**[00127]** In certain embodiments, provided herein is a method of increasing the amount of hemoglobin in a subject by administering an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). In certain embodiments, also provided herein is a method of increasing the amount of hemoglobin in a subject having thalassemia comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). Further provided is a method of increasing the amount of hemoglobin in subjects having non-transfusion-dependent thalassemia comprising administering an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor) to the subject. In certain embodiments, the provided methods increase hemoglobin concentration in the subject. In certain embodiments, the provided methods increase Hb concentration to a desired level, for example, above 10 g/dL, more preferably above 11 g/dL, more preferably above 12 g/dL, more preferably above 13 g/dL, or most preferably above 14 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 0.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 1.0 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 1.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 2.0 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 2.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 3.0 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 3.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 4.0 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 4.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 5.0 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 5.5 g/dL. In certain embodiments, the provided methods increase Hb concentration by at least about 6.0 g/dL. In certain embodiments, the increase in Hb concentration is determined from baseline at one or more assessment between week 1 and week 20 (e.g., between week 2 and week 15, between week 3 and week 15, and between week 4 and week 12) of treatment with an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in

the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). In certain embodiments, the provided methods increase Hb concentration as described above in female subjects having thalassemia (e.g., beta-thalassemia or non-transfusion-dependent thalassemia). In certain embodiments, the provided methods increase Hb concentration from baseline to about 12 g/dL in female subjects having thalassemia (e.g., beta-thalassemia or non-transfusion-dependent thalassemia). In certain embodiments, the provided methods increase Hb concentration as described above in male subjects having thalassemia (e.g., beta-thalassemia or non-transfusion-dependent thalassemia). In certain embodiments, the provided methods increase Hb concentration from baseline to about 13 g/dL in male subjects having thalassemia (e.g., beta-thalassemia or non-transfusion-dependent thalassemia).

**[00128]** In some aspects, provided herein are methods for treating hemolytic anemia in a subject in need thereof comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). Also provided is mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor), for use in treating hemolytic anemia in a subject in need thereof. Further provided is the use of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor), in the manufacture of a medicament for treating hemolytic anemia. In one aspect, the hemolytic anemia to be treated is hereditary and/or congenital hemolytic anemia, acquired hemolytic anemia, or anemia as part of a multi-system disease.

**[00129]** In some aspects, provided herein are methods for activating wild-type or mutant PKR in red blood cells in a subject in need thereof comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). Also provided is mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor), for use in activating wild-type or mutant PKR in red blood cells in a subject in need thereof. Further provided is the use of mitapivat or

a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor), in the manufacture of a medicament for activating wild-type or mutant PKR in red blood cells.

**[00130]** Mitapivat is an activator of PKR mutants having lower activities compared to the wild type, thus are useful for methods of the present disclosure. Such mutations in PKR can affect enzyme activity (catalytic efficiency), regulatory properties (modulation by fructose bisphosphate (FBP)/ATP), and/or thermostability of the enzyme. Examples of such mutations are described in Valentini et al, JBC 2002. Some examples of the mutants that are activated by the compounds described herein include G332S, G364D, T384M, R479H, R479K, R486W, R532W, R510Q, and R490W. Without being bound by theory, in certain embodiments, mitapivat affects the activities of PKR mutants by activating FBP non-responsive PKR mutants, restoring thermostability to mutants with decreased stability, or restoring catalytic efficiency to impaired mutants.

**[00131]** In certain embodiments, mitapivat increases the affinity of PKR to phosphoenolpyruvate (PEP). In certain embodiments, mitapivat restores the ability of RBCs to cover PEP and ADP to pyruvate and ATP.

**[00132]** In certain embodiments, provided herein are methods of reducing transfusion frequency of a subject with PKD comprising administering to the subject mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor). In certain embodiments, the transfusion frequency is reduced by at least 5% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 10% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 15% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 20% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 25% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 30% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 35% in the number of RBC units transfused over at least 15 weeks. In certain embodiments, the transfusion frequency is reduced by at least 40% in the number of RBC

units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 5% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 10% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 15% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 20% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 25% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 30% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 35% in the number of RBC units transfused over at least 20 weeks. In certain embodiments, the transfusion frequency is reduced by at least 40% in the number of RBC units transfused over at least 20 weeks.

**[00133]** In some aspects, provided herein are methods of evaluating the efficacy of mitavipat therapy (monotherapy or concomitant medications with a CYP3A4/5 inducer or a CYP3A4/5 inhibitor) in a subject, the method comprising: administering to the subject mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor); and acquiring a value for the level of mitapivat, the level of 2,3-diphosphoglycerate (2,3-DPG), the level of adenosine triphosphate (ATP), or the activity of PKR in the subject, to thereby evaluate the efficacy of the mitapivat therapy. In some aspects, the value for the level is acquired by analyzing the plasma concentration of mitapivat and optionally, the metabolite known to be formed by CYP3A4/5. In some aspects, the level of 2,3-DPG is acquired by analyzing the blood concentration of 2,3-DPG. In some aspects, the level of ATP is acquired by analyzing the blood concentration of ATP. In some aspects, the activity of PKR is acquired by analyzing the blood concentration of a $^{13}C$-label in the blood. In some aspects, the analysis is performed by sample analysis of bodily fluid. In some aspects, the bodily fluid is blood. In some aspects, the analysis is performed by mass spectroscopy. In some aspects, the analysis is performed by LC-MS.

**[00134]** In some aspects, provided herein are methods of treating a subject, the method comprising: administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof, in combination with or in the absence of an

effective amount of a secondary drug (*e.g.* a CYP3A4/5 inducer or a CYP3A4/5 inhibitor); and acquiring a value for the level of mitapivat and optionally its metabolite known to be formed by CYP3A4/5, the level of 2,3-diphosphoglycerate (2,3-DPG), the level of adenosine triphosphate (ATP), or the activity of PKR in the subject, to thereby treat the subject.

## EXEMPLIFICATIONS

**[00135]** As depicted in the Examples below, the drug-drug interactions between mitapivat sulfate and a CYP3A4/5 inducer (e.g. rifampin) or a CYP3A4/5 inhibitor (e.g. itraconazole) are studied. Mitapivat can be synthesized using the procedures described in International Patent Application Publication Nos. WO 2011/002817 and WO 2016/201227.

### ***Example 1: Mitapivat sulfate is primarily metabolized by CYP3A4***

**[00136]** CYP-enzyme phenotyping using human liver microsomes and recombinant CYP enzymes revealed that mitapivat sulfate was primarily metabolized by CYP3A4/5 (>90%), with minor contributions from CYP2C9, CYP2C8, and CYP1A2. There was evidence of metabolism-dependent inhibition of CYP2C19 (largely reversible) and CYP3A4 (largely irreversible). Mitapivat sulfate was found to be a substrate and inhibitor for P-gp but not for breast cancer resistance protein (BCRP) and was found to be a potential inducer of human CYP2B6 and CYP3A4. Mitapivat sulfate appeared to be a mild inhibitor of CYP2C8, CYP2C9, xYP2C19, CYP2D6, and CYP3A4/5 enzymes (testosterone 6β-hydroxylation), bile salts export pump (BSEP), organic anion transporting polypeptide (OATP)1B1, organic anion transporter (OAT)3, and organic cation transporter (OCT)2, and of uridine-5'-diphospho-glucuronosyltransferase (UGT) 1A3, 1A4, and 1A9. Mitapivat sulfate does not appear to be an inhibitor of multidrug resistance-associated protein (MRP)2, MRP3, OATP1B3, and OAT1. The metabolite was found to be a mild inhibitor of CYP2C9 and CYP2C19 and was not an inhibitor of P-gp or BCRP under tested concentrations.

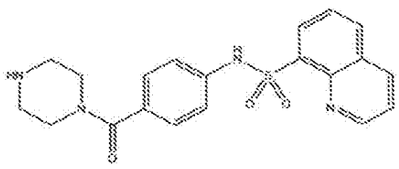
### Example 2: Drug-drug Interaction (DDI) Studies of Mitapivat Sulfate and a CYP3A4 Inducer or a CYP3A4 Inhibitor

**[00117]** Twenty-eight (28) healthy, adult male and female (non-childbearing potential) subjects were enrolled in the study in total; 14 subjects in each study part (Parts 1 and 2). A

minimum of 8 female subjects were enrolled in the study (i.e., a minimum of 4 female subjects per study part). Each subject participated in either Part 1 or Part 2, but not both.

**Part 1**

**[00118]** On Day 1 of Treatment Period 1, a single oral dose of 20 mg mitapivat sulfate was administered. Serial blood samples for plasma assay of mitapivat concentrations and its CYP3A4 metabolite, referred to herein as the "Metabolite" (structure below),



were collected from predose to 120 hours following administration of mitapivat sulfate. In Treatment Period 2, an oral dose of 200 mg itraconazole was administered once daily (QD) for 9 consecutive days (Day 1 through Day 9 of Treatment Period 2) with a single oral dose of 20 mg mitapivat sulfate coadministered on Day 5. Serial blood samples for plasma assay of mitapivat and the Metabolite concentrations were collected from predose to 120 hours following coadministration of mitapivat sulfate and itraconazole on Day 5.

**[00119]** In Treatment Period 1, mitapivat sulfate was administered orally with approximately 240 mL of water. In Treatment Period 2, on Days 1 to 4, itraconazole was administered alone immediately followed by approximately 220 mL of water, and on Day 5, itraconazole was administered first (no water) and was immediately followed by mitapivat sulfate administration with approximately 220 mL of water. Study drugs (mitapivat sulfate and itraconazole) were administered following an overnight fast of at least 10 hours on Day 1 of Treatment Period 1 (mitapivat sulfate only) and Day 5 of Treatment Period 2 (mitapivat sulfate and itraconazole), and subjects remained fasted for 4 hours after dosing. On all other dosing days, itraconazole was administered following a predose fast of at least 4 hours and subjects remained fasted for at least 2 hours after dosing.

**Part 2**

**[00120]** On Day 1 of Treatment Period 1, a single oral dose of 50 mg mitapivat sulfate was administered. Serial blood samples for plasma assay of mitapivat and the Metabolite

concentrations were collected from predose to 120 hours following administration of mitapivat sulfate. In Treatment Period 2, an oral dose of 600 mg rifampin was administered QD for 12 consecutive days (Day 1 through Day 12 of Treatment Period 2) with a single oral dose of 50 mg mitapivat sulfate coadministered on Day 8. Serial blood samples for plasma assay of mitapivat sulfate and the Metabolite concentrations were collected from predose to 120 hours following coadministration of mitapivat and rifampin on Day 8.

**[00121]** In Part 2, study drugs were administered with approximately 240 mL of water on all dosing days including the coadministration of mitapivat sulfate and rifampin on Day 8 of Treatment Period 2. Mitapivat sulfate and rifampin was administered following an overnight fast of at least 10 hours on Day 1 of Treatment Period 1 (mitapivat sulfate only) and Day 8 of Treatment Period 2 (both mitapivat sulfate and rifampin) and subjects remained fasted for 4 hours after dosing. On all other dosing days, rifampin was administered following a predose fast of at least 4 hours and subjects remained fasted for at least 2 hours after dosing. There was a washout period of 7 days between the mitapivat sulfate dose in Treatment Period 1 and the first itraconazole (Part 1) or rifampin (Part 2) dose in Treatment Period 2. All study drugs were consumed within 5 minutes for both Part 1 and Part 2.

**Test and Reference Products, Dose, Duration and Mode of Administration**

**Part 1**

**[00122]** Mitapivat sulfate (20 mg tablets) administered along with Itraconazole (also referred to as Sporanox®, 10 mg/mL oral solution, is considered as the test arm in this study. Mitapivat sulfate administered alone is considered as the reference arm in this study.
In Period 1, a single oral dose of 20 mg mitapivat sulfate was administered orally with approximately 240 mL of water. In Period 2, on Day 5 (coadministration of itraconazole and mitapivat sulfate), 200 mg itraconazole (20 mL of 10 mg/mL oral solution) was administered first (no water) and was immediately followed by 20 mg mitapivat sulfate (1 x 20 mg tablet) administration with approximately 220 mL of water. On all other dosing days, itraconazole was administered alone immediately followed by approximately 220 mL of water.
Each subject received 2 doses of mitapivat sulfate and 9 doses of itraconazole in Part 1.

**Part 2**

**[00123]** Mitapivat sulfate (50 mg tablets) administered along with Rifampin (also referred to as Rifadin®, 300 mg capsules) is considered as the test arm in this study.

Mitapivat sulfate (50 mg tablets) administered alone is considered as the reference arm in this study.

**[00124]** In Period 1, a single oral dose of 50 mg mitapivat sulfate was administered orally. In Period 2, subjects were administered 600 mg rifampin (2 x 300 mg capsules) QD on Day 1 to Day 12 inclusive, with 50 mg mitapivat sulfate (1 x 50 mg tablet) coadministered on Day 8. Study drugs were administered with approximately 240 mL of water on all dosing days including the coadministration of mitapivat sulfate and rifampin on Day 8 of Period 2.

**[00125]** Each subject received 2 doses of mitapivat sulfate and 12 doses of rifampin in Part 2.

**Duration of Treatment:**

**[00126]** The total duration of participation including the screening period for each subject was approximately 49 to 62 days for Part 1 and approximately 61 or 63 days in Part 2 of the study.

**[00127]** In each study part, Treatment Period 1 was approximately 6 days and Treatment Period 2 was approximately 12 days; a washout phase was 7 days between both treatment periods in each study part.

**Criteria for Evaluation of Pharmacokinetics**

**[00128]** In Parts 1 and 2, for mitapivat and the Metabolite, blood samples were obtained for all subjects to determine the plasma concentrations of mitapivat, and the Metabolite, before mitapivat sulfate dosing (0 hour) on Day 1 of Period 1 (Parts 1 and 2) and Day 5 (Part 1) or Day 8 (Part 2) of Period 2 at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 24, 48, 72, 96, and 120 hours postdose. Plasma concentrations of mitapivat and the Metabolite were determined using methods validated with respect to accuracy, precision, linearity, sensitivity, and specificity, with a lower limit of quantitation (LLOQ) of 0.5 ng/mL for both analytes. A noncompartmental PK approach was used to analyze individual plasma mitapivat and the Metabolite concentration time data (using Phoenix® WinNonlin® Version 7.0). The following PK parameters were calculated: $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$, $AUC_{0\text{-}24}$, $AUC_{\%extrap}$, Cmax, CL/F (mitapivat only), Tlast, Tmax, t½, and Vz/F (mitapivat only).

**Statistical Methods for Pharmacokinetics**

**[00129]** The plasma concentrations of mitapivat and the Metabolite were listed and summarized by treatment and collection time for all subjects in the PK Population. Plasma concentrations were presented with the same level of precision as received from the bioanalytical laboratory including sample size (n), arithmetic mean (Mean), standard deviation (SD), coefficient of variation (CV%), standard error of the mean (SEM), minimum, maximum, and median. Concentrations from excluded subjects were included in the plasma concentration tables, but were excluded from the summary statistics and noted as such in the tables. All BLQ values were presented as "BLQ" in the plasma concentration table listings and footnoted accordingly.

**[00130]** PK parameters were presented with a minimum of 3 significant figures. PK parameters were listed by subject, tabulated by treatment, and summarized using descriptive statistics (n, mean, SD, CV%, SEM, minimum, median, maximum, geometric mean (Geom. Mean) and geometric mean coefficient of variation (Geom. CV%). Time-based parameters (i.e., Tmax and t½) and treatment:reference AUC and Cmax ratios were presented to 2 decimals. Excluded subjects were included in the PK parameter table listings, but were excluded from the summary statistics and noted as such in the tables.

**[00131]** To evaluate the effect of itraconazole and rifampin on mitapivat and the Metabolite (primary objective), plasma mitapivat and the Metabolite PK parameters (Cmax, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}inf}$) were natural log (ln) transformed before analysis. The ln-transformed value of the PK parameters was analyzed using a linear mixed effect model for each study part separately with treatment as a fixed effect and subject as a random effect. The linear mixed effect model included treatment as a fixed effect and subject as a random effect. The inferential results (least-squares means [LSMs], difference between LSMs, and 90% confidence intervals [CIs] of the difference) were back transformed to the original scale. Geometric LSMs, geometric mean ratios (GMRs), and 90% CIs were presented. The GMR of the LSMs were calculated from the back-transformed difference between the treatment LSMs. The GMRs were expressed as a percentage relative to mitapivat sulfate given alone for both Part 1 and Part 2. The 90% CIs for the ratios were derived by back-transformation of the CIs obtained for the difference between the treatment LSMs. The CIs were expressed as a percentage relative to mitapivat sulfate given alone for both Part 1 and Part 2.

**[00132]** The median differences in Tmax for evaluation of the effect of itraconazole and rifampin on mitapivat and the Metabolite along with the range of the difference were

calculated using the non parametric Wilcoxon Signed Rank test, and the p-value was presented. Note that Tmax was not ln-transformed for these analyses.

**Pharmacokinetic Results**

**Part 1**

**[00133]** Mean plasma mitapivat and the Metabolite concentration-time profiles following 20 mg mitapivat sulfate administered alone (Period 1) or coadministered with multiple doses of 200 mg itraconazole (Period 2) on linear and semi-log scales are presented in Figures 1 and 2.

**Mitapivat**

**[00134]** Mitapivat plasma concentrations were detectable in all subjects at 0.5 hour (the first scheduled postdose sample) in both Periods 1 and 2. Mitapivat was still detectable up to the last sample time at 120 hours postdose in the majority of subjects in Period 1 and in all subjects in Period 2.

**[00135]** Measurable predose mitapivat concentrations were present in all but 2 subjects in Period 2 (two subjects were BLQ). As used herein, BLQ refers to below the limit of quantification. These predose concentrations were < 5% of the Cmax, so PK calculations were performed.

**[00136]** Mitapivat concentrations exhibited a characteristic multi-compartmental pharmacokinetic profile. When coadministered with itraconazole, mitapivat concentrations rose to a higher mean peak with a delay in time to peak by approximately 2 hours compared to when the drug was administered alone. The post-peak decline of the multi-compartmental behavior was slower after coadministration with itraconazole when compared to mitapivat administered alone. Overall, higher mitapivat concentrations were experienced for a longer time period when mitapivat was coadministered with itraconazole.

**The Metabolite**

**[00137]** When mitapivat was administered alone, the Metabolite plasma concentrations were detectable in all but 1 subject at 0.5 hour. The Metabolite remained detectable all but 1 subject up to 48 hours postdose, with quantifiable the Metabolite out to 72 hours in 3 subjects and 96 hours in 1 subject. In Period 2 (coadministration with itraconazole), the Metabolite

concentrations were delayed relative to administration of mitapivat alone. Concentrations were not measurable in all subjects until 1.5 hours postdose. Maximum concentrations were also reduced. However, the Metabolite residence was prolonged in the presence of itraconazole, with concentrations still detectable in all but 1 subject throughout the 120-hour sampling interval (in 1 subject the last measurable concentration was at 96 hours postdose).

**[00138]** The statistical comparisons of mitapivat PK parameters following 20 mg mitapivat sulfate administered alone (Period 1) or coadministered with multiple doses of 200 mg itraconazole (Period 2) PK parameters are summarized below in Table 1.

**Table 1**

**Summary of Statistical Comparisons of Plasma Mitapivat Pharmacokinetic Parameters Following 20 mg Mitapivat Sulfate Co-Administered With Multiple Doses of 200 mg Itraconazole Versus Administered Alone (Part 1)**

| | Mitapivat Sulfate + Itraconazole (Test) | | Mitapivat sulfate alone (Reference) | | | | |
|---|---|---|---|---|---|---|---|
| **Parameter (unit)** | **Geometric LSM** | **n** | **Geometric LSM** | **n** | **GMR** | **90% Confidence Interval** | **Intra-subject CV%** |
| $AUC_{0-t}$ (ng*hr/mL) | 8860 | 14 | 1885 | 14 | 4.6998 | 4.2519 - 5.1949 | 15.05 |
| $AUC_{0-inf}$ (ng*hr/mL) | 9475 | 14 | 1942 | 14 | 4.8780 | 4.4154 - 5.3890 | 14.97 |
| $C_{max}$ (ng/mL) | 704.3 | 14 | 412.5 | 14 | 1.7074 | 1.5815 - 1.8433 | 11.48 |
| Mitapivat sulfate alone: 20 mg mitapivat sulfate (1 x 20 mg tablet) at Hour 0 on Day 1 (Period 1)<br>Mitapivat sulfate + itraconazole: 200 mg itraconazole (20 mL of 10 mg/mL oral solution) QD on Days 1 to Day 9 inclusive (within ± 1 hour of dosing time on Day 1) with 20 mg mitapivat sulfate (1 x 20 mg tablet) coadministered at Hour 0 on Day 5 (Period 2)<br>Geometric least-squares means (LSMs) are calculated by exponentiating the LSMs from the linear mixed effect model.<br>Geometric Mean Ratio (GMR) = test / reference<br>Intra-subject CV% = 100 x (square root (exp[MSE]-1), where MSE = Residual variance from linear mixed effect model. | | | | | | | |

**[00139]** The coadministration of 200 mg itraconazole with 20 mg mitapivat sulfate increased mitapivat exposure relative to when mitapivat sulfate was administered alone. The geometric mean $AUC_{0-t}$ and $AUC_{0-inf}$ ratios of mitapivat in the presence and absence of itraconazole were 4.7 and 4.9, respectively. The geometric mean Cmax ratio of mitapivat in the presence and absence of itraconazole was 1.7. The intra-subject variability was low at approximately 15% for AUCs and 11% for Cmax.

**[00140]** The statistical comparisons of the Metabolite PK parameters following 20 mg mitapivat sulfate administered alone (Period 1) or coadministered with multiple doses of 200 mg itraconazole (Period 2) PK parameters are summarized below in Table 2.

**Table 2**

**Summary of Statistical Comparisons of Plasma Metabolite Pharmacokinetic Parameters Following 20 mg Mitapivat Sulfate Co-Administered With Multiple Doses of 200 mg Itraconazole Versus Administered Alone (Part 1)**

| | Mitapivat Sulfate + Itraconazole (Test) | | Mitapivat sulfate alone (Reference) | | | | |
|---|---|---|---|---|---|---|---|
| Parameter (unit) | Geometric LSM | n | Geometric LSM | n | GMR | 90% Confidence Interval | Intra-subject CV% |
| $AUC_{0-t}$ (ng*hr/mL) | 300.0 | 14 | 197.1 | 14 | 1.5218 | 1.4268 - 1.6231 | 9.65 |
| $AUC_{0-inf}$ (ng*hr/mL) | 338.6 | 14 | 216.2 | 14 | 1.5661 | 1.4784 - 1.6590 | 8.63 |
| $C_{max}$ (ng/mL) | 9.110 | 14 | 18.79 | 14 | 0.4849 | 0.4441 - 0.5295 | 13.20 |
| Mitapivat sulfate alone: 20 mg mitapivat sulfate (1 x 20 mg tablet) at Hour 0 on Day 1 (Period 1)<br>Mitapivat sulfate + itraconazole: 200 mg itraconazole (20 mL of 10 mg/mL oral solution) QD on Days 1 to Day 9 inclusive (within ± 1 hour of dosing time on Day 1) with 20 mg mitapivat sulfate (1 x 20 mg tablet) coadministered at Hour 0 on Day 5 (Period 2)<br>Geometric least-squares means (LSMs) are calculated by exponentiating the LSMs from the linear mixed effect model.<br>Geometric Mean Ratio (GMR) = test / reference<br>Intra-subject CV% = 100 x (square root (exp[MSE]-1), where MSE = Residual variance from linear mixed effect model. | | | | | | | |

**[00141]** The coadministration of 200 mg itraconazole with 20 mg mitapivat sulfate increased total Metabolite exposure relative to when mitapivat sulfate was administered alone. The geometric mean $AUC_{0-t}$ and $AUC_{0-inf}$ ratios of the Metabolite in the presence and absence of itraconazole were 1.5 and 1.6, respectively. The geometric mean Cmax ratio of the Metabolite in the presence and absence of itraconazole was 0.5. The intra-subject variability was low at approximately 9% to 10% for AUCs and 13% for Cmax.

**Part 2**

**[00142]** Mean plasma mitapivat and metabolite the Metabolite concentration-time profiles following 50 mg mitapivat sulfate administered alone (Period 1) or coadministered with multiple doses of 600 mg rifampin (Period 2) on linear and semi-log scales are presented in Figures 3 and 4.

**Mitapivat**

**[00143]** Mitapivat plasma concentrations were detectable in all subjects at 0.5 hour (the 1st scheduled postdose sample) when given alone or with rifampin. Mitapivat was still detectable up to the last sample time at 120 hours postdose in all but 1 subject when given alone had a BLQ concentration at 120 hours). When coadministered with rifampin, detectable

mitapivat concentrations were not present in any sample collections from any subject beyond 24 hours postdose (except 1 subject with detectable mitapivat at 48 hours).

**[00144]** Mitapivat concentrations increased to a lower and slightly earlier peak when coadministered with rifampin compared to when administered alone. The peak arithmetic mean mitapivat concentrations were observed at a nominal time of approximately 1 hour postdose for Period 1 and 0.5 hour postdose for Period 2. The mean concentrations of mitapivat then declined in a multi exponential manner for both periods; however, the post-peak initial decline in mitapivat concentrations was more rapid and to lower levels when coadministered with rifampin compared to dosing alone.

**The Metabolite**

**[00145]** The Metabolite plasma concentrations were detectable at 0.5 hour postdose in all subjects after mitapivat sulfate was given alone and in all but 1 subject (1 subject was reported as BLQ) when mitapivat sulfate was coadministered with rifampin. The Metabolite was still detectable in all but 4 subjects up to the last scheduled sampling time of 120 hours postdose when mitapivat sulfate was given alone. When mitapivat sulfate was coadministered with rifampin, The Metabolite remained detectable in all subjects up to 24 hours postdose, with quantifiable The Metabolite out to 48 hours in 6 subjects and 72 hours in 1 subject. No Metabolite concentrations were detectable in samples collected beyond 72 hours.

**[00146]** The peak arithmetic mean Metabolite concentrations were observed at a nominal time of approximately 2 hours postdose with mitapivat sulfate given alone and 1 hour when mitapivat sulfate was coadministered with rifampin. When mitapivat sulfate was given alone, higher mean concentrations The Metabolite were observed over the initial 1 hour postdose. Metabolite concentrations were higher when mitapivat sulfate was coadministered with rifampin beyond 1 hour. The mean concentrations of The Metabolite then declined in a multi exponential fashion for both treatments. Decline in Metabolite concentrations was more rapid when mitapivat sulfate was coadministered with rifampin.

**[00147]** The statistical comparisons of mitapivat PK parameters following 50 mg mitapivat sulfate administered alone (Period 1) or coadministered with multiple doses of 600 mg rifampin (Period 2) PK parameters are summarized below in Table 3.

**Table 3**

**Summary of Statistical Comparisons of Plasma Mitapivat Pharmacokinetic Parameters Following 50 mg Mitapivat Sulfate Co-Administered With Multiple Doses of 600 mg Rifampin Versus Administered Alone (Part 2)**

| | Mitapivat sulfate + rifampin (Test) | | Mitapivat sulfate alone (Reference) | | | | |
|---|---|---|---|---|---|---|---|
| Parameter (unit) | Geometric LSM | n | Geometric LSM | n | GMR | 90% Confidence Interval | Intra-subject CV% |
| $AUC_{0-t}$ (ng*hr/mL) | 486.4 | 14 | 5565 | 14 | 0.0874 | 0.0719 - 0.1062 | 29.73 |
| $AUC_{0-inf}$ (ng*hr/mL) | 496.4 | 14 | 5681 | 14 | 0.0874 | 0.0719 - 0.1062 | 29.84 |
| $C_{max}$ (ng/mL) | 292.4 | 14 | 1263 | 14 | 0.2316 | 0.1933 - 0.2773 | 27.45 |
| Mitapivat sulfate alone: 50 mg mitapivat sulfate (1 x 50 mg tablet) at Hour 0 on Day 1 (Period 1)<br>Mitapivat sulfate + rifampin: 600 mg rifampin (2 x 300 mg capsules) QD on Day 1 to Day 12 inclusive (within ± 1 hour of dosing time on Day 1) with 50 mg mitapivat sulfate (1 x 50 mg tablet) coadministered at Hour 0 on Day 8 (Period 2)<br>Geometric least-squares means (LSMs) are calculated by exponentiating the LSMs from the linear mixed effect model.<br>Geometric Mean Ratio (GMR) = test / reference<br>Intra-subject CV% = 100 x (square root (exp[MSE]-1), where MSE = Residual variance from linear mixed effect model. | | | | | | | |

**[00148]** The coadministration of 600 mg rifampin with 50 mg mitapivat sulfate decreased mitapivat exposure relative to when mitapivat sulfate was administered alone. The geometric mean $AUC_{0-t}$ and $AUC_{0-inf}$ ratios of mitapivat in the presence and absence of rifampin were 0.09. The geometric mean Cmax ratio of mitapivat in the presence and absence of rifampin was 0.23. The intra-subject variability was low at approximately 30% for AUCs and 27% for Cmax.

**[00149]** The statistical comparisons of Metabolite PK parameters following 50 mg mitapivat sulfate administered alone (Period 1) or co-administered with multiple doses of 600 mg rifampin (Period 2) PK parameters are summarized below in Table 4

**Table 4**

**Summary of Statistical Comparisons of Plasma Metabolite Pharmacokinetic Parameters Following 50 mg Mitapivat Sulfate Co-Administered With Multiple Doses of 600 mg Rifampin Versus Administered Alone (Part 2)**

| | Mitapivat sulfate + rifampin (Test) | | Mitapivat sulfate alone (Reference) | | | | |
|---|---|---|---|---|---|---|---|
| Parameter (unit) | Geometric LSM | n | Geometric LSM | n | GMR | 90% Confidence Interval | Intra-subject CV% |
| $AUC_{0-t}$ (ng*hr/mL) | 230.5 | 14 | 623.0 | 14 | 0.3700 | 0.3316 - 0.4129 | 16.50 |
| $AUC_{0-inf}$ (ng*hr/mL) | 244.9 | 14 | 662.6 | 14 | 0.3696 | 0.3321 - 0.4113 | 16.08 |
| $C_{max}$ (ng/mL) | 64.47 | 14 | 53.80 | 14 | 1.1983 | 1.0642 - 1.3492 | 17.86 |

| | Mitapivat sulfate + rifampin (Test) | | Mitapivat sulfate alone (Reference) | | | | |
|---|---|---|---|---|---|---|---|
| **Parameter (unit)** | **Geometric LSM** | **n** | **Geometric LSM** | **n** | **GMR** | **90% Confidence Interval** | **Intra-subject CV%** |
| Mitapivat sulfate alone: 50 mg mitapivat sulfate (1 x 50 mg tablet) at Hour 0 on Day 1 (Period 1)<br>Mitapivat sulfate + rifampin: 600 mg rifampin (2 x 300 mg capsules) QD on Day 1 to Day 12 inclusive (within ± 1 hour of dosing time on Day 1) with 50 mg mitapivat sulfate (1 x 50 mg tablet) coadministered at Hour 0 on Day 8 (Period 2)<br>Geometric least-squares means (LSMs) are calculated by exponentiating the LSMs from the linear mixed effect model.<br>Geometric Mean Ratio (GMR) = test / reference<br>Intra-subject CV% = 100 x (square root (exp[MSE]-1), where MSE = Residual variance from linear mixed effect model. | | | | | | | |

**[00150]** The coadministration of 600 mg rifampin with 50 mg mitapivat sulfate decreased the Metabolite exposure relative to when mitapivat sulfate was administered alone. The geometric mean AUC0-t and AUC0-inf ratios of the Metabolite in the presence versus absence of rifampin were 0.37. The geometric mean Cmax ratio of the Metabolite in the presence versus absence of rifampin was 1.2. The intra-subject variability was low at approximately 16% to 18% for AUCs and Cmax.

**Conclusions**

**[00151]** Total exposure of mitapivat increased in the presence of itraconazole compared to dosing of mitapivat sulfate alone.

**[00152]** Total exposure of mitapivat decreased in the presence of rifampin compared to dosing of mitapivat sulfate alone.

## Example 3. Development of a PBPK Model for Mitapivat Sulfate to Evaluate the Potential for Drug-Drug Interactions

### Part I

**[00153]** A physiologically based pharmacokinetic model (hereinafter "PBPK" model) for mitapivat sulfate based on the currently available in-vitro and clinical PK data is being developed. The developed PBPK model will be used to assess DDI liability with mitapivat sulfate acting as a victim of CYP3A4-mediated metabolism as well as a perpetrator of CYP and transporter mediated DDI.

**[00154]** Specifically, a PBPK model that includes a simple first order absorption model will be developed. Full PBPK and minimal PBPK distribution models will be evaluated, both

of which consider liver and intestinal metabolism. The fraction of mitapivat sulfate absorbed will be estimated from mass balance data from the [14C] human ADME study (Clinical Study AG348-C-009). In-vitro data will be used to assign the relative contribution of CYP3A4 to the clearance of mitapivat sulfate. $K_I$ and $k_{inact}$ values for mechanism based inhibition of CYP3A4 and CYP3A4 induction (Emax and EC50 ) data by mitapivat sulfate will be incorporated within the PBPK model to assess the net DDI effects at steady state. Once an mitapivat sulfate PBPK model has been verified against the available clinical data, a series of DDI simulations assessing mitapivat sulfate as a victim or perpetrator of DDI will be performed in healthy volunteers.

**Experiments with mitapivat sulfate as a victim**

**[00155]** The impact of the co-administration of the strong CYP3A4 inhibitor, itraconazole, on the PK of mitapivat sulfate will be determined based on the following single and multiple-dose administration of 5, 20 and 50 mg BID.

**[00156]** The impact of the co-administration of the strong CYP3A4 inhibitor, ketoconazole, on the PK of mitapivat sulfate will be determined based following single and multiple-dose administration of 5, 20 and 50 mg BID.

**[00157]** The impact of the co-administration of the moderate CYP3A4 inhibitor fluconazole on the PK of mitapivat sulfate will be determined based on the following single- and multiple dose administration of 5, 20 and 50 mg BID.

**[00158]** The impact of the co-administration of the mild CYP3A4 inhibitor cimetidine on the PK of mitapivat sulfate will be determined based on the following single- and multiple dose administration of 5, 20 and 50 mg BID.

**[00159]** The impact of the co-administration of the strong CYP3A4 inducer, rifampin, on the PK of mitapivat sulfate will be determined based on the following single- and multiple dose administration of 5, 20 and 50 mg BID.

**[00160]** The impact of the co-administration of the moderate CYP3A4 inducer efavirenz on the PK of mitapivat sulfate will be determined following single- and multiple dose administration of 5, 20 and 50 mg BID.

**Experiments with mitapivat sulfate as perpetrator**

**[00161]** The extent to which 5 to 50 mg BID mitapivat sulfate affects the systemic exposure of CYP3A4 substrate midazolam when co-administered with mitapivat sulfate will be determined.

**[00162]** The effect of 5 to 50 mg mitapivat sulfate on the systemic exposures of CYP2B6 substrate bupropion when co-administered will be determined. CYP2B6 induction (Emax and EC50 ) data by mitapivat sulfate will be incorporated within the PBPK model. Sensitivity analysis will be conducted to gauge the impact of the uncertainty around Emax and EC50 on the predicted DDIs.

**[00163]** The effect of 5 to 50 mg AG mitapivat sulfate BID on the systemic exposures of CYP2C9 substrate warfarin when co-administered will be determined. CYP2C9 induction (Emax and EC50) data by mitapivat sulfate will be incorporated within the PBPK model. Sensitivity analysis will be conducted to gauge the impact of the uncertainty around Emax and EC50 on the predicted DDIs.

**[00164]** The effect of 5 to 50 mg mitapivat sulfate BID on the systemic exposures of CYP2C8/CYP3A4 substrate repaglinide when co-administered will be determined. CYP2C8 induction (Emax and EC50) data by mitapivat sulfate will be incorporated within the PBPK model. Sensitivity analysis will be conducted to gauge the impact of the uncertainty around Emax and EC50 on the predicted DDIs.

**[00165]** The effect of 5 to 50 mg mitapivat sulfate BID on the systemic exposures of CYP2C19 substrate omeprazole when co-administered will be determined. CYP2C19 induction (Emax and EC50) data by mitapivat sulfate will be incorporated within the PBPK model. Sensitivity analysis will be conducted to gauge the impact of the uncertainty around Emax and EC50 on the predicted DDIs.

**[00166]** The effect of mitapivat sulfate (5 to 50 mg BID) on the systemic exposures of OATP1B1/OATP1B3 (rosuvastatin), P-gp (digoxin), metformin (MATE1 and OCT2), OAT3 (methotrexate) substrate drugs when co-administered will be determined. The respective IC50 values for inhibition of OATP1B1, P-gp, MATE1, OCT2 and OAT3 by mitapivat sulfate will be incorporated within the PBPK model. Sensitivity analysis will be performed to assess the impact of these parameters on the DDI liability of mitapivat sulfate.

**Part II**

**[00167]** The first order absorption component of the previously developed PBPK model will be extended using available pH solubility data to include the ADAM model. The recovery of mitapivat sulfate plasma concentration profiles after single and multiple dosing (up to 50 mg BID) to healthy volunteer subjects will be demonstrated. Once verified against the available clinical data, the modified PBPK model will be used to assess the impact of an increase in gastric pH on the pharmacokinetics of mitapivat sulfate via manipulation of system parameters (gastric pH).

**[00168]** While a number of embodiments have been described, the scope of this disclosure is to be defined by the appended claims, and not by the specific embodiments that have been represented by way of example. The contents of all references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated herein in their entireties by reference. Unless otherwise defined, all technical and scientific terms used herein are accorded the meaning commonly known to one with ordinary skill in the art.

## CLAIMS

WHAT IS CLAIMED IS:

1. A method of treating pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

2. A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

3. A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

4. The method of any one of claims 1-3, further comprising:

(a) monitoring the subject for an effect of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inducer; and

(b) in the event that an effect of the drug-drug interaction is present, adjusting the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and/or the effective amount of the CYP3A4/5 inducer being administered to the subject to reduce the effect.

5. The method of claim 4, further comprising:

(c) repeating (a) and (b) until there are no further detectable symptoms of the drug-drug interaction.

6. A method of treating pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

7. A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

8. A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

9. The method of any one of claims 6-8, further comprising:

(a) monitoring the subject for an effect of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inhibitor; and

(b) in the event that an effect of the drug-drug interaction is present, adjusting the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and/or the effective amount of the CYP3A4/5 inhibitor being administered to the subject to reduce the effect.

10. The method of claim 9, further comprising:

(c) repeating (a) and (b) until there are no further detectable symptoms of the drug-drug interaction.

11. The method of any one of claims 1-10, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and the effective amount of the CYP3A4/5 inducer (or inhibitor) sequentially.

12. The method of any one of claims 1-10, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and the effective amount of the CYP3A4/5 inducer (or inhibitor) concurrently.

13. The method of any one of claims 1-10, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and the effective amount of the CYP3A4/5 inducer (or inhibitor) sequentially and concurrently.

14. A method of treating pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

15. A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

16. A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer of cytochrome P450 3A4/5 (CYP3A4/5).

17. The method of any one of claims 14-16, wherein administration of a CYP3A4/5 inducer, if any, is terminated at a sufficient time interval prior to the administration of the effective amount of mitapivat or a pharmaceutically acceptable salt thereof such that there is no detectable symptom of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inducer.

18. A method of treating a condition associated with pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

19. A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

20. A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

21. The method of any one of claims 18-20, wherein administration of a CYP3A4/5 inhibitor, if any, is terminated at a sufficient time interval prior to the administration of the effective amount of mitapivat or a pharmaceutically acceptable salt thereof such that there is no detectable symptom of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inhibitor.

22. The method of any one of claims 1-21, comprising administering to the subject about 1 mg to about 2500 mg, or about 5 mg to 2500 mg, or about 30 mg to 2500 mg, or about 5 mg to about 700 mg, or about 15 mg to about 700 mg, about 5 mg to about 360 mg, about 5

mg to about 300 mg, about 50 mg to about 300 mg, about 120 mg to about 360 mg, about 120 mg to about 300 mg, about 5 mg to about 120 mg, about 5 mg, about 20 mg, about 50 mg, about 100 mg, about 120 mg , about 200 mg, about 300 mg, or about 360 mg of mitapivat or a pharmaceutically acceptable salt thereof.

23. The method of any one of claims 1-22, comprising administering to the subject about 1 mg to about 2500 mg of mitapivat or a pharmaceutically acceptable salt thereof.

24. The method of any one of claims 1-23, comprising administering to the subject about about 5 mg to 2500 mg of mitapivat or a pharmaceutically acceptable salt thereof.

25. The method of any one of claims 1-24, comprising administering to the subject about 5 mg to about 360 mg of mitapivat or a pharmaceutically acceptable salt thereof.

26. The method of any one of claims 1-25, comprising administering to the subject about 5 mg to about 300 mg of mitapivat or a pharmaceutically acceptable salt thereof.

27. The method of any one of claims 1-26, comprising administering to the subject about 50 mg to about 300 mg of mitapivat or a pharmaceutically acceptable salt thereof.

28. The method of any one of claims 1-26, comprising administering to the subject about 5 mg of mitapivat or a pharmaceutically acceptable salt thereof.

29. The method of any one of claims 1-27, comprising administering to the subject about 20 mg of mitapivat or a pharmaceutically acceptable salt thereof.

30. The method of any one of claims 1-27, comprising administering to the subject about 50 mg of mitapivat or a pharmaceutically acceptable salt thereof.

31. The method of any one of claims 1-27, comprising administering to the subject about 100 mg of mitapivat or a pharmaceutically acceptable salt thereof.

32. The method of any one of claims 1-31, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof once daily.

33. The method of any one of claims 1-31, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof twice daily.

34. The method of any one of claims 1-33, comprising administering to the subject a sulfate salt of mitapivat.

35. The method of any one of claims 1-5, 11-17 and 22-34, wherein the CYP3A4/5 inducer is a strong inducer of CYP3A4/5.

36. The method of any one of claims 1-5, 11-17 and 22-34, wherein the CYP3A4/5 inducer is a moderate inducer of CYP3A4/5.

37. The method of any one of claims 1-5, 11-17 and 22-34, wherein the CYP3A4/5 inducer is a mild inducer of CYP3A4/5.

38. The method of any one of claims 1-5, 11-17 and 22-34, wherein the CYP3A4/5 inducer is selected from the group consisting of aminoglutethimide, bexarotene, bosentan, carbamazepine, dexamethasone, efavirenz, fosphenytoin, griseofulvin, modafinil, nafcillin, nevirapine, oxcarbazepine, phenobarbital, phenytoin, primidone, rifabutin, rifampin, rifapentine, St. John's wort, avasimibe, dabrafenib, mifepristone, etravirine, armodafninil, glycerol phenylbutyrate, asunaprevir, rifunamide, oritavancin, clobazam, felbamate, eslicarbazepine acetate, and genistein.

.39. The method of any one of claims 1-5, 11-17, 22-34 and 38, wherein the CYP3A4/5 inducer is rifampin.

40. The method of any one of claims 1-5, 11-17, 22-34, 38 and 39, comprising administering about 600 mg of rifampin to the subject.

41. The method of any one of claims 6-13 and 18-34, wherein the inhibitor of CYP3A4/5 is a strong inhibitor of CYP3A4/5.

42. The method of any one of claims 6-13 and 18-34, wherein the inhibitor of CYP3A4/5 is a moderate inhibitor of CYP3A4/5.

43. The method of any one of claims 6-13 and 18-34, wherein the inhibitor of CYP3A4/5 is a mild inhibitor of CYP3A4/5.

44. The method of any one of claims 6-13 and 18-34, wherein the inhibitor of CYP3A4/5 is selected from the group consisting of toamiodarone, amprenavir, aprepitant, atazanavir, chloramphenicol, clarithromycin, conivaptan, cyclosporine, darunavir, dasatinib,

delavirdine, diltiazem, erythromycin, fluconazole, fluoxetine, fluvoxamine, fosamprenavir, imatinib, indinavir, isoniazid, itraconazole, ketoconazole, lapatinib, miconazole, nefazodone, nelfinavir, posaconazole, ritonavir, quinupristin, saquinavir, tamoxifen, telithromycin, troleandomycin, verapamil, voriconazole, grapefruit, Seville oranges, limes, pomelos, fruit juices, and vegetables from mustard green family.

45. The method of any one of claims 6-13, 18-34 and 44, wherein the inhibitor of CYP3A4/5 is itraconazole.

45. The method of any one of claims 6-13, 18-34, 44 and 45, comprising administering about 200 mg of itraconazole to the subject.

## ABSTRACT

Described herein are methods of treating pyruvate kinase deficiency (PKD), sickle cell disease or thalassemia with mitapivat or a pharmaceutically acceptable salt thereof, or use of the drug for the treatment of these conditions, in combination with or in the absence of with a secondary drug, such as an inducer or an inhibitor of cytochrome P450. Various doses and dosing regimens of mitapivat in monotherapy and in concomitant medications are described.

