval [CI], 25 to 53). After the removal of the 10 patients who had two non-missense mutations, a hemoglobin response occurred in 20 of 42 patients (48%; 95% CI, 32 to 64); with further removal of the 5 patients who were homozygous for R479H mutations, a hemoglobin response occurred in 20 of 37 patients (54%).

A relationship was observed between the level of pyruvate kinase protein in red cells at baseline and the hemoglobin response (Fig. 2B). The mean (±SD) residual level of pyruvate kinase protein in red cells at baseline was 59±33% among the patients who had a hemoglobin response and 14±21% among those without a hemoglobin response.

No definitive relationship was apparent between the randomly assigned dose and the likelihood of a hemoglobin response. The actual dose received by 80% of the patients with a hemoglobin response was 50 mg or less twice daily, whereas 15% of the patients with a hemoglobin response received an actual dose of 300 mg twice daily, which shows that a hemoglobin response can be achieved with a low dose of mitapivat (Table S4 in the Supplementary Appendix). For some patients with a hemoglobin response, the magnitude of the change in the hemoglobin level varied according to the dose. However, during the extension phase, 14 patients who were receiving twice-daily doses of more than 25 mg of mitapivat underwent a dose taper; the hemoglobin levels in 11 of these patients were maintained at the lower doses. One of these patients returned to a dose of 300 mg to sustain the hemoglobin response after a dose taper in the extension phase (see Table S1 in the Supplementary Appendix). In 4 patients who did not have a hemoglobin response, the twice-daily dose was increased from 50 mg to 300 mg; however, none of these patients met the definition of having had a hemoglobin response at the higher dose. In 9 patients, the dose of mitapivat was reduced owing to a rapid hemoglobin response.

In the patients with a hemoglobin response, directionally appropriate changes in other indicators of clinical activity over time (e.g., absolute reticulocyte count, indirect bilirubin, lactate dehydrogenase, and haptoglobin) provide additional evidence of decreased hemolysis (Fig. 3, and Table S8 in the Supplementary Appendix), changes that are consistent with the expected mechanism of action of mitapivat. Decreased indirect bilirubin levels suggest an independent effect of mitapivat on bilirubin metabolism.

### PHARMACOKINETICS AND PHARMACODYNAMICS

Mitapivat exposure in patients in this study was consistent with expectations on the basis of the phase 1 study in healthy volunteers (Table S9 in the Supplementary Appendix).[22] No difference in exposure was seen in patients on the basis of the hemoglobin response. In this study, there were no significant trends regarding whole-blood ATP or 2,3-diphosphoglycerate metabolite levels in patients with a hemoglobin response, a finding that contrasted with the results in healthy volunteers (Fig. S5 in the Supplementary Appendix). Changes in the average age of red cells associated with a reduction in the number of reticulocytes and reduced hemolysis could contribute to this observation, since reticulocytes have higher ATP levels than older blood cells.[25]

## DISCUSSION

Mitapivat is a small-molecule, allosteric activator of pyruvate kinase in red cells with a side-effect profile that allowed continued administration in 46 of 52 patients (88%) with pyruvate kinase deficiency in this phase 2 study. Treatment with mitapivat was associated with a rapid, clinically significant increase in the hemoglobin level in approximately half the treated patients. The hemoglobin response was sustained for up to 35 months with ongoing mitapivat administration and was associated with improvement in laboratory markers of hemolysis. This study establishes proof of concept for a molecular therapy targeting the underlying enzymatic defect of a hereditary enzymopathy.



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Table 2. **Adverse Events.***

| Adverse Event | Core Period | | | Core Period plus Extension Phase |
|---|---|---|---|---|
| | Mitapivat, 50 mg Twice Daily (N=27) | Mitapivat, 300 mg Twice Daily (N=25) | All Patients (N=52) | All Patients (N=52) |
| Summary of adverse events — no. of patients (%) | | | | |
| At least one adverse event | 26 (96) | 25 (100) | 51 (98) | 52 (100) |
| At least one adverse event of grade ≥3 | 4 (15) | 7 (28) | 11 (21) | 19 (37) |
| Grade 3 | 3 (11) | 6 (24) | 9 (17) | 16 (31)† |
| Grade 4 | 1 (4) | 1 (4) | 2 (4) | 3 (6)‡ |
| At least one serious adverse event | 5 (19) | 3 (12) | 8 (15) | 15 (29) |
| At least one adverse event leading to treatment discontinuation | 2 (7) | 2 (8) | 4 (8) | 6 (12)§ |
| Most common adverse events — no. of patients (%) | | | | |
| Headache | 9 (33) | 14 (56) | 23 (44)¶ | 24 (46) |
| Insomnia | 5 (19) | 16 (64) | 21 (40)‖ | 22 (42) |
| Nausea | 10 (37) | 10 (40) | 20 (38) | 21 (40) |
| Nasopharyngitis | 7 (26) | 2 (8) | 9 (17) | 16 (31) |
| Hot flush | 2 (7) | 7 (28) | 9 (17)** | 9 (17) |
| Arthralgia | 5 (19) | 3 (12) | 8 (15) | 9 (17) |
| Fatigue | 4 (15) | 4 (16) | 8 (15) | 9 (17) |
| Vomiting | 2 (7) | 5 (20) | 7 (13) | 9 (17) |
| Diarrhea | 3 (11) | 3 (12) | 6 (12) | 9 (17) |
| Influenza | 6 (22) | 1 (4) | 7 (13) | 9 (17) |
| Cough | 4 (15) | 4 (16) | 8 (15) | 8 (15) |
| Dizziness | 5 (19) | 2 (8) | 7 (13) | 8 (15) |
| Oropharyngeal pain | 3 (11) | 4 (16) | 7 (13) | 8 (15) |
| Pyrexia | 1 (4) | 5 (20) | 6 (12) | 8 (15) |
| Serious adverse events — no. of events (%) | | | | |
| Pharyngitis | 1 (4) | 1 (4) | 2 (4) | 2 (4) |
| Hemolytic anemia | 1 (4)†† | 1 (4)‡‡ | 2 (4) | 2 (4) |
| Cholelithiasis | 1 (4) | 0 | 1 (2) | 1 (2) |
| Hemolysis | 0 | 1 (4)‡‡ | 1 (2) | 1 (2) |
| Hypertriglyceridemia | 0 | 1 (4) | 1 (2) | 1 (2) |
| Influenza | 1 (4) | 0 | 1 (2) | 1 (2) |
| Osteoporosis | 1 (4) | 0 | 1 (2) | 1 (2) |
| Cellulitis | 0 | 0 | 0 | 1 (2) |
| Gastroenteritis | 0 | 0 | 0 | 1 (2) |
| Colitis | 0 | 0 | 0 | 1 (2) |
| Enteritis | 0 | 0 | 0 | 1 (2) |
| Mesenteric-vein thrombosis | 0 | 0 | 0 | 1 (2)§§ |
| Postprocedural hemorrhage | 0 | 0 | 0 | 1 (2)¶¶ |
| Ovarian cyst | 0 | 0 | 0 | 1 (2) |
| Left renal-cell carcinoma | 0 | 0 | 0 | 1 (2)‖‖ |
| Inguinal hernia | 0 | 0 | 0 | 1 (2) |



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Table 2. (Continued.)

* Adverse events are listed according to the number of patients who had the highest grade for a particular event. The most common adverse events occurred in at least 15% of the patients in the overall population.
† Grade 3 adverse events were colitis, diarrhea, dizziness, headache, hemolysis, hypertension, left renal-cell carcinoma, and postprocedural hemorrhage (in one patient each); hemolytic anemia and insomnia (in two patients each); and hypertriglyceridemia and nasopharyngitis (in three patients each).
‡ Grade 4 adverse events were hypertriglyceridemia, influenza, and mesenteric-vein thrombosis (in one patient each).
§ The adverse events that led to treatment discontinuation were an increase in the alanine aminotransferase level plus nonalcoholic steatohepatitis, hemolytic anemia, nausea plus pharyngitis, hypertriglyceridemia, left renal-cell carcinoma, and pleural effusion (in one patient each).
¶ Headache was transient and generally resolved within several days.
‖ Insomnia typically occurred within 14 days after the initiation of mitapivat, was self-resolving (generally in <7 days), and was not unexpected on the basis of off-target antagonistic or inverse agonist activity against the histamine H3 receptor.[23]
** Hot flush events were transient and generally reported within the first 7 days after treatment initiation and resolved without treatment within 3 days. Events did not correspond to changes in hormone levels or correlate with age or sex.
†† One patient had a progressive worsening of hemolysis and no hemoglobin response despite an increase in the dose of mitapivat to 300 mg twice daily and blood transfusions.
‡‡ After abrupt withdrawal of mitapivat, one patient had two serious adverse events, which were reported sequentially as hemolysis followed by hemolytic anemia.
§§ Mesenteric-vein thrombosis was reported in a patient who had a history of hepatic portal-vein thrombosis.
¶¶ One patient had persistent bleeding after tonsillectomy.
‖‖ One patient had an existing renal lesion before enrollment in the study. The renal-cell carcinoma did not grow at an abnormally rapid rate during the study, according to expert review. Nevertheless, it was assessed by the investigator as being associated with mitapivat.

Grade 3 or greater adverse effects that were considered by the investigator to be related to mitapivat were seen in 17% of patients who were treated daily for up to 35 months. The most commonly reported adverse events occurred soon after drug initiation and were transient. Doses of mitapivat that were significantly lower than the initial doses were associated with hemoglobin responses. Changes in sex hormone levels stayed mainly within normal ranges and did not correlate with adverse events or changes in bone mineral density.

The safety profile that was shown in this study provides equipoise for studies of mitapivat as a disease-altering therapy for patients with pyruvate kinase deficiency, who are likely to undergo therapy for years. The efficacy and safety of mitapivat continue to be studied in the extension phase as well as in two ongoing phase 3 studies (ClinicalTrials.gov numbers, NCT03559699 and NCT03548220). An exposure–response analysis of the pharmacokinetic, efficacy, and safety data from this study was used to determine the most effective strategy for individualizing doses in these trials, in which patients start at a low twice-daily dose of 5 mg, with two sequential steps for escalation to 20 mg and 50 mg on the basis of the achievement of a hemoglobin response without exceeding the upper limit of the normal range.

Preclinical data have shown that mitapivat activates pyruvate kinase activity in vitro across a broad spectrum of *PKLR* mutations, a finding that was consistent with the known binding site for mitapivat, which is distinct from the areas of the most common *PKLR* mutations.[21] In this study, mitapivat administration resulted in a robust and sustained hemoglobin response in patients with diverse *PKLR* genotypes, all of whom had at least one *PKLR* missense mutation. This result suggests that patients with at least one missense *PKLR* mutation are more likely than patients with two non-missense mutations to have a hemoglobin response to mitapivat. Most patients with pyruvate kinase deficiency have compound heterozygous *PKLR* alterations with at least one missense mutation. Therefore, mitapivat may have the potential to increase hemoglobin levels in the majority of patients with this disease.[5] This prediction requires prospective testing in patients across a broader range of genotypes and disease severity — patients who are being included in ongoing clinical trials. Patient-reported quality of life was not assessed in this phase 2 safety study, although such outcome measures are being evaluated in the ongoing phase 3 trials.

Since mitapivat directly binds and activates residual mutant red-cell pyruvate kinase enzyme, it is hypothesized that a minimal level of full-length red-cell pyruvate kinase protein may be required for pyruvate kinase activation by mitapivat. In this study, a hemoglobin response was associated with the residual level of pyruvate



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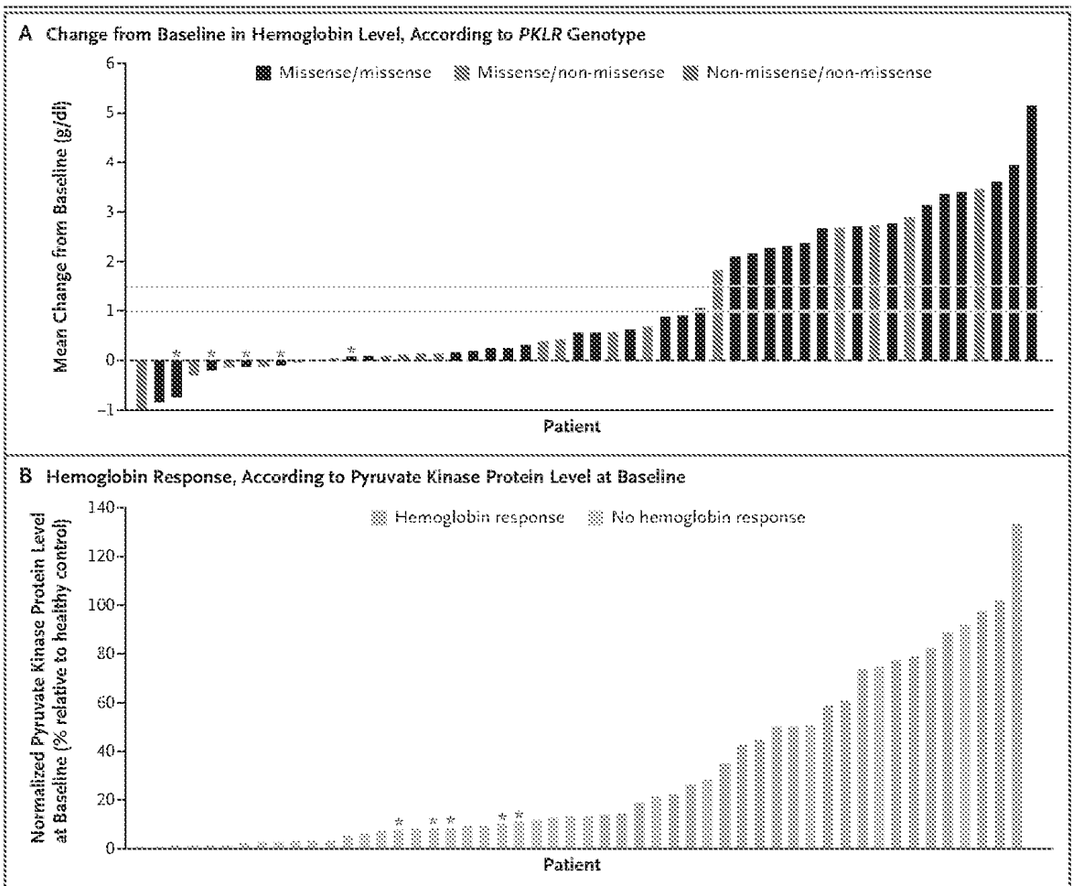



Figure 2. **Change in Hemoglobin According to *PKLR* Genotype and Hemoglobin Response According to Pyruvate Kinase Protein Level at Baseline.**

Panel A shows the mean change from baseline in the hemoglobin level during the 24 weeks of the core period, according to the patient's *PKLR* genotype category. Of the 52 patients who received mitapivat, 20 (38%) — all of whom had at least one missense mutation — had a mean increase of more than 1.0 g per deciliter in the hemoglobin level (indicated by a thick gray line); 19 patients had a mean increase of at least 1.5 g per deciliter (thin gray line) (Table S6 in the Supplementary Appendix). A mean increase of more than 1.0 g per deciliter in the hemoglobin level did not occur in any of the 5 patients who were homozygous for the R479H mutation (as indicated by an asterisk) or in any of the 10 patients who were homozygous for non-missense mutations (as indicated by a red bar). Panel B shows the patients who had a hemoglobin response (defined as an increase from baseline of >1.0 g per deciliter in the hemoglobin level at >50% of the assessments during the core period) and those who did not have a response, according to the baseline level of pyruvate kinase protein in red cells, a value that has been normalized to the hemoglobin level (an approximation of the total red-cell protein level) and to the pyruvate kinase protein level measured in a sample obtained from a healthy control (to allow for interassay comparisons). Patients with an increased baseline level of pyruvate kinase protein were more likely to have a hemoglobin response to mitapivat. However, since there were exceptions to this observation, the baseline level of pyruvate kinase protein is not a consistent predictor of hemoglobin response. Patients who were homozygous for the R479H mutation are indicated by asterisks. In 1 patient, the baseline level of red-cell pyruvate kinase protein was not available, so the day 15 sample was used. The data bars in Panels A and B are not aligned for each patient, so a 1:1 comparison of the individual data bars in the two panels is not possible.

kinase protein in red cells at baseline. This finding is consistent with previous ex vivo observations and provides further evidence that mitapivat is working by means of its proposed mechanism of action. Nevertheless, differing degrees of hemoglobin response in patients with equivalent



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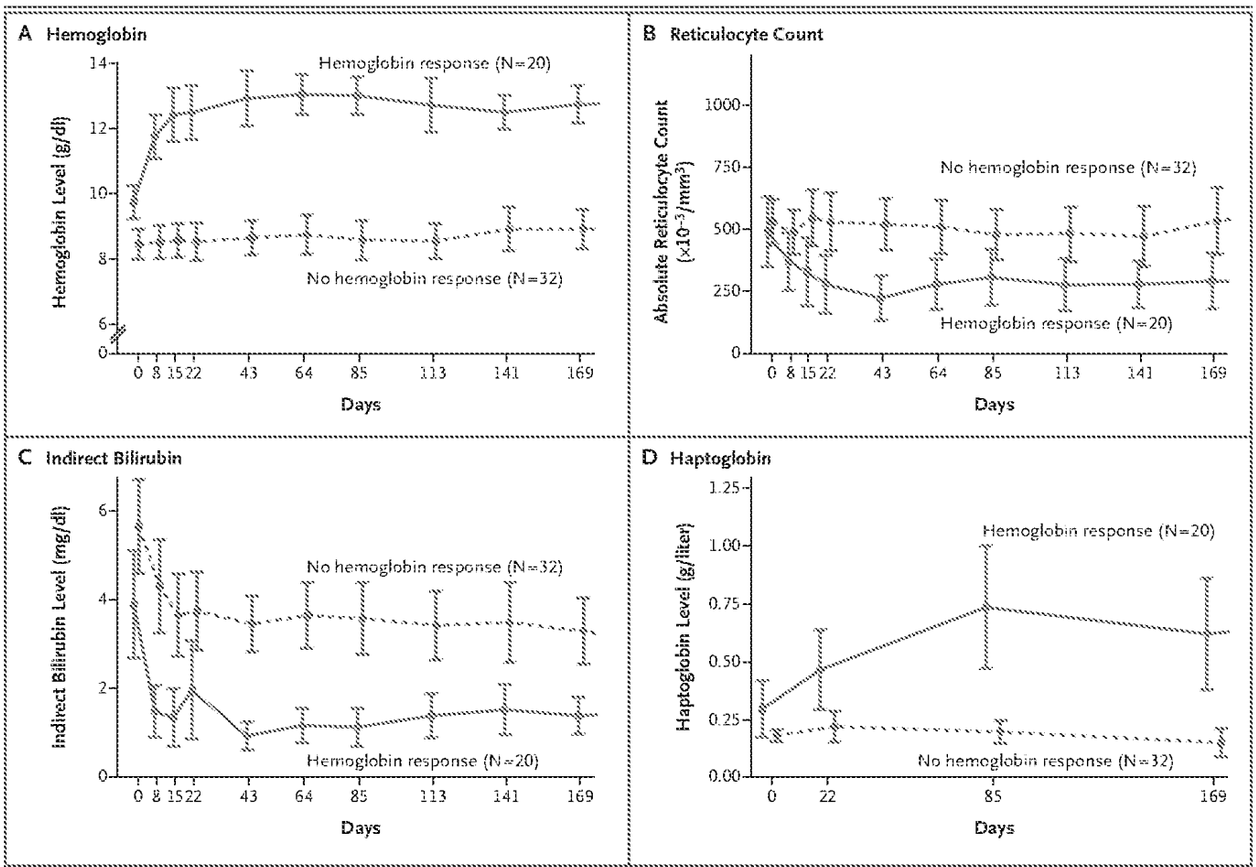



Figure 3. **Hematologic Measures in Patients with and Those without a Hemoglobin Response.**

Shown are the mean levels of hemoglobin (Panel A), the absolute reticulocyte count (Panel B), indirect bilirubin (Panel C), and haptoglobin (Panel D) during the 24-week core period, according to whether the patients had a hemoglobin response or no hemoglobin response. The hemoglobin response was sustained during nearly 3 years of follow-up in the extension phase (Fig. S4 in the Supplementary Appendix). The indirect bilirubin levels decreased in all the patients. However, the lactate dehydrogenase levels (Table S8 in the Supplementary Appendix) and reticulocyte counts changed only in the patients with a hemoglobin response. Thus, there may be an independent effect of mitapivat on bilirubin metabolism. The I bars indicate 95% confidence intervals. In Panel B, the values for the reticulocyte count per cubic millimeter are presented at 0.001 of the actual value. To convert the values for bilirubin to micromoles per liter, multiply by 17.1.

levels of red-cell pyruvate kinase protein suggest that this protein level is not a reliable predictor of hemoglobin response.

The data from this study indicate that the adverse-event profile for mitapivat at the adjusted dose aimed at increasing the hemoglobin level appears to allow for long-term administration of the drug. In addition to finding that 88% of the patients could continue to receive mitapivat without unacceptable adverse events, we showed that the subsequent pyruvate kinase activation resulted in clinically significant hemoglobin increases and improvement in hemolytic markers in approximately half the patients with pyruvate kinase deficiency, particularly those with a *PKLR* genotype that included at least one missense mutation.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

Supported by Agios Pharmaceuticals.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank all the patients, research nurses, study coordinators, and subinvestigators for their contributions to this study; David Nathan and Donna Neuberg of the Dana–Farber Cancer Institute and Karen Anderson of Agios Pharmaceuticals for their discussions; Susanne Vidot of Excel Medical Affairs for providing writing assistance; and all the Clinical and Translational Research Centers for their services during the study.

We gratefully acknowledge the dedication of the late Christian Rose to this research.



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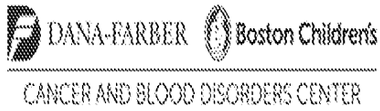
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# Clinical Research Progress in Pyruvate Kinase Deficiency

Rachael Grace, MD, MMSc

Dana-Farber/Boston Children's Cancer and Blood Disorders Center

Boston, MA

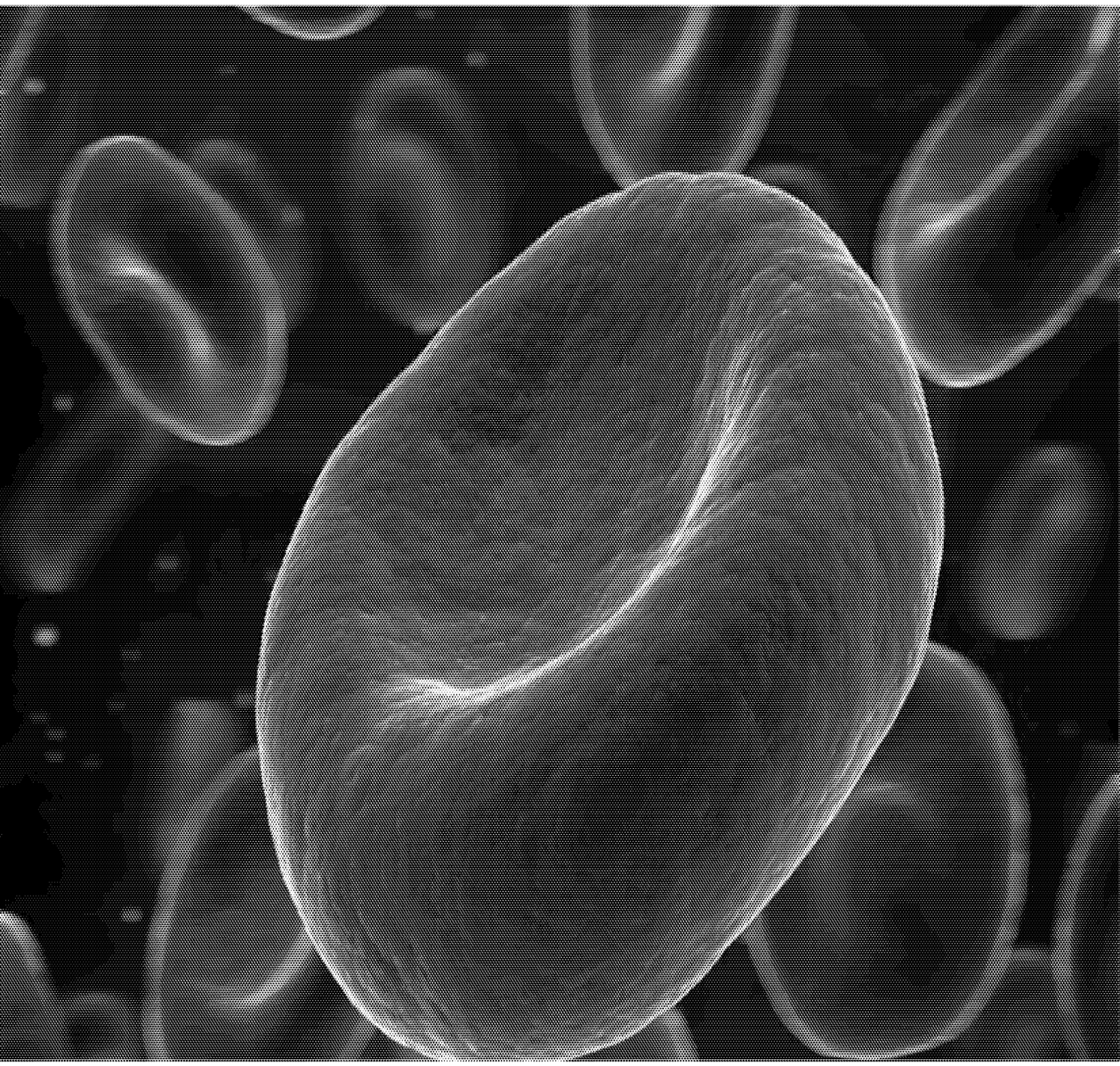




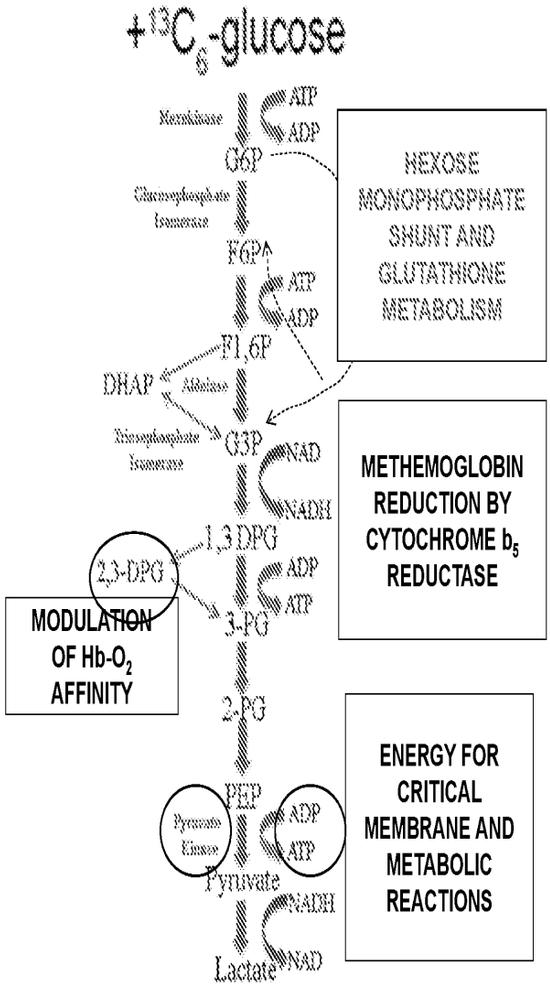

# Disclosures

- Agios Pharmaceuticals – research funding, scientific advisor

- Discussion of investigational use of AG-348 (mitapivat) for treatment of pyruvate kinase deficiency

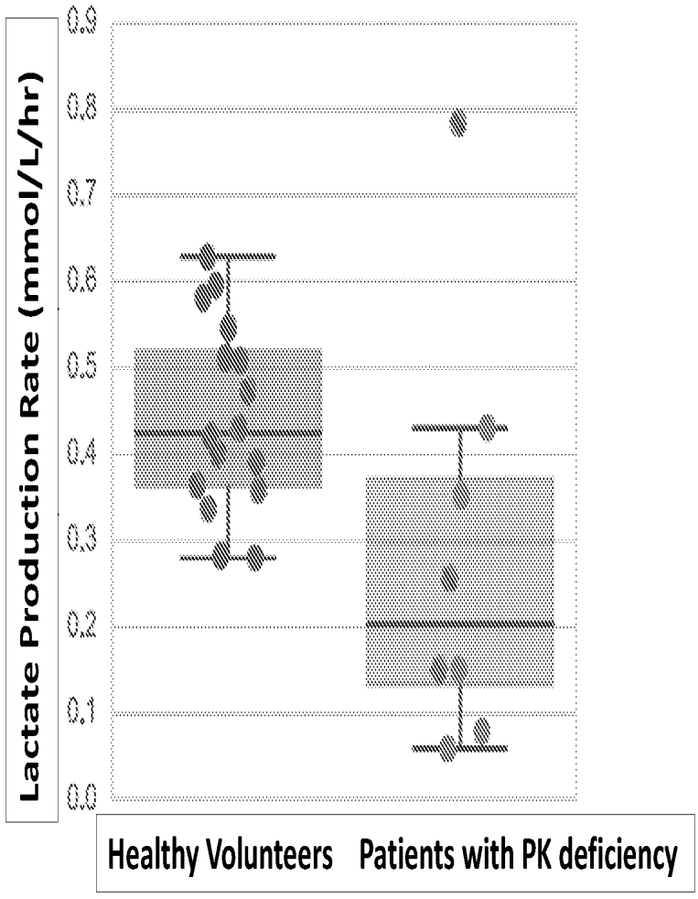


# Red Cell Metabolism




Grace and Glader, *Pediatr Clin N Am.* 2018; 579-595

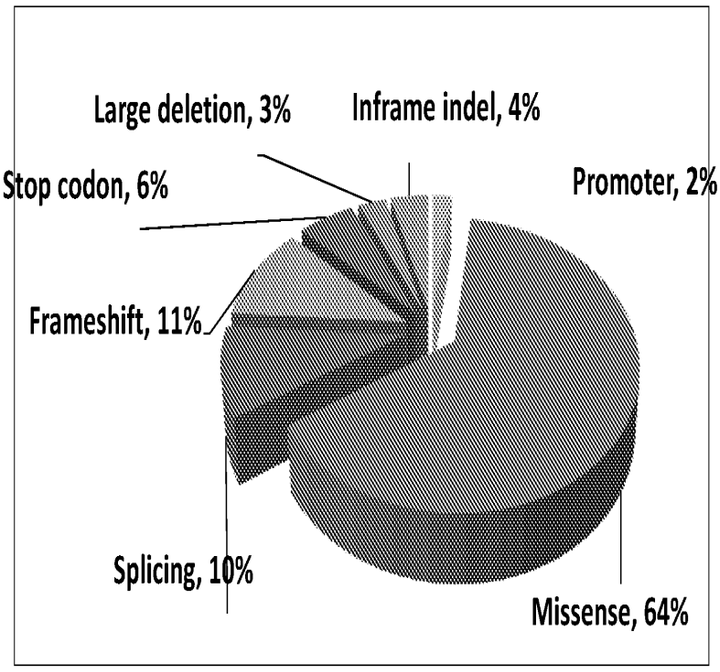
# RBC Glycolytic Flux




Chubukov et al, *American Society of Hematology Annual Meeting.* 2016.

# Epidemiology of PK deficiency

- Autosomal Recessive – Homozygotes and Compound Heterozygotes
- >300 mutations in the *PKLR* gene which cause:
  - Decreased catalytic activity
  - Protein instability
  - Decreased protein expression
- Prevalence: ~1/million



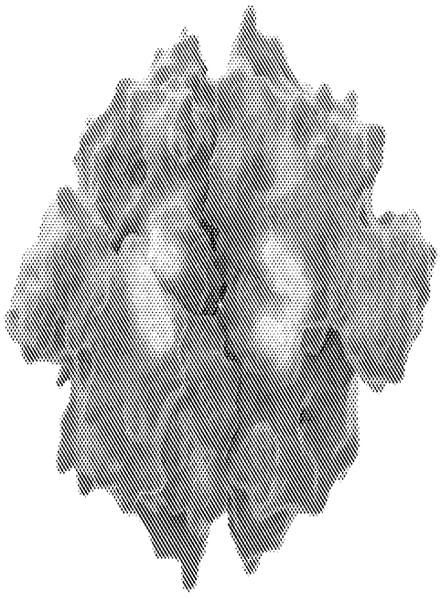

Beutler et al, *Blood* 2000; 3585-3588
Carey at al, *Blood* 2000; 4005-4006
Ayi et al, *NEJM* 2008; 1805-1810
Grace et al, *Blood* 2018; 2183-2192



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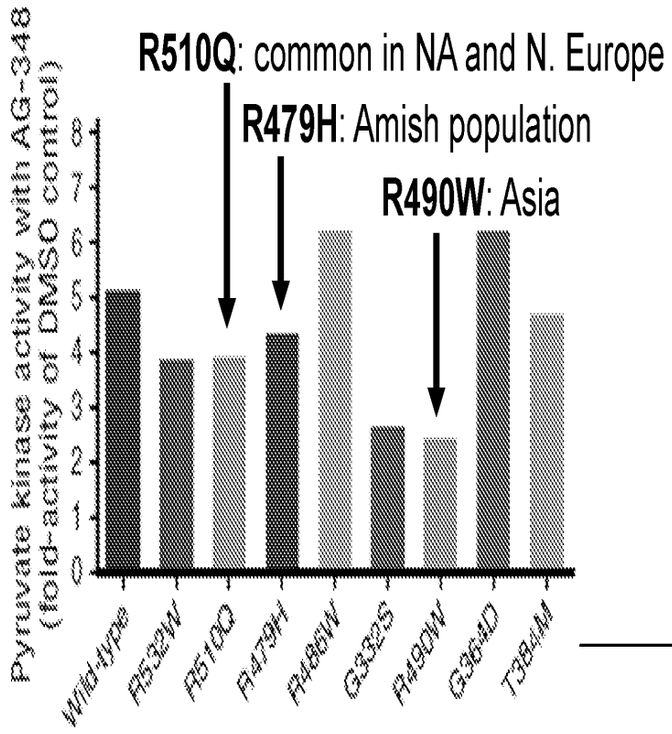
# AG-348 activates WT and mutant PK-R

- Allosteric activator of red cell pyruvate kinase (PK-R)
  - Potently activates wild type PK-R and a wide spectrum of recombinantly expressed PK-R mutants
  - Increases thermal stability and catalytic activity



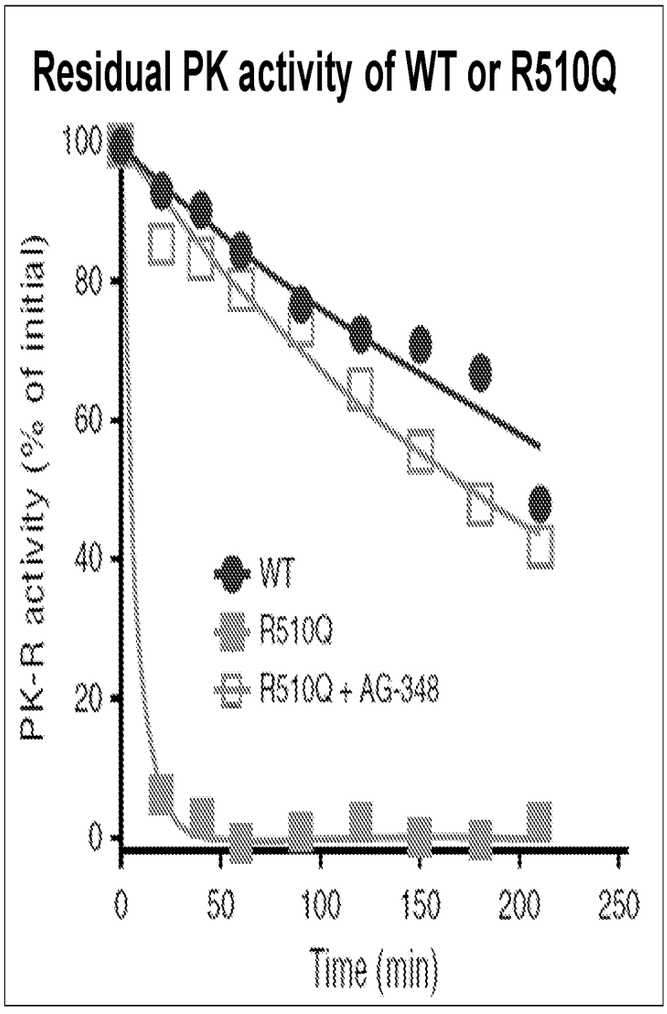
= AG-348 binding pockets

= areas of common defects




Kung et al. *Blood*. 2017; 1347-1356.

# AG-348 can stabilize mutant PK enzymes

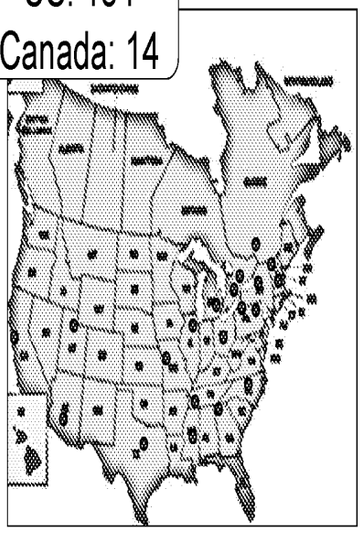



Kung C, et al. *Blood*. 2017; 1347-1356.

# PKD Natural History Study

- Observational Study: Clinical Information over 2 years
- Enrollment: n=254
- Ongoing registry: PEAK Registry: https://www.peakregistry.com/

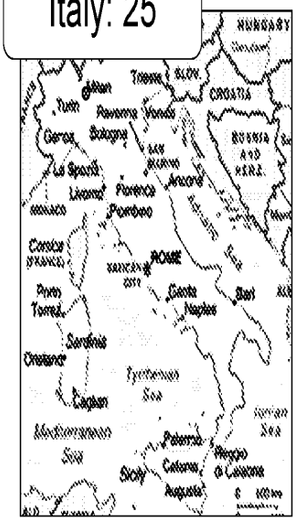
US: 154
Canada: 14



Germany: 31
Netherlands: 23
Czech Republic: 7




Italy: 25



# Symptoms/Complications in Newborns

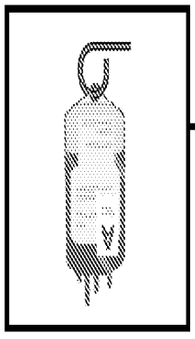
- Hydrops fetalis (4%)
- Intrauterine growth retardation (6%)
- Intrauterine transfusions (12%)
- Prematurity (15%)



- Hemolytic Anemia
- Hyperbilirubinemia:
  Phototherapy (93%)
  Exchange (46%)
- Blueberry muffin rash (<1%)
- Neonatal hepatic failure (<1%)

Grace et al, *Blood* 2018; 2183-2192

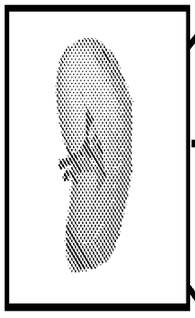
# Current Management



**Transfusions**

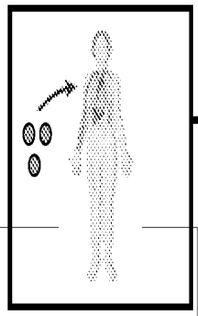
**Splenectomy in 59% (150/254)**

- Regularly transfused, then intermittent post-SPL 40%
- Intermittent Transfusions 38%
- Regular Transfusions pre/post SPL 14%
- Never transfused 21%
- Median age: 5 years (0.4-37.8)
- Median post-Hb: 8.8 g/dl (6.3-12)
- Median rise in Hb: 1.6 g/dl



**Other Splenectomy Outcomes**

- Continued regular transfusions 10%
- Overall rate of thrombosis 11%
- Post-splenectomy sepsis 7%



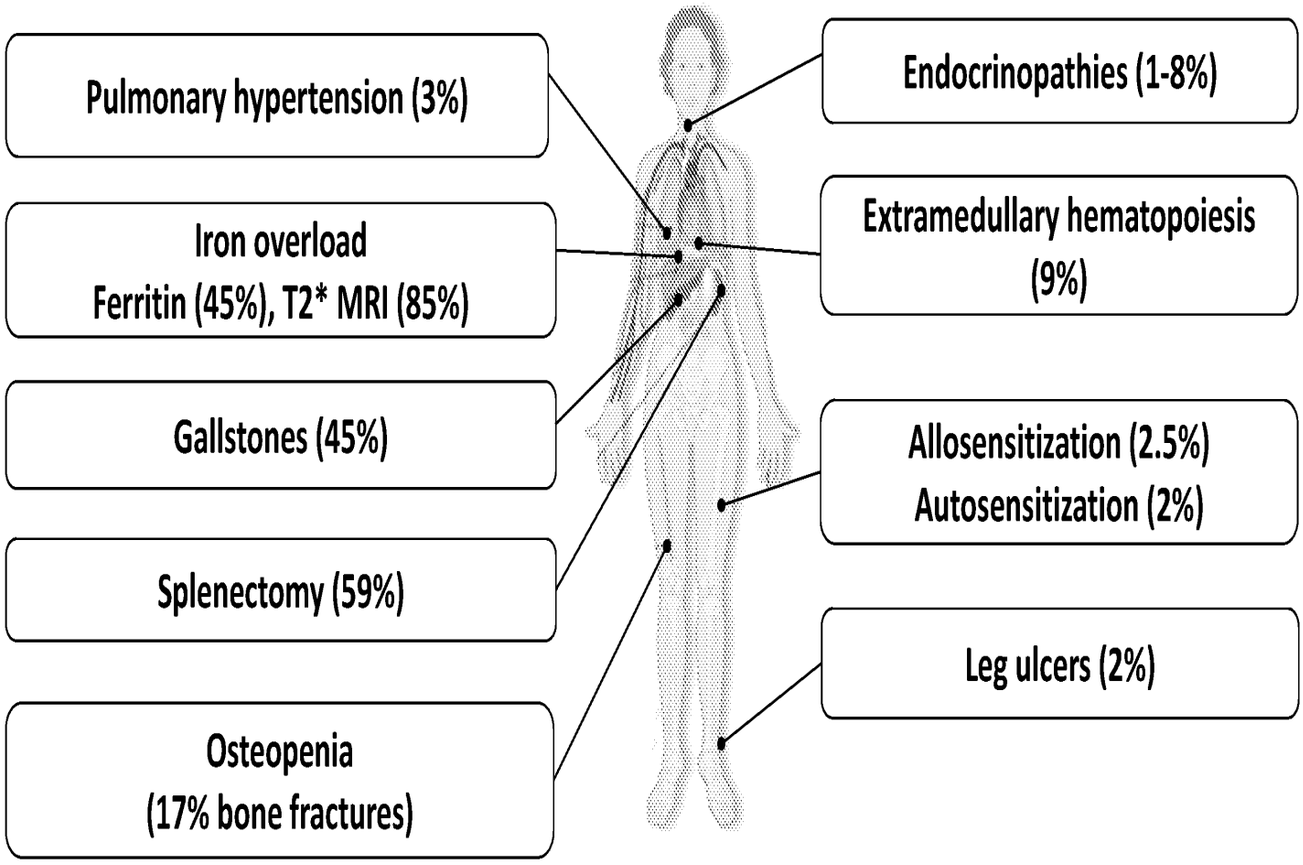
**Predictors of Hb Response**

**Stem Cell Transplant (n=16)**

- M/M or M/NM PKLR genotype
- Higher baseline hemoglobin
- Low indirect bilirubin
- Median follow up 2.3 y (2 mo-19 y)
- Grade 4 chronic GVHD 38%
- 2 year cumulative survival 74%

Grace et al, *Blood* 2018; 2183-2192 Van Straaten et al, *Haematologica* 2017; 103:e82-86

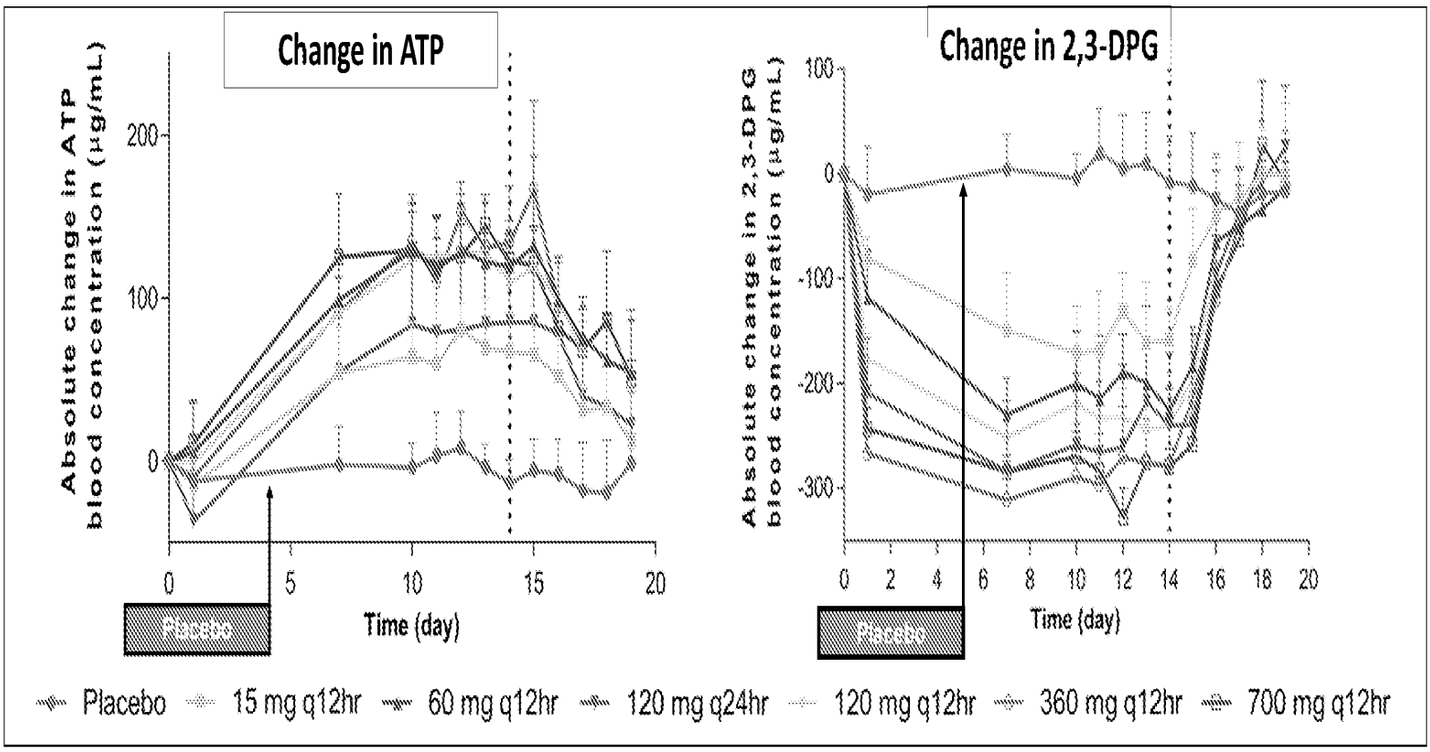
# Other Findings/Complications




Grace et al, *Blood* 2018; 2183-2192

# Healthy volunteer study:↑ RBC ATP and ↓ 2,3-DPG

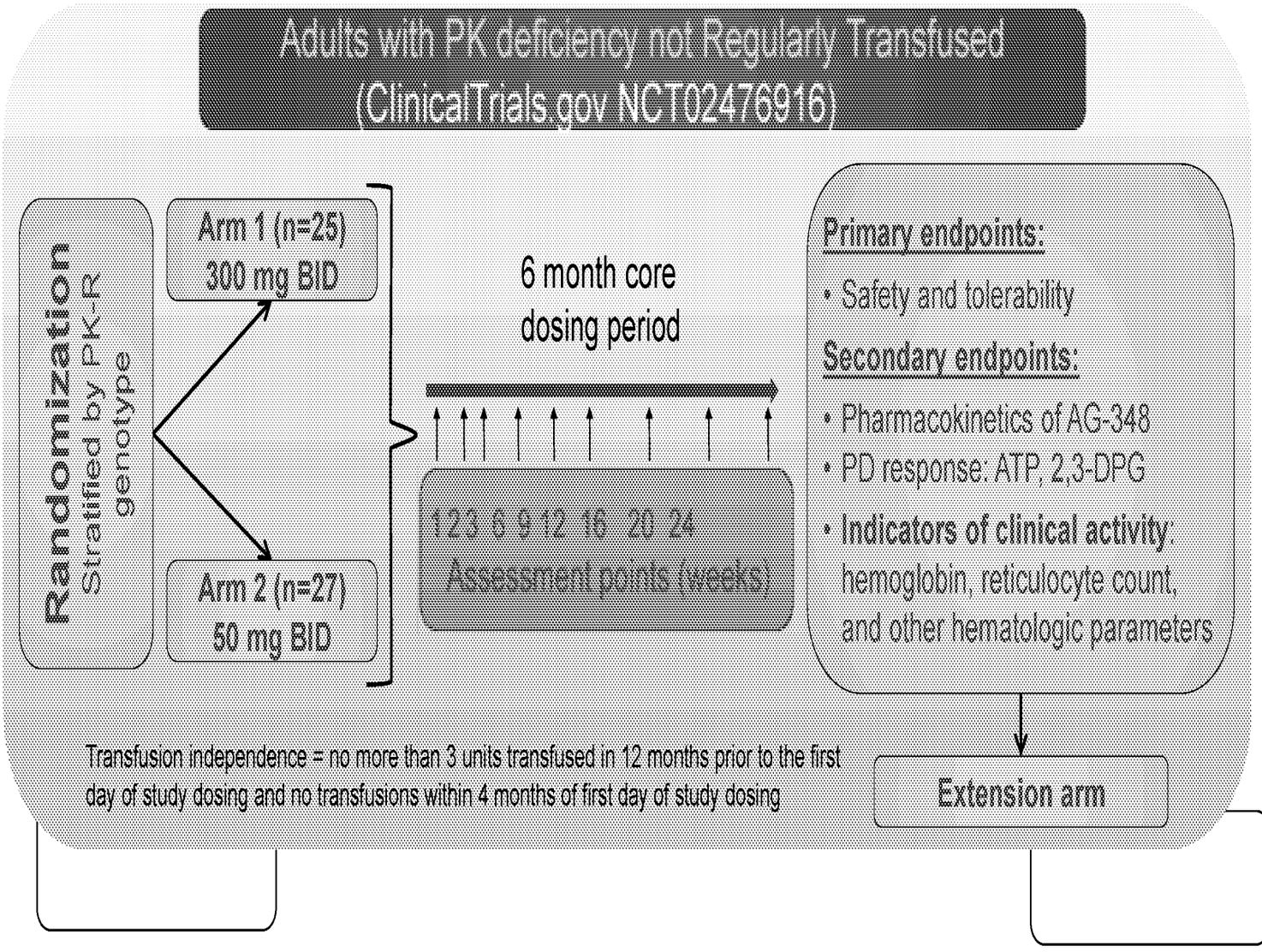
- Randomized, Placebo Controlled Multiple Ascending Dose Study
- Healthy Volunteers, 14 day AG-348 dosing (15-700 mg, n=48)




Yang H et al. *Clin Pharmacol Drug Dev* 2018.

# DRIVEPK: Completed Phase 2 Trial

**Open-label, global phase 2 AG-348 study: 14 centers in the US, Canada, and EU**



# DRIVEPK: Baseline Characteristics

| Characteristic | 50 mg BID n=27 | 300 mg BID n=25 |
|---|---|---|
| Male, n (%) | 18 (66.7) | 14 (56.0) |
| Age at randomization, median (range), y | 28 (18–58) | 40 (21–61) |
| Caucasian*, n (%) | 22 (81.5) | 21 (84.0) |
| Hb baseline, median (range), g/dL | 9.6 (6.9–12.3) | 8.6 (6.5–12.0) |
| Splenectomy, n (%) | 23 (85.2) | 20 (80.0) |
| Mutation category, n (%) | | |
| Missense / missense | 15 (55.6) | 17 (68.0) |
| Missense / non-missense | 6 (22.2) | 4 (16.0) |
| Non-missense / non-missense | 6 (22.2) | 4 (16.0) |

Grace et al, *American Society of Hematology Annual Meeting.* 2017.

# DRIVEPK: Safety Summary

- AG-348 was generally well tolerated; the majority of AEs were grade 1–2
- Most common AEs: headache, insomnia, nausea, and hot flush
- Mild reversible aromatase inhibition seen

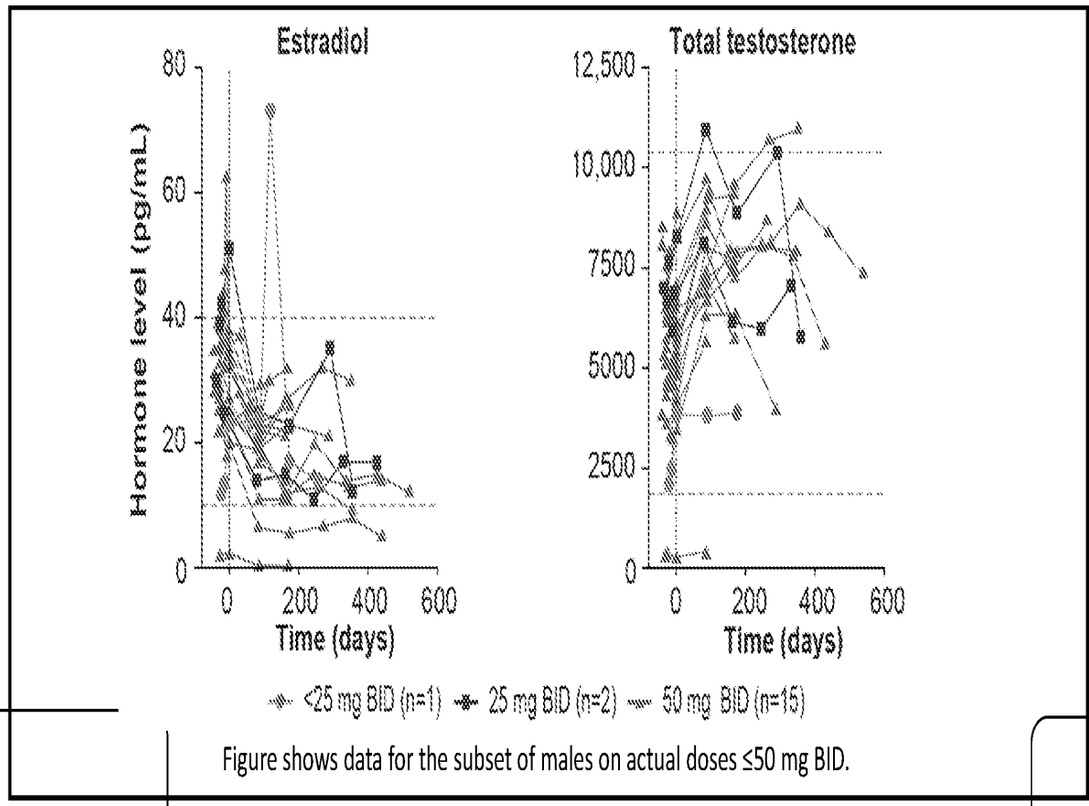


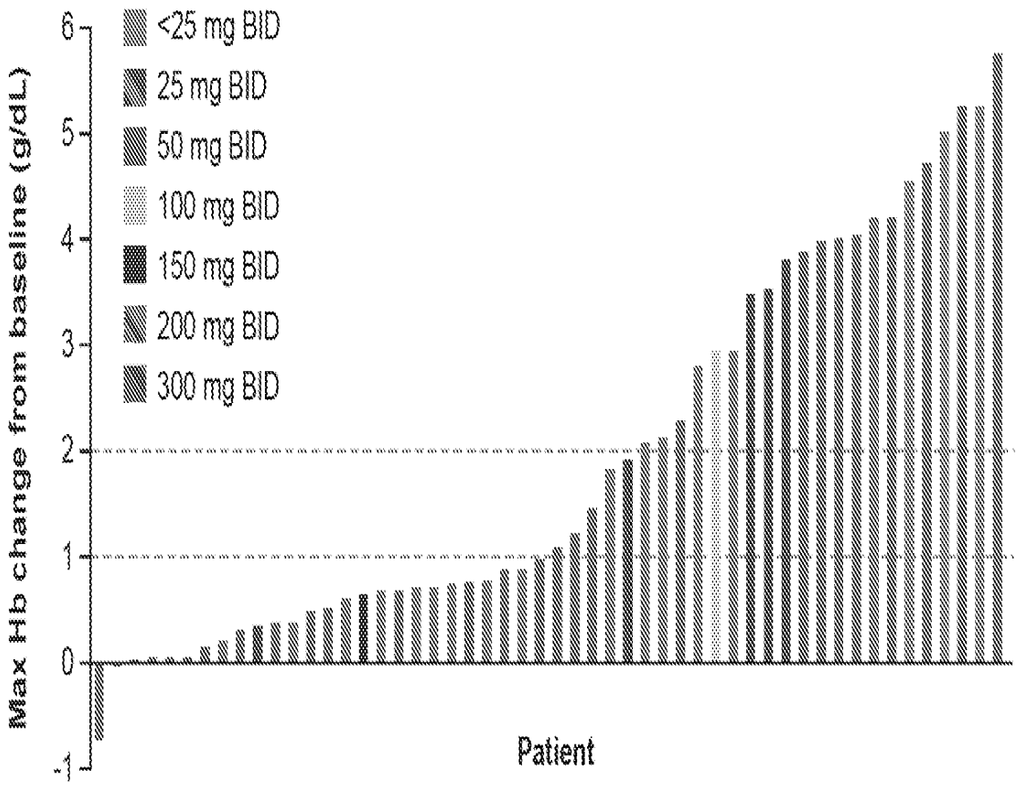

Figure shows data for the subset of males on actual doses ≤50 mg BID.

Grace et al, *American Society of Hematology Annual Meeting.* 2017.

# Maximum increase in hemoglobin

26 of 52 (50.0%) patients had a maximum Hb increase of >1.0 g/dL

- Of these patients, the mean maximum increase was 3.4 g/dL (range 1.1–5.8)



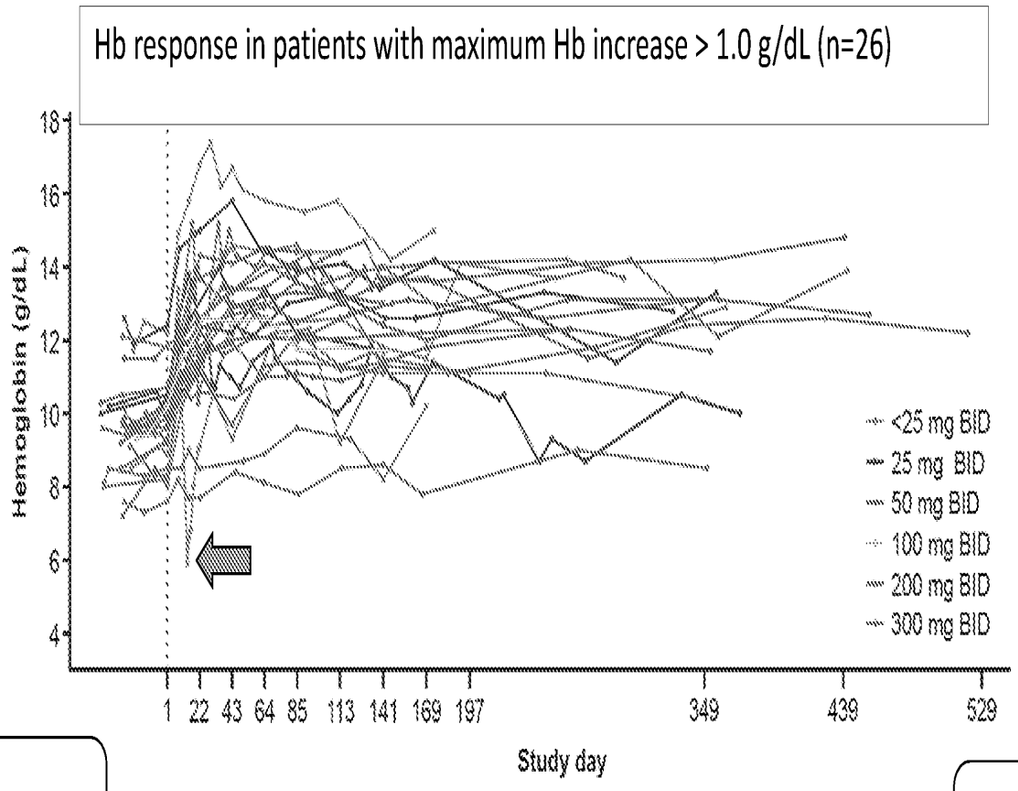

Baseline Hb: average of central assessments in the screening period

Data analyzed by the dose received for the longest duration in the Core period.

Grace et al, *American Society of Hematology Annual Meeting.* 2017.

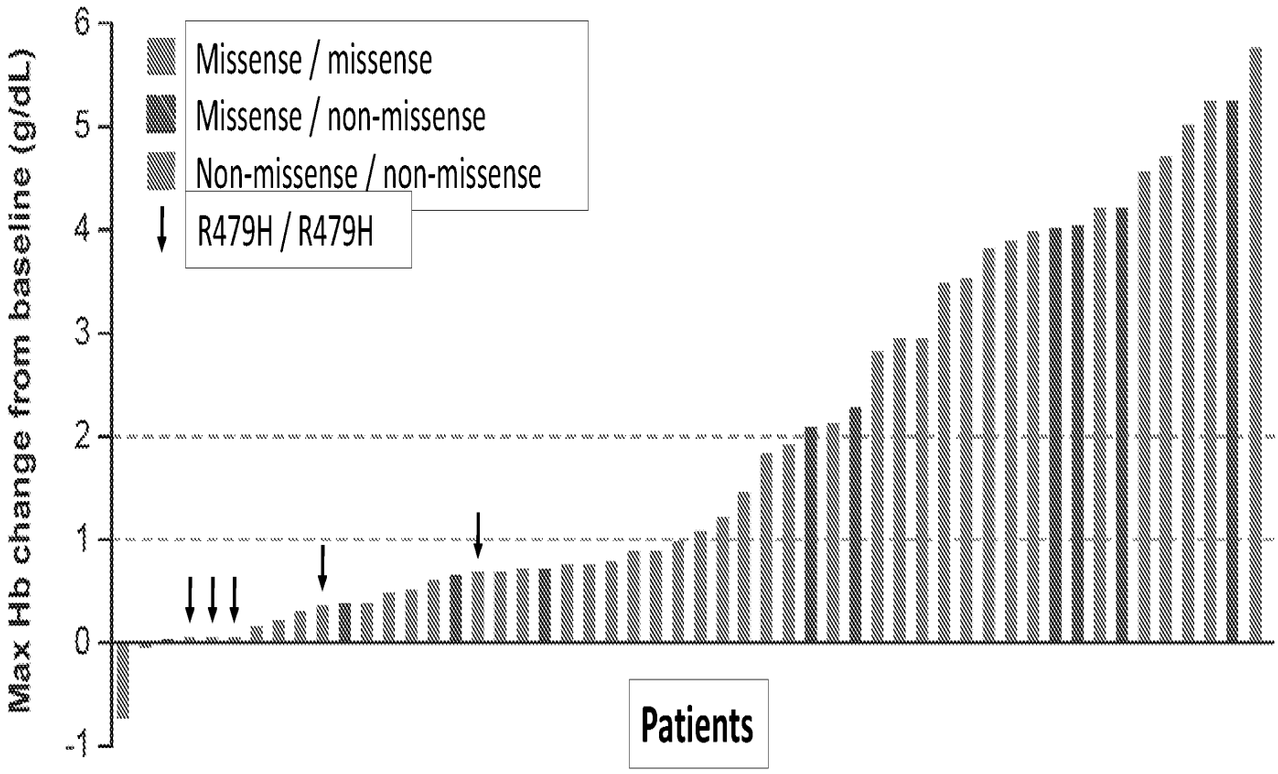
# Hb increases are rapid and sustained

- Days to the first Hb increase >1.0 g/dL (median, range): 10 (7–187)
- In 9 patients, the dose had to be held or reduced due to a rapid rise in Hb



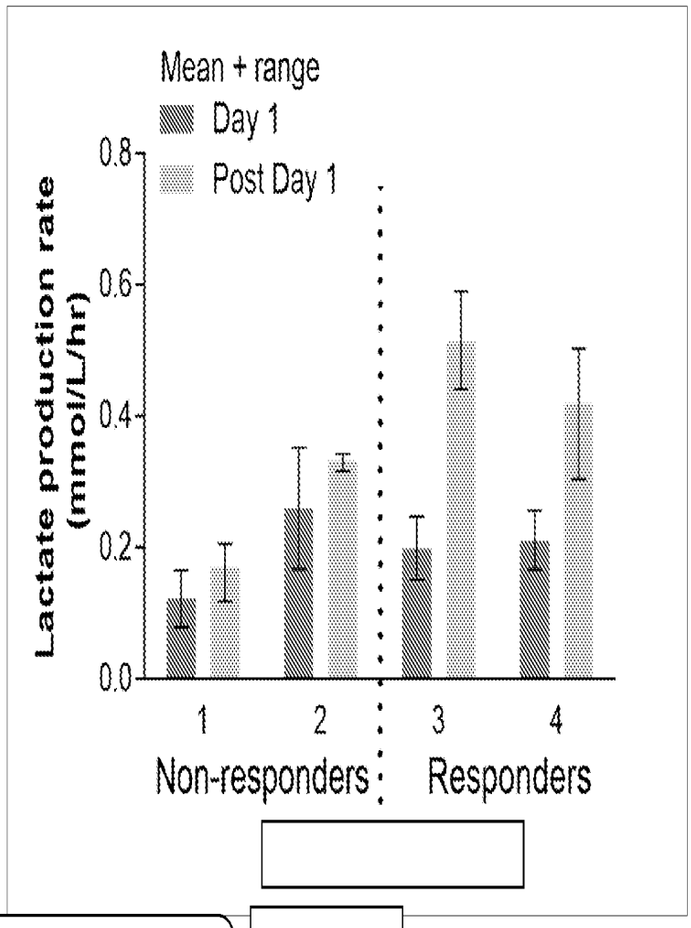

Grace et al, *American Society of Hematology Annual Meeting.* 2017.

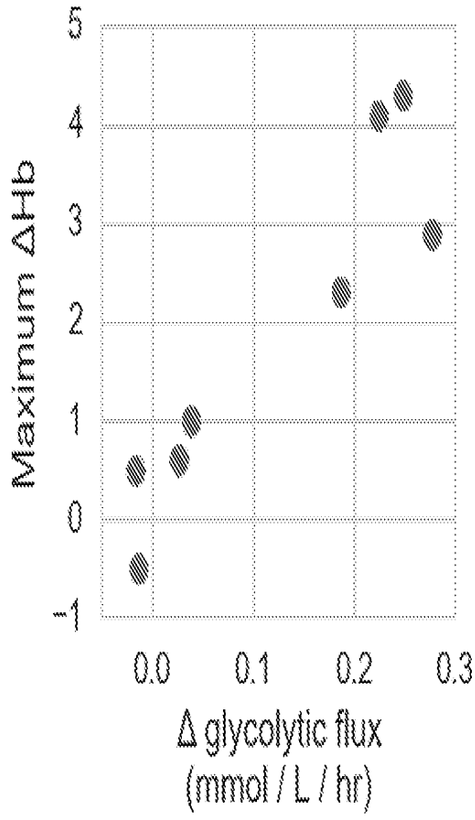
# Hemoglobin response & Genotype




Grace et al, *American Society of Hematology Annual Meeting*. 2017.

# Increased Glycolytic Flux in Responders




Chubukov V et al. *American Society of Hematology Annual Meeting,* 2016

# Summary and What's next?

- Hemoglobin response >1 g/dl in 50% of patients in the phase 2 study
  - Hb increase is linked to activation of the glycolytic pathway
  - Genotype and PK-R protein level are associated with hemoglobin response
- AG-348 is well tolerated but has an off target effect as a mild aromatase inhibitor
  - Lower doses than expected were associated with Hb response
- What's Next?
  - Continued development of PK activators:
    - Ongoing Phase 2 extension study and Phase 3 Adult Trials of AG-348
    - Discussions with regulators about possibility of AG-348 trial in children
    - Development of follow-on PK-R activators
  - PEAK Registry global efforts
  - Gene Therapy for PK deficiency

Min-Oo, G. et al., *Nature Genetics* 2003 (4): 357-62 Garcia-Gomez et al., *Molecular Therapy*, 2016

# Acknowledgements


| PKD NHS Investigator | Site | PKD NHS Investigator | Site |
|---|---|---|---|
| **Dr. Jenny Despotovic** | Texas Children's Hospital, TX | **Dr. Eduard van Beers** | UMC, Utrecht, Netherlands |
| **Dr. Melissa Rhodes** | University of Mississippi, MS | **Dr. Wilma Barcellini** | Ospedale Maggiore di Milano, Italy |
| **Dr. Rachael Grace** | Boston Children's Hospital, MA | **Dr. Stefan Eber** | University of Munich, Germany |
| **Dr. Christina Knoll** | Phoenix Children's Hospital, AZ | **Dr. Marcin Wlodarski** | Freiburg, Germany |
| **Dr. Bert Glader** | Stanford University, CA | **Dr. Joachim Kunz** | Heidelberg, Germany |
| **Dr. Holmes Morton** | Lancaster General Hospital, PA | **Dr. Nina Kollmar** | Kassal, Germany |
| **Dr. Peter Newburger** | University of Massachusetts, MA | **Dr. Suzanne Holzhauer** | Berlin, Germany |
| **Dr. Winifred Wang** | St Jude's Hospital, TN | **Dr. Vicky Breakey** | Hamilton, ON |
| **Dr. Jennifer Rothman** | Duke Children's Hospital, NC | **Dr. Yves Pastore** | Montreal, QC |
| **Dr. Heather Bradeen** | University of Vermont, VT | **Dr. Kevin Kuo** | Toronto, ON |
| **Dr. Hassan Yaish** | Salt Lake City, UT | **Dr. Yaddanapudi Ravindranath** | Wayne State, Detroit, MI |
| **Dr. Sujit Sheth** | Cornell, New York, New York | **Dr. Heng Wang** | DCC Clinic, Middlefield, OH |
| **Dr. Alexis Thompson** | Lurie Children's, Chicago, IL | **Dr. Melissa Rose** | Nationwide Hospital, Ohio |
| **Dr. Mukta Sharma** | Children's Mercy Hospital, MO | **Dr. Janet Kwiatkowski** | University of Pennsylvania, PA |
| **Dr. Dagmar Pospíšilová** | Palacky Univer., Czech Republic | **Dr. Satheesh Chonat** | Children's Healthcare of Atlanta, GA |

**DRIVEPK Investigator, Site**

**Dr. Bertil Glader,** Stanford University

**Dr. Sujit Sheth,** Weill Cornell – New York Presbyterian Hospital

**Dr. Hassan Yaish,** University of Utah

**Dr. Janet Kwiatkowski,** The Children's Hospital of Philadelphia

**Dr. D. Holmes Morton,** Central Pennsylvania Clinic

**Dr. Yaddanapudi Ravindranath,** Wayne State University School of Medicine

**Dr. Kevin Kuo,** University of Toronto – University Health Network

**Dr. D. Mark Layton,** Hammersmith Hospital

**Dr. Eduard van Beers,** Universitair Medisch Centrum Utrecht

**Dr. Frederic Galacteros,** Hôpital Henri Mondor

**Dr. Christian Rose,** Hôpital Saint Vincent de Paul

**Dr. Wilma Barcellini,** Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico

**PKD NHS Steering Committee:** Ellis Neufeld, Bert Glader, Alberto Zanella, D. Holmes Morton

**Boston Children's Hospital Research Team:** Kathryn Addonizio, Nolan Neu, Jill Falcone

**Laboratories:** Patrick Gallagher, Kimberly Lezon-Geyda, Paola Bianchi, Elisa Fermo

# Clinical Research Progress in a Rare Anemia: New Developments in Pyruvate Kinase Deficiency

Clinical Research Grand Rounds
Boston Children's Hospital
April 5, 2016

Rachael Grace, MD, MMSc
Assistant Professor, Harvard Medical School
Director, Hematology Clinic, Boston Children's Hospital, MA

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# Disclosures

- The PKD Natural History Study is funded by Agios Pharmaceuticals
- I am a Scientific Advisor for Agios Pharmaceuticals
- I will discuss an investigational agent, AG-348, in my presentation



Ex. 2001-0628

# Overview

- Brief overview of Pyruvate Kinase Deficiency (PKD)
- Discuss the PKD Natural History Study (NHS)
  - Initiating a NHS with industry in an orphan disease
  - Present data from the NHS
- Drug Development in PKD
  - Pre-clinical data
  - Data from Healthy Volunteer Studies
  - Adaptive trial design of the Phase II clinical trial

# OVERVIEW OF PYRUVATE KINASE DEFICIENCY

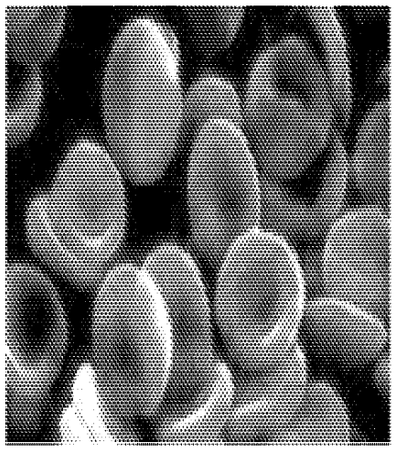
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# The Mature Erythrocyte

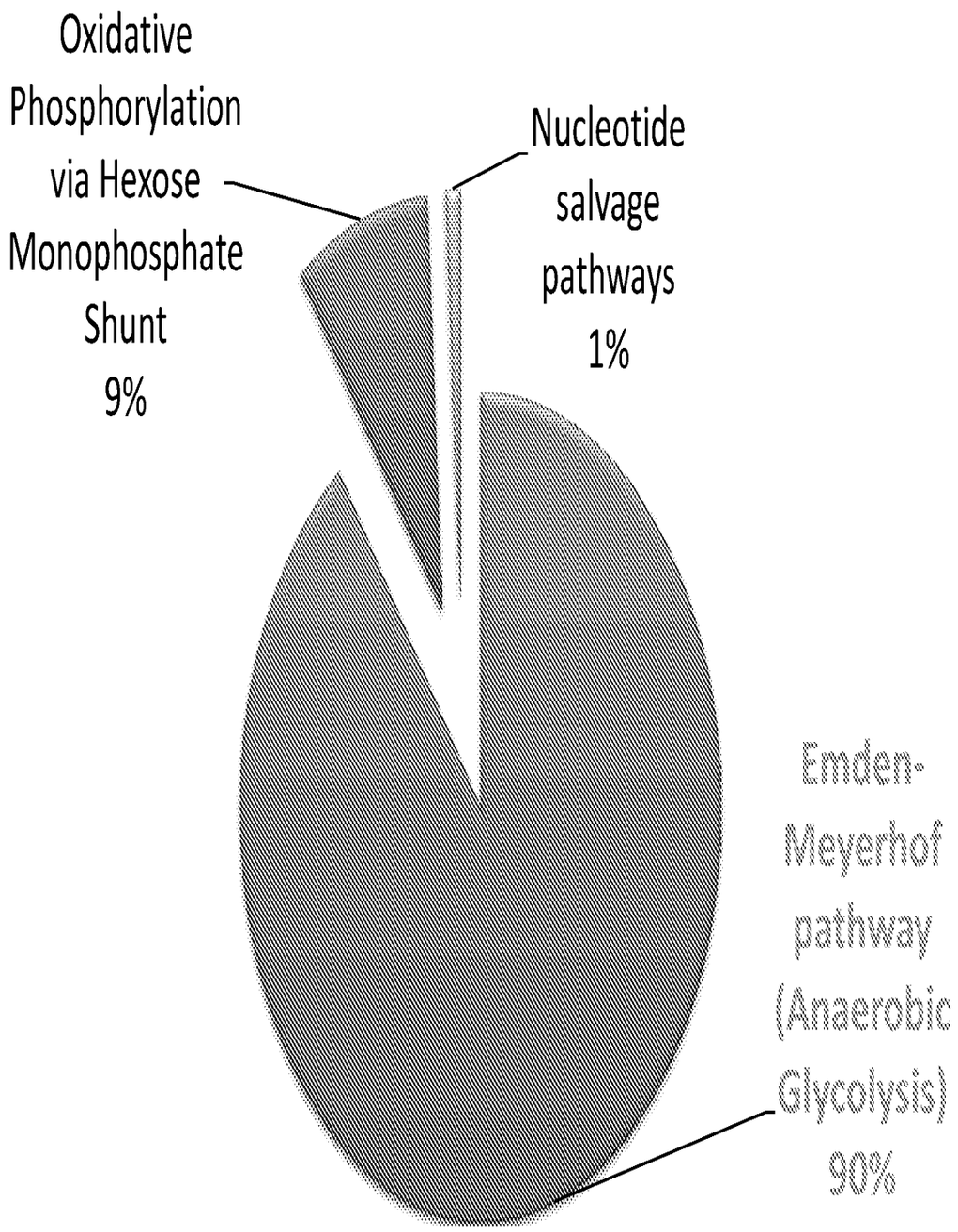
- Lacks a nucleus, DNA, RNA, and ribosomes
- Cannot synthesize RNA, DNA, or proteins
- Has no mitochondria
- Lacks an electron transport system for oxidative phosphorylation



→Erythrocyte must maintain an adequate ATP supply

- Pump cations against electrochemical gradient
- Maintain membrane phospholipids
- Protect hemoglobin and cell from oxidative damage

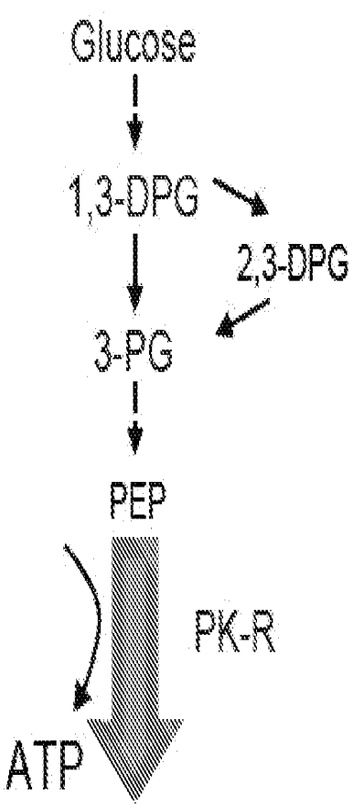
# How Does the Mature Erythrocyte Make Energy?

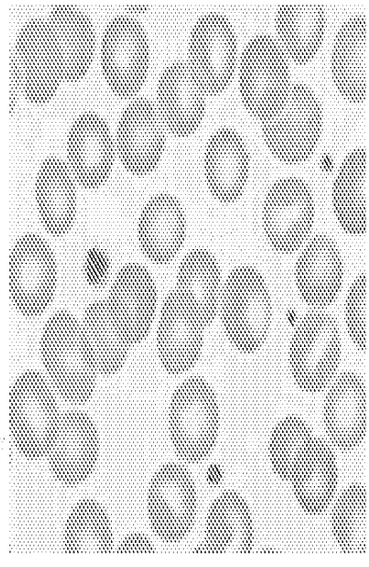



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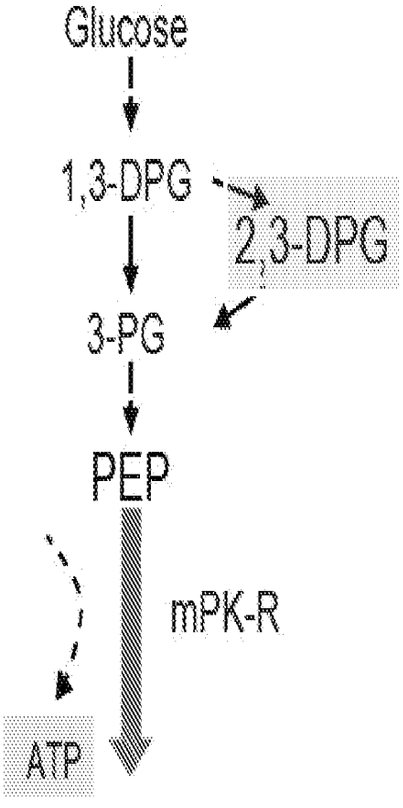
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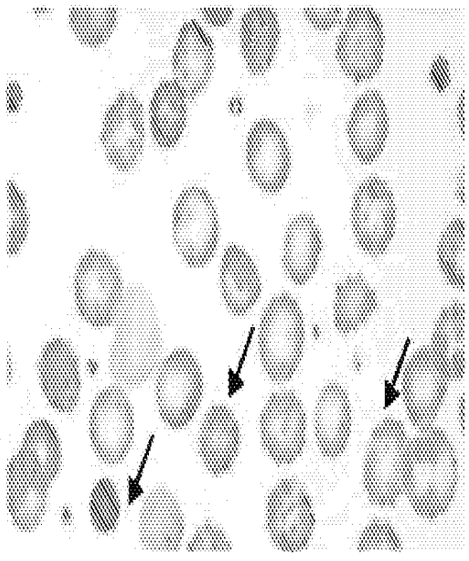
# Pyruvate Kinase Deficiency



Normal



Chronic hemolysis

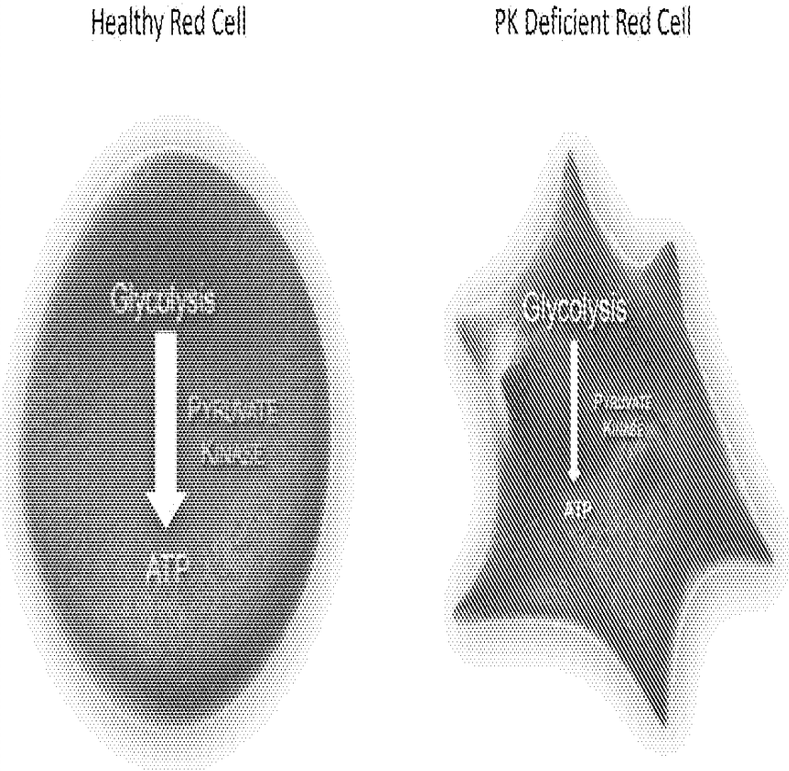
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# PK deficiency causes hemolysis

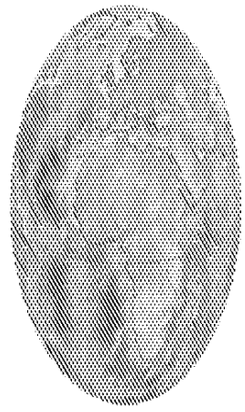
| Disease Overview | |
|---|---|
| Description | Rare genetic disease often presenting at birth as neonatal jaundice |
| Etiology | Caused by mutations in PK-LR gene coding for Erythrocyte Pyruvate Kinase |
| Clinical Presentation | Lifelong hemolytic anemia and associated morbidities |
| Diagnosis | PK enzyme activity and/or molecular analysis |




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# Epidemiology



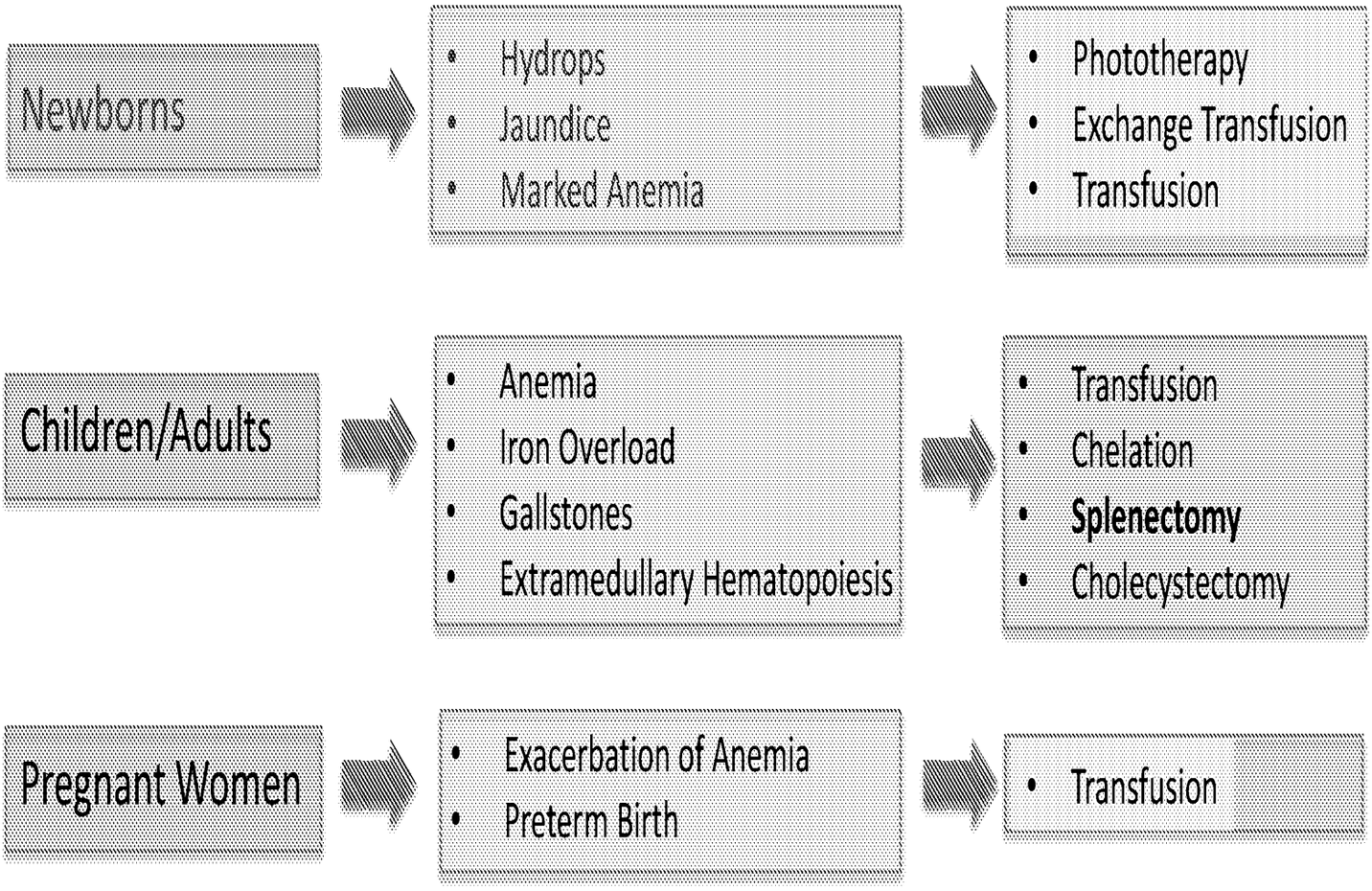
- >200 mutations described in the PKLR gene which cause:
  - Decreased catalytic activity
  - Protein instability
  - Decreased protein expression
- Autosomal Recessive – Homozygotes and Compound Heterozygotes
- Estimated Prevalence 3:100,000 to 1:20,000
  - Actual numbers of patients found is ~1/million,

    or 30x less common than might be predicted
  - Much more common among the Amish due to founder effect

Beutler et al, Blood 2000
Carey at al, Blood 2000

# Clinical Features and Treatment

Anemia of variable severity with reticulocytosis




Zanella et al, Blood Rev 2007

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# PKD NATURAL HISTORY STUDY

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# Initiating a Natural History Study

- Industry interest in this rare disease with significant unmet needs
- Natural History Study needed to inform drug development
    - Essential foundation of a clinical development program
- Critical for designing clinical trials → need drug development perspective in the design stage of the NHS
    - ➢What is the clinical spectrum and course?
    - ➢What is the standard of care for this disease?
    - ➢What assessments/disease measures are useful?
    - ➢What is a clinical efficacy endpoint?
    - ➢What is a clinically meaningful change in the endpoint?



Ex. 2001-0638

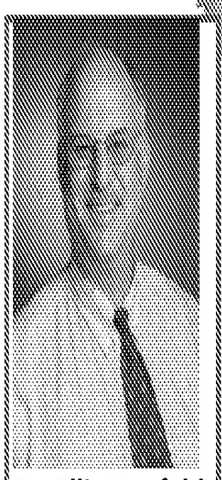
# Initiating a Natural History Study

Clinical interest in an improved understanding of PKD

- **Define Disease**: track all ages and follow over time
  - Clinical Phenotypes/Spectrum
  - Current standard of care
  - Biomarkers correlating with disease course
  - Identification of disease features/complications
  - Range, pace, and frequency of manifestations
  - Intra-patient variability
- **Improve Diagnosis**
- **Create Guidelines/Treat more effectively**

→ Most likely to succeed with a community effort and a plan to share data widely

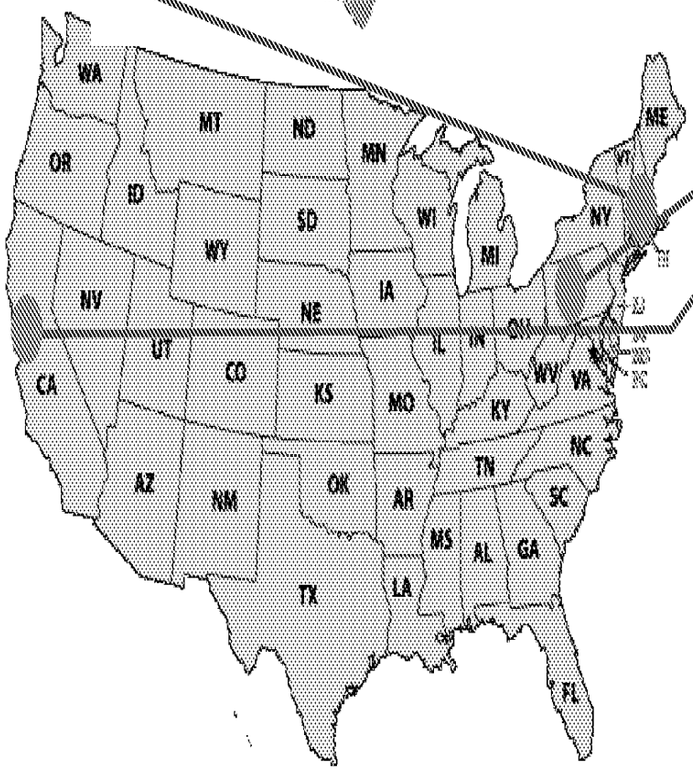
# Identify Respected Experts and Mentors

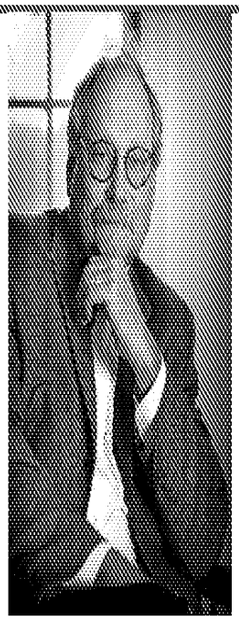


Dr. Ellis Neufeld
Boston, MA

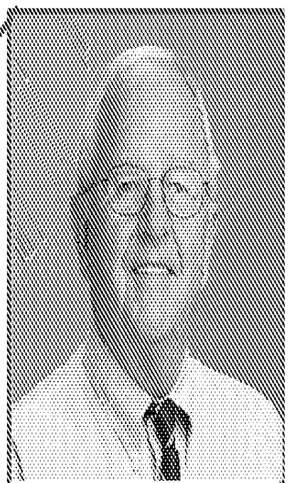


Professor Alberto Zanella
Milan, Italy



Dr. Holmes Morton
Belleville, Pennsylvania



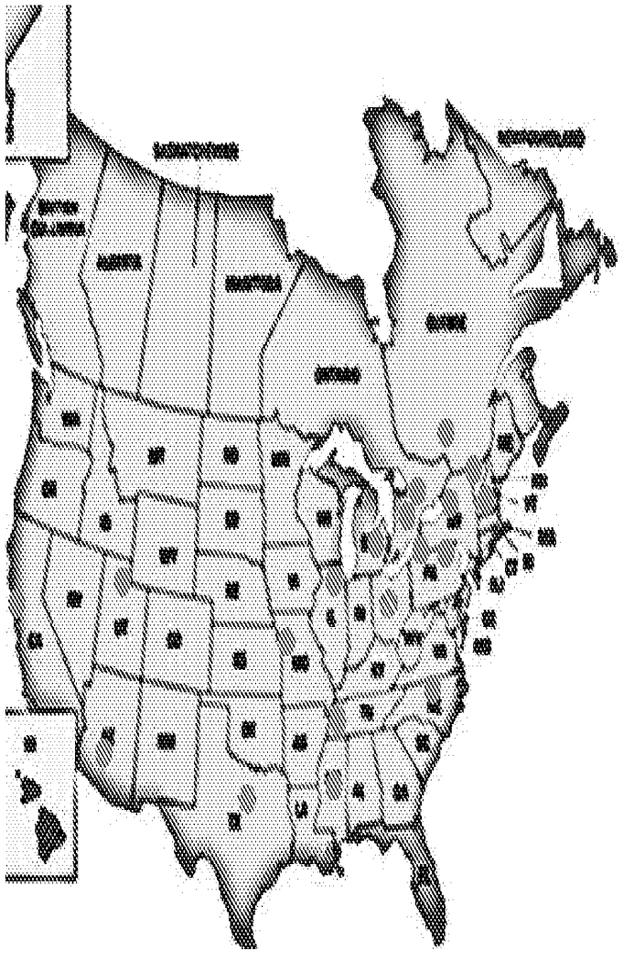
Dr. Bertil Glader
Stanford, California

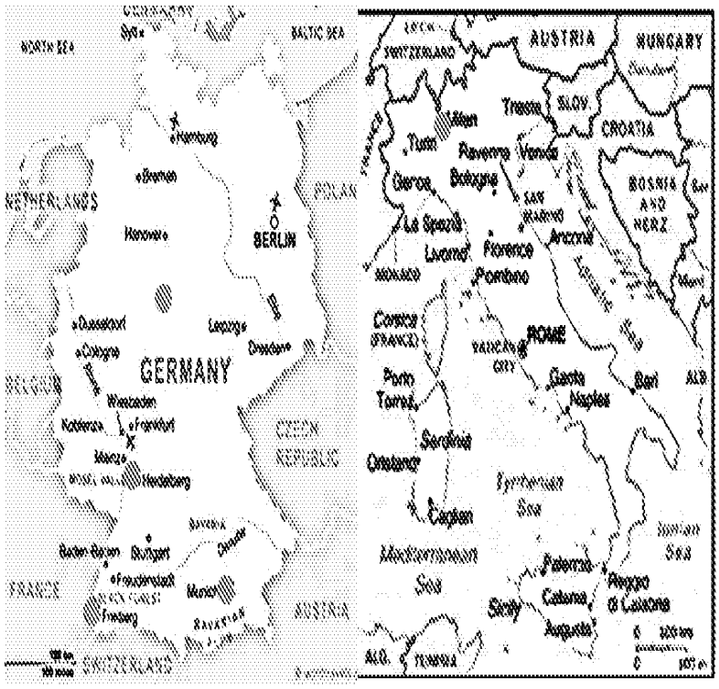
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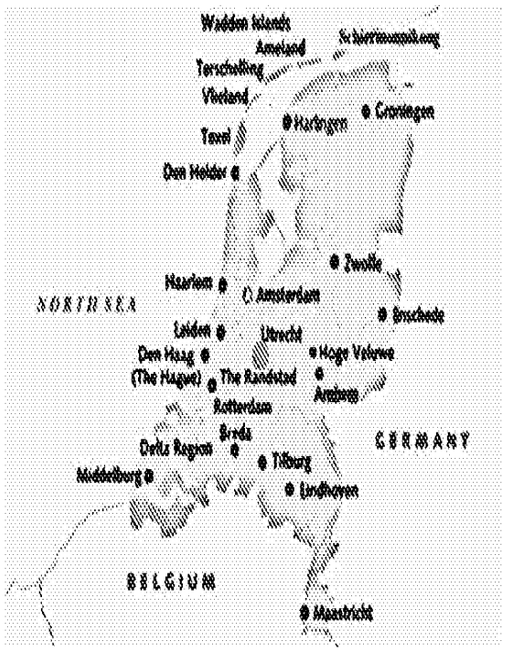
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# PKD Natural History Study Sites

- 18 sites in the United States
- 3 sites in Canada
- 6 sites in Europe






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# PKD Natural History Study

Observational Study - clinical information and surveys over 2 years
Blood sample for research genetic testing to confirm diagnosis

- **Coordinating Site:** Boston Children's Hospital
- **Sites:** United States: 18 sites
  Europe: 6 sites
  Canada: 3 sites
- **Laboratories:** Paola Bianchi, Elisa Fermo (Milan, Italy)
  Patrick Gallagher, Kimberly Lezon-Geyda (CT, USA)
- **Current Enrollment:** 190 participants to date, actively enrolling

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# PKD Natural History Study Investigators

| Site Investigator | Site | Site Investigator | Site |
|---|---|---|---|
| Dr. Jenny Despotovic | *Baylor Hospital, TX* | Dr. Eduard van Beers | *UMC, Utrecht, Netherlands* |
| Dr. Melissa Rhodes | *University of Mississippi, MS* | Dr. Wilma Barcellini | *Ospedale Maggiore di Milano, Italy* |
| Dr. Rachael Grace | *Boston Children's Hospital, MA* | Dr. Stefan Eber | *University of Munich, Germany* |
| Dr. Christina Knoll | *Phoenix Children's Hospital, AZ* | Dr. Marcin Wlodarski | *Freiburg, Germany* |
| Dr. Bert Glader | *Stanford University, CA* | Dr. Joachim Kunz | *Heidelberg, Germany* |
| Dr. Holmes Morton | *Lancaster General Hospital, PA* | Dr. Nina Kollmar | *Kassal, Germany* |
| Dr. Peter Newburger | *University of Massachusetts, MA* | Dr. Vicky Breakey | *Hamilton, ON* |
| Dr. Winifred Wang | *St Jude's Hospital, TN* | Dr. Yves Pastore | *Montreal, QC* |
| Dr. Jennifer Rothman | *Duke Children's Hospital, NC* | Dr. Kevin Kuo | *Toronto, ON* |
| Dr. Heather Bradeen | *University of Vermont, VT* | Dr. Yaddanapudi Ravindranath | *Wayne State, Detroit, MI* |
| Dr. Hassan Yaish | *Salt Lake City, UT* | Dr. Heng Wang | *DCC Clinic, Middlefield, OH* |
| Dr. John Chapin | *Cornell, New York, New York* | Dr. Melissa Rose | *Nationwide Hospital, Ohio* |
| Dr. Alexis Thompson | *Lurie Children's, Chicago, IL* | Dr. Janet Kwiatkowski | *University of Pennsylvania, PA* |
| Dr. Mukta Sharma | *Children's Mercy Hospital, MO* | | |

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# Clinical Research and the Pennsylvania Amish

- PKD NHS research team was invited to Belleville, Pennsylvania to a family's home for a gathering
- A community letter announced the gathering
- >50 members of the Amish community sat together in a barn overlooking the valley to discuss clinical care in PKD and the goals of the Natural History Study



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# Demographics

| | n (%); N=178 |
|---|---|
| Age at enrollment (median, range) | 19.9 y, 0.1-70.7 y |
| <18 years (n) | 83 (47%) |
| ≥18 years (n) | 95 (53%) |
| Gender | |
| Male | 80 (46%) |
| Race | |
| White | 168 (94%) |
| Hispanic | 14 (8%) |
| Amish | 54 (30%) |

# Diagnosis: Enzyme Assay vs DNA Analysis

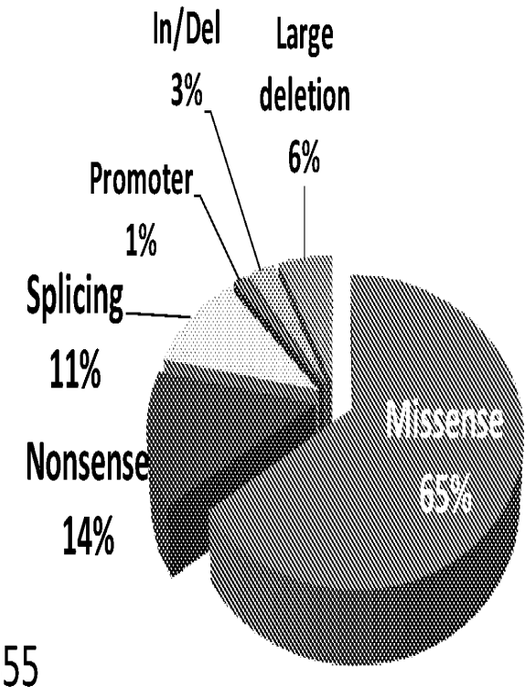
| Advantage | Enzyme Assay | DNA Analysis |
|---|---|---|
| Useful in Transfused Patients | No | Yes |
| Stability of Sample | Low | High |
| Finds all (most) mutations in a given enzyme | Yes | Variable |
| Suitable for Prenatal Diagnosis | No | Yes |
| Definite Yes or No result | Variable | Variable |

→ 10% of enrolled participants do not have two PKLR mutations

# PKD NHS: Diversity of Genotypes

65 different mutations identified

- The majority were missense mutations
- >20 mutations not previously described




Most Common Mutations

**1529 G>A**

Homozygous: n=3

Heterozygous: n=20

**1436 C>T**

Homozygous: n=55

**1456 C>T**

Homozygous: n=2

Heterozygous: n=17

**721 G>T**

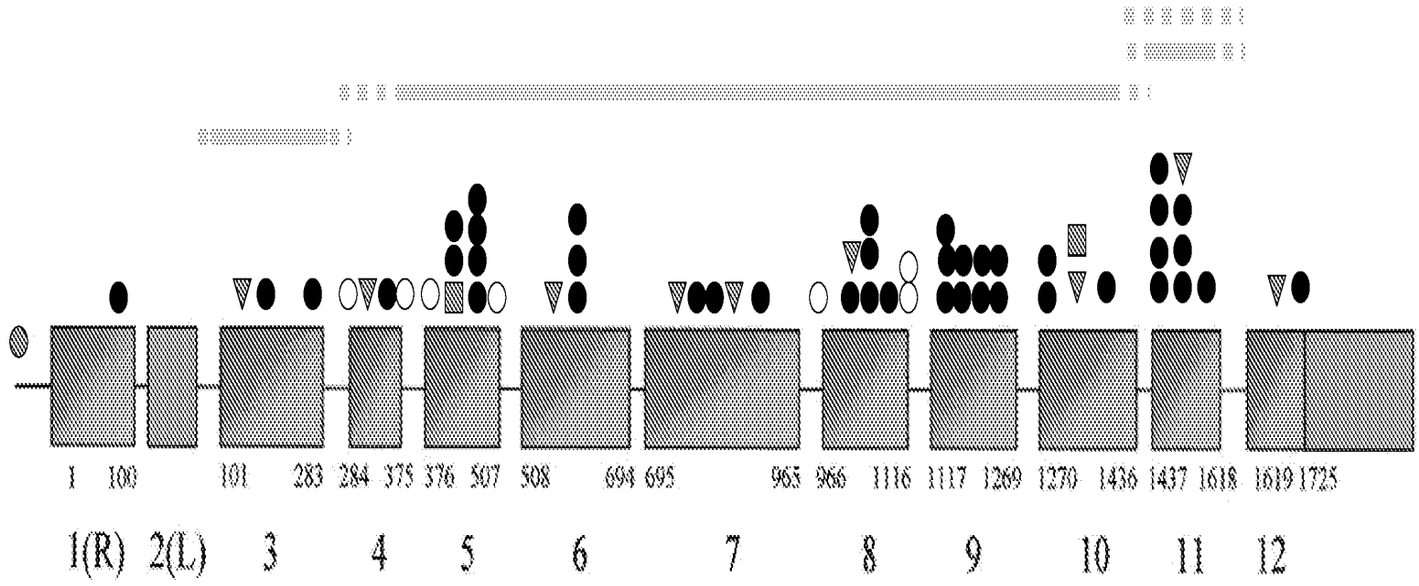
Homozygous: n=1

Heterozygous: n=13

Within the group of 1436 C>T homozygous patients, there was wide variability in the history of transfusions, baseline anemia, and history of gallstones.

Bianchi et al, ASH 2015

# PKD NHS: Diversity of Genotypes




| | | |
|---|---|---|
| ● | 42 | Missense |
| ▼ | 9 | Nonsense (6 Frameshift/3 Stop) |
| ○ | 7 | Splicing |
| ▩ | 2 | Inframe Insertion/Deletion |
| ◍ | 1 | Promoter |
| ▪▪▪▪ | 4 | Large Deletion |

Bianchi et al, ASH 2015

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# PK Deficiency Symptoms: Neonates

## Prenatal Complications in the Neonate with PKD

Complications prior to birth in 33% (55/166)

- Preterm Delivery 16% (n=26)
- Preterm Labor 12% (n=20)
- Prenatal Transfusion 12% (n=20)
- IUGR/Fetal Distress 6% (n=10)
- Hydrops 4% (n=7)

## Jaundice

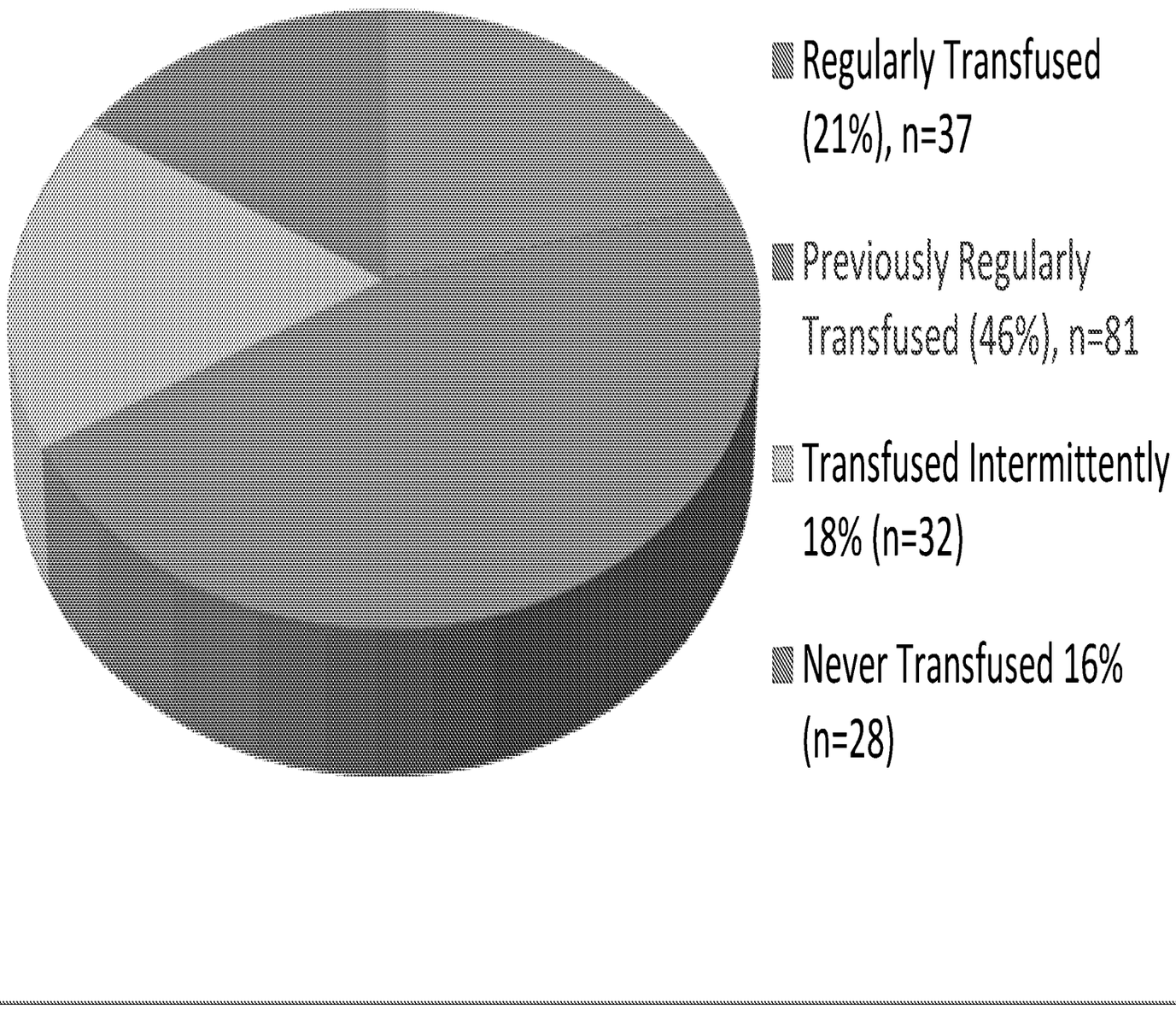


Jaundice in 88% of babies (n=144)

- Phototherapy 91%
- Exchange Transfusion 45%

➢ Need for exchange transfusion does not predict later clinical severity

# PK Deficiency Symptoms: Anemia




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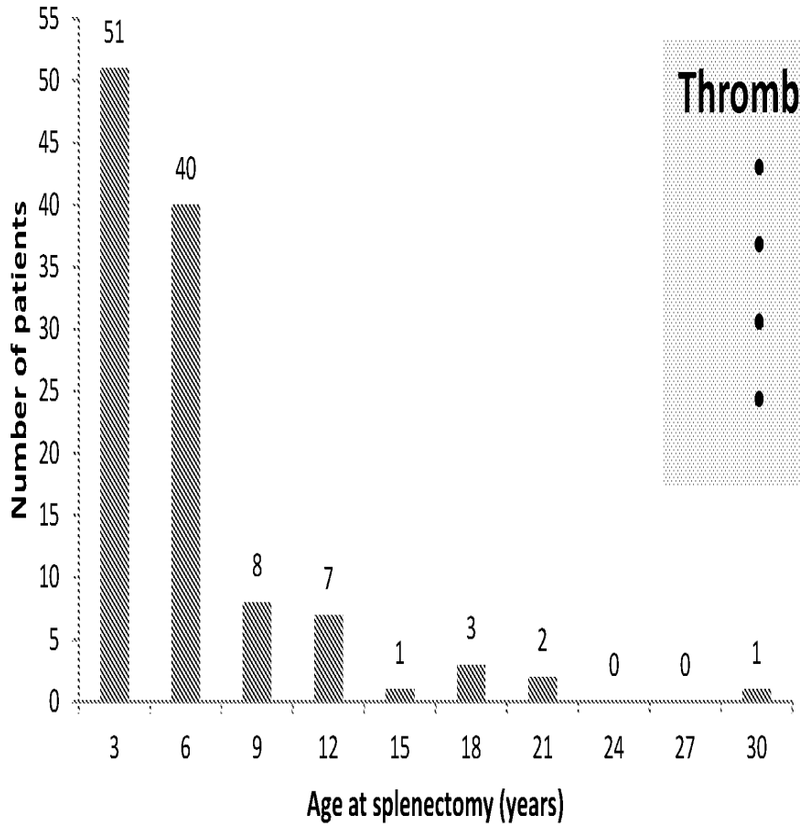
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# PK Deficiency Treatments: Splenectomy

The majority of patients had a splenectomy: 65% (115/178)

→ 15% remain transfusion dependent after splenectomy

Median age at splenectomy: 3.7 y (0.6-28.1)



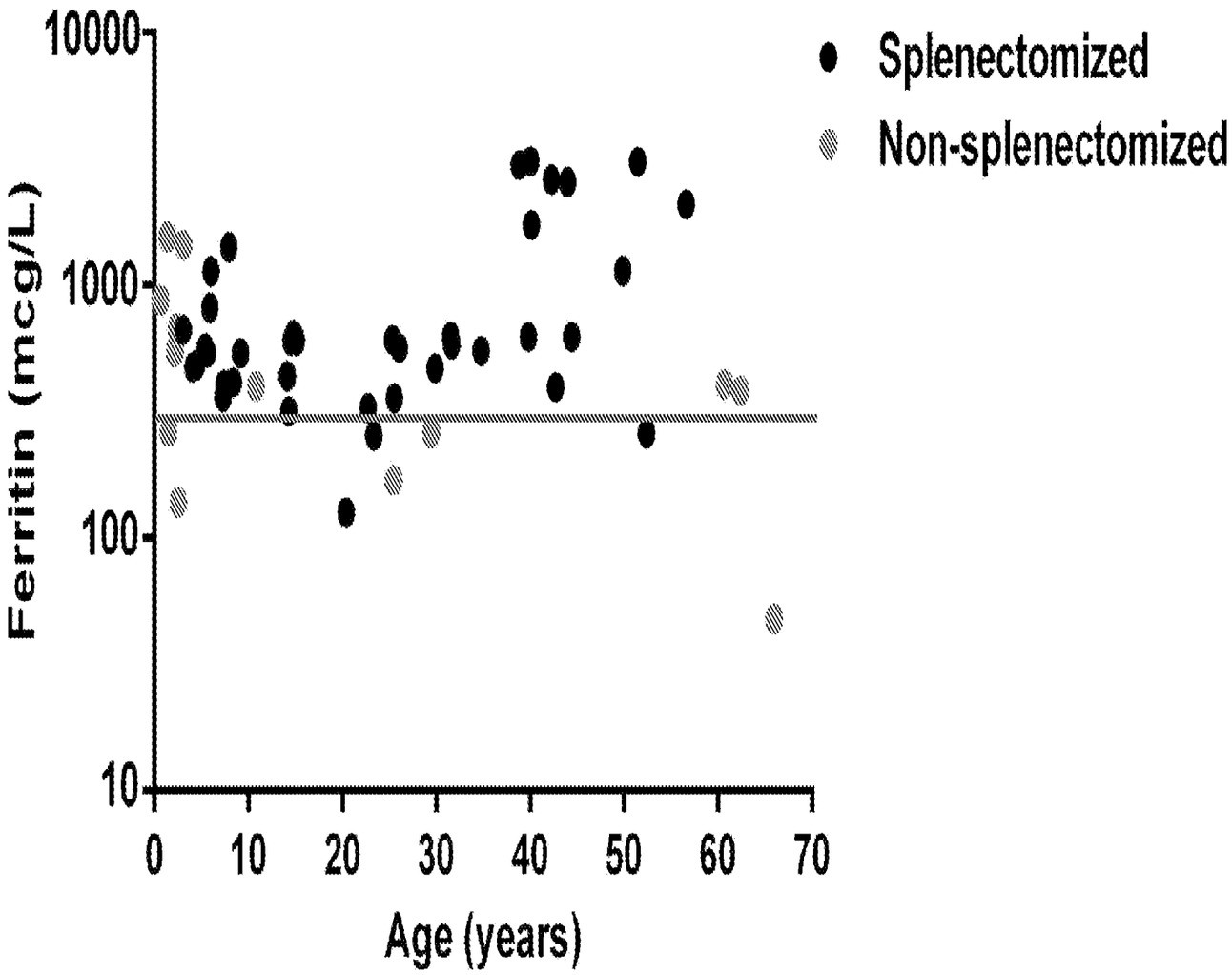

| Thrombotic Complications | 8% (9/115) |
|---|---|
| • Deep Vein Thrombosis | 7/9 |
| • Pulmonary Embolism | 2/9 |
| • Stroke | 2/9 |
| • Portal Vein Thrombosis | 1/9 |

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# PK Deficiency Complications: Iron Overload




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# PK Deficiency Complications: Iron Overload

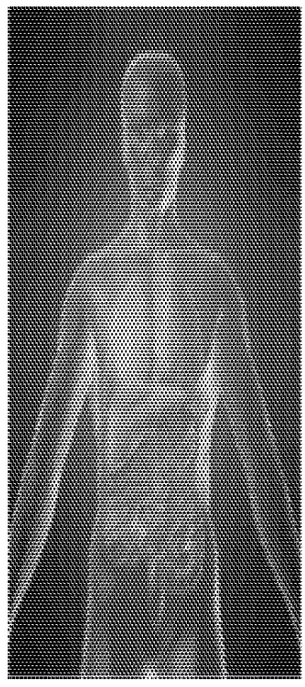
**Median Ferritin**:

| | |
|---|---|
| Currently transfused: | 1195 ng/ml (IQR 688-1733), n=32 |
| Historically transfused: | 606 ng/ml (IQR 410-1099), n=60 |
| Acute Transfusions/Never Transfused: | 321 ng/ml (IQR 160-570), n=36 |

- *Ferritin is higher in those who had a splenectomy even after adjusting for transfusion status.*

**MRI** was performed in 46% (n=81) of participants

- Hepatic T2* Median: 5.2 mg/g DW liver
  - 63% had hepatic iron overload (>4 mg/g DW liver)
  - *70% with no or few transfusions had hepatic iron overload*
- Cardiac T2* Median: 36.2 ms
  - 3% had cardiac iron overload (<20 ms)

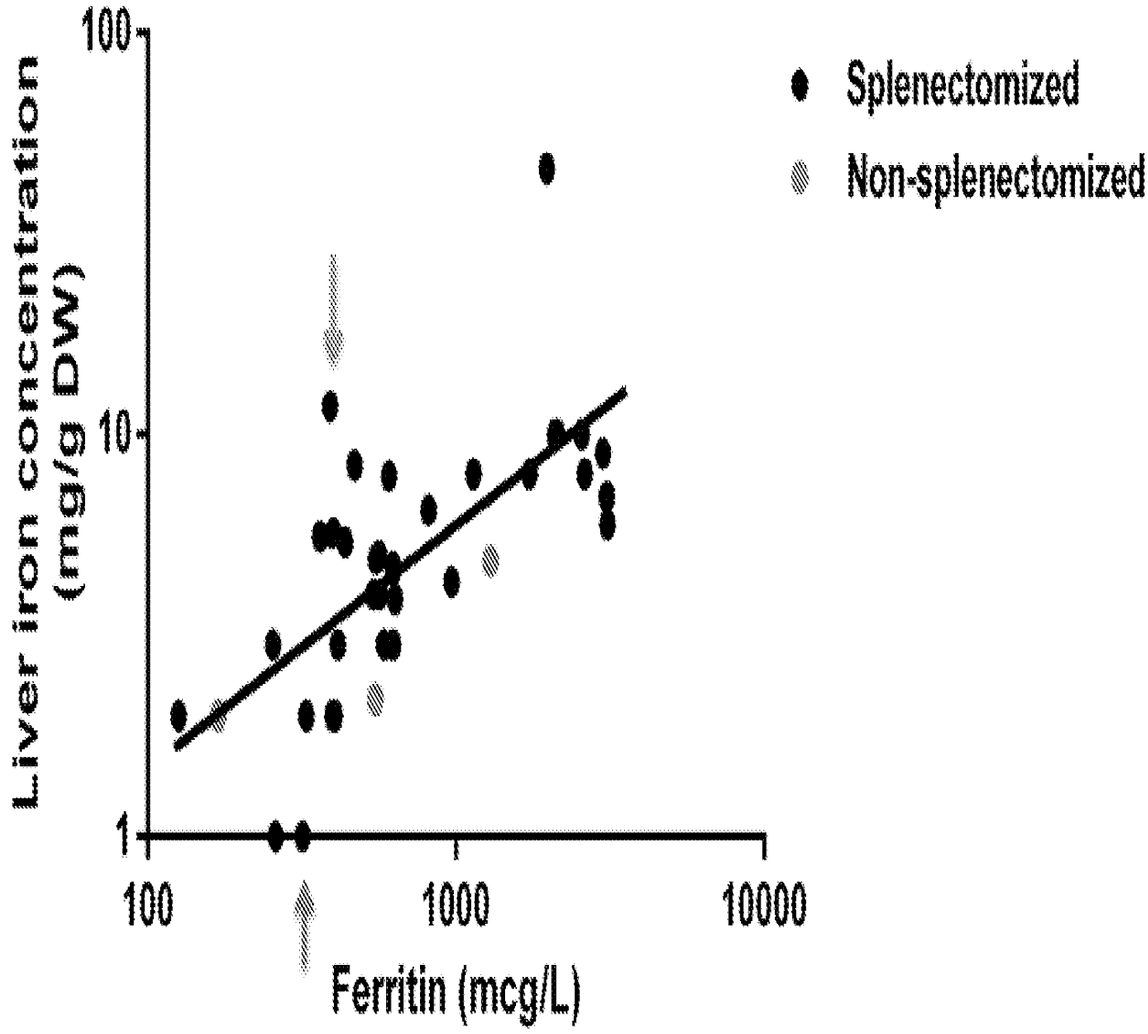


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# Iron Monitoring in PKD

Correlation of T2* MRI LIC with ferritin (mcg/L), $r^2$=0.6, p<0.05

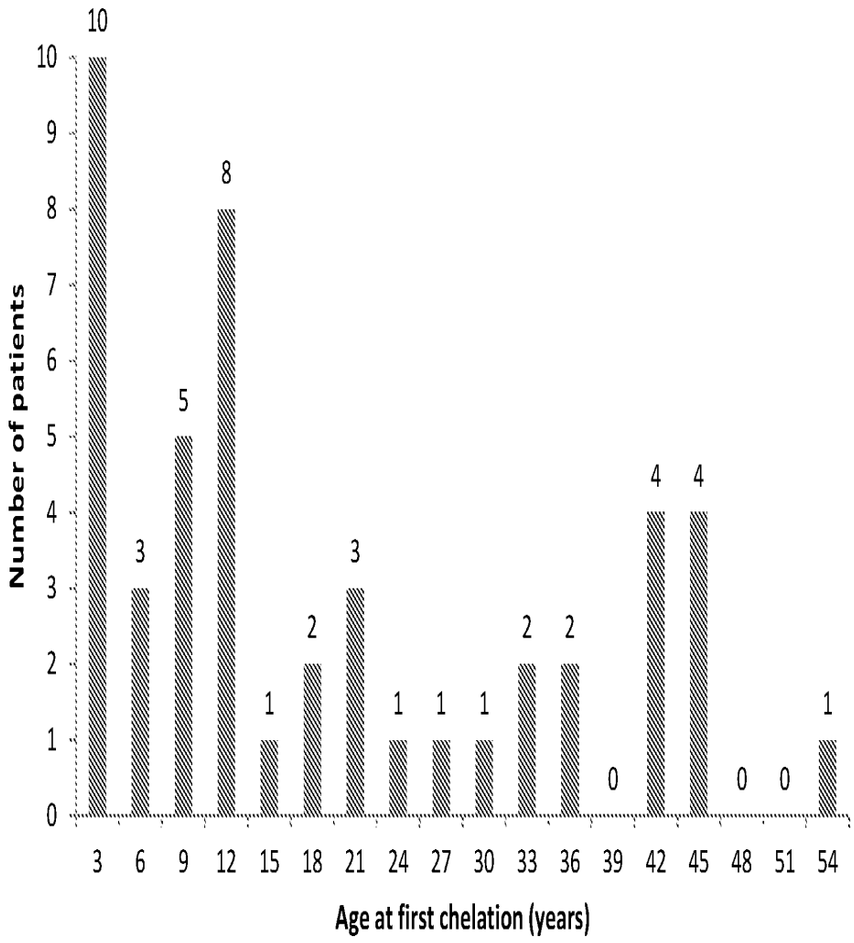



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# Treatment of Iron Overload

Age when chelation was first prescribed




28% (n=51) have received <u>Chelation Therapy</u>

- Median age of chelation: 11.7 y (1-54 y)

2% (n=4) have received <u>Phlebotomy</u> for chelation

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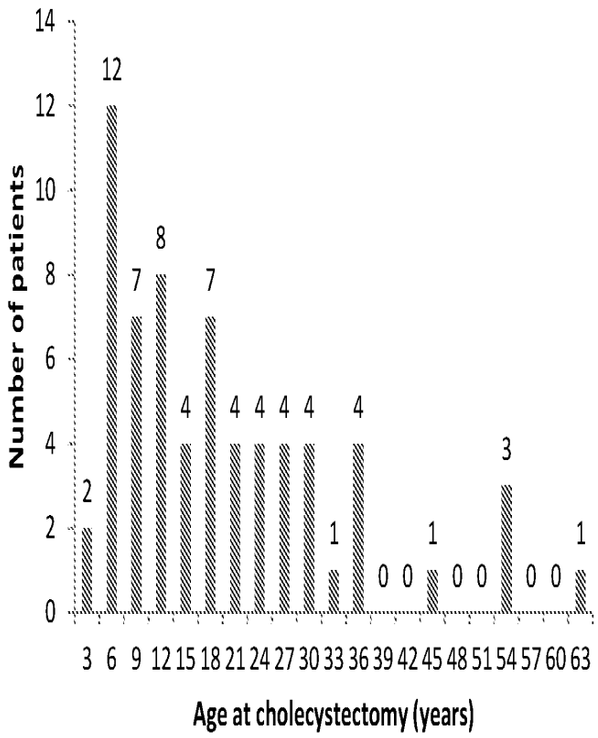
# PKD Deficiency: Symptoms and Complications

## Gallstones

Incidence of gallstones is 43% (n=75)

Median Age 14.5 y (range 2.2-60.4 y)

Cholecystectomy 77% occurred after splenectomy




## Extramedullary Hematopoiesis

Extramedullary Hematopoiesis: 12% (21/178)

| | |
|---|---|
| Hepatic | n=13 |
| Splenic | n=14 |
| Paraspinous | n=7 |
| Mediastinal | n=7 |

## Osteopenia

Bone Fractures: 17% (30/178)

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Ex. 2001-0656

# Define Disease: Classification of Clinical Severity

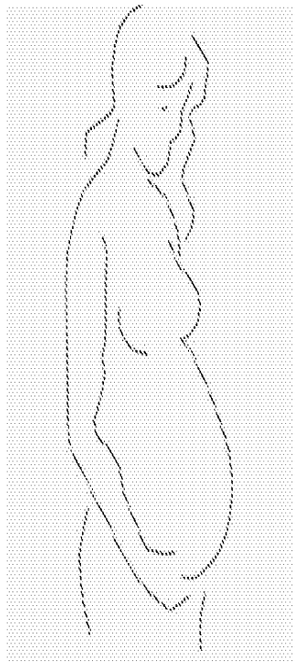
| | MILD | MILD-MODERATE | MODERATE-SEVERE | SEVERE | |
|---|---|---|---|---|---|
| **Severity Group Characteristics** | **Never Regularly Transfused** | **SPL, Less Anemic Hb >8.7 g/dl** | **SPL, More Anemic Hb ≤8.7 g/dl** | **Regularly Transfused after SPL** | **p-value** |
| **n** | 50 | 37 | 31 | 11 | |
| **Age at diagnosis Median (range)** | 2.0 (0-60.3) | 0 (0-10.0) | 0.1 (0-35.1) | 1.7 (0-13.8) | **0.04** |
| **Hb (g/dl) Median (range)** | 10.0 (6.5-14.2) | 9.7 (8.8-11.2) | 8.3 (6.3-8.7) | 8.6 (6.7-11.6) | NA |
| **Reticulocyte Count %, Median (range)** | 9.6 (1.6-82.9) | 28.1 (8.2-61.0) | 34.2 (5.4-61.3) | 20.9 (2.5-30.5) | **0.02** |
| **MCV (fL) Median (range)** | 100 (61-133) | 110 (93-133) | 115 (90-135) | 97 (85-100) | **<0.01** |
| **Cholecystectomy** | 15/50 (10%) | 3/37 (8%) | 14/31 (45%) | 10/11 (91%) | 0.01 |
| **History of chelation** | 5/50 (10%) | 3/37 (8%) | 14/31 (45%) | 10/11 (91%) | **<0.01** |
| **Ferritin (mg/ml) Median (range)** | 334 (17-3087) | 567 (126-3016) | 593 (139-2134) | 1420 (308-5630) | **0.03** |

# PK Deficiency in Pregnancy

31 women with 102 pregnancies:

- Pregnancy Outcomes:

| | |
|---|---|
| Normal Birth | 60% (61) |
| Miscarriage | 21% (21) |
| Preterm (<37 weeks) | 12% (12) |



- Assisted Reproduction was rare (2%)
- Transfusions during Pregnancy:

| | |
|---|---|
| Prior to pregnancy | 4% |
| During | 12% |
| After delivery | 21% |

# Adult Patient Reported Outcomes Surveys

- Participants complete surveys which cover a number of domains
  - EuroQoL-5D
    - Mobility, Self Care, Activities, Discomfort, and Anxiety/Depression
    - n=71
  - Functional Assessment of Cancer Therapy (FACT)-Anemia
    - Physical, Social/Family, Emotional, Functional, and Fatigue
    - n=71
  - Patient Reported Outcome Measurement Information System (PROMIS) – Fatigue
    - n=45

  - A latent variable regression model was used to assess the relationship between each domain and hemoglobin, gender, splenectomy status, and age



Ex. 2001-0659

# Adult Survey Results

- Associations with **splenectomy**:
  - More likely to work (FACT-An, p=0.004)
  - More likely to enjoy life, accept their illness, and be content with their lives (FACT-An, p=0.004)
- Associations with **higher hemoglobin levels**:
  - Increased ability to work (FACT-An, p<0.001)
  - Enhanced scores of emotional well-being (FACT-An, p=0.001)
  - Higher report of emotional/family support (FACT-An, p=0.001)
- Associations with **female gender**
  - Higher levels of fatigue (PROMIS, p=0.05)
- Associations with **older age**:
  - Higher levels of fatigue (EQ, p=0.02)
  - Lower physical health (p=0.01) and less emotional support (FACT-An, p=0.03)
  - Decreased ability to work (FACT-An, p<0.001)



Ex. 2001-0660

# Next Steps: Natural History Study

1. **Define Disease**:
   - Transfusion/Hemoglobin Phenotypes
   - Current standard of care
     - Splenectomy, Cholecystecomy
     - Iron Monitoring and Treatment
   - Identification of disease features/complications
     - Common Complications: Iron Overload, gallstones
     - Rare Complications: Bone Density, Thrombosis
   - Biomarkers: ATP, 2,3-DPG, PK activity
2. **Improve Diagnosis**
3. **Create Guidelines**

# DRUG DEVELOPMENT IN PKD

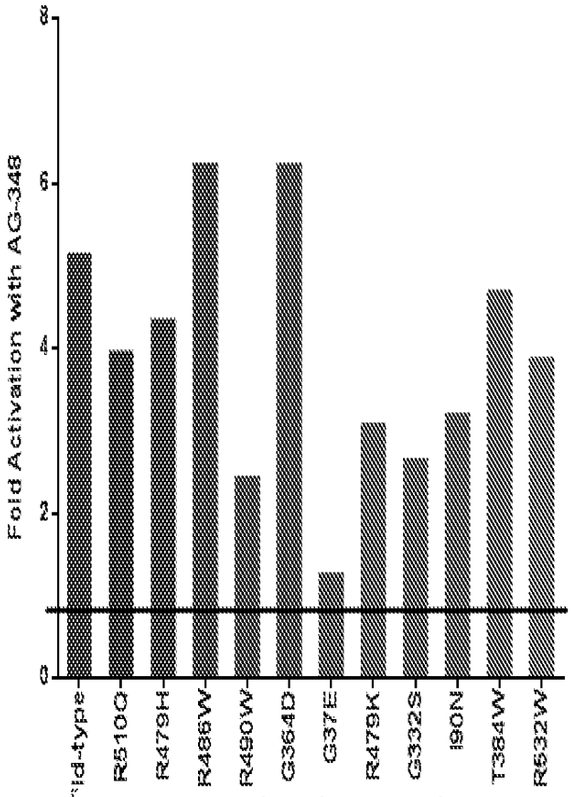
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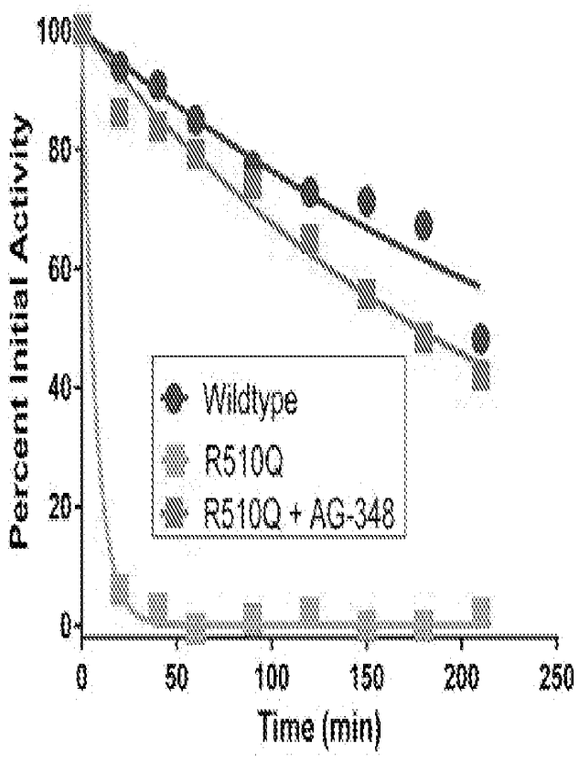
# AG-348 Preclinical Development

- Allosteric activator of red cell pyruvate kinase (PKR)
  - Potently activates wild type PKR and a wide spectrum of recombinantly expressed PKR mutants
  - Increases thermal stability and catalytic activity




= AG-348 binding pockets

= areas of common defects




Yang et al. ASH 2014

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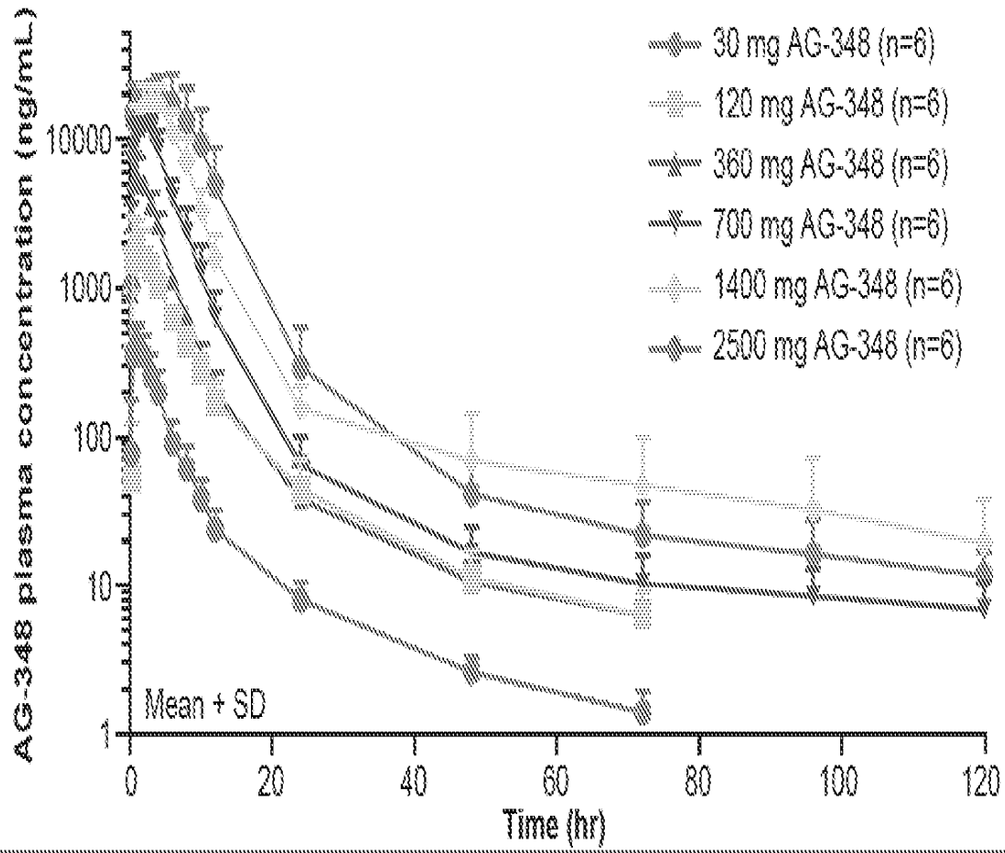
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# Healthy Volunteer Study: Single Ascending Dose (SAD) Study

## SAD (30-2500 mg, n= 48)

- 19/48 had at least 1 AE (Grade 1 or 2); nausea, headache
- Rapid absorption with low PK variability

Plasma concentration after a single dose



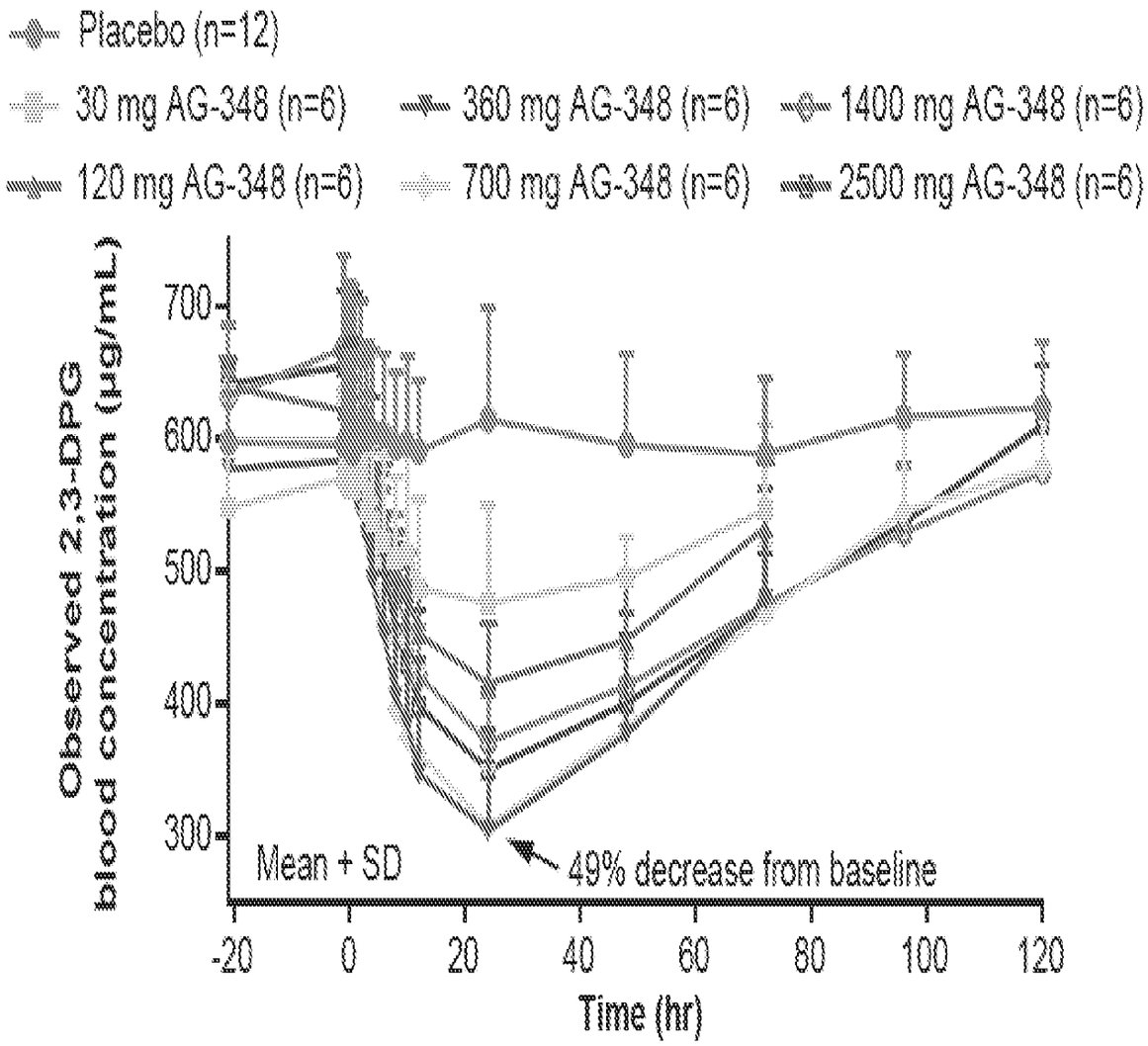

Yang et al. ASH 2014



Ex. 2001-0664

# SAD: Pharmacodynamic Endpoints

## 2,3-DPG after a single dose of AG-348



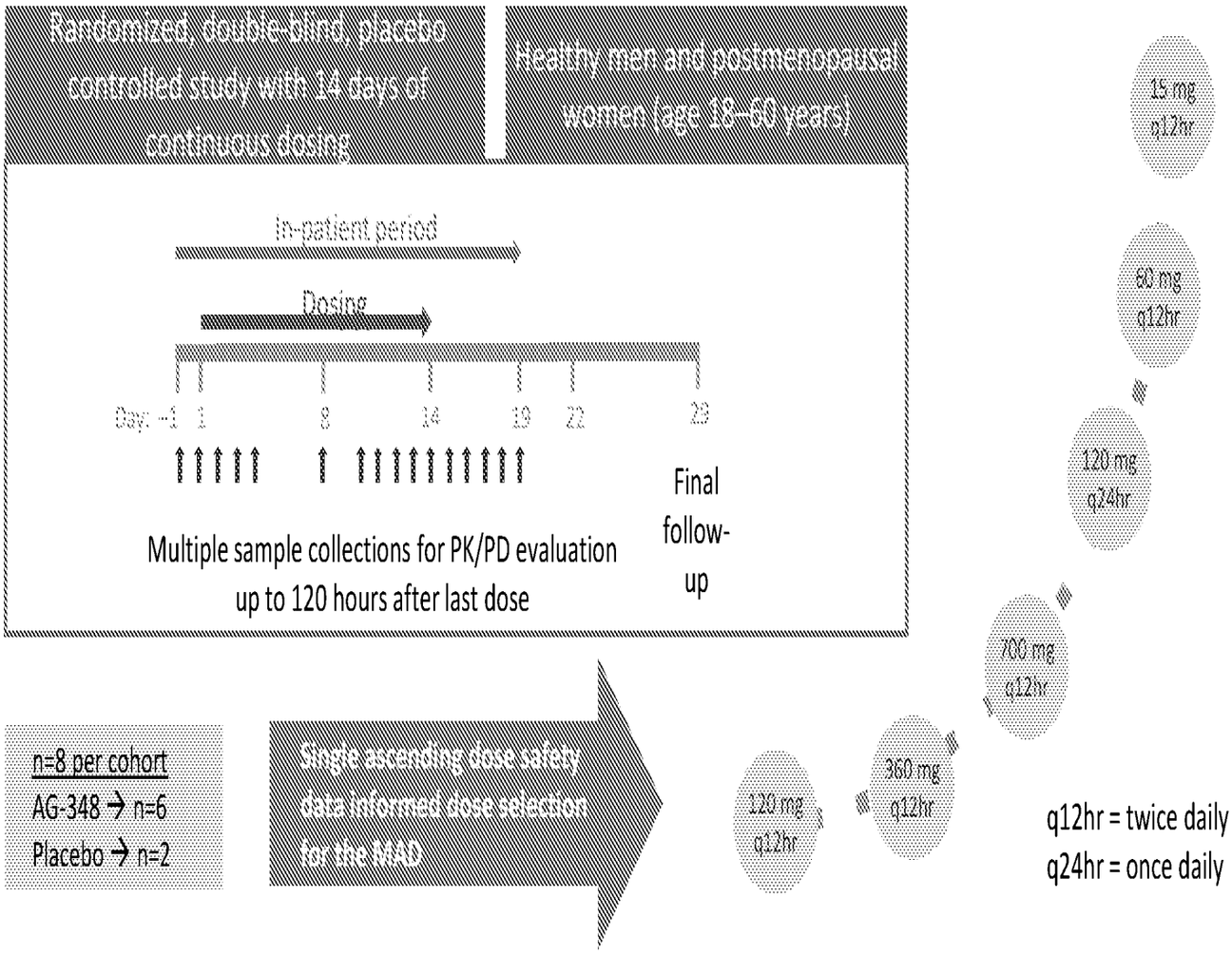

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# Healthy Volunteer Study: Multiple Ascending Dose (MAD) Study Design

**MAD: Establish the safety and tolerability and PD effects of AG-348**




PK/PD = pharmacokinetic/pharmacodynamic

NCT02149966

Yang et al. EHA 2015

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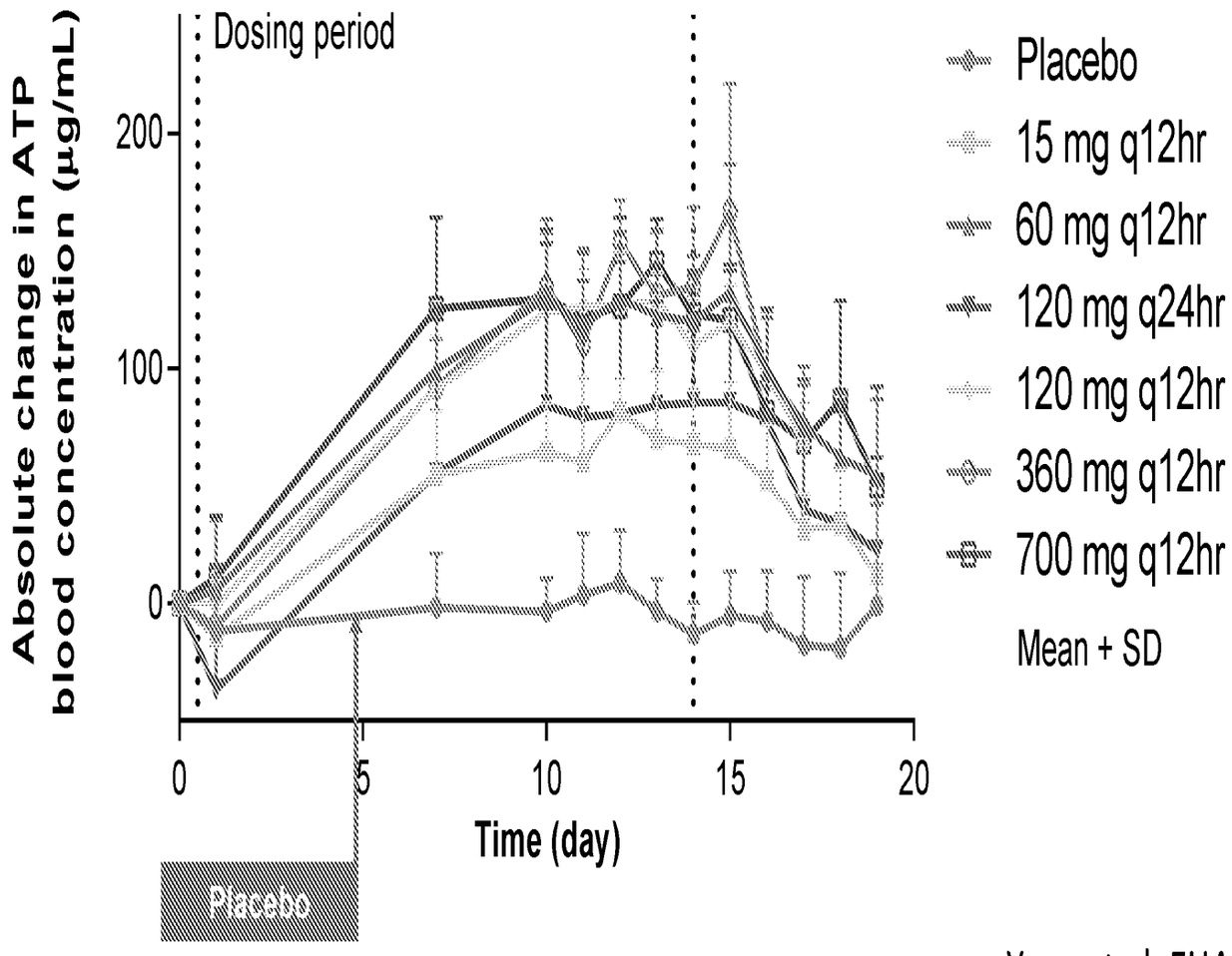
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Ex. 2001-0666

# MAD: Pharmacodynamic Endpoints

- Randomized, Placebo Controlled Multiple Ascending Dose Study
- MAD(15-700 mg, n=48)




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# MAD: Tolerability

- 14-day safety data provided a range of doses for consideration
- Drug-related events were Grade 1 and 2; one Grade 3 event at 700 mg q12h (increased LFTs – dose limiting toxicity)

| AE, n (%) | Placebo n=12 | 15–360 mg q12hr n=30 | 700 mg q12hr n=6 |
|---|---|---|---|
| Any AE | 4 (33) | 10 (33) | 6 (100) |
| Most common treatment-related AEs (≥2 subjects): | | | |
| Nausea | – | – | 5 (83) |
| Headache | 1 (8) | 1 (3) | 4 (67) |
| Vomiting | 1 (8) | – | 3 (50) |
| Feeling hot | 1 (8) | – | 3 (50) |
| Decreased appetite | – | 2 (7) | 1 (17) |
| Restlessness | – | – | 3 (50) |
| Fatigue | – | – | 2 (33) |
| Dizziness | 1 (8) | – | 2 (33) |
| Hyperhidrosis | 1 (8) | – | 2 (33) |
| Drug eruption | – | 2 (7) | – |
| Abdominal discomfort | 2 (17) | – | – |

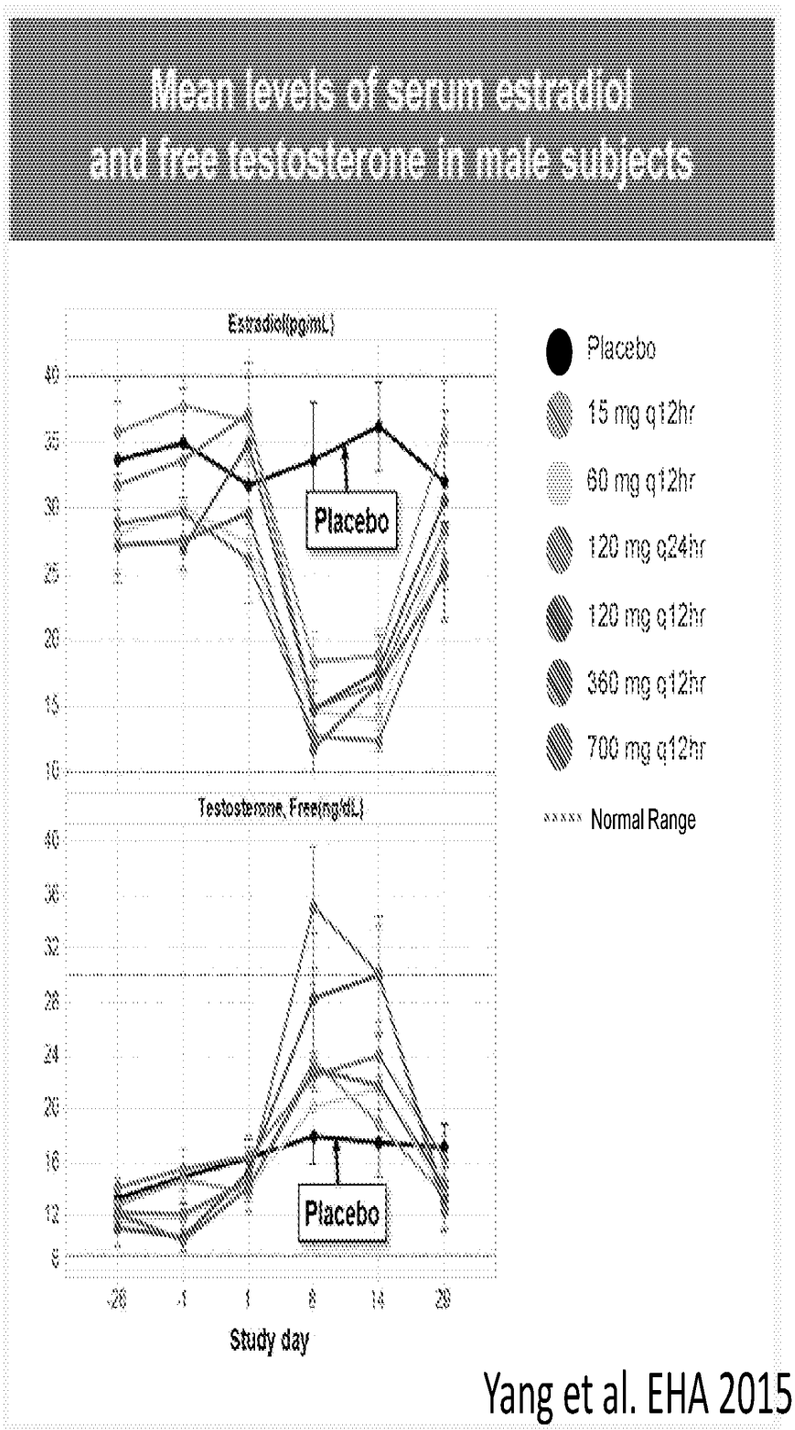
Yang et al. EHA 2015



Ex. 2001-0668

# Hormone Changes Seen with MAD Study: Reversible Aromatase Inhibition

- Changes in serum androgens and estrogens
- Most changes remained within normal ranges
- Levels recovered to baseline
- Endocrine/physiologic significance to be evaluated in Phase 2 study
- AG-519 in healthy volunteer studies now, no preclinical aromatase activity




Yang et al. EHA 2015

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DRIVEPK

# Phase 2 Proof of Concept Study

## Single Ascending Dose
## Multiple Ascending Dose

Completed AG-348
healthy volunteers studies

**Objectives:**

- ✓ Short-term safety
- ✓ Pharmacokinetics
- ✓ Pharmacodynamics

## Phase II Study: DRIVE PK

Ongoing study of AG-348 in transfusion-independent adults with PK deficiency

**Objectives:**

- ❑ Safety – chronic dosing
- ❑ Efficacy
- ❑ Pharmacokinetics
- ❑ Pharmacodynamics

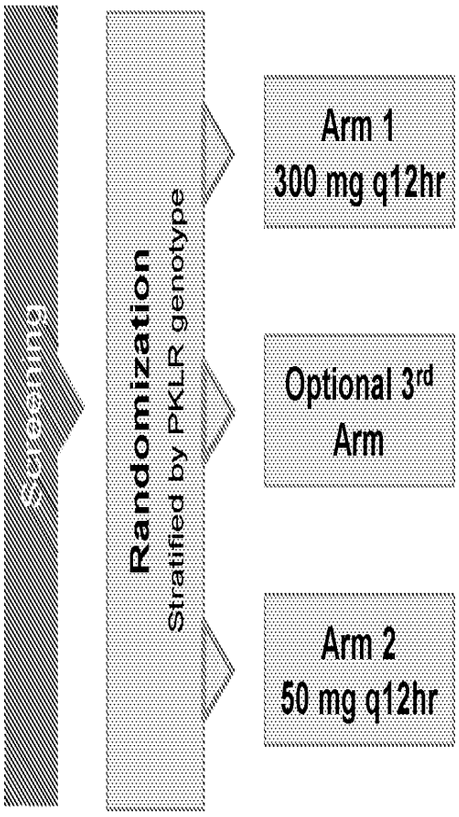
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# Global Phase 2 Study

DRIVEPK

Transfusion-independent PK-deficient adults n=25 in each arm




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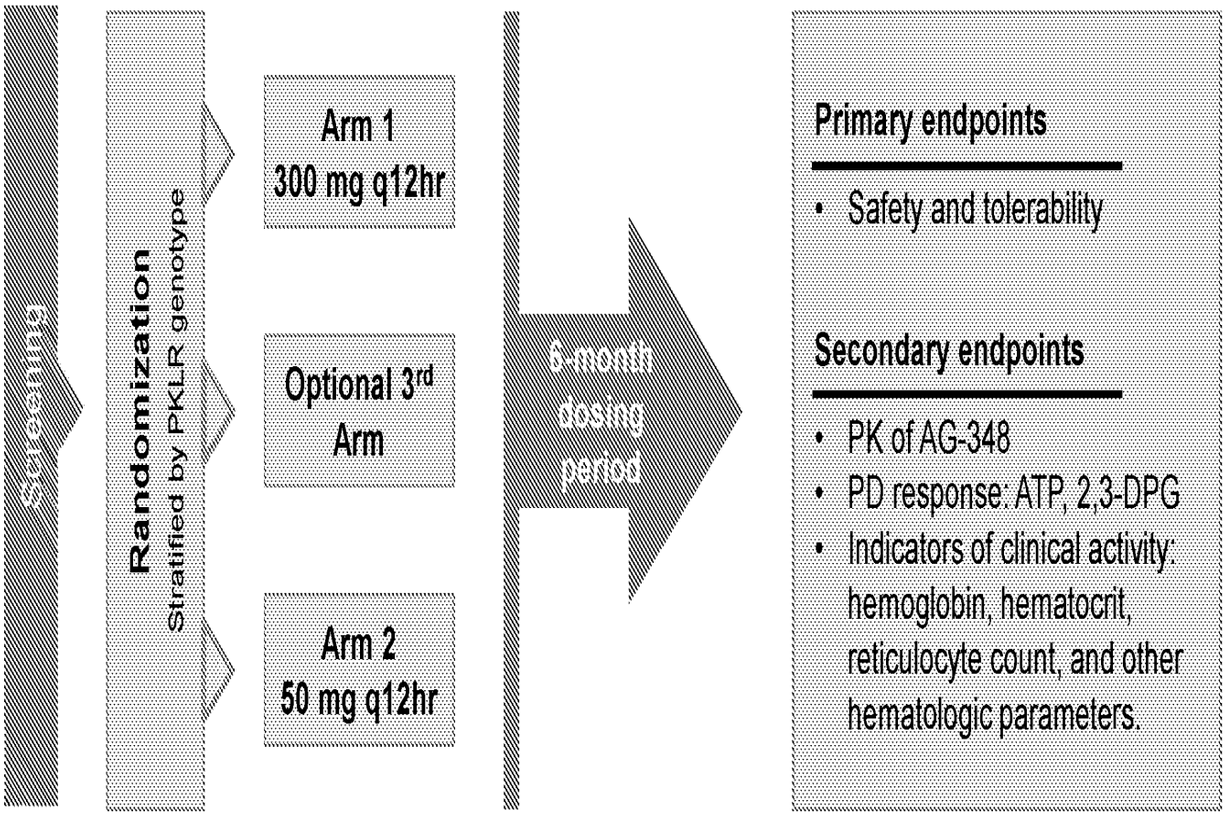
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# Global Phase 2 Study

DRIVEPK

Transfusion-independent PK-deficient adults n=25 in each arm




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# Summary

- Natural History Studies are the foundation of clinical development programs in rare diseases.
- The drug development perspective is integral early in the design stage of a Natural History Study.
- Collaboration is key to progress in rare diseases (industry, colleagues, patients).
- Our understanding of PK deficiency has improved and will continue to grow through the NHS.
- A new treatment approach for PKD is currently in clinical trials.