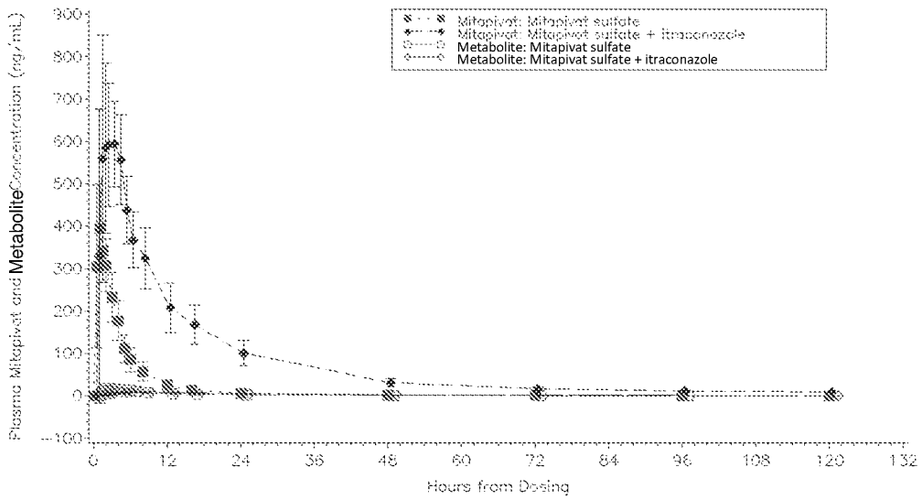

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
300
800
700
600
500
400
300
200
100
0
-100
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 1

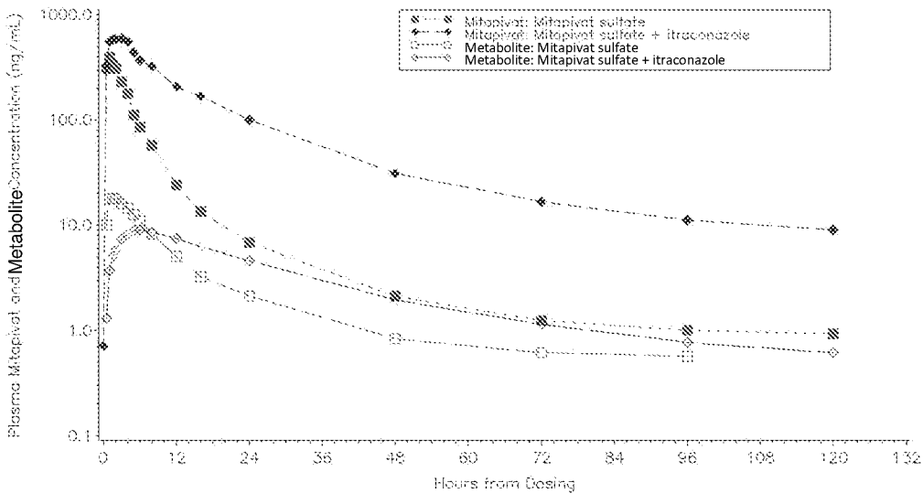

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 2

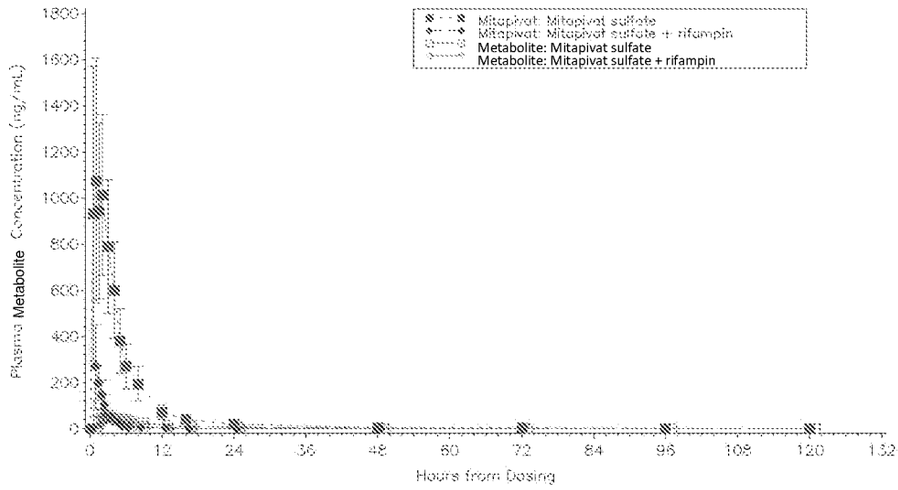

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 3

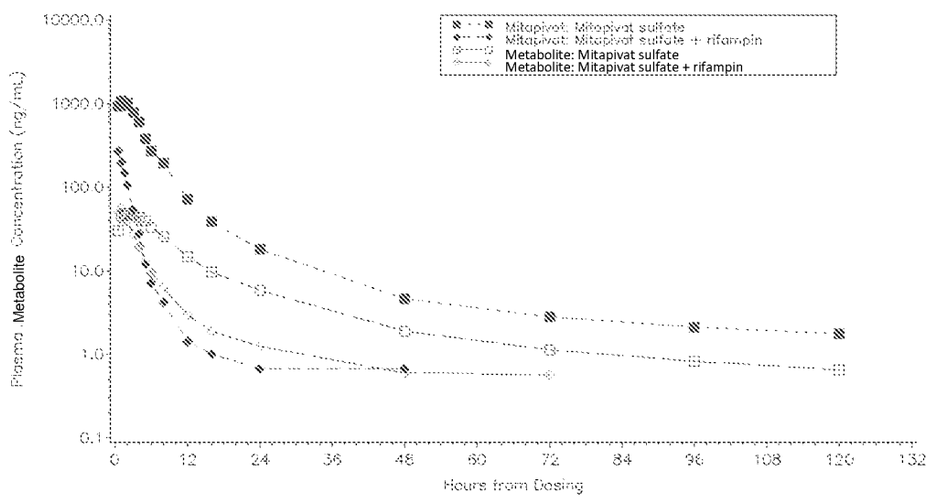

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
10000.0
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 4

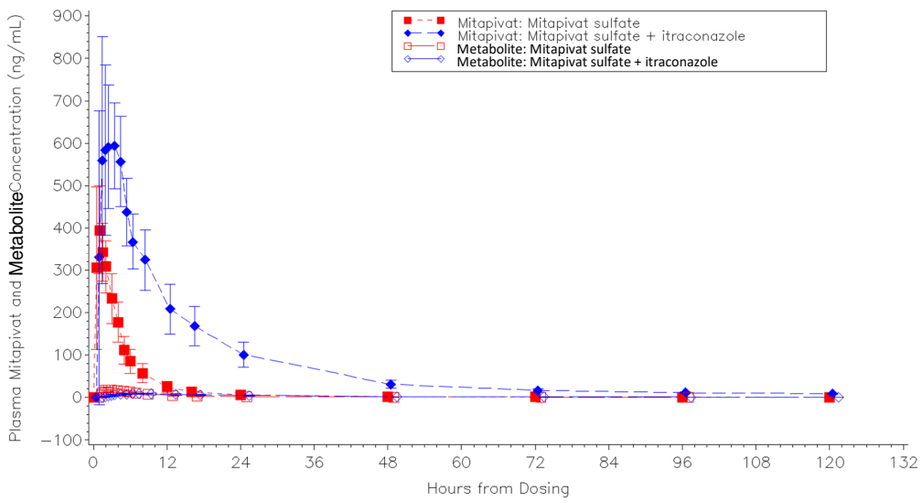

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
900
800
700
600
500
400
300
200
100
0
−100
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 1

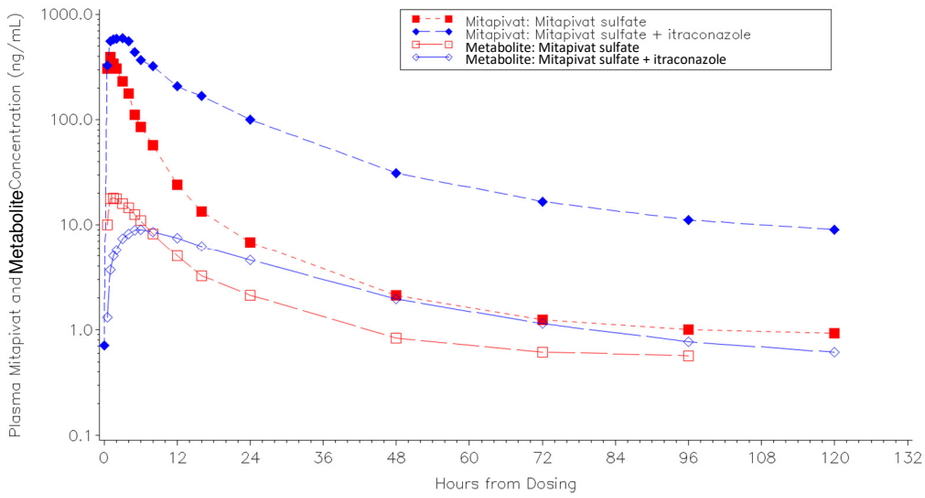

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 2

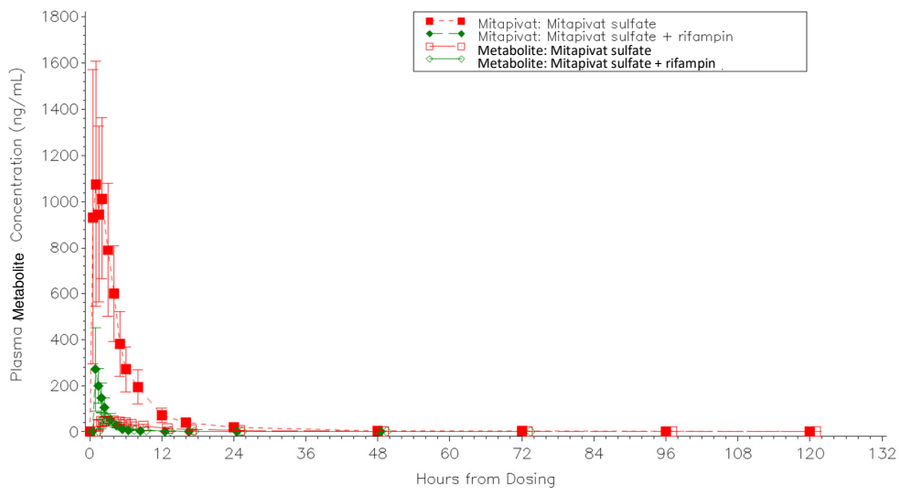

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 3

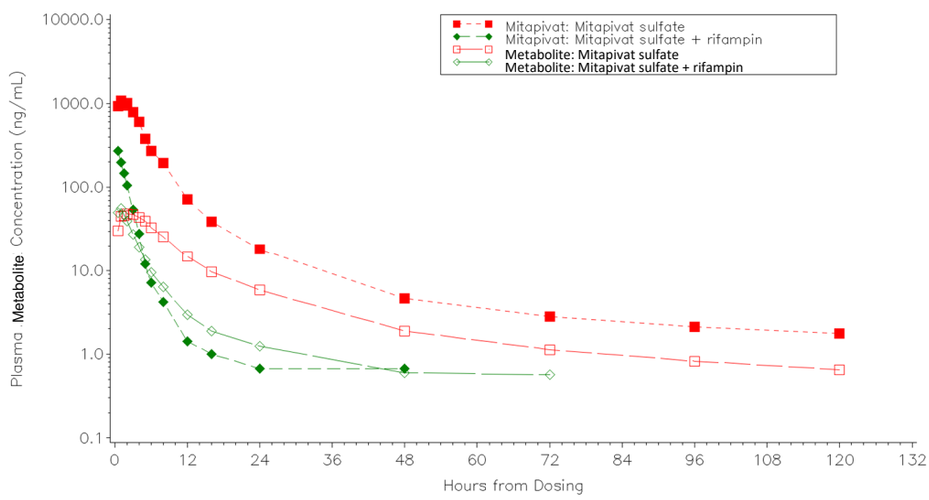

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
10000.0
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

Figure 4

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | |
| **Filing Date:** | |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Filer:** | Steven G. Davis/Erin Shea |
| **Attorney Docket Number:** | 128748-01502 |

Filed as Large Entity

**Filing Fees for Utility under 35 USC 111(a)**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| UTILITY APPLICATION FILING | 1011 | 1 | 300 | 300 |
| UTILITY SEARCH FEE | 1111 | 1 | 660 | 660 |
| UTILITY EXAMINATION FEE | 1311 | 1 | 760 | 760 |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **1720** |

DocCode – SCORE

# SCORE Placeholder Sheet for IFW Content

Application Number: 16900610 Document Date: 06/12/2020

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

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Form Revision Date: March 1, 2019

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 39763803 |
| **Application Number:** | 16900610 |
| **International Application Number:** | |
| **Confirmation Number:** | 1054 |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Customer Number:** | 86738 |
| **Filer:** | Mei Bai |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 128748-01502 |
| **Receipt Date:** | 18-JUN-2020 |
| **Filing Date:** | 12-JUN-2020 |
| **Time Stamp:** | 16:52:12 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Application Data Sheet | Application_Data_Sheet.pdf | 36097<br>1e5274daee40b8edb5f299dc0e48f80cb4cced15 | no | 6 |

**Warnings:**

| Information: | |
|---|---|
| This is not an USPTO supplied ADS fillable form | |
| **Total Files Size (in bytes):** | 36097 |

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**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

**Corrected Application Data Sheet**

**Inventor Information**

| | |
|---|---|
| Inventor Number:: | 1 |
| Given Name:: | Varsha |
| Middle Name:: | Venkatachalam |
| Family Name:: | Iyer |
| City of Residence:: | ~~Cambridge~~ Boston |
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| Country of Residence:: | US |
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| City of mailing address:: | Cambridge |
| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 02139 |

| | |
|---|---|
| Inventor Number:: | 2 |
| Given Name:: | Chandra |
| Middle Name:: | Agarwal |
| Family Name:: | Prakash |
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| State or Province of Residence:: | MA |
| Country of Residence:: | US |
| Street of mailing address:: | ~~88 Sidney Street~~ 41 Garnet Circle |

| | |
|---|---|
| City of mailing address:: | ~~Cambridge~~ North Andover |
| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | ~~02139~~ 01845 |

| | |
|---|---|
| Inventor Number:: | 3 |
| Given Name:: | Hua |
| Family Name:: | Yang |
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| State or Province of Residence:: | MA |
| Country of Residence:: | US |
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| City of mailing address:: | Cambridge |
| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 02139 |

**Correspondence Information**

| | |
|---|---|
| Correspondence Customer Number:: | 86738 |
| E-Mail address:: | docket@mccarter.com |

**Application Information**

| | |
|---|---|
| Application Number:: | ~~Not Yet Assigned~~ 16/900,610 |

| | |
|---|---|
| Filing Date:: | 06/12/20 |
| Application Type:: | NonProvisional |
| Subject Matter:: | Utility |
| CD-ROM or CD-R?:: | None |
| Sequence submission?:: | None |
| Computer Readable Form (CRF)?:: | No |
| Title:: | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| Attorney Docket Number:: | 128748-01502 |
| Request for Early Publication?:: | No |
| Request for Non-Publication?:: | Yes |
| Total Drawing Sheets:: | 4 |
| Small Entity?:: | No |
| Petition included?:: | No |
| Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2:: | No |

**Representative Information**

| | |
|---|---|
| Representative Customer Number:: | 86738 |

**Domestic Priority Information**

| Application:: | Continuity Type:: | Parent Application:: | Parent Filing Date:: | Prior Appl Status:: |
|---|---|---|---|---|
| This Application | Claims benefit of provisional | 62/861600 | 06/14/19 | Pending<br>Expired |

**Foreign Priority Information**

**Applicant Information**

| | |
|---|---|
| Applicant Number:: | 1 |
| Applicant Type:: | Assignee |
| Organization Name:: | Agios Pharmaceuticals, Inc. |
| Street of mailing address:: | 88 Sidney Street |
| City of mailing address:: | Cambridge |
| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 02139 |

**Assignee Information Including Non-Applicant Assignee Information**

## Signature:

| | | | |
|---|---|---|---|
| **NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**<br>This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.<br>See 37 CFR 1.4(d) for the manner of making signatures and certifications. | | | |
| **Signature** | /Mei Bai/ | Date (YYYY-MM-DD) | 2020-06-18 |
| Name | Mei Bai, Ph.D. | Registration Number | 64,507 |

PTO/SB/92 (07-16)
Approved for use through 11/30/2020. OMB 0561-0031
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   on _____ .
   Date

2. ☐ Facsimile transmitted to the United States Patent and Trademark Office, or

   OR

3. ☒ EFS-Web transmitted to the USPTO.

| /Mei Bai/ | June 18, 2020 |
|---|---|
| Signature | Date |
| Mei Bai, Ph.D. | (617) 449-6590 |
| Typed or printed name | Telephone number |

Note: Each paper must have its own certificate of mailing or transmission, or this certificate must identify each submitted paper:

Application Data Sheet (5 pages)

# File History Content Report

The following content is missing from the original file history record obtained from the United States Patent and Trademark Office. No additional information is available.

Document Date - 2020-06-12

Document Title - Applicant Communication Re: Entity Status Set to Undiscounted (Initial Default Setting or Status Change)

This page is not part of the official USPTO record. It has been determined that content identified on this document is missing from the original file history record.

Ex. 2001-0293

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/900,610 | 06/12/2020 | Varsha Venkatachalam Iyer | 128748-01502 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**FORMALITIES LETTER**

*OC000000117883336*

Date Mailed: 06/19/2020

# NOTICE TO FILE CORRECTED APPLICATION PAPERS

***Filing Date Granted***

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given **TWO MONTHS** from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - Numbers, letters, and reference characters on the drawings must measure at least 0.32 cm (1/8 inch) in height. See Figure(s) 1-4.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Additional claim fees of $ **8660** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

**SUMMARY OF FEES DUE:**

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **160** surcharge.
- $ **4140** for **9** independent claims over 3.

- $ **3700** for **37** total claims over 20.
- $ **820** for multiple dependent claim surcharge.
- $( **0**) previous unapplied payment amount.
- $ **8820** TOTAL FEE BALANCE DUE.

**Items Required To Avoid Processing Delays:**

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Varsha Venkatachalam Iyer
  Chandra Agarwal Prakash
  Hua Yang

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

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Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sabubeker/

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| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 16/900,610 | 06/12/2020 | 1629 | 1720 | 128748-01502 | 45 | 12 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**FILING RECEIPT**

OC000000117883333

Date Mailed: 06/19/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

**Please verify the accuracy of the data presented on this receipt.** If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

**Inventor(s)**

Varsha Venkatachalam Iyer, Cambridge, MA;
Chandra Agarwal Prakash, North Andover, MA;
Hua Yang, Acton, MA;

**Applicant(s)**

Agios Pharmaceuticals, Inc., Cambridge, MA;

**Power of Attorney:** None

**Domestic Priority data as claimed by applicant**

This appln claims benefit of 62/861,600 06/14/2019

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
*Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.*

**Permission to Access Application via Priority Document Exchange:** Yes

**Permission to Access Search Results:** Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

**If Required, Foreign Filing License Granted:** 06/18/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/900,610**

**Projected Publication Date:** Request for Non-Publication Acknowledged

**Non-Publication Request:** Yes

**Early Publication Request:** No

**Title**

MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450

**Preliminary Class**

514

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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---


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# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 40326935 |
| **Application Number:** | 16900610 |
| **International Application Number:** | |
| **Confirmation Number:** | 1054 |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Customer Number:** | 86738 |
| **Filer:** | Mei Bai |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 128748-01502 |
| **Receipt Date:** | 19-AUG-2020 |
| **Filing Date:** | 12-JUN-2020 |
| **Time Stamp:** | 16:41:35 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Preliminary Amendment | PAmend_Resp_to_NTFCAP.pdf | 41541<br>4e425356cd8a0d098f0274105d712b75477e243a | no | 8 |

**Warnings:**

| Information: | | | | | |
|---|---|---|---|---|---|
| 2 | Drawings-only black and white line drawings | Replacement_drawings.PDF | 143099<br>b5a707b67a89d7a1b4000e8120885ab3e7bdda72 | no | 4 |
| Warnings: | | | | | |
| Information: | | | | | |
| | | | Total Files Size (in bytes): | 184640 | |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: 08/19/2020 Signature: /Mei Bai/
(Mei Bai, Ph.D.)

Docket No.: 128748-01502
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Utility Application of:
Varsha Venkatachalam Iyer et al.

Application No.: 16/900,610 Confirmation No.: 1054

Filed: June 12, 2020 Art Unit: N/A

For: MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 Examiner: Not Yet Assigned

**PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115 AND RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS**

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

## INTRODUCTORY COMMENTS

In response to the Notice to File Corrected Application Papers mailed June 19, 2020, please amend the above-identified U.S. patent application as follows:

**Amendments to the Claims** are reflected in the listing of claims which begins on page 2 of this paper.

**Amendments to the Drawings** begin on page 6 of this paper and include an attached replacement sheet.

**Remarks/Arguments** begin on page 7 of this paper.

## AMENDMENTS TO THE CLAIMS

1. (Currently Amended) A method of treating pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

2. (Currently Amended) A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

3. (Currently Amended) A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof and an effective amount of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

4. (Currently Amended) The method of claim 1~~any one of claims 1-3~~, further comprising:

(a) monitoring the subject for an effect of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inducer or inhibitor; and

(b) in the event that an effect of the drug-drug interaction is present, adjusting the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and/or the effective amount of the CYP3A4/5 inducer or inhibitor being administered to the subject to reduce the effect.

5. (Original) The method of claim 4, further comprising:

(c) repeating (a) and (b) until there are no further detectable symptoms of the drug-drug interaction.

6-10. (Cancelled)

11. (Currently Amended) The method of claim 1~~any one of claims 1-10~~, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and the effective amount of the CYP3A4/5 inducer [[(]]or inhibitor[[)]] sequentially.

12. (Currently Amended) The method of claim 1~~any one of claims 1-10~~, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof and the effective amount of the CYP3A4/5 inducer [[(]]or inhibitor[[)]] concurrently.

13. (Cancelled)

14. (Currently Amended) A method of treating pyruvate kinase deficiency (PKD) in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

15. (Currently Amended) A method of treating sickle cell disease in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

16. (Currently Amended) A method of treating thalassemia in a subject, comprising administering to the subject an effective amount of mitapivat or a pharmaceutically acceptable salt thereof in the absence of an inducer or inhibitor of cytochrome P450 3A4/5 (CYP3A4/5).

17. (Currently Amended) The method of claim 14~~any one of claims 14-16~~, wherein administration of a CYP3A4/5 inducer or inhibitor, if any, is terminated at a sufficient time interval prior to the administration of the effective amount of mitapivat or a pharmaceutically acceptable salt thereof such that there is no detectable symptom of a drug-drug interaction between mitapivat or a pharmaceutically acceptable salt thereof and the CYP3A4/5 inducer or inhibitor.

18-21. (Cancelled)

22. (Currently Amended) The method of claim 1~~any one of claims 1-21~~, comprising administering to the subject about 1 mg to about 2500 mg, or about 5 mg to 2500 mg, or about 30

mg to 2500 mg, or about 5 mg to about 700 mg, or about 15 mg to about 700 mg, about 5 mg to about 360 mg, about 5 mg to about 300 mg, about 50 mg to about 300 mg, about 120 mg to about 360 mg, about 120 mg to about 300 mg, about 5 mg to about 120 mg, about 5 mg, about 20 mg, about 50 mg, about 100 mg, about 120 mg , about 200 mg, about 300 mg, or about 360 mg of mitapivat or a pharmaceutically acceptable salt thereof.

23-31. (Cancelled)

32. (Currently Amended) The method of claim 1~~any one of claims 1-31~~, comprising administering to the subject the effective amount of mitapivat or a pharmaceutically acceptable salt thereof once daily or twice daily.

33. (Cancelled)

34. (Currently Amended)The method of claim 1~~any one of claims 1-33~~, comprising administering to the subject a sulfate salt of mitapivat.

35. (Cancelled)

36. (Currently Amended)The method of claim 1~~any one of claims 1-5, 11-17 and 22-34~~, wherein the CYP3A4/5 inducer or inhibitor is a moderate inducer or inhibitor of CYP3A4/5.

37. (Currently Amended)The method of claim 1~~any one of claims 1-5, 11-17 and 22-34~~, wherein the CYP3A4/5 inducer or inhibitor is a mild inducer or inhibitor of CYP3A4/5.

38. (Currently Amended)The method of claim 1~~any one of claims 1-5, 11-17 and 22-34~~, wherein the CYP3A4/5 inducer is selected from the group consisting of aminoglutethimide, bexarotene, bosentan, carbamazepine, dexamethasone, efavirenz, fosphenytoin, griseofulvin, modafinil, nafcillin, nevirapine, oxcarbazepine, phenobarbital, phenytoin, primidone, rifabutin, rifampin, rifapentine, St. John's wort, avasimibe, dabrafenib, mifepristone, etravirine, armodafninil, glycerol phenylbutyrate, asunaprevir, rifunamide, oritavancin, clobazam, felbamate, eslicarbazepine acetate, and genistein.

39-43. (Cancelled)

44. (Currently Amended) The method of claim 1~~any one of claims 6-13 and 18-34~~, wherein the inhibitor of CYP3A4/5 is selected from the group consisting of toamiodarone, amprenavir, aprepitant, atazanavir, chloramphenicol, clarithromycin, conivaptan, cyclosporine, darunavir, dasatinib, delavirdine, diltiazem, erythromycin, fluconazole, fluoxetine, fluvoxamine, fosamprenavir, imatinib, indinavir, isoniazid, itraconazole, ketoconazole, lapatinib, miconazole, nefazodone, nelfinavir, posaconazole, ritonavir, quinupristin, saquinavir, tamoxifen, telithromycin, troleandomycin, verapamil, voriconazole, grapefruit, Seville oranges, limes, pomelos, fruit juices, and vegetables from mustard green family.

45-46. (Cancelled)

## AMENDMENTS TO THE DRAWINGS

Please replace 4 sheets of drawings with 4 sheets of replacement drawings.

Attachment: Replacement sheets



Ex. 2001-0306

## REMARKS

***Claim Status***

Upon entry of this amendment, claims 1-5, 11, 12, 14-17, 22, 32, 34, 36-38 and 44 will be pending. Claims 6-10, 13, 18-21, 23-31, 33, 35, 39-43, 45 and 46 are currently canceled. Claims 1-4, 11, 12, 14-17, 22, 32, 34, 36-38 and 44 are currently amended. Support for the amendments to the claims can be found in the specification and claims as originally filed. For example, support for the amendments to claims 1-4 can be found in original claims 6-9, respectively. Support for the amendments to claims 14-17 can be found in original claims 18-21, respectively. Support for the amendments to claim 32 can be found in original claim 33. Support for the amendments to claims 36 and 37 can be found in original claims 42 and 43, respectively. No new matter has been added.

***Response to File Corrected Application Papers***

Applicant has submitted herewith replacement drawings, in which numbers, letters, and reference characters on the drawings measure at least 0.32 cm (1/8 inch) in height.

In view the amendments submitted herewith, the total number of claims is 18. In addition, the multiple claim dependencies have been removed throughout the claims. Accordingly, the surcharge for multiple dependent claim is no longer applicable. In response to the Notice of Insufficiency, Applicant submits herewith $1380 additional claim fees for 3 independent claims in excess of 3.

If the Examiner believes that a telephone conversation with the Applicant's Agent would be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 449-6590.

Applicants believe no further fees are due with this response. However, if a fee is due, please charge our Deposit Account No. 50-4876, under Order No. 128748-01502 from which the undersigned is authorized to draw.

Dated: 08/19/2020

Respectfully submitted,

By_/Mei Bai/_____

Mei Bai, Ph.D.
Registration No.: 64,507
MCCARTER & ENGLISH, LLP
265 Franklin Street
Boston, Massachusetts 02110
(617) 449-6590
(617) 607-9200 (Fax)
Attorney/Agent For Applicant

Attachments

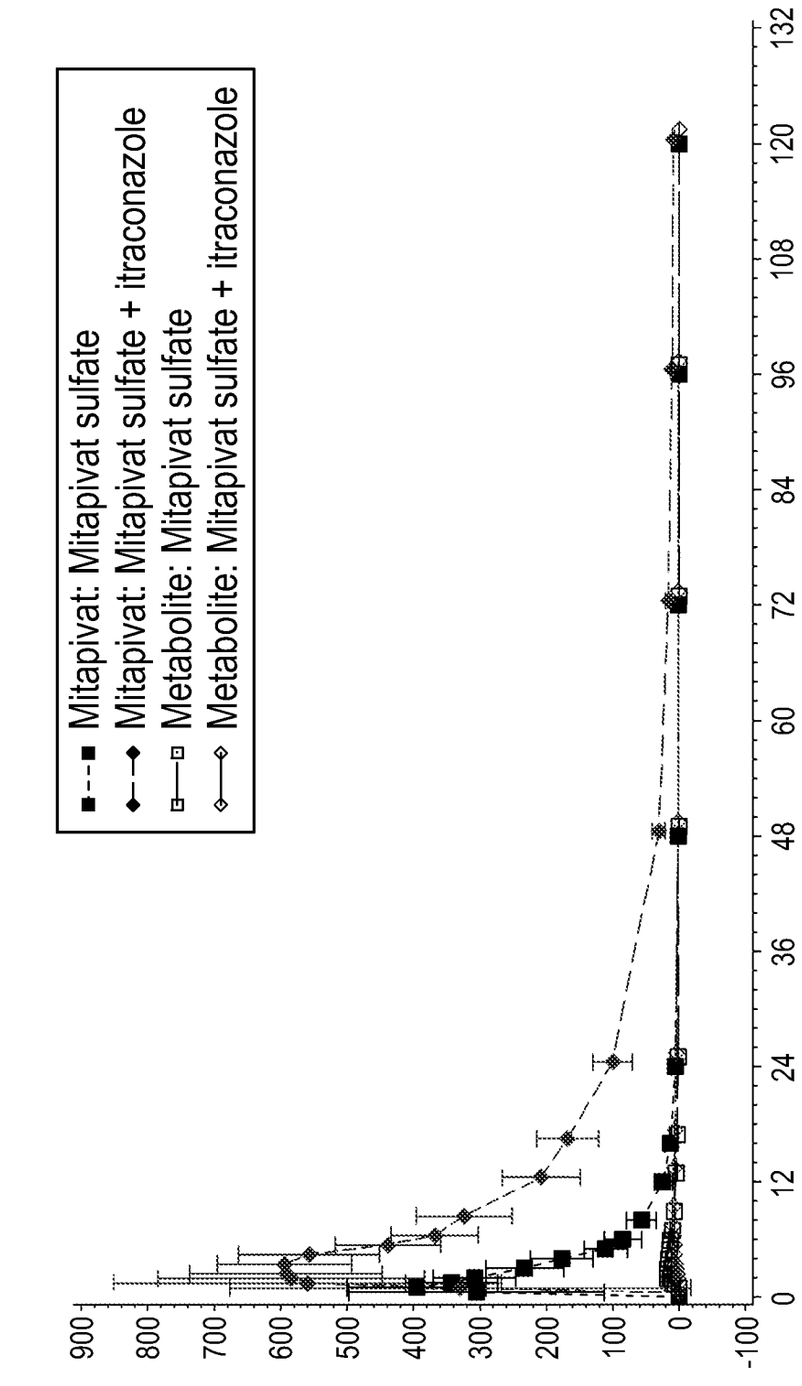

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
900
800
700
600
500
400
300
200
100
0
-100
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

FIG. 1

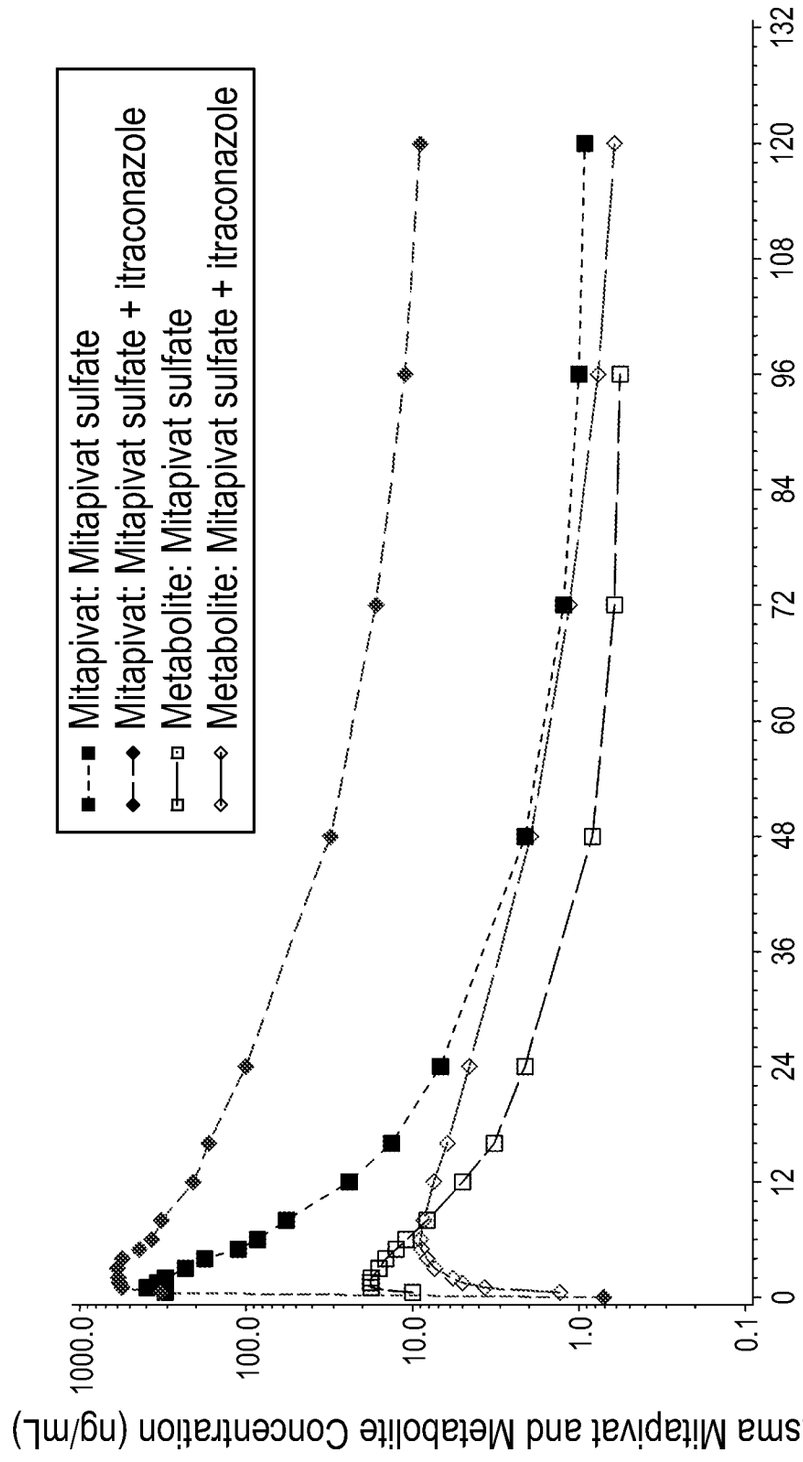

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + itraconazole
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + itraconazole
Plasma Mitapivat and Metabolite Concentration (ng/mL)
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

FIG. 2

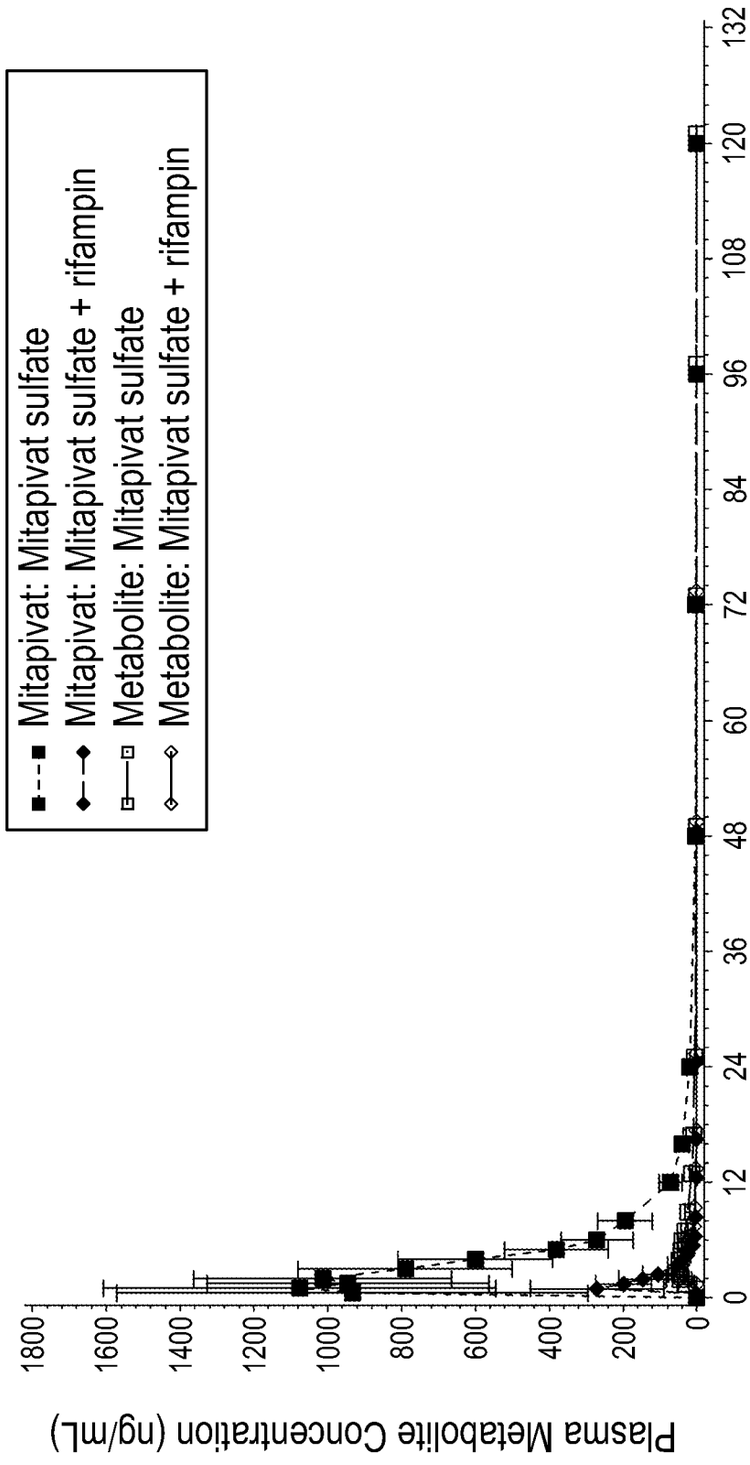

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
1800
1600
1400
1200
1000
800
600
400
200
0
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

FIG. 3

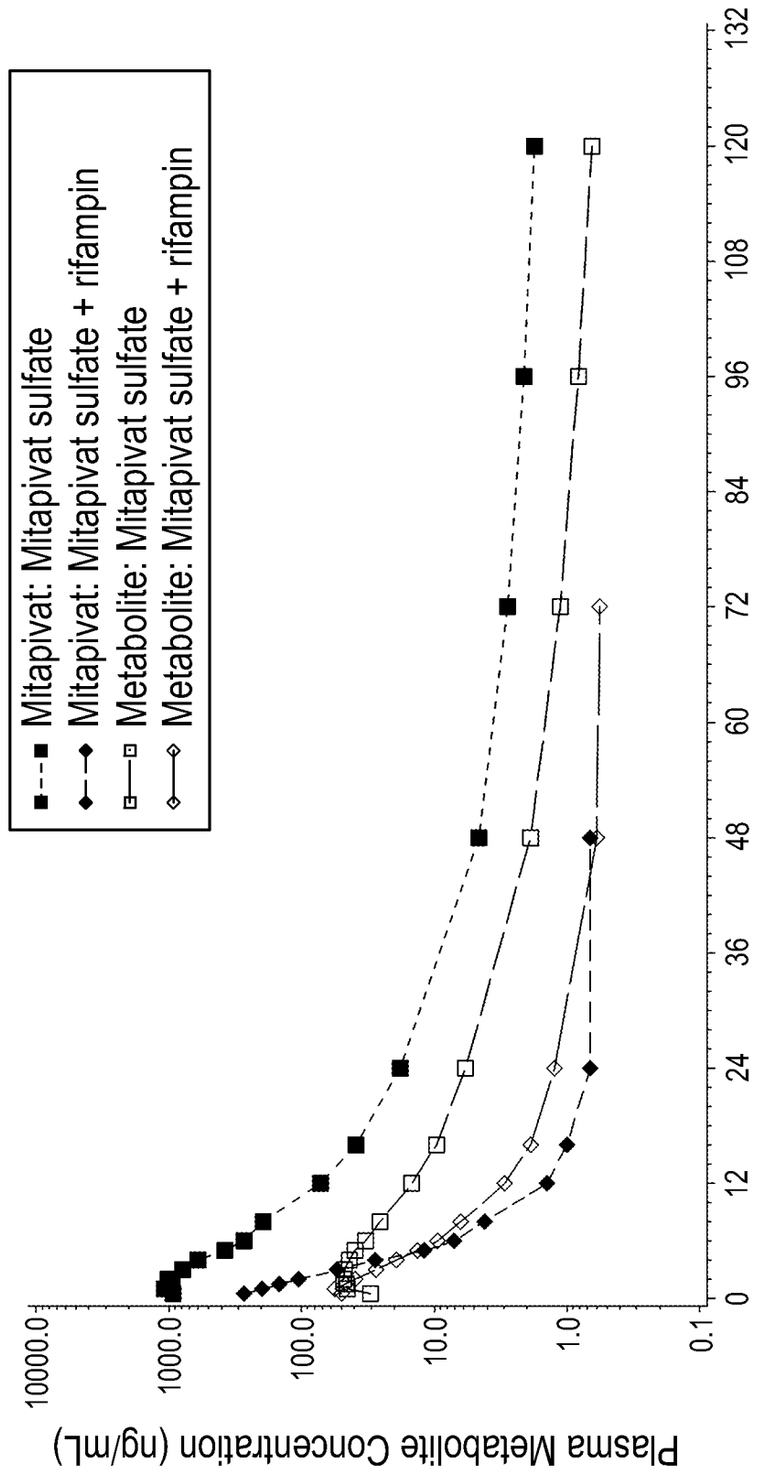

Mitapivat: Mitapivat sulfate
Mitapivat: Mitapivat sulfate + rifampin
Metabolite: Mitapivat sulfate
Metabolite: Mitapivat sulfate + rifampin
Plasma Metabolite Concentration (ng/mL)
10000.0
1000.0
100.0
10.0
1.0
0.1
0
12
24
36
48
60
72
84
96
108
120
132
Hours from Dosing

FIG. 4

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 40360569 |
| **Application Number:** | 16900610 |
| **International Application Number:** | |
| **Confirmation Number:** | 1054 |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Customer Number:** | 86738 |
| **Filer:** | Mei Bai |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 128748-01502 |
| **Receipt Date:** | 24-AUG-2020 |
| **Filing Date:** | 12-JUN-2020 |
| **Time Stamp:** | 12:59:37 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Application Data Sheet | Corrected_Application_Data_Sheet.pdf | 36051<br>bfbb36445d5107b4d5d034b27e2dea5010a8bc49 | no | 6 |

**Warnings:**

| Information: | |
|---|---|
| This is not an USPTO supplied ADS fillable form | |
| **Total Files Size (in bytes):** | 36051 |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

**Corrected Application Data Sheet**

**Inventor Information**

| | |
|---|---|
| Inventor Number:: | 1 |
| Given Name:: | Varsha |
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| Family Name:: | Yang |
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| State or Province of Residence:: | MA |
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| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | ~~02139~~ 01720 |

**Correspondence Information**

| | |
|---|---|
| Correspondence Customer Number:: | 86738 |
| E-Mail address:: | docket@mccarter.com |

**Application Information**

| | |
|---|---|
| Application Number:: | 16/900,610 |
| Filing Date:: | 06/12/20 |

| | |
|---|---|
| Application Type:: | NonProvisional |
| Subject Matter:: | Utility |
| CD-ROM or CD-R?:: | None |
| Sequence submission?:: | None |
| Computer Readable Form (CRF)?:: | No |
| Title:: | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| Attorney Docket Number:: | 128748-01502 |
| Request for Early Publication?:: | No |
| Request for Non-Publication?:: | No |
| Total Drawing Sheets:: | 4 |
| Small Entity?:: | No |
| Petition included?:: | No |
| Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2:: | No |

**Representative Information**

| | |
|---|---|
| Representative Customer Number:: | 86738 |

**Domestic Priority Information**

| Application:: | Continuity Type:: | Parent Application:: | Parent Filing Date:: | Prior Appl Status:: |
|---|---|---|---|---|
| This Application | Claims benefit of provisional | 62/861600 | 06/14/19 | Expired |

**Foreign Priority Information**

**Applicant Information**

| | |
|---|---|
| Applicant Number:: | 1 |
| Applicant Type:: | Assignee |
| Organization Name:: | Agios Pharmaceuticals, Inc. |
| Street of mailing address:: | 88 Sidney Street |
| City of mailing address:: | Cambridge |
| State or Province of mailing address:: | MA |
| Country of mailing address:: | US |
| Postal or Zip Code of mailing address:: | 02139 |

**Assignee Information Including Non-Applicant Assignee Information**

## Signature:

| | | | |
|---|---|---|---|
| **NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**<br>This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.<br>See 37 CFR 1.4(d) for the manner of making signatures and certifications. | | | |
| **Signature** | /Mei Bai/ | Date (YYYY-MM-DD) | August 24, 2020 |
| Name | Mei Bai, Ph.D. | Registration Number | 64,507 |

PTO/SB/92 (07-16)
Approved for use through 11/30/2020. OMB 0561-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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# Certificate of Mailing or Transmission under 37 CFR 1.8

I hereby certify that this correspondence is being:

1. ☐ Deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to:

   Commissioner for Patents
   P.O. Box 1450
   Alexandria, VA 22313-1450

   on _____ .
   Date

2. ☐ Facsimile transmitted to the United States Patent and Trademark Office, or

   OR

3. ☒ EFS-Web transmitted to the USPTO.

| /Mei Bai/ | August 24, 2020 |
|---|---|
| Signature | Date |
| Mei Bai, Ph.D. | (617) 449-6590 |
| Typed or printed name | Telephone number |

Note: Each paper must have its own certificate of mailing or transmission, or this certificate must identify each submitted paper:

Application Data Sheet (5 pages)

# United States Patent and Trademark Office

*Office of the Chief Financial Officer*

Document Code:WFEE

User :C46183

Sale Accounting Date:08/28/2020

| Sale Item Reference Number | Effective Date |
|---|---|
| 16900610 | 08/19/2020 |

| Document Number | Fee Code | Fee Code Description | Amount Paid | Payment Method |
|---|---|---|---|---|
| I20208RE41265617 | 1201 | INDEPENDENT CLAIMS IN EXCESS OF 3 | $1,380.00 | Deposit Account |
| I20208RE41265617 | 1051 | LATE FILING FEE FOR OATH OR DECLARATION | $160.00 | Deposit Account |

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/900,610 | 06/12/2020 | Varsha Venkatachalam Iyer | 128748-01502 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**INFORMAL NOTICE**

*OC000000119564427*

Date Mailed: 08/31/2020

## INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Varsha Venkatachalam Iyer
  Chandra Agarwal Prakash
  Hua Yang

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/erimando/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/900,610 | 06/12/2020 | Varsha Venkatachalam Iyer | 128748-01502 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**WITHDRAWAL NOTICE**

*OC000000119564418*

Date Mailed: 08/31/2020

## Letter Regarding a New Notice and/or the Status of the Application

If a new notice or Filing Receipt is enclosed, applicant may disregard the previous notice mailed on 08/24/2020. The time period for reply runs from the mail date of the new notice. Within the time period for reply, applicant is required to file a reply in compliance with the requirements set forth in the new notice to avoid abandonment of the application.

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web. https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If the reply is not filed electronically via EFS-Web, the reply must be accompanied by a copy of the new notice.

If the Office previously granted a petition to withdraw the holding of abandonment or a petition to revive under 37 CFR 1.137, the status of the application has been returned to pending status.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/erimando/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/900,610 | 06/12/2020 | Varsha Venkatachalam Iyer | 128748-01502 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**FORMALITIES LETTER**

*OC000000119403816*

Date Mailed: 08/24/2020

## NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

*Filing Date Granted*

The U.S. Patent and Trademark Office has received your reply on 08/19/2020 to the NOTICE TO FILE CORRECTED APPLICATION PAPERS (Notice) mailed 06/19/2020 and it has been entered into the application. The reply, however, is not a complete reply for the reason(s) listed below. A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application. Replies should be mailed to: Mail Stop Missing Parts, Commissioner for Patents, P.O.Box 1450, Alexandria VA 22313-1450.

**The period for reply continues to run from the date of the Notice mailed 06/19/2020.** Applicant must submit all required items and pay any fees required below within two months from the date of the NOTICE TO FILE CORRECTED APPLICATION PAPERS to avoid abandonment. If the reply is submitted after two months from the date of the NOTICE TO FILE CORRECTED APPLICATION PAPERS, extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

**Items Required to Avoid Abandonment:**

The required items noted below SHOULD be filed along with any items required above.

- Additional claim fees of $ **1380** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

**SUMMARY OF FEES DUE:**

The fee(s) required within **TWO MONTHS** from the date of the Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **160** surcharge.
- $ **1380** for **3** independent claims over 3.

- $( **0**) previous unapplied payment amount.
- $ **1540** TOTAL FEE BALANCE DUE.

**Items Required To Avoid Processing Delays:**

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Varsha Venkatachalam Iyer
  Chandra Agarwal Prakash
  Hua Yang

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/zabraha/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 16/900,610 | 06/12/2020 | 1629 | 3260 | 128748-01502 | 18 | 6 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**UPDATED FILING RECEIPT**

OC000000119584428

Date Mailed: 08/31/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

**Please verify the accuracy of the data presented on this receipt.** If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

**Inventor(s)**

Varsha Venkatachalam Iyer, Cambridge, MA;
Chandra Agarwal Prakash, North Andover, MA;
Hua Yang, Acton, MA;

**Applicant(s)**

Agios Pharmaceuticals, Inc., Cambridge, MA;

**Power of Attorney:** None

**Domestic Priority data as claimed by applicant**

This appln claims benefit of 62/861,600 06/14/2019

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
*Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.*

**Permission to Access Application via Priority Document Exchange:** Yes

**Permission to Access Search Results:** Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

**If Required, Foreign Filing License Granted:** 06/18/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/900,610**

**Projected Publication Date:** Request for Non-Publication Acknowledged

**Non-Publication Request:** Yes

**Early Publication Request:** No

**Title**

MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450

**Preliminary Class**

514

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

## PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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## Title 35, United States Code, Section 184

## Title 37, Code of Federal Regulations, 5.11 & 5.15

**GRANTED**

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

**NOT GRANTED**

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# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 40861224 |
| **Application Number:** | 16900610 |
| **International Application Number:** | |
| **Confirmation Number:** | 1054 |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Customer Number:** | 86738 |
| **Filer:** | Mei Bai |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 128748-01502 |
| **Receipt Date:** | 16-OCT-2020 |
| **Filing Date:** | 12-JUN-2020 |
| **Time Stamp:** | 15:06:09 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Oath or Declaration filed | Executed_DEC.PDF | 2711527<br>b9ad6eee48e500fd4977275cd8d02d654b685bc9 | no | 3 |

**Warnings:**

| Information: | | | | | |
|---|---|---|---|---|---|
| 2 | Power of Attorney | Executed_POA.PDF | 167910<br>19e6477b3e746aa43308ef265a9642c72f7c1317 | no | 1 |
| Warnings: | | | | | |
| Information: | | | | | |
| | | | Total Files Size (in bytes): | 2879437 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

## DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
|---|---|

As the below named inventor, I hereby declare that:

This declaration is directed to:

- [ ] The attached application, or
- [x] United States application or PCT international application number 16/900,610 filed on 06/12/2020.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby state that I have reviewed and understand the contents of the above identified specification,

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations § 1.56.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

**WARNING:**

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Varsha Venkatachalam Iyer Date (Optional): 27-August-2020

Signature: Varsha Iyer

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA/01 form for each additional inventor.

## DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| **Title of Invention** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
|---|---|

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 16/900,610 filed on 06/12/2020 .

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby state that I have reviewed and understand the contents of the above identified specification,

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations § 1.56.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

**WARNING:**

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Chandra Agarwal Prakash Date (Optional): _____

Signature: Chandra Prakash

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA/01 form for each additional inventor.

## DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
|---|---|

As the below named inventor, I hereby declare that

This declaration is directed to:

- [ ] The attached application, or
- [x] United States application or PCT international application number 16/900,610 filed on 06/12/2020

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby state that I have reviewed and understand the contents of the above identified specification,

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations § 1.56.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

**WARNING:**

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Hua Yang  Date (Optional): ST 07/31/2020

Signature:

Note: An application data sheet (PTO/AIA/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/SB/AIA/01 form for each additional inventor.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 03/31/2021. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

# POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

| Application Number | Filing Date |
|---|---|
| 16/900,610 | June 12, 2020 |

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 86738

**OR**

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

**Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:**

[X] The address associated with the above-mentioned Customer Number

**OR**

[ ] The address associated with Customer Number:

**OR**

| | | | | | |
|---|---|---|---|---|---|
| [ ] Firm or Individual Name | | | | | |
| Address | | | | | |
| City | | State | | Zip | |
| Country | | | | | |
| Telephone | | Email | | | |

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Agios Pharmaceuticals, Inc.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

**SIGNATURE of Applicant for Patent**

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

| | | | |
|---|---|---|---|
| Signature | | Date (Optional) | |
| Name | Rona A. Nardone | | |
| Title | Senior Director of Intellectual Property | | |

**NOTE:** Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted.

ME1 33620215v.1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/900,610 | 06/12/2020 | Varsha Venkatachalam Iyer | 128748-01502 |

**CONFIRMATION NO. 1054**

86738
MCCARTER & ENGLISH, LLP BOSTON
265 Franklin Street
Boston, MA 02110

**POA ACCEPTANCE LETTER**

*OC000000120740544*

Date Mailed: 10/21/2020

## NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 10/16/2020.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/mbeyene/

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 42425101 |
| **Application Number:** | 16900610 |
| **International Application Number:** | |
| **Confirmation Number:** | 1054 |
| **Title of Invention:** | MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 |
| **First Named Inventor/Applicant Name:** | Varsha Venkatachalam Iyer |
| **Customer Number:** | 86738 |
| **Filer:** | Mei Bai |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | 128748-01502 |
| **Receipt Date:** | 12-APR-2021 |
| **Filing Date:** | 12-JUN-2020 |
| **Time Stamp:** | 14:58:45 |
| **Application Type:** | Utility under 35 USC 111(a) |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Transmittal Letter | Information_Disclosure_Statement.pdf | 25427<br>d7d5e468cbd53e517a13c1a4c2cde48181de70dd | no | 2 |

**Warnings:**

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | IDS_Citation_by_Applicant_99_References.pdf | 109468<br>50557c5453a8c3a3073b6435b22acf6852caf5b0 | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 3 | Foreign Reference | WO2016201227A1.PDF | 2724324<br>9ac465b130e7202180b8650cd978fd69ff0ca8e1 | no | 70 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Non Patent Literature | Academic_Press_Practical_Process_Research_Development_223_2000.PDF | 162462<br>936801c17edce6b7dacc72253543f4e318ed921c | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Non Patent Literature | 20210212_AGI-PKR-16US_2015_Dec3_Abstract_DRIVE_PK_A_Phase_2_trial_of_AG-348_in_patients_wi.pdf | 133807<br>2c8029df5a1ba38a0e8536f87c35984ef64ec5f0 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Non Patent Literature | 20210209_AGI-PKR-16US_2015_Nov21_Abstract_Activator_treatment_for_pyruvate_kinase_deficien.pdf | 184522<br>b6c38597b13c9e2bd1939839ebe097a12599eeb0 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 7 | Non Patent Literature | Barbier_ESH_ENERCA_Presentation_1-29-2016.PDF | 3772382<br>10320ff24c72a14899c41421742e5c72c6ece09c | no | 20 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 8 | Non Patent Literature | 20210212_AGI-PKR-16US_2015_EHA_PyruvateKinaseDeficiency_ClincialDevelopmentUpdate.pdf | 565584<br>a0955a82e25571727 4706efdff151bebe517656d | no | 3 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |

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|---|---|---|---|---|---|
| 9 | Non Patent Literature | Berge_J_Pharmaceutical_Sciences_66_1_1977.PDF | 4476288<br>cf404ad9f149b2766b5f6cbe06686f31dd7cff6e | no | 19 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 10 | Non Patent Literature | Byrn_et_al.PDF | 786966<br>cfc367d59e5d4ba927a210ac52a28bf9f5eb1aff | no | 10 |
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| **Information:** | | | | | |
| 11 | Non Patent Literature | Caira_Topics_in_Current_Chemistry_198_163_1998.PDF | 9636998<br>985193b616a948807cf750544876390ba75040bc | no | 46 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 12 | Non Patent Literature | Chemical_Abstract_Service_Accession_No_2151847-10-6_2017.PDF | 64058<br>52c2f63767471f6eb2fc6a5271bdf896e547d21b | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 13 | Non Patent Literature | EHA_20_Poster_6-13-2015.PDF | 157505<br>0eab3991d99f5a9c4e3c24fc8d5a6597477abdeb | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
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| 15 | Non Patent Literature | ASH_58_Poster_12-5-2016.PDF | 741479<br>744462b372cf292865c7b44063b524ffd0277f1c | no | 1 |
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| 17 | Non Patent Literature | 20210212_AGI-PKR-16US_2014_Sep_29_Presentation_CPSA_-_PKR_presentation_-_Oct_2014_-_final.pdf | 3084698<br>0608216f0ef3ecf3fd9b6c0de036d069cfca601b | no | 22 |
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| 18 | Non Patent Literature | 20210212_AGI-PKR-16US_2017_Mar15_Presentation_Using_metabolism_to_support_drug_discovery.pdf | 6141645<br>61ea743fc6a23f76164174091eefa6a056550ac0 | no | 22 |
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| 19 | Non Patent Literature | EHA_SWG_Poster_11-7-2019.PDF | 505117<br>c4156157e5fd20cd70202cfdb669336502734038 | no | 1 |
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| 20 | Non Patent Literature | Grace_ASH_58_Presentation_12-4-2016.PDF | 2838855<br>7f6ad42d30b32cbe255b057080308fd381c3c715 | no | 14 |
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| 22 | Non Patent Literature | ASH_59_Poster_12-10-2017.PDF | 211251<br>8d52e5668e67b87ea2e4fbd61b776cb7d060af3c | no | 1 |
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| 23 | Non Patent Literature | 20210209_AGI-PKR-16US_2017_Dec9_Abstract_Results_update_from_the_DRIVE_PK_study.pdf | 155924<br>caf92220341e071df266f75bd36db1bcfc8221ad | no | 2 |
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| 24 | Non Patent Literature | ASH_61_LT_Poster_12-9-2019.PDF | 168966<br>c0f647f4e856804b26482c27cded9ddb19fe1403 | no | 1 |
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| 25 | Non Patent Literature | 20210212_AGI-PKR-16US_2019_Dec3_Abstract_Long-term_safety_and_efficacy_of_mitapivat_AG-34.pdf | 1789912<br>287c9902d86db1da059e0fd8376aebf5befc25ff | no | 5 |
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| 26 | Non Patent Literature | 20210212_AGI-PKR-16US_2017_Sep3_BritishBloodTransfusion_presentation.pdf | 1058063<br>752147fe67b6e14693ab46d3389b151ee14efc37 | no | 19 |
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| 27 | Non Patent Literature | 20210212_AGI-PKR-16US_2016_June9_Abstract_Effects_of_AG-348__a_pyruvate_kinase_activator__.PDF | 51057<br>43b656de7323875bdb5e7931b497f6a48bbffe11 | no | 1 |
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| 28 | Non Patent Literature | 20210212_AGI-PKR-16US_2016_June11_presentation_Effects_of_AG-348__a_pyruvate_kinase_activa.pdf | 1121706<br>d2fb405533c8fbda2cc39a37f57838e8da664db9 | no | 17 |
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| 30 | Non Patent Literature | 20210212_AGI-PKR-16US_2017_June22_Presentation_Effects_of_AG-348__a_pyruvate_kinase_activa.pdf | 701100<br>b51f4fddf41abc58de7668ae87320848ae63e40e | no | 17 |
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| 37 | Non Patent Literature | 20210203_AGI-PKR-16US_2017_July19_TREATMENT_OF_RARE_GENETIC_DISEASES.pdf | 5300422<br>e76094fb0b0acae1920fc1319f5f2da2b4a8647c | no | 45 |
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| 58 | Non Patent Literature | 20210212_AGI-PKR-16US_2019_Dec3_Abstract_Mitapivat_AG-348__an_oral_PK-R_activator__in_ad.pdf | 166043<br>6a1c93e56d01eb3989863bf8e7825c15f6294e0f | no | 2 |
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| 59 | Non Patent Literature | ASH_M2_61_Poster_12-8-2019.PDF | 204836<br>fb1c8f1b13b3595a9cc92197f939f0df66890533 | no | 1 |
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| 60 | Non Patent Literature | 20210203_AGI-PKR-16US_2020_June11_EHAP roof_of_concept_for_the_oral_pyruvate_kinase_activat.PDF | 65992<br>4d59a0302b47792ae3129d6acb1772e7c78c5e02 | no | 2 |
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Dated: April 12, 2021
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Docket No.: 128748-01502
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Utility Application of:
Varsha Venkatachalam Iyer et al.

Application No.: 16/900,610 Confirmation No.: 1054

Filed: June 12, 2020 Art Unit: 1653

For: MITAPIVAT THERAPY AND MODULATORS OF CYTOCHROME P450 Examiner: Not Yet Assigned

**INFORMATION DISCLOSURE STATEMENT (IDS)**

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Commissioner for Patents
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Dear Sir:

Pursuant to 37 C.F.R. §§ 1.56, 1.97, and 1.98, the attention of the United States Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of the above-identified application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 C.F.R. § 1.97(b)(3)).

In accordance with 37 C.F.R. § 1.98(a)(2)(ii), Applicant has not submitted copies of the U.S. patent and U.S. patent application. Applicant submits herewith copies of the foreign patent and non-patent literature in accordance with 37 C.F.R. § 1.98(a)(2).

In accordance with 37 C.F.R. § 1.97, Applicant hereby cites the following additional patent application directed to related subject matter:

| U.S. Application | Filing Date | Publication No. | Status | Atty. Docket No. |
|---|---|---|---|---|
| 16/765,456 | May 19, 2020 | 2020-0277279 | Published | 128748-00403 |

In accordance with 37 C.F.R. § 1.97(g), the filing of this Information Disclosure Statement shall not be construed as a representation that a search has been made. In accordance with 37 C.F.R. § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in this Information Disclosure Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

It is submitted that the Information Disclosure Statement is in compliance with 37 C.F.R. § 1.98, and the Examiner is respectfully requested to consider the listed references.

Applicant believes no fee is due with this response.

Dated: April 12, 2021

Respectfully submitted,

Electronic signature: /Mei Bai, Ph.D./
Mei Bai, Ph.D.
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PTO/SB/92 (07-16)
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| **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** | | | | **Application Number** | 16/900,610 |
| | | | | **Filing Date** | June 12, 2020 |
| | | | | **First Named Inventor** | Varsha Venkatachalam Iyer |
| ***(Use as many sheets as necessary)*** | | | | Art Unit | 1653 |
| | | | | Examiner Name | Not Yet Assigned |
| Sheet | 1 | of | 8 | Attorney Docket Number | 128748-01502 |

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| **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** | | | | Application Number | 16/900,610 |
| | | | | Filing Date | June 12, 2020 |
| | | | | First Named Inventor | Varsha Venkatachalam Iyer |
| *(Use as many sheets as necessary)* | | | | Art Unit | 1653 |
| | | | | Examiner Name | Not Yet Assigned |
| Sheet | 2 | of | 8 | Attorney Docket Number | 128748-01502 |

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| | | | | Examiner Name | Not Yet Assigned |
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

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CHAPTER 11

# *Tools for Purifying the Product: Column Chromatography, Crystallization and Reslurrying*

## 1. INTRODUCTION

The chemist has many options for purifying and isolating intermediates and final products. The operations preferred on scale differ from those routinely used in the laboratory, as discussed in Chapter 1. Deferred purification by telescoping should not be overlooked as a productivity-boosting option. Purification may include extractions and polish filtration performed as part of work-up (Chapter 10) and subsequent chromatography, crystallization, and/or reslurrying. Liquid products may be purified and isolated by distillation. Considering the drawbacks of chromatography on scale, chromatographic purifications are generally used only when reaction optimization and non-chromatographic means of purification prove inadequate to prepare high-quality product. Distillation on scale usually requires specialized equipment and is limited to relatively low molecular weight compounds that do not decompose during heating.

Isolation of solid intermediates and final product is often achieved by crystallization. Intermediates may be upgraded by crystallization, decreasing the burden on the purification of the final product. Under controlled conditions, crystallization generally provides the best purification of a product, and it may be essential



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for preparing API of the desired purity and polymorphic state. As crystallization on scale can be labor-intensive and equipment-intensive, care must be taken to institute a rugged crystallization process.

The following sections discuss chromatography on scale first, as compounds are usually crystallized after chromatographic purification. The theory of crystallization is described, followed by a discussion on morphic states and salt selection. Guidelines are presented to predict how readily a crystallization and isolation process can be performed on scale. Tips on washing a filter cake and drying are followed by a description of reslurrying as an optional alternative to recrystallizing a solid.

## II. PURIFICATION BY COLUMN CHROMATOGRAPHY

Column chromatography may be used for purification of compounds in the kilo lab and on larger scale. For the kilo lab, wide-diameter columns such as those described by Still for flash chromatography [1] are preferred. TLC can be used to select a preparative chromatographic eluant for flash chromatography, and chromatographic separations can also be examined on a small scale before valuable material is committed to large-scale chromatography.

**TIPS:**

- The key in efficiently using column chromatography is to develop a chromatographic system that maximizes the differences in elution times between the desired product and the impurities. The optimal separation occurs when the impurities do not move from the origin and the product has good mobility, e.g., moves half-way up the TLC plate. Under these conditions the solid phase is used as an absorbent, and a small amount of solvent can be used to separate the product from the impurities.
- To speed chromatographic operations, pressurize a chromatography column with an inert gas, or apply suction to the collection side of the column. Pressure from an inert gas should be applied with care, and applying suction is safer.
- Large-scale chromatography units are commercially available for preparative purifications.
- Preparative chromatography should always be carried out in well-ventilated areas. When using large amounts of electrostatic solvents, e.g., hexane, care should be taken to ground equipment and prevent fires due to electrostatic discharge. Heptane is safer than hexane (see Table 5.3).

Ex. 2001-0358

# DRIVE PK: A Phase 2 Trial of AG-348 in Patients with Pyruvate Kinase Deficiency

**Ann Barbier**[1*], Bruce Silver[2*], Elizabeth Merica[1*], Marvin Cohen[3*], Charles Kung[1*], Hua Yang, PhD[1*], Rachael F Grace[4], Bertil Glader, MD, PhD[5] and Sam Agresta, MD[1]

[1]Agios Pharmaceuticals, Cambridge, MA; [2]Bruce A Silver Clinical Science and Development, Dunkirk, MD; [3]MBC Pharma Solutions, Newtown, PA; [4]Harvard Medical School, Dana Farber Boston Children's Cancer and Blood Disorder Center, Boston, MA; [5]Stanford University, Lucile Packard Children's Hospital, Palo Alto, CA

INTRODUCTION: Pyruvate kinase (PK) deficiency is a congenital hemolytic anemia caused by deficient function of the PK enzyme. This condition is currently managed with supportive care, including transfusions, folate supplementation, splenectomy, and reducing iron overload, but there are no treatments specifically targeting the underlying metabolic defect. AG-348 is a novel, orally available, small molecule activator of wild-type and mutant red blood cell PK.

METHODS: Results of single-ascending dose (SAD) and multiple-ascending dose (MAD) studies in healthy adult volunteers (Yang H et al. *Blood* 2014;124[21]:4007; Yang H et al. *Haematologica* 2015;100[s1]:Abs S138) indicated good safety and tolerability and predictable pharmacokinetics at doses that demonstrated significant pharmacodynamic responses of increased whole blood adenosine triphosphate (ATP) and decreased 2,3-diphosphoglycerate (2,3-DPG) levels. In pre-clinical studies AG-348 inhibited the enzyme aromatase, and sex hormone profiles in the MAD study showed signs of aromatase inhibition.

RESULTS: DRIVE PK is a multicenter, international, randomized, open-label, two-arm dose ranging trial of AG-348 in adults with PK deficiency. DRIVE PK is currently open for enrollment. Eligible patients are randomized to either low dose (50 mg BID) or high dose (300 mg BID) arms and receive treatment for 6 months, with the option of extended treatment. Patients are stratified by genotype to allow for genotype-phenotype correlations. A third dose may be added based on the observations made in the two original arms. The primary objective is to evaluate the safety and tolerability of AG-348. Secondary objectives include evaluation of pharmacokinetics and pharmacodynamics, as measured by AG-348, ATP and 2,3-DPG levels. A range of biochemical markers of clinical benefit will be assessed, including hemoglobin levels, reticulocyte count, bilirubin, erythropoietin, ferritin, transferrin saturation, and haptoglobin. PK activity, glycolytic flux assay and PK protein levels in blood will be analyzed as exploratory endpoints. The trial population consists of adult patients with PK deficiency with hemoglobin levels at screening of ≤12.0 g/dL (men) or ≤11.0 g/dL (women), who have received no more than three units of red blood cells in the 12 months preceding the first dose of AG-348, and no transfusion within 4 months. Full inclusion/exclusion criteria can be found on www.clinicaltrials.gov, NCT02476916.

CONCLUSION: DRIVE PK, an ongoing phase 2 trial of the PK activator, AG-348, is the first interventional trial to target the underlying metabolic defect in patients with PK deficiency. This flexible trial with two dose arms, and potential for a third dose arm, is designed to evaluate safety and tolerability, pharmacokinetic and pharmacodynamic responses, as well as hematological markers of red blood cell metabolism.



Ex. 2001-0359

**Activator Treatment for Pyruvate Kinase Deficiency – Results from Phase 1 and Overview of Phase 2 trial**

Ann Barbier, MD, PhD, Vice President, Clinical Development, Rare Genetic Diseases.
Agios Pharmaceuticals, Cambridge

Pyruvate kinase (PK) deficiency is a rare genetic disorder of metabolism resulting in chronic hemolytic anemia, with comorbidities including iron overload and multi-organ dysfunction. Defective glycolysis in red blood cells leads to increases in the metabolic precursor, 2,3-diphosphoglycerate (2,3-DPG), and deficiency of the product, adenosine triphosphate (ATP). AG-348 is an oral allosteric activator of both wild type and multiple mutant PK-Rs.

**Aims:** To assess safety, tolerability, and pharmacokinetics/pharmacodynamics (PK/PD) of AG-348 in healthy volunteers and identify a dosing schedule for future trials in patients with PK deficiency.

**Methods:** A phase 1, single-center, randomized, double-blind, placebo-controlled MAD study (NCT02149966) was conducted in healthy men and women (18–60 years) in 6 sequential cohorts (each cohort: n=6 AG-348, n=2 placebo [P]). Subjects gave informed consent and received oral AG-348 at 15–700 mg twice daily (q12h) or 120 mg once daily (q24h) for 14 days with follow-up to Day 29. Adverse events (AEs), laboratory parameters, ECGs, and vital signs were monitored. Plasma AG-348 concentrations and whole blood 2,3-DPG and ATP levels were measured in serial blood samples for PK/PD assessment. Hormone levels were monitored due to pre-clinical data suggesting potential modulation.

**Results**: 48 subjects (42M/6F), mean age 41.5 (range, 25–60) years, enrolled. Final, un-blinded safety data showed ≥1 AE in 16/36 (44%) AG-348-treated and 4/12 (33%) Placebo subjects. Treatment-related AEs (≥1) were noted in 11/36 (31%) AG-348-treated and 3/12 (25%) Placebo subjects. All treatment-related AEs were mild or moderate in severity (only one grade 3 event) and often reversible despite continued dosing. The most frequent AG-348-related AEs were nausea and headache, 5/36 (14%) for each. Gastrointestinal AEs occurred in AG-348-treated subjects only at the highest dose, 700 mg q12h. One grade 3 AE occurred (AG-348 700 mg q12h, elevated liver function tests [LFTs], which resolved after treatment discontinuation). There were 4 AG-348 discontinuations: due to AEs in 2 subjects (grade 2 drug eruption, 60 mg q12h; grade 3 elevated LFTs, 700 mg q12h), and 2 subjects withdrew consent (both had grade 1/2 nausea and grade 1/1 vomiting; both at 700 mg q12h). The highest well-tolerated dose was 360 mg q12h (doses between 360 and 700 mg were not explored). Plasma AG-348 exposure was dose-dependent, with low to moderate variability in the PK parameters of AG-348 and its metabolite AGI-8702. There was a dose-dependent decrease in 2,3-DPG and increase in ATP with the effects plateauing at 360 mg q12h. Decrease in 2,3-DPG was robust after Dose 1, while the increase in ATP occurred gradually and was strongly evident at Day 8. Change from baseline in 2,3-DPG and ATP plateaued at ~300 µg/mL (~50% decrease) and ~175 µg/mL (~50% increase), respectively.

**Summary/Conclusion**: AG-348 showed a robust 2,3-DPG/ATP PD glycolytic profile at well-tolerated doses in healthy volunteers. The results support proceeding with a planned phase 2 trial in patients with PK deficiency.

# Financial Disclosure

| | |
|---|---|
| Name of Company | Agios Pharmaceuticals |
| Research support | |
| Employee | X |
| Consultant | |
| Major Stockholder | |
| Speakers' Bureau | |
| Scientific Advisory Bd. | |
| Other | |

# 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

**Ann Barbier[1] 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 European School of Hematology, Lisbon, January 2016

Ex. 2001-0362

# Disclosures

- This study was funded by Agios Pharmaceuticals
- Hua Yang, Elizabeth Merica, Yue Chen, Hyeryun Kim, Penelope A Kosinski, Charles Kung, Meredith Goldwasser, Luke Utley, Sam Agresta: Agios – employees and stockholders
- Marvin Cohen, Bruce Silver: Agios – consultants
- Ronald Goldwater: Parexel International – employee
- Editorial assistance was provided by Christine Tomlins, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios

# Key Points

- Pyruvate Kinase (PK) deficiency is a serious, rare, genetic disease.
- AG-348 is a PK-R activator intended to correct the glycolytic pathway defect in patients with PK deficiency.
- Healthy volunteer study has established a safety profile at pharmacodynamically active doses that may be relevant to patients with PK deficiency.
- DRIVE PK, a global phase 2 study in patients, is now open and enrolling

# PK Deficiency is a Severe Red Blood Cell Disease

## Disease overview

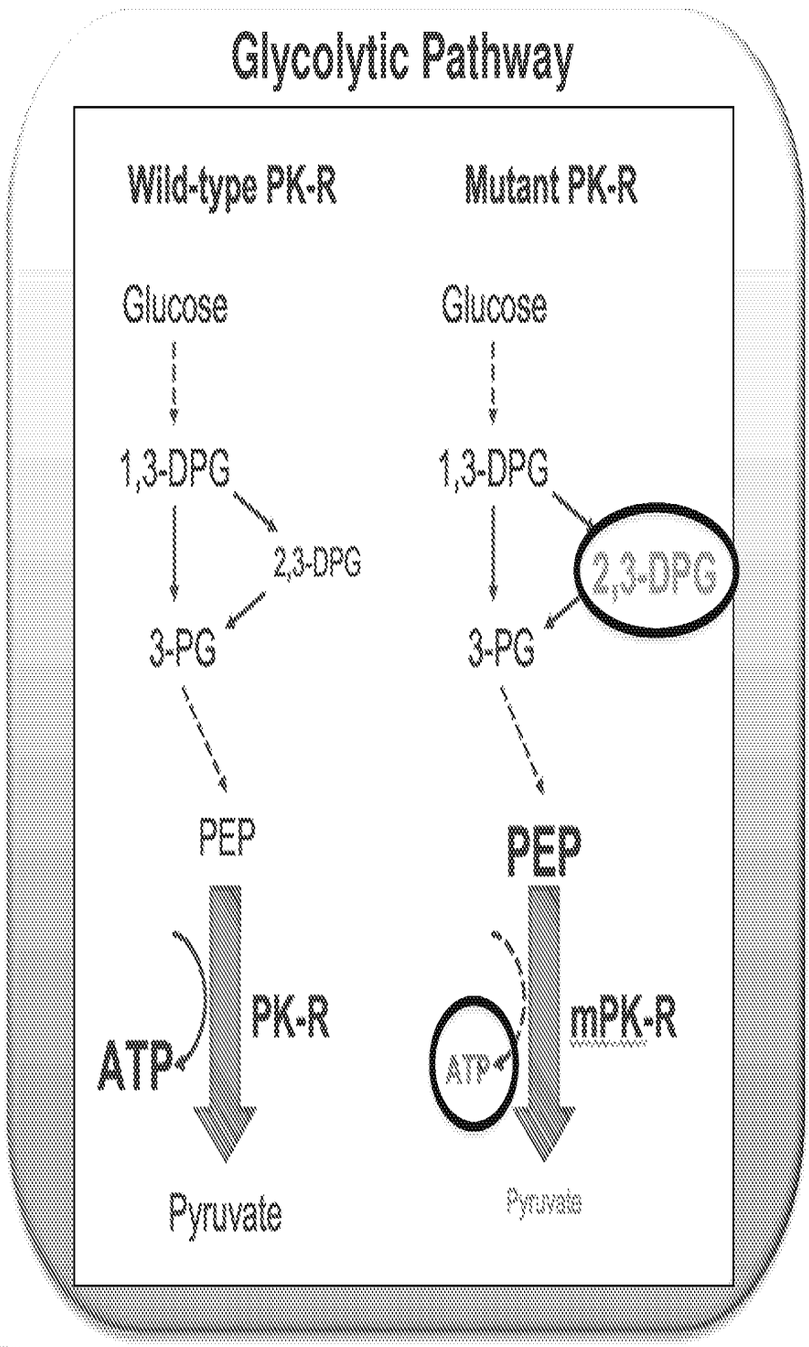
| | |
|---|---|
| Description | • Presents at birth or in childhood with severe hemolytic anemia<br>• Estimated 2,400 patients diagnosed in the US and EU |
| Etiology | • Caused by mutations in the *PK-LR* gene coding for erythrocyte pyruvate kinase (PK-R) |
| Clinical Presentation | • Lifelong hemolytic anemia<br>• Iron overload and jaundice<br>• Infection and aplastic anemia |
| Diagnosis/ Treatment | • PK-R enzyme activity and/or genetic testing<br>• Supportive treatment: transfusions, splenectomy, iron chelation |

Sources: Zanella A et al. *Blood Rev* 2007;21:217-31; Blood and Bone Marrow Pathology; Wintrobe's Clinical Hematology; Physician Interviews; Market Research



Ex. 2001-0365

# Mutant PK-R: ATP Deficiency in Red Blood Cells

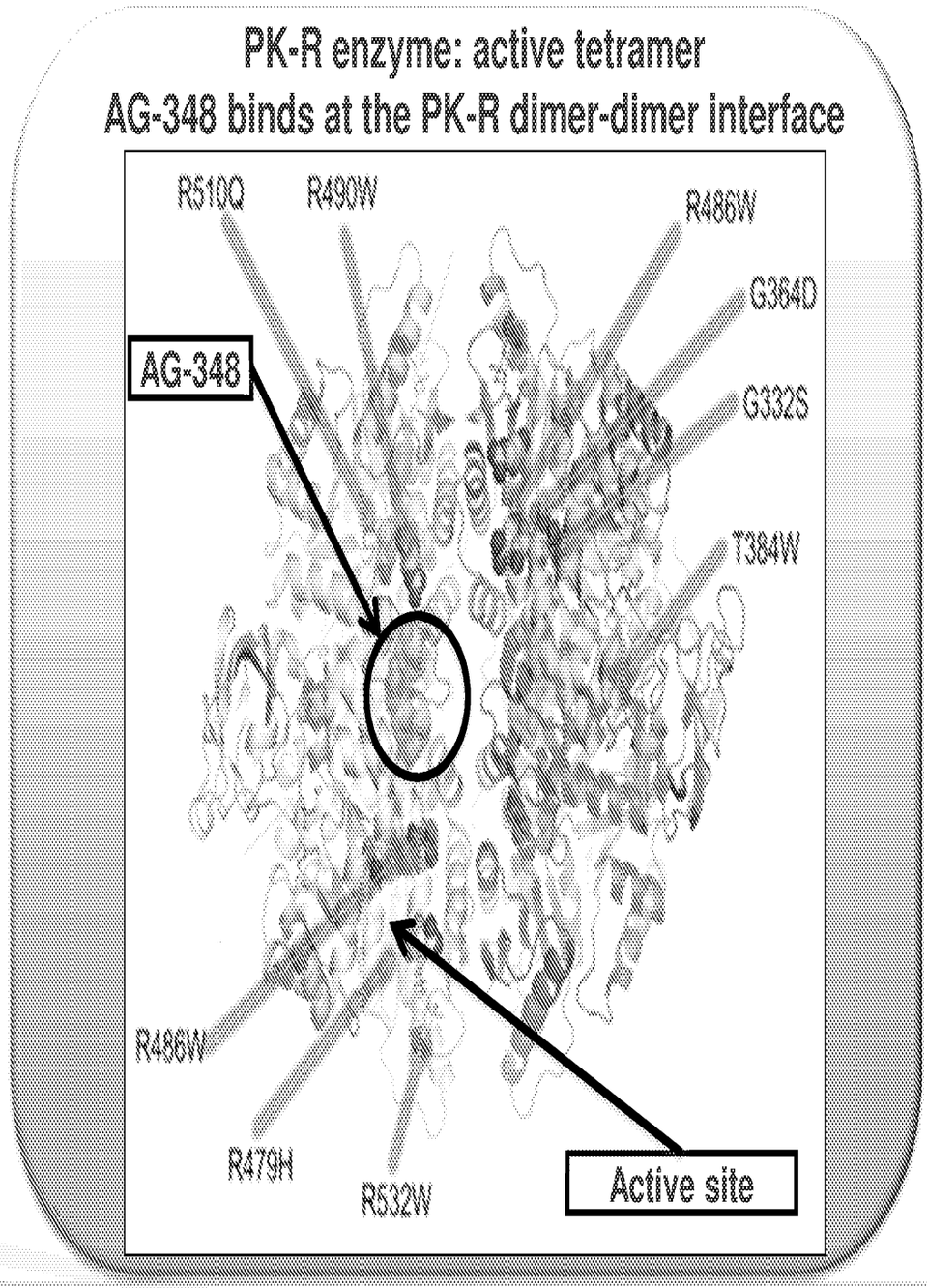



Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010



Ex. 2001-0366

# AG-348: Allosteric Activator of Wild-Type and Mutant PK-R

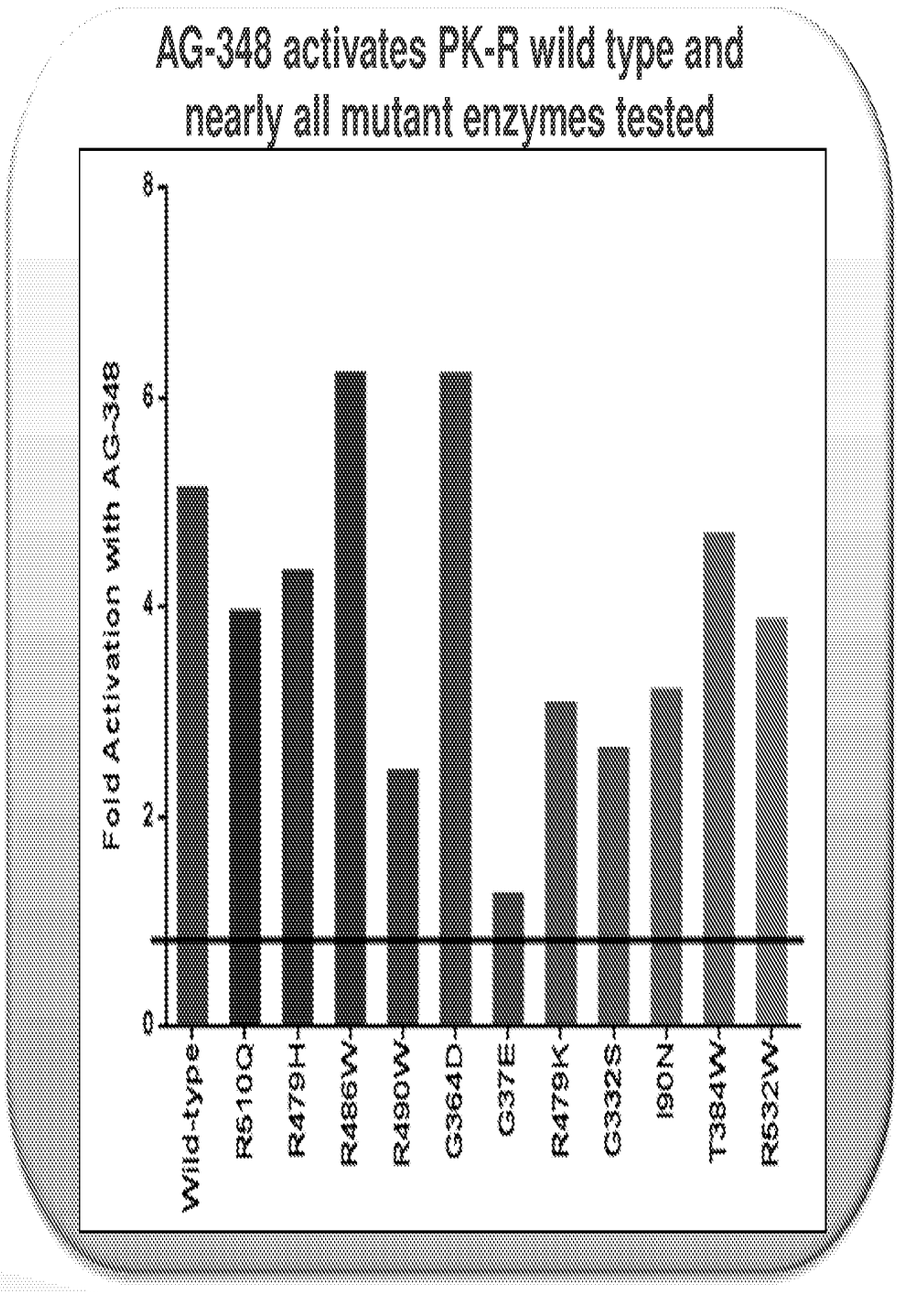



Kung C et al. 55th ASH Annual meeting 2013, Abstract 2180



Ex. 2001-0367

# AG-348 Activates PK-R Wild-Type and Mutant Subtypes

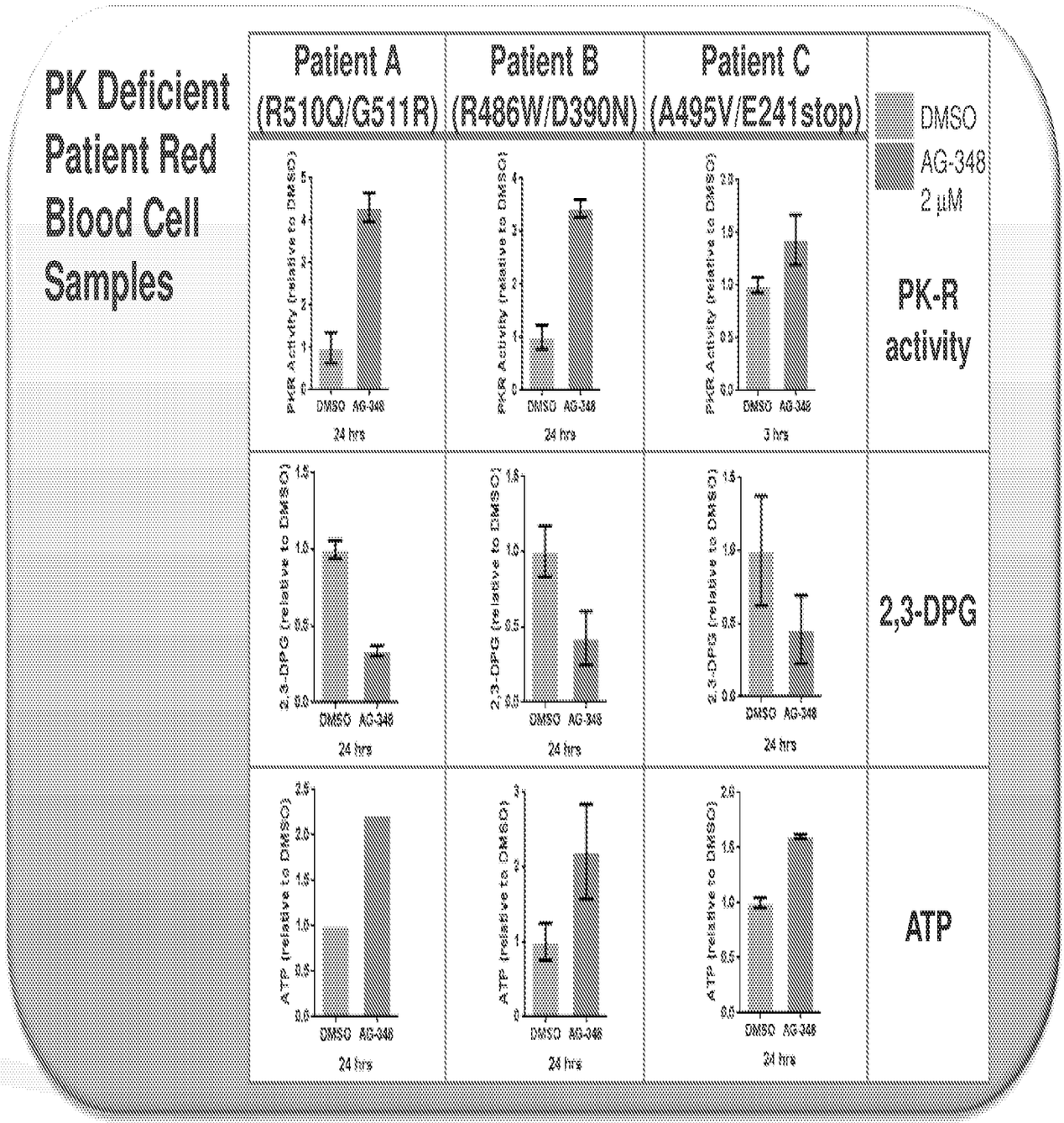



Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010



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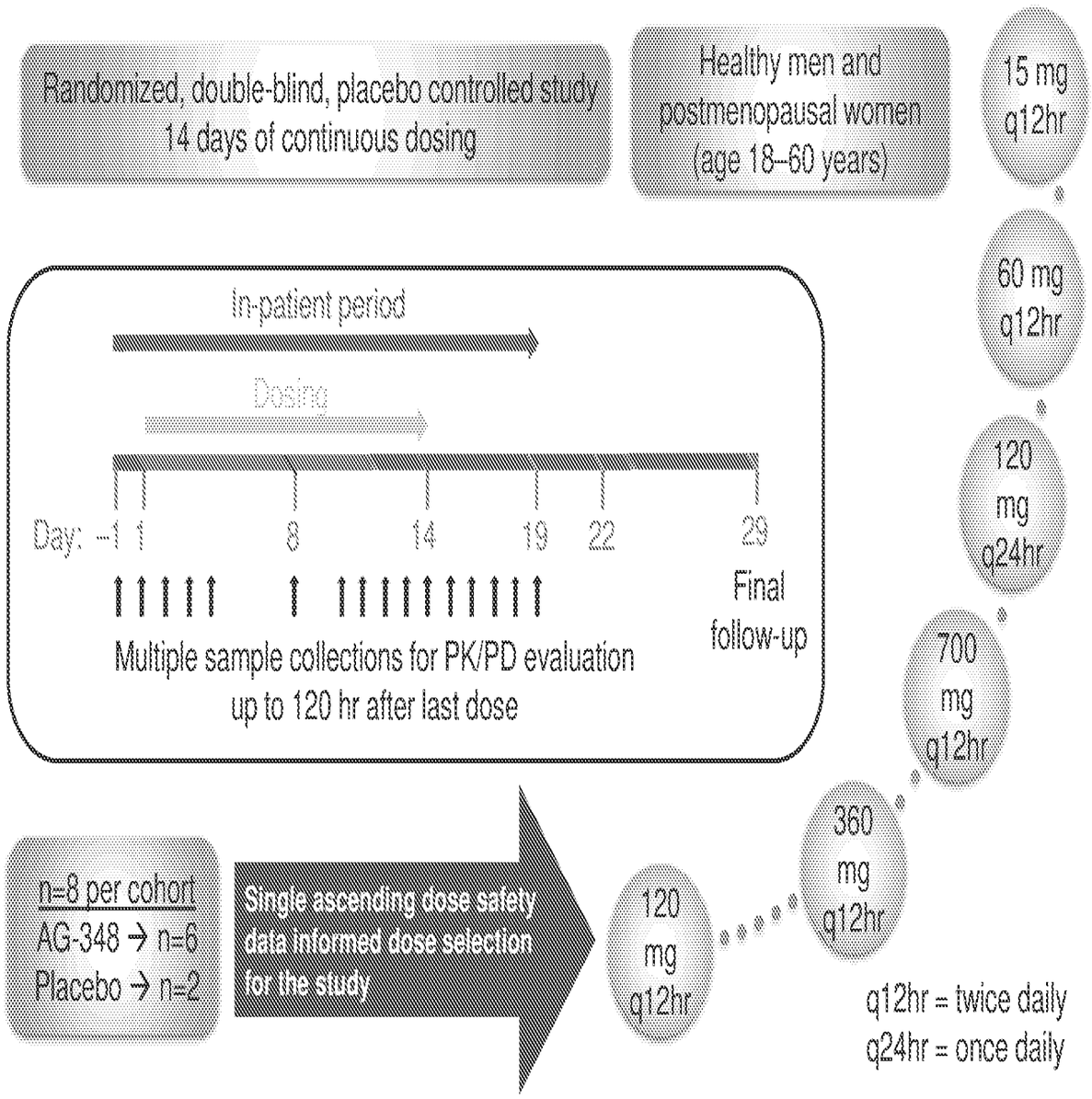
# AG-348 Restores the Glycolytic Pathway in Models




Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010

# Multiple Ascending Dose Study

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




PK/PD = pharmacokinetic/pharmacodynamic

NCT02149966



Ex. 2001-0370

# Demographics and Disposition

| Characteristic | Placebo n=12 | AG-348 n=36 |
|---|---|---|
| Men, n (%) | 9 (75) | 33 (92) |
| Women, n (%) | 3 (25) | 3 (8) |
| Age in years, mean (SD) | 37.8 (11.4) | 42.8 (12.2) |
| Race, n (%) | | |
| Asian | — | 1 (3) |
| Black | 6 (50) | 18 (50) |
| White | 6 (50) | 17 (47) |
| Body mass index in kg/m$^2$, mean (SD) | 27.4 (2.3) | 26.5 (2.0) |

- 12 placebo treated and 32 of 36 AG-348 treated patients completed study:
  - Two withdrew due to AEs: Drug eruption (60 mg q12hr), grade 3 liver function test abnormalities at the highest dose explored (700 mg q12hr)
  - Two withdrew consent due to nausea and vomiting at the highest dose explored (700 mg q12hr)

AE = adverse event; SD = standard deviation



Ex. 2001-0371

# AG-348 was well tolerated up to 360 mg oral q12 hr

- Drug-related events were grade 1 and 2
- One grade 3 event at 700 mg q12h (increased LFTs – DLT)

| 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) | — | — |

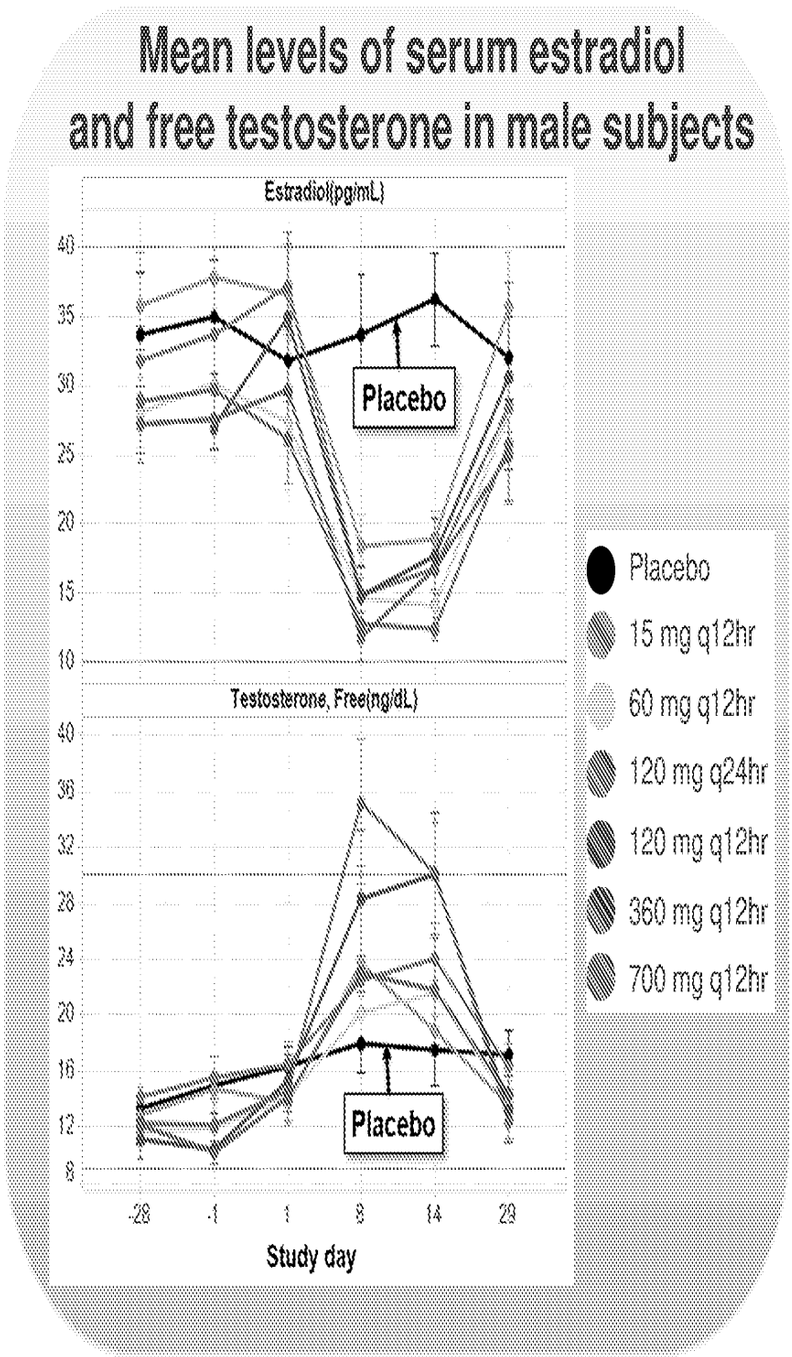
AEs were graded using National Cancer Institute Common Terminology Criteria, version 4.03



Ex. 2001-0372

# Hormone Changes: Reversible Aromatase Inhibition

- Changes in serum androgens and estrogens were observed
- Most changes remained within normal reference ranges for age and sex
- Levels recovered to baseline
- Endocrine/physiologic significance to be evaluated in phase 2 study



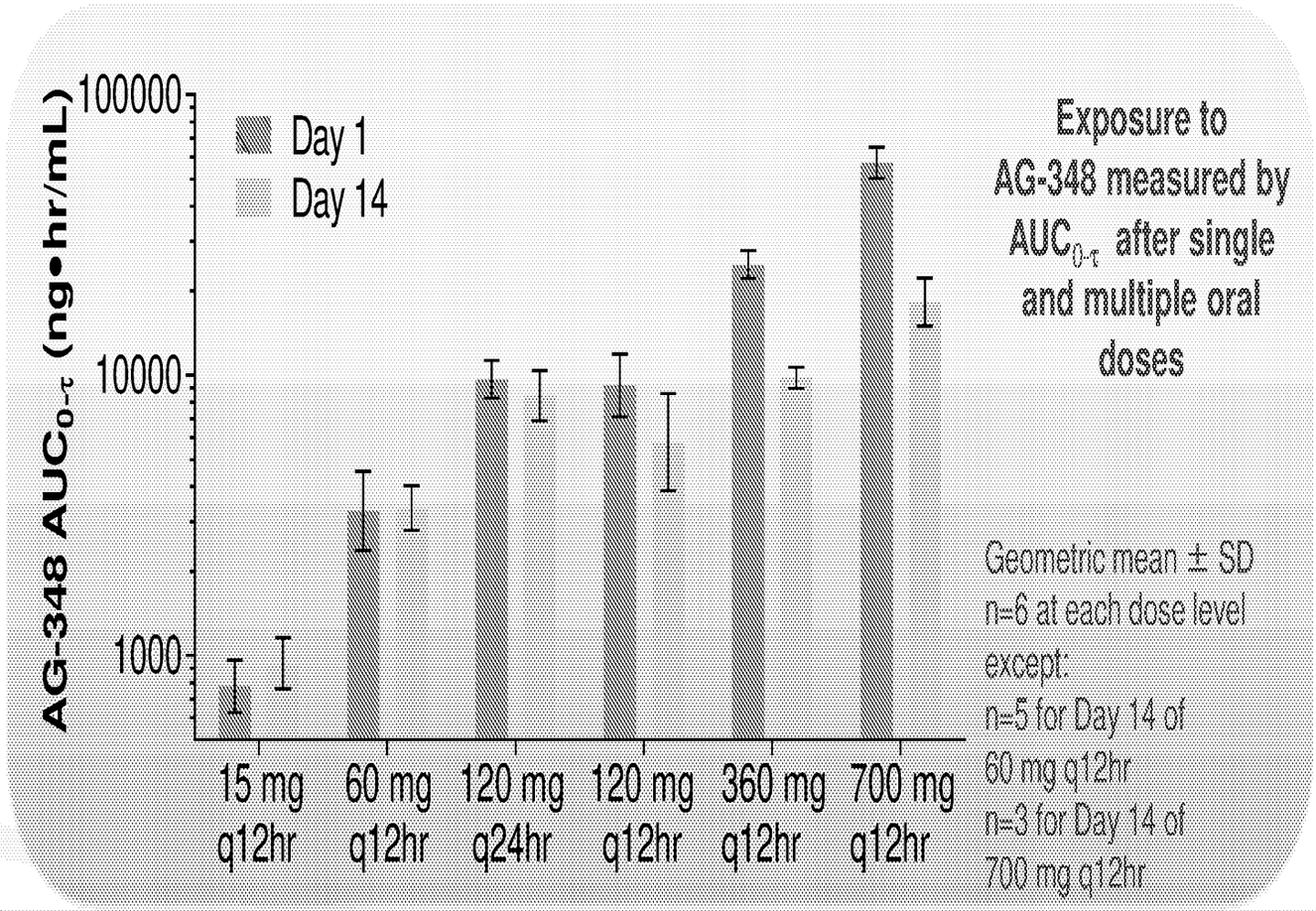



Ex. 2001-0373

# Pharmacokinetic and Pharmacodynamic Results

# Pharmacokinetics

- AG-348 displayed low to moderate pharmacokinetic variability
- Plasma exposure increased dose-proportionally
  - At higher doses, exposure at Day 14 was lower than on Day 1
  - AG-348 is a moderate inducer of CYP3A4, the main clearance pathway for AG-348



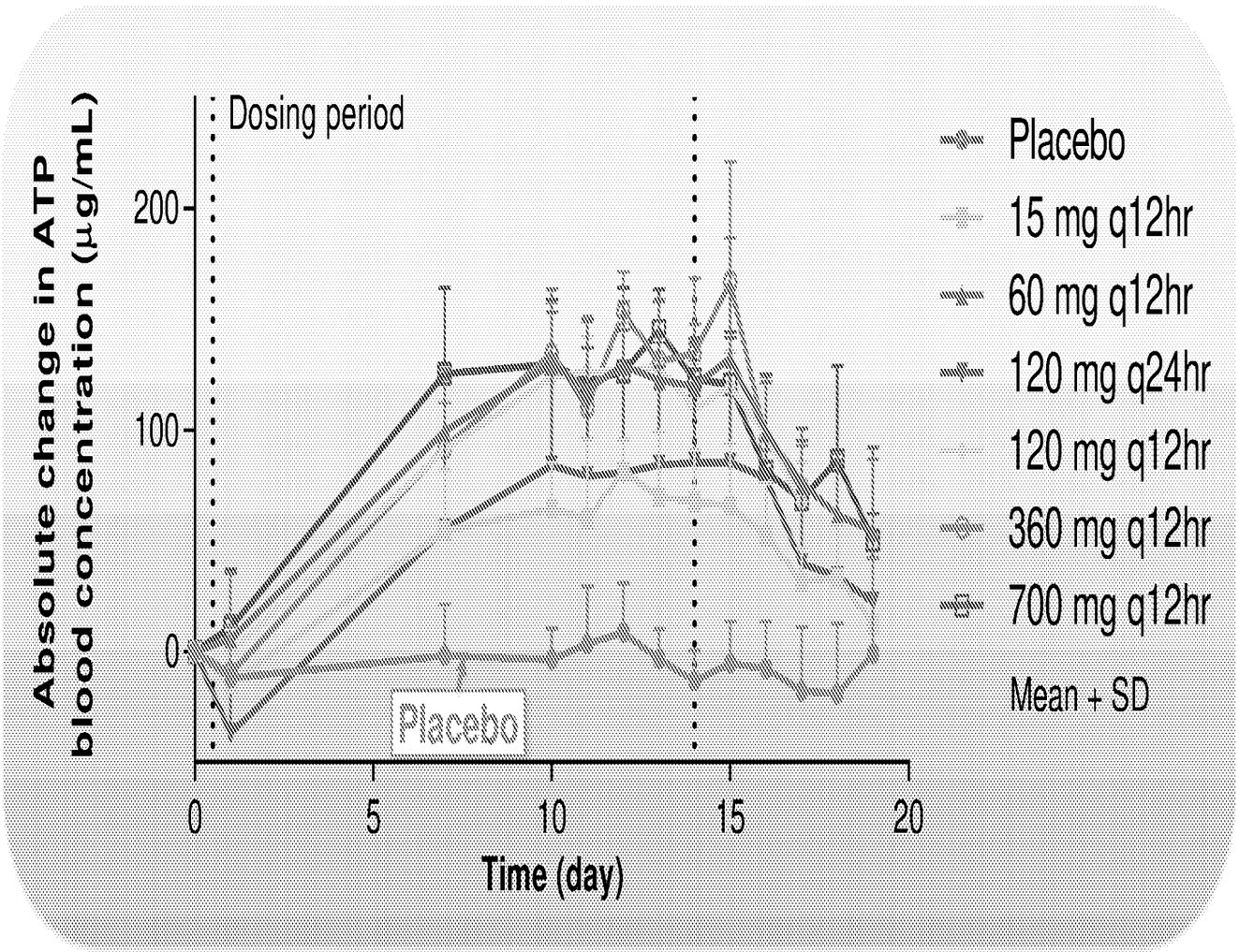

AUC0-τ = area under the concentration–time curve from 0 to the end of the dosing interval τ



Ex. 2001-0375

# Dose Dependent Increase in ATP

- A ~50% increase from Baseline was observed with ≥60 mg AG-348
- Levels remained elevated through 120 hours after the final dose



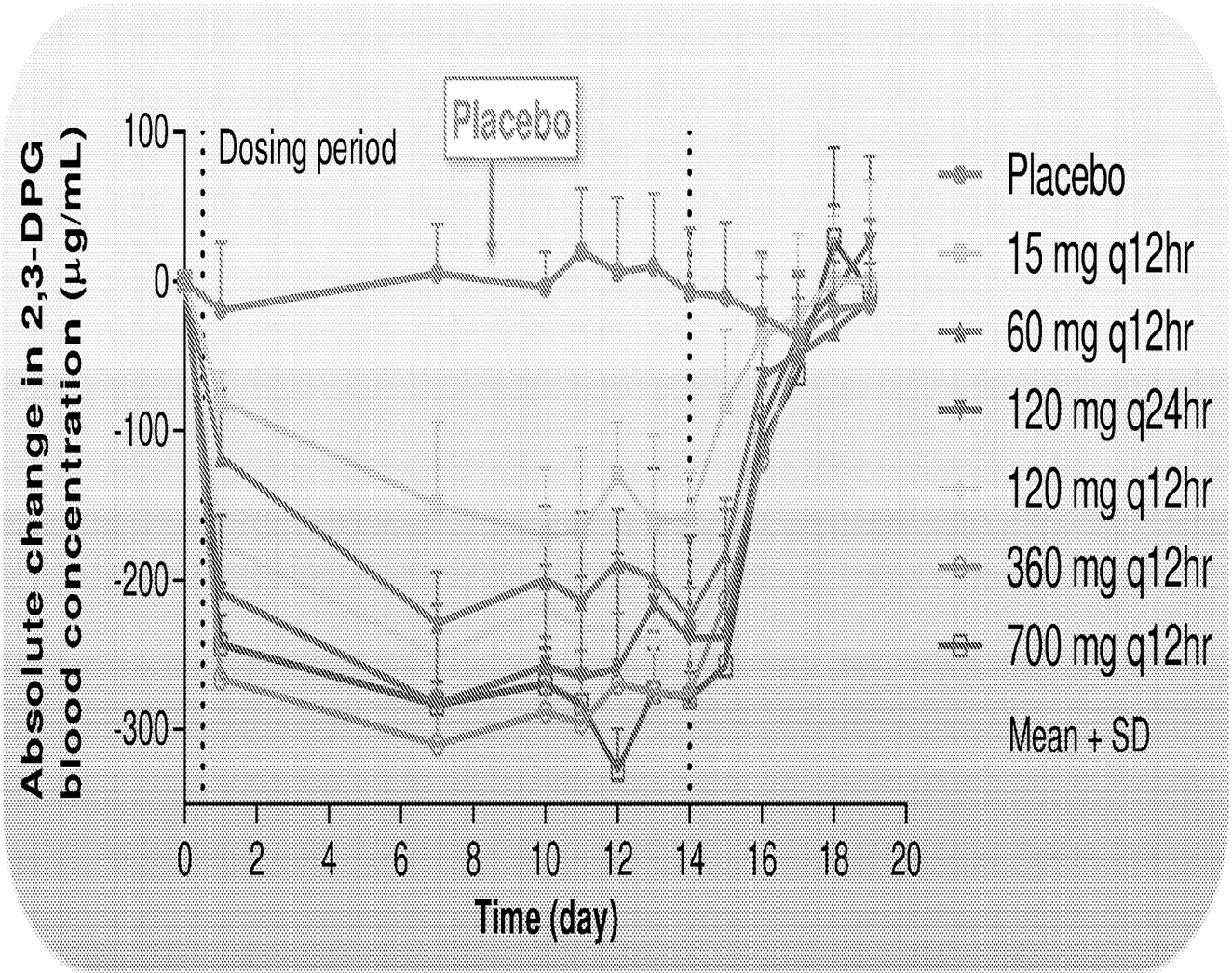

Day 0 = Baseline value. Day 14 is the final day of dosing



Ex. 2001-0376

# 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 hours after the final dose

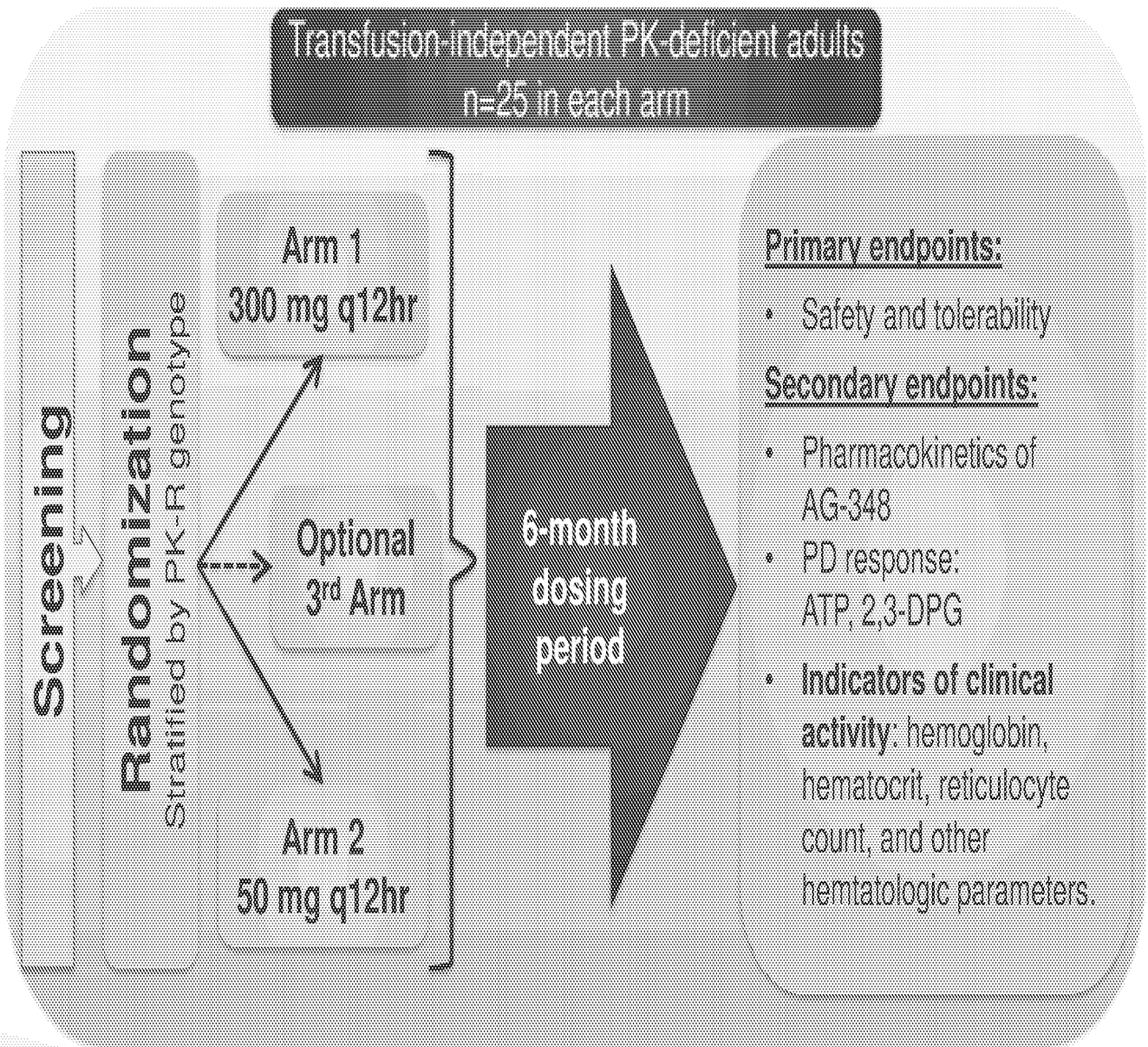



Day 0 = Baseline value. Day 14 is the final day of dosing



Ex. 2001-0377

# DRIVEPK




*Natural History Study collaboration with Boston Children's Hospital ongoing*



Ex. 2001-0378

# Summary and Conclusions

- PK deficiency is a serious hematologic disorder
- AG-348 is a novel, first-in-class, PK-R activator that is intended to restore the glycolytic pathway defect in patients with PK deficiency
- Healthy volunteer study:
  - Established a clear range of well tolerated doses over 14 days and demonstrated maximal PD effects
- PD data are consistent with preclinical and *ex-vivo* data demonstrating:
  - Sustained concomitant increases in ATP
  - Decreases in 2,3-DPG
- Phase II study is currently enrolling.



Ex. 2001-0379

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



Ex. 2001-0380

# Pyruvate Kinase Deficiency Clinical Development Update

11June, 2015
Wien, Austria

Ann Barbier, MD, PhD

1

## Novel First-in-Class Portfolio: Precision Medicine Approach

| | Research | Planned: Early Stage → Late Stage Clinical Development |
|---|---|---|
| **Development Programs** | | |
| AG-221 (IDH2m inhibitor) | | Advanced Hematologic Malignancies → Registration Program |
| | | Advanced Solid Tumors |
| AG-120 (IDH1m inhibitor) | | Advanced Hematologic Malignancies → Registration Program |
| | | Advanced Solid Tumors |
| AG-881 (pan-IDH inhibitor) | | Phase 1 |
| AG-348 (Pyruvate kinase (R) Activator) | | Phase 1 Volunteer Studies → Phase 2 in Patients |
| **Research Programs** | | |
| Cancer Metabolism | (Multiple Novel Targets) | |
| Rare Genetic Disorders of Metabolism | (Multiple Monogenic Diseases) | |

2

2

## PK Deficiency

- ***Estimated*** patient population in United States and Europe:
  - ~2400 diagnosed today
  - Largely undiagnosed
- Chronic hemolytic anemia caused by mutations in PK
- Medical management is challenging
  - Transfusions and splenectomy common to manage symptoms
  - Lifelong risks for chronic hemolysis and iron overload

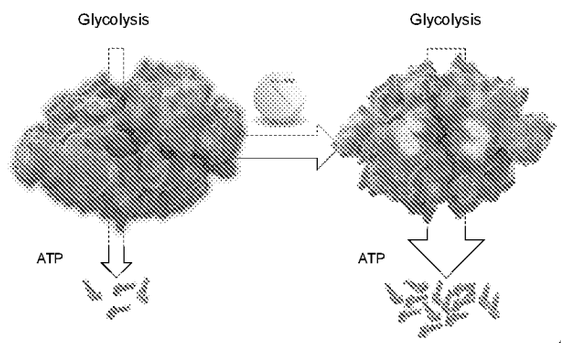
Source: Physician Interviews; Zanella, *Blood Rev.* 2007; 21(4):217; Market research

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## Two Mechanisms for AG-348 Action

*Increased catalytic efficiency*



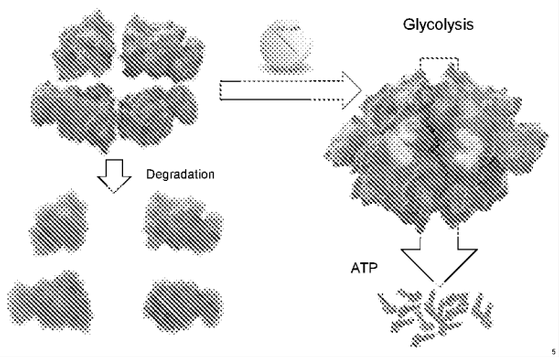

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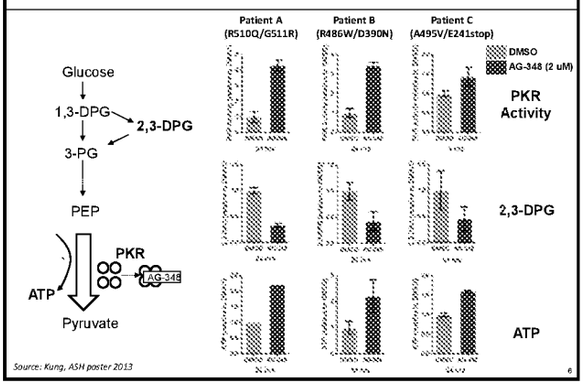
**Two Mechanisms for AG-348 Action**
*Increased protein stability*




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**Effect of AG-348 on metabolites in RBC from PK deficiency patients**



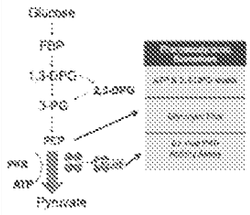

*Source: Kung, ASH poster 2013*

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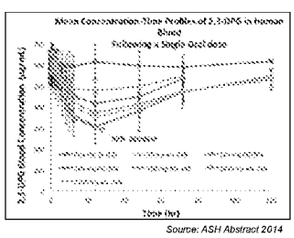
**2014 ASH Abstract: SAD and MAD Phase 1 Trials**
**Preliminary Data**

Robust Suite of Metabolic Biomarkers




*Source: Kung, ASH poster 2013*

2,3-DPG Metabolic Biomarker




*Source: ASH Abstract 2014*

7

7

AG-348: clinical development status for patients with PK deficiency

**Current status**

Phase 1

(Healthy volunteer clinical studies)

Single ascending dose escalation

Conclusions:
- Favorable safety profile
- No SAEs
- MTD not reached

Multiple ascending dose escalation

Conclusions:
- No SAEs in blinded analysis
- Enrollment complete
- Analysis ongoing

**Potential future directions**

Initiate global phase 2 trial in transfusion-independent adults with PK deficiency in first half 2015

Screening | AG-348 treatment: PK/PD markers | End of treatment

- Treatment dose/schedule decided by healthy volunteer studies
- Clinical end points:
  - Safety
  - Efficacy
    - Hemoglobin: stabilization and/or increase
    - Transfusion frequency and amount
  - Quality of life (end points evaluated in NHS)

Development in pediatric patients based on POC in adults

8

8

## Please also visit the following posters and talk

| Topic/Date | Lead/Presenting Author | Title | No. |
|---|---|---|---|
| AG-348: Oral Presentation<br>12 June, 12.00 – 12.15<br>Room Strauss 1 | Sam Agresta<br>(Agios; Boston, USA) | 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 | #S138 |
| Observational cohort: Poster Presentation<br>12 June, 17.15 – 18.45<br>Poster Area, Hall C | Rachael Grace<br>(Boston Children's Hospital; Boston, USA) | CATEGORIZATION OF CLINICAL SEVERITY IN PYRUVATE KINASE DEFICIENCY (PKD) IN AN INTERNATIONAL, OBSERVATIONAL COHORT | #P375 |
| AG-348: Poster Presentation<br>13 June, 17.15 – 18.45<br>Poster Area, Hall C | Yue Chen<br>(Agios; Boston, USA) | PRECLINICAL PHARMACOKINETIC / PHARMACODYNAMIC RELATIONSHIPS FOR AG-348, AN INVESTIGATIONAL SMALL-MOLECULE ACTIVATOR OF PYRUVATE KINASE | #P751 |
| Natural History Study: E-Poster Presentation | Rachael Grace<br>(Boston Children's Hospital; Boston, USA) | THE CLINICAL FEATURES AND TREATMENT OF IRON OVERLOAD IN PYRUVATE KINASE DEFICIENCY (PKD): DATA FROM THE PKD NATURAL HISTORY STUDY (NHS) | #E1466 |

*Abstracts are available online on the EHA conference website: http://learningcenter.ehaweb.org/eha/*

9

9

## THANK YOU

10

10

Journal of Pharmaceutical Sciences

JANUARY 1977
VOLUME 66 NUMBER 1

*REVIEW ARTICLE*

# Pharmaceutical Salts

STEPHEN M. BERGE *‡, LYLE D. BIGHLEY *, and DONALD C. MONKHOUSE *

**Keyphrases** □ Pharmaceutical salts—general pharmacy, physicochemical properties, bioavailability, pharmaceutical properties, toxicology, review □ Salts, pharmaceutical—general pharmacy, physicochemical properties, bioavailability, pharmaceutical properties, toxicology, review □ Physicochemical properties—dissolution, solubility, stability, and organoleptic properties of pharmaceutical salts, review □ Bioavailability—formulation effects, absorption alteration and pharmacokinetics of pharmaceutical salts, review □ Toxicology—pharmaceutical salts, review

CONTENTS

*Potentially Useful Salts* ..... 2
*Physicochemical Studies* ..... 4
Dissolution Rate ..... 5
Solubility ..... 7
Organoleptic Properties ..... 8
Stability ..... 9
Miscellaneous Properties ..... 10
*Bioavailability* ..... 10
Formulation Effects ..... 11
Absorption Alteration ..... 13
Pharmacokinetics ..... 13
*General Pharmacy* ..... 14
Pharmacological Effect ..... 14
Dialysis ..... 14
Miscellaneous ..... 14
*Toxicological Considerations* ..... 15
Toxicity of Salt Ion ..... 15
Toxicity of Salt Form ..... 15
*Conclusions* ..... 16
*References* ..... 16

The chemical, biological, physical, and economic characteristics of medicinal agents can be manipulated and, hence, often optimized by conversion to a salt form. Choosing the appropriate salt, however, can be a very difficult task, since each salt imparts unique properties to the parent compound.

Salt-forming agents are often chosen empirically. Of the many salts synthesized, the preferred form is selected by pharmaceutical chemists primarily on a practical basis: cost of raw materials, ease of crystallization, and percent yield. Other basic considerations include stability, hygroscopicity, and flowability of the resulting bulk drug. Unfortunately, there is no reliable way of predicting the influence of a particular salt species on the behavior of the parent compound. Furthermore, even after many salts of the same basic agent have been prepared, no efficient screening techniques exist to facilitate selection of the salt most likely to exhibit the desired pharmacokinetic, solubility, and formulation profiles.

Some decision-making models have, however, been developed to help predict salt performance. For example, Walkling and Appino (1) described two techniques, "decision analysis" and "potential problem analysis," and applied them to the selection of the most suitable derivative of an organic acid for development as a tablet. The derivatives considered were the free acid and the potassium, sodium, and calcium salts. Both techniques are based on the chemical, physical, and biological properties of these specific derivatives and offer a promising avenue for developing optimal salt forms.

Information on salts is widely dispersed throughout the pharmaceutical literature, much of which addresses the use of salt formation to prolong the release of the active component, thereby eliminating various undesirable drug properties (2–6). This review surveys literature of the last 25 years, emphasizing comparisons between the properties of different salt forms of the same compound. Included also is a discussion of potentially useful salt forms. Our purpose is twofold: to present an overview of the many different salts from which new drug candidates can be chosen and



Ex. 2001-0384

**Table I—FDA-Approved Commercially Marketed Salts**

| Anion | Percent[a] | Anion | Percent[a] |
|---|---|---|---|
| Acetate | 1.26 | Iodide | 2.02 |
| Benzenesulfonate | 0.25 | Isethionate[i] | 0.88 |
| Benzoate | 0.51 | Lactate | 0.76 |
| Bicarbonate | 0.13 | Lactobionate | 0.13 |
| Bitartrate | 0.63 | Malate | 0.13 |
| Bromide | 4.68 | Maleate | 3.03 |
| Calcium edetate | 0.25 | Mandelate | 0.38 |
| Camsylate[b] | 0.25 | Mesylate | 2.02 |
| Carbonate | 0.38 | Methylbromide | 0.76 |
| Chloride | 4.17 | Methylnitrate | 0.38 |
| Citrate | 3.03 | Methylsulfate | 0.88 |
| Dihydrochloride | 0.51 | Mucate | 0.13 |
| Edetate | 0.25 | Napsylate | 0.25 |
| Edisylate[c] | 0.38 | Nitrate | 0.64 |
| Estolate[d] | 0.13 | Pamoate (Embonate) | 1.01 |
| Esylate[e] | 0.13 | Pantothenate | 0.25 |
| Fumarate | 0.25 | Phosphate/diphosphate | 3.16 |
| Gluceptate[f] | 0.18 | Polygalacturonate | 0.13 |
| Gluconate | 0.51 | Salicylate | 0.88 |
| Glutamate | 0.25 | Stearate | 0.25 |
| Glycollylarsanilate[g] | 0.13 | Subacetate | 0.38 |
| Hexylresorcinate | 0.13 | Succinate | 0.38 |
| Hydrabamine[h] | 0.25 | Sulfate | 7.46 |
| Hydrobromide | 1.90 | Tannate | 0.88 |
| Hydrochloride | 42.98 | Tartrate | 3.54 |
| Hydroxynaphthoate | 0.25 | Teoclate[j] | 0.13 |
| | | Triethiodide | 0.13 |

| Cation | Percent[a] | Cation | Percent[a] |
|---|---|---|---|
| Organic: | | Metallic: | |
| Benzathine[k] | 0.66 | Aluminum | 0.66 |
| Chloroprocaine | 0.33 | Calcium | 10.49 |
| Choline | 0.33 | Lithium | 1.64 |
| Diethanolamine | 0.98 | Magnesium | 1.31 |
| Ethylenediamine | 0.66 | Potassium | 10.82 |
| Meglumine[l] | 2.29 | Sodium | 61.97 |
| Procaine | 0.66 | Zinc | 2.95 |

[a] Percent is based on total number of anionic or cationic salts in use through 1974. [b] Camphorsulfonate. [c] 1,2-Ethanedisulfonate. [d] Lauryl sulfate. [e] Ethanesulfonate. [f] Glucoheptonate. [g] *p*-Glycollamidophenylarsonate. [h] *N,N'*-Didehydroabietylethylenediamine. [i] 2-Hydroxyethanesulfonate. [j] 8-Chlorotheophyllinate. [k] *N,N'*-Dibenzylethylenediamine. [l] *N*-Methylglucamine.

to assemble data that will provide, for the student and practitioner alike, a rational basis for selecting a suitable salt form.

## POTENTIALLY USEFUL SALTS

Salt formation is an acid–base reaction involving either a proton-transfer or neutralization reaction and is therefore controlled by factors influencing such reactions. Theoretically, every compound that exhibits acid or base characteristics can participate in salt formation. Particularly important is the relative strength of the acid or base—the acidity and basicity constants of the chemical species involved. These factors determine whether or not formation occurs and are a measure of the stability of the resulting salt.

The number of salt forms available to a chemist is large; surveys of patent literature show numerous new salts being synthesized annually. Various salts of the same compound often behave quite differently because of the physical, chemical, and thermodynamic properties they impart to the parent compound. For example, a salt's hydrophobicity and high crystal lattice energy can affect dissolution rate and, hence, bioavailability. Ideally, it would be desirable if one could predict how a pharmaceutical agent's properties would be affected by salt formation.

Tables I and II list all salts that were commercially marketed through 1974. The list was compiled from all agents listed in "Martindale The Extra Pharmacopoeia," 26th ed. (7). Table I categorizes all salt forms approved by the Food and Drug Administration (FDA), while Table II lists those not approved by the FDA but in use in other countries. (Only salts of organic compounds are considered because most drugs are organic substances.) The relative frequency with which each salt type has been used is calculated as a percentage, based on the total number of anionic or cationic salts in use through 1974. Because of simple availability and physiological reasons, the monoprotic hydrochlorides have been by far the most frequent choice of the available anionic salt-forming radicals, outnumbering the sulfates nearly six to one. For similar reasons, sodium has been the most predominant cation.

Knowledge that one salt form imparts greater water solubility, is less toxic, or slows dissolution rate would greatly benefit chemists and formulators. In some cases, such generalizations can be made. Miller and Heller (8) discussed some properties associated with specific classes of salt forms. They stated that, in general, salt combinations with monocarboxylic acids are insoluble in water and lend themselves to repository preparations, while those of dicarboxylic acids confer water solubility if one carboxylic group is left free. Pamoic acid, an aromatic dicarboxylic acid, is an exception since it is used as a means of obtaining prolonged action by forming slightly soluble salts with certain basic drugs. Saias *et al.* (9) reviewed the use of this salt form in preparing sustained-release preparations. More recently, latentiation of dihydrostreptomycin (10)



Ex. 2001-0385

**Table II—Non-FDA-Approved Commercially Marketed Salts**

| Anion | Percent[a] |
|---|---|
| Adipate | 0.13 |
| Alginate | 0.12 |
| Aminosalicylate | 0.25 |
| Anhydromethylenecitrate | 0.13 |
| Arecoline | 0.13 |
| Aspartate | 0.25 |
| Bisulfate | 0.25 |
| Butylbromide | 0.13 |
| Camphorate | 0.13 |
| Digluconate | 0.13 |
| Dihydrobromide | 0.13 |
| Disuccinate | 0.13 |
| Glycerophosphate | 0.88 |
| Hemisulfate | 0.13 |
| Hydrofluoride | 0.13 |
| Hydroiodide | 0.25 |
| Methylenebis(salicylate) | 0.13 |
| Napadisylate[b] | 0.13 |
| Oxalate | 0.25 |
| Pectinate | 0.13 |
| Persulfate | 0.13 |
| Phenylethylbarbiturate | 0.13 |
| Picrate | 0.13 |
| Propionate | 0.13 |
| Thiocyanate | 0.13 |
| Tosylate | 0.13 |
| Undecanoate | 0.13 |

| Cation | Percent[a] |
|---|---|
| Organic: | |
| Benethamine[c] | 0.33 |
| Clemizole[d] | 0.33 |
| Diethylamine | 0.33 |
| Piperazine | 0.98 |
| Tromethamine[e] | 0.33 |
| Metallic: | |
| Barium | 0.33 |
| Bismuth | 0.98 |

[a] Percent is based on total number of anionic and cationic salts in use through 1974. [b] 1,5-Naphthalenedisulfonate. [c] *N*-Benzylphenethylamine. [d] 1-*p*-Chlorobenzyl-2-pyrrolidin-1′-ylmethylbenzimidazole. [e] Trishydroxymethylaminomethane.

using pamoic acid resulted in the formation of a delayed-action preparation. Numerous studies using pamoate salts are dispersed throughout the literature (11–15).

Alginic acid also has been used to prepare long-acting pharmaceuticals. Streptomycin alginate was prepared (16) and shown to be effective in sustained-release preparations. A striking example of a long-acting alginate salt is that of pilocarpine. When dispersed in sterile water and dried to a solid gel, this compound was found useful in the preparation of long-acting ophthalmic dosage forms (17). While liquid preparations of the alginate and hydrochloride salts possess similar miotic activity, studies showed that solid pilocarpine alginate flakes constricted pupil size more effectively and increased the duration of miosis significantly when compared with the liquid preparations. Solid dose pilocarpine may be more uniformly available, because it diffuses more slowly through the gel matrix which holds the drug in reserve. In contrast, drops of the commonly employed solution dosage form release the dose immediately to the conjunctival fluid.

Málek *et al.* (18) devised a unique way of prolonging action through salt formation; they showed that the distribution of several antibiotics could be markedly altered by merely preparing macromolecular salts. Since macromolecules and colloidal particles have an affinity for the lymphatic system, streptomycin, neomycin, viomycin, and streptothrycin were combined with high molecular weight compounds such as polyacrylic acids, sulfonic or phosphorylated polysaccharides, and polyuronic derivatives. Parenteral administration of these compounds produced low blood levels of the antibiotic for long periods, while lymph levels were high. (In comparison, streptomycin sulfate gave high blood levels but low lymph levels.) This alteration in distribution caused the streptomycin to prolong its passage through the body, since lymphatic circulation is quite slow.

The appropriate choice of a salt form has been found to reduce toxicity. It can be rationalized that any compound associated with the normal metabolism of food and drink must be essentially nontoxic. The approach of choosing organic radicals that are readily excreted or metabolized opened up a new class of substances from which to select a salt form. For example, certain salts of the strong base choline have proven to be considerably less toxic than their parent compound. The preparation and properties of choline salts of a series of theophylline derivatives were reported (19), and it was shown that choline theophyllinate possessed a greater $LD_{50}$ than theophylline or its other salts (20). It was postulated that this agent would be less irritating to the GI tract than aminophylline, because "its basic constituent, choline, is an almost completely nontoxic substance of actual importance to the physiologic economy." This evidence led to the preparation of choline salicylate (21) as an attempt to reduce the GI disturbances associated with salicylate administration. Clinical studies indicated that choline salicylate elicited a lower incidence of GI distress, was tolerated in higher doses, and was of greater benefit to the patient than was acetylsalicylic acid (aspirin).

Amino acids and acid vitamins also have been used as salt-forming agents. Based on the evidence that coadministration of amino acids with aminoglycoside antibiotics reduced their toxicity, a series of amino acid salts of dihydrostreptomycin was prepared (22). In all but one case, the acute toxicities of these salts were lower than the toxicity of the sulfate. The ascorbate and pantothenate also were synthesized and shown to be less toxic than the sulfate. Of the salts prepared, the ascorbate had the highest $LD_{50}$.

The vitamins most commonly used for forming salts exhibiting reduced toxicity are ascorbic and pantothenic acids. Keller *et al.* (23) were the first to use pantothenic acid as a means of "detoxifying" the basic streptomyces antibiotics. Parenteral administration of the pantothenates of streptomycin and dihydrostreptomycin had a significantly reduced incidence of acute neurotoxicity in cats as compared with the sulfates. Subsequent studies (24–28) supported this finding and showed that the pantothenates of neomycin and viomycin also are less toxic. The ascorbate of oleandomycin was synthesized and its pharmacological properties were reported (29). Upon intramuscular injection in rats, it produced less irritation than the phosphate.