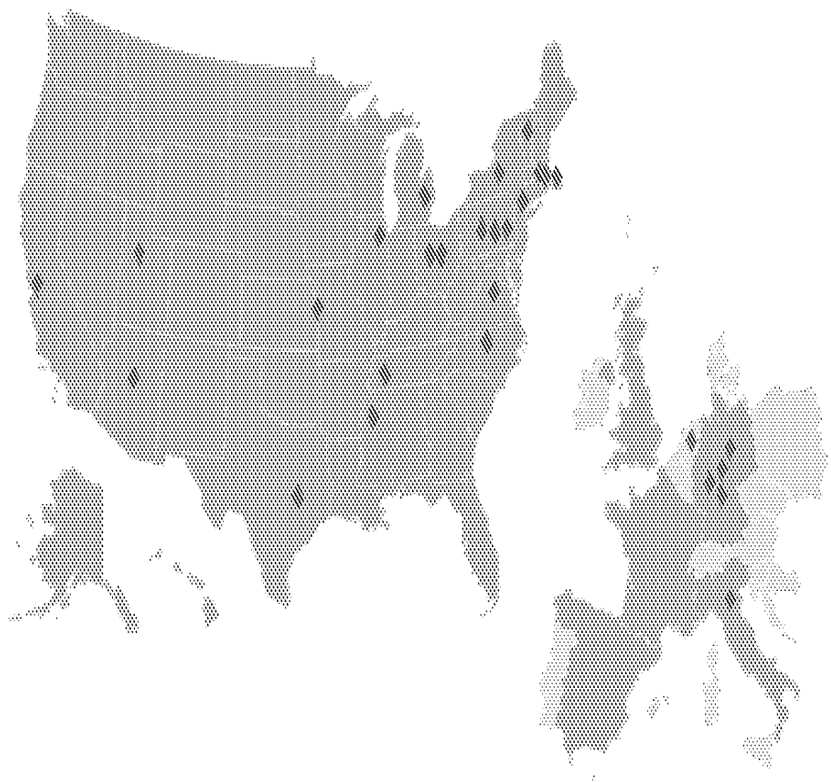
# Acknowledgements


## PKD NHS Boston Children's Team

Ellis Neufeld
Dongjing Guo
Krystle Benedict
Wendy London
Nolan Neu
Jill Falcone

## PKD NHS Laboratories

Patrick Gallagher
Paola Bianchi
Kimberly Lezon-Geyda
Elisa Fermo

## PKD NHS Investigators and Participants


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# Clinical Research Progress in a Rare Anemia: New Developments in Pyruvate Kinase Deficiency

April 25, 2017

Rachael Grace, MD, MMSc
Assistant Professor, Harvard Medical School
Dana-Farber Boston Children's Cancer and Blood Disorders Center
Boston, MA

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# Disclosures

- The PKD Natural History Study and DRIVEPK study are funded by Agios Pharmaceuticals
- I am a Scientific Advisor to Agios Pharmaceuticals
- I will discuss an investigational agent, AG-348, in my presentation



Ex. 2001-0676

# Overview

- Brief overview of Pyruvate Kinase Deficiency
- Initial Findings from the PKD Natural History Study
- Drug Development in PKD
  - Pre-clinical data
  - Data from Healthy Volunteer Studies
  - Initial Results of the Phase II clinical trial



Ex. 2001-0677

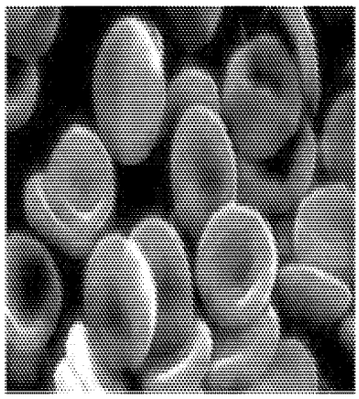
# OVERVIEW OF PYRUVATE KINASE DEFICIENCY

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# The Mature Erythrocyte

- Lacks a nucleus, DNA, RNA, and ribosomes
- Cannot synthesize RNA, DNA, or proteins
- Has no mitochondria
- Lacks an electron transport system for oxidative phosphorylation



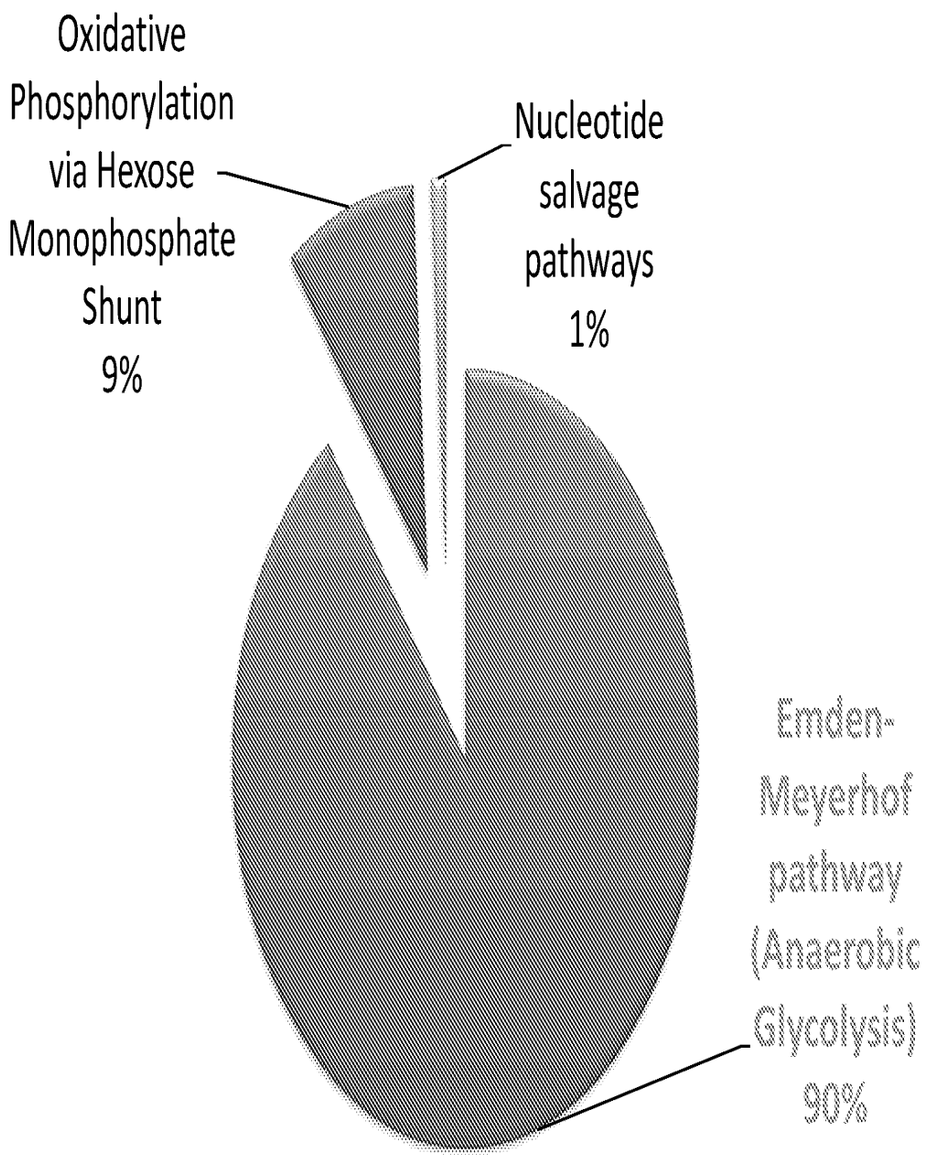
→Erythrocyte must maintain an adequate ATP supply

- Pump cations against electrochemical gradient
- Maintain membrane phospholipids
- Protect hemoglobin and cell from oxidative damage



Ex. 2001-0679

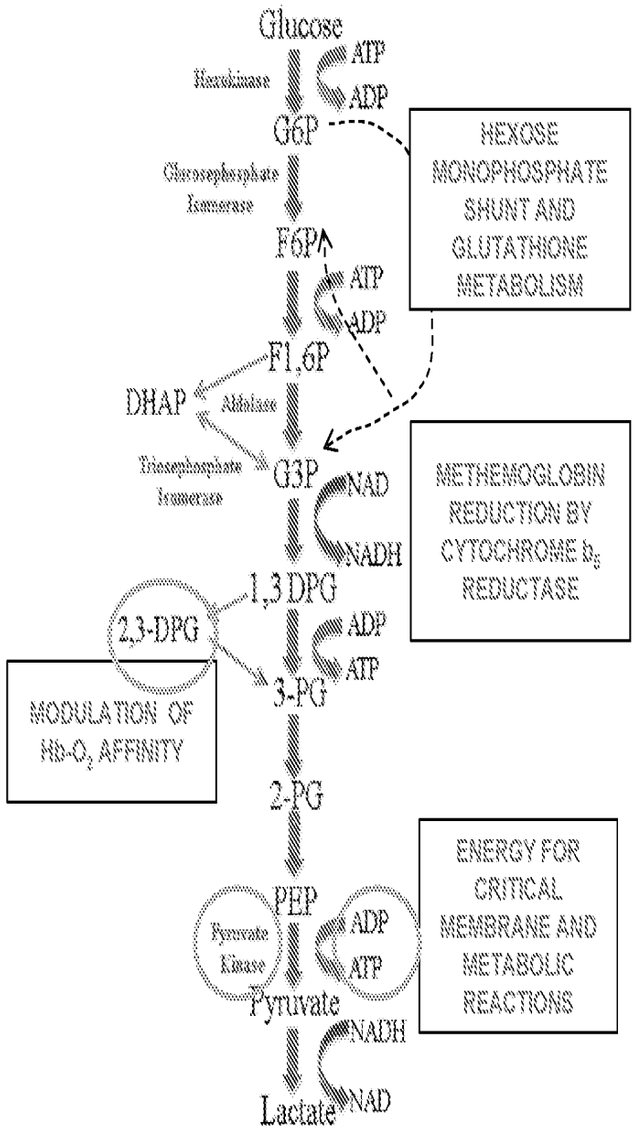
# How Does the Mature Erythrocyte Make Energy?



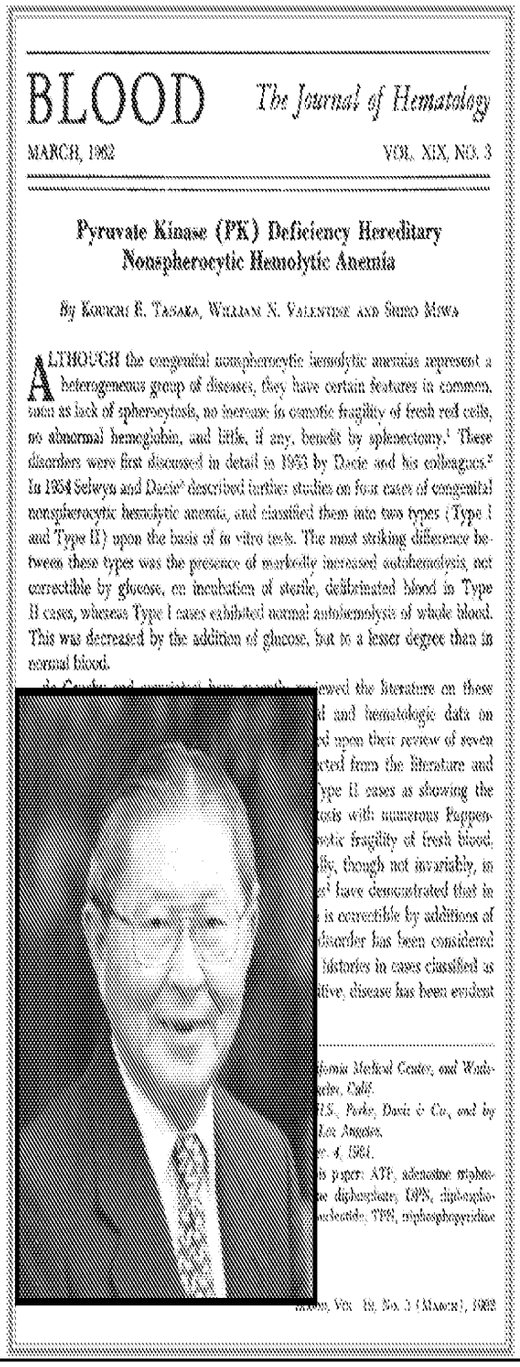



Ex. 2001-0680

# Red Cell Metabolism



# Pyruvate Kinase Deficiency



BLOOD *The Journal of Hematology*

MARCH, 1962 VOL. XIX, NO. 3

**Pyruvate Kinase (PK) Deficiency Hereditary Nonspherocytic Hemolytic Anemia**

*By* Kouichi K. Tanaka, William N. Valentine and Sumio Miwa

ALTHOUGH the congenital nonspherocytic hemolytic anemias represent a heterogeneous group of diseases, they have certain features in common, such as lack of spherocytosis, no increase in osmotic fragility of fresh red cells, no abnormal hemoglobin, and little, if any, benefit by splenectomy.[1] These disorders were first discussed in detail in 1953 by Dacie and his colleagues.[2] In 1958 Selwyn and Dacie[3] described further studies on four cases of congenital nonspherocytic hemolytic anemia, and classified them into two types (Type I and Type II) upon the basis of in vitro tests. The most striking difference between these types was the presence of markedly increased autohemolysis, not correctible by glucose, on incubation of sterile, defibrinated blood in Type II cases, whereas Type I cases exhibited normal autohemolysis of whole blood. This was decreased by the addition of glucose, but to a lesser degree than in normal blood.

...reviewed the literature on these ...l and hematologic data on ...d upon their review of seven ...cted from the literature and ...ype II cases as showing the ...osis with numerous Pappen... ...otic fragility of fresh blood. ...lly, though not invariably, in ...[3] have demonstrated that in ... is correctible by additions of ...disorder has been considered ...histories in cases classified as ...itive, disease has been evident

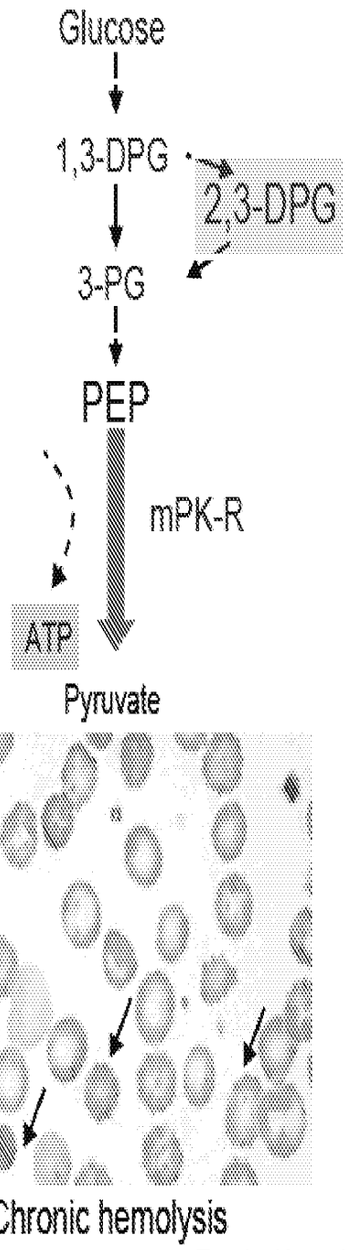
...Roma Medical Center, and Wads... ...ville, Calif.

...U.S. Parke, Davis & Co., and by ...Los Angeles.

...r 4, 1961.

...is paper: ATP, adenosine triphos... ...ne diphosphate; DPN, diphospho... ...nucleotide; TPN, triphosphopyridine

...BLOOD, VOL. XIX, NO. 3 (MARCH), 1962




Tanaka K, et al. *Blood*. 1962;19:267-295.

# Epidemiology

- >200 mutations described in the PKLR gene which cause:
  - Decreased catalytic activity
  - Protein instability
  - Decreased protein expression
- Autosomal Recessive – Homozygotes and Compound Heterozygotes
- Estimated Prevalence 3:100,000 to 1:20,000
  - Actual numbers of patients found is ~1/million,
    or 30x less common than might be predicted
  - Much more common among the Amish due to founder effect

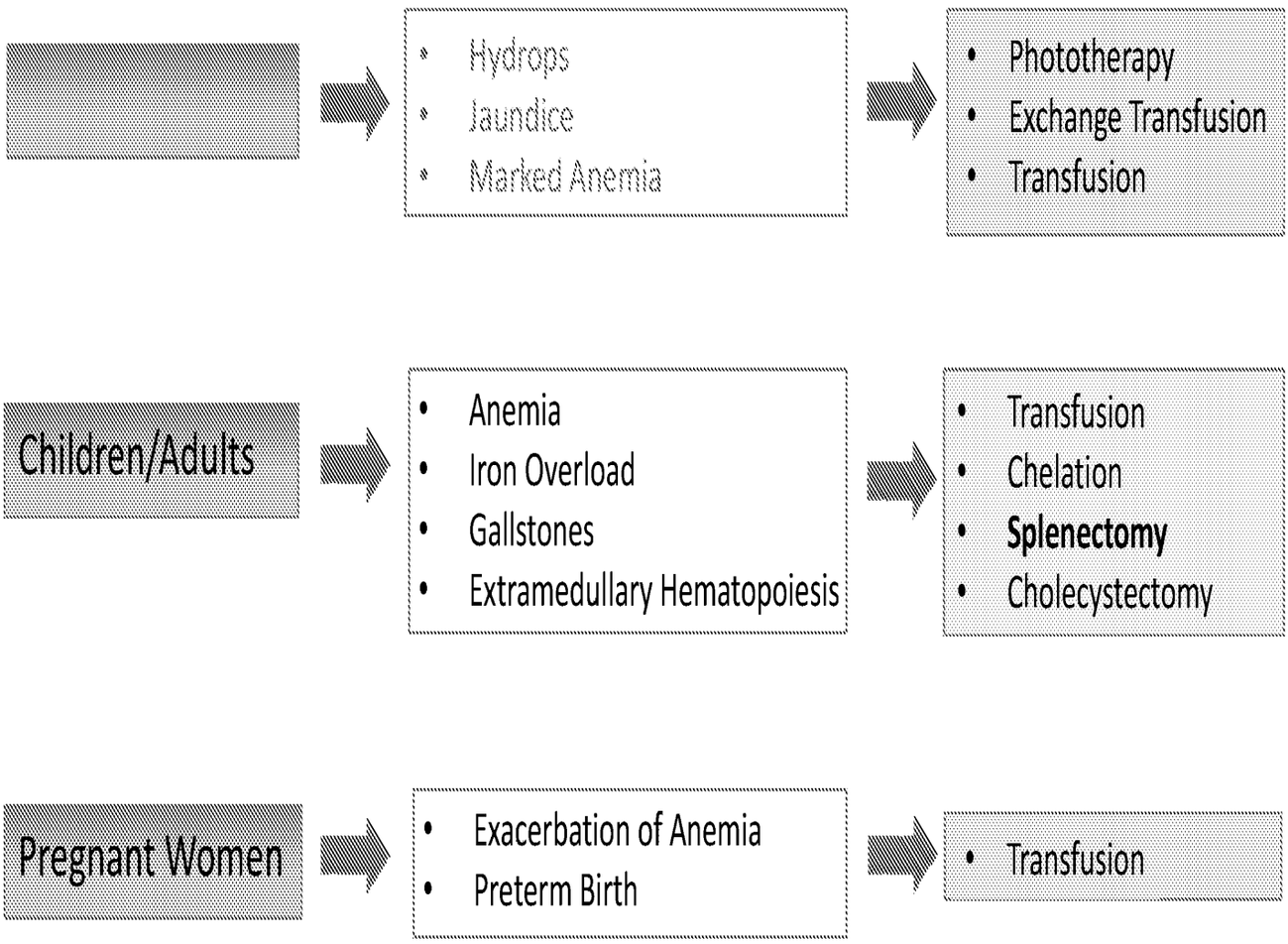
Beutler et al, Blood 2000
Carey at al, Blood 2000



Ex. 2001-0683

# Clinical Features and Treatment

Hemolytic anemia of variable severity with reticulocytosis




Zanella et al, Blood Rev 2007



Ex. 2001-0684

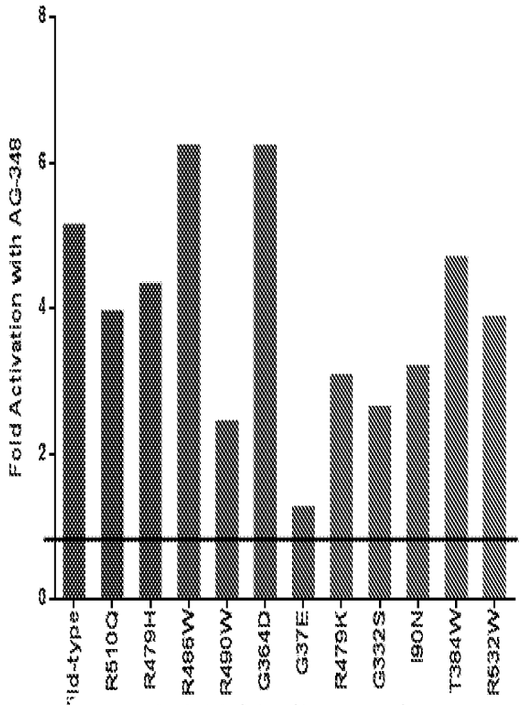
# PKD NATURAL HISTORY STUDY

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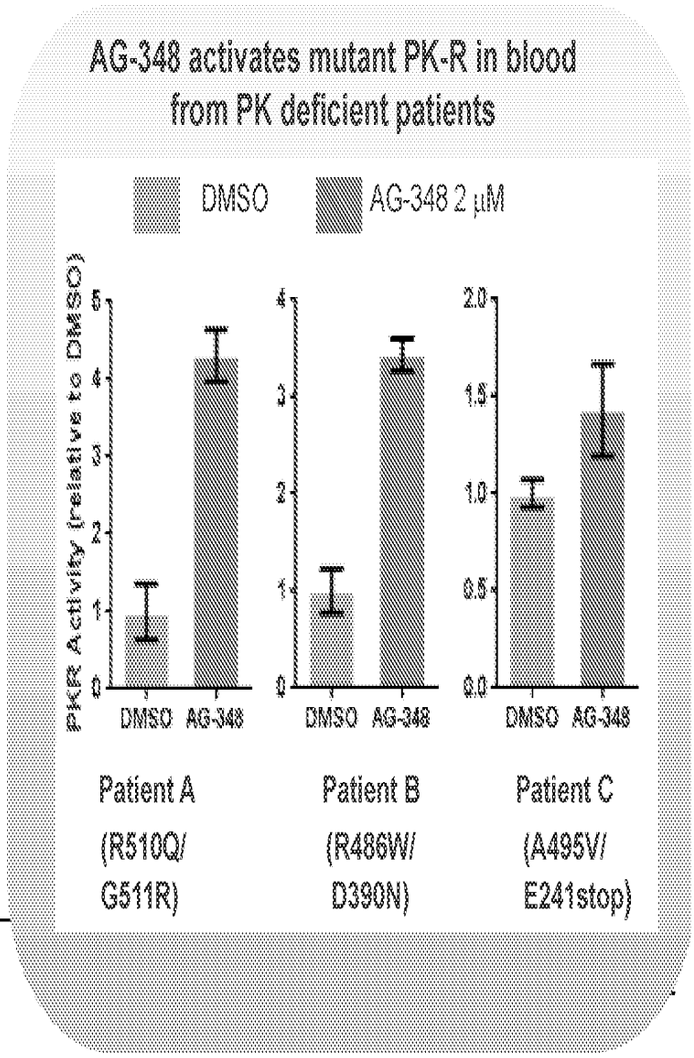
# AG-348 Preclinical Development

- Allosteric activator of red cell pyruvate kinase (PKR)
  - Potently activates wild type PKR and a wide spectrum of recombinantly expressed PKR mutants
  - Increases thermal stability and catalytic activity




= AG-348 binding pockets

= areas of common defects




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CANCER AND BLOOD DISORDERS CENTER

# Initiating a Natural History Study

- Industry interest in this rare disease with significant unmet needs
- Natural History Study needed to inform drug development
    - Essential foundation of a clinical development program
- Critical for designing clinical trials → need drug development perspective in the design stage of the NHS
    - What is the clinical spectrum and course?
    - What is the standard of care for this disease?
    - What assessments/disease measures are useful?
    - What is a clinical efficacy endpoint?
    - What is a clinically meaningful change in the endpoint?



Ex. 2001-0687

# Initiating a Natural History Study

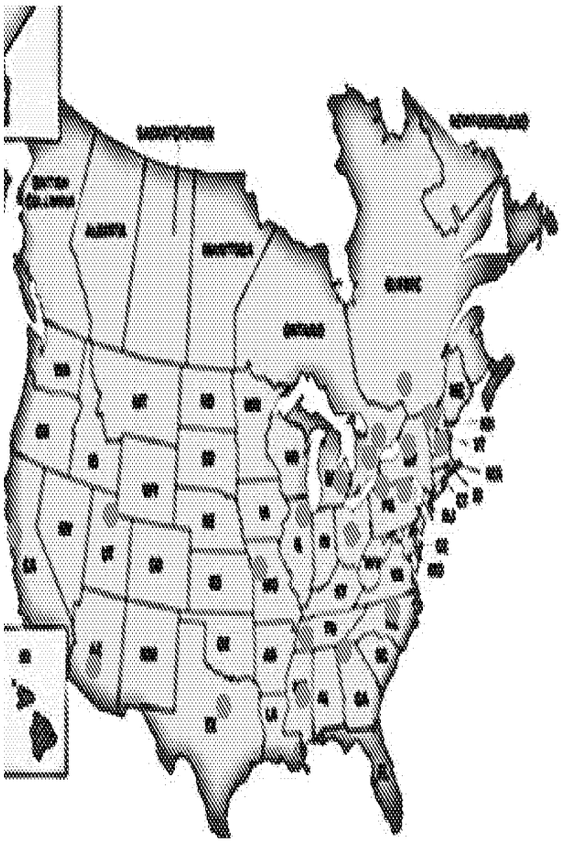
Clinical interest in an improved understanding of PKD

- **Define Disease**: track all ages and follow over time
  - Clinical Phenotypes/Spectrum
  - Current standard of care
  - Biomarkers correlating with disease course
  - Identification of disease features/complications
  - Range, pace, and frequency of manifestations
  - Intra-patient variability
- **Improve Diagnosis**
- **Create Guidelines/Treat more effectively**

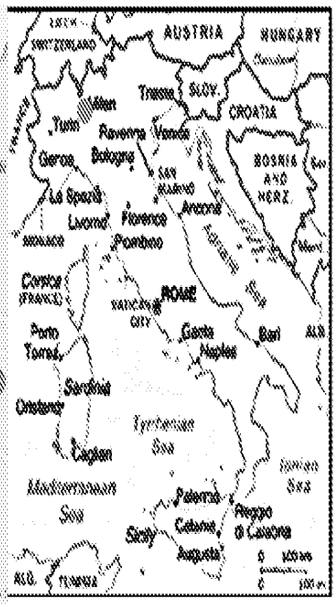


Ex. 2001-0688

# PKD Natural History Study Sites









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# PKD Natural History Study

Observational Study - clinical information and surveys over 2 years

- **Sites:** United States: 19 sites
  Europe: 6 sites
  Canada: 3 sites

- **Coordinating Site:** Boston Children's Hospital

- **Laboratories:** PKLR Testing
  Paola Bianchi, Elisa Fermo (Milan, Italy)
  Patrick Gallagher, Kimberly Lezon-Geyda (CT, USA)
  PK Enzyme Activity/Protein, RBC Metabolite Testing
  Bert Glader (Stanford, CA)
  Mayo Labs (Rochester, MN)
  QPS (Newark, DE)

- **Enrollment:** 255 participants, currently closed to enrollment



Ex. 2001-0690

# PKD Natural History Study Investigators

| Site Investigator | Site | Site Investigator | Site |
|---|---|---|---|
| Dr. Jenny Despotovic | *Baylor Hospital, TX* | Dr. Eduard van Beers | *UMC, Utrecht, Netherlands* |
| Dr. Melissa Rhodes | *University of Mississippi, MS* | Dr. Wilma Barcellini | *Ospedale Maggiore di Milano, Italy* |
| Dr. Rachael Grace | *Boston Children's Hospital, MA* | Dr. Stefan Eber | *University of Munich, Germany* |
| Dr. Christina Knoll | *Phoenix Children's Hospital, AZ* | Dr. Marcin Wlodarski | *Freiburg, Germany* |
| Dr. Bert Glader | *Stanford University, CA* | Dr. Joachim Kunz | *Heidelberg, Germany* |
| Dr. Holmes Morton | *Lancaster General Hospital, PA* | Dr. Nina Kollmar | *Kassal, Germany* |
| Dr. Peter Newburger | *University of Massachusetts, MA* | Dr. Suzanne Holzhauer | *Berlin, Germany* |
| Dr. Winifred Wang | *St Jude's Hospital, TN* | Dr. Vicky Breakey | *Hamilton, ON* |
| Dr. Jennifer Rothman | *Duke Children's Hospital, NC* | Dr. Yves Pastore | *Montreal, QC* |
| Dr. Heather Bradeen | *University of Vermont, VT* | Dr. Kevin Kuo | *Toronto, ON* |
| Dr. Hassan Yaish | *Salt Lake City, UT* | Dr. Yaddanapudi Ravindranath | *Wayne State, Detroit, MI* |
| Dr. Sujit Sheth | *Cornell, New York, New York* | Dr. Heng Wang | *DCC Clinic, Middlefield, OH* |
| Dr. Alexis Thompson | *Lurie Children's, Chicago, IL* | Dr. Melissa Rose | *Nationwide Hospital, Ohio* |
| Dr. Mukta Sharma | *Children's Mercy Hospital, MO* | Dr. Janet Kwiatkowski | *University of Pennsylvania, PA* |
| Dr. Dagmar Pospíšilová | *Palacky Univer., Czech Republic* | Dr. Satheesh Chonat | *Children's Healthcare of Atlanta, GA* |

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# Clinical Research and the Pennsylvania Amish



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# Demographics

| | | n (%); n=203 |
|---|---|---|
| Age at enrollment (median, range) | | 20.1 y, 0.1-69.9 y |
| | <18 years (n) | 92(45%) |
| | ≥18 years (n) | 111 (55%) |
| Gender | Male | 96 (47%) |
| Race | White | 189 (93%) |
| Hispanic | | 14 (7%) |
| Amish | | 55 (28%) |
| Site of Enrollment | US/Canada | 137 (68%) |
| | Europe | 66 (32%) |

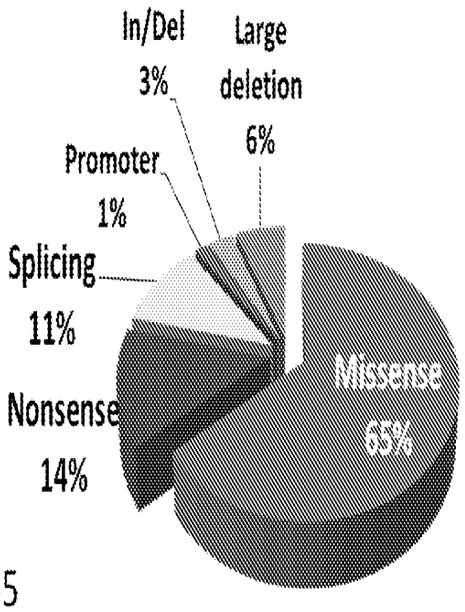


Ex. 2001-0693

# PKD NHS: Diversity of Genotypes

65 different mutations identified

- The majority were missense mutations
- >20 mutations not previously described




Most Common Mutations

**1529 G>A**

Homozygous: n=3

Heterozygous: n=20

**1436 C>T**

Homozygous: n=55

**1456 C>T**

Homozygous: n=2

Heterozygous: n=17

**721 G>T**

Homozygous: n=1

Heterozygous: n=13

Within the group of 1436 C>T homozygous patients, there was wide variability in the history of transfusions, baseline anemia, and history of gallstones.

Bianchi et al, ASH 2015

# Diagnosis: Enzyme Assay vs DNA Analysis

| Advantage | Enzyme Assay | DNA Analysis |
|---|---|---|
| Useful in Transfused Patients | No | Yes |
| Stability of Sample | Low | High |
| Finds most mutations in a given enzyme | Yes | Variable |
| Suitable for Prenatal Diagnosis | No | Yes |
| Definite Yes or No result | Variable | Variable |

→ Molecular analysis occurred before the study:

→ 34% from North America

→ 66% from European countries

→ 10% of enrolled participants do not have two PKLR mutations

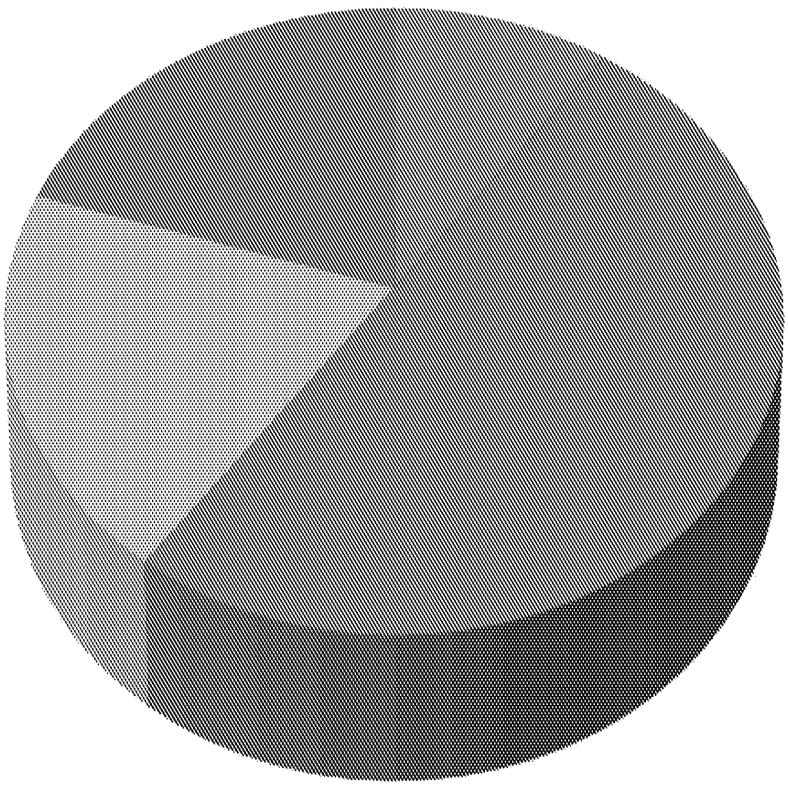
# Transfusion and Splenectomy Status: Children <5 y

| n=39 | | | |
|---|---|---|---|
| **Not Splenectomized, Regularly Transfused** | 59% (n=23) | | |
| | Transfusion Interval | 5.2 weeks | (3.1-13) |
| | Median pre-transfusion Hb | 7.8 g/dl | (5.5-10.7) |
| | Median volume tranfused | 13 cc/kg | (8.6-23) |
| **Not Splenectomized, Not Regularly Transfused** | 26% (n=10) | | |
| | Median Hb | 8.6 g/dl | (7.5-11.8) |
| | 5 had been transfused for acute triggers | | |
| | 5 had never been transfused | | |
| **Splenectomized** | 15% (n=6) | | |



Ex. 2001-0696

# PKD Transfusion Status



- Regularly Transfused Before and After Splenectomy (10%), n=21
- Regular Transfusions prior to Splenectomy, Intermittent Transfusions Post-Splenectomy (50%), n=88
- Transfused Intermittently, Never Regularly Transfused (20%), n=35
- Never Transfused (20%), n=34

| Hemolytic Triggers: | |
|---|---|
| Infections | 61% |
| Pregnancy | 51% |
| Stress | 32% |
| Other: | alcohol, menses |

# PKD and Splenectomy

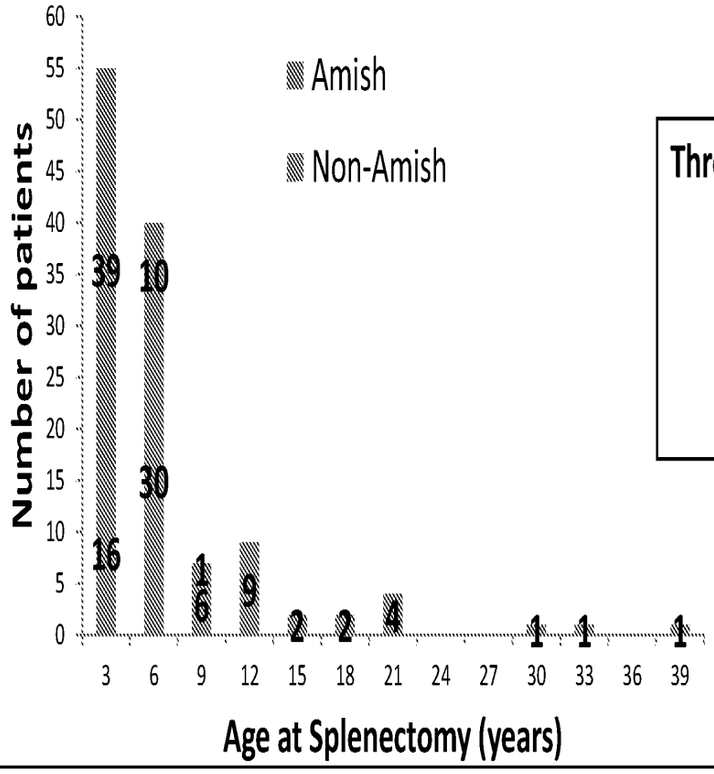
The majority of patients had a splenectomy: 63% (127/203)

Median age at splenectomy (non-Amish): 5.1 years (range: 0.5-37.8)

Median post-splenectomy Hb: 8.9 g/dl (IQR 8.1-9.9)

Median rise in Hb: 1.7 g/dl (n=60)

→ 10% remain transfusion dependent after splenectomy




| Thrombotic Complications | 9% (12/127) |
|---|---|
| • Deep Vein Thrombosis | 7/12 |
| • Pulmonary Embolism | 5/12 |
| • Stroke | 2/12 |
| • Portal Vein Thrombosis | 2/12 |

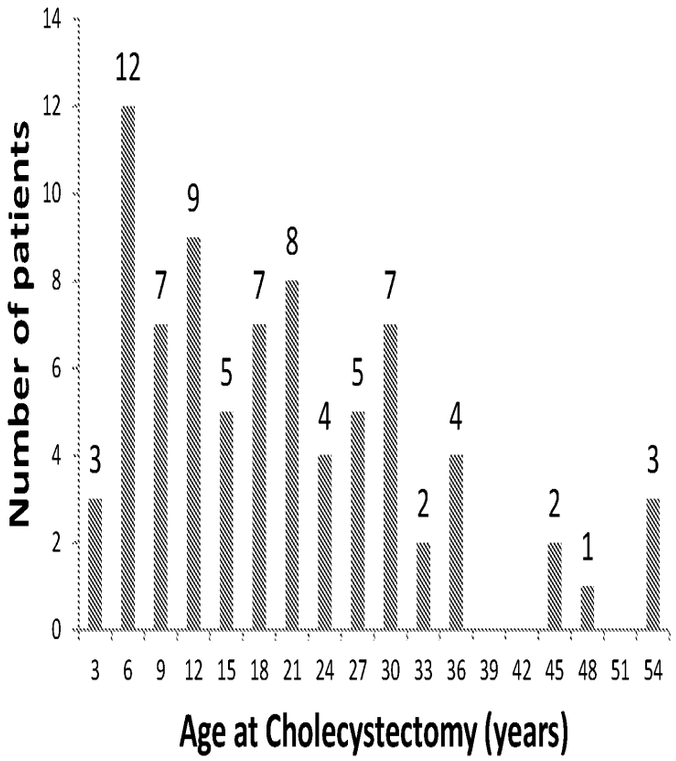
# PKD Complications: Gallstones

- Incidence of gallstones is 43% (n=89)

  Median Age 15.7 y (range 2.2-60.4 y)

  58% in children <18 years old

- Cholecystectomy was performed after splenectomy in 70%
- Of 106 who underwent splenectomy without a prior or simultaneous cholecystectomy, 50 (47%) went on to later require cholecystectomy



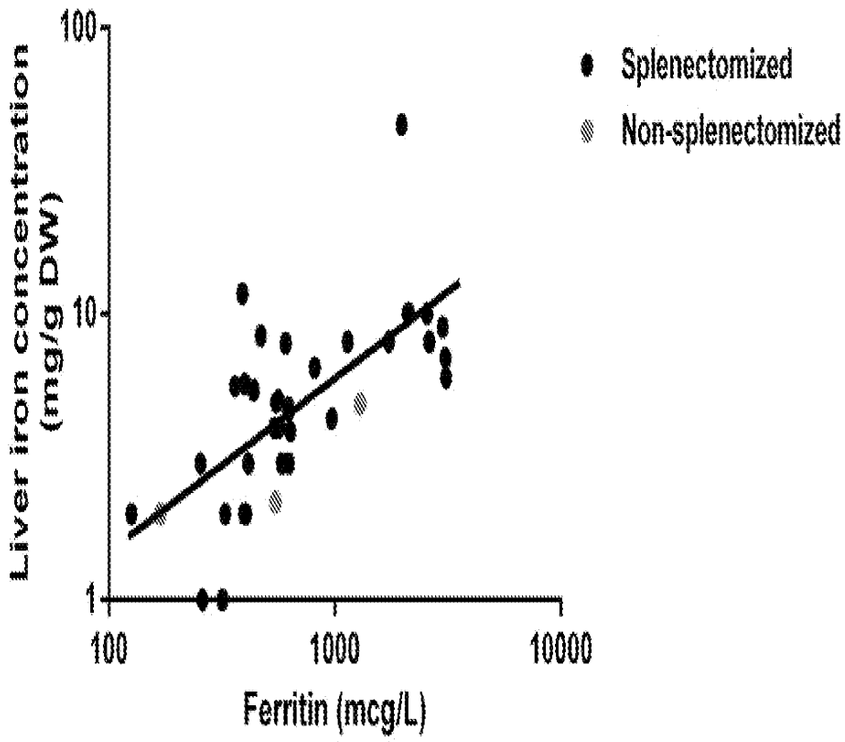



Ex. 2001-0699

# PKD Complications: Iron Overload

- At enrollment, 48% (70/147) had iron overload defined by ferritin >1000 ng/mL or on chelation in the prior 12 months
- Iron overload had been present at some point in 86% (95/110), as defined by LIC > 3 mg/g DW or ever on chelation

Correlation of T2* MRI LIC with ferritin (mcg/L), $r^2$=0.62, p<0.0001




*Of 29 with an LIC measurement and ferritin <1000 ng/mL, 15 (52%) had an LIC >3 mg/g DW*



Ex. 2001-0700

# Iron Status and Clinical Characteristics

| Baseline characteristics | Ferritin >1000 ng/ml or Received Chelation | | |
|---|---|---|---|
| | absent | present | p |
| N | 77 | 70 | |
| Female sex | 60% | 51% | 0.3 |
| Median Age at enrollment (y) (IQR) | 20.4 (6.5, 36.6) | 28.1 (14.0, 45.7) | 0.046 |
| Splenectomized (%) | 57% | 81% | 0.002 |
| Median Hemoglobin (g/dl) (IQR) | 9.2 (8.4, 10.2) | 8.6 (7.9, 9.1) | 0.004 |
| Median Bilirubin (mg/dl) (IQR) | 3.7 (2.4, 4.8) | 4.2 (2.8, 6.5) | 0.03 |
| Median Ferritin (ng/ml)(IQR) | 414 (255, 583) | 1225 (926, 2100) | <0.0001 |
| LIC (mg/g)(IQR) | 4.0 (2.0, 5.2) | 6.0 (4.4, 9.0) | 0.0009 |
| Median Transferrin saturation (%)(IQR) | 40% | 71% | 0.0003 |
| Chelation in 12 mo prior to enrollment | 0 | 58% | <0.0001 |
| Transfusion Status | | | |
| Never regularly transfused | 29 (38%) | 11 (16%) | <0.0001 |
| Transfusion independent, historically regularly transfused | 39 (51%) | 29 (41%) | |
| Transfusion dependent | 9 (11%) | 30 (43%) | |



Ex. 2001-0701

# PKD: Other Complications

| | |
|---|---|
| **Extramedullary Hematopoiesis** | 11%<br>Paraspinous n=8<br>Mediastinal n=7 |
| **Osteopenia/Bone Fractures** | 19% |
| **Leg Ulcers** | 1% |
| **Pulmonary Hypertension** | 3.5%<br>Median age: 40.1 years<br>S/p Splenectomy in 6/7 |
| **Endocrinopathies** | |
| **Growth Hormone Deficiency** | 2% |
| **Hypoparathyroidism** | 2% |
| **Thyroid Disease** | 5% |
| **Delayed Puberty** | 7% |
| **Myelodysplastic Syndrome** | 0% |
| **Allo-immunization** | 2.5% |
| **Auto-immunization** | 2% |



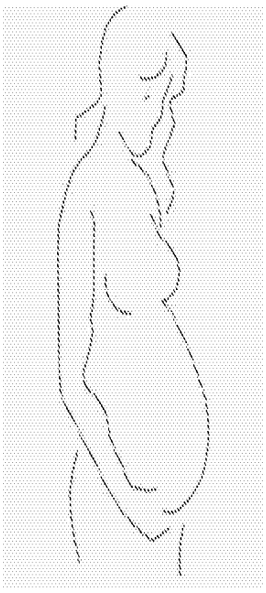
Ex. 2001-0702

# PK Deficiency in Pregnancy

31 women with 102 pregnancies:

- Pregnancy Outcomes:

| | |
|---|---|
| Normal Birth | 60% (61) |
| Miscarriage | 21% (21) |
| Preterm (<37 weeks) | 12% (12) |



- Assisted Reproduction was rare (2%)
- Transfusions during Pregnancy:

| | |
|---|---|
| Prior to pregnancy | 4% |
| During | 12% |
| After delivery | 21% |

# Next Steps: Natural History Study

1. **Define Disease**:
   - Transfusion/Hemoglobin Phenotypes
   - Current standard of care
     - Splenectomy, Cholecystecomy
     - Iron Monitoring and Treatment
   - Identification of disease features/complications
     - Common Complications: Iron Overload, gallstones
     - Rare Complications: Bone Density, Thrombosis
   - Biomarkers: ATP, 2,3-DPG, PK activity/protein
2. **Improve Diagnosis**
3. **Create Guidelines**



Ex. 2001-0704

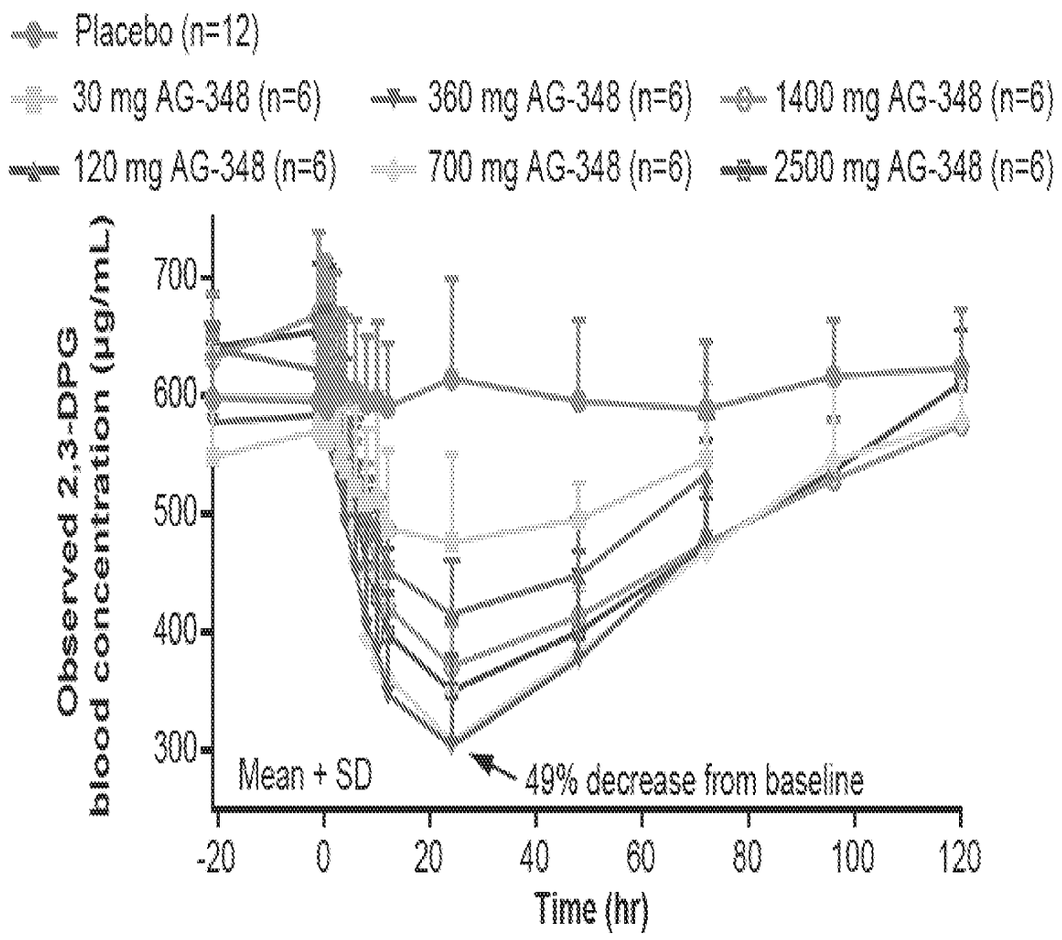
# DRUG DEVELOPMENT IN PKD



Ex. 2001-0705

# Healthy Volunteer AG-348 SAD Study: PD Endpoints

## 2,3-DPG after a single dose of AG-348

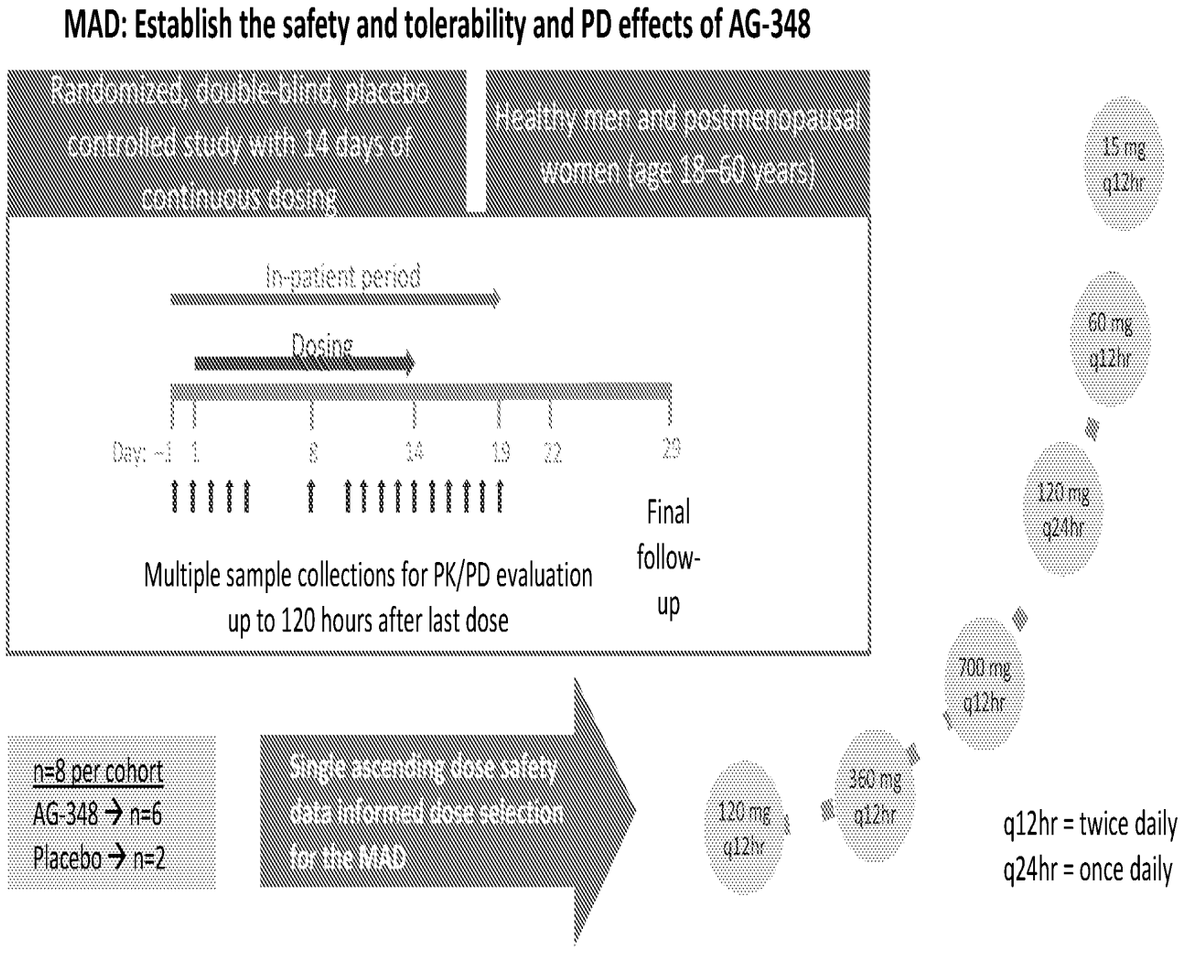



Yang et al. ASH 2014



Ex. 2001-0706

# Healthy Volunteer AG-348 MAD Design




PK/PD = pharmacokinetic/pharmacodynamic

NCT02149966

Yang et al. EHA 2015

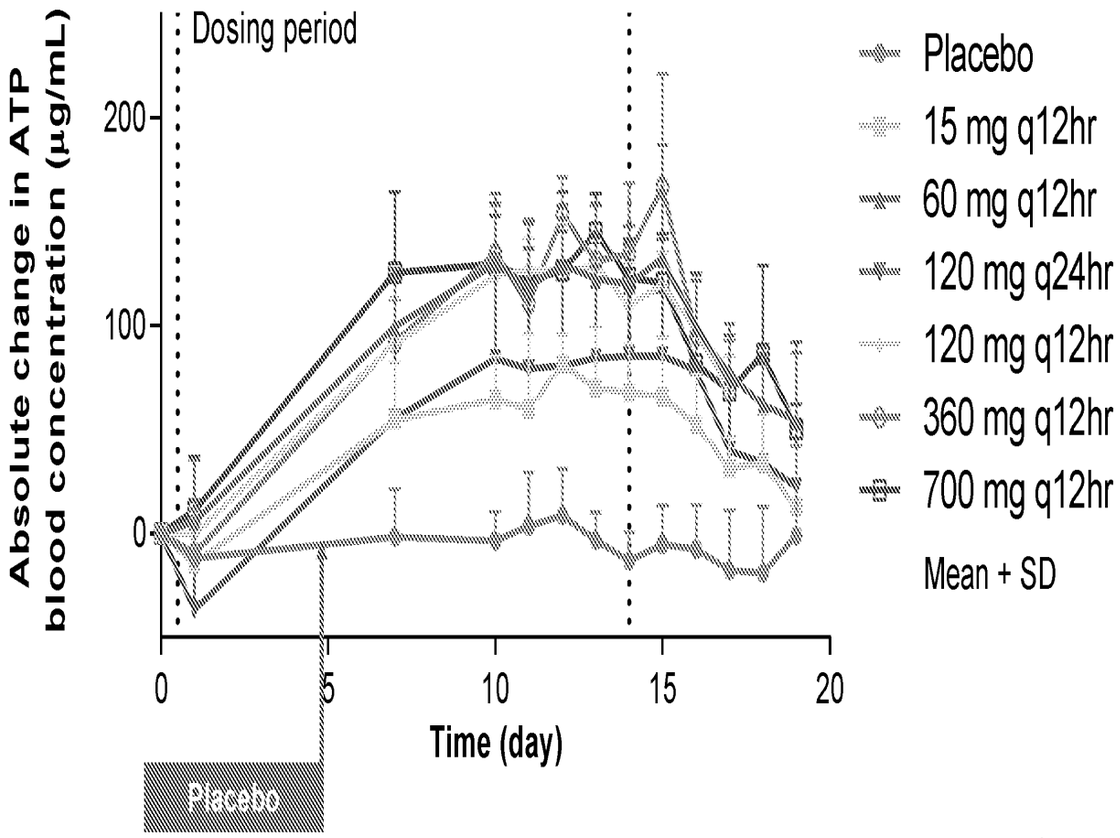
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# AG-348 MAD: Pharmacodynamic Endpoints

- Randomized, Placebo Controlled Multiple Ascending Dose Study
- MAD(15-700 mg, n=48)




Yang et al. EHA 2015

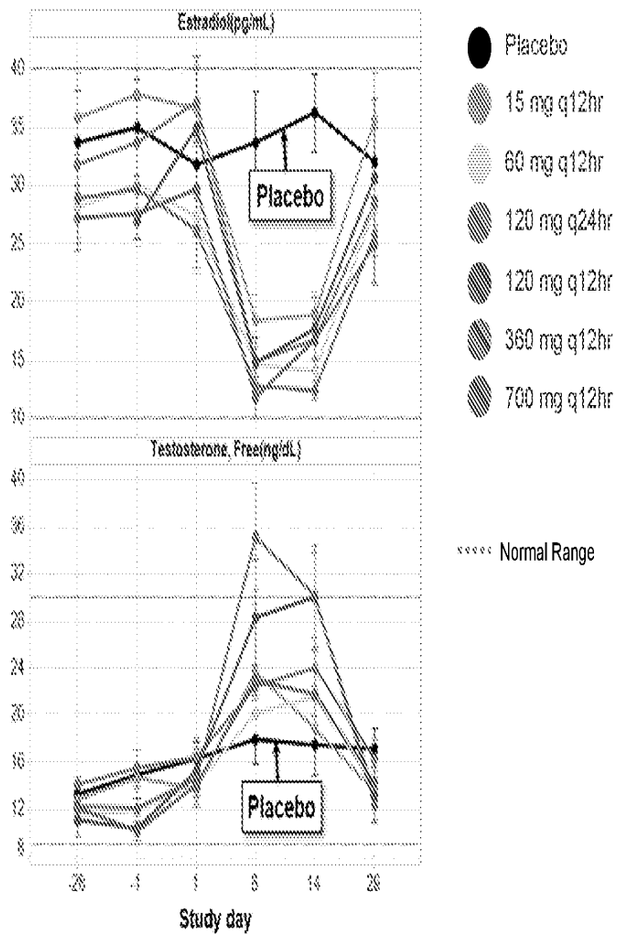


Ex. 2001-0708

# AG-348 MAD: Reversible Aromatase Inhibition

- Changes in serum androgens and estrogens
- Most changes remained within normal ranges
- Levels recovered to baseline when AG-348 was discontinued
- Endocrine/physiologic significance to be evaluated in Phase 2 study

**Mean levels of serum estradiol and free testosterone in male subjects**



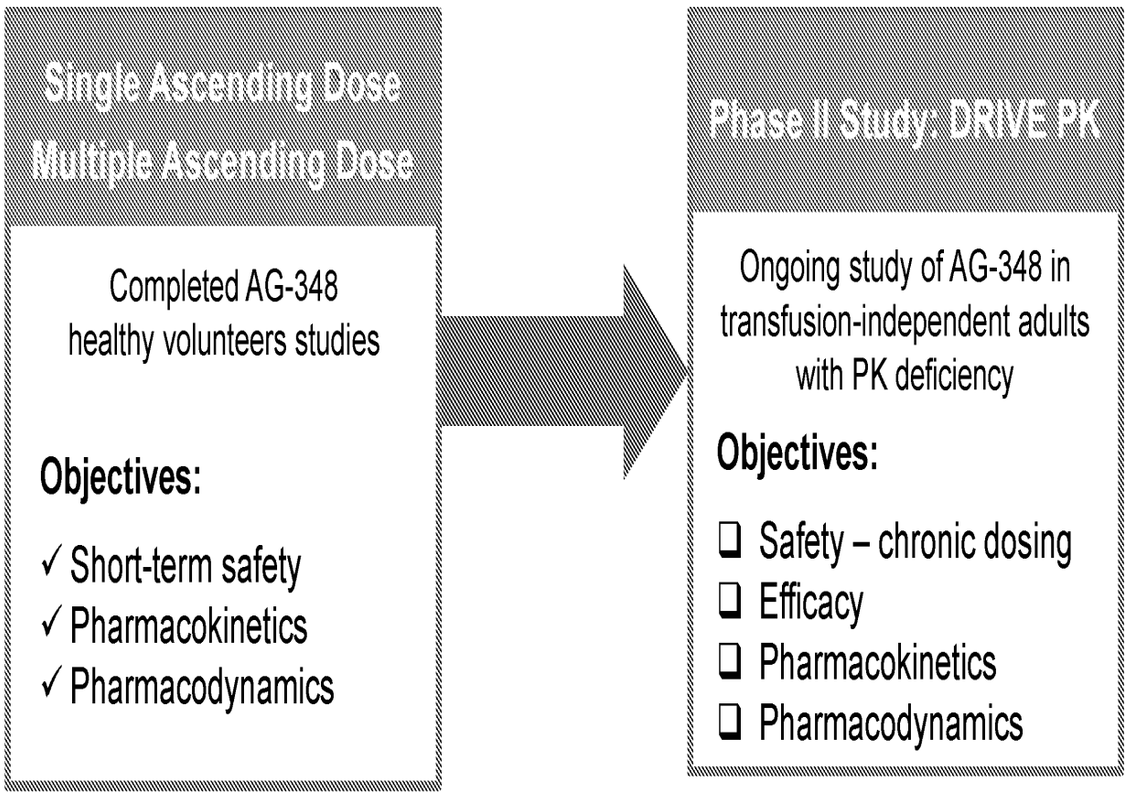

Yang et al. EHA 2015



Ex. 2001-0709

# Phase 2 AG-348 Proof of Concept Study

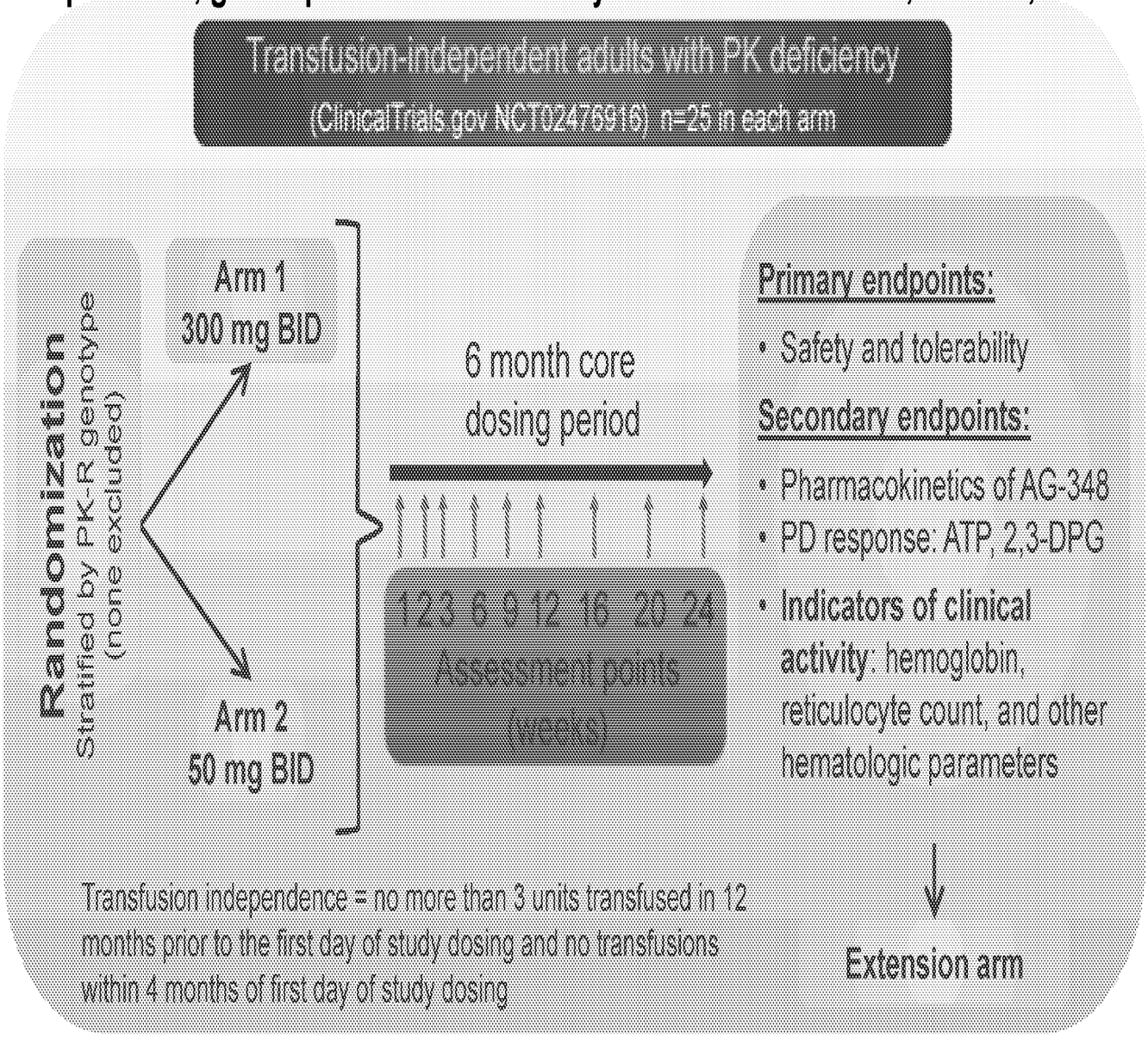
DRIVEPK



# Phase 2 Study design

DRIVEPK

## Open-label, global phase 2 AG-348 study: 14 centers in the US, Canada, and EU




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# DRIVEPK: Demographics and disposition

- Study initiated June 2015; data cut-off September 23, 2016
- Evaluable analysis set: ≥3 weeks of data (n=32)
- 13 patients ongoing in the core period (as of September 23, 2016)
  - Early discontinuations in core period: relocation (n=1), AEs (n=3)
- Of the 17 patients who completed the core period, 15 enrolled in the extension period

| Characteristics | 50 mg BID, n=17 | 300 mg BID, n=17 | Total, N=34 |
|---|---|---|---|
| Men/women, n | 11/6 | 9/8 | 20/14 |
| Age in years, mean (range) | 28.5 (19-45) | 37.0 (20-61) | 32.8 (19-61) |
| Race[a] white, n | 15 | 15 | 30 |
| Hemoglobin (Hb) baseline, g/dL, mean (SD, range) | 9.8 (1.41, 7.6–12.4) | 8.7 (1.37, 6.5–11.8) | 9.2 (1.47, 6.5–12.4) |
| Duration of treatment, weeks, median (range) | 24.7 (4.7–50.4) | 24.0 (2.4–44.4) | 24.4 (2.4–50.4) |
| Splenectomized, n | 14 | 14 | 28 |



Ex. 2001-0712

# DRIVEPK: AG348 Safety summary

- AG-348 was generally well tolerated; the majority of AEs were grade 1–2
- No grade 4 AEs or deaths
- SAEs (n=2): Grade 2 osteoporosis; hemolysis and anemia due to discontinuation of the drug after a rapid Hb response

| AEs, regardless of causality (occurring in >5 patients or assessed as Grade ≥3) | 50 mg BID n=17 | | 300 mg BID n=17 | | Total N=34 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n | 13 | 2 | 17 | 6 | 30 | 8 |
| Headache | 7 | 0 | 8 | 0 | 15 | 0 |
| Nausea | 7 | 0 | 7 | 0 | 14 | 0 |
| Insomnia | 3 | 1 | 10 | 1 | 13 | 2 |
| Fatigue | 3 | 0 | 3 | 0 | 6 | 0 |
| Vomiting | 2 | 0 | 4 | 0 | 6 | 0 |
| Hypertriglyceridemia | 0 | 0 | 4 | 3 | 4 | 3 |
| Anemia | 1 | 1[a] | 1 | 1[b] | 2 | 2 |
| Hypertension | 0 | 0 | 1 | 1 | 1 | 1 |
| Dizziness | 2 | 0 | 1 | 1 | 3 | 1 |
| Hemolysis | 0 | 0 | 2 | 1[b] | 2 | 1 |



Ex. 2001-0713

# Effect of AG-348 on hormones

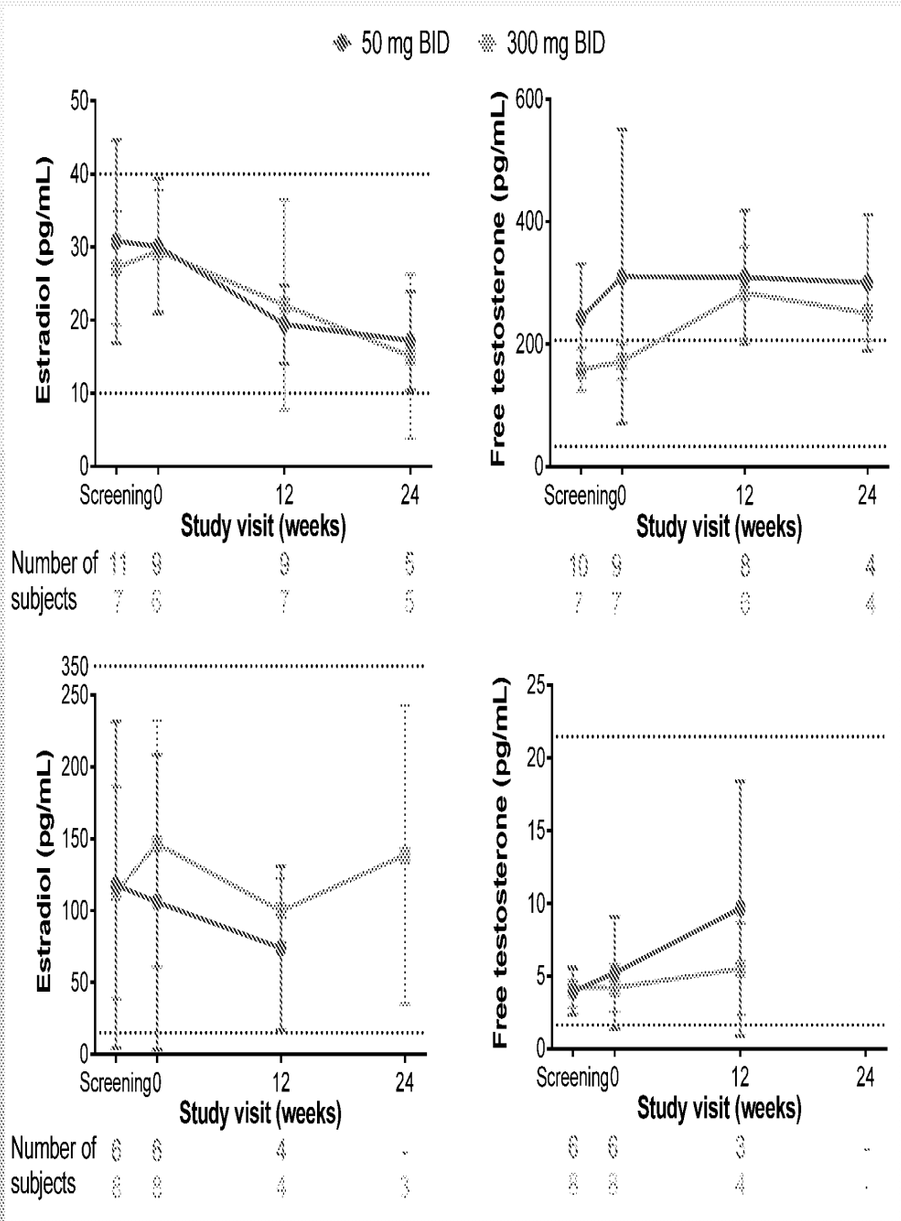
- Preliminary findings are consistent with aromatase inhibition by AG-348

Male

Hormone levels assessed at baseline, 12 and 24 weeks

Patients with available data shown

Female



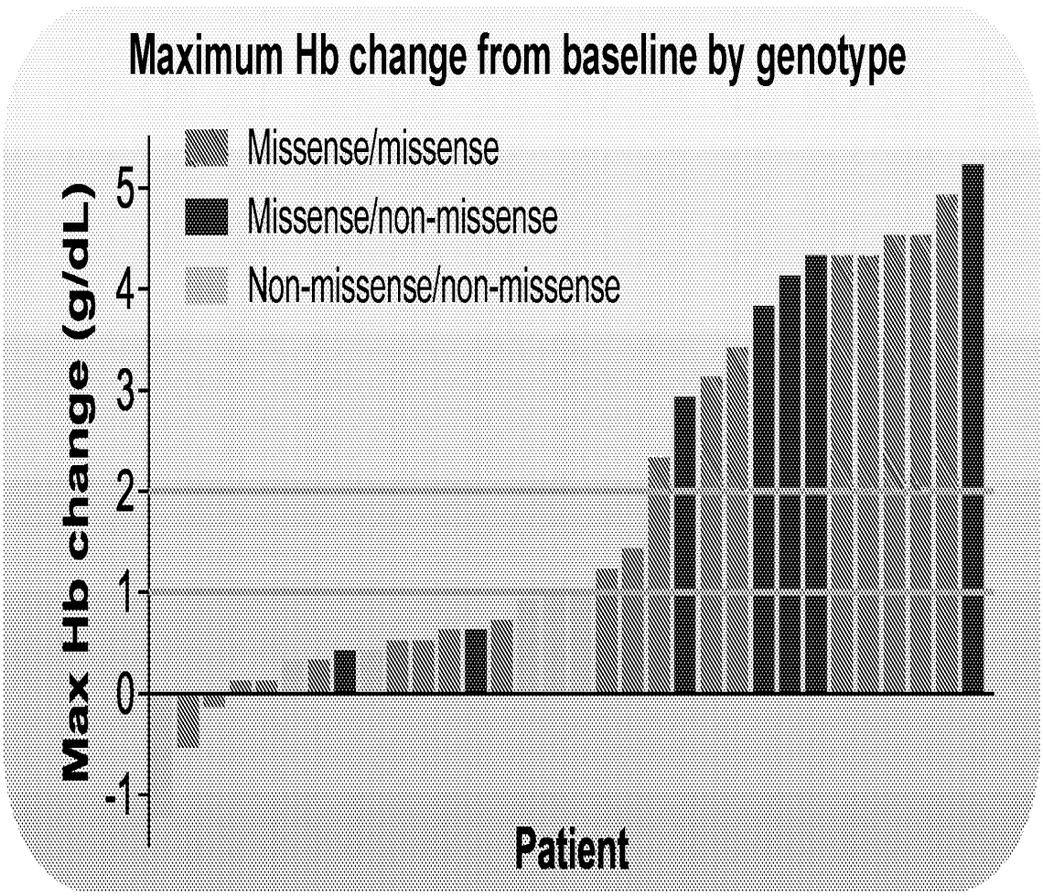

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# Clinical Activity Results

# Maximum increase in hemoglobin

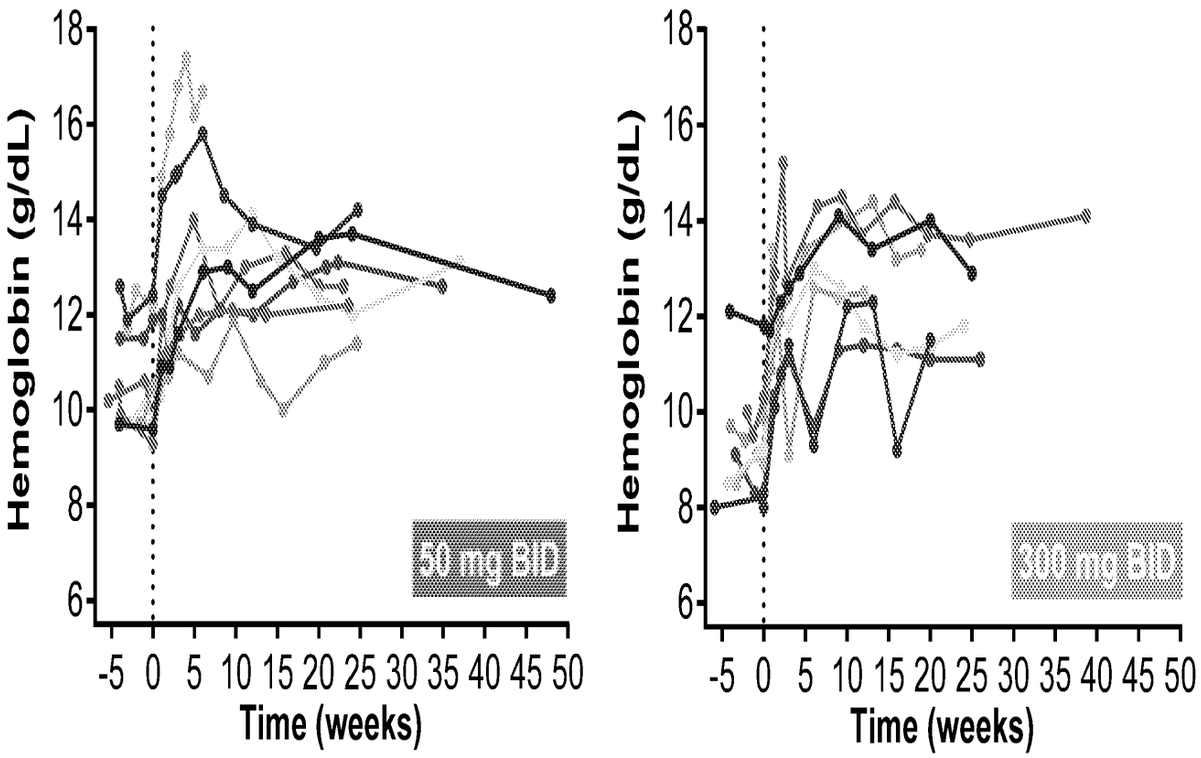
- 15 of 32 (47%) patients had a maximal increase in Hb >1.0 g/dL
  - 15/26 patients (58%) who had ≥1 missense mutation had a Hb response
- 5 patients homozygous for R479H were non-responders
- Hb response and response maintenance are seen across a range of 4 doses
  - Robust Hb responses led to dose decreases with maintained Hb



# Hb increases are rapid and sustained

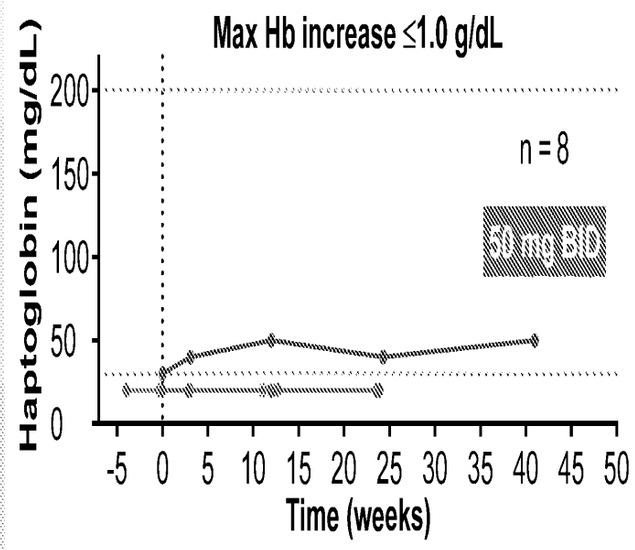
DRIVEPK

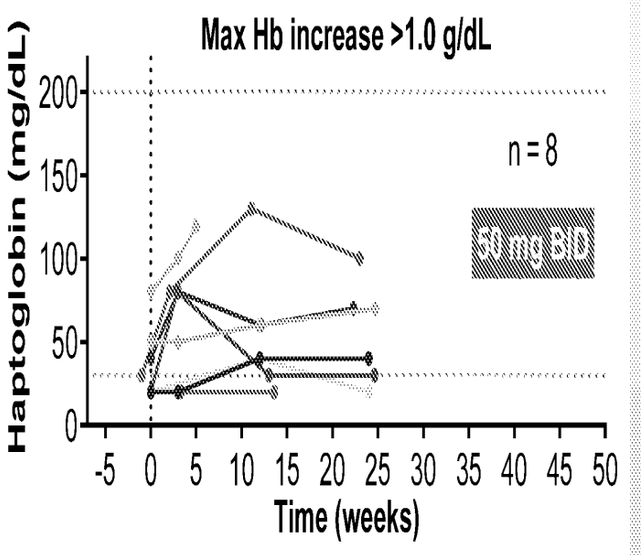
- In patients who had Hb increases >1.0 g/dL (n=15):
  - Median time to Hb increase >1.0 g/dL was 1.4 weeks (range, 1.1–21.0)
  - The mean maximum increase was 3.6 g/dL (range, 1.2–5.2)
- 10 patients had dose reductions: 5 due to rapid Hb increase[a]

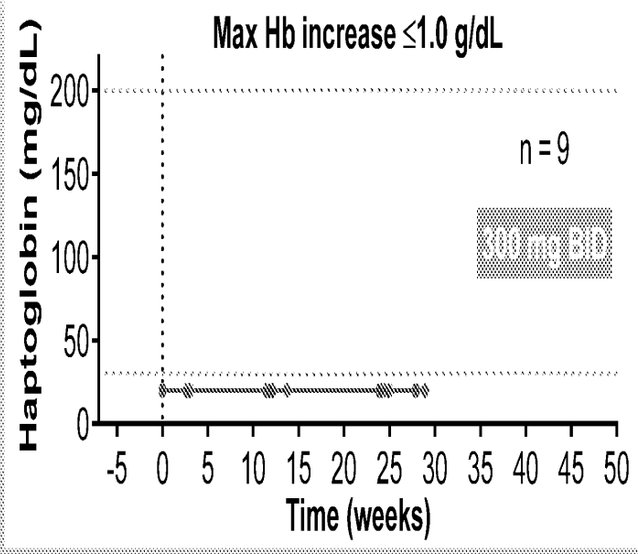


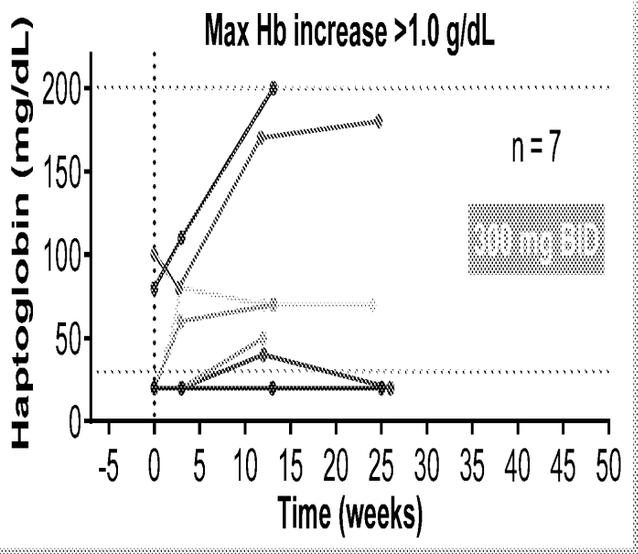

Responders n=15, each line represents an individual patient

# Haptoglobin levels Increase in Responders




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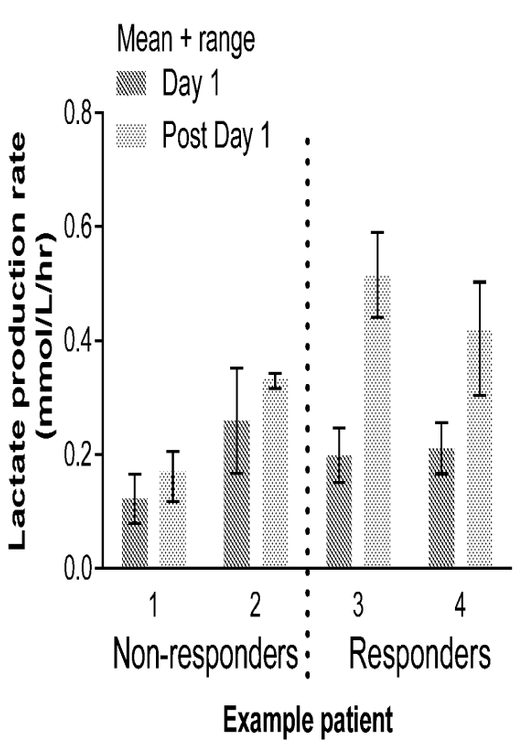
# Pharmacodynamic Results

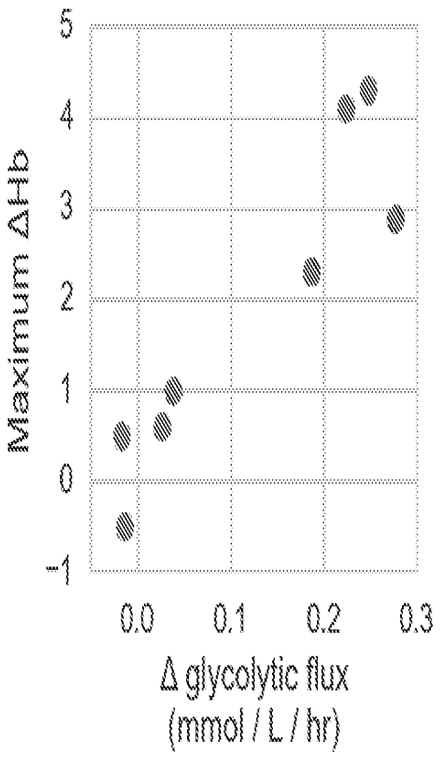
# Increased Glycolytic Flux in Responders

- Rate of metabolism of PK-R pathway was assessed in blood samples from a subset of patients pre and post treatment
- These data suggest a positive correlation between Hb change and change in glycolytic flux

Detailed metabolic characterization in blood from 8 patients

Glycolytic flux assessed *ex vivo* via incorporation of labelled glucose into the end product, lactate




Representative 4 patients shown here N=8

# DRIVE-PK conclusions to date

- AG-348 is a novel, first-in-class, PK-R activator in clinical testing as a potential disease-altering therapy to improve anemia in patients with PK deficiency

- Daily dosing with AG-348 for up to 6 months is well tolerated
  - Clinical significance of AG-348 aromatase inhibition is unclear

- AG-348 demonstrates clinically relevant rapid and durable increases in Hb in 47% of patients enrolled in the study
  - Hb increase is linked to activation of glycolytic pathway
  - Preliminary genotype-Hb response correlations were observed



Ex. 2001-0721

# Summary

- Natural History Studies are the foundation of clinical development programs in rare diseases.
- The drug development perspective is integral early in the design stage of a Natural History Study.
- Collaboration is key to progress in rare diseases (industry, colleagues, patients).
- Our understanding of PK deficiency has improved and will continue to grow through the NHS.
- The next clinical trials in PK deficiency will be opening soon.



Ex. 2001-0722

# Acknowledgements


## PKD NHS Boston Children's Team

Nolan Neu

Dongjing Guo

Wendy London

## PKD NHS Laboratories

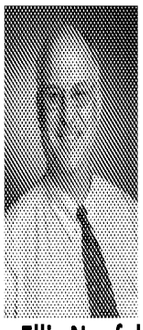
Patrick Gallagher

Paola Bianchi

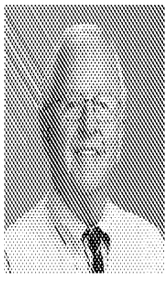
Kimberly Lezon-Geyda

Elisa Fermo

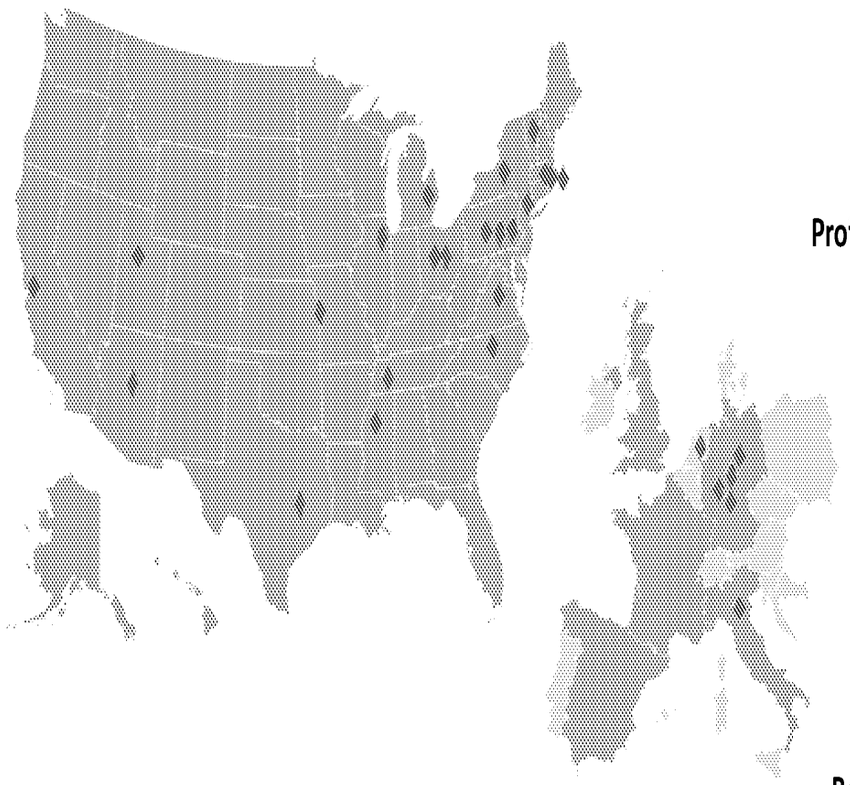
## PKD NHS Investigators and Participants

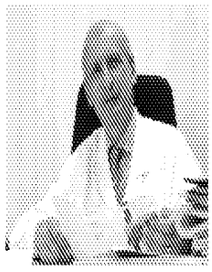


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Belleville, Pennsylvania

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# Acknowledgements


| *DRIVEPK Investigators* | *Sites* |
|---|---|
| Bert Glader | Stanford University |
| Sujit Sheth | Weill Cornell – New York Presbyterian Hospital |
| Hassan Yaish | University of Utah |
| Janet Kwiatkowski | The Children's Hospital of Philadelphia |
| Holmes Morton | Central Pennsylvania Clinic |
| Yaddanapudi Ravindranath | Wayne State University School of Medicine |
| Kevin Kuo | University of Toronto – University Health Network |
| Mark Layton | Hammersmith Hospital |
| Eduard van Beers | Universitair Medisch Centrum Utrecht |
| Frederic Galacteros | Hôpital Henri Mondor |
| Christian Rose | Hôpital Saint Vincent de Paul |
| Emmanuelle Bernit | Hôpital de la Timone |
| Wilma Barcellini | Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico |


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# TREATMENT OF RARE GENETIC DISEASES BY SMALL MOLECULE ALLOSTERIC ACTIVATORS

Stefan Gross, Ph.D.
Director, Enzymology and Biochemistry
Agios Pharmaceuticals



Ex. 2001-0725

# Treatment of Rare Genetic Diseases by Small Molecule Allosteric Activators

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- Introduction to Rare Genetic Diseases
- Progress towards treatment of CDG1a, the most prevalent congenital disorder of glycosylation
- Development of AG-348, an enhancer of pyruvate kinase activity for the treatment of patients with PKR-deficient hemolytic anemia



Ex. 2001-0726

# What are Rare Genetic Diseases?

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**Conventional Wisdom:**

Rare Genetic Diseases are incurable diseases caused by low-prevalence mutations in genomic DNA

RGDs are neither Rare nor truly Genetic

Although individually rare, their aggregate occurrence is common enough that as a disease class, they cause a significant proportion of global human suffering and are worthy of medical intervention

In a large fraction of RGDs, the primary biological insult is the result of metabolic disregulation caused by decrease-of-function or destabilizing mutations in enzymes. Small-molecule drug technology holds the potential to ameliorate pathogenic metabolism and improve patients' lives.



Ex. 2001-0727

# Although individually rare, RGDs are collectively not uncommon

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***Table 1.*** Frequency of Inherited Metabolic Disorders in the West Midlands 1999-2003.

| Condition | Total number of cases | Birth prevalence live births | *Upper 95% ci* | *Lower 95% ci* |
|---|---|---|---|---|
| PKU* | 25 | 1 in12 420 | 5008 | 33 784 |
| Amino acid (excluding PKU) | 58 | 1 in 5354 | 2943 | 9990 |
| Urea cycle defects | 14 | 1 in 22 179 | 6702 | 90 909 |
| Carbohydrate | 19 | 1 in 16 343 | 4509 | 52 910 |
| Organic acid | 39 | 1 in 7962 | 3837 | 17 301 |
| Glycogen storage | 21 | 1 in 14 786 | 5504 | 44 643 |
| Lysosomal storage | 60 | 1 in 5175 | 2874 | 9551 |
| Purine and pyrimidine† | 4 | 1 in 77 628 | 12 063 | 2 000 000 |
| Fatty acid oxidation | 24 | 1 in 12 938 | 5123 | 35 971 |
| Peroxisomal | 23 | 1 in 13 500 | 5244 | 38 462 |
| Mitochondrial | 63 | 1 in 4929 | 2776 | 8953 |
| Metals‡ | 11 | 1 in 28 228 | 7418 | 147 059 |
| Lipids and steroids¶ | 20 | 1 in15 526 | 5647 | 48 544 |
| Porphyrin and haem* | 1 | 1 in 31 0510 | 10 070 | 3 333 333 |
| Miscellaneous | 14 | 1 in 22 179 | 6702 | 90 909 |
| **Total (including PKU)** | **396** | **1 in 784** | **619** | **970** |

The incidence of inherited metabolic disorders in the West Midlands, UK

S Sanderson, A Green, M A Preece, H Burton

Arch Dis Child 2006;91:896–899. doi: 10.1136/adc.2005.091447

# Massachusetts Comprehensive Newborn Screening: 48 most common Metabolic Rare Genetic Diseases

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2-Methyl 3-hydroxy butyric aciduria (2M3HBA)
2-Methylbutyryl-CoA dehydrogenase deficiency (2MBG)
3-hydroxy-3-methyl glutaric aciduria (HMG)
3-Methylcrotonyl-CoA carboxylase Deficiency (3MCC)
3-Methylglutaconic aciduria (3MGA)
Argininemia (ARG)
Argininosuccinic acidemia (ASA)
Benign hyperphenylalaninemia (H-PHE)
Biotinidase deficiency (BIOT)
Carbamoylphosphate synthetase deficiency (CPS)
Carnitine palmitoyltransferase IA deficiency (liver) (CPT IA)
Carnitine palmitoyltransferase II deficiency (CPT II)
Carnitine uptake defect (CUD)
Carnitine: acylcarnitine translocase deficiency (CACT)
Citrullinemia (CIT)
Citrullinemia type II (CIT II)
Defects of biopterin cofactor biosynthesis (BIOPT BS)
Defects of biopterin cofactor regeneration (BIOPT Reg)
Dienoyl-CoA reductase deficiency (DE RED)
Galactokinase deficiency (GALK)
Galactose epimerase deficiency (GALE)
Galactosemia (GALT)
Glutaric acidemia type I (GAI)
Glutaric acidemia type II (GA2)
Homocystinuria (HCY)
Hypermethioninemia (MET)
Hyperornithinemia, Hyperammoninemia, Homocitrullinemia Syndrome (HHH):
Isobutyryl-CoA dehydrogenase deficiency (IBG)
Isovaleric acidemia (IVA)
Long-chain L--OH acyl-CoA dehydrogenase deficiency (LCHAD)
Malonic Acidemia (MAL)
Maple syrup disease (MSUD)
Medium/short-chain L-3-OH acyl-CoA dehydrogenase deficiency (M/SCHAD)
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)
Medium-chain ketoacyl-CoA thiolase deficiency (MCKAT)
Methylmalonic acidemia: cobalamin A, B (Cbl A,B)
Methylmalonic acidemia: cobalamin C,D (Cbl C,D)
Methylmalonic acidemia: mutase deficiency (MUT)
Multiple carboxylase deficiency (MCD)
Ornithine transcarbamylase deficiency (OTC)
Phenylketonuria (PKU)
Propionic acidemia (PROP)
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)
Tyrosinemia type I (TYR I)
Tyrosinemia type II (TYR II)
Tyrosinemia type III (TYR III)
Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD)
β-Ketothiolase deficiency (BKT)

Disease management in most cases is limited to diet restriction.

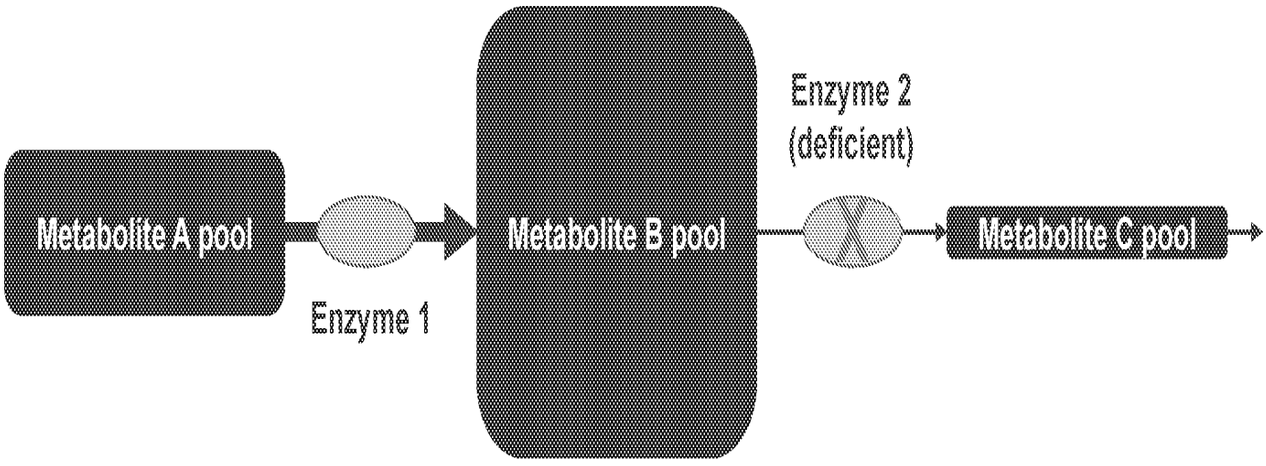


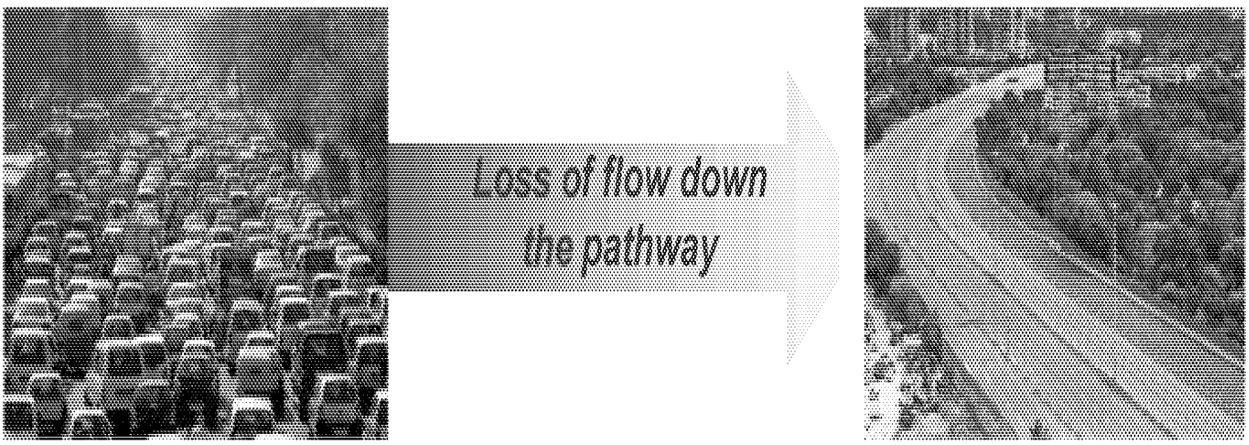
Ex. 2001-0729

# Rare Genetic Diseases due to defects in metabolic pathways

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*Disease results from the toxic buildup of "Metabolite B" and/or the deficit of "Metabolite C" or other downstream products*



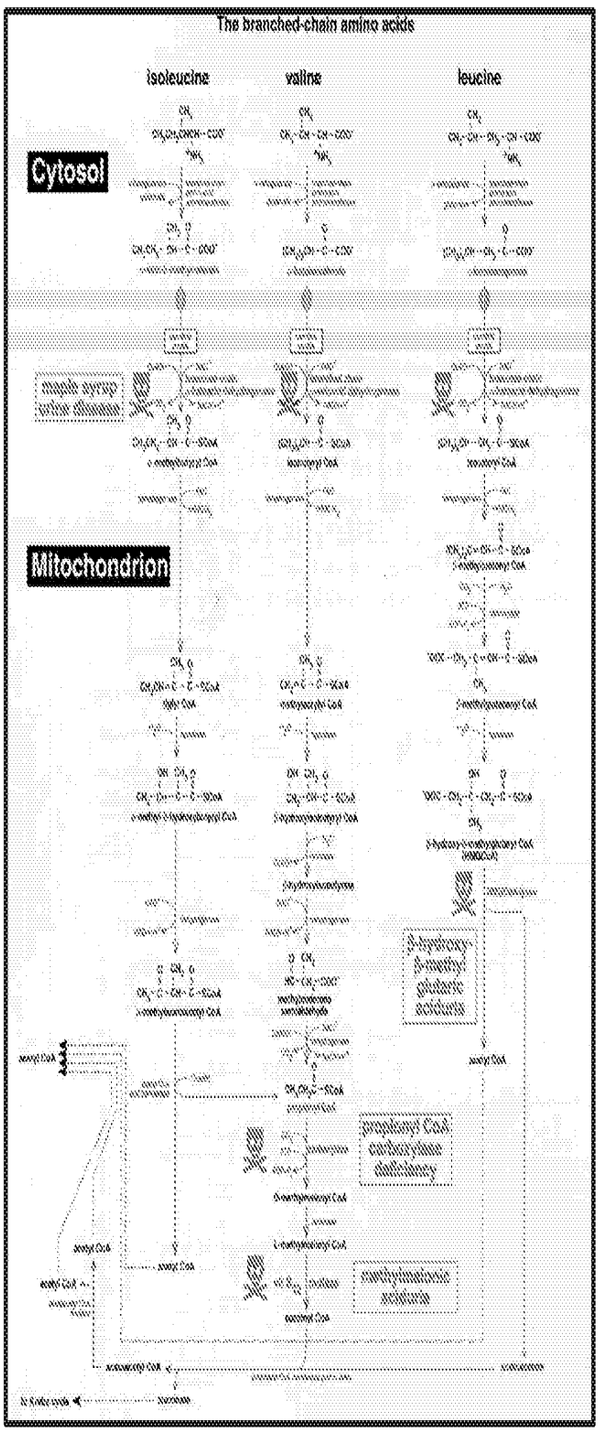






Ex. 2001-0730

# Multiple diseases may exist in any given metabolic pathway

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## Maple syrup urine disease

Deficiency of branched chain **α-ketoacid dehydrogenase complex** causes accumulation of branched-chain amino acids isoleucine, valine, and leucine and their corresponding α-ketoacids.

## β-hydroxyl-β-methyl glutaric aciduria

Autosomal recessive defect in **HMCoA lyase** of leucine catabolism and ketogenesis which is associated with hypoketotic hypoglycemia, hyperammonaemia, and metabolic acidosis

## Propionyl CoA carboxylase deficiency

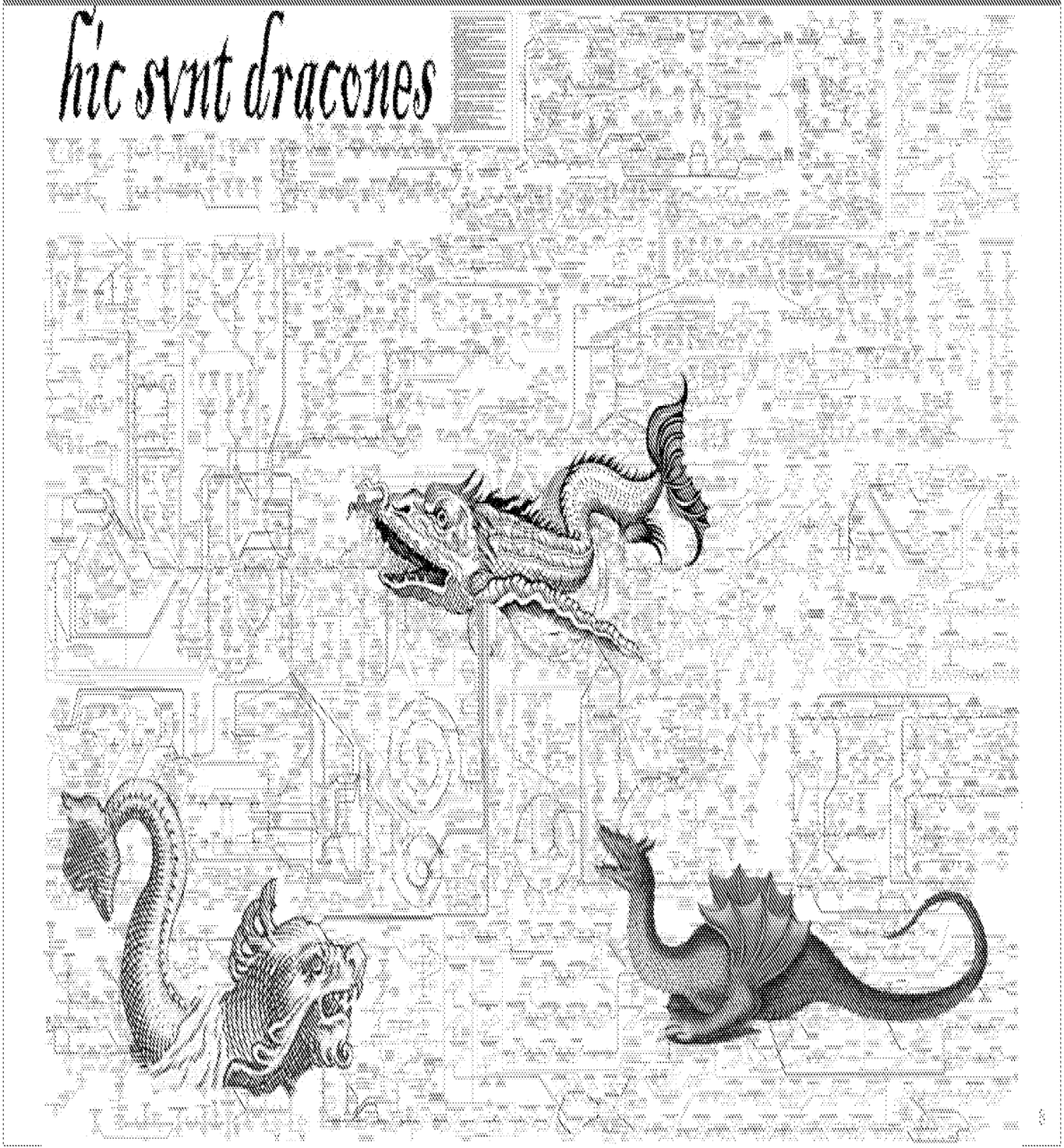
Presents early in the neonatal period with progressive encephalopathy, leading to mortality by secondary hyperammonemia, infection, cardiomyopathy, or basal ganglial stroke.

## Methylmalonic aciduria

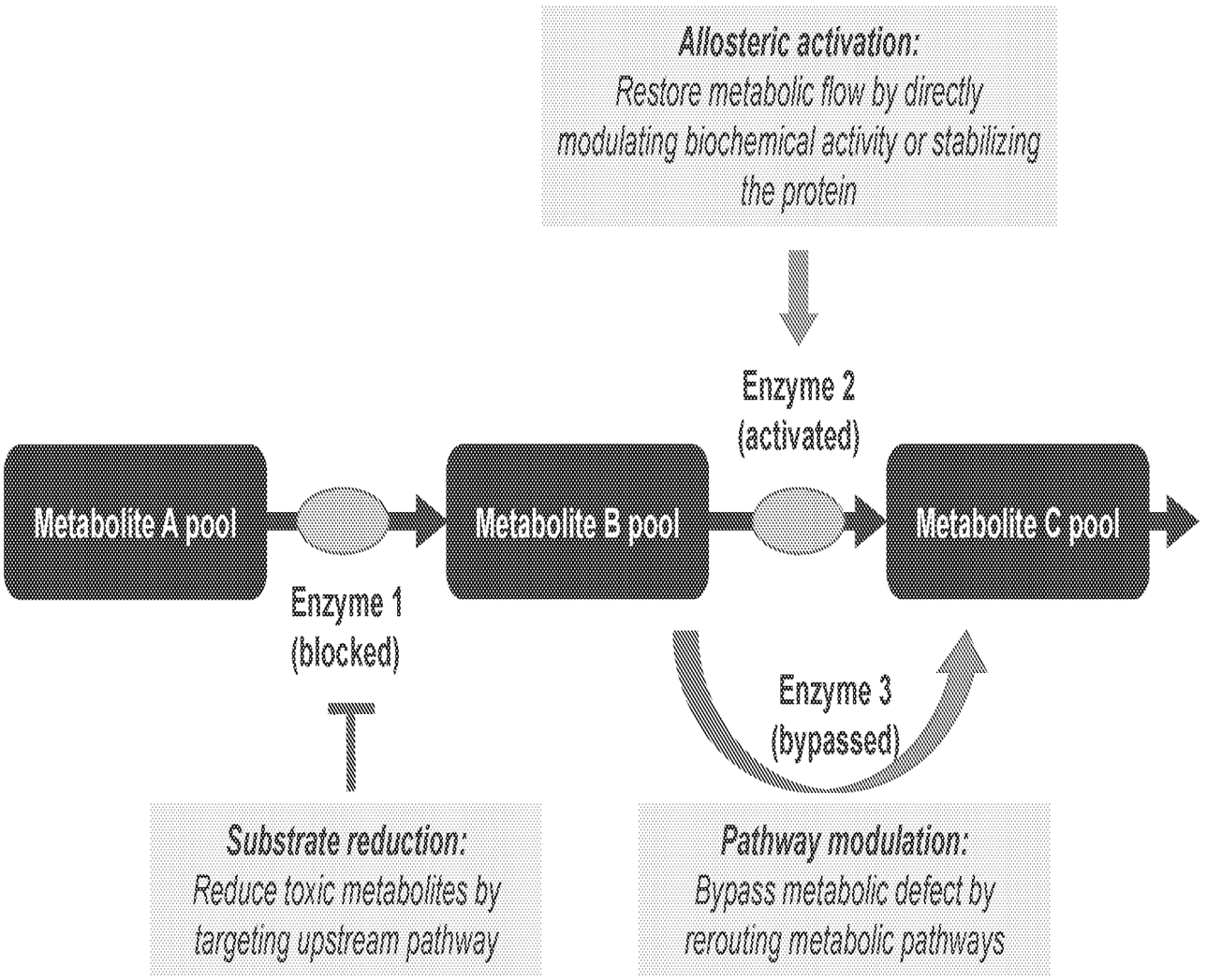
Caused by a deficiency of **L-methylmalonyl CoA reductase**. Symptoms include lethargy, delayed psychomotor development, seizures, acute encephalopathy. Most affected individuals die in infancy.

The human metabolome contains > 75,000 small molecules interconverted by > 5700 enzymes

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# Correcting metabolic defect with small molecules



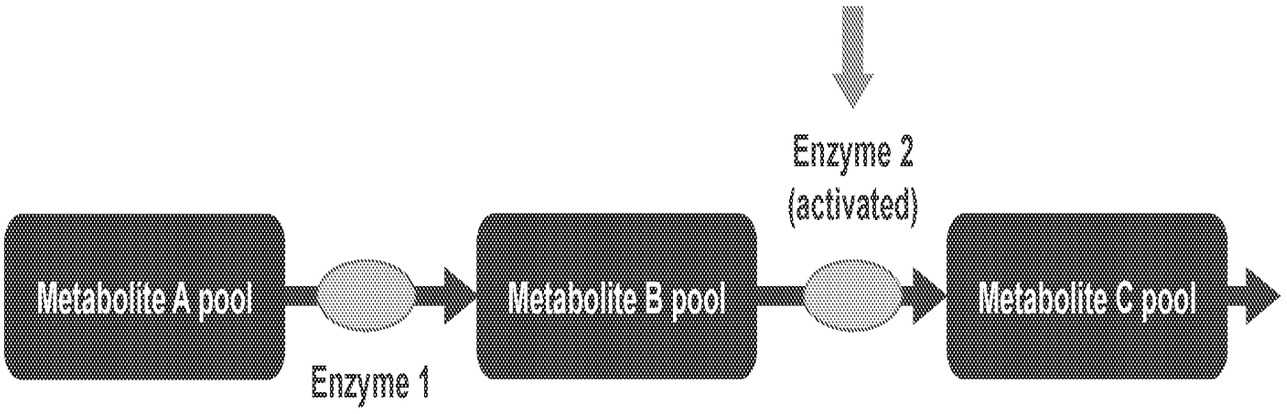


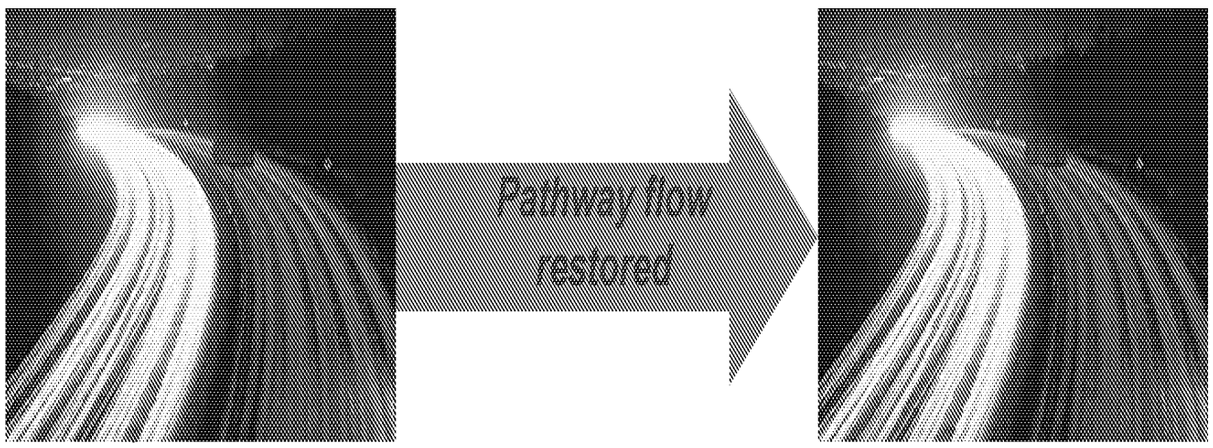

Ex. 2001-0733

# Correcting metabolic defect with small molecules

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***Allosteric activation:***
*Restore metabolic flow by directly modulating biochemical activity or stabilizing the protein*






Ex. 2001-0734

# It's very easy to design an enzyme inhibitor

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enzyme activators are a bit more challenging.....



Ex. 2001-0735

# Challenges unique to Metabolic RGDs Drug Discovery and Development

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**>600 RGDs described** featuring inherited mutation in single metabolic enzyme

Vast majority of RGDs **lack therapeutic options**

1. Most diseases are intracellular & CNS defects not amenable to protein Tx
2. Which diseases/pathways are amenable to small molecule Tx?
3. Diverse range of mutations, including loss of function
4. Complexity of metabolic enzymes as drug targets
5. Lack of disease models / understanding in rare diseases
6. Lack of defined clinical endpoints/regulatory pathways



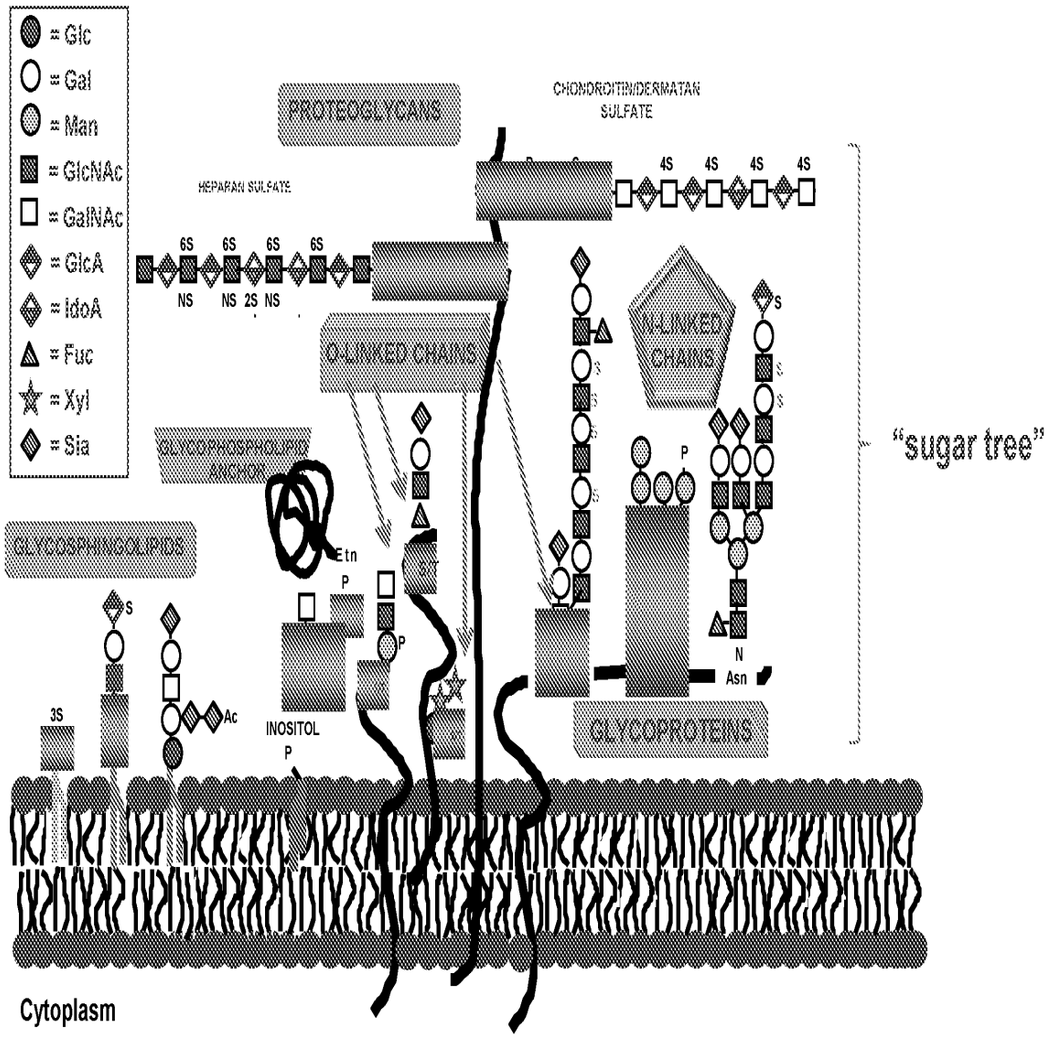
Ex. 2001-0736

# PROGRESS TOWARDS TREATMENT OF CDG1A

# Glycosylation:
# 2% of the genome devoted to making glycans

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Glycosylation impacts a broad spectrum of cellular functions, cell-cell interactions, and signaling




Glycosylation defects can occur at all phases of "sugar tree" biosynthesis (initiation, assembly, trimming, and elongation) → Congenital Disorders of Glycosylation" (CDG)

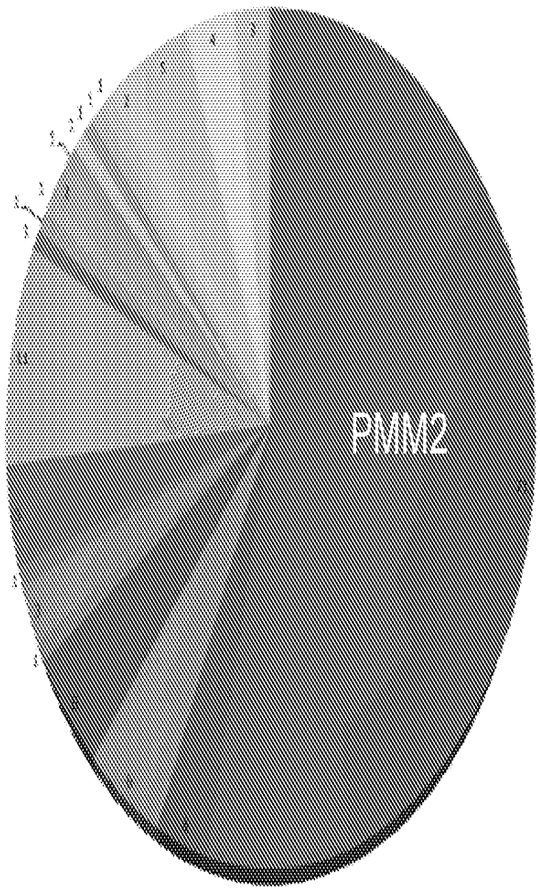


Ex. 2001-0738

# Phosphomannomutase-2 (PMM2) CDG is the most common type of CDG

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PMM2-CDG remains the most commonly diagnosed CDG, >54%.
132 confirmed cases of CDGs identified by Mayo Clinic in the past few years




- PMM2*
- MPI*
- ALG6*
- ALG3*
- DPM1*
- ALG12*
- ALG8*
- DPAGT1*
- ALG1*
- DOLK*
- ALG11*
- SRD5A3*
- DDOST*
- MOCS**
- COG7**
- COG1**
- COG8**
- COG5**
- SLC35A2**
- PGM1***
- ATP6V0A2

Transferrin and apoCIII (6min) LCMS analysis allows for the identification of CDG subtypes routinely at Mayo clinic (some combined with verification by enzyme activity and/or DNA sequence)

* Type I missing 1-2 sugar chains (unoccupied glycosylation sites), defective in glycosylation initiation/assembly. PMM2-CDG belongs to type I

** Type II abnormal chains; defective in glycan tree elongation or trimming

*** Mixed Type

*With permission from Raymond K et al Poster #483,International Congress of Inborn Errors of Metabolism Conference, Sept 4 2013*



Ex. 2001-0739

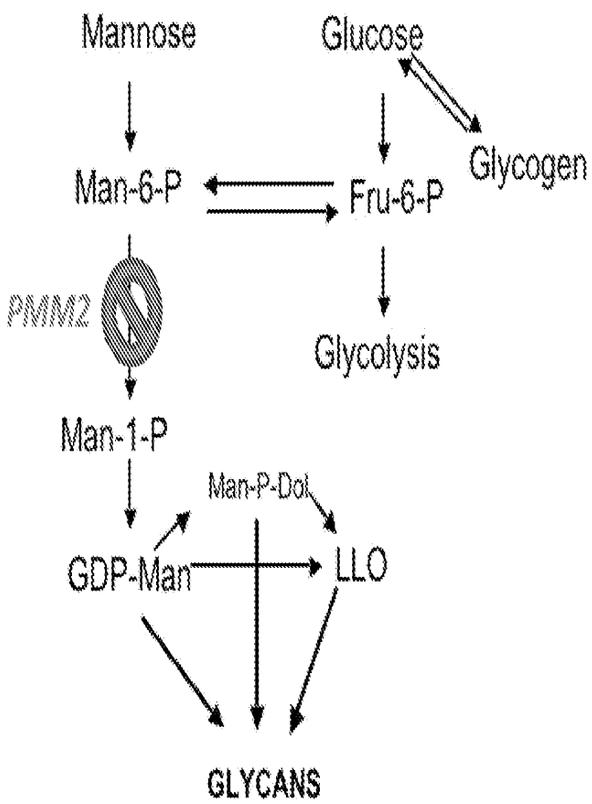
# PMM2-Congenital Disorders of Glycosylation
## *Deficiency in PMM2 (Phosphomannomutase 2)*

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### Disease Overview

- One of over 30 CDGs characterized by abnormal glycosylation of N-linked oligosaccharides
- Severely debilitating and often fatal disorder with onset in infancy & multi-systemic clinical manifestations:
  - Developmental delay
  - Cerebellar atrophy
  - Peripheral neuropathy
  - Stroke-like episodes or comatose episode
  - Epilepsy
  - Retinal pigmentary degeneration
  - Strabismus
  - Skeletal abnormalities, dysmorphic features
  - Hepatopathy
- Divided into three stages: infantile multisystem, late-infantile and childhood ataxia-intellectual disability, and adult stable disability
- No treatment available
- Reported incidence of 1 in 20,000-80,000 Caucasian births; >700 cases reported so far in literature but likely underdiagnosed

### Disease Pathway & Pathophysiology




*PMM2 defect leads to shortage of GDP-Mannose required to form oligosaccharides → abnormal glycosylation in a range of tissues*



Ex. 2001-0740

# PMM2-Congenital Disorders of Glycosylation
## *Deficiency in PMM2 (Phosphomannomutase 2)*

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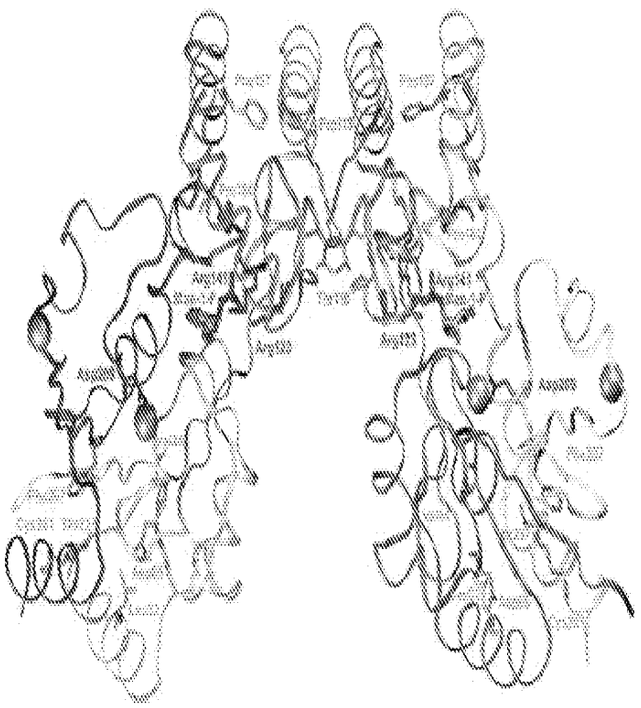
**Mutations & enzyme defects:**

- >100 mutations reported, but nearly 85% are missense mutations
- Most are compound heterozygous with residual PMM2 activity ranging from 5-40%
  - Those with >50% activity are asymptomatic

**Agios Therapeutic Approach**

- Allosteric activator approach: Rescue protein instability and PMM2 activity
  - Restoring activity to >50% of WT may be sufficient

**PMM2 crystal structure**



**Mutation effects**

- Green: Dimerization
- Red: Catalytic residues
- Yellow: Folding/stability

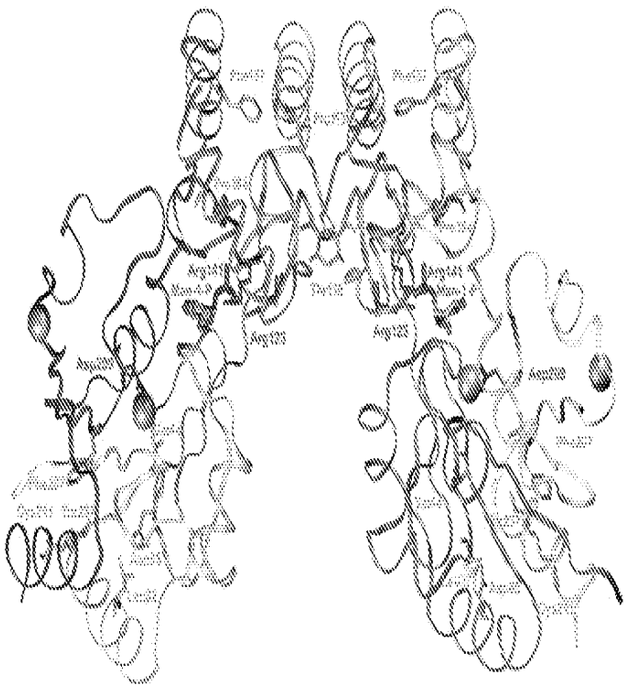
*Vega et al., JIMD (2011)*

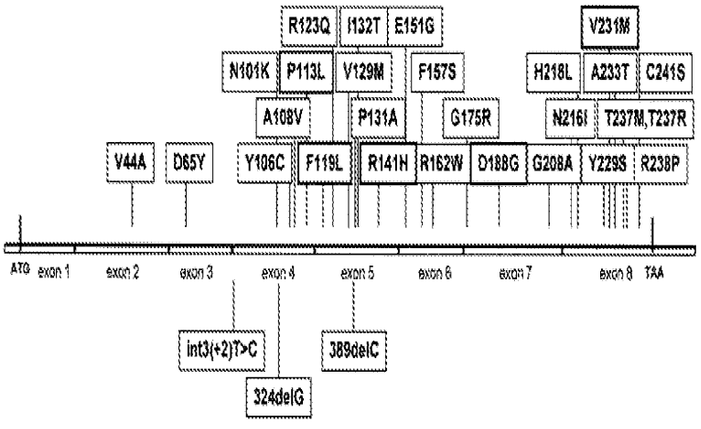


Ex. 2001-0741

# PMM2: A dimeric enzyme with allosteric AND substrate-level activation

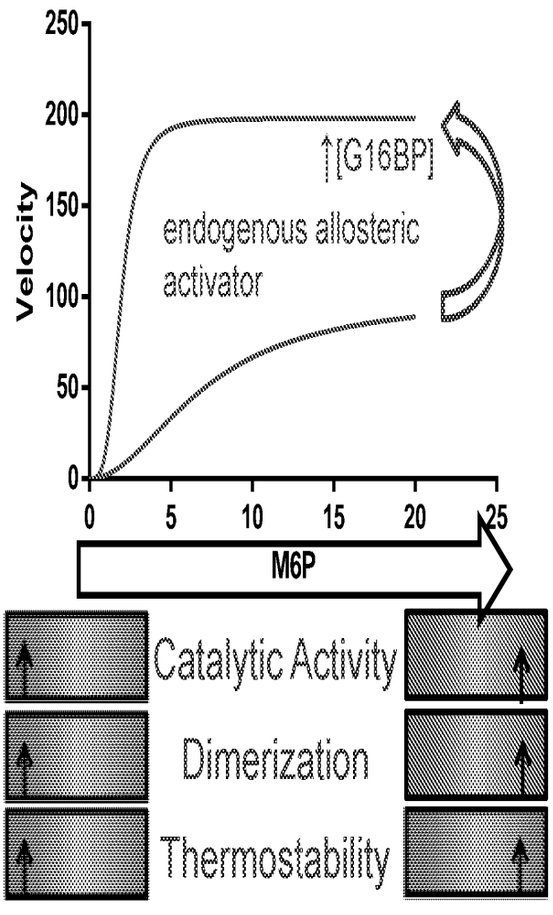
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- Mis-sense mutations affect the three axes of enzyme function to varying degrees
- Dimeric nature facilitates the possibility of both mutant homo- and hetero-zygotes



Ex. 2001-0742

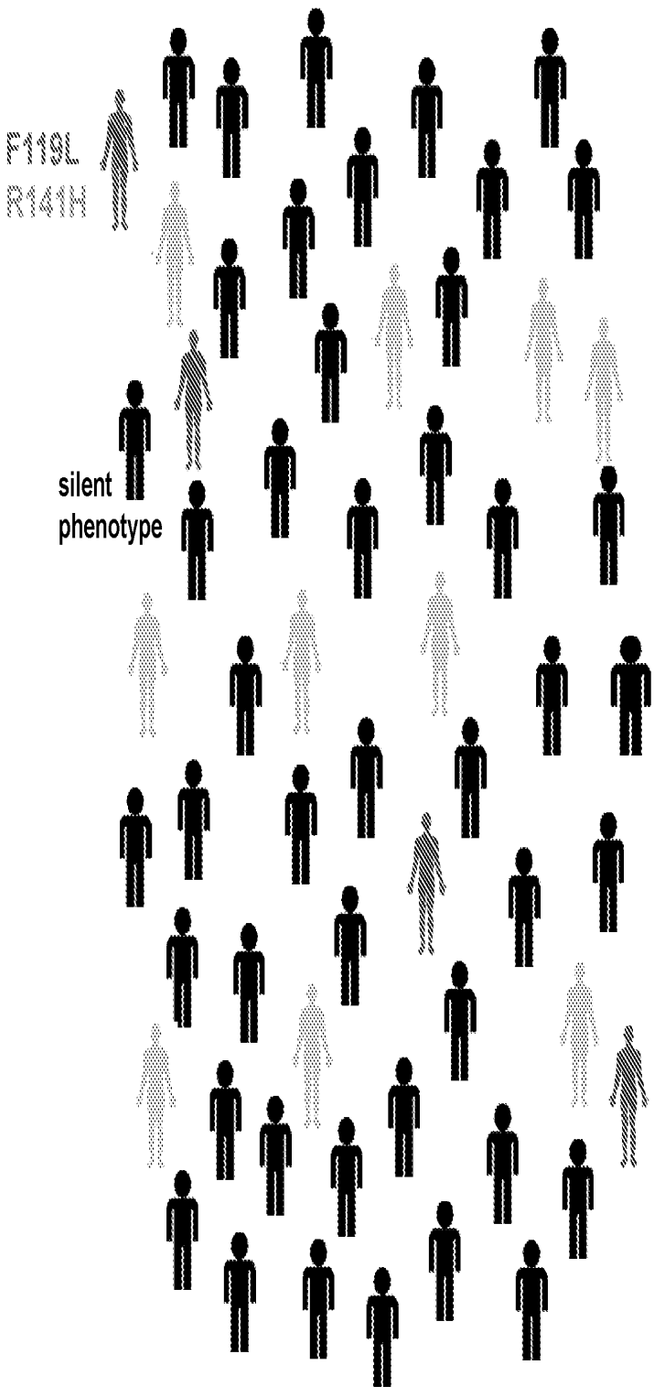
# PMM2 Homozygous Mutants: The Ghosts of Elsinore

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**Table 3** Expected and observed numbers of CDG1 patients with different genotypes. Frequencies, by counting, of F119L and R141H are 16/36=0.44 and 16/36=0.44, respectively

| Genotypes | Expected frequency | Expected number |
|---|---|---|
| F119L/F119L | $(0.44)^2$ | 3.5 |
| F119L/R141H | $(2\times0.44\times0.44)$ | 7.0 |
| R141H/R141H | $(0.44)^2$ | 3.5 |
| Other combinations[a] | 0.26 | 4.0 |

[a]F119L/other, R141H/other, and other/other.
$P = 0.0009$; $\chi^2 = 16.4$; df = 3.




ARTICLE

## Lack of Hardy-Weinberg equilibrium for the most prevalent *PMM2* mutation in CDG-Ia (congenital disorders of glycosylation type Ia)

Els Schollen[1], Susanne Kjaergaard[2], Eric Legius[1], Marianne Schwartz[2] and Gert Matthijs[1]

ORIGINAL PAPER

## Absence of homozygosity for predominant mutations in *PMM2* in Danish patients with carbohydrate-deficient glycoprotein syndrome type 1

Susanne Kjaergaard, Flemming Skovby and Marianne Schwartz

Lack of Homozygotes for the Most Frequent Disease Allele in Carbohydrate-Deficient Glycoprotein Syndrome Type 1A

Gert Matthijs,[1] Els Schollen,[1] Emile Van Schaftingen,[1] Jean-Jacques Cassiman,[1] and Jaak Jaeken[2]

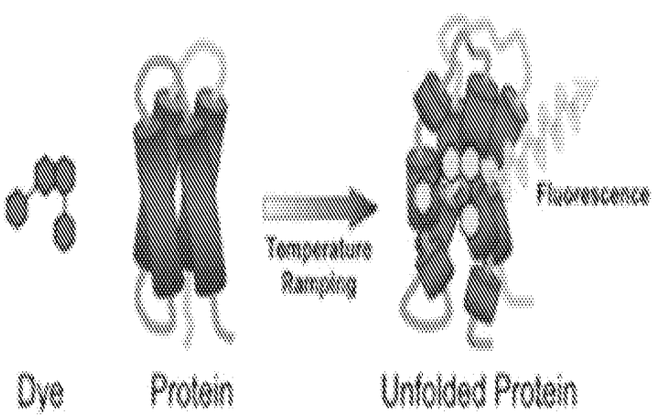


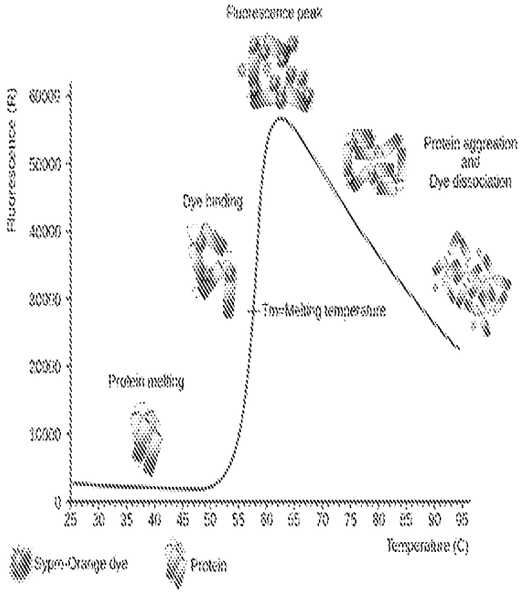
Ex. 2001-0743

# Biochemical Tools to Study PMM2

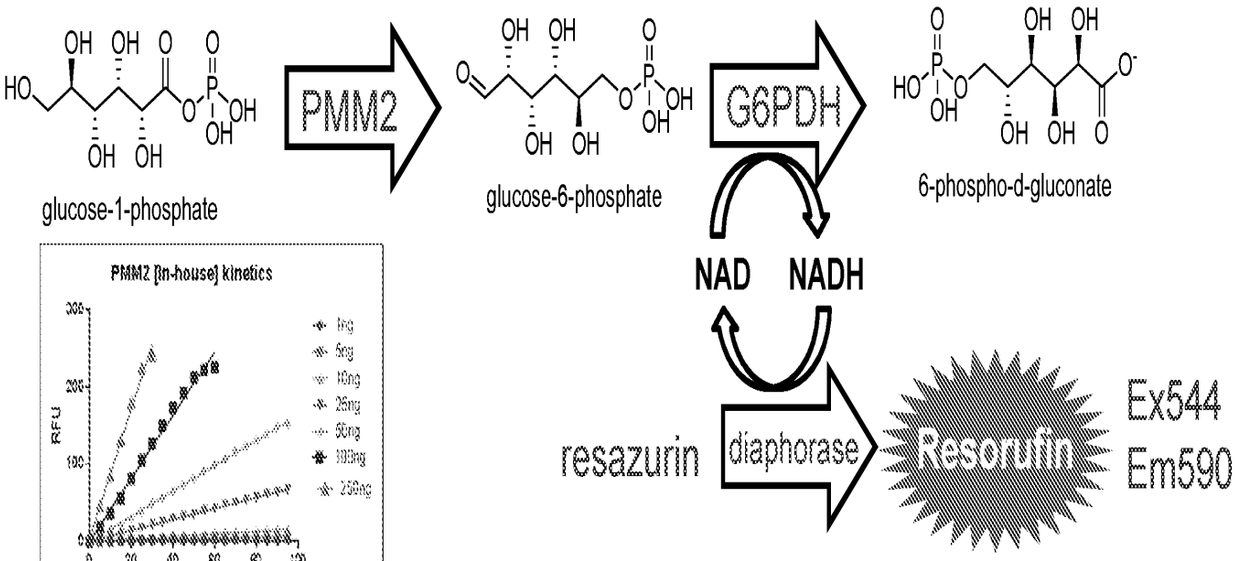
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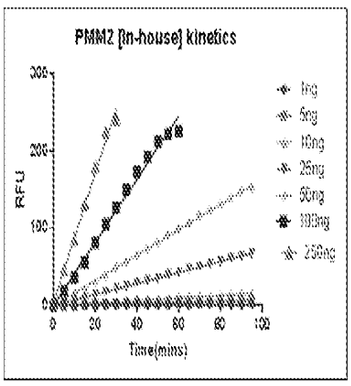
- Thermostability Assay




- Continuous Kinetic Assay with substrate analogue



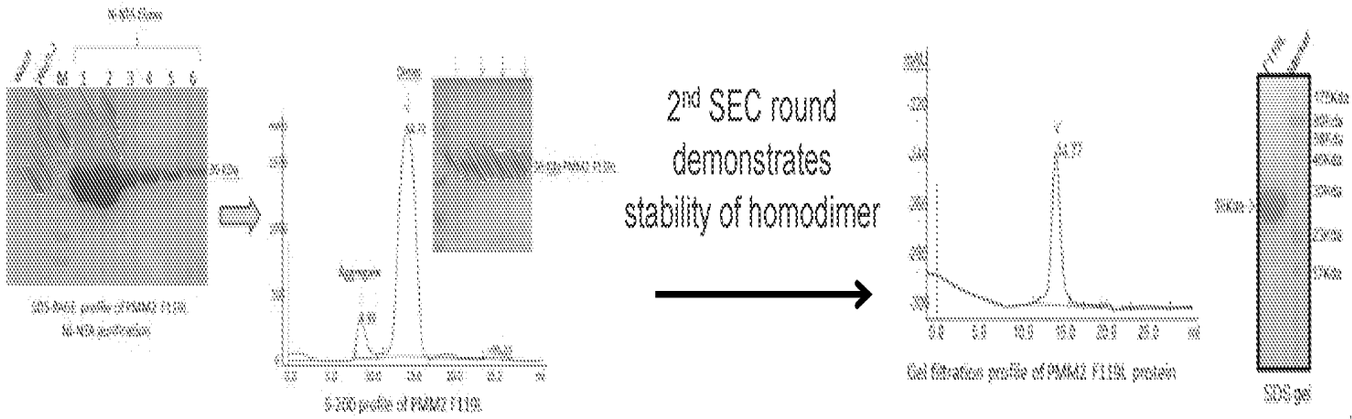





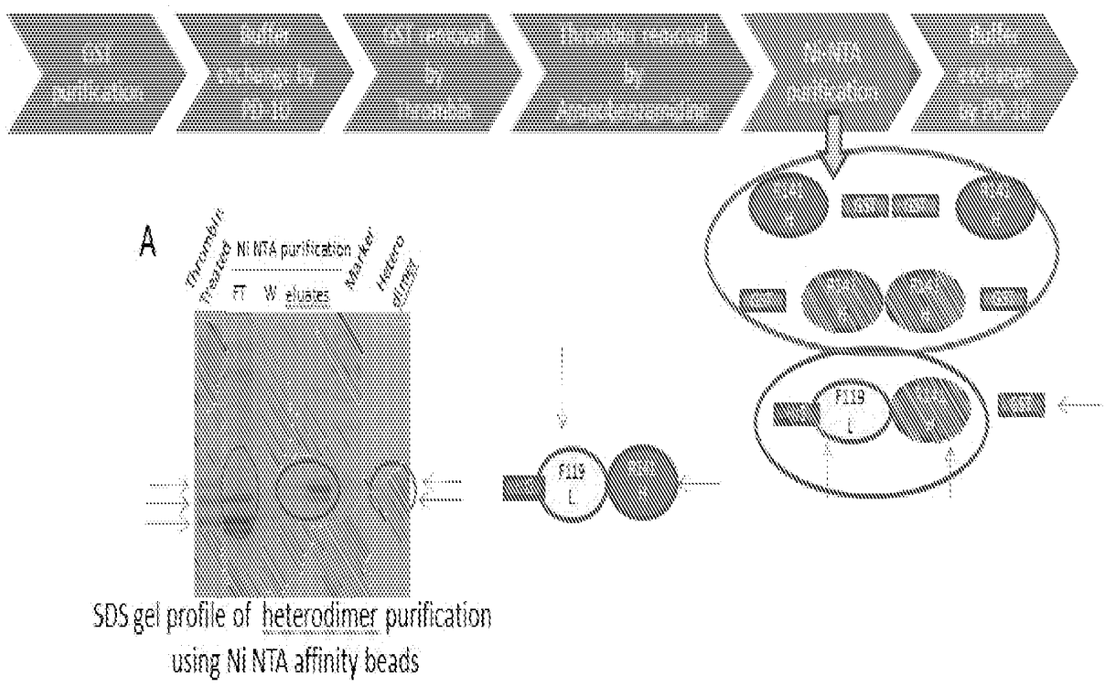

Ex. 2001-0744

# Production of Homodimer and Heterodimer PMM2

- WT, F119L, R141H produced in E. coli




- F119L/R141H heterodimer produced in E. coli



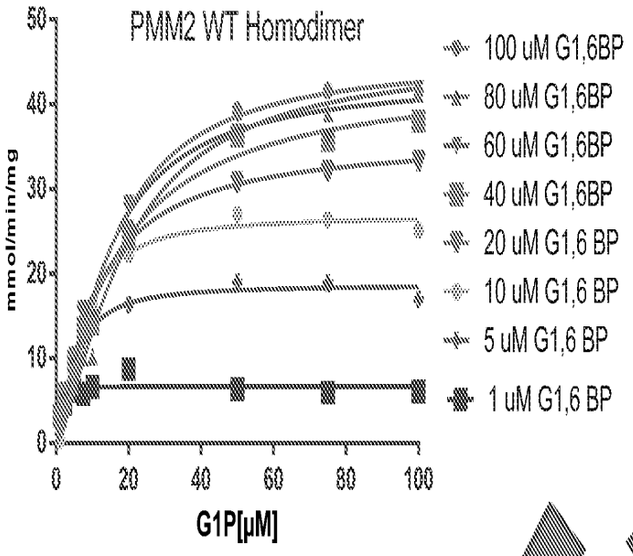

SDS gel profile of heterodimer purification using Ni NTA affinity beads



Ex. 2001-0745

# Kinetic characterization of wt PMM2

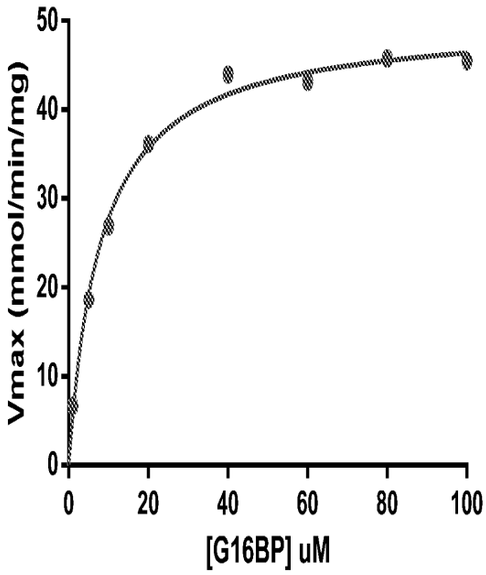
agios



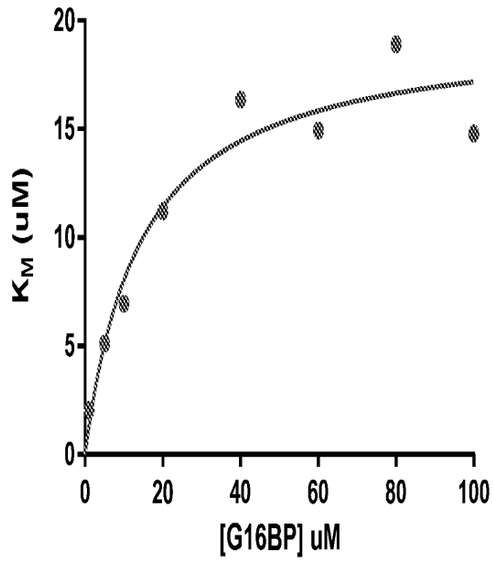

| G16 BP (µM) | Km G1P (µM) | Hill Slope | Vmax (µmol/min/mg x $10^{-1}$) | Kcat ($s^{-1}$) | Kcat/Km $M^{-1}s^{-1}$ X $10^{5}$ |
|---|---|---|---|---|---|
| 100 | 14.79 | 1.42 | 45.42 | 1.97 | 1.33 |
| 80 | 18.91 | 1.43 | 45.76 | 1.99 | 1.05 |
| 60 | 13.61 | 1.37 | 43.15 | 1.87 | 1.38 |
| 40 | 16.36 | 1.09 | 43.93 | 1.91 | 1.17 |
| 20 | 11.21 | 1.14 | 36.14 | 1.57 | 1.4 |
| 10 | 6.942 | 1.54 | 26.86 | 1.17 | 1.68 |
| 5 | 5.107 | 1.49 | 18.66 | 0.81 | 1.59 |
| 1 | 1.76 | 2.25 | 6.70 | 0.29 | 1.65 |

$K_{cat}/K_M$ is constant

**PMM2 Catalytic Efficiency as a function of activator**




**PMM2 Substrate Affinity as a function of activator**



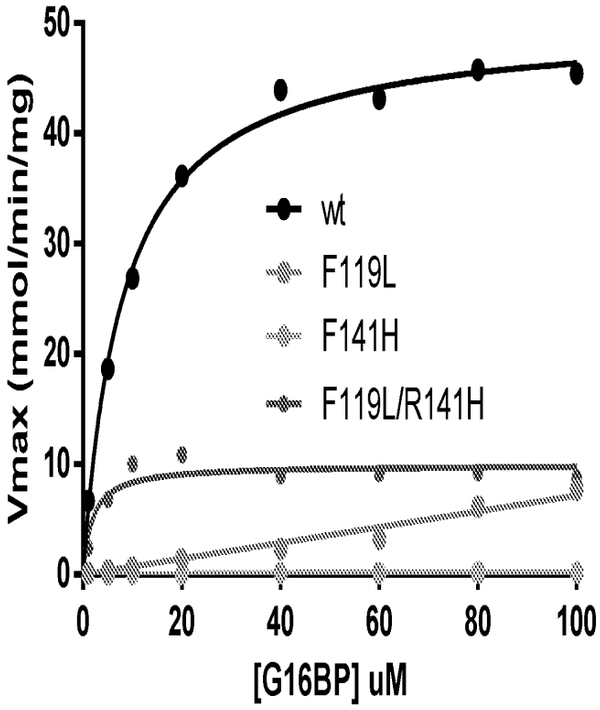



Ex. 2001-0746

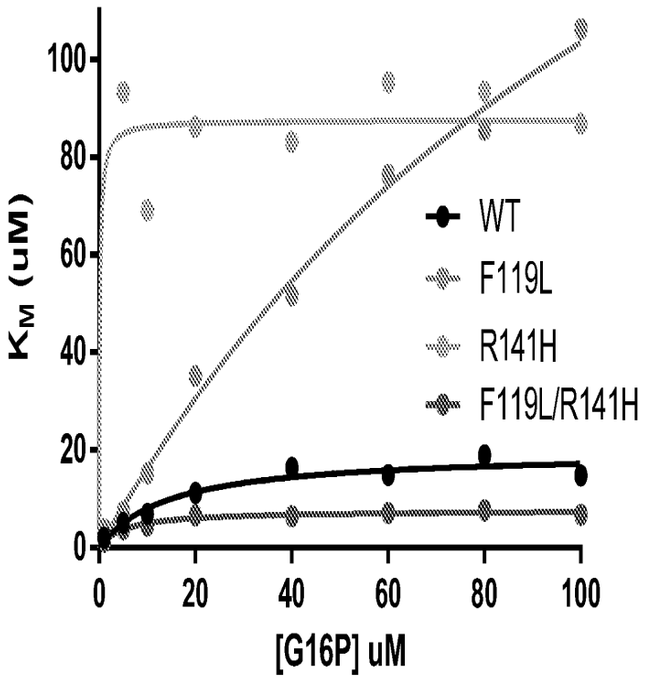
# Heterodimerization provides a distinct kinetic advantage for PMM2 mutant proteins

agios

**PMM2 Catalytic Efficiency as a function of activator**




**PMM2 Substrate Affinity as a function of activator**

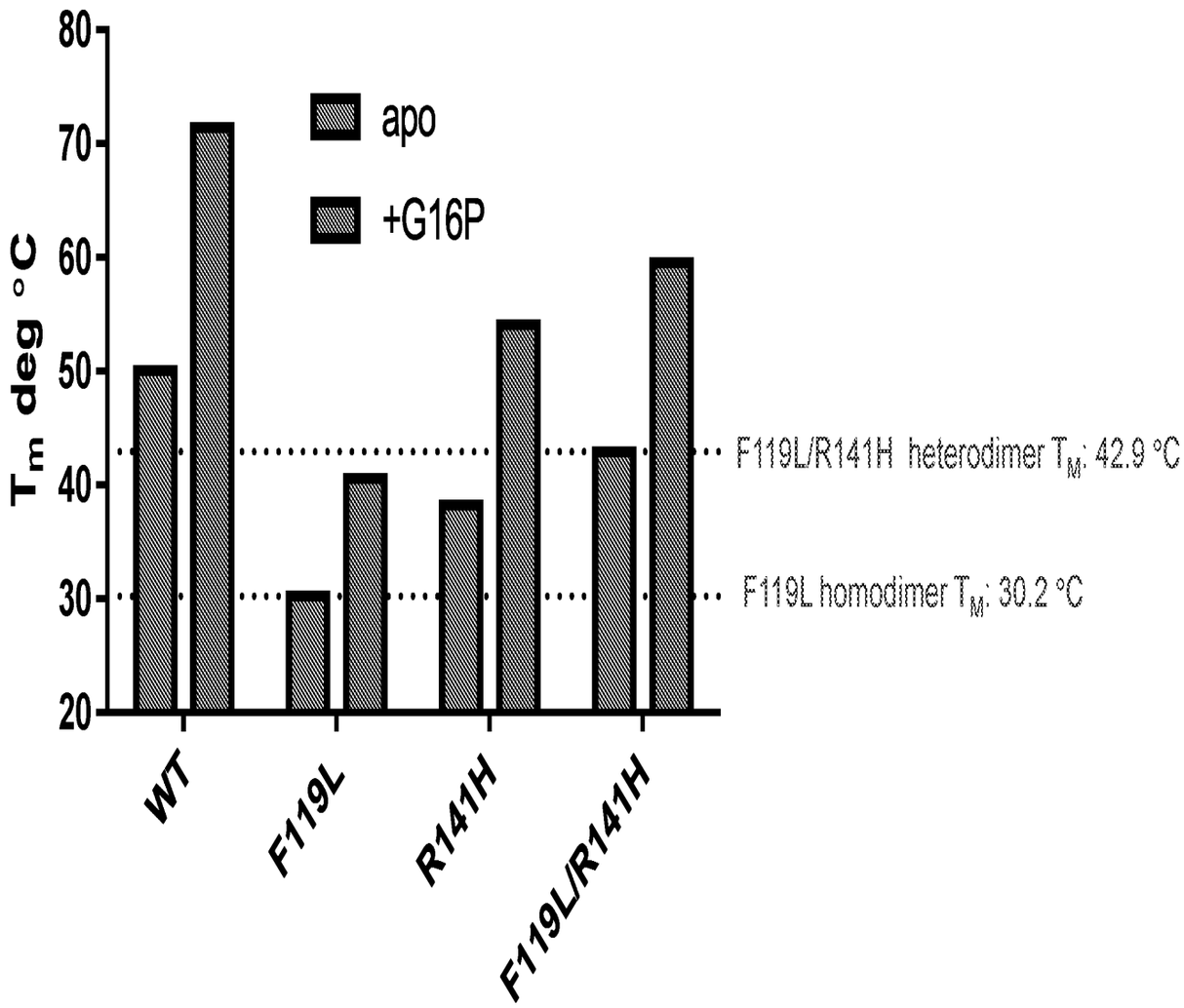



| Isozyme | $k_{cat}$ defect | $K_M$ defect |
|---|---|---|
| F119L/F119L | moderate | moderate |
| R141H/R141H | severe | severe |
| F119L/R141H | mild | mild |



Ex. 2001-0747

# Heterodimerization provides a distinct stability advantage for PMM2 mutant proteins




- The unstable F119L mutant is stabilized by association with the R141H mutant
- Both WT and mutant proteins exhibit ligand-induced stabilization when associated with the endogenous activator G16P.

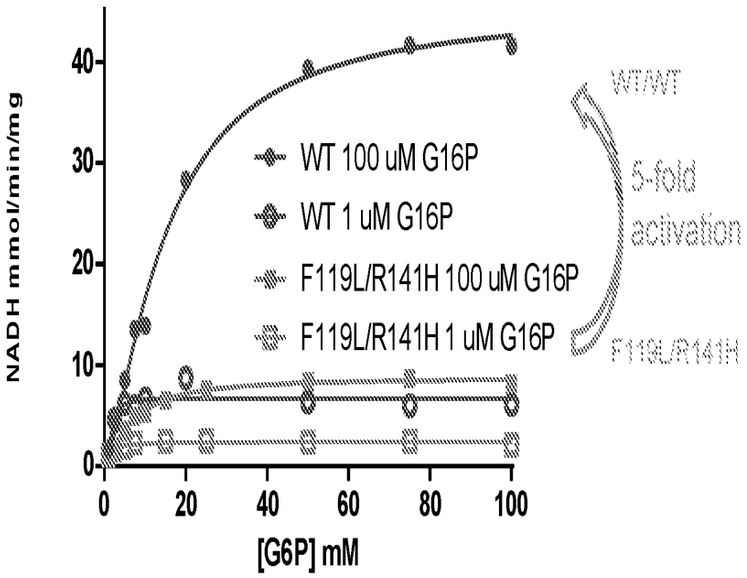


Ex. 2001-0748

# Feasibility and Strategy for Allosteric Activator of PMM2

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- **Identified** the biologically relevant F119L/R141H heterodimer as the target
- **Stability defects** in F119L homodimer can be improved by tight heterodimeric association with catalytically defective R141H
- **Catalytic deficiencies** in the R141H homodimer can be improved by tight heterodimeric association with the unstable F119L
- **Small molecule** metabolite G16P activation and stabilization suggests PMM2 is a drugable target for pharmacological intervention.




| | WT | F119L/R141H | WT | F119L/R141H |
|---|---|---|---|---|
| | 100 uM G16P | 100 uM G16P | 1 uM G16P | 1 uM G16P |
| Vmax | 45.4 | 8.77 | 6.71 | 2.39 |
| h | 1.4 | 1.37 | 2.25 | 1.25 |
| Khalf | 14.8 | 6.78 | 1.76 | 1.21 |

Rescue of the F119L/R141H heterodimer requires rather modest improvements in kinetic parameters

- Km defect is < 2-fold
- Kcat defect is < 6-fold

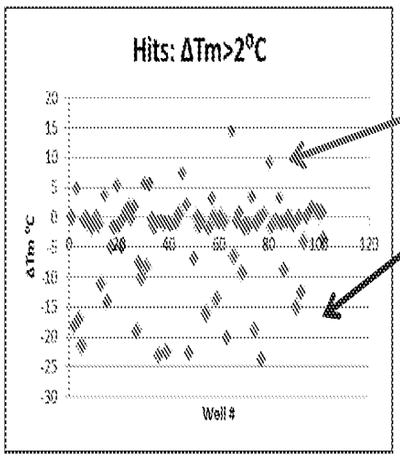


Ex. 2001-0749

# Screen for F119L/R141H stabilizers identifies candidate starting chemical equity

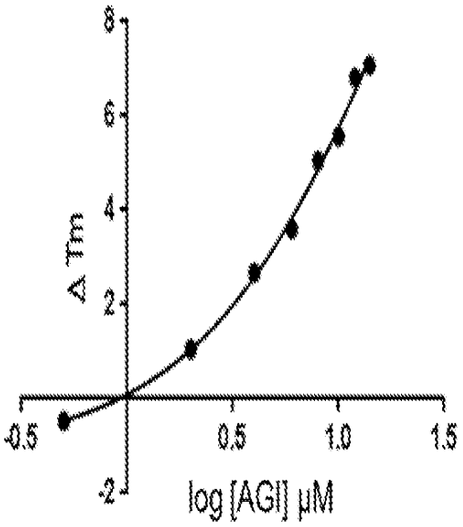
agios

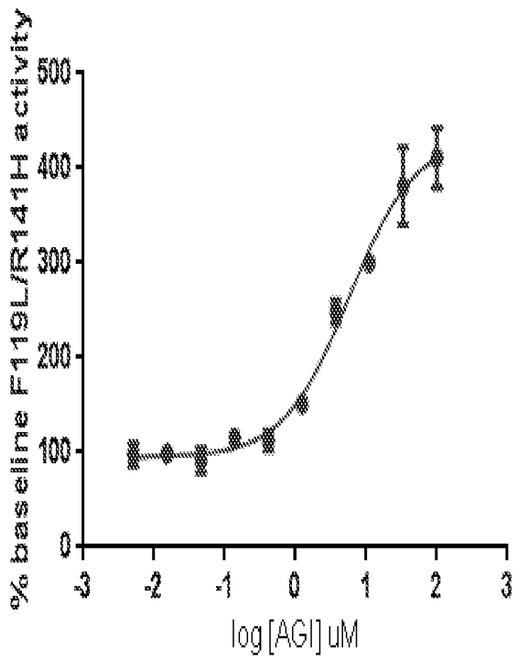
- Agios proprietary collection of low MW compounds and metabolites was screened for the ability to stabilize F119L/R141H heterodimer
- Stabilizing hits were tested for the ability to activate the enzyme




Stabilizing hits increase melting temperature

Destabilizing hits decrease melting temperature




Potential activator/stabilizer hits were identified. Progressibility still undetermined.



Ex. 2001-0750

# CDG/PMM Summary

agios

- Point mutations in PMM2 which cause kinetic defects or decreased protein stability are responsible for CDG Ia, the most common glycosylation disorder
- Lack of Hardy-Wienberg equilibrium in the population can be explained by embryonic lethality of homozygotic alleles producing highly defective proteins.
- The clinically observed F119L/R141H heterozygote produces a minimally active heterodimer through functional complementation between the thermolaible F119L and the catalytically defective R141H subunits
- POC for small molecule stabilizer/activator has been obtained by a fragment-based lead identification approach



Ex. 2001-0751

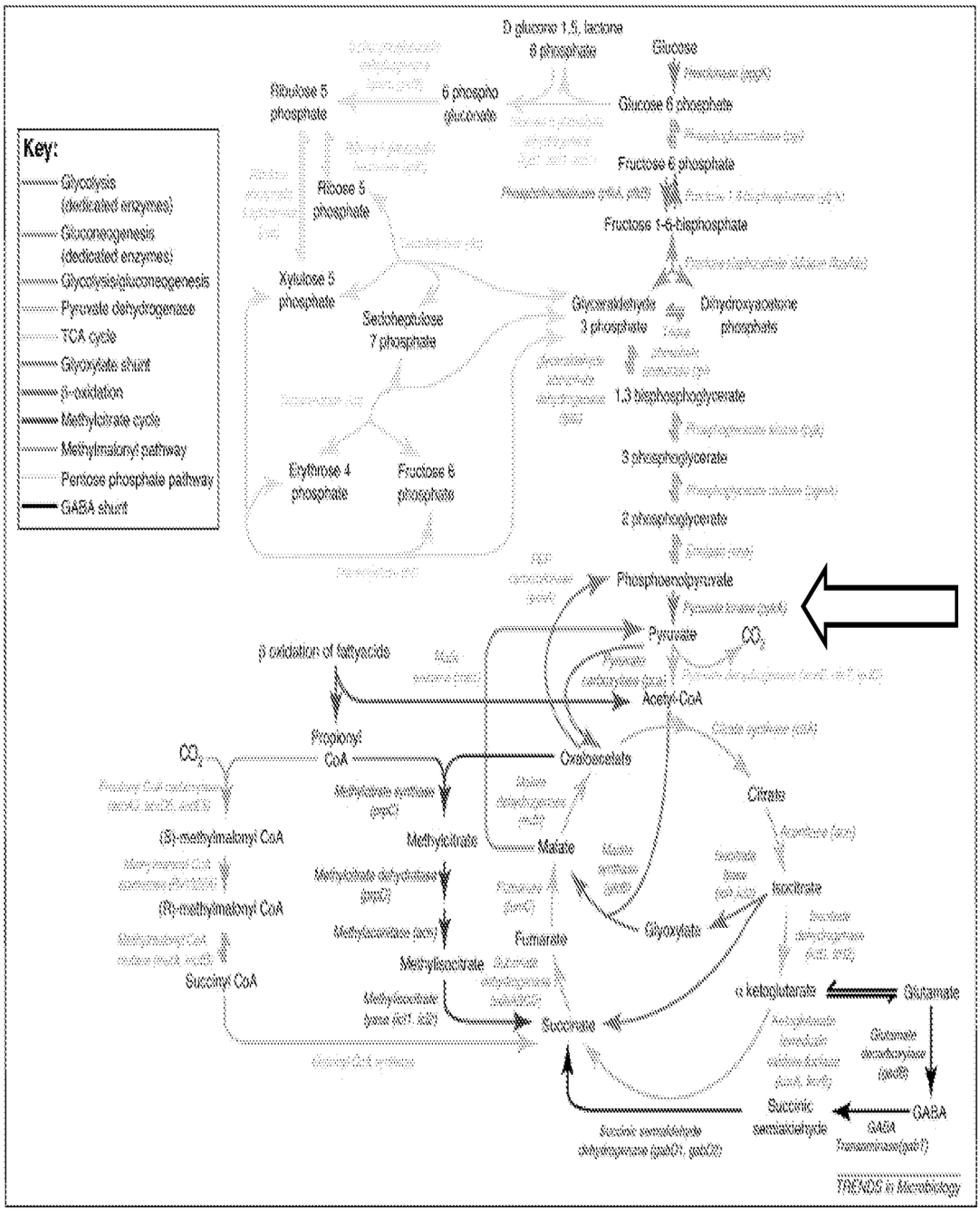
# AG-348, AN ENHANCER OF PYRUVATE KINASE ACTIVITY FOR THE TREATMENT OF PATIENTS WITH PKR-DEFICIENT HEMOLYTIC ANEMIA



Ex. 2001-0752

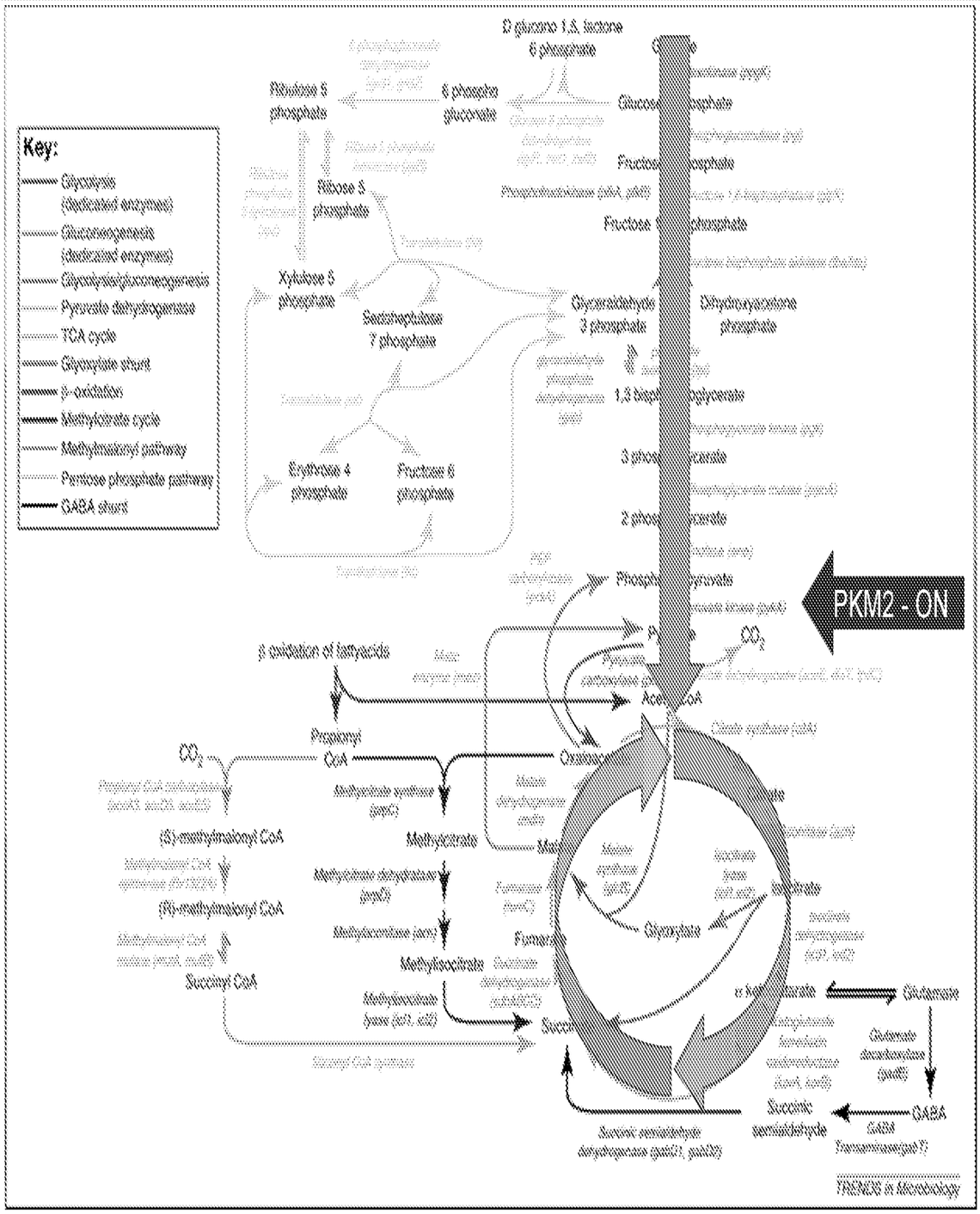
# Pyruvate kinase is a key metabolic node

agios



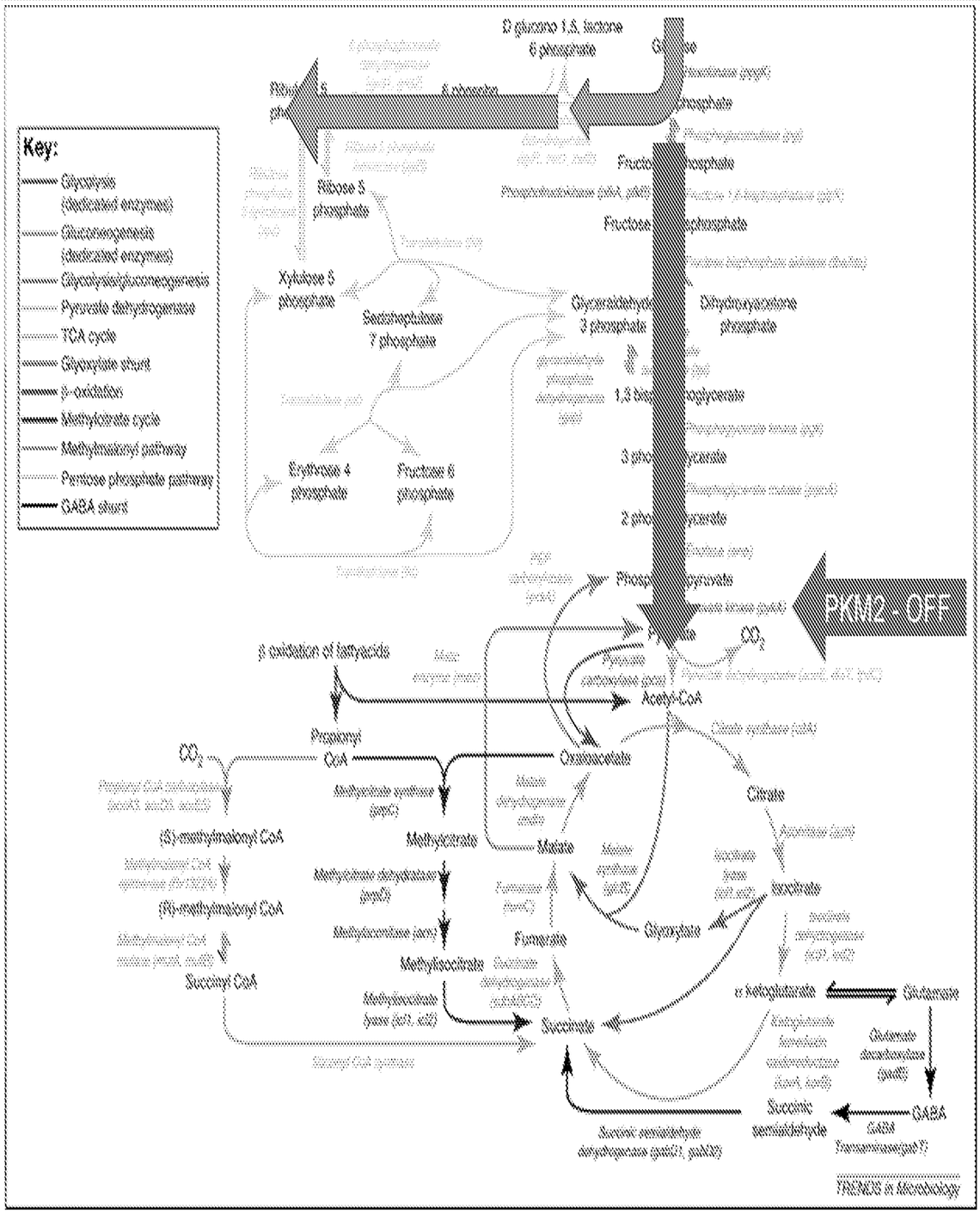
# PKM2 "on" directs carbon flow into TCA cycle for energy production

agios



# PKM2 "off" directs carbon flow into pentose phosphate shunt for nucleotide production

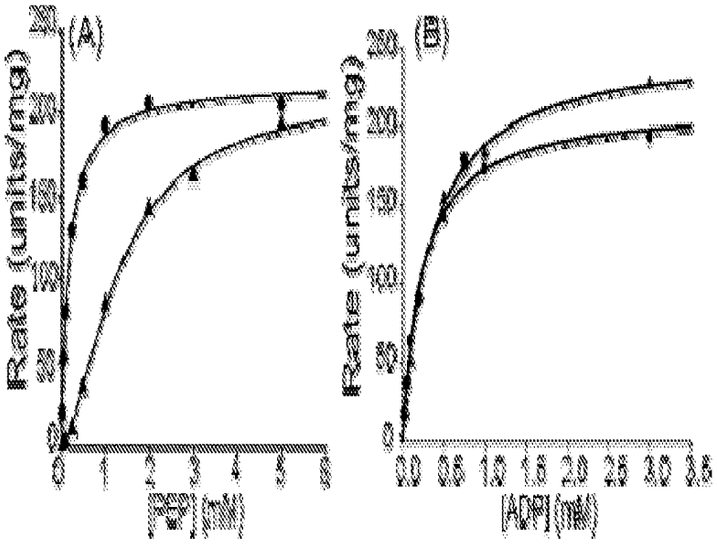
agios






Ex. 2001-0755

# PKM2 is allosterically activated by FBP




| FBP | $K_m$ (mM) | $V_{max}$ | $n_H$ |
|---|---|---|---|
| ADP + | 0.24 ± 0.03 | 214 ± 6 | 1.0[b] |
| - | 0.34 ± 0.02 | 253 ± 6 | 1.0[b] |
| PEP + | 0.17 ± 0.01 | 218 ± 4 | 1.0[b] |
| - | 2.1 ± 0.7 | 310 ± 37 | 1.6 ± 0.1[c] |

## Two consequences of activation:

- Increase in affinity for PEP but not ATP substrate
- Decrease in subunit co-operativity

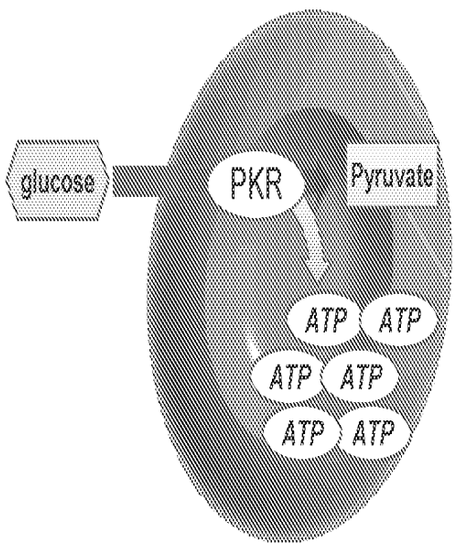


Ex. 2001-0756

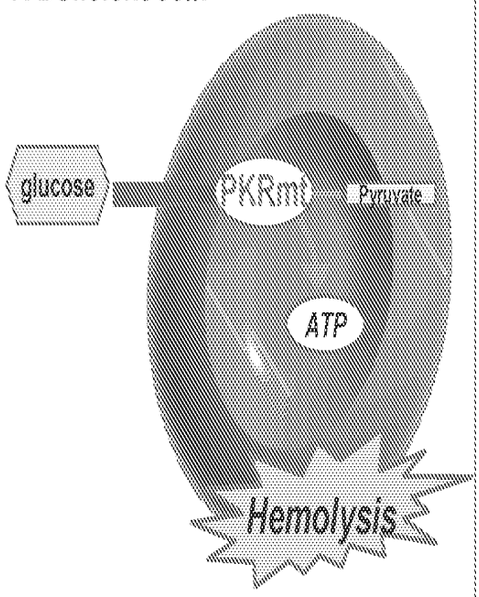
# Pyruvate Kinase Deficiency (PKD): IEM caused by metabolic defect of red blood cells

- Pyruvate Kinase Deficiency (PKD) is a rare form of hemolytic anemia
  - Caused by mutations in PKR
- Caused by mutations in PKR enzyme
  - Last step in glycolysis in red blood cells
  - Only energy (ATP)-generating pathway in red blood cells
- Patients have life-long anemia, transfusions, and iron overload
  - Splenectomy is common
- Therapeutic strategy: **Small molecule activation of mutant PKR**

Normal red blood cell:




PK Deficient cell:

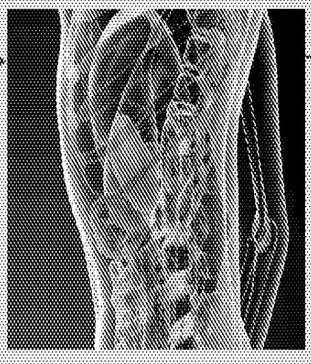
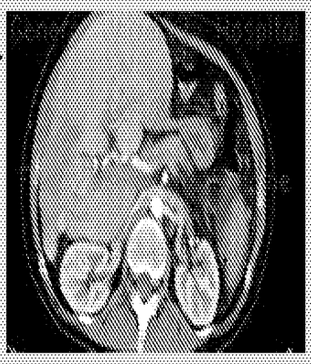





Ex. 2001-0757

# Disease Progression from Infants to Adults Demonstrates Lifetime of Burden

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| INFANTS | TODDLERS | CHILDREN | ADULTS |
|---|---|---|---|
|  |  | |  |
| Jaundice, severe anemia, exchange transfusions | Splenectomy only partially ameliorates hemolysis and leads to increased infection risk, antibiotic prophylaxis | | Iron overload leading to liver cirrhosis, cardiac and endocrine issues<br>Gall stones, cholecystectomy |

Ongoing Natural History Study captures retrospective/prospective clinical data, Quality of Life (QoL) measures and genetic diagnostic information.

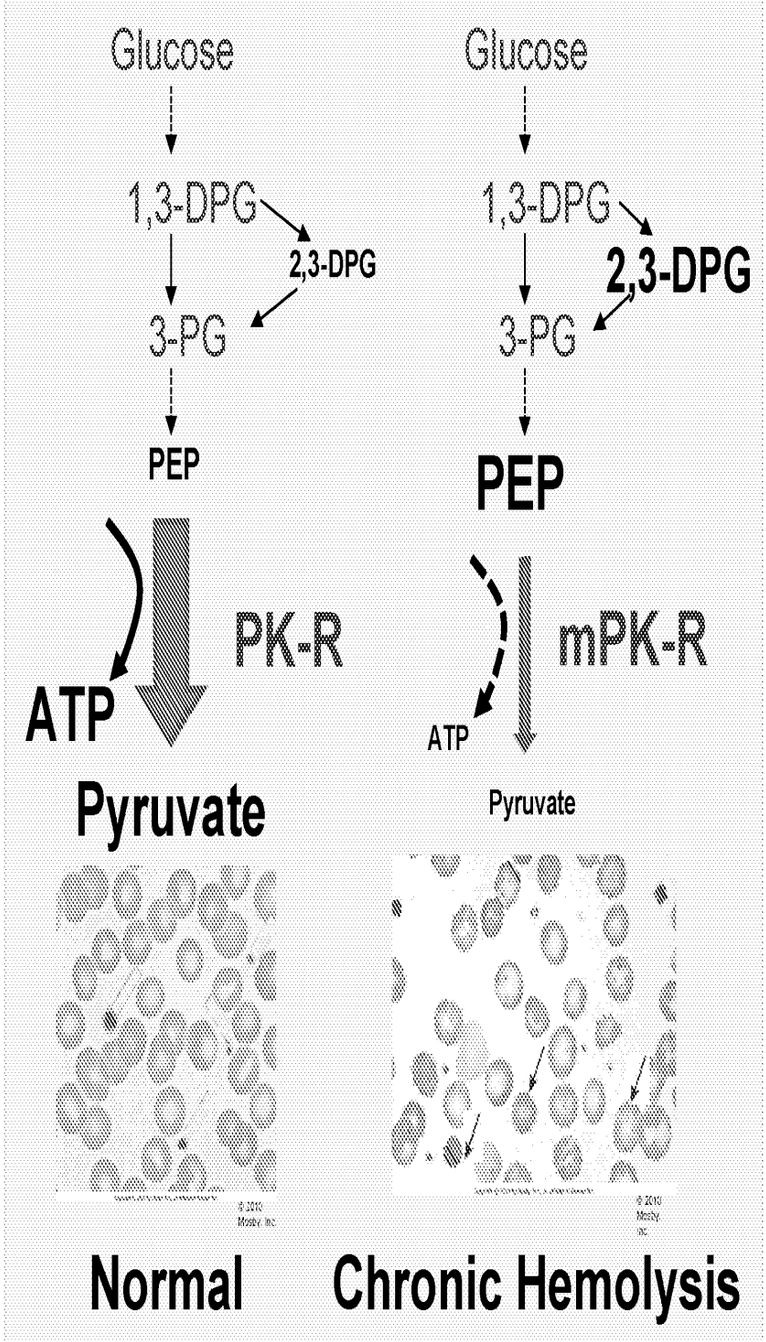
www.clinicaltrials.gov, NCT02053480

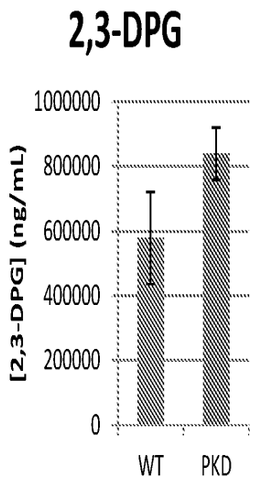


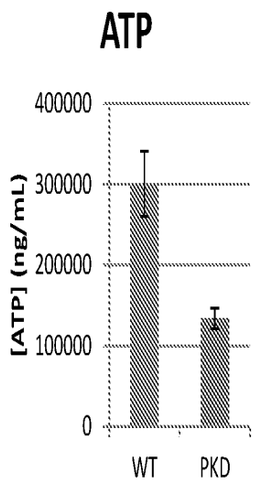
Ex. 2001-0758

# Metabolic Defects in PK Deficiency

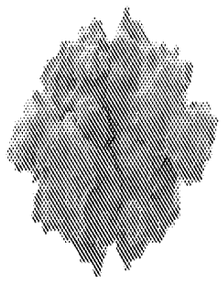
agios



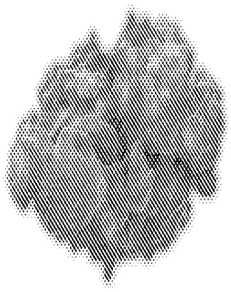
# Small molecule remediation of PKR deficiency



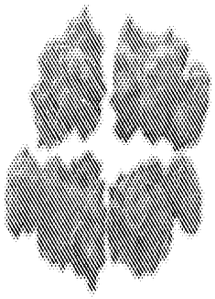
Normal PKR

Can we mimic or augment the effects of FPB activation?

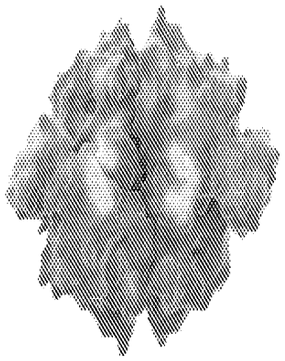
PKD mutations



or

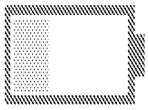




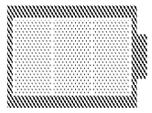


Reduced catalytic efficiency

Reduced protein stability



Energy (ATP) Deficiency



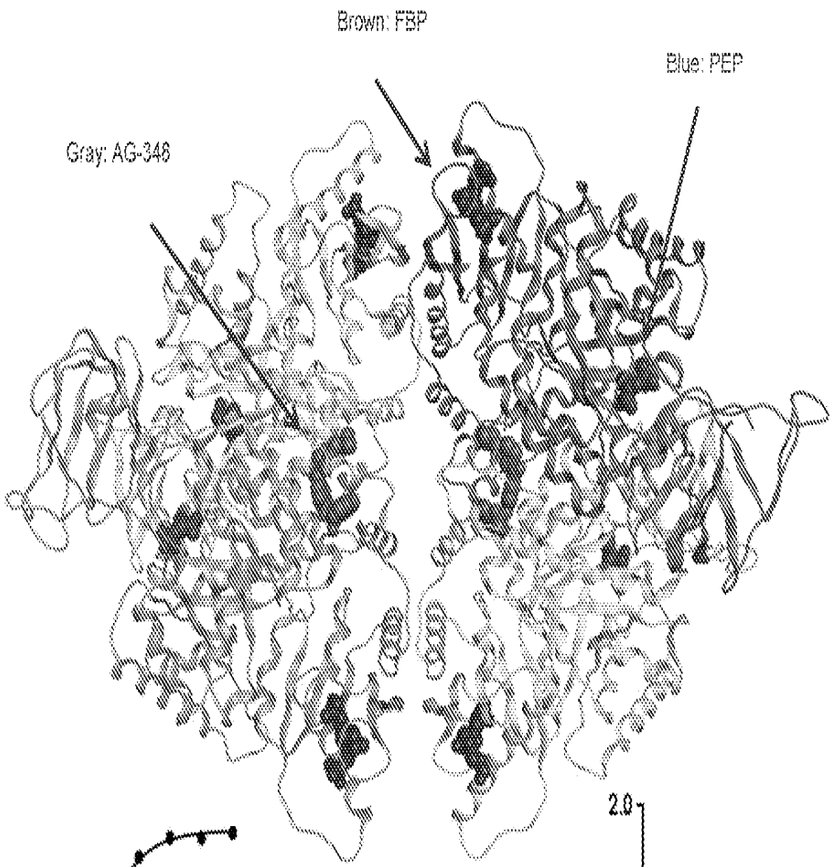
Increased ATP

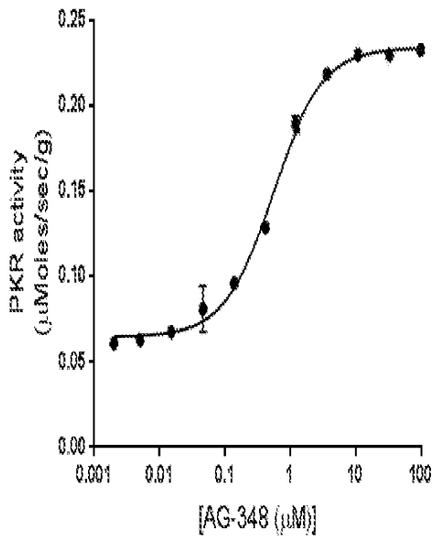


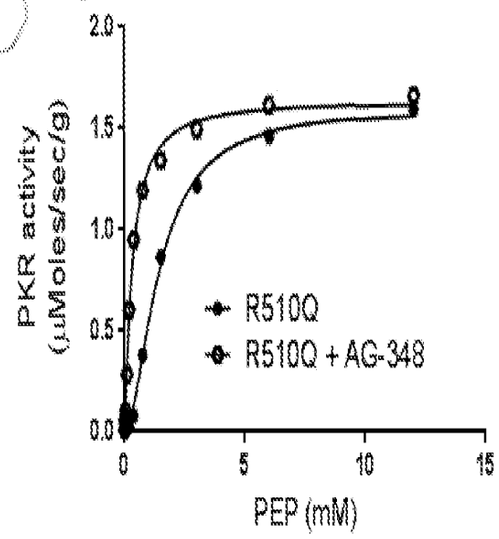
Ex. 2001-0760

# AG-348: a novel small-molecule activator of PK which mimics FBP activation

agios






Kung et al. Blood 2017 (publication in press)

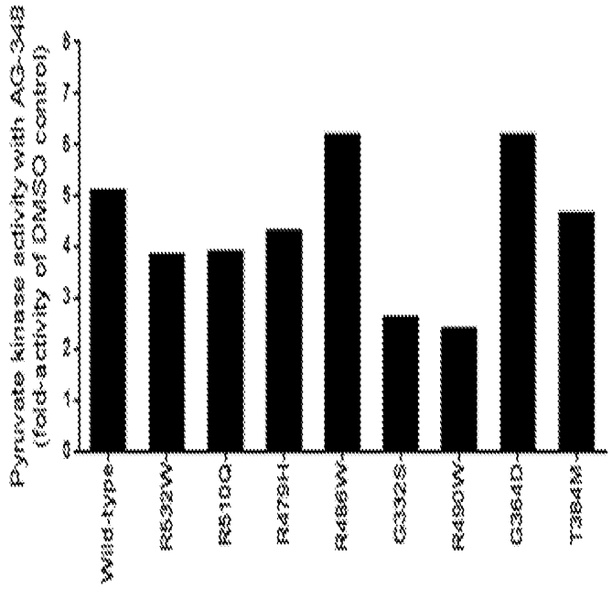


Ex. 2001-0761

# AG-348 activates a spectrum of recombinant mtPKR enzymes



PKR tetramer with sites of mtPKR enzymes tested in Figure 3B highlighted in red. For clarity, each mutation site is only shown in a single monomer

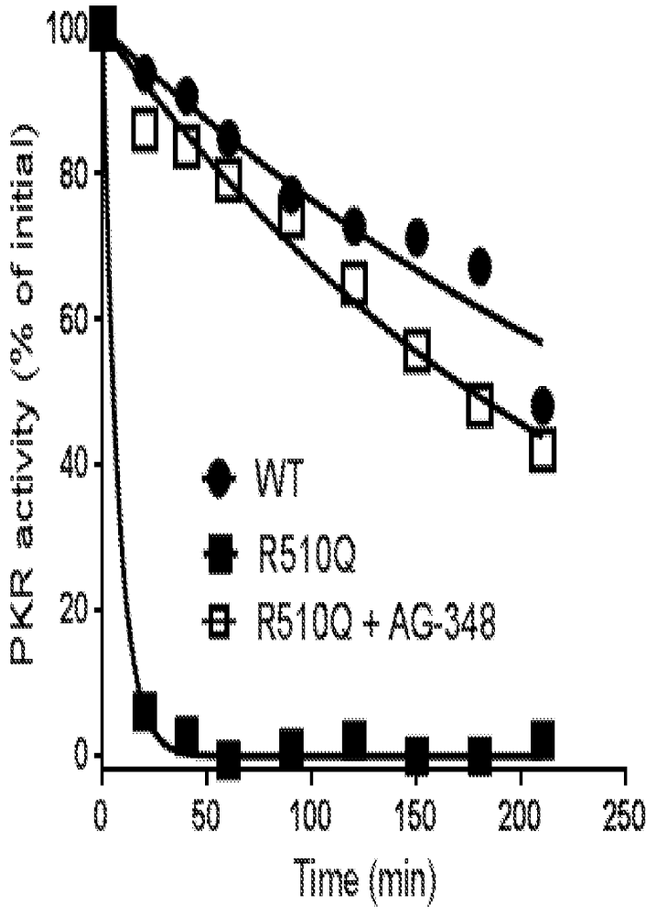



| AG-348 $AC_{50}$ (nM) | 13 | 9 | 55 | 16 | 59 | 22 | 14 | 41 | 26 |
|---|---|---|---|---|---|---|---|---|---|

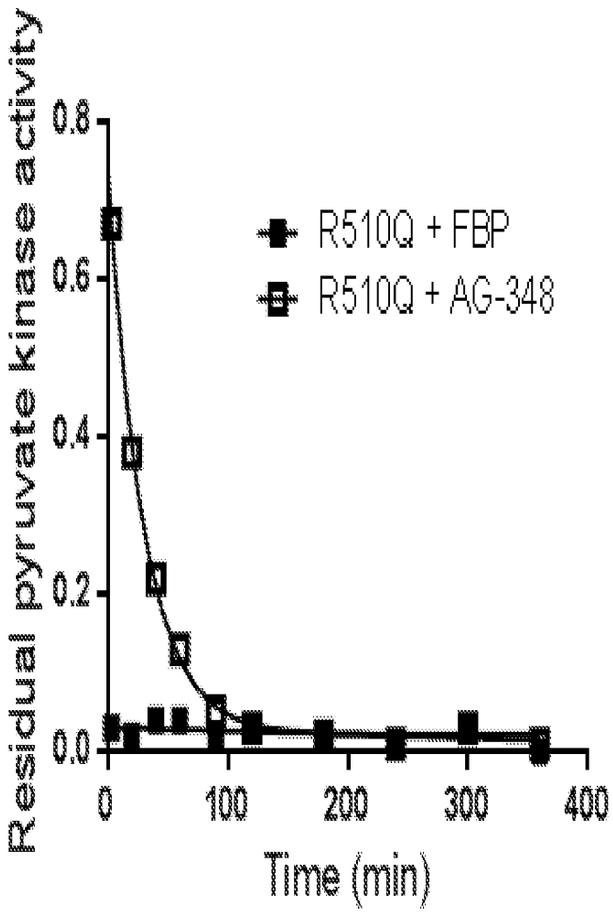
Kung et al. Blood 2017 (publication in press)

# AG-348 can stabilize PKR R510Q enzyme, and has slower off-rate than FBP *in vitro*

agios



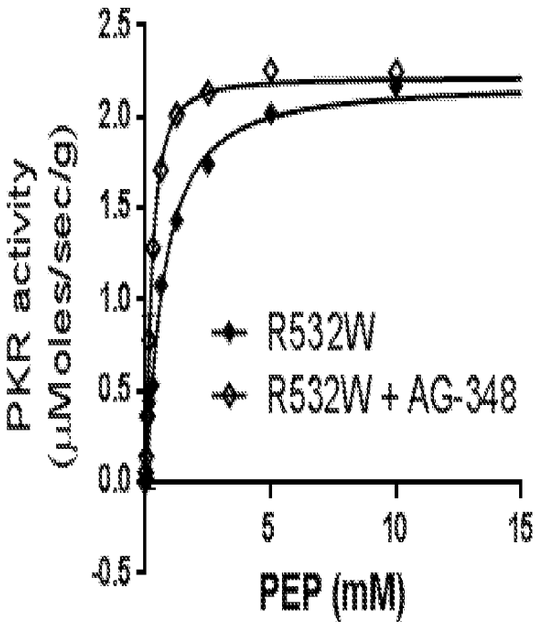

Plot of residual activity over time of WT or R510Q recombinant enzymes following incubation at 53° C

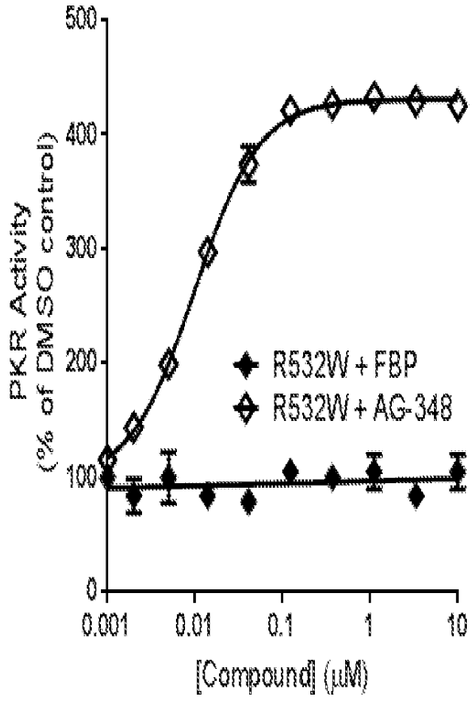



Off-rate measurement of AG-348 or FBP from recombinant R510Q enzyme

Kung et al. Blood 2017 (publication in press)

# AG-348 can activate mutant enzymes *in vitro* that abrogate activation by endogenous regulator FBP

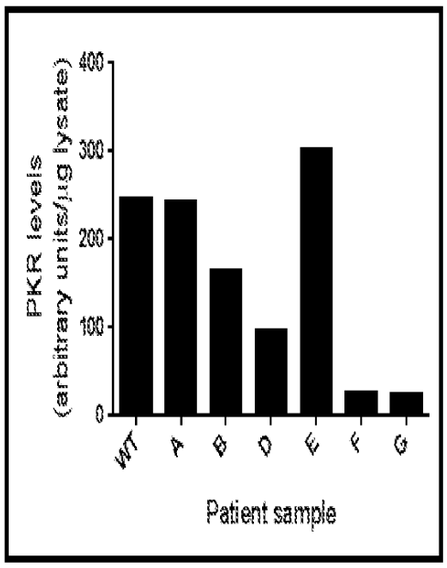


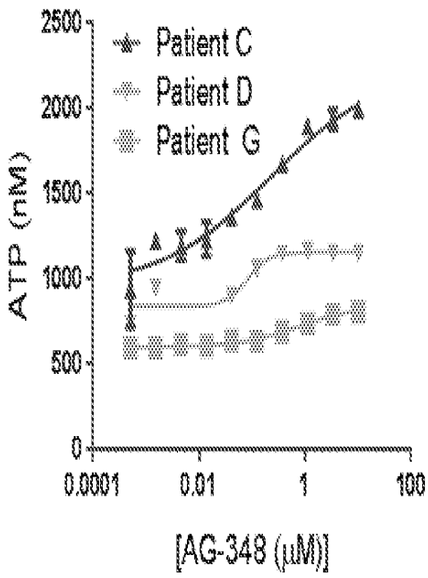



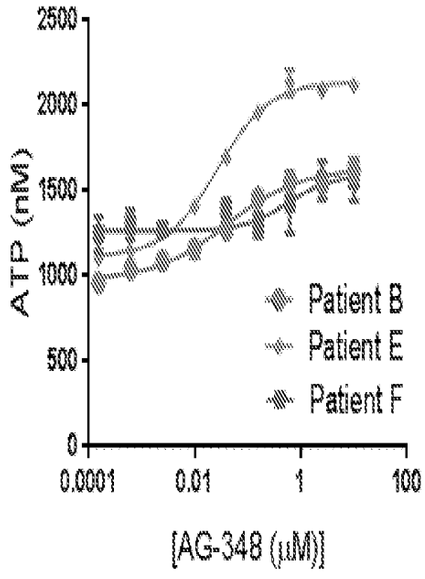

Kung et al. Blood 2017 (publication in press)

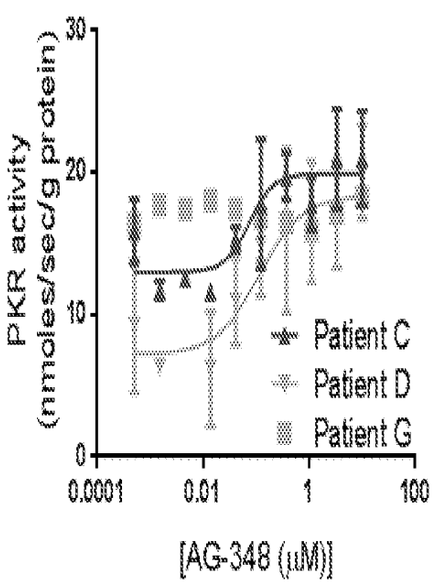
# AG-348 increases PKR activity and ATP levels in patient red cells *ex-vivo*

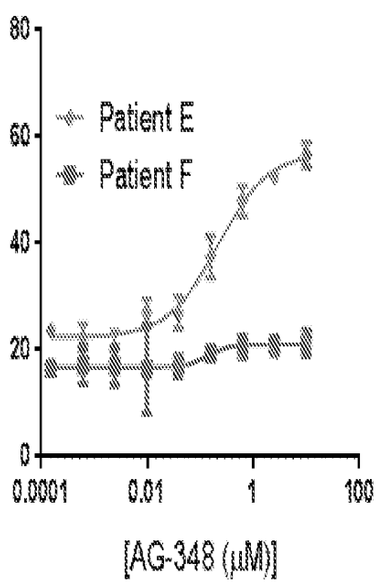
agios

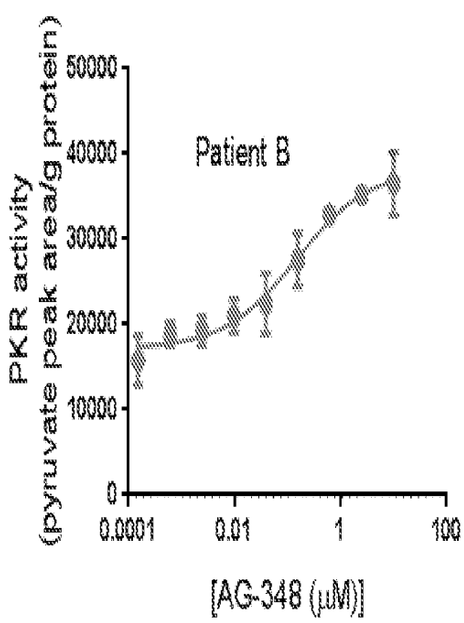



Kung et al. Blood 2017 (publication in press)

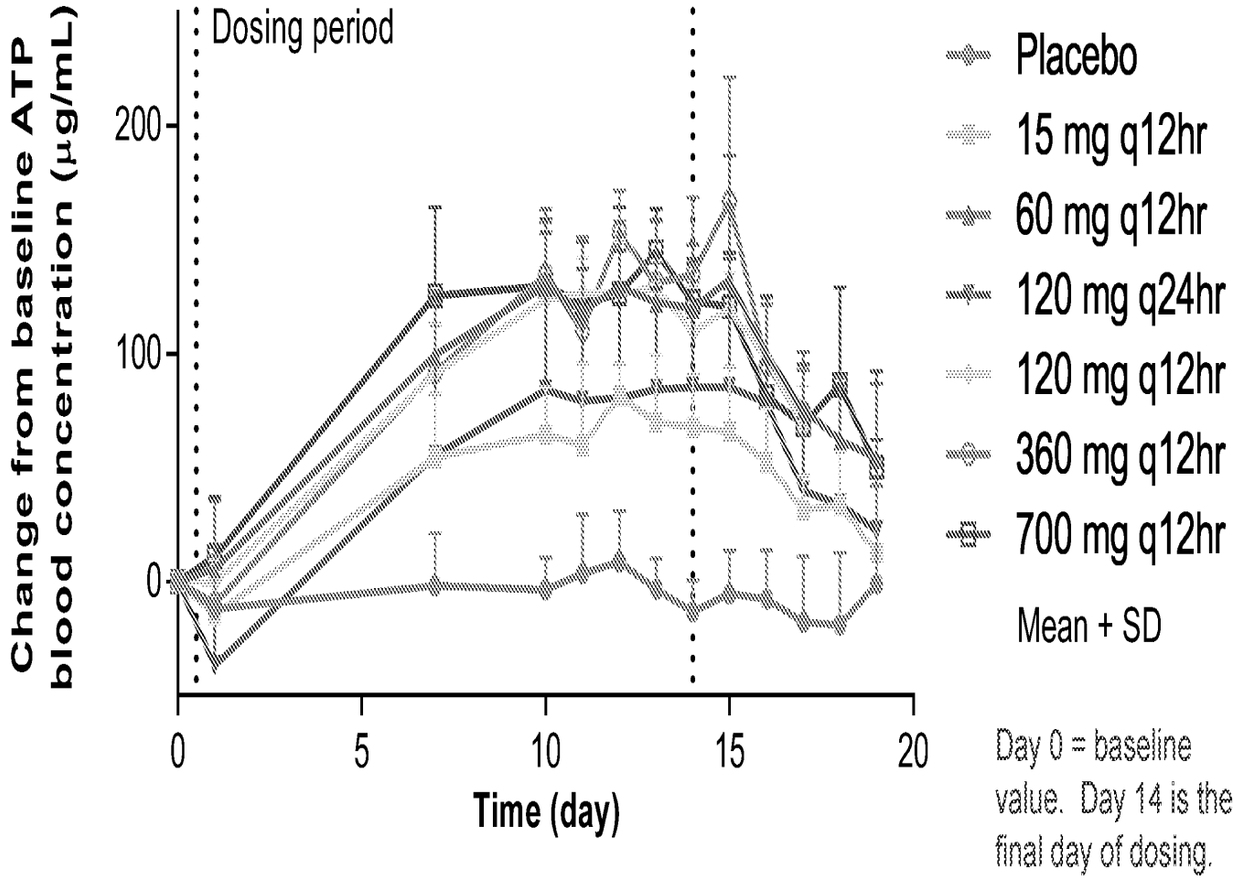


Ex. 2001-0765

# Robust dose-dependent *increase* in ATP in healthy volunteers' blood

agios

- A ~50% increase from baseline was observed with ≥120 mg AG-348
- Levels remained elevated through 120 hours after the final dose



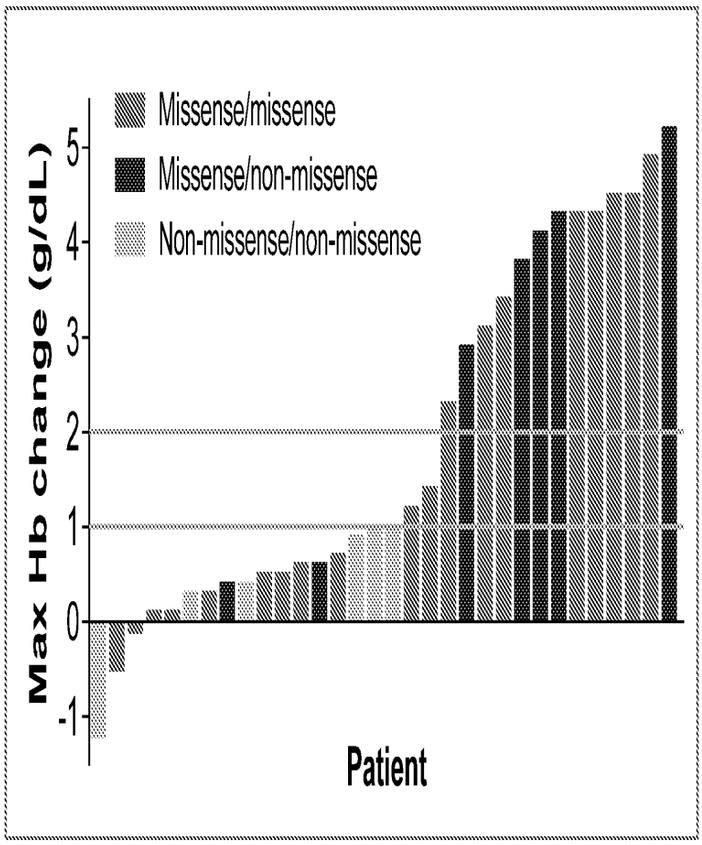

Data presented at ASH 2016, Yang et al.



Ex. 2001-0766

# Compelling Proof-of-Concept for AG-348, the First Disease Modifying Therapy for PK Deficiency

agios




52 patients enrolled;
17 completed first 24 weeks, 15 in extension
Results as of cut-off date of 23 September 2016

## DRIVE PK Learnings

- Robust hemoglobin increases in 15 / 32 patients; 15 / 26 patients with 1 or more missense mutation
- Responses are rapid and sustained; median time to response of 1.4 weeks
- Majority of responders seen at doses ≤50 mg BID and as low as 5 mg QD
- Well-tolerated beyond six months of dosing

Data presented at ASH 2016



Ex. 2001-0767

# Treatment of Rare Genetic Diseases by Small Molecule Allosteric Activators

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- Rare Genetic Diseases are individually rare, but as a disease class make an appreciable contribution to human morbidity
- Defective enzymes that are the cause of pathologic disregulated metabolsm are an emerging class of druggable targets
- In CDG1a, natural complementation of defective alleles and early lead ID efforts point toward the feasibility of small molecule activator/stabilizers
- AG-348, a small molecule activator/stabilizer with clinical POC, holds promise as the first disease-modifying treatment for PK-deficient hemolytic anemia



Ex. 2001-0768

# "Drug discovery is the most complicated team sport"



# TREATMENT OF RARE GENETIC DISEASES BY SMALL MOLECULE ALLOSTERIC ACTIVATORS

Stefan Gross, Ph.D.
Director, Enzymology and Biochemistry
Agios Pharmaceuticals



Ex. 2001-0770

# Treatment of Rare Genetic Diseases by Small Molecule Allosteric Activators

- Introduction to Rare Genetic Diseases
- Progress towards treatment of CDG1a, the most prevalent congenital disorder of glycosylation
- Development of AG-348, an enhancer of pyruvate kinase activity for the treatment of patients with PKR-deficient hemolytic anemia



Ex. 2001-0771

# What are Rare Genetic Diseases?

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**Conventional Wisdom:**

Rare Genetic Diseases are incurable diseases caused by low-prevalence mutations in genomic DNA

RGDs are neither Rare nor truly Genetic

Although individually rare, their aggregate occurrence is common enough that as a disease class, they cause a significant proportion of global human suffering and are worthy of medical intervention

In a large fraction of RGDs, the primary biological insult is the result of metabolic disregulation caused by decrease-of-function or destabilizing mutations in enzymes. Small-molecule drug technology holds the potential to ameliorate pathogenic metabolism and improve patients' lives through increasing enzyme activity.



Ex. 2001-0772

# Although individually rare, RGDs are collectively not uncommon

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***Table 1.*** Frequency of Inherited Metabolic Disorders in the West Midlands 1999-2003.

| Condition | Total number of cases | Birth prevalence live births | *Upper 95% ci* | *Lower 95% ci* |
|---|---|---|---|---|
| PKU* | 25 | 1 in12 420 | 5008 | 33 784 |
| Amino acid (excluding PKU) | 58 | 1 in 5354 | 2943 | 9990 |
| Urea cycle defects | 14 | 1 in 22 179 | 6702 | 90 909 |
| Carbohydrate | 19 | 1 in 16 343 | 4509 | 52 910 |
| Organic acid | 39 | 1 in 7962 | 3837 | 17 301 |
| Glycogen storage | 21 | 1 in 14 786 | 5504 | 44 643 |
| Lysosomal storage | 60 | 1 in 5175 | 2874 | 9551 |
| Purine and pyrimidine† | 4 | 1 in 77 628 | 12 063 | 2 000 000 |
| Fatty acid oxidation | 24 | 1 in 12 938 | 5123 | 35 971 |
| Peroxisomal | 23 | 1 in 13 500 | 5244 | 38 462 |
| Mitochondrial | 63 | 1 in 4929 | 2776 | 8953 |
| Metals‡ | 11 | 1 in 28 228 | 7418 | 147 059 |
| Lipids and steroids¶ | 20 | 1 in15 526 | 5647 | 48 544 |
| Porphyrin and haem* | 1 | 1 in 31 0510 | 10 070 | 3 333 333 |
| Miscellaneous | 14 | 1 in 22 179 | 6702 | 90 909 |
| **Total (including PKU)** | **396** | **1 in 784** | **619** | **970** |

The incidence of inherited metabolic disorders in the West Midlands, UK

S Sanderson, A Green, M A Preece, H Burton

Arch Dis Child 2006;91:896–899. doi: 10.1136/adc.2005.091447

# Massachusetts Comprehensive Newborn Screening: 48 most common Metabolic Rare Genetic Diseases

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2-Methyl 3-hydroxy butyric aciduria (2M3HBA)
2-Methylbutyryl-CoA dehydrogenase deficiency (2MBG)
3-hydroxy-3-methyl glutaric aciduria (HMG)
3-Methylcrotonyl-CoA carboxylase Deficiency (3MCC)
3-Methylglutaconic aciduria (3MGA)
Argininemia (ARG)
Argininosuccinic acidemia (ASA)
Benign hyperphenylalaninemia (H-PHE)
Biotinidase deficiency (BIOT)
Carbamoylphosphate synthetase deficiency (CPS)
Carnitine palmitoyltransferase IA deficiency (liver) (CPT IA)
Carnitine palmitoyltransferase II deficiency (CPT II)
Carnitine uptake defect (CUD)
Carnitine: acylcarnitine translocase deficiency (CACT)
Citrullinemia (CIT)
Citrullinemia type II (CIT II)
Defects of biopterin cofactor biosynthesis (BIOPT BS)
Defects of biopterin cofactor regeneration (BIOPT Reg)
Dienoyl-CoA reductase deficiency (DE RED)
Galactokinase deficiency (GALK)
Galactose epimerase deficiency (GALE)
Galactosemia (GALT)
Glutaric acidemia type I (GAI)
Glutaric acidemia type II (GA2)
Homocystinuria (HCY)
Hypermethioninemia (MET)
Hyperornithinemia, Hyperammoninemia, Homocitrullinemia Syndrome (HHH):
Isobutyryl-CoA dehydrogenase deficiency (IBG)
Isovaleric acidemia (IVA)
Long-chain L--OH acyl-CoA dehydrogenase deficiency (LCHAD)
Malonic Acidemia (MAL)
Maple syrup disease (MSUD)
Medium/short-chain L-3-OH acyl-CoA dehydrogenase deficiency (M/SCHAD)
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)
Medium-chain ketoacyl-CoA thiolase deficiency (MCKAT)
Methylmalonic acidemia: cobalamin A, B (Cbl A,B)
Methylmalonic acidemia: cobalamin C,D (Cbl C,D)
Methylmalonic acidemia: mutase deficiency (MUT)
Multiple carboxylase deficiency (MCD)
Ornithine transcarbamylase deficiency (OTC)
Phenylketonuria (PKU)
Propionic acidemia (PROP)
Short-chain acyl-CoA dehydrogenase deficiency (SCAD)
Tyrosinemia type I (TYR I)
Tyrosinemia type II (TYR II)
Tyrosinemia type III (TYR III)
Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD)
β-Ketothiolase deficiency (BKT)

Disease management in most cases is palliative or limited to diet restriction.

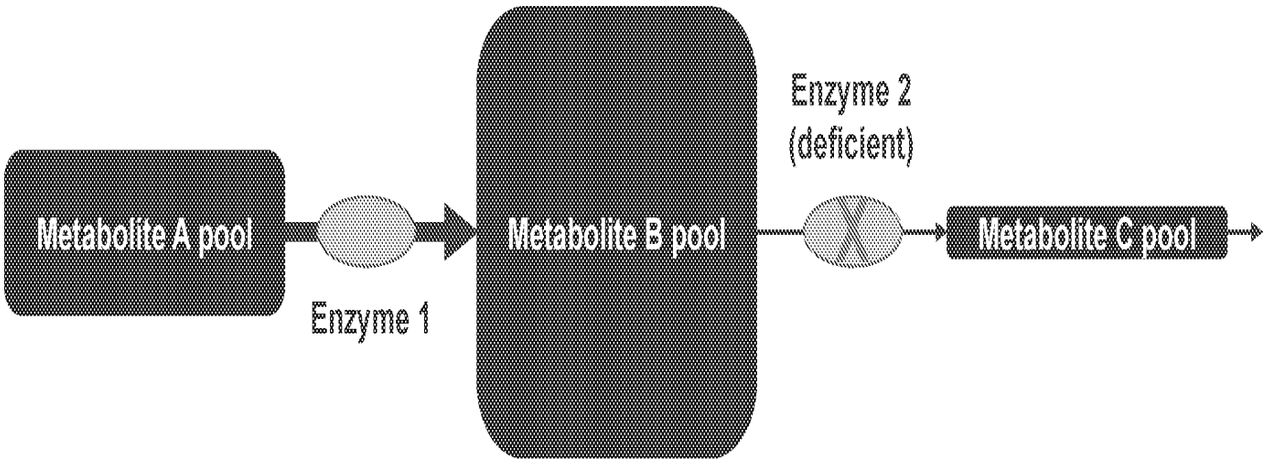


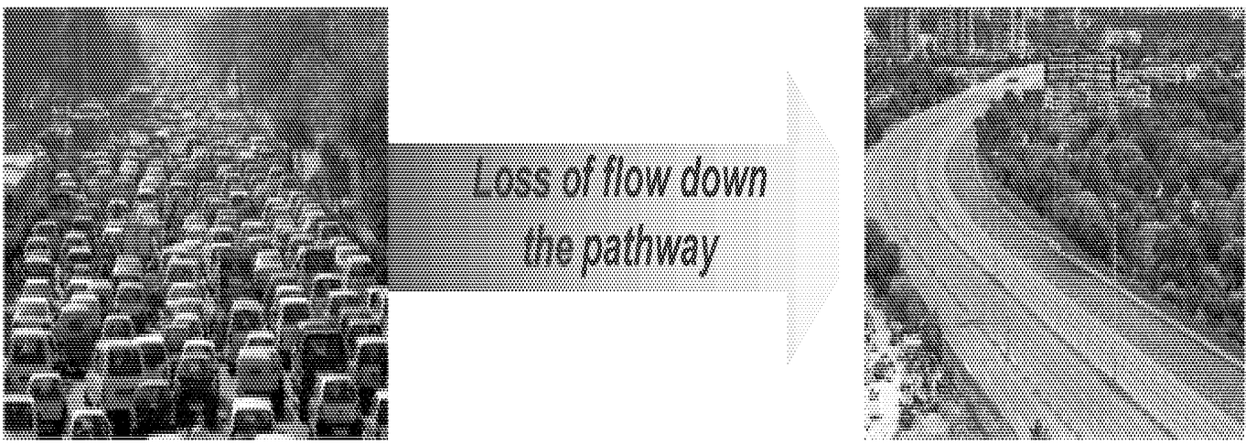
Ex. 2001-0774

# Rare Genetic Diseases due to defects in metabolic pathways

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*Disease results from the toxic buildup of "Metabolite B" and/or the deficit of "Metabolite C" or other downstream products*



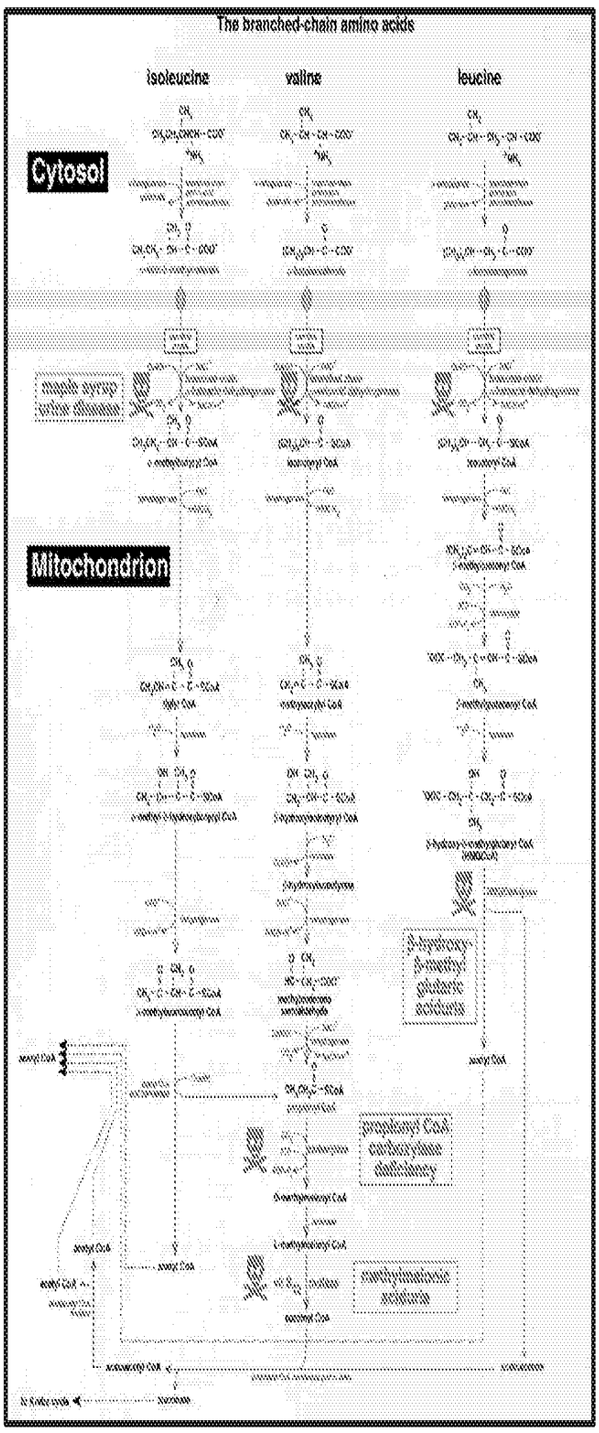






Ex. 2001-0775

# Multiple diseases may exist in any given metabolic pathway

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## Maple syrup urine disease

Deficiency of branched chain **α-ketoacid dehydrogenase complex** causes accumulation of branched-chain amino acids isoleucine, valine, and leucine and their corresponding α-ketoacids.

## β-hydroxyl-β-methyl glutaric aciduria

Autosomal recessive defect in **HMCoA lyase** of leucine catabolism and ketogenesis which is associated with hypoketotic hypoglycemia, hyperammonaemia, and metabolic acidosis

## Propionyl CoA carboxylase deficiency

Presents early in the neonatal period with progressive encephalopathy, leading to mortality by secondary hyperammonemia, infection, cardiomyopathy, or basal ganglial stroke.

## Methylmalonic aciduria

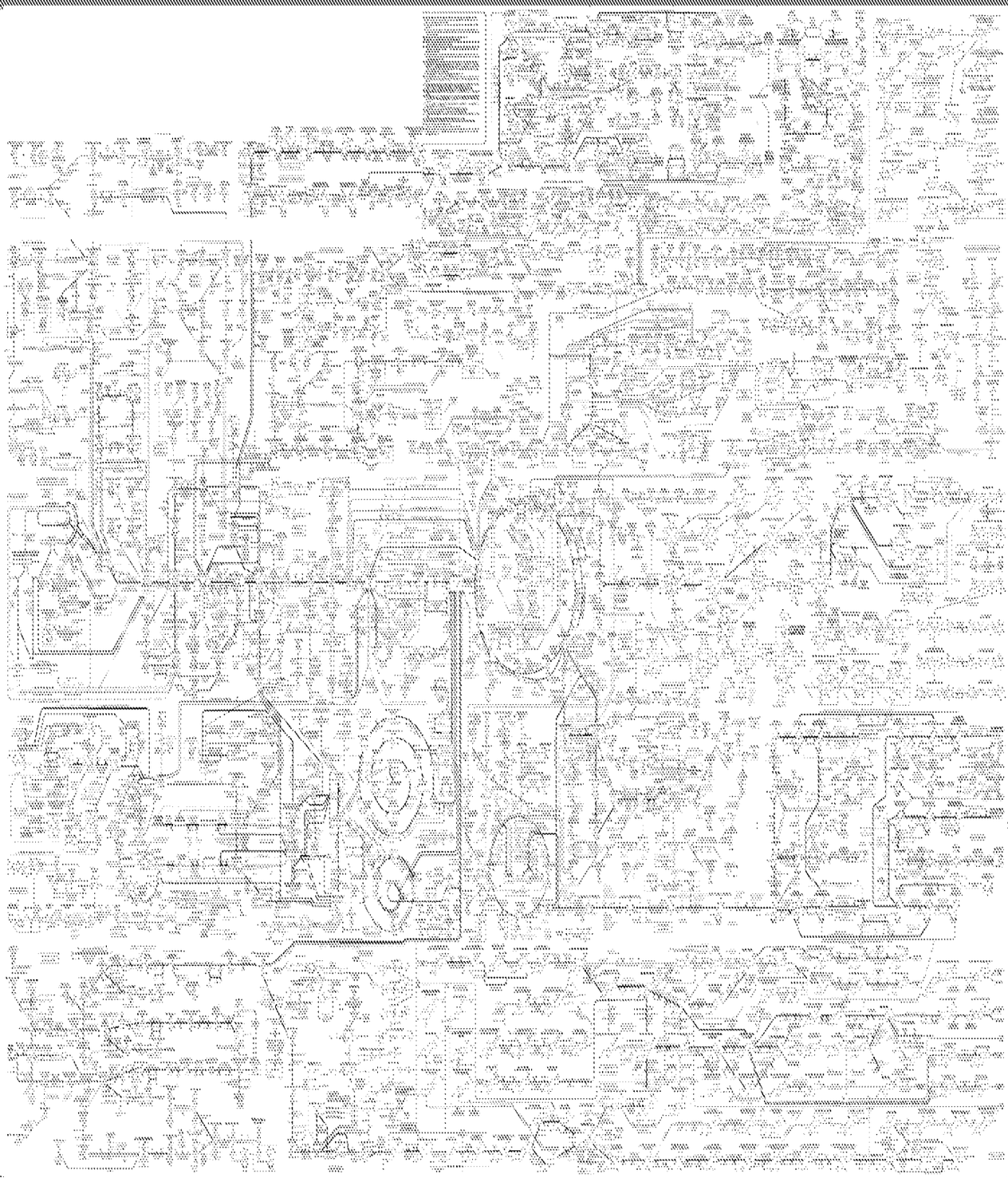
Caused by a deficiency of **L-methylmalonyl CoA reductase**. Symptoms include lethargy, delayed psychomotor development, seizures, acute encephalopathy. Most affected individuals die in infancy.



Ex. 2001-0776

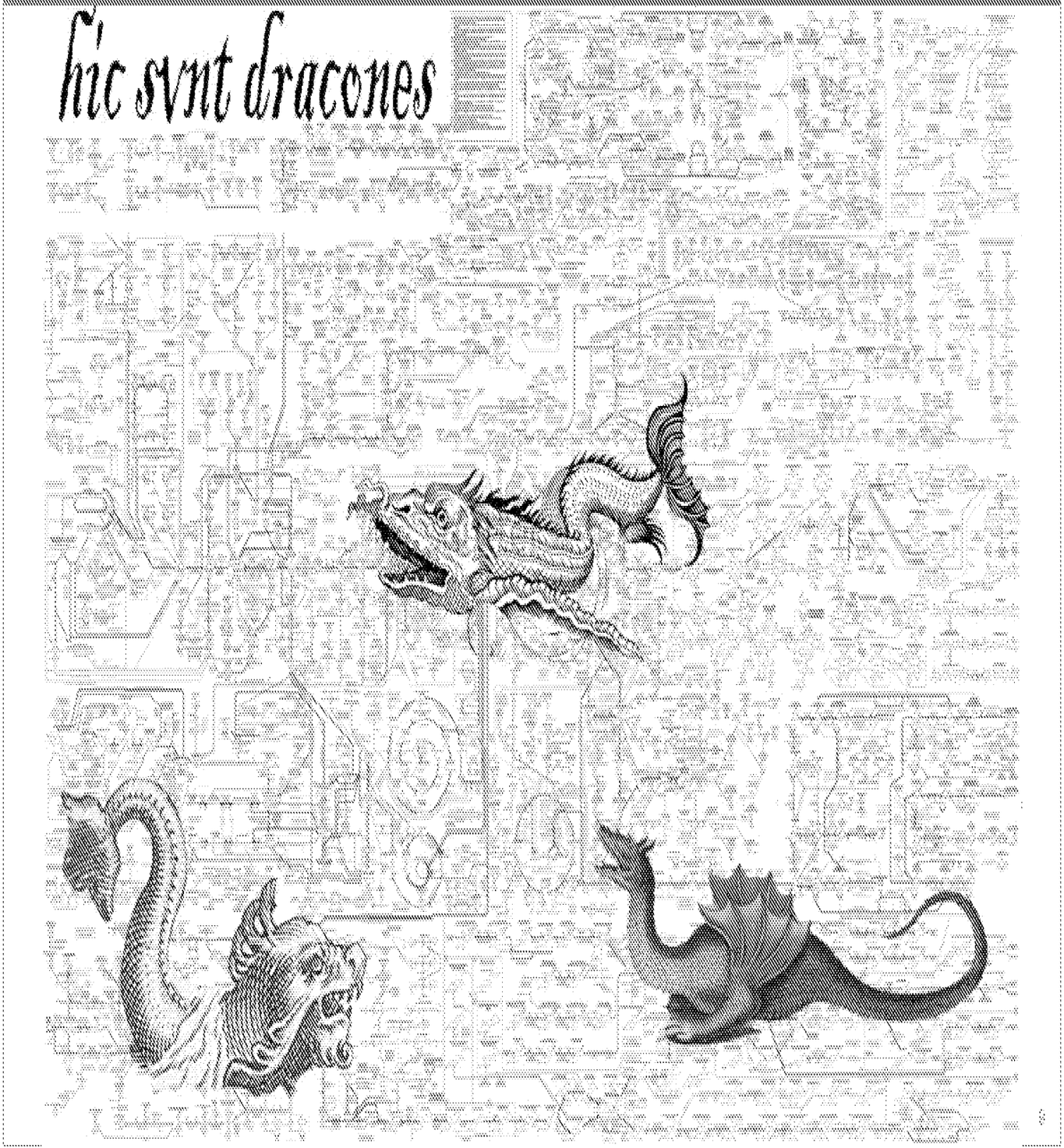
# The human metabolome contains > 75,000 small molecules interconverted by > 5700 enzymes

agios

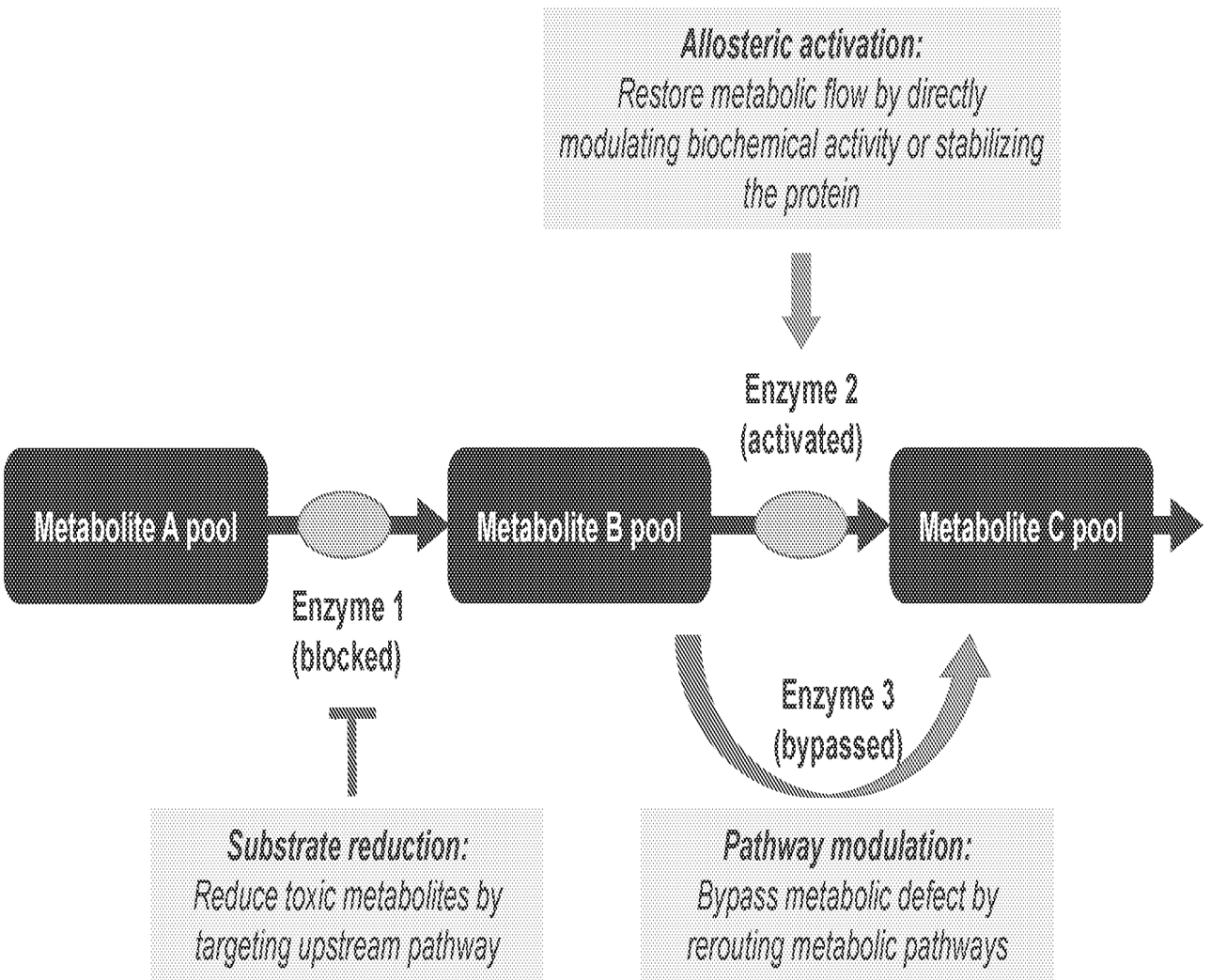


The human metabolome contains > 75,000 small molecules interconverted by > 5700 enzymes

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# Correcting metabolic defect with small molecules



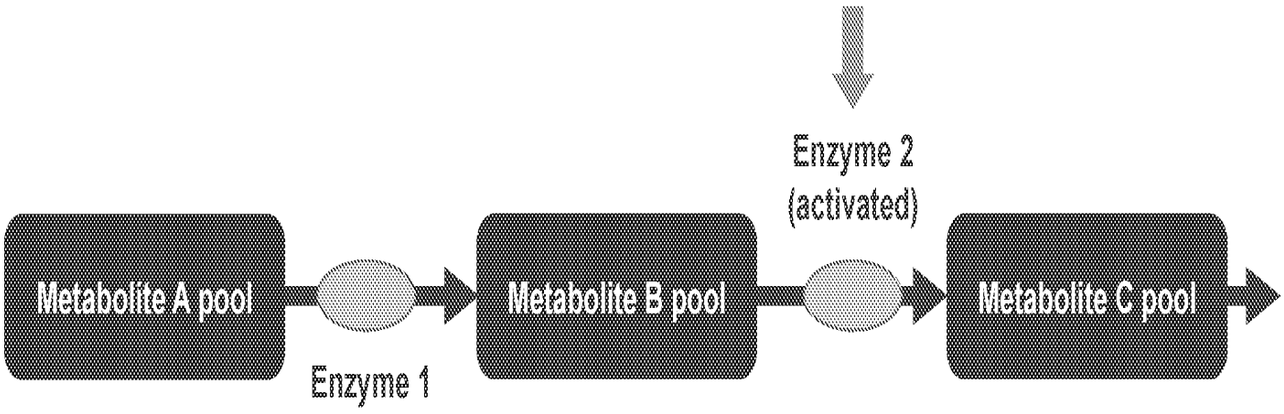


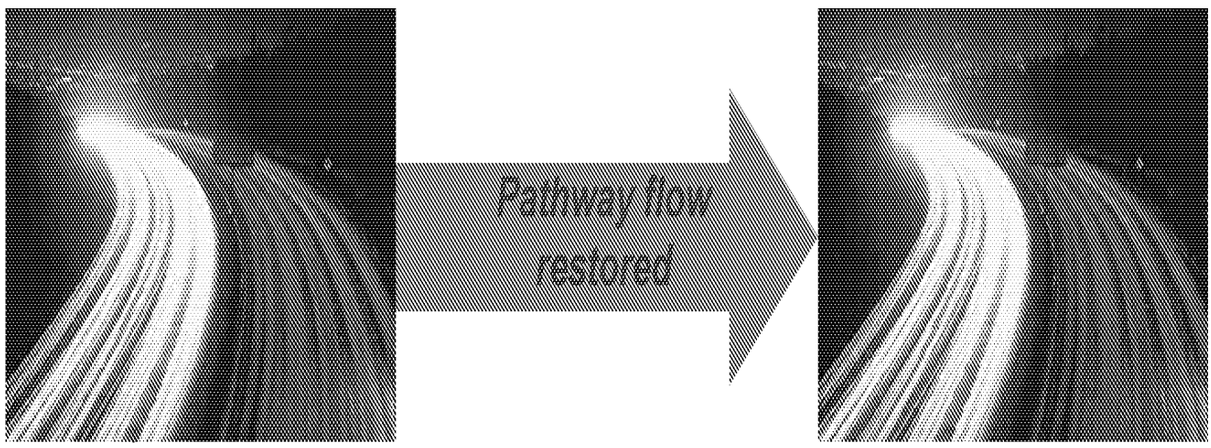

Ex. 2001-0779

# Correcting metabolic defect with small molecules

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***Allosteric activation:***
*Restore metabolic flow by directly modulating biochemical activity or stabilizing the protein*






Ex. 2001-0780

# It's very easy to design an enzyme inhibitor

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enzyme activators are a bit more challenging.....



Ex. 2001-0781

# Challenges unique to Metabolic RGDs Drug Discovery and Development

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**>600 RGDs described** featuring inherited mutation in single metabolic enzyme

Vast majority of RGDs **lack therapeutic options**

1. Most diseases are intracellular & CNS defects not amenable to protein Tx
2. Which diseases/pathways are amenable to small molecule Tx?
3. Diverse range of mutations, including loss of function
4. Complexity of metabolic enzymes as drug targets
5. Lack of disease models / understanding in rare diseases
6. Lack of defined clinical endpoints/regulatory pathways



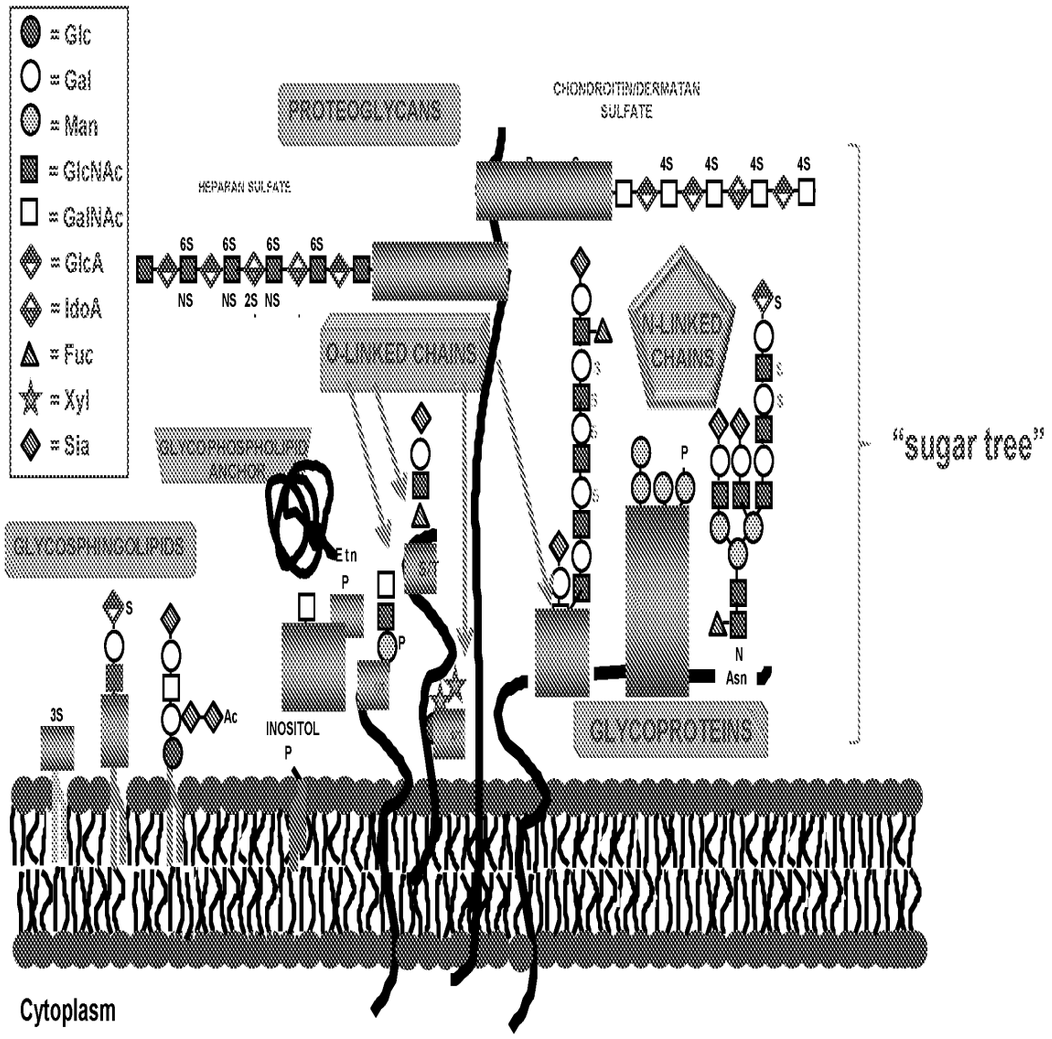
Ex. 2001-0782

# PROGRESS TOWARDS TREATMENT OF CDG1A

# Glycosylation:
# 2% of the genome devoted to making glycans

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Glycosylation impacts a broad spectrum of cellular functions, cell-cell interactions, and signaling

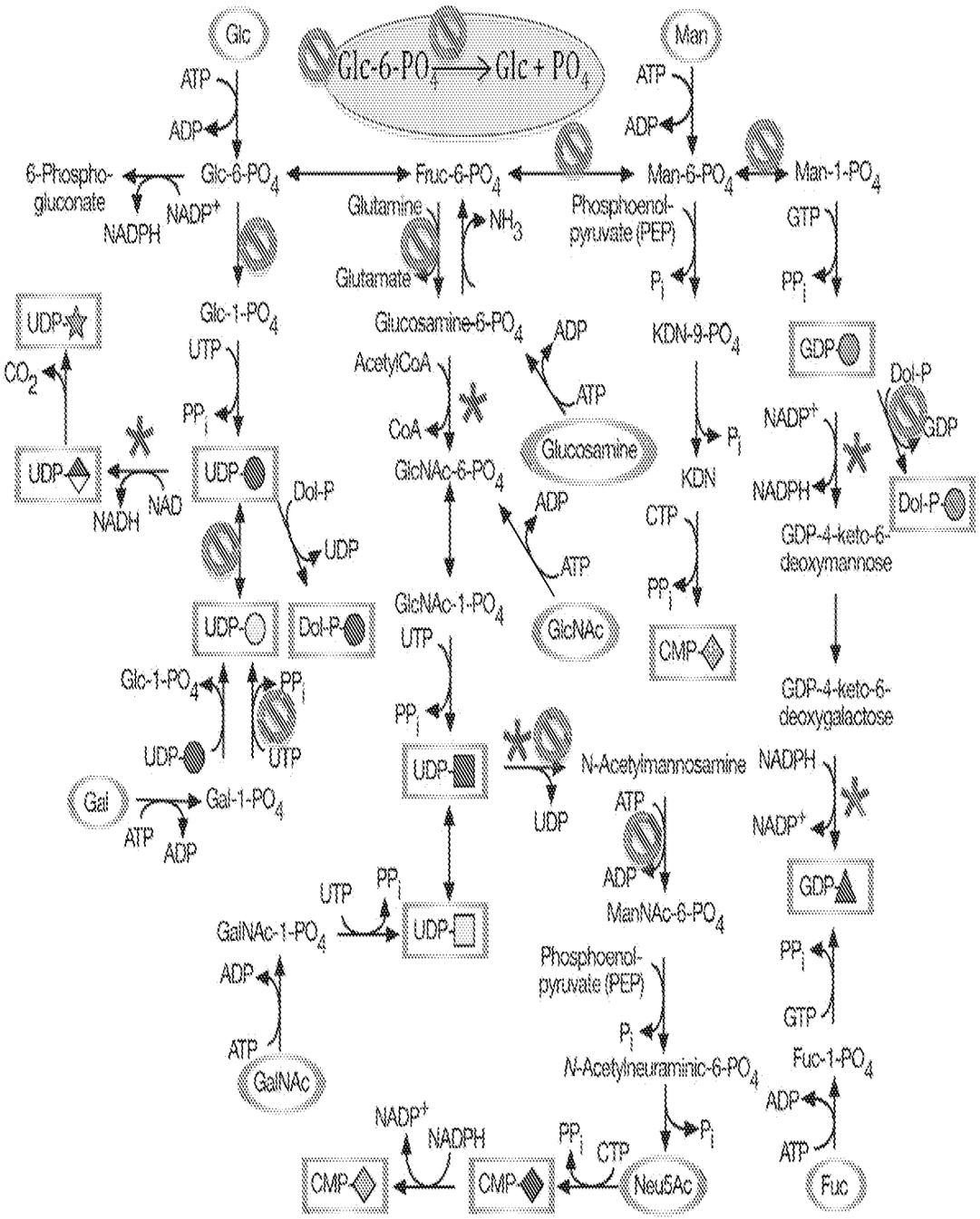



Glycosylation defects can occur at all phases of "sugar tree" biosynthesis (initiation, assembly, trimming, and elongation) → Congenital Disorders of Glycosylation" (CDG)



Ex. 2001-0784

# Monosaccharide metabolism in mammals and human glycosylation disorders.




Glycosylation Disorder

Hudson H. Freeze J. Biol. Chem. 2013;288:6936-6945

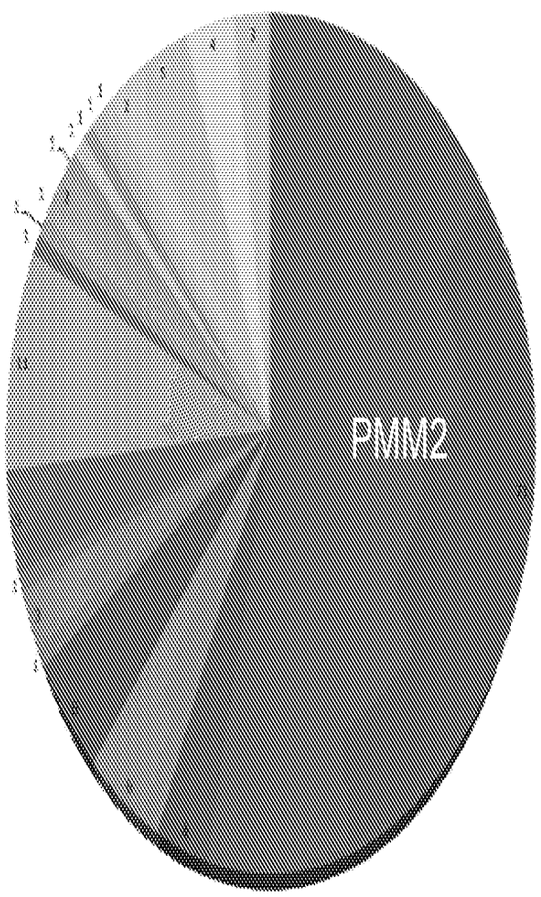


Ex. 2001-0785

# Phosphomannomutase-2 (PMM2) CDG is the most common type of CDG

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PMM2-CDG remains the most commonly diagnosed CDG, >54%.
132 confirmed cases of CDGs identified by Mayo Clinic in the past few years




- PMM2*
- MPI*
- ALG6*
- ALG3*
- DPM1*
- ALG12*
- ALG8*
- DPAGT1*
- ALG1*
- DOLK*
- ALG11*
- SRD5A3*
- DDOST*
- MOCS**
- COG7**
- COG1**
- COG8**
- COG5**
- SLC35A2**
- PGM1***
- ATP6V0A2

Transferrin and apoCIII (6min) LCMS analysis allows for the identification of CDG subtypes routinely at Mayo clinic (some combined with verification by enzyme activity and/or DNA sequence)

* Type I missing 1-2 sugar chains (unoccupied glycosylation sites), defective in glycosylation initiation/assembly. PMM2-CDG belongs to type I

** Type II abnormal chains; defective in glycan tree elongation or trimming

*** Mixed Type

*With permission from Raymond K et al Poster #483,International Congress of Inborn Errors of Metabolism Conference, Sept 4 2013*



Ex. 2001-0786

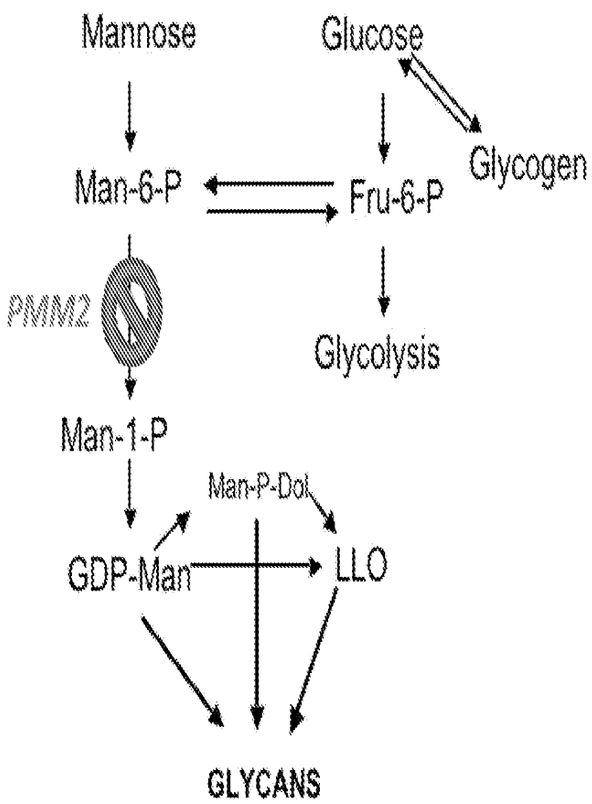
# PMM2-Congenital Disorders of Glycosylation
## *Deficiency in PMM2 (Phosphomannomutase 2)*

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### Disease Overview

- One of over 30 CDGs characterized by abnormal glycosylation of N-linked oligosaccharides
- Severely debilitating and often fatal disorder with onset in infancy & multi-systemic clinical manifestations:
  - Developmental delay
  - Cerebellar atrophy
  - Peripheral neuropathy
  - Stroke-like episodes or comatose episode
  - Epilepsy
  - Retinal pigmentary degeneration
  - Strabismus
  - Skeletal abnormalities, dysmorphic features
  - Hepatopathy
- Divided into three stages: infantile multisystem, late-infantile and childhood ataxia-intellectual disability, and adult stable disability
- No treatment available
- Reported incidence of 1 in 20,000-80,000 Caucasian births; >700 cases reported so far in literature but likely underdiagnosed

### Disease Pathway & Pathophysiology

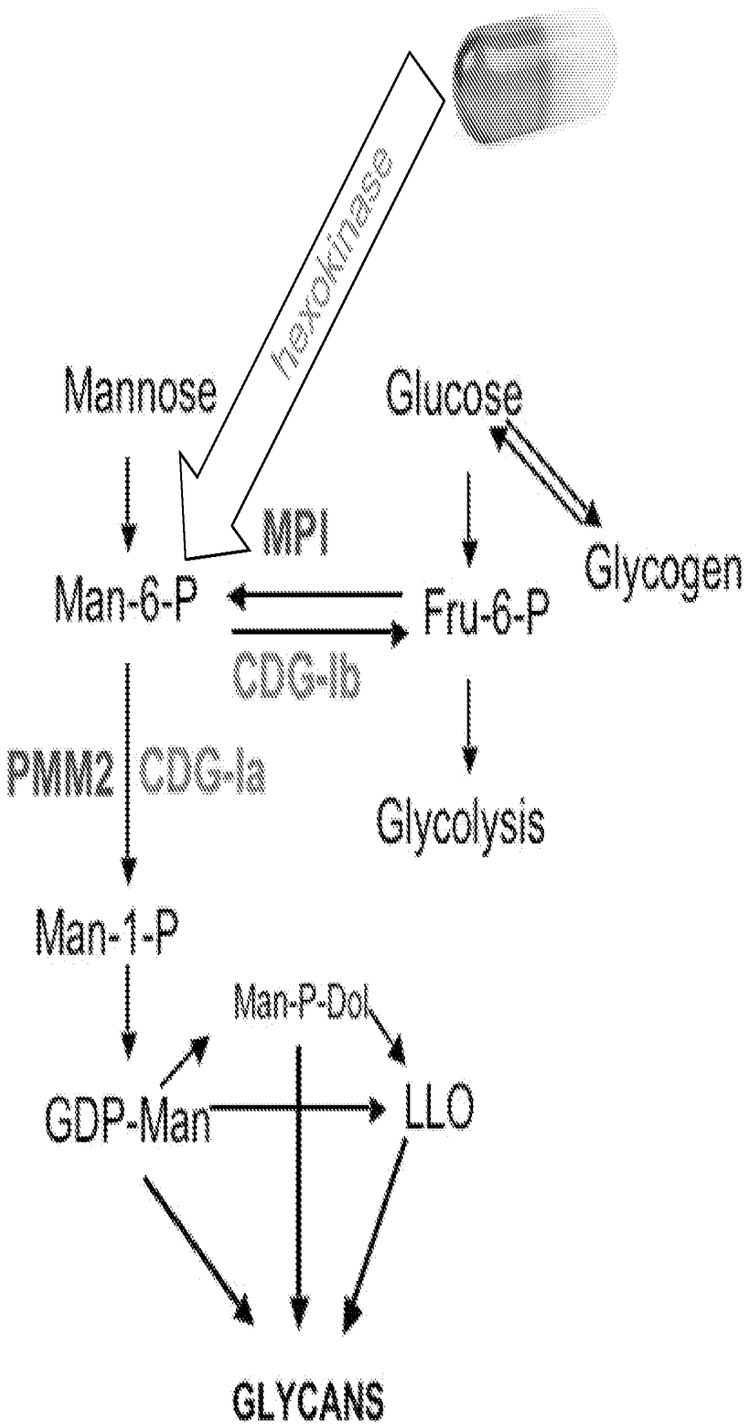



*PMM2 defect leads to shortage of GDP-Mannose required to form oligosaccharides → abnormal glycosylation in a range of tissues*



Ex. 2001-0787

# Mannose therapy: simple but ineffective in CDG1a




- MPI-deficient patients (CDG1b) benefit from alimentary addition of Man because it bypasses the defective step by allowing the formation of M6P via hexokinase
- Why don't CGD1a patients response to mannose therapy?
- PMM2-deficient patients (CDG1a) do not benefit due to cannibalization by MPI, converting M6P to F6P



Ex. 2001-0788

# Therapeutic Approaches for PMM2-deficient patients

agios

| Approach | Rationale | Advantages | Disadvantages |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
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Ex. 2001-0789

# PMM2-Congenital Disorders of Glycosylation
## *Deficiency in PMM2 (Phosphomannomutase 2)*

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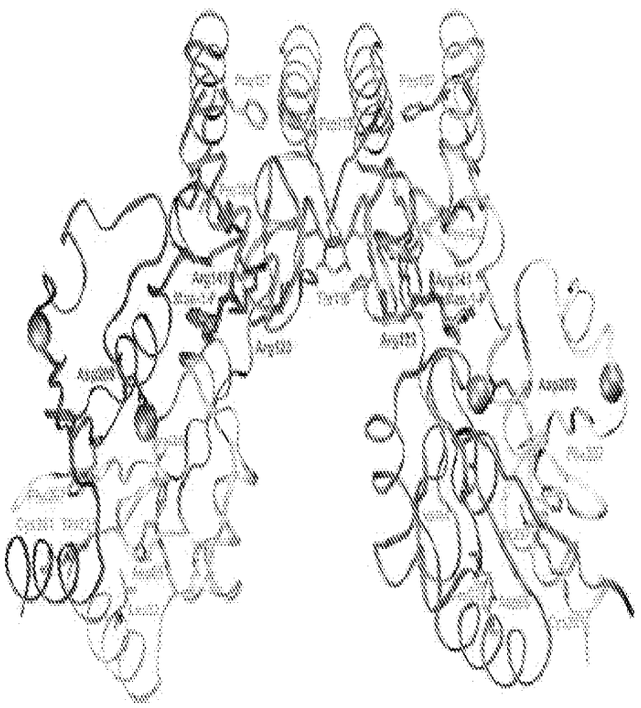
**Mutations & enzyme defects:**

- >100 mutations reported, but nearly 85% are missense mutations
- Most are compound heterozygous with residual PMM2 activity ranging from 5-40%
  - Those with >50% activity are asymptomatic

**Agios Therapeutic Approach**

- Allosteric activator approach: Rescue protein instability and PMM2 activity
  - Restoring activity to >50% of WT may be sufficient

**PMM2 crystal structure**



**Mutation effects**

- Green: Dimerization
- Red: Catalytic residues
- Yellow: Folding/stability

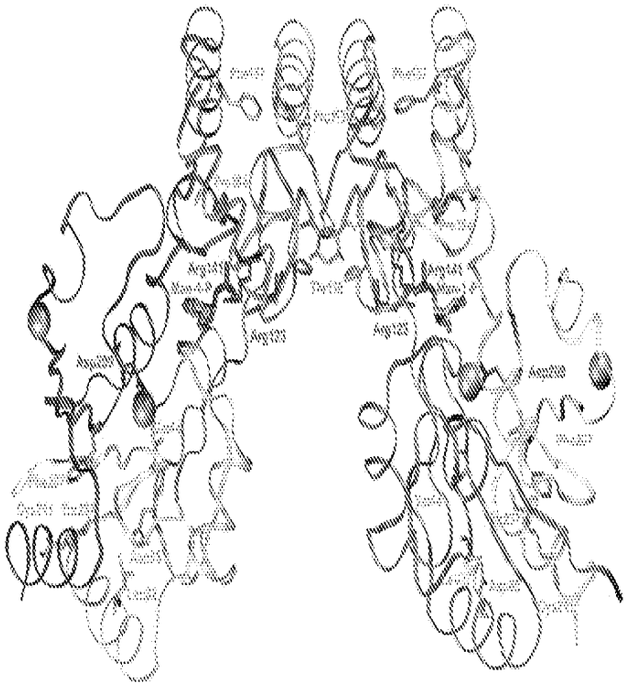
*Vega et al., JIMD (2011)*



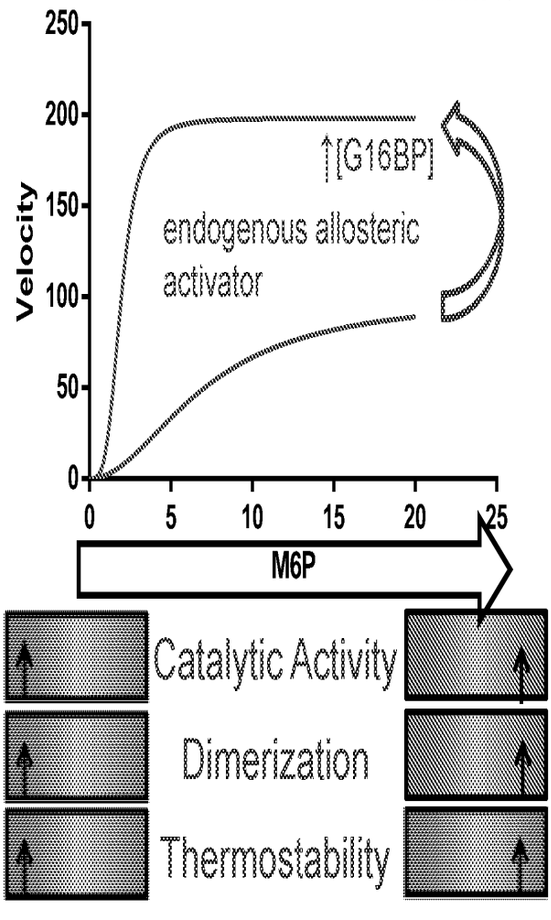
Ex. 2001-0790

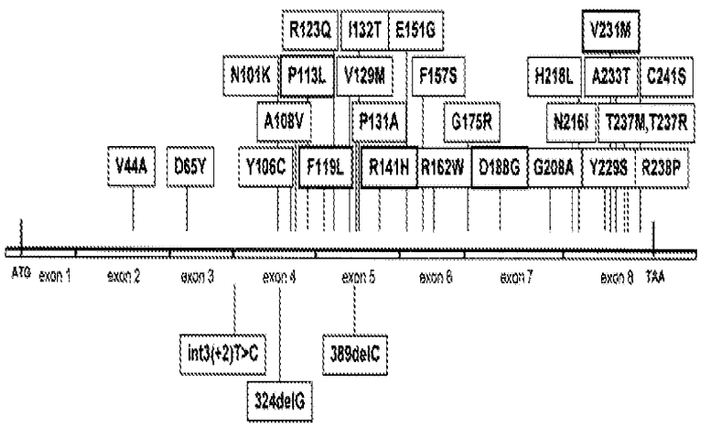
# PMM2: A dimeric enzyme with allosteric AND substrate-level activation

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- Mis-sense mutations affect the three axes of enzyme function to varying degrees
- Dimeric nature facilitates the possibility of both mutant homo- and hetero-zygotes



Ex. 2001-0791

# PMM2 Homozygous Mutants: The Ghosts of Elsinore

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**Table 3** Expected and observed numbers of CDG1 patients with different genotypes. Frequencies, by counting, of F119L and R141H are 16/36=0.44 and 16/36=0.44, respectively

| Genotypes | Expected frequency | Expected number |
|---|---|---|
| F119L/F119L | $(0.44)^2$ | 3.5 |
| F119L/R141H | $(2\times0.44\times0.44)$ | 7.0 |
| R141H/R141H | $(0.44)^2$ | 3.5 |
| Other combinations[a] | 0.26 | 4.0 |

[a]F119L/other, R141H/other, and other/other.
$P = 0.0009$; $\chi^2 = 16.4$; df = 3.

ARTICLE

**Lack of Hardy-Weinberg equilibrium for the most prevalent *PMM2* mutation in CDG-Ia (congenital disorders of glycosylation type Ia)**

Els Schollen[1], Susanne Kjaergaard[2], Eric Legius[1], Marianne Schwartz[2] and Gert Matthijs[1]

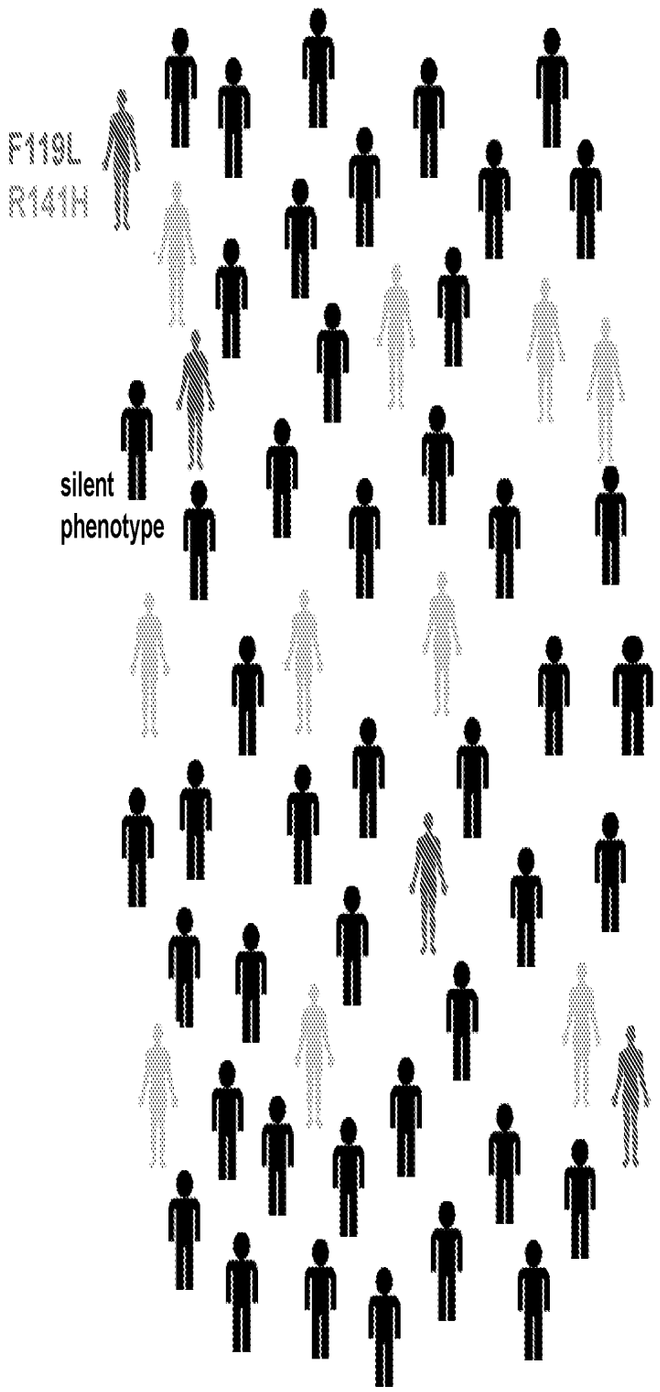
ORIGINAL PAPER

**Absence of homozygosity for predominant mutations in *PMM2* in Danish patients with carbohydrate-deficient glycoprotein syndrome type 1**

Susanne Kjaergaard, Flemming Skovby and Marianne Schwartz

**Lack of Homozygotes for the Most Frequent Disease Allele in Carbohydrate-Deficient Glycoprotein Syndrome Type 1A**

Gert Matthijs,[1] Els Schollen,[1] Emile Van Schaftingen,[1] Jean-Jacques Cassiman,[1] and Jaak Jaeken[2]




Agios Pharmaceuticals – CONFIDENTIAL

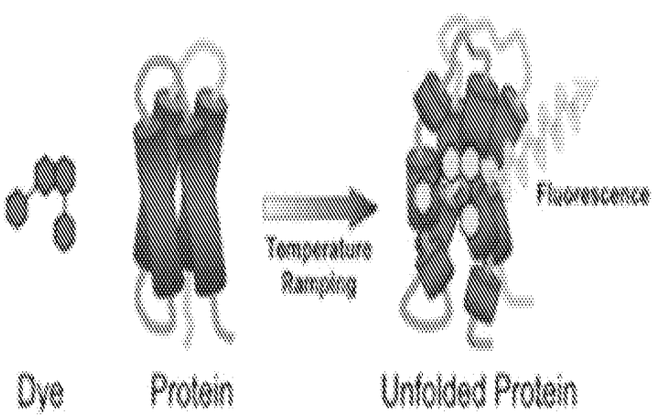


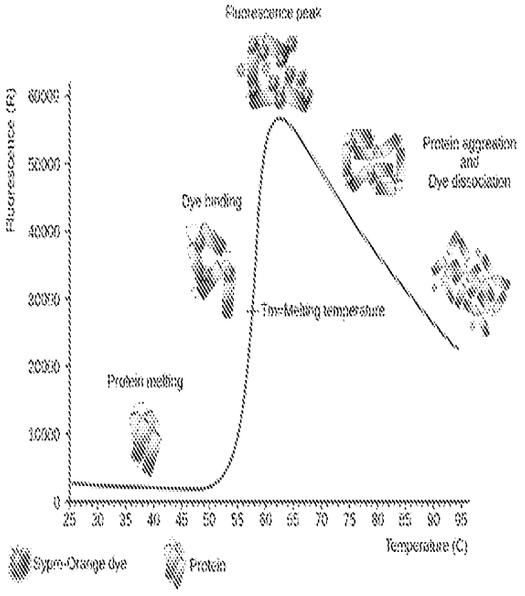
Ex. 2001-0792

# Biochemical Tools to Study PMM2

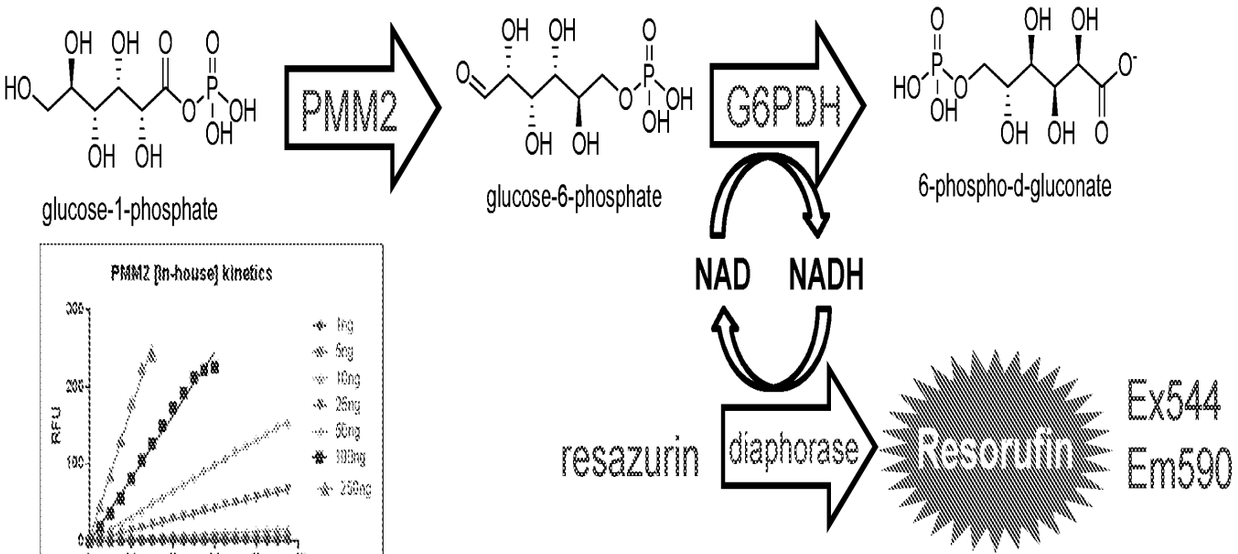
agios

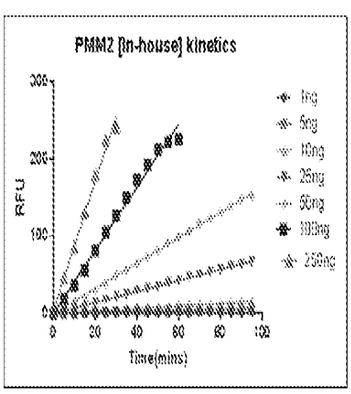
- Thermostability Assay




- Continuous Kinetic Assay with substrate analogue



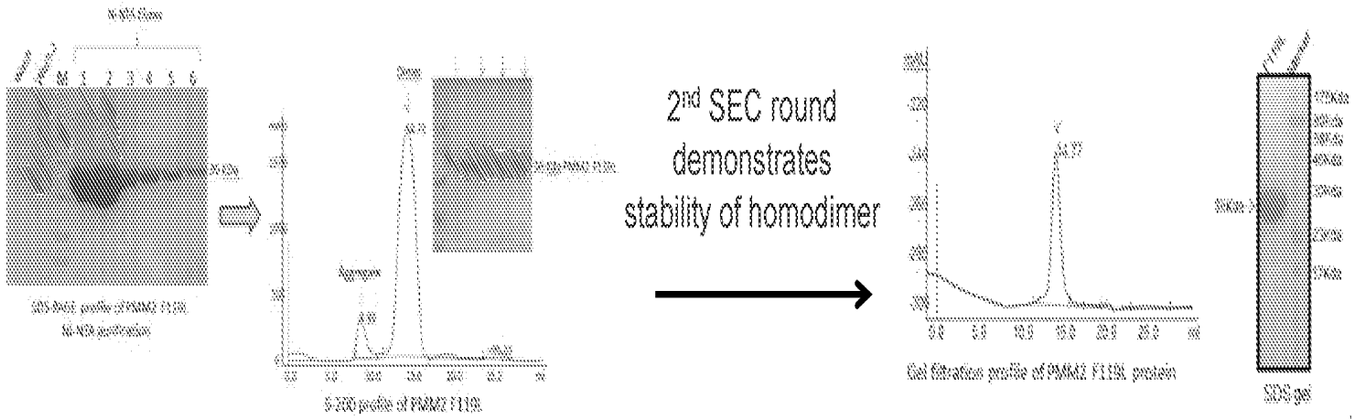





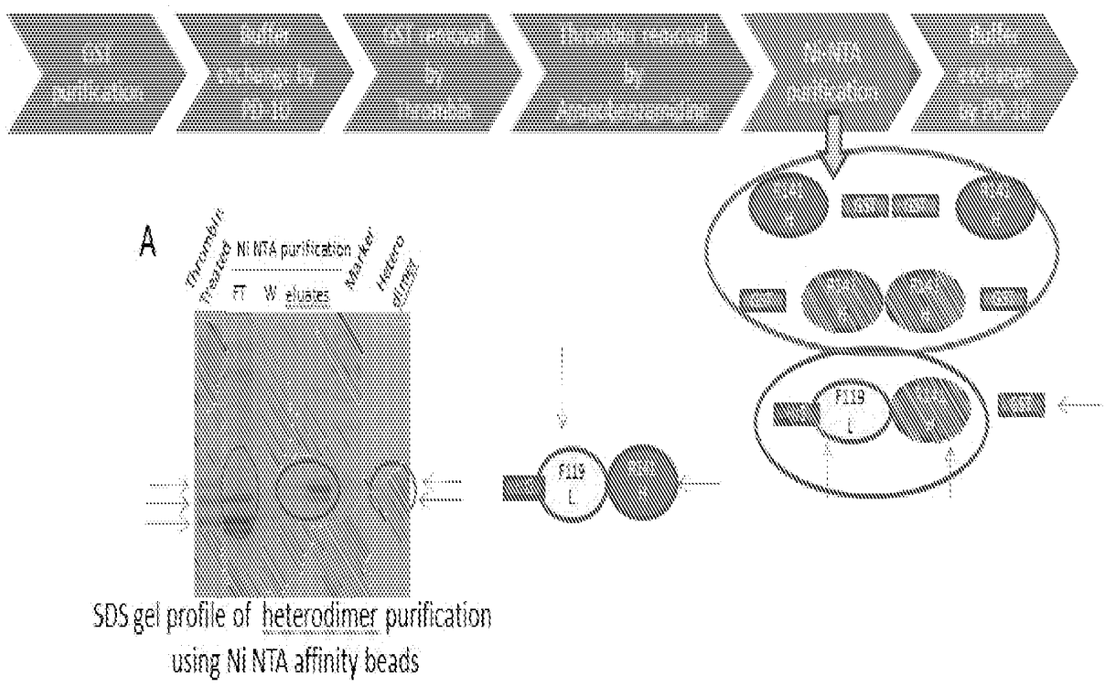

Ex. 2001-0793

# Production of Homodimer and Heterodimer PMM2

- WT, F119L, R141H produced in E. coli




- F119L/R141H heterodimer produced in E. coli



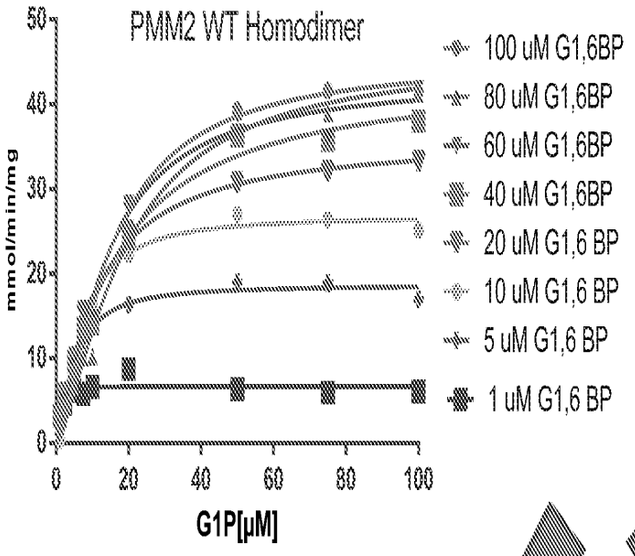

SDS gel profile of heterodimer purification using Ni NTA affinity beads



Ex. 2001-0794

# Kinetic characterization of wt PMM2

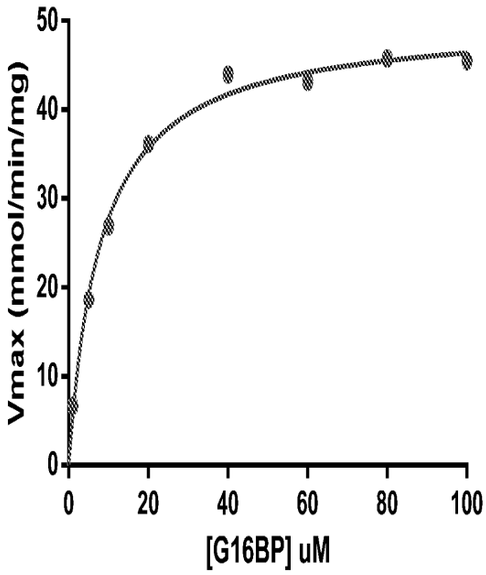
agios



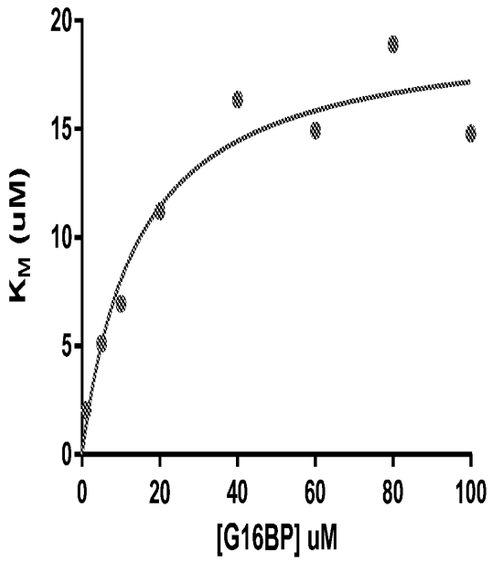

| G16 BP (µM) | Km G1P (µM) | Hill Slope | Vmax (µmol/min/mg x $10^{-1}$) | Kcat ($s^{-1}$) | Kcat/Km $M^{-1}s^{-1}$ X $10^{5}$ |
|---|---|---|---|---|---|
| 100 | 14.79 | 1.42 | 45.42 | 1.97 | 1.33 |
| 80 | 18.91 | 1.43 | 45.76 | 1.99 | 1.05 |
| 60 | 13.61 | 1.37 | 43.15 | 1.87 | 1.38 |
| 40 | 16.36 | 1.09 | 43.93 | 1.91 | 1.17 |
| 20 | 11.21 | 1.14 | 36.14 | 1.57 | 1.4 |
| 10 | 6.942 | 1.54 | 26.86 | 1.17 | 1.68 |
| 5 | 5.107 | 1.49 | 18.66 | 0.81 | 1.59 |
| 1 | 1.76 | 2.25 | 6.70 | 0.29 | 1.65 |

$K_{cat}/K_M$ is constant

**PMM2 Catalytic Efficiency as a function of activator**




**PMM2 Substrate Affinity as a function of activator**



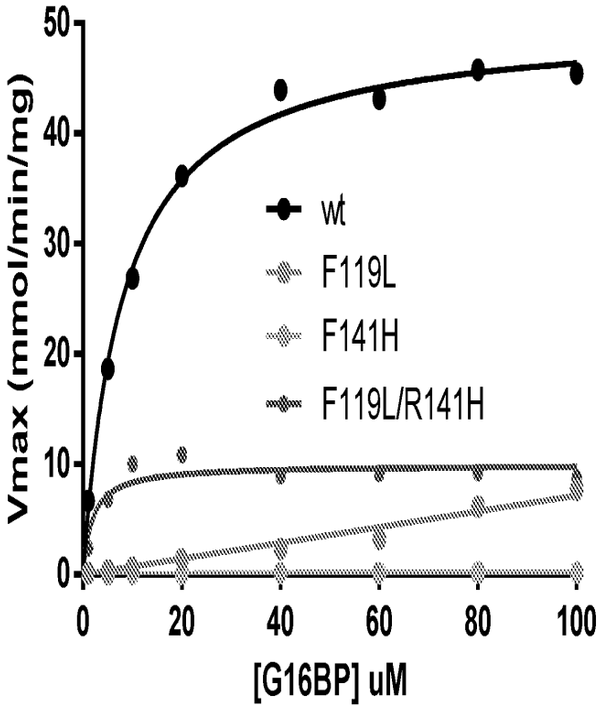



Ex. 2001-0795

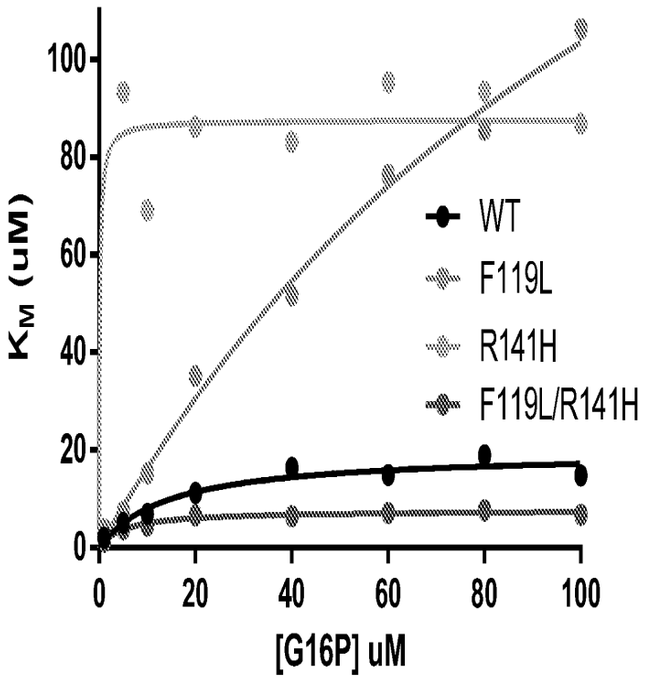
# Heterodimerization provides a distinct kinetic advantage for PMM2 mutant proteins

agios

**PMM2 Catalytic Efficiency as a function of activator**




**PMM2 Substrate Affinity as a function of activator**




| Isozyme | $k_{cat}$ defect | $K_M$ defect |
|---|---|---|
| F119L/F119L | moderate | moderate |
| R141H/R141H | severe | severe |
| F119L/R141H | mild | mild |

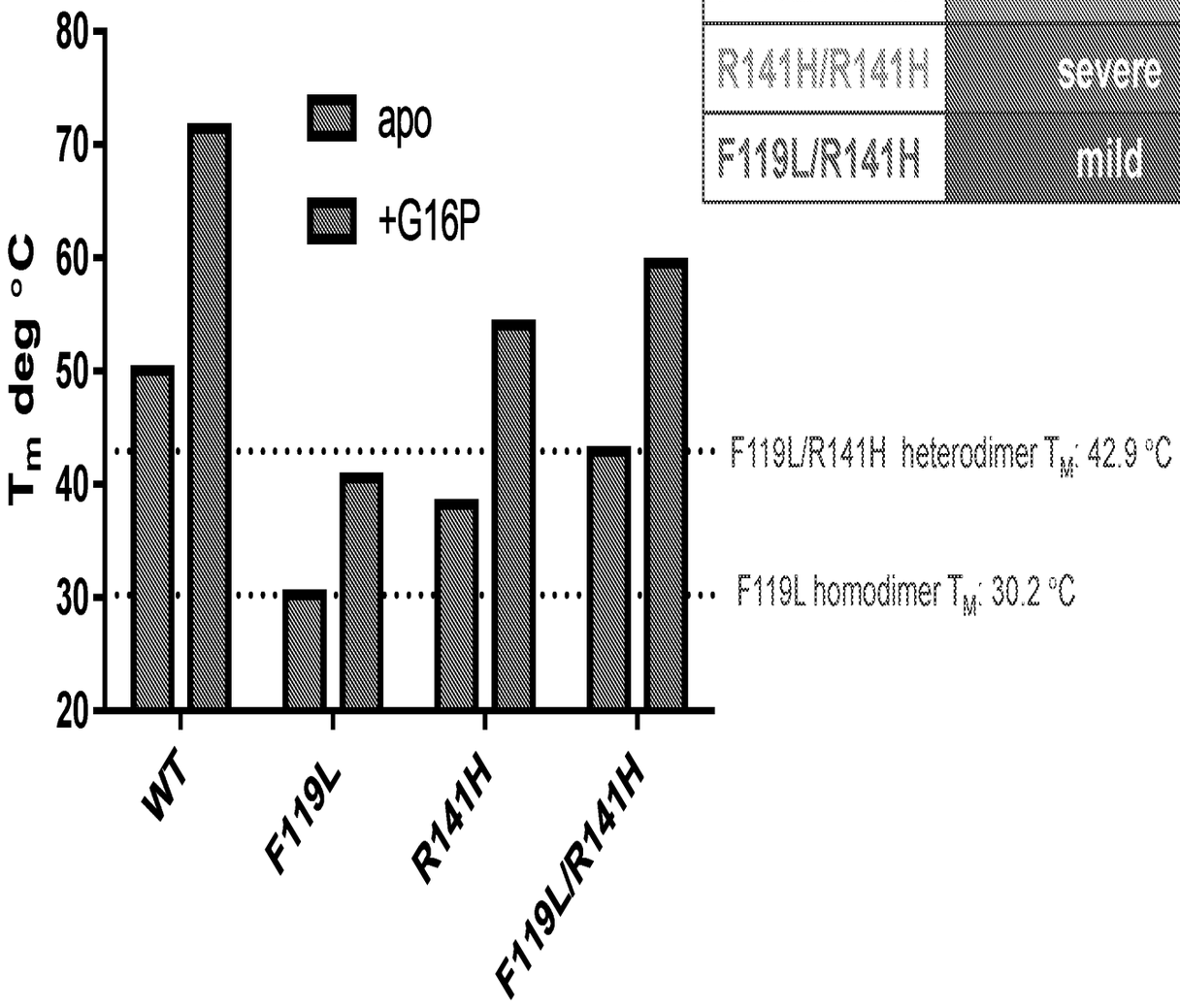


Ex. 2001-0796

# Heterodimerization provides a distinct stability advantage for PMM2 mutant proteins

agios

| Isozyme | Stability Defect |
|---|---|
| F119L/F119L | moderate |
| R141H/R141H | severe |
| F119L/R141H | mild |




- The unstable F119L mutant is stabilized by association with the R141H mutant
- Both WT and mutant proteins exhibit ligand-induced stabilization when associated with the endogenous activator G16P.

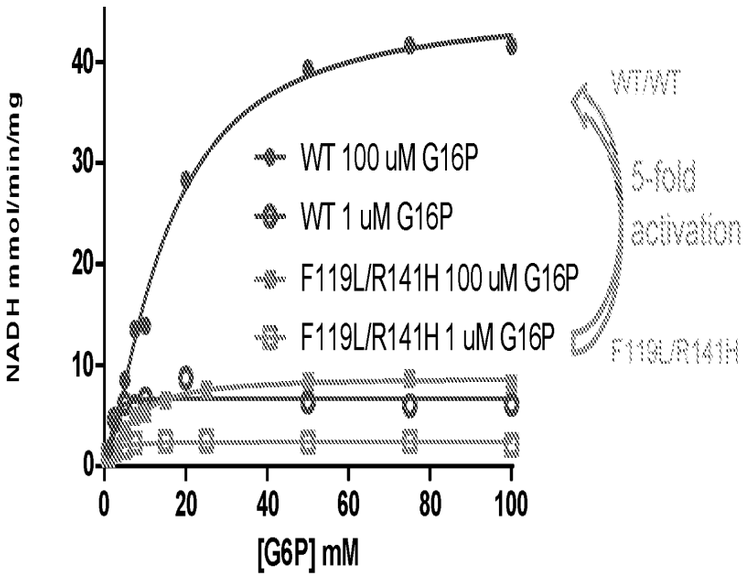


Ex. 2001-0797

# Feasibility and Strategy for Allosteric Activator of PMM2

agios

- **Identified** the biologically relevant F119L/R141H heterodimer as the target
- **Stability defects** in F119L homodimer can be improved by tight heterodimeric association with catalytically defective R141H
- **Catalytic deficiencies** in the R141H homodimer can be improved by tight heterodimeric association with the unstable F119L
- **Small molecule** metabolite G16P activation and stabilization suggests PMM2 is a drugable target for pharmacological intervention.




| | WT | F119L/R141H | WT | F119L/R141H |
|---|---|---|---|---|
| | 100 uM G16P | 100 uM G16P | 1 uM G16P | 1 uM G16P |
| Vmax | 45.4 | 8.77 | 6.71 | 2.39 |
| h | 1.4 | 1.37 | 2.25 | 1.25 |
| Khalf | 14.8 | 6.78 | 1.76 | 1.21 |

Rescue of the F119L/R141H heterodimer requires rather modest improvements in kinetic parameters

- Km defect is < 2-fold
- Kcat defect is < 6-fold

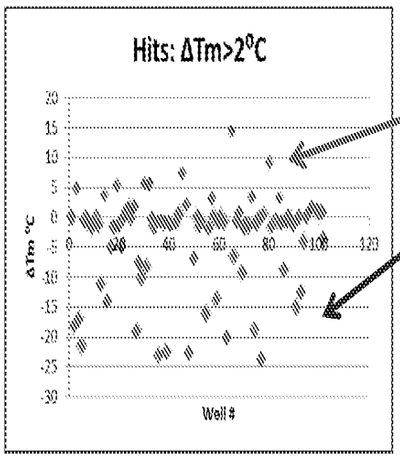


Ex. 2001-0798

# Screen for F119L/R141H stabilizers identifies candidate starting chemical equity

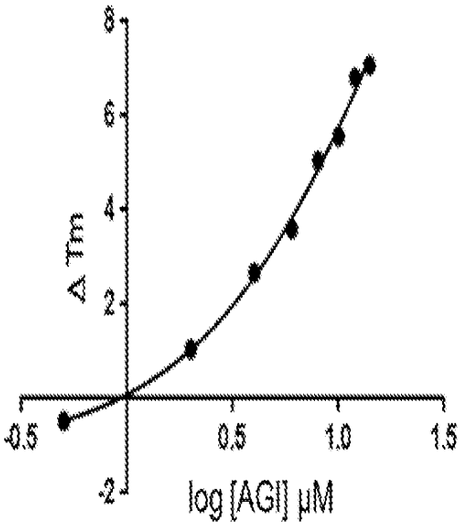
agios

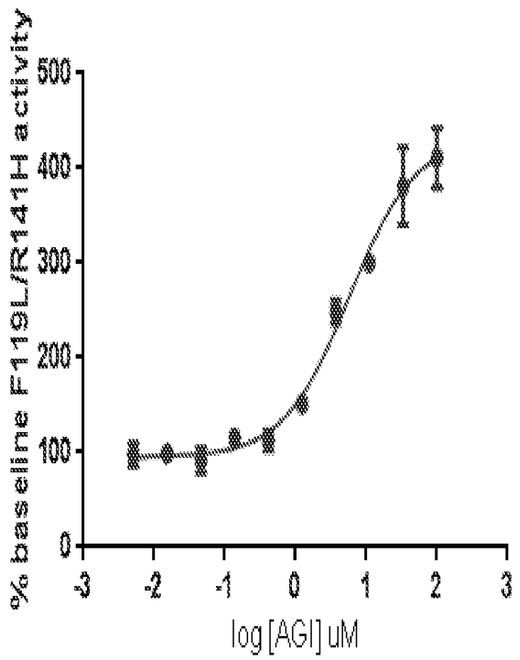
- Agios proprietary collection of low MW compounds and metabolites was screened for the ability to stabilize F119L/R141H heterodimer
- Stabilizing hits were tested for the ability to activate the enzyme




Stabilizing hits increase melting temperature

Destabilizing hits decrease melting temperature




Potential activator/stabilizer hits were identified. Progressibility still undetermined.



Ex. 2001-0799

# CDG/PMM Summary

agios

- Point mutations in PMM2 which cause kinetic defects or decreased protein stability are responsible for CDG Ia, the most common glycosylation disorder
- Lack of Hardy-Wienberg equilibrium in the population can be explained by embryonic lethality of homozygotic alleles producing highly defective proteins.
- The clinically observed F119L/R141H heterozygote produces a minimally active heterodimer through functional complementation between the thermolaible F119L and the catalytically defective R141H subunits
- POC for small molecule stabilizer/activator has been obtained by a fragment-based lead identification approach



Ex. 2001-0800

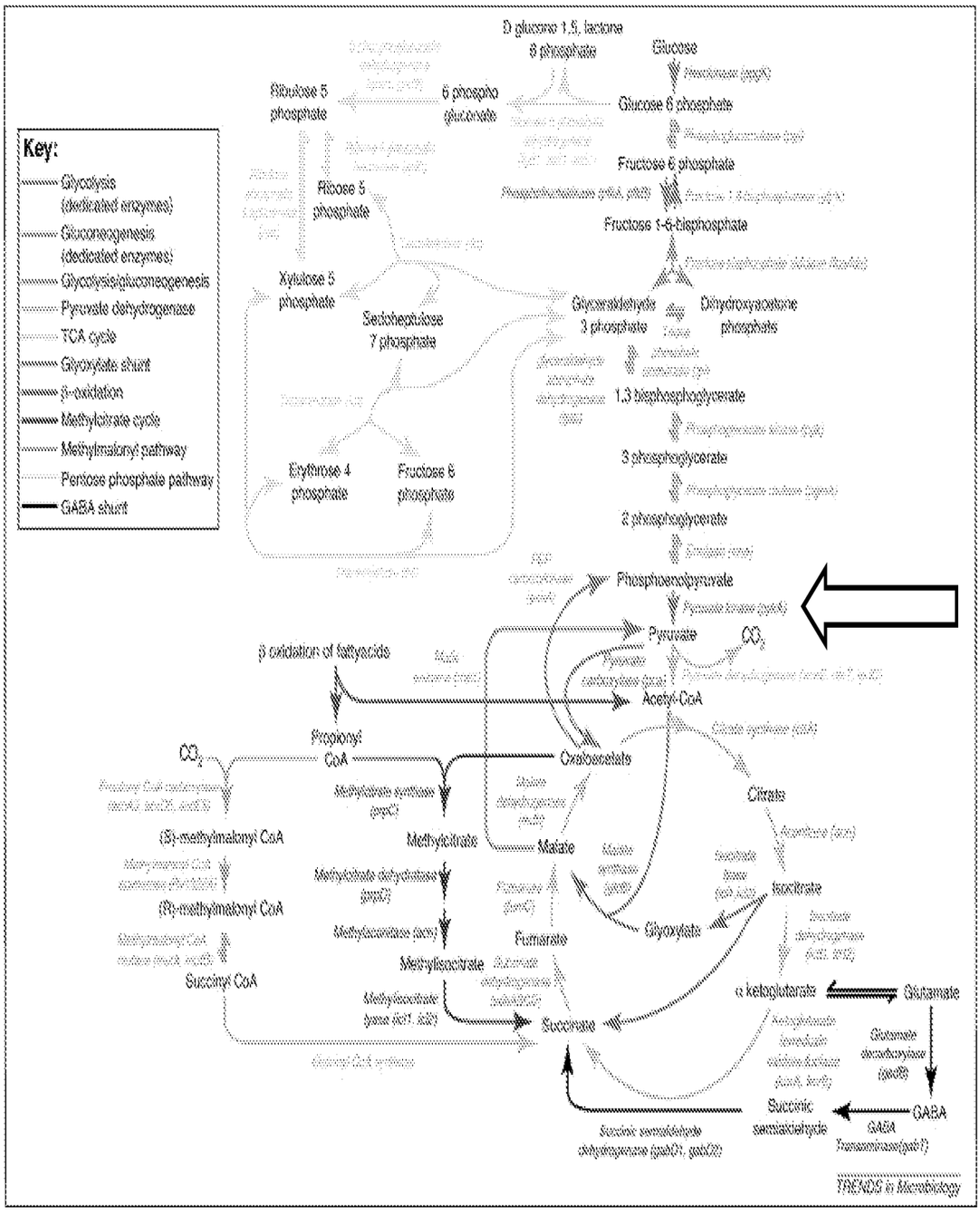
# AG-348, AN ENHANCER OF PYRUVATE KINASE ACTIVITY FOR THE TREATMENT OF PATIENTS WITH PKR-DEFICIENT HEMOLYTIC ANEMIA



Ex. 2001-0801

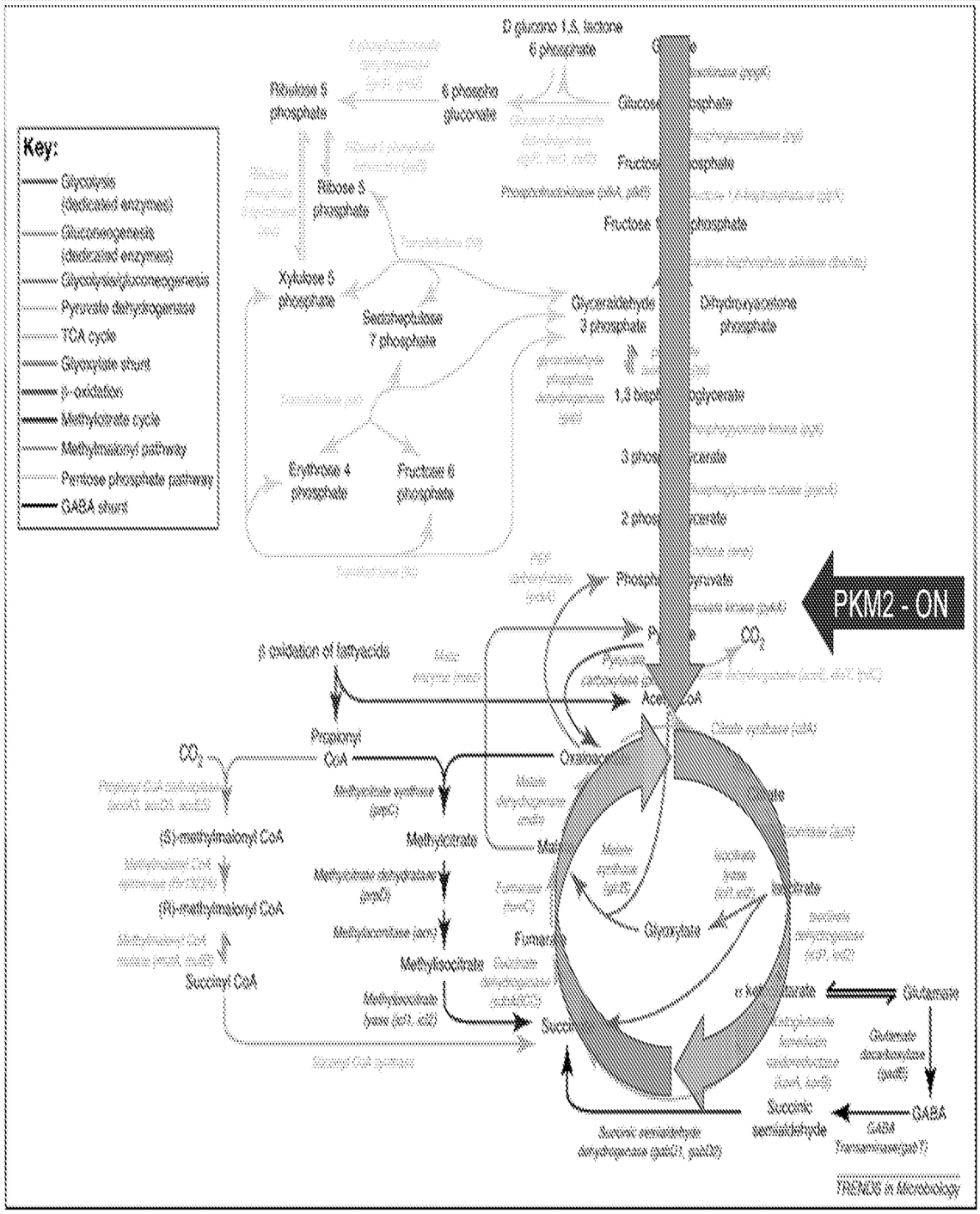
# Pyruvate kinase is a key metabolic node

agios



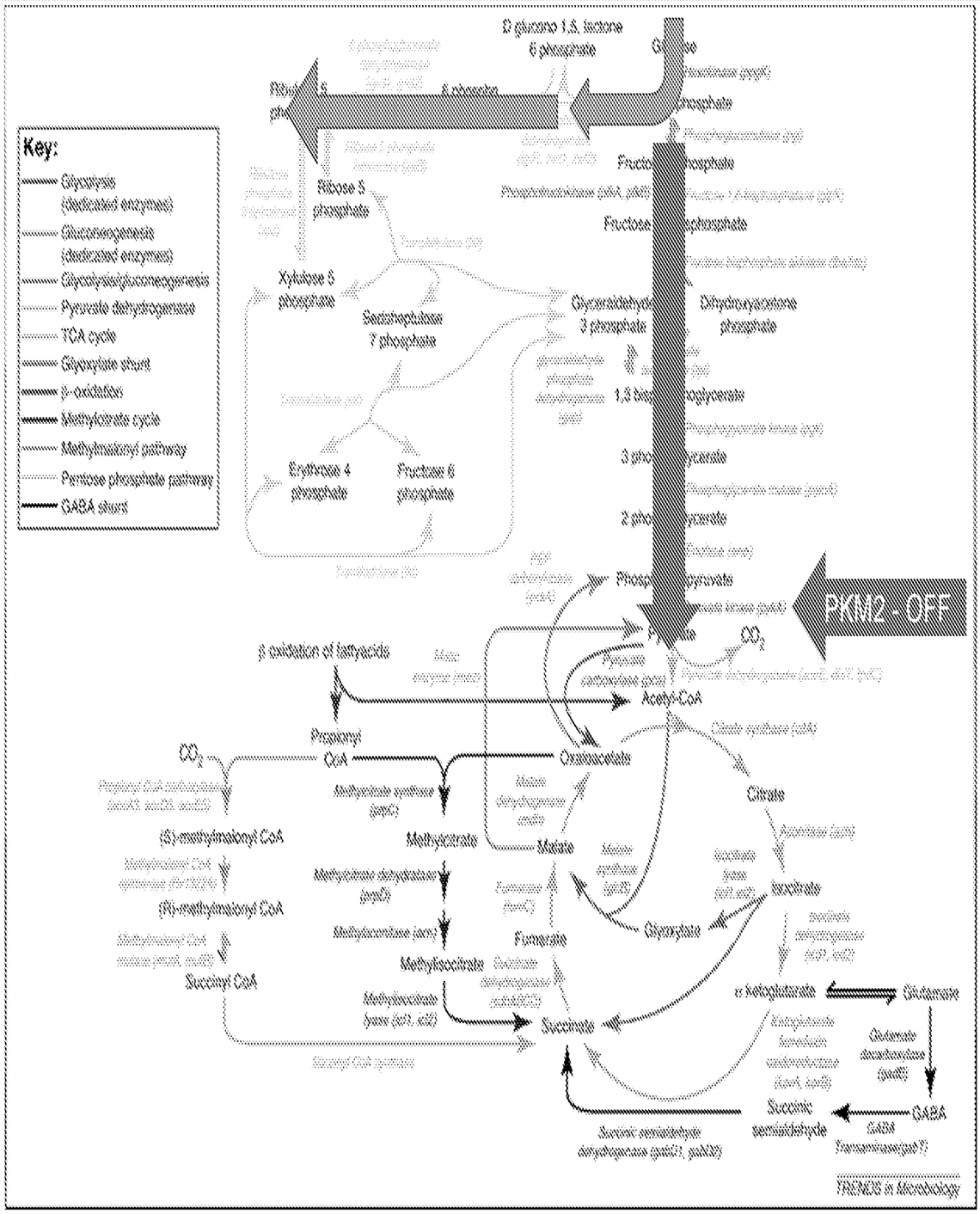
# PKM2 "on" directs carbon flow into TCA cycle for energy production

agios

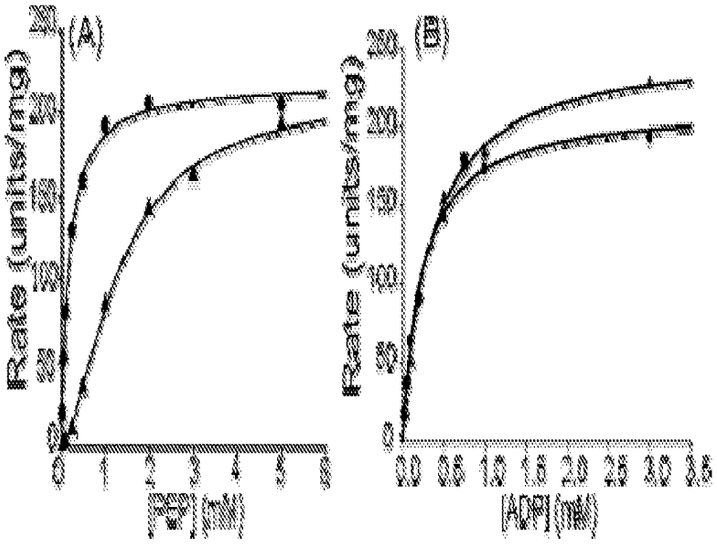


# PKM2 "off" directs carbon flow into pentose phosphate shunt for nucleotide production

agios



# PKM2 is allosterically activated by FBP




| FBP | $K_m$ (mM) | $V_{max}$ | $n_H$ |
|---|---|---|---|
| ADP + | 0.24 ± 0.03 | 214 ± 6 | 1.0[b] |
| - | 0.34 ± 0.02 | 253 ± 6 | 1.0[b] |
| PEP + | 0.17 ± 0.01 | 218 ± 4 | 1.0[b] |
| - | 2.1 ± 0.7 | 310 ± 37 | 1.6 ± 0.1[c] |

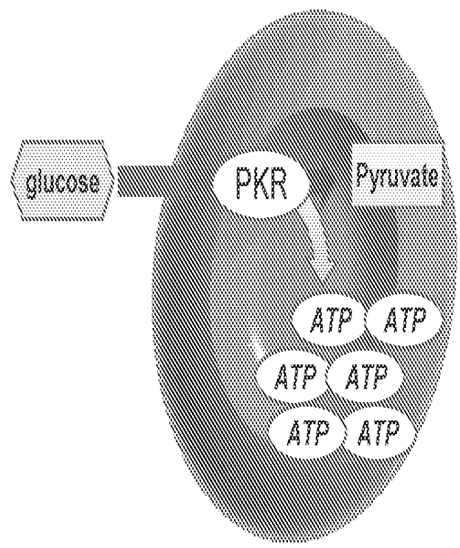
## Two consequences of activation:

- Increase in affinity for PEP but not ATP substrate
- Decrease in subunit co-operativity

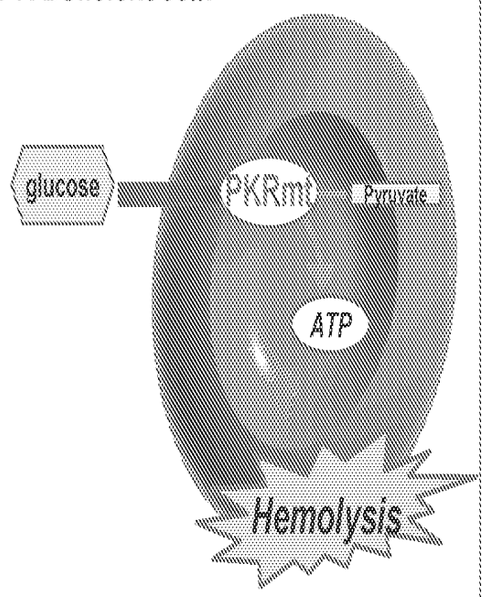
# Pyruvate Kinase Deficiency (PKD): IEM caused by metabolic defect of red blood cells

- Pyruvate Kinase Deficiency (PKD) is a rare form of hemolytic anemia
  - Caused by mutations in PKR
- Caused by mutations in PKR enzyme
  - Last step in glycolysis in red blood cells
  - Only energy (ATP)-generating pathway in red blood cells
- Patients have life-long anemia, transfusions, and iron overload
  - Splenectomy is common
- Therapeutic strategy: **Small molecule activation of mutant PKR**

Normal red blood cell:




PK Deficient cell:

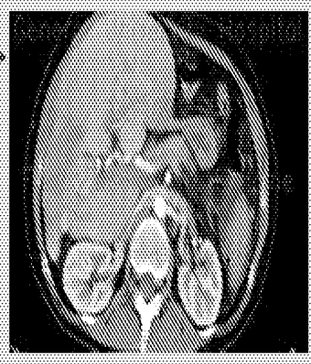





Ex. 2001-0806

# Disease Progression from Infants to Adults Demonstrates Lifetime of Burden

agios

| INFANTS | TODDLERS | CHILDREN | ADULTS |
|---|---|---|---|
|  |  | |  |
| Jaundice, severe anemia, exchange transfusions | Splenectomy only partially ameliorates hemolysis and leads to increased infection risk, antibiotic prophylaxis | | Iron overload leading to liver cirrhosis, cardiac and endocrine issues<br>Gall stones, cholecystectomy |

Ongoing Natural History Study captures retrospective/prospective clinical data, Quality of Life (QoL) measures and genetic diagnostic information.

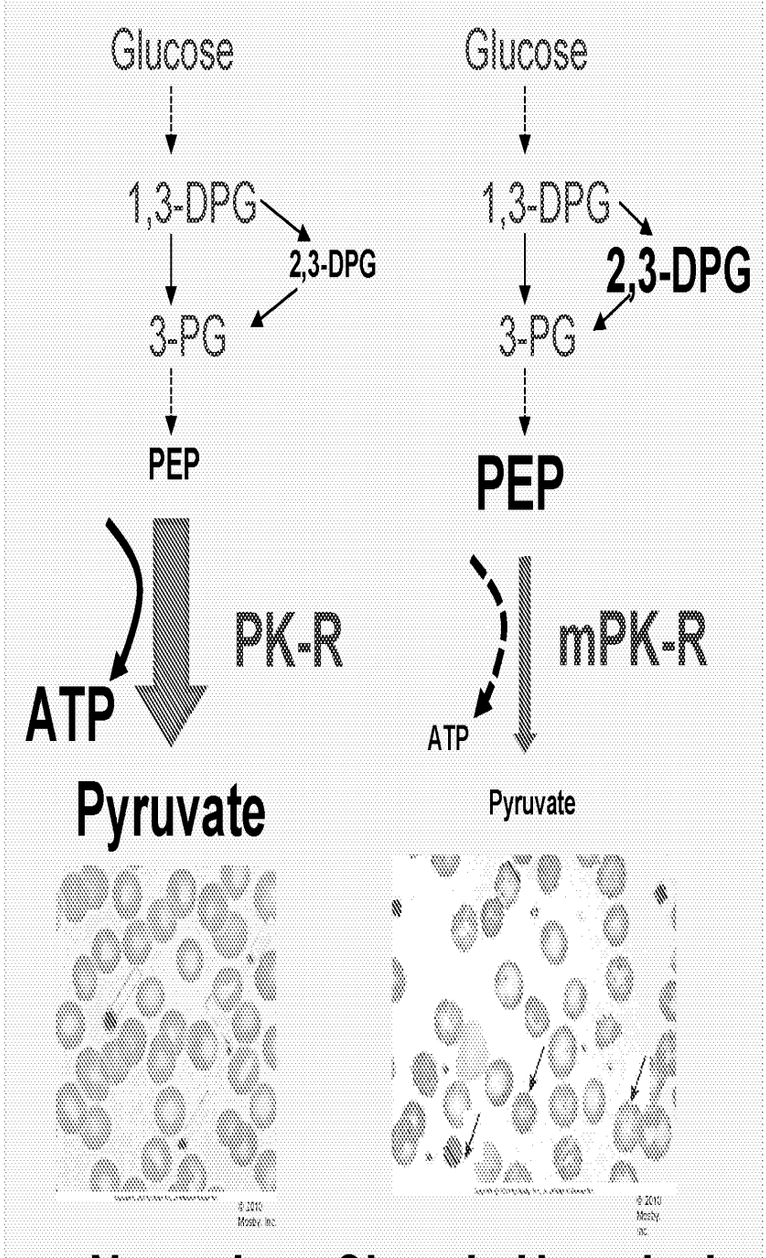
www.clinicaltrials.gov, NCT02053480

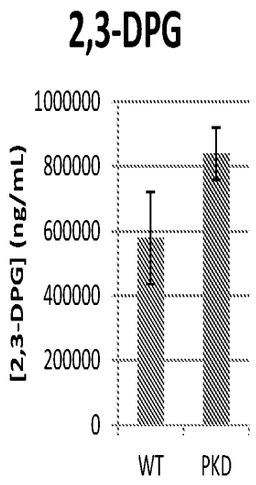


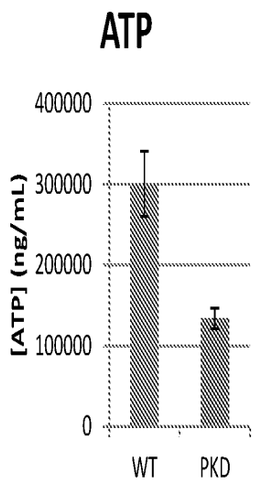
Ex. 2001-0807

# Metabolic Defects in PK Deficiency

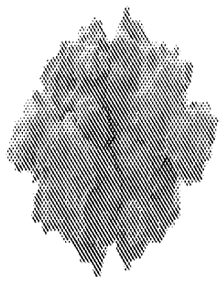
agios



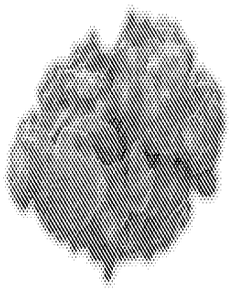
# Small molecule remediation of PKR deficiency



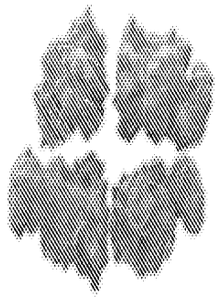
Normal PKR

Can we mimic or augment the effects of FPB activation?