*p*-Acetamidobenzoic acid, an innocuous metabolite of folic acid present in normal blood and urine, has been used in preparing salts. In particular, it yields stable salts with amines that otherwise tend to form hygroscopic products with conventional acid components (30).

Often the salt form is chosen by determining a salt



Ex. 2001-0386

component that will pharmacologically antagonize an unfavorable property or properties exhibited by the basic agent. Salts of *N*-cyclohexylsulfamic acid are an example of the practical application of this approach. *N*-Cyclohexylsulfamic acid salts, better known as cyclamates, have a characteristic sweet, pleasing taste. Although presently under investigation by the FDA for potentially carcinogenic properties, salts incorporating this compound can render unpleasant or bitter-tasting drugs acceptable. For example, the cyclamates of dextromethorphan and chlorpheniramine exhibit greatly improved bitterness thresholds compared to commonly occurring salts (31). Furthermore, their stability in aqueous solution was described as good when maintained at a pH not greater than 4.

*N*-Cyclohexylsulfamic acid salts of thiamine hydrochloride and lincomycin also have been synthesized. Thiamine *N*-cyclohexylsulfamate hydrochloride was reported to have a more pleasant taste than other thiamine salts while having an equal or greater stability (32). Lincomycin cyclamate, shown to possess an enhanced thermal stability over its hydrochloride, was prepared (33) to test the hypothesis that reduced lincomycin absorption in the presence of small quantities of cyclamates was due to a simple metathetic reaction. However, this assumption was found not to be true. An extensive study of the preparation and characterization of cyclamic acid salts of several widely used classes of drugs including antihistamines, antibiotics, antitussives, myospasmolytics, and local anesthetics was reported (34, 35).

Various salts of penicillin and basic amine compounds have been formulated in an effort to produce a long-acting, nonallergenic form of penicillin. Since antihistamines appear to mitigate the symptomatology of penicillin reactions in some patients, coadministration of the two has been advocated. The preparation of the benzhydralamine salt of penicillin was an attempt to produce a repository form of penicillin with antiallergic properties (36). Blood levels achieved with this salt were comparable to those of penicillin G potassium; however, its antiallergic properties were not evaluated. In fact, the investigators noted that antihistamines can actually cause sensitization at times and stated that "despite their occasionally favorable influence on the symptoms of penicillin sensitivity, they contribute directly to the potential of drug sensitivity when co-administered with penicillin."

Silver salts of sulfanilamide, penicillin, and other antibiotics have been prepared and represent cases where the species (ions) are complementary. When aqueous solutions of the salts were applied topically to burned tissue, they yielded the combined benefits of the oligodynamic action of silver and the advantages of the antibacterial agents (37).

The use of 8-substituted xanthines, particularly the 8-substituted theophyllines, as salt-forming agents was first reported in the preparation of a series of antihistamine salts (38–41). Synthesis of these xanthine salts was an attempt to find a drug to counteract the drowsiness caused by the antihistamines with the stimulant properties of the xanthines. When an electronegative group is introduced into the xanthine molecule at the 8-position, the electron-drawing capacity of the substituent results in the creation of an acidic hydrogen at position 7. Thus, these moderately strong acidic compounds can undergo salt formation with various organic bases.

The 8-halotheophyllines were the first group of xanthines studied as potential salt-forming agents. Since the report on the preparation of the 8-chlorotheophylline salt of diphenhydramine (42), synthesis of the 8-halotheophyllinates of a number of organic bases has been attempted. The 8-chlorotheophylline salts of quinine, ephedrine, and strychnine were prepared and characterized (43). These salts were less water soluble than the corresponding free alkaloidal bases. In a similar report, the 8-chlorotheophyllinates of three synthetic narcotics, meperidine, levorphanol, and metopon, were prepared (44).

Pharmacological and clinical studies involving the 8-bromotheophylline pyrilamine salt revealed the unusual diuretic properties associated with the 8-halotheophylline portion of the compound (45, 46). This finding initiated an investigation into the preparation of a soluble 8-bromotheophylline salt of high diuretic activity. With readily available amines, over 30 salts were synthesized and screened for diuretic activity (47). When tested against theophylline salts of the same amines, the 8-bromotheophyllinates showed greater activity in every case.

With the successful formation of 8-halotheophyllinates of organic bases, Morozowich and Bope (48) proposed that, if the halogen moiety was replaced with a more electronegative substituent such as a nitro group, a more acidic compound would be formed. Presumably, more stable salts would result and precipitation of the free xanthine derivative in the stomach would be less likely to occur. On this premise, they successfully prepared pharmacologically effective 8-nitrotheophyllinates of several pharmaceutically useful bases.

Duesel *et al.* (19), in their study of choline theophyllinate, prepared the 8-chloro-, 8-bromo-, and 8-nitrotheophylline salts of choline. Oral toxicity studies in mice showed that the $LD_{50}$ of the 8-nitrotheophyllinate was much greater than that of either 8-halotheophylline. In fact, it remained nonlethal at doses as high as 5 g.

Polygalacturonic acid, a derivative of pectin, has been used to prepare quinidine salts exhibiting reduced toxicity (49, 50). The compound possesses special demulcent properties and inhibits mucosal irritation. The rationale for use of this agent is to reduce the ionic shock to the GI mucosa resulting from the flood of irritating ions liberated by rapid dissociation of the conventional inorganic quinidine salts. Studies have shown that it is four times less toxic orally than the sulfate. This difference was attributed to the slower release of quinidine from the polygalacturonate.

Other compounds reported to be potentially useful as pharmaceutical salt forms are listed in Table III.

## PHYSICOCHEMICAL STUDIES

Biological activity of a drug molecule is influenced by two factors: its chemical structure and effect at a specific site and its ability to reach—and then be removed from—the site of action. Thus, a knowledge of the physicochemical properties of a compound that influence its absorption, distribution, metabolism, and excretion is essential for a complete understanding of the onset and duration of ac-



Ex. 2001-0387

**Table III—Potentially Useful Salt Forms of Pharmaceutical Agents**

| Salt-Forming Agent | Compound Modified | Modification | Reference |
|---|---|---|---|
| Acetylaminoacetic acid | Doxycycline | Solubility | 51 |
| *N*-Acetyl-L-asparagine | Erythromycin | Solubility, activity, stability | 52 |
| *N*-Acetylcystine | Doxycycline | Combined effect useful in pneumonia | 53 |
| Adamantoic acid | Alkylbiguanides | Prolonged action | 54 |
| Adipic acid | Piperazine | Stability, toxicity, organoleptic properties | 55 |
| *N*-Alkylsulfamates | Ampicillin | Absorption (oral) | 56 |
| | Lincomycin | Solubility | 57 |
| Anthraquinone-1,5-disulfonic acid | Cephalexin | Stability, absorption | 58 |
| Arabogalactan sulfate (arabino) | Various alkaloids | Prolonged action | 59, 60 |
| Arginine | Cephalosporins | Toxicity | 61 |
| | α-Sulfobenzylpenicillin | Stability, hygroscopicity, toxicity | 62 |
| Aspartate | Erythromycin | Solubility | 63 |
| Betaine | Tetracycline | Gastric absorption | 64 |
| Bis(2-carboxychromon-5-yloxy)alkanes | 7-(Aminoalkyl)theophyllines | Activity, prolonged prophylactic effect | 65 |
| Carnitine | Metformin | Toxicity | 66 |
| 4-Chloro-*m*-toluenesulfonic acid | Propoxyphene | Organoleptic properties | 67 |
| Decanoate | Heptaminol | Prolonged action | 68 |
| Diacetyl sulfate | Thiamine | Stability, hygroscopicity | 69 |
| Dibenzylethylenediamine | Ampicillin | Prolonged action | 70, 71 |
| Diethylamine | Cephalosporins | Reduced pain on injection | 72 |
| Diguaiacyl phosphate | Tetracycline | Activity | 73 |
| Dioctyl sulfisuccinate | Vincamine | Organoleptic properties | 74 |
| Embonic (pamoic) acid | Kanamycin | Toxicity | 75 |
| | 2-Phenyl-3-methylmorpholine | Toxicity | 76 |
| Fructose 1,6-diphosphoric acid | Tetracycline | Solubility | 77 |
| | Erythromycin | Solubility | |
| Glucose 1-phosphoric acid, glucose 6-phosphoric acid | Tetracycline | Solubility | 77 |
| | Erythromycin | Solubility | |
| L-Glutamine | Erythromycin | Solubility, activity, stability | 52 |
| Hydroxynaphthoate | Bephenium | Toxicity | 78 |
| 2-(4-Imidazolyl)ethylamine | Prostaglandin | Prolonged action | 79 |
| Isobutanolamine | Theophylline | Stability | 80 |
| Lauryl sulfate | Vincamine | Organoleptic properties | 81 |
| Lysine | α-Sulfobenzylpenicillin | Toxicity, stability, hygroscopicity | 62 |
| | Cephalosporins | | 61 |
| Methanesulfonic acid | Pralidoxime (2-PAM) | Solubility | 82 |
| *N*-Methylglucamine | α-Sulfobenzylpenicillin | Toxicity, stability, hygroscopicity | 62 |
| | Cephalosporins | Reduced pain on injection | 72 |
| *N*-Methylpiperazine | Phenylbutazone | Toxicity, faster onset of action | 83 |
| Morpholine | Cephalosporins | Reduced pain on injection | 72 |
| 2-Naphthalenesulfonic acid | Propoxyphene | Organoleptic properties | 84 |
| Octanoate | Heptaminol | Prolonged action | 68 |
| Probenecid | Pivampicillin | Organoleptic properties | 85 |
| Tannic acid | Various amines | Prolonged action | 86, 87 |
| Theobromine acetic acid | Propoxyphene | Activity | 88 |
| 3,4,5-Trimethoxybenzoate | Tetracycline | Organoleptic properties | 89 |
| | Heptaminol | Prolonged action | 68 |
| Trometharnine | Aspirin | Absorption (oral) | 90 |
| | Dinoprost (prostaglandin $F_{2\alpha}$) | Physical state | 91 |

tion, the relative toxicity, and the possible routes of administration (2).

In a review in 1960, Miller and Holland (92) stated that "different salts of the same drug rarely differ pharmacologically; the differences are usually based on the physical properties." In a subsequent review (93), Wagner expanded upon this statement, asserting that, although the nature of the biological responses elicited by a series of salts of the same parent compound may not differ appreciably, the intensities of response may differ markedly.

The salt form is known to influence a number of physicochemical properties of the parent compound including dissolution rate, solubility, stability, and hygroscopicity. These properties, in turn, affect the availability and formulation characteristics of the drug. Consequently, the pharmaceutical industry has systematically engaged in extensive preformulation studies of the physicochemical properties of each new drug entity to determine the most suitable form for drug formulation. Published information concerning such studies, however, is sparse. Preformulation studies have been outlined, and the influence of the salt form on the volatility and hygroscopicity of an agent under investigation was discussed (94).

In one such study, methylpyridinium-2-aldoxime (pralidoxime) salts were investigated (95). This study set out to prepare a salt with water solubility adequate to allow intramuscular injection of a low volume (2–3 ml) therapeutic dose. The original compound, the methiodide, had the disadvantages of limited aqueous solubility and high potential toxicity, since its high iodide content could result in iodism. On the basis of physiological compatibility, better water solubility, favorable stability, and relatively high percentage of oxime, the chloride salt of pralidoxime was selected for therapeutic administration; it was claimed that "the anion used to form the salt can confer physical properties of importance and significance for the formulation and administration of the compound" (95).

Some physicochemical properties of a series of mineral acid salts of lidocaine also were determined (96). While the hydrochloride and hydrobromide were more hygroscopic, they were more soluble in a number of solvents than the nitrate, perchlorate, phosphate, or sulfate salts.

**Dissolution Rate**—The dissolution rate of a pharmaceutical agent is of major importance to the formulator. In many cases, particularly with poorly soluble drugs, this characteristic best reflects the bioavailability of the com-



Ex. 2001-0388

pound. As a rule, a pharmaceutical salt exhibits a higher dissolution rate than the corresponding conjugate acid or base at an equal pH, even though they may have the same equilibrium solubility. The explanation for this result lies in the processes that control dissolution.

Dissolution can be described by a diffusion layer model[1] in terms of an equation developed by Nernst and Brunner (97):

$$\frac{dW}{dt} = \frac{DS}{h}(C_s - C) \qquad \text{(Eq. 1)}$$

where $W$ is the mass of the solute dissolved at time $t$, $dW/dt$ is the rate of mass transfer per unit time, $D$ is the solute molecule diffusion coefficient, $S$ is the surface area of the dissolving solid, $h$ is the diffusion layer thickness, $C$ is the concentration of the drug in the bulk solution at time $t$, and $C_s$ is the saturation solubility of the solute in the diffusion layer.

The driving force for dissolution in Eq. 1 is the difference between the saturation solubility of the drug and the concentration of the drug in the bulk fluid. If the drug is not rapidly absorbed after it dissolves, then $C$, the concentration in the bulk solution, approaches $C_s$ and the dissolution rate is retarded. When this occurs, absorption is "absorption rate" limited (or "membrane transport" limited). If the absorption rate is rapid (or if the absorption mass transfer coefficient is much larger than $DS/h$ of Eq. 1), however, $C$ becomes negligible compared to $C_s$ and dissolution occurs under "sink" conditions. Absorption is then said to be dissolution rate limited, which is what occurs with most poorly soluble drugs. In either case, an increase in $C_s$, as in salt formation, increases dissolution.

Salts often speed dissolution by effectively acting as their own buffers to alter the pH of the diffusion layer, thus increasing the solubility of the parent compound, $C_s$, in that layer over its inherent solubility at the pH of the dissolution medium. Hence, dissolution is controlled by solubility in the diffusion layer which, in turn, is determined by the pH of that layer. The influence of $K_{sp}$ on the solubility term, $C_s$, and dissolution rate, should an accumulation of ions be allowed to occur, will be treated later.

Nelson (98), in a study of theophylline salts, was the first to show the correlation between diffusion layer pH and dissolution rate. The major impact that this study had on the pharmaceutical sciences was its conclusion that, if other factors remained constant, the dissolution rate of a compound determined the rate of buildup of blood levels with time and the maximum levels obtained. Those salts of the acidic theophylline with high diffusion layer pH's had greater *in vitro* dissolution rates than those exhibiting a lower diffusion layer pH. And, indeed, the rank order of dissolution rates correlated well with clinically determined blood levels. Presumably, the higher pH in the diffusion layer retards hydrolysis of the salt, thereby maintaining the anionic charge of the theophyllinate ion. This report led to many additional studies which illustrate the influence of the salt form on dissolution and the beneficial effects of changing nonionized drugs into salts.

Juncher and Raaschou (99) demonstrated that the rank order of peak blood levels of penicillin V, obtained upon administration of three different salts and the free acid, was the same as the rank order of their rates of dissolution *in vitro*. While the investigators ascribed these differences to the solubility properties of the salts, their experiments actually compared dissolution rates, not solubilities. The relative order of dissolution rates and mean maximal blood levels was: potassium salt > calcium salt > free acid > benzathine salt.

Nelson (100) determined dissolution rates for several weak acids and their sodium salts in media whose pH's represented GI fluids. In all cases, the sodium salt dissolved more rapidly than the free acid. This finding resolved the misconception that absorption of drugs is related only to solubility in the appropriate medium; rather, solubility affects absorption only to the extent that it affects dissolution rate. Absorption of drugs is a dynamic process, and the ultimate solubility of a drug in fluid at absorption sites is of limited consequence since absorption prevents the attainment of saturated solutions. Therefore, dissolution rate, more than solubility, influences absorption since it is a preceding process.

In two subsequent studies, Nelson and coworkers further illustrated the effects of changing nonionized drugs into salts. A report concerning tolbutamide (101), a weak acid, showed that the initial dissolution rate of tolbutamide sodium was approximately 5000 times more rapid than the free acid in acidic media and 300 times more rapid in neutral media. This difference, measured *in vitro*, reflected the differences observed between the free acid and the salt when administered to human subjects. Oral administration of tolbutamide sodium produced an immediate drop in blood sugar comparable to that produced by intravenous injection of the salt, while the slowly dissolving tolbutamide produced a smooth, sustained fall in blood sugar (102).

Correlation of urinary excretion rates and dissolution rates of tetracycline and some of its acid salts also was demonstrated by Nelson (103). The salts that exhibited greater *in vitro* dissolution rates showed greater urinary excretion rates, indicating more rapid absorption.

Benet (104), in a discussion of the biopharmaceutical basis for drug design, referred to the influence of the salt form on dissolution. He compared the dissolution rates of tetracycline and tolbutamide and their salts, as reported in the studies previously cited, and explained why the rates differ at the pH's exhibited by physiological fluids.

Although salt formation usually increases the dissolution rate of a drug, studies with aluminum acetylsalicylate (105, 106), warfarin sodium (107), and benzphetamine pamoate (108) showed that administration of the salt *slowed* dissolution of the drug and subsequent absorption compared to the nonionized form. This decrease appeared to result from precipitation of an insoluble particle or film on the surface of the tablet. Such a phenomenon decreases the effective surface area and prevents deaggregation of the particles. Theoretical considerations of the processes controlling dissolution of an acid salt of a base (108) and the sodium salt of a weak acid (109, 110) in reactive media have been discussed.

Tablet processing and various formulation factors can decrease the dissolution rate of a salt in human gastric juice over its nonionized form (111). Granulation and tableting caused the dissolution rate of phenobarbital sodium to

[1] The authors recognize the existence of other models; this one was chosen simply for illustrative purposes.



Ex. 2001-0389

decrease but had the opposite effect on phenobarbital. Therefore, as a tablet dosage form, the dissolution rate of the sodium salt was slower than that of the free acid. These results were attributed to differences in the disintegrating properties of the tablets; in some instances, rapid dissolution may in fact be a problem for very soluble drugs.

Others have illustrated a phenomenon that decreases the dissolution rate of a salt below that of its nonionized form. Lin *et al.* (112) studied the relationship between salts and biological activity by dissolving three salts and the free base of an experimental antihypertensive in water, 0.1 *N* HCl, and pH 7.2 phosphate buffer. The dissolution rate of the monohydrochloride salt was lower than that of the free base in 0.1 *N* HCl and higher than the free base in both water and phosphate buffer. These authors ascribed this variation to the common ion effect and substantiated it experimentally. Although the biological activity of the monohydrochloride was greater than that of the free base, the implications of altered absorption characteristics on the activity of any other hydrochloride salt in GI fluids must be considered. Similar results also were reported for hydrochloride salts of some tetracyclines (113).

Some consideration must be given to the influence of salt formation on oral toxicity, which often reflects the relationship between the *in vivo* dissolution rate and the appearance of drug in the circulation (114, 115). Morozowich *et al.* (114) showed that the relative toxicities of a series of salts of a drug reflect the rate of absorption, providing the salt-forming agents themselves are relatively nontoxic. They stated that "when absorption is rate-limited by dissolution of the salt in the gastrointestinal tract, as will be the case with slowly soluble salts, the toxicity of a slowly dissolving salt will most probably be lower than that of a more rapidly dissolving salt." The implications of salt formation on toxicology will be discussed under *Toxicological Considerations*.

Several reviews dealt with the influence of the dissolution rate on drug availability and, in particular, salt effects (116, 117). Other reports illustrating the influence of salts and salt form on dissolution rate are listed in Table IV.

**Solubility**—Knowledge of the solubility characteristics of a pharmaceutical agent is essential, because solubility is usually an important factor in the pharmacokinetic profile, the chemical stability, and, ultimately, the formulation of the drug. As discussed previously, it is certainly a primary factor in controlling dissolution rates. The solubility of a compound depends basically upon the physical and chemical properties of the solute; *e.g.*, a lower melting point for a compound within a series reflects a decreased lattice energy, which would suggest a higher solubility. Solubility depends as well upon such elements as temperature, pressure, solvent properties (such as resulting pH), and, to a lesser extent, the state of subdivision of the solute.

An important solvent property which is often overlooked involves the common ion effect; in particular, hydrochloride salts of drugs often exhibit less than desirable solubility in gastric juice because of the abundance of chloride ions. The equilibrium involved is shown in Scheme I.

$$DH^+Cl^-_{(solid)} \overset{K_{sp}}{\rightleftharpoons} (DH^+)_{aq} + (Cl^-)_{aq}$$

*Scheme I*

Salt formation is perhaps one of the first approaches considered as a means of increasing a compound's water solubility. As with dissolution rates, however, salt formation does not always confer greater solubility. Liberally dispersed throughout the pharmaceutical literature are studies that compare the solubilities of different salt forms of the same compound with those of its free acid or base (Table IV). Selection of the salt form exhibiting the desired solubility properties is critical, since these properties often dictate the formulation characteristics of the drug.

**Table IV—Additional References on Salt Form and Dissolution Rate**

| Topic | Reference |
|---|---|
| Dissolution rate of mixtures of weak acids and tribasic sodium phosphate | 118 |
| Physiological availability and *in vitro* dissolution characteristics of some solid dosage formulations of aminosalicylic acid and its salts | 119 |
| Biopharmaceutics, rate of dissolution: chronological bibliography | 120 |
| Biopharmaceutics: rate of dissolution *in vitro* and *in vivo* | 121 |
| Dissolution tests and interpretation of anomalies observed in the dissolution process of sulfaquinoxaline based on salt formation | 122 |
| Influence of the dissolution rate of lithium tablets on side effects | 123 |
| Dissolution kinetics of drugs in human gastric juice | 124 |
| Comparison of dissolution and absorption rates of different commercial aspirin tablets | 125 |
| *In vitro* dissolution rates of aminorex dosage forms and their correlation with *in vitro* availability | 126 |

Phase solubility techniques were used to study the formation of complex salts of triamterene (127). The results indicated that the organic acid salts of basic drugs, such as amines, were more soluble in water than the corresponding inorganic (halide) salts. This consideration is important in the synthesis and selection of a salt form that will exhibit enhanced bioavailability and desirable formulation characteristics.

The hydrogen-ion concentration can significantly affect the solubility of a salt. Anderson (128) discussed the influence of pH on the solubility of pharmaceuticals. Mathematical relationships between pH and solubility of therapeutically useful weak acids and bases and their salts were given along with some considerations in the formulation of solutions of these particular agents.

An extensive study on the solubility interrelationships of the hydrochloride and free base of two pharmaceutically useful amines was reported (129). Mathematical equations describing the total solubility at an arbitrary pH in terms of the independent solubilities of the hydrochloride and free base species and the dissociation constant of the salt were derived and fitted to experimental data with good results. This report elucidated the point that, while the solubility of the amine hydrochloride generally sets the maximum obtainable concentration for a given amine, the solubility of the free base and the pKa determine the maximum pH at which formulation as a solution is possible (assuming that the desired concentration exceeds the free base solubility). Shifting the pH–solubility profile to higher pH values for formulation purposes may require increasing the solubility of the free base. This increase might be accomplished by using an appropriate cosolvent. Since the dissociation characteristics of carboxylic acids and other acidic organic species are similar to those of organic hydrochlorides, it is expected that the pH–solubility profiles



Ex. 2001-0390

of these organic acids, although reversed, can be characterized theoretically using the same treatment.

Several reports showed that the structure of an organic salt-forming radical influences the solubility of the resulting salt. The water solubilities of 16 salts (carboxylates, sulfates, sulfamates, and phosphates) of the weak base erythromycin were dependent on the size of the alkyl group of the acid (130). In a study with *N*-alkylsulfamates of lincomycin (66), a similar phenomenon was observed: solubility of these salts decreased as the size of the alkyl group attached to the acidic function increased.

Senior (131), in a study on the formulation and properties of the antibacterial chlorhexidine, determined the water solubilities of 35 salts and the free base. He found that inorganic salts had remarkably low solubilities while those of the lower aliphatic acids proved to be somewhat more soluble. Hydroxylation of the acid increased solubility, since salt formation with polyhydroxy acids, particularly the sugar acids, conferred extensive water solubility to the molecule.

Several investigators reported the influence of the solubility of a drug on its formulation and subsequent availability from the dosage form. In a discussion of the preparation and formulation of epinephrine salts in an aerosol system using liquefied gas propellants, Sciarra *et al.* (132) pointed out that the solubility characteristics of the agent are important in two respects. First, the solubility of the therapeutically active ingredient in the various propellants is an important consideration if the product is to be used for either local action in the lungs or systemic therapy. Second, the solubility of the drug in extracellular fluids plays an important role in selection of the compound. The bitartrate, malate, maleate, and fumarate salts of epinephrine were prepared and subjected to solubility and stability studies. While all salts had similar partition coefficients, the solubility of the maleate in several propellants and its stability in formulated aerosols made it the drug form of choice.

Ephedrine hydrochloride was more rapidly released than the free base from theobroma oil suppositories containing different surfactants (133). This enhanced rate of release (dialysis) was ascribed primarily to the greater aqueous solubility of the hydrochloride, which solubilized faster from the oil-in-water emulsion, whereas the ephedrine alkaloid base tended to remain behind in the oil phase.

The solubility of the active ingredient in ointment bases can dramatically influence its diffusion properties. A study of salicylic acid and its sodium salt showed that the diffusion of both was very low from hydrophobic bases, whereas the solubility of the drug significantly affected the diffusion from hydrophilic bases. The more soluble sodium salicylate diffused much faster from these latter bases than did salicylic acid (134).

Additional references on the relationship of salt form and solubility are listed in Table V.

**Table V—Additional References on Salt Form and Solubility**

| Topic | Reference |
|---|---|
| Influence of solubility on the rate of GI absorption of aspirin | 135 |
| Effect of dosage form upon the GI absorption rate of salicylates | 136 |
| Physical-chemical properties of polyene macrolide esters and their water-soluble salts | 137 |
| Isolation and reaction products of orotic acid and amines and their solubility in water | 138 |
| Solubility and stability of erythromycin salts | 139 |
| Studies on pharmaceutical preparations of orotic acid: water-soluble properties of orotic acid salts | 140 |
| Solubility of antibiotics in 24 solvents | 141, 142 |
| Solubility of antibiotics in 26 solvents | 143 |

**Organoleptic Properties**—Modern medicine requires that a pharmaceutical formulation be efficacious, safe, stable, and acceptable to the patient. Of primary importance in the development of oral dosage forms is taste acceptability. This factor presents no major problems when the drug is to be administered as a tablet or a capsule and swallowed as a unit but is clearly a prominent factor in patient acceptability when it is to be administered as a liquid, chewable tablet, or lozenge.

Since taste is a chemical sense, a substance must be dissolved if it is to elicit a taste sensation—either by taking it as a solution or by its dissolving in the saliva. Therefore, one method used to minimize undesirable organoleptic properties of pharmaceuticals involves the preparation of a poorly soluble salt form of the drug such that the saturation concentration is less than the taste threshold.

Erythromycin estolate (lauryl sulfate) has approximately one-twelfth the solubility of the free base, is tasteless, and is useful in the formulation of oral suspensions (144). A study on erythromycin salts showed that the bitterness level was dependent on two properties: (*a*) the water solubility of the salt, which is dependent on the size of the alkyl group attached to the acid function; and (*b*) the strength of the acid used to form the salt, *i.e.*, the stability of the salt (130). The stearyl sulfamate salt possessed the most desirable organoleptic properties.

Many problems concerned with formulation and stability of topical and oral pharmaceuticals containing bacitracin have been overcome by incorporating bacitracin into the formulation as its zinc salt. One distinct advantage over the parent compound is its lack of taste, caused by its relative insolubility. Thus, it is the preferred drug form for preparations where taste is a factor (145). Taste panel evaluations of the comparative bitterness of bacitracin zinc and bacitracin indicate that the taste of the zinc salt is more easily masked and that the presence of a bitterness-masking adjuvant, such as sucrose, increases the bitterness threshold ratio differences between the two compounds even further (146).

Propoxyphene napsylate, nearly water insoluble, is only slightly bitter to the taste as compared to the highly water-soluble hydrochloride (147) and can be readily formulated into a flavored aqueous suspension. The taste of these suspensions can be improved significantly by the addition of a common ion (sodium or calcium napsylate) to depress solubility further.

A newer approach to the improvement of drug palatability has been to form insoluble salts with ion-exchange resins. Several investigators described and tested the practical application of this method (148–150). Spross *et al.* (149) outlined the conditions necessary for improving the palatability of a drug by adsorbing it onto an ion-exchange resin without appreciably modifying its pharmacological effects. They found that: (*a*) the degree of drug release from the ion-exchange adsorbate depends on the



Ex. 2001-0391

equivalent quotient between the electrolytes in the surrounding fluid and the ionic drugs, (*b*) the amount of ions is far less in the saliva than in the gastric juice (the temporary electrolyte contents can be estimated at 0.05 mEq in the saliva and at 10 mEq in the gastric juice), and (*c*) the exchange rates should allow the equilibria to be attained within a fairly short period. Insoluble drug resinates formed between dextran gel[2] cation exchangers and several basic drugs were in many cases much more pleasant tasting than their parent compounds. Furthermore, release of the drug from the ion-exchange adsorbate was quite rapid and complete under conditions prevailing in the GI tract.

Similar findings were reported using a polymethacrylic acid ion-exchange resin (150). In addition, coating the adsorbate particles with a 4:1 ethylcellulose–hydroxypropyl methylcellulose mixture further reduced bitterness. While *in vitro* release from the uncoated resinate was rapid and complete, release from coated adsorbates varied with the extent of coating.

Another approach to improving the taste properties of pharmaceutical agents is to prepare a pleasant-tasting soluble salt of a poor-tasting parent drug. This approach often can be very difficult, however, since solubilization of the parent compound usually imparts its unpleasant taste to the preparation. Nevertheless, some success has been reported using the artificial sweeteners cyclamate sodium and saccharin.

As described earlier, formation of *N*-cyclohexylsulfamate salts of several drug substances has produced better tasting derivatives with enhanced solubility properties (31, 32). The physicochemical and toxicological properties of benzalkonium saccharinate and a series of saccharinates of other quaternary ammonium compounds were reported (151). While conventional quaternary ammonium compounds have a very bitter taste, their saccharin analogs are sweet.

Potassium salts frequently possess an unpleasant taste and a metallic aftertaste. The palatability of some potassium salts in flavored vehicles was reported (152); while the salts had similar taste thresholds at effective therapeutic levels, all potassium salts exhibited inferior palatability.

Table III includes several samples of other salts that exhibit an improved taste relative to their free acid or base forms.

**Stability**—The chemical and physical stability of a pharmaceutical must be known, because it can influence the choice of dosage form, the manufacturing and packaging, and the therapeutic efficacy of the final preparation. Systematic determination of the thermal stability, solution stability (at various pH's), and light sensitivity of a drug and its derivatives, both alone and in the presence of common additives, provides essential input toward selecting the most suitable derivative and dosage form.

Depending on the route of degradation, different salt forms impart different stability characteristics to the parent drug by various mechanisms. Most commonly used are sparingly soluble salts which, when used in the formulation of suspensions, reduce the amount of drug in solution and, hence, its degradation. Differences in hygroscopicity of several salts influence the stability of the drug in the dry state. In some cases, the salt-forming radical itself enhances the stability of the parent agent.

The stability of penicillin G and its salts has been widely studied due to the drug's therapeutic importance and its characteristic instability. Schwartz and Buckwalter (153) described some of the stability characteristics of this antibiotic, stating that, with present techniques, a solution of penicillin cannot be made stable for more than 2 weeks, even at refrigerator temperatures. They also discussed the use of suspensions of sparingly soluble amine salts in aqueous vehicles as a means of "allowing marketing of a 'ready-made' penicillin product." Procaine, benzathine, and hydrabamine salts are marketed, and their acceptable stability as aqueous suspensions is based mainly on their insolubility and the minimization of degradation in solution.

A theoretical treatment of the solubility of these salts was presented in which equations were derived for calculating the solubility as a function of pH and the pH of minimum solubility (154, 155). These equations are based on the mass action law and its relationship to the ionization constants of the amine and the penicillin and the solubility of the salt in water. Since the salt in solution is partially dissociated, further suppression of the solubility may be achieved by the common ion effect. Swintosky *et al.* (156) demonstrated this effect with penicillin G procaine by adding procaine hydrochloride to the preparation and further enhancing its stability. The 8-chlorotheophylline salt (or complex) of penicillin G was reported to be water soluble, yet stable in solution (157). Since 8-chlorotheophylline is acidic, it has been postulated that a buffer effect could account for the stabilization of this "salt."

While penicillin G procaine is more stable in aqueous vehicles, it is less thermally stable than the sodium or potassium salts, decomposing if heated much above 60°. The sodium and potassium salts are known to withstand heating up to 100° for 4 days with little loss in potency (158). This behavior might well be due to differences in melting points—*viz.*, 106° for penicillin G procaine and ~215° dec. for the potassium salt.

Since hydrolysis of penicillin is dependent on moisture content, preparations in which moisture is rigorously excluded are quite stable in the dry state. A study on the effect of moisture on penicillin salts found the calcium salt to be less hygroscopic than the sodium salt and, hence, more stable in moist atmospheres (159). Similarly, penicillin G potassium is also much less hygroscopic than penicillin G sodium and has become the preferred form for marketing in the dry state (160).

Several studies reported the relative stabilities of thiamine salts, particularly the hydrochloride and the mononitrate (161–163). The mononitrate is observed to be less hygroscopic and is accordingly much less water soluble than the hydrochloride. Investigations of various preparations including compressed tablets, multivitamin capsules, and dry-filled vitamin B complex capsules at various temperatures showed that the mononitrate was more stable than the hydrochloride (164, 165). The stabilities of numerous thiamine salts were studied in aqueous solution and in dry powder preparations with various excipients (166, 167). In aqueous solution, the resulting pH was the chief factor controlling hydrolysis and oxidative decomposition of thiamine salts; their stability as powder

[2] Sephadex.



Ex. 2001-0392

preparations was related to their aqueous solubility, with the sparingly soluble salts being more stable (and presumably less hygroscopic).

An orally administered drug must be stable in acidic solution because it generally must pass intact through the acidic environment of the stomach if it is to exhibit therapeutic blood levels. The advantage of erythromycin estolate over the free base lies in its low solubility in gastric juice, which enables it to be administered with food without any decrease in attained blood levels. The salt is more stable in the stomach because it remains undissolved. Therefore, it retains its potency even when exposed to acidic environments for extended periods (144).

In a study on the preparation and characterization of lincomycin cyclamate (33), it was noted that the cyclamate salt had an enhanced thermal stability over the hydrochloride. In a subsequent report (168), differential thermal analysis and thermogravimetric analysis showed that while the hydrochloride easily undergoes thermal degradation, the cyclamate anion confers a considerably greater thermal stability on the lincomycin moiety.

Mullins and Macek (169) showed that the physical and chemical stability of the calcium salt of novobiocin makes it the form of choice for the formulation of a liquid preparation of the antibiotic. The amorphous calcium novobiocin salt proved to be tasteless, yet fully biologically active and perfectly stable in aqueous suspension. Neither the sodium salt nor the free acid is suitable; the sodium salt cannot be formulated in a liquid due to its chemical instability, while the crystalline free acid is not absorbed from the GI tract. Amorphous novobiocin is absorbed but is metastable in solution and slowly converts to the unabsorbed crystalline form.

Other reports of alterations in stability characteristics due to salt formation are listed in Table VI.

**Miscellaneous Properties**—The salt form has been reported to influence other physicochemical properties of a drug substance. Studies illustrating the effect of the salt-forming radical on surface tension, deaggregation behavior, and ion-pair extraction have appeared.

The influence of the anion on the absorption processes of two charged species, dextromethorphan and tetracycline, was studied in the rat stomach (186, 187). A linear relationship existed between the rate of absorption from buffer solutions of the anions under investigation and their surface tensions. Thus, the absorption process was related to the surface activity of the various salts and not to their lipid solubilities. This change of surface activity with the buffer (or salt) species is similar to the results reported in a study of the surface activity of various phenothiazine salts (188).

The antibacterial chlorhexidine possesses surface activity. A study of the colloidal properties of some chlorhexidine salts showed that the counterion can affect the critical micelle concentration of a surface-active agent, and this effect was usually associated with a change in micellar size (189).

The deaggregation behavior of a relatively insoluble acid and its sodium salt was studied, and deaggregation was postulated to be a possible rate-limiting step in the absorption of a drug from a dosage form (190). While no direct comparisons of the two forms were made, inspection of the data shows that the deaggregation rate of the salt was considerably more rapid than that of the free acid in equivalent dosage forms. Therefore, if absorption is dependent on the dissolution rate, which in turn is dependent on the deaggregation rate, the salt should produce the highest and earliest blood levels. On the other hand, it is possible that hygroscopic (and deliquescent) salts can absorb atmospheric moisture, cause a sticky surface, and inhibit deaggregation.

**Table VI—Additional References on Salt Form and Stability**

| Topic | Reference |
|---|---|
| Stability of chlorhexidine solutions | 170 |
| Stability of chlorhexidine when autoclaved | 171 |
| Anhydrotetracycline and 4-epianhydrotetracycline in marketed tetracycline and aged tetracycline products | 172 |
| Solid-state stability of some crystalline vitamin A compounds | 173 |
| Physicochemical studies on the stability of penicillin salts | 174 |
| Light sensitivity of tetracyclines | 175 |
| Hygroscopic properties, thermostability, and solubility of oleandomycin salts | 176 |
| Stability of orotic acid and its amine salts in aqueous solution | 177 |
| Some factors influencing the stability of tablets (aspirin) | 178 |
| Stability of aqueous solutions of sodium aminosalicylate | 179 |
| Hygroscopic properties of various preparations of erythromycin | 180 |
| Physicochemical studies on the decomposition of aminosalicylic acid and its salts | 181 |
| Stabilities of aqueous solutions of 2-diethylaminoethyl-3-methyl-2-phenylvalerate hydrochloride and its methobromide | 182 |
| Investigation of some properties of penicillin G salts | 183 |
| Stability of ferrous iron tablets on storage | 184 |
| Stability of aspirin aluminum compounded with antacids | 185 |

Higuchi and coworkers presented an extensive study on the physicochemical basis of the ion-pair extraction of pharmaceutical amines. Distribution ratios of dextromethorphan (191) and chlorpheniramine (192) between an organic layer and water were highly dependent on the concentration and nature of the anion present. Less hydrophilic anions yielded more readily extractable ion-pairs. A study of the thermodynamic properties, enthalpy, free energy, and entropy, involved in the extraction equilibria of dextromethorphan ion-pairs indicated that the entropy change associated with transfer of the different anions between phases is the main controlling factor in the extraction process (193).

## BIOAVAILABILITY

Most drugs prescribed in the United States are administered in solid and polyphasic dosage forms. Consequently, dissolution of the drug must precede the absorption process. The simplest model that adequately describes this process is shown in Scheme II.

$$\text{solid drug} \xrightarrow[1]{\text{dissolution}} \text{dissolved drug} \xrightarrow[2]{\text{absorption}} \text{drug in circulation}$$

*Scheme II*

Since the dissolution rate is generally slow for drugs with poor solubility, Step 1 is frequently rate limiting in the overall absorption process. As a result, the onset, intensity, duration of pharmacological activity, and, hence, bioavailability are affected by changes in dissolution rate. As discussed previously, administering a salt of the parent drug often proves to be an effective means of altering dissolution rate and absorption.



Ex. 2001-0393

**Table VII—Additional References on Bioavailability and Formulation Effects**

| Topic | Reference |
|---|---|
| Effects of various substances on the absorption of tetracycline in rats | 197 |
| Effects of dosage form upon the GI absorption rate of salicylates | 136 |
| Determination of *in vivo* and *in vitro* release of theophylline aminoisobutanol in a prolonged-action system | 198 |
| Ion-exchange resin salts for oral therapy: carbinoxamine | 199 |
| Latentiation of dihydrostreptomycin by pamoate formation | 16 |
| Solid-state ophthalmic dosage systems in effecting prolonged release of pilocarpine in the cul-de-sac | 17 |
| Absorption of erythromycin: various pharmaceutical forms | 200 |
| Comparative study of the absorption of drugs from old and new rectal preparations | 201 |

**Formulation Effects**—Choice of the salt form of a drug may have a pronounced effect on the formulation of the parent compound. For example, Fenton and Warren (194) found there was no release of medicament from proflavine cream BPC, a water-in-oil emulsion containing 0.1% proflavine as the hemisulfate salt. They also investigated the release of various salts of proflavine with aliphatic carboxylic acids from water-in-oil cream emulsions. Salts formed with the water-soluble, oil-insoluble "lower" acids, such as formic and acetic acids, showed very poor release from a water-in-oil cream. The "higher" acid salts (*e.g.*, *n*-valeric, caproic, cyclohexanecarboxylic, and caprylic) all showed increased diffusion from similar emulsions since these salts are soluble in both water and oil. Their release was even greater from oil-in-water emulsions, however, in agreement with their preferential oil solubility. The *n*-valerate salt provided the most effective water-in-oil cream. The primary factor responsible for diffusion of proflavine from a water-in-oil cream is the low hydrophilic–lipophilic balance conveyed to the salt by the acid. This finding illustrates the desirability of carefully selecting the salt anion of a cationic drug in lieu of the nature of the dosage form.

Studies of the effect of formulation on the bioavailability of warfarin sodium relative to warfarin yielded interesting results (107, 195). Absorption of warfarin upon administration of the sodium salt as a lactose-base tablet was no better than that from a similar formulation of the free acid. In fact, absorption was further depressed when the salt was formulated with starch instead of lactose. Later results indicated that the *in vitro* water dissolution rate of a warfarin sodium tablet was 350 times that of a slowly dissolving warfarin tablet formulation, yet the latter exhibited rapid and complete absorption *in vivo*. The virtual insolubility of warfarin in acidic gastric fluids precluded its absorption from the stomach. However, the strongly acidic medium was necessary for tablet disintegration, which, in turn, was critical for absorption. Following initial exposure to 0.1 *N* HCl, *in vitro* dissolution of the warfarin tablet in pH 7.4 buffer was 14 times faster than that of the sodium salt, a result that explained the otherwise contradictory *in vivo* blood level data. Therefore, absorption was ultimately dependent upon gastric emptying rate and gastric pH, as long as the formulation disintegrated properly in the stomach.

The rectal absorption of aspirin, aspirin aluminum, and calcium carbaspirin from several suppository bases was studied in dogs (196). The absorption of aspirin aluminum from either cocoa butter or a polyethylene glycol base was poor. While the maximum salicylate levels produced by aspirin and calcium carbaspirin from the cocoa butter base occurred at a later time than from the other bases studied, minimal plasma levels were exhibited by a polysorbate 61 base formulation. The highest peak and largest area under the blood level curve were seen with calcium carbaspirin in a vegetable fatty acid glyceride base. The poor absorption of aspirin aluminum from suppositories was not unexpected since it is poorly soluble. Furthermore, as pointed out for aspirin aluminum tablets (105, 106), an insoluble aluminum compound may form on the surface of the dissolving drug, further impeding its dissolution rate and bioavailability.

Additional references on bioavailability and formulation effects are given in Table VII.

**Absorption Alteration**—Several years ago, clinicians claimed that certain salts of theophylline were therapeutically preferable to other salts or to the free acid (202–204). For example, Schluger *et al.* (204) found higher blood theophylline levels after administering uncoated tablets of theophylline ethylenediamine than were observed with enteric-coated tablets of choline theophyllinate. These results were at variance with the *in vivo* work of Gagliani *et al.* (202), who found that the oral ingestion of choline theophyllinate produced significantly higher blood levels than the ethylenediamine salt. This apparent discrepancy could be explained by the formulation effects of tablet coating, *etc.*, which was not discussed in the work of Gagliani *et al.* In another study, a slightly more rapid rise in blood concentration and a greater area under the curve were observed with theophylline isopropanolamine than with theophylline ethylenediamine (303). It was suggested that the difference was a result of the greater water solubility of the isopropanolamine salt.

The *in vitro* dissolution rates of the choline and isopropanolamine salts of theophylline have been observed to be three to five times greater than the ethylenediamine salt, depending on the dissolution medium (98). It has been suggested that these differences in dissolution rate are consistent with, and offer an explanation for, the clinical results.

In a comparative study of the absorption of ampicillin trihydrate and ampicillin potassium following oral administration (205), the potassium salt yielded 37% higher peak levels and a larger area under the curve. Only 36% of the administered ampicillin trihydrate was absorbed while 53% of the potassium salt was absorbed. Determining the percentage of each drug eliminated in the urine showed that 39% was eliminated following administration of the trihydrate and 52% from the potassium salt. The entire difference between the two drug forms was accounted for in the initial 4 hr postadministration.

Several studies compared blood levels obtained with erythromycin and its salts and esters. Erythromycin estolate produced blood levels that were severalfold higher than those obtained with erythromycin base or erythromycin stearate (206–208). These differences were found to persist in the fasting as well as nonfasting subjects (207), indicating that food did not appreciably alter the absorption of erythromycin estolate when given under single or



Ex. 2001-0394

multiple dosing (207). This finding is explained by the fact that this salt is acid stable and alkaline dissociable, permitting its passage through the acid of the stomach both in fasting and nonfasting subjects (209). Accordingly, the antibacterial activity remained essentially the same when this form of erythromycin was given, regardless of the state of fasting of the subject (209).

On first inspection, the higher serum levels attained with erythromycin estolate suggest that the salt form is more readily absorbed. Also critical to efficacy, however, is the volume of distribution of the drug, since extensive binding to plasma proteins can render a drug unavailable for activity at the biophase. Therefore, the significance of blood level data greatly depends on the measure of the free or unbound fraction of total antibiotics in the blood, which more directly indicates probable therapeutic benefit.