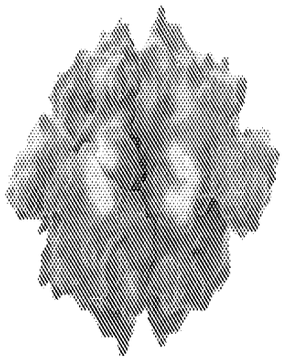
PKD mutations



or

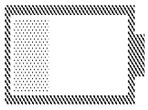


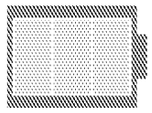




Reduced catalytic efficiency

Reduced protein stability





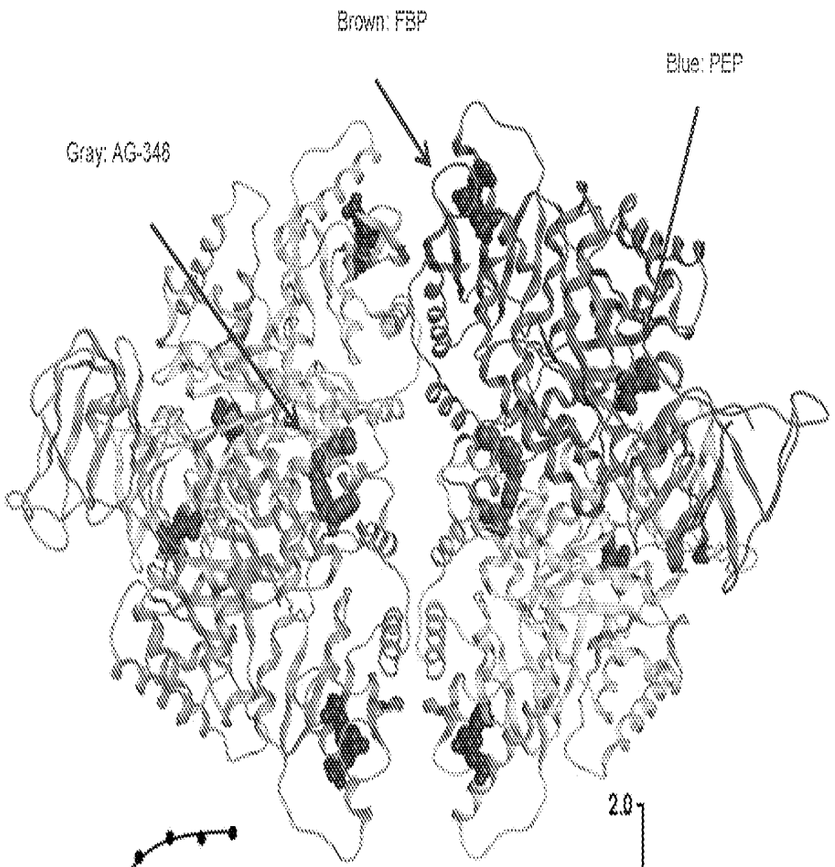
Energy (ATP) Deficiency

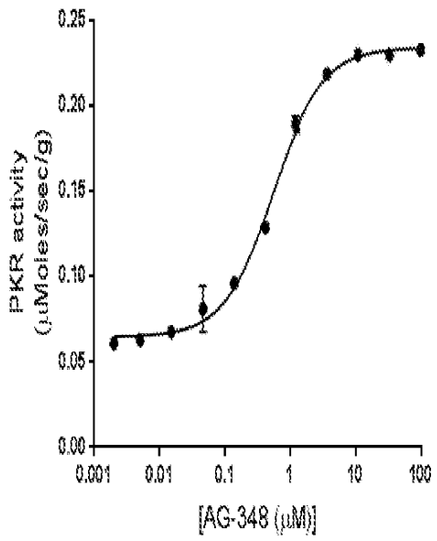
Increased ATP

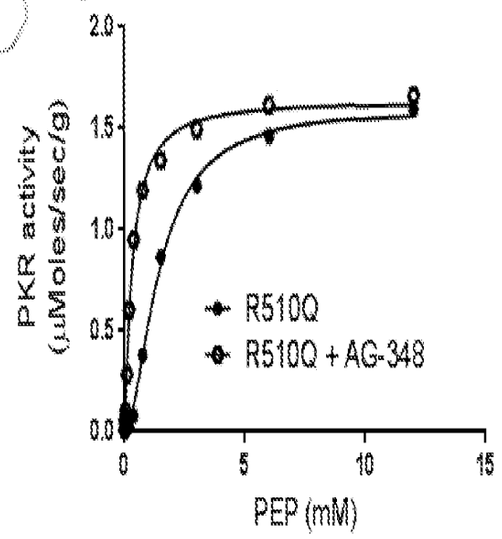


Ex. 2001-0809

# AG-348: a novel small-molecule activator of PK which mimics FBP activation



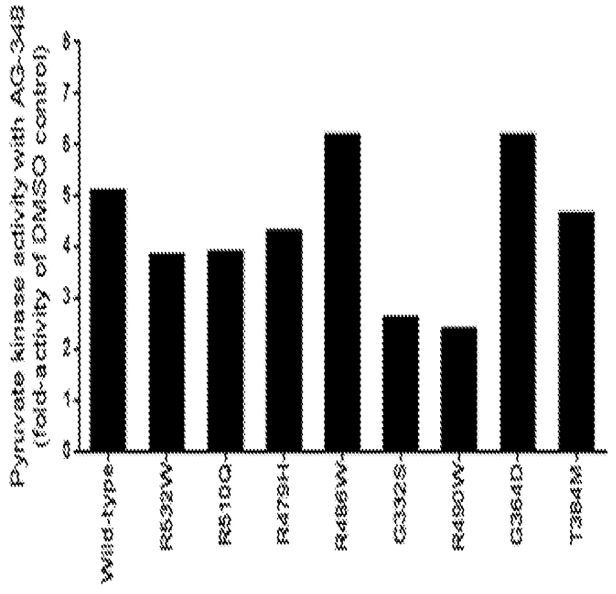




Kung et al. Blood 2017 (publication in press)

# AG-348 activates a spectrum of recombinant mtPKR enzymes



PKR tetramer with sites of mtPKR enzymes tested in Figure 3B highlighted in red. For clarity, each mutation site is only shown in a single monomer



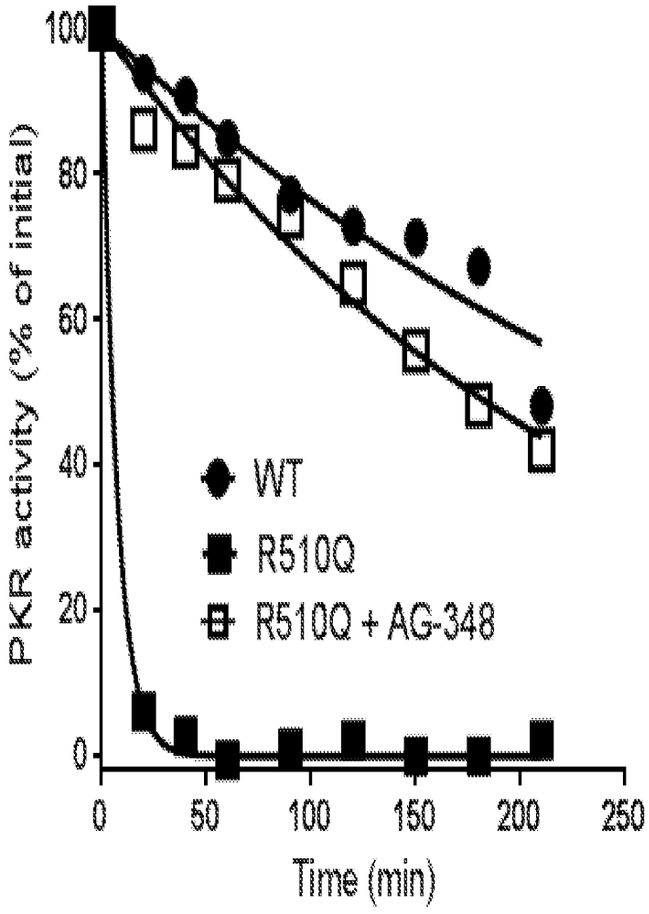

| AG-348 $AC_{50}$ (nM) | 13 | 9 | 55 | 16 | 59 | 22 | 14 | 41 | 26 |
|---|---|---|---|---|---|---|---|---|---|

Kung et al. Blood 2017 (publication in press)

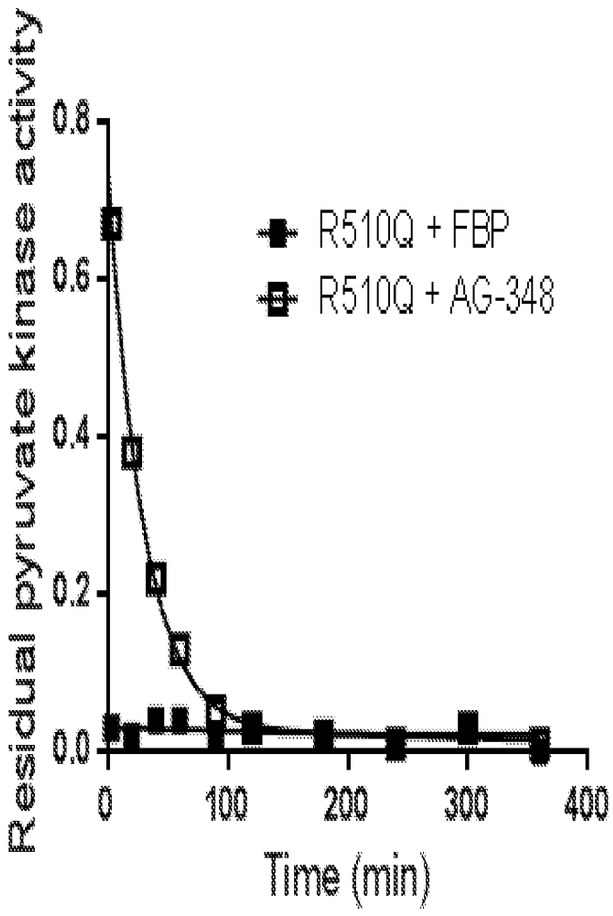


Ex. 2001-0811

# AG-348 can stabilize PKR R510Q enzyme, and has slower off-rate than FBP *in vitro*




Plot of residual activity over time of WT or R510Q recombinant enzymes following incubation at 53° C



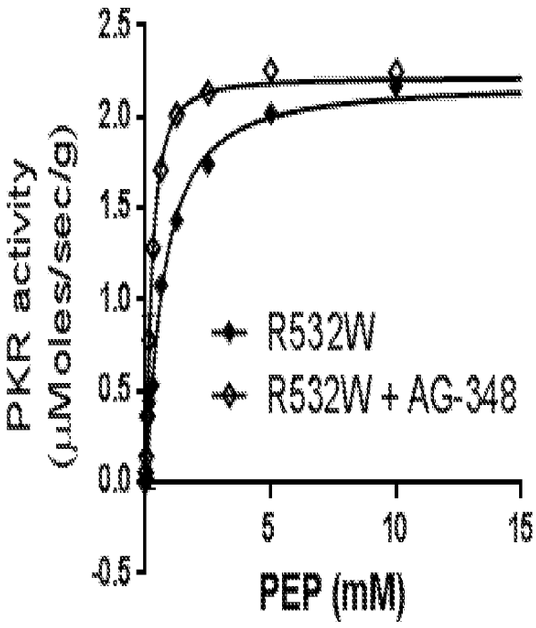

Off-rate measurement of AG-348 or FBP from recombinant R510Q enzyme

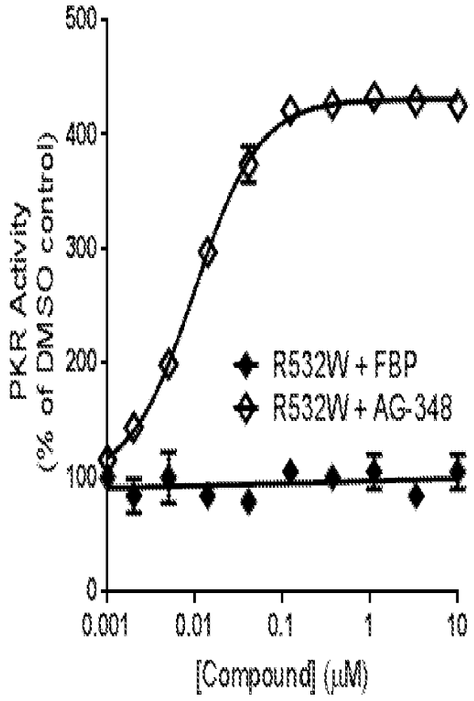
Kung et al. Blood 2017 (publication in press)



Ex. 2001-0812

# AG-348 can activate mutant enzymes *in vitro* that abrogate activation by endogenous regulator FBP

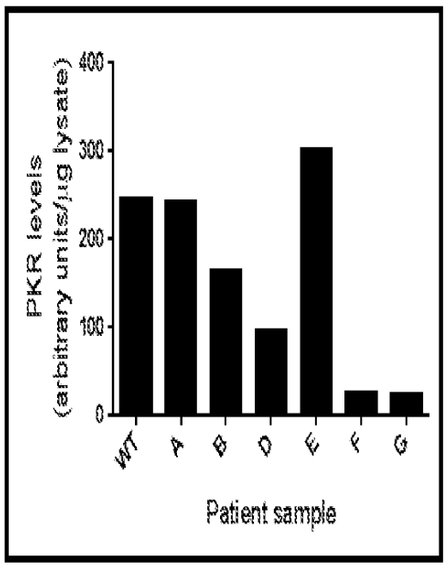


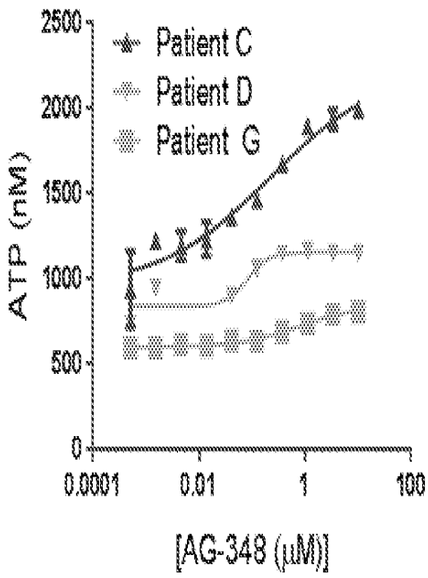



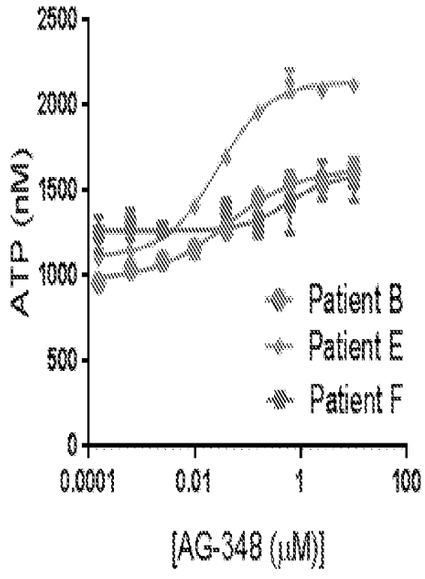

Kung et al. Blood 2017 (publication in press) 44

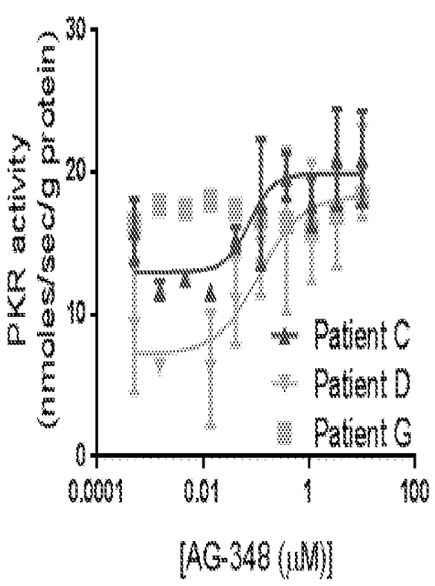
# AG-348 increases PKR activity and ATP levels in patient red cells *ex-vivo*

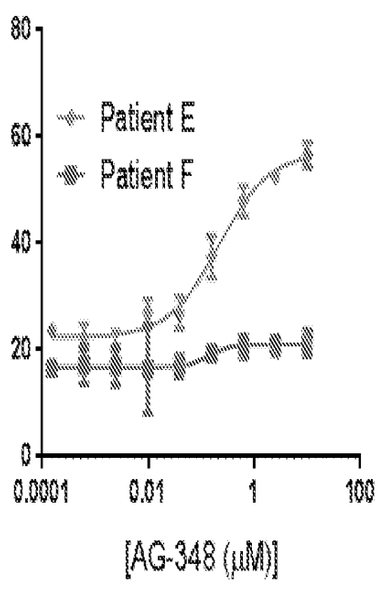
agios

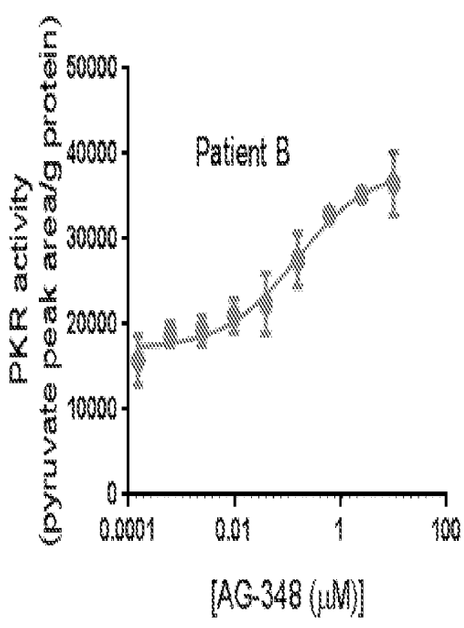


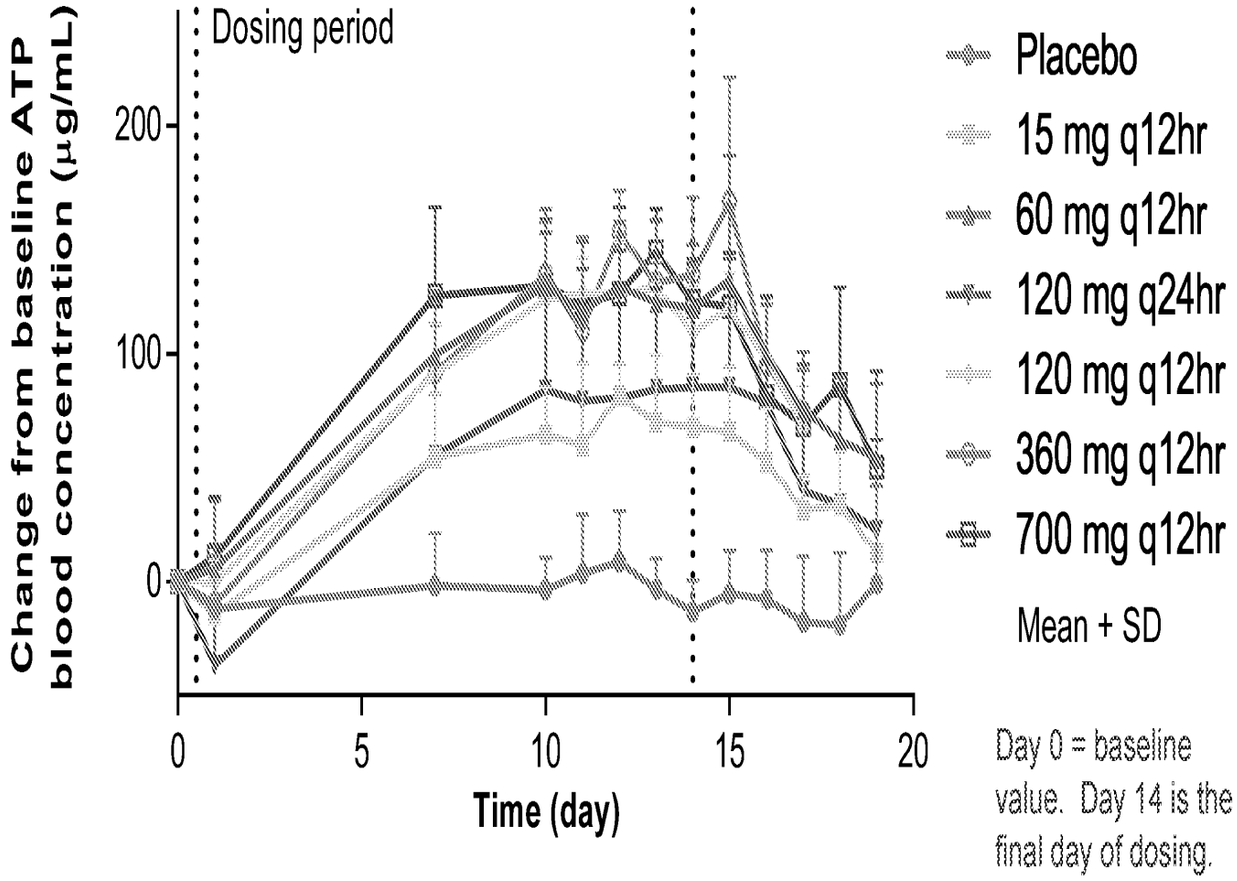




Kung et al. Blood 2017 (publication in press) 45

# Robust dose-dependent *increase* in ATP in healthy volunteers' blood

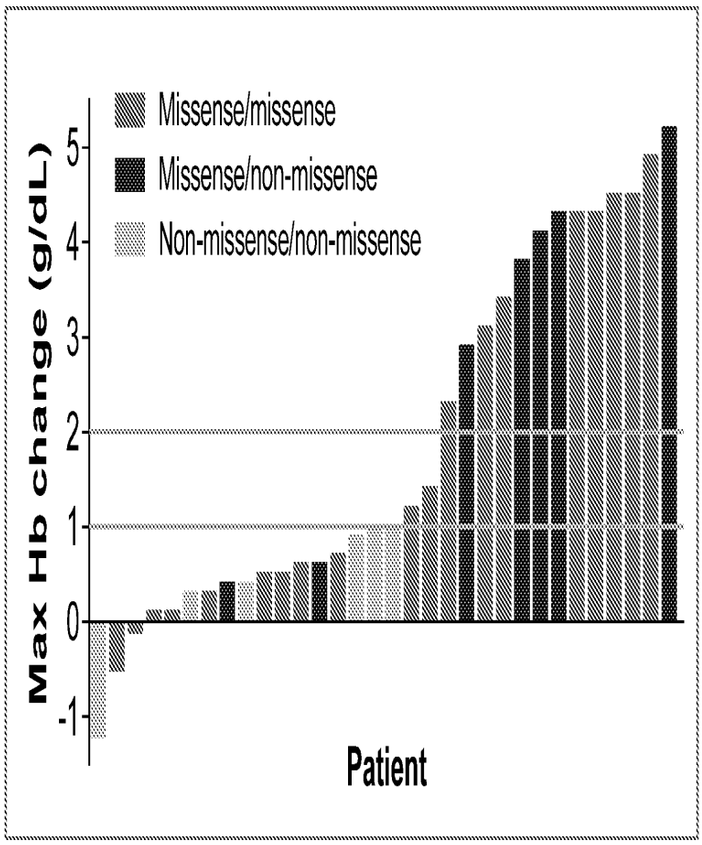
agios

- A ~50% increase from baseline was observed with ≥120 mg AG-348
- Levels remained elevated through 120 hours after the final dose




Data presented at ASH 2016, Yang et al.

# Compelling Proof-of-Concept for AG-348, the First Disease Modifying Therapy for PK Deficiency




52 patients enrolled;
17 completed first 24 weeks, 15 in extension
Results as of cut-off date of 23 September 2016

## DRIVE PK Learnings

Robust hemoglobin increases in 15 / 32 patients; 15 / 26 patients with 1 or more missense mutation

Responses are rapid and sustained; median time to response of 1.4 weeks

Majority of responders seen at doses ≤50 mg BID and as low as 5 mg QD

Well-tolerated beyond six months of dosing

Data presented at ASH 2016



Ex. 2001-0816

# Treatment of Rare Genetic Diseases by Small Molecule Allosteric Activators

agios

- Rare Genetic Diseases are individually rare, but as a disease class make an appreciable contribution to human morbidity
- Defective enzymes that are the cause of pathologic disregulated metabolsm are an emerging class of druggable targets
- In CDG1a, natural complementation of defective alleles and early lead ID efforts point toward the feasibility of small molecule activator/stabilizers
- AG-348, a small molecule activator/stabilizer with clinical POC, holds promise as the first disease-modifying treatment for PK-deficient hemolytic anemia



Ex. 2001-0817

# "Drug discovery is the most complicated team sport"



A publication of the American Pharmaceutical Association and the American Chemical Society

JOURNAL OF Pharmaceutical Sciences

January 1997
Volume 86, Number 1

REVIEW ARTICLE

# Characteristics and Significance of the Amorphous State in Pharmaceutical Systems

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Received April 26, 1996, from the [‡]Merck Frosst Canada Inc., Pointe Claire-Dorval, Quebec, H9R 4P8, Canada, and [†]School of Pharmacy, University of Wisconsin—Madison, Madison, WI 53706. Final revised manuscript received July 31, 1996. Accepted for publication August 1, 1996[x].

**Abstract** □ The amorphous state is critical in determining the solid-state physical and chemical properties of many pharmaceutical dosage forms. This review describes the characteristics of the amorphous state and some of the most common methods that can be used to measure them. Examples of pharmaceutical situations where the presence of the amorphous state plays an important role are presented. The application of our current knowledge to pharmaceutical formulation problems is illustrated, and some strategies for working with amorphous character in pharmaceutical systems are provided.

## Introduction

During the final stage of developing a synthetic procedure for a new drug entity, a great deal of emphasis is placed on obtaining material of high purity, and reproducibility in terms of its physical, chemical, and biological properties. Every effort is made to ensure a high degree of crystallinity, wherein the molecules have regular and well-defined molecular packing, and emphasis is also placed on whether or not the compound can exist in polymorphic or solvated crystal forms.[1] These forms can have different thermodynamic properties (*e.g.*, melting temperature, vapor pressure, solubility), and a knowledge of their existence is required to anticipate spontaneous changes in the properties of the solid during storage and/or handling of the material. It is also possible that upon isolation the material will be obtained in a fully or partially amorphous state.[2] The four most common means by which amorphous character is induced in a solid are shown in Figure 1. These are condensation from the vapor state, supercooling of the melt, mechanical activation of a crystalline mass (*e.g.*, during milling), and rapid precipitation from solution (*e.g.*, during freeze-drying or spray drying). Amorphous character is common with polymeric molecules used as excipients, and large peptides and proteins used as therapeutic agents, a it can also occur with small organic and inorganic molecul When a system consists of multiple components, as w pharmaceutical formulations, it is possible that amorpho solid-state solutions can form analogous to liquid solutio Water vapor can also be absorbed by an amorphous solid form an amorphous solid solution.

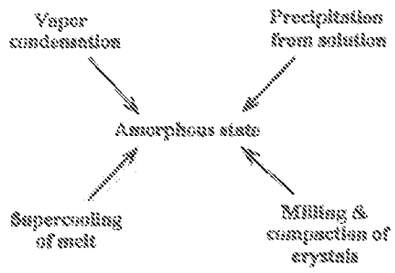



Figure 1—Schematic diagram of the most common ways in which amorph character is induced in a pharmaceutical system.

The three-dimensional long-range order that norma exists in a crystalline material does not exist in the amorphc state, and the position of molecules relative to one another more random as in the liquid state. Typically amorpho solids exhibit short-range order over a few molecular dime sions and have physical properties quite different from the of their corresponding crystalline states. In Figure 2 schematically plot the enthalpy ($H$) or specific volume ($V$) a solid substance as a function of its temperature. For crystalline material at very low temperatures we see a sm increase in enthalpy and volume with respect to temperatu indicative of a certain heat capacity ($C_p$) and thermal expa sion coefficient ($\alpha$). There is a discontinuity in both $H$ and at the melting temperature ($T_m$) representing the first-orc phase transition to the liquid state. Upon rapid cooling the melt the values of $H$ and $V$ may follow the equilibri line for the liquid beyond the melting temperature into

[x] Abstract published in *Advance ACS Abstracts*, October 1, 1996.

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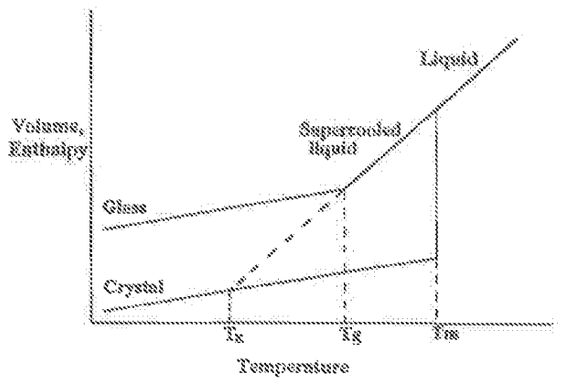



Figure 2—Schematic depiction of the variation of enthalpy (or volume) with temperature.

"supercooled liquid" region. On cooling further a change in slope is usually seen at a characteristic temperature known as the glass transition temperature ($T_g$). At $T_g$ the properties of the glassy material deviate from those of the equilibrium supercooled liquid to give a nonequilibrium state having even higher $H$ and $V$ than the supercooled liquid. As a result of its higher internal energy (*e.g.*, ≈25 kJ·mol$^{-1}$ for cephalosporins[8]) the amorphous state should have enhanced thermodynamic properties relative to the crystalline state (*e.g.*, solubility,[4] vapor pressure) and greater molecular motion. We would also expect amorphous systems to exhibit greater chemical reactivity[3] and to show some tendency to spontaneously crystallize, possibly at different rates below and above $T_g$.[5] From a pharmaceutical perspective we have an interesting situation. The high internal energy and specific volume of the amorphous state relative to the crystalline state can lead to enhanced dissolution and bioavailability,[4] but can also create the possibility that during processing or storage the amorphous state may spontaneously convert back to the crystalline state.[5]

In considering the importance of the amorphous state in pharmaceutical systems we must direct our attention to two main situations. In the first, a material may exist intrinsically in the amorphous state or it may be purposefully rendered amorphous and we would like to take advantage of its unique physical chemical properties. Under these circumstances we usually want to develop strategies to prevent physical and chemical instability of the amorphous sample. In the second case, we may be dealing with a crystalline material that has been inadvertently rendered amorphous during processing. This type of amorphous character usually exists predominately at surfaces at levels not easily detected and has the potential to produce unwanted changes in the physical and chemical properties of the system. In this situation we usually want to process the system so that the amorphous portions of the solid are converted back to the most thermodynamically stable crystalline state.

## Definition and Description of the Amorphous State

The rapid cooling of a liquid below its melting point ($T_m$) may lead to an amorphous state with the structural characteristics of a liquid, but with a much greater viscosity (Figures 2 & 3). The enthalpy and volume changes immediately below $T_m$ exhibit no discontinuity with those observed above $T_m$, so we consider this amorphous state to be an equilibrium "supercooled" liquid. This amorphous state is also called the "rubbery state" because of the macroscopic properties of amorphous solids in this region. We can further characterize this state by considering its rate and extent of molecul motions. The average time scale of molecular motions with a supercooled liquid is usually less than 100 s, the viscos is typically between $10^{-1}$ and $10^{12}$ Pa·s (Figure 3), and bo properties are strongly temperature dependent.[6–10] Cooli the supercooled liquid even further appears to reduce t molecular mobility of the material to a point at which t material is kinetically unable to attain equilibrium in the tir scale of the measurement as it loses its thermal energ resulting in a change in the temperature dependence of t enthalpy and volume. The temperature at which this occu is the experimentally observed glass transition temperatu ($T_g$). Below $T_g$ the material is "kinetically frozen" into thermodynamically unstable glassy state with respect to bo the equilibrium liquid and the crystalline phase, and a further reduction in temperature has only a small effect up its structure. Molecular motions in glasses typically occ over a period in excess of 100 s, and viscosities are usual greater than $10^{12}$ Pa·s.[6–10] Many of the physical properti of glassy amorphous materials (*e.g.*, thermal expansion coe ficient) are different from those of the corresponding supe cooled liquid above $T_g$.

The molecular processes which contribute to the gla transition are currently the subject of intensive research a debate. Whether the changes in thermodynamic properti (*e.g.*, specific heat, volume) that are seen during cooling ( reheating) are due to a real thermodynamic phase transiti or are of purely kinetic origin is a controversial issue, and theory has yet been proposed which accounts for all t observed experimental features. Several excellent revie which describe the current thinking in this field have be published.[6–8,10,11] Models based on statistical mechanical free volume theories are the simplest and most wide invoked. Polymer scientists, metallurgists, ceramists, e each have their preferred approaches with specific advantag for the materials and processes with which they are workin From Figure 2 it can be seen that the glass transition can considered to be a thermodynamic requirement for a supe cooled liquid since without such a transition the amorpho material would attain a lower enthalpy than the crystalli state at some critical temperature and would eventually atta a negative enthalpy. This critical temperature is known the Kauzmann temperature ($T_K$) and is thought to mark t lower limit of the experimental glass transition ($T_g$) and be the point at which the configurational entropy of the syste reaches zero.[9,10] Experimental studies of the glass transiti are complicated by the existence of many different modes molecular motion in most systems (*e.g.*, rotational or tras lational), changes in the scale and type of motions wi temperature, and cooperativity or coupling of molecul motions. One can only say for certain that at $T_g$ the mea molecular relaxation time ($\tau$) associated with the predomina molecular motions is about 100 s and that $T_g$ can be expecte to vary with experimental heating and cooling rates, samp molecular mass,[12,13] sample history, sample geometry,[14,15] a sample purity.[16] The experimental glass transition tempe ature is also influenced by the choice of technique used measure it because of the varying sensitivities of availab techniques to different types and speeds of molecular motion

The temperature dependence of molecular motions direct determines many important physical properties of amorpho materials, including the location of the glass transiti temperature and the ease of glass formation. This temper ture dependence is most frequently described using t empirical Vogel-Tammann-Fulcher (VTF) equation:[7,8,10]

$$\tau = \tau_0 \exp(DT_0/T-T_0) \qquad (1$$

where $\tau$ is the mean molecular relaxation time, $T$ is t

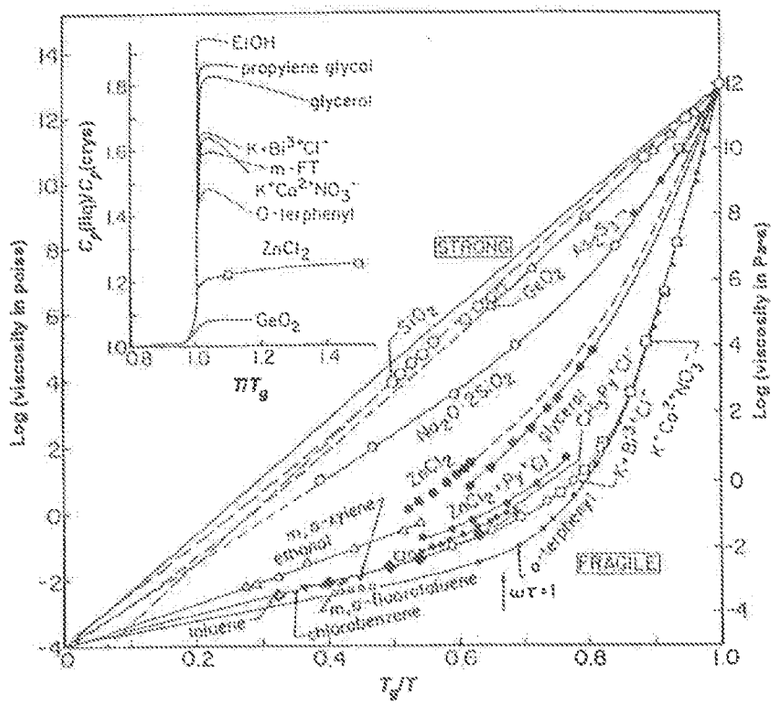



Figure 3—Molecular mobility (or viscosity) of amorphous materials as a function of normalized temperature above $T_g$.[7,8] Reprinted with permission from ref 8. Copyright 1995 American Association for the Advancement of Science.

temperature, and $\tau_0$, $D$, and $T_0$ are constants. The value of $T_0$ in the VTF equation is believed to correspond to the theoretical Kauzmann temperature ($T_K$), and $\tau_0$ can be related to the relaxation time constant of the unrestricted material.[7,8] When $T_0$ is 0, the familiar Arrhenius equation is obtained, and $D$ is directly proportional to the activation energy for molecular motion. When $T_0$ is greater than 0, there is a temperature dependent apparent activation energy. The Williams–Landel–Ferry (WLF) equation[17] describing the temperature dependence of viscosity ($\eta$) in polymers above $T_g$ is a special case of the VTF equation:

$$\eta = \eta_g \exp\{C_1(T - T_g)/(C_2 + (T - T_g))\} \qquad (2)$$

where $\eta_g$ is the mean viscosity at $T_g$ and $C_1$ and $C_2$ are constants. This equation can be derived from first principles based on polymer free volume theories. The constants $C_1$ and $C_2$ are found to be quite universal for a range of polymers[17] and are equivalent to $DT_0/(T_g - T_0)$ and $(T_g - T_0)$, respectively, in the VTF equation. The WLF equation has been shown to fit viscosity data for several small organic molecules using the universal constants,[18–20] making it useful for predicting the relaxation behavior or molecular mobility of amorphous pharmaceutical solids. However, it is important to recognize that this is not always the case and such predictions cannot always be assumed to be accurate.

Depending upon the magnitude and temperature dependence of the apparent activation energy for molecular motions near and above $T_g$ in supercooled liquids, it is possible to classify them as either "strong" or "fragile" amorphous systems (Figure 3).[7,8] A strong liquid typically exhibits Arrhenius-like changes in its molecular mobility with temperature and a relatively small change in heat capacity at $T_g$. Proteins are good examples of strong glass formers, with their changes in heat capacity at $T_g$ often being so small that they cannot be detected using standard calorimetry techniques.[21] A fragile supercooled liquid has a much stronger temperature depe dence of molecular mobility near $T_g$ and a relatively lar change in heat capacity at $T_g$ and will typically consist nondirectionally, noncovalently bonded molecules (*e.g.*, eth nol). The constant $D$ in the VTF equation is an indicator fragility, with low values (<10) corresponding to very frag glass formers and high values (>100) indicating strong gla forming tendencies. The value of $T_0$ in the VTF equation also linked to the fragility of the system with $(T_g - T_0) >$ typical of strong glass formers and $(T_g - T_0) <$ 50 usual fragile materials. A simple graphical means of ranki materials in terms of their strength/fragility is to plot l molecular mobility (or viscosity) as a function of the temp ature normalized to the experimental glass transition te perature (*e.g.*, Figure 3).[7,8] A "rule of thumb" for determini fragility without relaxation time data has also been propos based on the relative magnitudes of the melting and gl transition temperatures: strong, $T_m/T_g$ (in K) >1.8; fragi $T_m/T_g$ (in K) < 1.5).[7, 8] (Table 1). (See Note Added in Proof

The extent of departure of a *glass's* properties from eq librium is determined by its formation conditions, so we c presume the existence of multiple metastable glasses *bel* $T_g$ (Figure 2),[5,6] and even polyamorphic glasses that conv via first-order transitions.[22–24] As a result of this, the te perature dependence of molecular motions *below* the gl transition temperature is highly dependent upon the con tions under which the glass was formed.[18] This temperatu dependence is generally less extreme than above $T_g$ and m linear, with some authors proposing an Arrhenius-like re tionship. That molecular motions do occur below $T_g$ unquestionable, and the consequences of the relaxation "aging" of glassy materials have been widely reported. F example, Guo *et al.*[25] described effects upon the film-coat wa permeability and dissolution rate of film coated tablets, a Byron and Dalby[26] studied the effects of aging on the perm ability of poly(vinyl alcohol) films to a model water solu

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Table 1—Measured Physical Properties of Some Amorphous Pharmaceutical Materials[a]

| Material | $M_w$ | $T_m$ (K) | $T_g$ (K) | $T_g/T_m$ | $\Delta C_p$ ($J \cdot g^{-1} \cdot K^{-1}$) | $\rho_{crystal}$ ($kg \cdot m^{-3}$) | $\rho_{amorph}$ ($kg \cdot m^{-3}$) |
|---|---|---|---|---|---|---|---|
| Indomethacin | 358 | 433 | 320 | 1.37 | 0.466 | 1.39 | 1.32 |
| Sucrose | 342 | 459 | 348 | 1.50 | 0.544 | 1.59 | 1.43 |
| Lactose (anhydrous) | 342 | 486 | 393 | 1.27 | 0.472 | 1.50 | 1.48 |
| Trehalose (anhydrous) | 342 | 476 | 385 | 1.24 | 0.524 | 1.58 | 1.49 |
| Dextran | $\approx 6 \times 10^5$ | — | 498 | — | 0.400 | — | 0.92 |
| Poly(vinylpyrrolidone) | $\approx 1 \times 10^5$ | — | 458 | — | 0.260 | — | 1.28 |
| Water[b] | 18 | 273 | 136 | 2.01 | 0.160 | <0.95 | ≈0.95 |

[a] $M_w$ = molecular mass; $T_g$ = glass transition temperature; $T_m$ = melting temperature; $\Delta C_p$ = heat capacity change at $T_g$; $\rho$ = density. [b] Reference 154.

drug. The effects of aging are often detrimental, but they can also be used to improve a product's performance with a deliberate "annealing" process. This strategy is particularly useful when small amounts of amorphous character have been unintentionally introduced into a system by high-energy processing (see later).[27,28] The time scale of molecular motions in a glass is much longer than above $T_g$ ($\tau \gg 100$ s) and requires different experimental techniques for its study. In almost all cases the molecular relaxation processes that occur in glasses follow a nonexponential function. This nonexponentiality has been widely studied and modeled[29] and appears to be the result of a heterogeneous microstructure within glasses which leads to a distribution of types and rates of molecular motion under any given time and temperature conditions. The reader is referred to some excellent reviews for detailed information on the application of these models to glassy systems.[12,29] The empirical Kohlrausch–Williams–Watts (KWW) stretched exponential function is most often used to describe the distribution of molecular motions:[10]

$$\phi(t) = \exp\{-(t/\tau)^{\beta}\} \qquad (3)$$

where $\phi(t)$ is the extent of relaxation at time $t$, $\tau$ is the mean molecular relaxation time, and $\beta$ is a constant. A $\beta$ value of unity corresponds to a single relaxation time with exponential behavior. The smaller the value of $\beta$, the more the distribution of molecular motions deviates from a single exponential. $\beta$ has been shown to correspond to the strength/fragility of the material above $T_g$, but no similar relationship has yet been established below $T_g$. By fitting data to the KWW function it is possible to determine the mean molecular relaxation time ($\tau$) and $\beta$ for any well-defined glass.[30] A general means of ranking glasses in terms of the temperature dependence of molecular motions, similar to Angell's strong/fragile classification system above $T_g$, would be of great use to pharmaceutical materials scientists but has not yet been developed because of the greater complexities of the glassy state.

Perhaps the most important question relating to amorphous pharmaceutical systems is, *At what temperature do the molecular motions responsible for physical and chemical instabilities cease to become likely over the lifetime of that particular system?*[31] It has been suggested that this lower temperature limit might correspond to the Kauzmann temperature ($T_K$). Although this appears to be the case for some systems, there also appears to be an influence from the strength/fragility of the system, and also from whether or not the molecular motions that are responsible for the glass transition and any instabilities are identical.[30] Mean molecular relaxation times have been reported for several pharmaceutical glass formers as a function of temperature following enthalpy relaxation and thermomechanical relaxation experiments, and the temperature of negligible molecular mobility during a 3-year shelf life varied according to (i) the method used to assess the molecular motions and (ii) the identity of the glass former.[30] As yet there is no reliable means of predicting the temperature of negligible molecular mobility in amorphous solids, and thus a conservative approach is required when defining storage and processing conditions for amorphous pharmaceutical systems (see later).

The behavior of amorphous systems as defined in Figure is dependent upon the assumption of constant pressure a composition. Pressure effects upon amorphous materials ha not been widely studied but are likely to be significant wi effects on molecular packing potentially modifying the gla transition temperature, the thermal expansion behavior, a the strength/fragility of a supercooled liquid.[10,31,32] From practical perspective the glass transition temperature of system containing volatile components may only be expe mentally accessible at elevated pressures. For example, t widespread and significant plasticizing effects of sorbed wat vapor in high-$T_g$ amorphous polymers have only recently be fully realized because of advances in sample-handling metho which allow samples of varying water content to be sealed ambient temperature and then heated through $T_g$ without lo of their sorbed water vapor.[33] The properties of a glas amorphous solution prepared by lyophilization are also like to be significantly different from those of the same syste prepared at ambient pressure since the reduced pressu within a lyophilization chamber will affect the structure the amorphous cake that is formed and also the composit of the solution through the primary and secondary dryi processes. Angell *et al.*[34] have noted that for aqueous solutio the fragility of the supercooled solution is dependent upon t solute concentration in the solution. From the limited da available it can be concluded that some supercooled aqueo solutions become stronger as they become more dilute (*e.g.*, sugars), whereas others become more fragile (*e.g.*, electrolyte salts). The type of behavior observed appears to be linked the extent of hydrogen bonding in the aqueous solution. Th fragility of such mixed systems may also be related to th ideality of their mixing behavior. Simple mixing rules ha been used by many authors[35,36] to describe the variation the glass transition temperature with blend compositio however, the effects of nonidealities (*e.g.*, immiscibility, m lecular size differences, specific interactions, *etc.*) are oft significant. The simplest and most reliable approach for u with amorphous pharmaceutical materials appears to be modified Gordon–Taylor equation[35,36] which is based on fr volume theories with some simplifying assumptions. F simple two-component mixtures,

$$T_{g_{mix}} = (w_1 T_{g1} + K w_2 T_{g2})/(w_1 + K w_2) \qquad (4$$

where $T_g$ is the glass transition temperature, $w_1$ and $w_2$ a the weight fractions of components 1 and 2, and $K$ can b calculated from the densities ($\rho$) and glass transition tempe atures ($T_g$) of the components:

$$K = (T_{g1}\rho_1)/(T_{g2}\rho_2) \qquad (5$$

Similar equations can be readily derived for mixtures of mo than two components. A perfectly miscible system will displa a single sharp glass transition event. Immiscibility, incom patibility, or nonideality is often indicated by a poor fit to th

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theoretical equation, the appearance of more than one $T_g$, or "broadening" of the glass transition event. Deviations from ideal behavior can also be identified and their most likely causes assessed using the graphical approach of Schneider and co-workers.[36,37] Deviations usually occur over discrete composition ranges and often can be explained in terms of molecular size effects and the disappearing free volume of the high-$T_g$ component at lower temperatures and compositions.[38,39] Such an approach is analogous to percolation theories and has considerable potential for describing mixed amorphous systems. Simple solution theories also can be used to describe such systems and to provide a qualitative understanding of the important factors regulating the glass transition in pharmaceutical systems. For example, it is likely that, when a macromolecule is mixed in small amounts with an amorphous small molecule, it will introduce a considerable excess free volume to the system because of its much larger molecular size. In this situation the glass transition temperature of the mixture probably will not be elevated as much as predicted by theory. The addition of low levels of a small molecule to an amorphous macromolecular system probably will be much less disruptive. Both materials will make near ideal contributions to the overall free volume of the mixture, and in this instance the predictions of the mixing equations are likely to be quite accurate for at least the first 50 K change in $T_g$. This is very important since the presence of very low levels of low molecular weight contaminants or additives (including water vapor) is predicted and observed to have significant plasticizing effects on pharmaceutical glasses,[26] whereas the addition of low levels of high molecular mass additives often has minimal antiplasticizing effect.[29] It should be noted that the concept of a critical additive composition ($W_2$) at which a glassy macromolecular material is sufficiently plasticized by a low molecular weight penetrant that it transforms to a rubbery amorphous solid under ambient conditions has been described by several authors.[33,40]

Pharmaceutical solids rarely exist as 100% crystalline or 100% amorphous phases so it is necessary at this point to consider how partially crystalline or amorphous systems are likely to behave. The coexistence of two thermodynamically different states of a material will probably result in (i) significant and measurable structural heterogeneities and (ii) batch to batch variations in physical properties. The presence of one phase in another can act as a focal point for spontaneous phase transitions such as crystallization.[39,41,42] In addition, as each phase is intimately dispersed in the other, there may not be complete independence of their behavior. For example, the dispersion of crystalline drug in an amorphous carrier has been reported to alter the observed glass transition temperature of the amorphous phase.[43] For macromolecules there may even be molecules which are part of both crystalline and amorphous domains physically linking the two regions together. Partially ordered systems have traditionally been described using either "one-state" or "two-state" models.[4,28,31,32] In the two-state model, domains of material are assumed to be either 100% amorphous or 100% crystalline and they coexist side by side in a molecular mixture. This type of system can be simulated to some extent by making physical mixtures of reference samples of crystalline and amorphous materials.[3] The one-state model consists of domains which are truly partially crystalline and in which the molecules have formed a semiordered structure as a result of being restricted in their motion during crystallization, or following the disruption of a more perfect crystalline state. The one-state model seems intuitively more likely than the two-state model but raises many questions which cannot be readily answered by studying mixtures of the reference crystalline and amorphous materials. In metallic systems there is also a state known as the "nanocrystalline phase" which has properties intermediate

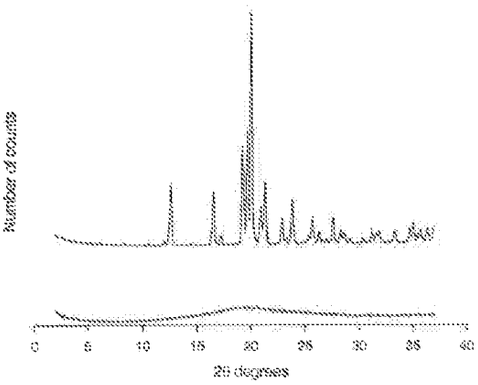


Figure 4—X-ray powder diffraction patterns for amorphous (bottom) and crystal (top) lactose.

to those of the amorphous and crystalline states,[44] and t concept of "glassy" or "plastic" crystals has recently be described.[45] Clearly the ability to distinguish between cr talline and amorphous states of a material and to be able quantify one phase in the presence of the other is critical the successful design and production of amorphous pharm ceutical systems.

## Characterization of the Amorphous State

Upon passing into the supercooled liquid state or throu the glass to rubber transition it is possible to observe chang in a multitude of material physical properties includi density, viscosity, heat capacity, X-ray diffraction, and dif sion behavior. Techniques which measure these propert (directly or indirectly) can be used to detect the presence an amorphous material (glass or rubber), and some of the methods are sensitive enough to allow quantification of t amount of molecular order or disorder (amorphous conte in a partially crystalline system.

As there is no long-range three-dimensional molecular or associated with the amorphous state, the diffraction electromagnetic radiation (*e.g.*, X-rays) is irregular compar to that in the crystalline state (Figure 4). Diffraction te niques are perhaps the most definitive method of detecti and quantifying molecular order in any system, and conv tional, wide-angle and small-angle diffraction techniques ha all been used to study order in systems of pharmaceuti relevance.[3,5,41] The specificity and accurate quantitati nature of these nondestructive techniques make them fi line choices for studying partially crystalline pharmaceuti materials. Conventional X-ray powder diffraction measu ments can be used to quantify non-crystalline material do to levels of about 5%[41] and with temperature and envir mental control can also be used to follow the kinetics of pha transformations, or to quantify the presence of a crystalli drug in an amorphous excipient matrix.[46] Small-angle X-r measurements have been used to study subtle structu (density) changes in polymers in the glassy state up annealing,[47] and neutron scattering is gaining wider use the characterization of short-range two-dimensional order amorphous materials.[48] It should be remembered that d fraction techniques only "see" molecular order, and th disorder is only implied.

The irregular arrangement of molecules in the amorpho state usually causes them to be spaced further than in crystal so that the specific volume is greater and the dens lower than that of the crystal, and we say that there is greater "free volume" (Figure 2). Highly accurate measu

ments of volume or density are difficult, and the magnitudes of differences involved are fairly small (Table 1), so such determinations are not a method of choice for characterizing amorphous pharmaceutical systems. The use of gas displacement pycnometry for quantifying the amorphous content of partially crystalline pharmaceutical systems has been described by Saleki-Gerhardt *et al.*,[47] and the accuracy achieved was about ±10%. Liquid displacement pycnometry has also been used to determine the crystallinity of several starch samples.[59] This approach has proved useful for quantifying low levels of disorder in crystalline pharmaceutical samples.[60] Precise dilatometric techniques are widely used for the study of amorphous polymers,[51] and these techniques could be useful for pharmaceutical systems; however, the methods are time-consuming and quite difficult to perform and so would not be suitable for routine use.

The most characteristic property of the amorphous state is its viscosity (approximately $<10^{12}$ Pa·s above $T_g$ and $>10^{12}$ Pa·s below $T_g$). The greater free volume and molecular disorder of an amorphous material compared to its crystalline counterpart result in the mechanical properties of amorphous systems (viscosity, elastic modulus) being much more like those of a liquid than those of a solid. In some supercooled liquids a breakdown of the Stokes–Einstein relationship between the viscosity and the rate of diffusion (or molecular mobility) has been observed.[52] It is thought that the breakdown of this fundamental relationship may be due to the decoupling of certain modes of molecular motion in the amorphous state.[52,53] Methods which are used to measure the viscosity of amorphous materials are quite specialized and include the bending of rods or curved fibers below $T_g$,[54] and the torsion pendulum and falling sphere methods above $T_g$.[55] Experimental difficulties are often quite significant, and thus such measurements are usually feasible only in a specially equipped research laboratory.

Diffusion-controlled processes (such as gas transport, self-diffusion, crystallization, and some chemical reactions) which are all closely linked to the viscosity of the amorphous matrix have been the subject of many studies of glassy and rubbery amorphous materials.[56] As a consequence of the greater free volume in amorphous materials, diffusive transport processes are usually significantly more rapid and isotropic than in crystals. Often different diffusion rates are observed above and below $T_g$, with significantly faster diffusion occurring above $T_g$. This phenomenon can be used to identify the glass transition temperature in some amorphous systems. The sensitivity of transport processes to small fractions of crystalline order has yet to be clearly defined, although it appears that ordered domains can slow down the rate of diffusion. There is an intuitive connection between the viscosity of an amorphous system and the rate of diffusion within it. The well-known theoretical models developed for the liquid state are not directly applicable to amorphous systems, but models specifically derived for amorphous systems are numerous, with the most popular being based on thermodynamic and polymer free volume theories.[56,57] It is very difficult to make general predictions about diffusion behavior in amorphous systems because of the large number of system specific variables which can have an effect upon transport properties. It is possible to rank the rates of diffusion in the order liquid > rubber > glass > crystal, assuming all other factors to be approximately equal. Transport properties of amorphous pharmaceutical materials are important because they can be exploited to control drug release in modified release dosage forms (*e.g.*, transdermal patches). The methods used to study transport phenomena in amorphous pharmaceutical systems include diffusion cells, NMR measurements,[57] and molecular dynamics simulations.[58]

The use of molecular probes (*e.g.*, fluorescent or phosphorescent) to determine the physical properties of amorphous materials is becoming quite common.[59,60,61] These techniques rely upon the regulation of a probe's mobility by the amorphous system under consideration. As with all probe-based techniques, the accuracy of the data obtained relies on the choice of a probe which does not alter the properties of the amorphous material while at the same time being sensitive to those same properties. Water, for example, will plasticize most amorphous materials, and its own mobility is necessarily affected by the state of its amorphous surroundings,[57] so it usually makes a very poor probe for amorphous systems. Phosphorescent probe studies at different temperatures with amorphous sucrose have shown changes in probe mobility around the calorimetric glass transition of the sugar,[59] and similar findings have been reported with sucrose–water, glycerol–water, and malto-oligosaccharide–water glasses using spin-probe electron spin resonance spectroscopy.[59,60,61] Quantification of the percent order and disorder is not currently possible using probe techniques, although probe mobility is likely to be affected by the presence of ordered regions in the matrix. Probe techniques have the potential advantage of providing molecular level information about the state of an amorphous material but because of their indirect nature require considerable care in their execution and expertise in data interpretation.

Spectroscopic techniques are valuable for the characterization of amorphous systems because of their high structural resolution. Nuclear magnetic resonance (NMR), Raman, infrared (IR) and electron spin resonance (ESR) studies of amorphous pharmaceutical systems have all been described.[57,62–64] These methods can be used to (i) determine the glass transition temperature, (ii) quantify the percent amorphous content, and (iii) determine mean molecular relaxation times as a function of temperature.[57–64] While there are some advantages in being able to follow the molecular mobility of specific chemical groups within a molecule (*e.g.*, resolution of different modes of motion), the interpretation of spectroscopic data is often quite complex. This can mean that the conclusions drawn are open to question unless supporting evidence can be obtained using complementary techniques.[58] Samples ranging from simple powders or solutions to entire dosage forms can be studied using these nondestructive techniques, and this makes them especially useful for characterizing pharmaceutical systems. Dielectric relaxation and dynamic mechanical spectroscopy have also been widely used to study amorphous pharmaceutical materials.[65–68] These techniques are especially sensitive to the glass transition event and to secondary thermal transitions which reflect lower order molecular motions. The assignment of molecular motions measured using these techniques to particular structural groups is possible only with simple systems, and differentiation between crystalline and amorphous phases is not usually quantitative. A relatively wide frequency range can be used and activation energies for transitions can be determined. In addition, time–temperature superpositioning may be used to predict the effects of conditions that are not experimentally attainable.[69] Relatively large samples (>100 mg) are required for these two techniques, and sample preparation can sometimes be difficult.

Thermal analytical methods have been widely used to characterize amorphous pharmaceutical systems, and several comprehensive reviews of their use have been published.[70,71] Most often thermal analytical techniques are simply used to determine the glass transition temperature of an amorphous drug or excipient. For example, Porter and Ridgway[72] used differential scanning calorimetry (DSC) to determine the glass transition temperature of several enteric coating polymers and correlated the results with a softening point measured using a simple indentation device. The glass transition temperatu

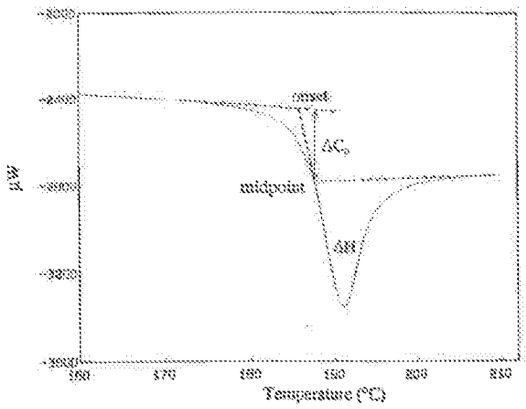



Figure 5—Glass transition of poly(vinylpyrrolidone) by DSC showing the onset and midpoint of the transition, the change in heat capacity at $T_g$ ($\Delta C_p$), and the enthalpy relaxation endotherm ($\Delta H$).

of mixtures of amorphous materials (*e.g.*, a film coating agent and a plasticizer) has also been determined as a function of mixture composition and regions of miscibility identified.[66,74,75] Thermal analytical methods can obviously be used to determine fundamental thermodynamic properties (*e.g.*, heat capacity, enthalpy changes) of the amorphous state. The heat capacity of an amorphous material is always higher than that of its crystalline state (Figure 2), and a change in the rate of heat capacity change with temperature defines its calorimetric glass transition temperature[63] (Figures 2 and 5). This change in heat capacity at the glass transition temperature ($\Delta C_p$) can be related to the fragility of the amorphous material (see earlier).[7,8] Glasses are thought to be frozen into an unstable state on normal experimental time scales, but on longer time scales they will slowly relax back to the metastable supercooled liquid state. The enthalpic changes associated with this process can be measured using DSC[30] and used to determine mean molecular relaxation times ($\tau$) for glassy materials. Such relaxation processes can also be studied by thermomechanical analysis (TMA) for some sample configurations.[66,76] Crystallization from the amorphous state can be induced by thermoanalytical techniques to produce an exothermic change whose magnitude is quantitatively related to the extent of crystallization occurring. This can be used to determine the initial percent crystallinity of a partially amorphous sample provided total crystallization is known to occur.[5,41] Thermal analytical methods can also be used to study the contributions of nucleation and crystal growth processes to the overall rate of crystallization.[77] The influences of the experimental variables used in thermal analytical methods (*e.g.*, sample size, heating rate, cooling rate) upon the results obtained with amorphous materials have been widely studied and can have very profound effects upon the type and quality of data obtained.[78] Great caution is required in the application of thermal analytical techniques to characterize the thermodynamically unstable *glassy* amorphous state. The variance of the glass transition temperature with heating and/or cooling rate can be used to determine the activation energy for the glass transition, which can then be used to characterize the strength/fragility of an amorphous system.[7,8] The use of more sophisticated thermal analytical techniques such as modulated DSC and thermally stimulated current methods[79,80] has great merit in the characterization of the amorphous state but is beyond the scope of this review. For a full description of these evolving techniques the reader is referred to the current thermal analysis literature.[30–32]

Vapor sorption by amorphous and crystalline pharmaceutical materials is usually quite different and thus can be used to precisely distinguish between them.[5,27,68] Typically, crystalline materials *adsorb* vapors in small quantities at the surfaces, or take up larger stoichiometric quantities to form solvates. In contrast, amorphous materials *absorb* vapors in relatively large amounts (100% by weight is not uncommon). Vapor sorption in glassy and rubbery amorphous systems has been widely studied experimentally and modeled extensively, particularly with polymers.[64,65] The models most applicable to pharmaceutical materials are based on solution theory and assume no specific interactions between sorbent and sorbate.[65] Rubbery amorphous materials usually sorb considerably greater quantities of vapor than their respective glasses, and this phenomenon can be used to identify the state of an amorphous material.[33] Many sorbents plasticize the amorphous material into which they absorb, and thus a critical partial vapor pressure and corresponding system composition exist at which the glass to rubber transition is induced at ambient temperature.[68,69] Temperature effects on vapor sorption tend to be small, usually with an increase in level of sorption with decreasing temperature which is consistent with an exothermic solution model.[65] Water vapor sorption has been most widely used in the study of amorphous drugs and excipients, and the predominant techniques are the vacuum microbalance and desiccator/saturated salt solution gravimetric methods.[68,87,88] Volumetric and potentiometric methods have also been used.[89,90] The sensitivity of water vapor sorption techniques to amorphous material is of the order of a few weight percent,[41] and thus these techniques are the preferred means of studying pharmaceutical systems containing low levels (<10%) of amorphous material. The plasticizing effects of water vapor on amorphous pharmaceutical materials have been described in some detail[33,68,88] and can potentially induce unwanted effects such as spontaneous phase transitions and lyophile collapse.[45,91] Isothermal microcalorimetric techniques can be used in conjunction with water vapor sorption methods to study such phase transitions from the amorphous state.[31] The influence of additives on the kinetics of amorphous to crystalline phase transition in lactose have been investigated,[92] as has the physical stability of amorphous drugs.[93] Typically the amorphous content of a partially crystalline material can be quantified and the enthalpies of sorption and crystallization determined.

Without water vapor sorption capabilities microcalorimetry can be used in a non-isothermal mode to detect glass transition events and secondary transitions in amorphous pharmaceutical solids. It has also been used in several studies to detect physical and chemical instabilities in formulations containing amorphous drugs or excipients.[94] Isothermal solution calorimetry has been successfully used to identify and quantify the degree of crystallinity in several pharmaceutical systems.[5] Microcalorimetry has the advantage of great thermal sensitivity, which can be very useful for studying weak secondary transitions in amorphous systems such as proteins. It has the disadvantage of being chemically unspecific, and thus the processes occurring in the calorimeter have to be independently verified using other analytical techniques. Microcalorimetric instrumentation is widely available, and many pharmaceutical research and development groups are using the technique routinely to study amorphous materials.

In summary, there are many precise and accurate methods suitable for studying and characterizing amorphous pharmaceutical materials in all their configurations, including final dosage forms. As with any scientific method, the use of multiple complimentary techniques which measure the properties most relevant to the problem or material under consideration is preferred. The most notable difference between the techniques described is in their ability to quantify the amount of order and disorder in partially amorphous systems.

Table 2—Water Content and Glass Transition Temperature of the Amorphous Fraction of a Partially Crystalline Sucrose Sample Containing Different Amounts of Water

| Total Amount of Water (%) | Amorphous Content (%) | Water Content of Amorphous Fraction (g of $H_2O$/100 g of solid) | Glass Transition Temp (°C) |
|---|---|---|---|
| 0.0 | 100 | 0 | 74 |
| 0.1 | 5.0 | 2 | 47 |
| | 2.5 | 4 | 25 |
| | 1.0 | 10 | −17 |
| 0.5 | 5.0 | 10 | −17 |
| | 2.5 | 20 | −62 |
| | 1.0 | 50 | −91 |

and in their ability to monitor molecular level processes over a wide range of temperatures (ideally above and below $T_g$).

## Pharmaceutical Significance of the Amorphous State

Typically, the pharmaceutical scientist concerned with the use of drugs and excipients in solid dosage forms must deal with those factors that influence manufacturability, stability, bioavailability, and therapeutic performance. Although in the majority of cases drug entities come to the development stage in crystalline form, there are situations where they exist in a partially or fully amorphous form.[1] In some cases it may simply be impossible to crystallize the material by available crystallization techniques. Processing (*e.g.*, milling, lyophilization, granulating, drying) might introduce a certain level of amorphous structure to an otherwise highly crystalline material.[82,41] The amorphous state may also be introduced deliberately to enhance the biopharmaceutical properties of the product. For example, for a crystalline drug with very poor aqueous solubility, the formation of a coamorphous mixture with a water soluble additive can provide an opportunity to enhance dissolution and perhaps bioavailability.[4,83] Additionally many excipients come to the formulator in a fully or partially amorphous state (*e.g.*, microcrystalline cellulose, starch, poly(vinylpyrrolidone)), and other excipients may be purposefully rendered amorphous to enhance functionality (*e.g.*, spray-dried lactose).

Since molecules in the amorphous state exist in a higher energy state than in the crystalline state, we would expect that properties requiring certain levels of molecular mobility (*e.g.*, permeability) would be influenced by the presence of amorphous structure and by the relationship of the operating temperature to $T_g$ and $T_m$. Small amounts of absorbed water can plasticize amorphous solids[39] so we would also expect relative humidity to be an important factor influencing the solid-state properties of amorphous systems. It has been shown that, if a system consists of a small amount of amorphous material in a crystalline matrix, the preferential absorption of water vapor into the amorphous structure will result in a local water content greatly amplified relative to the total water content, with a correspondingly greater plasticizing effect on the amorphous structure[37] (Table 2).

In considering some examples of the importance of the amorphous state in determining the solid-state properties of pharmaceuticals, we will examine three broad areas: crystallization, chemical degradation, and mechanical responses to stress (*e.g.*, powder compression in tabletting). In all of these cases a critical factor is the rate at which molecules in the solid state undergo rotational or translational motions. As discussed previously (see eqs 1 and 2) molecular mobility changes significantly with temperature, additive concentration, and water content, particularly in the vicinity of $T_g$. The ultimate challenge is to relate the time scales required f various physical and chemical solid-state processes to the ti scales expected for molecular mobility under any given set environmental conditions. A few illustrations of the state our knowledge in applying these concepts to pharmaceutic systems are given below.

**Crystallization**—Since molecules in the amorphous sta are thermodynamically metastable relative to the crystalli state, the potential for crystallization during handling a storage is always present. Evidence exists to show that su crystallization can be responsible for phenomena such postcompression hardening of tablets,[36,57] lyophilized ca collapse,[58] and particle aggregation in dry powder inhalers It would appear important to be able to anticipate conditio that give rise to such crystallization and to be able to inhi it if so desired.

Crystallization from the amorphous state is primari governed by the same factors that determine crystallizati from the melt. Hence we can illustrate the important facto to be considered by observing the general equations f homogeneous nucleation from the melt.[100] The rate of nucl ation $I$ is expressed as

$$I = A \exp \frac{-(\Delta G_a + \Delta G^*)}{kT} \quad (3$$

where $\Delta G_a$ = the activation energy for transport across t nucleus–amorphous matrix interface, $\Delta G^*$ = the free energ change for nucleation with a critical nucleus of radius $r$, $A$ a constant, $k$ is the Boltzmann constant, and $T$ is t temperature, and where for a spherical nucleus it can shown that

$$\Delta G^* = \frac{16\Pi\Delta g^3}{3\Delta G_v^2} \quad (4$$

where $\Delta G_v$ = the difference in free energy per unit volu between the crystalline and amorphous forms, $G_c - G_a$, a $\Delta g$ = the interfacial free energy per unit area at the nucleus amorphous matrix interface. Further, it can be shown th

$$\Delta G_v = (G_c - G_a) \approx -(\Delta H_f)(\Delta T_r) \quad (5$$

where $\Delta H_f$ = heat of fusion, $\Delta T_r = (1 - T_c/T_m)$ = degree supercooling, $T_m$ = melting temperature, and $T_c$ = crysta lization temperature.

From these equations we can see that for any amorphou system the temperature at which optimal nucleation, a hence crystallization, should occur will depend primarily the degree of supercooling below $T_m$ and where the tempe ature lies relative to $T_g$. The closer to $T_g$, the greater t degree of supercooling and the lower the molecular mobilit As described by Jolley[101] and schematically shown in Figu 6, the enhanced tendency for supercooling caused by lowerin the temperature and increased viscosity that accompanies th reduction in temperature should give rise to a maxim crystallization rate at some temperature between $T_g$ and $T$ Indeed a non-isothermal DSC scan of amorphous sucro (Figure 7) shows a crystallization exotherm somewhere b tween the $T_g$ and $T_m$.[41] It also has been shown that t crystallization temperature for amorphous sugars is reduc by the presence of absorbed water in direct proportion to t plasticizing effects of water on $T_g$ and raised by the incorpor tion of high $T_g$ additives, again demonstrating the importan of molecular mobility in this process.[39] This has led to general conclusion that crystallization from the amorphou state over practical time scales can be prevented by keepin the operating temperature below $T_g$[101,102] by reducing t

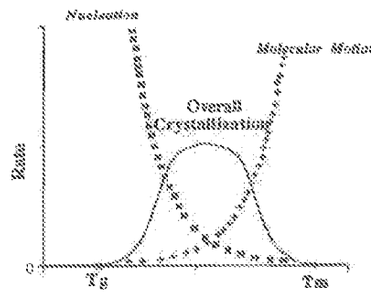



Figure 6—Schematic depiction of the parameters controlling crystallization from the amorphous state. Adapted from ref 101.

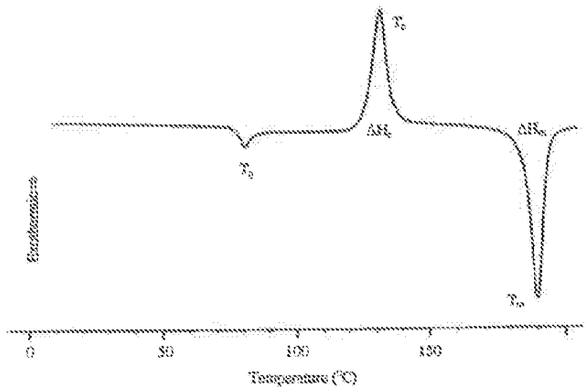



Figure 7—DSC scan of amorphous sucrose showing the glass transition temperature ($T_g$), the crystallization exotherm ($T_c$, $\Delta H_c$), and the melting endotherm ($T_m$, $\Delta H_m$). Adapted from refs 41 and 42.

water content or by raising the $T_g$ of the system using additives with high $T_g$ values[103] (*e.g.*, PVP with a $T_g$ of about 180 °C[56]). If a small amount of amorphous structure is introduced into a crystalline sample by processing[8,4,104] and this is deemed to be undesirable, raising the operating temperature above $T_g$, or exposing the sample to elevated relative humidity so as to lower $T_g$, can "anneal" the sample back to a completely crystalline state.

An important question with regard to crystallization is, *At how low a temperature must an amorphous sample be stored to provide typical 3–5 year shelf-life stability?* In recent studies with dry amorphous indomethacin having a $T_g$ of about 50 °C it was shown that complete crystallization occurred within a few weeks at temperatures as low as 20 °C, but that reducing the temperature to refrigerator temperature provided periods of stability of a year or more.[5] An analysis of relaxation times for indomethacin below its $T_g$, using enthalpic relaxation measurements[105] and a fit of these data to the VTF equation (see eq 1) revealed a value of $T_0$ equal to about 267 °C. Assuming that this corresponds to the temperature at which essentially all important molecular motions would cease,[102] it was concluded that storage in a freezer (<267 °C) should provide at least 3–5 year shelf-life stability. An alternative means of stabilizing an amorphous drug would have been to raise its $T_g$ by coplasticizing with a high-$T_g$ glass former such as PVP.[102] These strategies have been advocated by many workers, but a clear understanding of the molecular basis for such stabilizing effects has not yet been achieved.[106–109] More work of this type is still required, and for further information the interested reader is referred to some general reviews related to crystallization from the amorphous state.[5,27,38,41,110–112]

**Chemical Reactivity**—Chemical degradation of drugs in the solid state, particularly at elevated temperatures and relative humidities, is a fairly common occurrence with drugs exhibiting a susceptibility for degradation when in solution. Presumably, in the amorphous state the reacting molecules have sufficient free volume and molecular mobility to react. We would expect a comparison of reaction rates of crystalline and amorphous forms of a drug under otherwise identical conditions to reveal greater rates with the amorphous form than with the crystalline forms, and this has been shown to be the case for a number of systems.[113,114] It is possible that a particularly solid state reaction would require a high level of positional specificity between reacting species and that it would occur most readily in a highly ordered crystalline state. In such cases reactivity in the amorphous or liquid state would be reduced relative to that in the crystalline state.[115] In the case of insulin it has been reported that some degradation pathways occur more readily in the amorphous state while in one particular reaction requiring such specific positioning the crystalline state is more reactive.[117]

If enhanced molecular mobility in the amorphous state is the major factor in controlling reaction rates, we would expect dependence of reactivity on free volume resulting in non-Arrhenius kinetics.[118] Thus an equation that combines activational energetics with free volume considerations has been derived:[119]

$$k = A' \exp\left[-\left(\frac{V^*}{V_f}\right) - \left(\frac{E_a}{kT}\right)\right] \quad (4)$$

where $k$ is the rate constant, $A'$ a pre-exponential factor, $E_a$ is the energy of activation, $V_f$ is the free volume, and $V^*$ is the critical free volume for the molecular motion required for reactivity. For systems with high $V_f$ (well above $T_g$ or in the liquid state) the rate constant should follow Arrhenius kinetics, while at lower temperatures closer to and below $T_g$ free volume should be an important factor. As an example of the first case we can consider a recent study of degradation of indomethacin in the liquid state (above $T_m$) and just below $T_m$ in the amorphous state (about 100 °C above $T_g$), where it was shown that the rate constants fit the Arrhenius equation with no discontinuity as the sample changed from the amorphous solid to the melt.[118] If free volume factors are important, we would expect the non-Arrhenius behavior to parallel the effects of temperature on relaxation rates, as reflected in the VTF or WLF equations described earlier (eqs 1 and 2). Depending on the fragility of the amorphous state we might expect greater or lesser deviations from Arrhenius behavior. The situation is complicated by the fact that a system studied over a limited temperature range will exhibit apparent linearity on an Arrhenius plot. Consequently, to test for temperature dependence in such amorphous systems it is best to use as wide a temperature range as possible and to work under conditions in the vicinity of $T_g$ where free volume effects change significantly. Such a situation seems to exist in the case of the nonenzymatic browning reaction that typically occurs between reducing sugars and amines where it has been shown that reaction rates correlate with $T - T_g$ (as in eqs 1 and 2) in a number of cases.[120] To date in the pharmaceutical literature there has been only one published study of an amorphous system that appears to follow WLF-like chemical kinetics. Roy *et al.*[121] measured the rates of three different types of chemical degradation for lyophilized samples of a *Vinca* alkaloid–antibody conjugate and showed excellent correlation of reaction rates with $T - T_g$ for three temperatures and three water contents (different values of $T_g$). It is important to recognize that some reactions in the amorphous state can still take place below $T_g$ and the

attempts to scale reaction kinetics to $T_g$ may be inappropriate. In such cases the value of $T_0$ in the VTF equation (see eq 1) may be more physically meaningful. More studies covering a wide range of temperatures and using other models are required to address this important issue if we ultimately expect to be able to predict chemical kinetic behavior in such systems.

**Mechanical Properties**—In the processing and handling of solid pharmaceuticals there are a number of situations where rheological or mechanical properties are critical for product manufacturability, stability, and performance. Typically most crystalline materials tend to exhibit high levels of elasticity and brittleness upon exposure to an external stress. By contrast molecules in the amorphous state tend to exhibit varying degrees of viscoelasticity, depending on their temperature relative to $T_g$. Such viscoelastic behavior provides solids with the ability to flow under conditions of mechanical stress and to provide a number of important excipient functionalities. Relief of mechanical stresses through flow would appear to be important in creating tablet bonds after compression of powders[97,122] and in preventing mechanical failure of polymeric film coats on tablets because of stress relaxation.[123] The use of plasticizers, including water, to lower the $T_g$ of various cellulosic and acrylic polymers utilized in film coating is based on this principle, as is the use of spray-dried lactose (about 15% amorphous content) or microcrystalline cellulose (about 30% amorphous content) as direct compression tabletting excipients. To emphasize the importance of the amorphous state in these latter situations it should be pointed out that the water content of such direct compression excipients must be held at a certain level to provide optimal compression properties. For example, a critical level of about 4–6% water provides a significant change in the viscoelastic properties of microcrystalline cellulose[124] and is the level below which direct compact properties are lost. Similarly spray drying crystalline sucrose with 3% maltodextrin produces a direct compression material with properties strongly dependent on the water content of the maltodextrin[125,126] presumably because of its plasticizing effects on the amorphous component.

## Strategies for Dealing with Partially Amorphous Pharmaceutical Systems

Let us consider a new drug entity or excipient that contains irregular particles which are not birefringent under cross-polarized light. The material also exhibits nonstoichiometric water uptake in excess of that expected for surface adsorption on a crystalline surface (*e.g.*, >0.01%)[84,104] and upon exposure to elevated temperature and relative humidity appears to undergo chemical degradation in a manner similar to its behavior in solution or in the melt. Such characteristics should immediately suggest that the material may have considerable amorphous character.[41,85] At this point it would be useful to check on the history of the processes used to prepare the material: How was it crystallized? Did it undergo any milling that might have led to the creation of amorphous character? Were there any additives present or processing conditions that might stabilize or favor the amorphous state? To further assess the characteristics of this system we can take two approaches. First, we can attempt to predict the properties of an amorphous state based on characteristics of the 'crystalline' sample; and second, we can attempt to prepare a completely amorphous form of the drug and carry out direct measurements of its properties.

In the first case we can begin by measuring the crystal melting temperature ($T_m$). This can then be used to estimate the glass transition temperature based on the fact that the mean ratio of the melting temperature to the glass transition temperature ($T_m/T_g$) in kelvin for pharmaceutical materia is approximately 1.36[70,75] (Table 1). An estimate of t Kauzmann temperature ($T_K$) (or the point of 'negligib molecular mobility) can also be made as $T_m/2$ or $T_g - 50$ The heat capacity change at $T_g$ ($\Delta C_p$) can be estimated fro the presumably known chemical structure[127–129] or from t knowledge that for many materials (especially polymers) t product of $\Delta C_p$ and $T_g$ is approximately 100 kJ·g$^{-1}$. Likewi the product of $T_g$ and the change in thermal expansivity $T_g$ ($\Delta\alpha$) is approximately 0.113 leading to an estimate f $\Delta\kappa$.[130,131] The viscosity of most glasses is between $10^9$ a $10^{13}$ Pa·s at $T_g$ and above $T_g$ often varies with temperatu according to the WLF equation (with so-called univers constants) so a preliminary estimate of molecular mobility any temperature above $T_g$ could be made. A prediction of t fragility of the amorphous material also could be made fro the estimated $\Delta C_p$ and $T_m/T_g$ values,[7,8,132] and from th knowledge a more accurate assessment of the temperatu dependence of molecular mobility and viscosity above $T_g$ cou be attempted.[133] Assuming ideal behavior, the plasticizi effect of any absorbed water vapor (or other additiv penetrant) on the amorphous material can be estimated usi simple mixing rules.[36] This should allow the determinati of the critical plasticizer content at which the glass to rubb transition is induced at ambient or other operating tempe rature. If it is possible to obtain a purely amorphous sampl direct characterization of this form can then be carried ou Amorphous reference samples can usually be produced b either quench-cooling the melt in liquid nitrogen, lyophiliz tion, or spray drying. Other than powder X-ray diffracta measurements for assessing lack of crystallinity, the mo useful techniques for the early evaluation of amorphous soli are differential scanning calorimetry and water vapor sorpti analysis. A non-isothermal DSC scan of a dry amorpho material can be used to measure $T_g$, $\Delta C_p$, $T_c$ (non-isotherm recrystallization temperature), and $T_m$ (Figure 6). From the parameters characterization of the amorphous material c be carried out in a reasonable level of detail. Questions th can be answered include the following: What is the gla transition temperature relative to the ambient temperatur What is $T_g$ relative to $T_m$, and thus what is the fragility the compound? Does the amorphous material spontaneous crystallize upon heating above $T_g$, and if so, at what tempe rature? Can crystallization be induced by scratching t surface of the sample, or by exposing to water vapor? All these questions may be addressed using only a small quanti (a few milligrams) of material. The answers to these questio can be compared to those predicted using the "rules of thum given earlier and any deviations from typical amorpho behavior identified. Water vapor sorption by the amorpho material should be measured next at room temperature ov a wide range of relative humidities. Since it has been sho that water vapor absorption varies very little over the te perature range 5–60 °C for many systems,[36] this data can taken to be representative of water uptake at most tempe ratures experienced by a pharmaceutical solid. The use of automated sorption microbalance apparatus[97] should allo such measurements to be performed with only a few m ligrams of material. If water absorbed into the solid is ab to cause crystallization over a short time scale, this will reflected by a spontaneous loss of the absorbed water at son critical relative humidity. This will give an indication of wh water content (or humidity) will be required to eliminate t amorphous content of any sample, or below what wat content one must store amorphous samples to avoid cryst lization. These experiments can be followed by more preci measurements of $T_g$ vs water content by DSC to provide more exact indication of how water might act as a plasticiz and what water content and relative humidity are requir

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to induce the glass to rubber transition at ambient temperature. Comparisons with the behavior predicted by simple mixing rules could also be made in order to obtain an understanding of the nature of amorphous solid−water interactions.[26,27,28] Thus, from a few simple calculations and measurements, it is possible to determine many of the physical properties of the amorphous form of a new material, and to assess its likely behavior in a pharmaceutical dosage form. Further detailed physicochemical characterization with the techniques described earlier will obviously be desirable, as will accelerated stability tests and biopharmaceutical studies.

## Conclusions

The amorphous state is critical in determining the behavior and properties of many pharmaceutical formulations. Through an appreciation of the properties of the amorphous state it is possible to define means with which to characterize and work with such molecularly disordered pharmaceutical systems. In cases where amorphous character is desirable in a pharmaceutical formulation, it may be stabilized using strategies based on an understanding of the thermodynamic and kinetic properties of amorphous systems. In circumstances where amorphous character is undesirable, approaches are available so that disorder can be minimized and conversion of amorphous material to the most stable crystalline state can be promoted.

## Note Added in Proof

While this manuscript was in press, it was pointed out that another reference predates refs 7 and 8 in reference to the rule of thumb proposed for determining fragility: Slade, L.; Levine, H. *Adv. Food Nutr. Res.* **1995**, *38*, 103−269.

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## Acknowledgments

The authors wish to recognize helpful discussions with S. Kiranidhi, V. Andronis, and E. Y. Shalaev. Constructive commen and suggestions from the referees of this manuscript are al acknowledged. G.Z. thanks the Purdue/Wisconsin Joint Program Molecular Mobility in Pharmaceutical Solids for financial suppo

JS9601896

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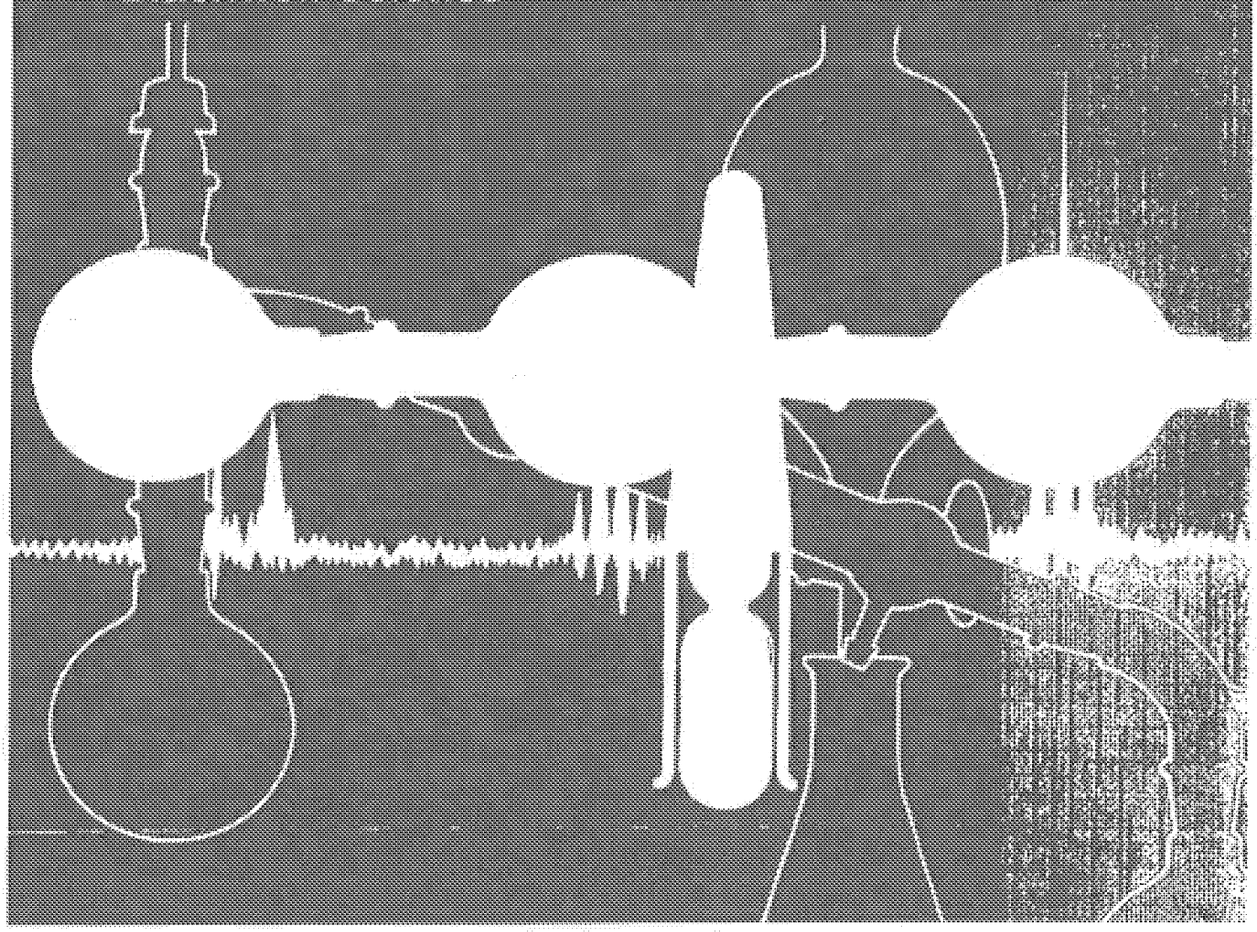
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# EXPERIMENTAL ORGANIC CHEMISTRY

## PRINCIPLES AND PRACTICE

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Blackwell Science



Ex. 2001-0831

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Editorial offices:
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25 John Street, London WC1N 2BL
23 Ainslie Place, Edinburgh EH3 6AJ
238 Main Street, Cambridge
Massachusetts 02142, USA
54 University Street, Carlton
Victoria 3053, Australia

Other Editorial Offices:
Arnette Blackwell SA
1, rue de Lille, 75007 Paris
France

Blackwell Wissenschafts-Verlag GmbH
Kurfürstendamm 57
10707 Berlin, Germany

Blackwell MZV
Feldgasse 13, A-1238 Wien
Austria

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First published 1989
Reprinted 1990, 1992, 1994, 1995

Set by Times Graphics, Singapore
and printed and bound in Great Britain by
The Bath Press, Avon

DISTRIBUTORS

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Blackwell Science Pty Ltd
54 University Street
Carlton, Victoria 3053
(*Orders*: Tel: (03) 347 5552)

British Library
Cataloguing in Publication Data

Harwood, Laurence
Experimental organic chemistry.
1. Organic chemistry. Experiments
I. Title II. Moody, Christopher
547.00724

ISBN 0-632-02016-4
ISBN 0-632-02017-2 pbk

Library of Congress
Cataloging-in-Publication Data

Harwood, Laurence
Experimental organic chemistry.
Includes index.
1. Chemistry, Organic—Laboratory manuals. I. Moody, Christopher. II. Title.
QD261.H265 1989 547'.0078 88-7680
ISBN 0-632-02016-4
ISBN 0-632-02017-2

Ex. 2001-0832

The most important factor in drying organic solutions is the choice of drying agent. Ideally the solid drying agent should be totally insoluble in organic solvents, inert to a wide range of organic compounds (including solvents) and able to take up water quickly and efficiently to give a hydrated form which is an easily filterable solid. The most commonly used drying agents are listed in Table 3.5, which gives information on their *capacity* (how much water they can take up), *speed* (rate of water uptake), *efficiency* (how dry they leave the solution) and *applicability* (suitability for different classes of compound). Clearly the choice will depend on a number of factors, the most crucial of which is the nature of the organic compound that is dissolved in the solvent, and that is ultimately to be isolated. As a good general purpose drying agent, magnesium sulfate finds the widest use.

It is important to note that drying agents that are suitable for drying *organic solutions* are not usually appropriate for drying *organic solvents* for use with moisture-sensitive compounds (see pp. 77–87). The drying of organic solvents is an entirely separate problem that is referred to on p. 79, and dealt with specifically in Appendix 2.

## Crystallization

The simplest and most effective technique for the purification of solid organic compounds is crystallization. Crystalline compounds are easy to handle, their purity is readily assessed (Chapter 4) and they are often easier to identify than liquids or oils. Crystals can be obtained in one of three ways: from the melted solid on cooling, by sublimation (pp. 154–155) or from a supersaturated solution. The last method is by far the most common in the organic laboratory.

### Crystallization of Organic Compounds

A general plan for the purification of an organic compound by *crystallization* is shown in Figure 3.37. The process involves five stages: dissolution, filtration, crystallization, collection of the crystals and drying the crystals. The purity of the crystals can then be determined (Chapters 4 and 5), and if necessary further purification by *recrystallization* can be carried out. Before discussing each of the stages in the process in detail, we should briefly consider how crystallization succeeds in purifying compounds at all.

*How crystallization works*

The technique involves dissolving the impure solid in the minimum volume of a hot solvent and filtering to remove insoluble impurities. The resulting hot saturated solution of the compound, together with any soluble impurities, is set aside to cool slowly, whereupon crystals of pure compound will separate from solution. The solution remaining after crystallization is usually known as the *mother liquor*. Why are the crystals

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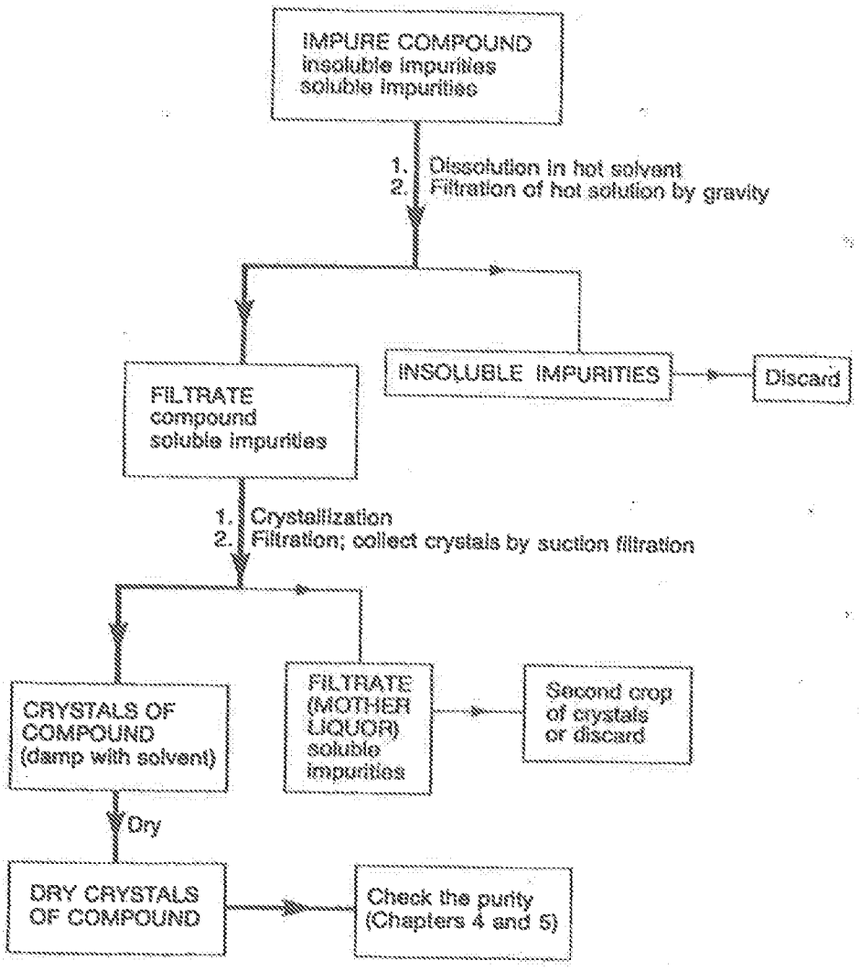



**Figure 3.37.** Plan for purification of an organic compound by crystallization.

pure? The process of crystallization is an equilibrium: molecules in solution are in equilibrium with those in the crystal lattice. Since a crystal lattice is highly ordered, other different molecules, such as impurities, will be excluded from the lattice and will return to the solution. Therefore only molecules of the required compound are retained in the crystal lattice and the impurities will remain in solution. For a crystallization to be successful, *the solution must be allowed to cool slowly*, so that the crystals are formed slowly, and the equilibrium process which excludes the impurities is allowed to operate. If a solution is cooled rapidly, impurity molecules will be trapped or included in the rapidly growing crystal lattice. This rapid

*Slow cooling*

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formation of solid material from solution is *precipitation*, and is not the same as crystallization.

At this stage it should be pointed out that crystallization does not always work. Substances which are grossly impure will often refuse to crystallize, and in these cases some preliminary purification by another technique, such as extraction (pp. 114–125) or chromatography (pp. 155–205), may be necessary.

### Dissolution

The first problem is to dissolve the impure substance in a suitable solvent. The ideal solvent for a crystallization should not react with the compound, should be fairly volatile so that it is easy to remove from the crystals, should have a boiling point that is lower than the melting point of the compound to be crystallized, should be nontoxic and nonflammable, but most important of all, the compound should be very soluble in hot solvent and insoluble in cold solvent. In many cases, particularly when crystallizing known compounds, you will know what solvent to use because the literature or your laboratory text will tell you. In other cases, you will have to decide what solvent to use. Choosing a solvent for crystallization is not always easy, but organic chemists tend to follow the rule that 'like dissolves like'. So, for the crystallization of nonpolar substances such as hydrocarbons, use a nonpolar solvent such as hexane or light petroleum. Compounds containing polar groups such as OH are best crystallized from polar OH containing solvents such as ethanol. Indeed polar solvents are often preferred for other compounds because they tend to give better crystals. Some suggestions for crystallization solvents for the most common classes of organic compound, arranged in order of increasing polarity, are given in Table 3.6.

*Like dissolves like*

If the crystallization solvent is not known for certain, do not commit all your solid and attempt to dissolve it up. Rather, carry out some preliminary solubility tests. To do this, place a small quantity of the solid

*Test the solubility*

**Table 3.6. Suggested solvents for crystallization.**

| Class of compound | Suggested solvents |
|---|---|
| Hydrocarbons | Light petroleum, hexane, cyclohexane, toluene |
| Ethers | Ether, dichloromethane |
| Halides | Dichloromethane, chloroform |
| Carbonyl compounds | Ethyl acetate, acetone |
| Alcohols, acids | Ethanol |
| Salts | Water |

Ex. 2001-0835

*Use a hood if the solvent is toxic*

*Use a steam bath for heating flammable solvents*

(*ca.* 20 mg or the amount that fits on the tip of a micro-spatula) in a small test tube — an ignition tube or a 10 × 75 mm test tube is ideal — and add a few drops of solvent to the tube. If the substance dissolves easily in cold solvent, try again with a different solvent. If the substance is insoluble in cold solvent, warm the tube on a steam or water bath, and if the substance remains insoluble, add more solvent with continued heating. If the compound still refuses to dissolve, try again with a different solvent. Once you have found a solvent that dissolves the compound when hot, you need to check that the solid will separate again on cooling. Place the tube in a beaker of ice–water, and leave it to stand for a minute or two. If a solid forms on cooling, the solvent is probably suitable for crystallization of the bulk material. With experience, these preliminary solubility tests can be carried out quickly, and provide a satisfactory guide to the choice of crystallization solvent.

*Always keep a seed crystal*

Once you have found a suitable solvent, you are ready to dissolve up the solid for crystallization. Before doing so, it is a good idea to weigh the solid, if you have not already done so, so that the recovery of material from the crystallization process can be determined. If the substance is already crystalline, do not dissolve all of it. Always retain a few crystals in case they are needed for seeding purposes (see p. 132). Large crystals are often difficult to dissolve, and should be ground up before adding the crystallization solvent.

*Mixed solvents for crystallization*

If a suitable crystallization solvent cannot be found, then you may have to use a *mixed solvent system*. A mixed solvent system is a pair of miscible solvents, chosen so that one of them (the good solvent) dissolves the compound readily, and the other (the poor solvent) does not. For example, many moderately polar organic compounds are soluble in ether, but not in light petroleum, and therefore a mixture of the two solvents may be suitable for crystallization. There are two schools of thought on how to carry out a crystallization using mixed solvents. One method is to dissolve the solid in the minimum volume of hot good solvent, add the poor solvent dropwise until the solution starts to become slightly turbid or cloudy, and then set the solution aside to crystallize. The second method is to suspend the solid in hot poor solvent, and then add the good solvent dropwise with continued heating until the solid *just* dissolves; then set the solution aside as before. Typical mixed solvent systems that often work quite well include ether–light petroleum, dichloromethane–light petroleum, ether–acetone and ethanol–water. If possible choose a system in which the good solvent is the lower boiling solvent. One final word of warning: the use of mixed solvents often encourages *oiling out* (see p. 132), and therefore crystallization from a single solvent is preferred.

### Filtration

Once your compound is in solution in a hot solvent, the solution should be filtered to remove any insoluble material. This material may be an

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insoluble impurity or by-product or may simply be pieces of extraneous material such as dust, glass or paper. The solution should be filtered under gravity through a fluted filter paper into an Erlenmeyer flask using the technique described in pp. 74–75.

*Use hand protection for hot filtration*

In some cases the solution of your organic compound will be strongly colored by impurities. This is not a problem provided that the colored impurities remain in solution. However, occasionally they are adsorbed by the crystals as they form, to give an impure, colored product. Luckily the fact that such impurity molecules are easily adsorbed can be used to remove them from solution. This process is usually known as *decolorization*, and involves treating the hot solution with activated charcoal, often known as decolorizing carbon or under the tradename Norit®. To decolorize a solution add a small quantity of activated charcoal, usually about 2% by weight of the sample, to the hot, but not boiling, solution. If the solution is at or close to its boiling point, the addition of the finely divided charcoal will cause it to boil over. Continue to heat the solution containing the charcoal for about 5–10 min with occasional swirling or stirring. By this time the impurity molecules responsible for the color should have been adsorbed by the charcoal, and filtration of the mixture should give a decolorized solution of the organic compound. The filtration can be carried out under gravity through a fluted filter paper, although a second filtration may be necessary to remove all the fine particles of charcoal.

*Decolorization*

*Use a steam bath for flammable solvents*

### Crystallization and What To Do if No Crystals are Formed

Having filtered your hot solution into an Erlenmeyer flask, cover the flask with a watch glass to prevent contamination by atmospheric dust, and then set it aside so that the solution can cool slowly. The rate of cooling determines the size of the crystals, rapid cooling favoring the formation of a lot of small crystals, and slow cooling encouraging the growth of fewer, but much larger, crystals. A convenient compromise between speed of crystallization and crystal quality is to allow the hot solution to cool to room temperature by placing the flask on a surface such as glass or cork that does not conduct the heat away too quickly. The *rate* of crystallization is usually greatest at about 50 °C below the melting point of the substance, and maximum formation of crystals occurs at about 100 °C below the melting point. Once the crystals have formed, it is usually a good idea to cool the solution from room temperature to about 0 °C by placing the Erlenmeyer in an ice bath. This will ensure that the maximum amount of crystals are obtained. It is not usually good practice to cool the solution below 0 °C, unless there are special problems in getting crystals to form in the first place (see p. 132), because this results in condensation of water vapor into the solution unless special precautions are taken.

What do you do when no crystallization occurs after cooling the solution to room temperature? You should attempt to induce crystalliza-

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*Seeding*

*Scratching*

*Cooling*

tion by one of the following methods. Add a seed crystal which was saved from the original material before dissolution. This will provide a nucleus on which other crystals can grow. If this fails, try scratching the side of the flask with a glass rod. This is thought to produce micro-fragments of glass which then serve as nuclei to induce crystallization. If this fails, try cooling the flask in an acetone–solid $CO_2$ bath (see p. 104), and then scratch the side of the flask as the solution warms up to room temperature. If the substance still refuses to crystallize, it probably means that you have too much solvent; the excess solvent should be boiled off (**hood — check for flames in the vicinity**), and the reduced volume of solution should be set aside again until crystallization occurs.

*Oiling out*

The final problem that may be encountered in crystallization is the separation of the substance as an oil rather than as crystals. This is known as *oiling out*, and usually occurs when the compound is very impure or when it has a melting point that is lower than the boiling point of the solvent. Even if the oil eventually solidifies, the compound will not be pure, and the material should be redissolved by heating the solution. You may need to add a little more solvent at this stage, or more good solvent if mixed solvents are being used. Indeed, crystallization from a slightly more dilute solution may prevent oiling out. Slower cooling also favors the formation of crystals rather than oils. If the compound completely refuses to crystallize, the chances are that it is too impure, and it should be purified by some other means such as chromatography.

### Collecting the Crystals

After crystallization the crystals are separated from the *mother liquor* by suction filtration, a technique which has already been discussed in detail on pp. 75–77. After filtration, the crystals should be washed with a little fresh solvent. Remember that if the crystallization has been performed using mixed solvents, the wash solvent should be the same mixture.

*Second crop will be less pure*

The mother liquor from the crystallization (which is now the filtrate) may still contain a significant quantity of your organic product. In this case a second batch of crystals, known as the *second crop*, can often be obtained by concentrating the mother liquor by boiling off some of the solvent (**hood — check for flames in the vicinity**) and then allowing the solution to cool and crystallize as before. However, be warned, the second crop is usually less pure than the first simply because the impurities were concentrated in the mother liquor during the first crystallization. Do not combine the two crops of crystals until you have checked the purity of each batch.

### Drying the Crystals

After filtration and washing, the crystals should be dried to constant weight. Techniques for drying solids are discussed in pp. 136–138.

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# Population pharmacokinetics of AG-348 in healthy volunteers and adult patients with pyruvate kinase deficiency

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## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare, congenital, hereditary disease causing lifelong hemolytic anemia.[1]
- PK deficiency is caused by a mutation in the *PKLR* gene that results in reduced enzyme activity of the red-cell PK isoform (PK-R), which leads to defective glycolysis.[2]
- AG-348 is a first-in-class small molecule allosteric activator of PK-R[3] that has demonstrated an acceptable safety profile and has been shown to induce clinically relevant and sustained increases in hemoglobin levels in patients with PK deficiency.[4,5]
- A population pharmacokinetic model including a covariate model has been developed to support dose recommendations for planned clinical trials.

## OBJECTIVES

- Characterize the population pharmacokinetics of AG-348 in healthy volunteers and in patients with PK deficiency.
- Identify factors that may explain the pharmacokinetic variability of AG-348.
- Simulate AG-348 exposure at different doses in healthy volunteers and patients with PK deficiency.

## METHODS

Study design and population

- This analysis was conducted using plasma pharmacokinetic data, dosing information, demographics, and liver and renal function at baseline from 132 healthy volunteers and 52 adult patients with PK deficiency from four clinical trials (**Table 1**).
- The 132 healthy volunteers received either a single oral dose or multiple oral doses (14 days) of AG-348 at dose levels ranging from 5 to 2500 mg once daily (QD) or twice daily (BID).
- The 52 patients with PK deficiency were initially randomized to AG-348 treatment of 50 mg BID or 300 mg BID. Dose modifications were made for a variety of reasons (e.g. adverse events, hemoglobin level exceeding the midpoint of the normal range), resulting in a variety of dosing regimens ranging from 5 mg QD to 300 mg BID for up to 24 weeks.

Population pharmacokinetic analysis

- Population pharmacokinetic modeling and simulation were performed using NONMEM 7.4.1.
- SAS 9.4, R Studio, Pirana, and Perl-speaks-NONMEM were used for data preparation, model development, and post-modeling processing.
- A structural model was developed. Statistically significant covariates were retained in the model using a stepwise method with a forward inclusion criterion ($\alpha$=0.05, dOFV=3.84) and a backward elimination criterion ($\alpha$=0.001, dOFV=10.83).
- The final model was used to simulate AG-348 exposure at 50 mg BID and 300 mg BID in patients with PK deficiency.

Table 1. Summary of the studies included in this analysis

| Study | Dose levels | No. of subjects | Sampling scheme |
|---|---|---|---|
| Phase 1 single ascending dose (SAD) study in healthy volunteers (NCT02148198) | 30, 120, 365, 700, 1400, and 2500 mg single dose | 36 | Pre-dose and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, and 72 hr post dose<br>Additional sampling 96 and 120 hr post dose for 700, 1400, and 2500 mg dose levels |
| Phase 1 multiple ascending dose (MAD) study in healthy volunteers (NCT02148796) | 15, 60, 150, 300, and 700 mg BID or 14 days<br>1050 mg QD for 14 days | 36 | Day 1: Pre-dose and 0.25, 0.5, 1, 2, 4, 8, and 12 hr post morning dose; additional sampling 24 hr post morning dose for 150 mg BID dose level<br>Day 14: Pre-dose and 0.25, 0.5, 1, 2, 4, 6, 10, 24, 48, and 72 hr post morning dose; additional sampling 96 and 120 hr post morning dose for 15 mg BID, 60 mg BID, 300 mg BID, and 1050 mg QD dose levels<br>Pre-morning dose on Day 2, 3, 4, 8, 15, 16, 17, 18, 21, and 28 |
| DRIVE PK phase 2 study in patients with PK deficiency: Core period (NCT02476916) | 150 and 300 mg BID for up to 24 weeks (doses were reduced and varied) | 52 | Intensive sampling for up to 12 subjects:<br>Day 1 and Day 15: Pre dose and 0.5, 1, 2, 4, 8, and 12 hr post dose<br>Pre dose on Day 22, 43, 64, 85, 113, 141, and 169<br>Trough sampling for the remaining subjects:<br>Pre dose on Day 1, 15, 22, 43, 64, 85, 113, 141, and 169 |
| Phase 1 clinical pharmacology study in healthy volunteers (NCT03026556) | 5, 55, and 220 mg single dose | 60 | Pre dose and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, and 72 hr post dose |

*Subjects who received placebo were not included. †Data from def subjects were not included. ‡Trough concentration at BID dosing. §Trough concentration on QD dosing. ¶12 optional and 16 non-fasted subjects each dose level.

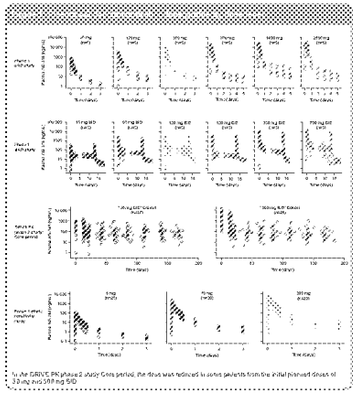
## RESULTS

Table 2. Demographics

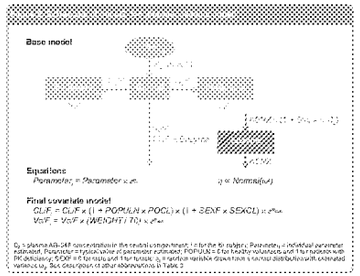
| Characteristic | Healthy volunteers (N=132) | Patients with PK deficiency (N=52) |
|---|---|---|
| Male, n (%) | 101 (76.5) | 32 (61.5) |
| Age, years | 36 (18–55) | 36 (18–60) |
| Ethnicity, n (%)<br>Hispanic<br>Non-Hispanic<br>Not reported | 20 (15.2)<br>112 (84.8)<br>0 (0.0) | 0 (0.0)<br>47 (90.4)<br>5 (9.6) |
| Race, n (%)<br>White<br>Black<br>Asian<br>Not reported or other | 41 (31.1)<br>55 (41.7)<br>31 (23.5)<br>5 (3.8) | 43 (82.7)<br>1 (1.9)<br>3 (5.8)<br>5 (9.6) |
| Weight, kg | 77.9 (47.9–109.6) | 76.0 (41.3–111.0) |
| Body mass index, kg/m² | 25.7 (18.0–31.6) | 25.5 (15.2–37.3) |
| Alanine aminotransferase (ALT), U/L | 23.0 (9.0–51.0) | 22.5 (8.0–104.0) |
| Albumin, g/L | 42.0 (33.0–51.0) | 46.0 (40.0–52.0) |
| Alkaline phosphatase (ALP), U/L | 62.5 (31.0–102.0) | 69.0 (43.0–173.0) |
| Aspartate aminotransferase (AST), U/L | 28.0 (16.0–54.0) | 36.5 (15.0–154.0) |
| Total bilirubin, mg/dL | 0.5 (0.2–1.0) | 4.6 (2.1–14.0) |
| Blood urea nitrogen (BUN), mg/dL | 4.6 (1.5–7.9) | 4.8 (2.6–8.3) |
| Creatinine, µmol/L | 74.7 (35.4–122.0) | 65.0 (28.0–93.0) |
| eGFR, mL/min/1.73 m² | 102.1 (60.5–160.4) | 105.9 (51.6–172.4) |

Values are median (range) unless otherwise indicated.
‡Five subjects (NCT02148198, NCT02148666, and NCT02150668: Phase study NCT02476916: Missing sex and baseline eGFR = estimated glomerular filtration rate calculated by CTS = Agios's creatinine standard; = 32.0 if (Sex = 1 and SCR ≤ 0.7) or (Sex = 2 and SCR ≤ 0.5); = 1 if (Sex = 1 and SCR > 0.7) or (Sex = 2 and SCR > 0.5); = 0.743 (if female).

- Time courses of plasma AG-348 concentration by dose level are shown in **Figure 1**.



- The plasma pharmacokinetics of AG-348 were described by a semimechanistic three-compartment disposition model, including absorption lag time, first-order absorption, first-order elimination, and an enzyme turnover model to describe the autoinduction of AG-348 (**Figure 2**).



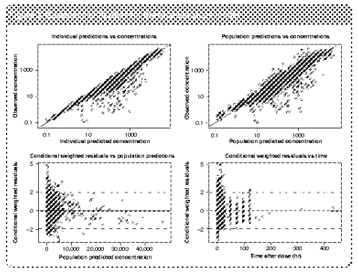

$C_p$ = plasma AG-348 concentration in the central compartment; i = for the ith subject; Parameter_i = individual parameter estimate; Parameter_TV = typical value of parameter estimate; POPULN = 0 for healthy volunteers and 1 for subjects with PK deficiency; SEXF = 0 for males and 1 for females; $\eta_i$ = random variable describing individual deviation from typical value; $\omega_i$ = normally distributed with estimated variance; Sex described in other abbreviations in Table 2.

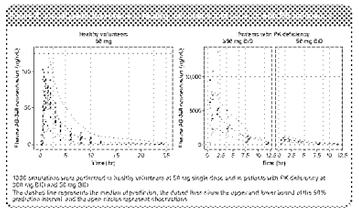
- A stepwise covariate analysis was performed.
- Age, ethnicity, race, and body mass index had no effect on apparent plasma clearance of AG-348.
- No correlations were found between apparent plasma clearance of AG-348 and baseline hepatic function indicators (ALT, albumin, ALP, AST, total bilirubin) or baseline renal function indicators (BUN, creatinine, eGFR).
- Disease status (healthy volunteers vs patients) had a statistically significant influence on plasma AG-348 clearance.
  - Patients with PK deficiency had an 2% (95% CI 34.3, 48.1) lower clearance than healthy volunteers.
- Sex had a mild effect, with a statistically borderline significant influence on plasma AG-348 clearance.
  - Male subjects had 19.9% (95% CI 6.9, 32.9) lower clearance than female subjects.
- The final model parameters are summarized in **Table 3**.
- The final covariate model describes the data well (**Figures 3** and **4**).

Table 3. Estimates of the population pharmacokinetic parameters

| Parameter | Description | Typical value (% RSE) | Inter-individual variability (% RSE) | Residual variability (% RSE) |
|---|---|---|---|---|
| $K_a$ | Absorption lag time | ALAG1, hr | 0.22 FIX* (–) | – | – |
| $K_a$ | First-order absorption rate | $K_a$, hr⁻¹ | 1.98 (2.4) | 3.59–3.90 | 25.7 (6.1) |
| POCL | Percentage of clearance increase in patients compared with healthy volunteers | POCL | –48.2% (8.5) | –48.1% to –34.3% | – |
| SEXCL | Percentage of clearance increase in females compared with males | SEXCL | 19.6% (33.3) | 6.9%–30.4% | – |
| CL/F | Apparent clearance of central compartment | CL/F, L/hr | 12.7 (3.7) | 11.8–14.6 | 22.5 (5.0) |
| Vc/F | Apparent volume of central compartment | Vc/F, L | 35.9 (3.7) | 33.5–38.6 | 0.2 (63.3) |
| Q/F | Apparent intercompartmental clearance between central compartment and peripheral compartment 1 | Q/F, L/hr | 5.11 (5.8) | 5.84–6.24 | 83.7 (5.1) |
| $V_p$/F | Apparent volume of peripheral compartment 1 | $V_p$/F, L | 53.5 (4.4) | 49.8–122.0 | – |
| Q/F | Apparent intercompartmental clearance between central compartment and peripheral compartment 2 | Q/F, L/hr | 2.99 (12.3) | 2.58–3.59 | – |
| $V_p$/F | Apparent volume of peripheral compartment 2 | $V_p$/F, L | 13.4–5.35 | 11.9–14.9 | – |
| KENZ | Rate constant for first-order drug-metabolizing enzyme degradation | KENZ, hr⁻¹ | 0.203 (14.6) | 0.182–0.304 | 7a.8 (2a.8) |
| Steepb | Slope of interaction for 2009 effect on enzyme sole-elimination | Steepb (=1899 nghr/mL)* | 1.98 (2.9) | 1.59–2.27 | – |
| Residual variability | Proportional, %CV | 32.5% (2.5) | – | – |
| | Additive, SD | 2.15 (3.8) | – | – |

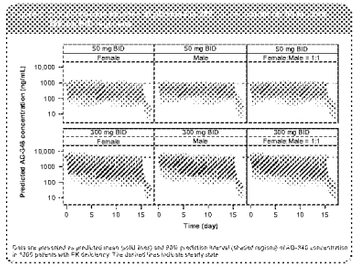
Grey box indicates covariate that had a statistically significant influence on apparent AG-348 clearance. †ALAG1 was not estimated but fixed at 0.22 h according to the likelihood profiling analysis. ‡SD = standard deviation; RSE = relative standard error



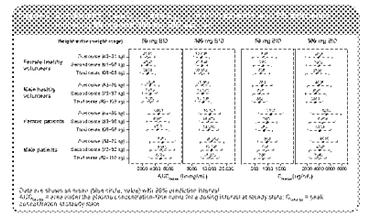

1000 simulations were performed in healthy volunteers at 50 mg single dose and in patients with PK deficiency at 300 mg BID and 50 mg BID.
The dashed line represents the median of prediction, the dotted lines show the upper and lower bound of the 90% prediction interval, and the open circles represent observations.

- In simulated concentration-time profiles, steady state is reached at approximately 10–14 days following 50 mg BID and 300 mg BID dose regimens (**Figure 5**).
- Although sex was found to have a statistically significant mild effect on apparent clearance, simulated pharmacokinetic profiles are similar between males and females, suggesting that the effect of sex on the pharmacokinetics of AG-348 is not likely to be clinically relevant.




Data are presented as predicted mean (solid lines) and 90% prediction interval (shaded regions) of AG-348 concentration in 1000 patients with PK deficiency. The dashed lines indicate steady state.

- Simulation results indicate (**Figure 6**):
  - AG-348 exposure at steady state is lower in healthy volunteers than in patients.
  - Sex has little to no effect on exposure at steady state.
  - Body weight has little to no effect on exposure at steady state.




Data are shown as mean (95% CI), except with 90% prediction interval.
$AUC_{tau,ss}$ = area under the plasma concentration-time curve for a dosing interval at steady state; $C_{trough,ss}$ = trough concentration at steady state.

## LIMITATIONS

- Caution should be taken when interpreting the effect of disease status (healthy volunteers vs patients) on clearance owing to the limited pharmacokinetic data obtained in the patient trial (NCT02476916).
- Additional data from future clinical trials, when available, will be used to evaluate the significance of this difference.

## CONCLUSIONS

- This is the first time AG-348 pharmacokinetics and covariates have been characterized in both healthy volunteers and patients with PK deficiency.
- This cross-study analysis provided a quantitative understanding of the pharmacokinetic characteristics of AG-348 and identified factors explaining the variability of the pharmacokinetic characteristics.
- AG-348 and pharmacokinetics were described by a three-compartment disposition model, including absorption lag time, first-order absorption, first-order elimination, and an enzyme turnover model to describe the autoinduction of AG-348.
- The influence of sex on clearance was mild and of statistically borderline significance. It is unlikely to be clinically significant given that drug exposure was similar between males and females in terms of $C_{max,ss}$ and $AUC_{tau,ss}$ derived from Monte Carlo simulations.
- The effect of disease status (healthy volunteers vs patients) and sex on clearance will be evaluated further when additional data are available from future clinical trials.
- This population pharmacokinetic model serves as the foundation for exposure-response / exposure-safety analyses, which will be used to support the clinical dose recommendation and clinical trial design in the future.

Acknowledgments

We would like to thank the healthy volunteers and patients who agreed to participate in these studies.

Disclosures

This study was funded by Agios Pharmaceuticals, Inc.
HJ, VI, LH, HM, HY, ML, KL: Agios – employment and stockholder. AJB: Agios – employment and stockholder at time of study.
Editorial assistance was provided by Susanne Vidal, PhD, Excel Medical Affairs, Horsham, UK, and supported by Agios.

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Presented at the American Society for Clinical Pharmacology and Therapeutics (ASCPT) Annual Meeting, March 21–24, 2018, Orlando, FL, USA

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# POPULATION PHARMACOKINETICS OF AG-348 IN HEALTHY VOLUNTEERS AND ADULT PATIENTS WITH PYRUVATE KINASE DEFICIENCY

**Author Block: H. Jia**, V. Iyer, A.J. Barbier, L. Hua, H. Mangus, H. Yang, M. Liang, K. Le;
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**Abstract:**

**BACKGROUND:** Pyruvate kinase (PK) deficiency is a rare, congenital, autosomal recessive hemolytic anemia caused by deficiency of the red cell PK isoform (PK-R). AG-348 is a first-in-class, small molecule allosteric activator of PK-R currently in clinical trials.

**METHODS:** Population pharmacokinetic and covariate analyses of AG-348 were performed using data from 72 healthy volunteers (HVs) and 52 adult PK deficiency patients following single and multiple oral doses (30-2500 mg per day in HVs; 5-600 mg per day in PK deficiency patients). Full data for patients were not available until 09Nov2017.

**RESULTS:** Plasma AG-348 pharmacokinetics were well described by a semi-mechanistic 3-compartment disposition model, including first-order absorption, first-order elimination and an enzyme-turnover model to describe the auto-induction of AG-348. Sex and population (HVs vs. patients) were found to be statistically significant on plasma AG-348 clearance. Body weight was significantly correlated with central volume of distribution. Model-predicted clearance was 40.6% (95% CI: 32.2-48.8%) lower in patients with PK deficiency than in HVs. Female subjects had 38.6% (95% CI: 17.2-60%) higher clearance than male subjects.

**CONCLUSION:** This is the first time AG-348 pharmacokinetics have been characterized in PK deficiency patients. This cross-study analysis provided a quantitative understanding of the pharmacokinetic characteristics of AG-348 and identified factors explaining its variability. The clinical relevance of the covariate relationship between sex and clearance will be further evaluated when combined with exposure-response analysis to guide dose recommendations and study design of pivotal trials.

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Author Disclosure Information:

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# Clinical development of a novel oral activator of red cell pyruvate kinase for the treatment of pyruvate kinase deficiency

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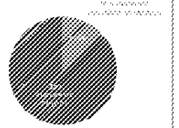
## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare chronic hemolytic anemia caused by mutations in the *PKLR* gene and the resulting reduced red cell PK (PK-R) activity.
- The clinical development program for AG-348, a novel activator of PK-R and investigational treatment for PK deficiency, is summarized.
- A phase 3 program consisting of two studies in adults is currently enrolling patients, as is a registry study in patients of all ages.

### PK deficiency: disease overview[1,2]

- **Epidemiology:** Under-recognized hereditary hemolytic anemia; Heterogeneous disease with variable severity among all ages; The most common form of non-spherocytic hemolytic anemia
- **Pathophysiology:** Caused by mutations in the *PKLR* gene coding for PK-R, resulting in defective glycolysis and decreased red blood cell lifespan
- **Clinical manifestations:** Lifelong hemolytic anemia; Iron overload and jaundice; Infection risk post splenectomy
- **Diagnosis and management:** PK-R enzyme activity and genetic testing; Supportive treatment: transfusions, splenectomy, iron chelation

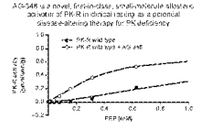
### PK deficiency: mutation type[1]



Types of *PKLR* mutations found in 193 unrelated cases in the PK deficiency natural history study

### AG-348[3]

AG-348 is a novel, first-in-class, small-molecule allosteric activator of PK-R in clinical testing as a potential disease-altering therapy for PK deficiency




AG-348 binding enhances the affinity of PK-R for its substrate phosphoenolpyruvate (PEP)

### PK-R tetramer[4]

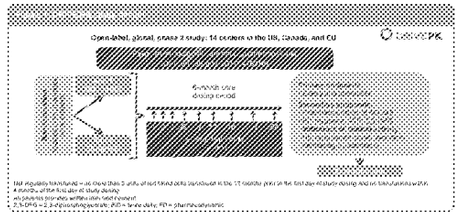


Active PK-R is a tetramer; mutations (green) decrease the enzyme activity

AG-348 (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

## METHODS

- DRIVE PK (NCT02476916) is an ongoing, open-label, phase 2 study (**Figure 1**).
- Key eligibility criteria: adult patients with PK deficiency who are not regularly transfused; hemoglobin (Hb) ≤12.0 g/dL (♂ male) or ≤11.0 g/dL (♀ female).




Hb response is defined as an increase from baseline in Hb of ≥1.0 g/dL and a mean increase from baseline in Hb of ≥1.0 g/dL, demonstrated at both visits scheduled 2 weeks apart between the 12-week point and the last day of study dosing, and not sustained within 4 weeks of the last day of study dosing.
2,3-DPG = 2,3-diphosphoglycerate; BID = twice daily; PD = pharmacodynamic

## RESULTS

### DRIVE PK: preliminary safety summary[5]

- AG-348 was generally well tolerated.
- The majority of adverse events (AEs) were grade 1–2.

Table 1. Most common AEs regardless of causality or grade occurring in ≥10% of patients

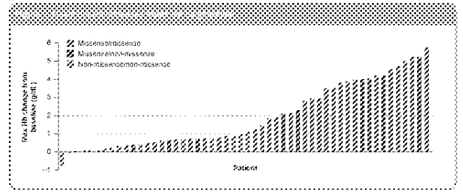
| | Cohort 1 (n=6) | | Cohort 2 (n=25) | | Total (n=31) | |
|---|---|---|---|---|---|---|
| | All grades, n (%) | Grade ≥3, n (%) | All grades, n (%) | Grade ≥3, n (%) | All grades, n (%) | Grade ≥3, n (%) |
| Patients experiencing ≥1 AE, n (%) | 26 (86.7) | 6 (20.0) | 25 (100.0) | 7 (28.0) | 31 (96.1) | 13 (26.0) |
| Headache | 15 (50.0) | 0 | 14 (56.0) | 0 | 29 (46.2) | 0 |
| Insomnia | 6 (20.0) | 0 (0.0) | 16 (64.0) | 0 (0.0) | 22 (42.3) | 0 (0.0) |
| Nausea | 10 (37.0) | 0 | 10 (40.0) | 0 | 20 (39.5) | 0 |
| Viral upper respiratory tract infection | 5 (20.0) | 0 | 7 (16.0) | 1 (4.0) | 12 (23.1) | 1 (1.9) |
| Arthralgia | 5 (16.7) | 0 | 4 (16.0) | 0 | 9 (17.3) | 0 |
| Hot flush | 2 (7.4) | 0 | 7 (28.0) | 0 | 9 (17.3) | 0 |
| Cough | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Diarrhea | 4 (14.8) | 0 (0.0) | 4 (16.0) | 0 | 8 (15.4) | 1 (1.9) |
| Dizziness | 5 (18.5) | 0 | 3 (12.0) | 0 (0.0) | 8 (15.4) | 0 (0.0) |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Influenza | 2 (25.0) | 0 (0.0) | 3 (12.0) | 0 | 5 (15.4) | 0 (0.0) |
| Anemia[a] | 2 (33.3) | 0 | 3 (12.0) | 0 | 5 (16.1) | 0 |

Adverse events coded using Medical Dictionary for Regulatory Activities (MedDRA) version 19.0.
Other grade 3 AEs not included in the table: cystitis (n=2), dysarthria, viral infection, pharyngitis (n=2), hypertriglyceridemia (n=2), hypertensive crisis (n=2), hypokalemia (n=2), gastrointestinal hemorrhage (n=2), hyperuricemia (n=2).
[a]Related to study drug, as assessed by the investigator.

- The safety profile was consistent over the duration of treatment (median 37.5 weeks).
- Treatment-related AEs leading to discontinuation (n=4):
  - Hemolytic anemia: hypertriglyceridemia, pharyngitis and nausea; pleural effusion.
- There were 14 serious AEs in 11 patients.
  - Five treatment related serious AEs in four patients: anemia, hypertriglyceridemia, osteoporosis, withdrawal hemolysis followed by anemia.
- Effect of AG-348 on sex hormones:
  - Modest changes from baseline in sex hormone levels were observed in males at planned pivotal trial dose levels (550 mg BID).
  - Data are consistent with mild aromatase inhibition.
  - Most sex hormone values remained within normal limits in females; interpretation is confounded by variability in menopausal status and contraceptive use.

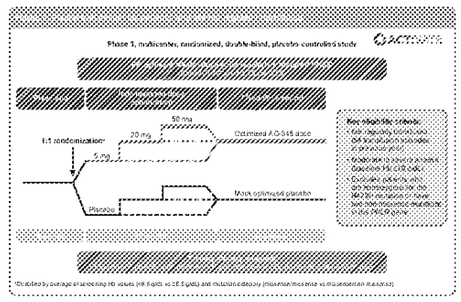
### DRIVE PK: efficacy (core period)[5]

- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL (**Figure 2**).
- The mean maximum increase in Hb was 3.4 g/dL in patients with an Hb increase >1.0 g/dL.
- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range, 7–137 days).
- The dose had to be held or reduced owing to a rapid rise in Hb in nine patients.




## PHASE 3 STUDIES ARE ENROLLING PATIENTS

- The safety and efficacy data from the DRIVE PK study support the development of AG-348 in patients with PK deficiency.
- Two separate phase 3 clinical trials will examine the safety and efficacy of AG-348 in adult patients (**Figure 3**, **Figure 4**).




Durability is defined by average of preceding Hb values (Hb 6 g/dL or ≥1 g/dL) and mutation category (missense/missense vs missense/non-missense)

## PYRUVATE KINASE DEFICIENCY REGISTRY

- The Peak Registry (NCT03481738) is a global, longitudinal, observational study for adult and pediatric patients with PK deficiency. This study is enrolling patients.
- Goals of the Peak Registry:
  - Collect and aggregate longitudinal data (minimum 2 years, up to 9 years) from patients with PK deficiency who have been diagnosed via genetic analysis (all ages) worldwide (up to 20 countries).
  - Promote further understanding of PK deficiency disease parameters, e.g. transfusion dependency, treatment practices, disease burden.

The Peak Registry

### Acknowledgements

We would like to thank the patients and the dedicated physicians who have worked on these Agios programs to better understand PK deficiency and develop potential treatments for this rare genetic disease.

### Disclosures

These studies are funded by Agios Pharmaceuticals, Inc.

BWJ, KMA, M-HJ, and CB: Agios – employment and stockholder.

Editorial assistance was provided by Suzanne Vidot, PhD, Excel Medical Affairs, Horsham, UK, and supported by Agios.

### References

1. Grace RF et al. *Am J Hematol* 2015;90:825-30.
2. Percy MJ et al. *Blood Cells Mol Dis* 2007;39:169-84.
3. Grace RF et al. *Blood* 2016;131:2183-92.
4. Kung C et al. *Blood* 2017;130:1347-56.
5. Grace RF et al. *59th ASH Annual Meeting* 2017: Poster 2194.

Presented at the National Organization for Rare Disorders (NORD) Rare Summit 2018, October 13–15, 2018, Washington, DC, USA

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**Abstract for submission to the National Organization for Rare Disorders (NORD) Rare Summit 2018**
**Oct 15–16, 2018, Washington, DC**

**Authors:** Bryan W. Jones, Karen M. Anderson, Marie-Hélène Jouvin, Chris Bowden
**Affiliation:** Agios Pharmaceuticals, Inc., Cambridge, MA, USA.
**Presenting author:** Bryan W. Jones

# Clinical development of a novel oral activator of red cell pyruvate kinase for the treatment of pyruvate kinase deficiency

**Keyword:** Pyruvate kinase deficiency, congenital hemolytic anemia

**Objective:** Pyruvate kinase (PK) deficiency is a rare chronic hemolytic anemia caused by mutations in the *PKLR* gene and resulting reduced red cell PK (PK-R) activity. The clinical development program for AG-348, a novel activator of PK-R and investigational treatment for PK deficiency, will be summarized.

**Method:** DRIVE PK is an ongoing, open-label, phase 2 study in which adult patients with PK deficiency who do not receive regular blood transfusions were randomized to receive either 50 mg or 300 mg of AG-348 twice daily (BID).

**Results:** AG-348 was administered over a 6-month Core Treatment Period followed, if eligible, by an Extension Period (ongoing). Dose adjustments were allowed. In all, 52 patients were randomized and treated; the median treatment duration at the time of this analysis was 37.5 weeks (range, 12.9–92.4 weeks). Forty-three patients (83%) completed the Core Period; 9 patients did not complete the Core Period due to either an adverse event (AE, n=4: pharyngitis/nausea, hemolysis, hypertriglyceridemia, pleural effusion), investigator decision (n=2), or patient withdrawal of consent (n=3). The most common AEs were headache (46%, n=24), insomnia (42%, n=22), and nausea (38%, n=20). As previously demonstrated, increased testosterone levels from baseline consistent with aromatase inhibition were observed, with values predominantly remaining within normal range. There were 5 treatment-related serious AEs reported in 4 patients (hemolysis followed by anemia, anemia, osteoporosis, hypertriglyceridemia). Twenty-six patients (50%) had a maximum hemoglobin (Hb) increase >1 g/dL during the Core Period, with a mean maximum Hb

increase of 3.4 g/dL (range, 1.1–5.8 g/dL) in these patients. In most cases, Hb increases were rapid and sustained, and were observed across doses from 5 mg to 300 mg BID. Patients with at least 1 missense mutation were more likely to have an Hb increase. Biochemical markers of hemolysis (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who had a maximum Hb increase >1.0 g/dL. Two phase 3 studies are currently enrolling: the ACTIVATE-T (NCT03559699) and ACTIVATE (NCT03548220) studies in adult patients who do, or do not, receive regular blood transfusions, respectively. Details of these studies will be provided, as will details of the ongoing Peak Registry (NCT03481738) study currently enrolling patients of all ages to further define the clinical characteristics of PK deficiency.

**Conclusion:** AG-348 is a novel, first-in-class activator of the red cell PK enzyme in clinical testing for PK deficiency. The ongoing phase 2 DRIVE PK study has completed enrollment; some patients continue to receive AG-348 treatment in the Extension Period of the study. Treatment across a range of AG-348 doses has been generally well tolerated, and has resulted in relevant, durable increases in Hb with concomitant improvements in markers of hemolysis. These data support further exploration of AG-348 as the first disease-altering therapy for PK deficiency. A phase 3 program consisting of two studies in adults is currently enrolling patients, as is a registry study in patients of all ages.

**Character counts** (including spaces)
Title: 122
Key Word: 55
Objective: 294
Method: 211
Results: 1923
Conclusion: 684



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# ACTIVATE-T: a phase 3, open-label study to evaluate the efficacy and safety of AG-348 in regularly transfused adults with pyruvate kinase deficiency

Marie-Hélène Jouvin[1], Chris Bowden[1], Rachael F Grace[2], John Porter[3]

[1]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [2]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA; [3]Department of Haematology, University College London Cancer Institute, London, UK

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ACTIVATE-T

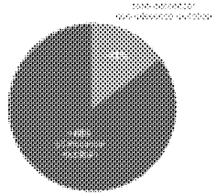
## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare, hereditary, hemolytic anemia.
- AG-348 is being developed as a treatment for PK deficiency and has been tested in phase 1 and 2 (DRIVE PK) studies.
- A phase 3 study (ACTIVATE-T) is currently open and enrolling patients.

### PK deficiency: disease overview[1-3]

| | |
|---|---|
| Prevalence | • Under-recognized hereditary disease<br>• Heterogeneous disease with variable severity among all ages<br>• Often presents in childhood |
| Genetics | • Caused by mutations in the *PKLR* gene coding for red-cell PK (PK-R), resulting in defective glycolysis and decreased red blood cell lifespan |
| Clinical manifestations | • Lifelong hemolytic anemia<br>• Iron overload and jaundice<br>• Infection risk post splenectomy |
| Diagnosis and treatment | • PK-R enzyme activity and genetic testing<br>• Supportive treatment: transfusions, splenectomy, iron chelation |

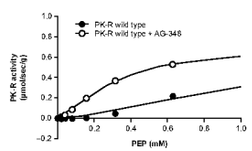
### PK deficiency: mutation type[2]




Types of *PKLR* mutations found in 193 unrelated cases in the PK deficiency natural history study

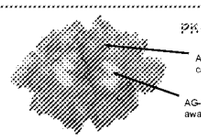
### AG-348[4]

AG-348 is a novel, first-in-class, small-molecule allosteric activator of PK-R in clinical testing as a potential disease-altering therapy for PK deficiency




AG-348 binding enhances the affinity of PK-R for its substrate phosphoenolpyruvate (PEP)
(recombinant enzyme data shown)

### PK-R tetramer[1]



Active PK-R is a tetramer; mutations (green) decrease the catalytic activity

AG-348 (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

### PK-deficient patients who are regularly transfused

- These patients represent a small subset of the overall population of patients with PK deficiency.
  - 23/198 (12%) adults in the PK deficiency natural history study are regularly transfused.[3]
- Heterogeneous transfusion practices in PK deficiency:
  - No universally accepted transfusion guidelines
  - Transfusion frequency can vary from once per week to a few times per year
  - Transfusions administered at regularly scheduled intervals vs when hemoglobin (Hb) nadir reached, in addition to ad hoc/on demand (e.g. infection, patient feeling tired)
  - Patients requiring ≥8 transfusions/year are likely to require transfusions to maintain an acceptable Hb level.

### AG-348 in PK deficiency

#### DRIVE PK study design[4]

- Phase 2, open-label, dose-ranging study (NCT02476916).
- Main eligibility criteria: adult patients with PK deficiency who are not regularly transfused; Hb ≤12.0 g/dL (if male) or ≤11.0 g/dL (if female).
- Main endpoints:
  - Primary: safety – adverse events (AEs), serum sex hormones, laboratory parameters, bone mineral density
  - Secondary: efficacy – Hb, markers of hemolysis, erythropoietin, markers of iron metabolism, pharmacokinetics, pharmacodynamics.
- Patients randomized to initial AG-348 dose of 50 mg twice daily (BID) or 300 mg BID.
- Core period (first 6 months) completed; extension period (4 years) ongoing.

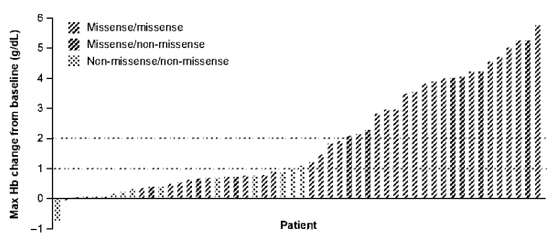
#### DRIVE PK cumulative safety summary[5]

- AG-348 was generally well tolerated.
- The majority of AEs were grade 1–2.
- The safety profile was consistent over the duration of treatment (median 37.5 weeks).
- Treatment-related AEs leading to discontinuation (n=4):
  - Hemolytic anemia, hypertriglyceridemia, pharyngitis and nausea, pleural effusion.
- There were 14 serious AEs in 11 patients.
  - Five treatment-related serious AEs in four patients: anemia, hypertriglyceridemia, osteoporosis, withdrawal hemolysis followed by anemia.
- Effect of AG-348 on sex hormones:
  - Modest changes from baseline in sex hormone levels were observed in males at planned pivotal trial dose levels (≤50 mg BID).
  - Data are consistent with mild aromatase inhibition.
  - Most sex hormone values remained within normal limits in females; interpretation is confounded by variability in menopausal status and contraceptive use.

#### DRIVE PK efficacy (core period)[5]

- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL (**Figure 1**).
- The mean maximum increase in Hb was 3.4 g/dL in patients with an Hb increase >1.0 g/dL.
- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range, 7–187 days).
- The dose had to be held or reduced owing to a rapid rise in Hb in nine patients.

**Figure 1: Maximum Hb increase from baseline by patient in the DRIVE PK study**




## ACTIVATE-T STUDY

### Summary

- The safety and efficacy data from the DRIVE PK study support the development of AG-348 in patients with PK deficiency.
- ACTIVATE-T is an open-label trial to evaluate the efficacy (reduction in transfusion burden) and safety of AG-348 in the ultra-rare population of adult patients with PK deficiency who are regularly transfused (**Figure 2**).
- An independent data monitoring committee will review the study data periodically and provide safety oversight.

### Study status

- ACTIVATE-T is currently open and enrolling patients at participating sites globally.
- The additional ACTIVATE study is expected to open in June 2018, and is a phase 3, multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of AG-348 in adult patients with PK deficiency who are not regularly transfused.

### PK deficiency global registry

The Peak Registry

- Patients who are not eligible for the ACTIVATE-T trial may be enrolled in the Peak Registry (NCT03481738).
- Goals of the Peak Registry:
  - Collect and aggregate longitudinal data (minimum 2 years, up to 9 years) from patients with PK deficiency who have been diagnosed via genetic analysis (all ages) worldwide (up to 20 countries).
  - Promote further understanding of PK deficiency disease parameters, e.g. transfusion dependency, treatment practices, Hb correlation with disease burden (refine/redefine and substantiate understanding based on data).

### ACTIVATE-T study design: Part 1 and Part 2

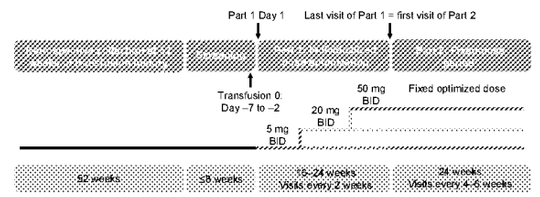
**Key inclusion criteria**

- ≥18 years of age
- Documented presence of at least two mutant alleles in *PKLR*, of which at least one is a missense mutation (based on genotype–Hb response correlations in DRIVE PK; see **Figure 1**)
- ≥6 transfusion episodes in the past 52 weeks
- Adequate organ function

**Key exclusion criteria**

- Homozygous for the R479H mutation or two non-missense mutations in *PKLR*
- History of transfusions occurring, on average, more frequently than once every 3 weeks during the past 52 weeks
- Significant medical condition that confers an unacceptable risk to participating in the study, and/or that could confound the interpretation of data

- Approximately 15–20 regularly transfused patients will be enrolled




[a]Patients who remain on the study until the end of Part 2 may be eligible for an extension study

Screening
- Collection of transfusion history:
  - Dates of all transfusions
  - Pretransfusion Hb for ≥80% of transfusions
  - Number of blood units transfused for all transfusions
- Transfusion history information used to:
  - Determine eligibility
  - Calculate mean transfusion frequency, which will be used to guide the timing of AG-348 dose-level increases
  - Calculate individual transfusion trigger (TT), mean of pretransfusion Hb
  - Calculate mean number of blood units (MNU) transfused per transfusion
  - Function as historical control data for the analysis

Part 1: Individual dose escalation period
- All patients start on 5 mg BID
- Dose can be increased from 5 to 20 mg BID and, subsequently, from 20 to 50 mg BID
- Dose should be increased if patient does not experience a reduction in transfusion frequency after 1–2 transfusions

Part 1: Fixed dose period
- Patient receives AG-348 at their optimized dose, with no planned adjustment for 24 weeks
- Dose can be reduced for safety reasons on a case-by-case basis throughout the study

Transfusion management Part 1 and Part 2
- Patient transfused when their Hb reaches individual TT (±0.5 g/dL or ±0.31 mmol/L)
- Patient transfused with their MNU

Part 2: Extension period
- For patients who have experienced significant benefit from AG-348

**Main endpoints**

**Primary**
- Reduction in transfusion burden (defined as a reduction of ≥33% in the number of red blood cell units transfused during the 24 weeks of the fixed-dose period compared with the historical transfusion burden standardized to 24 weeks)

**Secondary**
- Safety

**Exploratory**
- Liver iron status
- Health-related quality of life
- Pharmacokinetics
- Pharmacodynamics (levels of PK-R)

**Statistics**
- Sample size driven by feasibility (15–20 patients)
- With a sample size of 20, the power of the study will be 58% to detect a response rate of 30% compared with a null rate of 10%, based on a two-sided Fisher's exact test at the 0.05 significance level

Disclosures
This study is funded by Agios Pharmaceuticals, Inc.
M-HJ and CB: Agios – employment and stockholder. RFG: Agios – scientific advisor, research funding. JP: None disclosed.
Editorial assistance was provided by Susanne Vidot, PhD, Excel Medical Affairs, Horsham, UK, and supported by Agios.

Acknowledgements
We would like to thank the patients taking part in the PK deficiency studies.

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1. Grace RF et al. *Am J Hematol* 2015;90:825-30. 2. Percy MJ et al. *Blood Cells Mol Dis* 2007;39:189-94. 3. Grace RF et al. *Blood* 2018;131:2183-92. 4. Kung C et al. *Blood* 2017;130:1347-56. 5. Grace RF et al. *59th ASH Annual Meeting* 2017: Poster 2194.

Scan code to receive PDF file of the poster or visit http://bit.ly/2jnyes

Presented at the 23rd Congress of the European Hematology Association (EHA), 14–17 June 2018, Stockholm, Sweden

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# ACTIVATE-T: A PHASE 3, OPEN-LABEL STUDY TO EVALUATE THE EFFICACY AND SAFETY OF AG-348 IN REGULARLY TRANSFUSED ADULTS WITH PYRUVATE KINASE DEFICIENCY

Author(s): Marie-Hélène Jouvin, Chris Bowden

EHA Learning Center. Jouvin M. Jun 15, 2018; 214857

**Marie-Hélène Jouvin**

All contributions

Abstract: PF285

Type: Poster Presentation

Presentation during EHA23: On Friday, June 15, 2018 from 17:30 - 19:00

Location: Poster area

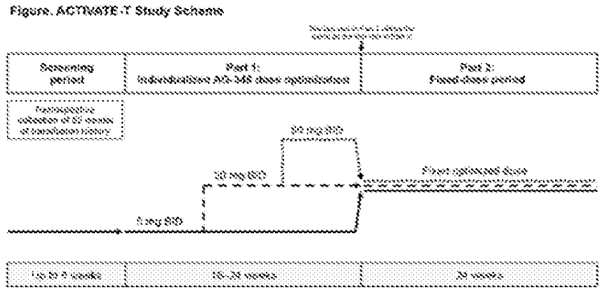
Background
Pyruvate kinase (PK) deficiency is an under-recognized hereditary disease resulting in lifelong hemolytic anemia. PK deficiency is caused by mutations in the *PKLR* gene that lead to reduced red cell PK (PK-R) enzyme activity, resulting in defective glycolysis and decreased lifespan of red blood cells. AG-348 is a novel, first-in-class, oral, small molecule allosteric activator of PK-R under clinical testing as the first targeted disease-altering therapy for PK deficiency. In the DRIVE PK study (NCT02476916; a phase 2, open-label, dose-ranging trial in adults with PK deficiency who are not regularly transfused) twice-daily (BID) dosing with AG-348 for >6 months was well tolerated and induced rapid, durable responses (Grace RF et al. ASH 2017). As of July 14, 2017, 26 (50%) of 52 enrolled subjects had a maximum hemoglobin (Hb) increase of >1 g/dL. Among these 26 subjects, the mean maximum Hb increase was 2.4 g/dL and 25 (96%) had at least one missense *PKLR* mutation. The ACTIVATE-T study is designed to assess the efficacy and safety of AG-348 for the first time in regularly transfused subjects with PK deficiency.

Aims
To report the design of the planned ACTIVATE-T study.

Methods
ACTIVATE-T is a global, multicenter, open-label study to evaluate the efficacy and safety of AG-348 in regularly transfused adults with PK deficiency. Approximately 15–20 adults with PK deficiency who are regularly transfused will be enrolled and treated with AG-348. The study will comprise an 8-week screening period, in which each subject's complete transfusion history from the prior 52 weeks will be documented, followed by a 16- to 24-week dose optimization period, and a 24-week fixed dose period (Figure). During the dose optimization period, each subject will undergo individualized AG-348 dose optimization. All subjects will start on a dose of 5 mg BID, which may be increased twice over the course of 16–14 weeks (from 5 to 20 mg BID and from 20 to 50 mg BID). In the fixed dose period, each subject will receive AG-348 at their optimized dose for 24 weeks. Key inclusion criteria include written informed consent, regular transfusion (≥6 transfusion episodes in the previous year), and adequate organ function. Subjects who are homozygous for the R479H mutation or have two non-missense mutations (without the presence of another missense mutation) in *PKLR* will be excluded. An additional key exclusion criterion is an average transfusion frequency of more than once every 3 weeks in the previous year. During the study, subjects will be transfused when their Hb reaches their individual transfusion trigger calculated from their transfusion history. The primary endpoint is the proportion of subjects who achieve a reduction in transfusion burden, defined as a reduction of ≥33% in the number of red blood cell units transfused during the 24 weeks of the fixed-dose period compared with the historical transfusion burden standardized to 24 weeks. Secondary endpoints include safety. An independent data monitoring committee will meet at least once to review the study data. The ACTIVATE-T study will be initiated globally in the first half of 2018.

Results
Not yet available.

**Figure. ACTIVATE-T Study Scheme**




Conclusion
AG-348 is a novel, first-in-class PK-R activator undergoing clinical testing in subjects with PK deficiency. The ACTIVATE-T study of AG-348 in regularly transfused subjects with PK deficiency will start enrollment in 2018.

Session Topic: 29. Enzymopathies, membranopathies and other anemias

Keyword(s): Clinical Trial, Hemolytic anemia, Inherited disease, Targeted therapy

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Ex. 2001-0846

# ACTIVATE: a phase 3, randomized, multicenter, double-blind, placebo-controlled study of AG-348 in adults with pyruvate kinase deficiency who are not regularly transfused

Marie-Hélène Jouvin[1], Chris Bowden[1], Eduard van Beers[2]

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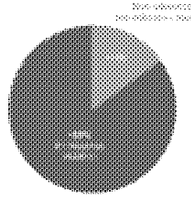
## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare, hereditary, hemolytic anemia.
- AG-348 is being developed as a treatment for PK deficiency and has been tested in phase 1 and 2 (DRIVE PK) studies.
- A phase 3 study (ACTIVATE) is anticipated to open in June 2018.

### PK deficiency: disease overview[1-4]

| | |
|---|---|
| Prevalence | • Under-recognized hereditary hemolytic anemia<br>• Heterogeneous disease with variable severity among all ages<br>• The most common form of nonspherocytic hemolytic anemia |
| Etiology | • Caused by mutations in the *PKLR* gene coding for red-cell PK (PK-R), resulting in defective glycolysis and decreased red blood cell lifespan |
| Clinical manifestations | • Lifelong hemolytic anemia<br>• Iron overload and jaundice<br>• Infection risk post splenectomy |
| Diagnosis and management | • PK-R enzyme activity and genetic testing<br>• Supportive treatment: transfusions, splenectomy, iron chelation |

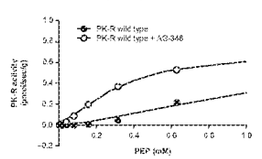
### PK deficiency: mutation type[3]




Types of *PKLR* mutations found in 183 unrelated cases in the PK deficiency natural history study

### AG-348[4]

AG-348 is a novel, first-in-class, small-molecule allosteric activator of PK-R in clinical testing as a potential disease-altering therapy for PK deficiency




AG-348 binding enhances the affinity of PK-R for its substrate phosphoenolpyruvate (PEP)
(recombinant enzyme data shown)

### PK-R tetramer[4]



Active PK-R is a tetramer; mutations (green) decrease the enzyme activity

AG-348 (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

### AG-348 in PK deficiency

#### DRIVE PK study design[4]

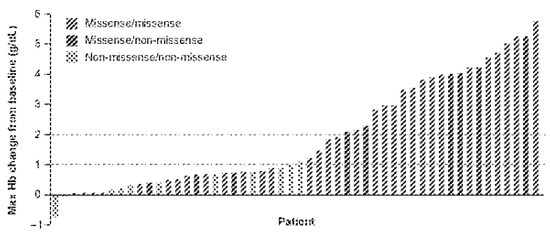
- Phase 2, open-label, dose-ranging study (NCT02476916).
- Main eligibility criteria: adult patients with PK deficiency who are not regularly transfused; hemoglobin (Hb) ≤12.0 g/dL (if male) or ≤11.0 g/dL (if female).
- Main endpoints:
  - Primary: safety – adverse events (AEs), serum sex hormones, laboratory parameters, bone mineral density
  - Secondary: efficacy – Hb, markers of hemolysis, erythropoietin, markers of iron metabolism, pharmacokinetics, pharmacodynamics.
- Patients randomized to initial AG-348 dose of 50 mg twice daily (BID) or 300 mg BID.
- Core period (first 6 months) completed; extension period (4 years) ongoing.

#### DRIVE PK cumulative safety summary[4]

- AG-348 was generally well tolerated.
- The majority of AEs were grade 1–2.
- The safety profile was consistent over the duration of treatment (median 37.5 weeks).
- Treatment-related AEs leading to discontinuation (n=4):
  - Hemolytic anemia, hypertriglyceridemia, pharyngitis and nausea, pleural effusion.
- There were 14 serious AEs in 11 patients.
  - Five treatment-related serious AEs in four patients: anemia, hypertriglyceridemia, osteoporosis, withdrawal hemolysis followed by anemia.
- Effect of AG-348 on sex hormones:
  - Modest changes from baseline in sex hormone levels were observed in males at planned pivotal trial dose levels (≤50 mg BID).
  - Data are consistent with mild aromatase inhibition.
  - Most sex hormone values remained within normal limits in females; interpretation is confounded by variability in menopausal status and contraceptive use.

#### DRIVE PK efficacy (core period)[4]

- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL (**Figure 1**).
- The mean maximum increase in Hb was 3.4 g/dL in patients with an Hb increase >1.0 g/dL.
- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range, 7–187 days).
- The dose had to be held or reduced owing to a rapid rise in Hb in nine patients.




## ACTIVATE STUDY

### Summary

- The safety and efficacy data from the DRIVE PK study support the development of AG-348 in patients with PK deficiency.
- ACTIVATE is a phase 3, multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of AG-348 in adult patients with PK deficiency who are not regularly transfused (NCT03548220; **Figure 2**).
- An independent data monitoring committee will review the study data periodically and provide safety oversight.

### Study status

- ACTIVATE is expected to open in June 2018.

### PK deficiency global registry

- Patients who are not eligible for the ACTIVATE trial may be enrolled in the Peak Registry (NCT03451738).
- Goals of the Peak Registry:
  - Collect and aggregate longitudinal data (minimum 2 years, up to 9 years) from patients with PK deficiency who have been diagnosed via genetic analysis (all ages) worldwide (up to 20 countries)
  - Promote further understanding of PK deficiency disease parameters, e.g. transfusion dependency, treatment practices, Hb correlation with disease burden (refine/redefine and substantiate understanding based on data).

The Peak Registry

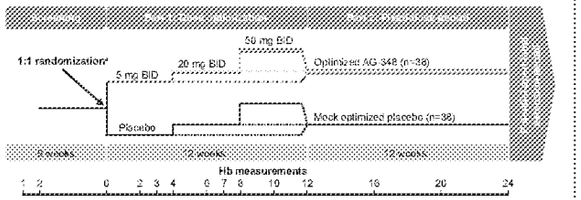
### Key inclusion criteria

- ≥18 years of age
- Documented presence of at least two mutant alleles in *PKLR*, of which at least one is a missense mutation (based on genotype–Hb response correlations in DRIVE PK; see **Figure 1**)
- Hb concentration ≤10.0 g/dL, regardless of sex
- ≤4 transfusion episodes in the 12-month period up to the first day of study treatment, and no transfusions in the 3 months prior to the last dose
- Adequate organ function

### Key exclusion criteria

- Homozygous for the R479H mutation or two non-missense mutations in *PKLR*
- Significant medical condition that confers an unacceptable risk to participating in the study and/or that could confound the interpretation of data

### ACTIVATE study design




*Stratified by average of screening Hb values (<9.5 g/dL vs ≥9.5 g/dL) and mutation category (missense/missense vs missense/non-missense)

Screening:
- Eligible patients randomized to AG-348 or placebo

Part 1: Double-blind dose optimization period:
- All patients start on 5 mg BID AG-348 or matched placebo
- Dose can be increased from 5 to 20 mg BID and, subsequently, from 20 to 50 mg BID
- Dose should be increased if patient does not experience an increase in Hb of ≥1.5 g/dL or could benefit from a greater increase in Hb

Part 2: Blinded dose period:
- Patient receives AG-348 or matched placebo at their optimized dose, with no planned adjustment for 12 weeks
- Dose can be reduced for safety reasons but it cannot be raised during this period

Planned interim analyses:
- For patients randomized to AG-348 who have experienced significant benefit from AG-348
- For all patients randomized to placebo

### Main endpoints

**Primary**
- Hb response, defined as a ≥1.5 g/dL increase in Hb concentration from baseline that is sustained at two or more scheduled assessments at Weeks 16, 20, and 24 during the fixed-dose period

**Secondary**
- Average change from baseline at Weeks 16, 20, and 24 in Hb concentration
- Average change from baseline at Weeks 16, 20, and 24 in markers of hemolysis
- Average change from baseline at Weeks 16, 20, and 24 in markers of hematopoietic activity
- Change from baseline in HRQoL PRO scores, PK Deficiency Diary and PK Deficiency Fatigue Assessment
- Change from baseline at Week 24 in iron markers
- Safety: AEs, laboratory parameters, bone mineral density
- AG-348 pharmacokinetics
- Relationship between AG-348 pharmacokinetics and safety parameters

**Exploratory**
- Relationship between AG-348 pharmacokinetics and indicators of clinical activity
- Effects of AG-348 on pharmacodynamic markers of PK deficiency
- Transfusions
- Liver iron content, as quantified by magnetic resonance imaging

### Statistics

**Sample size**
- Assuming a response rate of 35% in the active arm and 5% in the placebo arm, 75 patients (38 per arm) are needed to have 90% power to detect a treatment effect in Hb response rate, based on a two-sided Fisher's exact test at the 0.05 significance level.

**Primary efficacy analysis**
- Patient's Hb response status will be analyzed using a logistic regression model that will analyze Hb response (yes vs no) as a dependent variable and treatment as an independent variable, adjusting for stratification factors, including screening Hb concentrations (<9.5 vs ≥9.5 g/dL) and *PKLR* mutation category (missense/missense vs missense/non-missense).

HRQoL, PRO = health-related quality of life; patient-reported outcome.

**Disclosures**
This study is funded by Agios Pharmaceuticals, Inc.
M-HJ and CB: Agios – employment and stockholder. EvB: Agios – advisory board member.
Editorial assistance was provided by Helen Varley, PhD, CMPP, Excel Medical Affairs, Horsham, UK, and supported by Agios.

**Acknowledgements**
We would like to thank the patients taking part in the PK deficiency studies.

**References**
1. Grace RF, et al. *Am J Hematol* 2015;90:825-30. 2. Perng MJ, et al. *Blood Cells Mol Dis* 2007;39:189-94. 3. Grace RF, et al. *Blood* 2018;131:2183-92. 4. Kung C, et al. *Blood* 2017;130:1047-56. 5. Grace RF, et al. 59th *ASH Annual Meeting* 2017. Poster 2194.

Scan code to access PDF file of the poster or visit [redacted]/abs/83

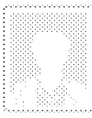
Presented at the 23rd Congress of the European Hematology Association (EHA), 14–17 June 2018, Stockholm, Sweden

Ex. 2001-0847

# ACTIVATE: A PHASE 3, RANDOMIZED, MULTICENTER, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY OF AG-348 IN ADULTS WITH PYRUVATE KINASE DEFICIENCY WHO ARE NOT REGULARLY TRANSFUSED

Author(s): Marie-Hélène Jouvin, Chris Bowden

EHA Learning Center Jouvin M. Jun 15, 2018; 214855



Marie-Hélène Jouvin

Abstract: PF382

Type: Poster Presentation

Presentation during EHA23: On Friday, June 15, 2018 from 17:30 - 18:00

Location: Poster area

Background

Pyruvate kinase (PK) deficiency is an under-recognized hereditary disease resulting in lifelong hemolytic anemia. PK deficiency is caused by mutations in the *PKLR* gene that lead to reduced red cell PK (PK-R) enzyme activity, resulting in defective glycolysis and decreased lifespan of red blood cells. AG-348 is a novel, first-in-class, oral, small molecule allosteric activator of PK-R under clinical testing as the first targeted disease-altering therapy for PK deficiency. In the DRIVE PK study (NCT02476916, a phase 2, open-label, dose-ranging trial in adults with PK deficiency who are not regularly transfused), twice daily (BID) dosing with AG-348 for >5 months was well tolerated and induced rapid, durable responses (Grace PF et al. ASH 2017). As of July 14, 2017, 26 (50%) of 52 enrolled subjects had a maximum hemoglobin (Hb) increase of ≥1 g/dL. Among these 26 subjects, the mean maximum Hb increase was 3.4 g/dL, and 25 (96%) had at least one missense *PKLR* mutation.

Aims

To report the design of the planned ACTIVATE study of AG-348.

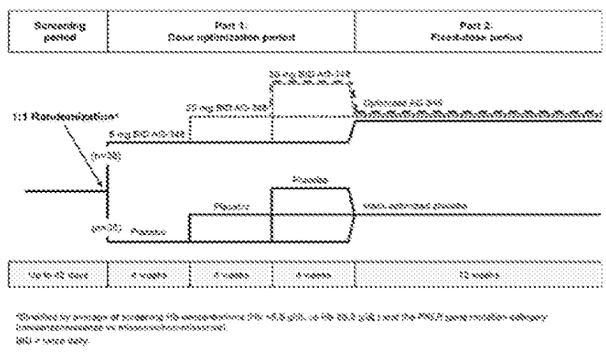
Methods

ACTIVATE is a phase 3, multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of AG-348 in adult subjects with PK deficiency who are not regularly transfused. The study will consist of a screening period of up to 42 days, a 12-week dose optimization period, and a 12-week fixed-dose period (Figure). During the dose optimization period, AG-348 or matched placebo will be titrated up to each subject's individually optimized dose, with an initial dose of 5 mg BID for all subjects and two potential sequential dose increases (from 5 to 20 mg BID and from 20 to 50 mg BID), depending on safety and Hb change. Approximately 76 adults with PK deficiency who are not regularly transfused will be randomized in a 1:1 ratio to AG-348 (administered orally, BID) or matched placebo. Key inclusion criteria include: written informed consent; not regularly transfused (≤4 transfusion episodes in previous year and no transfusions in the 3 prior months); baseline Hb ≤10 g/dL; adequate organ function. Subjects who are homozygous for the R479H mutation or have two non-missense mutations (without the presence of another missense mutation) in *PKLR* will be excluded. The primary endpoint is the Hb response, defined as the proportion of subjects who achieve a ≥1.5 g/dL increase in Hb sustained over at least two of the last three visits in the fixed-dose period. Key secondary endpoints include change in Hb, markers of hemolysis, hematopoietic activity, health-related quality of life, and safety. An independent data monitoring committee will review the study data periodically and provide safety oversight. The ACTIVATE study will be initiated globally in the first half of 2018.

Results

Not yet available.

**Figure. ACTIVATE Study Schema**




*Stratified by average of screening Hb concentrations (PK <5.8 g/dL or Hb 5.8-9.0 g/dL) and the PKLR gene mutation category (homozygous for R479H or non-missense/non-missense).
BID = twice daily.

Conclusion

AG-348 is a novel, first-in-class PK-R activator undergoing clinical testing in subjects with PK deficiency. The ACTIVATE study of AG-348 in subjects with PK deficiency who are not regularly transfused will start enrollment in 2018.

Session topic: 29. Enzymopathies, membranopathies and other anemias

Keyword(s): Clinical Trial, Hemolytic anemia, Phase III, Targeted therapy

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Ex. 2001-0850

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Category: 2. Erythrocyte / Erythroid Disorders / Bone Marrow Failure
Title of Abstract:
AG-348, A PYRUVATE KINASE ACTIVATOR, FOR PYRUVATE KINASE DEFICIENCY: RESULTS FROM THE DRIVE PK STUDY
Abstract Body:

Background: Pyruvate kinase (PK) deficiency is the 2nd most frequent congenital hemolytic anemia due to a red cell enzyme defect, and causes variable phenotypes ranging from mild anemia to transfusion-dependent hemolytic anemia. Current supportive treatments have a risk of complications. AG-348 is a first-in-class, oral, small molecule allosteric activator of red cell PK. Aim: To report preliminary data from the ongoing open-label DRIVE PK study of AG-348 in adults with PK deficiency (NCT02476916). Methods: Non-transfusion dependent (<=3 units RBCs in the 12 mo before and none in the 4 mo before first dose) patients (pts) were randomized to AG-348 50 mg or 300 mg orally twice daily (BID) for 6 mo (Core Period), and can continue treatment for 2 more yrs (Extension). Results: As of 18Jan2017, the enrollment has been completed with 52 pts; 23 remain in the Core Period and of 24 completing the Core, 21 entered the Extension (20 still on treatment). Pts are on doses ranging from <25 mg BID to 300 mg BID. At the last data cutoff (23Sep2016; N=34), AG-348 had an acceptable adverse event profile; headache (44%), nausea (41%) and insomnia (38%) were the most frequent. Of 32 evaluable pts, 15 (47%) had a maximal increase in hemoglobin (Hb) >1 g/dL. Updates on all 52 pts enrolled will be provided. Conclusions: Prolonged daily dosing with AG-348 is well tolerated and results in clinically relevant, durable Hb increases, highlighting its potential to be the first disease-altering treatment for PK deficiency. Preparations for a registry of Japanese pts with PK deficiency are ongoing.



Ex. 2001-0851

OS3-9B-1

# AG-348, a pyruvate kinase activator, for pyruvate kinase deficiency: Results from the DRIVE PK study

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*Presented at the 79th Annual Meeting of the Japanese Society of Hematology, 20–22 October 2017, Tokyo, Japan*

Ex. 2001-0852

# Disclosures

- This study was funded by Agios Pharmaceuticals Inc.
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- Frédéric Galactéros – study investigator
- Christian Rose: Agios – study investigator
- Wilma Barcellini: Agios – study investigator, no other disclosures
- D Holmes Morton – study investigator
- Eduard van Beers – study investigator
- Hassan Yaish – study investigator
- Yaddanapudi Ravindranath – study investigator
- Kevin HM Kuo – study investigator
- Sujit Sheth – study investigator
- Janet L Kwiatkowski: Agios – study investigator; Agios, Shire, Sideris, and Ionis Pharmaceuticals – consultant
- Bruce Silver, Marvin Cohen: Agios – consultants
- Bertil Glader: Agios – study co-principal investigator and scientific advisor
- Charles Kung, Hua Yang, Penelope A Kosinski, Lei Hua, Ann J Barbier: Agios – employees and stockholders
- Editorial assistance was provided by Christine Ingleby, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios

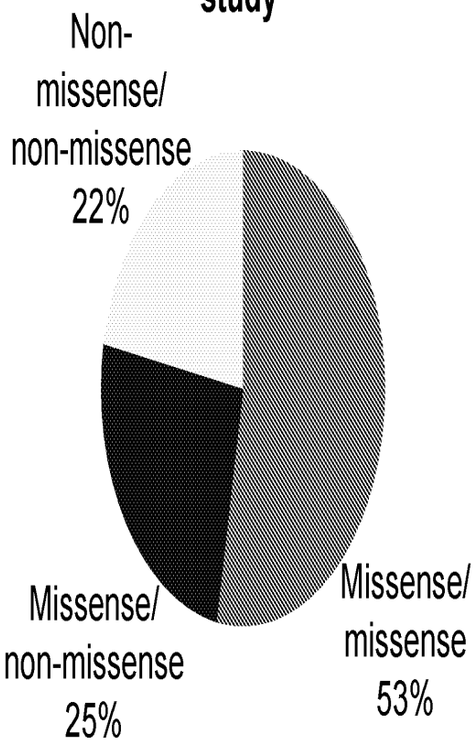


Ex. 2001-0853

# Pyruvate kinase (PK) deficiency: a severe congenital anemia

| | |
|---|---|
| **Description** | • Presents in childhood with severe hemolytic anemia |
| **Etiology** | • Caused by mutations in the *PK-LR* gene coding for erythrocyte pyruvate kinase (PK-R) |
| **Disease burden** | • Lifelong hemolytic anemia<br>• Iron overload and jaundice<br>• Infection risk post splenectomy |
| **Diagnosis/ treatment** | • PK-R enzyme activity and/or genetic testing<br>• Supportive treatment: transfusions, splenectomy, iron chelation |

**Type of *PK-LR* mutations found in 74 unrelated cases enrolled in the PK deficiency natural history study**




Source: Grace R et al. *Am J Hematol* 2015;90(9):825-30; Bianchi P et al. 57th ASH Annual Meeting 2015, Abstract 3337

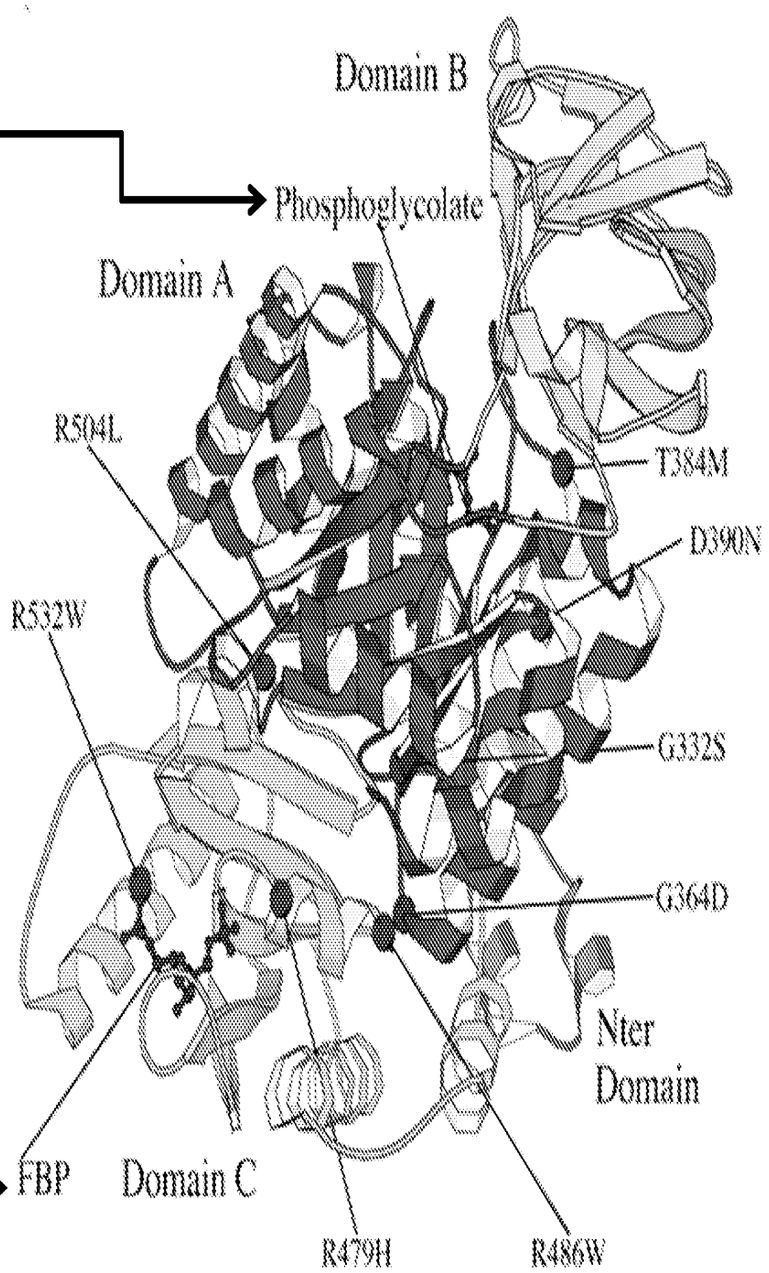


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# Tertiary structure of human PK-R

Active site (PEP binding)

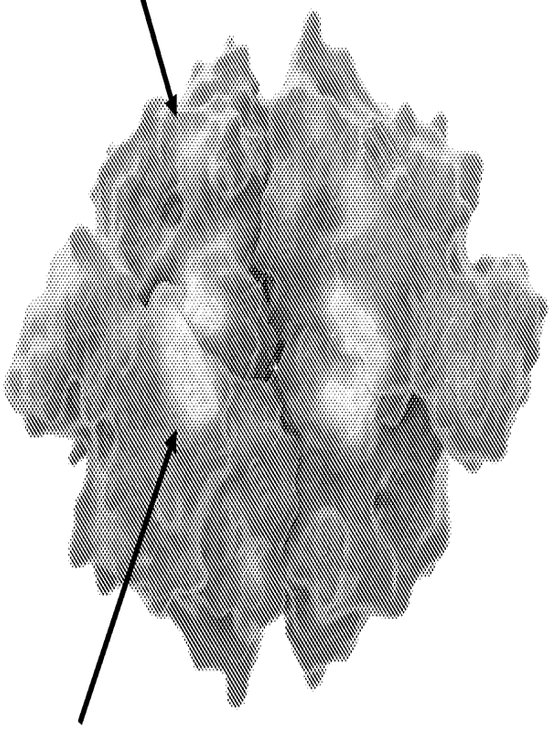
Allosteric activation site (FBP binding)




Valentini, G. et al. J. Biol. Chem 277: 23807-23814, 2002

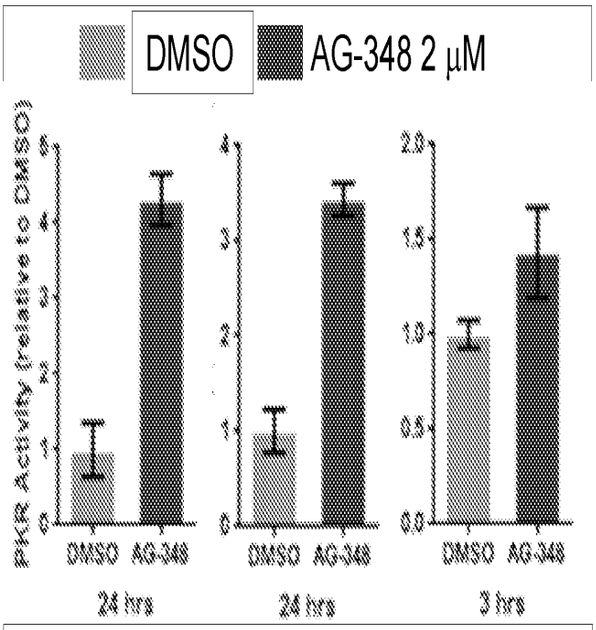
# AG-348 activates mutant PK-R

Active PK-R is a tetramer; mutations (green) decrease the catalytic activity



AG-348 (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

AG-348 activates mutant PK-R in blood from PK deficient patients




| Patient A (R510Q/ G511R) | Patient B (R486W/ D390N) | Patient C (A495V/ E241stop) |
|---|---|---|

Kung C et al. 55th ASH Annual Meeting 2013, Abstract 2180; Kung C et al. 56th ASH Annual Meeting 2014, Abstract 4010

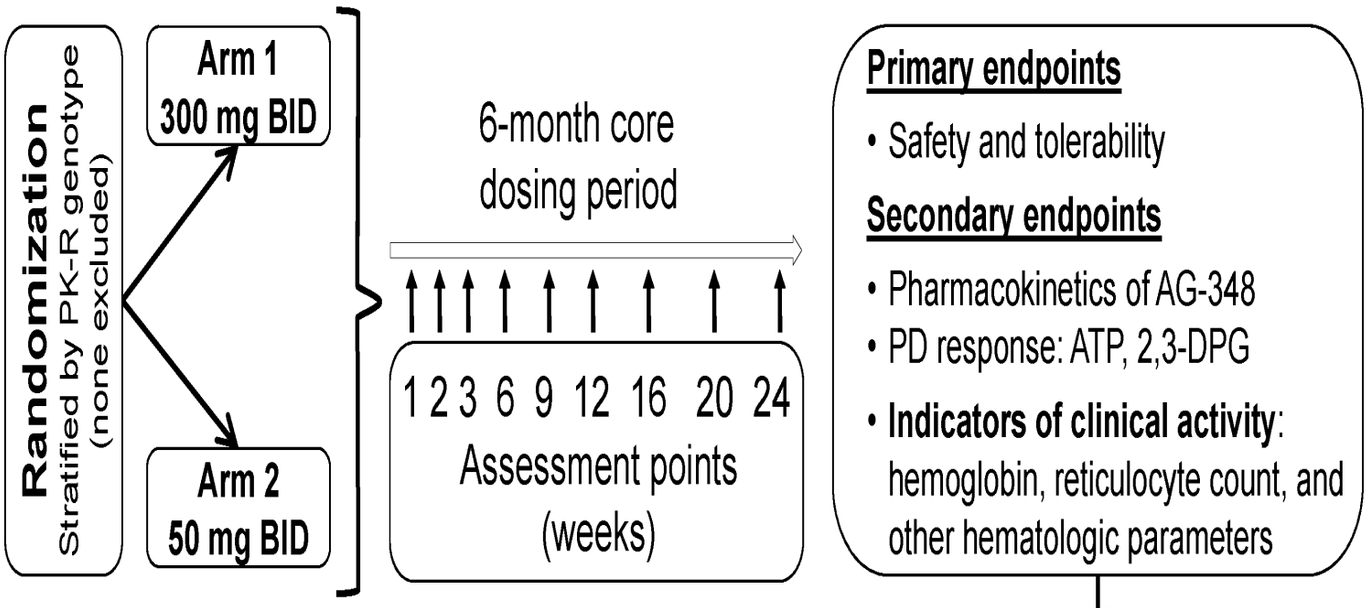


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# Study design

## Open-label, global phase 2 study: 14 centers in the US, Canada, and EU

Transfusion-independent PK-deficient adults
(ClinicalTrials.gov NCT02476916) n=25 in each arm




Transfusion independence = no more than 3 units of red blood cells transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

Fully enrolled as of November 2016

All patients provided written informed consent. 2,3-DPG = 2,3 diphosphoglycerate; BID = twice daily; PD = pharmacodynamic



Ex. 2001-0857

# Demographics

| Characteristics[a] | 50 mg BID n=27 | 300 mg BID n=25 | Total N=52 |
|---|---|---|---|
| Men, n (%) | 18 (66.7) | 14 (56.0) | 32 (61.5) |
| Age at randomization in years, median (range) | 29 (18–58) | 40 (21–62) | 34 (18–62) |
| Race white[b], n (%) | 22 (81.5) | 20 (80.0) | 42 (80.8) |
| Hemoglobin (Hb) baseline, median (range) | 9.6 (6.9–12.3) | 8.6 (6.5–12.0) | 8.9 (6.5–12.3) |
| Duration of treatment, weeks, median (range) | 23.0 (13.0–76.9) | 26.3 (12.9–70.9) | 24.9 (12.9–76.9) |
| Splenectomized, n (%) | 23 (85.2) | 20 (80.0) | 43 (82.7) |
| Iron chelation prior to enrolment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (76.0) | 38 (73.1) |

[a]Data cut-off March 27, 2017; [b]Not reported in 3 patients, 3 patients were Asian, and 4 were "other"



Ex. 2001-0858

# Safety summary

- AG-348 was generally well tolerated
- The majority of adverse events (AEs) were grade 1–2
- Treatment-related AEs leading to discontinuation, n=3
  - Chest discomfort/pleural effusion, pharyngitis/nausea, anemia
- 13 serious AEs in 10 patients
  - Six drug-related SAEs in 5 patients: Withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia; pharyngitis
    - Grade 4 hypertriglyceridemia at Week 24, resolved upon AG-348 discontinuation (Grade 1 at baseline)

| Patient disposition by study period | Ongoing, N | Completed, N | Discontinued[a], N |
|---|---|---|---|
| Core | 15 | 29[b] | 8 |
| Extension | 21 | 0 | 3 |

[a]Reasons for discontinuation: AEs (3), investigator decision (4), and withdrew consent (4)

[b]Five subjects completing the Core period did not enter the Extension

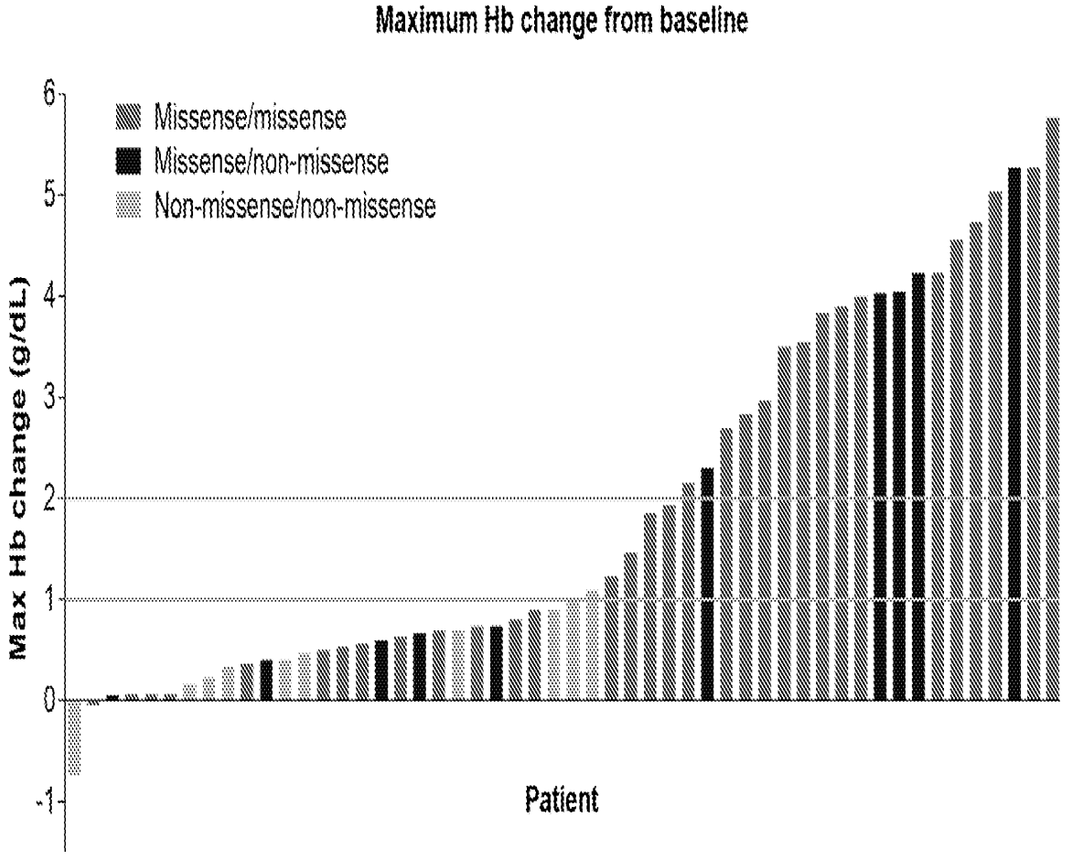


Ex. 2001-0859

# Clinical activity results

# Maximum Hb increase observed by genotype

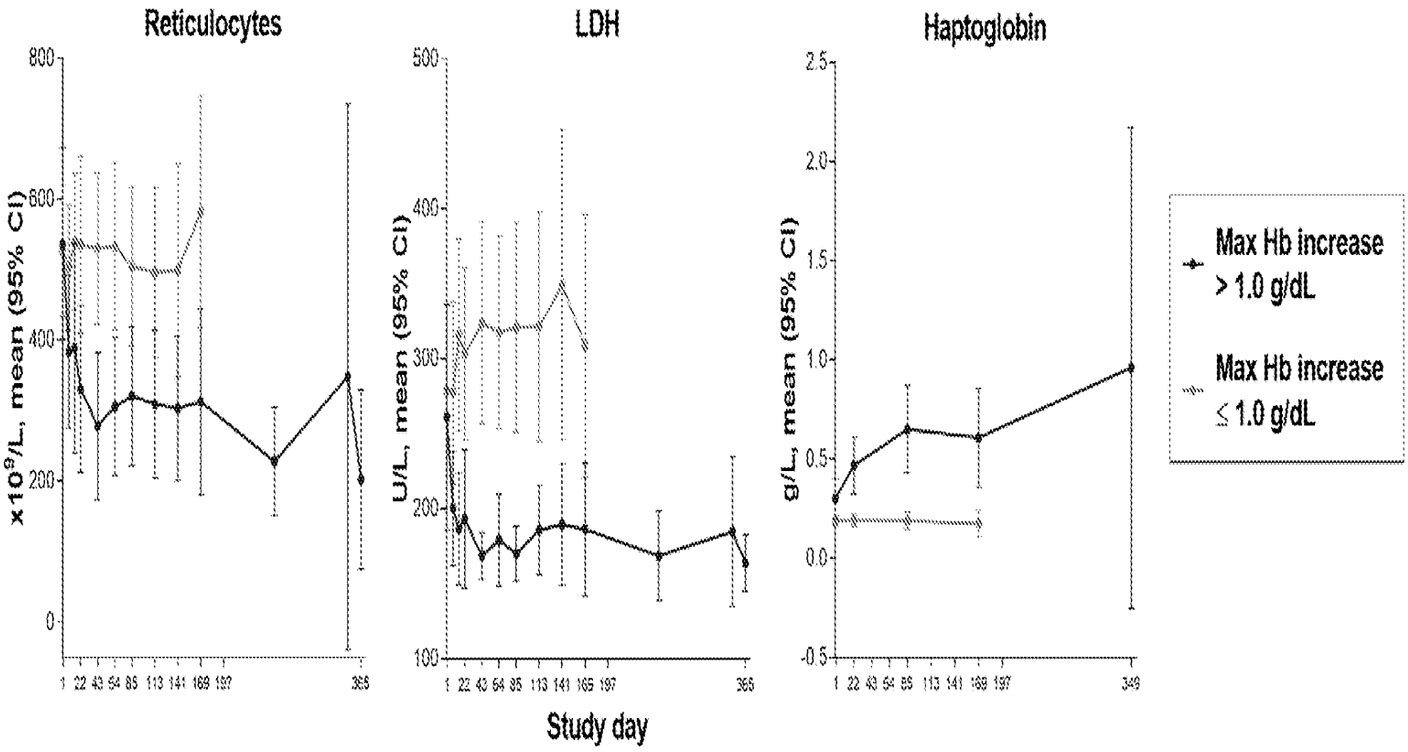
- 25/52 (48%) patients had a maximum increase in Hb >1.0 g/dL
  - 24/42 (57%) patients who had ≥1 missense mutation had Hb increase >1.0 g/dL




The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)

# Hemolysis markers improved in patients with Hb increase of >1.0 g/dL

- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin



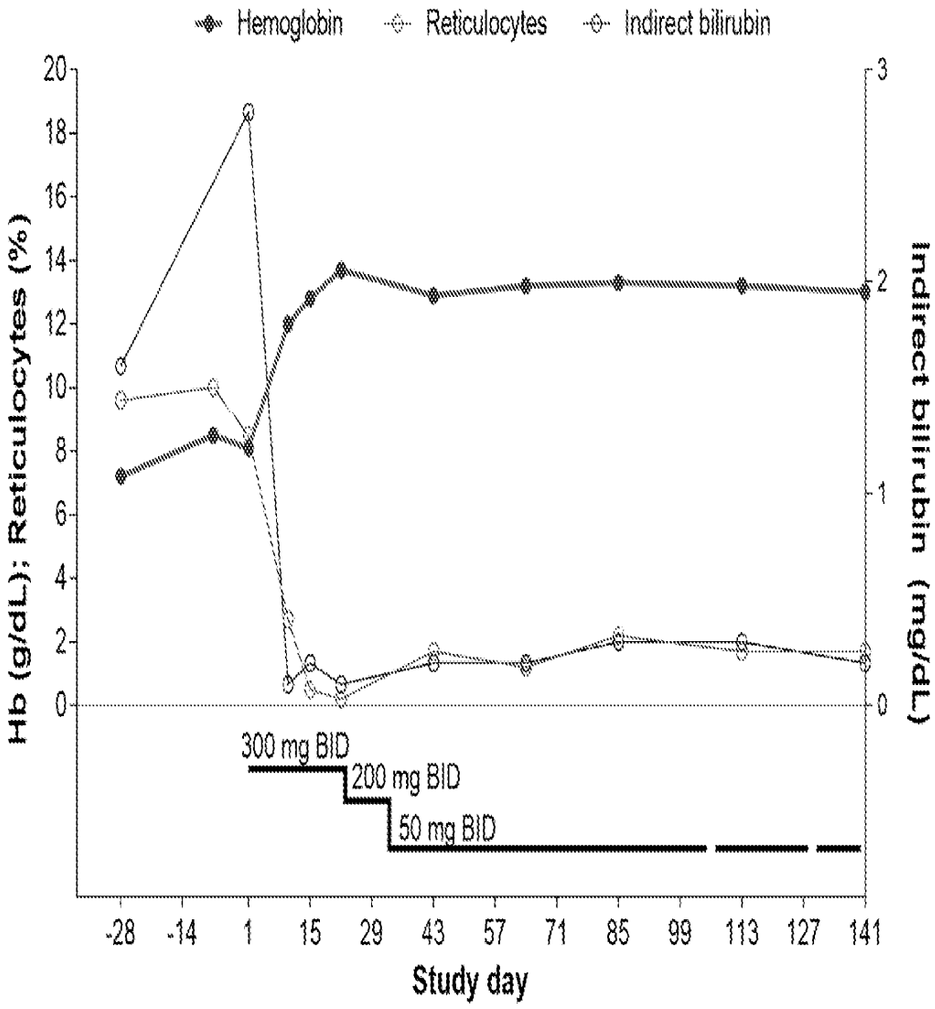

Only visits with ≥5 subjects included. Figure shows central laboratory results. LDH = lactate dehydrogenase



Ex. 2001-0862

# Hb response with improvements in hemolysis parameters: single subject experience with AG-348

- Female 35 years of age, white, missense/missense genotype (T384M/R479H)






Ex. 2001-0863

# DRIVE PK conclusions

- AG-348 is a novel, first-in-class PK-R activator in clinical testing as a potential disease-altering therapy for patients with PK deficiency
- Chronic daily dosing with AG-348 is well tolerated
  - Clinical significance of AG-348 aromatase inhibition is unclear
  - One grade 4 serious AE of elevated triglycerides was observed
- 25 of 52 (48%) subjects had a maximum Hb increase of >1.0 g/dL
  - Responses are rapid in onset and durable
  - Other parameters (reticulocytes, LDH, and haptoglobin) indicate decreased hemolysis in responders
  - Some genotype–Hb response correlations were observed
- These data highlight the potential of AG-348 to be the first disease-altering treatment for patients with PK deficiency, providing a rationale for pivotal studies



Ex. 2001-0864

# Acknowledgments


- We would like to thank the patients who agreed to participate in this study
- We would also like to thank all the clinical research sites and study investigators:
  - Boston Children's Hospital; Rachael F Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet L Kwiatkowski
  - Central Pennsylvania Clinic; D Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin HM Kuo
  - Hammersmith Hospital; D Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Hôpital Henri Mondor; Frédéric Galactéros
  - Hôpital Saint Vincent de Paul; Christian Rose
  - Hôpital de la Timone; Emmanuelle Bernit
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
- We would like to thank Drs Ellis Neufeld and David Nathan for helpful discussions
- This clinical study was funded by Agios Pharmaceuticals

Editorial support was provided by Christine Ingleby, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios




Ex. 2001-0865

# Global PK deficiency registry summary

Note to Prof Kanno: You may wish to present this new slide providing brief details of the new global registry study.

| | |
|---|---|
| **Scope** | • Observational<br>• Longitudinal<br>  • 9 year (7 year enrollment, minimum 2 year follow-up)<br>• Global<br>  • Up to 60 sites in 20 countries |
| **Population** | • All ages<br>• Confirmed diagnosis of PK deficiency via genetic testing |
| **Size** | • Approximately 500 patients |
| **Primary Objective** | • To develop an understanding of the longitudinal clinical implications of the disease, including:<br>  • Natural history<br>  • Treatments and outcomes<br>  • Variability of clinical care<br>  • Disease burden |
| **Contact Information** | • Agios Medical Information – MedInfo@agios.com |



Ex. 2001-0866

Blood. 2017 Sep 14; 130(11): 1347–1356.
Prepublished online 2017 Jul 31.
doi: 10.1182/blood-2016-11-753525: 10.1182/blood-2016-11-753525

PMCID: PMC5609468
PMID: 28760888

# AG-348 enhances pyruvate kinase activity in red blood cells from patients with pyruvate kinase deficiency

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Received 2016 Nov 24; Accepted 2017 Jul 3.

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## Key Points

- AG-348 is a small-molecule allosteric activator of WT red cell pyruvate kinase as well as mutant enzymes associated with hemolytic anemia.
- Activity in vitro, in mice, and in red blood cells suggests it may address the underlying molecular pathology in PK deficiency patients.

## Abstract

Pyruvate kinase (PK) deficiency is a rare genetic disease that causes chronic hemolytic anemia. There are currently no targeted therapies for PK deficiency. Here, we describe the identification and characterization of AG-348, an allosteric activator of PK that is currently in clinical trials for the treatment of PK deficiency. We demonstrate that AG-348 can increase the activity of wild-type and mutant PK enzymes in biochemical assays and in patient red blood cells treated ex vivo. These data illustrate the potential for AG-348 to restore the glycolytic pathway activity in patients with PK deficiency and ultimately lead to clinical benefit.

## Introduction

Pyruvate kinase (PK) deficiency is a glycolytic enzymopathy first described 50 years ago[1-4] that is characterized by a life-long chronic hemolytic anemia with severe comorbidities. Treatment is generally supportive with common interventions including splenectomy and blood transfusions.[5,6] Thus, there is an unmet medical need for novel therapies that address the underlying basis for PK deficiency.

Mammals have 2 PK genes, encoding the PK muscle (*PKM*) and PK liver and red blood cell (*PKLR*) forms.[7] *PKLR* controls the expression of the red blood cell (PK-R) or liver (PKL) isoforms from tissue-specific promoters. Loss-of-function mutations in the *PKLR* gene cause PK deficiency, with clinical

symptoms apparently confined to red blood cells (RBCs), although the vast majority of mutations associated with PK deficiency affect both PK-R and PKL. PK-R catalyzes the final and irreversible step in glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate, with concomitant formation of the energy carrier molecule adenosine triphosphate (ATP; Figure 1A). The importance of glycolysis in RBCs is highlighted by the fact that mutations in nearly every glycolytic enzyme result in hemolytic anemia.[8] This is because mature RBCs lack mitochondria and rely almost exclusively on glycolysis to generate ATP.

PK-R 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).[9] Hundreds of mutations, most of which are single nucleotide missense mutations, have been described, which can have deleterious effects on PK-R catalytic activity, protein stability, or protein expression.[6] For example, the R486W mutation, present in ~25% of patients with PK deficiency in southern Europe,[10] is thought to affect the catalytic efficiency of the enzyme, and the R510Q mutation, found in ~40% of northern European patients with PK deficiency, significantly destabilizes the active tetrameric species.[11] PK deficiency is an autosomal recessive disease, and patients are either homozygous, or more commonly, compound heterozygous for 2 mutant PK-R (mtPK-R) alleles.[12]

A variety of experimental therapies, including gene therapy,[13-15] have been proposed for PK deficiency[5,16,17]; however, there are no approved drugs that directly target mutated PK-R.

We sought to address the underlying cause of PK deficiency by identifying a molecule that could induce and stabilize the active *R*-state of the PK-R tetramer, thus directly restoring PK activity to the mutant enzyme(s). Although PK-R is already subject to endogenous activation by FBP,[9] this is insufficient to overcome the deleterious effects of mutations in patients with PK deficiency. We and others have previously described small-molecule allosteric activators of the PKM2 isoform of PK that bind in a pocket distinct from the FBP binding site.[18,19] We asked whether we could identify a small-molecule agonist that targeted the analogous pocket in PK-R.

Here, we describe the mechanism of action and cellular effects of AG-348, a first-in-class small-molecule allosteric activator of PK-R. We demonstrate that AG-348 is a potent activator of wild-type (WT) PK-R in biochemical assays, in RBCs, and in vivo in mice. Moreover, as the genetic heterogeneity of PK deficiency dictates that a small-molecule therapy should enhance the activity of a spectrum of mtPK-R enzymes to maximize potential clinical benefit, we show that AG-348 is a pan-activator of several mtPK-R enzymes associated with PK deficiency and that ex vivo treatment of RBCs from patients with PK deficiency results in increased PK-R activity and changes in metabolism consistent with enhanced glycolysis. These data support the hypothesis that treatment of patients with PK deficiency with AG-348 may correct the underlying pathology of this disease.

## Methods

### Biochemistry

WT and mtPK-R proteins were expressed in the *E. coli* strain BL21 (Invitrogen, Carlsbad, CA) as an *N*-terminal His6-fusion, purified after refolding, and active fractions were snap-frozen in liquid nitrogen and stored at −80°C. PK-R activity was detected spectrophotometrically using a coupled enzyme system with lactate dehydrogenase. Reaction conditions were optimized for each individual mtPK-R to have the largest difference in activity following stimulation with FBP (see supplemental Methods, available on the *Blood* Web site). Compounds were dissolved in relevant vehicle (typically dimethyl sulfoxide [DMSO]) and were preincubated with PK-R proteins for 30 min before reaction initiation. Fold-change was defined as the ratio of activity between AG-348 versus DMSO treatment under the specific assay conditions. For thermostability studies, following compound incubation at room temperature, reactions were shifted to

53°C and aliquots taken over time to assess residual activity. For off-rate measurements, compounds were incubated at 10 × $AC_{50}$ (concentration at which activity reaches 50% of maximum) for 30 min before dilution into activity buffer and assessment of residual activity over time.

### Cell assays

Peripheral blood was obtained from a commercial source (Research Blood Components, Brighton, MA) or from patients following each institution's relevant institutional review board protocol (Boston Children's Hospital, Boston, MA; Tufts Medical Center, Boston, MA; and St. Jude Children's Research Hospital, Memphis, TN). RBCs were isolated via Percoll centrifugation and resuspended in phosphate-buffered saline containing 1% glucose, 170 mg/L adenine, and 5.25 g/L mannitol. For activity and ATP assays, following addition of AG-348 (0.1% final DMSO concentration), cells were incubated at 37°C overnight. ATP was measured using CellTiterGlo (Promega, Madison, WI), and PK-R activity was assessed from the lysate using the coupled enzyme assay described above. For some experiments, PK-R activity was measured using direct detection of pyruvate using mass spectrometry. PK-R protein levels were quantitated using a MesoScale assay (antibodies from Abcam, Cambridge, UK: ab89071; and from Aviva, London, UK: OAGA00912).

For additional details and methods, please see the full supplemental Methods section.

## Results

AG-348 is a quinolone sulfonamide (*N*-(4-(4-(cyclopropylmethyl)piperazine-1-carbonyl)phenyl)quinoline-8-sulfonamide; Figure 1B) related to a family of activators of PKM2 that we have previously described.[19] We identified AG-348 through a series of medicinal chemistry efforts optimizing both compound activity against PK-R and molecular properties consistent with an investigational medicine for use in humans.

We recombinantly expressed and purified WT PK-R enzyme and evaluated its activity upon AG-348 treatment. AG-348 is a potent activator of PK-R, stimulating activity to ~5-fold of DMSO control with a concentration that increases activity by 50% ($AC_{50}$) of 29 ± 17 nM (n = 108; Figure 1C). Examination of key kinetic parameters reveals that AG-348 increases the catalytic efficiency ($k_{cat}/K_M$) of WT PK-R, primarily by increasing the affinity for the substrate PEP, as reflected in the change of $K_M$ for PEP following AG-348 treatment (Figure 1D; Table 1). AG-348 also is an activator of PKM2 and PKL (supplemental Figure 1).

To understand the molecular basis of PK-R activation by AG-348, we solved the crystal structure of AG-348 bound to PK-R to 2.75 Å resolution using the molecular replacement method. The overall cocomplex structure is in the active *R*-state also observed with FBP-bound PK-R[9,20]; however, AG-348 is bound in a pocket at the dimer–dimer interface (Figure 1E), distinct from the FBP-binding domain. The AG-348 binding pocket is deeply buried within the PK-R tetramer and occluded from the solvent; this could suggest a postbinding conformational change to the final *R*-state.

Although the coverage of electron density obtained for the compound was incomplete, it was sufficient to allow us to model 1 copy of the ligand in 1 pocket and to model 2 copies in the other site. With the symmetric nature of the binding pocket, each ligand establishes the same interactions (Figure 1F). The quinoline moiety sits in a flat and mainly apolar surface defined by residues Phe69, Leu70, and Leu73 from chain A and Phe69, Tyr433, and Leu437 from chain B. One of the 2 oxygen atoms of the sulfonamide accepts a hydrogen bond from the backbone oxygen of Tyr433. The nitrogen of the sulfonamide establishes a hydrogen bond with the backbone oxygen of Leu396, but this interaction is likely to be weak because closer analysis of the electron density does not allow a clear placement for this atom. The cyclopropyl ring of AG-348 sits in the other copy of the quinoline pocket. The electron density

does not allow an easy placement of the 2 remaining rings; therefore, they were modeled in the pocket using low-energy conformations. There are no known mutations associated with PK deficiency that affect the amino acids listed above that make contact with AG-348.[21]

Next, we evaluated the effects of AG-348 on PK activity in intact human RBCs. We purified RBCs from whole blood from healthy donors and incubated them with AG-348 overnight. RBCs were then spun down, washed, and lysed by freeze thawing, and the lysate was evaluated for PK-R activity. AG-348 treatment increases PK-R activity in a dose-dependent manner to ~2.5-fold of DMSO control (average 275% ± 56%) with an $AC_{50}$ of 62 ± 35 nM (Figure 2A). The observation of increased PK-R activity even after washing and lysing the RBCs implies that AG-348 binds with slow-off kinetics, consistent with the binding mode observed in the crystal structure.

Although the PK-R activity assay demonstrates that AG-348 can enter the RBC and bind to the PK-R enzyme, the rate of metabolism through the PK step in RBCs is highly regulated and potentially influenced by a host of factors, including FBP and PEP concentrations.[22] For example, if the intracellular concentration of PEP was maintained at millimolar concentrations, rather than the actual physiological concentration of ~10 μM,[23,24] PK-R would be maximally activated, and there would be no further effect of AG-348 in the cell setting (Figure 1D). Thus, we tested whether activation of PK-R by AG-348 is capable of promoting glycolytic metabolism in intact RBCs, both in vitro and in vivo, where these factors would be fully accounted for. First, we measured the levels of ATP in DMSO control– or AG-348–treated RBCs. In RBCs isolated from different donors, AG-348 consistently increased ATP levels in a dose-dependent manner by an average of 60% over DMSO control with an $AC_{50}$ of 10.9 ± 7 nM (n = 102; Figure 2B).

Next, we characterized the effects of AG-348 treatment on RBC metabolism in vivo in mice. C57/BL6 mice were dosed twice daily by oral gavage with AG-348 (1 mg/kg, 10 mg/kg, 50 mg/kg, and 150 mg/kg) for 7 days. At time points up to 72 h following the last dose, groups of mice (n = 4) were sacrificed, and plasma and whole blood samples were collected to evaluate drug exposure and pharmacodynamic markers, including levels of ATP, 2,3-diphosphoglycerate (2,3-DPG), and PK-R activity. Analysis of AG-348 exposure in the plasma showed dose-proportional increases in maximal concentration ($C_{max}$) and the area under the curve (AUC) during each dosing period ($AUC_{0\text{-}12\ hrs}$; Figure 2C). Treatment with AG-348 was associated with dose-dependent changes in all 3 pharmacodynamic markers (Figure 2D-F; supplemental Figure 2). PK-R activity levels were increased up to ~2-fold over vehicle with residual activity detectable up to 36 h after the last dose at the 150 mg/kg dose level (Figure 2D). There was a concomitant increase in ATP levels, with an ~40% increase in the $AUC_{0\text{-}12\ hrs}$ (Figure 2E). The upstream glycolytic intermediate 2,3-DPG was reduced accordingly by ~20% (Figure 2F). The changes in levels of ATP and 2,3-DPG are substantial, given that these metabolites are among the most abundant intracellular metabolites in RBCs, present at millimolar concentrations.[22] These data confirm that AG-348 can activate PK-R in vivo and that pharmacological activation of PK-R results in dramatic changes in RBC metabolism.

Valentini et al previously described the properties of PK-R mutants that they noted spanned distinct subdomains of the enzyme.[20] We recombinantly expressed and purified several of these (G332S, G364D, T384M, R479H, R486W, R532W) as well as additional mtPK-Rs, including the commonly found R510Q enzyme (Figure 3A). Kinetic analyses (Table 1) reveal deficiencies in both the affinity for PEP substrate ($K_M$) and the maximal velocity ($V_{max}$) in comparison with the WT enzyme. This leads to overall reduced enzyme efficiency ($k_{cat}/K_M$) for the panel of mutants analyzed (range, 8% to 50% of WT $k_{cat}/K_M$; Table 1), except for R510Q (181% of WT $k_{cat}/K_M$) where the mutational defect is known to be in protein stability.[11] Physiological PEP concentration[23,24] (supplemental Figure 3) in both normal (~10 μM) and PK-deficient (~30 μM) RBCs is far below $K_M$ values (>1 mM) for WT or mtPK-R enzymes, so the PK-R enzyme reaction should not approach $V_{max}$ in the cellular setting. Therefore, in kinetic terms, increasing

enzyme efficiency ($k_{cat}/K_M$) with respect to PEP substrate is predicted to be the most effective mode of action to compensate for glycolytic deficiency. Indeed, in this panel of mutants, if $k_{cat}/K_M$ of the most severe mutant G322S could be increased by 6-fold, the result should theoretically be equivalent to 50% of WT PK-R activity (the approximate level of activity found in asymptomatic carriers of PK deficiency).

We preincubated each mtPK-R enzyme with AG-348 for 30 min and repeated the same kinetic characterization. We observed enhanced activity of all mutant enzymes tested with a ≥2-fold change in activity in comparison with baseline (Figure 3B). With the exception of G332S, $k_{cat}/K_M$ of the activated mtPK-R exceeded 50% of baseline WT enzyme efficiency (Table 1). In each case, the greatest contributor to the enhanced activity came from an increased affinity to PEP and not a change in $V_{max}$ (Table 1; representative graphs shown in Figure 3C and supplemental Figure 4A).

Activation of mtPK-R enzymes by AG-348 was dose dependent (representative data shown in Figure 3D and supplemental Figure 4B), with $AC_{50}$ values ranging from 9 to 59 nM (Figure 3B). A compelling example of AG-348's unique mechanism of action is illustrated by the R532W mutation, which renders PK-R insensitive to stimulation by FBP (Figure 3D). In contrast, AG-348 is able to activate the enzyme ≥4-fold with an $AC_{50}$ of 9 nM, consistent with the fact that it occupies a distinct allosteric-binding pocket from that of FBP.

Next, we examined whether binding to AG-348 could confer heightened stability on mtPK-R enzymes. We examined the thermostability of WT and R510Q enzymes by subjecting them to incubation at 53°C for up to 4 h, with and without AG-348 pretreatment. The WT enzyme retains ~50% residual activity at the conclusion of the assay, whereas the R510Q enzyme loses ≥90% of its activity within 10 min, with a calculated half-life ($t_{1/2}$) ~2% of that of the WT enzyme (Figure 3E). After preincubation of R510Q with AG-348 for 30 min at room temperature, the enzyme demonstrated markedly enhanced stability, with a $t_{1/2}$ ~70% of the WT enzyme ($t_{1/2\ (WT)}$ = 258 min, $t_{1/2\ (R510Q)}$ = 5 min, $t_{1/2\ (R510Q+AG-348)}$ = 177 min; Figure 3E). We further evaluated the binding characteristics of AG-348 with the R510Q enzyme by measuring the off-rate of the compound, in comparison with the endogenous activator FBP. The off-rate of FBP is too fast to be measurable, whereas AG-348 has a significantly slower off-rate, with a $t_{1/2}$ of ~20 min (Figure 3F).

Collectively, these experiments demonstrate that AG-348 enhances the activity of a spectrum of mtPK-R enzymes associated with PK deficiency. AG-348 has the potential to act both by enhancing the efficiency of the residual enzyme that is present and by increasing the $t_{1/2}$ of unstable mtPK-R tetramers.

Next, we evaluated the effect of AG-348 on RBCs from PK-deficient patients. Patient peripheral blood samples were obtained from local hospitals (samples A, B, C, D, and G, usually within several hours post–blood draw) or shipped via overnight courier (samples E and F). Minimal data were provided with these deidentified patient samples, except that it was understood that the patients had not received recent transfusions. We sequenced the *PKLR* gene in each patient sample and confirmed the presence of 2 mtPK-R alleles (Figure 4A). We also used antibody-based capture to quantitate the total levels of PK-R protein in the blood. A range of PK-R protein levels was observed (Figure 4B), from close to WT control to nearly undetectable (samples F and G).

PK-deficient RBCs are characterized by changes in metabolism associated with defective glycolysis, including a build-up of the upstream glycolytic intermediate 2,3-DPG and PK-R substrate PEP and deficiency in the PK-R product ATP.[23] We extracted metabolites from the blood of patient A as well as that of WT controls and observed differences supporting the presence of a constriction of glycolysis at the PK step (supplemental Figure 5A). In particular, ATP levels were reduced, and 2,3-DPG levels were elevated in patient A. PEP levels were found to be elevated ~3-fold (estimated at ~30 μM) in whole blood from patient A and separately quantitated to be ~37 μM in patient G (supplemental Figure 3), in agreement with previously reported results,[23] although metabolic comparisons between blood samples

have a number of potential confounding factors, including differences in sample handling (supplemental Figure 5B-C) and the composition of blood (eg, the presence of reticulocytes and immature RBCs in PK-deficient blood).[25]

To determine the effect of AG-348 on PK-R activity and glycolysis metabolites, we purified RBCs from peripheral blood of patients with PK deficiency and incubated them with AG-348. In comparison with DMSO control, time-dependent decreases in relative levels of 2,3-DPG and PEP were seen in RBCs from patients A and B (Figure 4C; supplemental Figure 6A). Exposure of patient RBCs encompassing a range of genotypes to AG-348 led to dose-dependent increases in PK activity and ATP levels (Figure 4D-E; supplemental Figure 6B) at potencies similar to those observed in WT cells (Figure 4F). The effects seen in RBCs from patients F and G were less than those in the other samples (Figure 4F). Because AG-348 depends on physically binding to the PK-R protein to activate it and effect changes in cellular metabolism, these results are consistent with and likely due to the extremely low levels of residual PK-R protein present (Figure 4B).

As RBC metabolite levels can change significantly post–blood draw,[26] we arranged for rapid transport of blood from patient A to our laboratory (within 30 min), along with a time-matched WT control. AG-348 treatment increased PK-R activity and ATP levels to within the range of baseline levels in WT control cells (Figure 4G), although WT cells had further increased ATP and PK-R activity after AG-348 exposure. Collectively, these data demonstrate enhanced glycolytic pathway activity induced by AG-348 in PK-deficient RBCs that contain a variety of mutations.

## Discussion

The exact mechanism of extravascular hemolysis in PK deficiency is unknown, but it is likely to be related to the unique metabolic requirements of RBCs as they traverse the circulatory system. During passage through the hypoxic tissue in the spleen, mature erythrocytes and even mitochondria-containing reticulocytes depend on PK-R to generate ATP. Nathan et al[27] elegantly demonstrated that PK-deficient reticulocytes had reduced lifespan through selective destruction in the spleen using radiolabeling of cell populations enriched by age. Experiments using agents such as cyanide and salicyate that can affect oxygen-dependent mitochondrial metabolism suggest that ATP depletion in PK-deficient cells leads to increased RBC dehydration through potassium efflux driven by increased activity of the Gardos channel.[28-30] Given the genetic heterogeneity and broad spectrum of clinical presentation found in PK deficiency, it is likely that multiple factors influence the nature of hemolysis within individual patients. For instance, direct evidence for ineffective erythropoiesis has been found in splenic tissue from patients with PK deficiency.[31] Nonetheless, the common feature found in patients with PK deficiency are genetic defects in the *PKLR* gene.

Here we have described the discovery and detailed biochemical, biophysical, cellular, and pharmacological characterization of AG-348, a novel small-molecule allosteric activator of both WT PK-R as well as mtPK-R enzymes associated with PK deficiency. We show that AG-348 binding induces the active *R*-state of the PK-R tetramer and that even in the absence of any deficiency of PK activity, activation of PK-R by AG-348 has profound effects on RBC metabolism in vivo in mice. These experiments show that AG-348 can increase PK activity in RBCs beyond the level achievable from the native effector molecule FBP and support its potential to correct defective metabolism in PK deficiency.

The relationship between activation of mtPK-R by AG-348 and physiological response (eg, increased red cell life span) is under investigation and will have to be determined with clinical studies. In our best estimation using recombinant enzymes and patient RBCs, AG-348's ability to increase $k_{cat}/K_M$ of mtPK-R enzymes to approximately that of the unstimulated WT activity is anticipated to result in a biological response. Ultimately, efficacious activation levels could be further dependent on a number of factors including PK-R protein concentration. It is also conceivable that PK-deficient cells might have adapted to

their low PK-R activity levels and are thus poised to benefit from any additional stimulation. It is unlikely that full WT activity would have to be restored to see clinical benefit, because PK deficiency carriers have PK-R enzyme activity and ATP levels that are intermediate between patients with PK deficiency and WT controls and are asymptomatic.[32]

Our ex vivo experiments in PK-deficient RBCs demonstrate that AG-348 increases ATP levels and PK activity and thus may help address the energy deficiency that leads to the reduced lifespan of these cells ( Figure 4D-E) or RBC precursors such as reticulocytes. Combined with the observation that glycolytic intermediates upstream of PK-R are reduced (Figure 4C), these results suggest that AG-348 treatment can induce a metabolic state more similar to WT RBCs than to baseline.

Importantly, AG-348's activity in cells from patients A, B, and E (each being compound heterozygous for 2 different mtPK-R enzymes) demonstrate that the molecule is able to activate the mix of mtPK-R homotetrameric and heterotetrameric species presumably present in those RBCs. It may be sufficient to activate 1 of the 2 mutant alleles found in patients, as is illustrated in the case of patients C and D, in which 1 of the alleles is a severe truncation that is unlikely to contribute to the residual enzyme activity ( Figure 4A). Given the genetic complexity of PK deficiency, it is probable that some mutant enzymes that do not respond to AG-348 will be found, perhaps in residues that govern the allosteric action mediated by AG-348.

AG-348 shows maximal activity in cells in which there are adequate levels of PK-R (Figure 4B,F; supplemental Figure 6C). In the ongoing natural history study for PK deficiency (ClinicalTrials.gov NCT02053480), ~20% of non-Amish patients are estimated to have a genotype that predicts near-complete loss of functional PK-R protein expression, which would likely preclude activity of AG-348.[33] For patients with low levels of PK-R caused by decreased protein stability (eg, R510Q), AG-348 may not effectively extend the lifespan of RBCs in the peripheral blood. However, our biochemical and structural analyses suggest the possibility that AG-348 might act on a precursor RBC to stabilize and extend the $t_{1/2}$ of the newly synthesized mtPK-R enzyme. Further studies to explore this concept using ex vivo models of erythropoiesis[34] are ongoing.

These data support the hypothesis that AG-348 may restore RBC glycolytic pathway activity in vivo and correct the underlying pathology of PK deficiency. Early data from a phase 2 study of AG-348 in patients with PK deficiency demonstrated that increases in hemoglobin are possible during treatment with AG-348.[35] Additional analysis of these emerging data, and correlative studies with the experiments described in this article, should increase understanding of the mechanism of AG-348 and its potential to provide clinical benefit to patients. The ability of AG-348 to restore, at least in part, the function of mtPK-Rs offers the possibility that other enzymopathies might also be treated using a similar allosteric activator approach.

Several groups have found that ATP depletion leads to significant changes in RBC membrane structure and integrity in WT erythrocytes.[36,37] Given that AG-348 can increase the activity of the WT PK-R enzyme, AG-348 could possibly be beneficial in RBC diseases that are characterized by increased energy stress, even if only secondary to the underlying basis of the disease. For instance, β-thalassemia is a hemoglobinopathy characterized by the presence of excess α-hemoglobin chains, which are cleared by ATP-dependent proteolytic mechanisms.[38] It will be interesting to further investigate these possibilities in ex vivo studies on patient RBCs as well as in mouse models of disease.

## Supplementary Material

The online version of this article contains a data supplement.

## Acknowledgments

The authors thank Boston Children's Hospital (especially Ellis Neufeld), Tufts Medical Center and the Floating Hospital for Children (especially Cathy Rosenfield and Furha Cossor), and St. Jude Children's Research Hospital (especially Kerri Nottage) for assistance in obtaining patient samples for research purposes. Editorial assistance was provided by Christine Tomlins of Excel Scientific Solutions and was supported by Agios Pharmaceuticals.

## Footnotes

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## Authorship

Contribution: C.K. designed experiments, prepared figures, and wrote the paper; J.H., P.A.K., G.C., Y.C., G.H., C.H., S.G., B.D., Y.S., and K.J. designed and performed experiments and prepared figures; Z.L., Z.G., G.Y., H.T., C.F., Y.X., and X.L. performed experiments and analyzed data; F.S., J.P.-M., H.Y., S.-S.M.S., S.B., L.S., and L.D. guided the research; and all authors critically revised the manuscript and approved the final version.

Conflict-of-interest disclosure: C.K., J.H., P.A.K., G.C., G.H., Y.C., C.H., S.G., Y.S., K.J., B.D., S.B., S.-S.M.S., H.Y., J.P.-M., F.S., L.S., and L.D. are or were full-time employees of Agios Pharmaceuticals. Work conducted at Shanghai ChemPartner Ltd. was funded by Agios Pharmaceuticals.

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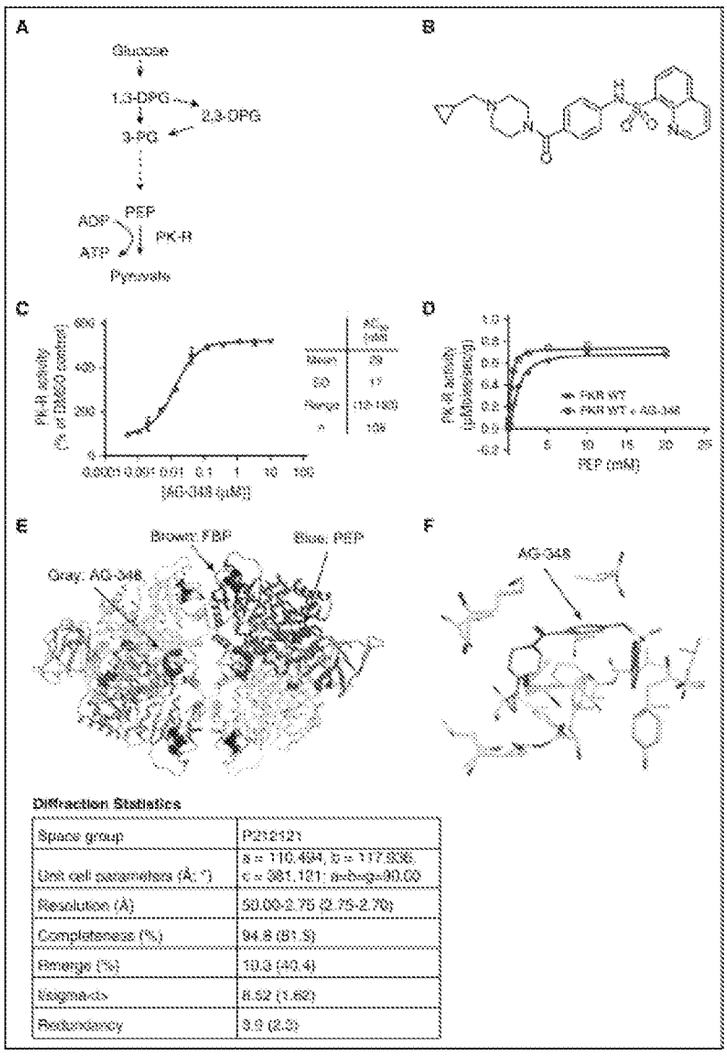
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## Figures and Tables

Figure 1.




**Diffraction Statistics**

| | |
|---|---|
| Space group | P212121 |
| Unit cell parameters (Å; °) | a = 110.484, b = 117.836, c = 381.181; α=β=γ=90.00 |
| Resolution (Å) | 50.00-2.75 (2.75-2.70) |
| Completeness (%) | 94.8 (81.5) |
| Rmerge (%) | 10.3 (40.4) |
| I/sigma<I> | 8.52 (1.62) |
| Redundancy | 3.9 (2.3) |

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**Structure and function of PK-R and AG-348.** (A) Simplified schematic of glycolysis. (B) Chemical structure of AG-348. (C) Activity of recombinant PK-R enzyme incubated with indicated concentrations of AG-348 (PEP = 0.065 mM). Mean, standard deviation, range, and number of experimental replicates are indicated in the column on right-hand side of panel. (D) Plot of activity of recombinant WT PK-R enzyme stimulated with PEP with or without preincubation by AG-348 (5 μM); the average of 3 technical replicates is shown. (E) Ribbon diagram of the cocomplex crystal structure of PK-R tetramer bound to AG-348 (shown as space-filling model in gray). PEP (blue) and FBP (brown) also shown as space-filling models. Diffraction statistics are shown below the diagram. (F) Model of AG-348 in PK-R allosteric- binding pocket illustrating key interactions. All error bars are standard deviations. ADP, adenosine diphosphate; PG, phosphoglyceric acid.

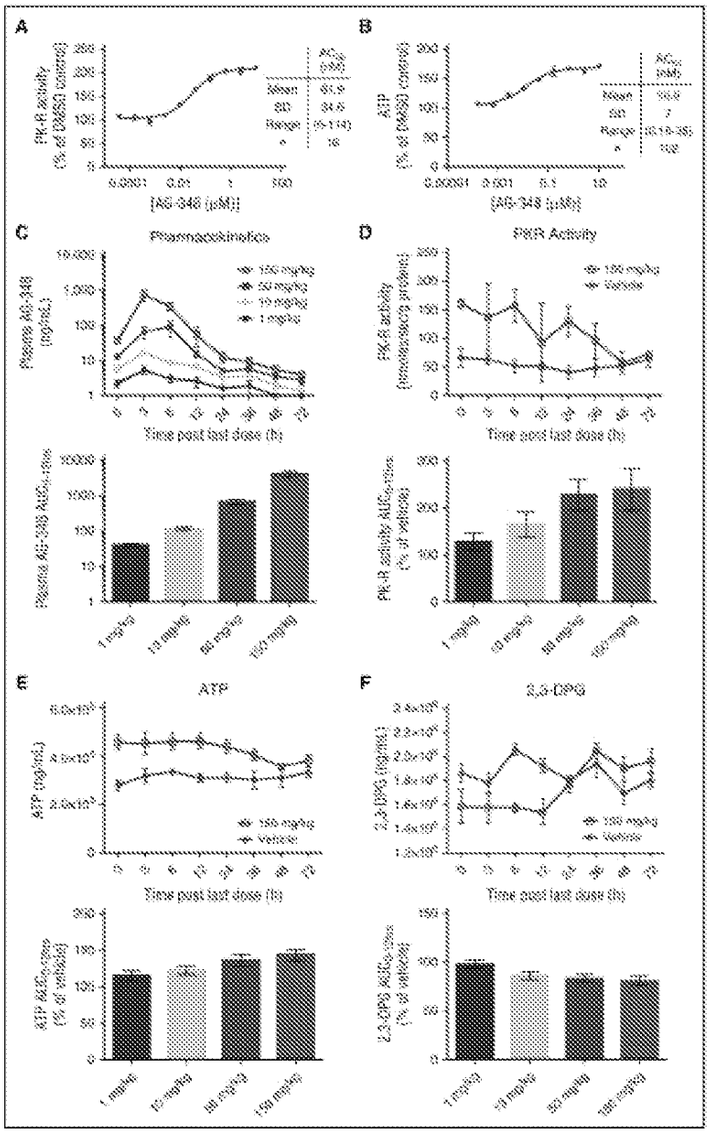
Table 1.

Kinetic parameters from recombinant PK-R enzymes measured with or without treatment with AG-348

| Enzyme | No compound | | | | 5 µM AG-348 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $V_{max}$, µmol/s/mg | PEP $K_M$, mM | $k_{cat}$, 1/s | $k_{cat}/K_M$ | $V_{max}$, µmol/s/mg | PEP $K_M$, mM | $k_{cat}$, 1/s | $k_{cat}/K_M$ | % of WT PK-R $k_{cat}/K_M$ |
| WT | 0.69 | 1.13 | 42.5 | 37.6 | 0.74 | 0.32 | 45.7 | 142.0 | 378 |
| R532W | 0.22 | 1.73 | 13.6 | 7.9 | 0.21 | 0.42 | 12.9 | 30.4 | 81 |
| R510Q | 1.60 | 1.45 | 98.8 | 68.1 | 1.64 | 0.32 | 101.5 | 317.3 | 844 |
| R479H | 0.50 | 1.65 | 30.9 | 18.7 | 0.53 | 0.55 | 32.4 | 58.5 | 156 |
| R486W | 0.34 | 1.45 | 21.2 | 14.6 | 0.39 | 0.25 | 24.2 | 98.4 | 262 |
| G332S | 0.14 | 2.73 | 8.3 | 3.0 | 0.15 | 1.11 | 9.4 | 8.5 | 23 |
| R490W | 0.37 | 1.52 | 22.8 | 15.0 | 0.39 | 0.51 | 24.2 | 47.6 | 127 |
| G364D | 0.16 | 2.14 | 10.1 | 4.7 | 0.22 | 0.55 | 13.5 | 24.7 | 65 |
| T384M | 0.11 | 1.42 | 6.9 | 4.8 | 0.12 | 0.37 | 7.2 | 19.3 | 51 |

$k_{cat}$, first-order rate constant; $k_{cat}/K_M$, measure of enzymatic efficiency[39]; $K_M$, substrate concentration to achieve half maximal velocity; $V_{max}$, maximum velocity.

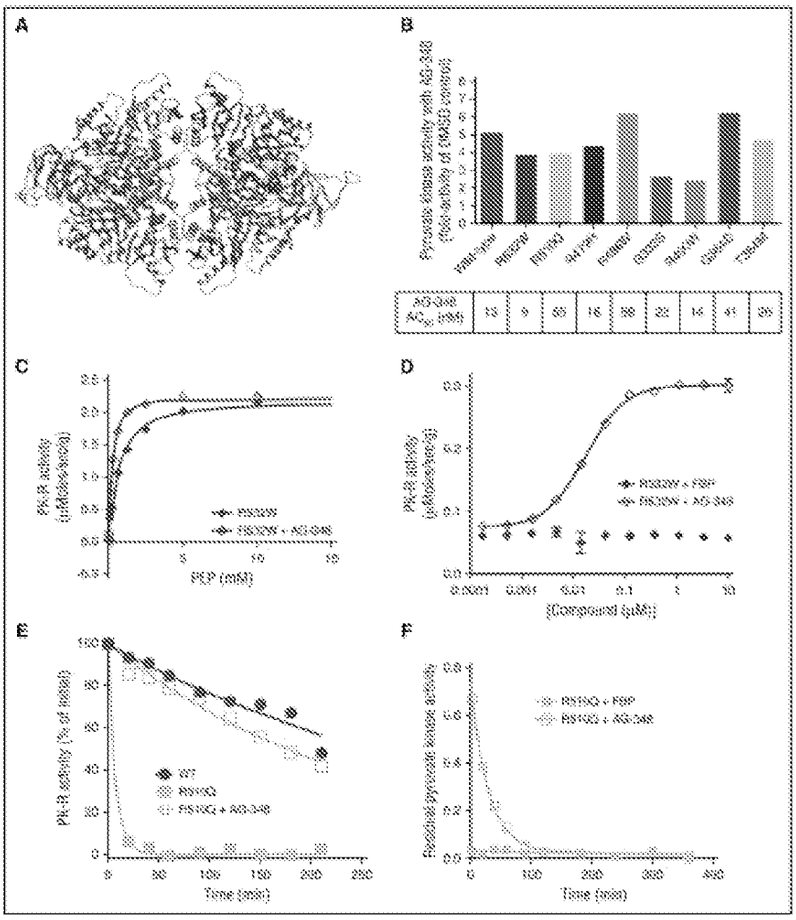
Figure 2.




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**AG-348 activates WT PK-R in RBCs from healthy donors and in mice.** (A) WT PK-R activity from RBCs from healthy donors incubated overnight with indicated concentrations of AG-348 (PEP = 0.1 mM). Mean, standard deviation, range, and number of experimental replicates are indicated in the column on right-hand side of panel. (B) ATP levels of healthy-donor RBCs incubated overnight with indicated concentrations of AG-348. (C) Concentration of AG-348 measured in plasma of mice dosed with AG-348 twice daily for 7 days at indicated dose levels. The bar graph depicts the calculated $AUC_{0\text{-}12\ hours}$ at each dose level. (D) PK-R activity in RBCs from mice dosed with AG-348 as described in panel C (PEP = 0.1 mM). For clarity, only the 150 mg/kg dose level is shown here. See supplemental Figure 2 for full data plots. The bar graph depicts the calculated $AUC_{0\text{-}12\ hours}$ at each dose level. (E) ATP levels and calculated $AUC_{0\text{-}12\ hours}$ in whole blood from mice dosed with AG-348 as described in panel D. (F) 2,3-DPG levels and calculated $AUC_{0\text{-}12\ hours}$ in whole blood from mice dosed with AG-348 as described in panel D. For clarity, only the 150 mg/kg dose level is shown in panels E and F. All error bars are standard deviations.

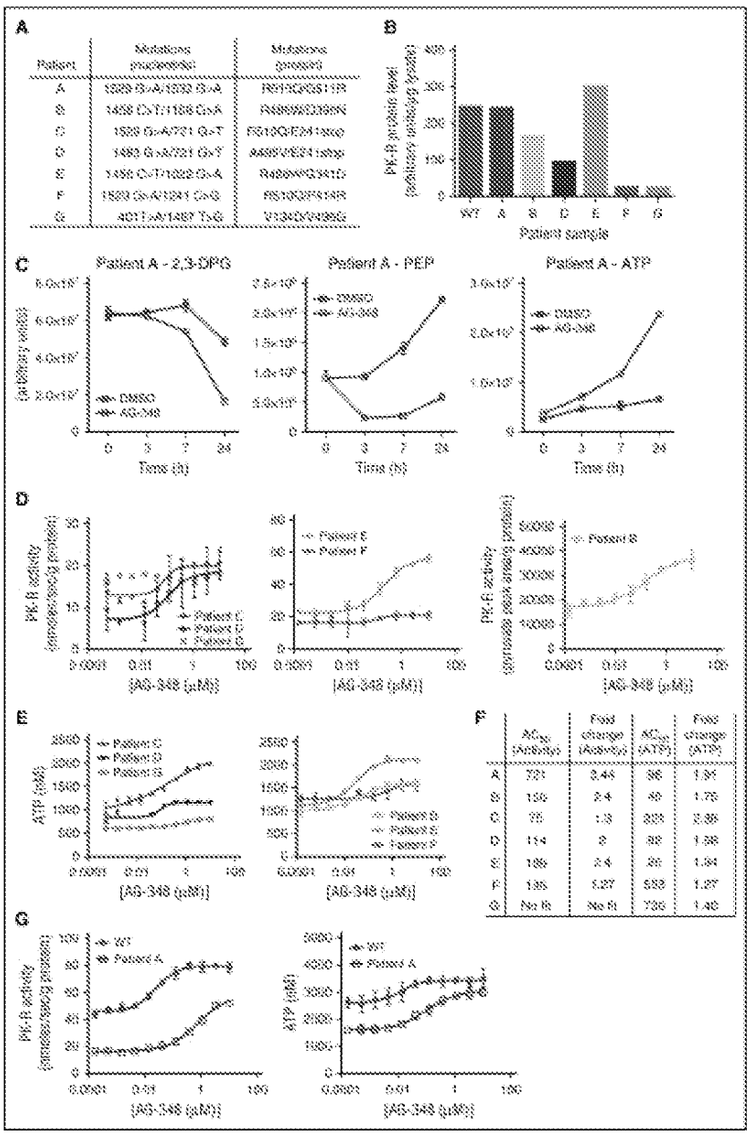
Figure 3.




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**AG-348 activates a spectrum of recombinant mtPK-R enzymes.** (A) PK-R tetramer with sites of mtPK-R enzymes tested in Figure 3B highlighted in red. For clarity, each mutation site is only shown in a single monomer. (B) Fold activation and $AC_{50}$ values of AG-348 treatment of mtPK-R enzymes (AG-348 = 10 μM, PEP, as listed in the supplemental table). (C) Activity of recombinant R532W mtPK-R enzyme stimulated with PEP with or without preincubation with AG-348 (AG-348 = 5 μM). (D) Activity of recombinant R532W mtPK-R enzyme incubated with indicated concentration of FBP or AG-348 (PEP = 0.05 mM). Panels C and D show the average of 3 technical replicates. (E) Residual activity over time of WT or R510Q recombinant enzymes following incubation at 53°C (AG-348 = 5 μM, PEP = 2 mM). (F) Off-rate measurement of AG-348 or FBP (both at 5 μM final assay concentration, PEP = 2 mM) from recombinant R510Q enzyme. All error bars are standard deviations.

Figure 4.




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**AG-348 activation of mtPK-R in RBCs from patients with PK deficiency.** (A) Genotypes of patients with PK deficiency described in these studies. (B) PK-R protein levels measured in patients with PK deficiency or WT controls. Data were not available from this assay for patient C, but a Western blot showing approximate protein levels in this patient is shown in supplemental Figure 6C. (C) Levels of 2,3-DPG, PEP, and ATP in RBCs from patient A at indicated times following treatment with DMSO or AG-348. (D) PK-R activity in PK-deficient RBCs incubated with AG-348 for 24 h (PEP = 0.5 mM). All PK-R activity measurements used the coupled enzyme spectrometric assay, except in the case of patient B, for whom activity was assessed by direct measurement of pyruvate formation using liquid chromatography followed by tandem mass spectrometry as described in the supplemental Methods. (E) ATP levels in PK-deficient RBCs incubated with AG-348 for 24 h. For panels D and E, please note that patient samples E and F were received via overnight courier post–blood draw, whereas other patient samples were received on the same day as blood draw, but potentially up to 8 h post–blood draw (supplemental Figure 5B-C). (F) Table showing AG-348 $AC_{50}$ and fold-change observed in PK-R activity and

ATP levels from indicated patient samples. $AC_{50}$ values shown are in nanomolar. Fold-change (in relation to DMSO control) PK-R activity and ATP graphs are shown in supplemental Figure 6B. (G) PK-R activity (left) and ATP levels (right) in RBCs from patient A or a WT control (both prepared ~30 min post–blood draw), incubated with AG-348 for 24 h. Panels D, E, and G show the average of 3 technical replicates. All error bars are standard deviations.

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# AG-348 Activation of Pyruvate Kinase in Vivo Enhances Red Cell Glycolysis in Mice

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Pyruvate kinase deficiency (PKD) is an autosomal recessive enzymopathy that is the most common cause of hereditary nonspherocytic hemolytic anemia (HNSHA). PKD is a rare disease characterized by a life-long chronic hemolysis with severe co-morbidities. It is hypothesized that insufficient energy production to maintain red cell membrane homeostasis promotes the chronic hemolysis. Treatment is generally palliative, focusing on the resultant anemia, and there are no approved drugs that directly target mutated pyruvate kinase.

AG-348 is an allosteric activator of the red cell isoform of pyruvate kinase (PKR) that has recently entered Phase I clinical trials in normal healthy volunteers. AG-348 increases the catalytic efficiency and enhances the protein stability of a spectrum of recombinantly expressed PKR mutant proteins that have been associated with PKD. PKD red cells are characterized by changes in metabolism associated with defective glycolysis, including a build-up of the upstream glycolytic intermediate 2,3-DPG and deficiency in the PKR product adenosine triphosphate (ATP). PKR flux, e.g. the rate of carbon flow through the PKR enzyme reaction, was examined in PKD patient or wild type (WT) donor blood samples by incubation of whole blood with a stable isotope tracer, [U-13C6]-glucose. At various time points after the addition of [U-13C6]-glucose, metabolism was quenched and metabolites were extracted. Metabolite pool sizes and 13C label incorporation into glycolytic intermediates were monitored by LC/MS. The rate of label incorporation was found to be significantly slower in PKD patient red cells, consistent with decreased glycolytic activity. Treatment of PKD red cells with AG-348 ex-vivo induces changes in metabolism consistent with increased glycolytic activity including reduced 2,3-DPG levels, increased ATP levels, and increased PKR enzyme activity levels.

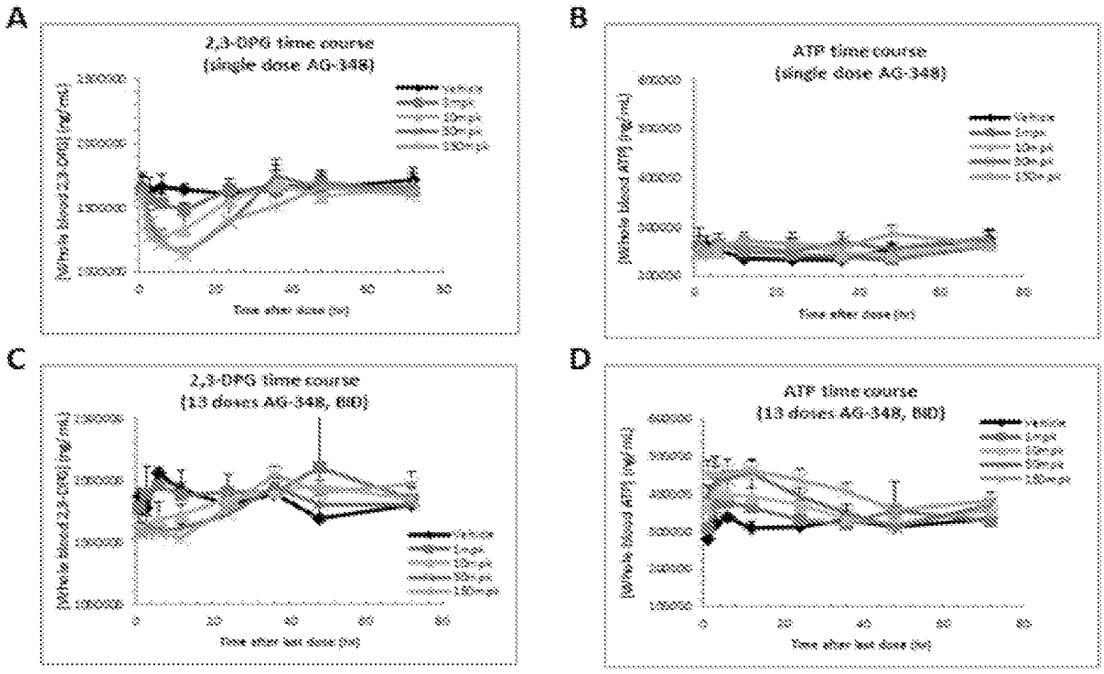
The effect of AG-348 on red cell metabolism in vivo was evaluated in mice. C57/BL6 mice were dosed by oral gavage either with a single dose, or with multiple doses (BID) of AG-348 for 7 days. Dose levels tested were 1 mpk, 10 mpk, 50 mpk, and 150 mpk. Following the last dose, mice were bled to evaluate drug exposure and pharmacodynamic markers including 2,3-DPG and ATP levels, and PKR activity. AG-348 was demonstrated to be a well-behaved compound, with dose-proportional increase in exposure, both in the single-dose and multiple dose studies. A single dose of AG-348 resulted in a dose-dependent increase in PKR activity levels, concomitant with reduction in 2,3-DPG levels. There were no significant changes in ATP levels after a single administration of AG-348. In the multiple-dose studies, similar changes in PKR activity and 2,3-DPG levels were observed. In contrast to the single-dose study, ATP levels were observed to be robustly increased in a dose-dependent manner.

The effect of AG-348 on PKR flux was assessed in whole blood from mice treated with AG-348. C57BL/6 mice were dosed by oral gavage with AG-348 (150 mg/kg twice daily [BID]) for 3 days. Whole blood was incubated with [U-13C6]-glucose and the metabolite pool sizes and rate of 13C label incorporation into glycolytic intermediates were assessed. The data were subsequently analyzed using a mathematical model to quantify flux through the PKR reaction and it was determined that AG-348 treatment significantly increased flux through the PKR reaction.

Collectively, these data demonstrate that AG-348 not only potently binds to and activates the PKR enzyme in vivo, but this enzyme activation induces enhanced glycolytic pathway activity in red cells that results in profound changes in cellular metabolism, as reflected in dramatically increased ATP levels and reduced 2,3-DPG levels. As AG-348 has similar potency against the WT PKR enzyme as against tested mutant PKR enzymes in vitro, these data support the hypothesis that AG-348 treatment may similarly enhance glycolytic activity in PKD patients and thus correct the underlying pathology of PKD.



Ex. 2001-0886




**Legend: A)** 2,3-DPG levels in whole blood from C57/BL6 mice treated with a single dose of AG-348 **B)** ATP levels in whole blood from mice treated as in **A** **C)** C57/BL6 mice were dosed with AG-348 for 7 days, BID (13 total doses). 2,3-DPG levels were measured in whole blood. **D)** ATP levels following the last dose in whole blood from mice treated as in **C**. Error bars shown are standard deviation.



Ex. 2001-0887

# Genotype-response correlation in DRIVE PK, a phase 2 study of AG-348 in patients with pyruvate kinase deficiency

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## INTRODUCTION

- Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by mutations in the *PKLR* gene, leading to a deficiency of the glycolytic enzyme red cell PK (PK-R) (**Figure 1**).[1,2]
- AG-348 is an orally available small-molecule allosteric activator of PK-R that activates the wild type (WT) and a range of mutant PK-R enzymes associated with PK deficiency (**Figure 2**).[3,4]
- Increased PK-R activity and ATP levels in patient red blood cells treated with AG-348 *ex vivo* may be linked to *PKLR* genotype and/or PK-R protein level (**Figure 3**).[4]
- In a phase 2 clinical study of patients with PK deficiency (DRIVE PK; NCT02476916), 26 of 52 patients (50%) experienced a maximum Hb increase of >1.0 g/dL (mean maximum increase, 3.4 g/dL; range, 1.1–5.8 g/dL), including 25 of 42 patients (59.5%) with at least one missense mutation (**Figure 4**).[5]
  - In most cases, Hb increases were rapid and sustained, and seen across a wide dose range from 5 to 300 mg twice daily (BID) (**Figure 5**).
  - Hemolysis markers (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who experienced a maximum Hb increase of >1.0 g/dL.
  - Hb increases were observed in patients with a variety of *PKLR* mutations, and increases were associated with the presence of at least one missense mutation.
- Because PK deficiency is a genetically heterogeneous disease, with over 200 described mutations, we sought to understand in greater detail the molecular parameters associated with Hb increases in patients treated with AG-348.

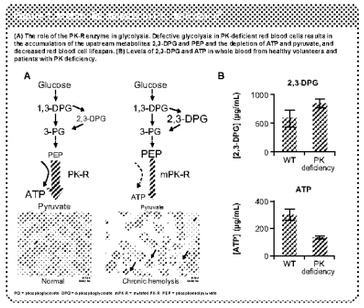
## OBJECTIVE

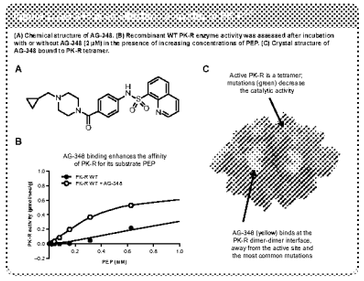
- To analyze the relationship between Hb increase and patient genotype, biochemical response to AG-348 treatment, and baseline PK-R protein level.

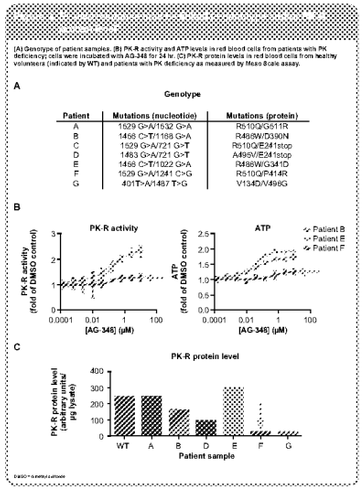
## METHODS

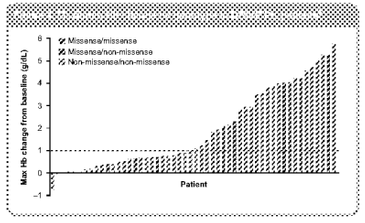
- Whole blood samples were collected from patients with PK deficiency enrolled in the phase 2 DRIVE PK study.
- Patient genotypes were determined by Centogene AG (http://www.centogene.com).
- Levels of PK-R protein were quantitated using a Meso Scale assay as described previously (antibodies from Abcam, Cambridge, UK [ab89071] and Aviva Systems Biology, London, UK [OAGA00912]).[4]
  - The signal was normalized to a reference control sample from a subject without PK deficiency.
  - For PK-R protein-level testing, the sample was obtained on Day 0 prior to the initiation of AG-348 treatment, except in a single patient for whom the sample from Day 15 was used.
- Patient consent was received for all testing procedures.

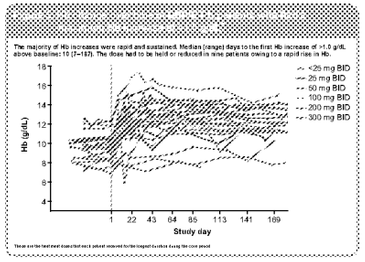
## RESULTS

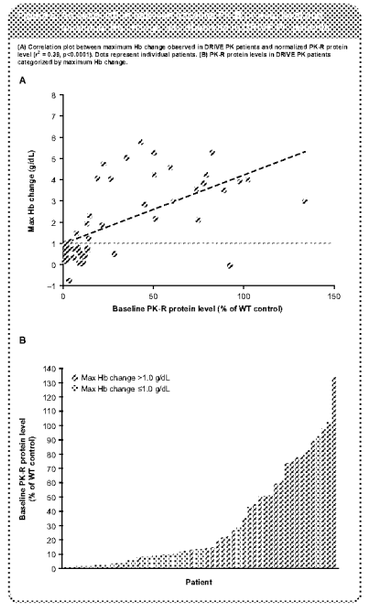


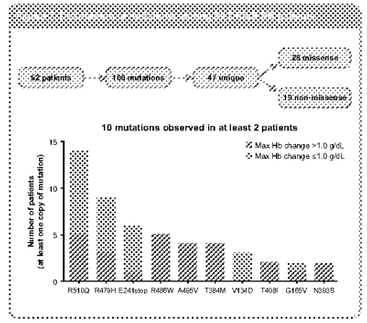



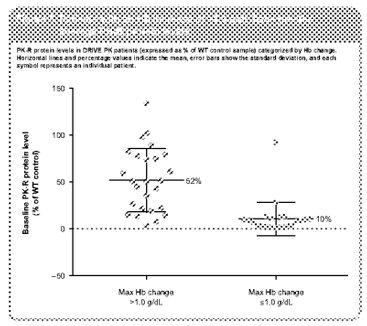



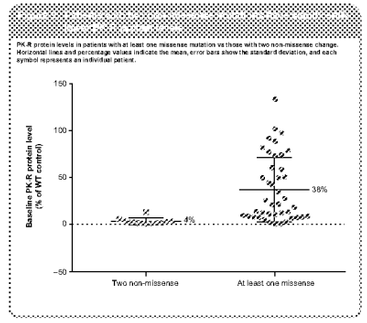



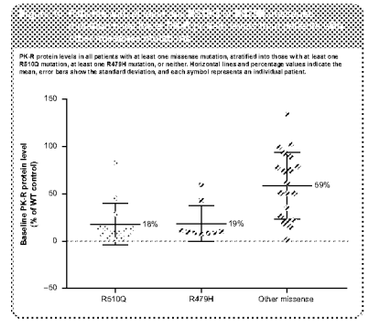




## SUMMARY AND CONCLUSIONS

- A statistically significant correlation was observed between baseline PK-R protein level and Hb increases in patients with PK deficiency treated with AG-348.
- This correlation is evidence that AG-348 is working via its proposed mechanism of action of stimulating the residual activity of the mutant enzyme.
- Although neither genotype nor PK-R protein level could predict Hb increases with absolute precision, some trends were observed:
  - Patients with two non-missense mutations had lower protein levels than those with at least one missense mutation.
  - Patients with R479H or R510Q mutations had lower protein levels than patients with other missense mutations.
- These preliminary findings will be examined further in the ongoing phase 3 studies of AG-348 (NCT03548220 and NCT03559699).

### Acknowledgments

We would like to thank the patients taking part in this study.

### Disclosures

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CK, PAK, HM, LH, GC, MM, KS, M-HJ, and CB: Agios – employment and stockholder. RFG: Agios – advisory board and research funding. BG: Agios – advisory board.

Editorial assistance was provided by Susanne Vidot, PhD, Excel Medical Affairs, Horsham, UK, and supported by Agios.

### References

1. Grace RF et al. *Am J Hematol* 2015;90:825-30.
2. Grace RF et al. *Blood* 2018;131:2183-92.
3. Kung C et al. *55th ASH Annual Meeting* 2013: Abstr 2180.
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Presented at the 60th American Society of Hematology (ASH) Annual Meeting, December 1–4, 2018, San Diego, CA, USA

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# Genotype–Response Correlation in DRIVE PK, a Phase 2 Study of AG-348 in Patients with Pyruvate Kinase Deficiency

Charles Kung, Penelope A.KosinskiHeidiMangusLeiHua, Gary Connor, Michelle Mobilia, Karen Sullivan, Sasha Frye, Marie-Hélène Jouvin, Rachael F. Grace, Bertil Glader, and Chris Bowden

Blood 2018 132:3621; doi: https://doi.org/10.1182/blood-2018-99-111166

Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by mutations in the *PKLR* gene, leading to a deficiency of the glycolytic enzyme red cell PK (PK-R). AG-348 is an orally available small-molecule allosteric activator of PK-R that activates the wild type and a range of mutant PK-R enzymes associated with PK deficiency (Kung et al. *Blood* 2017;130:1347-56).

In a phase 2 clinical study in patients with PK deficiency (DRIVE PK, NCT02476916), 26 of 52 patients (50%) experienced a maximum hemoglobin (Hb) increase of >1.0 g/dL (mean maximum increase, 3.4 g/dL; range, 1.1-5.8 g/dL). In most cases, Hb increases were rapid and sustained, and seen across a wide dose range from 5 to 300 mg twice daily. Hemolysis markers (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who experienced a maximum Hb increase of >1.0 g/dL. Hb increases were observed in patients with a variety of *PKLR* mutations and were associated with the presence of at least 1 missense mutation.

As PK deficiency is a genetically heterogeneous disease, with over 200 described mutations, we sought to understand in greater detail the molecular parameters that were associated with Hb increases in AG-348-treated patients. Here, we have analyzed the relationship between Hb increase and patient genotype, biochemical response to AG-348 treatment, and baseline PK-R protein level. The results are consistent with the proposed mechanism of action of
AG-348 through binding and activation of residual mutant PK-R enzyme.

Of the 106 *PKLR* mutations in the 52 subjects (2 subjects carried 3 mutations), there were 47 unique mutations, including 28 missense and 19 non-missense. Of these mutations, only 10 were present in at least 2 patients, highlighting the intrinsic limitations of findings from this small sample size. That notwithstanding, among these 10 mutations, we observed that some were more likely to be associated with Hb increases (e.g. increases were observed in 5/5 patients with at least 1 R486W mutation and 4/4 patients with at least 1 T384M mutation). Other mutations, including V134D (0/3), E241stop (1/6), and R510Q (5/14), were associated with a lack of Hb increase. Notably, 1/10 patients who had 2 non-missense mutations and 0/5 patients who were homozygous for the R479H "Amish" mutation had Hb increases.

We have previously reported that, in biochemical assays, AG-348 could efficiently activate recombinantly expressed R479H and R510Q mutant enzymes. Although both of these are technically missense mutations, it is well understood that R510Q results in severely decreased protein stability (Wang et al. *Blood* 2001;98:3113-20), and the R479H mutation, occurring at the splice junction between 2 exons, can deleteriously affect mRNA splicing

(van Wijk et al.*Br J Haematol* 2004;125:253-63). Therefore, as AG-348 works by directly binding and stimulating the activity of residual mutant PK-R enzyme, we explored the hypothesis that PK-R protein levels in patients with these mutations might be too low to allow for adequate target engagement at clinically tested doses.

Using antibody-based capture and detection, we quantitated baseline PK-R protein levels of DRIVE PK subjects. Analysis of these data revealed that patients with Hb increases had, on average, higher PK-R protein levels (49% of reference control compared to 13% in patients without Hb increases). All 10 patients with non-missense/non-missense mutations had nearly undetectable PK-R protein levels (average of 3.7% of reference control), consistent with and confirming the predicted effect of these mutations on protein expression. Baseline PK-R protein levels were also lower in patients with at least 1 R510Q (18% of reference control) or R479H (19% of reference control) mutation compared to patients with other missense mutations (59% of reference control).

Taken together, these analyses demonstrate that Hb increases upon AG-348 treatment are associated with the presence of at least 1 missense PK-R mutant enzyme with residual protein for AG-348 to bind and activate. These data strongly suggest that the hematological effects of AG-348 come from the proposed mechanism of action of restoration of glycolytic pathway activity in PK-deficient red cells. The genetic complexity of PK deficiency, combined with the relatively small sample size, limit extrapolation of these findings to the entire PK deficiency patient population, but may help to inform future studies.

Disclosures

**Kung**:*Agios:* Employment, Equity Ownership. **Kosinski**:*Agios:* Employment, Equity Ownership. **Mangus**: *Agios:* Employment, Equity Ownership. **Hua**:*Agios:* Employment, Equity Ownership. **Connor**:*Agios:* Employment, Equity Ownership. **Mobilia**:*Agios:* Employment, Equity Ownership. **Sullivan**:*Agios* : Employment, Equity Ownership. **Frye**: *Agios* : Employment, Equity Ownership. **Jouvin**:*Agios* : Employment, Equity Ownership. **Grace**:*Agios Pharmaceuticals* : Membership on an entity's Board of Directors or advisory committees;*Agios Pharmaceuticals* : Research Funding;*Agios Pharmaceuticals* : Consultancy. **Glader**:*Agios* : Consultancy, Research Funding. **Bowden**: *Agios* : Employment, Equity Ownership.

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# Genotype-response correlation in DRIVE PK, a phase 2 study of mitapivat (AG-348) in patients with pyruvate kinase deficiency

Charles Kung[1], Penelope A Kosinski[1], Heidi Mangum[1], Lei Hua[1], Michelle Mobilia[1], Karen Sullivan[1], Marie-Hélène Jouvin[1], Rachael F Grace[2], Bertil Glader[3], Chris Bowden[1]

[1]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [2]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA; [3]Stanford University School of Medicine, Palo Alto, CA, USA

## INTRODUCTION

- Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by mutations in the *PKLR* gene, leading to a deficiency of the glycolytic enzyme red cell PK (PK-R) (**Figure 1**).[1,2]
- Mitapivat (AG-348) is an orally available small-molecule allosteric activator of PK-R that activates the wild type (WT) and a range of mutant PK-R enzymes associated with PK deficiency (**Figure 2**).[3,4]
- Increased PK-R activity and ATP levels in patient red blood cells treated with mitapivat *ex vivo* may be linked to *PKLR* genotype and/or PK-R protein level (**Figure 3**).[4]
- In a phase 2 clinical study of patients with PK deficiency (DRIVE PK, NCT02476916), 26 of 52 patients (50%) experienced a maximum hemoglobin (Hb) increase of >1.0 g/dL (mean maximum increase, 3.4 g/dL; range, 1.1–5.8 g/dL), including 25 of 42 patients (59.5%) with at least one missense mutation (**Figure 4**).[5]
  - In most cases, Hb increases were rapid and sustained, and seen across a wide dose range from 5 to 300 mg twice daily (BID) (**Figure 5**).
  - Hemolysis markers (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who experienced a maximum Hb increase of >1.0 g/dL.
  - Hb increases were observed in patients with a variety of *PKLR* mutations, and increases were associated with the presence of at least one missense mutation.
- Because PK deficiency is a genetically heterogeneous disease, with >200 described mutations, we sought to understand in greater detail the molecular parameters associated with Hb increases in patients treated with mitapivat.

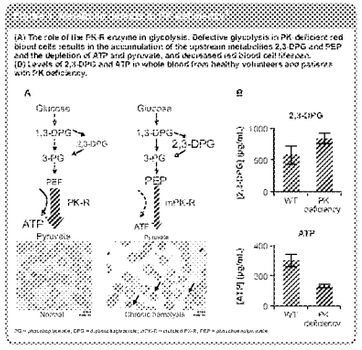
## OBJECTIVE

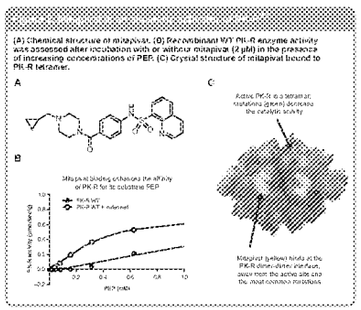
- To analyze the relationship between Hb increase and patient genotype, biochemical response to mitapivat treatment, and baseline PK-R protein level.

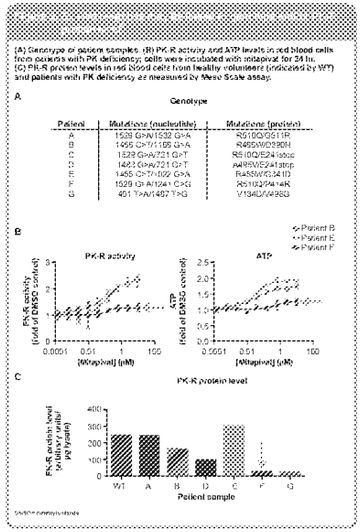
## METHODS

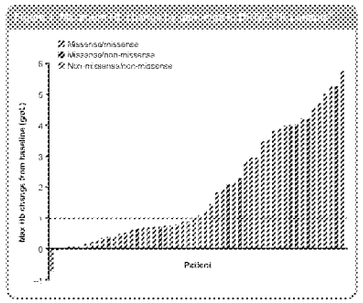
- Whole blood samples were collected from patients with PK deficiency enrolled in the phase 2 DRIVE PK study.
- Patient genotypes were determined by Centogene AG (*http://www.centogene.com*).
- Levels of PK-R protein were quantitated using a Meso Scale assay as described previously (antibodies from Abcam, Cambridge, UK [ab89071] and Aviva Systems Biology, London, UK [OAGA00812]).[4]
  - The signal was normalized to a reference control sample from a subject without PK deficiency.
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- Patient consent was received for all testing procedures.

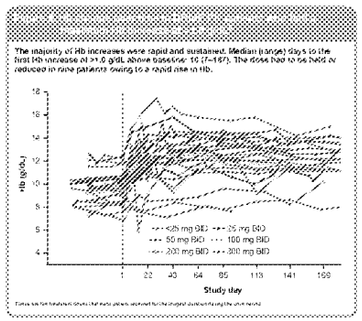
## RESULTS

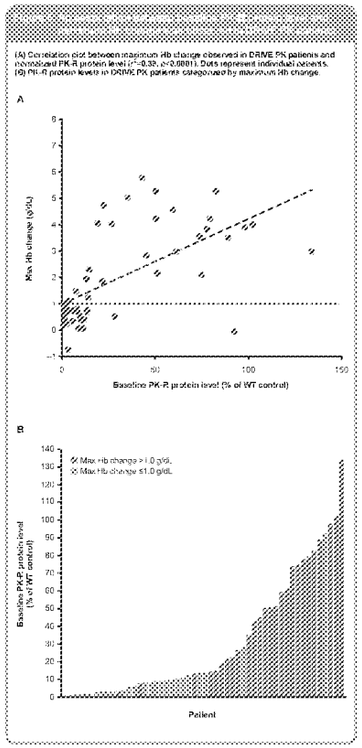


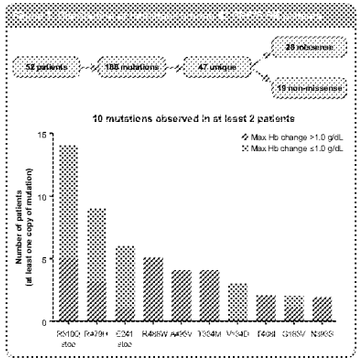



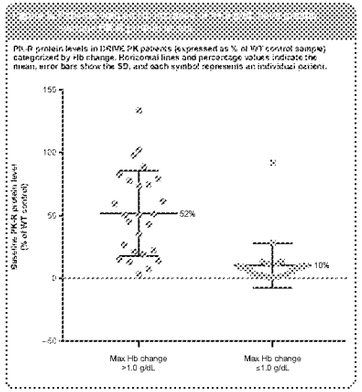



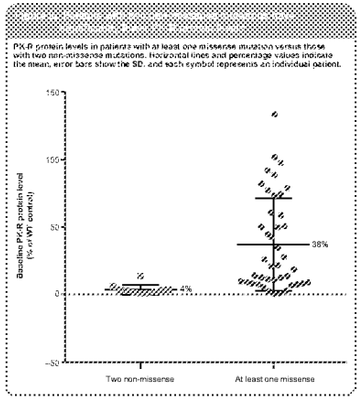



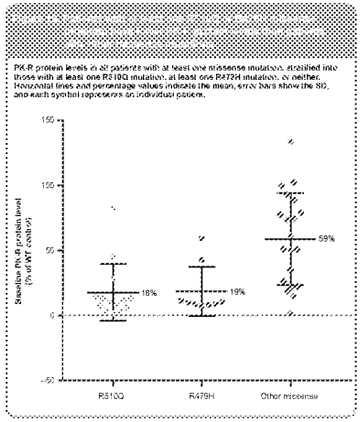




## SUMMARY AND CONCLUSIONS

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- This correlation is evidence that mitapivat is working via its proposed mechanism of action of stimulating the residual activity of the mutant enzyme.
- Although neither genotype nor PK-R protein level could predict Hb increases with absolute precision, some trends were observed:
  - Patients with two non-missense mutations had lower protein levels than those with at least one missense mutation.
  - Patients with R479H or R510Q mutations had lower protein levels than patients with other missense mutations.
- These preliminary findings will be examined further in the ongoing phase 3 studies of mitapivat (NCT03548220 and NCT03559699).

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Editorial assistance was provided by Christine Ingleby, PhD, CMPP, Excel Medical Affairs, Horsham, UK, and supported by Agios.

*References*

1. Grace RF et al. *Am J Hematol* 2015;90:825-30
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Presented at the 24th Congress of the European Hematology Association (EHA), June 13–16, 2019; Amsterdam, the Netherlands

Ex. 2001-0891

# Abstract Submission

***28. Enzymopathies, membranopathies and other anemias***

EHA-2087

## GENOTYPE-RESPONSE CORRELATION IN DRIVE PK, A PHASE 2 STUDY OF MITAPIVAT (AG-348) IN PATIENTS WITH PYRUVATE KINASE DEFICIENCY

Charles Kung* [1], Penelope A. Kosinski[1], Heidi Mangus[1], Lei Hua[1], Michelle Mobilia[1], Karen Sullivan[1], Marie-Hélène Jouvin [1], Rachael F. Grace[2], Bertil Glader[3], Chris Bowden[1]

[1]Agios Pharmaceuticals, Inc., Cambridge, [2]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, [3] Stanford University School of Medicine, Palo Alto, United States

**Does the study abide by applicable national and international regulations and guidelines, including but not limited to ethical committees, data protection and privacy regulations, informed consent and off-label use of drugs?:** Yes

**Background:** Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by mutations in the *PKLR* gene, leading to a deficiency of the glycolytic enzyme red cell PK (PK-R). Mitapivat (AG-348) is an oral, small-molecule, allosteric activator of PK-R that activates the wild type and a range of mutant PK-R enzymes associated with PK deficiency. In a phase 2 study in 52 patients (pts) with PK deficiency (DRIVE PK, NCT02476916), 50% of pts experienced a maximum hemoglobin (Hb) increase of >1.0 g/dL (mean maximum increase, 3.4 g/dL; range, 1.1–5.8 g/dL). Hb increases were observed in patients with a variety of *PKLR* mutations and were associated with the presence of ≥1 missense mutation.

As PK deficiency is genetically heterogeneous (>200 described mutations), we sought to better understand the molecular parameters associated with Hb increases in mitapivat-treated pts.

**Aims:** To analyze the relationship between Hb increase and pt genotype, biochemical response to mitapivat, and baseline PK-R protein level.

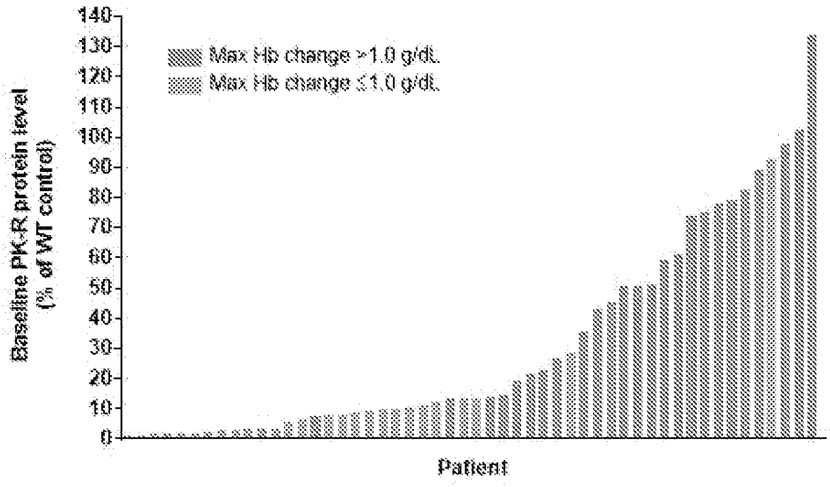
**Methods:** Whole blood samples were collected from pts enrolled in DRIVE PK. Genotypes were determined by Centogene AG. Baseline PK-R protein was quantitated by antibody-based capture and detection using a Meso Scale assay and the signal normalized to a control sample from a subject without PK deficiency.

**Results:** Of 106 *PKLR* mutations in the 52 pts (2 pts carried 3 mutations), there were 47 unique mutations, including 28 missense and 19 non-missense. Of these, only 10 were present in ≥2 pts. Of these 10 mutations, some were more likely to be associated with Hb increases (e.g. increases were observed in 5/5 pts with ≥1 R486W mutation and 4/4 pts with ≥1 T384M mutation). Other mutations, including V134D (0/3), E241stop (1/6), and R510Q (5/14), were more likely to be associated with a lack of Hb increase. Notably, 1/10 pts who had 2 non-missense mutations and 0/5 pts homozygous for the R479H "Amish" mutation had Hb increases. We have previously reported that, in biochemical assays, mitapivat could efficiently activate recombinantly expressed R479H and R510Q mutant enzymes. Although both of these are technically missense mutations, it is well understood that R510Q results in severely decreased protein stability, and the R479H mutation, occurring at the splice junction between 2 exons, can deleteriously affect mRNA splicing. Therefore, as mitapivat works by directly binding and stimulating the activity of residual mutant PK-R enzyme, we explored the hypothesis that PK-R protein levels in pts with these mutations might be too low to allow for adequate target engagement at clinically tested doses.

Pts with Hb increases had, on average, higher baseline PK-R protein levels (49% of control vs 13% in pts without Hb increases). All 10 pts with non-missense/non-missense mutations had nearly undetectable PK-R protein levels (average of 3.7% of control), consistent with and confirming the predicted effect of these mutations on protein expression. Baseline PK-R protein levels were also lower in pts with ≥1 R510Q (18% of control) or R479H (19% of control) mutation than in pts with other missense mutations (59% of control).

**Image/Pictures:**

Figure. An association between baseline PK-R protein level and maximum Hb change was observed in DRIVE PK patients




**Summary/Conclusion:** These analyses demonstrate that Hb increases upon mitapivat treatment are associated with the presence of ≥1 missense PK-R mutant enzyme with residual protein for mitapivat to bind and activate. These data strongly suggest that the hematologic effects of mitapivat come from the proposed mechanism of action of restoration of glycolytic pathway activity in PK-deficient red cells.

**Keywords**: Clinical trial, Genotype, Hemolytic anemia, Red cell



# Mechanism of action of the pyruvate kinase activator mitapivat (AG-348) in hematological disease

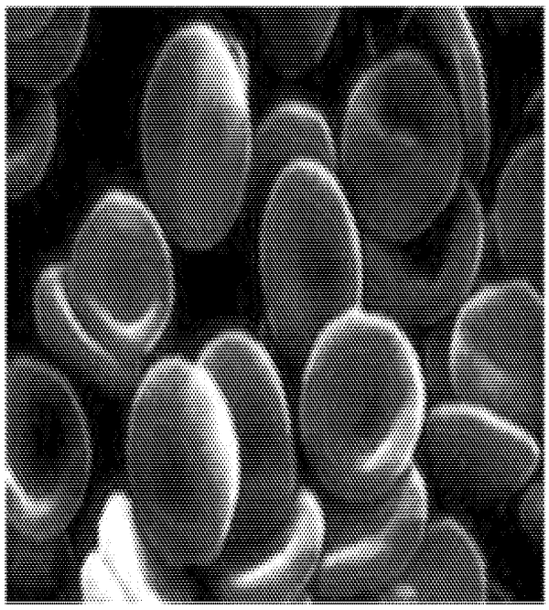
## Red Cells Gordon Conference

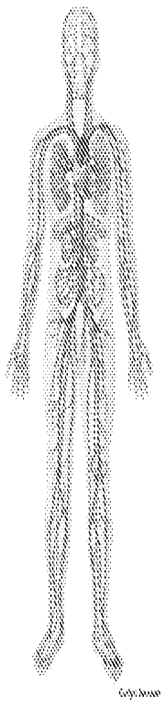
7-17-2019

Charles Kung
Agios Pharmaceuticals

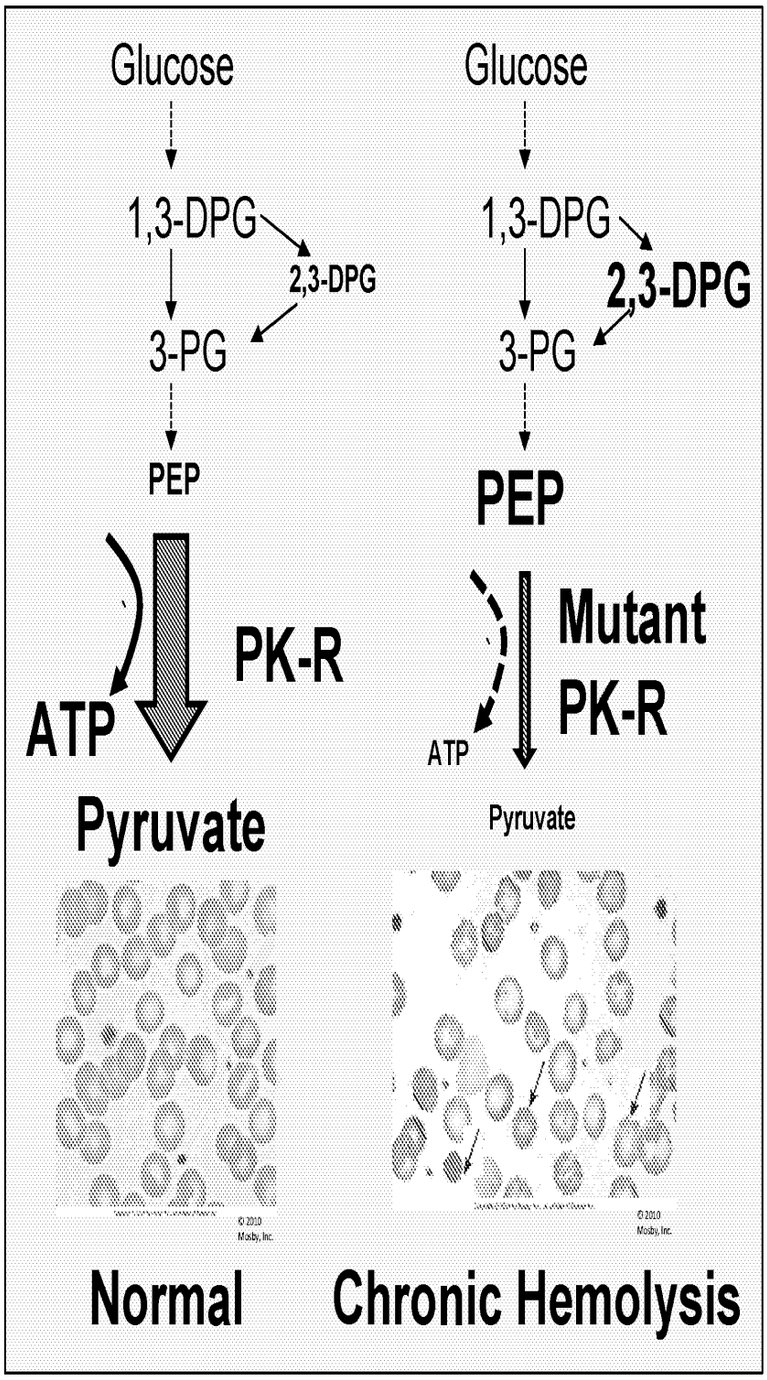
# The red blood cell is a highly specialized cell type that relies on glycolysis for energy production

- Loaded with hemoglobin to deliver oxygen to body organs
  - Lacks a nucleus, DNA, RNA, and ribosomes (no new protein synthesis)
  - Has no mitochondria (reticulocytes have some residual)
- Erythrocytes must maintain an adequate ATP supply **through glycolysis**
- After survival for ~120 days in circulation, normally engulfed by macrophages in spleen

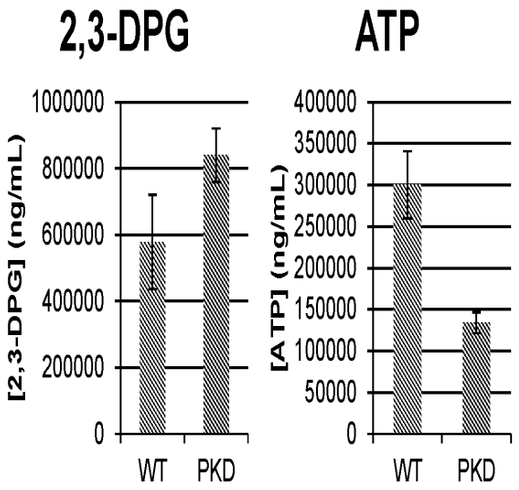




# PK deficiency is caused by defective glycolysis in red blood cells




**Metabolite levels measured in human whole blood samples**




WT n = 96

PK deficiency n = 7

Kung et al., ASH 2014
Abstract #4010

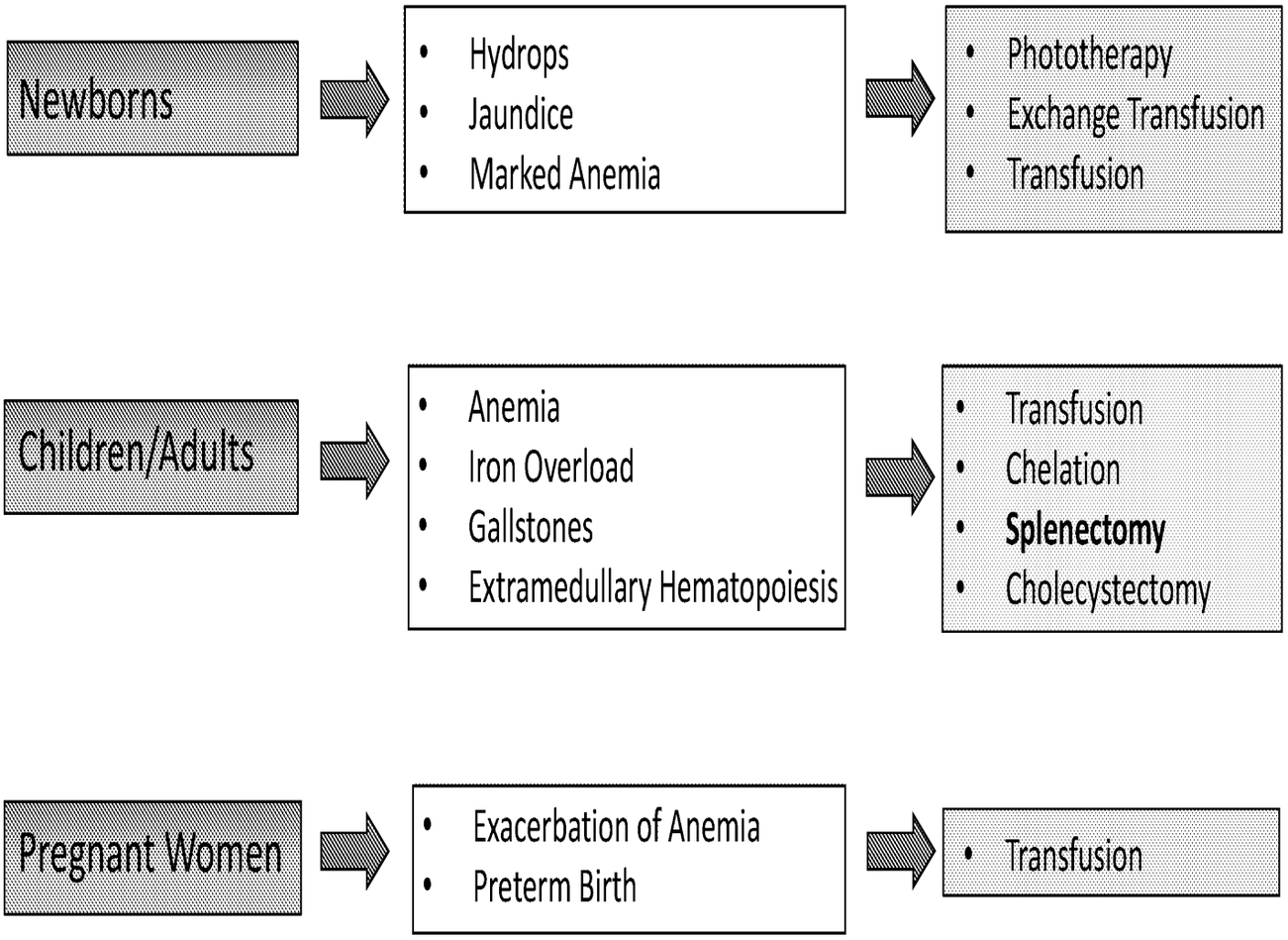


Ex. 2001-0896

# Pyruvate kinase deficiency

## Clinical Features and Treatment

Hemolytic anemia of variable severity with reticulocytosis



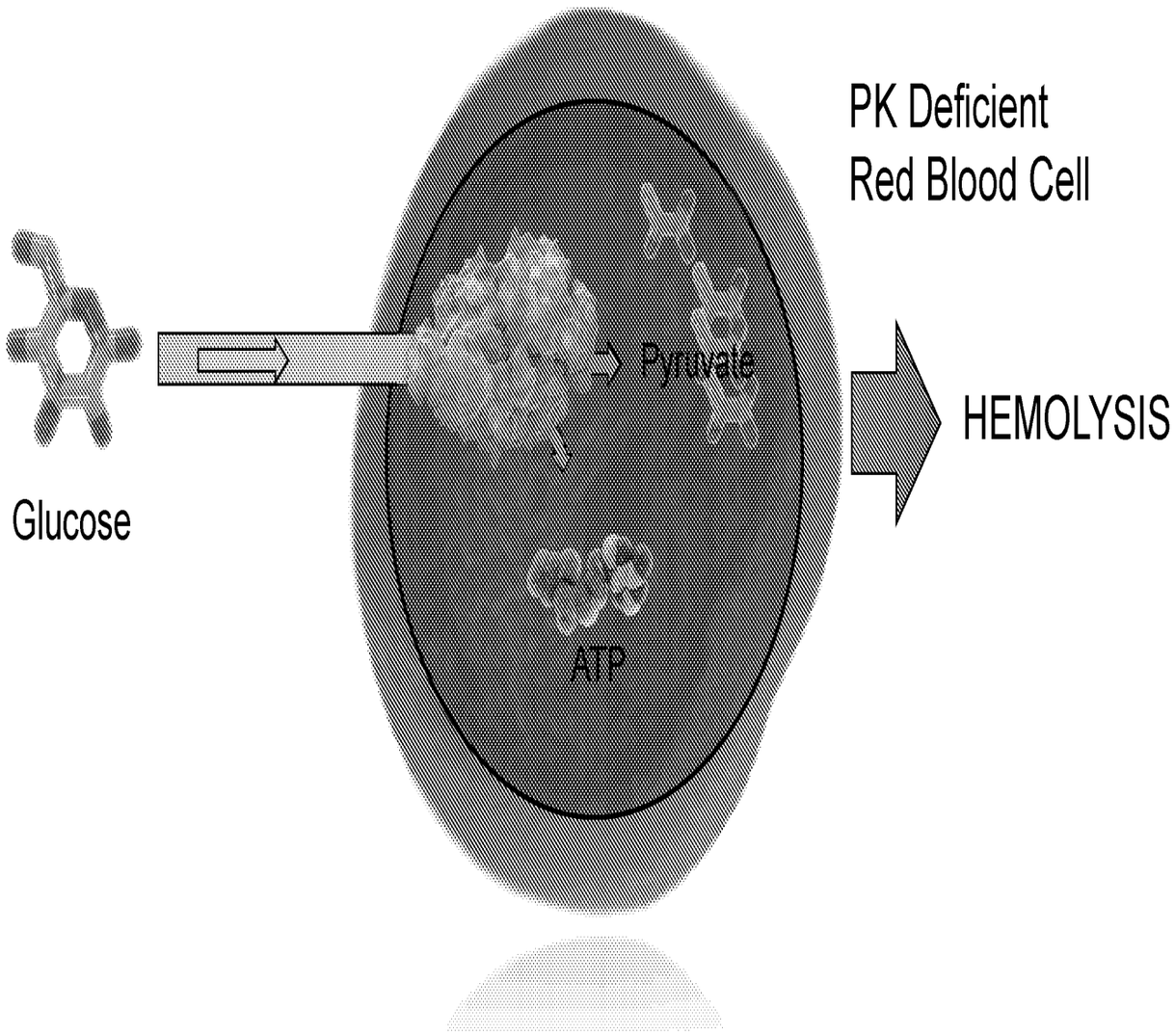

Zanella et al. *Blood Rev* 2007;21:217



Ex. 2001-0897

# Therapeutic Approach to Treating PK Deficiency

## Allosteric modulators of PK-R to treat PK deficiency

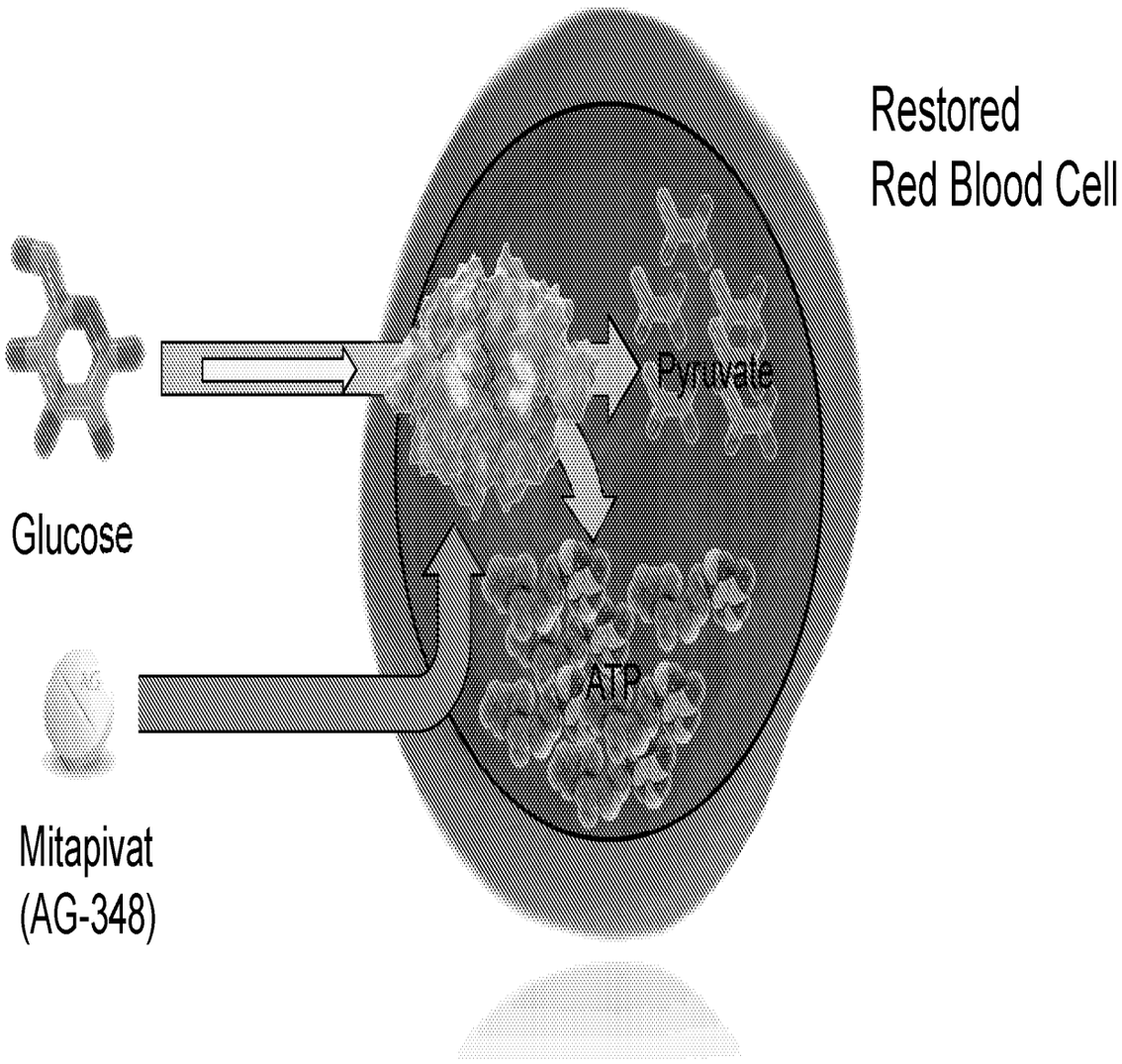





Ex. 2001-0898

Therapeutic Approach to Treating PK Deficiency

# Allosteric modulators of PK-R to treat PK deficiency

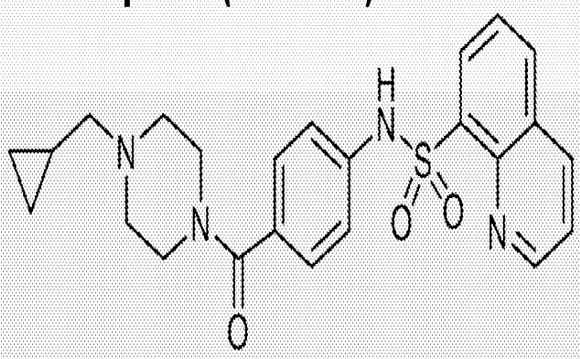


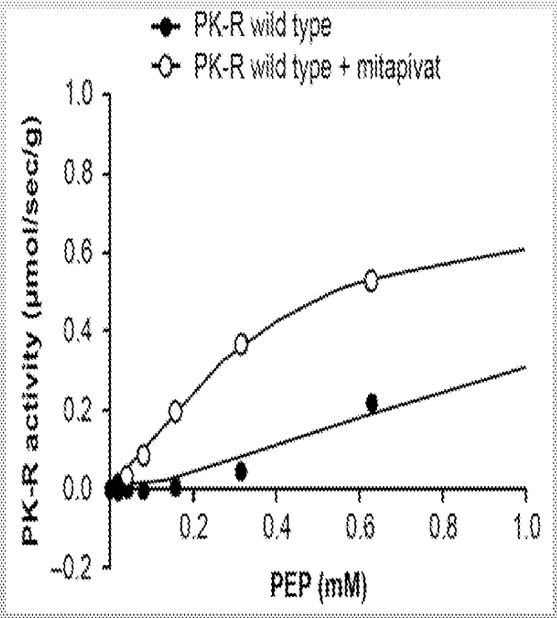



Ex. 2001-0899

# Mitapivat is an allosteric activator of wild-type and mutant PK-R

**Mitapivat (AG-348)**

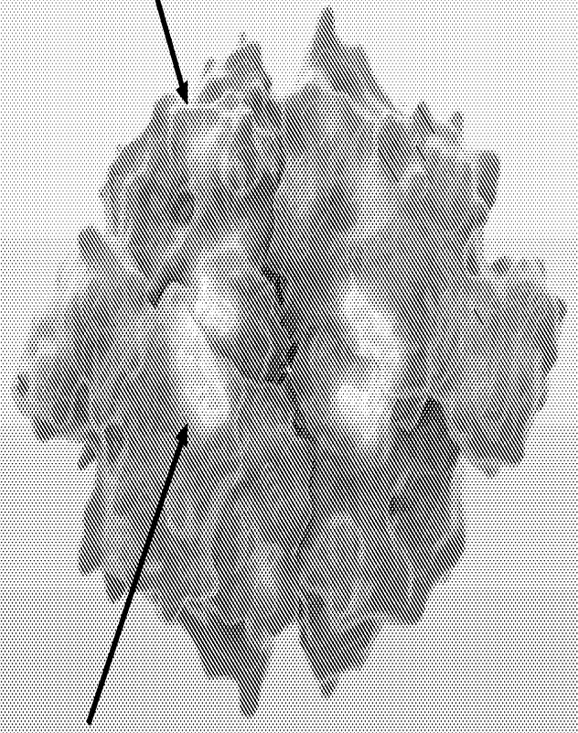





**Mitapivat binding enhances the affinity of PK-R for its substrate PEP**

(recombinant enzyme)

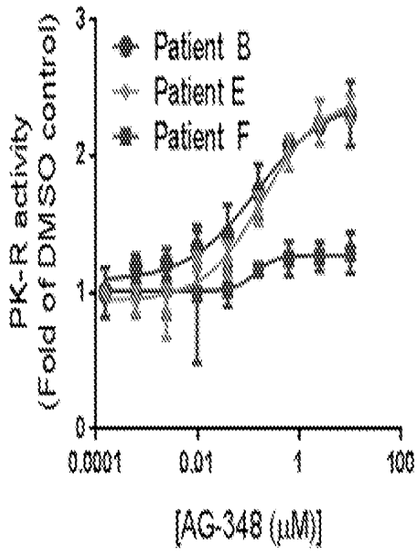
**Active PK-R is a tetramer; mutations (green) decrease the catalytic activity**



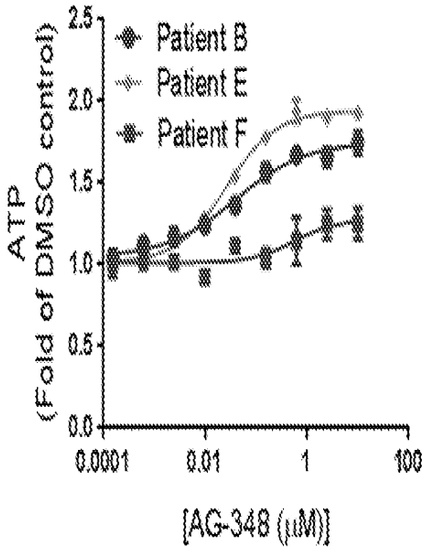
**Mitapivat (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations**

# Ex-vivo treatment with mitapivat can increase PK-R activity and ATP in PK deficiency patient red cells

**PK-R Activity**




**ATP**



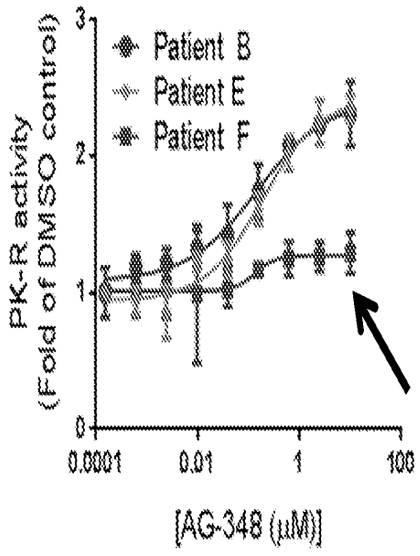

PK deficiency patient red cells were treated for 24 hrs with mitapivat at indicated concentrations



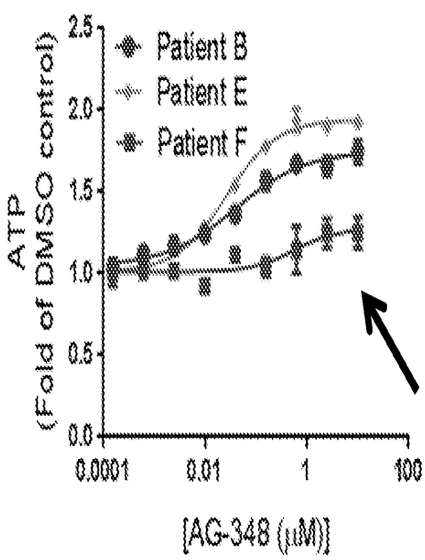
Kung et al., *Blood* 2017;130(11):1347

# Ex-vivo response may be linked to genotype and/or PK-R protein level

## PK-R Activity




## ATP

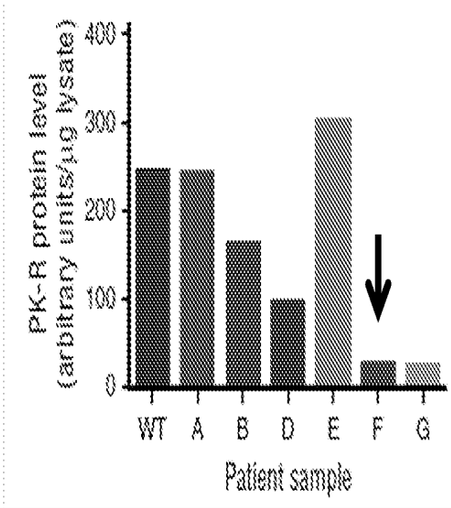



PK deficiency patient red cells were treated for 24 hrs with mitapivat at indicated concentrations

## Genotype

| Patient | Mutations (nucleotide) | Mutations (protein) |
|---|---|---|
| A | 1529 G>A/1532 G>A | R510Q/G511R |
| B | 1456 C>T/1168 G>A | R486W/D390N |
| C | 1529 G>A/721 G>T | R510Q/E241stop |
| D | 1483 G>A/721 G>T | A495V/E241stop |
| E | 1456 C>T/1022 G>A | R486W/G341D |
| F | 1529 G>A/1241 C>G | R510Q/P414R |
| G | 401T>A/1487 T>G | V134D/V496G |

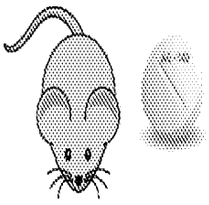
## PK-R protein level



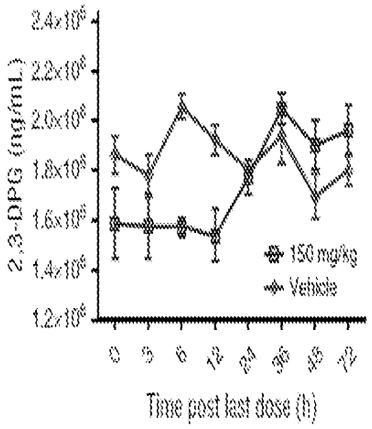



Kung et al., *Blood* 2017;130(11):1347

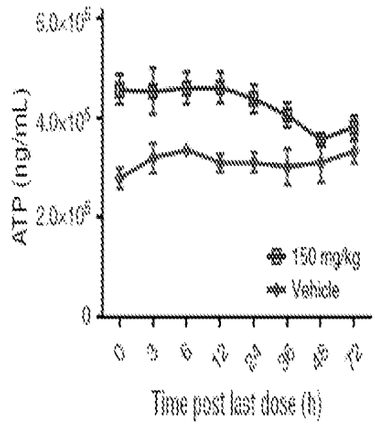
# Robust change in 2,3-DPG/ATP markers observed in mice and human healthy volunteer clinical studies



2,3-DPG

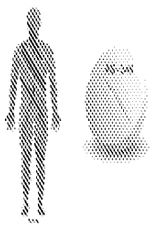


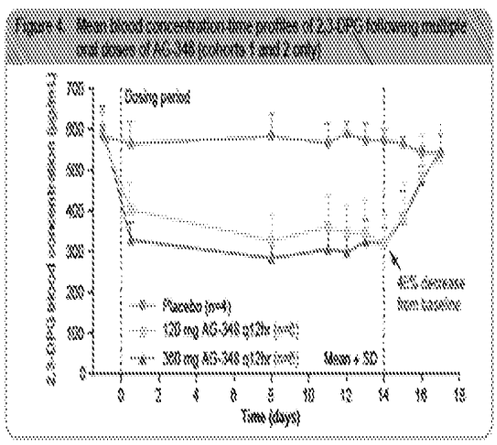

ATP




7 days of twice daily dosing

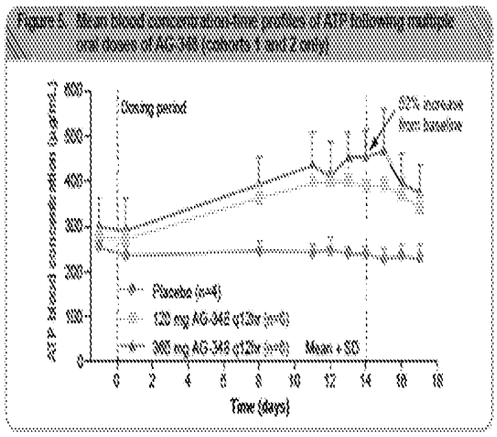
Kung et al., *Blood* 2017;130(11):1347






2,3-DPG

- There were modest increases in blood ATP levels after single AG-348 doses in the SAD study.
- There were substantial increases in ATP levels on Days 8–14 of the MAD study in cohorts 1 and 2, and levels remained elevated through 72 hr after the last dose (Figure 5).




ATP

Final data in Yang et al., *Clin Pharmacol Drug Dev* 2019;8(2):246



Ex. 2001-0903

# Effects of AG-348, a pyruvate kinase activator, in patients with pyruvate kinase deficiency: Updated results from the DRIVE PK study

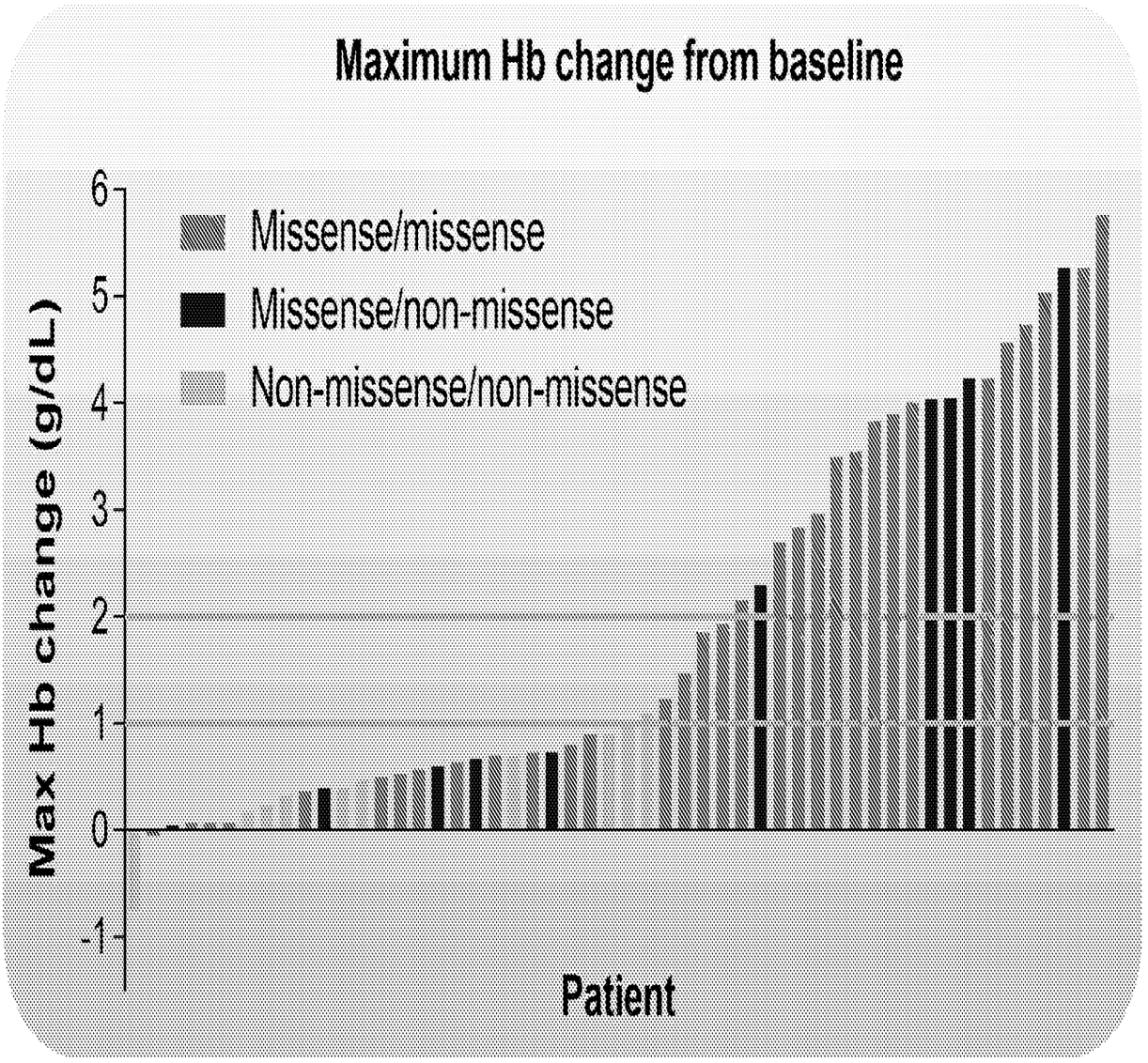
Rachael F Grace*[1], D Mark Layton[2], Frédéric Galactéros[3], Christian Rose[4], Wilma Barcellini[5], D Holmes Morton[6], Eduard van Beers[7], Hassan Yaish[8], Yaddanapudi Ravindranath[9], Kevin HM Kuo[10], Sujit Sheth[11], Janet L Kwiatkowski[12], Bruce Silver[13], Charles Kung[14], Marvin Cohen[15], Hua Yang[14], Penelope A Kosinski[14], Lei Hua[14], Ann Barbier[14], Bertil Glader [16]

*[1]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, MA USA; [2]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, UK; [3]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, France; [4]Hôpital Saint Vincent de Paul, Lille, France; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, [6]Central Pennsylvania Clinic, Belleville, PA, USA, [7]Universitair Medisch Centrum Utrecht, Utrecht, Netherlands, [8]University of Utah, Salt Lake City, UT, USA [9]Wayne State University School of Medicine, Children's Hospital of Michigan, Detroit, MI, USA; [10]University of Toronto, Toronto, Canada; [11]Weill Cornell Medical College, New York, NY, USA; [12]Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA, USA;[13]Bruce A Silver Clinical Science and Development, Dunkirk, MD, USA; [14]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [15] MBC Pharma Solutions, Newtown, PA, USA; [16]Stanford University School of Medicine, Palo Alto, CA, USA*

*Presented at the 22nd Congress of the European Hematology Association, 22-25 June 2017, Madrid, Spain*

# Maximum hemoglobin (Hb) increase observed by genotype

- 25/52 (48%) patients had a max increase in Hb >1.0 g/dL
  - 24/42 (57%) patients who had ≥1 missense mutation had Hb increase >1.0 g/dL
  - 5/5 patients homozygous for R479H did not have Hb increase

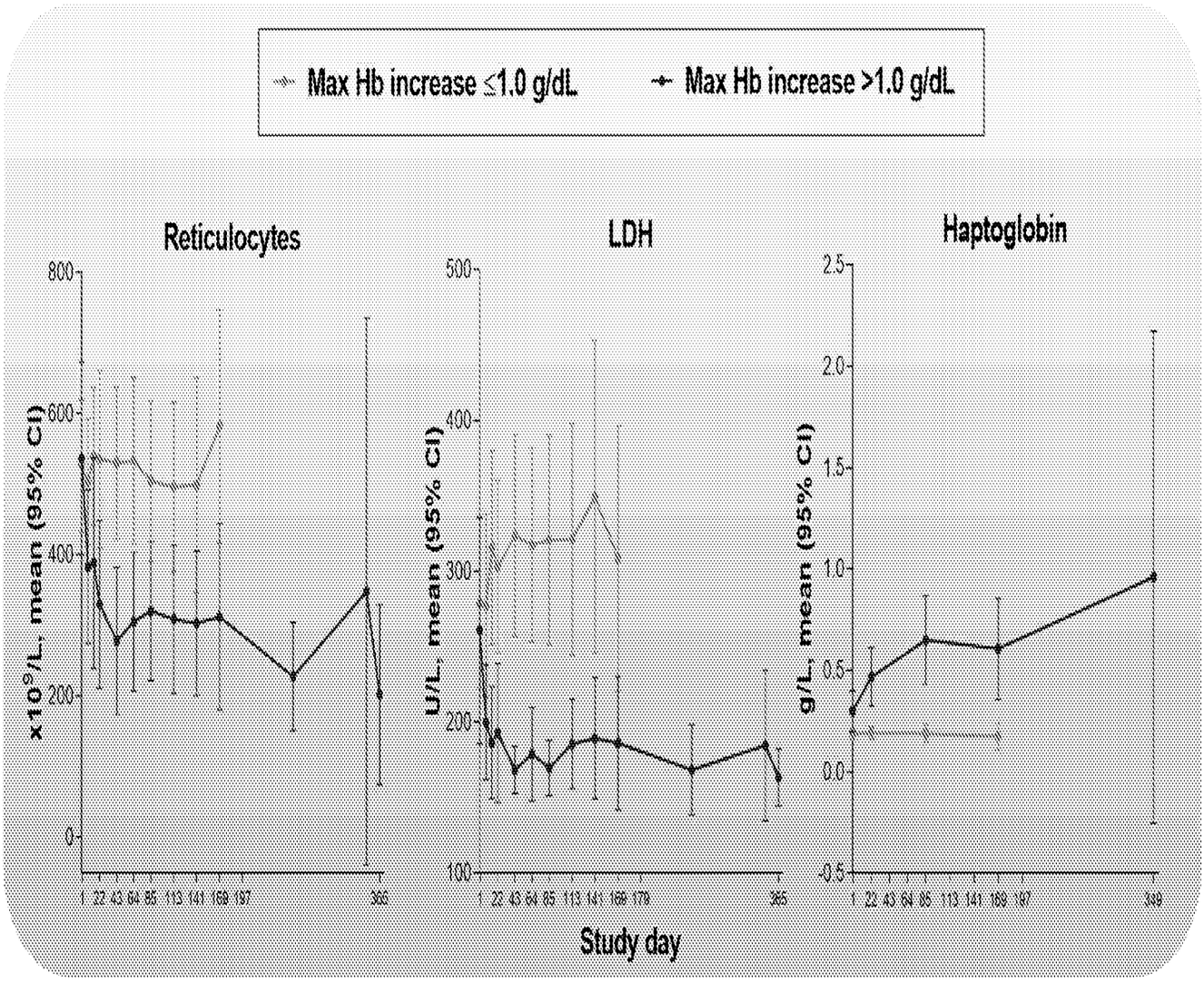





Ex. 2001-0905

# Hemolysis markers improve in patients with Hb increase of >1.0 g/dL

- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin

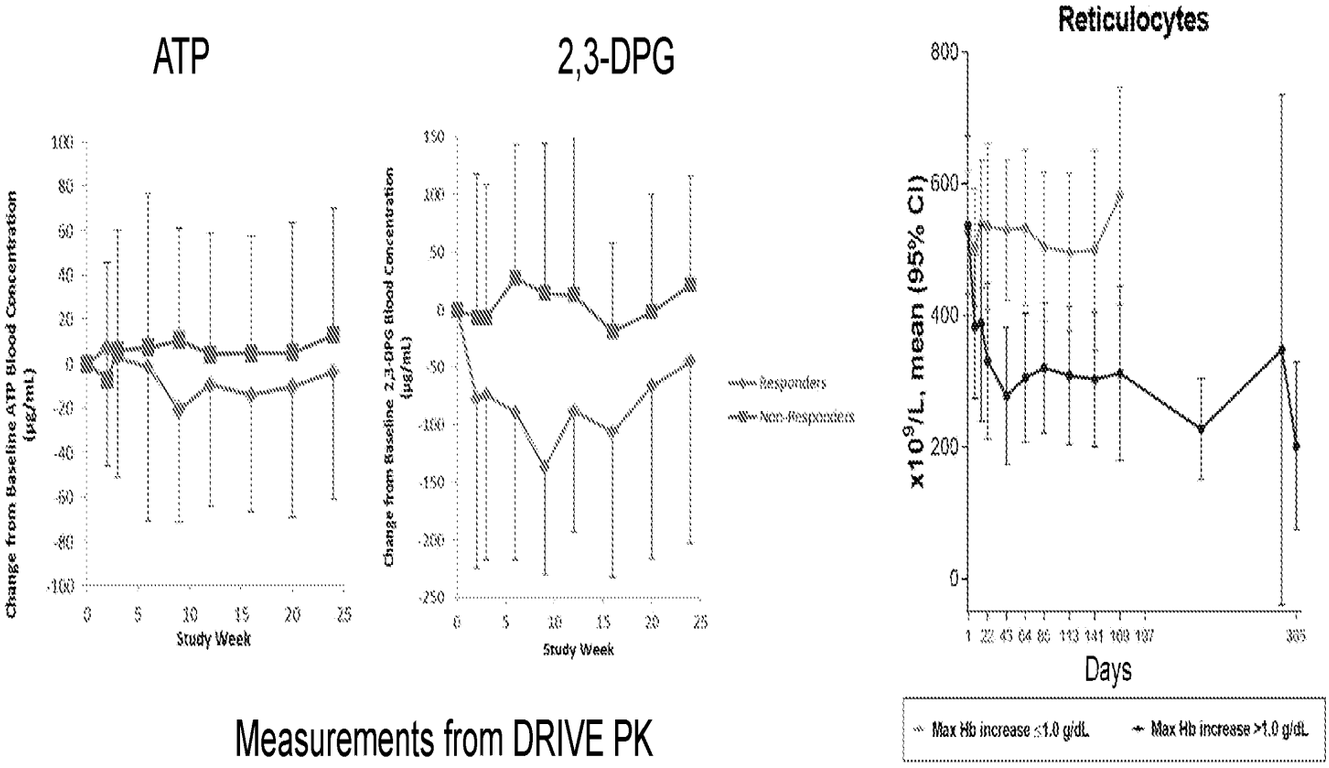





Ex. 2001-0906

# Changes in ATP or 2,3-DPG in PK deficiency patients are not sufficiently predictive of Hb response

2,3-DPG Pharmacodynamics from AG519-C-001 125 mg MAD Cohort

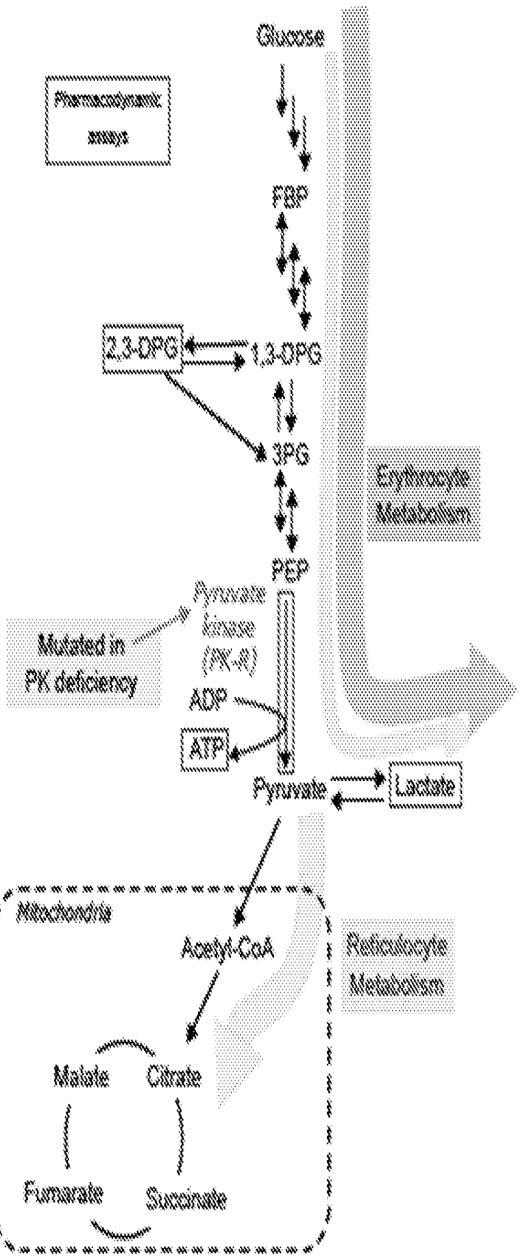


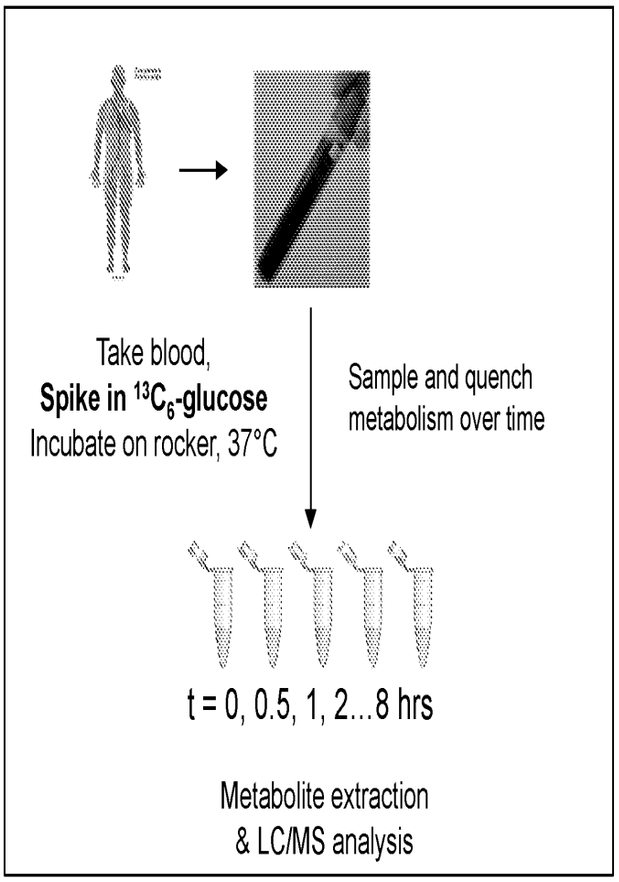

Measurements from DRIVE PK

- There is minimal difference in mean change from baseline ATP blood concentration for all responders compared to all non-responders
- There is a small decrease in mean change from baseline 2,3-DPG blood concentration for all responders compared to all non-responders, but standard deviations overlap
- Hb responders have a very different composition of blood (decreased reticulocytes, etc.) that may potentially help explain these results

14 Hb responder defined as maximum Hb increase >1.0 g/dL

# Tracer studies in PK deficiency patient blood samples



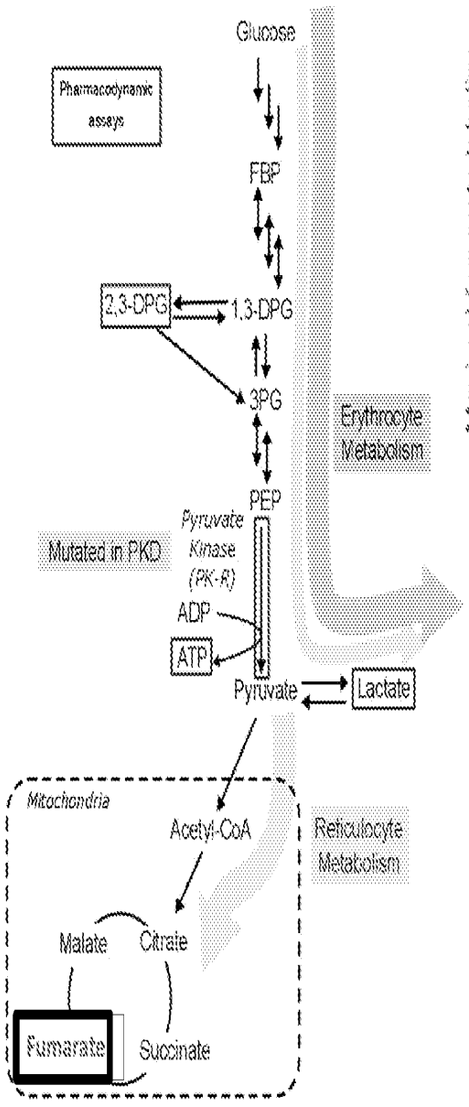



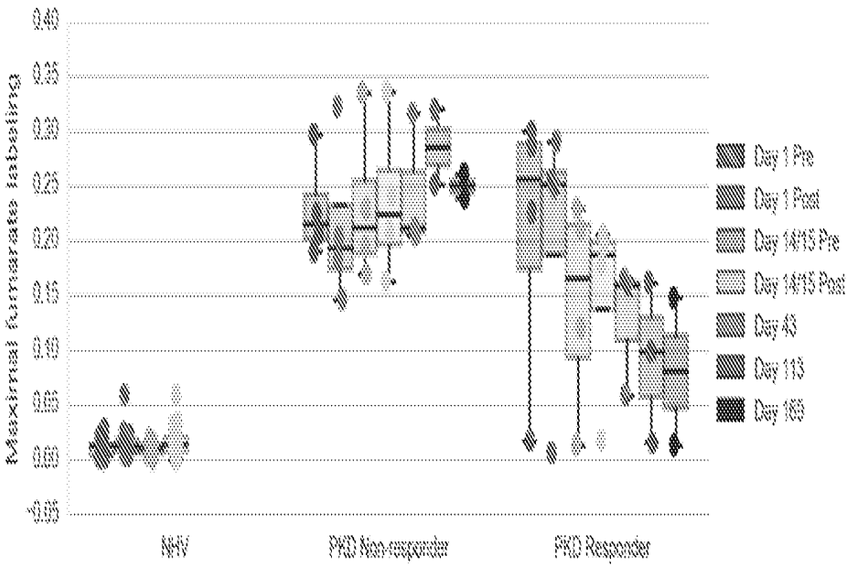

Flux through the PK-R pathway was assessed by measurement of total lactate production as well as $^{13}C$-glucose isotopomer tracing studies in blood samples taken from a subset of DRIVE PK subjects pre and post-treatment



Chubukov et al., ASH 2016 Abstract #2452

# Hb response is associated with changes in labeling into TCA cycle intermediates



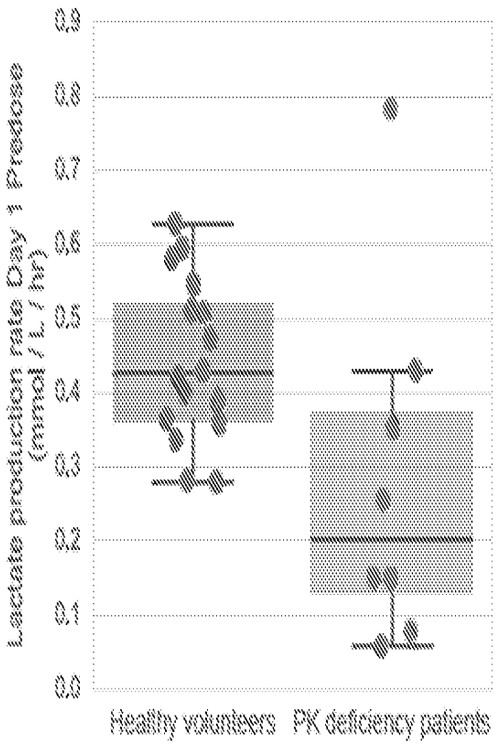



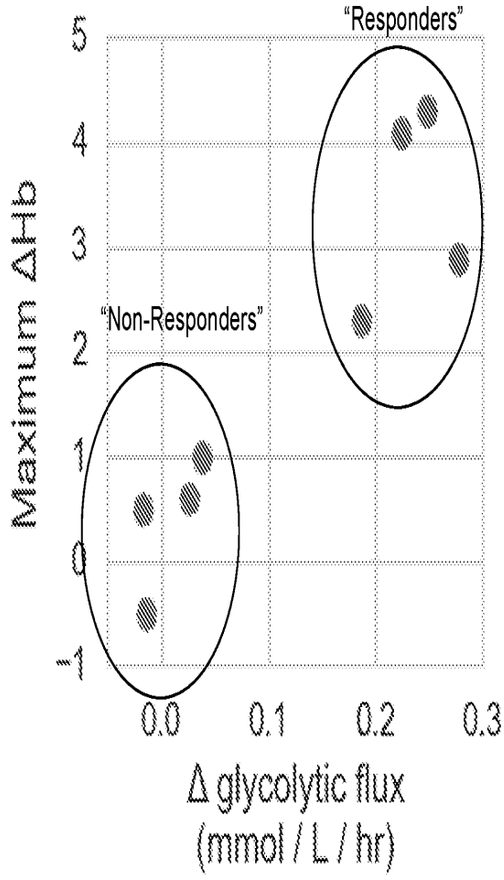

- Labeling into fumarate is readily measured in PK deficiency patients but not healthy volunteers
- Trend for labeling to be reduced in mitapivat responders
- "World's most expensive reticulocyte assay" –Dr. David Nathan



Chubukov et al., ASH 2016 Abstract #2452

# Patients with Hb increases also had increased glycolytic flux in peripheral blood



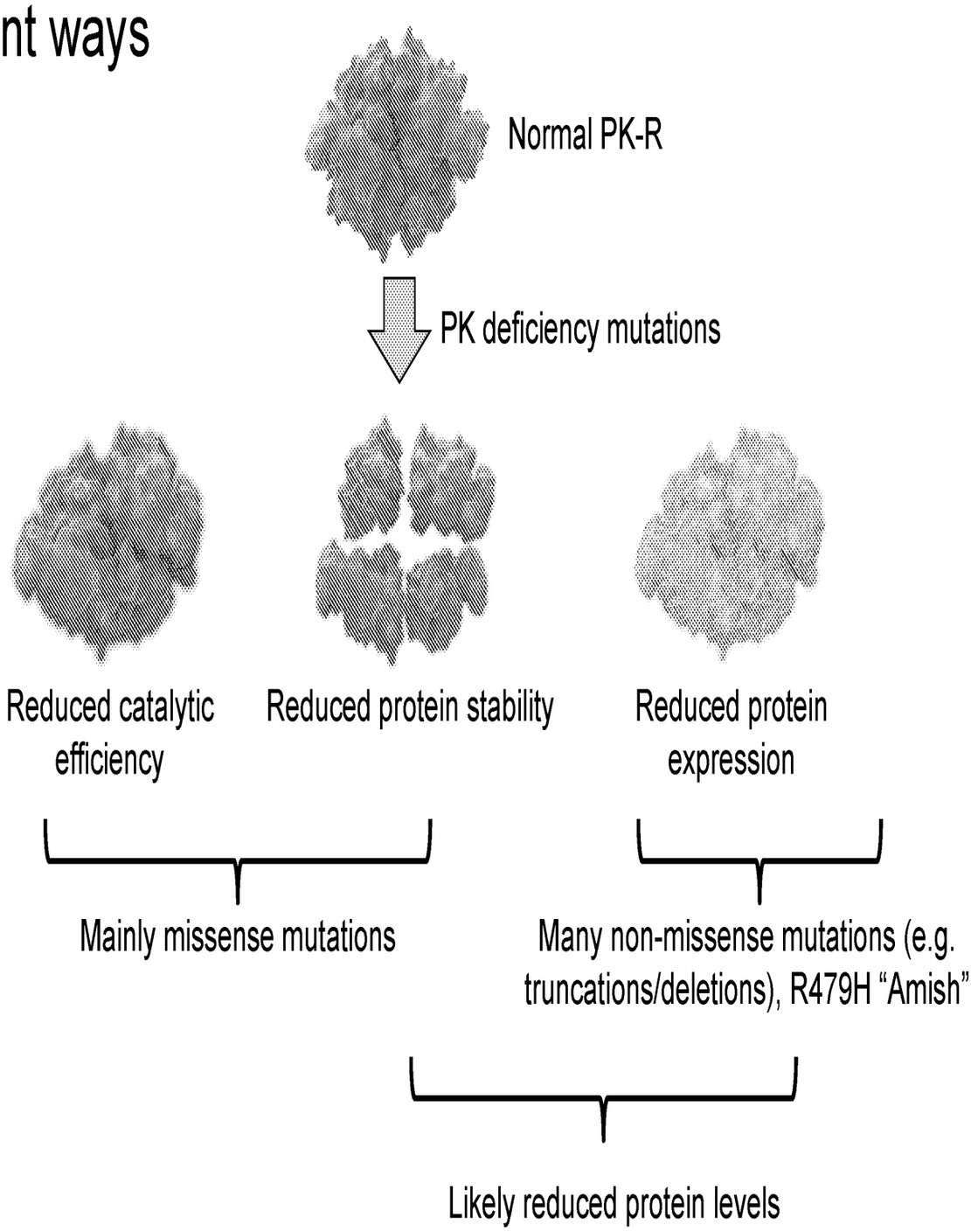

Glycolytic flux lower in PK deficiency patient blood samples at baseline

A positive correlation between increased Hb and increased glycolytic flux was observed

Hb responder defined as maximum Hb increase >1.0 g/dL

Chubukov et al., ASH 2016 Abstract #2452

# Pyruvate kinase mutations can affect PK-R activity in different ways

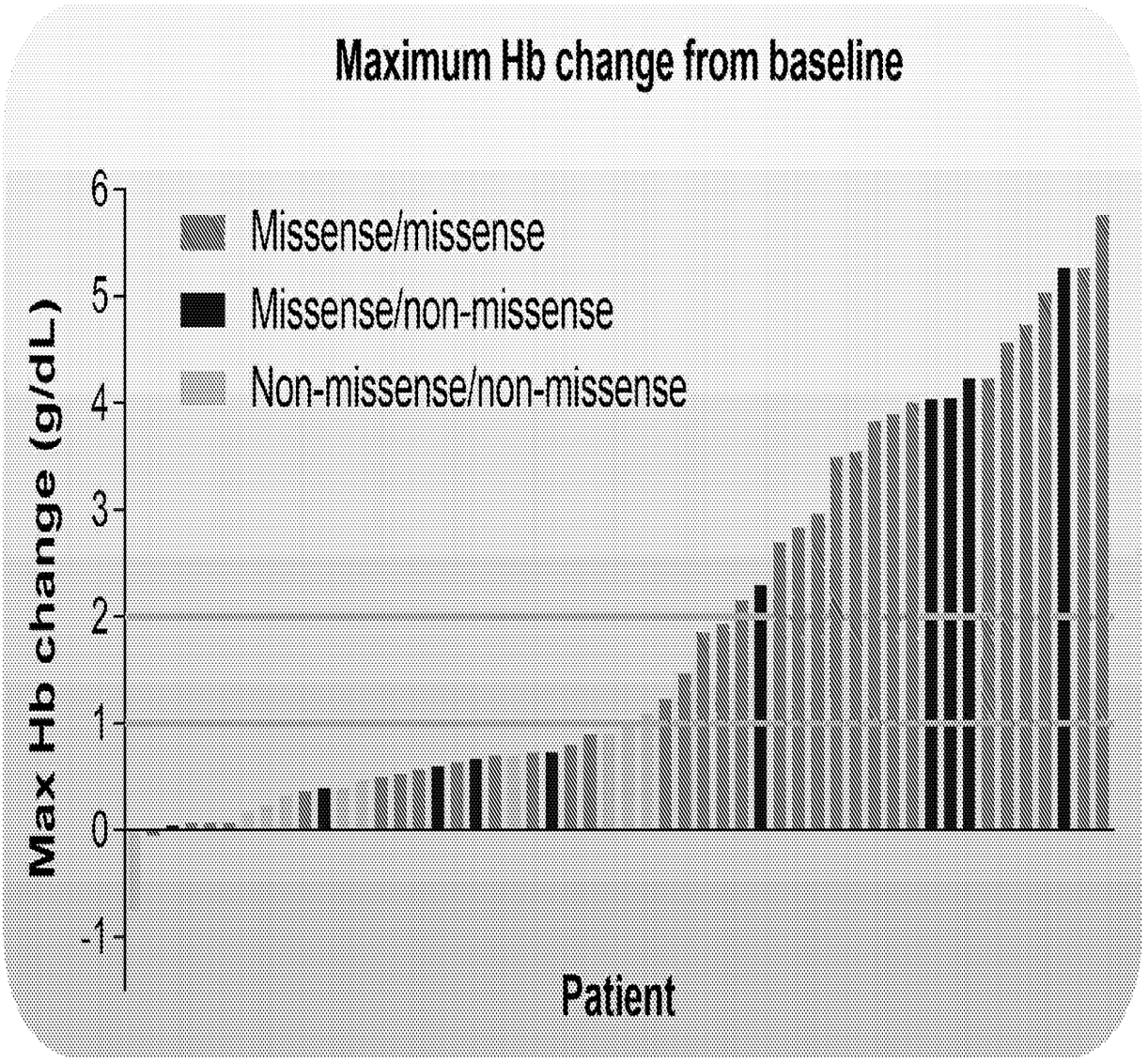





Ex. 2001-0911

# Maximum hemoglobin (Hb) increase observed by genotype

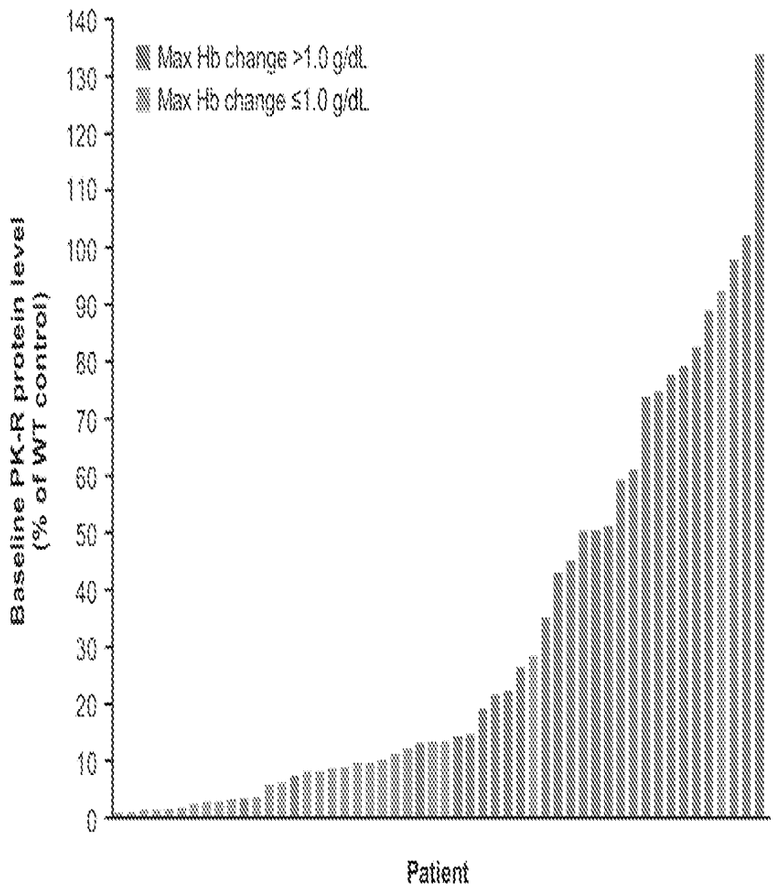
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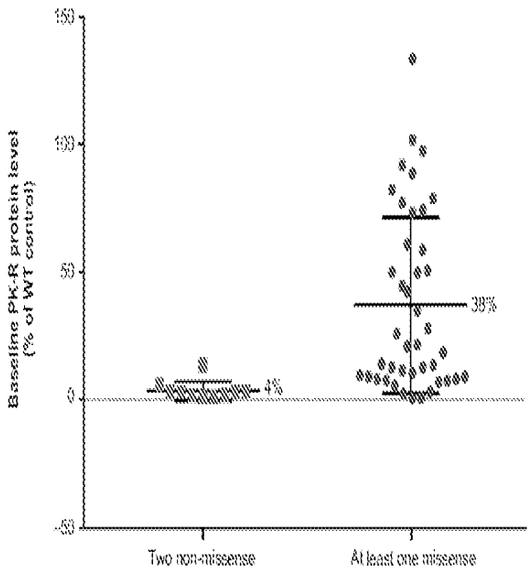





Ex. 2001-0912

# Correlation between PK-R baseline protein level and Hb response in DRIVE PK




- A striking but not absolute correlation between PK-R protein levels and Hb response
- Consistent with mitapivat mechanism of action through stimulation of residual PK-R enzyme activity



Kung et al., ASH 2018 Abstract #3621

Ex. 2001-0913

# Ongoing phase 3 trials in PK deficiency




## ACTIVATE

- **~80 patients** treated for 6 months
- **Primary endpoint:** portion of patients achieving 1.5 g/dL Hb increase over multiple visits

Non-regularly transfused adult patients, placebo controlled

## ACTIVATE-T

- **~20 patients**, minimum of 6 transfusions 1 year before enrollment
- **Primary endpoint:** reduction in transfusion burden over 6 months compared to patient's transfusion history

Regularly transfused adult patients, open-label



Ex. 2001-0914

# Genetic partial pyruvate kinase deficiency can influence phenotype of other hematological diseases

Patient with one HbS allele and one PKD allele presents with severe sickle disease

Sickle cell disease in a carrier with pyruvate kinase deficiency

Nazeer Alli[1], Marius Coetzee[2], Vernon Louw[2], Ben van Rensburg[2], Gerrit Rossouw[2], Lisa Thompson[3], Serge Pissard[4] and Swee Lay Thein[3]

Alli et al., *Hematology* 2008 Dec;13(6):369-72

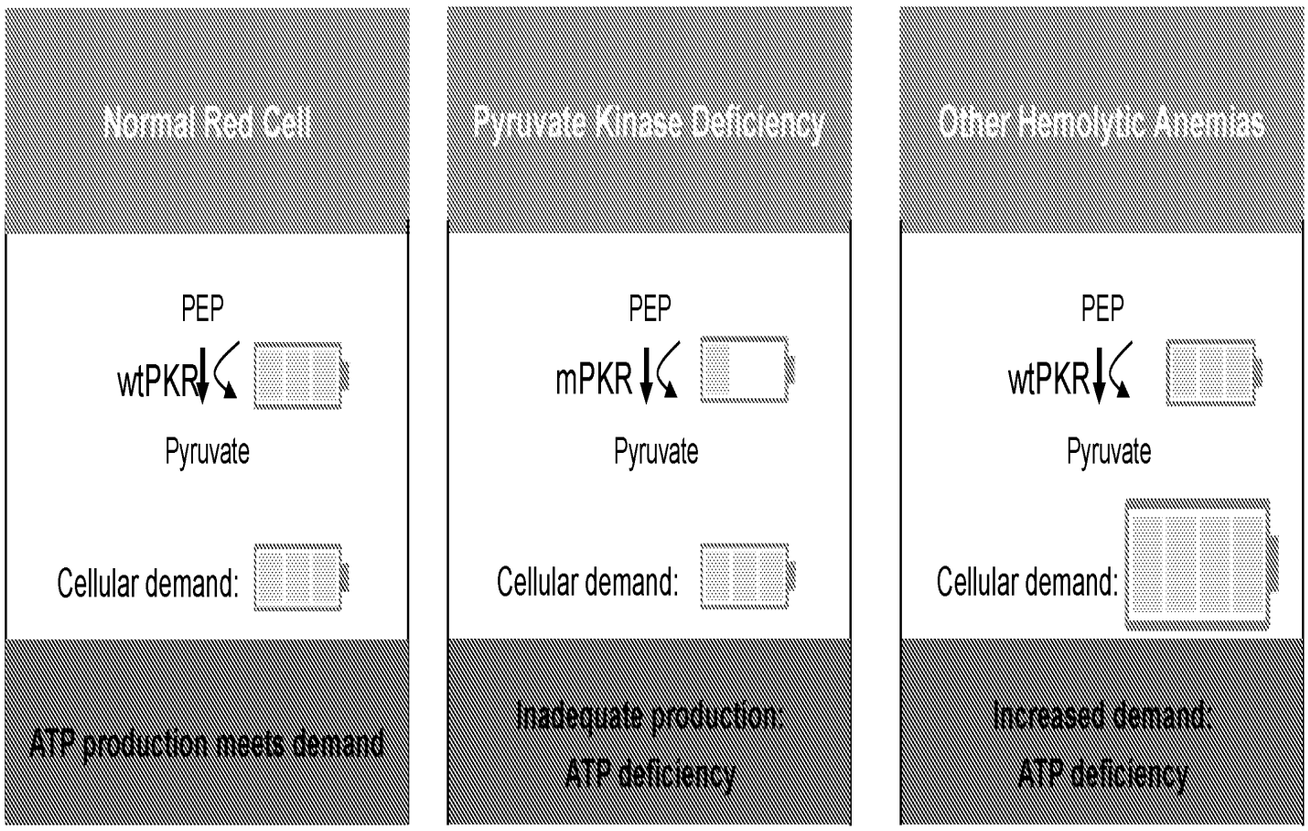
Single allele of PKD results in severe manifestation of hereditary spherocytosis

Partial pyruvate kinase deficiency aggravates the phenotypic expression of band 3 deficiency in a family with hereditary spherocytosis

Rob van Zwieten,[1] Brigitte A. van Oirschot,[2] Martijn Veldthuis,[1] Johannes G. Dobbe,[3] Geert J. Streekstra,[3] Wouter W. van Solinge,[2] Roger E.G. Schutgens,[3] and Richard van Wijk[2]*

van Zwieten et al., *Am J Hematol* 2015; Mar;90(3):E35-9

# Can PK activators be used for other hematological diseases? (I)




Hypothesis: Stimulation of glycolytic metabolism by PK activation may serve as a compensatory mechanism for the underlying cause of hemolytic anemia



Ex. 2001-0916

# Can PK activators be used for other hematological diseases? (II)

**Mutations in Krüppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression**

Vip Viprakasit,[1] Supachai Ekwattanakit,[2,3] Suchada Riolueang,[3] Nipon Chalaow,[3] Chris Fisher,[4] Karen Lower,[5] Hitoshi Kanno,[6] Kalaya Tachavanich,[1] Sasithorn Bejrachandra,[7] Jariya Saipin,[7] Monthana Juntharaniyom,[8] Kleebsabai Sanpakit,[1] Voravarn S. Tanphaichitr,[3] Duantida Songdej,[4] Christian Babbs,[9] Richard J. Gibbons,[4] Sjaak Philipsen,[9] and Douglas R. Higgs[4]

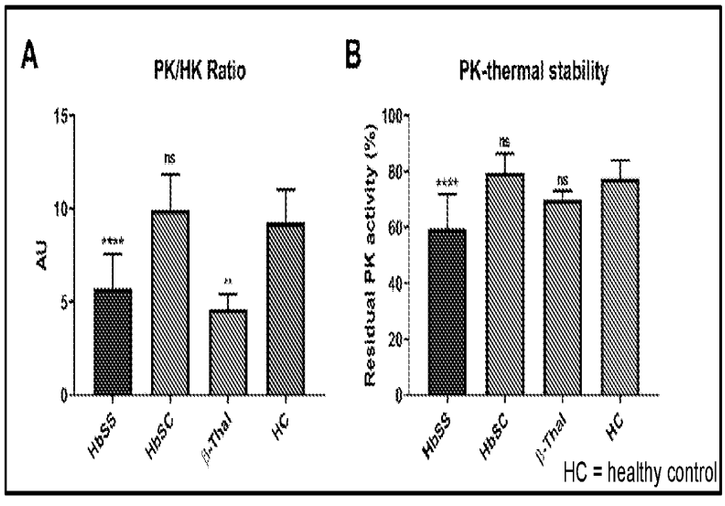
Mutations in KLF lead to decreased PK activity

Viprakasit et al., *Blood* 2014;123(10):1586

**Hereditary spherocytosis is associated with decreased pyruvate kinase activity due to impaired structural integrity of the red blood cell membrane**

HS cells have relatively decreased PK activity

Andres et al., *Br J Haematol* 2019; doi: 10.1111/bjh.16084




Sickle cell and thalassemic red blood cells have reduced PK/HK activity ratio and reduced PK stability

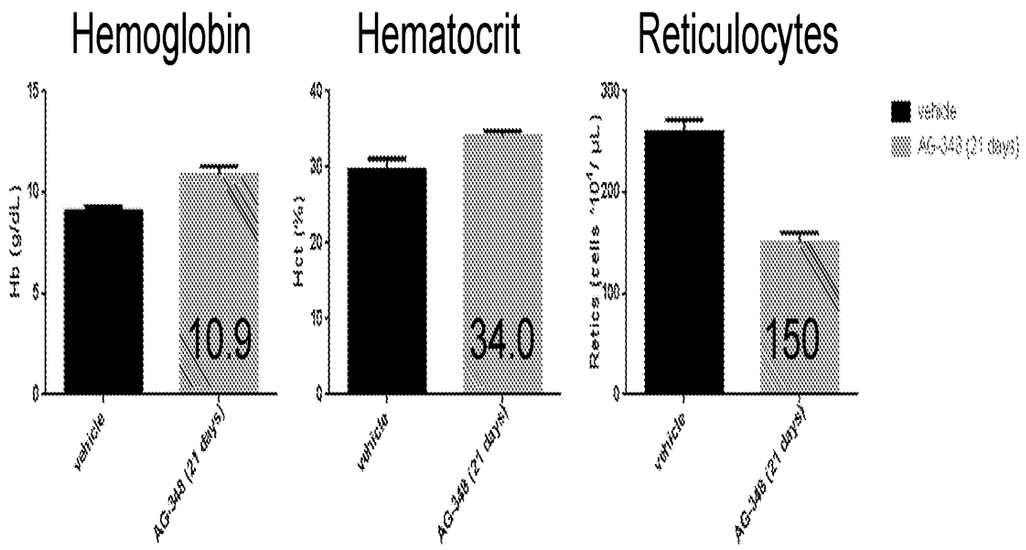
Rab et al., EHA 2019 Abstract #PS1519

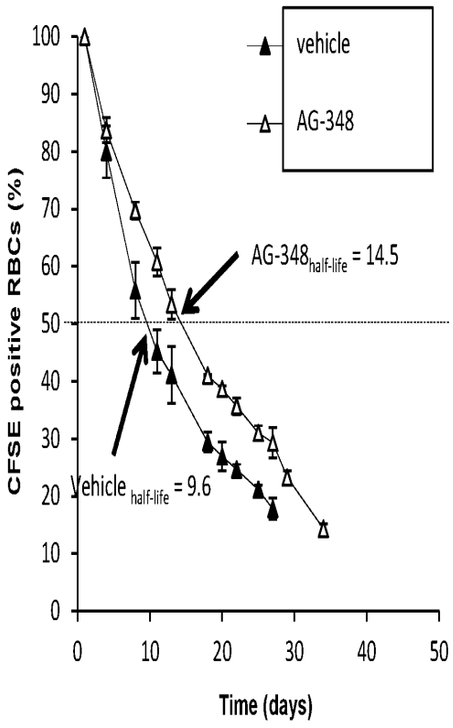
**Hypothesis: Pathophysiology of hemolytic anemia may lead to reduced pyruvate kinase activity**



Ex. 2001-0917

# Mitapivat treatment improves hematological parameters and RBC lifespan in th3/+ thalassemia mouse model

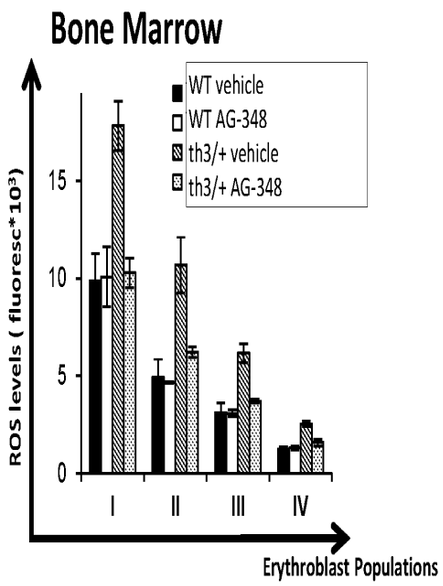


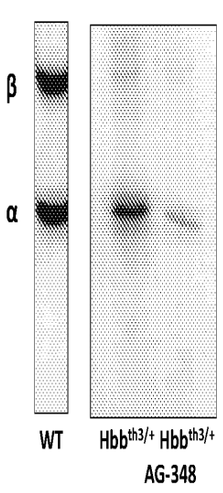



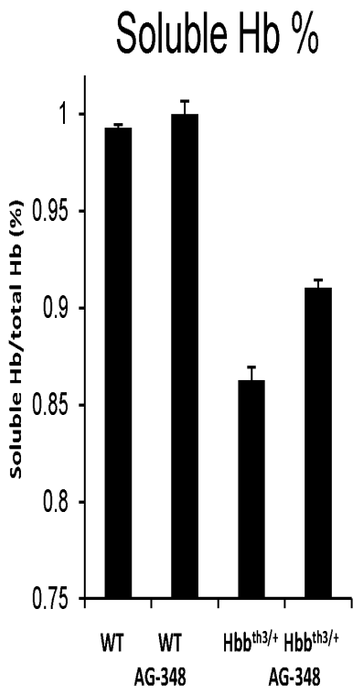

- Mitapivat increases Hb by ~2 g/dL and reduces reticulocyte count after 21 days of treatment in th3/+ beta-thalassemia mouse model
- ~50% increase in lifespan of peripheral RBCs

Matte et al., EHA 2016 Abstract #S135
Lucia de Franceschi, University of Verona

# AG-348 treatment results in reduced ROS levels in bone marrow and increased soluble Hb



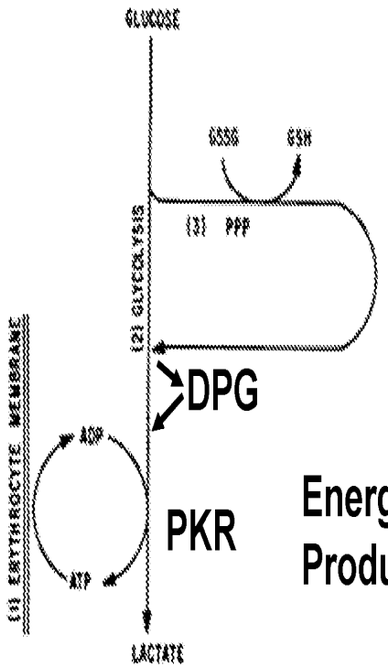




- ROS levels in bone marrow reduced nearly to baseline levels seen in WT cells
- Mechanistic work ongoing but may be related to ATP-dependent clearance of excess alpha-globin chains

ROS = reactive oxygen species

Matte et al., EHA 2016 Abstract #S135
Lucia de Franceschi, University of Verona

# How might mitapivat help a thalassemic red cell?




**Protection from oxidative stress (NADPH)**

**DPG**

**PKR**

**Energy Production (ATP)**

**Fig 1.** Principal groups of hereditary hemolytic anemias are those due to abnormalities of (1) the erythrocyte membrane, (2) the glycolytic pathway, and (3) glutathione metabolism and the pentose phosphate pathway. The interrelationship of these pathways is presented in this highly simplified diagram. ADP = adenosine diphosphate; ATP = adenosine triphosphate; PPP = pentose phosphate pathway; GSSG = oxidized glutathione; GSH = reduced glutathione.