Wiegand and Chun (210) showed that, despite the higher blood levels attained with erythromycin estolate (after correction for half-life differences), the stearate salt produced seven times more free drug in serum than did the estolate salt. This finding explained the higher total tissue levels observed on administration of the stearate. They attributed the difference to a greater serum protein binding of the intact erythromycin estolate, proving that its higher serum levels did not necessarily reflect more efficient absorption.

Marked differences have been observed following oral administration of various salts of penicillin. Penicillin G potassium has been compared with penicillin G benzathine (benzethacil) (211, 212), penicillin G hydrabamine (213), penicillin V (214), penicillin G procaine (212), and penicillin G ammonium (215). As anticipated, penicillin G potassium produced higher and earlier blood levels than penicillin G benzathine (211, 212). Furthermore, tablets of penicillin G potassium buffered with sodium citrate yielded higher peak levels than unbuffered penicillin G potassium. While the absorption of buffered tablets was apparently not significantly affected by food intake, the unbuffered tablets yielded lower average levels and irregular absorption under similar conditions.

When penicillin G potassium was compared with penicillin G hydrabamine (213), a less soluble salt, blood levels similar to those produced by penicillin G benzathine were observed. When equivalent doses were administered, the penicillemia that occurred with penicillin G hydrabamine was only one-third or one-fourth as great and was of shorter duration than that produced by penicillin G potassium.

Rudolfsen *et al.* (212) found that peak concentrations of penicillin G potassium were three to four times those obtained with penicillin G procaine and five to six times those of penicillin G benzathine, indicating a more rapid rate of absorption. The therapeutic action was related to the maximum concentration attained, but it also depended on the persistence of penicillemia, which was greater with penicillin G potassium than with the other two compounds. The authors suggested that the lower blood levels attained with the relatively insoluble penicillin G benzathine were caused by its destruction in the GI tract prior to absorption. This suggestion seems unlikely, however, since the drug should not degrade very rapidly as an undissolved suspension.

Because of its superior stability in gastric juice, penicillin V produces higher blood penicillin levels than corresponding doses of penicillin G. As a result, extensive investigations have been conducted with various salts of penicillin V (99, 214–220). For instance, tablets, capsules, and oral suspensions of penicillin V acid produced significantly higher blood concentrations than comparable penicillin G preparations (214). In the same study, the average serum levels produced by the benzathine salt of penicillin V were significantly higher than comparable doses of penicillin G benzathine for the first 4 hr but lower thereafter, again illustrating the value of using an insoluble salt to prolong blood levels of an acid-unstable compound. In another study (215), penicillin V acid was shown to produce higher and more prolonged plasma concentrations than either penicillin G potassium or ammonium, whose properties were comparable.

Plasma levels were correlated with dissolution rates of various forms of penicillin V (99). As solid dosage forms, the readily soluble potassium and calcium salts produced earlier and higher blood levels in fasting subjects than either the free acid or its benzathine salt. On the other hand, when the potassium and benzathine salts were administered orally as solutions, absorption was the same, implying that the poor solubility of the benzathine salt was responsible for the inferior blood levels obtained from its solid dosage forms. Other studies found that, while the potassium salt is 40% better absorbed than the free acid in fasting subjects, both forms produce therapeutic levels when administered with food (218).

Experiments with fistulated dogs indicated that penicillin V is absorbed primarily from the stomach. Therefore, it is not surprising that the potassium salt should show higher blood levels on oral administration since it is the most soluble salt in gastric pH (216). In accordance with this observation, it was also reported that the benzathine salt exhibited higher serum levels in patients with gastric achlorhydria (pernicious anemia) than in patients with normal gastric function (219). Differences in gastric emptying time may also explain this result.

Several studies compared the absorption of tetracycline and its salts (103, 221, 222). For example, serum concentrations in dogs and humans showed that a phosphate complex salt of tetracycline was absorbed more rapidly and gave higher blood levels during the first 6–8 hr than did tetracycline hydrochloride (221). The total amount of drug absorbed was about twice as great with the former compound.

Another study (222) suggested that tetracycline base produced higher blood levels than tetracycline hydrochloride. However, a subsequent investigation (223) showed that, in the absence of adjuvants or fillers, tetracycline hydrochloride and tetracycline base were absorbed equally well. Results obtained in this same study indicated that tetracycline hydrochloride encapsulated with citric acid produced higher serum concentrations than tetracycline hydrochloride mixed with hexametaphosphate or the phosphate complex salt of tetracycline.

In a study comparing urinary excretion rates and *in vitro* dissolution, the absorption of tetracycline and tetracycline phenolsulfonaphthaleinate was rate limited by their dissolution rates, whereas tetracycline hydrochloride absorption was rate limited by the absorption process itself (103).



Ex. 2001-0395

Several lincomycin salts were studied for their comparative availability (224). In particular, the blood levels obtained with the relatively water-insoluble hexadecylsulfamate salt were compared to those of the hydrochloride following oral administration. Higher and extended whole blood and serum concentrations were obtained in mice, rats, and dogs with the hexadecylsulfamate. However, subcutaneously administered lincomycin did not produce significantly different fractions absorbed, regardless of which salt was administered. It is not known whether the greater area under the curve with oral administration of lincomycin hexadecylsulfamate is due to greater absorption from the GI tract, slower renal clearance, or greater enterohepatic circulations.

Salts of streptomycin, neomycin, viomycin, and streptothrycin have been formed with: (*a*) polyacrylic acids, (*b*) sulfonic or phosphorylated polysaccharides, and (*c*) natural polycarboxyl acids from a series of polyuronic substances and polysaccharide derivatives containing carboxyl groups (18). The report indicated that these salts were absorbed from the injection locus primarily by the lymph system. Blood levels from the salts were generally lower but were maintained for a longer time than the equivalent amount of antibiotic alone, and higher concentrations of longer duration may actually be produced in the lymphatic drainage.

The influence of salt formation on the onset and duration of pharmacological activity also was illustrated with tolbutamide and several of its salts (101). Following oral administration, the sodium salt produced a rapid decrease in blood sugar level followed by a rapid recovery. By contrast, the free acid of tolbutamide caused a slow and prolonged drop in blood sugar level, a preferred effect since the chance of hypoglycemic shock would be lessened. This finding also illustrates the often overriding influence of the actual disease state on the choice of drug form.

Additional references on the implications of salt formation on absorption are listed in Table VIII.

**Pharmacokinetics**—Because of the various new properties that are usually imposed on a compound by salt formation, the pharmacokinetics of the drug necessarily change as a function of these properties.

For example, a pharmacokinetic evaluation comparing ampicillin sodium and potassium with ampicillin trihydrate was performed after oral administration to beagle dogs (243). The absorption rate constants of the sodium and potassium salts, which were similar, proved significantly greater than the rate constant of ampicillin trihydrate, resulting in earlier, higher peak concentrations with two to three times higher serum concentrations during the 1st hr. Yet, any differences between the fraction absorbed for the three products were not statistically significant. Apparently, although dissolution of the ampicillin trihydrate was the rate-limiting step in its absorption, the overall extent of bioavailability remained unaffected.

An interesting study of the biliary excretion of erythromycin base and erythromycin estolate was reported (244). The biliary excretion of erythromycin base was high, while that of erythromycin estolate was much lower; preferential secretion of erythromycin in the bile could partially account for the lower serum levels exhibited by the base. However, the proportion of the ingested dose secreted in the bile was small, and the total amounts involved were not sufficient to account entirely for the differences in serum concentrations attained. Undoubtedly, the protein binding studies of Wiegand and Chun (210) (discussed under *Absorption Alteration*) more satisfactorily explain the difference in serum concentrations.

**Table VIII—Additional References on Bioavailability and Absorption Alteration**

| Topic | Reference |
|---|---|
| Blood levels produced by three theophylline-containing elixirs | 225 |
| Naproxen oral absorption characteristics | 226 |
| Effect of food on absorption of a new form of erythromycin propionate | 227 |
| Effect of the anion on the absorption of tetracycline from the rat stomach | 186 |
| Blood levels following oral administration of different preparations of novobiocin | 228 |
| Absorption of iopanoic acid and its sodium salt | 229 |
| Oral absorption of secobarbital (quinalbarbitone) and its sodium salt | 230 |
| Absorption rate of barbiturates in humans | 231 |
| Morphine and atropine mucate | 232 |
| Excretion of bephenium salts in urine of human volunteers | 233 |
| Polymethylene bis(isothiuronium) salts: antituberculosis activity | 234 |
| Prolonged antitussive action of a resin-bound noscapine preparation | 235 |
| Pharmacology of sulfapyridine and sulfathiazole | 236 |
| Evaluation of plasma concentrations of propoxyphene utilizing a hybrid principal component analysis of variance technique: equimolar doses | 237 |
| Antryride, a new trypanocidal drug | 238 |
| Pralidoxime methanesulfonate: plasma levels and pharmacokinetics after oral administration to humans | 239 |
| Intestinal absorption of pralidoxime and other aldoximes | 240 |
| Blood plasma levels and elimination of salts of pralidoxime (2-PAM) in humans after oral administration | 241 |
| Enhancement of GI absorption of a quaternary ammonium compound by trichloroacetate | 242 |

Often, salt formation can be used to modify drug absorption and dose tolerance favorably. For example, aminosalicylic acid exhibits a short half-life and, therefore, requires large and frequent doses which may cause gastric irritation. Consequently, different chemical forms such as salts have been prepared (119, 245–247) to reduce the incidence of gastric irritation, increase absorption, and prolong blood levels.

Aminosalicylic acid is an interesting example in other ways; considerable confusion about this drug exists because many fail to recognize its nonlinear pharmacokinetics. Several definitive studies were reported regarding the absorption of the acid and its sodium, potassium, and calcium salts from solution, suspension, and tablet formulations (245, 246). Comparison of the relative bioavailabilities of aminosalicylic acid suspended in water and its salts dissolved in water showed that, while differences in rate of absorption were found to exist, absorption of both the acid and its salts was essentially complete. Absorption of the free acid from tablets reached only 77% of the dose, whereas that of the tableted salts was rapid and complete.

Regardless of formulation, the area under the plasma concentration–time curve of unmetabolized drug from free acid administration was less than that for the salts. This result was attributed to concentration-dependent metabolism during absorption: when the rate of absorption is high, the metabolic processes become saturated and more unmetabolized drug remains in the blood; conversely,



Ex. 2001-0396

**Table IX—Additional References on Bioavailability and Pharmacokinetics**

| Topic | Reference |
|---|---|
| Pharmacodynamics of fosfomycin (phosphonomycin) after intravenous administration to humans | 248 |
| Pharmacodynamics of phosphonomycin after oral administration to humans | 249 |
| Comparative studies on distribution, excretion, and metabolism of $^{3}$H-hydroxyzine and its $^{14}$C-methiodide in rats | 250 |
| Pharmacokinetics of ampicillin trihydrate, ampicillin sodium, and dicloxacillin sodium following intramuscular injection | 251 |
| Physiological disposition of fenoprofen in humans; pharmacokinetic comparison of calcium and sodium salts administered orally | 252 |

when the absorption rate is low, as for the free acid, a higher percentage of drug is metabolized.

Additional references regarding bioavailability and pharmacokinetics are presented in Table IX.

## GENERAL PHARMACY

**Pharmacological Effect**—Chlorpromazine hydrochloride and quaternary chlorpromazine chloride were investigated with respect to their effects on the central nervous system (CNS) (253). The quaternized compound was less potent and more toxic in rodents than the parent tertiary compound.

Naloxone, an effective opiate antagonist, is generally used as the hydrochloride salt; however, the drug has a very short duration of action. The mucate salt was prepared to extend its duration of action, since mucic acid is only slightly soluble in water (254). In a test on the blocking of morphine activity in mice, however, the mucate salt did not differ in duration from the hydrochloride. These investigators assumed the same receptor site for naloxone as for morphine and, since Heron's (232) work suggested that the receptor had a greater affinity for morphine mucate than for the free base, it also should have a greater affinity for naloxone mucate. The results disproved this hypothesis. Furthermore, this theory implies that intact salt reaches the receptor, which is highly unlikely, regardless of whether the drug is administered as a solution or as a suspension.

A series of salts of 9-aminoacridine and its derivatives was prepared and screened for antifungal and antibacterial activity (255–257). By using salts of fatty acids, the antifungal action was found to parallel the length of the carbon chain of the anion, with maximal activity occurring with acridine caproate, undecylate, and undecylenate (where undecylenic acid also exhibits some intrinsic antifungal activity) (255). This result appears reasonable, because these salts would be more lipid soluble and could be expected to pass through the cell wall of the infecting organism more readily, probably as an ion-pair.

The efficacy of bases or salts as topical anesthetics for relieving cutaneous itch, burning, and pain in unbroken skin has also been examined (258). In these experiments, itching and pricking were induced by an alternating current of low amperage and voltage applied to the skin or by exposure of the skin to UV light. Interestingly, aqueous solutions of salts of the local anesthetics did not alleviate itching or burning in any of the subjects, although saturated solutions of their bases in a mixture of water, 40% alcohol, and 10% glycerol were claimed to be effective. Such transport phenomena across the stratum corneum are often dependent on the polarity of the drug and vehicle and on the binding of the drug to keratin.

**Table X—Additional References on General Pharmacy and Pharmacological Effect**

| Topic | Reference |
|---|---|
| Differential excretion of bromide and chloride ions and its role in bromide retention | 259 |
| Pharmacological study of calcium methionate | 260 |
| Synthesis and *in vitro* fungistatic evaluation of some *N*-substituted amides and amine salts of sorbic acid | 261 |
| Antiamebic studies on clamoxyquin [5-chloro-7-[[(3-diethylaminopropyl)amino]methyl]-8-quinolinol] *in vitro* and in experimentally infected animals | 262 |
| Adjunctive value of oral propiylaxis with the oximes pralidoxime (2-PAM) lactate and pralidoxime methanesulfonate to therapeutic administration of atropine in dogs poisoned by inhaled sarin vapor | 263 |
| Pralidoxime (2-hydroxyiminomethyl-*N*-methylpyridinium) methanesulfonate and atropine in the treatment of severe organophosphate poisoning | 264 |
| Efficacy and limitations of oxime–atropine treatment of organophosphorus anticholinesterase poisoning | 265 |
| Antitussive activity of enoxolone (glycyrrhetinic acid) and its derivatives | 266 |
| Pharmacological properties of glycyrrhetinic acid hydrogen succinate (disodium salt) | 267 |
| Ganglionic blocking activity of diastereomeric dimethylaminobornyl acetates and their methiodides | 268 |
| A new potent nonnarcotic antitussive, 1-methyl-3-[bis(2-thienyl)methylene]piperidine: pharmacology and clinical efficacy | 269 |

**Dialysis**—Dialysis through a cellophane membrane of the hydrochloride or sodium salts has been studied with several drugs (270). In many cases, it appeared that the ionic form of the drug was bound to the membrane whereas the nonionized form was not. Ephedrine hydrochloride presented an interesting example, however, since it dialyzed considerably faster than its corresponding base. It was theorized that the chloride ion dialyzed rapidly, enhancing the rate of dialysis of the ephedrine ion. Accordingly, when chloride ion was present on both sides of the membrane, the observed rate of dialysis for the ephedrine ion was comparable to the ephedrine base.

The diffusion of sodium chloride through a lipoprotein interface was very slow, especially if calcium chloride was present on both sides of the interface (271). In the presence of low concentrations of choline chloride or carbamylcholine chloride, the diffusion of sodium chloride is more rapid. Apparently, choline salts are able to increase the permeability of the lipoprotein to salts, which may relate to the physiological action of choline salts.

**Miscellaneous**—Release rates were determined for aminophylline, ephedrine alkaloid, and ephedrine hydrochloride from theobroma oil suppositories containing nonionic surfactants (133). While surfactants with hydrophilic–lipophilic balance (HLB) values less than 11 only minimally affected release rate, rates increased with surfactants of HLB values greater than 11. Under optimal conditions, aminophylline was faster than ephedrine hydrochloride which, in turn, was superior to the ephedrine base.

Willis and Banker (272) reported on the formation of polymer–drug salts as an approach to the physicochemical design of dosage forms. Poly(methyl vinyl ether/maleic



Ex. 2001-0397

anhydride) salts of methapyrilene were prepared and tested with the free base for *in vitro* dissolution and dialysis. Their dissolution and dialysis rates were not appreciably different from the free drug or its hydrochloride salt. Various poly(methyl vinyl ether/maleic anhydride) hemiester salts of methapyrilene exhibited substantially slower release than the polymer ether salts, hydrochloride salt, or free base forms. Polymer–drug salts thus appear to have promise.

A series of metallic salts of edetic (ethylenediaminetetraacetic) acid were tested *in vitro* to determine their effect on blood coagulation (273). The results showed that only the dipotassium and disodium salts had any effect on coagulation. It was theorized that the lack of anticoagulant activity resulted from an almost complete suppression of ionization of the heavy metal salts.

Interesting research regarding the angina-preventive effect of some chromone-2-carboxylate salts showed a direct correlation between biological activity and pKa of the salt-forming amines (274).

Lin *et al.* (112) investigated the relationship between salt form and biological activity of a given antihypertensive. While the intrinsic dissolution rates of the dihydrochloride and disulfate salts were many fold greater than the monohydrochloride, the hypotensive potencies of the salts did not differ significantly from one another in an anesthetized dog study. Yet, when administered to renal hypertensive dogs, the dihydrochloride and disulfate salts produced greater hypotensive effects than did the monohydrochloride.

## TOXICOLOGICAL CONSIDERATIONS

**Toxicity of Salt Ion**—Any discussion regarding the toxicity of salts of a drug must consider the pharmacological properties of the cation or anion used to form the salt as well as those of the free drug, since any of these may produce toxic effects. The toxicology of several ions that are commonly used to form salts and that are relevant to this review were discussed in depth (275).

Toxicity from ingestion of calcium salts of drugs is rare. If hypercalcemia occurs, however, calcium deposits in the kidney can bring on a reduction of renal function. The principal toxic effects of lithium also involve the kidneys. When small amounts of lithium are taken, no apparent damage occurs; yet large amounts of the metal can lead to irreversible damage. An apparent correlation was observed between lithium dosage and sodium intake (276). When lithium dosage was low or sodium intake was high, rats were able to excrete all lithium given and sustained a reversible polyuria. Conversely, if large amounts of lithium were administered to the rats or if their sodium intake was lowered, they incurred irreversible kidney damage. Ammonium ion, although it serves a major role in maintenance of the acid–base balance of the body, can be toxic in high concentrations and initiate CNS derangements.

Sulfate ions given orally tend to be minimally absorbed and may act as a laxative. The nitrate ion is irritating to the GI tract, causing nausea and gastric distress. Also, intestinal bacteria may convert the nitrate ion to nitrite which oxidizes hemoglobin to methemoglobin. The citrate ion, an intermediary in carbohydrate metabolism, can form a soluble complex with calcium which is poorly dissociable and rarely causes any toxic reactions. While tartrate ions are usually absorbed minimally from the GI tract, high concentrations reaching the circulation can cause renal damage.

Acetate and lactate ions are normal metabolites and appear to be well tolerated in relatively large amounts. Iodide and bromide ions can produce conditions known as iodism and bromidism, respectively. Bromide intoxication occurs quite frequently, since bromides are used as ingredients in some nonprescription preparations (277–280). Bromide is slowly excreted by the kidney (its half-life is 12 days) and tends to accumulate when taken for prolonged periods or when used by patients with decreased renal function (277).

**Toxicity of Salt Form**—Provided the salt-forming agents are nontoxic, the relative toxicities of a series of salts of a compound are often observed to reflect directly their absorption rates. For example, the toxicities of dibromide, dichloride, diiodide, and dimethylsulfate salts of quinapyramine[3], a trypanocidal drug, were determined (238). The sparingly soluble halogen salts were much less toxic subcutaneously or intramuscularly than the freely soluble dimethylsulfate, yet all salts showed about equal toxicity upon intravenous administration. The difference in toxicities obviously resulted from rapid absorption of the methylsulfate compared to the slowly absorbed, poorly soluble halogen salts. Similar reasoning has been used to explain the acute oral toxicity of propoxyphene hydrochloride in rodents, which is twice that of equimolar doses of the napsylate salt (281).

Several salts of benzphetamine and etryptamine were prepared as potential sustained-release formulations (114). The water solubility, *in vitro* dissolution rates at pH 1.0 and 7.2, and the median lethal times ($LT_{50}$) were determined for each salt. Both the $LT_{50}$ and $LD_{50}$ (determined on only a few salts) increased as the *in vitro* dissolution rate at pH 7.2 decreased. While dissolution at pH 1.0 did not correlate well with toxicity, the $LT_{50}$'s were inversely related to the square root of the dissolution rates at pH 7.2.

Toxicity studies comparing iopanoic acid, a cholecystographic contrast medium, with its sodium salt (115) showed that the salt form has 10-fold greater toxicity. The $LD_{50}$'s of the free acid and the salt were 22 and 2.32 g/kg, respectively. It was postulated that the free acid precipitated from the sodium salt upon its reaction with gastric hydrochloric acid. The fine, amorphous particles of precipitated acid had a greatly increased surface area and, therefore, dissolved more rapidly than even fine crystals of the free acid. The faster and more complete drug absorption then resulted in increased toxicity.

Salts exhibiting greater water solubility than their parent compounds or less soluble salts are not always more toxic. For example, various inorganic and organic ions were used to prepare salts of methyl pyridinium-2-aldoxime that would have greater water solubility and would eliminate undesirable side effects due to the iodide ion (95). Even though the aqueous solubility of the majority of these salts was many times greater than the iodide, their toxicity on a molar basis was not significantly different, with the exception of the dihydrogen phosphate salt which was 15%

[3] Antrycide.



Ex. 2001-0398

**Table XI—Additional References on Toxicological Considerations**

| Topic | Reference |
|---|---|
| Toxicity and absorption of 2-sulfanilimidopyridine and its soluble sodium salt | 285 |
| Sorbic acid as a fungistatic agent for foods: harmlessness of sorbic acid as a dietary component | 286 |
| Toxicity and distribution of erythromycin | 287 |
| Further toxicological studies with erythromycin | 288 |
| Pharmacology and toxicology of erythromycin estolate | 289 |
| Erythromycin propionate (propionylerythromycin): a review of 20,525 case reports for side-effect data | 290 |
| New class of antibiotic salts of reduced toxicity | 22 |
| GI intolerance to oral iron preparations | 291 |
| Comparative toxicology of iron compounds | 292 |
| Influence of the dissolution rate of lithium tablets on side effect | 123 |
| Toxicity and tissue distribution studies on the hydrochloride, bismuth iodide complex, and a resinate of emetine | 293 |
| Bacitracin zinc in pharmaceutical preparations | 145 |
| New approach to quaternary ammonium compounds | 151 |
| Pharmacology of choline theophyllinate | 294 |

more toxic. Further research with oximes revealed that other salts are also toxic (282).

GI bleeding is a common toxic effect of aspirin for a large percentage of the population. Consequently, a search was initiated for an aspirin derivative that would not induce GI blood loss (283). All compounds prepared, however, including the sodium and calcium salts, caused GI hemorrhage with a severity similar to aspirin.

Polyene antibiotics are potent antifungal agents but bear considerable toxicity. Even though the methyl ester hydrochlorides of these compounds are more soluble, they retain almost all of their antifungal activity and, more significantly, show a uniform decrease in toxicity compared to their parent compounds (284).

Additional references on toxicological considerations of salt formation are given in Table XI.

## CONCLUSIONS

Salt formation is a means of altering the physical, chemical, and biological characteristics of a drug without modifying its chemical structure. Clearly, the salt form can have a dramatic influence on the overall properties of the parent compound. At present, selecting a salt form that exhibits the desired combination of properties is a difficult semiempirical choice. Pharmaceutical scientists now recognize these facts and are beginning to study the effects of different salt forms on the physicochemical properties, bioavailability, and toxicity of drug substances.

Although now only a few generalizations are available to predict the effect of particular salt forms on the characteristics of a drug, perhaps in time it will be possible to evolve increasingly more powerful generalizations regarding the effect of a salt on the properties of its parent compound. In addition, we predict that polymer–drug salts will have a revolutionary effect on future trends in drug therapy, particularly in the areas of reducing drug toxicity and in controlling the release profile of novel drug delivery systems.

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*Review*

# Pharmaceutical Solids: A Strategic Approach to Regulatory Considerations

**Stephen Byrn,[1,4] Ralph Pfeiffer,[1] Michael Ganey,[2,3] Charles Hoiberg,[2] and Guirag Poochikian[2]**

***Purpose.*** This review describes a conceptual approach to the characterization of pharmaceutical solids. ***Methods.*** Four flow charts are presented: (1) polymorphs, (2) hydrates, (3) desolvated solvates, and (4) amorphous forms. ***Results.*** These flow charts (decision trees) are suggested as tools to develop information on pharmaceutical solids for both scientific and regulatory purposes. ***Conclusions.*** It is hoped that this review will lead to a more direct approach to the characterization of pharmaceutical solids and ultimately to faster approval of regulatory documents containing information on pharmaceutical solids.

KEY WORDS: polymorph; hydrate; amorphous form; desolvated solvate.

Interest in the subject of pharmaceutical solids stems in part from the Food and Drug Administration's (FDA's) drug substance guideline that states "appropriate" analytical procedures should be used to detect polymorphic, hydrated, or amorphous forms of the drug substance. These guidelines suggest the importance of controlling the crystal form of the drug substance. The guideline also states that it is the applicant's responsibility to control the crystal form of the drug substance and, if bioavailability is affected, to demonstrate the suitability of the control methods.

Thus, while it is clear that the New Drug Application (NDA) should contain information on solid state properties, particularly when bioavailability is an issue, the applicant may be unsure about how to scientifically approach the gathering of information and perhaps what kind of information is needed. This review is intended to provide a strategic approach to remove much of this uncertainty by presenting concepts and ideas in the form of flow charts rather than a set of guidelines or regulations. This is especially important because each individual compound has its own peculiarities which require flexibility in approach. The studies proposed herein are part of the Investigational New Drug (IND) process.

Solid drug substances display a wide and largely unpredictable variety of solid state properties. Nevertheless, application of basic physicochemical principles combined with appropriate analytical methodology can provide a strategy for scientific and regulatory decisions related to solid state behavior in the majority of cases. By addressing *fundamental* questions about solid state behavior at an early stage of drug development, both the applicant and the FDA are in a better position to assess the possible effects of any variations in the solid state properties of the drug substance. The resulting early interaction of the parties with regard to this area would not only tend to ensure uniformity of the materials used throughout the clinical trials but also fully resolve solid state issues before the critical stages of drug development. A further benefit of these scientific studies is the development of a meaningful set of solid state specifications which critically describe the solid form of the drug substance. These specifications would thus also facilitate the approval of a change in supplier or chemical process.

Our approach in this review is to suggest a sequence for collecting data on a drug substance that will efficiently answer specific questions about solid state behavior in a logical order. In "difficult" cases, perhaps where mixtures of forms must be dealt with, or other unusual properties are encountered, the suggested sequences would still have to be followed as a first stage in this investigation.

We have chosen to present this approach in the form of a series of decision trees, or flow charts (algorithms), one for each of the most common solid state forms. The charts are accompanied by examples from the literature representing the kind of data that would be useful in supporting the various decisions.

Decision trees provide conceptual frameworks for understanding how the justification for different crystal forms might be presented in the drug application. Industry may wish to use these decision trees as a strategic tool to organize the gathering of information early in the drug development process. Put another way, these decision trees provide a thought process that will lead to development of the most

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appropriate analytical controls. One should also note that it is the responsibility of the industry to select the appropriate test or tests to identify the phase of the solid and determine its relevant pharmaceutical properties. This approach is superior to simply performing a broad range of tests without regard to their relevance.

We should point out that, from a regulatory standpoint, if a company can establish a specification/test to ensure production of a well defined solid form of the drug substance, then it is not necessary to do all of the physical/chemical testing outlined in the decision trees. From a scientific standpoint, however, such an approach is risky since new forms may appear unpredictably during various stages of the development process. The appearance of these new forms usually slows the drug approval process and makes planning difficult.

Four decision trees are described in the sections that follow: Polymorphs; Hydrates (Solvates); Desolvated Solvates; and Amorphous Forms. Polymorphs exist when the drug substance crystallizes in different crystal packing arrangements all of which have the same elemental composition (Note that hydrates can exist in polymorphs). Hydrates exist when the drug substance incorporates water in the crystal lattice in either stoichiometric or nonstoichiometric amounts. Desolvated solvates are produced when a solvate is desolvated (either knowingly or unknowingly) and the crystal retains the structure of the solvate. Amorphous forms exist when a solid with no long range order and thus no crystallinity is produced. It is apparent that the appropriate flow chart can only be determined after the solid has been characterized using some of the tests described in the first decision point of the decision trees/flow charts (i.e. X-ray powder diffraction, elemental analysis, etc.). If there is no interest in marketing or producing an amorphous form or desolvated solvate at any stage in the process, then the corresponding flow charts do not need to be addressed. As already mentioned, it is advisable to investigate the drug substance for the existence of polymorphs and hydrates since these may be encountered at any stage of the drug manufacturing process or upon storage of the drug substance or dosage form.

All of the flow charts end (see for example Figure 1) with an indication of the types of controls which will be required based on whether a single morphic form or a mixture will be produced as the drug substance. Although this ending provides a simplistic view of a very complicated process of selecting appropriate controls, it is included to illustrate the consequence of the decisions made with regard to the drug substance. The reader should realize that the actual selection of the appropriate control could be the subject of another review which might contain another set of flow charts or decision trees.

## POLYMORPHS

The flow chart/decision tree for polymorphs is shown in Figure 1. It outlines investigations of the formation of polymorphs, the analytical tests available for identifying polymorphs, studies of the physical properties of polymorphs and the controls needed to ensure the integrity of drug substance containing either a single morphic form or a mixture.

### A. Formation of Polymorphs—Have Polymorphs Been Discovered?

The first step in the polymorphs decision tree is to crystallize the substance from a number of different solvents in order to attempt to answer the question: Are polymorphs possible? Solvents should include those used in the final crystallization steps and those used during formulation and processing and may also include water, methanol, ethanol, propanol, isopropanol, acetone, acetonitrile, ethyl acetate, hexane and mixtures if appropriate. New crystal forms can often be obtained by cooling hot saturated solutions or partly evaporating clear saturated solutions. The solids produced are analyzed using X-ray diffraction and at least one of the other methods. In these analyses, care must be taken to show that the method of sample preparation (i.e. drying, grinding) has not affected the solid form. If the analyses show that the solids obtained are identical (e.g. have the same X-ray diffraction patterns and IR spectra) then the answer to the question "Are polymorphs possible?" is "No",

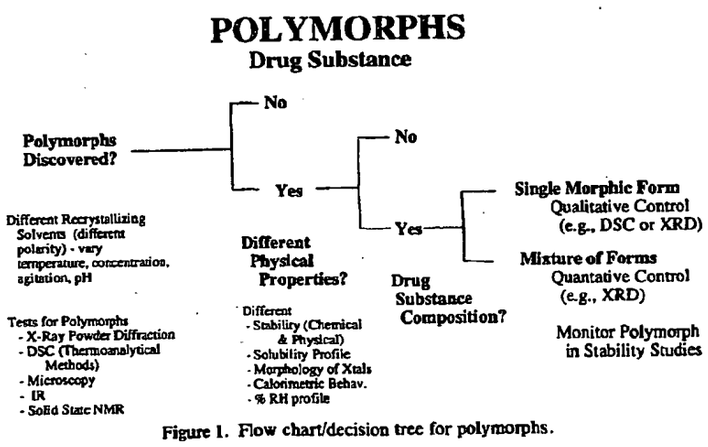



Figure 1. Flow chart/decision tree for polymorphs.

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and further research is not needed. The work of Miyamae *et al.* serves as a good example of solid state studies of a drug substance which exists as polymorphs (1). Powder diffraction showed that there were two crystal forms (see Figure 2).

These workers also carried out single crystal analysis of the two crystal forms of the compound. The structures are shown in Figure 3. While such studies are not required, and indeed sometimes not possible, they provide an unequivocal confirmation of the existence of polymorphs. Moreover, once the single crystal structure of a phase has been determined, it is possible to calculate the corresponding X-ray powder pattern. This provides an irrefutable standard for identifying the phase by that method.

The DSC thermal curves of the two forms are slightly different, as shown in Figure 4 and thus may not be the preferred way of differentiating these polymorphs.

The IR spectra of the two polymorphs are quite similar(1), and IR does not appear to be a powerful method for differentiating the crystal forms in this case. Thus, for 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine, powder diffraction appears to be the best method for differentiating the two forms.

Solid-state NMR is another powerful technique for analyzing different crystal forms (2,3). Figure 5 shows the solid-state C-13 NMR spectra of Forms I and II of prednisolone. Differences in the positions of the two resonances in the 120 ppm range clearly differentiate the two forms. In principle, solid state NMR is an absolute technique in which the signal intensity is proportional to the number of nuclei provided appropriate conditions are met. In addition, solid state NMR is a bulk technique which is not very sensitive to surface changes. This method appears to be very sensitive and will undoubtedly be used more often in the future as a tool to detect different crystal forms. However, with present technology, errors in solid-state quantitative studies may be rather large.

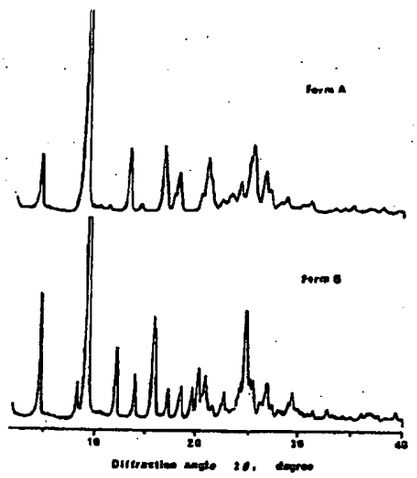



Figure 2. Powder X-ray diffraction patterns of the polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine (1).

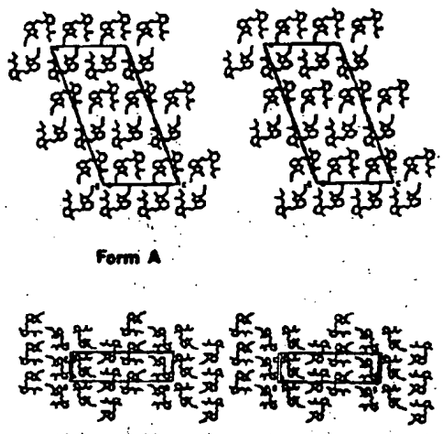



Figure 3. Stereoscopic drawings of the crystal packing of both polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine viewed along the shortest axis (Form A, b-axis; Form B, a-axis) (1).

### B. Do the Polymorphs Have Different Physical Properties?

If polymorphs exist then it is necessary to examine the physical properties of the different polymorphs that can affect dosage form performance (bioavailability and stability) or manufacturing reproducibility. The properties of interest are solubility profile (intrinsic dissolution rate, equilibrium solubility), stability (chemical and physical), and crystal morphology (including both shape and particle size), calorimetric behavior, and %RH profile. If there are no discernible differences between these physico-chemical properties, then the answer to the second question in the decision tree, "Different physical properties?" is "No."

The variable physical properties of several drugs with different polymorphs are reported in the literature. For example, the dissolution profiles of the polymorphs of chloramphenicol are significantly different (4). In addition, van't Hoff solubility analysis has been used to elucidate the dif-

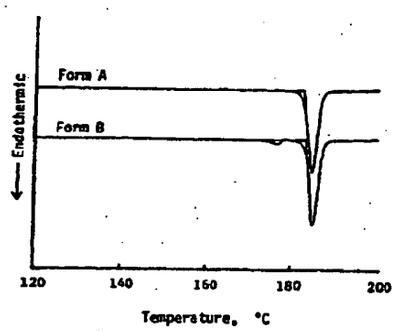



Figure 4. DSC thermal curves of the polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine (1). These curves show that Form A melts whereas Form B undergoes a small endothermic transition and then melts at the same temperature as Form A.

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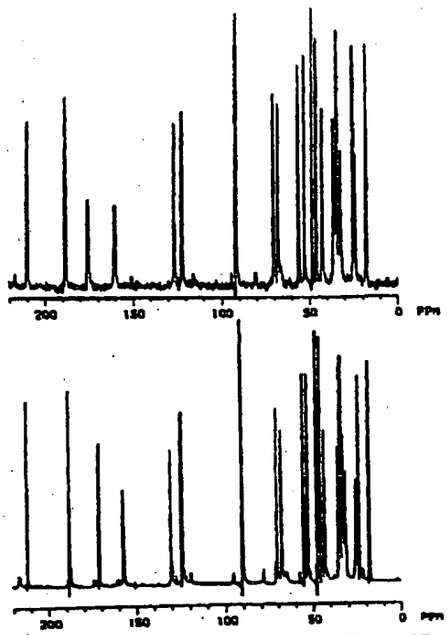



Figure 5. Solid state NMR of the two Crystal Forms of Prednisolone (2).

ferent solubilities of two polymorphs of methyl prednisolone(5). This method involves determining the equilibrium solubility of each polymorph at various temperatures. The log of the equilibrium solubility is then plotted vs 1/T. This should give straight lines for each polymorph and the temperature at which the curves intersect is the transition temperature. This technique does not work if the polymorphs interconvert.

For balance, it is important to point out that there are also cases where polymorphs exist but they have virtually identical dissolution properties(6).

### C. Drug Substance Control

The important question lies in the properties that differ among polymorphs and whether those properties affect the dosage form performance (i.e., quality or bioavailability). If they do then from a regulatory standpoint it is appropriate to establish a specification/test (e.g. powder X-ray diffraction or IR) to ensure the proper form is produced. From a production standpoint, it is important to develop a process that reproducibly produces the desired polymorph.

If mixtures of forms cannot be avoided, then quantitative control is needed to ensure that a fixed proportion of forms is obtained. Furthermore, the method of analyzing for the proportion of forms would have to be validated. Also, the proportion of forms would have to remain within stated limits through the retest date of the drug substance and potentially throughout the shelf life of the product; a difficult requirement if the forms interconvert. Thus, the way to avoid a substantial amount of work in this area is to select a single solid form for production. Usually, this would be the most physically stable form when their bioavailabilities are not significantly different. Selection of the most stable from would, of course, insure that it there would be no conversion into other forms.

Powder diffraction is often a useful method to determine the percentages of polymorphs in a mixture; however, the detection limit is variable from case to case and can be as high as 15%. Matsuda (7) carried out a mixture analysis of phenylbutazone polymorphs. Diffraction lines disappear and appear as the ratio of the crystal forms change. Some of these calibration curves developed from this analysis are almost horizontal, meaning that any given mixture gives the same line intensity in this mixture range. However, other calibration curves are sloped and would appear to allow a reasonable analysis. It is fair (although Matsuda did not carry out an estimate) to estimate the errors in this analysis as ±15%.

Tanninen and Ylirussi (8) used computer curve fitting to carry out a mixture analysis of prazosin. In this particular case, they reported a highly accurate analysis, and, in fact, showed a calibration curve that could detect 0.5% of one form in another. This is obviously a highly accurate mixture analysis by powder diffraction and shows the power of this method for some applications. However, this analysis required extreme care in sample preparation and may be more difficult to carry out in a production setting where particle size may not be controlled. Similar comments apply to the analysis of mixtures by IR, where the accuracy and precision may also vary considerably from case to case. Given the analytical problems in dealing with mixtures of forms, it may generally be simpler to develop a method to prepare only one crystal form.

In summary, it is important to determine whether polymorphs are present and to solve any problems before pivotal clinical studies are initiated.

### D. Determination of the Polymorph Present in the Drug Product

In cases where stability or bioavailability issues exist, the solid form present in the drug product should be investigated, if possible.

For bulk drug substances, X-ray powder diffraction and other techniques can identify the polymorph; however, solid state NMR appears to be the best method for the study of the drug substance in the dosage form (2, 3). Solid-state NMR study of three commercial products containing prednisolone showed that the products A and B contain Form I, whereas product C contains Form II.. This analysis was possible even though these tablets contain approximately 95 mg of excipients and 5 mg of drug. There are numerous cases, often involving complex mixtures or low dose products, where solid state NMR (and, in fact, any technique) will not be sensitive enough to identify the polymorph present in the drug product. However, the safety and efficacy is, of course, controlled by the potency assays and by the physical tests (e.g., dissolution).

## HYDRATES (SOLVATES)

The flow chart/decision tree for hydrates (solvates) is shown in Figure 6. It outlines investigations of the formation

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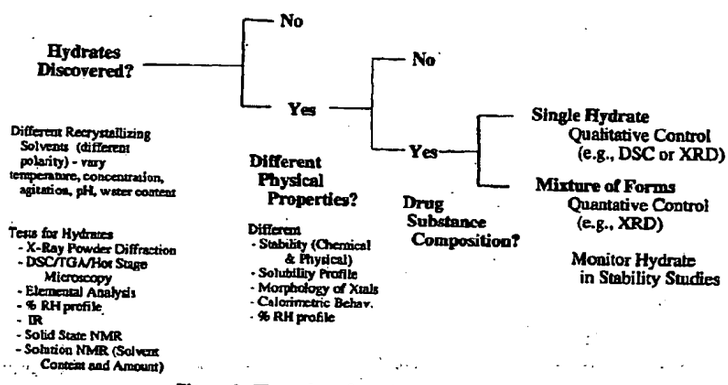



Figure 6. Flow chart for solvates or hydrates.

of hydrates (solvates), the analytical tests available for hydrates (solvates), studies of the physical properties of hydrates (solvates) and the controls needed to ensure the integrity of drug substance containing either a single morphic form or a mixture.

### A. Have Hydrates (Solvates) Been Discovered?

The flow chart for hydrates (solvates) (Figure 7) is applied after the preliminary crystallizations have been completed. These are essentially the same as in the polymorph decision tree but, in addition, should include solvent-water mixtures in order to maximize the chance for hydrate formation. These experiments can be guided by the moisture uptake (% RH) studies. Any solids that indicate a significant change in water content as indicated by the % RH-moisture profile should also be examined. The resulting solid phases are preferably characterized by a combination of methods—two for phase identity and two to reveal composition and stoichiometry.

With a very few exceptions, the structural solvent contained in marketed crystalline drug products is water. It is nevertheless often desirable to characterize other solvated crystalline forms of a drug for several reasons: they may be the penultimate form used to crystallize the final product and thus require controlled characterization; they may form if the final crystallization from solvents, especially mixed solvents, is not well controlled; they may be the actual crystallized form of a final product that is desolvated during a final drying step; they may be the form used in recovery for subsequent rework. The relevance of these points will vary from case to case, but for the present discussion we shall treat the subject of solvates in its broadest form.

Examples taken from the literature serve to illustrate the kind of data that proves useful in characterizing solvated crystal forms. For example, a recent report from our laboratory showed that IR and solid state NMR was useful for the identification of the different crystal forms of dirithromycin(9). TGA is another powerful method for the analysis of solvates. For example, one early study showed that TGA could differentiate three different hydrated salts of fenoprofen(10). Combined with IR or other methods, TGA is an unequivocal method for the verification of the existence of solvates. In addition, TGA is a good method for looking at mixtures of solvated and unsolvated crystal forms, and probably can be developed into an analytical method for determining the ratios of solvated and unsolvated forms.

DSC is also a good method for detecting solvates since there is usually heat change involved in desolvation, especially for hydrates(11). Specifically, DSC by itself does not prove the existence of a solvate, but once other analytical

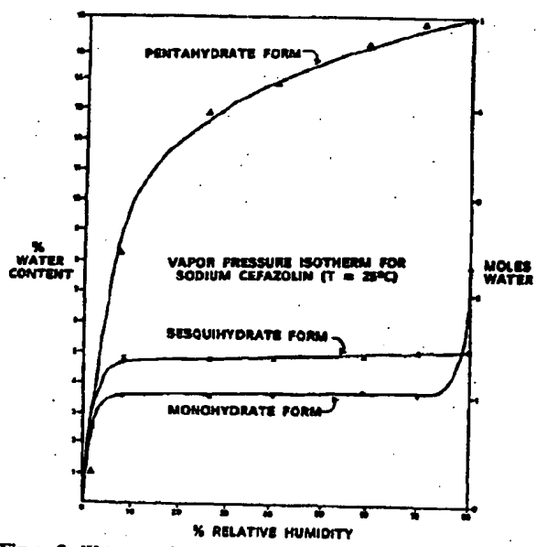



Figure 7. Water uptake vs percent relative humidity for sodium cefazolin.

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data from TGA, NMR, etc. are available, DSC becomes a good method for analyzing solvates and determining a percentage of solvates present.

The three solvates of ethynylestradiol (0.5 acetonitrile, 1.0 methanol, 0.5 water) provides another interesting example (12). These solvates have different cell parameters and are crystallographically completely distinct materials. The hemihydrate was obtained from an organic solvent which is not completely miscible with water but was saturated with water. In fact, it is known that crystallization from water-immiscible solvents containing small but slightly different proportions of water can produce different hydrates of a substance.

The DSC/TGA of the three ethynylestradiol solvates(12) are unique and in this case it may be possible to develop DSC/TGA into an analytical procedure for determining the proportions of each solvate. The DSC in some of these traces appears to show a melt and recrystallization corresponding to the loss of solvent of crystallization. However, the exact interpretation of this is not possible without either a DSC microscope or interrupting the tracing to analyze the sample at various temperatures. The methanolate appears to lose solvent in two equal steps, indicating that there may also be a hemimethanolate of this compound. Again, confirmation of this would require interrupting the heating and analyzing the substance after the first solvent loss has occurred. In addition, the DSC/TGA traces suggest that all of the forms are converted to an anhydrous form which then melts at a higher temperature. Thus, interrupting any one of these thermal curves just prior to the final melt could reveal a new form that gives the powder pattern for the anhydrate. Unfortunately, no data of this type is provided in the case cited.