Ex. 2001-0920

# How might mitapivat help a thalassemic red cell?

Excess globin chains are degraded via the proteasome and other ATP-dependent pathways

**Protein Quality Control During Erythropoiesis and Hemoglobin Synthesis**

Eugene Khandros[a] and Mitchell J. Weiss[b]

[a]Cell and Molecular Biology Graduate Group, The Combined Degree Program, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

[b]Associate Professor of Pediatrics, Division of Hematology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

Khandros & Weiss, *Hematol Oncol Clin North Am* 2010;24(6):1071

**ATP-dependent Proteolysis of Hemoglobin α Chains in β-Thalassemic Hemolysates Is Ubiquitin-dependent***

(Received for publication, May 2, 1988)

Joseph R. Shaeffer

*From the Center for Blood Research and the Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115*

Shaeffer, *J Biol Chem* 1988;263(27):13663

Hypothesis: increased ATP production stimulated by mitapivat boosts α-chain proteolysis, resulting in increased fitness for RBC precursors in the bone marrow



Ex. 2001-0921

# Ongoing phase 2 study in thalassemia patients




- ~20 non-transfusion dependent adults
- Evaluating 50 and 100 mg BID
- Primary endpoint: hemoglobin response (1.0 g/dL increase over baseline at 12 weeks)
- Goal to achieve proof of concept in 2019

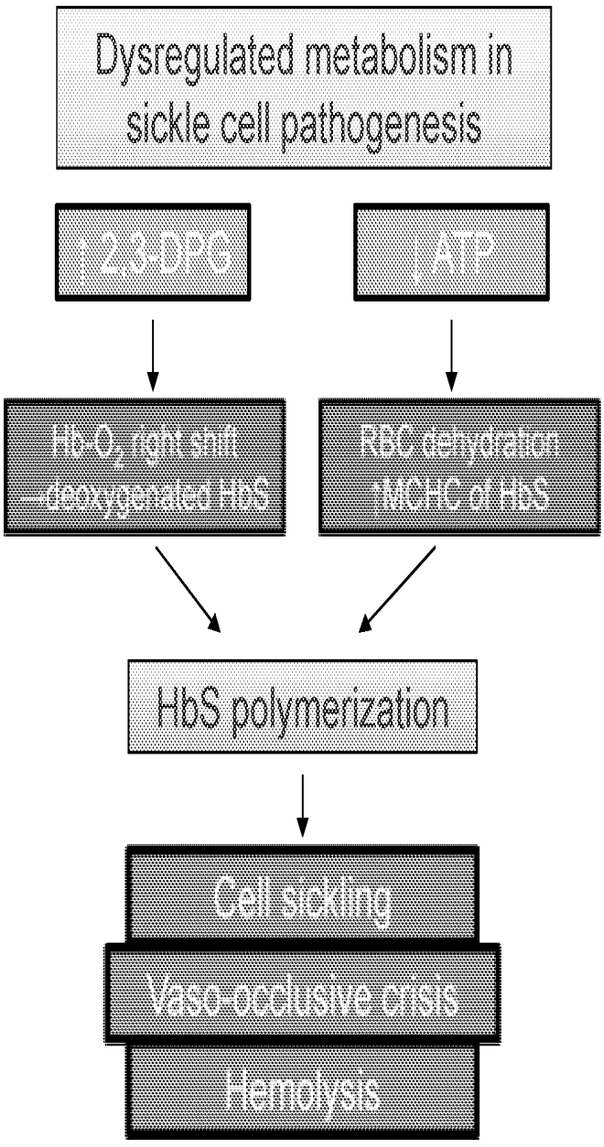


BID = twice daily

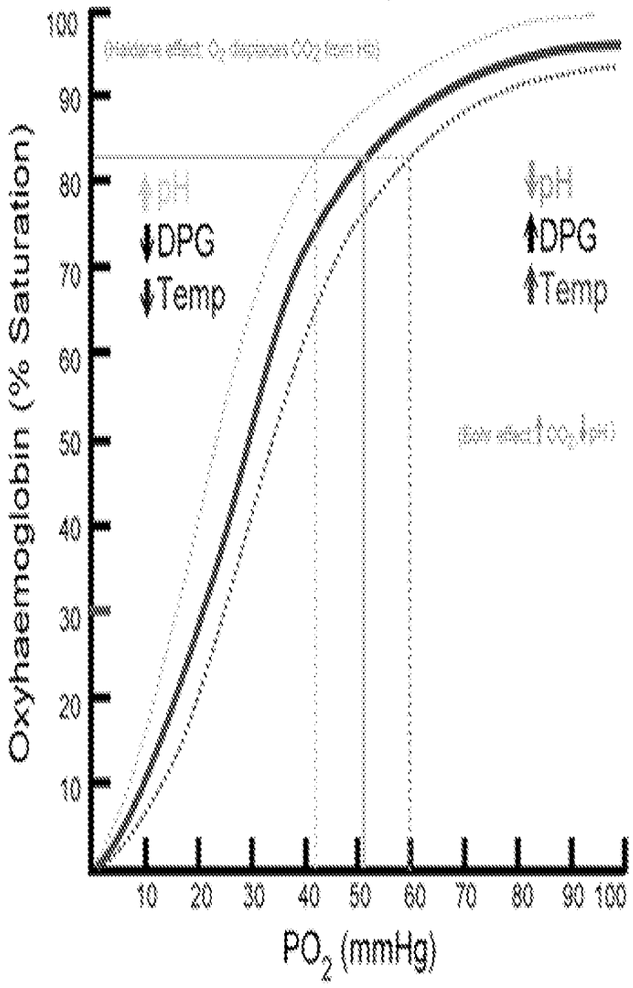
Clinicaltrials.gov: NCT03692052

Ex. 2001-0922

# Therapeutic hypothesis for mitapivat: Modulation of 2,3-DPG and ATP levels will reduce HbS polymerization




Hb-$O_2$ affinity curve right-shifted with increased 2,3-DPG




Clinical study in sickle cell patients to initiate in 2019 (NCT04000165)

- Collaboration with NIH



Ex. 2001-0923

# Summary

- Mutations in pyruvate kinase cause hemolytic anemia
- Mitapivat (AG-348) is an allosteric activator of pyruvate kinase that can activate WT and a spectrum of mutant PK-R enzymes
- A phase 2 study with mitapivat showed that ~50% of patients with pyruvate kinase deficiency experienced an increase in Hb of >1.0 g/dL
- Increases in Hb in patients treated with mitapivat were associated with increased glycolytic flux and a higher baseline level of PK-R protein
- Stimulating glycolytic pathway activity through WT PK-R activation may be an effective compensatory pathway in hemoglobinopathies and other hemolytic anemias

# Thank you!

- All of my colleagues at Agios Pharmaceuticals
- Collaborating scientists from around the world
- All clinical investigators and staff
- All healthy volunteers and patients who have participated in clinical trials for AG-348





blood

MENU

112.THALASSEMIA AND GLOBIN GENE REGULATION | NOVEMBER 13, 2019

# Mitapivat (AG-348), an Oral PK-R Activator, in Adults with Non-Transfusion Dependent Thalassemia: A Phase 2, Open-Label, Multicenter Study in Progress

Kevin H. M. Kuo, MDMSc,FRCPC, D. Mark Layton, MB BS, Katrin Uhlig, MD MS, Megan Lynch, MSN, NP, Li Liu, PhD, Elliott P. Vichinsky, MD

Check for updates

*Blood* (2019) 134 (Supplement_1): 2249.

https://doi.org/10.1182/blood-2019-123113

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**Background:** Thalassemia is a group of inherited blood disorders in which genetic mutation(s) in the α- and/or β-globin locus lead to excess precipitation of β- or α-globin, respectively, and compromised red blood cell (RBC) survival. The condition is characterized by ineffective erythropoiesis and peripheral hemolysis, with resultant anemia. Adenosine triphosphate (ATP) supply appears to be insufficient in thalassemic RBCs to maintain RBC membrane fitness and clearance of globin precipitates. Mitapivat (AG-348) is an oral, small-molecule, allosteric activator of the RBC-specific form of pyruvate kinase (PK-R). PK-R is a key enzyme for maintaining energy homeostasis in RBCs, as they rely almost exclusively on the process of glycolysis to generate ATP. In healthy adults, mitapivat activates wild-type PK-R and increases ATP levels in RBCs.

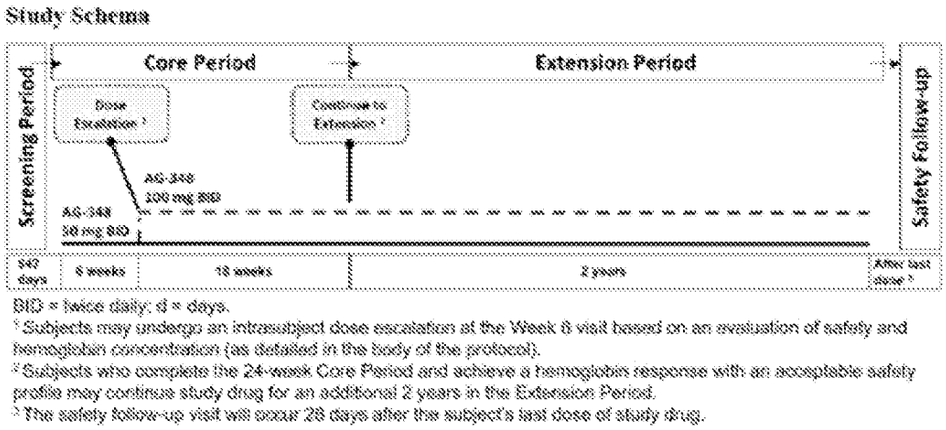
In adults with PK deficiency who were not regularly transfused, oral mitapivat was well tolerated and induced rapid, durable hemoglobin (Hb) increases (NCT02476916). In the $Hbb^{th/3+}$ mouse model of β-thalassemia, mitapivat increased ATP levels; reduced markers of ineffective erythropoiesis; and improved anemia, RBC survival, and indices of iron overload. These data support the hypothesis that increased ATP synthesis mediated via PK-R activation by mitapivat may improve the survival of thalassemic RBCs in the bone marrow and/or peripheral circulation, and thus represents a novel mechanism to treat patients with thalassemia. Here we present the design of a phase 2, open-label

study designed to test this hypothesis and assess the proof of concept of mitapivat in patients with thalassemia.

**Methods:** This phase 2, multicenter, open-label study (NCT03692052) is evaluating the efficacy, safety, pharmacokinetics, and pharmacodynamics of mitapivat in adults with non-transfusion-dependent thalassemia (NTDT). Four sites in North America and the United Kingdom are enrolling patients. The study consists of a 24-week core period followed by a 2-year extension period (Figure). Approximately 17 subjects with NTDT, i.e., β-thalassemia with or without α-globin gene mutations, HbE β-thalassemia, or α-thalassemia (HbH disease), will be enrolled. Non-transfusion dependence is defined as ≤5 units of RBCs transfused in the preceding 24 weeks and no transfusions in the 8 weeks prior to the first day of study drug. The hemoglobin inclusion criterion is ≤10.0 g/dL, which was increased from ≤9.0 g/dL in a recent protocol amendment to better reflect the clinical representation in the NTDT patient population.

All eligible patients will receive an initial mitapivat dose of 50 mg twice daily (BID). At the week 6 visit the dose may be increased to 100 mg BID, depending on safety and Hb response. The primary endpoint is the proportion of subjects who achieve an Hb response, defined as an increase in Hb of ≥1.0 g/dL from baseline at any time between week 4 and week 12 (inclusive). Key secondary and exploratory endpoints include changes in Hb and markers of hemolysis; hematopoietic activity; iron metabolism and iron overload; and assessments of safety and pharmacokinetics. The study is currently enrolling.

## Figure

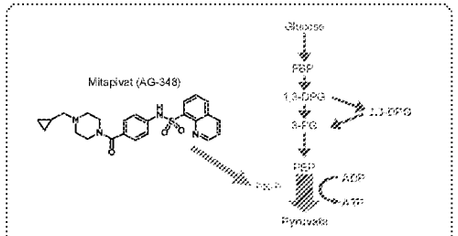


# Mitapivat (AG-348), an oral PK-R activator, in adults with non–transfusion-dependent thalassemia: A phase 2, open-label, multicenter study in progress

Kevin HM Kuo[1], D Mark Layton[2], Katrin Uhlig[3], Megan Lynch[3], Charles Kung[3], Li Liu[3], Elliott P Vichinsky[4]

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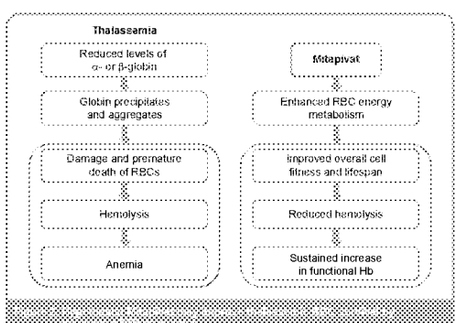
## BACKGROUND




ADP = adenosine diphosphate; ATP = adenosine triphosphate; DPG = diphosphoglycerate; TDP = Pyruvate 1,4-bisphosphate; PEP = phosphoenolpyruvate; PG = phosphoglycerate; PK-R = red cell pyruvate kinase

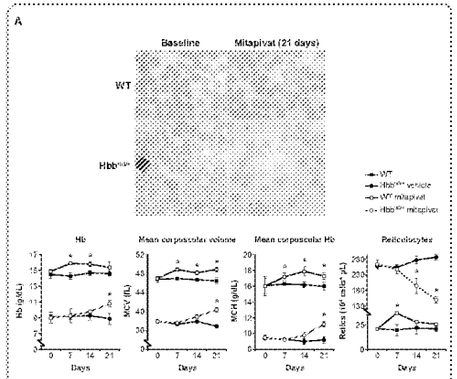
- Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis.
- PK catalyzes the conversion of phosphoenolpyruvate to pyruvate and generates two adenosine triphosphate (ATP) molecules per molecule of glucose (**Figure 1**).[1]
- PK-R is the form of PK present in red blood cells (RBC), where glycolysis is the primary energy production pathway.[1]
- Mitapivat sulfate (mitapivat; AG-348) is an orally available, small-molecule allosteric activator of wild-type (WT) and mutant PK-R.[2]
- In a phase 1 study in healthy volunteers, mitapivat increased blood ATP levels.[3]
- In a phase 2 study in adult patients with PK deficiency who were not regularly transfused, oral mitapivat was well tolerated and induced rapid, sustained hemoglobin (Hb) increases.[4]

## HYPOTHESIS



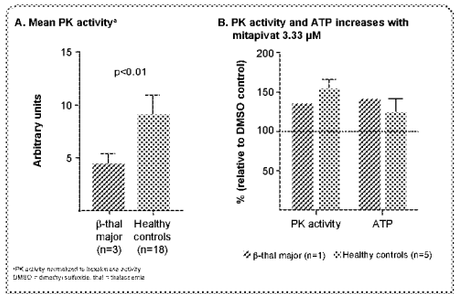

- Owing to imbalanced globin chain production, thalassemic RBCs have increased ATP demand to maintain cell fitness (**Figure 2**).[5,6]
- Activation of WT PK-R in thalassemic RBCs may enhance glycolysis and increase RBC ATP levels, leading to improved cell fitness and survival.

## SUPPORTING DATA




*p<0.05 compared with WT
†p<0.05 compared with vehicle (V)
Hbb^th/+ = heterozygous mouse model of β-thalassemia; Retics = reticulocytes

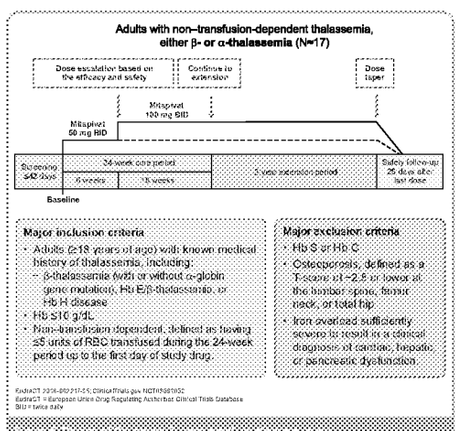
- β-thalassemic mice (Hbb$^{th/+}$) treated with mitapivat for up to 2 months showed (**Figure 3**)[2]:
  - Improvements in Hb and other hematological parameters (**A**)
  - Reduction in circulating erythroblasts, suggesting improvements in ineffective erythropoiesis (**B**)
  - Reductions in spleen weight (**C**), *ERFE* expression (**D**), and markers of apoptosis (**E**).




[a]PK activity normalized to baseline rate activity
DMSO = dimethyl sulfoxide; thal = thalassemia

- In RBCs from regularly transfused patients with β-thalassemia, PK activity was decreased compared with RBCs from healthy controls (**Figure 4A**).[19]
- *Ex vivo* treatment with mitapivat increased PK activity and ATP levels in RBCs from healthy controls and patients with β-thalassemia (**Figure 4B**).[19]

## PHASE 2 TRIAL DESIGN




ClinicalTrials.gov Identifier: NCT03692052
EudraCT = European Union Drug Regulating Authorities Clinical Trials Database
SAD = single ascending dose; MAD = multiple ascending dose

- A phase 2, open-label, multicenter study is ongoing to assess the efficacy, safety, pharmacokinetics, and pharmacodynamics of mitapivat in non–transfusion-dependent thalassemia (NCT03692052; **Figure 5**).
- The study is currently enrolling at four sites in the US, Canada, and the UK.

### *Core period*

- All eligible patients will receive an initial mitapivat dose of 50 mg BID.
- At Week 6, patients may receive dose escalation to mitapivat 100 mg BID on the basis of safety and Hb levels.
- Patients will not receive dose escalation if:
  - They have achieved an Hb increase from baseline to 12 g/dL (women) or 13 g/dL (men); and/or
  - They have experienced any grade ≥3 treatment-emergent adverse events (AEs) deemed related to study drug.

### *Extension period*

- Patients who complete the 24-week core period and achieve Hb response with an acceptable safety profile may continue mitapivat treatment for an additional 2 years in the extension period following confirmation by the sponsor's medical monitor.

### *Key study endpoints*

#### *Primary*

- **Hb response:** ≥1.0 g/dL increase in Hb from baseline in at least one assessment (Weeks 4–12).

#### *Secondary*

- **Mean change from baseline Hb** between Weeks 12 and 24.
- **Sustained Hb response:** ≥1.0 g/dL increase in Hb at two or more evaluable assessments (Weeks 12–24).
- For subjects who did not reach the primary endpoint, **delayed Hb response** of ≥1.0 g/dL increase at one or more assessments after Week 12.
- **AEs**, **serious AEs**, and AEs leading to dose reduction, interruption, or discontinuation.
- **Markers of hemolysis:** reticulocyte count, bilirubin, lactate dehydrogenase, and haptoglobin.
- **Markers of erythropoietic activity:** nucleated RBC, erythropoietin, and soluble transferrin receptor.

#### *Exploratory*

- Additional markers of erythropoietic activity: growth differentiation factor −15 and −11, non–transferrin-bound iron, and erythroferrone (iron panel also includes: iron, serum ferritin, total iron binding capacity, transferrin saturation, hepcidin, and C-reactive protein).
- Markers of oxidative stress: urinary 8-isoprostane, methylmalonic acid, and total homocysteine.
- Pharmacokinetics/pharmacodynamics: ATP, 2,3-DPG, PK-R activity, PK-R protein levels, and PK-R flux assay.

#### *Statistics*

- With a total of 17 patients enrolled, the study would have 80% power to reject a 30% null response rate at a one-sided 0.05 type I error rate if the true response rate were 60%.

## SUMMARY

- Mitapivat is an oral, small-molecule allosteric activator of WT and mutant PK-R
- Thalassemic RBCs have reduced ATP levels.
- Mitapivat may improve RBC survival in thalassemia by increasing ATP production.
- Mitapivat increased ATP and improved RBC parameters in a mouse model of β-thalassemia, and increased ATP levels *ex vivo* in human β-thalassemia RBCs.
- An ongoing, phase 2, open-label, multicenter study examines the effect of mitapivat on Hb in non–transfusion-dependent patients with thalassemia.
- The study is currently enrolling at four sites in the US, Canada, and the UK.

*Acknowledgments*
We would like to thank the patients who agreed to participate in this study, and Lucia De Franceschi (University of Verona–AOUI Verona) and Richard van Wijk (University Medical Center Utrecht) for use of their research on this poster.

*Disclosures*
This study was funded by Agios Pharmaceuticals, Inc.
KHMK: Agios, Aceleo, Bluebird Bio, Celgene, Pfizer – consultant; Avalon Novartis – consultant, honoraria; Bioverativ – data safety monitoring board member; DML Agios, Novartis – consultant and advisory board member; Celyad – data safety monitoring board member; KU, ML, CK, and LL: Agios – employment and stockholder; EPV: GBT, Pfizer, Novartis, Bluebird Bio, Agios – consultant and research funding.
Editorial assistance was provided by Mark Painter, Cardi Medical Affairs, Fairfield, CT, USA, and supported by Agios.

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*Presented at the 61st American Society of Hematology (ASH) Annual Meeting, December 7–10, 2019, Orlando, FL, USA*

Ex. 2001-0928

**Results:** Of the 336 pts randomized as part of the BELIEVE trial, 103 had genotype $\beta^0/\beta^0$, 87 had genotype $\beta^0/\beta^+$, 84 had genotype $\beta^+/\beta^+$, 9 had ≥1 unmutated β (includes β/β, $\beta^+/\beta$, $\beta^0/\beta$), and 52 had HbE/β-thalassemia.
The primary endpoint (≥33% reduction in RBC transfusion burden in Weeks 13–24, with a reduction of ≥2 units) was achieved with luspatercept vs PBO treatment by 9/68 (13.2%) vs 2/35 (5.7%; P = 0.271) b0/b0 pts, 15/59 (25.4%) vs 1/28 (3.6%; P = 0.015) b0/b+ pts, 16/58 (27.6%) vs 0/26 (P = 0.004) b+/b+ pts, and 6/31 (19.4%) vs 2/21 (9.5%; P = 0.341) HbE/b-thalassemia pts.
In the luspatercept arm, ≥33% reduction in transfusion burden during any 24 weeks was achieved by 26/68 (38.2%) $b^0/b^0$ pts (vs 1/35 [2.9%] PBO; P<0.0001), 23/59 (39.0%) b0/b+ pts (vs 0/28 PBO; P = 0.0001), 27/58 (46.6%) b+/b+ pts (vs 0/26 PBO; P<0.0001), and 12/31 (38.7%) HbE/b-thalassemia pts (vs 1/21 PBO [4.8%]; P = 0.007) (May 11, 2018 cutoff). With luspatercept treatment, 7/68 (10.3%) $b^0/b^0$ pts (vs 1/35 [2.9%] PBO; P = 0.193), 9/59 (15.3%) b0/b+ pts (vs 0/28 PBO; P = 0.031), 10/58 (17.2%) b+/b+ pts (vs 0/26 PBO; P = 0.028), and 8/31 (25.8%) HbE/b-thalassemia pts (vs 0/21 PBO; P = 0.013) achieved ≥50% reduction in transfusion burden during any 24 weeks.
Least squares mean change (standard error) in RBC units transfused from baseline in Weeks 13–24 in luspatercept vs PBO arms, respectively, were −0.48 (0.205) vs 0.85 (0.288) for $b^0/b^0$, −0.72 (0.273) vs 0.74 (0.395) for $b^0/b^+$, −0.90 (0.253) vs 0.71 (0.354) for $b^+/b^+$, and −0.69 (0.416) vs 0.26 (0.469) for HbE/b-thalassemia pts.
Grade ≥3 treatment-emergent adverse events (TEAEs; ≥1) were reported in 18/68 (26.5%), 17/58 (29.3%), 14/58 (24.1%), and 14/31 (45.2%) luspatercept-treated $b^0/b^0$, $b^0/b^+$, $b^+/b^+$, and HbE/b-thalassemia pts, respectively. The incidence of specific adverse events was consistent with the overall population.
**Summary/Conclusion:** Although response rates were lower in pts with the most severe disease ($\beta^0/\beta^0$), clinically meaningful reductions in transfusion burden were observed across genotypes.

## S296 IMPROVEMENT IN ERYTHROPOIESIS IN PATIENTS WITH TRANSFUSION-DEPENDENT B-THALASSEMIA FOLLOWING TREATMENT WITH BETIBEGLOGENE AUTOTEMCEL (LENTIGLOBIN FOR B-THALASSEMIA) IN THE PHASE 3 HGB-207 STUDY

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**Background:** Transfusion-dependent β-thalassemia (TDT) is a severe genetic disease requiring lifelong red blood cell (RBC) transfusions and regular iron chelation. Transfusions temporarily relieve anemia, but do not restore normal erythropoiesis. The goal of betibeglogene autotemcel (beti-cel; LentiGlobin for β-thalassemia; autologous CD34+ cells encoding $\beta^{A\text{-}T87Q}$-globin gene) is to correct ineffective erythropoiesis and enable lifelong production of functional adult hemoglobin (Hb), thereby enabling patients to achieve transfusion independence.
**Aims:** To assess the change in ineffective erythropoiesis following treatment with beti-cel in the ongoing phase 3 HGB-207 study (Northstar-2; NCT02906202) in patients with TDT and non-$\beta^0/\beta^0$ genotypes.
**Methods:** Patients received G-CSF and plerixafor to mobilize CD34+ cells which are collected via apheresis. Cells are then transduced with BB305 lentiviral vector and infused into patients following single-agent, pharmacokinetic-adjusted, busulfan myeloablation. The primary endpoint is transfusion independence (TI; weighted average Hb ≥9 g/dL without RBC transfusions for ≥12 months). Improvement of ineffective erythropoiesis was also evaluated. Patients are followed for 2 years and offered participation in a long-term follow-up study. Data are presented as median (min-max).
**Results:** As of 12 June 2019, 21 patients were treated and followed for 11.6 (0.9–26.3) months. The median age at enrollment was 15 (8–34) years; 6 patients were <12 years old. All patients engrafted. Ten patients with sufficient follow-up were evaluable for TI and 9 of these patients achieved transfusion independence for an ongoing duration of 15.2 (12.1–21.3) months. The weighted average Hb during TI was 12.2 (11.4–12.8) g/dL.
At Month 12, the 9 patients who achieved transfusion independence had improved bone marrow cellularity compared to baseline (44% vs 11% normocellular) and improved myeloid:erythroid ratios (baseline: 1:7.3–1:1.4; Month 12: 1:1.6–1.9:1). Seven of 9 patients had reticulocyte counts within normal range vs 44% (4/9) at baseline. Soluble transferrin receptor levels decreased from 171.8 (66–235) nmol/L at baseline to 49.4 (34–68) nmol/L with 78% (7/9) of patients within normal range at last follow-up (vs 44% [4/9] at baseline). Erythropoietin levels in patients achieving TI decreased 61.4% from 33.2 (10.8–73.4) U/L at baseline (n = 9) to 12.8 (4.9–21.8) U/L at Month 12 (n = 7). Finally, the hepcidin:ferritin ratio improved from 0.01 (0.00–0.06) at baseline (n = 9) to 0.03 (0.01–0.4) at Month 12 (n = 9).
Post-infusion non-hematologic grade ≥3 treatment-emergent adverse events (AEs) reported in ≥3 patients included stomatitis (n = 12), febrile neutropenia (n = 7), epistaxis (n = 3), pyrexia (n = 3), and veno-occlusive liver disease (n = 3). Adverse events considered related to beti-cel by the investigator included thrombocytopenia (n = 2, one event was serious), abdominal pain (n = 1), and pain in extremity (n = 1). There were no deaths and no evidence of insertional oncogenesis or clonal dominance. Updated results with outcomes from all 23 patients treated in the HGB-207 study will be presented.
**Summary/Conclusion:** Following treatment with betibeglogene autotemcel (beti-cel; LentiGlobin for β-thalassemia) gene therapy, 9/10 patients with TDT and non-$\beta^0/\beta^0$ genotypes achieved transfusion independence. These patients showed improvements in erythropoiesis, suggesting that the the bone marrow is in process of normalizing. The treatment regimen comprised of conditioning and beti-cel infusion had a tolerability profile consistent with the known effects of busulfan myeloablation.

## S297 PROOF OF CONCEPT FOR THE ORAL PYRUVATE KINASE ACTIVATOR MITAPIVAT IN ADULTS WITH NON–TRANSFUSION-DEPENDENT THALASSEMIA: INTERIM RESULTS FROM AN ONGOING, PHASE 2, OPEN-LABEL, MULTICENTER STUDY

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**Background:** Thalassemias are characterized by compromised red blood cell (RBC) survival due to mutations in the genes encoding α- and/or β-globins. Imbalanced globin chain production results in excess β- or α-globin precipitation, reduced RBC fitness, and ineffective erythropoiesis and hemolysis. ATP levels are reduced in thalassemic RBCs, despite increased energy demands to support clearance of globin precipitates and maintenance of cell membrane integrity. Mitapivat sulfate (AG-348) is a first-in-class, small-molecule, oral activator of RBC pyruvate kinase (PK-R), a key enzyme regulating ATP production via glycolysis. Glycolysis is the primary means of RBC energy generation. Increasing ATP synthesis via PK-R activation by mitapivat may improve thalassemic RBC fitness and survival, thus ameliorating anemia and other clinical sequelae. In a phase 2 study in patients (pts) with PK deficiency caused by mutations in PK-R, mitapivat increased hemoglobin (Hb) by >1.0 g/dL in 50% of pts (Grace et al. *NEJM* 2019;381:933). Studies in mouse β-thalassemia models and healthy adults have suggested benefits from activating wild-type PK-R.
**Aims:** To report interim results from an ongoing study of PK-R activation by mitapivat in adults with non–transfusion-dependent thalassemia (NTDT).
**Methods:** This open-label, phase 2 study (EudraCT 2018-002217-35/NCT03692052) evaluates the efficacy, safety, pharmacokinetics, and pharmacodynamics of mitapivat in adults with NTDT in a 24-week core treatment period plus optional 2-year extension. Key inclusion criteria: presence of β-thalassemia ± α-globin gene mutations, HbE



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β-thalassemia, or α-thalassemia (HbH disease); Hb ≤10.0 g/dL; ≤5 RBC units transfused in the preceding 24 weeks and none in the 8 weeks prior to study drug. Pts receive mitapivat 50 mg orally twice daily (BID) for 6 weeks; dosage may increase at week 6 to 100 mg BID, depending on safety. Primary endpoint: proportion of pts who achieve a Hb response (increase of ≥1.0 g/dL from baseline at any time between weeks 4–12, inclusive). Key secondary/exploratory endpoints include sustained or delayed Hb response; markers of hemolysis; hematopoietic activity; iron metabolism and overload; safety and pharmacokinetics.

**Results:** Nine pts (5 female), all with β-thalassemia, had received mitapivat as of 14Nov2019 (data cut). Mean age was 46 years (range 31–57) and mean baseline Hb was 7.54 g/dL (SD 1.35). All pts received 100 mg BID at week 6; at data cut, 8 pts had completed 12 weeks of treatment and were evaluable for the primary endpoint. Seven of these (87.5%, 90% CI 52.9, 99.4) achieved a Hb increase from baseline of ≥1.0 g/dL after a mean of 3.5 weeks (range 1.4–7.1). The mean Hb increase of responders was 1.76 g/dL (range 0.9–3.3) during weeks 4–12. Responders showed directionally favorable changes in markers of erythropoiesis and hemolysis. The safety profile was consistent with previously published mitapivat studies. There were no serious adverse events (AEs), and no pts discontinued owing to AEs. AEs occurring in ≥2 pts were insomnia, diarrhea, dyspepsia, fatigue, dizziness, cough, headache, upper respiratory tract infection, nasal congestion, and back pain. Additional pts have received mitapivat since the data cut, including pts with α-thalassemia; updated results will be presented.

**Summary/Conclusion:** These data establish proof of concept that activation of wild-type PK-R by mitapivat has potential clinical benefit in thalassemia, and support its further investigation as an oral treatment for pts with β- and α-thalassemia. This study is also the first drug trial aimed at treating anemia in α-thalassemia.

## S298 A HEPCIDIN MIMETIC, PTG-300, DEMONSTRATES PHARMACODYNAMIC EFFECTS INDICATING REDUCED IRON AVAILABILITY IN TRANSFUSION-DEPENDENT BETA-THALASSEMIA SUBJECTS

A. Lal[1], E. Voskaridou[2], P. Flevari [2], A. Taher[3], L. P. Chew[4], F. Valone[5], S. Gupta[5], V. Viprakasit[6]

*[1]University of California San Francisco, Oakland, United States, [2]Laiko General Hospital, Athens, Greece, [3]Chronic Care Center, Beirut, Lebanon, [4]Sarawak, General Hospital, Kutching, Malaysia, [5]Protagonist Therapeutics, Newark, United States, [6]Mahidol University, Bangkok, Thailand*

**Background:** β-thalassemia is an inherited hemoglobinopathy characterized by ineffective erythropoiesis and anemia. Restriction of iron availability by hepcidin mimetics is a potentially novel approach to treating β-thalassemia as evidenced in preclinical models. This study is designed to extend the findings that constraining iron availability improves endogenous hemoglobin synthesis observed in the preclinical models of β-thalassemia.

**Aims:** The purpose of this study was to assess the safety, tolerability, pharmacodynamics and efficacy of PTG-300, a hepcidin mimetic, in transfusion-dependent (TD) β-thalassemia subjects.

**Methods:** TD β-thalassemia subjects were eligible if they received at least 6 units of RBC in the 24 weeks prior to screening with no transfusion free period > 45 days. Subjects received weekly injections of PTG-300 subcutaneously for 16 weeks. Cohorts of 6 subjects planned were initiated in staggered fashion at PTG-300 doses of 3, 10, 20, 40, 80 mg weekly and 40 mg twice weekly. Doses could be increased after 8 weeks if a ≥20 percent reduction in transfusions was not achieved. In the first two weeks of dosing, blood samples were collected on Days 0, 1, 4, 7, 8, 11, and 14 and analyzed for the pharmacodynamic parameters of serum iron and transferrin saturation (TSAT). Thirty-five subjects were dosed after signing consent forms.

**Results:** PTG-300 was well-tolerated. Treatment-emergent adverse events (TEAE) were mostly mild to moderate in severity and were typical of subjects with TD β-thalassemia. TEAE were not dose-related and did not prevent cohort dose escalation. The most frequent TEAE was transient injection site erythema in 5 out of 35 subjects (14.3%). As shown in Table 1 below, there was a dose-related reduction in serum iron parameters with the maximum effect one day after dosing. Iron parameters largely returned to baseline 7 days post weekly dosing of PTG-300. Twice weekly dosing at 40 mg resulted in prolonged suppression of iron parameters.

Table 1. Mean Serum Iron and TSAT in the three highest dose cohorts

| | 40 mg Weekly (n = 7) | | 80 mg Weekly (n = 6) | | 40 mg Twice a Week (n = 6) | |
|---|---|---|---|---|---|---|
| Day | Iron (ug/dL) | TSAT (%) | Iron (ug/dL) | TSAT (%) | Iron (ug/dL) | TSAT (%) |
| 0 (pre-dose) | 242.4 | 95.3 | 237.0 | 99.5 | 173.5 | 77.0 |
| 1 | 134.1 | 54.1 | 102.7 | 44.2 | 64.8 | 29.7 |
| 7 (pre-dose) | 155.7* | 67.3* | 163.0 | 67.5 | 98.2 | 44.2 |
| 8 | 84.0 | 30.7 | 57.2* | 22.0* | 55.8 | 24.7 |
| 14 (pre-dose) | 169.6 | 73.7 | 189.3 | 73.3 | 68.8 | 30.2 |

*n = 6 for 40 mg/week on Day 7; n = 5 for 80 mg/week on Day 8.

Clinical responses to PTG-300, pre-specified as 20% or greater reduction in transfusions, were observed and the response rates are dose-related. The clinical meaningfulness of these reductions in transfusion still needs to be further evaluated.

**Summary/Conclusion:** Though preliminary, these results demonstrate the pharmacodynamic activity of PTG-300 in reducing TSAT and serum iron in subjects with TD β-thalassemia. It is too early to determine whether PTG-300 has clinically meaningful activity in subjects with transfusion-dependent β-thalassemia.

## S299 HEMATOPOIETIC STEM CELLS WITH ERYTHROID SIGNATURE IN BETA-THALASSEMIA

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**Background:** Beta-thalassemia (Bthal) is a genetic disorder due to mutations in the ß-globin gene, leading to a reduced or absent production of HbA, which interferes with erythroid cell maturation and limits normal red cell production. Patients are affected by severe anemia, hepatosplenomegaly, and skeletal abnormalities due to rapid expansion of the erythroid compartment in bone marrow (BM) caused by ineffective erythropoiesis. In a classical view of hematopoiesis, the blood cell lineages arise via a hierarchical scheme starting with multipotent stem cells that become increasingly restricted in their differentiation potential through oligopotent and then unipotent progenitors. In human, novel purification strategies based on differential expression of CD49f and CD90 enrich for long-term (49f+) and short-term (49f–) repopulating hematopoietic stem cells (HSCs), with distinct cell cycle properties, but similar myeloid (My) and lymphoid (Ly) potential. In this view, it has been proposed that erythroid (Ery) and megakaryocytic (Mk) fates branch off directly from CD90-/49f– multipotent progenitors (MPPs). Recently, a new study suggested that separation between multipotent (Ery/My/Ly) long-term repopulating cells (Subset1, defined as CLEC9A$^{high}$CD34$^{low}$) and cells with only My/Ly and no Ery potential (Subset2, defined as CLEC9A$^{low}$CD34$^{high}$) occurs within the phenotypic HSC/MPP and CD49f+ HSCs compartment.

**Aims:** A general perturbed and stress condition is present in the thalassemic BM microenvironment. Since its impact on the hematopoietic cell subpopulations is mostly unknown, we will investigate which model of hematopoiesis/erythropoiesis occurs in Bthal. Moreover, since Beta-Thalassemia is an erythropoietic disorder, it could be considered as a disease model to study the '*erythroid branching*' in the hematopoietic hierarchy.

**Methods:** We defined by immunophenotype and functional analysis the lineage commitment of most primitive HSC/MPP cells in patients affected by this pathology compared to healthy donors (HDs). Furthermore, in order to delineate the transcriptional networks governing hematopoiesis in Beta-thalassemia, RNAseq analysis was performed on sorted hematopoietic subpopulations from BM of Bthal patients and HDs.

**Results:** Differences in the primitive compartment were observed with an increased proportion of multipotent progenitors in Bthal patients compared to HDs. The Subset1 compartment is actually endowed with an enhanced Ery potential.

Focusing on progenitors (CD34$^+$CD38$^+$) and using a new sorting scheme that efficiently resolved My, Ery, and Mk lineage fates, we quantified the new My (CD71$^-$BAH1$^{-/+}$) and Ery (CD71$^+$ BAH1$^{-/+}$) subsets and found a reduction of Ery subset in Bthal samples. We can hypothesize that the



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# Proof of concept for the oral pyruvate kinase activator mitapivat in adults with non–transfusion-dependent thalassemia: Interim results from an ongoing, phase 2, open-label, multicenter study

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S297: New therapeutic approaches for thalassemia

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# Disclosures

This study was funded by Agios Pharmaceuticals, Inc. We would like to thank the patients taking part in this study.

**Kevin HM Kuo** – Agios, Apellis, Bluebird Bio, Celgene, Pfizer – consultant; Alexion, Novartis – consultant, honoraria; Bioverativ – data safety monitoring board member; Pfizer – research support. **D Mark Layton** – Agios, Novartis – consultant and advisory board member; Cerus – data safety monitoring board member. **Ashutosh Lal** – none. **Hanny Al-Samkari** – Agios, Dova, Moderna – consultant; Agios, Dova, Amgen – research funding. **Feng Tai, Megan Lynch,** and **Katrin Uhlig** – Agios – employment and stockholder. **Elliot P Vichinsky** – GBT, Pfizer, Novartis, Bluebird Bio, Agios – consultant and research funding

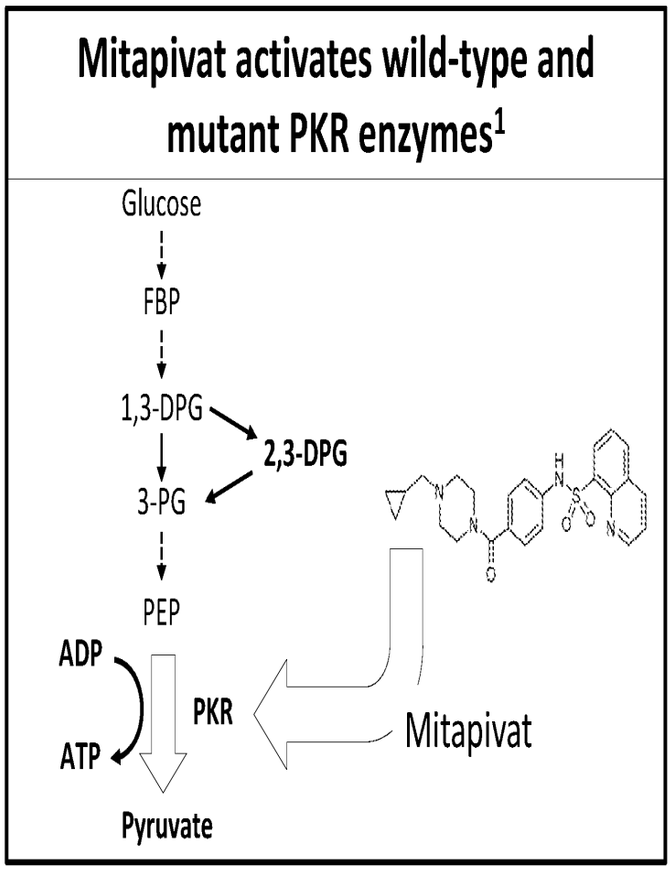
Editorial assistance was provided by Christine Ingleby, PhD, CMPP, Excel Medical Affairs, Horsham, UK, and supported by Agios

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# Mitapivat

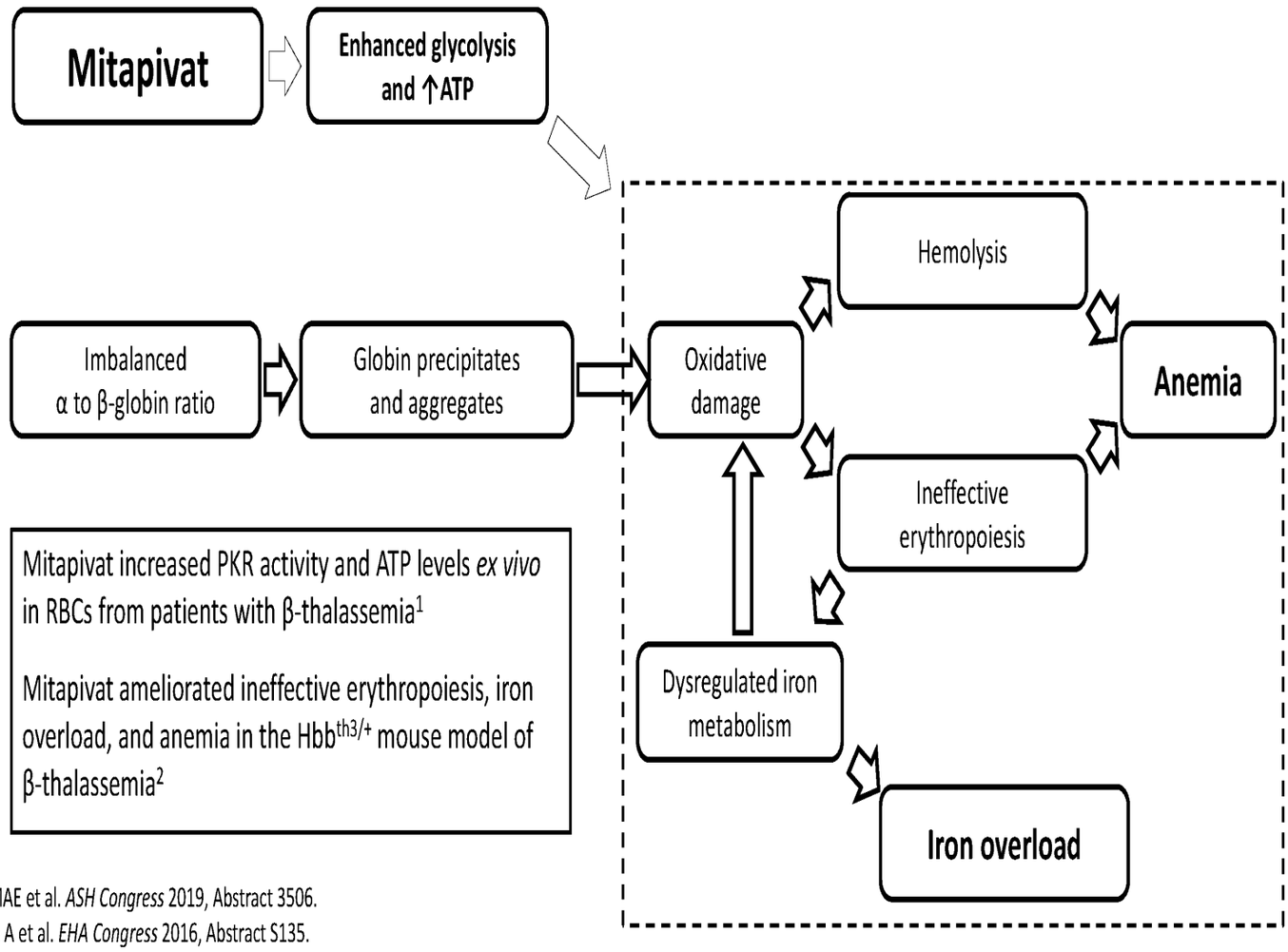
- Mitapivat (AG-348) is an investigational, first-in-class, oral, small-molecule allosteric activator of pyruvate kinase (PK)R[1], which catalyzes the final step of glycolysis in RBCs[2]
- Mitapivat increased RBC ATP levels by 60% in healthy volunteers[3]
- In a phase 2 study in adult patients with PK deficiency, BID dosing with mitapivat:
  - Increased hemoglobin (Hb) by > 1.0 g/dL in 50% of patients[4]
  - Was well tolerated for up to 42 months[5]




ATP = adenosine triphosphate; BID = twice daily; RBC = red blood cell

**1.** Kung C et al. *Blood* 2017;130:1347. **2.** Valentini G et al. *J Biol Chem* 2002;277:23807. **3.** Yang H et al. *Clin Pharmacol Drug Dev* 2019;8:246.
**4.** Grace RF et al. *NEJM* 2019;381:933. **5.** Grace RF et al. *EHA Congress* 2020, Abstract EP1561

# Hypothesis: Mitapivat mechanism in thalassemia




Mitapivat increased PKR activity and ATP levels *ex vivo* in RBCs from patients with β-thalassemia[1]

Mitapivat ameliorated ineffective erythropoiesis, iron overload, and anemia in the $Hbb^{th3/+}$ mouse model of β-thalassemia[2]

**1.** Rab MAE et al. *ASH Congress* 2019, Abstract 3506.
**2.** Matte A et al. *EHA Congress* 2016, Abstract S135.

# Study design: Open-label, phase 2, multicenter study

## Key Inclusion Criteria

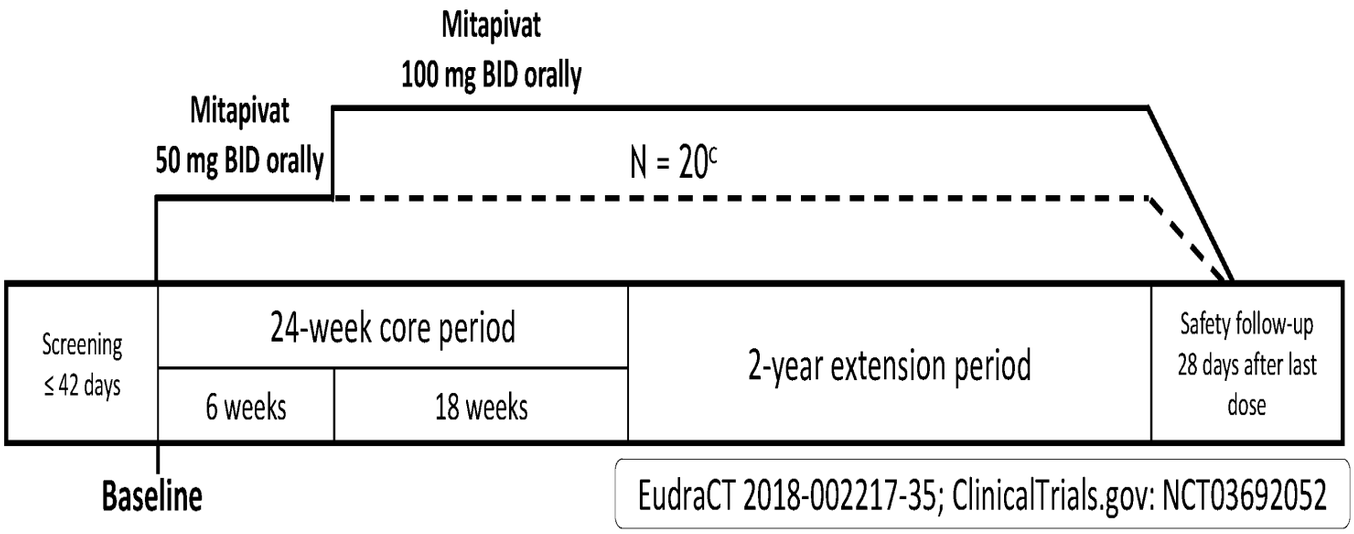
- β-thalassemia ± α-globin gene mutations, HbE β-thalassemia, or α-thalassemia (HbH disease)
- Hb ≤ 10.0 g/dL
- Non–transfusion-dependent[a]

## Primary Endpoint[b]

- Hb response, defined as increase of ≥ 1.0 g/dL from baseline at any time between weeks 4–12, inclusive

## Secondary/Exploratory Endpoints

- Sustained Hb response; delayed Hb response; markers of hemolysis; hematopoietic activity; safety



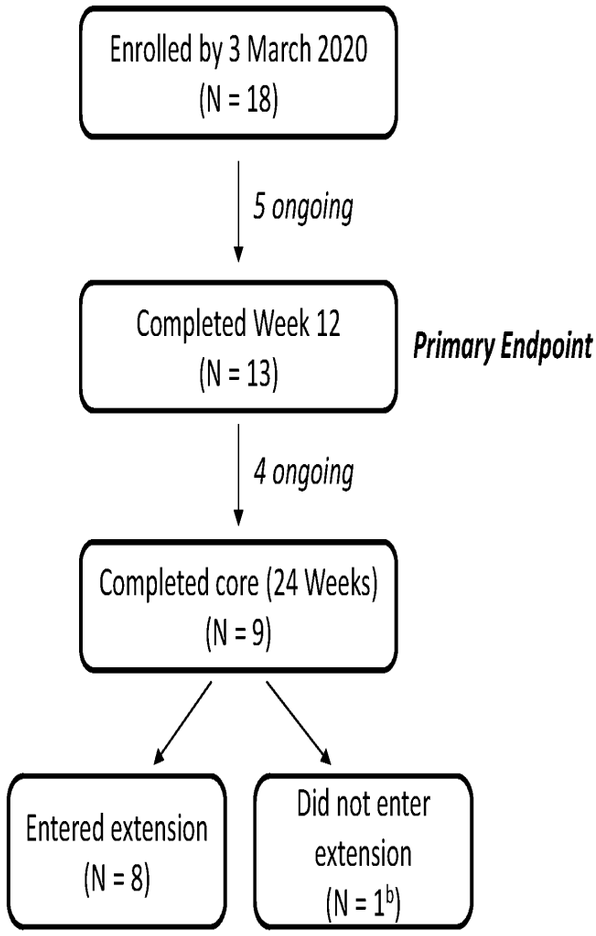

[a]≤ 5 RBC units transfused in the preceding 24 weeks and none in the 8 weeks prior to study drug. [b]With the originally planned sample size of 17 patients enrolled, the study would have 80% power to reject a ≤ 30% response rate at a one-sided 0.05 type 1 error rate. [c]Fully enrolled. BID = twice daily

# Demographics and disposition

| Baseline characteristics | Total (N = 18) |
|---|---|
| Median (range) duration of treatment, weeks | 20.6 (1.1–50.0) |
| Male/female, n | 5/13 |
| Age at informed consent, median (range), years | 43.5 (29–67) |
| Race, n (%) Asian | 9 (50.0) |
| White | 4 (22.2) |
| Native Hawaiian or other Pacific Islander | 1 (5.6) |
| Other[a] | 4 (22.2) |
| Thalassemia type, n (%) α | 5 (27.8) |
| β | 13 (72.2) |
| Hb baseline, median (range), g/dL | 8.43 (5.6–9.8) |
| Indirect bilirubin, median (range), mg/dL | 1.17 (0.31–5.52) |
| Lactate dehydrogenase, median (range), U/L | 249 (126–513) |
| Erythropoietin, median (range), mU/mL | 70.5 (15–11,191) |

Splenectomy and prior transfusions were reported in two patients each at baseline

[a]Includes patients who reported more than one category, and one not reported. [b]Investigator decision



# Key efficacy results

**Primary endpoint was met in 92.3% of patients**

| Endpoint | Genotype | N/N | % | 90% CI |
|---|---|---|---|---|
| Hb responders during weeks 4–12 among those who completed 12 weeks | All | 12/13 | 92.3 | 68.4, 99.6 |
| | α | 4/4 | 100 | 47.3, 100 |
| | β | 8/9 | 88.9 | 57.1, 99.4 |
| Hb responders during weeks 12–24 among those who completed 24 weeks | β[a] | 8/9 | 88.9 | 57.1, 99.4 |
| Sustained responders: primary response and ≥ 2 Hb responses during weeks 12–24 | β[a] | 7/8 | 87.5 | 52.9, 99.4 |

Hb responder defined as a ≥ 1.0 g/dL Hb increase from baseline at least once

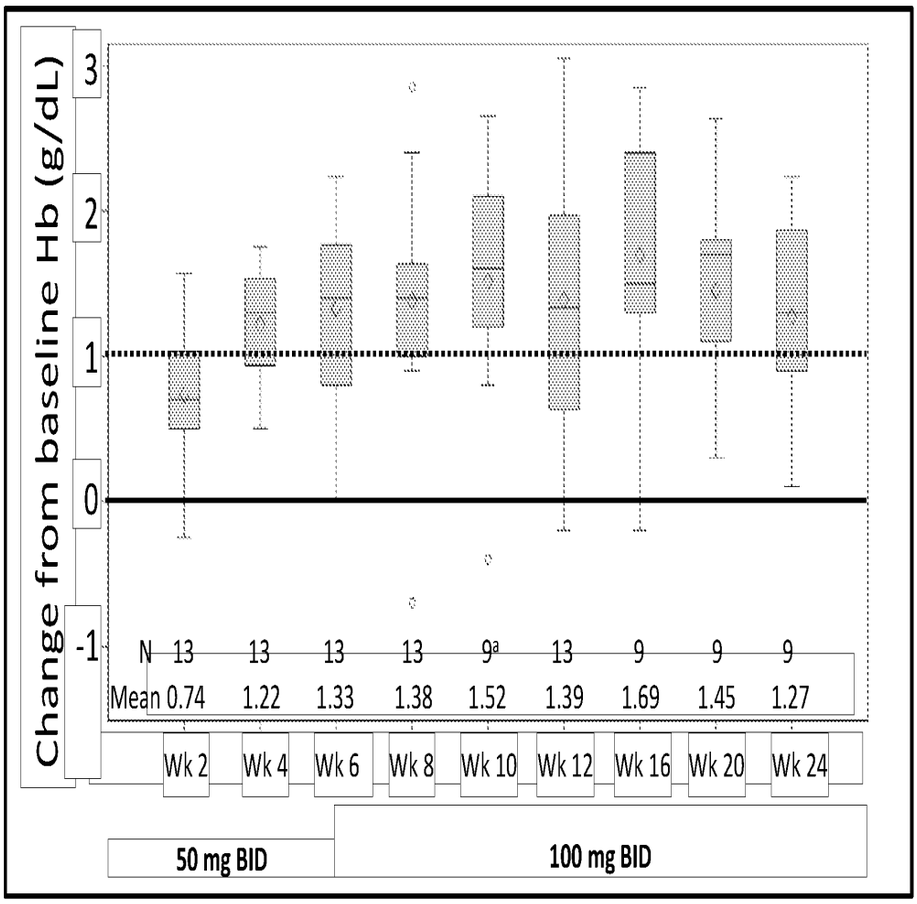
[a]Only patients with β-thalassemia had completed 24 weeks of treatment at the time of datacut

# Hemoglobin change from baseline

| Patient population | N | Weeks | Mean (SD) change from baseline Hb, g/dL |
|---|---|---|---|
| All patients | 13 | 4–12 | 1.34 (0.7) |
| α-thalassemia | 4 | 4–12 | 1.17 (0.4) |
| β-thalassemia | 9 | 4–24 | 1.43 (0.8) |

**Median (range) time to Hb increase of ≥ 1 g/dL among responders was 3.1 (1.4–7.1) weeks**

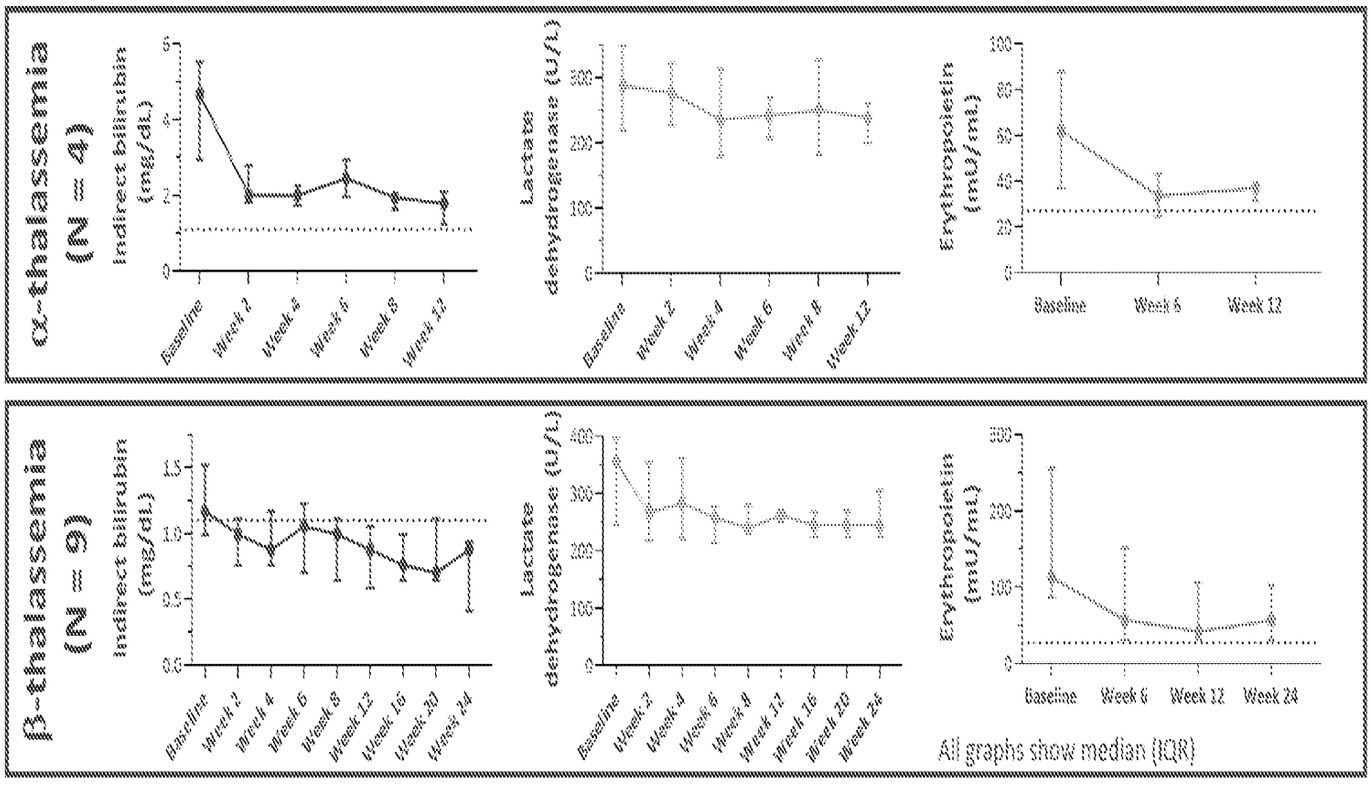
# Hemoglobin change over time




Solid blue line indicates baseline, dashed blue line indicates Hb change required for primary endpoint. Boxes represent interquartile range, lines indicate medians, diamonds indicate means, whiskers and outliers (circles) calculated with Tukey's method. [a]4 patients were not evaluated at Week 10 due to a protocol amendment eliminating this visit
Wk = week

# Markers of hemolysis and erythropoiesis

- The improvements in these markers correlated with the Hb increases




Dashed lines indicate upper limit of normal range. For α-thalassemia: N = 4 for lactate dehydrogenase and erythropoietin, for indirect bilirubin N = 3 at baseline, weeks 2, 8 and 12, and N = 2 at weeks 4 and 6; for β-thalassemia: N = 9 for erythropoietin, for lactate dehydrogenase N = 9 at baseline, weeks 6, 8, 12, and 20 and N = 8 at weeks 2, 4, 16, and 24, for indirect bilirubin N = 9 at baseline and N = 7 at the remaining times. IQR = interquartile range (25$^{th}$–75$^{th}$ centiles)

# ATP change with mitapivat

- Mean ATP percent increase from baseline was similar to that previously observed with mitapivat in healthy volunteers[1]

| Treatment dose | Visit | Mean (CV%) ATP change from baseline in blood, % |
|---|---|---|
| 50 mg | Week 6 (n = 9) | 82.7 (85.8) |
| 100 mg | Week 8 (n = 12) | 76.8 (62.7) |
| 100 mg | Week 12 (n = 12) | 86.7 (68.7) |
| 100 mg | Week 24 (n = 5) | 92.3 (71.6) |

**1.** Yang H et al. *Clin Pharmacol Drug Dev* 2019;8:246.

CV% = coefficient of variation, percent

# Safety summary[a]

| | Total (N = 18) |
|---|---|
| Patients with any AE, n (%) | 13 (72.2) |
| Patients with any related AE, n (%) | 11 (61.1) |
| Patients with AEs by maximum severity, n (%) | |
| Grade 1 | 4 (22.2) |
| Grade 2 | 7 (38.9) |
| Grade 3[b] | 2 (11.1) |

AEs coded using MedDRA, version 22.0

[a]As of datacut of 3 March 2020

[b]Neither were judged related

- There were no serious adverse events (AEs) and no AEs leading to treatment discontinuation as of the datacut
- Dose escalation to 100 mg BID was well tolerated and not associated with an increase in AEs
- Reported in one patient each:
  - AE leading to treatment interruption (grade 3, postural vertigo, not related)
  - AE leading to treatment modification (grade 2, bloating and heartburn, related)
- Post-datacut, one serious AE of renal dysfunction was reported, which resolved upon treatment discontinuation (grade 3, judged related by investigator)

# Most common AEs[a]

| AEs in ≥ 2 patients, number of patients (%) | Total (N = 18) |
|---|---|
| Insomnia | 8 (44.4) |
| Dizziness | 5 (27.8) |
| Cough | 4 (22.2) |
| Dyspepsia | 4 (22.2) |
| Fatigue | 4 (22.2) |
| Headache | 4 (22.2) |
| Nasal congestion | 4 (22.2) |
| Nausea | 4 (22.2) |
| Upper respiratory tract infection | 4 (22.2) |
| Abdominal distension | 3 (16.7) |
| Diarrhea | 3 (16.7) |
| Ocular icterus | 3 (16.7) |
| Oropharyngeal pain | 3 (16.7) |
| Pain | 3 (16.7) |
| Abdominal pain upper | 2 (11.1) |
| Back pain | 2 (11.1) |
| Pain in extremity | 2 (11.1) |
| Pyrexia | 2 (11.1) |
| Rash | 2 (11.1) |

- The safety profile was consistent with prior studies in healthy volunteers and patients with PK deficiency

[a]As of datacut of 3 March 2020
AEs coded using MedDRA, version 22.0

# Conclusions

- This is the first clinical study evaluating PKR activation as a therapeutic option in α- and β-thalassemia, and is the first drug trial aimed at treating α-thalassemia
- Proof-of-concept was demonstrated
  - > 90% of patients met the primary endpoint showing a clinically significant Hb increase
  - All four α-thalassemia patients and eight of nine β-thalassemia patients were responders
  - A sustained Hb response was observed over time in patients with longer follow-up
  - Improvements in markers of hemolysis and erythropoiesis were consistent with mitapivat's mechanism of action
- Mitapivat was generally well tolerated; the safety profile was consistent with previous studies

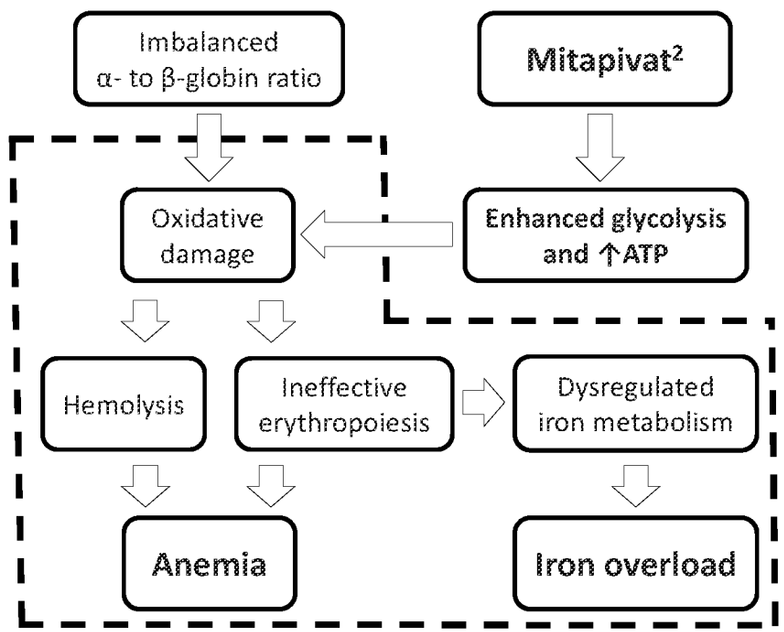
***These data indicate that activation of wild-type PKR by the oral agent mitapivat improved Hb and associated markers of hemolysis and erythropoiesis in patients with both α-and β-thalassemia, and that further investigation is warranted. Pivotal trials are in development.***

## S297: Proof of concept for the oral pyruvate kinase activator mitapivat in adults with non–transfusion-dependent thalassemia: Interim results from an ongoing, phase 2, open-label, multicenter study[1]

Kevin HM Kuo, D Mark Layton, Ashutosh Lal, Hanny Al-Samkari, Feng Tai, Megan Lynch, Katrin Uhlig, Elliot P Vichinsky

- 92.3% (12/13) of patients met the primary endpoint of a hemoglobin (Hb) increase of ≥ 1.0 g/dL from baseline at any time between weeks 4–12:
  - 4/4 patients with α-thalassemia and 8/9 with β-thalassemia met the primary endpoint
  - A sustained Hb response was observed over time in patients with longer follow-up
  - Mean change from baseline Hb in all patients (n = 13) was 1.34 g/dL
- Improvements in markers of hemolysis and erythropoiesis were demonstrated
- Mitapivat was generally well tolerated and the safety profile was consistent with previous studies[3–5]

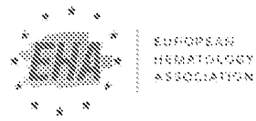
Proof of concept in α- and β-thalassemia was demonstrated, warranting further investigation in pivotal trials




**1.** EudraCT 2018-002217-35; ClinicalTrials.gov NCT03692052. **2.** Kung C et al. *Blood* 2017;130:1347. **3.** Yang H et al. *Clin Pharmacol Drug Dev* 2019;8:246. **4.** Grace RF et al. *NEJM* 2019;381:933. **5.** Grace RF et al. *EHA Congress* 2020, Abstract EP1561.

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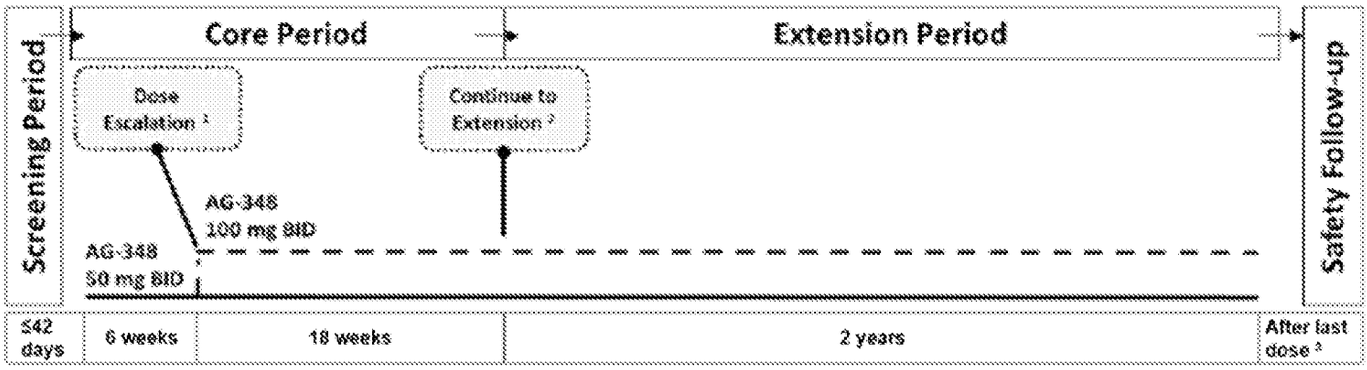
**Title**

An ongoing, open-label, multicenter study with the oral pyruvate kinase activator mitapivat (AG-348) in adults with non-transfusion-dependent thalassemia

**Case**

**Background**: Thalassemia is a group of hereditary blood disorders associated with hemolysis and ineffective erythropoiesis. Genetic mutation(s) in the α- and/or β-globin locus lead to excess precipitation of β- or α-globin, respectively, and compromise the survival of red blood cells (RBC). Adenosine triphosphate (ATP) supply appears to be insufficient in thalassemic RBCs to maintain RBC membrane integrity and clearance of globin precipitates. Mitapivat (AG-348) is an oral, small-molecule, allosteric activator of the RBC- form of pyruvate kinase (PK-R). PK-R is a key enzyme for maintaining energy homeostasis in RBCs, as they rely almost exclusively on the process of glycolysis to generate ATP. In healthy adults, mitapivat activates wild-type PK-R and increases ATP levels in RBCs. In adults with PK deficiency who do not receive regular transfusions, mitapivat was well tolerated and induced rapid, sustained hemoglobin (Hb) increases with improvement in markers of hemolysis (NCT02476916). In the $Hbb^{th/3+}$ mouse model of β-thalassemia, mitapivat increased ATP levels; reduced markers of ineffective erythropoiesis; and improved anemia, RBC survival, and indices of iron overload. These data support the hypothesis that increased ATP synthesis mediated via PK-R activation by mitapivat may improve the survival of thalassemic RBCs in the bone marrow and/or peripheral circulation, and thus represents a novel mechanism for potential treatment of patients with thalassemia. Here we present the design of a phase 2, open-label study designed to test this hypothesis in patients with thalassemia.
**Methods**: This phase 2, multicenter, open-label study (EudraCT 2018-002217-35 and NCT03692052) is evaluating the efficacy, safety, pharmacokinetics, and pharmacodynamics of mitapivat in adults with non-transfusion-dependent thalassemia (NTDT). One site each in Canada, the United Kingdom, and the United States are currently enrolling patients. The study consists of a 24-week core period followed by a 2-year extension period (Figure). Approximately 17 subjects with NTDT, i.e., β-thalassemia with or without α-globin gene mutations, HbE β-thalassemia, or α-thalassemia (HbH disease), will be enrolled. Non-transfusion dependence is defined as ≤5 units of RBCs transfused in the preceding 24 weeks and no transfusions in the 8 weeks prior to the first day of study drug. The hemoglobin inclusion criterion is ≤10.0 g/dL. All eligible patients will receive an initial mitapivat dose of 50 mg twice daily (BID). At the week 6 visit the dose may be increased to 100 mg BID, depending on safety and Hb response. The primary endpoint is the proportion of subjects who achieve an Hb response, defined as an increase in Hb of ≥1.0 g/dL from baseline at any time between week 4 and week 12 (inclusive). Key secondary and exploratory endpoints include changes in Hb and markers of hemolysis; hematopoietic activity; iron metabolism and iron overload; and assessments of safety and pharmacokinetics. The study is currently enrolling.

## Study Schema




BID = twice daily; d = days.
[1] Subjects may undergo an intrasubject dose escalation at the Week 6 visit based on an evaluation of safety and hemoglobin concentration (as detailed in the body of the protocol).
[2] Subjects who complete the 24-week Core Period and achieve a hemoglobin response with an acceptable safety profile may continue study drug for an additional 2 years in the Extension Period.
[3] The safety follow-up visit will occur 28 days after the subject's last dose of study drug.

**References**

This trial is registered at EudraCT 2018-002217-35 and at clinicaltrials.gov NCT03692052.

## Topic

Thalassemias

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### Co-authors

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# An Ongoing, Open-label, Multicenter Study With the Oral Pyruvate Kinase Activator Mitapivat (AG-348) in Adults With Non–Transfusion-Dependent Thalassemia

Kevin H. M. Kuo[1], D. Mark Layton[2], Katrin Uhlig[3], Megan Lynch[3], Li Liu[3], Elliott Vichinsky[4]

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# Affiliations and disclosures

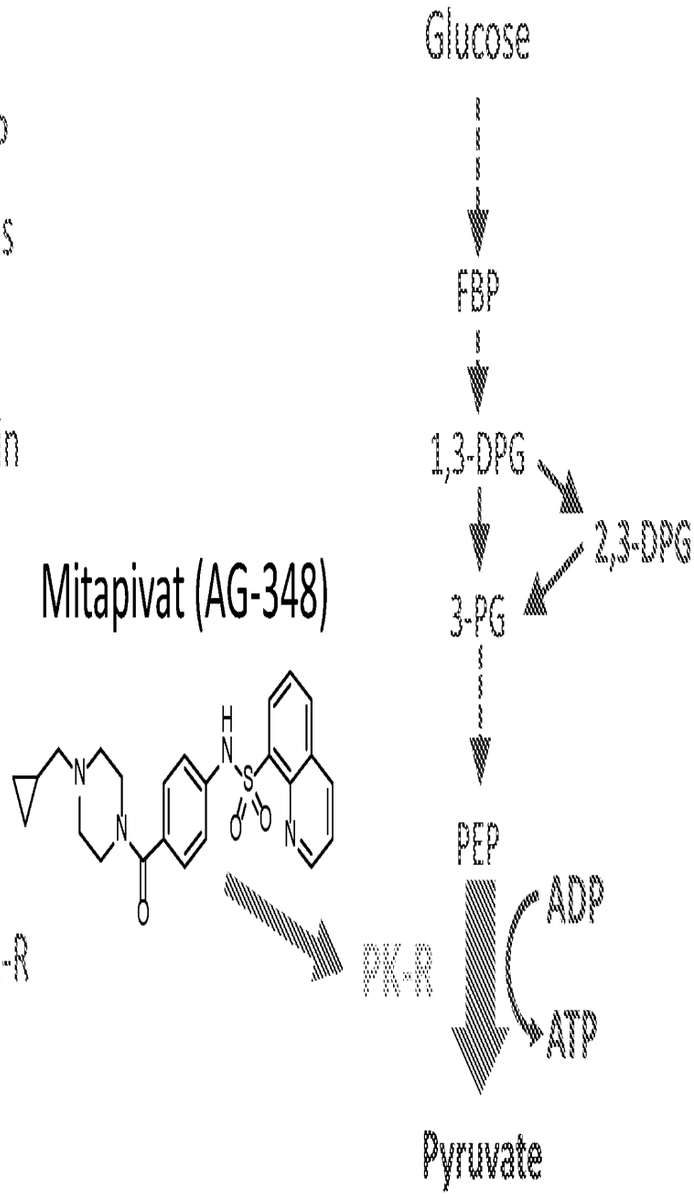
This study is funded by Agios Pharmaceuticals, Inc.

- Kevin H. M. Kuo: Toronto General Hospital, University Health Network, Toronto, ON, Canada. **Agios, Apellis, Bluebird Bio, Celgene, Pfizer – consultancy; Alexion, Novartis – consultancy, honoraria; Bioverativ – data safety monitoring board**

- Editorial assistance was provided by Mark Poirier, Excel Medical Affairs, Fairfield, CT, USA, and supported by Agios

# Pyruvate kinase (PK) and the PK activator mitapivat

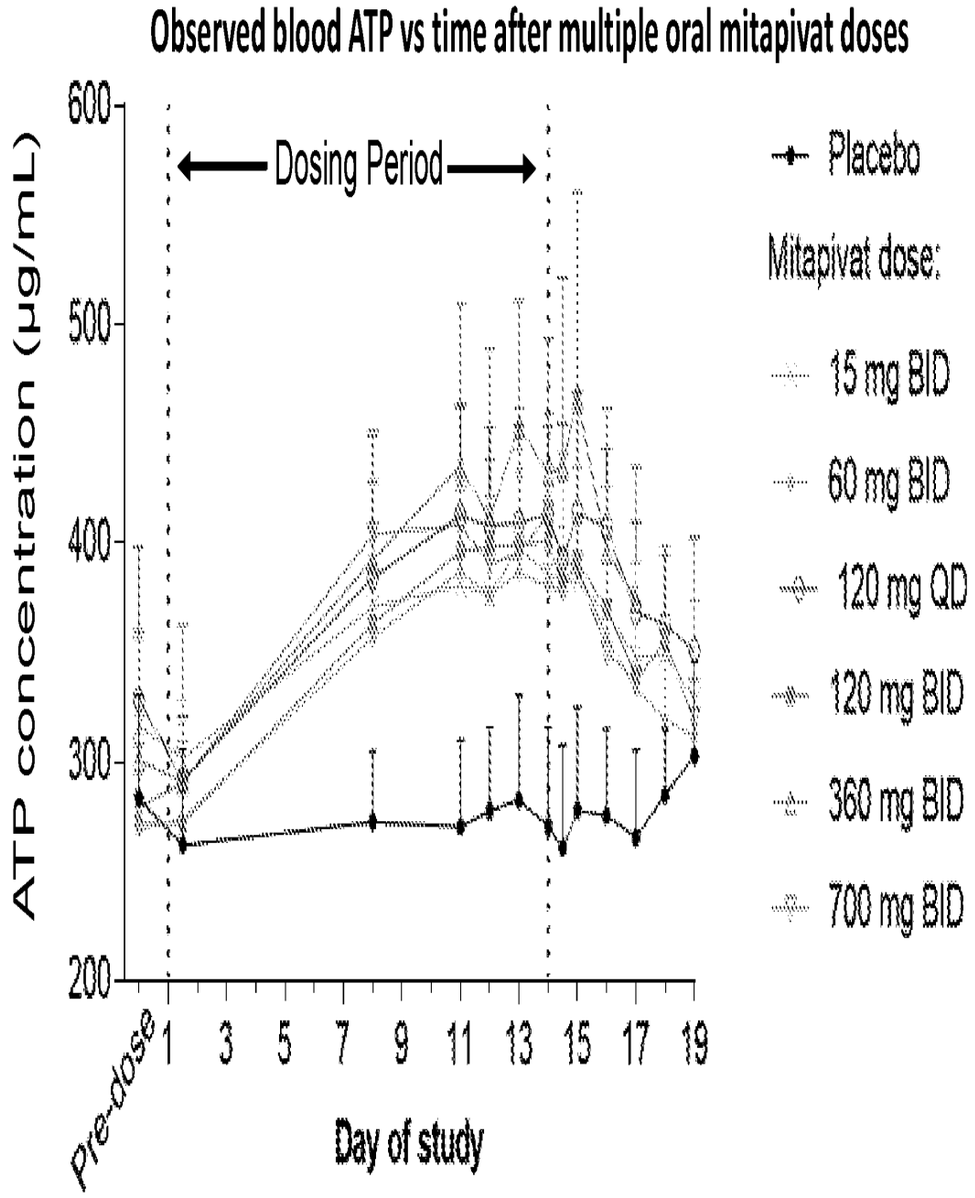
- PK is a key regulatory enzyme of glycolysis
- PK catalyzes the metabolic conversion of PEP and ADP to pyruvate and ATP, respectively, as the final step in glycolysis[1]
- PK-R is the form of pyruvate kinase present in red blood cells (RBC), where glycolysis is the primary energy production pathway[1]
- Mitapivat sulfate is an orally available small-molecule allosteric activator of PK-R[2]
- Mitapivat activates wild-type and mutant PK-R enzymes[2]




ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPG, diphosphoglycerate; FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate.

1. Valentini G et al. *J Biol Chem.* 2002;277:23807-14. 2. Kung C et al. *Blood* 2017;130:1347-56.

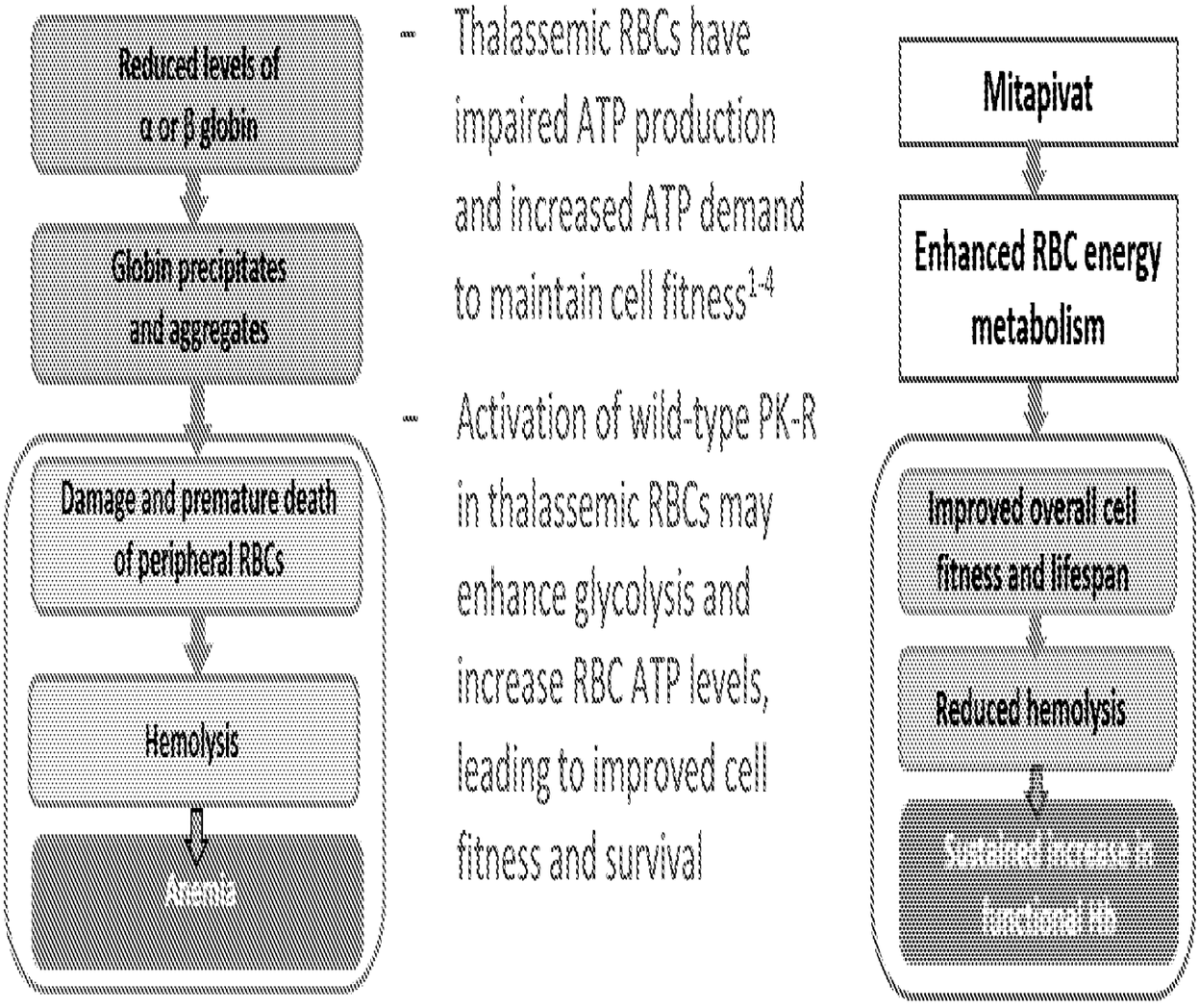
# Mitapivat increases RBC ATP levels in healthy volunteers




ATP, adenosine triphosphate; BID, twice daily; QD, once daily; RBC, red blood cell.

Yang H et al. *Clin Pharmacol Drug Dev.* 2019;8:246-59.

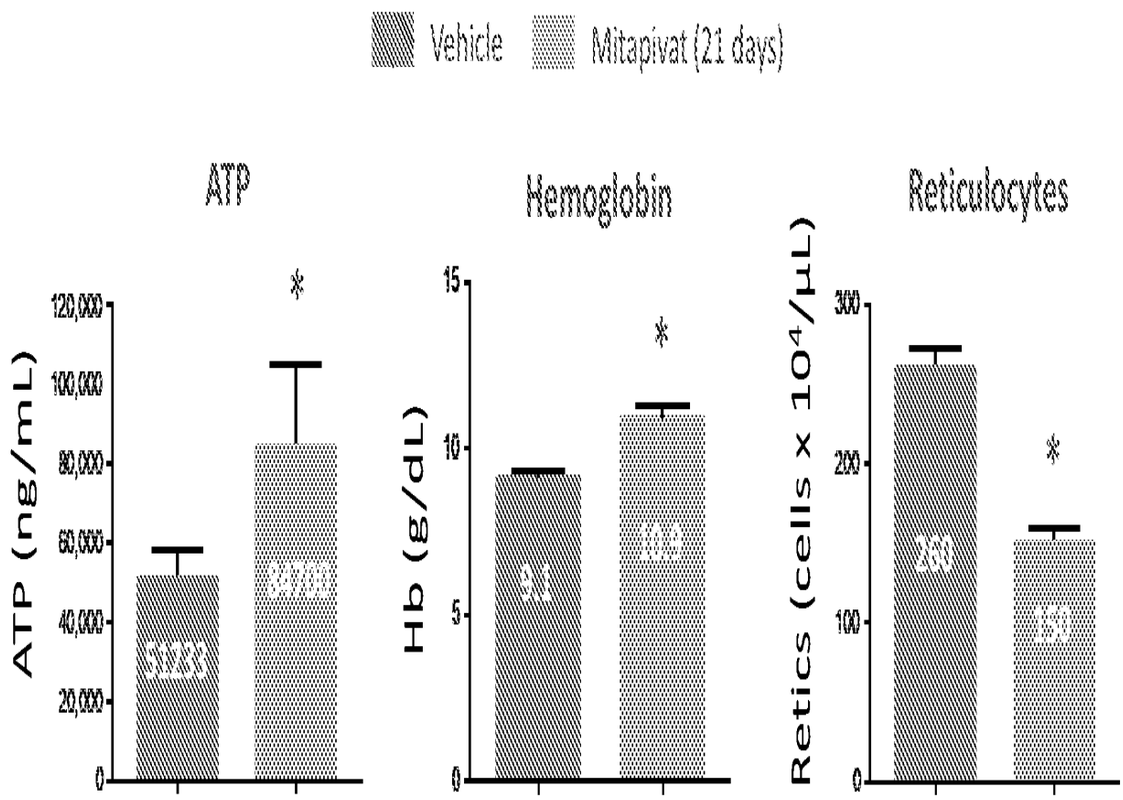
# Hypothesis: Mitapivat may improve thalassemic RBC survival by increasing ATP production




ATP, adenosine triphosphate; RBC, red blood cell.

1. Ting YL et al. *Br J Haematol.* 1994;88:547-54. 2. Chakraborty I et al. *Arch Med Res.* 2012;43:112-6. 3. Gunn RB et al. *J Clin Invest.* 1972;51:1043-50. 4. Scott GL, et al. *Br J Haematol.* 1970;18:13-28.

# Mitapivat increases ATP and improves RBC parameters in $Hbb^{th3/+}$ mice




- Mitapivat increases hemoglobin (Hb) by 1.8 g/dL and reduces reticulocyte count by 40% after 21 days of oral treatment in th3/+ β-thalassemia mouse model[1]
- Mitapivat reduces signs and markers of ineffective erythropoiesis and liver iron overload (data not shown)

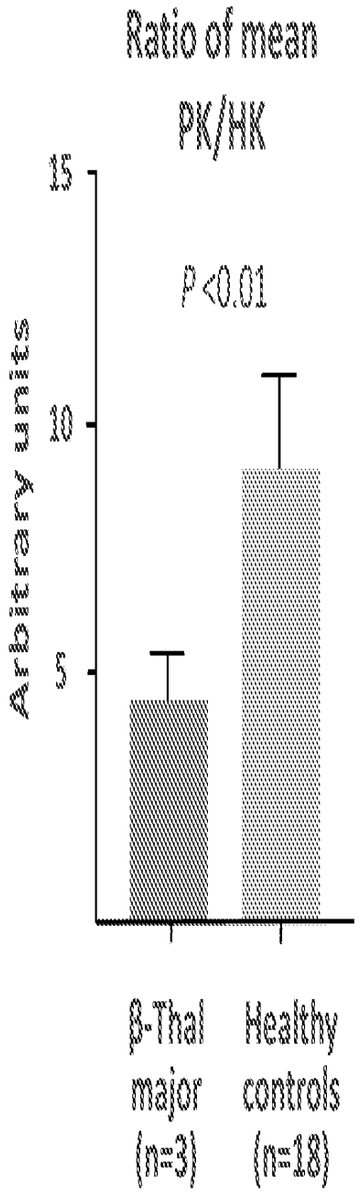
*$P$ <0.05 compared to vehicle

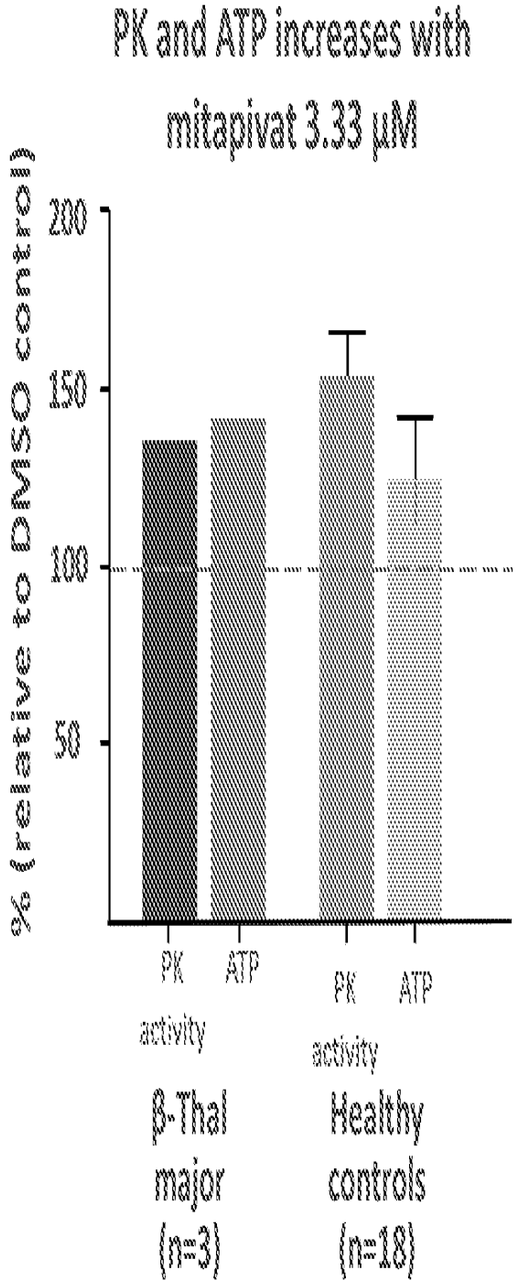
ATP, adenosine triphosphate; $Hbb^{th3/+}$, heterozygote mouse model of β-thalassemia.

1. Matte A et al. EHA 2016 Presentation, Abstract #S135.

# Mitapivat increases PK activity and ATP levels in thalassemic RBC

- In RBCs from patients with β-thalassemia,* PK activity (relative to hexokinase [HK] activity) is decreased compared with activity in RBCs from healthy controls[1]
- *Ex vivo* treatment with mitapivat increases PK activity and ATP levels in RBCs from both healthy controls and patients with β-thalassemia[1]



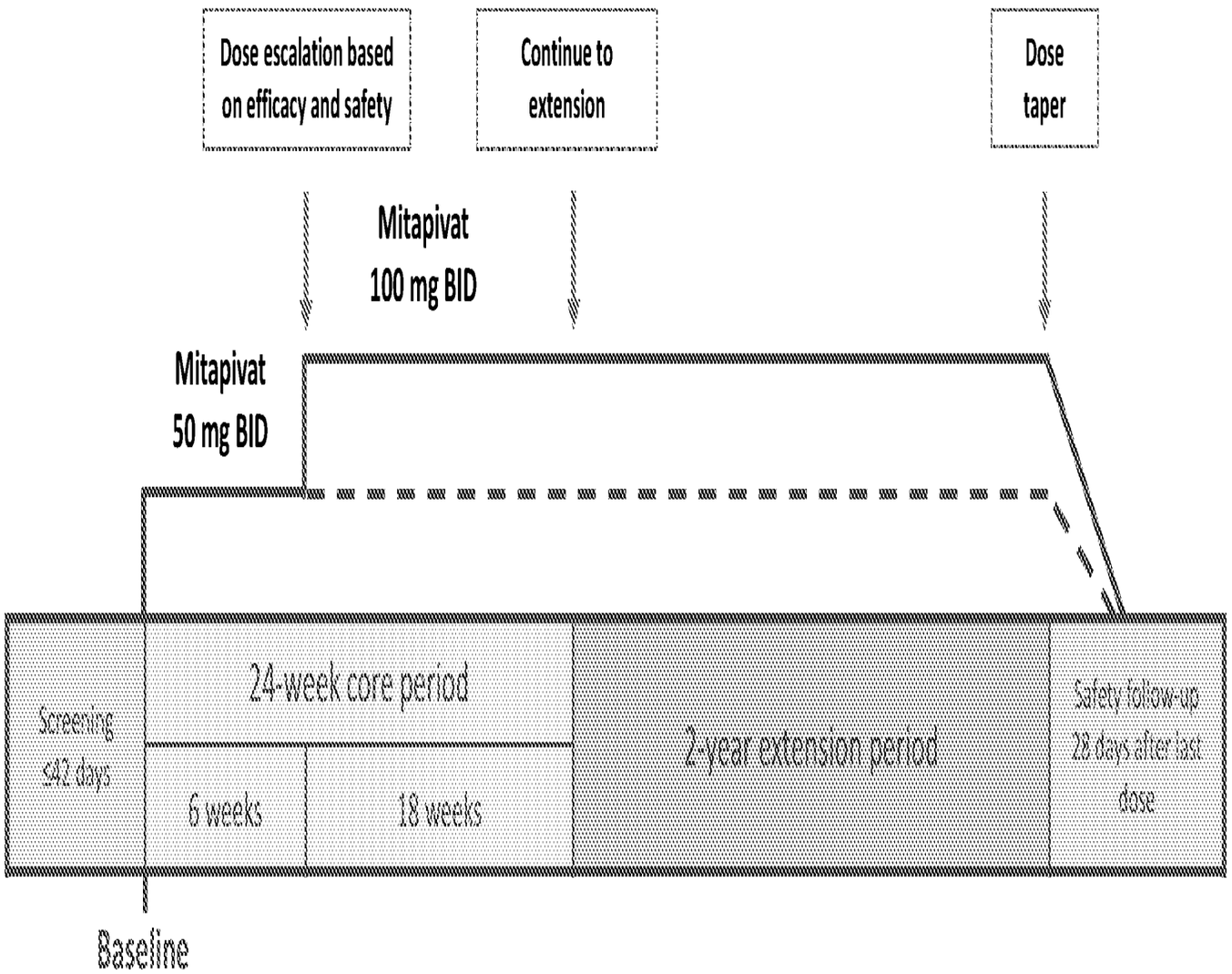




*3 patients with β-thalassemia major (regularly transfused), 18 healthy controls.

1. Rab MAE et al. Abstract: PS1519. 24th EHA Congress. Amsterdam, The Netherlands. June 13-16, 2019.

# Design of a phase 2, open-label, multicenter study

## Adults with non-transfusion-dependent thalassemia; β- or α-thalassemia (N ~ 17)




EudraCT 2018-002217-35; ClinicalTrials.gov NCT03692052; BID, twice daily

# Major eligibility criteria

- Adults (≥18 years) with known medical history of thalassemia, including:
  - β-thalassemia intermedia,
  - HbE β-thalassemia,
  - α-thalassemia (HbH disease), or
  - β-thalassemia with mutations of 1 or more α genes
- Hb ≤10 g/dL
- Non-transfusion dependent
  - ≤5 units RBC transfused during the 24 weeks prior to the first day of study drug
- Exclusions:
  - Hb S or Hb C forms of thalassemia
  - Iron overload sufficiently severe to result in a clinical diagnosis of cardiac, hepatic or pancreatic dysfunction

# Key study endpoints

## Primary

- **Hb response** (HR): ≥1.0 g/dL increase in Hb concentration from baseline at 1 or more assessments from Weeks 4 to 12

## Secondary

- Mean **change from baseline in Hb** concentrations over continuous 12 weeks interval (W12–24)
- **Sustained Hb response:** ≥1.0 g/dL increase in Hb at ≥2 evaluable assessments (W12–24)
- **Delayed Hb response**: subject has not achieved an HR, but has ≥1.0 g/dL increase in Hb concentration at ≥1 assessments after Week 12
- Type, incidence, severity, and relationship to treatment of **adverse events** (AEs) and serious AEs, and AEs leading to dose reduction, interruption, or discontinuation
- **Mitapivat pharmacokinetics**

# Summary

- Mitapivat is an oral small-molecule allosteric activator of PK-R
- Activation of PK-R by mitapivat in healthy volunteers increases RBC ATP levels
- Mitapivat may improve thalassemia RBC survival by increasing ATP production
- Mitapivat increases ATP and improves RBC parameters in a mouse model of β-thalassemia, and increases ATP levels *ex vivo* in human β-thalassemia RBCs
- A phase 2, open-label, multicenter proof-of-concept study is ongoing to assess the efficacy, safety, pharmacokinetics, and pharmacodynamics of mitapivat in non-transfusion-dependent thalassemia
- The study is currently enrolling at three sites in the United States, Canada and the United Kingdom

# Results update from the DRIVE PK study: Effects of AG-348, a pyruvate kinase activator, in patients with pyruvate kinase deficiency

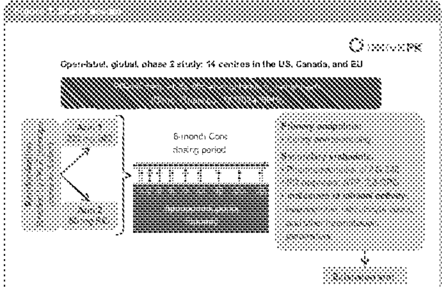
## BACKGROUND

- Pyruvate kinase (PK) deficiency is an under-recognized hereditary disease caused by mutations in the *PKLR* gene, which results in lifelong haemolytic anaemia.[1,2]
- Acute and chronic complications of supportive care (e.g. transfusions, splenectomy, or iron chelation) may additionally burden patients with PK deficiency.

## OBJECTIVE

- To report updated data from the ongoing DRIVE PK study (ClinicalTrials.gov NCT02476916), an open-label dose-ranging trial of AG-348 in adults with PK deficiency who are not receiving regular blood transfusions.

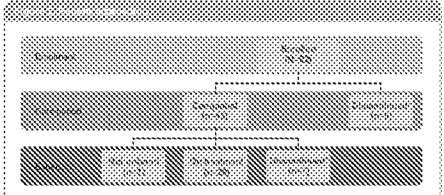
## METHODS




Not regularly transfused = no more than 3 units of red blood cells transfused in the 12 months prior to the first day of study dosing and no transfusions within 4 months of the first day of study dosing
All patients provided written informed consent
2,3-DPG = 2,3-diphosphoglycerate; BID = twice daily; PD = pharmacodynamic

- Enrolment is complete as of November 2016.
- Data cutoff: July 14, 2017.
- Cumulative safety results are summarized for the Core + Extension periods by randomized treatment group (50 mg BID, 300 mg BID, and overall).
- Clinical activity and sex hormone levels were analysed by the dose received for the longest duration in the Core period.
- Dose changes were allowed per protocol for various reasons:
  - Dose decrease: adverse events (AEs) and/or haemoglobin (Hb) exceeding the midpoint of the normal range (male: >15.0 g/dL; female: >13.5 g/dL).
  - Dose increase: lack of Hb response.

## RESULTS



*AEs (n=4), investigator decision (n=2), patient withdrew consent (n=3)
†Investigator decision (n=4), patient withdrew consent (n=1), lack of response (n=2)

Table 1. Demographic characteristics

| | | | |
|---|---|---|---|
| Male, n (%) | 18 (66.7) | 14 (56.0) | 32 (61.5) |
| Age at randomization, median (range), years | 28 (18–58) | 40 (21–61) | 34 (18–61) |
| White*, n (%) | 22 (81.5) | 21 (84.0) | 43 (82.7) |
| Hb baseline, median (range), g/dL | 9.9 (6.9–12.3) | 8.9 (6.5–12.0) | 8.9 (6.5–12.3) |
| Splenectomy, n (%) | 23 (85.2) | 20 (80.0) | 43 (82.7) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (76.0) | 38 (73.1) |
| Mutation category, n (%) | | | |
| Missense/missense | 15 (55.6) | 17 (68.0) | 32 (61.5) |
| Missense/non-missense | 6 (22.2) | 4 (16.0) | 10 (19.2) |
| Non-missense/non-missense | 6 (22.2) | 4 (16.0) | 10 (19.2) |
| Iron chelation prior to enrolment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Duration of AG-348 treatment, median (range), weeks | 34.6 (13.0–92.4) | 38.7 (12.9–86.4) | 37.5 (12.9–92.4) |

*Other races: not reported (n=3), Asian (n=3), other (n=3)

### Cumulative safety summary

- AG-348 was generally well tolerated.
- The majority of AEs were grade 1–2.
- Treatment-related AEs leading to discontinuation (n=4).
  - Haemolytic anaemia, hypertriglyceridaemia, pharyngitis/nausea, pleural effusion.
- There were 14 serious AEs in 11 patients.
  - Five treatment-related serious AEs in four patients: anaemia, hypertriglyceridaemia, osteoporosis, withdrawal haemolysis followed by anaemia.

| | | | | | | |
|---|---|---|---|---|---|---|
| Patients experiencing ≥1 AE, n (%) | 26 (96.3) | 8 (29.6) | 25 (100.0) | 7 (28.0) | 51 (98.1) | 15 (28.8) |
| Headache | 10 (37.0) | 0 | 14 (56.0) | 0 | 24 (46.2) | 0 |
| Insomnia | 6 (22.2) | 1 (3.7) | 16 (64.0) | 1 (4.0)* | 22 (42.3) | 2 (3.8) |
| Nausea | 10 (37.0) | 0 | 10 (40.0) | 0 | 20 (38.5) | 0 |
| Viral upper respiratory tract infection | 8 (29.6) | 0 | 4 (16.0) | 1 (4.0) | 12 (23.1) | 1 (1.9) |
| Arthralgia | 5 (18.5) | 0 | 4 (16.0) | 0 | 9 (17.3) | 0 |
| Hot flush | 2 (7.4) | 0 | 7 (28.0) | 0 | 9 (17.3) | 0 |
| Cough | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Diarrhoea | 4 (14.8) | 1 (3.7) | 4 (16.0) | 0 | 8 (15.4) | 1 (1.9) |
| Dizziness | 5 (18.5) | 0 | 3 (12.0) | 1 (4.0)* | 8 (15.4) | 1 (1.9) |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Influenza | 7 (25.9) | 1 (3.7) | 1 (4.0) | 0 | 8 (15.4) | 1 (1.9) |
| Vomiting | 3 (11.1) | 0 | 5 (20.0) | 0 | 8 (15.4) | 0 |

AEs graded using National Cancer Institute Common Terminology Criteria, version 4.03
Other grade ≥3 AEs not included in the table: colitis (n=1), mesenteric vein thrombosis (n=1), pharyngitis (n=2), hypertriglyceridaemia* (n=3), haemolytic anaemia* (n=2), haemolysis* (n=1), post-procedural haemorrhage (n=1), hypertension (n=1)
*Related to study drug as assessed by the investigator

### Effect of AG-348 on sex hormones

- Modest changes from baseline in sex hormone levels were observed in males at planned pivotal trial dose levels (≤50 mg BID).
  - Data are consistent with mild aromatase inhibition.
- Most sex hormone values remained within normal limits in females (data not shown).
  - Interpretation is confounded by variability in menopausal status and contraceptive use.

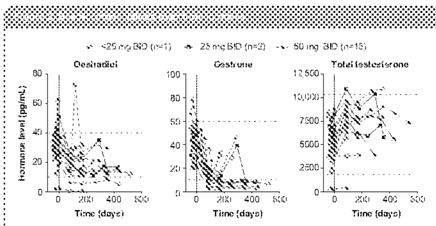
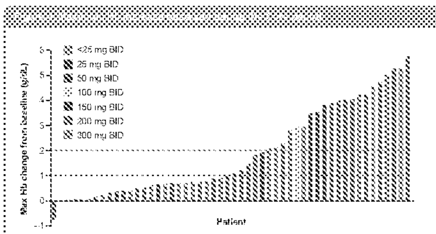


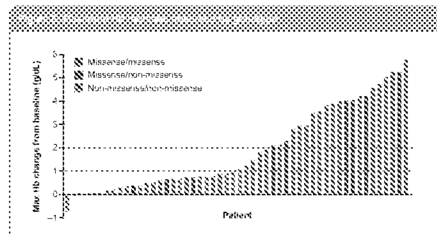

Figure shows data for the subset of males on actual doses ≤50 mg BID. Normal reference upper and lower limits shown as horizontal dashed lines

### Clinical activity

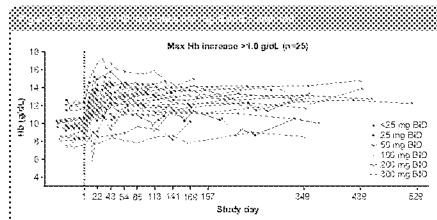
- 26 of 52 (50.0%) patients had a maximum Hb increase of >1.0 g/dL.
  - The mean maximum increase was 3.4 g/dL (range 1.1–5.8 g/dL).
- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL.




The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)



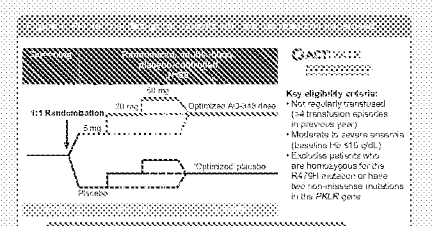

- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range 7–187 days).
  - Median baseline Hb in patients who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.3–12.3 g/dL) versus 8.0 g/dL (range 6.5–10.1 g/dL) in patients who did not.
- In nine patients, the dose had to be held or reduced due to a rapid rise in Hb.




## CONCLUSIONS

- AG-348 is a novel, first-in-class, PK-R activator in clinical testing as a potential disease-altering therapy for patients with PK deficiency.
- Chronic daily dosing with AG-348 is well tolerated.
  - Consistent safety profile over the duration of treatment (median 37.5 weeks).
  - Ongoing follow-up will continue to assess the clinical impact of mild aromatase inhibition.
- Patients who respond to AG-348 have rapid and durable responses.
  - 26 of 52 (50%) patients had a maximum Hb increase of >1.0 g/dL.
  - The mean maximum increase in Hb was 3.4 g/dL in patients with an Hb increase >1.0 g/dL.
  - Genotype–Hb response correlations informed eligibility criteria for pivotal trials.
- Pivotal trials in adults with PK deficiency are now open and enrolling:

ACTIVATE N=80 ACTIVATE-T N=20





Acknowledgements

We would like to thank the patients who agreed to participate in this study, and Drs Ellis Neufeld and David Nathan for helpful discussions.

Disclosures

This study was funded by Agios Pharmaceuticals, Inc.

DML: Agios – advisory committee; Cerus – advisory committee; Novartis – advisory committee. RFG: Agios – advisory committee, research funding. FG: Addmedica – advisory committee. WB: Agios – honoraria, research funding; Alexion – honoraria. Novartis – honoraria. DHM – no relevant financial relationships to disclose. EJvB: Agios – consultancy. HMY: Baxalta, Bayer, Octapharma, CSL Behring – consultancy, advisory committee; Baxalta – honoraria, advisory committee. YR: Agios – study investigator. KHMK: Abfero – scientific collaboration, Agios – consultancy, study investigator; Alexion – consultancy, honoraria, study investigator. Celgene – consultancy, study investigator; Novartis – consultancy, honoraria; Pfizer – study investigator; Phoenicia Biosciences – scientific collaboration. SS: ApoPharma, Bluebird Bio, Celgene, Novartis – consultancy. JLK: Agios – consultancy, honoraria; Apopharma – research funding; Bluebird Bio – advisory committee, research funding; Ionis – consultancy, honoraria. Novartis – research funding. BAS: Agios – consultancy. MC: Agios – consultancy. CK, HY, PAK, LH: Agios – employment and stockholder. AJB: Agios – employment and stockholder at time of study. BG: Agios – advisory committee.

Editorial assistance was provided by Helen Varley, PhD, Excel Medical Affairs, Horsham, UK, and supported by Agios.



References

1. Grace RF et al. *Am J Hematol* 2015;90:825-30.
2. Percy MJ et al. *Blood Cells Mol Dis* 2007;39:189-194.


Scan code to receive PDF file of the poster or visit http://bit.ly/2vtAsAY


Presented at the British Blood Transfusion Society (BBTS) Annual Conference, October 3–5, 2018, Brighton, UK




***Welcome Mark Layton***

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## Results update from the DRIVE PK study: Effects of AG-348, a pyruvate kinase activator, in patients with pyruvate kinase deficiency

BACKGROUND: Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by mutations in the PKLR gene, leading to a deficiency of



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the glycolytic enzyme red cell PK (PK-R). Current treatments are supportive only. AG-348 is an orally available small-molecule allosteric activator of PK-R.

AIM: To report updated data from the ongoing DRIVE PK study (NCT02476916), an open-label, dose-ranging trial of AG-348 in non-transfusion-dependent adults with PK deficiency.

METHODS: Patients were randomized to 50 mg or 300 mg AG-348 orally twice daily (BID) for a 6-month Core Period; completers could enter a 4-year Extension. Non-transfusion-dependence was defined as ≤3 units of red blood cells transfused in the 12 months preceding the first dose and no transfusions within 4 months preceding the first dose. Here, we report data based on the cutoff date July 14, 2017.

RESULTS: Enrollment completed in November 2016 (n=52; median age 34 years; 62% men; 83% white). As of July 14, 2017, 43 patients had completed the Core Period (9 discontinued); 36 completers entered the Extension: 29 ongoing, 7 had discontinued. Median (range) treatment duration was 37.5 (12.9–92.4) weeks. Due to protocol-specified dose modifications, patients received doses from 5 mg once daily to 300 mg BID. The most common adverse events (AEs; >20% patients) were headache (46%), insomnia (42%), nausea (39%), and viral upper respiratory tract infection (23%). Testosterone levels increased because of known aromatase inhibition; most values remained within the normal range. Five drug-related serious AEs occurred in 4 patients (withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia). Of 52 patients, 26 (50%) experienced a maximum hemoglobin (Hb) increase >1.0 g/dL (mean maximum increase 3.4 g/dL, range 1.1–5.8 g/dL). Hb increases were mostly rapid and sustained, and seen across a wide dose range. Most patients with an Hb increase >1.0 g/dL had ≥1 missense mutation.

CONCLUSIONS: AG-348 is a novel, first-in-class PK-R activator in clinical testing for PK deficiency. Chronic daily dosing with AG-348 has been generally well tolerated and has resulted in clinically relevant, durable increases in Hb. These data support the potential of AG-348 as the first disease-altering therapy for PK deficiency.

**Layton M** [1] , Grace R [2] , Galactéros F [3] , Rose C [4] , Barcellini W [5] , Morton H [6] , van Beers E [7] , Yaish H [8] , Ravindranath Y [9] , Kuo K [10] , Sheth S [11] , Kwiatkowski J [12] , Silver B [13] , Kung C [14] , Cohen M [15] , Yang H [14] , Kosinski P [14] , Hua L [14] , Barbier A [14] , Glader B [16]
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[2] Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston MA, USA



Ex. 2001-0961

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[4] Hôpital Saint Vincent de Paul, Lille, France
[5] Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[6] Central Pennsylvania Clinic, Belleville PA, USA
[7] Van Creveldkliniek, University Medical Centre Utrecht, University of Utrecht, Utrecht, the Netherlands
[8] University of Utah, Salt Lake City UT, USA
[9] Wayne State University School of Medicine, Children's Hospital of Michigan, Detroit MI, USA
[10] University of Toronto, Toronto ON, Canada
[11] Weill Cornell Medical College, New York NY, USA
[12] Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia PA, USA
[13] Bruce A Silver Clinical Science and Development, Dunkirk MD, USA
[14] Agios Pharmaceuticals, Inc., Cambridge MA, USA
[15] MBC Pharma Solutions, Newtown PA, USA
[16] Stanford University School of Medicine, Palo Alto CA, USA

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Ex. 2001-0962




# NEW DRUGS FOR ENZYMATIC DEFECTS

Mark Layton
Imperial College London

EHA-SWG Scientific Meeting
Anemias

February 2-4, 2017
Barcelona

Disclosures: Agios(Speakers' bureau), Alexion, Novartis (Advisory board)

Ex. 2001-0963

# Current treatment options

- Transfusion

  - Decision guided by symptoms or growth delay

- Splenectomy

  - Transfusion dependent/intolerant of anaemia

  **Substantial benefit in some enzymatic defects *e.g.* PK and GPI**

- Avoidance of oxidants in deficiencies of G6PD, PK, GPI and rarer glutathione defects

- Haemopoietic stem cell transplantation(HSCT)

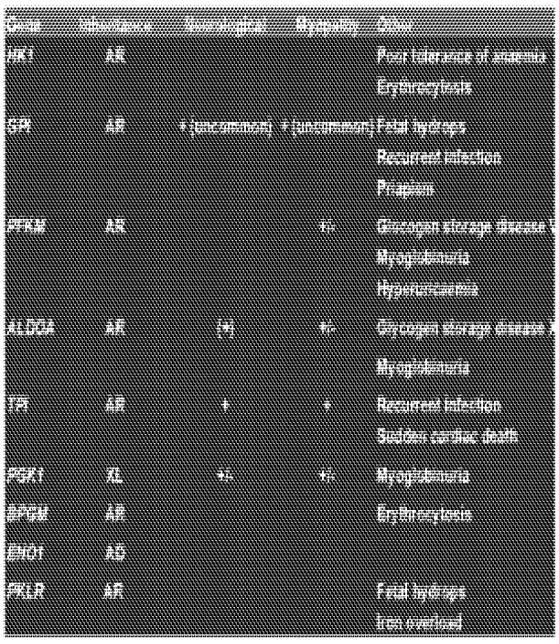
  - 5 year old boy with transfusion dependent PK deficiency (HLA-matched sibling donor). *Tanphaichitr et al, Bone Marrow Transplant, 2000*

  - Nomyeloablative HSCT leads to remission of haemolysis in PK-deficient Basenji dogs. *Zaucha et al, Biol Blood Marrow Transplant, 2001*

  - Benefit of HSCT appears to be confined to correction of haemolysis in multisystem disorders *e.g.* TPI deficiency

- Management of secondary complications (cholelithiasis, iron overload, osteopenia/osteoporosis).

**Hepatic siderosis (Perl's stain)**



| Gene | Inheritance | Haematological | Myopathy | Other |
|---|---|---|---|---|
| HK1 | AR | | | Poor tolerance of anaemia |
| | | | | Erythrocytosis |
| GPI | AR | + (uncommon) | + (uncommon) | Fetal hydrops |
| | | | | Recurrent infection |
| | | | | Priapism |
| PFKM | AR | | +/- | Glycogen storage disease |
| | | | | Myoglobinuria |
| | | | | Hyperuricaemia |
| ALDOA | AR | (+) | +/- | Glycogen storage disease |
| | | | | Myoglobinuria |
| TPI | AR | + | + | Recurrent infection |
| | | | | Sudden cardiac death |
| PGK1 | XL | +/- | +/- | Myoglobinuria |
| BPGM | AR | | | Erythrocytosis |
| ENO1 | AR | | | |
| PKLR | AR | | | Fetal hydrops |
| | | | | Iron overload |

**No effective therapy For extra-haematological manifestations**

# First principles

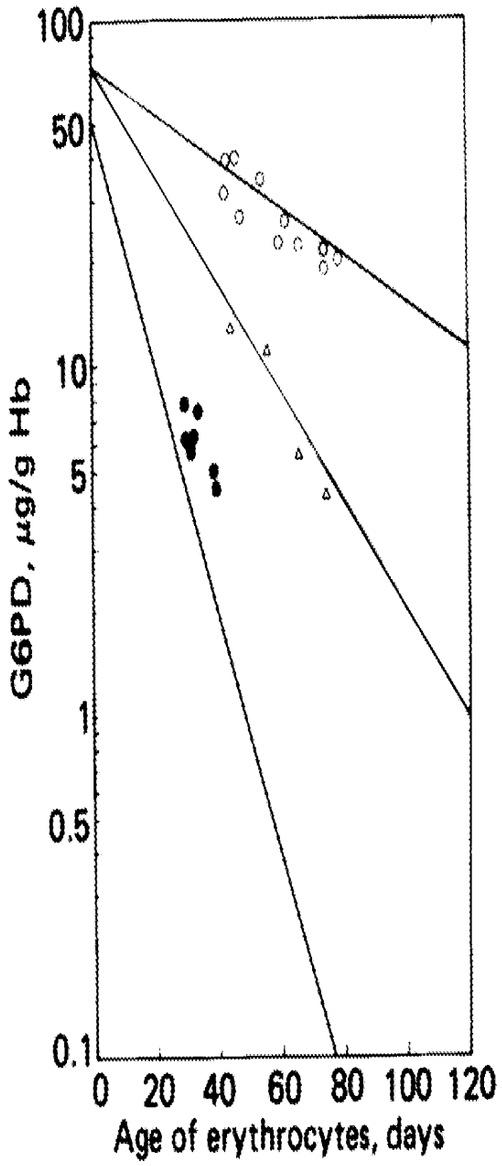
ACTIVITY OF MOST RED CELL ENZYMES DECREASES EXPONENTIALLY AS RED CELLS AGE

MAJORITY OF RED CELL ENZYME DEFICIENCIES ARE CAUSED BY MISSENSE MUTATIONS

G6PD 91%

PK 67%

Source HGMD®




Morelli *et al*. Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency. *Proc. Nati. Acad. Sc. USA* 1978;**75**:1979

# Strategies for treatment of inherited enzyme deficiencies

REPLACE MUTANT ENZYME

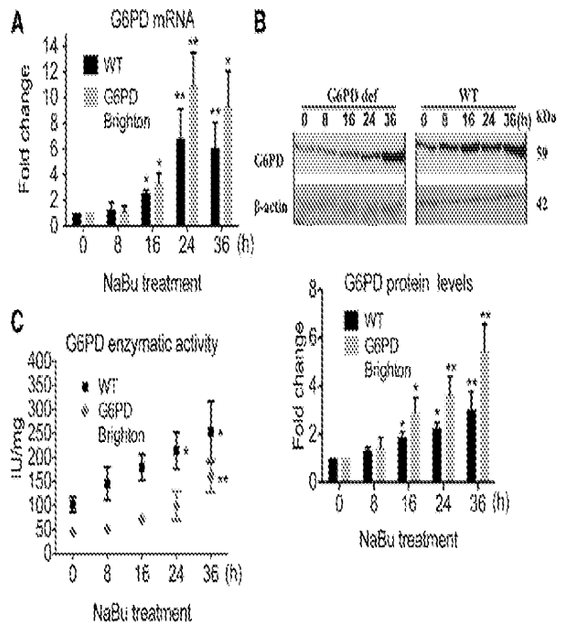
- Enzyme replacement therapy
- Stem cell therapy *e.g.* HSCT
- Gene therapy/editing

ENHANCE CATALYTIC ACTVITY

- Enzyme induction
- Pharmacological chaperone
- Allosteric activation

METABOLIC MODIFICATION

- Substrate depletion
- Scavenging of toxic metabolites




Transcriptional and epigenetic basis for restoration of G6PD enzyn activity in human G6PD-deficient cells.
Makarona *et al*. Blood, 2014

# Phase 1 Multiple Ascending Dose Study of the Safety, Tolerability and Pharmacokinetics/Pharmacodynamics of AG-348, a First-in-Class Allosteric Activator of Pyruvate Kinase-R, in Healthy Subjects

**Hua Yang[1], Elizabeth Merica[1], Yue Chen[1], Marvin Cohen[2], Ronald Goldwater[3], Hyeryun Kim[1], Penelope A Kosinski[1], Charles Kung[1], Meredith Goldwasser[1], Bruce Silver[4], Luke Utley[1], Sam Agresta[1]**

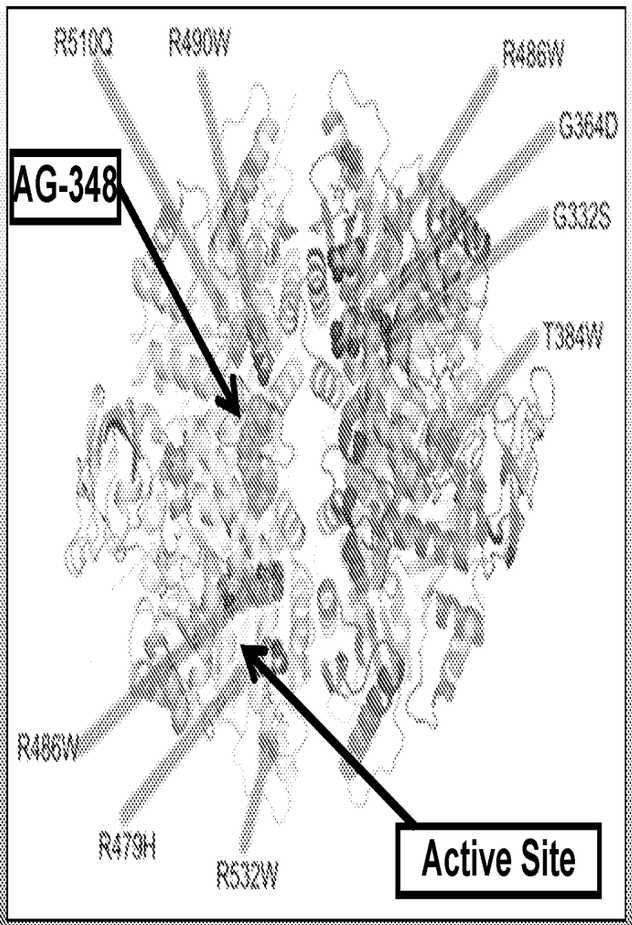
*[1]Agios Pharmaceuticals, Cambridge, MA, USA; [2]MBC Pharma Solutions, Newtown, PA, USA; [3]Parexel International, Baltimore, MD, USA; [4]Bruce A Silver Clinical Science and Development, Dunkirk, MD, USA*

*Presented at the 20th Congress of the European Hematology Association, 2015, Vienna, Austria*

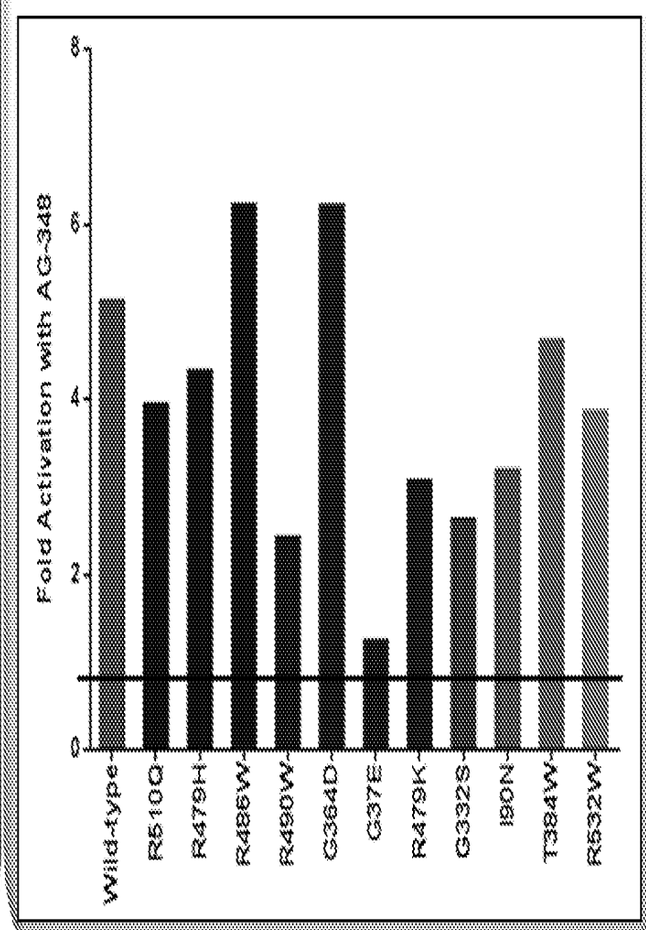
Ex. 2001-0967

# AG-348: Allosteric Activator of Wild Type & Mutant PK-R

**PK-R enzyme: active tetramer. AG-348 binds at the PK-R dimer-dimer interface**

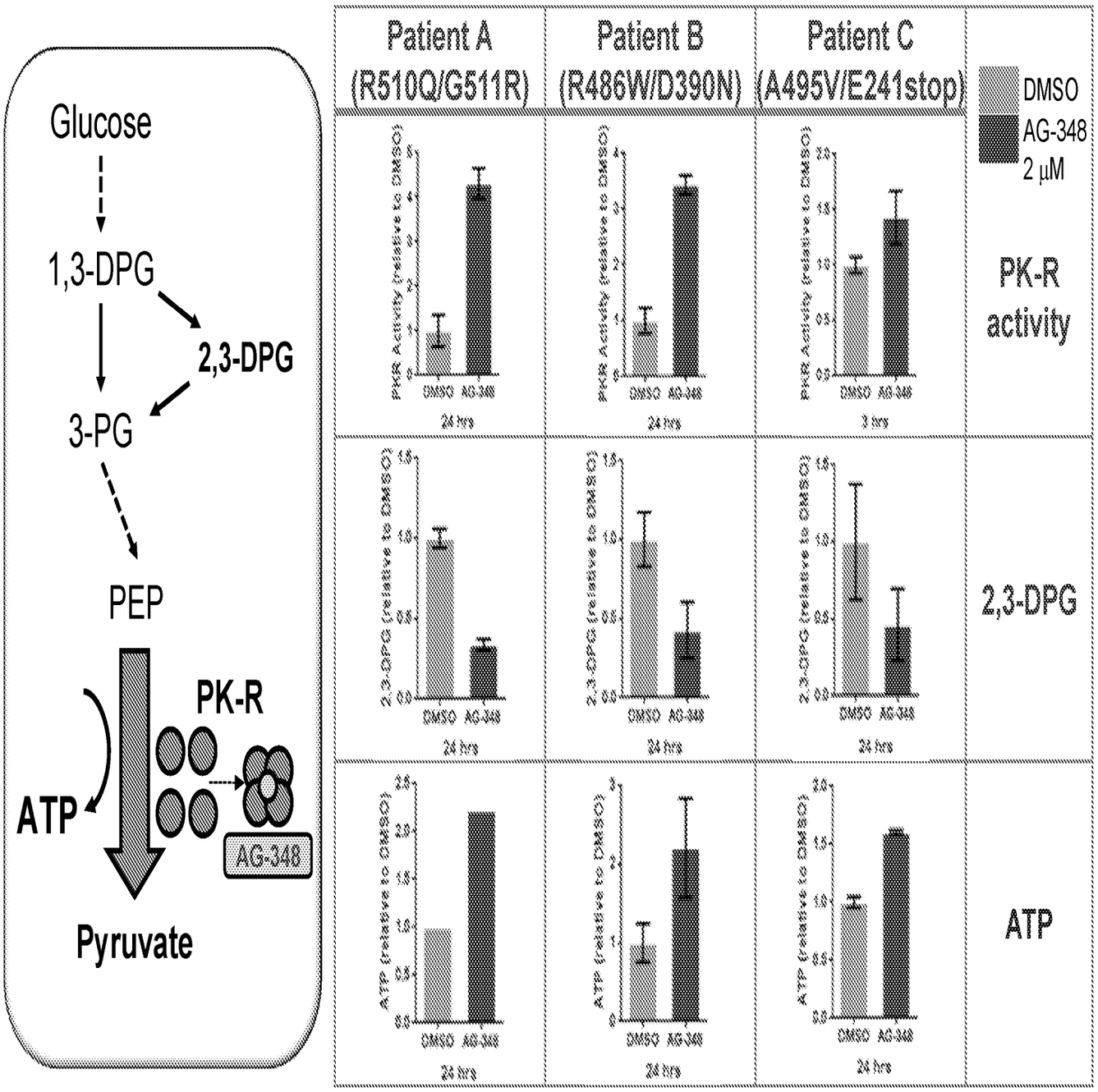



**AG-348 activates PK-R wild type and nearly all mutant enzymes tested**




*Source: Kung, ASH poster 2013*

# AG-348 Restores Glycolysis In Vitro

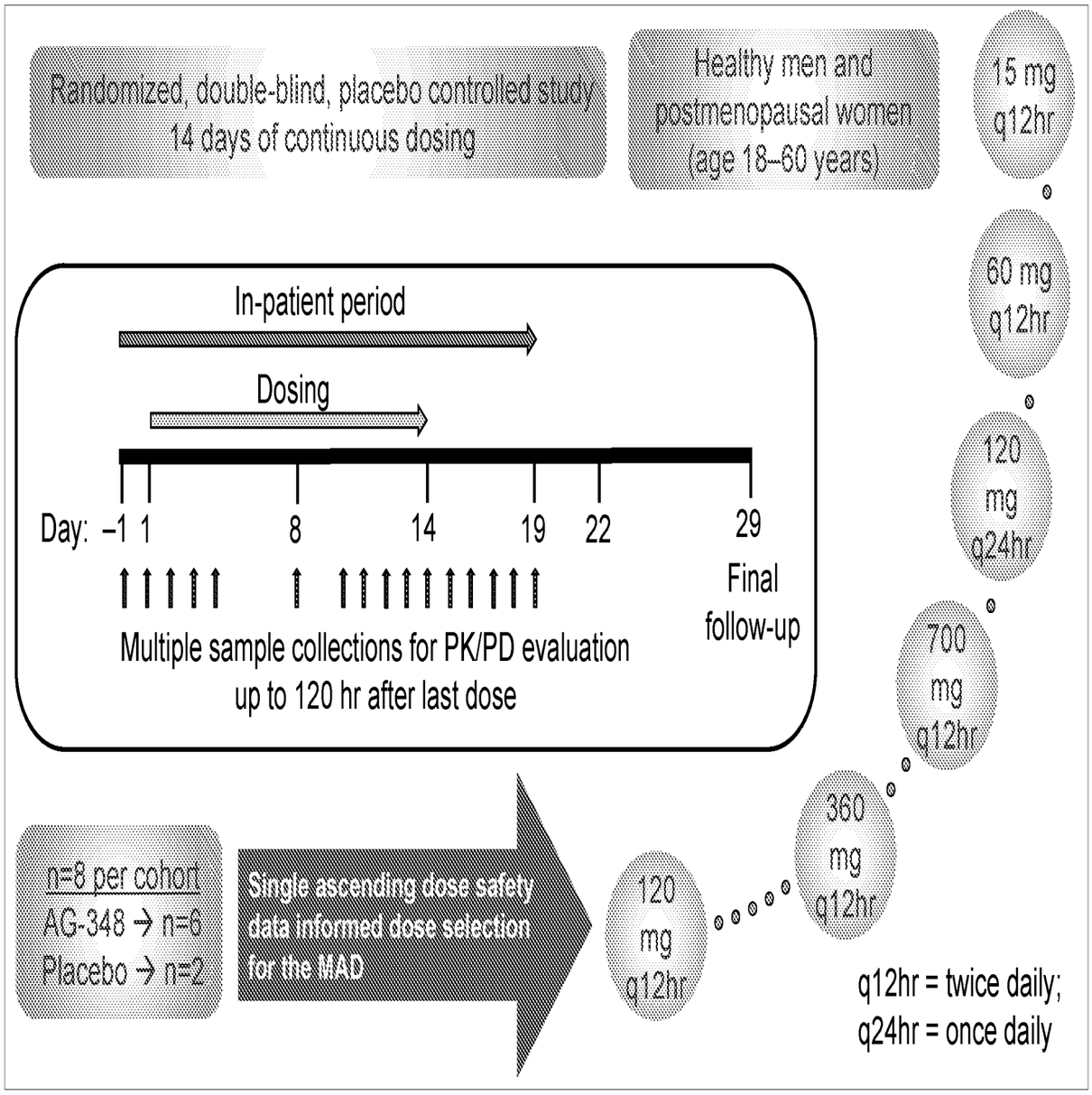



Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010

ATP = adenosine triphosphate; DPG = disphosphoglycerate; PEP = phosphoenolpyruvate; PG = phosphoglycerate

# Multiple Ascending Dose Study

## MAD: Establish the Safety and Tolerability, and PD Effects of AG-348



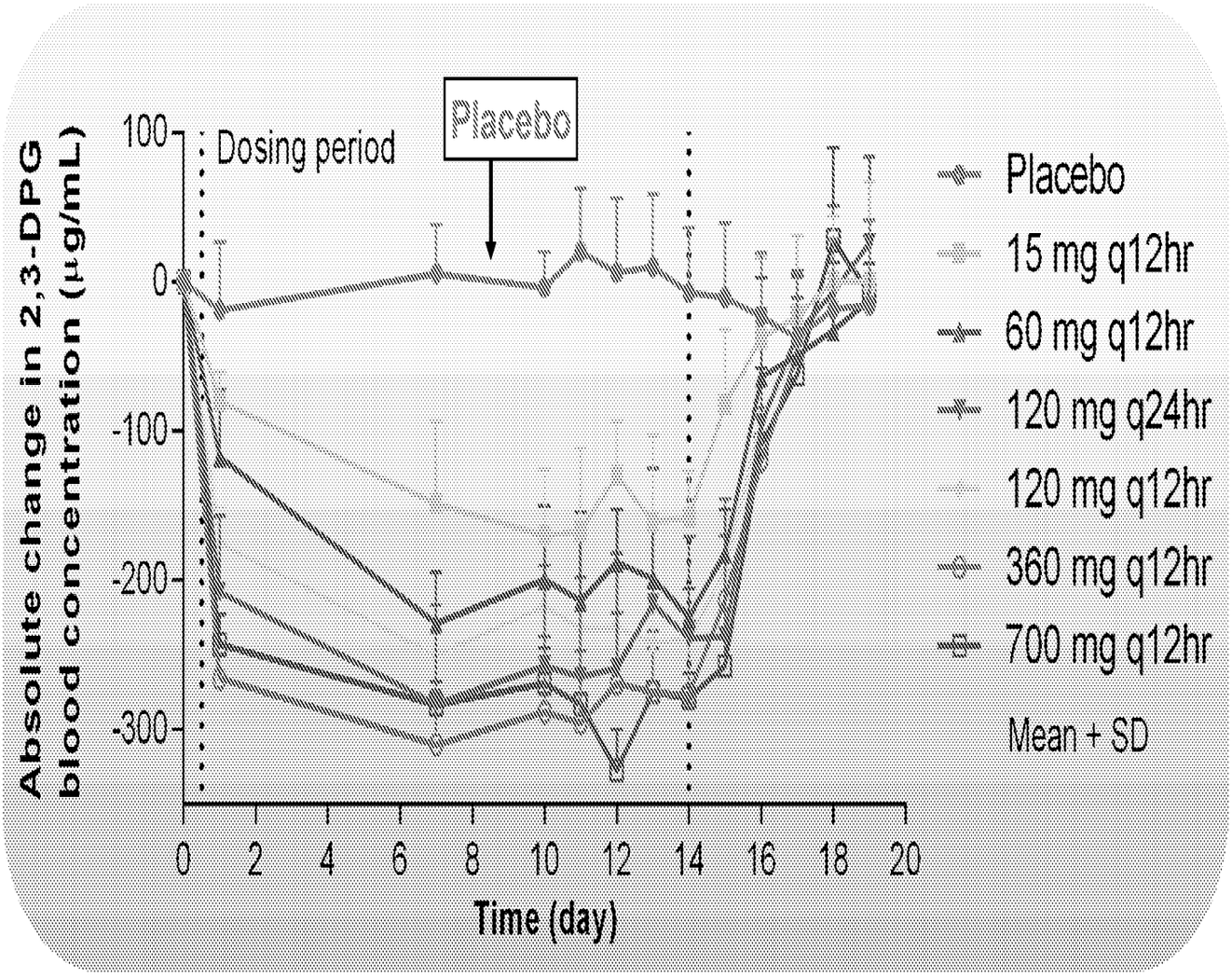

PK/PD = pharmacokinetic/pharmacodynamic



Ex. 2001-0970

# Dose Dependent Decrease in 2,3-DPG

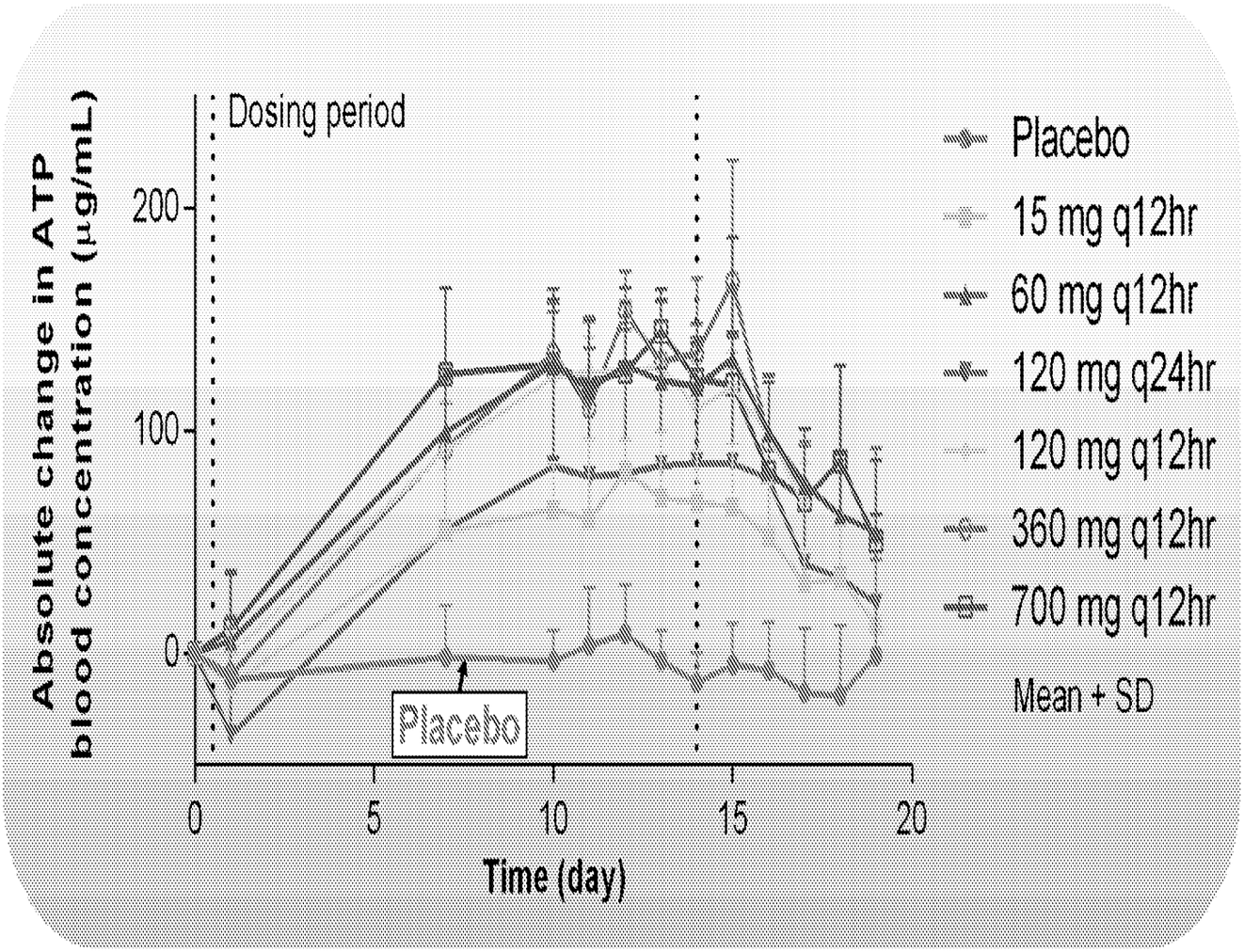
- A ~50% decrease from baseline was observed with ≥120 mg AG-348
- The concentration returned to baseline 72 hr after the final dose




Day 0 = Baseline value. Day 14 is the final day of dosing

# Dose Dependent Increase in ATP

- A ~50% increase from baseline was observed with ≥60 mg AG-348
- Levels remained elevated through 120 hr after the final dose




Day 0 = Baseline value. Day 14 is the final day of dosing



Ex. 2001-0972

# Effects of AG-348, a Pyruvate Kinase Activator, on Anaemia and Haemolysis in Patients with Pyruvate Kinase (PK) Deficiency: Data from the DRIVE PK Study

Rachael F Grace[1], D Mark Layton[2], Christian Rose[3], D Holmes Morton[4], Hassan Yaish[5], Eduard Van Beers[6], Kevin Kuo[7], Wilma Barcellini[8], Frédéric Galactéros[9], Yaddanapudi Ravindranath[10], Janet L Kwiatkowski[11], Bruce Silver[12], Charles Kung[13], Marvin Cohen[14], Hua Yang[13], Jeffrey Hixon[15], Victor Chubukov[13], Penelope A Kosinski[13], Lee Silverman[13], Lenny Dang[13], Huansheng Xu[13], Ann J Barbier[13], Bertil Glader[16]

*[1]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, MA; [2]Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom; [3]Hôpital Saint Vincent de Paul, Lille, France; [4]Central Pennsylvania Clinic, Belleville, PA; [5]University of Utah, Salt Lake City, UT; [6]Universitair Medisch Centrum Utrecht, Utrecht, Netherlands; [7]University of Toronto, Toronto, ON, Canada; [8]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [9]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, France; [10]Wayne State University School of Medicine - Children's Hospital of Michigan, Detroit, MI; [11]Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA; [12]Bruce A Silver Clinical Science and Development, Dunkirk, MD; [13]Agios Pharmaceuticals, Inc., Cambridge, MA; [14]MBC Pharma Solutions, Newtown, PA; [15]formerly at Agios, now at KSQ Therapeutics, Cambridge, MA; [16]Stanford University School of Medicine, Palo Alto, CA.*

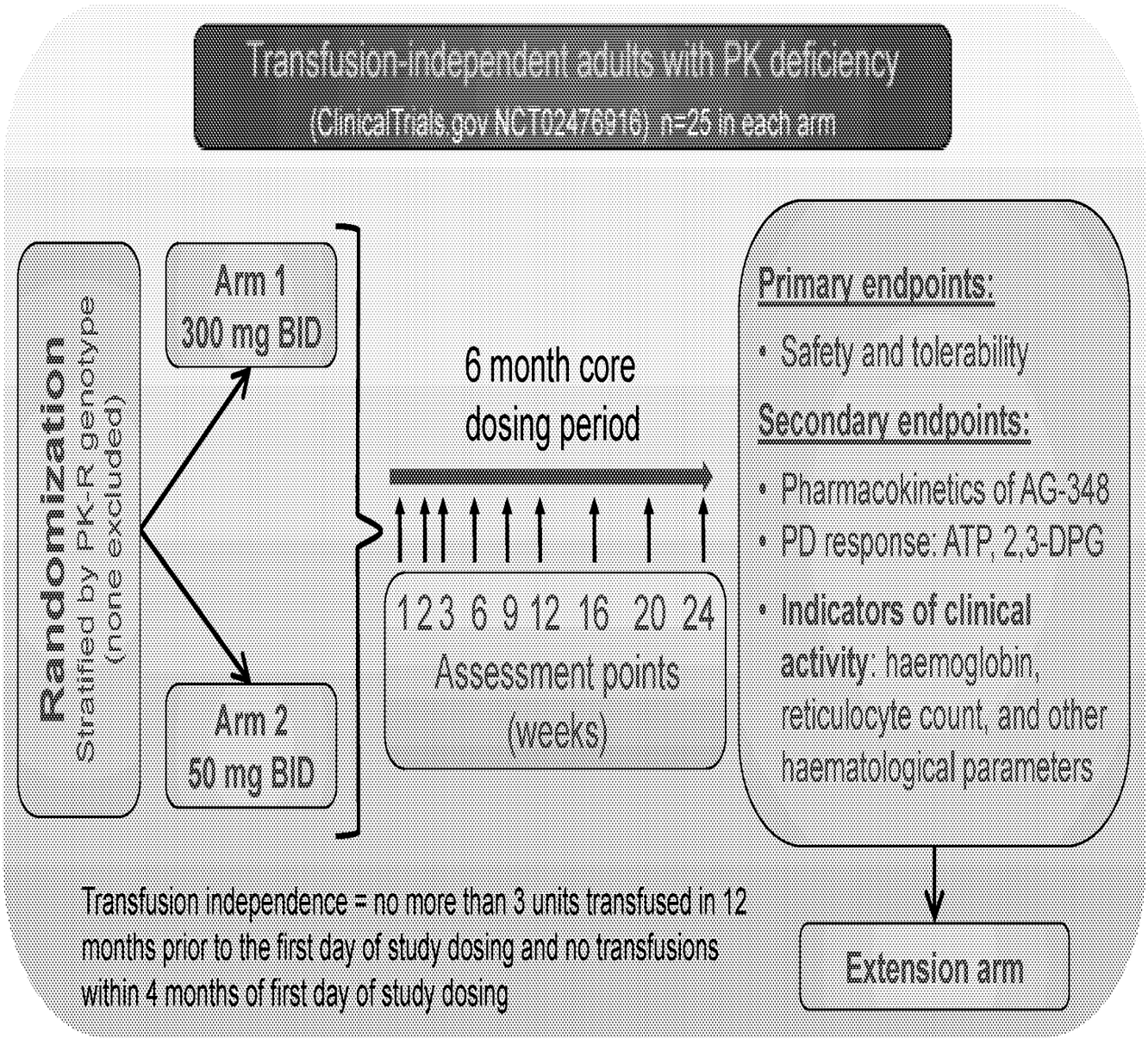
Presented at the 58th Annual Meeting and Exposition of American Society of Hematology, December 4, 2016, San Diego, CA

Ex. 2001-0973

# Study design

DRIVEPK

## Open-label, global phase 2 study: 14 centres in the US, Canada, and EU




All patients provided written informed consent. BID = twice daily; PD = pharmacodynamic



Ex. 2001-0974

# Demographics and disposition

- Study initiated June 2015; data cut-off September 23, 2016
- Evaluable analysis set: ≥3 weeks of data (n=32)
- Safety analysis set: received at least 1 dose of AG-348 (n=34)
- 13 patients ongoing in the core period (as of September 23, 2016)
  - Early discontinuations in the core period due to: relocation (n=1), AEs (n=3)
- Of the 17 patients who completed the core period, 15 enrolled in the extension period
- 1 patient discontinued in extension period due to physician decision (lack of efficacy)

| Characteristics | 50 mg BID, n=17 | 300 mg BID, n=17 | Total, N=34 |
|---|---|---|---|
| Male/Female, n | 11/6 | 9/8 | 20/14 |
| Age in years, mean (range) | 28.5 (19-45) | 37.0 (20-61) | 32.8 (19-61) |
| Race[a] white, n | 15 | 15 | 30 |
| Haemoglobin (Hb) baseline, g/dL, mean (SD, range) | 9.8 (1.41, 7.6–12.4) | 8.7 (1.37, 6.5–11.8) | 9.2 (1.47, 6.5–12.4) |
| Duration of treatment, weeks, median (range) | 24.7 (4.7–50.4) | 24.0 (2.4–44.4) | 24.4 (2.4–50.4) |
| Splenectomized, n | 14 | 14 | 28 |

[a]Not reported in 2 patients, 2 patients were Asian; AE = adverse event; SD = standard deviation



Ex. 2001-0975

# Safety summary

- AG-348 was generally well tolerated; the majority of AEs were grade 1–2
  - No grade 4 AEs or deaths
  - 2 patients experienced serious AEs: Grade 2 osteoporosis; haemolysis and anaemia due to discontinuation of the drug after a rapid Hb response (patient continued in the study)
  - 3 patients discontinued treatment due to AEs
  - DXA scan data (n=17) show high variability and are inconclusive

| AEs, regardless of causality (occurring in >5 patients or assessed as Grade ≥3) | 50 mg BID n=17 | | 300 mg BID n=17 | | Total N=34 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n | 13 | 2 | 17 | 6 | 30 | 8 |
| Headache | 7 | 0 | 8 | 0 | 15 | 0 |
| Nausea | 7 | 0 | 7 | 0 | 14 | 0 |
| Insomnia | 3 | 1 | 10 | 1 | 13 | 2 |
| Fatigue | 3 | 0 | 3 | 0 | 6 | 0 |
| Vomiting | 2 | 0 | 4 | 0 | 6 | 0 |
| Hypertriglyceridaemia | 0 | 0 | 4 | 3 | 4 | 3 |
| Anaemia | 1 | 1[a] | 1 | 1[b] | 2 | 2 |
| Hypertension | 0 | 0 | 1 | 1 | 1 | 1 |
| Dizziness | 2 | 0 | 1 | 1 | 3 | 1 |
| Haemolysis | 0 | 0 | 2 | 1[b] | 2 | 1 |

[a]Grade 3 anaemia, not a serious AE. [b]Grade 3 withdrawal haemolysis and anaemia in 46-year-old woman due to abrupt drug withdrawal after a very fast Hb response

AEs were graded using National Cancer Institute Common Terminology Criteria, version 4.03. Hb = haemoglobin; DXA = Dual energy X-ray absorptiometry



Ex. 2001-0976

# Effect of AG-348 on hormones

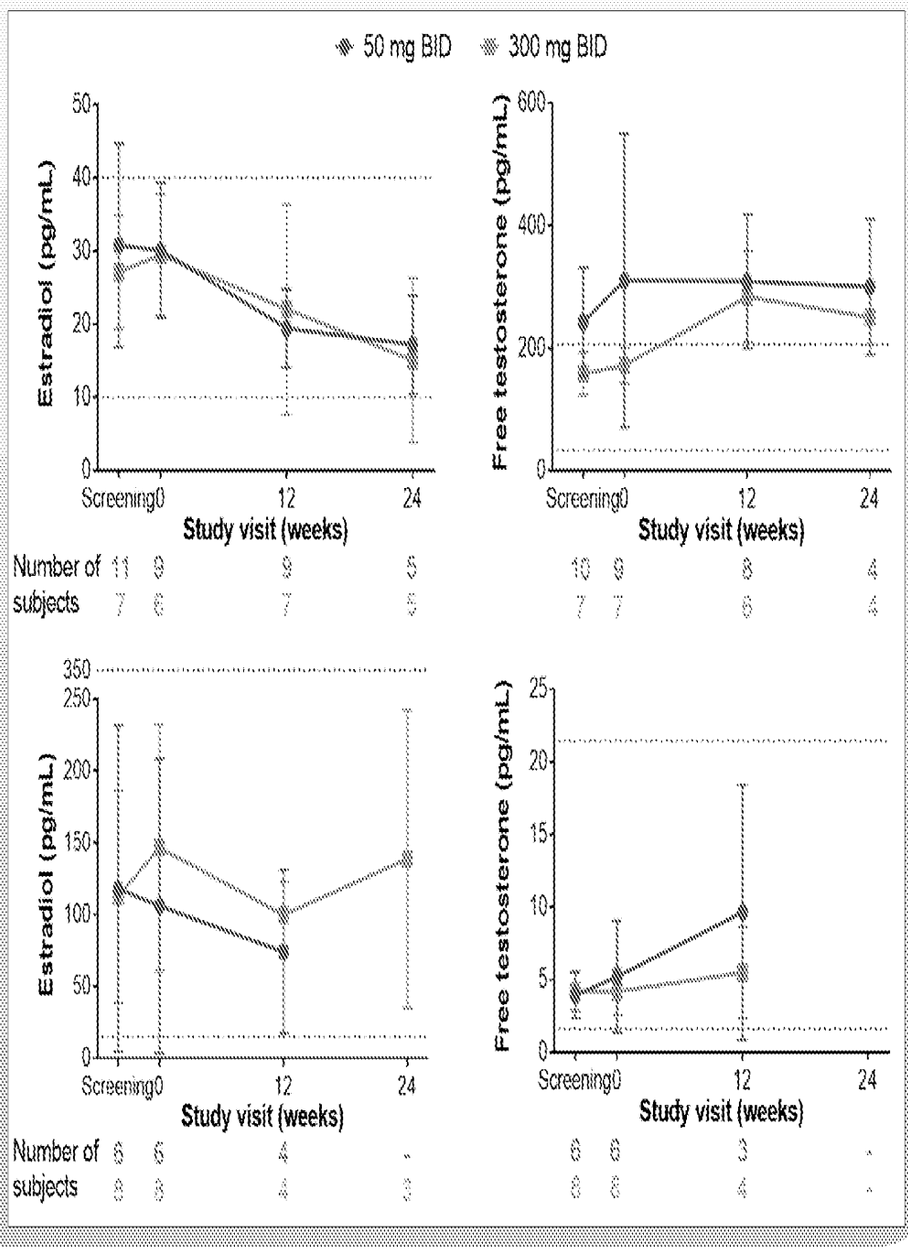
- Preliminary findings are consistent with aromatase inhibition by AG-348

Male

Hormone levels assessed at baseline, 12 and 24 weeks

Patients with available data shown

Female




Normal reference low and high limits shown as horizontal dotted lines



Ex. 2001-0977

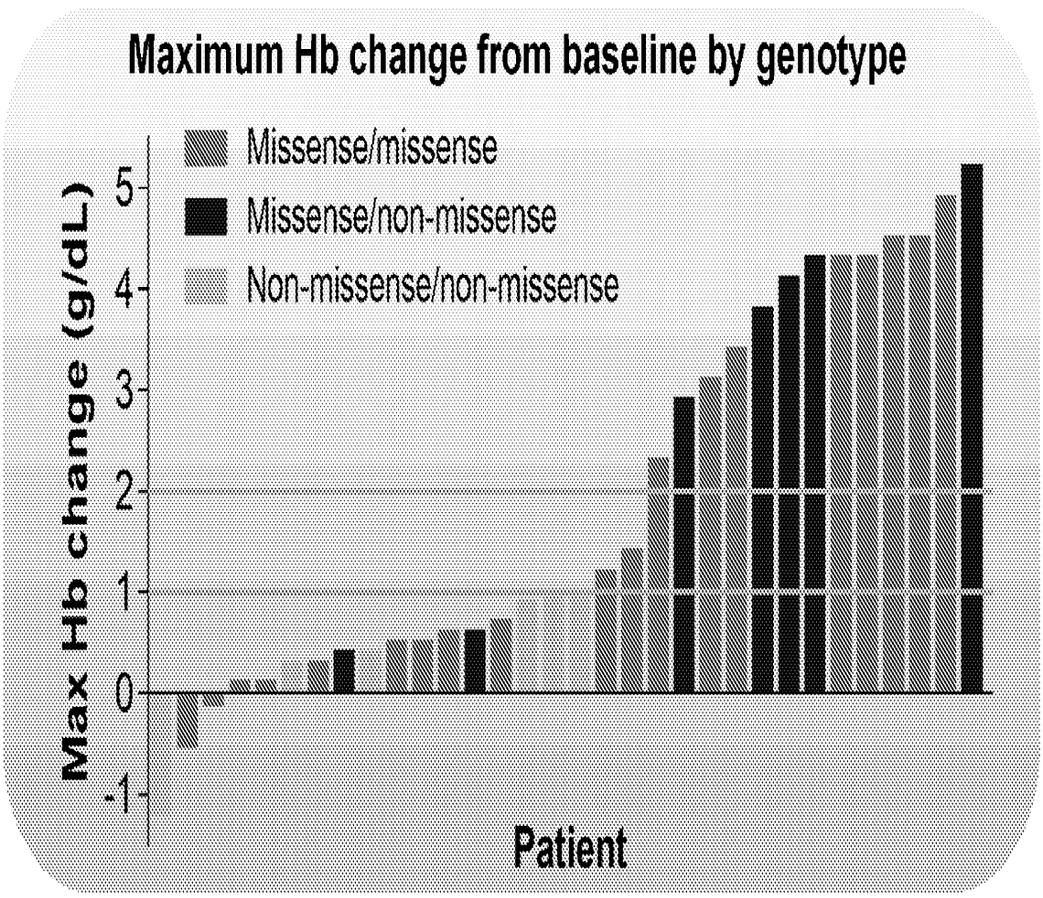
# Clinical Activity Results



Ex. 2001-0978

# Maximum increase in haemoglobin (Hb)

- 15 of 32 (47%) patients had a maximal increase in Hb >1.0 g/dL
  - 15 of 26 patients (58%) who had ≥1 missense mutation had a Hb response
- 5 patients homozygous for R479H (Amish mutation) were non-responders
- Hb response and response maintenance are seen across a range of 4 doses
  - Robust Hb responses led to dose decreases with maintained Hb



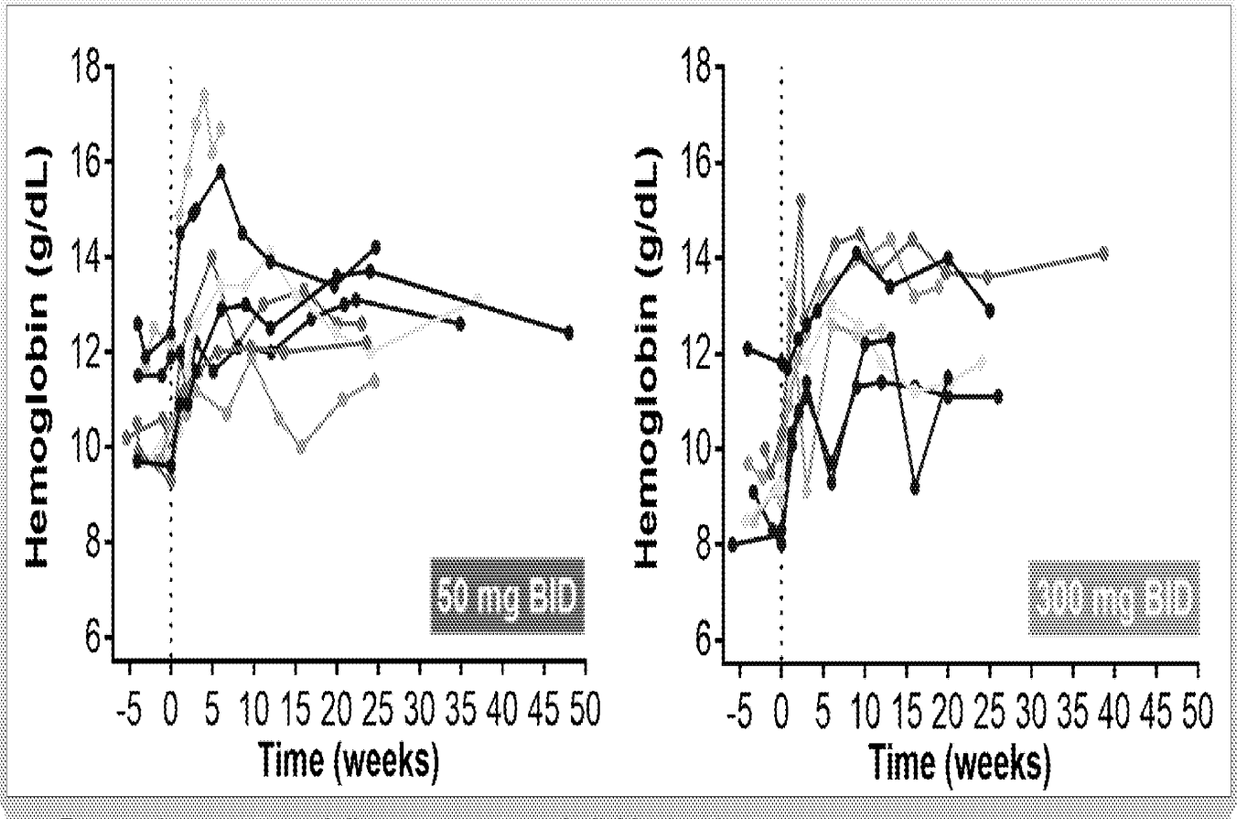

Evaluable analysis set, ≥3 weeks of data



Ex. 2001-0979

# Hb increases are rapid and sustained

- In patients who had Hb increases >1.0 g/dL (n=15):
  - Median time to Hb increase >1.0 g/dL was 1.4 weeks (range, 1.1–21.0)
  - The mean maximum increase was 3.6 g/dL (range, 1.2–5.2)
- 10 patients had dose reductions: 5 due to rapid Hb increase[a]



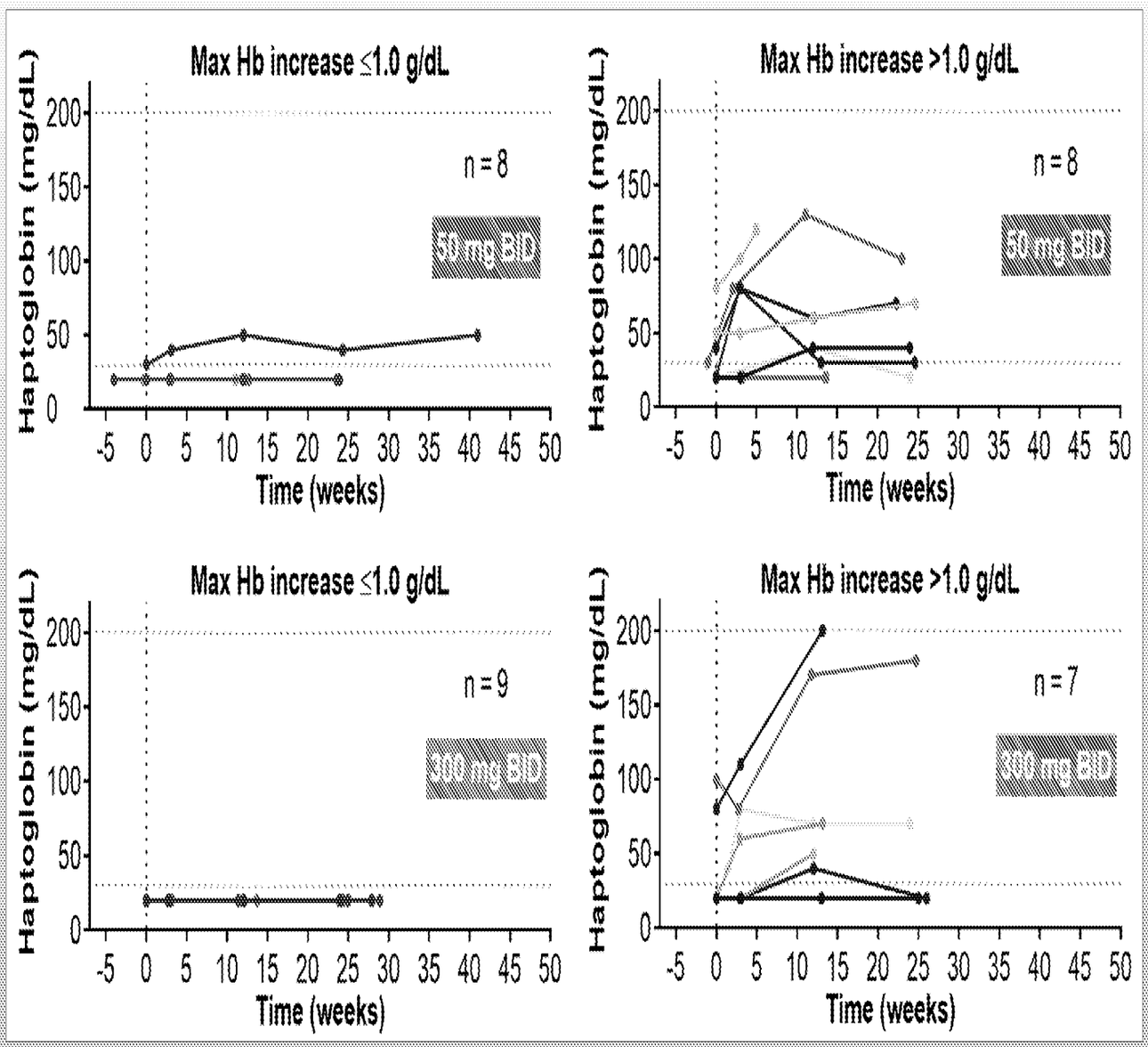

Responders n=15, each line represents an individual patient

[a]Other dose reductions due to: AEs (n=3), self-reduction due to fatigue (n=1), taper prior to discontinuation (n=1)



Ex. 2001-0980

# Haptoglobin levels increase in responders, indicating decreased haemolysis




Normal reference low and high limits shown as horizontal dotted lines



Ex. 2001-0981

# Pharmacodynamic Results



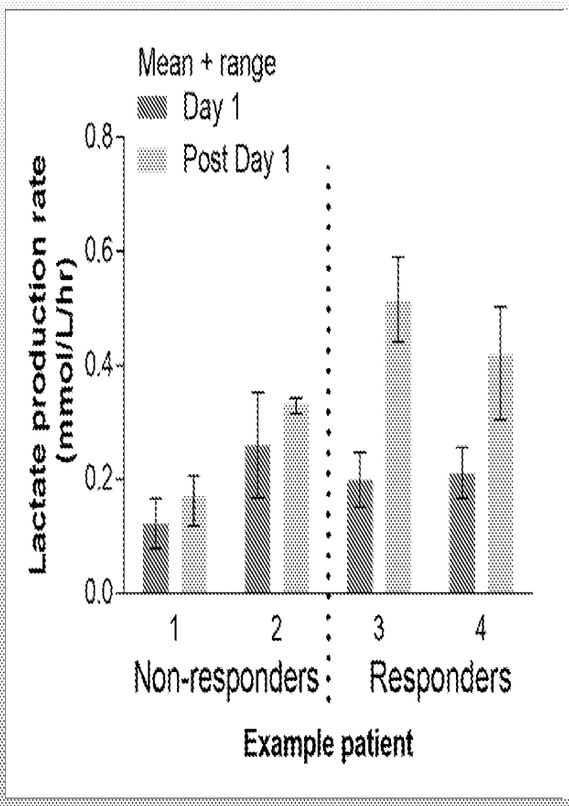
Ex. 2001-0982

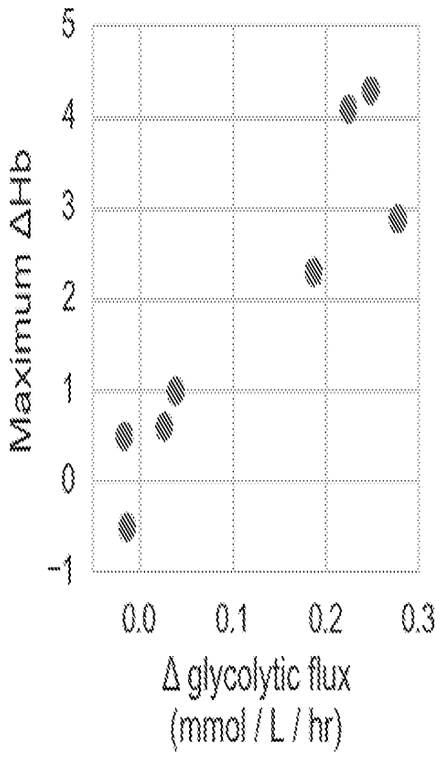
# Patients with Hb response showed increased rate of glycolysis in peripheral blood

- Glycolytic metabolism was assessed in blood samples from a subset of patients pre and post treatment
- These data suggest a positive correlation between Hb change and change in glycolytic flux

Detailed metabolic characterization in blood from 8 patients

Glycolytic flux assessed *ex vivo* via incorporation of $^{13}C$ labelled glucose into the end product, lactate




Representative 4 patients shown here

N=8



Ex. 2001-0983

# DRIVE-PK conclusions

- AG-348 is a novel, first-in-class, PK-R activator in clinical testing as a potential disease-modifying therapy to improve anaemia in patients with PK deficiency
- Daily dosing with AG-348 for up to 6 months is well tolerated
  - Clinical significance of AG-348 aromatase inhibition is unclear
- AG-348 demonstrates clinically relevant rapid and durable increases in Hb in 47% of patients enrolled in the study
  - Hb increase is linked to activation of glycolytic pathway
  - Preliminary genotype-Hb response correlations were observed
- These data highlight the potential of PK-R activators as the first disease-modifying treatment for patients with PK deficiency



Ex. 2001-0984

# Acknowledgment


- Clinical research sites and study investigators:
  - Boston Children's Hospital
  - Stanford University Medical Centre
  - University of Utah
  - Weill Cornell – New York Presbyterian Hospital
  - The Children's Hospital of Philadelphia
  - Central Pennsylvania Clinic
  - Wayne State University School of Medicine
  - University of Toronto – University Health Network
  - Hammersmith Hospital
  - Universitair Medisch Centrum Utrecht
  - Hôpital Henri Mondor
  - Hôpital Saint Vincent de Paul
  - Hôpital de la Timone
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico
- Agios Pharmaceuticals

Our patients who agreed to participate in this study

# Population pharmacokinetics and pharmacodynamics of AG-348 in healthy human volunteers guide dose selection for the treatment of pyruvate kinase deficiency

Kha Le[1], Marvin Cohen[2], Yue Chen[1], Hyaryun Kim[1], Bruce Silver[2], Sam Agresta[1], Elizabeth Merical[1], Charles Kung[1], Penelope Kosinski[1], Lee Silverman[1], Scott Biller[1], Hua Yang[1]
[1]Agios Pharmaceuticals, Cambridge, MA, USA; [2]MBC Pharma Solutions, Newtown, PA, USA; [3]Bruce A Silver Clinical Science and Development, Dunkirk, MD, USA

## BACKGROUND

- Pyruvate kinase (PK) deficiency is a glycolytic enzymopathy that results in non-spherocytic hemolytic anemia with a variable clinical presentation, ranging from mild or fully compensated forms to life-threatening neonatal anemia and life-long chronic hemolytic anemia associated with severe, debilitating co-morbidities.
- PK deficiency is caused by mutations in the *PKLR* gene, which in the red blood cell (RBC) results in defective pyruvate kinase isoform R (PK-R).
- PK-R catalyzes the final, irreversible step in glycolysis, the process on which mature RBCs rely almost exclusively to generate the energy carrier molecule adenosine triphosphate (ATP).
- PK-deficient RBCs and their progenitors are characterized by changes in metabolism associated with defective glycolysis, including a build-up of phosphoenolpyruvate (PEP) and 2,3-diphosphoglycerate (2,3-DPG), and lowered ATP levels.
- AG-348 is an orally bioavailable, allosteric activator of PK-R.
- Biochemical experiments demonstrate that AG-348 is a potent, pan-activator of many PK-R alleles associated with PK deficiency.
- Treatment of PK-deficient patient RBCs *ex vivo* with AG-348 results in increased ATP levels, and reductions in PEP and 2,3-DPG, consistent with pharmacological activation of the PK-R enzyme.[1]
- It is hypothesized that intervention with AG-348 restores glycolytic pathway activity and normalizes RBC metabolism *in vivo*.[1]
- This analysis integrates the pharmacokinetic and pharmacodynamic (PK/PD) properties of AG-348 in healthy human volunteers using population PK/PD modeling and simulation.

## OBJECTIVES

- To support the phase 2 dose selection through:
  - Characterization of non-linear and time-dependent pharmacokinetic properties of AG-348.
  - Characterization of longitudinal biomarker response (ATP and 2,3-DPG) as a function of plasma AG-348 exposure.
  - Prediction of steady state biomarker response of selected phase 2 doses.

## METHODS

- PK/PD data from two phase 1, single-center, randomized, double-blind, placebo-controlled dose escalation studies were included in this analysis.
- A total of 96 healthy volunteers received either placebo, or a single dose[2] (NCT02108106) or multiple doses[3] (14 days, NCT02149966) of AG-348.
- AG-348 dose level ranged from 15–2500 mg given once (QD) or twice (BID) daily.
- Rich sampling was performed on Day 1 (for single and multiple dose studies) and Day 14, with sparse sampling in between (for multiple dose study).
- Blood was collected from all patients to assess AG-348 pharmacokinetics, and for determination of levels of ATP and 2,3-DPG.
- PK/PD modeling using a non-linear mixed effects approach was performed to understand the pharmacokinetics of AG-348 and PK/PD relationship of AG-348 to 2,3-DPG and ATP in humans.
- Population simulations using the final model were performed to examine the dose-exposure-biomarkers relationship with AG-348 dosed twice daily at various levels (15–2500 mg).
- Modeling analysis and simulations were performed using NONMEM 7.3.

### Model Structure

$$1.\quad \frac{dA_1}{dt} = -k_a \frac{A_1}{1+\alpha_1 \cdot A_1}$$

$$2.\quad \frac{dA_2}{dt} = k_a \cdot \frac{A_1}{1+\alpha_1 \cdot A_1} - k_{23} \cdot A_2 + k_{32} \cdot A_3 - k_{24} \frac{A_2}{1+A_2} + k_{42} A_4 - k_e \cdot A_2 \cdot (1+A_5)$$

$$3.\quad \frac{dA_3}{dt} = k_{23} \cdot A_2 - k_{32} \cdot A_3$$

$$4.\quad \frac{dA_4}{dt} = k_{24} \frac{A_2}{1+A_2} - k_{42} \cdot A_4$$

$$5.\quad \frac{dA_5}{dt} = k_{PDO} \frac{A_2/V_c}{1+\alpha_2 \cdot A_2/V_c} - k_{dAg} \cdot A_5$$

$$6.\quad \frac{dA_6}{dt} = k_{PDO} \frac{A_2/V_c}{1+\alpha_2 \cdot A_2/V_c} - k_{dAg2} \cdot A_6$$

$$7.\quad \frac{dA_7}{dt} = BL\_ATP \cdot k_{testA} \left[1 + \frac{E_{maxA} \cdot A_2/V_c}{EC_{50,A} + A_2/V_c}\right] - A_7 \cdot k_{outA}$$

$$8.\quad \frac{dA_8}{dt} = BL\_23DPG \cdot k_{outB} - A_8 \cdot k_{outB} \left[1 + \frac{E_{maxB} \cdot A_2/V_c}{EC_{50,B} + A_2/V_c}\right]$$

## RESULTS

### Pharmacokinetics

- The pharmacokinetics of AG-348 were described by a 3-compartmental model with first order absorption (**Figures 1** and **2**, and **Table 1**).

Table 1. Model parameters

| | | | | | |
|---|---|---|---|---|---|
| **Pharmacokinetic parameters** | | | | | |
| $k_a$ | 1/d | 28.80 | 36 | 44 | 26 |
| $k_e$ | 1/d | 11.79 | 17 | 43 | 24 |
| $V_c$ | L | 22.74 | 17 | 78 | 46 |
| $k_{23}$ | 1/d | 5.49 | 32 | | |
| $k_{32}$ | 1/d | 9.45 | 15 | | |
| $k_{24}$ | 1/d | 70.77 | 47 | 30 | 30 |
| $k_{42}$ | 1/d | 0.26 | 36 | 89 | 43 |
| $\alpha_1$ | Dimensionless | 0.002 | 18 | | |
| $K_{m1}$ | ng/mL | 0.29 | 27 | | |
| $k_{deg}$ | 1/d | 0.09 | 20 | | |
| $K_{m2}$ | ng/mL | 706.55 | 65 | | |
| $k_{deg2}$ | 1/d | 3.21 | 30 | | |
| $\alpha_2$ | Dimensionless | 0.01 | 67 | | |
| $\theta_3$ | Dimensionless | 0.42 | 39 | | |
| **Pharmacodynamic parameters** | | | | | |
| $BL_{ATP}$ | ng/mL | 0.27 | 2 | | |
| $EC_{50,A}$ | ng/mL | 0.95 | 30 | | |
| $E_{max,A}$ | Dimensionless | 0.62 | 8 | | |
| $k_{outA}$ | 1/d | 0.22 | 55 | | |
| $BL_{23DPG}$ | ng/mL | 0.81 | 1 | | |
| $EC_{50,B}$ | ng/mL | 0.11 | 55 | | |
| $E_{max,B}$ | Dimensionless | 1.41 | 9 | | |
| $k_{outB}$ | 1/d | 0.88 | 8 | | |

RSE, relative standard error; η, inter-individual variability; $k_a$, absorption rate constant; $k_e$, elimination rate constant; $V_c$, central volume of distribution; $k_{23}$, $k_{32}$, $k_{24}$, $k_{42}$, rate constants of transfer between compartments; $\alpha_1$, non-linear absorption constant; $\alpha_2$, non-linear elimination constant; $k_{deg}$, $k_{deg2}$, first-order rate constant for ATP and 2,3-DPG production and degradation; $k_{PDO}$, zero-order production rate constant; $BL_{ATP}$, baseline concentration of ATP; $BL_{23DPG}$, baseline concentration of 2,3-DPG; $k_{outA}$, ATP production rate constant; $k_{outB}$, 2,3-DPG production rate constant; $EC_{50,A}$, half maximal ATP response concentration; $EC_{50,B}$, half maximal 2,3-DPG response concentration; $E_{max,A}$, maximum ATP response; $E_{max,B}$, maximal 2,3-DPG response

- Rate of absorption appears to be dependent on dose (**Equations 1** and **2**).
- A decrease in plasma exposure of AG-348 with multiple dosing was observed (at 120 mg BID and higher), which can be described with a semi-mechanistic auto-induction model (**Equations 2** and **5**, **Figure 2A**).
  - Preclinical data suggested that AG-348 is a moderate inducer of CYP3A4, the major metabolic pathway of AG-348.
- A complex distribution pattern with time was suggested by the model (**Equation 2**).
- Magnitude of CYP3A4 enzyme induction was dose dependent (**Figure 2A**).
- In the model, 90% maximum CYP3A4 enzyme induction level was reached at 12–19 days following 15–1400 mg dosing (**Figure 3B**).
- Expected time courses of plasma AG-348 in healthy adults for the selected phase 2 doses are shown in **Figure 3A**.

**Figure 1. Model schematic**

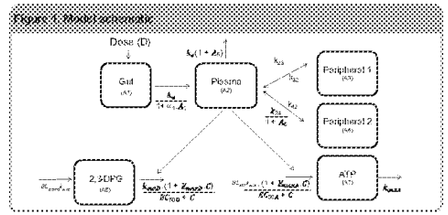



**Figure 2. Model-fit: Rich study predictions of (A) Pharmacokinetics (B) Pharmacodynamic biomarkers**

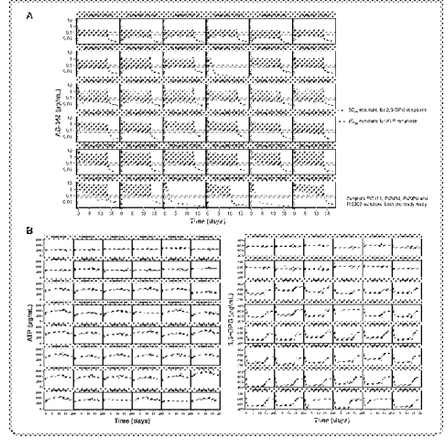
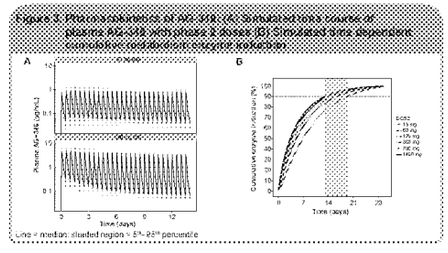


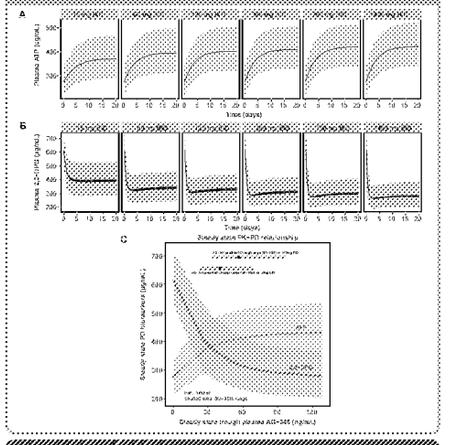

**Figure 3. Pharmacokinetics of AG-348: A. Simulated time course of plasma AG-348 with phase 2 doses (D) B. Simulated time dependent cumulative enzyme induction (%)**




### Pharmacodynamics

- The longitudinal time course of ATP and 2,3-DPG can be described by an indirect response model (**Figures 1** and **2**, **Equations 7** and **8**).
  - Plasma AG-348 stimulates rate of production ($K_{in}$) and degradation ($K_{out}$) of ATP and 2,3-DPG, respectively, in accordance with the hypothesized mechanism of action of AG-348
- Model diagnostics confirmed performance of the PK/PD model (**Figure 2B**).
- ATP and 2,3-DPG reached steady state at approximately 2 weeks of BID dosing (**Figure 4A, B**).
- 50 mg and 300 mg BID dosing is expected to cover the majority of the dynamic range of biomarker response (from 53–58% to 80–90% maximal, **Figure 4C**), supporting the selection of these doses for the ongoing, phase 2, dose-ranging study.

**Figure 4. Pharmacodynamic biomarker response to AG-348: Simulated time course of (A) ATP and (B) 2,3-DPG in response to AG-348 treatment (C) Simulated steady state relationship between plasma AG-348 and ATP/2,3-DPG levels**




## CONCLUSIONS

- This study represents the first comprehensive longitudinal analysis of AG-348 and its pharmacodynamic activity in humans.
- This integrated PK/PD model, incorporating time-varying PK/PD properties, forms the basis for understanding the exposure-response relationship in the ongoing phase 2 and future clinical studies of AG-348, as well as providing guidance on dosing selection to optimize the treatment of PK deficiency.

### Acknowledgments
We would like to thank the volunteers who took part in the clinical studies.

### References
1. Kung C et al. Blood 2013;122:2180.
2. Yang H et al. Blood 2014;124:4207.
3. Yang H et al. Haematologica 2015;100(s1):S138.

### Disclosures
The clinical studies and this analysis were funded by Agios Pharmaceuticals.
KL, YC, HK, SA, EM, CK, PK, LS, SB, HY: Agios – employment and stockholder; MC, BS: Agios – consultant.
Editorial assistance was provided by Christine Tomkins, PhD, Excel Scientific Solutions, Horsham, UK and supported by Agios.

Presented at the 57th American Society of Hematology Annual Meeting and Exposition, December 5–8, 2015, Orlando, FL

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# Population Pharmacokinetics and Pharmacodynamics of AG-348 in Healthy Human Volunteers Guide Dose Selection for the Treatment of Pyruvate Kinase Deficiency

**Kha Le**[1*], Marvin Cohen[1,2*], Yue Chen[1*], Hyeryun Kim[1*], Bruce Silver[3*], Sam Agresta[1], Elizabeth Merica[1*], Charles Kung[1*], Penelope Kosinski[1*], Lee Silverman[1*], Scott Biller[1*] and Hua Yang[1*]

[1]Agios Pharmaceuticals, Cambridge, MA; [2]MBC Pharma Solutions, Newtown, PA; [3]Bruce A Silver Clinical Science and Development, Dunkirk, MD

INTRODUCTION: Pyruvate kinase (PK) deficiency is a glycolytic enzymopathy that results in non-spherocytic hemolytic anemia with a variable clinical presentation, ranging from mild or fully compensated forms to life-threatening neonatal anemia and life-long chronic hemolytic anemia associated with severe, debilitating co-morbidities. PK deficiency is caused by mutations in the *PKLR* gene, which in the red blood cell (RBC) results in defective pyruvate kinase isoform R (PK-R). PK-R catalyzes the final, irreversible step in glycolysis, the process on which mature RBCs rely almost exclusively to generate the energy carrier molecule adenosine triphosphate (ATP). PK-deficient RBCs and their progenitors are characterized by changes in metabolism associated with defective glycolysis, including a build-up of phosphoenolpyruvate (PEP) and 2,3-diphosphoglycerate (2,3-DPG), and lowered ATP levels. AG-348 is an orally available, allosteric activator of PK-R. It is hypothesized that intervention with AG-348 restores glycolytic pathway activity and normalizes RBC metabolism *in vivo* (Kung C et al. *Blood*, 2013). Biochemical experiments demonstrate that AG-348 is a potent pan-activator of many PK-R alleles associated with PK deficiency. Treatment of PK-deficient patient RBCs *ex vivo* with AG-348 results in increased ATP levels, and reductions in PEP and 2,3-DPG, consistent with pharmacological activation of the PK-R enzyme. This analysis integrates the pharmacokinetic and pharmacodynamic (PK/PD) properties of AG-348 in healthy human volunteers using population PK/PD modeling and simulation.

METHODS: PK/PD modeling using a non-linear mixed effects approach was performed to understand the pharmacokinetics of AG-348 and PK/PD relationship of AG-348 to 2,3-DPG and ATP in humans. The PK/PD model integrated data from two phase 1, single-center, randomized, double-blind, placebo-controlled, dose escalation studies (one single and one 14-day multiple ascending dose) that enrolled a total of 96 healthy volunteers (Yang H et al. EHA Learning Center, 2015). AG-348 dose level ranged from 15-2500 mg given once (QD) or twice (BID) daily. Blood was collected from all patients to assess AG-348 pharmacokinetics, and for determination of levels of ATP and 2,3-DPG. Population simulations using the final model were performed to examine the dose-exposure-biomarkers relationship at various dose levels and duration of dosing.

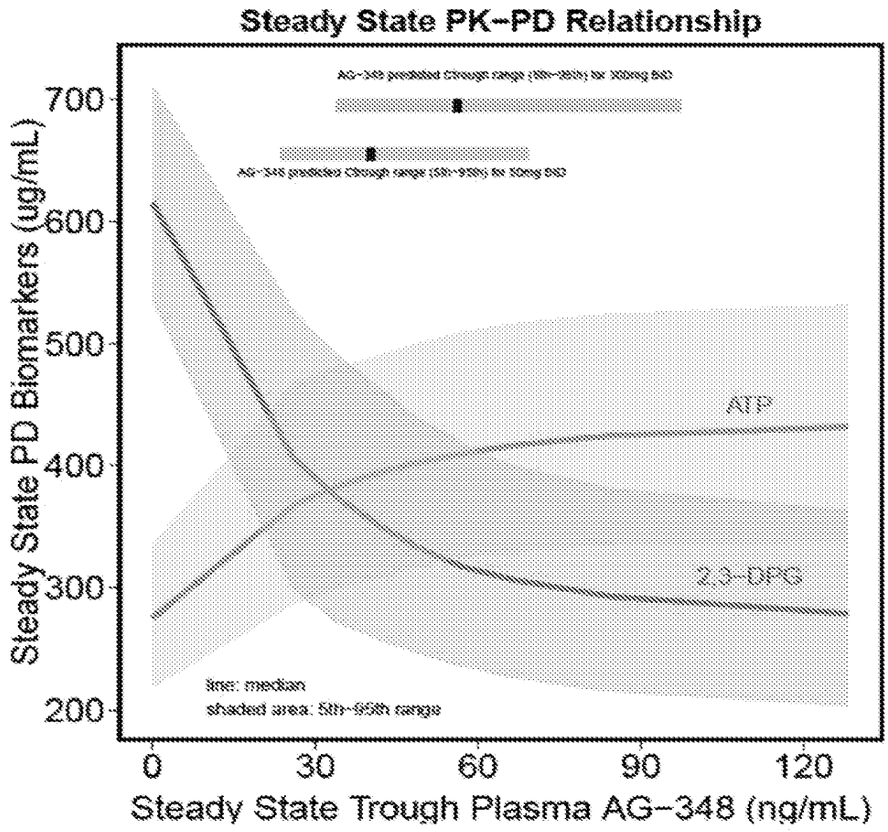
RESULTS: AG-348 showed rapid absorption following oral administration. Plasma exposure of AG-348 increased in a dose-proportional manner following a single dose. A three-compartmental model with a non-linear absorption compartment and a saturable induced enzyme compartment best described the pharmacokinetics of AG-348. Time-varying clearance was added to describe the observed decrease in exposure over time with multiple dosing; this is consistent with pre-clinical data that AG-348 is a moderate inducer of CYP3A4, the major oxidation pathway of AG-348. The multiple-dose data were well described by a semi-mechanistic autoinduction model with an indirect model and a saturable induction compartment. The PK/PD relationship between plasma AG-348 to ATP and 2,3-DPG showed best fit with a turnover model where the drug effect was described by an $E_{max}$ model. Model simulations predicted maximum enzyme induction and PD response 3 weeks after the first dose following BID dosing. Population PK/PD simulations further supported the choice of 50 mg and 300 mg BID doses for the phase 2 study (**Fig 1** and **2**). The proposed current model incorporating PK/PD data over a wide range of AG-348 exposures and time-varying changes in clearance provides a useful tool for prediction of AG-348 pharmacokinetics that can be used to optimize AG-348 dosing for PK deficiency treatment. Furthermore, the population PK/PD model of AG-348 to ATP and 2,3-DPG biomarkers in healthy volunteers provides a good foundation to facilitate the analysis and understanding of patient data in the ongoing phase 2 study.

CONCLUSION: This study represents the first comprehensive longitudinal analysis of AG-348 and its PD activity in humans. This integrated PK/PD model, incorporating time-varying PK/PD properties, forms the basis for



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understanding the exposure-response relationship in the ongoing phase 2 and future clinical studies of AG-348, as well as providing guidance on dosing selection to optimize the treatment of PK deficiency.



# Mitapivat (AG-348) in adults with pyruvate kinase deficiency who are regularly transfused: A phase 3, open-label, multicenter study (ACTIVATE-T) in progress

Megan Lynch[1], Lei Hua[1], Chris Mix[1], John Porter[2]

[1]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [2]University College London Hospitals, London, UK

## BACKGROUND

### PK deficiency: disease overview[1-3]

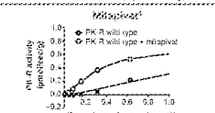
- Under-recognized hereditary disease
- Heterogeneous disease with variable severity among all ages
- Caused by mutations in the *PKLR* gene coding for red cell PK (PK-R), resulting in defective glycolysis and decreased red blood cell lifespan
- Lifelong hemolytic anemia
- Iron overload, jaundice, and other complications
- Infection and thrombosis risk post splenectomy
- PK-R enzyme activity and genetic testing
- Supportive treatment: transfusions, splenectomy, iron chelation

PK deficiency: mutation type[4]



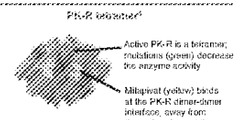
Types of *PKLR* mutations found in 193 unrelated cases in the PK deficiency natural history study

Mitapivat[5]




Mitapivat binding increases the affinity of PK-R for its substrate, phosphoenolpyruvate (monomeric enzyme unit shown)

PK-R structure[6]




### *Pyruvate kinase (PK) deficiency*

- PK deficiency is a rare, congenital, hemolytic anemia (**Figure 1**).
- Mitapivat sulfate (mitapivat; AG-348) is a novel, first-in-class, small-molecule allosteric activator of red-cell PK (PK-R). It is being developed as a potential treatment for PK deficiency and has been tested in phase 1 and 2 (DRIVE PK) studies.[7,8]

### *Mitapivat in PK deficiency*

#### *DRIVE PK study*

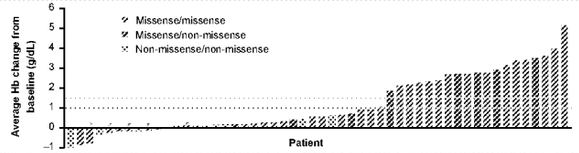
- Phase 2, open-label, dose-ranging study in adult patients with PK deficiency who are not regularly transfused (NCT02476916); hemoglobin (Hb) ≤12.0 g/dL (if male) or ≤11.0 g/dL (if female)[8]:
  - Primary endpoint: safety and side-effect profile of mitapivat
  - Secondary endpoints: Hb, markers of hemolysis, pharmacokinetics, and pharmacodynamics
  - Patients (N=52) were randomized to an initial mitapivat dose of 50 mg twice daily (BID) or 300 mg BID; subsequent dose changes were permitted on the basis of safety, tolerability, and Hb response
  - Core period (first 6 months) completed; extension period is ongoing (see Poster 3512 for extension study data).

#### *Cumulative safety summary*

- Mitapivat was generally well tolerated during the core study and an ongoing extension as of data cutoff (August 31, 2018; median treatment duration of 16.4 months [range, 3–35]).[8]
  - All patients experienced at least one adverse event (AE), of which the majority were grade 1 or 2; 19 of 52 patients (37%) experienced a grade 3 or 4 AE.
  - Six treatment-related AEs led to discontinuation (an increase in alanine aminotransferase level plus nonalcoholic steatohepatitis, hemolytic anemia, nausea plus pharyngitis, hypertriglyceridemia, left renal-cell carcinoma, and pleural effusion).
  - There were 18 serious AEs in 15 patients.

#### *Efficacy (core study)*

- 26 of 52 patients (50%) had a maximum Hb increase from baseline of >1.0 g/dL.
  - Among these patients, the mean maximum increase in Hb was 3.4 g/dL (range, 1.1–5.8).
  - Median time until first observed Hb increase of >1.0 g/dL was 10 days (range, 7–187).
  - All patients who had a mean Hb increase from baseline of >1.0 g/dL had at least one missense mutation (**Figure 2**).




A mean increase of 1.0 g/dL in the Hb level did not occur in any of the five patients who were homozygous for the R479H mutation (as indicated by asterisks). The blue gray line indicates a mean increase of +1.0 g/dL in the Hb level and the light gray line indicates a mean increase of 0.0 g/dL.

From the New England Journal of Medicine, Grace RF, Rose C, Layton DM, et al. Safety and efficacy of splenectomy for pyruvate kinase deficiency. 381(10):933-944. Copyright © 2019 Massachusetts Medical Society. Reprinted with permission from the Massachusetts Medical Society.

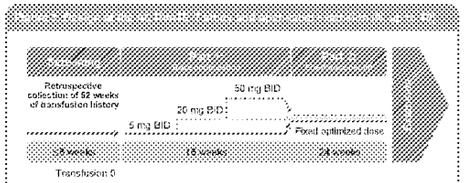
## ACTIVATE-T STUDY (PHASE 3)

### Summary

- ACTIVATE-T is a global, multicenter, open-label study to evaluate the efficacy and safety of mitapivat in adult patients with PK deficiency who are regularly transfused (NCT03559690; **Figure 3**).

### Current status

- This phase 3 study in adult patients with PK deficiency is enrolling patients at sites in North America, Europe, and Asia (**Figure 4** and **Table 1**).




**Key inclusion criteria**

- ≥18 years of age
- Documented presence of at least two mutant alleles in *PKLR*, of which at least one is a missense mutation (based on genotype–Hb response correlations in DRIVE PK; see **Figure 2**)
- Six or more transfusion episodes in the past 52 weeks
- Adequate organ function

**Key exclusion criteria**

- Homozygous for the R479H mutation or has non-missense mutations in *PKLR*
- History of transfusions occurring on average more frequently than once every 2 weeks in the past 52 weeks
- Significant medical condition that confers an unacceptable risk for participating in the study and/or that could confound the interpretation of data

*Screening*

- Transfusion history information used to:
  - Calculate mean transfusion frequency
  - Calculate individual transfusion trigger: mean (±0.5 g/dL or ±0.31 mmol/L) of a patient's collected historical pretransfusion Hb values
  - Calculate mean number of blood units (MNU) transfused per transfusion
  - Function as historical control data for the analysis.

*Part 1: Individualized dose optimization period*

- All patients start on 5 mg BID mitapivat.
- Following safety assessments the dose can be increased from 5 to 20 mg BID and subsequently from 20 to 50 mg BID.
- Maximal Hb increases are sought while maintaining an acceptable safety profile.

*Part 2: Fixed-dose period*

- Patients receive mitapivat at their optimized dose, with no planned adjustment for 24 weeks.
- Doses can be reduced or suspended for safety reasons at any time during the study.
- Patients are transfused with their MNU when their Hb reaches their individual transfusion trigger.

*Extension study*

- Patients completing the study may have the opportunity to enroll in an ongoing open-label extension study (AG348-C-011; EudraCT 2018-003459-36 and NCT03853798) in which all participants will receive mitapivat for up to 192 weeks.
  - Objective: evaluate the long-term safety, tolerability, and efficacy of treatment with mitapivat in participants who were previously enrolled in ACTIVATE-T or its companion study, ACTIVATE.

*Key endpoints*

*Primary*

- Reduction in transfusion burden, defined as a reduction of ≥33% in the number of red blood cell units transfused during the 24 weeks of the fixed-dose period compared with the historical transfusion burden standardized to 24 weeks.

*Key secondary*

- Number of red blood cell units transfused during the study
- Number of transfusion episodes during part 2
- Proportion of patients who become transfusion free.
- Proportion of patients who achieve Hb levels in the normal range in part 2.

*Other secondary*

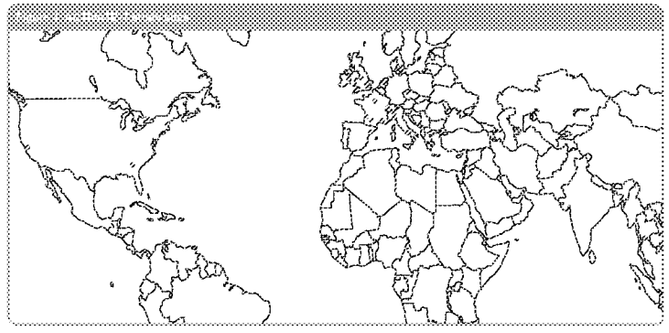
- The type, incidence, severity, and relationship to study treatment of AEs and serious AEs.
- Change from baseline in patient-reported health-related quality-of-life scores.
- AEs leading to treatment dose reduction, treatment interruption, and treatment discontinuation.
- Pharmacokinetic parameters of mitapivat.

*Statistics*

- Sample size is driven by feasibility (up to 40 patients).
- With a sample size of 20, the power of the study will be 88% to detect a response rate of 30% compared with a null rate of 10%, based on a two-sided Fisher's exact test at the 0.05 significance level.

Table 1. Active sites as of October 2018

| Country | Participating Sites |
|---|---|
| Canada | Toronto General Hospital, ON |
| Czech Republic | Ústav Hematologie a Krevní Transfuze, Prague |
| Denmark | Herlev and Gentofte University Hospital |
| France | AP-HP – Hôpital Henri Mondor, Créteil; AP-HM – Hôpital de la Timone, Marseille; CHU de Bordeaux – Hôpital Saint-Andre; IUCT – Institut Universitaire du Cancer de Toulouse |
| Ireland | St James's Hospital, Dublin |
| Italy | Ospedali Galliera di Genova; IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano; A.O.U. Seconda Università Degli Studi di Napoli; Azienda Ospedaliera di Rilievo Nazionale Antonio Cardarelli, Napoli |
| Netherlands | University Medical Center Utrecht |
| Spain | Hospital Universitari Germans Trias i Pujol, Barcelona |
| Switzerland | Centre Hospitalier Universitaire Vaudois, Lausanne |
| Thailand | Faculty of Medicine Siriraj Hospital – Mahidol University, Bangkok |
| UK | Imperial College Healthcare NHS Trust, London; University College London Hospitals NHS Foundation Trust; Addenbrooke's Hospital, Cambridge; Manchester Royal Infirmary |
| USA | Massachusetts General Hospital, Boston, MA; Children's Healthcare of Atlanta/Emory University, Atlanta, GA; University of Washington, Seattle, WA; University of California San Francisco Benioff Children's Hospital |



**Acknowledgments** We would like to thank the investigators and patients taking part in the PK deficiency studies. Disclosures: This study is funded by Agios Pharmaceuticals, Inc. ML, LH, and CM: Agios – employment and stockholder; JP: no conflict of interest to disclose. Editorial assistance was provided by Mark Poirier, Excel Medical Affairs, Pickfield, CT, USA, and supported by Agios.

**References** 1. Grace RF et al. *Am J Hematol* 2015;90:826-30. 2. Perry MJ et al. *Blood Cells Mol Dis* 2007;38:164-94. 3. Grace RF et al. *Blood* 2018;131:2183-92. 4. Kung C et al. *Blood* 2017;130:1347-56. 5. Yang H et al. *Clin Pharmacol Drug Dev* 2019;8:344-59. 6. Grace RF et al. *N Engl J Med* 2018;381:933-44.

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Presented at the 61st American Society of Hematology (ASH) Annual Meeting, December 7–10, 2019, Orlando, FL, USA

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# Mitapivat (AG-348) in Adults with Pyruvate Kinase Deficiency Who Are Regularly Transfused: A Phase 3, Open-Label, Multicenter, Study (ACTIVATE-T) in Progress

Megan Lynch, MSN, Lei Hua, PhD, Chris Mix, MD, John B. Porter, MA MDFRCP FRCPath

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*Blood* (2019) 134 (Supplement_1): 3526.

https://doi.org/10.1182/blood-2019-123107

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**Background:** Pyruvate kinase (PK) deficiency is an under-recognized hereditary disease that causes lifelong hemolytic anemia. Mutations in the *PKLR* gene lead to reduced red cell PK (PK-R) enzyme activity, resulting in defective glycolysis and decreased lifespan of red blood cells. Mitapivat (AG-348) is a novel, first-in-class, oral, small-molecule allosteric activator of PK-R under clinical testing as the first targeted, disease-altering therapy for PK deficiency. In the DRIVE PK study (NCT02476916; a phase 2, open-label, dose-ranging trial in adults with PK deficiency who are not regularly transfused), twice-daily (BID) dosing with mitapivat for >6 months was well tolerated and induced rapid, durable responses (Grace et al. *ASH* 2017). As of July 14, 2017, 26 (50%) of 52 enrolled subjects had a maximum hemoglobin (Hb) increase of >1 g/dL. Among these 26 subjects, the mean maximum Hb increase was 3.4 g/dL, and 25 (96%) had ≥1 missense *PKLR* mutation. Based on these findings, mitapivat has entered phase 3 clinical development. The design of the ongoing phase 3 ACTIVATE-T study and its extension study are reported here, with an update on country/site activation and key early learnings on trial logistics in PK deficiency.

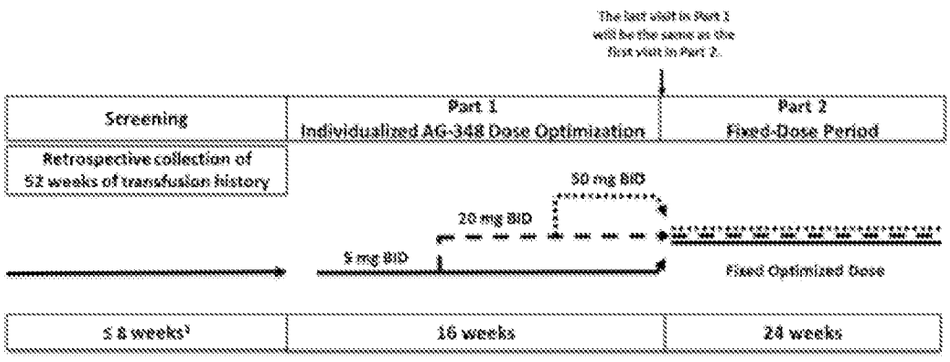
**Methods:** ACTIVATE-T is a global, multicenter, open-label study (NCT03559699) to evaluate the efficacy and safety of mitapivat in regularly transfused adults with PK deficiency. Regularly transfused is defined as ≥6 transfusion episodes in the previous year. Subjects who are homozygous

for the R479H mutation or have 2 non-missense mutations (without the presence of another missense mutation) in *PKLR* will be excluded, as will subjects with an average transfusion frequency of more than once every 3 weeks in the previous year.

The study comprises an 8-week screening period, during which each subject's complete transfusion history from the prior 52 weeks is documented, followed by a 16-week dose optimization period and a 24-week fixed-dose period (Figure). During the dose optimization period, each subject will undergo individualized mitapivat dose optimization. All subjects will start on a dose of 5 mg BID, which may be increased twice over the course of 16 weeks (from 5 to 20 mg BID and from 20 to 50 mg BID). In the fixed-dose period, each subject will receive mitapivat at their optimized dose for 24 weeks. During the study, subjects will be transfused when their Hb reaches or falls below their individual transfusion trigger calculated from their transfusion history. The primary endpoint is the proportion of subjects who achieve a reduction in transfusion burden, defined as a reduction of ≥33% in the number of red blood cell units transfused during the 24 weeks of the fixed-dose period compared with the historical transfusion burden standardized to 24 weeks. Secondary endpoints include safety.

All subjects who complete the study will have the opportunity to enroll in an open-label extension study (NCT03853798) in which all participants will receive mitapivat for up to 192 weeks. The ACTIVATE-T trial is enrolling globally.

Figure. ACTIVATE-T Study Schema



# THE PYRUVATE KINASE ACTIVATOR AG-348 IMPROVES MURINE β-THALASSEMIC ANEMIA AND CORRECTS INEFFECTIVE ERYTHROPOIESIS

A Matte[1], E. Beneduce[1], A. Siciliano[1], P. A. Kosinski[2], A. Janin[3], C. Lebouef[3], A. Iolascon[4], L. De Falco[4], L. Dang[2], C. Kung[2], L. De Franceschi[1]

[1]Department of Medicine, University of Verona-AOUI Verona, Verona; Italy; [2]Agios Pharmaceuticals, Inc., Cambridge, MA, USA [3]Inserm, U1165, Université Paris 7- Denis Diderot, AP-HP, Hôpital Saint-Louis, F-75010, Paris, France; [4]CEINGE and Dept. of Biochemistry, University of Naples, Naples; Italy

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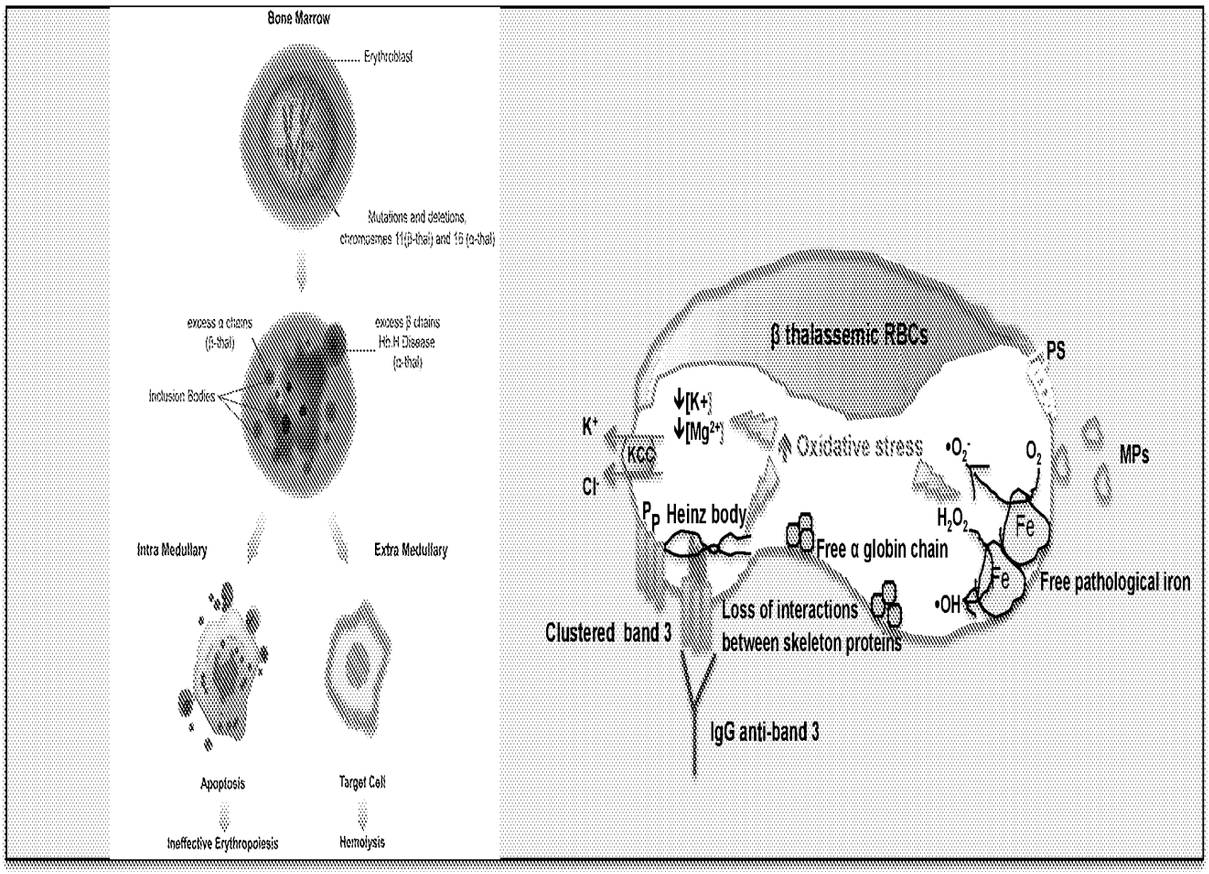
# β-Thalassemic Syndromes are Worlwide Distributed Hereditary Red Cell Disorder and is a Model of Pathological Erythropoiesis

- 7% of global population is a carrier for severe hemoglobinopathies and β thalassemias are one of the most common inherited red cell disorders

- β thalassemias are characterized by absent or reduced synthesis of β globin chains, resulting in accumulation of free α-chain and free pathological heme.

Weatherall DJ et al. Ann Human Biol 32: 117, 2005; Weatherall DJ Bull WHO 79: 704, 2001; Rund D et al. NEJM 353: 1135, 2005

# In β thalassemic Syndromes is due to both Ineffective Erythropoiesis and Reduced RBC Survival in the Peripheral Circulation




In β -thalassemias, the intra-cellular accumulation of free α -globin chains, free pathological iron and free heme result in high ROS production.

Ginzburg Y et al. Blood 118: 4321, 2011; De Franceschi L et al. Oxidative Medicine and Cellular Longevity 2013, Matte A ARS 23: 1284, 2015

# Potentiation of Endogenous Anti-oxidant Systems as Novel Therapeutic Strategy in β-thalassemia

- In β-thalassemia, exogenous anti-oxidants have been largely studied to limit ROS cytotoxity and ineffective erythropoiesis

- Potentiation of endogenous anti-oxidant systems might be a novel interesting therapeutic strategy to face chronic and severe oxidative stress such as in β-thalassemia.

Franco SS et al. Haematologica 99: 267, 2014; Matte A ARS 23: 1284, 2015; Rund D et al. NEJM 353: 1135, 2005; Macari ER et al. Blood 117: 5987, 2011; Schmidt HHHW et al. ARS 23:1130, 2015

# ATP is important for activity of anti-oxidant systems and low ATP levels have been reported in β-thal RBCs

- In RBCs, ATP is generated through glycolysis and pyruvate kinase-R is involved in the final step of ATP production

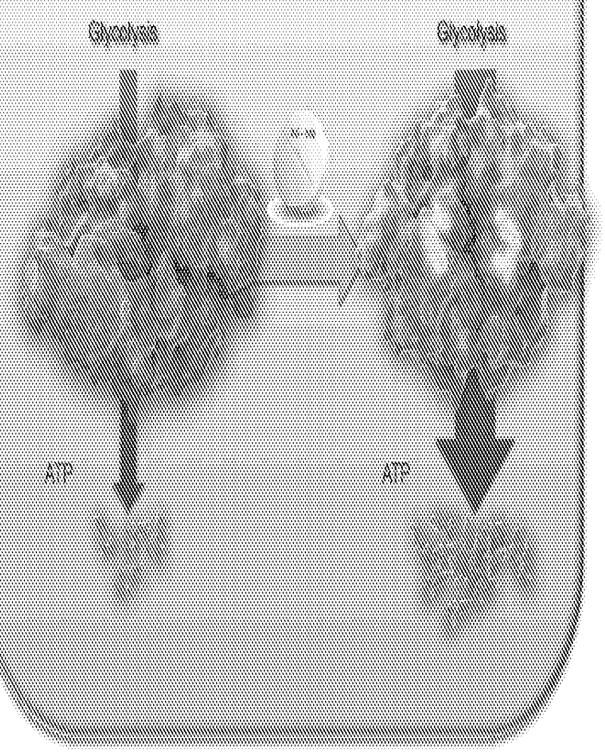
- β-thal intermedia RBCs show 3-fold faster glucose metabolism compared to controls

- Under oxygenation/deoxygenation condition, β-thal major and β-thal /HbE RBCs show reduced ATP content compared to healthy RBCs

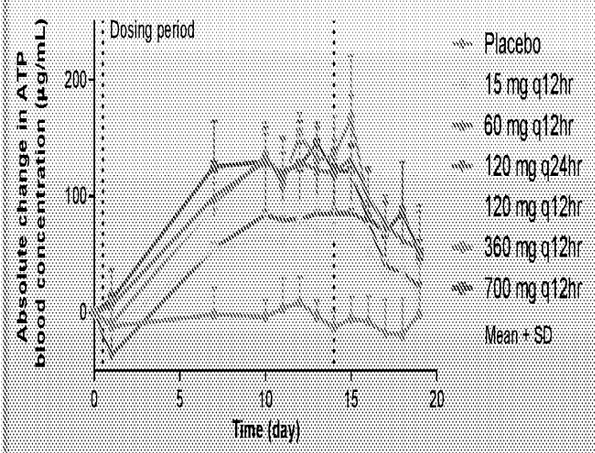
Ting YL et al. BIH 88: 347, 1994; Chakrabarty I et al. Arch Med Res 2012

# AG-348: Allosteric activator of the red cell isoform of pyruvate kinase (PK-R)

## AG-348 binds at PK-R enzyme dimer-dimer interface to promote active tetramer




## AG-348 increases ATP levels in healthy volunteers (NCT02149966)




AG-348 is currently in Phase 2 testing in patients with pyruvate kinase deficiency (NCT02476916)

Kung C et al. 55th ASH Annual meeting 2013, Abstract 2180 Presented at EHA, 2015, Abstract S138



Ex. 2001-0997

# Ineffective erythropoiesis is present in PK Deficiency

- **PK deficiency is the most common hereditary abnormality in glycolytic pathway, resulting in a large clinical spectrum of hemolytic anemia**

- **Abnormalities in erythropoiesis,** (dyserythropoiesis and ineffective erythropoiesis) **have been described in <u>few patients with PK deficiency</u> as well as in a <u>PK mutated mouse model</u>, suggesting a possible role of PK in erythroid maturation events**

Zanella A et al. 13: 57, 2000; Aizawa S et al. AJH 74: 68, 2003; Aizawa S et al. Exp Hematol 33: 1292, 2005; Hajja MA et al Ped Blood Cancer 61: 1463, 2014

# Study Aim

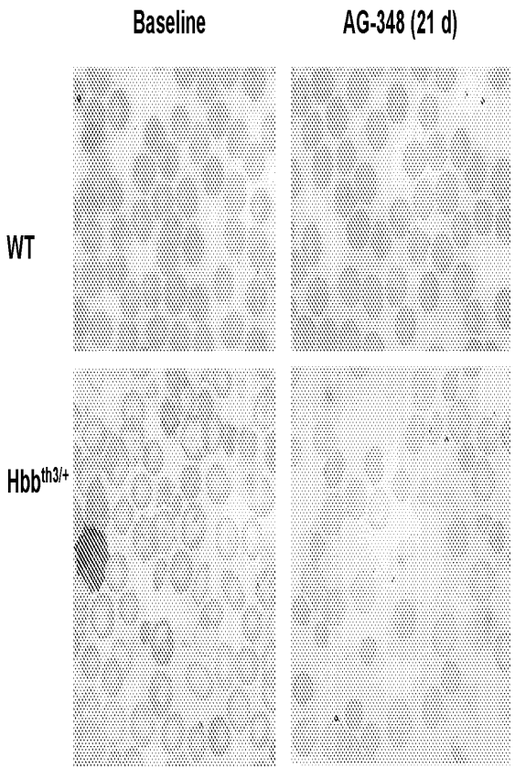
To evaluate the impact of AG-348 on anemia and ineffective erythropoiesis in a mouse model of β thal intermedia

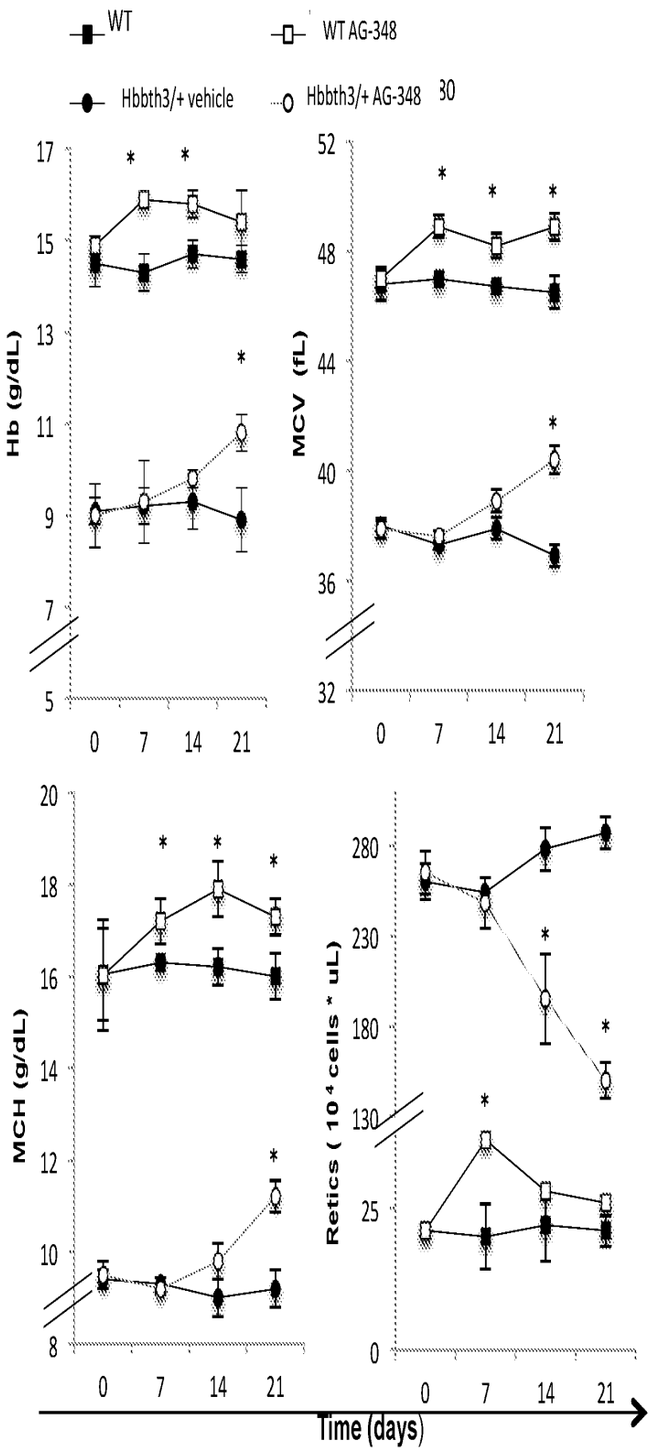
# Study Design

- $Hbb^{3th/+}$ mice were used as model of b-thalassemia intermedia and C57B6/2J as WT controls. Two month-old females were treated with either vehicle or AG-348 at 50 mg/kg bid by oral gavage.
- Hematological parameters, red cell indices and reticulocytes were determined.
- Flow cytometric analysis of erythroid precursors from bone marrow and spleen was performed using the CD44 and TER-119.
- Annexin-V positivity of orthochromatic erythroblasts was assessed.
- Histological analysis of spleen and liver was carried independently by two pathologists.
- RT-PCR analysis (including hepcidin and erythroferrone) and immunoblot analysis were carried out on sorted erythroid precursors and on liver cells.

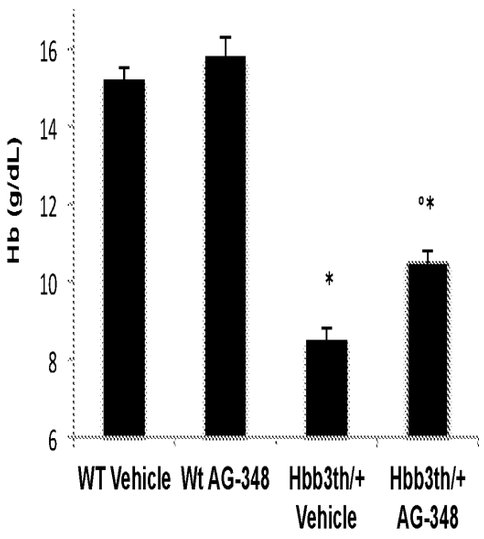
Franco SS et al. Haematologica 99: 267, 2014; Liu J et al. Blood 121: e43, 2013; Mattè A et al. Antioxid Redox Signal 23: 1284, 2015

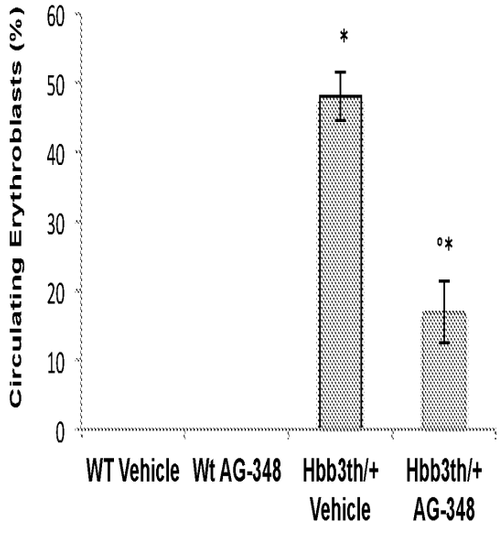
# AG-348 treatment significantly ameliorates anemia in a mouse model of β-thalassemia



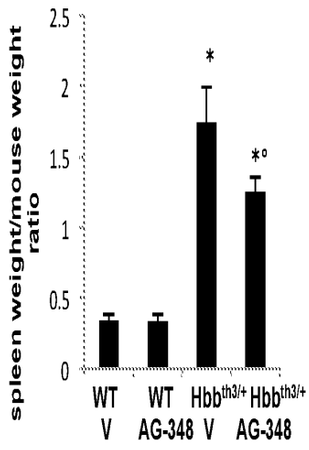
# Long-term (7 weeks treatment) with AG-348 showed increased Hb and reduction in circulating erythroblasts

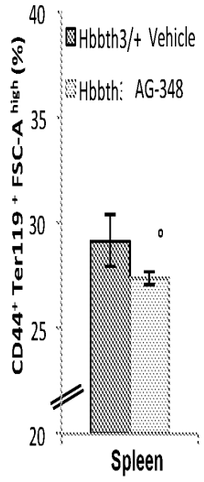


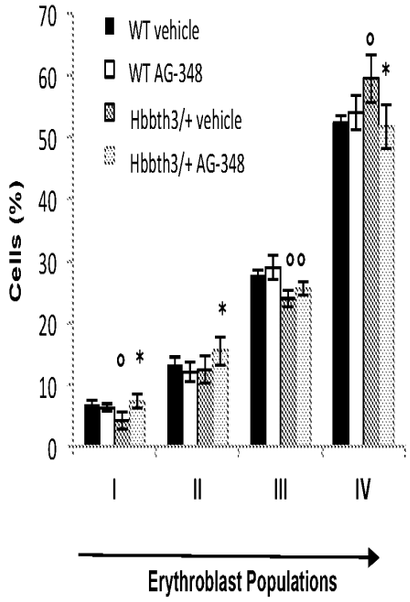



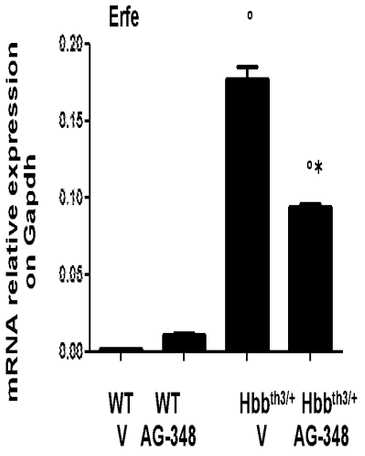

*p< 0.05 compared to WT
°p<0.05 compared to vehicle (V)

# In β-thalassemic mice, AG-348 treatment ameliorates the ineffective erythropoiesis and is associated with decreased *Erfe* expression

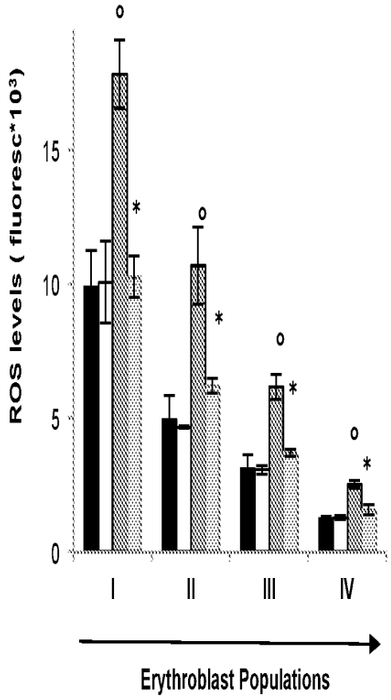


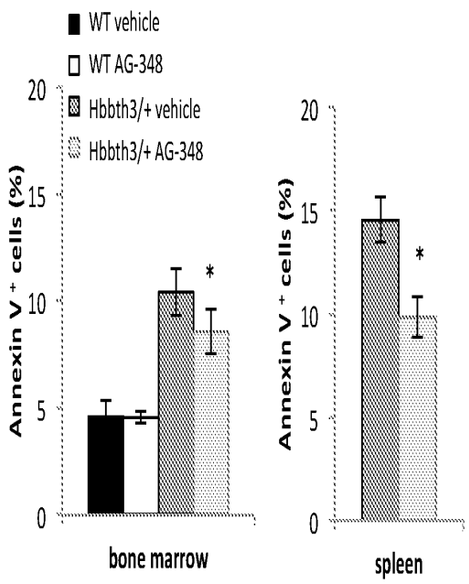



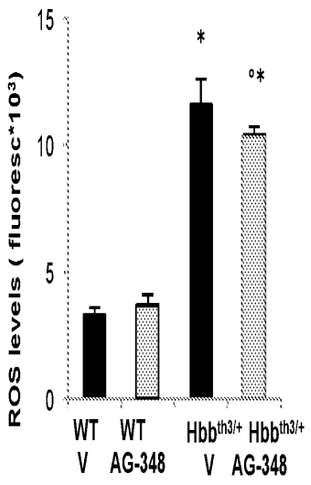
# In β-thalassemic Mouse Erythroid Precursors, AG-348 Treatment Reduces ROS Levels and the Amount of Apoptotic Orthochromatic E.

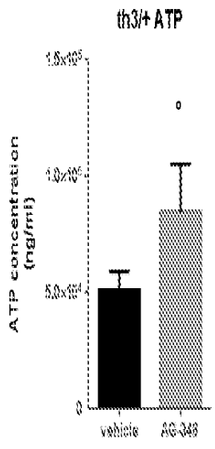


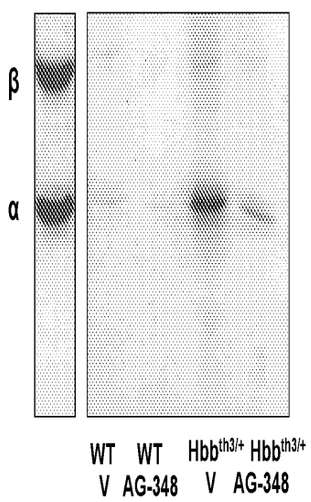



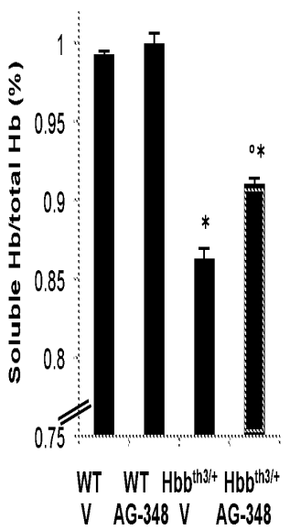

*p< 0.05 compared to WT
°p<0.05 compared to vehicle (V)

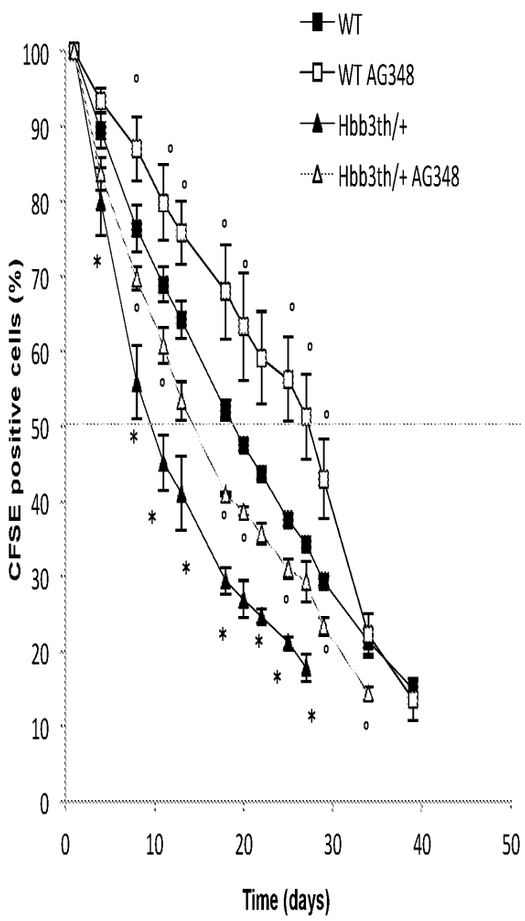
# In β-thalassemic mice, AG-348 treatment reduces ROS production and alpha chain aggregates in RBCs and increased RBC survival



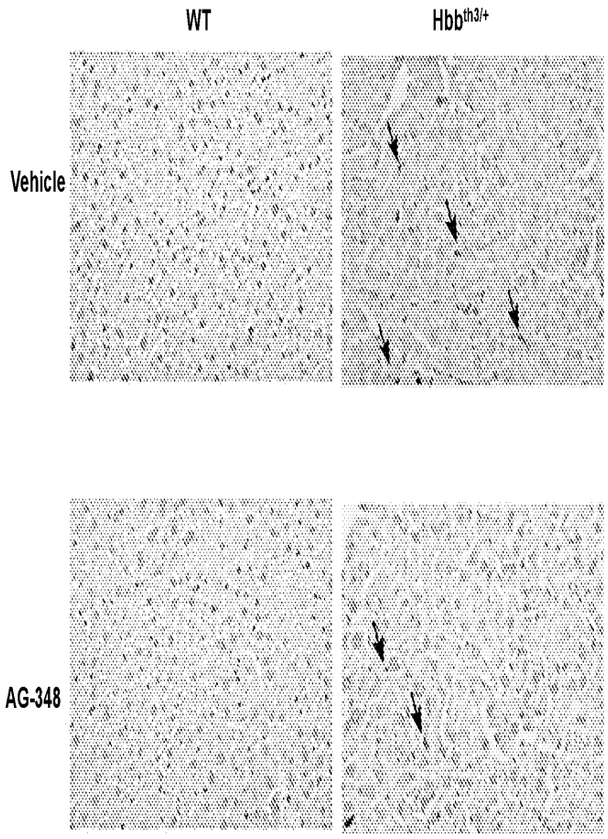



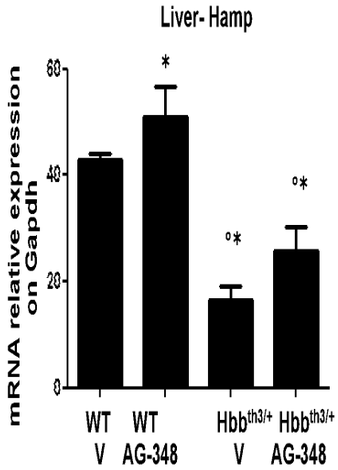




*p< 0.05 compared to WT
°p<0.05 compared to vehicle (V)

# β-thalassemic AG-348 treated mice showed reduced liver iron overload and increased Hepcidin expression




*p< 0.05 compared to WT
°p<0.05 compared to vehicle (V)

# Conclusions

✧ In β thalassemic mice, AG-348:

- Reduces ineffective erythropoiesis, extramedullar erythropoiesis, Erfe expression and ROS levels
- Increases Hb levels, reduces reticulocyte count and circulating erythroblasts
- Significantly increases RBC survival
- Reduces liver iron overload and increases Hamp

✧ AG-348 might represent a novel therapeutic approach in clinical management of anemia in β thalassemic syndromes.




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# Abstract Submission

***25. Red blood cells and iron - Biology***

EHA-3228

## THE PYRUVATE KINASE ACTIVATOR AG-348 IMPROVES MURINE B-THALASSEMIC ANEMIA AND CORRECTS INEFFECTIVE ERYTHROPOIESIS

alessandro matte[1], elisabetta beneduce[1], angela siciliano[1], penelope kosinski[2], anne janin[3], christophe lebouef[3], achille iolascon[4], luigia de falco[4], lenny dang[2], charles kung[2], Lucia De Franceschi*[1]

[1]medicine, University of Verona, verona, Italy, [2]development, agios, Cambridge, United States, [3]pathology, universite, Paris, France, [4]ceinge, University, naples, Italy

**Background:** β-thalassemias (β-thal) are worldwide distributed red cell disorders, characterized by ineffective erythropoiesis and reduced red blood cell (RBC) lifespan. Increased levels of reactive oxygen species (ROS) have been reported to play a key role in anemia of β thal, targeting both erythropoiesis and circulating RBCs. Pyruvate kinase (PK) is an important enzyme in the glycolytic pathway, responsible for conversion of phosphoenolpyruvate to pyruvate, with concomitant formation of the energy carrier adenosine triphosphate (ATP). As mature erythrocytes lack mitochondria, they rely almost exclusively on glycolysis to generate ATP, as well as the interlinked pentose phosphate pathway shunt to generate the reducing agent NADPH. The possible impact of PK activity on erythropoiesis is supported by evidence of ineffective erythropoiesis in human subjects with PK deficiency as well as mouse models of PK deficiency. The PK activator AG-348 has been evaluated in Phase I trials in healthy human subjects (NCT02149966) and is currently in Phase II studies in PK deficiency patients (NCT02476916). In the Phase I studies, AG-348 was shown to decrease levels of an upstream metabolite 2,3-diphosphoglycerate (2,3-DPG) and increased levels of ATP in whole blood, consistent with *in vivo* activation of PK.

**Aims:** To evaluate the impact of AG-348 on anemia and ineffective erythropoiesis in a mouse model of β thal intermedia.

**Methods:** Mouse strains C57B6/2J, as wildtype (WT) controls, and $Hbb^{th3/+}$, as a mouse model of β-thal intermedia, were used. Female mice aged between 2-3 months were treated with either vehicle or AG-348 at 50 mg/kg bid by oral gavage. Hematologic parameters, RBC indices, morphology, and reticulocyte count were evaluated at baseline, 7, 14, 21 days of treatment. Mouse erythropoiesis was studied using the CD44/TER119 gating strategy by FACS. ROS levels and the amount of Annexin-V+ cells were evaluated in erythroblast populations. Liver iron accumulation was evaluated by Pearl's staining and expression of liver hepcidin was measured by RT-PCR.

**Results:** In $Hbb^{3th/+}$ mice, 21 days of AG-348 treatment was associated with (i) a marked amelioration of anisopoykylocytosis; (ii) significantly increased Hb levels, MCV and MCH; (iii) a significant reduction in circulating erythroblasts (Es) and reticulocyte count; and (iv) reduction of ROS levels in circulating RBCs. In addition, AG-348 significantly decreased the amount of membrane precipitated α-globin chains and increased the amount of soluble Hb compared to the vehicle treated $Hbb^{3th/+}$ group. Consistent with these findings, we observed (i) a reduction of extramedullar erythropoiesis as indicated by both a decrease in spleen weight/mouse weight ratio and total Es (CD44TER119 Fsc high cells); (ii) a significant increase in pro-Es and basophilic Es, associated with reduction in orthochromatic Es; (iii) a reduction in ROS levels of $Hbb^{3th/+}$ Es and the amount of apoptotic orthochromatic Es compared to vehicle treated $Hbb^{3th/+}$ mice, suggesting an amelioration of β thal ineffective erythropoiesis.. The amelioration of ineffective erythropoiesis was paralleled by a reduction in liver iron overload and up-regulation of hepcidin mRNA in liver from AG-348 treated $Hbb^{3th/+}$ mice.

**Summary/Conclusion:** Our data show that the PK activator AG-348 beneficially affects ineffective erythropoiesis in a mouse model of β thal and might represent a novel therapeutic tool in clinical management of anemia in β thalassemic syndromes.

**Keywords**: Red blood cell, Thalassemia

# Clinical development of a novel oral activator of red cell pyruvate kinase for the treatment of pyruvate kinase deficiency

Leanne Pladson, Bryan W Jones, Chris Mix, Karen M Anderson, Chris Bowden

Agios Pharmaceuticals, Inc., Cambridge, MA, USA

Scan code to view poster online

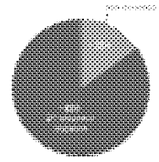
## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare chronic hemolytic anemia caused by mutations in the *PKLR* gene and the resulting reduced red cell PK (PK-R) activity.
- The clinical development program for mitapivat (AG-348), a novel activator of PK-R and investigational treatment for PK deficiency, is summarized.
- A phase 3 program consisting of two studies in adults is currently enrolling patients, as is a registry study in patients of all ages.

### PK deficiency: disease overview[1-3]

- **Epidemiology:** Under-recognized hereditary hemolytic anemia; Heterogeneous disease with variable severity among all ages; The most common form of nonspherocytic hemolytic anemia
- **Pathophysiology:** Caused by mutations in the *PKLR* gene coding for PK-R, resulting in defective glycolysis and decreased red blood cell lifespan
- **Clinical manifestations:** Lifelong hemolytic anemia; Iron overload and jaundice; Infection risk post splenectomy
- **Diagnosis and treatment:** PK-R enzyme activity and genetic testing; Supportive treatment: transfusions, splenectomy, iron chelation

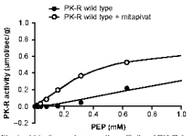
### PK deficiency: mutation type[2]




Types of *PKLR* mutations found in 193 unrelated cases in the PK deficiency natural history study

### Mitapivat (AG-348)[4]

Mitapivat is a novel, first-in-class, small-molecule allosteric activator of PK-R in clinical testing as a potential disease-altering therapy for PK deficiency




Mitapivat binding enhances the affinity of PK-R for its substrate phosphoenolpyruvate (PEP) (recombinant enzyme data shown)

### PK-R tetramer[4]



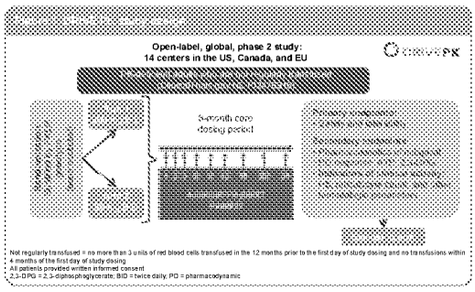
Active PK-R is a tetramer; mutations (green) decrease the enzyme activity

Mitapivat (yellow) binds at the PK-R dimer-dimer interface, away from the active site and the most common mutations

## METHODS

- DRIVE PK (NCT02476916) is an open-label, phase 2 study; the core period is complete and the extension period is ongoing (**Figure 1**).
- Key eligibility criteria: adult patients with PK deficiency who are not regularly transfused; hemoglobin (Hb) ≤12.0 g/dL (if male) or ≤11.0 g/dL (if female).

### DRIVE PK study design




Not regularly transfused = no more than 3 units of red blood cells transfused in the 12 months prior to the first day of study dosing and no transfusions within 4 months of the first day of study dosing
All patients provided written informed consent
2,3-DPG = 2,3-diphosphoglycerate; BID = twice daily; PD = pharmacodynamic

## RESULTS

### DRIVE PK cumulative safety summary[5]

- Mitapivat was generally well tolerated.
- The majority of adverse events (AEs) were grade 1–2.

Table 1. Most common AEs regardless of causality or grade (occurring in ≥15% of patients)

| | Starting dose (n=29) | | Dose ≥ 50 mg BID (n=29) | | Total (N=51) | |
|---|---|---|---|---|---|---|
| Preferred term | All patients n (%) | Grade ≥3 n | All patients n (%) | Grade ≥3 n | All patients n (%) | Grade ≥3 n |
| Patients experiencing ≥1 AE, n (%) | 28 (96.3) | 9 (29.6) | 25 (100.0) | 7 (28.0) | 51 (98.1) | 15 (28.9) |
| Headache | 10 (37.0) | 0 | 14 (56.0) | 0 | 24 (46.2) | 0 |
| Insomnia | 6 (22.2) | 1 (3.7) | 16 (64.0) | 1 (4.0)* | 22 (42.3) | 2 (3.9) |
| Nausea | 10 (37.0) | 0 | 10 (40.0) | 0 | 20 (38.5) | 0 |
| Viral upper respiratory tract infection | 8 (29.6) | 0 | 4 (16.0) | 1 (4.0) | 12 (23.1) | 1 (1.9) |
| Arthralgia | 5 (18.5) | 0 | 4 (16.0) | 0 | 9 (17.3) | 0 |
| Hot flush | 2 (7.4) | 0 | 7 (28.0) | 0 | 9 (17.3) | 0 |
| Cough | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Diarrhea | 4 (14.8) | 1 (3.7) | 4 (16.0) | 0 | 8 (15.4) | 1 (1.9) |
| Dizziness | 5 (18.5) | 0 | 3 (12.0) | 1 (4.0)* | 8 (15.4) | 1 (1.9) |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Influenza | 7 (25.9) | 1 (3.7) | 1 (4.0) | 0 | 8 (15.4) | 1 (1.9) |
| Vomiting | 3 (11.1) | 0 | 5 (20.0) | 0 | 8 (15.4) | 0 |

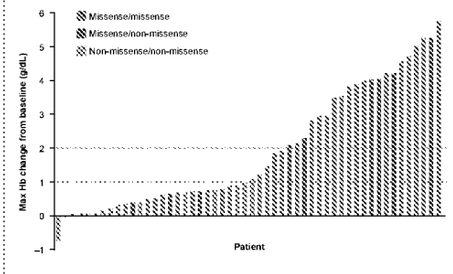
As of July 14, 2017
AEs graded using National Cancer Institute Common Terminology Criteria, version 4.03
Other grade ≥3 AEs not included in the table: colitis (n=1), mesenteric vein thrombosis (n=1), pharyngitis (n=2), hypertriglyceridemia* (n=2), hemolytic anemia* (n=2), hemolysis* (n=1), postprocedural hemorrhage (n=1), hypertension (n=1)
*Related to study drug as assessed by the investigator

- The safety profile was consistent over the duration of treatment (median 37.5 weeks).
- Treatment-related AEs leading to discontinuation (n=4):
  - Hemolytic anemia, hypertriglyceridemia, pharyngitis and nausea, pleural effusion.
- There were 14 serious AEs in 11 patients.
  - Five treatment-related serious AEs in four patients: anemia, hypertriglyceridemia, osteoporosis, withdrawal hemolysis followed by anemia.
- Effect of mitapivat on sex hormones:
  - Modest changes from baseline in sex hormone levels were observed in males at planned pivotal trial dose levels (≤50 mg BID).
  - Data are consistent with mild aromatase inhibition.
  - Most sex hormone values remained within normal limits in females; interpretation is confounded by variability in menopausal status and contraceptive use.

### DRIVE PK efficacy (core period)[5]

- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL (**Figure 2**).
- The mean maximum increase in Hb was 3.4 g/dL in patients with an Hb increase >1.0 g/dL.
- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range, 7–187 days).
- The dose had to be held or reduced owing to a rapid rise in Hb in nine patients.

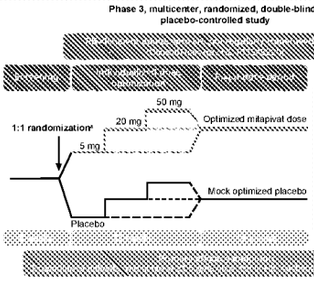
### Max Hb change from baseline by genotype




## PHASE 3 STUDIES ARE ENROLLING PATIENTS

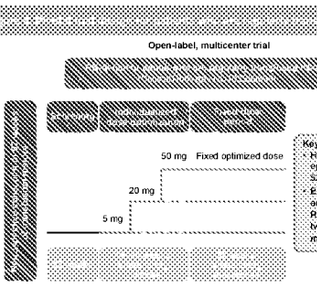
- The safety and efficacy data from the DRIVE PK study support the development of mitapivat in patients with PK deficiency.
- Two separate phase 3 clinical trials will examine the safety and efficacy of mitapivat in adult patients (**Figure 3**, **Figure 4**).

### Phase 3, multicenter, randomized, double-blind, placebo-controlled study




*Stratified by average of screening Hb values (<8.5 g/dL vs ≥8.5 g/dL) and mutation category (missense/missense vs missense/non-missense)

### Open-label, multicenter trial




## PYRUVATE KINASE DEFICIENCY REGISTRY

The Peak Registry

- The Peak Registry (NCT03481738) is a global, longitudinal, observational study for adult and pediatric patients with PK deficiency. This study is enrolling patients.
- Goals of the Peak Registry:
  - Collect and aggregate longitudinal data (minimum 2 years, up to 9 years) from patients with PK deficiency who have been diagnosed via genetic analysis (all ages) worldwide (up to 20 countries).
  - Promote further understanding of PK deficiency disease parameters, e.g. transfusion dependency, treatment practices, disease burden.

Acknowledgments
We would like to thank the patients and their dedicated physicians who have worked on these Agios programs to better understand PK deficiency and develop potential treatments for this rare genetic disease.

Disclosures
These studies are funded by Agios Pharmaceuticals, Inc.
LP, BWJ, CM, KMA, and CB: Agios – employment and stockholder
Editorial assistance was provided by Christine Ingleby, PhD, CMPP, Excel Medical Affairs, Horsham, UK, and supported by Agios.

References
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Presented at the Red Cells Gordon Research Conference (GRC), July 14-19, 2019, Newport, RI, USA

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**Abstract for submission to the Red Cells Gordon Research Conference (GRC)**
**Jul 14–19, 2019, Newport, RI**

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## Clinical development of a novel oral activator of red cell pyruvate kinase for the treatment of pyruvate kinase deficiency

**Objective:** Pyruvate kinase (PK) deficiency is a rare chronic hemolytic anemia caused by mutations in the *PKLR* gene and the resulting reduced red cell PK (PK-R) activity. The clinical development program for mitapivat (AG-348), a novel activator of PK-R and investigational treatment for PK deficiency, will be summarized.

**Methods:** DRIVE PK is an ongoing, open-label, phase 2 study in which adult patients with PK deficiency who do not receive regular blood transfusions were randomized to receive either 50 mg or 300 mg of mitapivat twice daily (BID).

**Results:** Mitapivat was administered over a 6-month Core Treatment Period followed, if eligible, by an Extension Period (ongoing). Dose adjustments were allowed. In all, 52 patients were randomized and treated; the median treatment duration at the time of this analysis was 37.5 weeks (range, 12.9–92.4 weeks). Forty-three patients (83%) completed the Core Period; 9 patients did not complete the Core Period due to either an adverse event (AE, n=4: pharyngitis/nausea, hemolysis, hypertriglyceridemia, pleural effusion), investigator decision (n=2), or patient withdrawal of consent (n=3). The most common AEs were headache (46%, n=24), insomnia (42%, n=22), and nausea (38%, n=20). As previously demonstrated, increased testosterone levels from baseline consistent with aromatase inhibition were observed, with values predominantly remaining within normal range. There were 5 treatment-related serious AEs reported in 4 patients (hemolysis followed by anemia, anemia, osteoporosis, hypertriglyceridemia). Twenty-six patients (50%) had a maximum hemoglobin (Hb) increase >1 g/dL during the Core Period, with a mean maximum Hb increase of 3.4 g/dL (range, 1.1–5.8 g/dL) in these patients. In most cases, Hb increases

were rapid and sustained, and were observed across doses from 5 mg to 300 mg BID. Patients with at least 1 missense mutation were more likely to have an Hb increase. Biochemical markers of hemolysis (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who had a maximum Hb increase >1.0 g/dL. Two phase 3 studies are currently enrolling: the ACTIVATE-T (NCT03559699) and ACTIVATE (NCT03548220) studies in adult patients who do, or do not, receive regular blood transfusions, respectively. Details of these studies will be provided, as will details of the ongoing Peak Registry (NCT03481738) study currently enrolling patients of all ages to further define the clinical characteristics of PK deficiency.

**Conclusion:** Mitapivat is a novel, first-in-class activator of the red cell PK enzyme in clinical testing for PK deficiency. The ongoing phase 2 DRIVE PK study has completed enrollment; some patients continue to receive mitapivat treatment in the Extension Period of the study. Treatment across a range of mitapivat doses has been generally well tolerated, and has resulted in relevant, durable increases in Hb with concomitant improvements in markers of hemolysis. These data support further exploration of mitapivat as the first disease-altering therapy for PK deficiency. A phase 3 program consisting of two studies in adults is currently enrolling patients, as is a registry study in patients of all ages.



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Ferrata Storti Foundation

Haematologica 2021
Volume 106(1):238-249

# 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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## ABSTRACT

Pyruvate kinase (PK) deficiency is a rare hereditary disorder affecting red blood cell (RBC) glycolysis, causing changes in metabolism including a deficiency in adenosine triphosphate (ATP). This affects red cell homeostasis, promoting premature removal of RBC from the circulation. In this study, we characterized and evaluated the effect of AG-348, an allosteric activator of PK that is currently in clinical trials for treatment of PK deficiency, on RBC and erythroid precursors from PK-deficient patients. In 15 patients, *ex vivo* treatment with AG-348 resulted in increased enzymatic activity in all patients' cells after 24 hours (h) (mean increase: 1.8-fold; range: 1.2-3.4). ATP levels increased (mean increase: 1.5-fold; range: 1.0-2.2) similar to control cells (mean increase: 1.6-fold; range: 1.4-1.8). Generally, PK thermostability was strongly reduced in PK-deficient RBC. *Ex vivo* treatment with AG-348 increased residual activity from 1.4- to >10-fold more than residual activity of vehicle-treated samples. Protein analyses suggest that a sufficient level of PK protein is required for cells to respond to AG-348 treatment *ex vivo*, as treatment effects were minimal in patient cells with very low or undetectable levels of PK-R. In half of the patients, *ex vivo* treatment with AG-348 was associated with an increase in RBC deformability. These data support the hypothesis that drug intervention with AG-348 effectively up-regulates PK enzymatic activity and increases stability in PK-deficient RBC over a broad range of PKLR genotypes. The concomitant increase in ATP levels suggests that glycolytic pathway activity may be restored. AG-348 treatment may represent an attractive way to correct the underlying pathologies of PK deficiency. (AG-348 is currently in clinical trials for the treatment of PK deficiency. Registered at *clinicaltrials.gov identifiers: NCT02476916, NCT03853798, NCT03548220, NCT03559699*).

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Received: September 25, 2019.

Accepted: January 23, 2020.

Pre-published: January 23, 2020.

https://doi.org/10.3324/haematol.2019.238865

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## Introduction

Pyruvate kinase (PK) deficiency is a rare hereditary disorder affecting red blood cell (RBC) glycolysis, resulting in life long chronic hemolytic anemia.[1] It is the most common cause of chronic hereditary non-spherocytic hemolytic anemia, although its exact prevalence is unknown.[2-4] PK deficiency is an autosomal recessive disease that occurs worldwide. It is caused by mutations in *PKLR*, the gene encoding the RBC and liver specific isoforms PK-R and PK-L, respectively.[5,6] Humans express two additional PK isoforms, PK-M1 and PK-M2, both encoded by the *PKLR* gene. PK-M2 is expressed in early fetal tissues and most adult tissues, including early stage



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erythroblasts, whereas expression of PK-M1 is confined to muscle.

To date, more than 370 mutations have been reported in PKLR associated with PK deficiency (*van Wijk, manuscript in preparation*). Most of these mutations are missense mutations encoding single amino acid changes that affect the enzyme's structure, stability or catalytic function.[7,8] Only a few mutations occur relatively frequently, implying that the majority of patients harbor a unique combination of mutations.[9]

Pyruvate kinase is an allosterically regulated homotetrameric enzyme that catalyzes the conversion of phosphoenol pyruvate (PEP) to pyruvate in the final step of glycolysis, thereby producing energy in the form of adenosine triphosphate (ATP). PK-deficient RBC are characterized by changes in metabolism associated with defective glycolysis, including a build-up of the upstream metabolite 2,3-disphosphoglycerate (2,3-DPG) and deficiency in the PK product ATP. Since RBC rely totally on glycolysis for ATP production it is hypothesized that insufficient energy production affects red cell homeostasis, promoting premature removal of PK-deficient RBC from the circulation by the spleen. Increased RBC dehydration resulting from increased activity of the Gardos channel, and consequent potassium efflux;[10-12] as well as ATP, depletion-mediated changes in RBC membrane integrity have been proposed as mechanisms triggering this premature clearance.[13,14] In addition, ineffective erythropoiesis may contribute to the pathophysiological consequences of PK deficiency.[15-17]

Clinically, PK deficiency manifests as chronic hemolytic anemia of highly variable clinical severity. Patients may present with severe neonatal anemia requiring exchange transfusion, whereas at the other end of the spectrum patients are diagnosed later in life with fully compensated hemolysis. The ongoing Pyruvate Kinase Deficiency Natural History Study showed that, apart from anemia, the most frequently reported complications were iron overload and gallstones. However, other complications such as aplastic crises, osteoporosis, extramedullary hematopoiesis, post-splenectomy sepsis, pulmonary hypertension, and leg ulcers are not uncommon. Typically, worsening of hemolysis occurs during infections, stress, and pregnancy.[18]

Current treatment for PK deficiency is generally supportive, with splenectomy, blood transfusion and iron chelation as the main therapies, focusing on the anemia and iron overload state.[19] Splenectomy is effective for the majority of PK-deficient patients, in that it reduces transfusion burden and improves baseline hemoglobin levels post splenectomy by an average of 1.6 g/dL. Fourteen percent of patients who underwent splenectomy, however, continued to require regular transfusions.[18] Curative treatment possibilities include stem cell transplantation (SCT) or experimental gene therapy.[20,21]

Recently, it was shown that AG-348 is an allosteric activator of PK-R. This small molecule directly targets PK-R by binding in a pocket at the dimer-dimer interface, distinct from the allosteric activator fructose-1,6-bisphosphate binding domain (FBP). This induces the active R-state conformation of the PK-R tetramer, resulting in enhanced activity of both wild type and mutant PK-R.[22] Phase I studies in healthy volunteers demonstrated glycolytic pathway activation upon treatment with AG-348 demonstrating its potential for treating PK deficiency.[23] Data from a phase II study in PK deficiency patients treated with AG-348 showed that approximately half of treated subjects experienced a rise in hemoglobin (Hb).[24]

In this study, we further evaluated the effect of *ex vivo* treatment with AG-348 on PK deficient red cells with a broad range of patient genotypes, including effects on enzyme activity and ATP levels. In particular, for the first time we report the effects of AG-348 on PK thermostability in red cell lysates, PK-R protein level, RBC deformability and the effect of AG-348 during *in vitro* PK-deficient erythropoiesis.

## Methods

This study was approved by the ethical committee of the University Medical Center Utrecht (Protocol 14-571/M).

### Metabolic profiling

Frozen whole blood samples were extracted with hot 70% ethanol, dried and resuspended in water. Relative abundance of central carbon metabolites was performed by ultra performance liquid chromatography-mass spectrometry (UPLC-MS) with high resolution accurate mass detection on a QExactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific).[25] Peak identification and integration was carried out with Maven software.[26] Quantitative analysis of ATP and 2,3-DPG was performed as described previously.[27]

### Pyruvate kinase activity, protein levels, and thermostability

Red blood cells were purified using microcrystalline α-cellulose columns.[28,29] PK and hexokinase activity was measured as described.[29,29] PK thermostability was measured as described[30] after 0, 5, 10, 20, 40 and 60 minutes (min) of incubation at 53°C.

Pyruvate kinase activity after *ex vivo* treatment with AG-348 was measured using low substrate (0.5 mM). PK-R protein levels were determined by Mesoscale Assay (MesoScale Discovery) as described[22] using goat anti-PKLR antibody (Aviva) and mouse anti-PKLR antibody (Abcam). SULFO-TAG goat anti-mouse (Mesoscale Discovery) was used as detection antibody. Protein level was determined by normalizing light intensity of the SULFO-TAG electrochemiluminescence to lysate protein concentration.

Western blot of PK-R and PK-M2 was performed as described[31] using polyclonal antibodies against PK-L[32] and monoclonal antibodies against PK-M2 (PodiCeps). Alexa Fluor® 680-conjugated goat anti-mouse and goat anti-rabbit antibodies (Li-Cor, Invitrogen) were used as detection antibodies.

### Ex vivo treatment with AG-348

Purified RBC or whole blood of patients and controls were incubated for 3, 6 and 24 h at 37°C in presence or absence of AG-348 in phosphate-buffered saline containing 1% glucose, 170 mg/L adenine, and 5.25 g/L mannitol (AGAM, pH 7.40). RBC were incubated with different dosages of AG-348 (up to 10 μM) for up to 24 h, at 37°C. After 6 and 24 h, PK-R activity and ATP levels (CellTiterGlo; Promega, Madison, WI, USA) were measured. For thermostability, RBC lysates were incubated for 2 h with AG-348.

### Osmoscan and deformability

Osmoscan and deformability measurements were performed using the Lorca (RRMechatronics).[33,34] AGAM buffer was added to whole blood (1:10), and supplemented with either AG-348 (20 M) or DMSO (untreated). Samples were incubated for 24 h at 37°C before measurements.



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### AG-348 treatment during *in vitro* erythropoiesis

Erythroid cells were produced from peripheral blood mononuclear cells (PBMC).[35] For proliferation, cells were cultured 10 days in CellQuin medium (Sanquin), supplemented with Stem Cell Factor (100 ng/mL, Amgen), erythropoietin (EPO) (2 U/mL, EPREX), dexamethasone (1 µM, Sigma), and 1 ng/mL IL-3 (R&D Systems). After 10 days, cells were reseeded (2E6 cells/mL) in CellQuin medium supplemented with EPO (10 U/mL, Eprex), heparine (5 U/mL, Leo Pharmacy), human AB plasma (3%, Sigma). At day 5, CellQuin was replaced by RetQuin medium (Sanquin). AG-348 (2 µmol/L) was added at days 0, 1, 3-10 during proliferation and days 0, 2, and 4 during differentiation.

### 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 was carried out or a Kruskal-Wallis test, followed by Dunn's tests for *post-hoc* analysis. Pearson's correlation was used to determine correlations of laboratory parameters with enzyme activity measurements.

## Results

Fifteen adult, transfusion independent, PK-deficient patients were enrolled for this study (median age: 44.0 years; range: 20.0-51.5). PK deficiency was confirmed by demonstrating compound heterozygosity or homozygosity for mutations in *PKLR* (Sanger sequencing). Whole blood from 15 healthy volunteers was used as control samples.

Baseline patients' characteristics displayed varying degrees of anemia (Table 1). In addition, most patients had strongly elevated reticulocyte counts, a prominent feature of PKD patients, in particular after splenectomy.[11] Most patients showed reduced red cell PK activity (Figure 1A) and reduced PK thermal stability (Figure 1C). Since the activity of many red cell enzymes is red cell age-dependent,[36] we also compared PK activity to hexokinase (HK) activity (Table 1 and Figure 1B). This PK/HK ratio was clearly decreased in all patients, indicating a relatively strong decrease in activity of PK (r=-0.677, $P<0.01$) (Figure 1B). When causative mutations where classified as missense (M) or non-missense (NM) a genotype to phenotype correlation was identified.[18] All four patients without splenectomy were of the M/M genotype, which is in line with the lower likelihood of splenectomy in this group.[18] Similarly, these patients had generally higher Hb levels and lower reticulocyte counts (Table 1).

### PK-R protein levels correlate with pyruvate kinase activity

Protein levels of PK-R were measured by both western blot and Meso Scale Discovery (MSD). PK-R protein levels were variable between patients (Figure 1C and D). In general, PK-activity correlated well positively with PK-R protein levels. The most notable exception was patient 4. RBC from this patient contained very low levels of protein, as measured by both western blot and ELISA, but repeated measurements showed high levels of PK activity (Table 1 and Figure 1A, D and E). Low levels of PK-R protein in this patient could result from combined instability of the p.Arg510Gln[37] mutant as well as the truncated p.(Arg488*) PK-R nonsense mutant. In line with this, PK thermostability was also strongly reduced in this patient (Figure 1C). However, the reason for the high PK activity in this patient remains unknown.

Increased PK-M2 expression, that could perhaps compensate for the loss in PK-R38 activity, was not detected except for patient 3 and, to a lesser degree, in RBC from patient 1 (Figure 1D).

### Pyruvate kinase-deficient red blood cells show glycolytic intermediates levels that are consistent with decreased pyruvate kinase activity

Metabolic profiling of PKD patients was performed by liquid chromatography-mass spectrometry (LC-MS/MS) on whole blood. Consistent with a decrease in PK activity at the final step of glycolysis, levels of PEP were significantly increased ($P<0.0001$) whereas levels of pyruvate and ATP were significantly decreased (both $P<0.0001$) (Figure 2). In addition, dihydroxyacetone phosphate (DHAP) and 2,3-DPG levels were increased ($P<0.001$ and $P<0.0001$), indicative of retrograde accumulation of upstream glycolytic intermediates.

### Ex vivo incubation with AG-348 increases pyruvate kinase activity and adenosine triphosphate in a dose-dependent manner

*Ex vivo* treatment of PK-deficient and healthy control RBC with increasing dose of AG-348 showed an increase in PK-activity and ATP levels. Illustrative PK activity dose response curves of two patients are shown in Figure 3A and B. Incubations with 10 µM AG-348 showed a mean 1.3-fold increase (range: 1.0-2.0) in PK activity in PK-deficient RBC after 6 h ($P<0.0001$) and of 1.8 (range: 1.2-3.4) after 24 h ($P<0.0001$) (Figure 3E). Similar values were obtained from control cells: 1.4 after 6 h (range: 0.9-1.9; $P<0.01$) and 2.3 (range: 1.2-7.1) after 24 h ($P<0.0001$).

Adenosine triphosphate levels increased accordingly in a dose-dependent manner (Figure 3C and D). AG-348 treatment significantly increased mean ATP levels in PK-deficient RBC to 1.4-fold (range: 1.0-2.3) after 6 h ($P<0.01$) and 1.5-fold (range: 1.0-2.2) after 24 h ($P<0.0001$). The mean increase in ATP levels in control cells was 1.5-fold (range: 1.3-1.7) after 6 h ($P<0.001$) and 1.6-fold (range: 1.4-1.8) after 24 h ($P<0.0001$). We observed no differences in PK activity and ATP response between PK-deficient patients with M/M and M/NM genotypes (Table 1 and Figure 3E and F).

Since glycolysis in RBC is known to be regulated by band 3,[39] we verified RBC band 3 expression levels by western blot analysis. We observed no differences in band 3 expression levels of PK-deficient patients compared to healthy controls (*Online Supplementary Figure S1*).

Collectively, these results are consistent with those of previous reports,[22] and illustrate the ability of AG-348 to stimulate PK activity in patient samples with a range of genotypes.

### Ex vivo treatment with AG-348 restores pyruvate kinase thermostability

At baseline, PK-R thermostability was strongly reduced in all PK-deficient patients (Figure 4C). Whereas RBC from healthy controls showed a mean residual activity of 81% (range: 64-108%) after incubation at 53°C for 60 min, the mean residual PK-R activity in PK-deficient patients was significantly decreased under these conditions to only 30% (range: 6-54%) ($P<0.0001$). This confirms earlier observations[36] and shows that PK thermostability is a



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Table 1. Genotypes, amino acid change, splenectomy status and laboratory parameters are shown for all 15 pyruvate kinase (PK)-deficient patients included in this study.

| ID | Sex | PKLR mutation | Amino acid change | Genotype | Splenectomy | Hb (g/dl) | Ret. (%) | PK (U/gHb) | HK (U/gHb) | PK/HK ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| P1 | F | c.1121T>C/c.1456C>T | p.(Leu374Pro)/p.(Arg486Trp) | M/M | Yes | 9.7 | 10.0 | 9.0 | 5.0 | 1.8 |
| P2 | M | c.331G>A/c.1456C>T | p.(Gly111Arg)/p.(Arg486Trp) | M/M | No | 11.2 | 7.0 | 3.1 | 1.7 | 1.8 |
| P3 | M | c.1178A>G/c.1456C>T | p.(Asn393Ser)/p.(Arg486Trp) | M/M | Yes | 9.4 | >35.0 | 16.9 | 6.2 | 2.7 |
| P4 | F | c.1462C>T/c.1529G>A | p.(Arg488*)/p.(Arg510Gln) | M/NM | Yes | 6.6 | >34.0 | 15.9 | 7.4 | 2.1 |
| P5 | F | c.1121T>C/c.1706G>A | p.Leu374Pro)/p.(Arg569Gln) | M/M | No | 10.9 | 7.0 | 2.1 | 1.5 | 1.4 |
| P6 | F | c.307+1G>A/c.1436G>A | p.[=; 0]/p.(Arg479His) | M/NM | Yes | 7.7 | 75.0 | 1.9 | 6.0 | 0.3 |
| P7 | M | c.376-2A>C/c.1529G>A | p.(Glu125_Tyr126ins32; Tyr126Alafs*83)/p.(Arg510Gln) | M/NM | Yes | 8.0 | 55.0 | 1.7 | 5.6 | 0.3 |
| P8 | F | c.331G>A/c.1456C>T | p.(Gly111Arg/p.(Arg486Trp) | M/M | No | 11.9 | 5.0 | 2.2 | 1.4 | 1.6 |
| P9 | M | c.331G>A/c.1492C>T | p.(Gly111Arg)/p.(Arg498Cys) | M/M | Yes | 10.3 | 40.0 | 2.5 | 4.1 | 0.6 |
| P10 | M | c.1154G>A/c.1529G>A | p.(Arg385Lys)/p.(Arg510Gln) | M/M | No | 13.4 | 15.0 | 2.7 | 2.0 | 1.3 |
| P11 | M | c.721G>T/c.1529G>A | p.(Glu241*)/p.(Arg510Gln) | M/NM | Yes | 8.2 | 85.0 | 2.2 | 6.1 | 0.4 |
| P12 | M | c.142_159del/c.494G>T | p.(Thr48_Thr53del)/p.(Gly165Val) | M/NM | Yes | 12.9 | 20.0 | 10.0 | 3.5 | 2.8 |
| P13 | M | c.1269G>A/c.1654G>A | p.([Met373_Ala423del; 0]) /p.(Val552Met) | M/NM | Yes | 9.7 | 55.0 | 1.8 | 5.8 | 0.3 |
| P14 | F | c.401T>A/c.1529G>A | p.(Val134Asp)/p.(Arg510Gln) | M/M | Yes | 9.6 | 60.0 | 1.2 | 5.2 | 0.2 |
| P15 | M | c.1529G>A/c.1529G>A | p.(Arg510Gln)/p.(Arg510Gln) | M/M | Yes | 2.8 | 40.0 | 1.6 | 2.8 | 0.6 |
| Contr. | | | | | No | 14.1 (1.3) | 1.18 (0.3) | 12.7 (2.3) | 1.1 (0.2) | 11.9 (2.1) |
| | | | **Reference range values:** | | | M: 13.8-17.2 F: 11.9-15.5 | 1.0-2.0 | 9.2-16.9 | 0.9-1.4 | 9.0-16.6 |

Enzyme activity of PK was decreased or normal. Hexokinase (HK) activity was almost always increased due to reticulocytosis. PK/HK ratio was strongly decreased in all cases, indicative of PK deficiency. Mean values (standard deviation) of 15 healthy controls are shown. Reference range values of the UMC Utrecht population are also included. M: male; F: female; Hb: hemoglobin; Ret.: reticulocytes; PK: pyruvate kinase; U: units; H:, hexokinase; M/M: missense/missense; M/NM: missense/nonmissense.

common feature of many different mutant forms of PK-R, and not just restricted to known unstable variants like p.Arg510Gln.

Treating PK-deficient RBC *ex vivo* with AG-348 (2 μM) prior to this assay showed a clear effect on stability of the enzyme (Figure 4C; illustrative examples of RBC from patients 12 and 9 are shown in Figure 4A and B). *Ex vivo* treatment with AG-348 lead to a mean residual PK activity of 58% (range: 0-115%; $P<0.001$) in RBC from PK-deficient patients and 106% in controls (range: 99-111%; $P=0.13$). We observed no differences in PK thermostability response between PK-deficient patients with M/M and M/NM genotypes (Table 1 and Figure 4C).

### Effect of *ex vivo* AG-348 treatment on deformability of pyruvate kinase-deficient red blood cells

Because RBC deformability is partly ATP dependent,[40,41] we investigated the effect of *ex vivo* AG-348 treatment on this important RBC functional property by osmotic gradient ektacytometry (osmoscan) and deformability measurements. Figure 5A shows osmoscan curves of the 15 healthy control samples (gray) and that of patient 7 (red) as an example. At baseline, maximum deformability ($EI_{max}$) of PK-deficient RBC was significantly decreased compared to controls, although the difference was small ($P<0.01$) (Figure 5D). Similarly, Omin and Ohyper, reflecting RBC surface-to-volume ratio and hydration status,[42] respectively, were significantly different in PK-deficient patients ($P=0.039$ and 0.047) (Figure 5C and E). This implies that PK-deficient RBC are somewhat less deformable than normal RBC, possibly due to a slightly lower surface-to-volume ratio and a slight increase in RBC hydration.

When PK-deficient patients were divided according to genotype (Table 1), there was a significant difference between M/M and M/NM genotypes, indicating that a more severe genotype results in lower maximal RBC deformability (EImax, $P=0.039$) (Figure 5B).

After 24 h of *ex vivo* treatment with AG-348, an improvement in RBC deformability was observed in about half of the PK-deficient patients (Figure 5F, patients 1, 3, 4, 6, 7, 8, and 9) compared to their untreated RBC. The effect of these changes were similar in magnitude to the baseline difference in deformability observed between PK-deficient RBC and control RBC (Figure 5C). Taken in aggregate, however, there was no overall significant improvement of deformability.

### Effect of *ex vivo* AG-348 treatment on PK-R-deficient erythroid development

During erythropoiesis, PK-M2 levels are gradually replaced by PK-R[43,44] and AG-348 has been described to also activate PK-M2.[28] Because PK deficiency has been associated with ineffective erythropoiesis,[15-17] we next investigated the effect of *ex vivo* AG-348 treatment on erythroid development from four PK-deficient patients and controls. Morphologically there were no major abnormalities in erythroid development between healthy controls or PK-deficient patients (Figure 6A). This was supported by there being no difference in erythroid surface marker expression[35] (*Online Supplementary Figure S2A*). There was



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no difference in erythroid differentiation with or without AG-348 treatment. This was observed in both healthy control cells and in PK-deficient cells (*Online Supplementary Figure S2B and C*). In presence of AG-348 (2 μM), we did note, however, a small increase in cell number in both normal controls and patients after proliferation for 10 days, although the difference was not significant ($P$=0.059) (Figure 6B).

Western blot analysis of PK-R and PK-M2 protein levels of healthy control samples clearly showed an increase in expression of PK-R and a decrease in PK-M2 during differentiation (*Online Supplementary Figure S3*). The decrease in PK-M2 was also observed in all patient samples, whereas a clear increase in PK-R expression was seen only in samples from patients 7 and 15 (*Online Supplementary Figure S3*). PK-R protein levels were low or inconclusive in patients 3 and 10. Presence of AG-348 during *ex vivo* erythropoiesis was associated with higher levels of PK-R in patients 7 and 15. AG-348 was not seen to have any effect on PK-M2 expression.

On the level of enzymatic activity, the PK/HK ratio from cultured red cells was higher in presence of AG-348 in

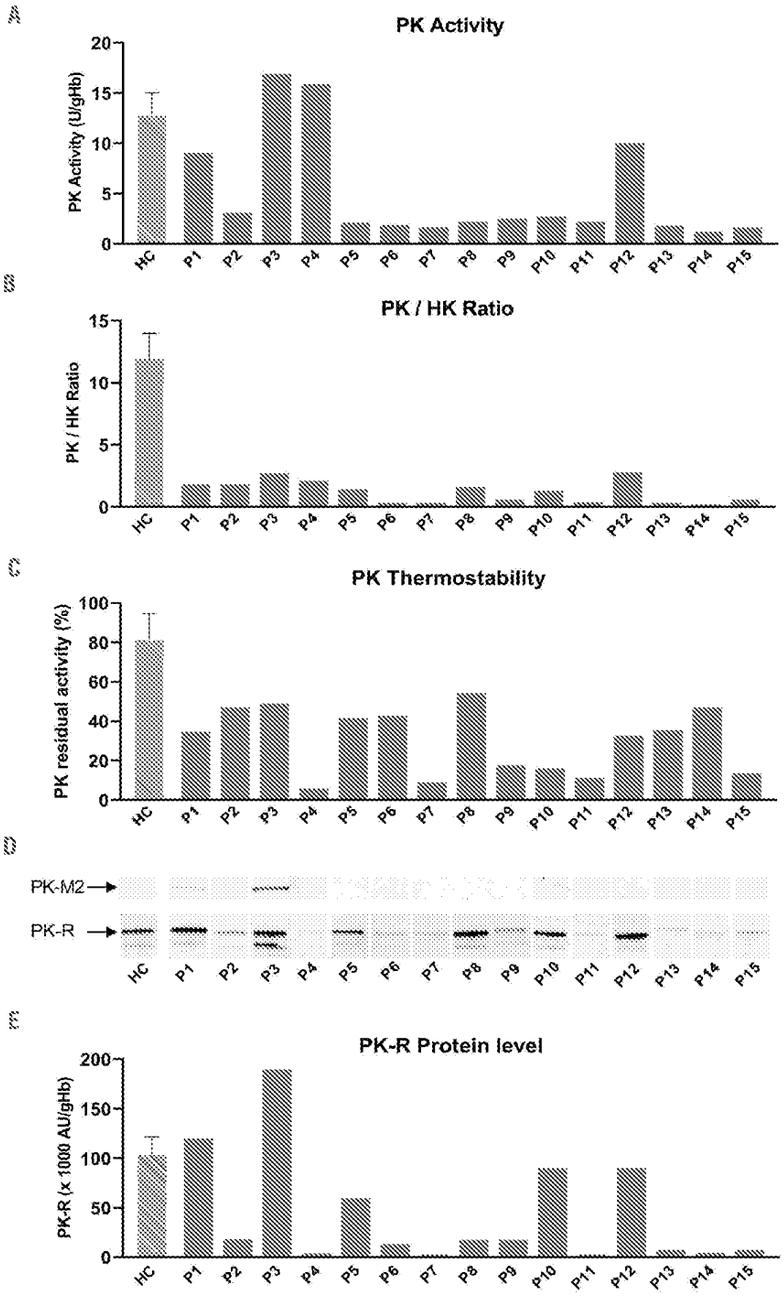



Figure 1. Baseline levels of pyruvate kinase (PK) activity, PK/Hexokinase (HK) ratio, PK thermostability, and PK-R protein levels in PK-deficient patients and controls. (A) PK activity of PK deficient patients (red) and healthy controls (gray). (B) PK/HK ratio in patients (red) and healthy controls (gray), as a means to evaluate PK activity in the presence of high number of reticulocytes, reflected by increased HK activity. (C) PK thermostability of patients (red) and controls (gray). (D) PK-R protein levels of patients (P#) and controls (HC) measured by western blot. (E) PK-R protein levels of patients (red) and controls (gray) measured by Meso Scale Discovery. ECL: electrochemiluminescence.



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both patients and healthy controls, although this effect did not reach statistical significance ($P$=0.064) (Figure 6C).

## Discussion

In this study, we characterized and evaluated the effect of AG-348, a recently reported first-in-class allosteric activator of PK-R[22,23] that is currently in clinical trials for the treatment of PK deficiency (*clinicaltrials.gov identifiers: NCT02476916, NCT03853798, NCT03548220, NCT03559699*). Our findings indicate that *ex vivo* treatment with AG-348 effectively increases PK-R activity over a broad range of PKLR genotypes as assessed by enzymatic activity as well as cellular ATP levels. Our findings are consistent with those previously reported by Kung *et al.*,[22] and further extend those findings through the evaluation of a substantially larger cohort of PK-deficient blood samples, including a comprehensive baseline metabolomics analysis, and assessment for the first time of the effects of PK deficiency on parameters such as red cell deformability and *ex vivo* expansion of erythroid progenitors.

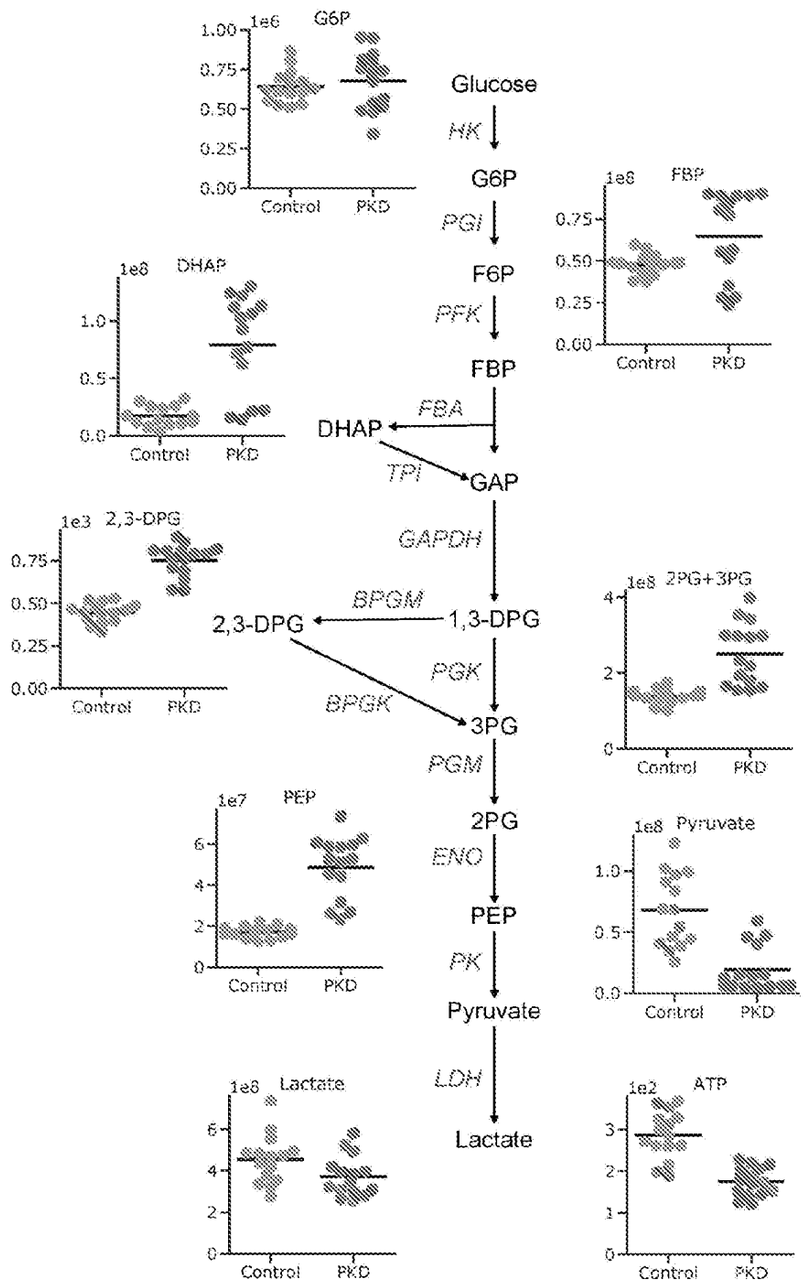


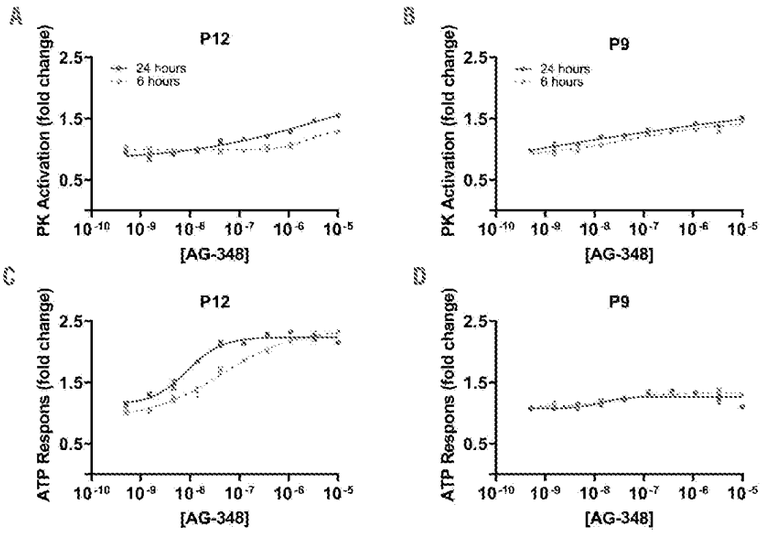

Figure 2. Pyruvate kinase (PK)-deficient patients show glycolytic intermediates levels that are consistent with decreased PK activity. Upstream intermediates of PK of PK-deficient patients (red) are significantly increased compared to values of health controls (gray), with, for example, an almost 2-fold increase in 2,3-DPG. Downstream targets, pyruvate and adenosine triphosphate (ATP), are significantly decreased compared to healthy controls. PKD: pyruvate kinase deficiency; G6P: glucose-6-phosphate; DHAP: dihydroxyacetone phosphate; 2,3-DPG: 2,3-diphosphoglycerate; PEP: phosphoenol pyruvate; FBP: fructose biphosphate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; GAP: glyceraldehyde phosphate; 1,3-DPG: 1,3-diphosphoglycerate; HK: hexokinase; PGI: phosphogluco isomerase; PFK: phosphofructokinase; FBA: fructose biphosphate aldolase; TPI: triosephosphate isomerase; GAPDH: glyceraldehyde phosphate dehydrogenase; BPGM: bisphosphoglycerate mutase; PGK: phosphoglycerate kinase; BPKG: bisphosphoglycerate kinase; PGM: phosphoglycerate mutase; ENO: enolase; LDH: lactate dehydrogenase.



Ex. 2001-1019

Our cohort shows an overall mean 1.8-fold increase in PK activity in response to *ex vivo* treatment with AG-348 (range: 1.2-3.4) (Figure 3E). This is very similar to the previously reported mean fold increase of 2.1 (range: 1.3-3.4). Similarly, the 1.5-fold increase in ATP levels we observed in PK-deficient RBC (range: 1.0-2.2) was comparable to previously reported results (mean fold increase: 1.7; range: 1.3-2.4).

PK thermostability was found to be significantly improved upon *ex vivo* treatment with AG-348 even though there was a high variability in response among the different genotypes (Figure 4). An interesting subset was composed of six PK-deficient patients (patients 4, 7, 10, 11, 14, and 15) who had the common PK-R mutation c.1529G>A p.(Arg510Gln), previously shown to have little defect in catalytic activity but to be highly unstable. Five of these



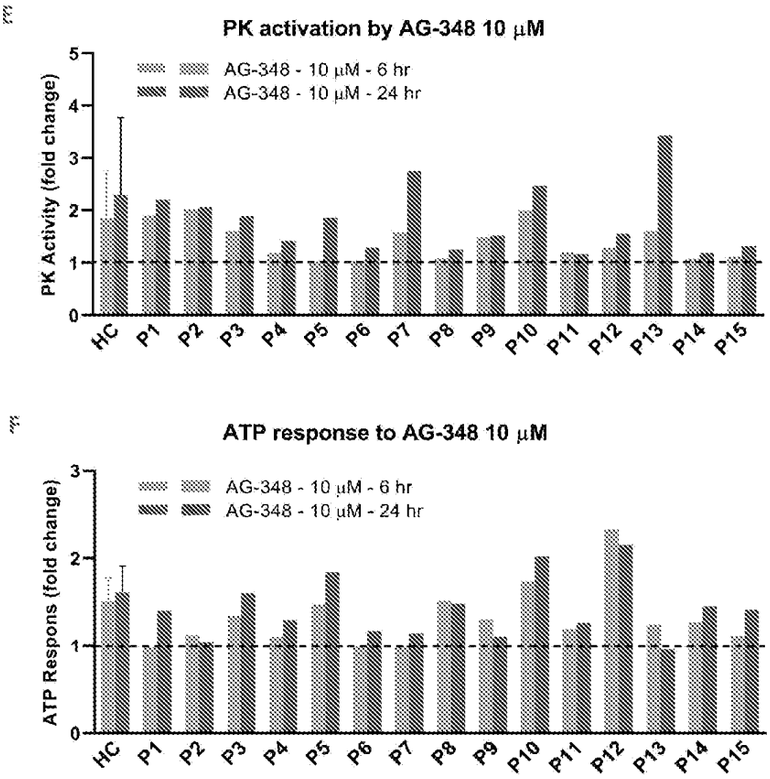




Figure 3. *Ex vivo* AG-348 treatment of red blood cells (RBC) increase pyruvate kinase (PK) activity and adenosine triphosphate (ATP) levels in a dose dependent manner. (A and B) Representative patient dose response curves of PK activity, expressed as fold activation compared to incubation without AG-348, as measured in RBC of patients 12 (A) and 9 (B) after 6- (turquoise) and 24-hour (h) (dark green) incubation with AG-348. (C and D) representative patient ATP dose response curves, expressed as fold activation compared to incubation without AG-348, as measured in RBC of patients 12 (C) and 9 (D) after 6- (turquoise) and 24-h (dark green) incubation with AG-348. (E) PK activation in RBC of 15 PK deficient patients after 6- (turquoise) and 24-h (dark green) incubation with 10 µM AG-348. Mean PK fold activation of healthy controls after 6- (light gray) and 24-h (dark gray) incubation with AG-348 10µM is shown on the left of the graph. (F) ATP response (fold activation) of 15 PK deficient patients after 6- (turquoise) and 24-h (dark green) incubation with 10 µM AG-348. Mean ATP response of healthy controls is shown on the left of the graph (gray bars). Error bars represent standard deviation. Dashed turquoise line represents 6-h incubation with AG-348, solid dark green line represents 24-h incubation.





Ex. 2001-1020

patients had unmeasurably low PK activity in the thermostability test, and the activity of four could be restored by pre-incubating RBC with AG-348 (Figure 4, patients 7, 10, 11 and 14). These four patients carried the p.(Arg510Gln) mutation in trans to a splice site mutation (patient 7), a missense mutation (patients 10 and 14), and a nonsense mutation (patient 11). A significant outlier was patient 15, who was homozygous for p.(Arg510Gln), suggesting the possibility that it may be more difficult to stabilize this mutation in the homozygous condition. Another outlier was patient 4, who had p.(Arg510Gln) paired with the nonsense mutation p.(Arg488*) yet had a relatively modest loss of activity in the thermostability test. Collectively these data illustrate that AG-348 has the potential to stabilize a diverse range of different mutant PK-R molecules *in vitro*, but also demonstrate the complex variability that is introduced by the compound heterozygous state of most patients.

One surprising result was that the effects of AG-348 were variable across the different patient samples, and that overall increases in PK activity, ATP and thermostability were not observed together in some patients. For example, patient 13 shows a clear response in terms of PK activity after AG-348 treatment for 24 h whereas the ATP response was present at 6 h and back to baseline by 24 h. However, we did observe that residual PK-R protein levels at baseline are important for the response to AG-348. When grouping patients according to their response in PK activity or ATP levels (i.e., >1.5-fold *vs.* <1.5-fold response), PK-R protein levels at baseline (Figure 1E) were found to be higher in the patients that showed a >1.5-fold increase upon *ex vivo* treatment with AG-348 (Figure 7). This suggests that the amount of PK-R protein is an important determinant for the *in vitro* activity of AG-348, and is consistent with its mechanism of action *via* direct binding and stimulation of mutant enzyme.

While the *in vitro* assessments as presented in this study provide clear evidence that AG-348 can stimulate the activity of mutant PKR enzymes in a variety of contexts, the central question is whether changes in these parameters would be associated with increased likelihood of clinical response to AG-348. Recently, data from the phase II study of AG-348 in PKD patients have been published.[24] Analysis of those results suggests some parallels between our data and the response observed in treated patients. For example, the clinical manuscript demonstrates that higher baseline PK-R protein levels in patients is associated with Hb increases, which parallels the findings described here. Similarly to the effects on thermostability noted above in patients with the p.(Arg510Gln) mutation, such patients in phase II had variable Hb responses.[24]

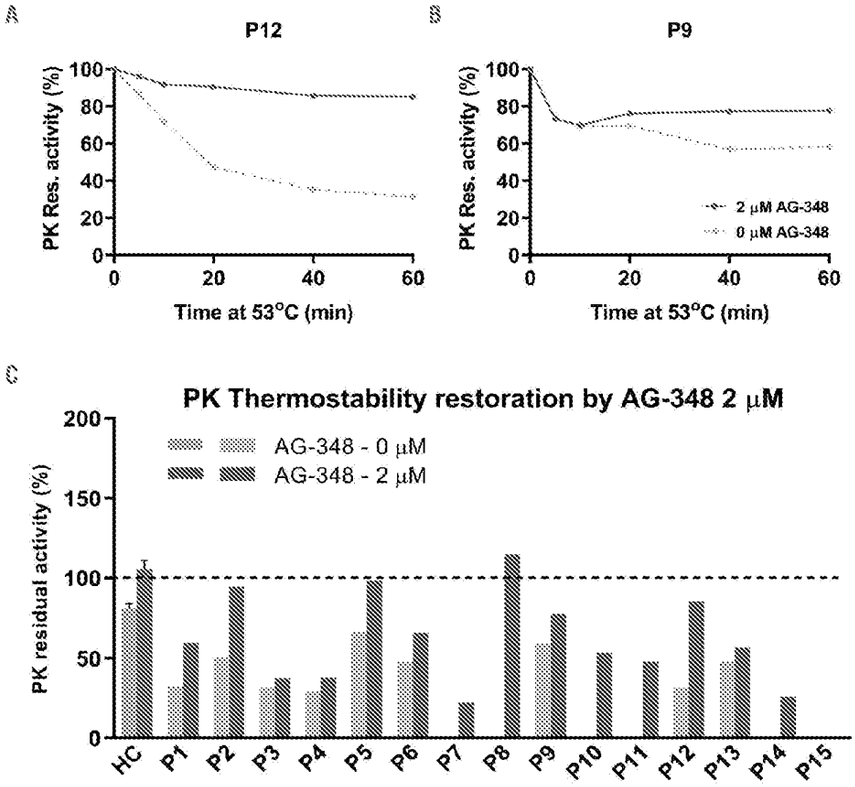



Figure 4. Pyruvate kinase (PK) thermostability is restored upon ex vivo treatment with AG-348. (A and B) Representative PK-deficient (PKD) patients' PK thermostability of red blood cell (RBC) lysates of patients 12 (A) and 9 (B) pre-incubated in absence (dashed line) or presence (solid line) of 2 µM AG-348 for 2 hours (h) at 37°C. For PK thermostability, residual PK activity (%) is measured after 5, 10, 20, 40 and 60 minutes (min) of heat treatment at 53°C. (C) Residual PK activity (%) after 60 min of heat treatment of 15 PKD pre-incubated in absence (light turquoise) and presence (dark turquoise bars) of 2 µM AG-348. Levels are normalized to baseline levels of PK-activity before heat treatment. Absence of bars indicate that PK residual activity was below the detection limit. Error bars represent standard deviation.



Ex. 2001-1021

Here, for the first time, we report on the effects of AG-348 on *ex vivo* produced PK-R deficient RBC. In presence of AG-348 we observed a trend towards a slightly improved proliferation of erythroid progenitor cells in both healthy controls and PK-deficient patients of various PKLR genotypes (Figure 5B). Further studies are warranted to establish this more firmly, but it could indicate that, apart from acting on mature RBC in the circulation, AG-348 could have a potential beneficial effect on both normal and PK-deficient early stage erythroid development. In addition, we show that *ex vivo* produced (mutant) PK-R can be activated to a similar degree as (mutant) PK-R from RBC from patients and healthy controls (Figure 6C). This was particularly evident for patients 7 and 15, who also showed the most pronounced effect of AG-348 on PK-R protein levels (*Online Supplementary Figure S3*).

At the same time, based on cell count, morphology, and erythroid differentiation markers, we did not observe major differences between normal and PK-deficient *ex vivo* erythroid proliferation. This is in contrast with previous reports that suggest ineffective erythropoiesis is a pathophysiological feature of PK deficiency, based on increased numbers of apoptotic early erythroid progenitor cells in the spleen of one PK-deficient patient,[16] and similar findings in the spleen from a mouse model of PK deficiency.[15] Regarding this observed discrepancy between our findings and previous studies, a possible confounder is that we

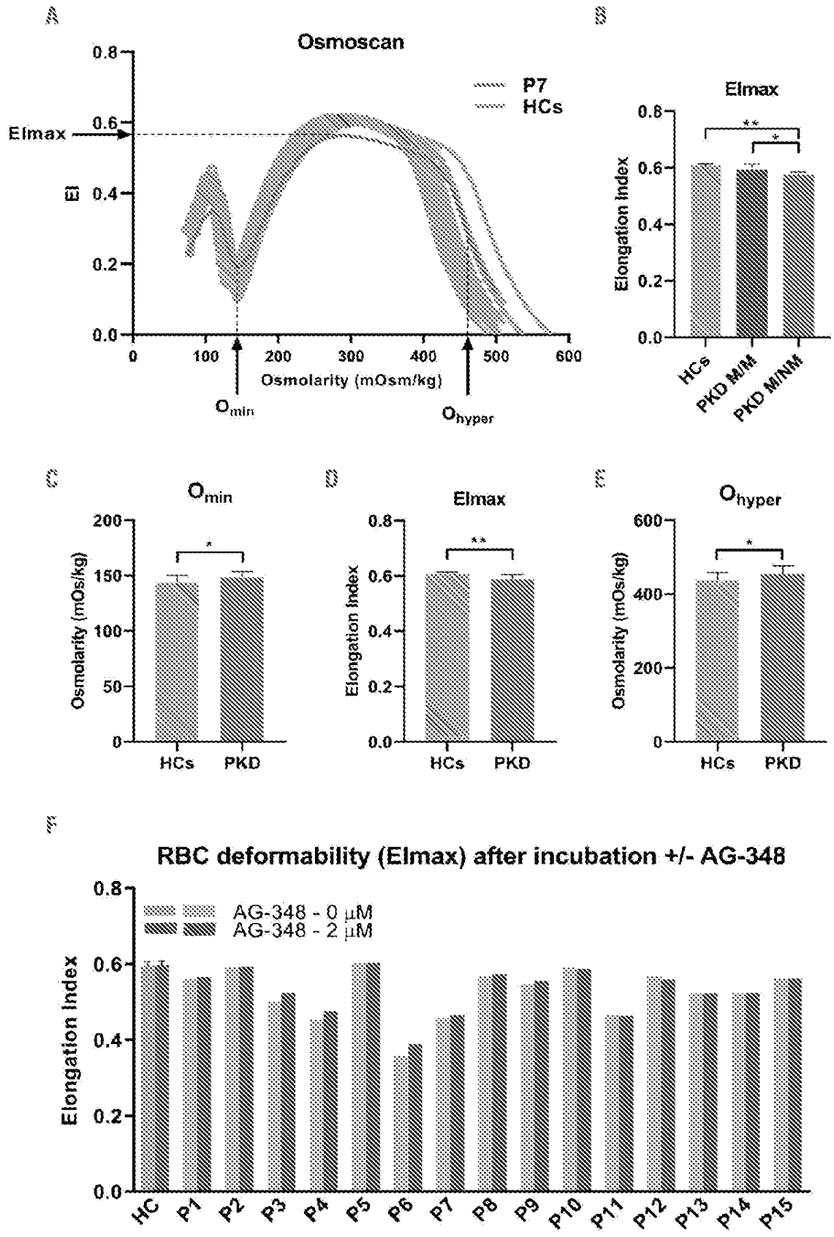



Figure 5. Red blood cell (RBC) deformability in pyruvate kinase (PK)-deficient patients is slightly decreased. (A) Osmoscan curve of patient 7 (red) and 15 healthy controls (HC) (gray). (B) Maximum deformability (Elmax) is slightly decreased in PK deficiency compared to HC. When grouped according to genotype, i.e., missense/missense M/M *versus* missense/non-missense (M/NM), there is a significant difference, indicating that a more severe genotype results in lower maximal RBC deformability. (C) Omin is slightly increased in PKD patients compared to HC. (D) Elmax is slightly decreased in PKD patients compared to HC. (E) Ohyper is slightly increased in PKD patients compared to HC. (F) After 24 hours of ex vivo treatment with 20 µM AG-348 in about half of the PKD patients an improvement in RBC deformability was observed (patients 1, 3, 4, 6, 7, 8, and 9) compared to their untreated RBC. Error bars represent standard deviation. $^{**}P<0.01$, $^{*}P<0.05$.



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grew erythroid cells from a fixed number of PBMC. PBMC composition will likely vary between patients and controls, in particular the number of $CD34^+$ cells. This may influence the eventual outcome of cellular proliferation. More importantly, genotype-phenotype correlations could explain these conflicting results as different genotypes result in different complications,[16] indicating that different mutations could result in a large variability in the occurrence of ineffective hematopoiesis.

A limitation of this study is the absence of a functional assay that could directly measure the effect of ATP increase. We did observe an improvement in RBC deformability as measured by osmotic gradient ektacytometry after *ex vivo* treatment with AG-348 in half of the

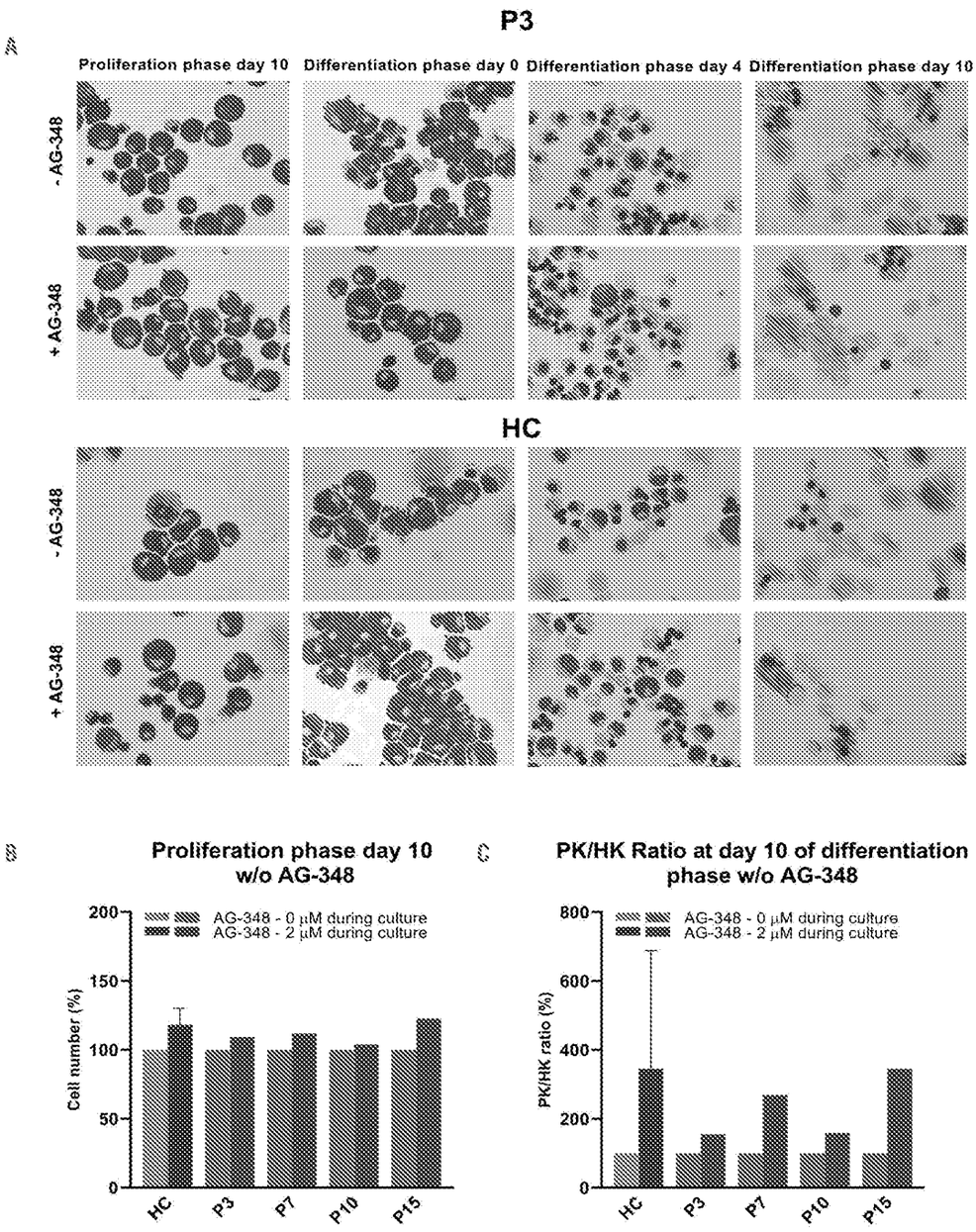



Figure 6. *Ex vivo* treatment with AG-348 does not evidently affect pyruvate kinase (PKD) *ex vivo* erythroid proliferation and differentiation. (A) Morphology of *ex vivo* cultured erythroid cells of a PKD patient (P3) and a healthy control (HC) at the final stage of proliferation (day 10) and on various days during differentiation (days 0, 4 and 10). (B) *Ex vivo* erythroid proliferation (cell numbers in %) was slightly higher when cells were cultured in presence of 2 µM AG-348 (dark purple) compared to 0 µM AG-348 (light purple). This was also observed for HC. (C) PK/HK ratio of erythroid cells of PKD patients (purple) and HC (gray) cultured in presence (dark) or absence (light) of 2 µM AG-348 respectively was increased compared to cells cultured without AG-348. W/o: with/without. Error bars represent standard deviation.



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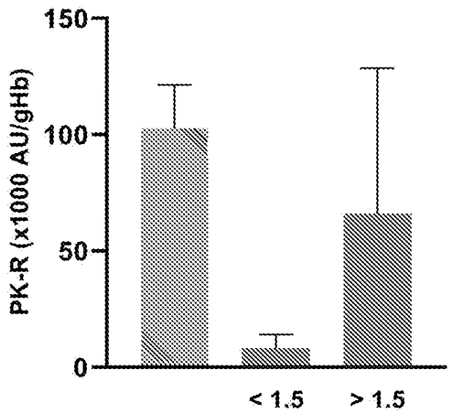



Figure 7. Pyruvate kinase (PK)-deficient red cells with higher PK-R protein levels are more likely to show an increase in adenosine triphosphate (ATP) levels and/or PK-R activity after *ex vivo* treatment with AG-348. Base line PK-R protein levels in patients showing a response in PK activity or ATP levels (i.e., >1.5-fold response vs. <1.5-fold response, red bars) upon *ex vivo* treatment with AG-348 PK-R levels of healthy controls are shown in gray. ECL: electrochemiluminescense; gHb: gram hemoglobin. Error bars represent standard deviation.

patients (Figure 5F). However, this technique likely underestimates the effect of ATP increase by AG-348. Although ektacytometry is an important and established method in the diagnosis of RBC membrane and hydration disorders,[33,34,45,46] it does have some limitations. Ektacytometry reflects the passive deformation of RBC in contrast to filter-based assays or microfluidics which forces RBC to actively deform; the latter is thought to be more dependent on ATP.[14,41] This could explain the finding of only a small correlation with ATP levels and deformability measured in RBC concentrates intended for transfusion.[40]

Our results show only a slight difference in RBC deformability between PK-deficient patients and healthy controls, with the lowest deformability observed in M/NM patients (Figure 5B). This is in line with recent findings[45] using the same technique, but contrasts with earlier findings based on a filtration-based assay.[47] We also observed normal deformability in the splenectomized PK-deficient patients compared to a small decrease in Elmax in non-splenectomized patients, which correlated inversely to reticulocyte count (r=-0.740, *P*=0.0016). These results are in line with previous studies that show that reticulocytes of PK-deficient patients are preferentially removed by the spleen,[11] probably due to their compromised deformability as a result of insufficient ATP levels.[47] It has been suggested that such a decreased deformability could be due to the decreased hydration as a result of $K^+$ efflux through PIEZO1-mediated $Ca^{2+}$ influx and activation of Gardos channels.[12] However, the osmoscan data show no signs of dehydration of RBC (i.e., decreased Ohyper), which is clearly measurable on RBC of patients with hereditary xerocytosis, where dehydration is thought to occur through the same mechanism.[48]

Collectively, the data presented in this study support the hypothesis that drug intervention with AG-348 effectively up-regulates PK-R enzymatic activity and increases stability in PK-deficient RBC over a broad range of PKLR genotypes. The concomitant increase in ATP levels suggests that the glycolytic pathway activity may be restored. AG-348 treatment may, therefore, represent an attractive way to correct the underlying pathologies of PK deficiency.