DSC analysis of solvates should be carried out using either an open pan or a pan with a pin-prick; otherwise, unusual and variable results will be obtained because the solvent is not provided a way of escape from the pan. One advantage of using an open pan for DSC is that it reproduces the conditions under which the TGA is performed.

Comparison of the ethinylestradiol powder diffraction patterns clearly establishes that these solvates are different crystal forms as would be expected from the single crystal data(12). In summary, DSC, TGA, and powder diffraction are all good methods for analysis of the different crystal forms of ethinylestradiol.

Figure 7 shows a percent relative humidity versus water uptake study of the type recommended by the USP committee on water(13) In this case, there are two hydrates which are relatively well behaved insofar as they are completely hydrated at about 10% relative humidity and remain uniformly hydrated throughout a wide humidity range. On the other hand, the so-called pentahydrate, which really is only a pentahydrate at very high humidity, changes water content considerably as the relative humidity is changed. The USP committee on moisture specifications recommended that moisture uptake vs relative humidity studies should be routinely performed on all drug substances and excipients (13).

### B. Do the Hydrates (Solvates) Have Different Physical Properties?

The physical properties of hydrates are often quite different from the anhydrate form. Figure 8 shows the dissolution profile of theophylline hydrate and anhydrate. This figure shows that the anhydrate reaches a much higher solubility in water, and on extended exposure recrystallizes to the less soluble hydrate. Such differences must be further examined for possible effects on bioavailability.

In our laboratory we have described the different crystal forms of hydrocortisone-21-tertiary butylacetate(14). Our studies showed that the nonstoichiometric ethanolate is oxygen-sensitive and, of course, would show different physical properties from the stoichiometric ethanolate and the other solvates. Prednisolone tertiary-butylacetate also exists as a nonstoichiometric hydrate which is oxygen sensitive(15). Thus, these are cases where different crystal forms have different chemical stability, although there may be no significant differences in solubility.

### C. Mixtures of Polymorphs and Hydrates

Other drug substances exist as both polymorphs and solvates. For example, furosemide exists in two polymorphs, two solvates, and an amorphous form (16, 17). The polymorphs are enantiotropically related, which means that at one temperature one polymorph is more stable, but at a different temperature the other polymorph is physically more stable. That is, plots of solubility versus temperature cross for the two polymorphs. In addition, the different crystal forms have different photostability (chemical stability in light) and moreover have different dissolution rates. Thus, there are significant differences in both chemical and physical properties.

The five different forms, or modifications of furosemide, give clearly different powder patterns. Thus, powder diffraction is a good method for analysis of these different forms. There are similarities between the IR spectra of the five different forms but there are also some significant differences, and expansion and careful analysis could lead to an FT/IR method for analysis of these different forms. IR would probably be a useful method for analysis at least for pairs of these compounds. However, it is not clear whether IR could be used to determine the percentages of several different forms in a more complex mixture. The DSC and TGA of the different forms are significantly different. As expected, the solvates show weight loss in the TGA.

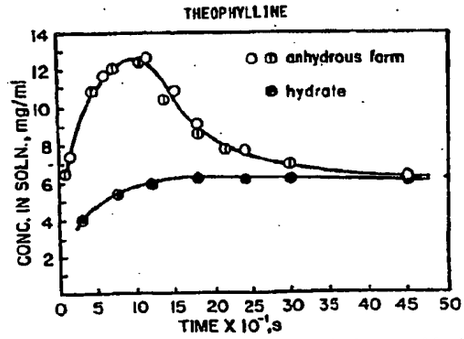



Figure 8. The dissolution-time curves for anhydrous and hydrated theophylline in water at 25°. The two types of open circles represent successive experiments (18).

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The interconversion of the different forms of furosemide have been analyzed and a diagram constructed. Such a diagram can be experimentally difficult when so many pairs of crystal forms must be studied for possible interconversions and under different conditions. It is clear from this diagram that many of the forms of furosemide can be converted to form I. This study is one of the most complete reports of solvates and polymorphs available in the literature and serves as a model for studies of such systems for regulatory submissions.

### D. Determination of the Hydrate Present in the Drug Product

Another important area is the analysis of the material which is produced after wet granulation of a substance which can form hydrates. We are aware of cases where the bulk drug substance is manufactured and stored as the anhydrate. However, upon wet granulation, there is a conversion (either partial or complete) to a hydrate. Subsequent drying is sometimes not adequate to convert the substance back to the anhydrate, and a hydrate or a mixture of hydrate and anhydrate remain. The formation of a hydrate and its subsequent drying can result in a change in particle size of the drug substance (19). It may also be possible to cause transformations during other processing steps. It is thus recommended that if wet granulation or processing that subjects the drug to even brief changes in temperature or pressure (e.g. milling or compression) is contemplated, then extensive studies of the ability to convert the drug substance to a new crystal form be carried out by mimicking the processing step in the laboratory.

## DESOLVATED SOLVATES

The term "desolvated solvates" refers to compounds that are crystallized as solvates but undergo desolvation prior to analysis. Often these "desolvated solvates" retain the structure of the solvate with relatively small changes in the lattice parameters and atomic coordinates, but no longer contain the solvent. In addition, desolvated solvates are apt to be less ordered that their crystalline counterparts. These forms are particularly difficult to characterize properly since analytical studies indicate that they are unsolvated materials (anhydrous crystal forms) when, in fact, they have the structure of the solvated crystal form from which they were derived. Several observations may give clues that one is dealing with a desolvated form: (1) The form can be obtained from only one solvent; (2) On heating, the form converts to a structure known to be unsolvated; and (3) The form has a particularly low density compared to other forms of the same substance. Experiments that help to clarify whether an apparently solvent free modification is a desolvated form or a true anhydrate include: (1) Single crystal X-ray structure determination in the presence of mother liquor from the crystallization; (2) comparison of the X-ray powder diffraction patterns and solid state NMR spectra of the solvated and desolvated crystal forms; and (3) determination of the vapor pressure isotherm by varying the vapor pressure of the specific solvent involved. A desolvated form will often take up stoichiometric amounts of the relevant solvent. In addition, crystals of the form directly isolated from the crystallizing medium will show a plateau in their isotherm as the vapor pressure of the solvent is reduced.

Figure 9 shows the flow chart used to address regulatory issues involving desolvated solvates. It is similar to the polymorphs flow chart except that the first question involves determining whether a solvate was formed initially and then desolvated, perhaps by "air drying." The remaining questions are identical to the polymorphs flow chart.

Figure 10 shows the behavior of three different crystal forms of the same antibiotic. One crystal form takes up about two waters of hydration and further analysis indicated that it was the dimethanolate which had been desolvated by drying. The second crystal form takes up approximately one water and was originally the monomethanolate which had been desolvated by drying. The third crystal form also takes up

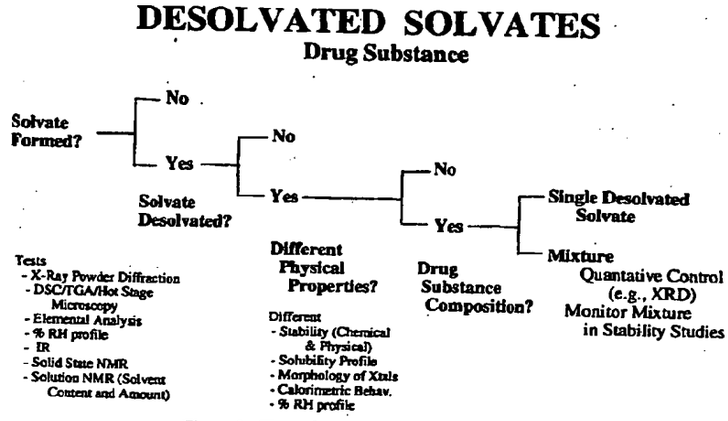



Figure 9. Flow chart for desolvated solvates.

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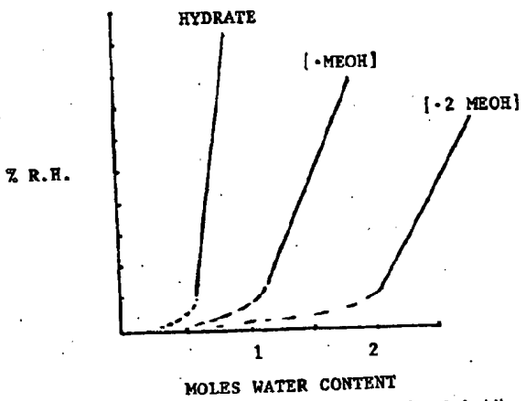



Figure 10. Water sorption by three crystal forms of cephaloridine. The brackets indicate the crystal form produced by desolvating the designated methanolate.

about one molecule of water and is the 0.75 hydrate typically obtained from water solution.

## AMORPHOUS FORMS

Amorphous forms are of substantial interest because they usually are much more soluble than their crystalline counterparts. Indeed, there are cases where the amorphous forms is the only solid form that has adequate bioavailability. The initial question with this flow chart (Figure 11) is similar to the previous ones: "Are amorphous forms possible?" Amorphous forms can be prepared in different ways, for example, by spray drying or by freeze drying. One can test whether an amorphous form has been produced by using one of the methods listed. X-ray powder diffraction and microscopy are the two primary methods for determining whether an amorphous form has been produced. Powder diffraction is an excellent method for determining the existence of an amorphous form since they usually exhibit a broad hump between 2 and 20° 2θ. An amorphous form is expected to have no peaks in the powder diffraction pattern. The USP test for the presence of an amorphous form involves determining, by microscopy, whether the material lacks birefringence. IR and solid-state NMR may be useful for detecting amorphous forms since the amorphous nature of the solid sometimes results in broad lines, or in NMR, altered relaxation times. The next question on the flow chart is: "Do the amorphous forms have different physical properties?" The answer to this question will almost certainly be "Yes." Three differences from crystalline forms may generally be expected: 1) Amorphous forms would have greater solubility, 2) Amorphous forms take up water more extensively, and 3) Amorphous forms are sometimes less chemically stable. Another key question for an amorphous form is: "Does it crystallize, and how and when?" This question is very important since inadvertent crystallization can greatly affect the solubility and dissolution rate, and lead to other failures in formulation. Attempts to purposely cause amorphous forms to crystallize can provide information on the parameters involved in crystallization of amorphous forms. Specific questions include: (1) "Does the amorphous form crystallize upon exposure to heat and/or humidity?," and (2) "What other factors (e.g. mechanical pressure and seeding) can lead to the crystallization of the amorphous forms?"

The amorphous form of any substance can be partly characterized by the glass transition temperature, $T_g$ (11). When heated to a temperature above $T_g$, the solid transforms from a glassy state to a more fluid-like rubbery state. The corresponding increased molecular mobility greatly raises the likelihood of two adverse events: (1) Crystallization and subsequent decreased solubility; and (2) Reduced chemical stability in the more reactive amorphous solid. Amorphous solids are also often prone to absorb moisture and this water sorption reduces the glass transition temperature further. The weight of water required to reduce the glass transition

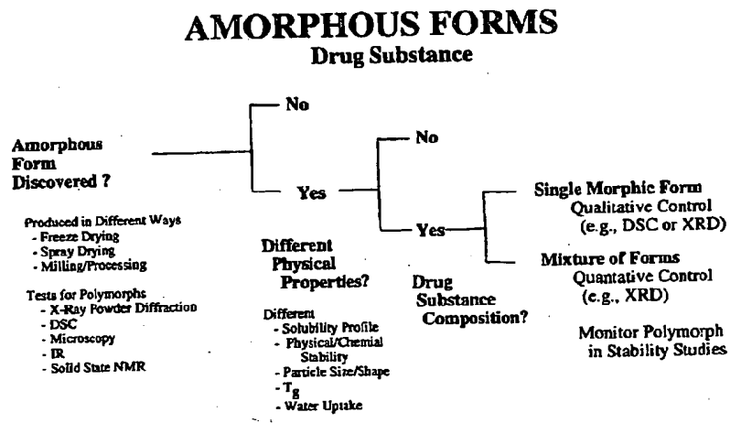



Figure 11. Flow chart for amorphous solids.

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temperature to room temperature is of obvious interest and is termed $W_g$. Table I shows a series of interesting studies on amorphous forms of some common pharmaceuticals.

The table compares the glass transition temperatures ($T_g$) of a number of pharmaceutical solids with the melting temperatures ($T_m$). It is interesting that the average ratio of the glass transition temperature to the melting temperature is about 0.70. This table provides a simple rule of thumb which allows the prediction of the glass transition temperature of pharmaceuticals from the known melting point. Crystallization and other solid-state phenomena, such as degradation reactions, as we have said, would be more likely to occur at temperatures above the glass transition temperature. For stability, one might, therefore, wish to prepare amorphous forms only for drugs which have a $T_g$ well above room temperature.

Amorphous indomethacin crystallizes upon standing at room temperature(Figure 12). Obviously, formulations containing amorphous indomethacin are at significant risk to crystallize and thus become less soluble. This has to some extent hampered preparing more bioavailable indomethacin dosage forms.

Quantitative analysis of mixtures of amorphous and crystalline forms provides some challenges. Cefixamine trihydrate is the subject of some early research in this area. This antibiotic, upon grinding, became a mixture of crystalline and amorphous forms. A calibration curve based upon analyzing the height of a selected powder X-ray peak was constructed and used to determine the crystallinity versus grinding time for this system. It is clear that powder diffraction provides a way to estimate the amount of amorphous cefiximine. These studies show that milling and other similar processing steps can create amorphous material and that this process may be detectable. As with wet granulation where transitions to hydrated forms can occur, processing of the drug substance can promote the formation of amorphous drug.

Pikal has compared the analysis of mixtures of crystalline and amorphous forms of several antibiotics by powder diffraction and calorimetry (20). His studies indicate that calorimetry can be a more accurate method for analysis of percent crystallinity but are complicated by water sorption. Zografi and co-workers (unpublished results) have developed a powerful method for the determination of low percentages of amorphous material based on the general propensity of amorphous materials to sorb moisture.

Table I. Pharmaceuticals Forming Glasses Above Room Temperature (21)

| Pharmaceutical | $T_g$ (K) | $T_m$ (K) | $T_g/T_m$ |
|---|---|---|---|
| Cholecalciferol | 296 | 352 | 0.84 |
| Sulfisoxazole | 306 | 460 | 0.67 |
| Stilbestrol | 308 | 439 | 0.70 |
| Phenobarbital | 321 | 443 | 0.72 |
| Quinidine | 326 | 445 | 0.73 |
| Salicin | 333 | 466 | 0.71 |
| Sulfathiazole | 334 | 471 | 0.71 |
| Sulfadimethoxine | 339 | 465 | 0.73 |
| Dehydrocholic acid | 348 | 502 | 0.69 |
| 17β-Estradiol | 351 | 445 | 0.80 |

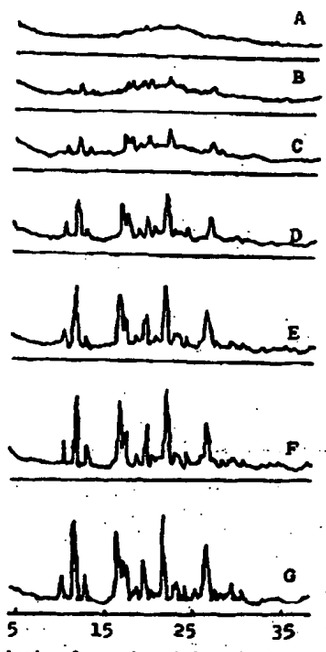



Figure 12. Behavior of amorphous indomethacin upon standing: A, at start; B 24 h; C, 48 h; D, 7d; E, 14d; F, 30d; G, 67d (22).

## SUMMARY

Four flow charts which describe approaches to regulatory issues involving pharmaceutical solids have been developed. These flow charts are for the different types of solids generally encountered (polymorphs, solvates, desolvated solvates, and amorphous forms). It is hoped that these flow charts will guide the solid-state research needed to prepare a comprehensive regulatory submission on the physicochemical properties of a pharmaceutical. It is also hoped that this review has provided enough information to allow the generation of results and information necessary to prepare a drug substance submission that will be quickly approved.

## ACKNOWLEDGMENTS

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Ex. 2001-0412

# Crystalline Polymorphism of Organic Compounds

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Crystal polymorphism is encountered in all areas of research involving solid substances. Its occurrence introduces complications during manufacturing processes and adds another dimension to the complexity of designing materials with specific properties. Research on polymorphism is fraught with unique difficulties due to the subtlety of polymorphic transformations and the inadvertent formation of pseudopolymorphs. In this report, a summary of thermodynamic, kinetic and structural considerations of polymorphism is presented. A wide variety of techniques appropriate to the study of organic crystalline polymorphism and pseudopolymorphism is then surveyed, ranging from simple crystal density measurement to observation of polymorphic transformations using variable-temperature synchrotron X-ray diffraction methods. Application of newer methodology described in this report is yielding fresh insights into the nature of the crystallization process, holding promise for a deeper understanding of the phenomenon of polymorphism and its practical control.

**Keywords:** Crystal polymorphism, Pseudopolymorphism, Crystallization.

1 **Introduction** . . . . . 164

2 **Crystal Polymorphism – Theoretical Principles and Practical Implications** . . . . . 165

2.1 Background – The Role of Polymorphism in the Production of Materials . . . . . 165
2.2 Crystallization and Polymorphic Transformations – Thermodynamic and Kinetic Considerations . . . . . 166
2.3 Polymorphism – Structural Considerations . . . . . 171

3 **Methodology for the Study of Crystal Polymorphism** . . . . . 177

3.1 Review of Preparative Methods . . . . . 177
3.2 Review of Investigative Methods . . . . . 180
3.3 Polymorphic Systems and Polymorphic Changes – Case Studies . . . 198

4 **Towards Control of Polymorphism** . . . . . 199

4.1 The Need for Polymorphic Control . . . . . 199
4.2 Current Strategies and Prognosis for Crystal Engineering . . . . . 200

5 **References** . . . . . 204

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## 1 Introduction

The protean nature of a chemical substance, reflected in its ability to crystallize in different structural arrangements (polymorphs), has since its discovery [1] been a source of both fascination and frustration for chemists. At a given temperature and pressure, only one polymorphic form of a substance is thermodynamically stable, all other forms being metastable. Since the rate of transformation of metastable polymorphs to the stable one may be slow, it is quite common to encounter several polymorphs of a single compound under normal laboratory conditions. Organic compounds tend to form different polymorphs owing to weak, non-directional intermolecular interactions which exist in the solid state. When a compound can be isolated in different polymorphic modifications, each with the potential of possessing unique properties (solubility, density, melting point, enthalpy of fusion, chemical reactivity, electrical conductivity, to name a few), the chemist, pharmaceutical chemist, or chemical engineer is presented with a degree of flexibility of choice for a particular application. Balanced against this flexibility, however, are the considerable practical difficulties that can arise, both in ensuring reproducible preparation of a specific polymorph and, during the lifetime of its application, preventing its spontaneous transformation to an undesirable form. Since free energy differences between polymorphic forms of a given substance are generally around a few kJ $mol^{-1}$ [2] and the process of crystallization is affected by many physical parameters (e.g. nature of the solvent, cooling and stirring rates, temperature, pressure, presence of impurities), minor variations in preparative conditions can tip the balance in favour of crystallization of a polymorph which is not necessarily the thermodynamically stable one. This element of unpredictability in the outcome of the crystallization process has serious implications for solids design in crystal engineering [3], where the required specificity of molecular organization in the crystalline state is crucial.

Various aspects of organic crystalline polymorphism and its occurrence in the fine chemicals, pharmaceuticals and other industries have been the subjects of several recent reviews. With varying degrees of overlap, these reviews can be roughly grouped into the following, according to their focus: thermodynamic and kinetic aspects [4–7], structural aspects [2, 3, 8–17], methodology [18–27], the crystallization process [28–34] and polymorphic control [35, 36]. The reader is referred to the above for a comprehensive view of what is a rather pervasive phenomenon in chemistry and whose pursuit is currently enjoying an upsurge of interest from solid-state researchers [5].

This report describes some recent developments in the understanding of the thermodynamic, kinetic and structural aspects of organic crystal polymorphism with an emphasis on the application of newer methodology used for its study, since this is one of the areas in which significant progress has been made in recent years. Numerous examples of polymorphic systems are described to illustrate the applications of both older and newer techniques for their investigation. These include studies of pseudopolymorphism manifested by hydrates and solvates of the parent organic molecule. Finally, the crucial question of

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control in polymorphism is briefly addressed with a view to illustrating current strategies and their implications for the design of solids.

## 2 Crystal Polymorphism – Theoretical Principles and Practical Implications

### 2.1 Background – The Role of Polymorphism in the Production of Materials

Many of the inconsistencies encountered in product performance in the chemical, chemical engineering, pharmaceutical, food and related industries can be attributed to polymorphism. An important example is inconsistent behaviour of drug substances upon dissolution which may have a direct influence on bioavailability. This arises because different polymorphic forms of the same drug may have solubilities which differ by an order of magnitude [24]. Inadvertent production of the 'wrong' polymorph at the crystallization stage following synthesis or at any of the intermediate processing stages can therefore result in pharmaceutical dosage forms which are either ineffective or toxic [37, 38]. Spontaneous polymorphic transformations mediated by solvents is common [4] and liquid preparations of metastable drugs frequently lose their effectiveness due to precipitation of less soluble, thermodynamically more stable polymorphs or pseudopolymorphs. A case in point is the antiprotozoal agent metronidazole benzoate which, when stored as an aqueous suspension below 38 °C is metastable, leading to precipitation and growth of the insoluble monohydrate [39, 40].

Dunitz and Bernstein [5] have recently documented several cases of "vanishing" polymorphs. These are usually metastable forms which, despite their thermodynamic instability, may have crystallized preferentially due to more rapid nucleation. Such metastable forms may persist and be used for many years before being "displaced", when a thermodynamically more stable form is prepared. Attempts to regenerate the original polymorph are frequently met with failure. Specific compounds with such a history include e.g. 1,2,3,5-tetra-*O*-acetyl-$\beta$-D-ribofuranose, benzocaine picrate and xylitol. This disturbing phenomenon extends to pseudopolymorphs. A previously known monohydrate of the antibiotic ampicillin has not been obtained since the appearance of the trihydrate [24]. A possible explanation for this behaviour is that after minute particles of the stable polymorph enter the environment, they eventually become widely disseminated ("planetary seeding" [5]) and serve as nuclei promoting crystallization of their own kind exclusively.

Manufacturing processes including crystallization scale-up, drying, heating, compression and milling can induce polymorphic transformations [24] and it follows that careful quality control is necessary at all stages to monitor undesirable changes. Systematic investigation of a compound to determine whether it is prone to polymorphism, as well as the nature of the polymorphism (enantiotropic or monotropic) [23], is routine practice in pharmaceutical pre-formulation studies. Identification of the different polymorphic forms of a drug substance, determination of their chemical and physical properties, thermodynamic

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stabilities, and temperatures and rates of interconversion are essential for ensuring drug preparations with reproducible behaviour [24]. Already, legislation requiring drug manufacturers to provide information relating to the occurrence (or apparent absence) of polymorphism in their products has been introduced [41]. Demonstrating the absence of a tendency to polymorphism is not easy; most substances when investigated for a sufficiently long time will reveal more than one polymorph [42].

Successful preparation of crystals of organic compounds having special properties (e.g. second-harmonic generation, metallic conductivity) may hinge on polymorphism, only one polymorph of the compound in question displaying the desired property. Bernstein has recently described representative systems which clearly illustrate the relationship between a polymorphic structure (a crystal architecture characterised by well-defined molecular interactions) and the unique physical properties which that structure confers on the solid material [13].

These remarks serve to emphasise some of the more important practical implications and consequences of polymorphism. Overcoming the problems encountered requires a deeper understanding of the processes of nucleation, crystal growth and polymorphic transformation. Several recent studies relating to these topics are reviewed in the next section.

## 2.2 Crystallization and Polymorphic Transformations – Thermodynamic and Kinetic Considerations

Crystallization of a specific polymorph from a melt, solution or vapour, commences with nucleation, i.e. the formation of a critical "embryonic" nucleus which is the structural blueprint for subsequent development and growth of the macroscopic crystal. The factors determining nucleation rate (e.g. the associated Gibbs free energy of activation, molecular volume, interfacial energy) generally differ for polymorphs of the same substance [4]. Since, in a supersaturated solution, nuclei of all possible polymorphs of the dissolved substance may be imagined to exist [36], the outcome of crystallization is kinetically complicated by competitive nucleation processes. Thermodynamic considerations of polymorphic crystallization include Ostwald's law of stages [4, 43], according to which, at high supersaturation, the first form which crystallizes is the thermodynamically least stable (most soluble) form. This form subsequently dissolves and transforms into a more stable one. The cycle continues until only the thermodynamically stable (least soluble) polymorph remains. The practical implication is that it should be possible to isolate the different polymorphs of a given compound at different levels of solution supersaturation and hence exercise some control over the crystallization process.

As regards polymorphic transformations in general, two types are distinguished, namely enantiotropic and monotropic [23]. These can be described in terms of the Gibbs free energy G, which has a minimum value for the thermodynamically stable phase of a polymorphic system and larger values for metastable phases and is such that the polymorph with the higher entropy will tend

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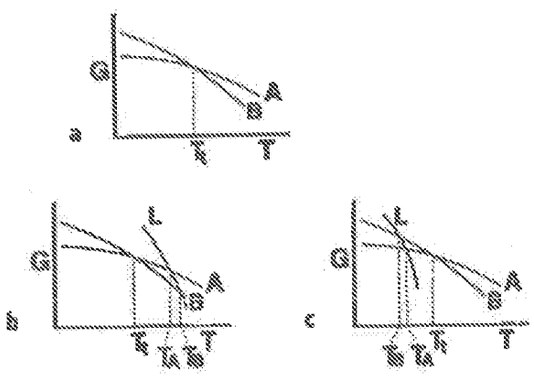



Fig. 1a–c. Gibbs free energy vs temperature for: a a dimorphic system, exhibiting; b enantiotropy; c monotropy

to become the stable form at higher temperature (species B in Fig. 1a). Above $T_t$ (the transition temperature), B is the stable polymorph while A is metastable and vice versa at temperatures below $T_t$. In an enantiotropic system (Fig. 1b), the free energy curve for the common liquid phase L intersects the A and B curves at $T > T_t$. In this case, the lower melting form (A) is stable at $T < T_t$, the higher melting form is stable at $T > T_t$, and the transition between the two forms is in principle reversible. Since transition temperatures in practice are often in the range 20–200 °C, one practical implication of enantiotropy is that conversion of one polymorph into another may be favoured during routine manufacturing processes [24]. On the other hand, for a system displaying monotropy (Fig. 1c), curve L intersects those for A and B below $T_t$ and the higher melting form (A) is always the thermodynamically stable one. Thus, below the melting point, only one form is stable and the other metastable. In practice, if a desired metastable polymorph is obtained during manufacture, it can revert to the stable polymorph under suitable conditions (e.g. in suspension, via solvent-mediation, or during compression). It follows that to prepare a specific polymorph and be aware of its possible fate during handling, it is advantageous to know the transition temperatures and thermodynamic stabilities of all the forms that may appear in the system [24].

The general considerations above highlight the importance of nucleation and the role of environmental conditions (e.g. solvent, temperature) in the crystallization of polymorphs as well as their interconversions. These areas continue to be the subject of intense interest especially in the context of polymorphic control in crystallization.

Some fundamental aspects of the nucleation process have been investigated by molecular dynamics (MD) methods. In a recent review [44] the advantages and limitations of molecular cluster models in simulating the dynamics of nucleation and phase changes have been discussed. In this approach, molecular dynamic simulations are correlated with experimental nucleation rates extracted from electron diffraction patterns of molecular supersonic jets. The dynamics of freezing of ammonia, $CCl_4$ and water, and the phase transformations of *t*-butyl chloride have been analysed. A useful feature of the MD computational

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approach is visual representation of phase transformations. Figure 2 illustrates MD-derived images of a crystalline cluster of 188 molecules of *t*-butyl chloride at various stages of freezing. MD simulations show that when sufficiently supercooled, the tetragonal phase spontaneously transforms to a lower temperature, ordered monoclinic phase. Diffraction patterns computed from the MD molecular packing were consistent with experimental neutron powder patterns for this phase.

Other investigators [45] have recently designed a numerical model to describe nucleation and growth of polymorphs with the aim of calculating the temporal sequences of precipitation and phase transformation in metastable solutions of polymorphic substances. Another group has recently modelled the formation and aggregation of polymorphs in continuous precipitation [46]. Consideration was given to the simultaneous growth and agglomeration of two different polymorphs as well as the case of nucleation of a single polymorph which subsequently transforms into a second one. The results indicated that the ratio of the nucleation rates, the ratio of the growth rates, and the aggregation tendencies determined polymorphic product composition as well as particle size distributions. This study is important since simultaneous precipitation of different polymorphs is encountered frequently in industrial crystallizations.

A mathematical phase-field model for the kinetics of isothermal polymorphic crystallization has recently been proposed [47], according to which crystallization involves rapid relaxation of the metastable state followed by nucleation and growth of the polycrystalline phase. Computer simulations were used to obtain results which could be tested experimentally using X-ray scattering experiments. Growth rates of different polymorphic polymers have also been investigated [48]. Simultaneous development of spherulites of different polymorphs occurs at different rates under isothermal conditions. From observation of interspherulitic boundaries between the $\alpha$- and $\gamma$-forms of polypivalolactone,

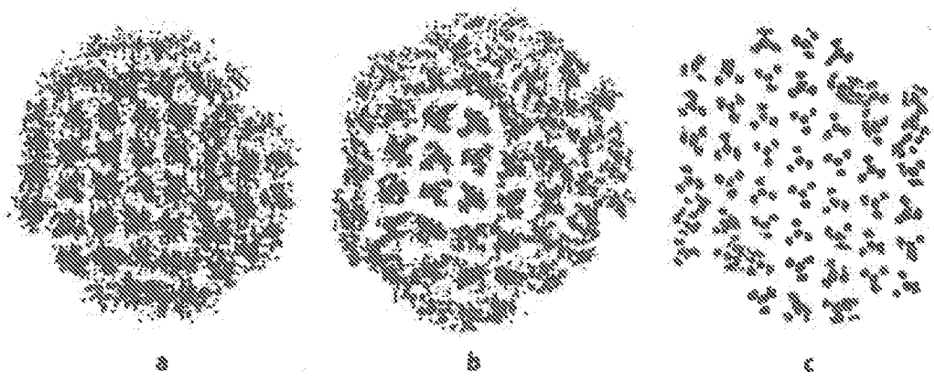



Fig. 2a–c. Images of a crystalline cluster of *t*-butyl chloride molecules at various stages of cooling, looking down the threefold molecular axis: a orientationally disordered tetragonal phase at 130 K; b nucleus of monoclinic phase growing in tetragonal phase at 80 K; c ordered monoclinic phase at 50 K after transformation. Surface molecules tend to be disordered at all temperatures. (Reprinted with permission from [44], copyright 1995 American Chemical Society)

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their relative growth rates could be determined. The thermodynamics and kinetics of crystallization of large molecules from solution have been discussed [49]. Modelling of the crystallization of protein molecules indicated that the concepts and numerics of colloid stability theory are appropriate.

Recent studies of polymorphic transformations in organic crystals mediated by melt, solution and interface have been reviewed [4]. Interface-mediated transformation has only recently been recognized as a distinct mode of polymorphic transformation. It involves nucleation and growth of a new polymorph through mass transfer across the interface connecting single crystals of two different polymorphs and it differs from solid-solid transformations in that a microscopic solution layer is required as an interface. Transformations mediated by melt, solution and interface are usually more rapid than solid-solid transitions; for the latter, the activation energy is larger due to the fact that nucleation and growth of the new phase within a second phase involve diffusion and structural rearrangement at the reaction interface. The fundamental thermodynamic relationships governing polymorphic solid state transitions have been reviewed [6]. It has also been pointed out that the mechanisms of polymorphic transitions in molecular crystals are largely unknown, though order-disorder transitions are understood in reasonable detail [5]. An important technique for studying solid-solid transformations is thermal analysis and a review of the basic thermodynamic principles for interpreting thermal analysis data for both polymorphic and pseudopolymorphic systems has appeared [23]. Kinetic and thermodynamic aspects of the thermal decomposition of inclusion compounds (a special class of pseudopolymorphs) have also recently been discussed [50].

Theoretical and experimental studies of the role of solvent on polymorphic crystallization and phase transformations abound in the literature of the last few years and some pertinent examples are described here. For solvent-mediated transformations, the driving force is the difference in solubility between different polymorphs. An important earlier paper on the kinetics of such phase transformations [51] described a model featuring two kinetic processes in solid to solid phase changes via a solution phase, namely dissolution of the metastable phase and growth of the stable one.

The effect of solvent on the crystallization of polymorphs has recently been investigated [43] using as a model compound the antibacterial sulphathiazole whose four known polymorphs are well characterised. The study, whose express intention was to test the Ostwald law, involved crystallization of the pure polymorphic forms of the drug, solubility measurements and crystallizations from various solvent systems. Systematic variation of supersaturation was employed in an attempt to crystallize each of the four forms of sulphathiazole, as predicted by the Ostwald law. Solubility studies showed Form I to be the most soluble form, followed in order by Forms II, IV and III. The supersaturation crystallizations using acetone, acetone-$CHCl_3$ (3:2), *n*-propanol and water, revealed that only the acetone-$CHCl_3$ system yielded results in accord with theory, Forms I, III and IV being isolated from it by varying the supersaturation. Crystallization from *n*-propanol, for example, yielded only Form I at all supersaturation levels. Thus, the finding that some solvents selectively favour the crystallization of a

Ex. 2001-0419

particular form (or forms) indicated that, while supersaturation is an important factor determining crystallization of polymorphs, the solvent may play a dominating role which is not thermodynamic in nature, but rather kinetic. The proposed mechanism, namely selective adsorption of different solvent molecules on specific faces of particular polymorphs, is consistent with that suggested earlier [52] for the effects of additives and solvents on crystal morphology. Such specific adsorption might result in inhibition of nucleation of certain polymorphs or retardation of their growth, allowing other, thermodynamically less favoured, polymorphs to crystallize instead.

The mechanism of a solvent-mediated transformation may change with complete change of solvent, or more subtly, when a gradual change in polarity is effected by dilution of the original solvent with another. The effects of the solvent systems water [53] and ethanol/water [54] on the crystallization of L-histidine polymorphs have been investigated. In aqueous solution at the isoelectric point, it was found that both the A and B polymorphs precipitate with a nearly constant ratio over a wide concentration range. However, slow transformation from B to A (which does not occur in the absence of solvent) was observed and pure A could eventually be isolated. This transformation involves smooth growth of the stable A polymorph and dissolution of the metastable form B. The ratio of the rate constants for the appearance of A and the dissolution of B indicated a growth-controlled mechanism for the transformation. In subsequent experiments investigating the effect of added ethanol, however, it was found that the fraction of polymorph A in precipitates decreased rapidly with increasing volume fraction of ethanol in the mixture, and pure B could be obtained when this fraction was 0.4. An explanation for the change in growth mechanism with added ethanol, based on the decreased concentration of polymorph A, was proposed [54].

This type of behaviour is not confined to polymorphs but may extend to pseudopolymorphic forms such as hydrates and solvates. A recent case of solvent-mediated phase transformation involved polymorphic and pseudopolymorphic forms of thiazole carboxylic acid [55], where the transformation is again sensitive to the composition of the mixed solvent. Three forms of the compound are known, an anhydrous form, a 0.5 hydrate, and a 1.5 hydrate. In 50–80% solutions (% = vol.% MeOH-$H_2O$), transformation of the 1.5 hydrate to the 0.5 hydrate was observed while transformation to the anhydrous form occurred in 85–100% solutions. No transformation occurred in 0–30% solutions. Detailed study of a solvent-mediated polymorphic transition has also been carried out for the antiulcerative agent cimetidine [56] for which seven polymorphic forms are known. An important feature of this study was the systematic use of seed crystals to induce crystallization at different supersaturation ratios.

The possibility of relating solvent effects to polymorphic crystallization at the molecular level may be realized when the individual crystal structures of the polymorphs are known. An analysis of this kind was carried out for the anti-inflammatory drug piroxicam [57] which was found to crystallize as the $\alpha$-polymorph from proton donor and basic solvents, but as the $\beta$-polymorph from non-polar solvents. On the assumption that crystallization of the drug requires

Ex. 2001-0420

release of solvent molecules from acceptor and donor sites on the molecule, it was possible to reconcile the observed intermolecular hydrogen bonding arrangements in the crystals (infinite chains in $\alpha$-, cyclic dimers in $\beta$-) with probable sites of solvation on the piroxicam molecule in solvents of different polarities. In this way, formation of the $\alpha$- and $\beta$-polymorphs from different solvent systems could be rationalised. Combination of polymorphic crystal structural data with appropriately detailed solvation models could be a useful adjunct to the existing methods of rationalising or predicting the outcome of polymorphic crystallization from solution.

## 2.3 Polymorphism – Structural Considerations

Dunitz has recently developed the theme of the crystal as an ordered supramolecular entity [2]. From this perspective, different polymorphic modifications of a given compound may thus be regarded as "supramolecular isomers". In discussing the possible structural arrangements occurring in crystals of polymorphs, a convenient distinction may be made [3] between rigid molecules (e.g. planar chloro-aromatics) and those with conformational flexibility (e.g. *N*-benzylideneanilines). In the former case, different polymorphic structures frequently display common features such as similar intermolecular directional contacts, layer stacking and unit cell dimensions which are related in a simple way. Conformational polymorphism, the existence of different conformers of a flexible molecule in the various crystal structures [58], may be expected to occur when the conformational energy minima differ by less than about 8 kJ $mol^{-1}$. If the energy barriers separating these minima are sufficiently low, these conformers may co-exist in solution and slight variations in crystallization conditions may lead to their individual isolation as conformational polymorphs [11]. Systems of this type have been exploited to study both the influences of the crystalline environment on molecular conformation as well as the properties of molecules which depend strongly on conformation [13]. An example of conformational polymorphism in which the structural arrangements are dictated by non-directional van der Waals forces only is shown in Fig. 3. The molecule in question is probucol, a drug used to control blood-cholesterol levels. Here, intermolecular hydrogen bonding between hydroxyl groups in the crystals is prevented owing to intramolecular steric crowding of these groups by neighbouring *t*-butyl substituents. The molecules adopt distinctly different conformations in the two polymorphs [59], the more symmetrical conformation approaching point symmetry $C_{2v}$.

Figure 4 shows representative hydrogen bonded (N-H···O, C-H···O) layers of planar nitrofurantoin molecules occurring in the $\alpha$- and $\beta$-polymorphs [60] which are triclinic and monoclinic, respectively. In this system, the molecular conformations in the two polymorphs are indistinguishable but the symmetries of their intermolecular hydrogen bonding schemes differ significantly. The common molecular conformation shown here occurs in five modifications (two polymorphs and three pseudopolymorphs) of this compound [61]. The three-dimensional crystal structures of the polymorphs result from close stacking

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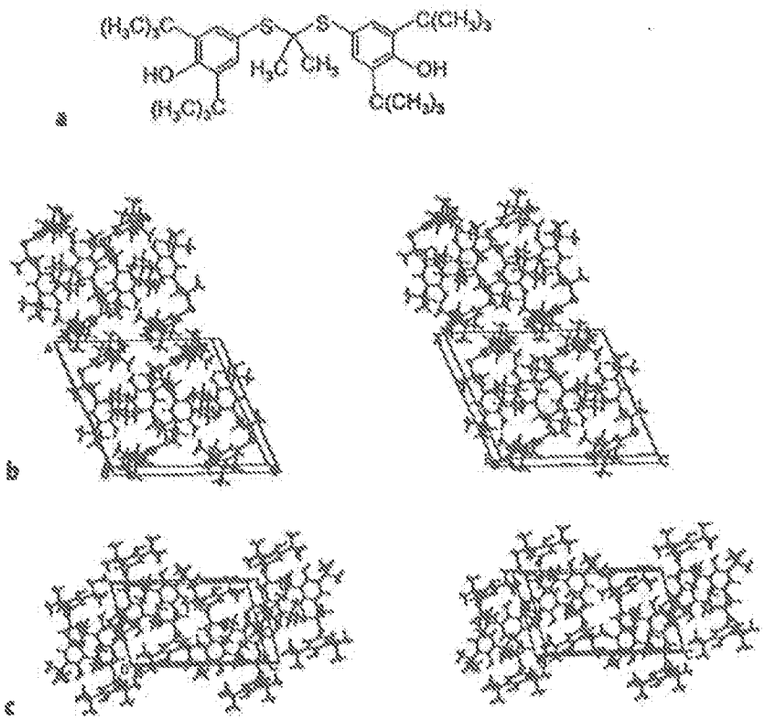



Fig. 3. a Molecular structure of probucol; b stereoview of the crystal structure of Form I; c stereoview of the crystal structure of Form II. (Adapted from [59] with permission)

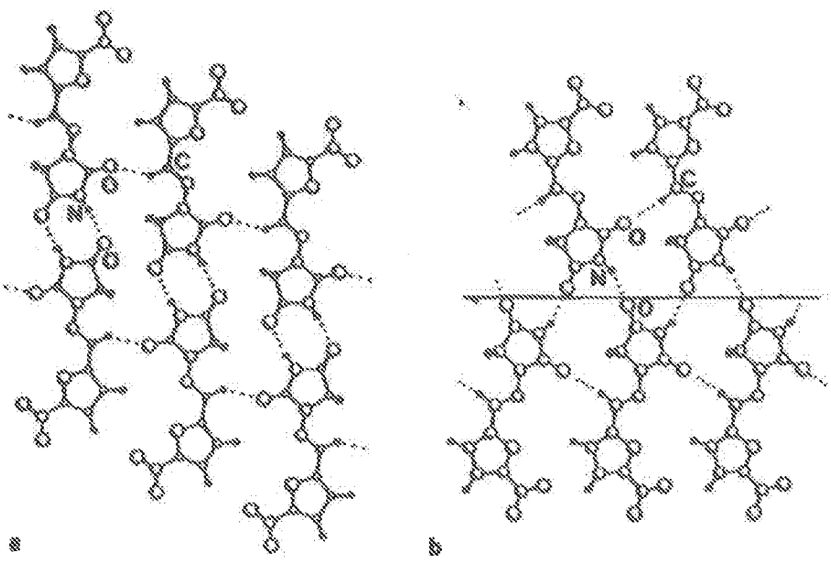



Fig. 4 a, b. Hydrogen bonded layers in nitrofurantoin polymorphs: a $\alpha$-form; b $\beta$-form (Adapted from [60] with permission)

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(~3.2 Å) of the respective layers shown in Fig. 4. This example conveys an idea of the variations in polymorphic structural arrangements that are possible with molecules containing hydrogen bonding functionalities.

During the last few years, the development of graph set analysis [16] has greatly facilitated the visualisation and comparison of polymorphic structures. Here, hydrogen bonding networks are classified as belonging to one of four distinct patterns, each specified by a designator (G in general): intramolecular (S), chains (C), rings (R), or other finite patterns (D). Further specification of the number of donor (d) and acceptor (a) atoms as well as the total number of atoms (n) comprising the pattern yields a concise and informative description of the hydrogen bonding arrangement, $G_d^a(n)$. A simple illustration of the use of graph set descriptors is given in Fig. 5 for a polymorph of thalidomide (space group C2/c, Z=8) [62]. Alternating hydrogen bonded ring motifs exist in this polymorphic structure as a result of bifurcated hydrogen bonding involving the N-H group. In contrast, the other known racemic modification of thalidomide (space group $P2_1/n$, Z=4) [63] contains only centrosymmetric dimers of the type $R_2^2(8)$. Application of graph set analysis to three polymorphs of iminodiacetic acid [64] has led not only to facile comparison of the crystal structures, but has also provided a basis for concise description of the polymorphic transformations occurring in that system. Thus, e.g. transformation of one polymorph of the acid into another is simply described as a conversion of $R_2^2(4)$ into $R_4^2(8)$. It has been pointed out that graph set analysis of hydrogen bonded systems also provides a means for seeking systematic correlations between the resultant patterns that demonstrate "hydrogen bond pattern functionality" [16]. This concept may be useful in the prediction of crystal structures as well as for the design of materials with desired supramolecular features. Extension of the graph set approach to encompass intermolecular atom-atom interactions other than hydrogen bonding for the classification of polymorphic crystal structures is envisaged as a natural and desirable development and more widespread use of this classification can be expected in future.