### Disclosures

*RW and EJB are consultant for Agios Pharmaceuticals.*

### Contributions

*CK, BAO, PAK and RW designed the experiments; BAO, SS, KJ, VC and PAK recruited, and collected samples and/or performed experiments; PAK, JH, LD and CK provided AG-348; MAER and RW analyzed the data and wrote the manuscript with input and revisions of BAO, PAK, GP, VC, WWS, EJB and CK.*

### Acknowledgments

*The authors would like to thank all the PK-deficient patients who participated in this study.*

### Funding

*This research has been supported by AGIOS Pharmaceuticals, and in part by an unrestricted grant provided by RR Mechatronics.*

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Ex. 2001-1025

# Decreased Activity and Stability of Pyruvate Kinase in Hereditary Hemolytic Anemia: a Potential Target for Therapy by AG-348 (Mitapivat), an Allosteric Activator of Red Blood Cell Pyruvate Kinase

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## Background

Reactive oxygen species (ROS) play an important role in the complex and multifactorial pathophysiology of hereditary hemolytic anemia like sickle cell disease (SCD), β-thalassemia and hereditary xerocytosis (HX). Increased intracellular levels of oxidative stress disrupt normal cell functioning and may contribute to premature red blood cell (RBC) clearance from the circulation. Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis, the cell's main source of energy. Because PK is very sensitive to redox balance we hypothesized that increased levels of oxidative stress in SCD, β–thalassemia and HX impairs proper enzyme function, thereby compromizing RBC energy metabolism. This may contribute to disease pathophysiology.

## Aim

To investigate if secondary deficiency of PK is common in SCD, thalassemia, and HX, and to investigate if PK in these disorders is able to respond to treatment with the allosteric PK activator AG-348 (mitapivat).

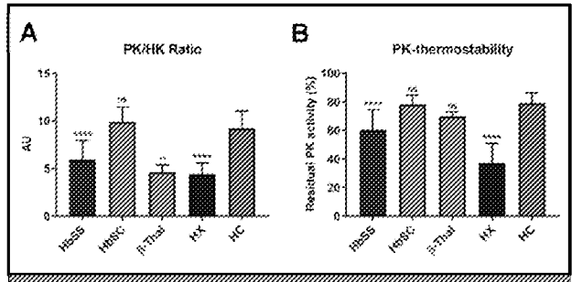



Figure 1 Pyruvate kinase (PK) enzymatic activity and thermostability. (A) Mean ratio of PK/hexokinase (HK) enzymatic activity in red blood cells (RBC) of patients with homozygous sickle cell disease (HbSS), compound heterozygosity for HbS and HbC (HbSC), β-thalassemia major (β-Thal), hereditary xerocytosis (HX) and healthy controls (HC). (B) Mean values of PK-thermostability in RBCs of patients with HbSS, HbSC, β-Thal, HX and HC. Error bars represent standard deviation. **** p<0.0001, *** p<0.001, ns p>0.05

## Methods

Enzymatic activities of red cell PK and hexokinase (HK) were measured together with PK-thermostability in order to assess relative PK activity and enzyme stability. Purified RBCs were incubated with AG-348 (3.33 µM) for 24 hours after which PK activity and ATP response was measured. RBCs of SCD patients were also analyzed with the oxygenscan, a newly developed method that characterizes individual sickling behavior by oxygen gradient ektacytometry (Rab et al, Am J Hematol, 2019). Individual tendency to sickle is reflected by Point-of-Sickling (PoS) that indicates the specific $pO_2$ at which RBCs start to sickle during deoxygenation under shear stress.

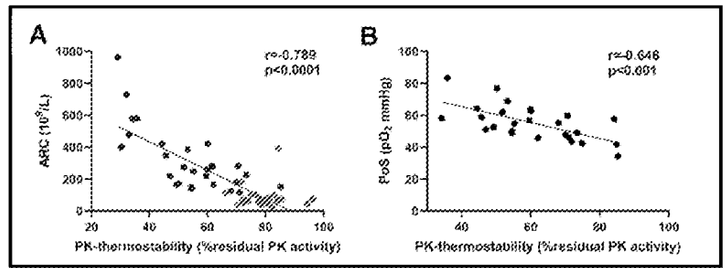



Figure 2 Correlation of PK-thermostability with absolute reticulocyte count (ARC) and point of sickling (PoS). (A) Correlation of PK-thermostability with ARC in patients with sickle cell disease (HbSS, HbSC and HbS/β-thalassemia). (B) Correlation of PK-thermostability with PoS in patients with HbSS, HbSC, β-thal, hereditary xerocytosis (HX) and healthy controls (HC). PK-thermostability and PoS: 1 each.

## Results

Thirty-eight patients and 21 healthy controls (HC) were included. The patient cohort consisted of patients homozygous for HbS (HbSS, n=26), patients compound heterozygous for HbS and HbC (HbSC, n=4), β-thalassemia major (regularly transfused, n=3), and hereditary xerocytosis (n=5). Patients showed reticulocytosis and, in line with this, a concomitant increase in HK activity. In contrast however, relative PK activity was decreased significantly compared to HK in HbSS, β-thalassemia and HX patients, but not in HbSC patients (Figure 1A). PK-thermostability was significantly decreased compared to HCs in HbSS patients and patients with HX (Figure 1B). In HbSC and β-thalassemia patients, PK-thermostability was comparable to HC. PK thermostability strongly correlated with absolute reticulocyte count (ARC), indicating that patients displaying the highest degree of PK instability had the highest reticulocyte count (Figure 2A). This suggests that in general, a higher degree of PK instability is associated with more severe anemia due to a high hemolytic rate.

## Results (2)

In SCD patients, PK-thermostability inversely correlated with PoS, indicating that decreased PK stability is associated with sickling at higher pO2 ($r=-0.646$, $p<0.001$, Figure 2B). When purified RBCs were incubated with 3.33µM of the allosteric PK-activator AG-348, an increase in PK activity was seen in all patients and HCs, with a mean increase of 122% in HbSS (range 111-139%, n=6), 137% in β-thalassemia (n=1), 163% in HX (range 152-174%, n=2) and 143% in HC (range 113-173%, n=9, Figure 3A). Accordingly, ATP-levels increased in all patients and HCs, with a mean increase of 133% in HbSS (range 125-141%, n=5), 144% patient with β-thalassemia (n=1), 121% in HX (range 112-129, n=3), and 132% in HCs (range 101-149%, n=9, Figure 3B).

## Conclusion

PK enzyme activity and stability is compromised in patients with various forms of hereditary hemolytic anemia. This implies that PK stability and, hence, compromised red cell metabolism could contribute to the complex pathophysiology of these diseases. In SCD patients, reduced PK-thermostability is associated with higher PoS, which we previously have shown to be associated with more severe disease (Rab et al, Am J Hematol, 2019, ASH 2019 abstract ID128870). This is confirmed by the correlation of decreased PK-thermostability with increased ARC as presented in this study. Current studies are in progress to further substantiate the underlying mechanism(s) involved, and to investigate whether AG-348 may ameliorate clinical features such as hemolysis, sickling tendency and iron overload.

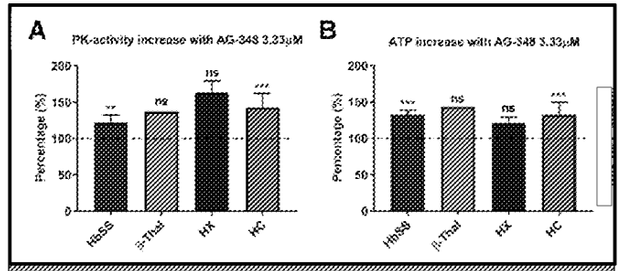



Figure 3 Effect of incubation of RBC with AG-348 on PK activity and ATP levels. (A) Mean values of PK activity after incubation with 3.33µM AG-348 in HbSS, β-Thal and HX. (B) Mean values of ATP increase after incubation with 3.33µM AG-348. Error bars represent standard deviation. **** p<0.0001, *** p<0.001, ns not significant

Ex. 2001-1026

blood

MENU

101.RED CELLS AND ERYTHROPOIESIS, STRUCTURE AND FUNCTION, METABOLISM, AND SURVIVAL, EXCLUDING IRON | NOVEMBER 13, 2019

# Decreased Activity and Stability of Pyruvate Kinase in Hereditary Hemolytic Anemia: A Potential Target for Therapy By AG-348 (Mitapivat), an Allosteric Activator of Red Blood Cell Pyruvate Kinase

Minke A.E. Rab, MD, Brigitte A. van Oirschot, Stephanie van Straaten, MD PhD, Bart J. Biemond, MD PhD, Jennifer Bos, Penelope A. Kosinski, MS, Charles Kung, PhD, Eduard J. van Beers, MD PhD, Richard van Wijk, PhD

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*Blood* (2019) 134 (Supplement_1): 3506.

https://doi.org/10.1182/blood-2019-129996

Split-Screen Share Tools

**Background:** Reactive oxygen species (ROS) play an important role in the complex and multifactorial pathophysiology of hereditary hemolytic anemia like sickle cell disease (SCD), β-thalassemia and hereditary xerocytosis (HX). Increased intracellular levels of oxidative stress disrupt normal cell functioning and may contribute to premature red blood cell (RBC) clearance from the circulation. Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis, the cell's main source of energy. Because PK is very sensitive to redox balance we hypothesized that increased levels of oxidative stress in SCD, β-thalassemia and HX impairs proper enzyme function, thereby compromizing RBC energy metabolism. This may contribute to disease pathophysiology.

**Aims:** To investigate if secondary deficiency of PK is common in SCD, thalassemia, and HX, and to investigate if PK in these disorders is able to respond to treatment with the allosteric PK activator AG-348 (mitapivat).

**Methods:** Enzymatic activities of red cell PK and hexokinase (HK) were measured together with PK-thermostability in order to assess relative PK activity and enzyme stability. Purified RBCs were incubated with AG-348 (3.33μM) for 24 hours after which PK activity and ATP response was



Ex. 2001-1027

measured. RBCs of SCD patients were also analyzed with the oxygenscan, a newly developed method that characterizes individual sickling behavior by oxygen gradient ektacytometry (Rab et al, Am J Hematol, 2019). Individual tendency to sickle is reflected by Point-of-Sickling (PoS) that indicates the specific $pO_2$ at which RBCs start to sickle during deoxygenation under shear stress.

**Results:** Thirty-eight patients and 21 healthy controls (HC) were included. The patient cohort consisted of patients homozygous for HbS (HbSS, n=26), patients compound heterozygous for HbS and HbC (HbSC, n=4), β-thalassemia major (regularly transfused, n=3), and hereditary xerocytosis (n=5).

Patients showed reticulocytosis and, in line with this, a concomitant increase in HK activity. In contrast however, relative PK activity was decreased significantly compared to HK in HbSS, β-thalassemia and HX patients, but not in HbSC patients (Figure 1A). PK thermostability was significantly decreased compared to healthy controls in HbSS patients and patients with HX (Figure 1B). In HbSC and β-thalassemia patients, PK-thermostability was comparable to HC. PK thermostability strongly correlated with absolute reticulocyte count (ARC), indicating that patients displaying the highest degree of PK instability had the highest reticulocyte count (Figure 1C). This suggests that in general, a higher degree of PK instability is associated with more severe anemia due to a high hemolytic rate. In SCD patients, PK-thermostability inversely correlated with PoS, indicating that decreased PK stability is associated with sickling at higher $pO_2$ ($r=-0.646$, $p<0.001$, Figure 1F).

When purified RBCs were incubated with 3.33μM of the allosteric PK-activator AG-348, an increase in PK activity was seen in all patients and HCs, with a mean increase of 122% in HbSS (range 111-139%, n=6), 137% in β-thalassemia (n=1), 163% in HX (range 152-174%, n=2) and 143% in HC (range 113-173%, n=9, Figure 1E). Accordingly, ATP-levels increased in all patients and HCs, with a mean increase of 133% in HbSS (range 125-141%, n=5), 144% patient with β-thalassemia (n=1), 121% in HX (range 112-129, n=3), and 132% in HCs (range 101-149%, n=9, Figure 1E).

**Conclusion:** PK enzyme activity and stability is compromised in patients with various forms of hereditary hemolytic anemia. This implies that PK stability and, hence, compromised red cell metabolism could contribute to the complex pathophysiology of these diseases. In SCD patients, reduced PK-thermostability is associated with higher PoS, which we previously have shown to be associated with more severe disease (Rab et al, Am J Hematol, 2019, ASH 2019 abstract ID128870). This is confirmed by the correlation of decreased PK-thermostability with increased reticulocyte count as presented in this study.



Ex. 2001-1028

Current studies are in progress to further substantiate the underlying mechanism(s) involved, and to investigate whether AG-348 may ameliorate clinical features such as hemolysis, sickling tendency and iron overload.

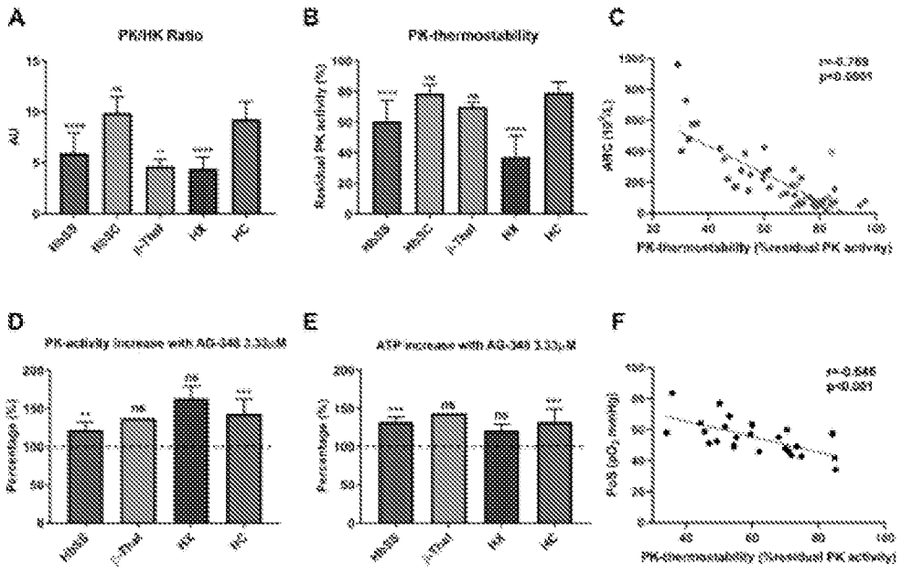



Figure 1. (A) Mean values of PK/HK ratio in homozygous SCD (HbSS), HbSC, β-Thalassemia (β-Thal), hereditary xerocytosis (HX) patients and healthy controls (HC). (B) Mean values of PK-thermostability in HbSS, HbSC, β-Thal, HX and HC. (C) Linear correlation of absolute reticulocyte count (ARC) and PK-thermostability (SS dark red dots, SC orange dots, β-Thal turquoise dots, HX blue dots, HC grey dots). (D) Mean values of PK activity increase after incubation with AG-348 3.33µM in HbSS, β-Thal patients, HX and HC. (E) Mean values of ATP increase after incubation with AG-348 3.33µM. (F) Linear correlation of Point of Sickling (PoS) and PK-thermostability in SCD. Error bar represents SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05., ns, not significant.

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## Disclosures

**Rab:***RR Mechatronics:* Research Funding. **Bos:***RR Mechatronics:* Research Funding. **Kosinski:***Agios Pharmaceuticals, Inc:* Employment, Other: Stakeholder. **Kung:***Agios Pharmaceuticals, Inc:* Employment, Other: Stakeholder. **van Beers:***Agios Pharmaceuticals, Inc.:* Membership on an entity's Board of Directors or advisory committees, Research Funding; *Novartis:* Consultancy, Research Funding; *Pfizer:* Research Funding; *RR Mechatronics:* Research Funding. **van Wijk:***Agios Pharmaceuticals:* Consultancy, Research Funding; *RR Mechatronics:* Research Funding.

**Topics:** anemia, hemolytic, congenital, erythrocytes, hypokinesia, pyruvate kinase, transcriptional activation, thalassemia, human placental lactogen, enzymes, hemoglobin, sickle, xerocytosis

## Author notes

*Asterisk with author names denotes non-ASH members.

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# Decreased activity and stability of pyruvate kinase in sickle cell disease and thalassemia: a potential target for therapy

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## INTRODUCTION

Reactive oxygen species (ROS) play an important role in the complex and multifactorial pathophysiology of sickle cell disease (SCD) and thalassemia. In SCD, auto-oxidation of red blood cell (RBC) hemoglobin produces high levels of ROS that exceed anti-oxidant capacity. In β–thalassemia, auto-oxidation of unpaired α-globins produces high levels of ROS. Increased intracellular levels of oxidative stress disrupt normal cell functioning and may contribute to premature clearance from the circulation. Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis, the cell's only source of energy. Because PK is very sensitive to redox balance we hypothesized that increased levels of oxidative stress in hemoglobinopathies impairs proper enzyme function and thereby compromises RBC energy metabolism. Such a secondary deficiency of PK may contribute to disease pathophysiology.

## OBJECTIVE

To investigate if secondary deficiency of PK is common in SCD and thalassemia, and to investigate if PK in these disorders is able to respond to treatment with the allosteric PK activator AG-348.

## METHODS

Enzymatic activities of red cell PK and hexokinase (HK) were measured together with PK thermal stability (residual activity of PK after one hour of heating at 53 degrees Celcius) in order to assess relative PK activity and enzyme stability. For ex vivo treatment with AG-348 RBC lysates were incubated with 3.33 uM AG-348 for 2 hours, after which PK thermal stability, PK activity and ATP response was measured. To asses the effect of AG-348 on cellular function RBCs of SCD patients were analyzed with the oxygenscan, a newly developed method that characterizes individual sickling behavior[1].

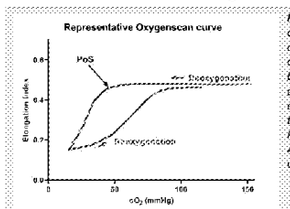



*Figure 1. Representative oxygenscan curve. An oxygenscan starts with deoxygenation followed by reoxygenation. Point of Sickling (PoS) embodies the oxygen tension when the first RBCs start to sickle. Adapted from Rab et al with permission.*

## RESULTS (1)

Twenty-eight patients and 18 healthy controls (HC) were included. The patient cohort consisted of patients with HbSS (n=16), HbS/β-thalassemia (n=1), β-thalassemia major (n=3, regularly transfused), and HbSC (n=3). Patients showed reticulocytosis and a concomitant increase in HK activity. However, PK activity was relatively decreased significantly compared to HK in SCD and β-thalassemia patients, but not in HbSC patients (Figure 2A). PK thermal stability also was significantly decreased in SCD patients compared to healthy controls. In HbSC and β-thalassemia patients PK-thermal stability was comparable to HC (Figure 2B). When purified RBCs were incubated with 3.33uM of the allosteric PK-activator AG-348 an increase in PK activity was seen in all patients and HCs, with a median of 122% in SCD (range 120-138%, n=3), 127% in β-thalassemia and 142% in HC (range 130-166%, n=5, Figure 2D).

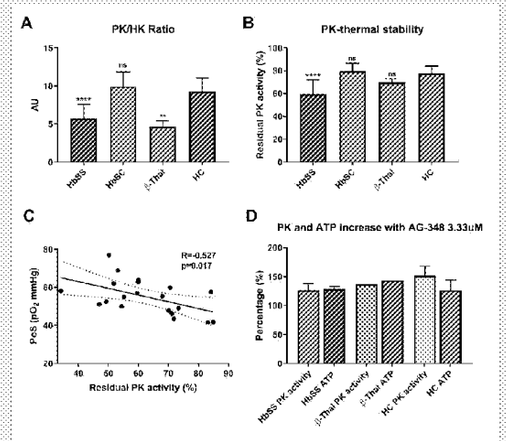



*Figure 2. (A) Mean values of PK/HK ratio in homozygous SCD (HbSS), HbSC, β-Thalassemia patients and healthy controls (HC). (B) Mean values of PK-thermal stability in HbSS, HbSC, β-Thal and HC. (C) Linear correlation of point-of-sickling (PoS) and PK-thermal stability. (D) Mean values of PK activity increase and ATP increase after incubation with AG-348 3.33uM in HbSS, β-Thal patients and HC. Error bar represent SD. ****p<0.0001, ** p<0.01, *p<0.05., ns, not significant. Dashed lines represent 95% confidence intervals.*

## RESULTS (2)

Concomitant with the increase in PK activity, ATP-levels increased in all patients and HCs, with a median of 126% in SCD (range 121-129%, n=3), 146% in 1 patient with β-thalassemia, and 122% in HCs (range 100-148%). In SCD patients PK-thermal stability inversely correlated significantly with oxygenscan parameters, in particular the point-of-sickling (PoS). This indicates that RBCs tend to sickle at a higher $pO_2$ when PK is more unstable. Interestingly, the 3 patients with most stable PK are HbSC patients (2) and a HbS/dβ-thalassemia patient with HbF levels of 35% (Figure 2C).

## CONCLUSIONS

PK enzyme activity and stability is compromised in patients with SCD and β-thalassemia. In SCD, reduced PK thermal stability is associated with higher PoS, which is associated with more severe disease. Current studies to further substantiate the underlying mechanism(s) and to investigate whether AG-348 may ameliorate pathophysiological features such as hemolysis and sickling tendency are in progress.

## ACKNOWLEDGEMENTS

We would like to thank all the patients that participate in this study.

## REFERENCES

1. Rab MAE, van Oirschot BA, Bos J, Merkx TH, van Wesel ACW, Abdulmalik O, et al. Rapid and reproducible characterization of sickling during automated deoxygenation in sickle cell disease patients. Am J Hematol. 2019;94(February):575–84.

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Ex. 2001-1030

# DECREASED ACTIVITY AND STABILITY OF PYRUVATE KINASE IN SICKLE CELL DISEASE AND THALASSEMIA: A POTENTIAL TARGET FOR THERAPY

**Author(s): Minke Rab, Brigitte van Oirschot, Stephanie van Straaten, Bart J. Biemond, Jennifer Bos, Penelope Kosinski, Charles Kung, Eduard van Beers, Richard van Wijk**

**Jun 15, 2019; 267136**

**Abstract:** PS1519

**Type:** Poster Presentation

**Presentation during EHA24:** On Saturday, June 15, 2019 from 17:30 - 19:00

**Location:** Poster area

**Background**
Reactive oxygen species (ROS) play an important role in the complex and multifactorial pathophysiology of sickle cell disease (SCD) and thalassemia. In SCD, auto-oxidation of red blood cell (RBC) hemoglobin produces high levels of ROS that exceed anti-oxidant capacity. In β–thalassemia, auto-oxidation of unpaired α-globins produces high levels of ROS. Increased intracellular levels of oxidative stress disrupt normal cell functioning and may contribute to premature clearance from the circulation. Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis, the cell's only source of energy. Because PK is very sensitive to redox balance we hypothesized that increased levels of oxidative stress in hemoglobinopathies impairs proper enzyme function and thereby compromizes RBC energy metabolism. This may contribute to disease pathophysiology.

**Aims**
To investigate if secondary deficiency of PK is common in SCD and thalassemia, and to investigate if PK in these disorders is able to respond to treatment with the allosteric PK activator AG-348.

**Methods**
Enzymatic activities of red cell PK and hexokinase (HK) were measured together with PK thermal stability (residual activity of PK) in order to assess relative PK activity and enzyme stability. RBC lysates were incubated with AG-348 (3.33 uM) for 2 hours after which PK thermal stability, PK activity and ATP response was measured. Red blood cells of SCD patients were also analyzed with the oxygenscan, a newly developed method that characterizes individual sickling behavior (Rab et al, Am J Hematol, 2019).

**Results**
Twenty-eight patients and 18 healthy controls (HC) were included. The patient cohort consisted of patients with HbSS (n=16), HbS/β-thalassemia (n=1), β-thalassemia major (n=3, regularly transfused), and HbSC (n=3). Patients showed reticulocytosis and a concomitant increase in HK activity. However, PK activity was relatively decreased significantly compared to HK in SCD and β-thalassemia patients, but not in HbSC patients (Figure 1A). PK thermal stability also was significantly decreased in SCD patients compared to healthy controls. In HbSC and β-thalassemia patients PK-thermal stability was comparable to HC (Figure 1B). When purified RBCs were incubated with 3.33uM of the allosteric PK-activator

AG-348 an increase in PK activity was seen in all patients and HCs, with a median of 122% in SCD (range 120-138%, n=3), 127% in β-thalassemia and 142% in HC (range 130- 166%, n=5, Figure 1D). Accordingly, ATP-levels increased in all patients and HCs, with a median of 126% in SCD (range 121-129%, n=3), 146% in 1 patient with β-thalassemia, and 122% in HCs (range 100-148%). In SCD patients PK-thermal stability inversely correlated significantly with oxygenscan parameters, in particular the point-of-sickling (PoS). This indicates that RBCs tend to sickle at a higher pO when PK is more unstable. Interestingly, the 3 patients with most stable PK are HbSC patients (2) and a HbS/dβ-thalassemia patient with HbF levels of 35% (Figure 1C).

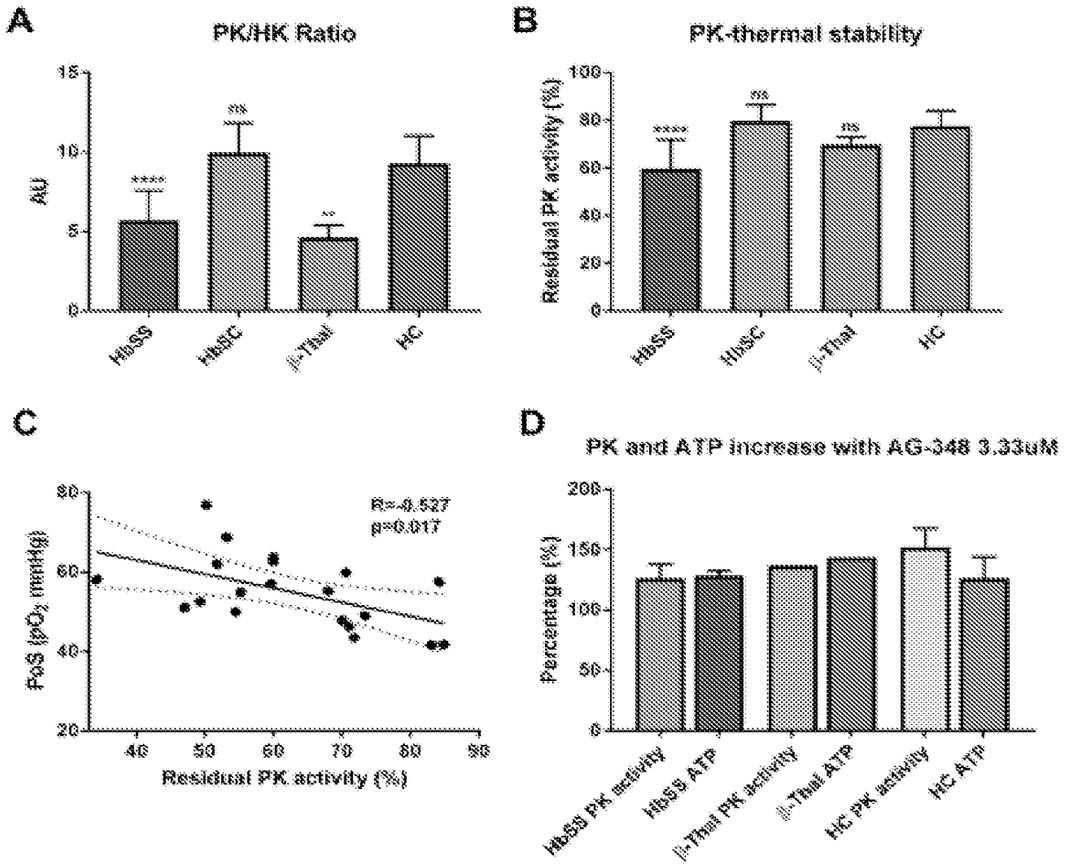



**Figure 1. (A)** Mean values of PK/HK ratio in homozygous SCD (HbSS), HbSC, β-Thalassemia patients and healthy controls (HC). **(B)** Mean values of PK-thermal stability in HbSS, HbSC, β-Thal and HC. **(C)** Linear correlation of point-of-sickling (PoS) and PK-thermal stability. **(D)** Mean values of PK activity increase and ATP increase after incubation with AG-348 3.33uM in HbSS, β-Thal patients and HC. Error bar represent SD. ****p<0.0001, ** p<0.01, *p<0.05., ns, not significant. Dashed lines represent 95% confidence intervals.

## Conclusion

PK enzyme activity and stability is compromised in patients with SCD and β-thalassemia. In SCD, reduced PK thermal stability is associated with higher PoS, which is associated with more severe disease. Current studies are in progress to further substantiate the underlying mechanism(s), and to investigate whether AG-348 may ameliorate pathophysiological features such as hemolysis and sickling tendency.

**Session topic:** 26. Sickle cell disease

# Pyruvate Kinase Deficiency

# Allosteric activator of Pyruvate Kinase First Results: DRIVE PK study

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*[1]Hôpital Saint Vincent de Paul, Université Catholique, Lille, France; [2]Genetic Diseases Unit of Globule Rouge, CHU Henri Mondor, Créteil, France; [3]Hammersmith Hospital, Imperial College Healthcare, United Kingdom; [4]Universitair Medisch Centrum Utrecht, Netherlands; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [6]Hôpital de la Timone, Marseille, France; [7]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, USA; [8]Stanford University School of Medicine, Palo Alto, CA; [9]Agios Pharmaceuticals, Inc., Cambridge, MA.*

Ile de Ré: Club du globule rouge et du fer 2017

# Pyruvate Kinase Deficiency

**Description**

- Severe congenital hemolytic anemia
- Neonatal + - late
- Prevalence [1 - 4]

**Etiology**

- PKLR gene mutations coding for erythrocyte pyruvate kinase (PK-R)

**Complications**

- Chronic anemia
- Jaundice, iron overload
- Post-splenectomy risks

**Diagnosis / Treatment**

- Enzyme activity and/or genetic analysis
- Supportive care: transfusions, splenectomy, iron chelation

**Study F: code PK *S* *Pissard***

**70 patients**

1st month diagnosis n = 41

Fatal n = 2

median age = 21 years

- **severe (n = 40):** Hb below 8 g/dL,
- **Moderate (n = 20):** Hb between 8 and 10 g/dL,
- **Mild (n = 10):** Hb greater than 10 g/dL,

Transfusion dependent 1/3

Splenectomy n = 44

Chelates 1/3

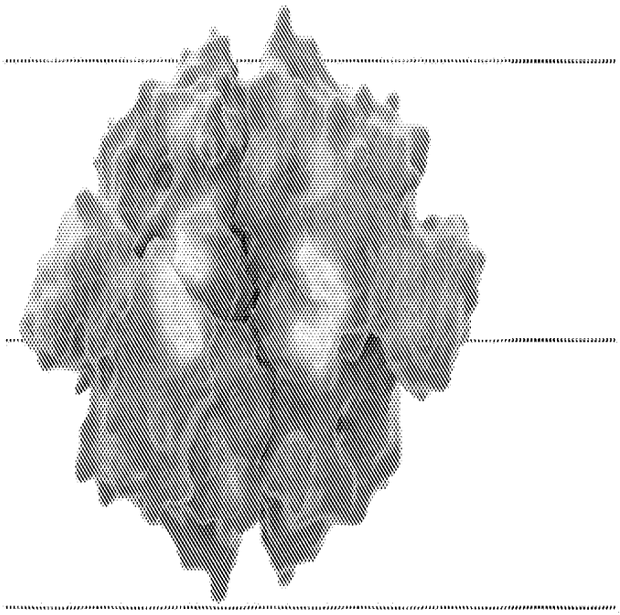
Grace R et al. *Am J Hematol* 2015;90(9):825-30; [1]Mohrenweiser HW *PNAS* 1981;78(8):5046-50; [2]Carey PJ et al. *Blood* 2000;96(12):4005-6; [3]Beutler E & Gelbart T *Blood* 2000;95(11):3585-8; [4]deMedicis et al. *Hum Hered* 1992;42(3):179-83



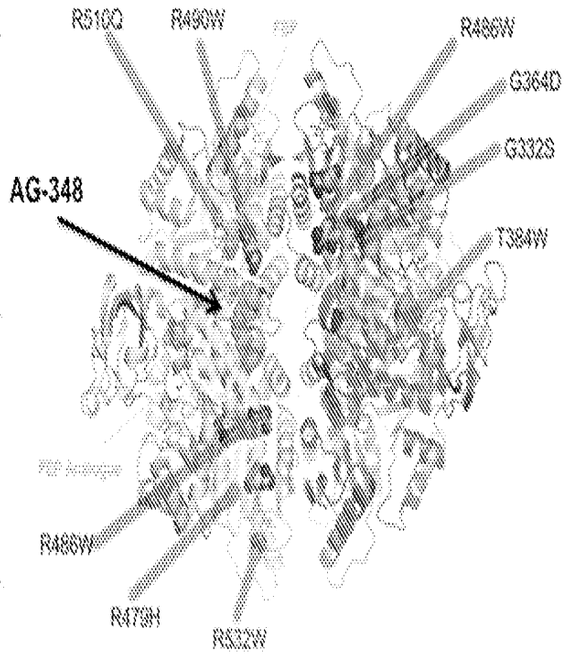
Ex. 2001-1034

# AG-348: an allosteric activator of pyruvate kinase (mutant/wild)

Pyruvate kinase is a tetramer;
Mutations (green) decrease catalytic activity



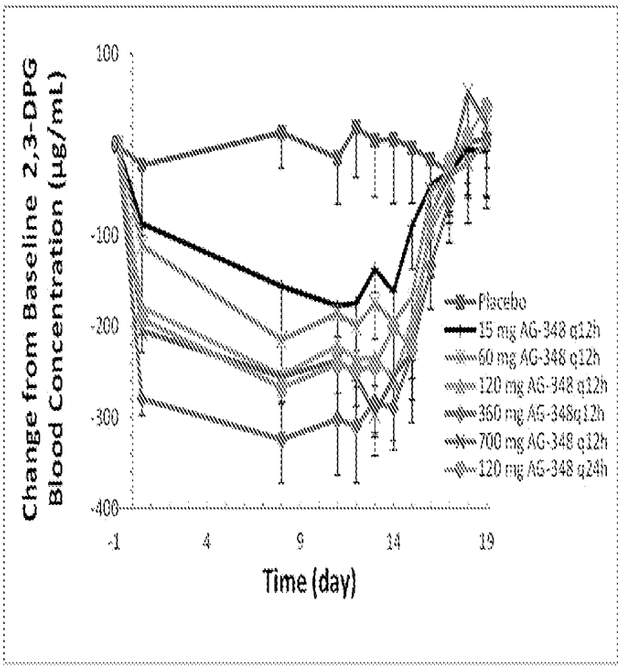
AG-348 (yellow) binds to the enzyme (dimer/dimer interface)

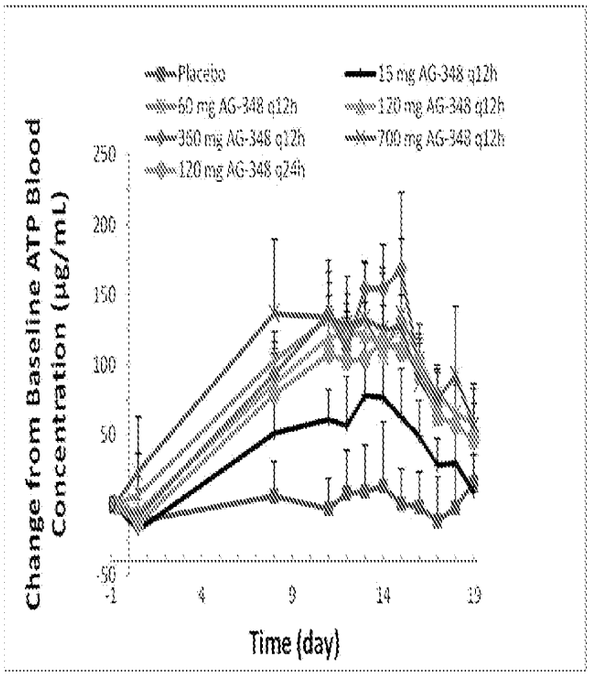





Ex. 2001-1035

# Variations 2,3-DPG and blood ATP according to AG-348 doses (dose escalations): healthy volunteer subjects




- Efficacy
- Tolerance

Yang H et al. *Blood* 2014;124[21]:4007; Yang H et al. *Haematologica* 2015;100[s1]: Abs S138)



Ex. 2001-1036

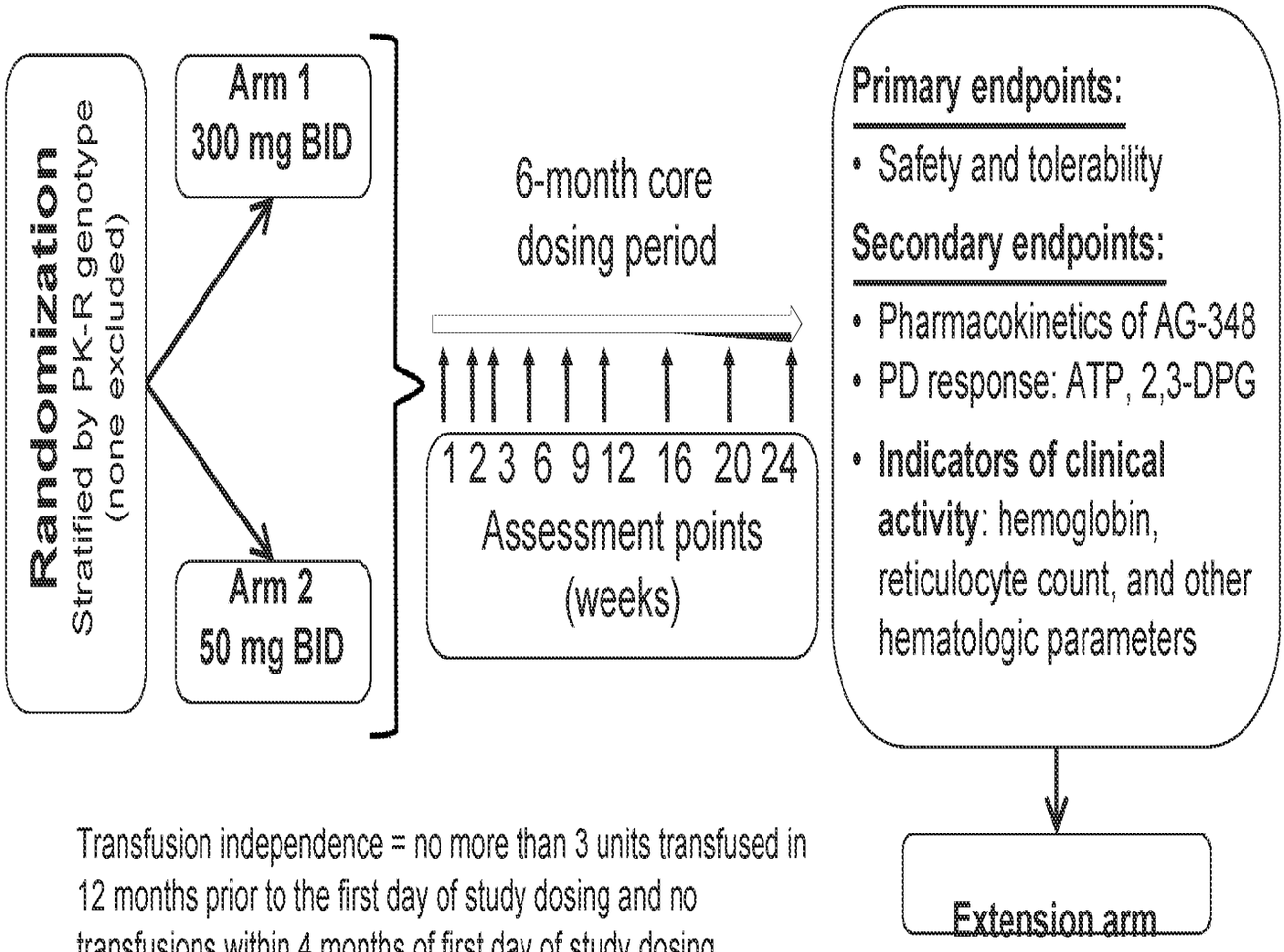
# Study design

DRIVEPK

## Open Label Phase 2 study: 14 centers in the US, Canada, EU

Transfusion-independent adults with PK deficiency

(ClinicalTrials.gov NCT02476916) n=25 in each arm




Transfusion independence = no more than 3 units transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

All patients provided written informed consent. BID = twice daily; PD = pharmacodynamic



Ex. 2001-1037

# Demographics

| Characteristics[a] | 50 mg BID, n=27 | 300 mg BID, n=25 | Total, N=52 |
|---|---|---|---|
| Men, n (%) | 18 (66.7) | 14 (56.0) | 32 (61.5) |
| Age at randomization in years, median (range) | 29 (18, 58) | 40 (21, 62) | 34 (18, 62) |
| Race white[b], n (%) | 22 (81.5) | 20 (80.0) | 42 (80.8) |
| Hemoglobin (Hb) baseline, median (range) | 9.6 (6.9, 12.3) | 8.6 (6.5, 12.0) | 8.9 (6.5, 12.3) |
| Duration of treatment, weeks, median (range) | 23.0 (13.0, 76.9) | 26.3 (12.9, 70.9) | 24.9 (12.9, 76.9) |
| Splenectomized, n (%) | 23 (85.2) | 20 (80.0) | 43 (82.7) |
| Iron chelation prior to enrollment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (76.0) | 38 (73.1) |

[a]Data cut-off March 27, 2017; [b]Not reported in 3 patients, 3 patients were Asian, and 4 were "other"



Ex. 2001-1038

# Safety summary

- AG-348 was generally well tolerated
- The majority of adverse events (AEs) were grade 1–2
- Treatment related AEs leading to discontinuation, n=3
  - Chest discomfort/pleural effusion, pharyngitis/nausea, anemia
- 13 serious AEs in 10 patients
  - Six drug-related SAEs in 5 patients: Withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia; pharyngitis
    - Grade 4 hypertriglyceridemia at Week 24, resolved upon AG-348 discontinuation (Grade 1 at baseline)

| Patient disposition by study period | Ongoing, N | Completed, N | Discontinued[a], N |
|---|---|---|---|
| Core | 15 | 29[b] | 8 |
| Extension | 21 | 0 | 3 |

[a]Reasons for discontinuation: AEs (3), investigator decision (4), and withdrew consent (4)

[b]Five subjects completing the Core period did not enter the Extension



Ex. 2001-1039

# Safety summary: Most common AEs

| AEs, regardless of causality (occurring in >5 patients) | 50 mg BID n=27 | | 300 mg BID n=25 | | Total N=52 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n (%) | 25 (92.6) | 7 (25.9) | 25 (100.0) | 5 (20.0) | 50 (96.2) | 12 (23.1) |
| Headache | 9 (33.3) | 0 | 14 (56.0) | 0 | 23 (44.2) | 0 |
| Insomnia | 5 (18.5) | 1 (3.7) | 15 (60.0) | 1 (4.0) | 20 (38.5) | 2 (3.8) |
| Nausea | 10 (37.0) | 0 | 9 (36.0) | 0 | 19 (36.5) | 0 |
| Viral upper respiratory tract infection | 7 (25.9) | 0 | 2 (8.0) | 0 | 9 (17.3) | 0 |
| Arthralgia | 5 (18.5) | 0 | 3 (12.0) | 0 | 8 (15.4) | 0 |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Back pain | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Cough | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Dizziness | 4 (14.8) | 0 | 3 (12.0) | 1 (4.0) | 7 (13.5) | 1 (1.9) |
| Vomiting | 3 (11.1) | 0 | 4 (16.0) | 0 | 7 (13.5) | 0 |
| Hot flush | 1 (3.7) | 0 | 6 (24.0) | 0 | 7 (13.5) | 0 |
| Diarrhea | 3 (11.1) | 0 | 3 (12.0) | 0 | 6 (11.5) | 0 |
| Influenza | 5 (18.5) | 1 (3.7) | 1 (4.0) | 0 | 6 (11.5) | 1 (1.9) |

Grade 3 AEs not reported in previous slide or table above: colitis (n=1), thrombosis (n=1), pharyngitis (n=1), post procedural hemorrhage (n=1), hypertension (n=1)

AEs graded using National Cancer Institute Common Terminology Criteria, version 4.03



Ex. 2001-1040

# Dose titration in DRIVE PK

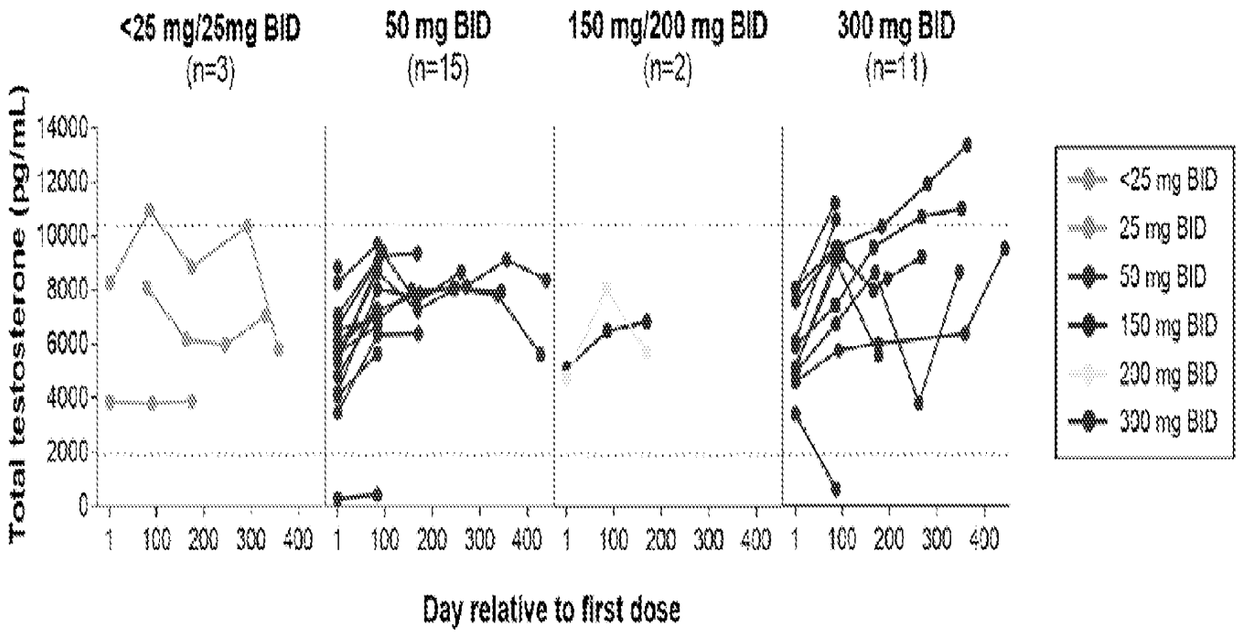
- Patients randomized to 50 mg or 300 mg BID AG-348 starting dose
  - Dose decreased:
    - AEs (e.g., insomnia)
    - Hb exceeding midpoint of normal range (Male: Hb >15.0 g/dL; Female>13.5 g/dL)
  - Dose increased:
    - Lack of Hb response
- Range of doses (5 mg QD–300 mg BID) used
- Efficacy and steroid hormone levels analyzed by the dose received for the longest duration in the Core period



Ex. 2001-1041

# Effect of AG-348 on hormones: total testosterone in men

- Preliminary findings are consistent with aromatase inhibition by AG-348 across multiple dose levels in men
  - Most testosterone values remained within the normal range
  - DEXA scan data inconclusive
  - Longer follow up required to assess clinical impact

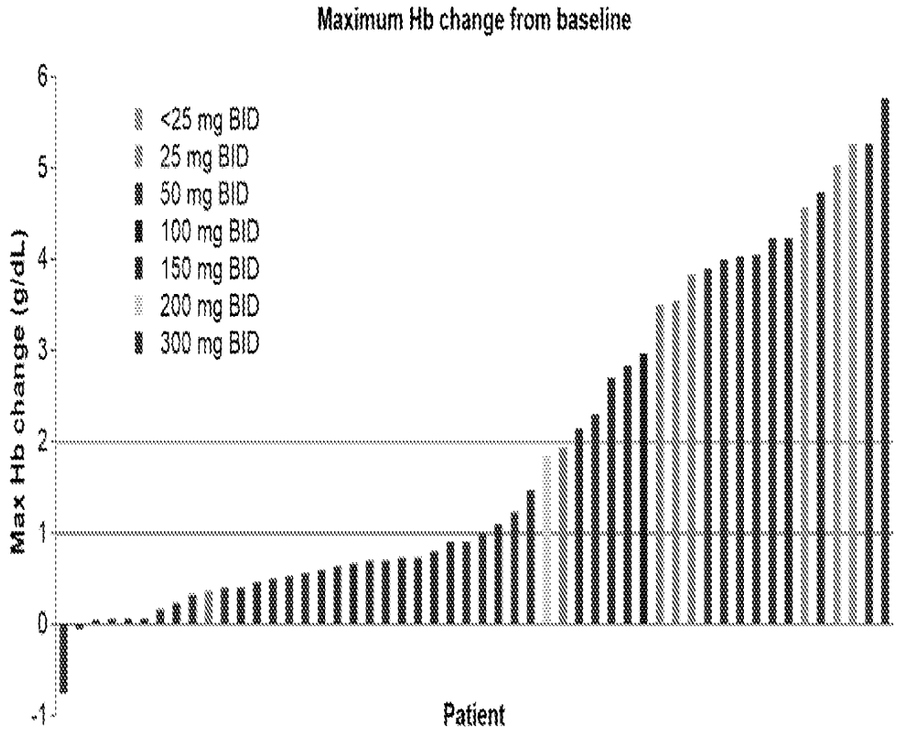



Normal reference low and high limits shown as horizontal dotted lines; DEXA = dual energy X-ray absorptiometry

# Clinical activity results

# Maximum Hb increase observed during the Core period

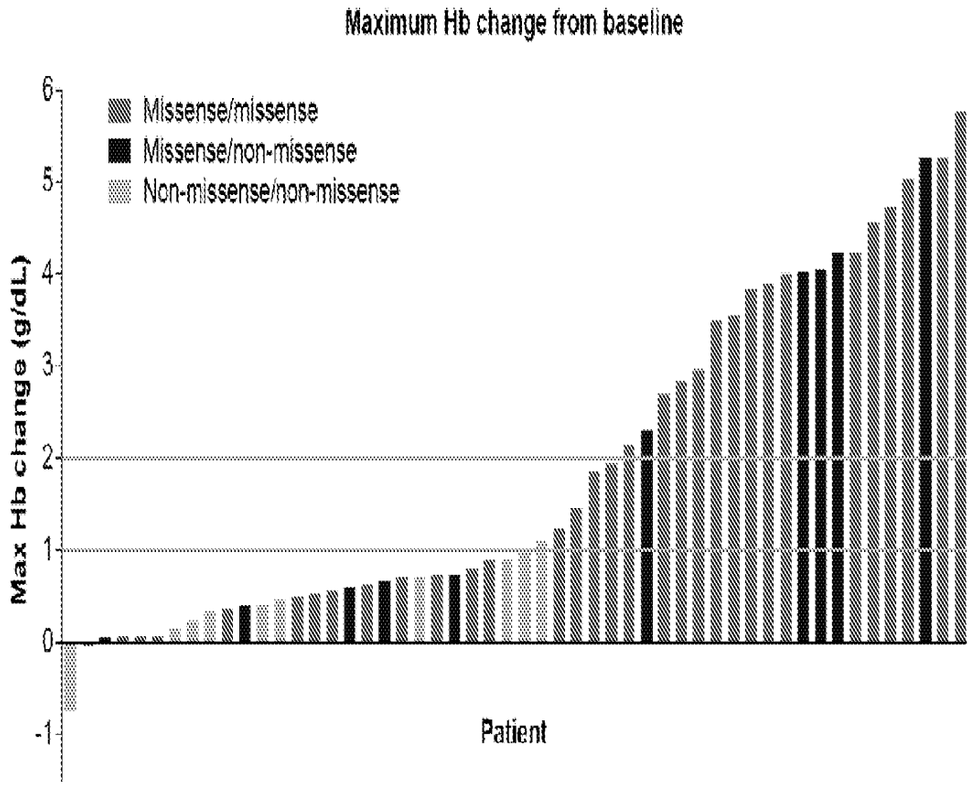
- 25/52 (48%) patients had a maximum Hb increase of >1.0 g/dL
  - The mean maximum increase was 3.5 g/dL (range 1.1-5.8 g/dL)




The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)

# Maximum Hb increase observed by genotype

- 25/52 (48%) patients had a max increase in Hb >1.0 g/dL
  - 24/42 (57%) patients who had ≥1 missense mutation had Hb increase >1.0 g/dL



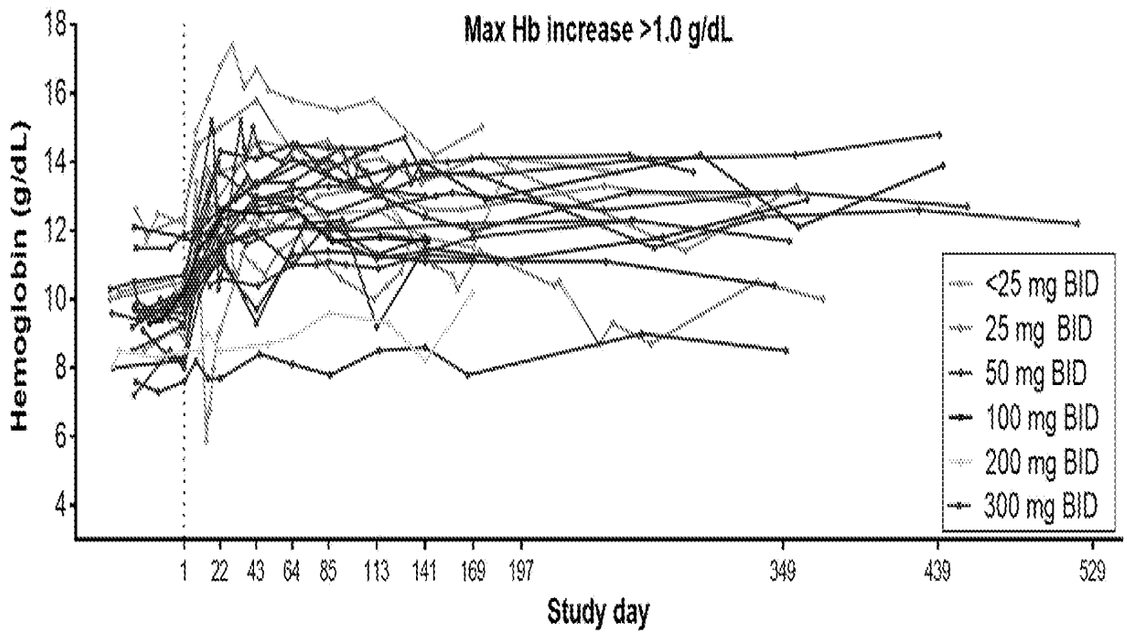

The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)



Ex. 2001-1045

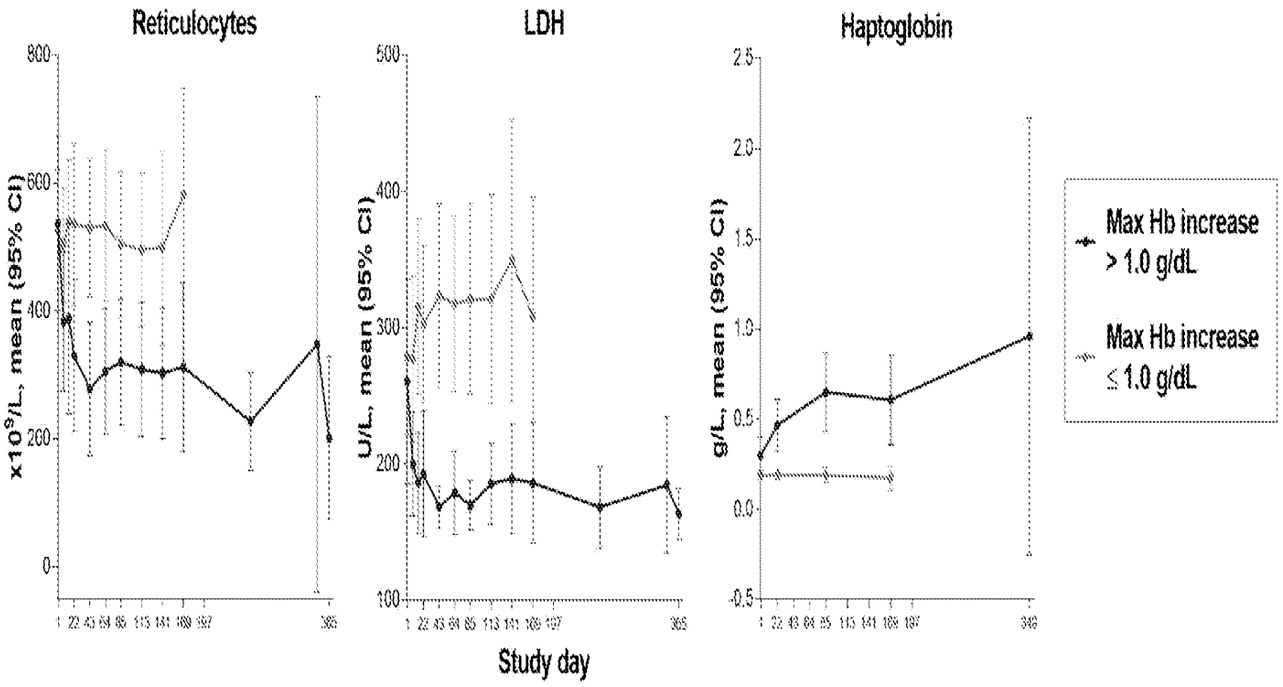
# Majority of Hb increases are rapid and sustained

- Median time to the first observation of a Hb increase >1.0 g/dL above baseline was 10 days (range 7–141 days)
  - Median baseline Hb in subjects who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.5–12.3 g/dL) vs. 8.0 g/dL (range 6.5–10.1) in subjects who did not
- In 8 patients, the dose had to be held or reduced due to rapid rise in Hb



# Hemolysis markers improve in patients with Hb increase of >1.0 g/dL

- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin



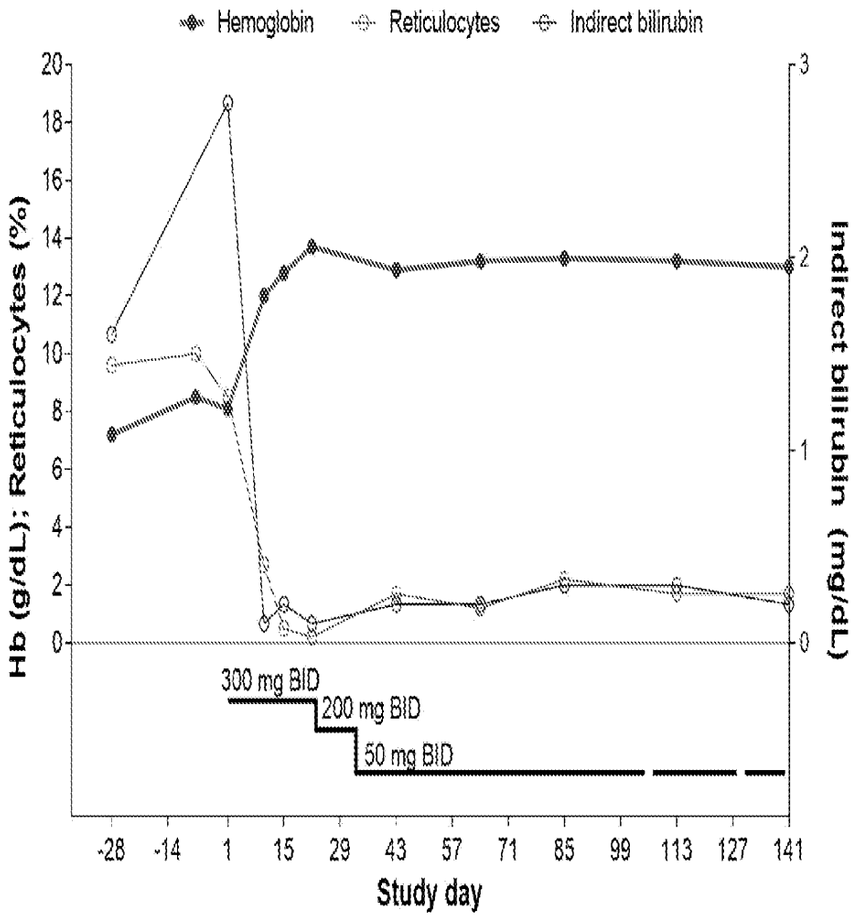

Only visits with ≥5 subjects included. Figure shows central laboratory results. LDH = lactate dehydrogenase



Ex. 2001-1047

# Hb response with improvements in hemolysis parameters: Single subject experience with AG-348

- 35 yo female, white, missense/missense genotype (T384M/R479H)






Ex. 2001-1048

# DRIVE-PK Conclusions

- AG-348 is a new molecule, first in its category, capable of activating the pyruvate kinase enzyme in deficient patients
- A daily dose is well tolerated up to 6 months
  - The significance of the anti-aromatase effect is not well understood as of this date
  - Grade 4 hypertriglyceridemia
- AG-348 is capable of increasing hemoglobin in a rapid and sustained and significant manner (>1g/dl) in close to 48% of cases.
  - The increase is related to a decrease in hemolysis
  - Preliminary correlation between response and genotype
- Phase III studies



Ex. 2001-1049

# Future Trials

## AG-348 Pivotal Program Design

| Design Element | Non-Transfusion Dependent | Regularly Transfused |
|---|---|---|
| **Patient Population** | • Non-transfusion dependent adults<br>• Baseline hemoglobin of ≤10.0 grams per deciliter<br>• Excludes patients with two non-missense mutations and those homozygous for R479H | • Regularly transfused adults<br>• Excludes patients with two non-missense mutations and those homozygous for R479H |
| Size | • 80-100 patients | • 20 patients |
| **Dose** | • Dose titration up to optimal hemoglobin response (5, 20 or 50 mg twice daily) | • Dose titration up to optimal hemoglobin response (5, 20 or 50 mg twice daily) |
| Endpoints | • Hemoglobin response<br>• Patient-reported outcomes (PRO) | • Reduction in transfusion burden<br>• Patient-reported outcomes (PRO) |
| **Control** | • Placebo controlled | • Reduction of transfusion burden compared to patients' baseline |



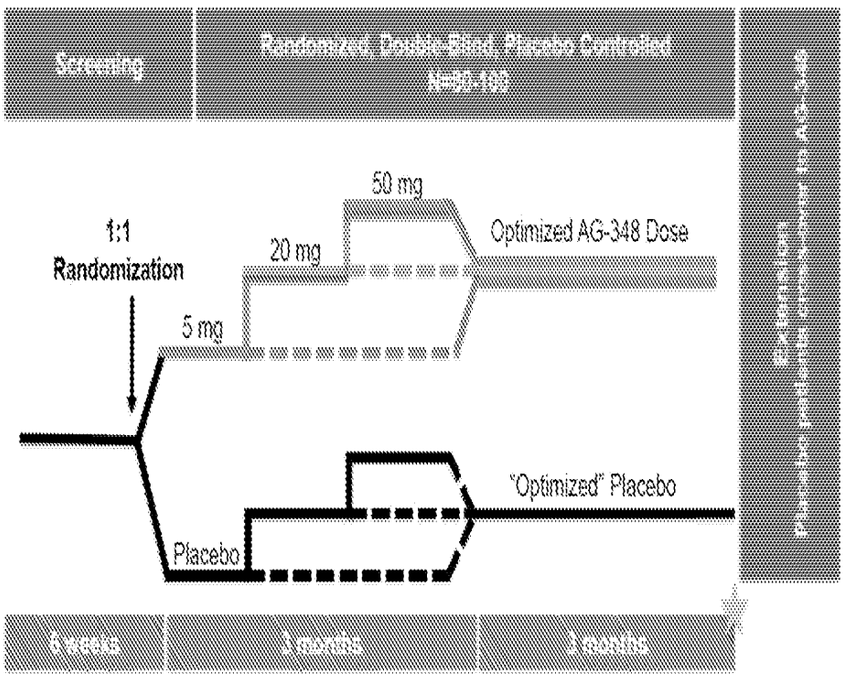
**Beginning 2018**



Ex. 2001-1050

# Trials: Non-transfusion dependent

- Adults
- 80 to 100 pts
  - Having at least one missense
    R479H excluded
- Cross-over

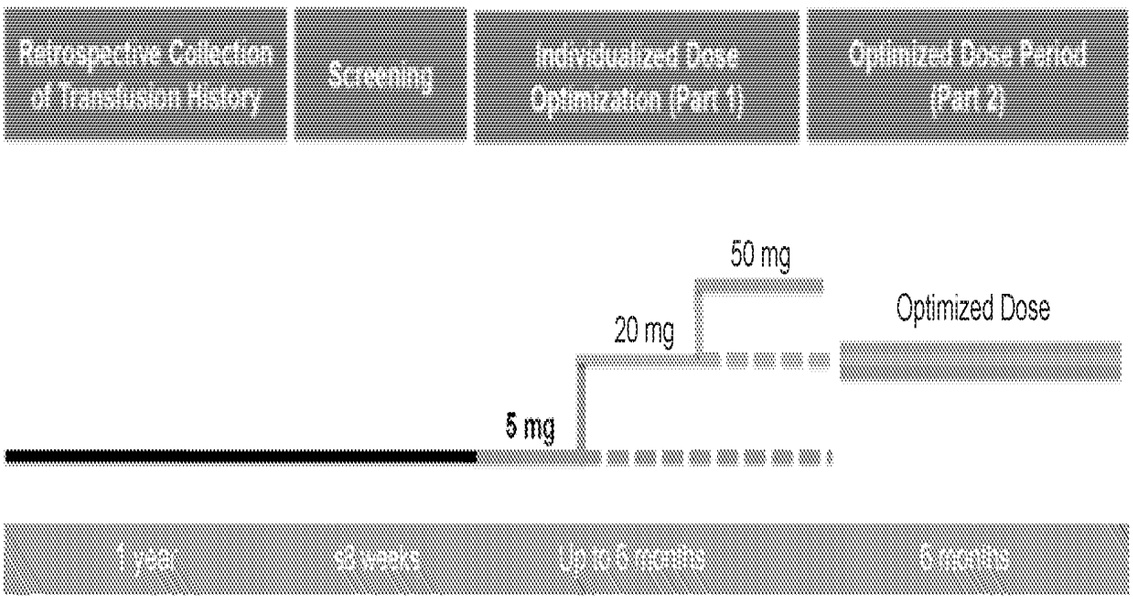



Primary Efficacy Endpoint: Proportion of patients who achieve at least a 1.5 g/dL increase in hemoglobin sustained over multiple visits

**Beginning 2018**

# Trials: transfusion-dependent

## Trial Design for Regularly Transfused Patients




Approximately 20 regularly transfused patients who have required a minimum of 6 transfusions over the year preceding enrollment

Primary Endpoint: Reduction in transfusion burden over a 6 month period compared to the patient's transfusion history

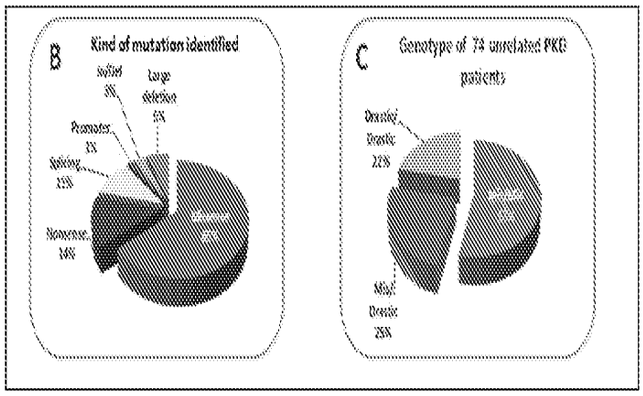


Ex. 2001-1052

# Record - Other treatments

- Register PK code
  - What conclusions can be drawn?

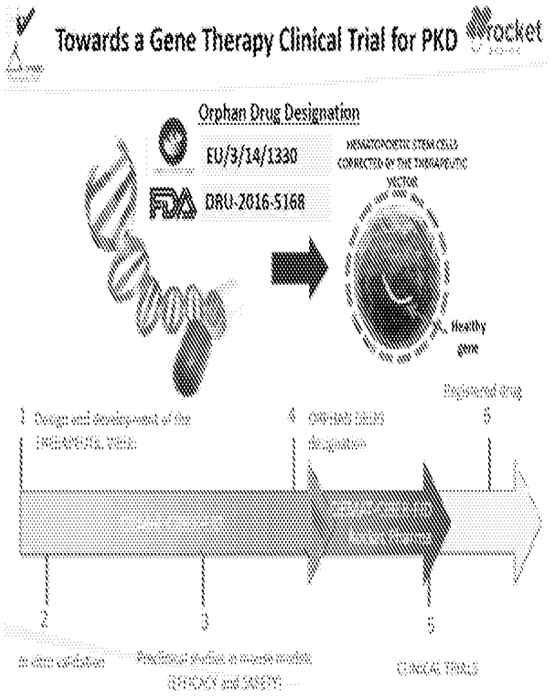
  International registration project




*Bianchi P, ASH. 2015.*

- Allograft

- Gene therapy






Ex. 2001-1053

# Conclusions

- The Drive PK study has shown that AG-438 is capable of increasing hemoglobin in a rapid, sustained and significant manner (>1g/dl) in close to one in two patients in connection with a decrease in hemolysis and, more than the dose, genotype is the principal response factor (update by ASH).
- Tolerance is satisfactory (anti-aromatase effect to be monitored)
- Potential and value of enzyme activating agents as treatment modifying the course of the disease allowing the initiation of randomized registration studies for transfusion-dependent and non-transfusion-dependent patients.
- Need to pursue national and international registries of Rare Diseases



Ex. 2001-1054

# Acknowledgments

Patients who agreed to participate

- All of the Code PK clinicians, Serge Pissard, MCGRE sector, Club Du Globule Rouge et du Fer
- (Serge Pissard, L Garcon, M Micheau, L Pascal)
- Participating Sites:
  - **Hôpital Henri Mondor, Creteil; Fréderic Galacteros**
  - **Hôpital Saint Vincent de Paul, Lille; Christian Rose**
  - **Hôpital de la Timone; Marseille, Emmanuelle Bernit**
  - Hammersmith Hospital; Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
  - Boston Children's Hospital; Rachael Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet Kwiatkowski
  - Central Pennsylvania Clinic; Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin Kuo



Ex. 2001-1055

# Déficit en Pyruvate Kinase

# Activateur Allosterique de la Pyruvate Kinase
# Premiers résultats : étude DRIVE PK

Christian Rose[1], Frédéric Galactéros[2] , D Mark Layton[3], Eduard Van Beers[4], Wilma Barcellini[5], Emmanuelle Bernit[6], Rachael F Grace[7], Bertil Glader[8], Charles Kung[9], Ann Barbier[9],

*[1]Hôpital Saint Vincent de Paul, Université Catholique, Lille, France; [2]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, France; [3]Hammersmith Hospital, Imperial College Healthcare, United Kingdom; [4]Universitair Medisch Centrum Utrecht, Netherlands; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [6]Hôpital de la Timone, Marseille, France; [7]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, USA; [8]Stanford University School of Medicine, Palo Alto, CA; [9]Agios Pharmaceuticals, Inc., Cambridge, MA.*

Ile de Ré : Club du globule rouge et du fer 2017

# Le déficit en Pyruvate kinase

**Description**

- Anémie hémolytique congénitale sévère
- Néonatal + - tard
- Prévalence [1–4]

**Etiologie**

- Mutations gène *PKLR* codant pour la pyruvate kinase érythrocytaire (PK-R)

**Complications**

- Anémie chronique
- Ictère , surcharge en fer
- Risques post-splénectomie

**Diagnostic / Traitement**

- Activité Enzymatique et/ou analyse génétique
- Soins de support :transfusions, splénectomie, chélation du fer

**Etude F: code PK *S Pissard***

**70 patients**

Diagnostic 1 er mois n= 41

Fatale n=2

âge médian = 21 ans

- **sévère (n=40) :** Hb en dessous 8 g/dL,
- **Modéré (n=20) :** Hb entre 8 et10 g/dL,
- **Faible (n=10) :** Hb sup à 10 g/dL,

Transfusion dépendant 1/3

splénectomie n=44

Chélates 1/3

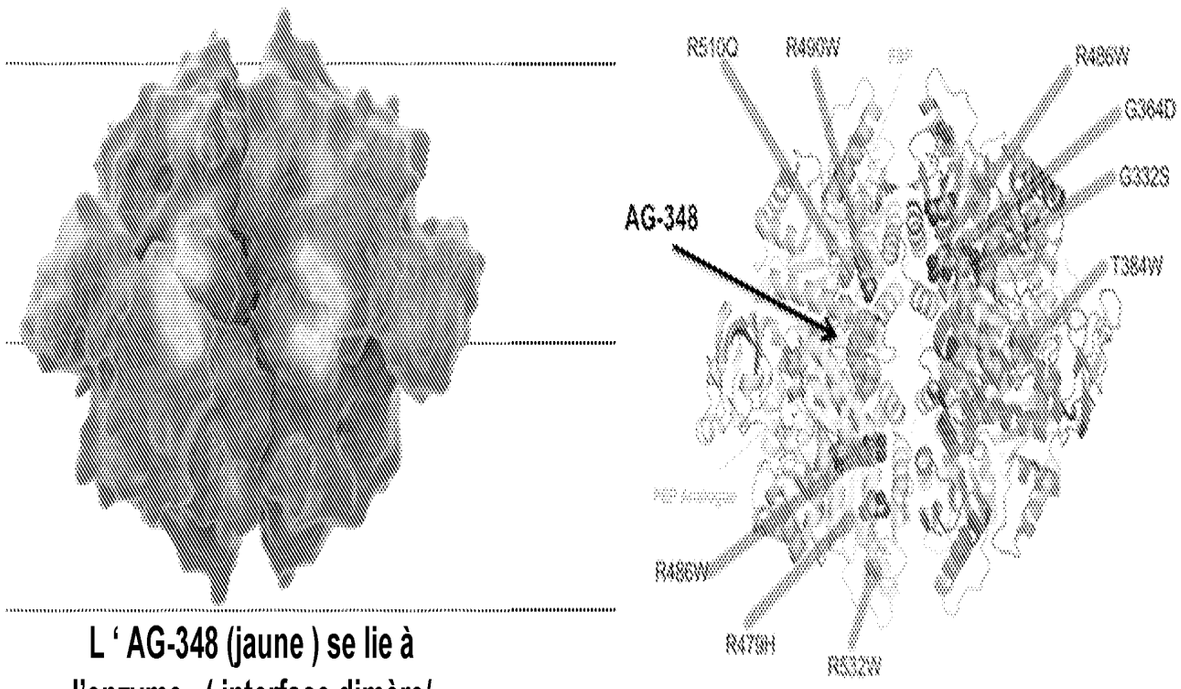
Grace R et al. *Am J Hematol* 2015;90(9):825-30; [1]Mohrenweiser HW *PNAS* 1981;78(8):5046-50; [2]Carey PJ et al. *Blood* 2000;96(12):4005-6; [3]Beutler E & Gelbart T *Blood* 2000;95(11):3585-8; [4]deMedicis et al. *Hum Hered* 1992;42(3):179-83



Ex. 2001-1057

# AG-348: un activateur allosterique de la pyruvate kinase ( mutant /sauvage)

**La pyruvate kinase est un tétramère;**
**Les mutations (vert ) diminuent l'activité catalytique**



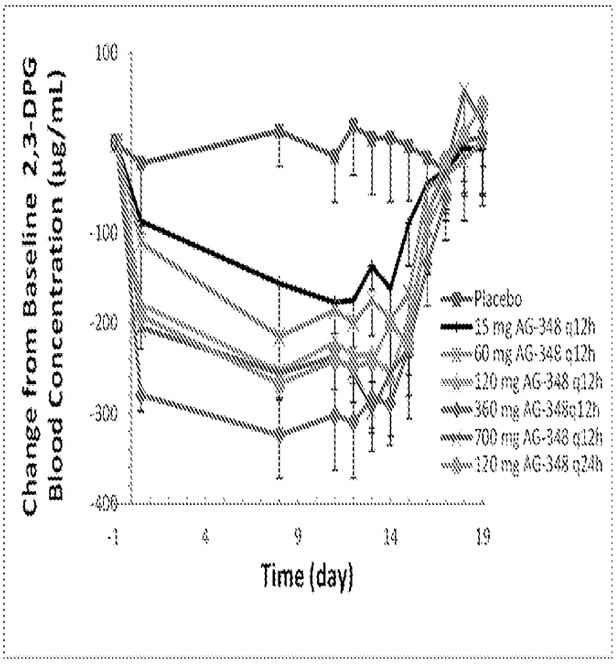

**L ' AG-348 (jaune ) se lie à l'enzyme ( interface dimère/ dimère)**

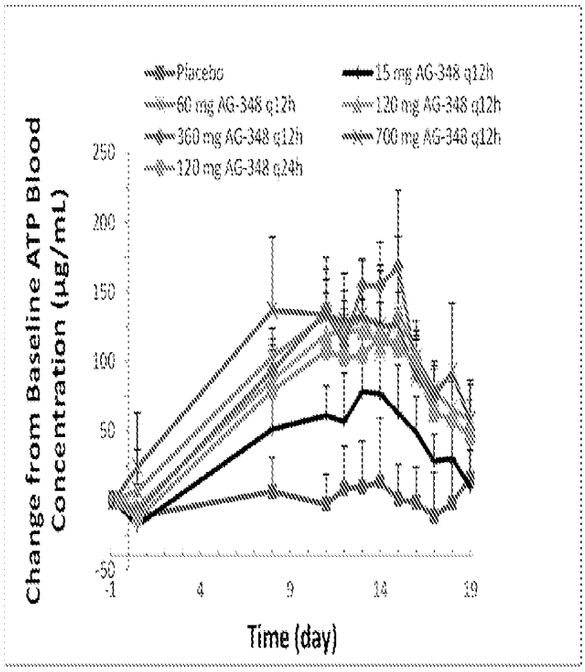
C Kung, *Blood* 2014 124:4010



Ex. 2001-1058

# Variations 2 3 DPG et ATP sanguins selon doses AG-348 ( escalades de doses): sujets volontaires sains




- Efficacité
- Tolérance

Yang H et al. *Blood* 2014;124[21]:4007; Yang H et al. *Haematologica* 2015;100[s1]:Abs S138)



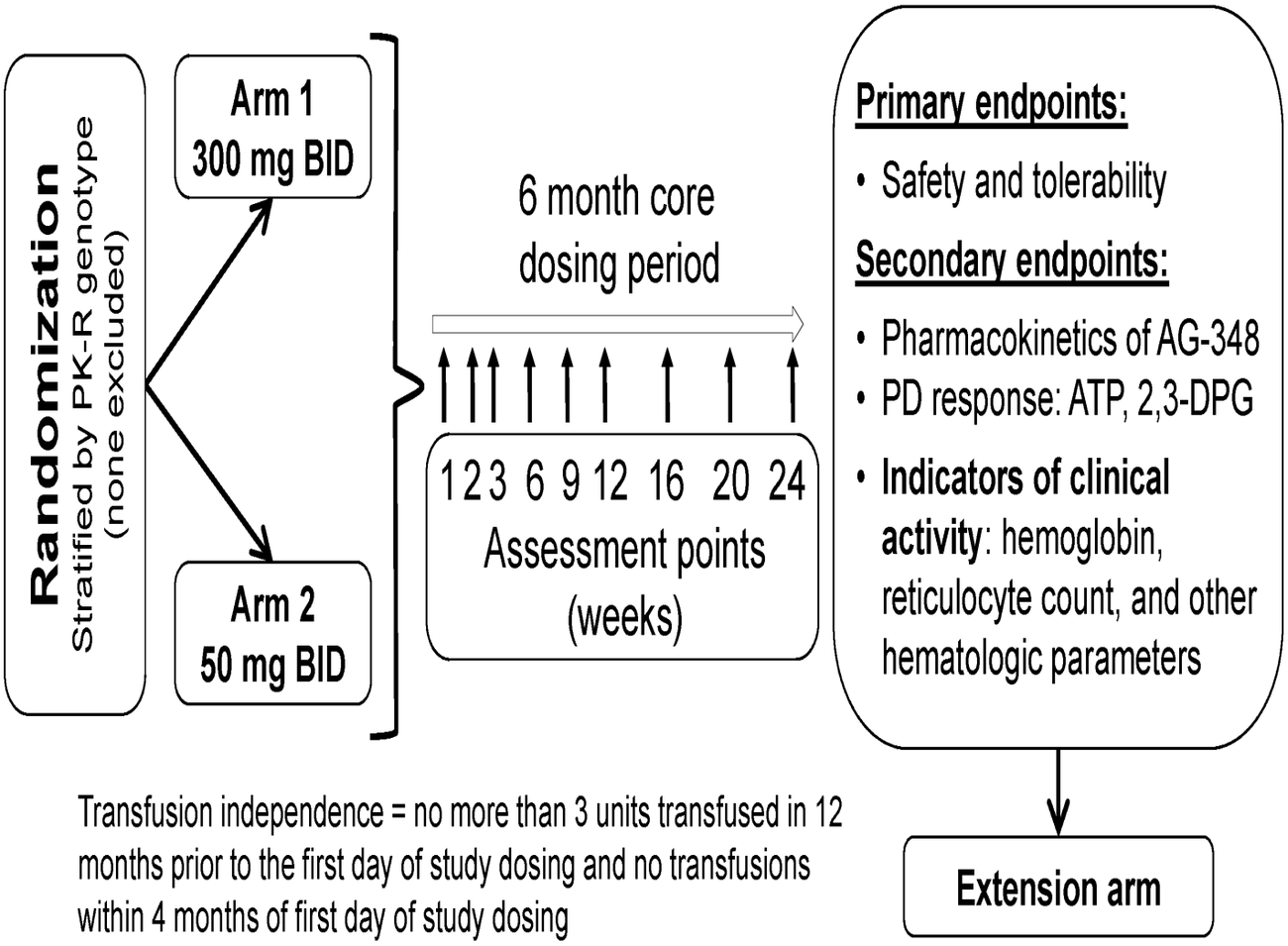
Ex. 2001-1059

# Design de l'étude

**Etude ouverte de phase 2 : 14 centres US, Canada, UE**

Transfusion-independent adults with PK deficiency

(ClinicalTrials.gov NCT02476916) n=25 in each arm




Transfusion independence = no more than 3 units transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

All patients provided written informed consent. BID = twice daily; PD = pharmacodynamic



Ex. 2001-1060

# Demographics

| Characteristics[a] | 50 mg BID, n=27 | 300 mg BID, n=25 | Total, N=52 |
|---|---|---|---|
| Men, n (%) | 18 (66.7) | 14 (56.0) | 32 (61.5) |
| Age at randomization in years, median (range) | 29 (18, 58) | 40 (21, 62) | 34 (18, 62) |
| Race white[b], n (%) | 22 (81.5) | 20 (80.0) | 42 (80.8) |
| Hemoglobin (Hb) baseline, median (range) | 9.6 (6.9, 12.3) | 8.6 (6.5, 12.0) | 8.9 (6.5, 12.3) |
| Duration of treatment, weeks, median (range) | 23.0 (13.0, 76.9) | 26.3 (12.9, 70.9) | 24.9 (12.9,76.9) |
| Splenectomized, n (%) | 23 (85.2) | 20 (80.0) | 43 (82.7) |
| Iron chelation prior to enrolment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (76.0) | 38 (73.1) |

[a]Data cut-off March 27, 2017; [b]Not reported in 3 patients, 3 patients were Asian, and 4 were "other"



Ex. 2001-1061

# Safety summary

- AG-348 was generally well tolerated
- The majority of adverse events (AEs) were grade 1–2
- Treatment related AEs leading to discontinuation, n=3
  - Chest discomfort/pleural effusion, pharyngitis/nausea, anemia
- 13 serious AEs in 10 patients
  - Six drug-related SAEs in 5 patients: Withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia; pharyngitis
    - Grade 4 hypertriglyceridemia at Week 24, resolved upon AG-348 discontinuation (Grade 1 at baseline)

| Patient disposition by study period | Ongoing, N | Completed, N | Discontinued[a], N |
|---|---|---|---|
| Core | 15 | 29[b] | 8 |
| Extension | 21 | 0 | 3 |

[a]Reasons for discontinuation: AEs (3), investigator decision (4), and withdrew consent (4)

[b]Five subjects completing the Core period did not enter the Extension



Ex. 2001-1062

# Safety summary: Most common AEs

| AEs, regardless of causality (occurring in >5 patients) | 50 mg BID n=27 | | 300 mg BID n=25 | | Total N=52 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n (%) | 25 (92.6) | 7 (25.9) | 25 (100.0) | 5 (20.0) | 50 (96.2) | 12 (23.1) |
| Headache | 9 (33.3) | 0 | 14 (56.0) | 0 | 23 (44.2) | 0 |
| Insomnia | 5 (18.5) | 1 (3.7) | 15 (60.0) | 1 (4.0) | 20 (38.5) | 2 (3.8) |
| Nausea | 10 (37.0) | 0 | 9 (36.0) | 0 | 19 (36.5) | 0 |
| Viral upper respiratory tract infection | 7 (25.9) | 0 | 2 (8.0) | 0 | 9 (17.3) | 0 |
| Arthralgia | 5 (18.5) | 0 | 3 (12.0) | 0 | 8 (15.4) | 0 |
| Fatigue | 4 (14.8) | 0 | 4 (16.0) | 0 | 8 (15.4) | 0 |
| Back pain | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Cough | 4 (14.8) | 0 | 3 (12.0) | 0 | 7 (13.5) | 0 |
| Dizziness | 4 (14.8) | 0 | 3 (12.0) | 1 (4.0) | 7 (13.5) | 1 (1.9) |
| Vomiting | 3 (11.1) | 0 | 4 (16.0) | 0 | 7 (13.5) | 0 |
| Hot flush | 1 (3.7) | 0 | 6 (24.0) | 0 | 7 (13.5) | 0 |
| Diarrhea | 3 (11.1) | 0 | 3 (12.0) | 0 | 6 (11.5) | 0 |
| Influenza | 5 (18.5) | 1 (3.7) | 1 (4.0) | 0 | 6 (11.5) | 1 (1.9) |

Grade 3 AEs not reported in previous slide or table above: colitis (n=1), thrombosis (n=1), pharyngitis (n=1), post procedural hemorrhage (n=1), hypertension (n=1)

AEs graded using National Cancer Institute Common Terminology Criteria, version 4.03



Ex. 2001-1063

# Dose titration in DRIVE PK

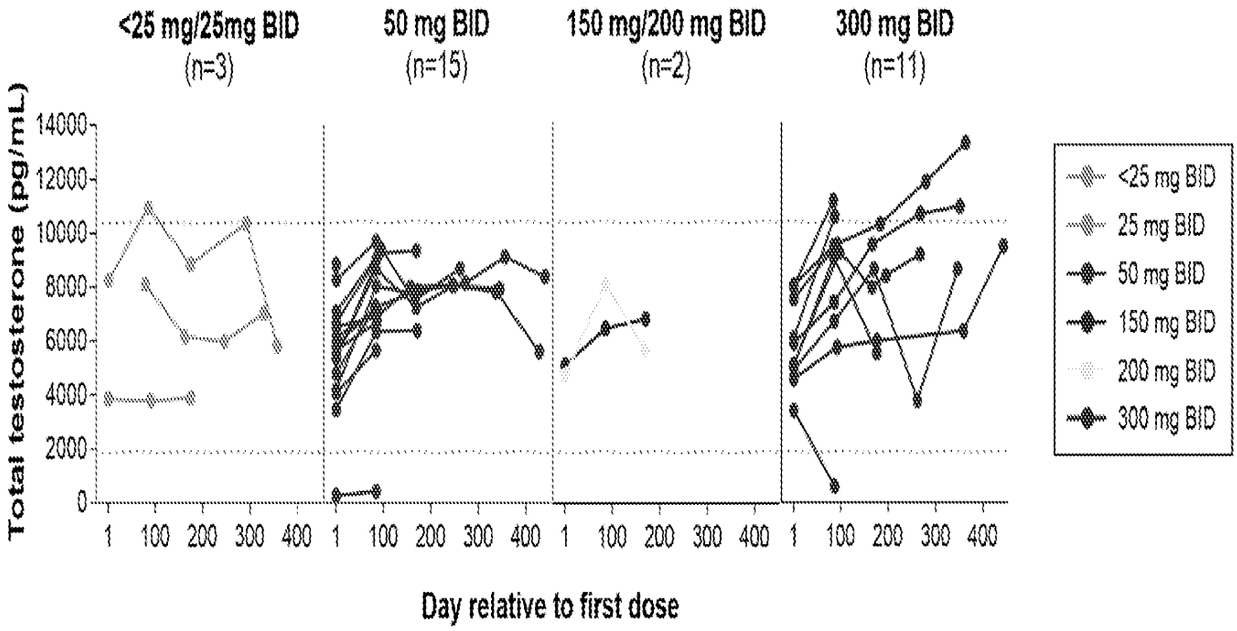
- Patients randomized to 50 mg or 300 mg BID AG-348 starting dose
  - Dose decreased:
    - AEs (e.g. insomnia)
    - Hb exceeding midpoint of normal range (Male: Hb >15.0 g/dL; Female>13.5 g/dL)
  - Dose increased:
    - Lack of Hb response
- Range of doses (5 mg QD–300 mg BID) used
- Efficacy and steroid hormone levels analyzed by the dose received for the longest duration in the Core period



Ex. 2001-1064

# Effect of AG-348 on hormones: total testosterone in men

- Preliminary findings are consistent with aromatase inhibition by AG-348 across multiple dose levels in men
  - Most testosterone values remained within the normal range
  - DEXA scan data inconclusive
  - Longer follow up required to assess clinical impact




Normal reference low and high limits shown as horizontal dotted lines; DEXA = dual energy X-ray absorptiometry

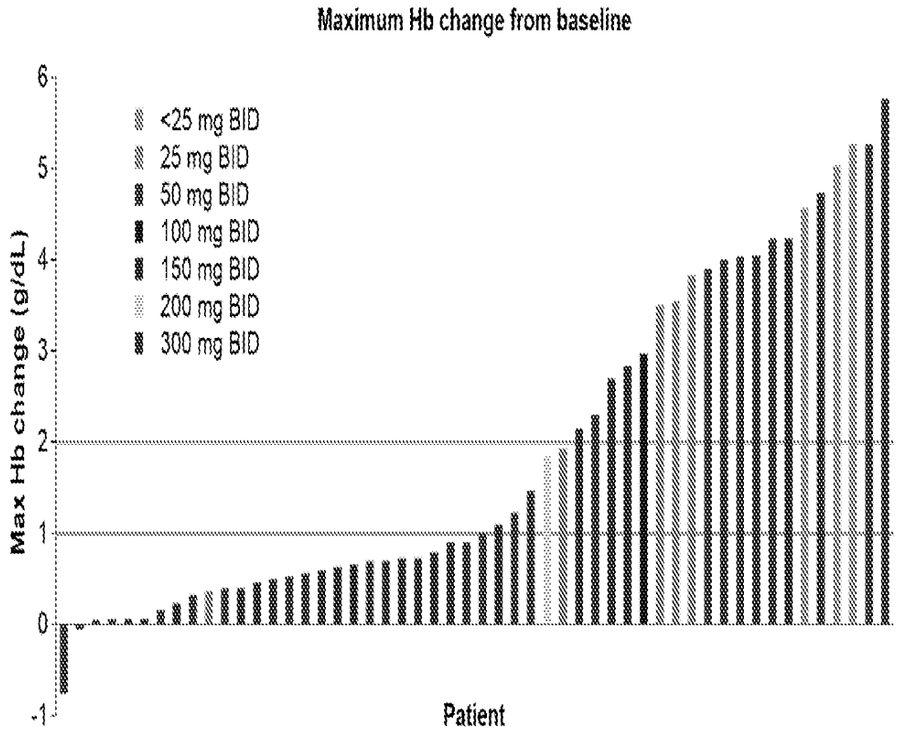


Ex. 2001-1065

# Clinical activity results

# Maximum Hb increase observed during the Core period

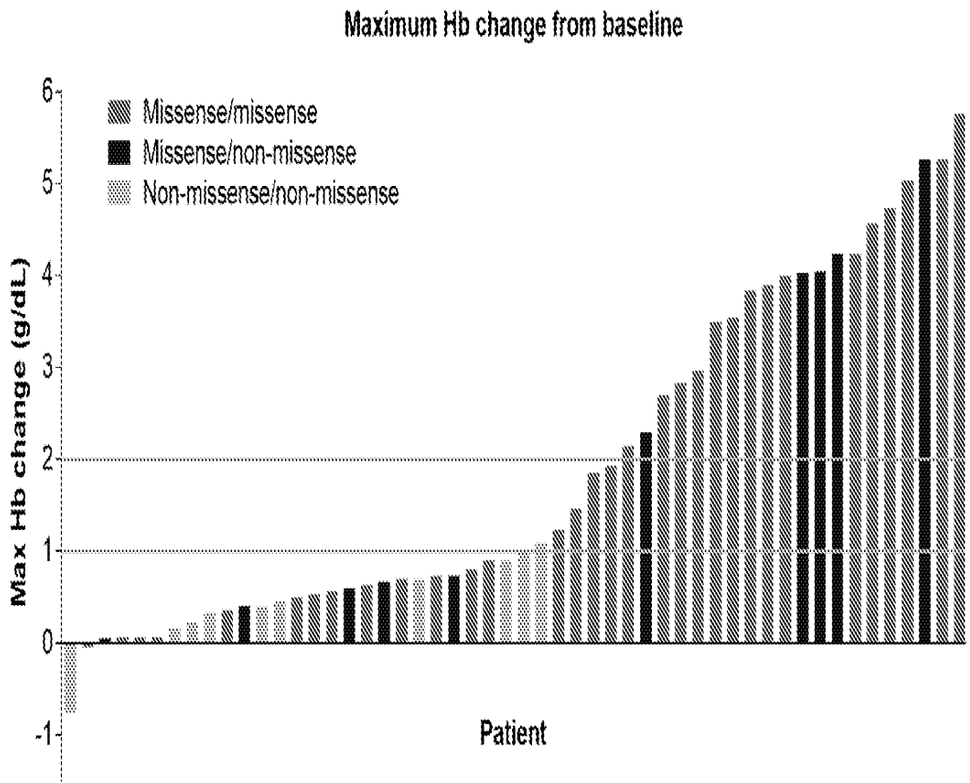
- 25/52 (48%) patients had a maximum Hb increase of >1.0 g/dL
  - The mean maximum increase was 3.5 g/dL (range 1.1-5.8 g/dL)




The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)

# Maximum Hb increase observed by genotype

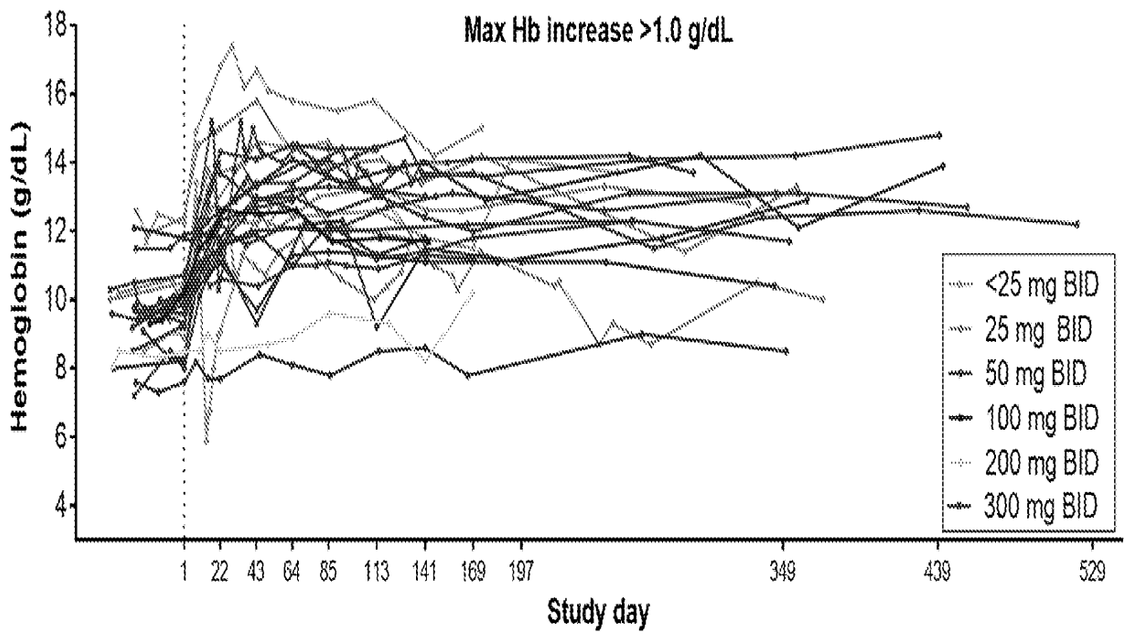
- 25/52 (48%) patients had a max increase in Hb >1.0 g/dL
  - 24/42 (57%) patients who had ≥1 missense mutation had Hb increase >1.0 g/dL




The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)

# Majority of Hb increases are rapid and sustained

- Median time to the first observation of a Hb increase >1.0 g/dL above baseline was 10 days (range 7–141 days)
  - Median baseline Hb in subjects who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.5–12.3 g/dL) vs. 8.0 g/dL (range 6.5–10.1) in subjects who did not
- In 8 patients, the dose had to be held or reduced due to rapid rise in Hb

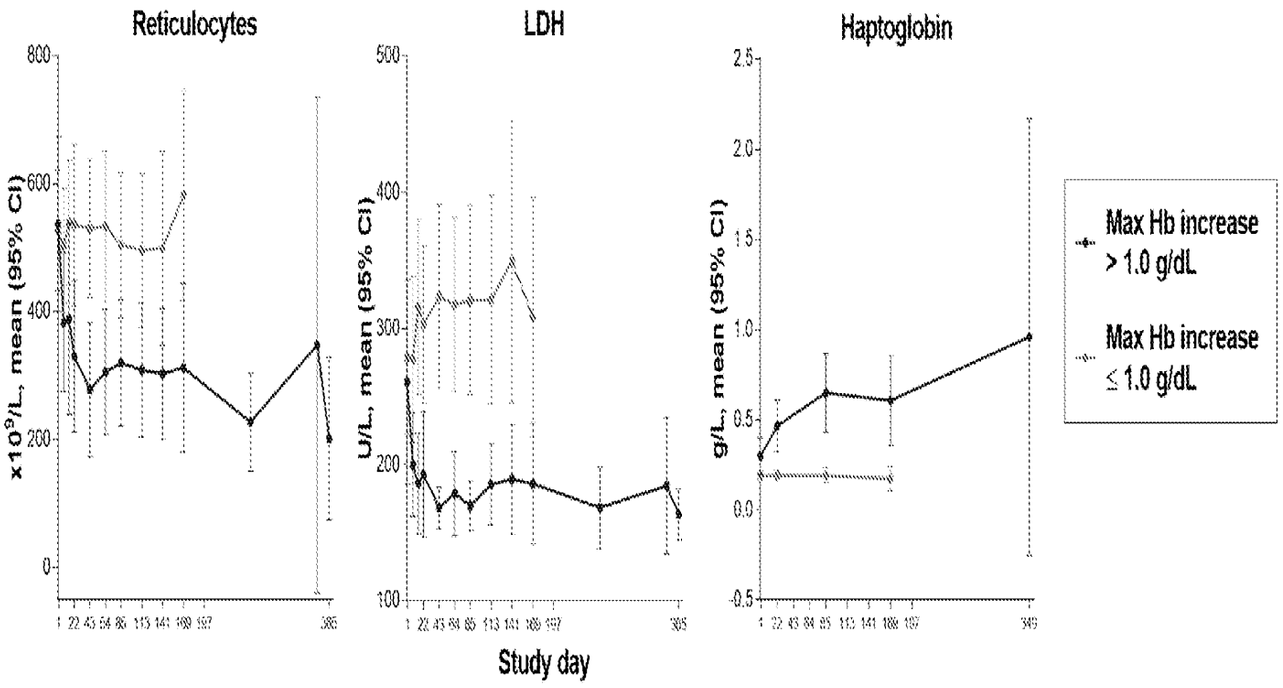





Ex. 2001-1069

# Hemolysis markers improve in patients with Hb increase of >1.0 g/dL

- Rapid decreases in reticulocytes and LDH in the first weeks of dosing
- Steady increase in haptoglobin



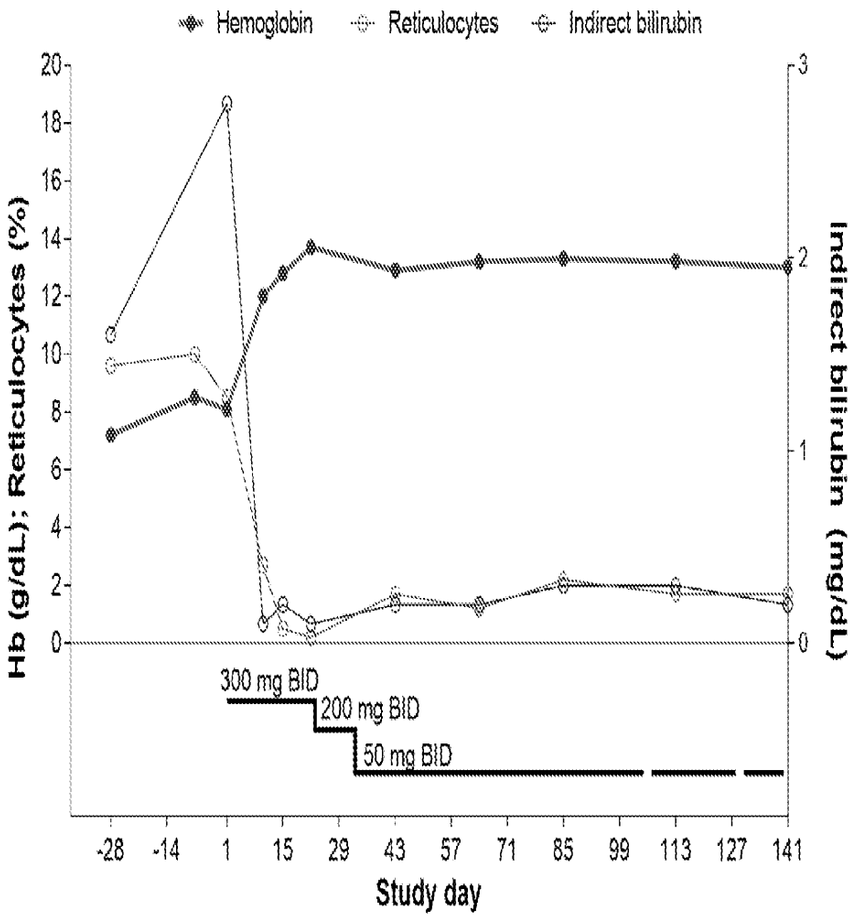

Only visits with ≥5 subjects included. Figure shows central laboratory results. LDH = lactate dehydrogenase



Ex. 2001-1070

# Hb response with improvements in hemolysis parameters: Single subject experience with AG-348

- 35 yo female, white, missense/missense genotype (T384M/R479H)






Ex. 2001-1071

# DRIVE-PK Conclusions

- L'AG-348 est une nouvelle molécule, première dans sa catégorie , capable d'activer l'enzyme de la pyruvate kinase chez les patients déficients
- Une dose quotidienne jusque 6 mois est bien tolérée
  - La signification de l'effet anti aromatase n'est pas précis à ce jour
  - Une hypertriglycéridémie de grade 4
- AG-438 est capable d'augmenter l'hémoglobine de manière rapide et durable et significative (>1g/dl) dans 48% des cas.
  - L'augmentation est liée à une diminution de l'hémolyse
  - Corrélation préliminaires entre réponse et génotype
- Etudes phase III



Ex. 2001-1072

# Futurs Essais

## AG-348 Pivotal Program Design

| Design Element | Non-Transfusion Dependent | Regularly Transfused |
|---|---|---|
| **Patient Population** | • Non-transfusion dependent adults<br>• Baseline hemoglobin of ≤10.0 grams per deciliter<br>• Excludes patients with two non-missense mutations and those homozygous for R479H | • Regularly transfused adults<br>• Excludes patients with two non-missense mutations and those homozygous for R479H |
| **Size** | • 80-100 patients | • ~20 patients |
| **Dose** | • Dose titration up to optimal hemoglobin response (5, 20 or 50 mg twice daily) | • Dose titration up to optimal hemoglobin response (5, 20 or 50 mg twice daily) |
| **Endpoints** | • Hemoglobin response<br>• Patient-reported outcomes (PRO) | • Reduction in transfusion burden<br>• Patient-reported outcomes (PRO) |
| **Control** | • Placebo controlled | • Reduction of transfusion burden compared to patients' baseline |



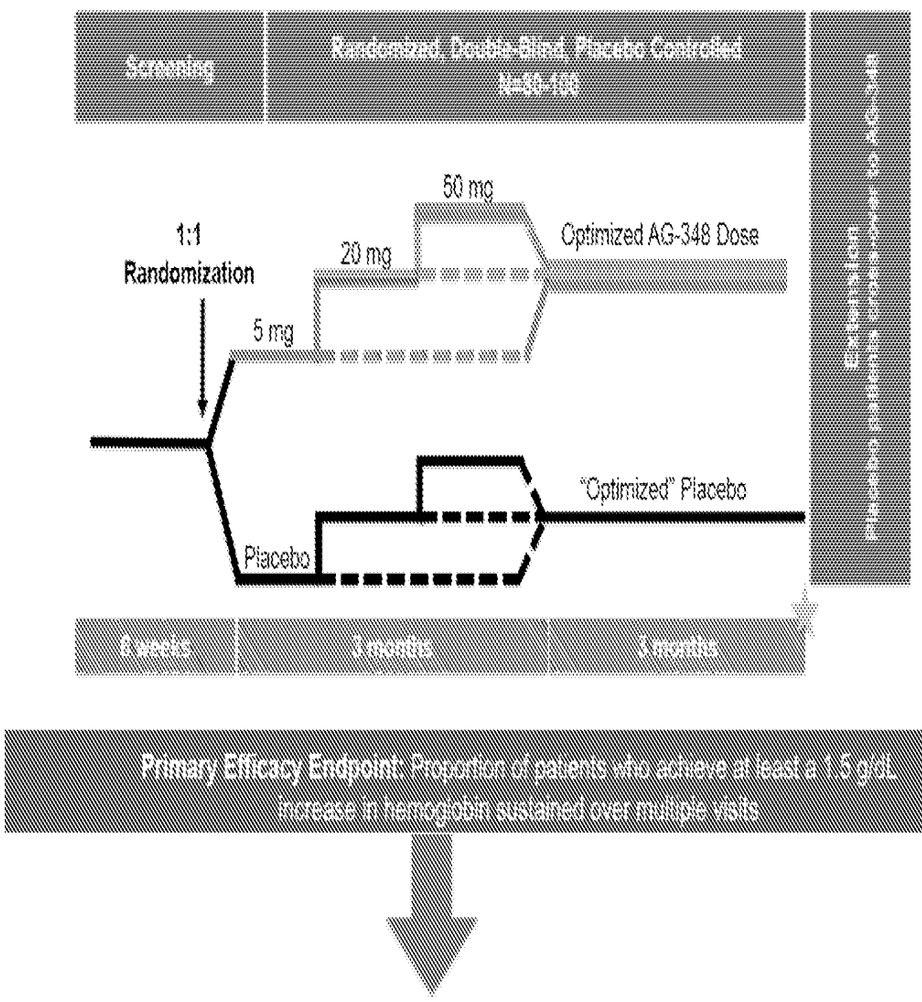
**Début 2018**



Ex. 2001-1073

# Essais: Non transfusion dépendant

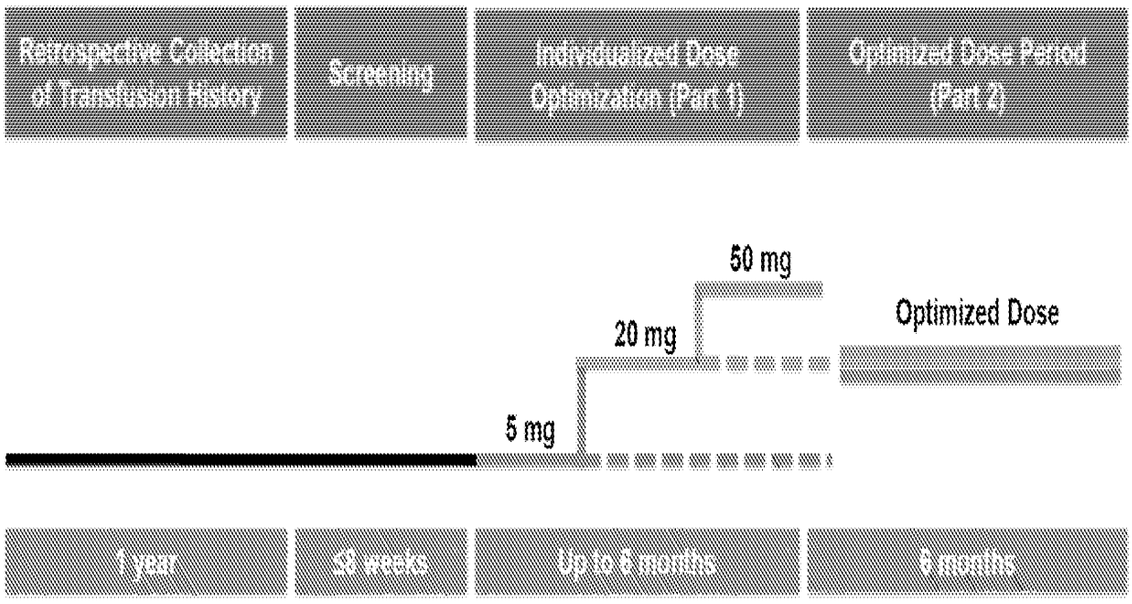
- **Adultes**
- **80 à 100 pts**
  - **Avoir au moins une faux sens Exclu R479H**
- **Cross over**




**Début 2018**

# Essais: transfusion dépendant

## Trial Design for Regularly Transfused Patients




Approximately 20 regularly transfused patients who have required a minimum of 6 transfusions over the year preceding enrollment

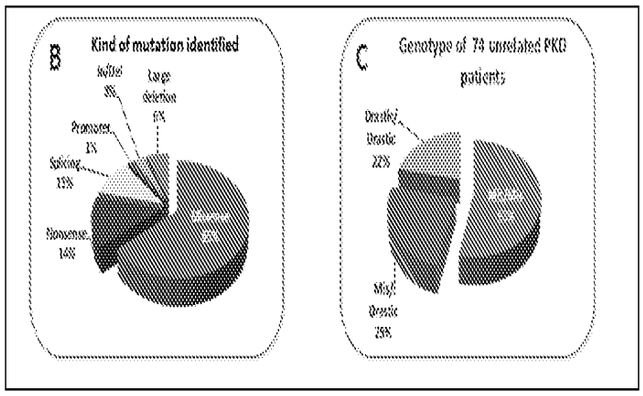
**Primary Endpoint:** Reduction in transfusion burden over a 6 month period compared to the patient's transfusion history



Ex. 2001-1075

# Registre - Autres traitements

- Registre code PK
  - Comment conclure ?

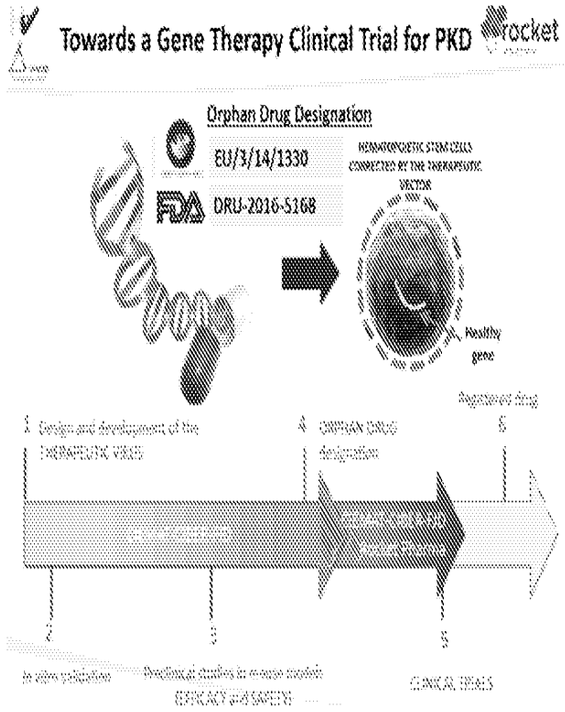



Projet Registre international

*Bianchi P, ASH . 2015.*

- Allogreffe

- Thérapie génique



# Conclusions

- L' étude Drive PK  a montrer que l' AG-438  est capable d'augmenter l'hémoglobine de manière rapide et durable et significative (>1g/dl) chez près d'un malade sur 2 en lien avec une diminution de l'hémolyse et plus que la dose le  génotype est le facteur principal d e réponse  ( actualisation à l'ASH)
- La tolérance est  satisfaisante ( effet anti aromatase à surveiller)
- Potentiel et l'intérêt des agents activateurs de l'enzyme comme traitement modifiant l'évolution de la maladie autorisant l'initiation d' études randomisées  d'enregistrement pour patients transfusion dépendant et non transfusion dépendant .
- Nécessité de poursuite registre nationaux et internationaux maladies Rares



Ex. 2001-1077

# Remerciements


Patients qui ont accepté de participer

- Tous les cliniciens Code PK Serge Pissard , filière MCGRE, Club Du Globule Rouge et du Fer
- ( Serge Pissard , L Garcon, M Micheau, L Pascal)
- Sites Participants :
  - **Hôpital Henri Mondor, Creteil ; Fréderic Galacteros**
  - **Hôpital Saint Vincent de Paul, Lille ; Christian Rose**
  - **Hôpital de la Timone; Marseille , Emmanuelle Bernit**
  - Hammersmith Hospital; Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
  - Boston Children's Hospital; Rachael Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet Kwiatkowski
  - Central Pennsylvania Clinic; Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin Kuo

- **Agios Pharmaceuticals**




Ex. 2001-1078

# Efficacy of a pyruvate kinase activator (AG-348) on anemia and hemolysis parameters during a pyruvate kinase deficiency: results of the DRIVE PK study

C. Rose (1); K. Debbache (2); M. Layton (3); E. Van Beers (4); W. Barcellini (5);
E. Bernit (6); R. Grace (7); B. Glader (8); C. Kung (9); H. Xu (10);
A. Barbier (11); F. Galacteros (12)
(1) Onco hématologie, Université Catholique de Lille, Hop St Vincent de Paul, Lille; (2) Red blood cell genetic diseases unit, CHU Henri Mondor, Créteil; (3) Hammersmith Hospital, Imperial Healthcare Trust, London, United Kingdom; (4) Hematology, University Medical Center Utrecht, Utrecht, Netherlands; (5) Hematology, Fondazione IRCCS Ca' Granda Ospedale
Maggiore Policlinico, Milan, Italy; (6) Service de médecine interne, Hôpital de la Timone, Marseille;
(7) Hematology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, United States; (8) Hematology, Stanford Medical School, Stanford, United States; (9) Biochemistry, Agios Pharmaceuticals, Cambridge, United States; (10) Biochemistry, Agios Pharmaceuticals, Cambridge, United States; (11) Medical, Agios Pharmaceuticals, Cambridge, United States; (12) Centre de référence des syndromes drépanocytaires majeurs, hôpital henri mondor, ap-hp; upec, IMRB-U955, Equipe 2 émergente: Transfusion and red blood cell diseases, Institut Mondor, Créteil

Number: **000189**
Presenter: **C. Rose**
Structure: **Structure 1**
Subject: **Red Blood Cells and Iron**
List of key words:
- Red blood cells and Iron > Hemolytic anemia

Young Researchers Prize **No**
Conflict of interest: **no**
Transfer of rights commitment
Updated on: **Thursday, January 5, 2017 08:45**

## Introduction

Pyruvate kinase (PK) deficiency is responsible for congenital hemolytic anemia of variable severity due to an enzyme deficiency of the glycolytic pathway. AG-348 is a small molecule available by oral pathway that is an allosteric activator of the enzyme. In vitro, AG-438 drives a 2-3-DPG decrease and an increase in ATP in healthy volunteers and has demonstrated, ex vivo, that it is capable of activating and protecting a wide variety of mutated enzymes. We present the results of an international study of doses of AG-438 (NCT02476916) in patients affected by PK deficiency.

## Patients and methods

The inclusion criteria were: patients with confirmed PK deficiency at the molecular level who were non-transfusion dependent (defined as fewer than 3 erythrocyte concentrates in the year preceding inclusion and none in the preceding 4 weeks). After consent, patients were randomized to receive either 50 mg or 300 mg, 2 times per day by mouth for 6 months. Evaluations were conducted weekly for the first 3 weeks, then every 3 weeks until the 3rd month, then monthly.

## Results

As of this date, the forecast 52 patients were included, but only 34 could be evaluated at this time for tolerance, and 32 for efficacy. Tolerance: the most frequent secondary effects were: headaches n = 15, nausea n = 14, insomnia n = 13. 2 serious side effects; grade 2 osteoporosis n = 1 and worsened anemia and hemolysis after suddenly discontinuing the drug in one patient who had a rapid and significant response on hemoglobin; he was later able to resume treatment. No grade 4, nor deaths, were reported. Only 4 patients interrupted the treatment: one due to moving and 3 due to secondary effects, including one case of worsened anemia. The serum sex hormone levels were measured at baseline, 12 and 24 weeks: an increase in free and total testosterone, as well as a decrease in estradiol and estrone were noted, confirming a previously known anti-aromatase action by AG-348 . In 32 patients who received more than 3 weeks of treatment; 15 patients (47%) had an increase in hemoglobin level (Hb) > 1.0 g/dL (range: 1.2–5.2 g/dL; median: 3.6 g/dL). Hb responses were observed as early as 2 weeks post-treatment and continued during treatment. Of the 32 patients who received more than 3 weeks of treatment: 6 had 2 nonsense mutations and none of them had an increase in Hb > 1.0 g/dL. Conversely, among the 26 patients with at least one missense mutation, 15 (58%) had an increase in Hb > 1.0 g/dL

## Conclusion

AG-348 is a novel first-in-class molecule, capable of activating the PK enzyme in PK deficient patients. AG-438 is well tolerated and has demonstrated efficacy; it is able to significantly increase hemoglobin (>1g/dl) in close to 60% of patients with a missense mutation. These data illustrate the potential and interest of enzyme-activating agents as a treatment that modifies the course of the disease in patients with PK deficiency.

## Efficacité d'un activateur de la pyruvate kinase (AG -348) sur l'anémie et les paramètres d'hémolyse au cours du déficit en pyruvate kinase : résultats de l'étude DRIVE PK

C. Rose (1) ; K. Debbache (2) ; M. Layton (3) ; E. Van Beers (4) ; W. Barcellini (5) ; E. Bernit (6) ; R. Grace (7) ; B. Glader (8) ; C. Kung (9) ; H. Xu (10) ; A. Barbier (11) ; F. Galactéros (12)
(1) Onco hématologie, Université Catholique de Lille,Hop St Vincent de Paul, Lille; (2) 2unité des maladies génétiques du globule rouge, CHU Henri Mondor, Créteil; (3) Hammersmith hospital, Imperial Healthcare Trust, Londres, Royaume Uni; (4) Hematology, University Medical Center Utrecht, Utrecht, Pays-Bas; (5) Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italie; (6) Service de médecine interne, Hôpital de la Timone, Marseille; (7) Hematology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, États-Unis; (8) Hematology, Stanford Medical School, Stanford, États-Unis; (9) Biochemistry, Agios Pharmaceuticals, Cambridge, États-Unis; (10) Biochemstry, Agios Pharmaceuticals, Cambridge, États-Unis; (11) Medical, Agios Pharmaceuticals, Cambridge, États-Unis; (12) Centre de référence des syndromes drépanocytaires majeurs, hôpital henri mondor, ap–hp; upec, IMRB-U955, Equipe 2 émergente: Transfusion et maladies du globule rouge, Institut Mondor, Créteil

Numéro : **000189**
Orateur : **C. Rose**
Structure : **Structure 1**
Thème : **Globules rouges et Fer**
Liste de mots-clés :
- Globules rouges et Fer > Anémie hémolytique

Prix Jeunes Chercheurs : **Non**
Conflit d'intêret : **non**
☑ Engagement de cession de droits
Mis à jour le : **jeudi 5 janvier 2017 08:45**

**Introduction**
Le déficit en pyruvate kinase (PK) est responsable d'une anémie hémolytique congénitale de sévérité variable dû à un déficit de l'enzyme de la voie glycolytique. L'AG-348 est une petite molécule disponible par voie orale qui est un activateur allostérique de l'enzyme. In vitro, l'AG-438 entraine une diminution 2-3-DPG et une augmentation de l'ATP chez les volontaires sains et a démontré ex vivo qu'elle est capable d'activer et de protéger une large variété d'enzymes mutées. Nous présentons les résultats d'une étude internationale de dose de l'AG-438 (NCT02476916) chez les patients atteints de déficit en PK.

**Patients et méthodes**
Les critères d'inclusion étaient : patients atteints de déficit en PK confirmé sur le plan moléculaire et non transfusion dépendant (défini par moins de 3 concentrés érythrocytaires dans l'année précédant l'inclusion et aucun dans les 4 semaines précédant). Après consentement, les patients étaient randomisés pour recevoir soit 50 mg ou 300 mg, 2 fois par jour per os pour 6 mois. L'évaluation était hebdomadaire les 3 premières semaines puis toutes les 3 semaines jusqu'au 3eme mois puis mensuelle.

**Résultats**
A ce jour les 52 patients prévus ont été inclus mais seul 34 sont pour le moment évaluables pour la tolérance et 32 pour l'efficacité. Tolérance : les effets secondaires les plus fréquents étaient : céphalées n=15, nausée n=14, insomnie n=13. 2 effets secondaires sérieux ;ostéoporose de grade 2 n=1 et une aggravation de l'anémie et de l'hémolyse après arrêt brutal de la drogue chez un patient qui a eu une réponse rapide et importante sur l'hémoglobine , il a pu reprendre le traitement ensuite . Aucun grade 4 ni décès n'étaient rapportés. Seul 4 patients ont interrompus le traitement : un pour cause de déménagement et 3 pour effets secondaires dont un cas pour aggravation de l'anémie. Le taux sérique des hormones sexuelles étaient mesurées à l'inclusion, 12 et 24 semaines : une augmentation de la testostérone libre et totale ainsi qu'une diminution de l'estradiol et de l'estrone étaient signales confirmant une action anti aromatase par l'AG-348 précédemment connue. Chez 32 patients ayant reçus plus de 3 semaines de traitement ; 15 patients (47%) avaient une augmentation du taux d' hémoglobine (Hb) >1.0 g/dL (range: 1.2–5.2 g/dL; médiane: 3.6 g/dL). Les réponses sur l'Hb étaient observées dès les 2 semaines du traitement et se poursuivaient sous traitement. Sur les 32 patients ayant reçus plus de 3 semaines de traitement: 6 avaient 2 mutations non-sens et aucun d' eux n' a eu une augmentation de l' Hb >1.0 g/dL. A l'inverse, parmi les 26 patients avec au moins une mutation *missens*, 15 (58%) ont eu une augmentation de l'Hb > 1.0 g/dL.

**Conclusion**
L'AG-348 est une nouvelle molécule, première dans sa catégorie , capable d'activer l'enzyme de la PK chez les patients déficients en PK . L'AG-438 est bien tolérée et a démontré une efficacité ; elle est capable d'augmenter l'hémoglobine de manière significative (>1g/dl) chez près de 60% des patients ayant une mutation *missens*. Ces données illustrent le potentiel et l'intérêt des agents activateurs de l'enzyme comme traitement modifiant l'évolution de la maladie chez les patients porteurs d'un déficit en PK.

# Efficacy of a pyruvate kinase activator (AG-348) on anemia and hemolysis parameters during pyruvate kinase deficiency: results of the DRIVE PK study

Christian Rose[1], Karima Debbache[2], D Mark Layton[3], Eduard Van Beers[4], Wilma Barcellini[5], Emmanuelle Bernit[6], Rachael F Grace[7], Bertil Glader[8], Charles Kung[9], Ann J Barbier[9], Frédéric Galacteros[10]

[1]Hôpital Saint Vincent de Paul, Université Catholique, Lille, France; [2]Red Blood Cell Genetic Diseases Unit, CHU Henri Mondor, Créteil, France; [3]Hammersmith Hospital, Imperial College Healthcare, United Kingdom; [4]Universitair Medisch Centrum, Utrecht, Netherlands; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [6]Hôpital de la Timone, Marseille, France; [7]Dana-Farber Boston Children's Cancer and Blood Disorders Center, Boston, USA; [8]Stanford University School of Medicine, Palo Alto, CA; [9]Agios Pharmaceuticals, Inc., Cambridge, MA

SFH 2017

Ex. 2001-1081

# Pyruvate Kinase Deficiency

**Description**

- Severe Congenital hemolytic anemia
- Neonatal +- late
- Prevalence [1 - 4]

**Etiology**

- PKLR gene mutations coding for erythrocyte pyruvate kinase (PK-R)

**Complications**

- Chronic anemia
- Jaundice, iron overload
- Post-splenectomy risks

**Diagnosis / Treatment**

- Enzyme activity and/or genetic analysis
- Supportive care: transfusions, splenectomy, iron chelation

**Study F: code PK**

**70 patients**

1st month diagnosis n = 41

Fatal n = 2

Median age = 21 years

- **severe (n = 40):** Hb below 8 g/dL,
- **Moderate (n = 20):** Hb between 8 and 10 g/dL,
- **Mild (n = 10):** Hb greater than 10 g/dL,

Transfusion dependent 1/3

Splenectomy n = 44

Chelates 1/3

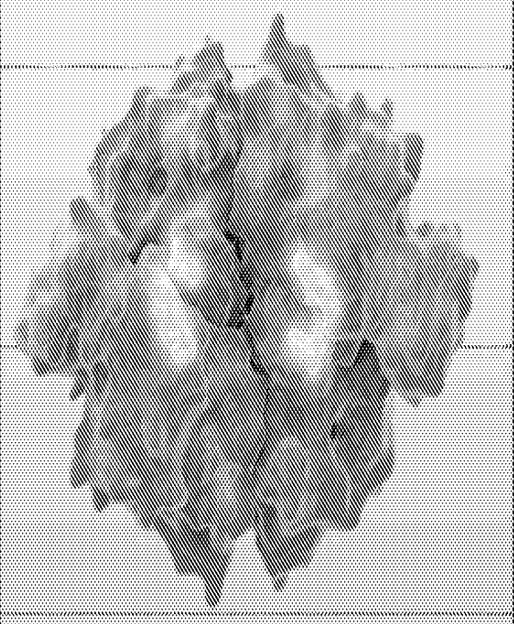
Grace R et al. *Am J Hematol* 2015;90(8):826-30, [2] Mohrenweiser HW *PNAS* 1981;78(6):3546-50, [3] Carey PJ et al. *Blood* 2000;96(12):4005-6, [4] Beutler E & Gelbart T *Blood* 2000;95(11):3585-8, [5] deMeftes et al. *Hum Hered* 1992;42(3):179-83



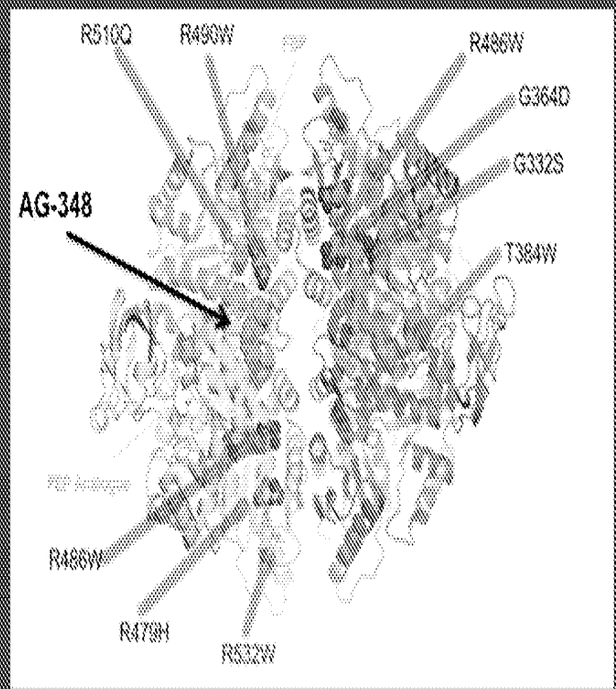
Ex. 2001-1082

# AG-348: an allosteric activator of pyruvate kinase (mutant/wild)

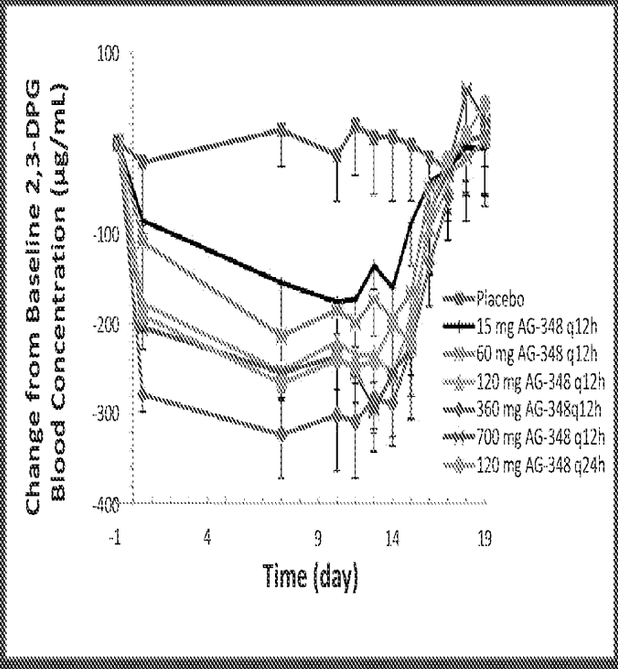
Pyruvate kinase is a tetramer; Mutations (green) reduce catalytic activity

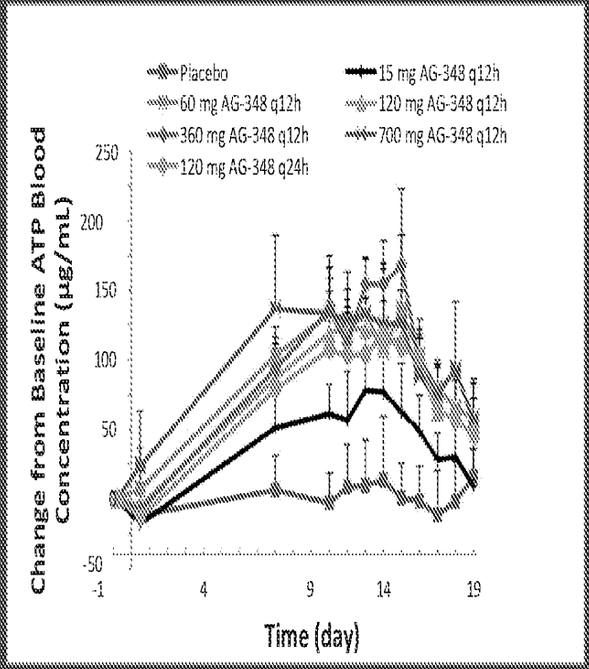


AG-348 (yellow) binds to the enzyme (dimer/dimer interface)



# Variations 2,3-DPG and blood ATP according to AG-348 doses (dose escalations): healthy volunteer subjects




- Efficacy
- Tolerance



Ex. 2001-1084

# Study design

DRIVEPK

**Open phase 2 study: 14 centers in the US, Canada, EU**

**Transfusion-independent adults with PK deficiency**

(ClinicalTrials.gov NCT02476916) n=25 in each arm

**Randomization**
Stratified by PK-R genotype
(none excluded)

**Arm 1**
**300 mg BID**

**Arm 2**
**50 mg BID**

6-month core dosing period

1 2 3 6 9 12 16 20 24
Assessment points (weeks)

**Primary endpoints:**

- Safety and tolerability

**Secondary endpoints:**

- Pharmacokinetics of AG-348
- PD response: ATP, 2,3-DPG
- **Indicators of clinical activity**: hemoglobin, reticulocyte count, and other hematologic parameters

**Extension arm**

Transfusion independence = no more than 3 units transfused in 12 months prior to the first day of study dosing and no transfusions within 4 months of first day of study dosing

All patients provided written informed consent. BID = twice daily; PD = pharmacodynamic

# Population

- Study initiated June 2015; All patient enrolled n=52, data cut-off September 23, 2016
- Evaluable analysis set: ≥3 weeks of data (n=32)
- Safety analysis set: received at least 1 dose of AG-348 (n=34)
- 13 patients ongoing in the core period (as of September 23, 2016)
  - Early discontinuations in the core period due to: relocation (n=1), AEs (n=3)
- Of the 17 patients who completed the core period, 15 enrolled in the extension period
- 1 patient discontinued in extension period due to physician decision (lack of efficacy)

| Characteristics | 50 mg BID, n=17 | 300 mg BID, n=17 | Total, N=34 |
|---|---|---|---|
| Men/women, n | 11/6 | 9/8 | 20/14 |
| Age in years, mean (range) | 28.5 (19-45) | 37.0 (20-61) | 32.8 (19-61) |
| Race=white, n | 15 | 15 | 30 |
| Hemoglobin (Hb) baseline, g/dL, mean (SD, range) | 9.8 (1.41, 7.6–12.4) | 8.7 (1.37, 6.5–11.8) | 9.2 (1.47, 6.5–12.4) |
| Duration of treatment, weeks, median (range) | 24.7 (4.7–50.4) | 24.0 (2.4–44.4) | 24.4 (2.4–50.4) |
| Splenectomized, n | 14 | 14 | 28 |

Not reported in 2 patients. 2 patients were Asian. AE = adverse event. SD = standard deviation



Ex. 2001-1086

# Safety and Tolerance

- AG-348 was generally well tolerated; the majority of AEs were grade 1–2
  - No grade 4 AEs or deaths
  - 2 patients experienced serious AEs: Grade 2 osteoporosis; hemolysis and anemia due to discontinuation of the drug after a rapid Hb response (patient continued in the study)
  - 3 patients discontinued treatment due to AEs
  - DXA scan data (n=17) show high variability and are inconclusive

| AEs, regardless of causality (occurring in ≥5 patients or assessed as Grade ≥3) | 50 mg BID n=13 | | 300 mg BID n=17 | | Total N=34 | |
|---|---|---|---|---|---|---|
| | All grades | Grade ≥3 | All grades | Grade ≥3 | All grades | Grade ≥3 |
| Patients experiencing at least 1 AE, n | 13 | 2 | 17 | 6 | 30 | 8 |
| Headache | 7 | 0 | 8 | 0 | 15 | 0 |
| Nausea | 7 | 0 | 7 | 0 | 14 | 0 |
| Insomnia | 3 | 1 | 10 | 1 | 13 | 2 |
| Fatigue | 3 | 0 | 3 | 0 | 6 | 0 |
| Vomiting | 2 | 0 | 4 | 0 | 6 | 0 |
| Hypertriglyceridemia | 0 | 0 | 4 | 3 | 4 | 3 |
| Anemia | 1 | 1[a] | 1 | 1[b] | 2 | 2 |
| Hypertension | 0 | 0 | 1 | 1 | 1 | 1 |
| Dizziness | 2 | 0 | 1 | 1 | 3 | 1 |
| Hemolysis | 0 | 0 | 2 | 1[b] | 2 | 1 |

[a]Grade 3 anemia, not a serious AE. [b]Grade 3 withdrawal hemolysis and anemia in 48-year old woman due to abrupt drug withdrawal after a very fast Hb response

AEs were graded using National Cancer Institute Common Terminology Criteria, version 4.02. Hb = hemoglobin; DXA = Dual energy X-ray absorptiometry



Ex. 2001-1087

# Effect of AG-348 on hormonal status

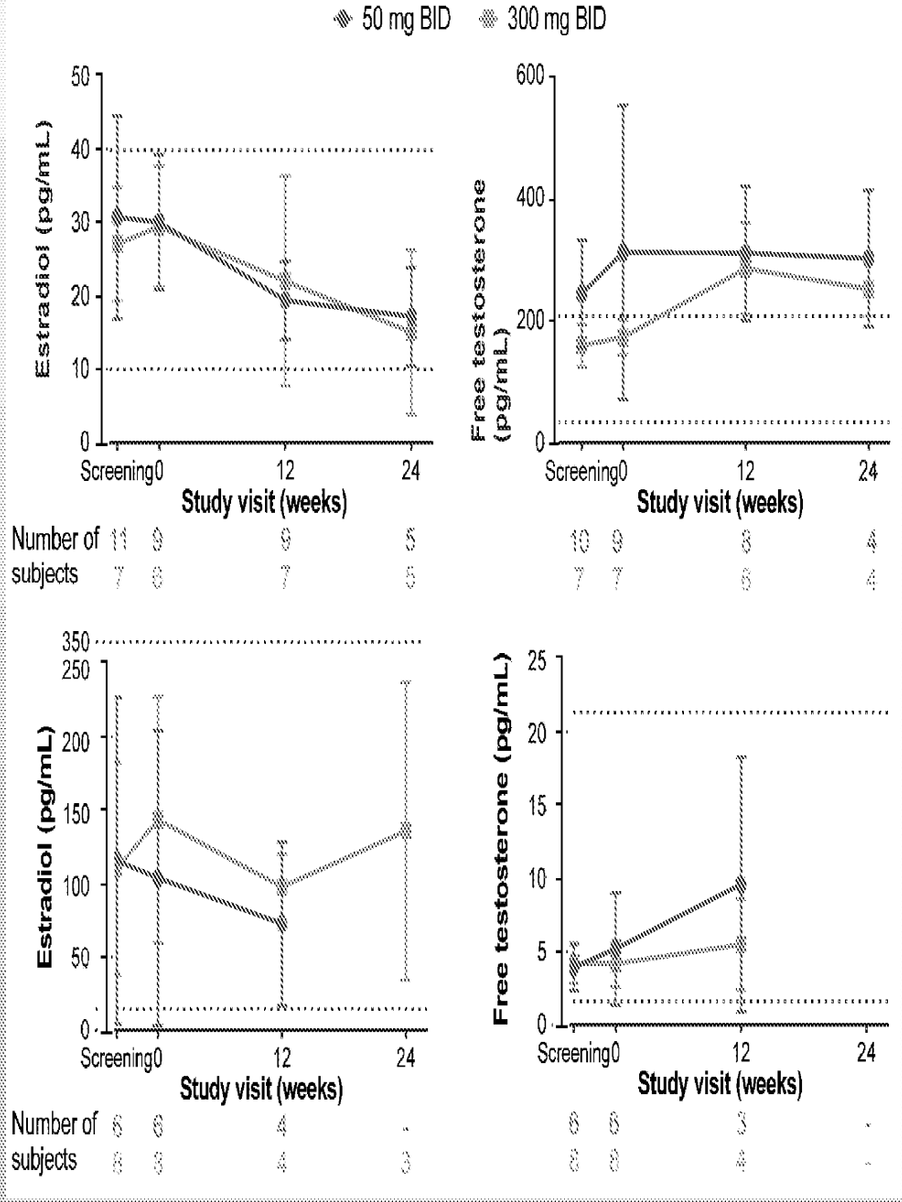
- Preliminary results are in agreement with an anti-aromatase effect

Male

Hormone levels assessed at baseline, 12 and 24 weeks

Patients with available data shown

Female




Normal reference low and high limits shown as horizontal dotted lines



Ex. 2001-1088

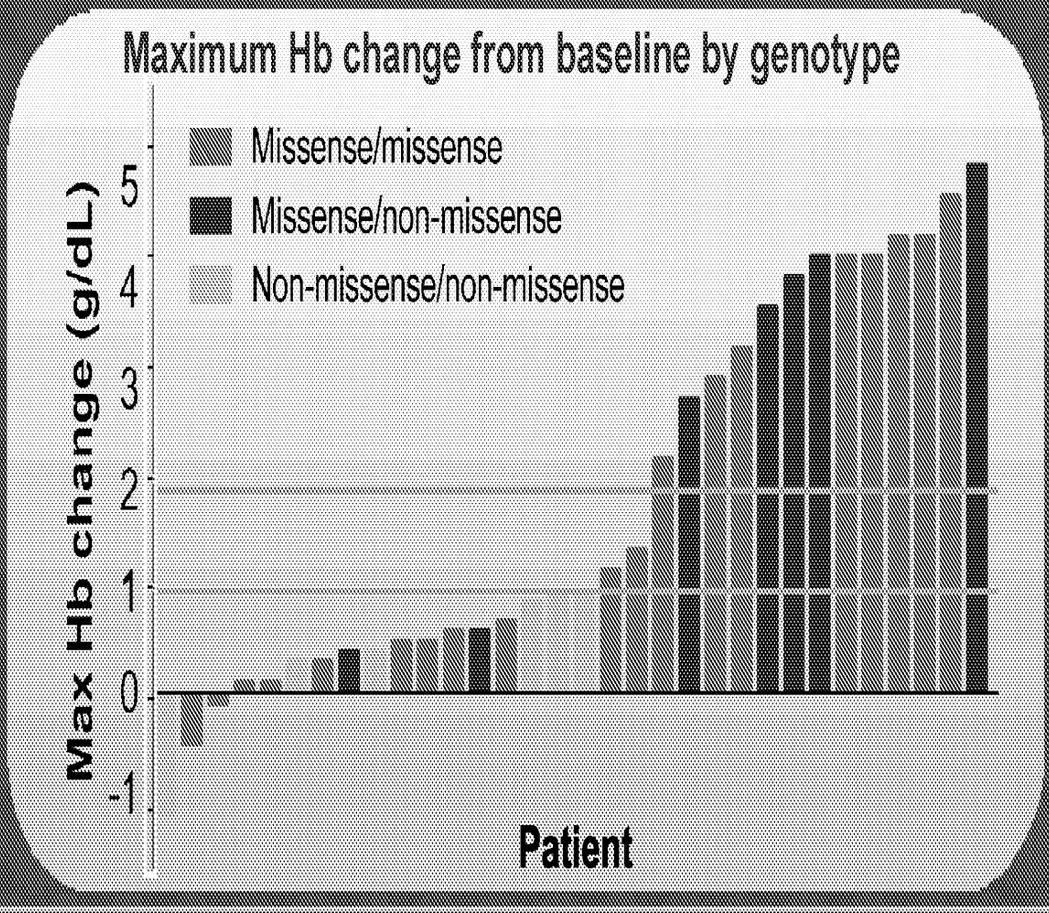
# Clinical Activity Results



Ex. 2001-1089

# Increase in hemoglobin (Hb)

- 15 of 32 (47%) patients had a maximal increase in Hb >1.0 g/dL
  - 15 of 26 patients (58%) who had ≥1 missense mutation had a Hb response
- 5 patients homozygous for R479H (mis/mis; Amish) were non-responders
- Hb response and response maintenance are seen across a range of doses
  - Robust Hb responses led to dose decreases with maintained Hb



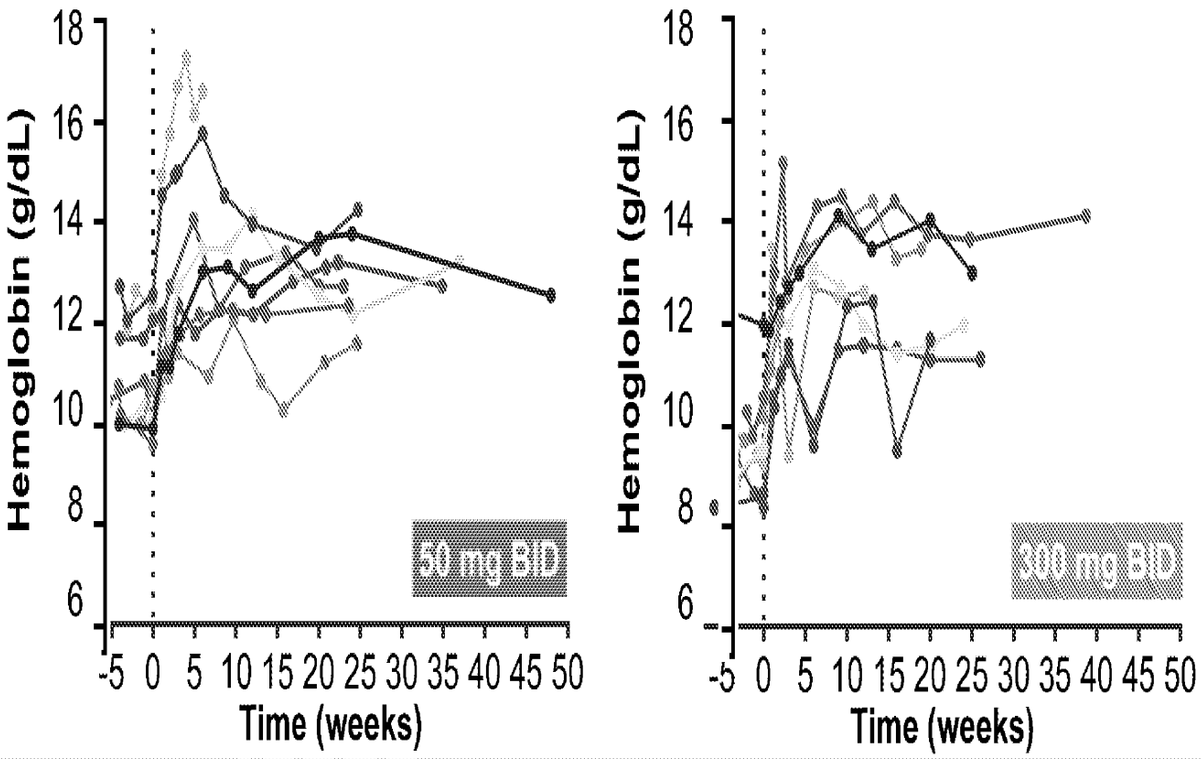

Evaluable analysis set, ≥3 weeks of data



Ex. 2001-1090

# Rapid and sustained increase in Hb

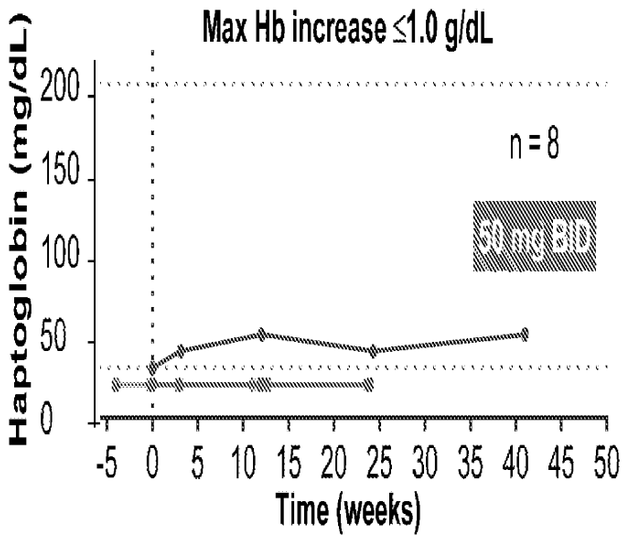
- In patients who had Hb increases >1.0 g/dL (*n*=15):
  - Median time to Hb increase >1.0 g/dL was 1.4 weeks (range, 1.1–21.0)
  - The mean maximum increase was 3.6 g/dL (range, 1.2–5.2)
- 10 patients had dose reductions: 5 due to rapid Hb increase[a]

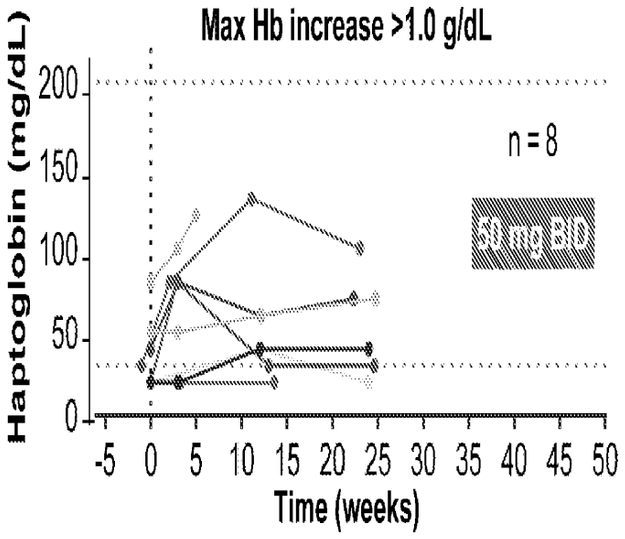


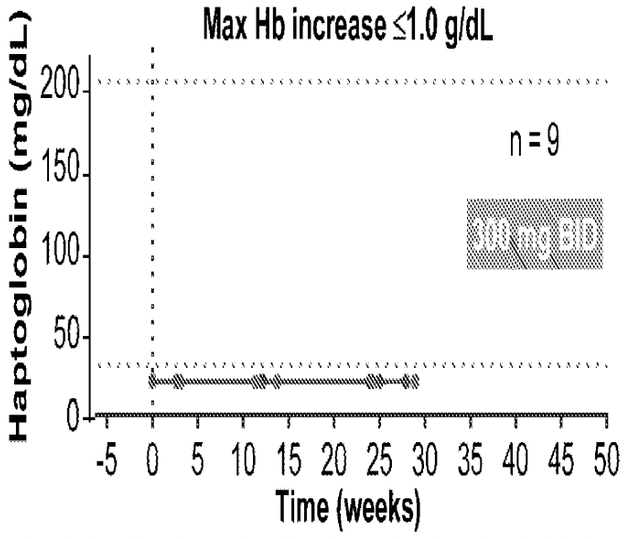

Responders n=15, each line represents an individual

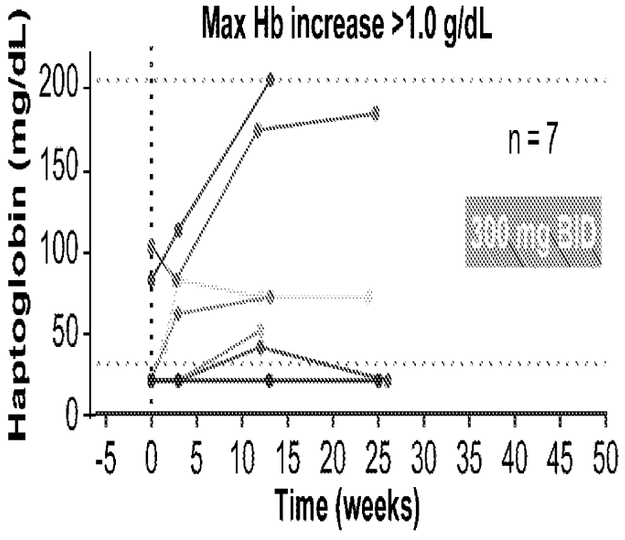
[a]Other dose reductions due to AEs (n=3), self-reduction due to fatigue (n=1), taper prior to discontinuation (n=1)

# The rate of Haptoglobin increases in responders indicating a decrease in hemolysis




Normal reference low and high limits shown as horizontal dotted lines

# Pharmacodynamic results



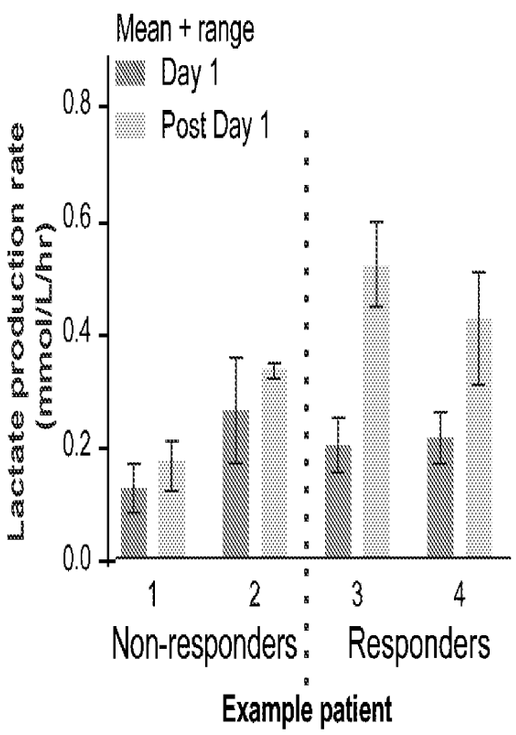
Ex. 2001-1093

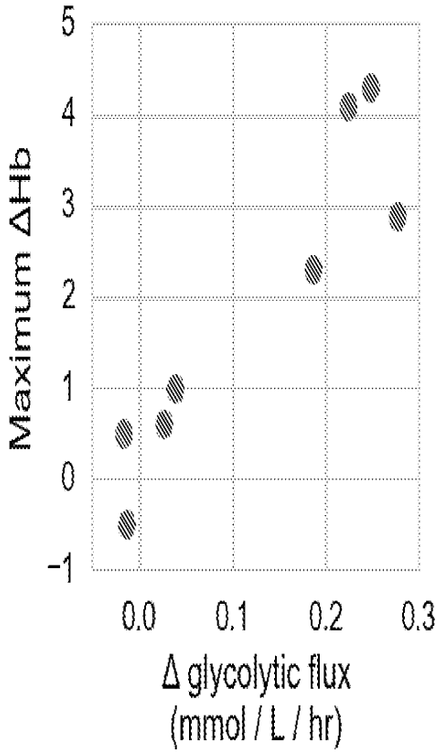
## An increase in hemoglobin correlated to an improvement in the glycolysis pathway

- Rate of metabolism of PK-R pathway was assessed in blood samples from a subset of patients pre and post treatment
- These data suggest a positive correlation between Hb change and change in glycolytic flux

Detailed metabolic characterization in blood from 8

Glycolytic flux assessed *ex vivo* via incorporation of labelled glucose into the end product, lactate




Representative 4 patients shown here N=8



Ex. 2001-1094

# DRIVE-PK Conclusions

- AG-348 is a novel molecule, first in its category, capable of activating the pyruvate kinase enzyme in deficient patients
- A daily dose is well tolerated up to 6 months
  - The significance of the anti-aromatase effect is not well understood as of this date
- AG-348 is capable of increasing hemoglobin in a rapid and sustained and significant manner (>1g/dl) in close to 47% of patients.
  - The increase is related to an action on the glycolysis pathway
  - Preliminary correlation between response and genotype
- These data illustrate the potential and interest of agents activating the enzyme as a treatment that modifies the course of the disease.



Ex. 2001-1095

# Acknowledgments


- Patients who agreed to participate
- All of the Code PK clinicians, MCGRE sector, Club Du Globule Rouge et du Fer
- ,
- Participating Sites:
  - **Hôpital Henri Mondor, Creteil; Frederic Galacteros**
  - **Hôpital Saint Vincent de Paul, Lille; Christian Rose**
  - **Hôpital de la Timone, Marseille, Emmanuelle Bernit**
  - ( L Garcon, M Micheau, L Pascal)
  - Hammersmith Hospital; Mark Layton
  - Universitair Medisch Centrum Utrecht; Eduard van Beers
  - Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico; Wilma Barcellini
  - Boston Children's Hospital; Rachael Grace
  - Stanford University Medical Centre; Bertil Glader
  - University of Utah; Hassan Yaish
  - Weill Cornell – New York Presbyterian Hospital; Sujit Sheth
  - The Children's Hospital of Philadelphia; Janet Kwiatkowski
  - Central Pennsylvania Clinic; Holmes Morton
  - Wayne State University School of Medicine; Yaddanapudi Ravindranath
  - University of Toronto – University Health Network; Kevin Kuo
- Agios Pharmaceuticals




Ex. 2001-1096

# Efficacité d'un activateur de la pyruvate kinase (AG-348) sur l'anémie et les paramètres d'hémolyse au cours du déficit en pyruvate kinase : résultats de l'étude DRIVE PK

Christian Rose[1], Karima Debbache[2], D Mark Layton[3], Eduard Van Beers[4], Wilma Barcellini[5], Emmanuelle Bernit[6], Rachael F Grace[7], Bertil Glader[8], Charles Kung[9], Ann J Barbier[9], Frédéric Galactéros[2]

[1]Hôpital Saint Vincent de Paul, Université Catholique, Lille, France; [2]Unité des Maladies Génétiques du Globule Rouge, CHU Henri Mondor, Créteil, France; [3]Hammersmith Hospital, Imperial College Healthcare, United Kingdom; [4]Universitair Medisch Centrum Utrecht, Netherlands; [5]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [6]Hôpital de la Timone, Marseille, France; [7]Dana Farber Boston Children's Cancer and Blood Disorders Center, Boston, USA; [8]Stanford University School of Medicine, Palo Alto, CA; [9]Agios Pharmaceuticals, Inc, Cambridge, MA

SFH 2017

Ex. 2001-1097

# Le déficit en Pyruvate kinase

**Description**

- Anémie hémolytique congénitale sévère
- Néonatal + - tard
- Prévalence [1–4]

**Etiologie**

- Mutations gène *PKLR* codant pour la pyruvate kinase érythrocytaire (PK-R)

**Complications**

- Anémie chronique
- Ictère , surcharge en fer
- Risques post-splénectomie

**Diagnostic / Traitement**

- Activité Enzymatique et/ou analyse génétique
- Soins de support :transfusions, splénectomie, chélation du fer

**Etude F: code PK**

**70 patients**

Diagnostic 1 er mois n= 41

Fatale n=2

âge médian = 21 ans

- **sévère (n=40) :** Hb en dessous 8 g/dL,
- **Modéré (n=20) :** Hb entre 8 et10 g/dL,
- **Faible (n=10) :** Hb sup à 10 g/dL,

Transfusion dépendant 1/3

splénectomie n=44

Chélates 1/3

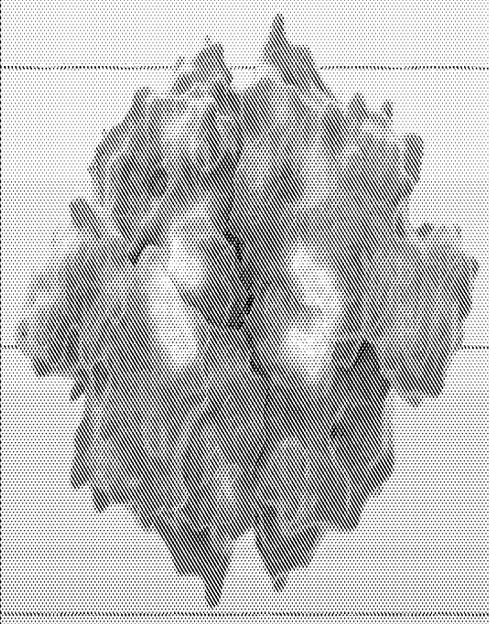
Grace R et al. *Am J Hematol* 2015;90(9):825-30. [1]Mohrenweiser HW *PNAS* 1981;78(8):5046-50. [2]Carey PJ et al. *Blood* 2000;96(12):4005-6. [3]Beutler E & Gelbart T *Blood* 2003;95(11):3585-3. [4]deMeditois et al. *Hum Hered* 1992;42(3):179-83.



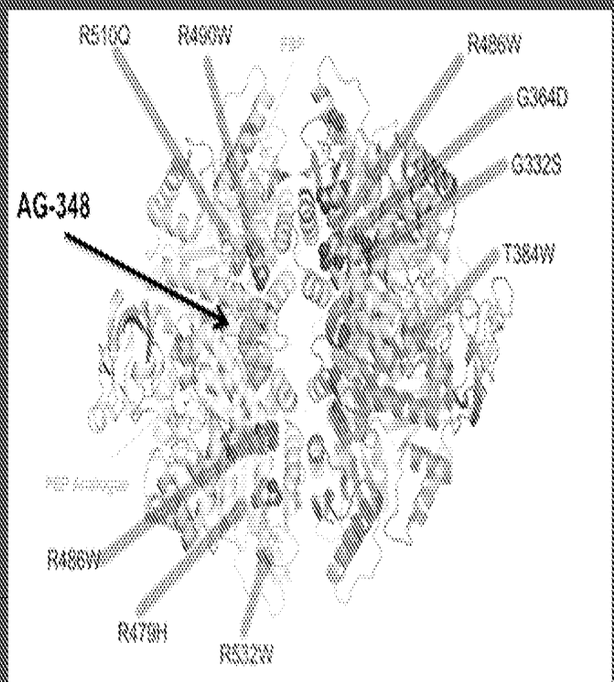
Ex. 2001-1098

# AG-348: un activateur allosterique de la pyruvate kinase ( mutant /sauvage)

La pyruvate kinase est un tétramère;
Les mutations (vert ) diminuent l'activité catalytique



L ' AG-348 (jaune ) se lie à l'enzyme ( interface dimère/ dimère)






Ex. 2001-1099