Elucidation of detailed polymorphic structural features and structural changes accompanying polymorphic transformations relies heavily on the single crystal X-ray diffraction technique. The inability to produce single crystals in

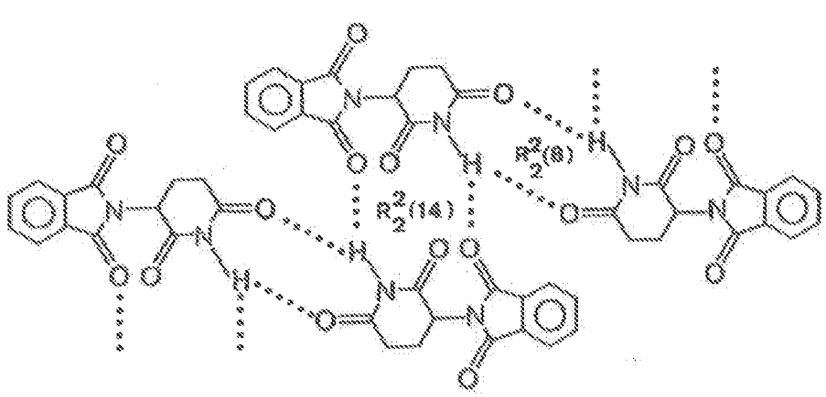



Fig. 5. Graph set notation for hydrogen bonded ring motifs in a polymorph of thalidomide

Ex. 2001-0423

the laboratory (especially for metastable polymorphs) has hindered progress in research on polymorphism. If, however, a polymorph is available in a powdered crystalline form and a computational method exists for predicting possible three-dimensional crystal structures, then the latter can be used to generate computed X-ray powder patterns for comparison with the experimental pattern. While the scope of the present review does not permit detailed exposition of the approaches and algorithms employed in crystal structure prediction, a summary of some recent developments, especially as they relate to polymorphism, is in order. The studies of polymorphism and crystal structure prediction are indeed two facets of the same topic [65]. Additional powerful motivations for predicting crystal structures include a better understanding of the crystallization process as well as the design of new solid materials. The reader is referred to several recent treatments of crystal structure prediction [66–70]. Comments on some of these methods and their applications follow.

In the atom-atom potential (AAP) method as implemented in the program PROMET [66], symmetry operations (e.g. a screw axis, centre of inversion) appropriate to the chosen space group, are applied to a molecule in fixed conformation (possibly optimised previously by a molecular mechanics calculation) to generate molecular clusters. Following a search for the most stable clusters on the potential hypersurface (calculated using empirical atom-atom potentials), translation is applied to generate one or more periodic structures. Packing energies are computed and acceptable structures are optimised. This is a very useful means of generating a series of polymorphic structures for a given molecule. In this approach, consideration is usually given to only the most populous space groups (93% of organic molecules being confined to 18 space groups [71]) thereby running a small risk of an incorrect choice. If an experimental X-ray powder pattern of the material is available, the correct crystal structure may be identified by comparison with computed powder patterns from the candidate structures generated by PROMET. In a study using this procedure [72] several literature cases were selected, for each of which the crystal structure of one polymorph had been determined, and mention was made of the existence of a second polymorph whose crystals were unsuitable for complete structural elucidation by X-ray analysis. Only unit cell and space group data were available for these undetermined polymorphs. Each starting molecular conformation was assumed to be the same as that in the corresponding fully characterised polymorph and was submitted to the PROMET procedure. In all cases, satisfactory structures were generated, with predicted unit cell data in good agreement with the experimental ones and with packing energies in the expected ranges. These results represent authentic crystal structure prediction, assisted by partial X-ray data. The method is therefore an alternative to direct methods of structural solution and also implies that, provided cell and space group information can be acquired, full structure determination without diffraction data is feasible. The authors of this study are less optimistic as regards true ab initio crystal structure prediction for numerous reasons, among them that packing energies for polymorphs of the same compound are always very similar, rendering the choice of the correctly computed structure difficult in the absence of other data, and that the occurrence of polymorphs containing mole-

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cules in different conformations is common. The point was also made that the correct energy ordering of polymorphic structures by computational methods may bear no relation to the experimental situation, where kinetic factors may determine which polymorph actually crystallizes under given conditions.

The AAP approach, with some variations, has recently been applied with varying degrees of success to the prediction of several other structures including, in order of increasing molecular complexity, high pressure solid phases of benzene [73], the three polymorphs of sulfanilamide [74], and the low and high temperature phases of poly(*p*-hydroxybenzoic acid) [75].

An ab initio molecular packing analysis procedure (program mpa) which avoids any prior assumption of space group symmetry has recently been described [76]. Only the molecular structure and the force field are required as inputs and the program finds intermolecular energy minima for packed arrangements for any given number of molecules comprising the asymmetric unit. Space group symmetry operations are predicted in this procedure and successful application to the crystal structures of urea and benzene were reported. It is significant that the energy minimisation for the urea structure converged to the correct space group ($P\bar{4}2_1m$) which has a frequency of occurrence of only 0.39 %.

Quantum mechanical methods have also been applied to crystal structure prediction. A recent example involved the use of ab initio crystal field methods with the SM (supermolecule) model and the PC (point charge) model applied to the three known polymorphs of glycine [77]. Comparison of the optimised structures with published X-ray structures for these forms indicated that the quantum-mechanically based SM model employing a 15-molecule cluster produced results in better agreement with experiment than the PC model which describes the crystal environment purely electrostatically.

Despite the varying degrees of success attainable by such computational methods, a survey of the recent literature on this subject seems to indicate that crystal structure prediction by theoretical methods is more rapid, has greater chances of success, and consumes far fewer computing resources when coupled with other techniques which provide additional experimental data for the crystal in question. (Examples of such combined studies are discussed in Sect. 3.2). At the same time, it can be argued that successful ab initio crystal structure prediction (i.e. assuming only the molecular structure as given) by whatever means possible in the future, would represent a very significant advance in the understanding of the fundamentals of the crystallization process. Regarding the feasibility of crystal structure prediction in general, some philosophical and technical points have been discussed, together with excellent practical recommendations for a programme of experimental and theoretical studies for elucidating the basic principles of organic solid-state chemistry [78]. This is seen as a prerequisite to the solution of the problem of crystal structure prediction.

Some new insights into the nature of organic crystal polymorphism have been gleaned from a recent systematic analysis [79] of data for polymorphic structures retrieved from the Cambridge Structural Database. A total of 345 crystal structures were reduced to 163 clusters (a cluster referring to a group of two or more polymorphs of the same compound). These clusters comprised 147

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with 2, 13 with 3, and 3 with 4 partners. Differences in molecular properties (P) (e.g. density, molecular volume, packing coefficient, AAP-calculated packing energies and other thermodynamic properties) were computed between cluster members (i, j) as $\Delta P = P_j - P_i$ or $\Delta P = 100(P_j - P_i)/P_j$ $(j > i)$ for a total of 204 data points. Both monovariate and bivariate statistical analyses were performed on the data, yielding several revealing trends and correlations. Histograms of differences in properties between polymorphic pairs, shown in Fig. 6a–c, indicate respectively that differences in crystal packing energies, densities and lattice vibrational entropies for polymorphs are rather small while Fig. 6d reveals that an appreciable proportion (actually 18%) of polymorphs have $Z' > 1$ (where $Z'$ is the number of molecules in the crystal asymmetric unit). Some further important conclusions drawn from this study are as follows: both calculated and experimental values for the relative stability of crystal polymorphs are currently subject to large uncertainties; polymorphs with $Z' > 1$ are as stable, or even more stable than those with $Z' = 1$; higher crystal density is, as expected, found to

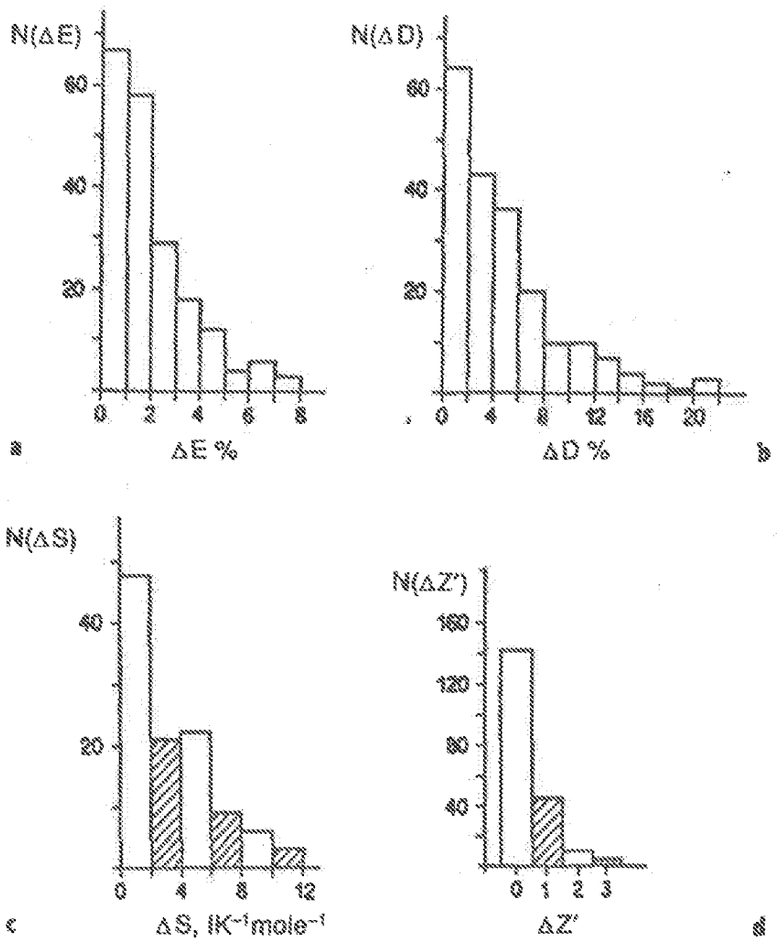



Fig. 6a–d. Histograms of differences in properties between polymorphs: a ΔE (packing energy, %); b ΔD (density, %); c ΔS (lattice-vibrational entropy, J $K^{-1}$ $mol^{-1}$); d $\Delta Z'$ (no. of molecules in the asymmetric unit). (Reprinted with permission from [79], copyright 1995, American Chemical Society)

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correlate with higher packing energy. Finally, from the observation that 24 % of the polymorphic pairs analysed comprised both a centrosymmetric and a non-centrosymmetric partner, it was concluded that the link between molecular properties and centrosymmetry of crystals is evidently weak. The reader is referred to this seminal study [79] for the full exposition of the above conclusions and their justification, as well as further perceptive observations on the phenomenon of polymorphism.

## 3 Methodology for the Study of Crystal Polymorphism

### 3.1 Review of Preparative Methods

Research on the polymorphism of a new molecular entity normally commences with experimental screening which can indicate the occurrence of more than one crystalline form of the substance. An inexpensive method of such testing is hot stage microscopy (HSM), which has been used very extensively and effectively by a leading proponent [80] for many years to provide preliminary indications of the presence of crystalline polymorphic and pseudopolymorphic (solvated), as well as glassy (amorphous) forms, all of which may have practical utility. Pseudopolymorphic forms are molecular adducts containing solvent of crystallization and have been classified [37] as stoichiometric solvates and non-stoichiometric inclusion compounds possessing channel, layer or cage (clathrate) structures. Procedures for detecting the existence of multiple forms by HSM have been outlined [37, 81]. These include, for example, the observation of solid-solid transformation upon heating the substance, observation of transformation (spontaneous or mechanically induced) following the freezing of a melt, and detection of gas evolution as bubbles from pseudopolymorphs immersed in silicone oil during heating (indicative of a solid → gas + solid transformation). Once the existence of multiple forms is established, practical methods for the preparation of specific forms on a larger scale may be explored. Frequently, recrystallization of the compound from solvents or solvent mixtures spanning a wide polarity range is effective in producing several of the different forms in sufficient quantity for complete characterisation by the analytical methods to be discussed. Most pseudopolymorphs are prepared by crystallization of the parent organic compound from the respective solvent, whereupon the latter becomes incorporated in the new crystal. Recrystallization from a mixed solvent system may yield a pseudopolymorph containing either or both solvents. Exposure of the parent organic compound to vapours may also result in the formation of pseudopolymorphs (as occurs e.g. when anhydrous drugs react with atmospheric water to form hydrates). This is exemplified by the drug indomethacin, for which four polymorphic forms (I–IV) have been identified [82]. Crystallization of the commercially available Form I from over fifty solvents yielded Forms I, II, IV and mixtures of these, as well as a number of pseudopolymorphs [83]. To ensure subsequent reproducibility in the preparation of specific forms by crystallization, careful attention to the details of solvent purity, degree of solu-

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tion agitation, temperature, supersaturation and the rate of cooling of the solution is necessary. Detailed methods for isolating metastable polymorphs from the melt or from solution have been reviewed [81].

Desolvation of a pseudopolymorphic form by controlled heating leads to the formation of a polymorph or a mixture of polymorphs of the parent compound, hence providing an additional route to isolation of such species. For example, for indomethacin quoted above, twelve pseudopolymorphs with the general formula indomethacin · $(solvent)_n$ ($n = 0.2 - 1.1$) were isolated [83] and their products of desolvation were characterised. Whereas mixtures of Forms I and II resulted from heating the benzene, $CCl_4$, $CHCl_3$ and toluene pseudopolymorphs, pure Form II was obtained from desolvation of the acetone pseudopolymorph. The most soluble (and pharmaceutically desirable) polymorph, Form IV, was obtained in a pure state by desolvation of the pseudopolymorph containing methanol.

A technologically very important and potentially beneficial feature of some polymorphs obtained in this way is the possibility that they may acquire altered rheological or other properties (flowability, texture, particle size distribution, compressibility) when compared with the same polymorphic crystalline forms obtained by direct crystallization from solution. Two examples of considerable pharmaceutical relevance may be cited, namely those of lactose and paracetamol. The lactose used as an excipient in pharmaceutical tablets and capsules is $\alpha$-lactose monohydrate. It was found that thermal dehydration of this species or desiccation of $\alpha$-lactose containing methanol yielded a stable product with superior binding properties and excellent flowability [84]. Tablets prepared by compaction of the stable product had an overall porosity nearly equal to those of tablets prepared with the original materials. The very poor compression abilities of paracetamol prompted an investigation of solvation/desolvation as a process for preparing pure paracetamol with improved properties [85]. A crystalline hemisolvate of paracetamol was prepared by cooling a hot saturated solution of the drug in dioxane. Desolvation of this species yielded pure paracetamol with significantly improved technological properties including flowability, die filling, and hardness/pressure profile. A crucial point is that this improvement was not attributable to the production of a new polymorphic form of the drug; the X-ray powder diffraction pattern of the desolvated material was the same as that of the commercially available, monoclinic polymorph of paracetamol. However, detailed examination with scanning electron microscopy revealed that desolvation produces material with an unusually porous, sintered-like texture which lends itself to compression more readily than other forms of the drug. Interestingly, solvation/desolvation using other oxygen-donor solvents (e.g. acetone, cyclohexanone) yielded paracetamol in the same (monoclinic) form but failed to produce paracetamol crystals with the required texture. An earlier review on polymorphism [37] lists several drug pseudopolymorphs whose desolvation leads to significant particle-size reduction (or micronization) of the ensuing polymorphic crystallites. This usually results in improved dissolution and tabletting properties of the polymorph.

Mechanical grinding and compression of compounds represent another possible route to polymorphs. In the former case, the local pressures induced by

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the mechanical stress may initiate the transformation of the original polymorph into another crystalline form. From an industrial viewpoint, grinding and compression are attractive processes, being relatively inexpensive and requiring no solvents. In addition, complete polymorphic conversion may be effected in very short times (several minutes in some cases). Recent examples include the production of polymorphic Form I of sulphathiazole by planetary ball-milling of Form III [86], the transformation of metastable Form I of caffeine into the stable Form II by either grinding or compression [87] and production of Form I of probucol by manual trituration of Form II [59]. The role of grinding in the design of pharmaceutical dosage forms has been investigated and the effects of increased specific surface areas and enhanced solubilities following mechanical action have been noted [88]. The same group reported the effects of environmental temperature and compression energy on the polymorphic transformations of the antidiabetic chlorpropamide [89] and discussed the relation between the polymorphic transformation pathway during grinding and the physicochemical properties of bulk powders of cephalexin, chloramphenicol palmitate and indomethacin [90]. Local temperature variations during grinding may also play a role in effecting polymorphic transformation and it is desirable to separate the two influences. This was recently achieved with cortisone acetate by cryogrinding at 78 K, during which the monoclinic form transformed to an orthorhombic form in ten minutes purely by mechanical effects [91].

Prolonged mechanical grinding of a crystalline compound may produce material in an amorphous (or glassy) state which, due to the lack of long-range internal order of the constituent molecules, displays a broad melting temperature range and a diffuse X-ray diffraction pattern. A discussion of polymorphism without reference to amorphism would represent serious neglect of an important aspect of phase behaviour. The amorphous state represents the thermodynamically least stable form of the compound, which consequently has a tendency to revert to a more stable form. The amorphous material is also the most soluble form of the compound and this property is used to advantage in pharmaceutical preparations in cases where the solubility of the crystalline form of the drug is low, leading to poor systemic absorption. A well known example is the antibacterial novobiocin acid, for which the solubility of the amorphous (and therapeutically active) form is ten times that of the crystalline (inactive) form [92]. However, use of an amorphous form in a suspension may require addition of another component to suppress spontaneous transformation to a thermodynamically more stable form. For novobiocin acid preparations, the additives methylcellulose and polyvinylpyrrolidone are successful in this respect. It seems likely that the amorphous state may attain even greater practical significance in view of the recent reference to "amorphous polymorphism" [14], i.e. the existence of more than one distinct amorphous phase of the same substance. This phenomenon, which has been studied by computer simulation, evidently occurs in substances where the thermodynamic behaviour of the liquid state exhibits liquid-liquid phase separation or a tendency towards it.

In concluding this discussion of the methods of preparing polymorphs, several examples may be quoted of observations or procedures which, owing to their novelty or confinement to only one particular compound, cannot be

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described as general. However, since systematic study of the factors which might be involved in effecting or affecting polymorphic transformation in these cases could in principle find wider application, brief mention of such examples is made here. The effect of explosion on polymorphs of chitin and chitosan has recently been studied [93]. Explosion of $\alpha$-chitin resulted in no change, but explosion of hydrated chitosan of low crystallinity yielded a product with increased crystallinity as well as a small amount of the anhydrous phase. The effect of an electric field on polymorphic transformation in certain classes of compounds may be a phenomenon warranting systematic investigation. Strong temperature-dependence of the $\beta \rightarrow \alpha$ polymorphic transition of isotactic polypropylene (containing additives) has been observed when the system is exposed to an electric field [94]. The effects of neutron-irradiation on the kinetics of polymorphic phase transitions for several inorganic compounds have been reported [95], but similar studies using organic compounds as substrates are lacking. It has, however, been demonstrated that ionizing radiation can induce a polymorphic transformation in an organic compound. A polycrystalline sample of the $\beta$-polymorph of a diacetylene nitroxide has been reported to undergo transition to the $\alpha$-polymorph on irradiation with CuK$\alpha$ or $^{60}$Co sources [96]. X-ray powder diffraction was used to monitor this phase tranformation.

Finally, the role of serendipity in producing polymorphs may be mentioned. Two recent cases involved unsuccessful attempts to produce molecular complexes by reaction of two components in solution, resulting instead in the precipitation of crystals of one component in a desirable polymorphic form. Attempts to grow crystals of methotrexate by various techniques failed [97]. However, tetragonal crystals of the compound were obtained from a solution containing methotrexate and thymidine, prepared for the purpose of obtaining a co-crystal of these components. Similarly, attempted complexation between 5-sulphamethoxydiazine and *p*-aminosalicylic acid failed, the solution of these species in a 1:1 molar ratio producing instead large crystals of Form II of the sulphonamide [98]. Interestingly, this polymorph of the drug is the biologically most active form and is usually crystallized from ethanol followed by rapid cooling of the solution to –12 °C. This yields small particles with little or no geometrical form [99]. A possible explanation for the crystallization of Form II during attempts to produce the complex has been discussed [98].

### 3.2
### Review of Investigative Methods

Having outlined the methods of preparation of polymorphs and pseudopolymorphs, this report now focuses on the methodology used to study these forms. The discussion commences with a survey of some well established and still widely used techniques, each of which is illustrated by one or more applications. This is followed by a survey of newer methodology which is being used to probe organic crystal polymorphism.

A wide spectrum of analytical techniques may be used to characterize polymorphs and pseudopolymorphs in terms of their structure, spectral energies, thermodynamic stabilities, kinetics of transformation and solubility behaviour.

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The choice of analytical and physicochemical methods for the characterization of polymorphs is dictated by the need to measure properties which ultimately depend on the different internal arrangements of the same molecules in these phases. When pseudopolymorphs are also considered, the range of suitable analytical techniques is significantly broadened owing to the presence in the crystal of the solvating molecule and the possibility of analysing the physical and chemical changes which may accompany both formation and decomposition of pseudopolymorphs.

Some remarks on the use of white- and polarized-light microscopy serve as an appropriate introduction to this section. With the advent of more sophisticated analytical methods, the use of microscopy has, to some extent, fallen into neglect. This is unfortunate, since investment of relatively little time and effort in studying a crystal of a polymorph or pseudopolymorph by microscopy can be invaluable, enabling one to assess the overall quality of a recrystallization, to detect crystal faults, fractures and macroscopic inclusions, and obtain preliminary information which will facilitate subsequent X-ray examination. Detection of different crystal habits (acicular, tabular, bladed, plate-like, prismatic) using white-light microscopy is not necessarily indicative of polymorphism since crystal habit depends on crystallization conditions and may vary widely for a given polymorph. However, this method is useful for distinguishing polymorphs having different colours in reflected or transmitted light. Pseudopolymorphs which undergo pseudomorphosis (i.e. loss of solvent on removal from their mother liquor) tend to form opaque, microcrystalline masses which are also discernible by ordinary microscopy. Addition of a polarizing attachment allows distinction between optically isotropic crystals and anisotropic crystals as well as the measurement of refractive indices [100]. Optically isotropic crystals belong to the cubic system and have a single value for their refractive index. The vast majority of crystalline organic compounds are optically anisotropic, having multiple refractive indices and displaying numerous optical effects which may be used to differentiate polymorphic forms. Anisotropic crystals reveal themselves by producing variable interference colours as well as regular extinction of plane-polarized light on rotation of the microscope stage. They may be uniaxial (characterized by two principal refractive indices and belonging to the trigonal, tetragonal or hexagonal systems) or biaxial (with three refractive indices and belonging to the triclinic, monoclinic or orthorhombic systems). Measurement of these refractive indices is certainly a means of identifying a polymorph unequivocally, but is seldom done for this purpose. The uniaxial or biaxial nature of the crystal is easily determined from observation of the respective characteristic interference figure when the crystal is viewed with condensed (conoscopic) light. Taken together, extinction directions, crystal morphology and uniaxial or biaxial character can facilitate the identification of a new polymorph, as exemplified by the following case from our laboratory. Carbamazepine commonly crystallizes in the monoclinic system with a prismatic habit [101]. Microscopic examination of crystal batches obtained by recrystallization of the drug from a wide range of solvents confirmed the predominance of this form. However, crystals obtained from tetrahydrofuran were acicular, yielding extinction parallel to the needle-axis and presenting a uniaxial interference figure.

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Measurement of the interfacial angles of a crystal section both microscopically and using an optical goniometer yielded values of 120 ± 1°. Thus, a new polymorph belonging to either of the trigonal or hexagonal systems was unequivocally identified. This was confirmed by subsequent X-ray photography which revealed a trigonal space group [102].

Owing to its vast depth of field, scanning electron microscopy (SEM) is widely used for observing the texture, morphology and surface features of both powders and large single crystals of polymorphs. The high vacuum used for sample observation precludes study of pseudopolymorphs containing volatile solvents but SEM micrographs of polymorphs are useful for purposes of identification provided that crystallization conditions and SEM sampling methods are carefully controlled.

Crystal density is an important technological parameter since it affects the flow properties of bulk solids. Due to the different bulk densities of polymorphs and their abilities to retain solvent, different isolation strategies are required in industry [24]. Furthermore, if mixtures of different solid phases are present in a sample (e.g. in a powdered pharmaceutical formulation), differences in component densities may lead to a heterogeneous product during processing due to phase segregation. Distinguishing polymorphs of the same compound by density measurement (determined by flotation or gas displacement pycnometry) is difficult because, as shown in Fig. 6b, differences in the densities of such species seldom exceed 5% and the experimental error of routine measurements is typically 2%. However, the latter can be reduced if special precautions are taken during flotation measurements, especially with regard to eliminating occluded air. Under these conditions, anomalously high or low measured densities may be useful indicators of the presence of pseudopolymorphs. Thus, e.g., a measured density of 1.30(1) g $cm^{-3}$ for a crystal of doxylamine succinate obtained from ethyl acetate was sufficiently different from that of polymorphic Form I ($\rho = 1.21(1)$ g $cm^{-3}$) to indicate the presence of a pseudopolymorph. Subsequent X-ray analysis showed the crystal to have the unexpected composition (doxylamine succinate)$_2$ · (succinic acid) [103].

Among the thermal methods of analysis, thermogravimetric analysis (TGA), differential thermal analysis (DTA) and differential scanning calorimetry (DSC) have been used extensively to quantify thermal events accompanying controlled heating of polymorphs and pseudopolymorphs [18, 23]. Figure 7 shows combined TGA and DSC traces for two pseudopolymorphs of nitrofurantoin [61], containing respectively *N,N*-dimethylformamide (DMF) and dimethylsulphoxide (DMSO).

In TGA, the sample (~5–10 mg) is heated at a predetermined rate and the weight is recorded as a function of temperature. This technique cannot distinguish polymorphs of a given organic compound, but for pseudopolymorphs which lose their included solvents prior to melting or decomposition of the parent ("host") compound, the percentage weight loss may be accurately measured and used to calculate the stoichiometry of the pseudopolymorph. Data recorded from the TGA traces shown in Fig. 7 indicated a nitrofurantoin : DMF stoichiometric ratio of 1 : 1 and a nitrofurantoin : DMSO ratio of 2 : 1. A TGA trace may reflect simple one-step weight loss of included solvent or more complex

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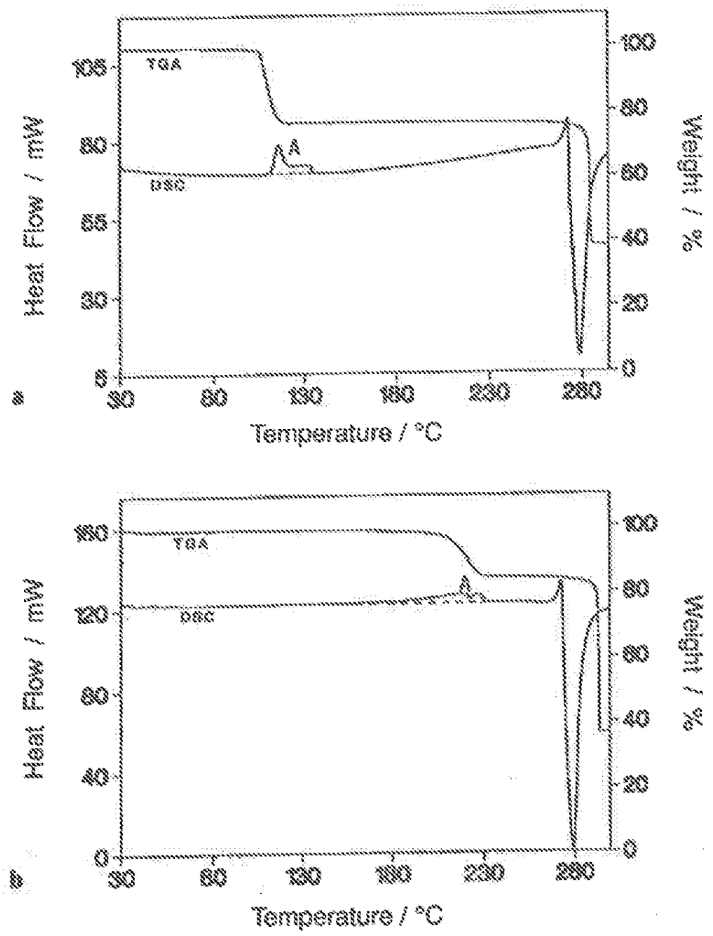



**Fig. 7a, b.** Combined TGA-DSC traces for pseudopolymorphs of nitrofurantoin containing: a DMF; b DMSO (Reprnted with permission from [61], copyright 1996, Gordon and Breach Publishers)

multi-step weight losses. Coupled with TGA, evolved gas analysis (EGA) using IR or mass spectrometry is an important means of identifying gaseous products [18] and may be used to quantify both the pyrolysis products from thermal degradation of polymorphs as well as solvents released from desolvation of pseudopolymorphs. Another important application of the TGA method is the determination of the activation energy for desolvation of a pseudopolymorph from traces recorded at varying heating rates [104]. Its application to pseudopolymorphs of succinylsulfathiazole [105] and tenoxicam [106], and to inclusion compounds of synthetic hosts [50] have recently been described.

In DTA, the sample temperature ($T_s$) is compared with that of a reference compound ($T_r$) as a function of increasing temperature. The resulting plot of $\Delta T$ ($= T_s - T_r$) vs T may display endothermic peaks corresponding to desolvation (for pseudopolymorphs) or fusion (for polymorphs) and exothermic peaks representing recrystallization or decomposition processes. In the related DSC technique, the difference in energy inputs into a compound and a reference substance is plotted against T during a controlled temperature programme. In

Ex. 2001-0433

theory, the area under the curve in a DSC trace is directly proportional to the enthalpy change for the thermal event, but special precautions are necessary to obtain accurate values for this quantity [18]. An alternative method for determining enthalpies of desolvation of pseudopolymorphs, based on direct vapour pressure measurement of the liberated solvent as a function of temperature, has been described [50]. The DSC traces in Fig. 7 display endotherms (peaks A, above the baseline) whose temperature ranges correlate well with those of the TGA traces, confirming that these peaks represent desolvation processes. The bimodal nature of these endotherms is difficult to interpret but evidently reflects complex desolvation mechanisms. The difference in the DSC temperature ranges for loss of DMF (100–125 °C) and loss of DMSO (160–235 °C) is striking. These pseudopolymorphs were subsequently shown by single crystal X-ray analysis to contain their respective solvent guest molecules in constricted channels formed by the nitrofurantoin host framework; each DMF molecule is bound to one drug molecule by a single hydrogen bond, whereas each DMSO molecule forms two hydrogen bonds with neighbouring drug molecules, thus providing a possible explanation for the significantly higher onset temperature for the loss of DMSO. In this connection, the use of the simple parameter $T_{os} - T_b$ ($T_{os}$ = extrapolated DSC desolvation onset temperature, $T_b$ = boiling point of the guest solvent) has been proposed as a possible measure of the relative thermal stabilities of inclusion compounds [50]. For both pseudopolymorphs referred to in Fig. 7, desolvation produced the $\beta$-polymorph, as confirmed by the characteristic sharp fusion endotherm at 273 °C, followed by a decomposition exotherm peaking at 279 °C.

Recent reviews of thermoanalytical methods and their application to polymorphic systems [18, 23] include descriptions of modern instrumentation, discussions of the influence of instrumental and sample parameters on measured data, as well as guides to the interpretation of DSC traces. One review [23] includes a summary of the thermodynamic rules established by Burger [107] for distinguishing monotropic and enantiotropic transitions. It is worth emphasising that in the absence of supporting techniques such as hot stage microscopy (HSM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR), errors in the interpretation of DSC data may result. Reference to HSM was made earlier in connection with its use in screening recrystallized batches of a compound to identify polymorphs and pseudopolymorphs. Apart from its ability to yield melting points and transition temperatures for polymorphic changes or glass transitions, HSM is an invaluable adjunct to the DSC technique in that visual recording of thermal events (e.g. desolvation, recrystallization, sublimation) may corroborate assignments of endothermic or exothermic peaks appearing in DSC traces. As pointed out recently [108], visual observation can sometimes be more sensitive indicators of phase changes than DSC measurements. The report describes the construction and use of the device shown in Fig. 8, which allows simultaneous DSC measurement and microscopic observations with a video-camera. A second camera is used to give a direct readout of the varying sample temperature.

Solid-state infrared (IR) spectroscopy in the spectral range 400–4000 $cm^{-1}$ has been used extensively to distinguish different polymorphic forms as well as

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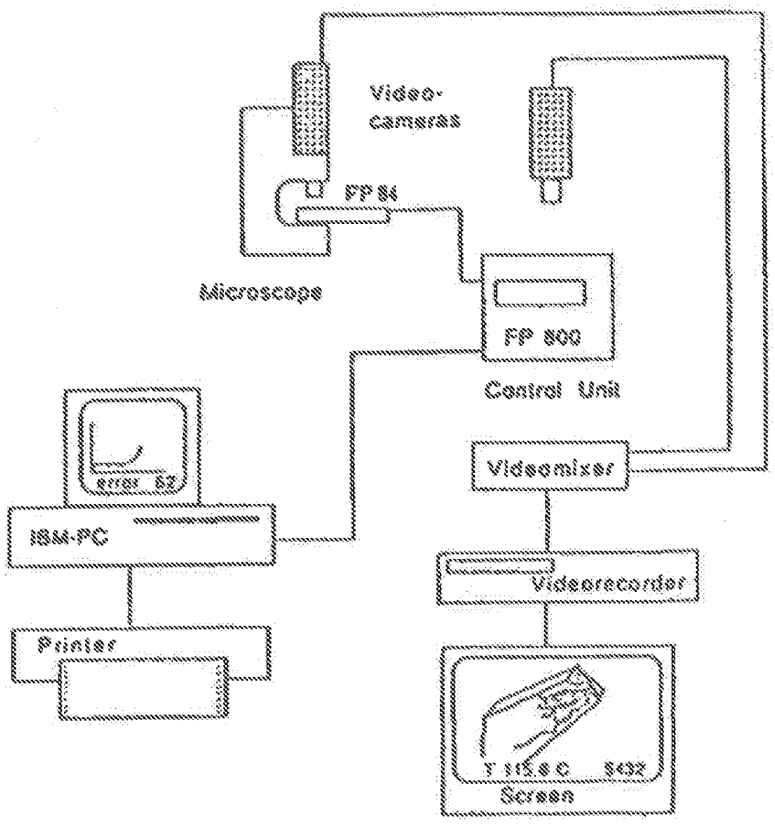



Fig. 8. Schematic description of an apparatus for simultaneous visual and DSC observations using the Mettler Thermosystem FP800 (Reprinted with permission from [108])

different pseudopolymorphs of the same compound. To ensure transparency to IR radiation, samples are usually prepared by trituration of crystalline material and suspension in Nujol or by pressing a co-ground mixture of the compound with KBr into a disk. However, since complete polymorphic transformation can sometimes be effected even by gentle manual grinding, it is advisable to confirm the existence of polymorphs by an independent method prior to a detailed IR analysis. Differences in molecular conformation and crystal packing features for different polymorphs of an organic compound manifest themselves in IR spectra chiefly by frequency shifts and splittings in, e.g., N-H, O-H and C=O absorption peaks (when these functional groups are present) due to differences in hydrogen bonding arrangements. The goal of IR analysis is the deduction of polymorphic structural differences from such spectral effects, but detailed interpretation is often limited and it is somewhat easier to rationalise these effects retrospectively if single crystal X-ray data can be obtained for the various forms.

This is illustrated by the following examples from our laboratory. A study of the literature on the compound sulphamerazine revealed that its polymorphism was controversial. The crystal structure of the drug recrystallized from acetone had been shown to be orthorhombic, Pbca with $Z=8$ [109]. Centrosymmetric hydrogen bonded (N-H···N) dimers exist in the crystal structure as shown

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schematically in Fig. 9. We obtained crystals of a second polymorph of sulphamerazine [110] by recrystallization from methanol and established the space group as $Pn2_1a$, $Z=8$. This form contains the same hydrogen bonded motif as shown in Fig. 9, but it is pseudo-centrosymmetric. The two polymorphs are distinguishable from IR spectra, the form containing the centrosymmetric dimer (with only one unique N-H···N hydrogen bond) yielding only one IR peak (3453 $cm^{-1}$) assignable to $\nu_{as}NH$ whereas that containing the pseudo-centrosymmetric dimer (with two distinct N-H···N hydrogen bonds) displayed a doublet (3493, 3478 $cm^{-1}$).

We recently reported FT-IR spectral data for seven crystalline forms of the androgen dehydroepiandrosterone [111], showing that differences in both fine structure and in the locations and intensities of major absorption bands can be used to distinguish them. X-ray crystal structures of three forms were determined [112], Form I (a polymorph), Form S1 (a 4:1 hydrate) and Form S4 (a methanol half-solvate). Form I (space group $P2_1$, $Z=4$) contains infinite chains of steroid molecules linked head-to-tail (shown schematically in Fig. 10) by two crystallographically distinct intermolecular O-H···O=C hydrogen bonds, consistent with two observed O-H stretching bands (3501, 3458 $cm^{-1}$). Doublets for the O-H absorption bands observed also in both Forms S1 and S4 can be correlated with distinct O-H ···O hydrogen bonds observed by X-ray analysis, and specifically, with unusual "flip-flop" hydrogen bonding in Form S4. In this study, attempts to determine the thermodynamic relationships between the various polymorphic forms were based partly on the application of Burger's IR rule [107] which relates IR band positions to relative stabilities.

The use of IR-spectra to distinguish polymorphs from pseudopolymorphs of the same parent compound, or to distinguish pseudopolymorphs containing different solvents, is relatively straightforward, since solvent molecules which are commonly incorporated in pseudopolymorphic crystals (e.g. water, ketones, alcohols) will exhibit their own characteristic absorption bands. IR spectroscopy was used to distinguish polymorphs of the antidiabetic drug glibenclamide from its solvates with pentanol and toluene [113]. Pseudopolymorphs of the

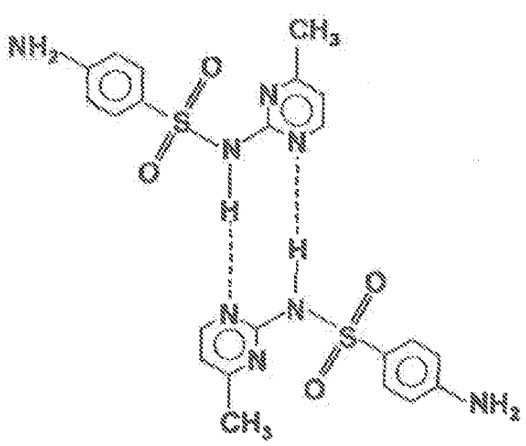



**Fig. 9.** Schematic representation of the hydrogen bonded dimer occurring in two polymorphs of sulphamerazine

Ex. 2001-0436

compound gossypol (a toxic constituent of cotton-seed) obtained from three different solvents showed characteristic differences in their IR spectra in the 3500 $cm^{-1}$ region [114]. Pseudopolymorphs of a host containing the same guest in different molar ratios are also known. An alicyclic diol of the helical tubuland family [115] forms two crystallographically distinct lattice inclusion complexes with 1,2-dichlorobenzene having host-guest ratios of 4:1 and 3:1 [116]. These were readily distinguished from their IR spectra in the fingerprint region.

The spectral techniques available for the characterization of polymorphs and solvates have been reviewed [27] and the merits of FT-IR spectroscopy for the acquisition of high-quality spectra have been stressed. The review also lists drug substances whose polymorphism has been investigated by IR-spectroscopy. Another recent review on the use of the IR method to investigate polymorphism is based on spectra for a derivative of sulphadoxine as a model compound [21]. Dynamic aspects of polymorphism are also amenable to study by IR spectroscopy. The effect of hydrogen bonding on the lattice dynamics, polymorphism, mesomorphism and phase tranformations of long-chain carboxylic acids as gauged from IR spectra has recently been reported [117].

A relatively new trend is the application of near-IR spectroscopy for compound identification and quantitation in many industrial environments including chemical processing and food science. The advantages of near-IR over conventional IR spectroscopy for the investigation of polymorphs have been described [118]. A fast, sensitive pattern recognition method for identifying a polymorph and determining its quality using near-IR spectra has been developed [119]. Pattern recognition methods involve training a computer to recognize spectra of acceptable samples of a material and to reject unacceptable ones by multivariate statistical analyses. This study showed that the method described can discriminate between the desired polymorph and other crystalline forms, the statistical results indicating that levels as low as 2% of an adulterating polymorph may be reliably detected. The authors maintain that the near-IR method, which can be automated, provides a reliability comparable to that of XRD for polymorphic identification but is likely to be less costly to implement.

There is a growing interest in the use of Raman spectroscopy for studying polymorphism and pseudopolymorphism [27]. The Raman technique has recently been used to distinguish two triclinic polymorphs of terephthalic acid [120] and, in combination with IR spectroscopy, to study the conformational polymorphism of 1-bromopentane [121].

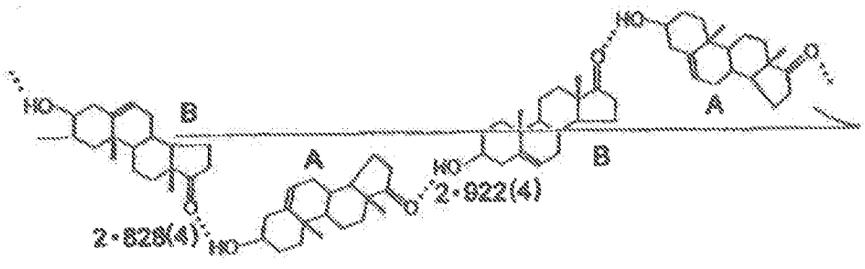



Fig. 18. Head-to-tail hydrogen bonding in Form I of dehydroepiandrosterone. A and B are crystallographically distinct and distances are in Å

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The use of white-light microscopy to identify differently coloured polymorphs was mentioned above. Colour polymorphism may be quantified using *UV/vis electronic spectroscopy (diffuse reflectance, fluorescence methods)*. Colour differences for polymorphs of the same compound may originate from differences in charge transfer interactions or different molecular conformations in the crystals. Pseudopolymorphs may also be distinguished using fluorescence spectroscopy since they may display characteristic absorption maxima as well as fluorescence intensities. Systems which exhibit colour polymorphism have been reviewed [27].

Implicit in the examples cited above to illustrate applications of various analytical methods for studying polymorphism is the definitive role of single crystal X-ray diffraction in providing unequivocal evidence for the very existence of these forms. Different polymorphs of a given compound have, by definition, non-equivalent crystal structures and the method of choice for detecting such non-equivalence is single crystal X-ray analysis. In many instances, the differences between polymorphs are so subtle that they can be distinguished only by this method [79]. This technique goes well beyond merely detecting different forms, providing in addition a wealth of structural information for each species at a level of detail and precision currently unsurpassed by other analytical techniques. Furthermore, as illustrated by several of the examples above, detailed knowledge of the molecular parameters and crystal packing features obtained by this method provides a very reassuring basis for the interpretation of results gleaned from other analytical techniques. For these reasons, much effort is expended by researchers in growing single crystals of polymorphs and pseudopolymorphs with adequate quality for complete X-ray structural elucidation.

For routine crystallographic studies of polymorphs or pseudopolymorphs containing small and medium-sized molecules, the methodology of crystal structure determination is well established and the reader is referred to standard monographs on the subject [122, 123]. However, a few points on current routine methodology are worth emphasising in order to place some of the newer developments (to be discussed later) in perspective.

Existing standard methods employ single crystals usually no smaller than 0.1–0.5 mm, automated X-ray intensity data-collection (typically with a four-circle diffractometer) and standard crystallographic software packages for data-processing, structure solution and refinement. Room-temperature data-collection is common, despite the gain in structural definition possible with cryostatic devices for cooling the crystal. Low-temperature analyses are sometimes carried out on pseudopolymorphs in which the included solvent molecules tend to have excessive thermal motion or are disordered at room-temperature. The advent of fast detectors (organic scintillators, image-plates, multiwire area detectors) and more powerful structure determination packages has greatly increased the speed with which crystal structures can be solved and refined [124].

In the absence of single crystals of suspected polymorphic forms, X-ray powder diffraction (XDP) serves as the primary test for non-equivalence of crystal structures, the XDP pattern of such a species being unique [25]. However, since

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sample preparation involves grinding in a mortar to an average particle size ≤ 100 μm to minimise preferred orientation effects, the possibility of a pressure-induced phase transformation during sample preparation must be considered. The XRD pattern of an unground sample of the material may be checked as a precautionary measure. Patterns are generally recorded on samples with mass range 200–500 mg using automatic diffractometers with strip chart output. The method is generally non-destructive, allowing recovery of the sample. Film methods (e.g. the Debye-Scherrer technique) require a few mg of material only and are still frequently used when only small amounts of polymorphic forms can be isolated. According to the guidelines specified in the US Pharmacopeia [125], agreement between a sample and a reference standard should be within the calibrated precision of the diffractometer for diffraction angle (typically a reproducibility of ± 0.10–0.20° in 2θ) with permissible relative intensity variations of up to 20%.

A recent analytical study stresses the growing need, prompted partly by legislatory requirements, to differentiate polymorphs and to quantify polymorphic mixtures in pharmaceutical production [126]. The compounds benzil and benzoic acid were chosen as a model system for the development of an XRD protocol which could be extended to the quantification of mixtures of drug polymorphs. The study involved the evaluation of sample thickness, the determination of preferred orientation effects, optimum milling conditions and the construction of diffraction intensity-composition calibration curves for mixtures of benzil and benzoic acid. Since the composition of such mixtures can be accurately determined by an independent method, namely HPLC, validation of the quantification of mixtures by the XRD protocol was possible. It was concluded that the protocol is accurate for the model system to within a few percent. It is desirable that the general validity of the approach suggested be tested on a range of real polymorphic systems.

The information contained in an XRD pattern of a powdered material is a significantly condensed version of that obtained by single crystal X-ray analysis and it is a routine matter to reconstruct the XRD pattern of a polymorph by computation, using as input the space group data, refined atomic coordinates, atomic thermal parameters and unit cell data which have been obtained from a single crystal X-ray study of that species. Some years ago, it was recommended [127] that computation of the XRD pattern of a new polymorph should always be carried out if single crystal data become available. The computed pattern, being free of experimental aberrations, serves as the best reference pattern for the polymorph in question. We have followed this practice and can strongly endorse its usefulness, not only for the purpose of identifying the polymorphic form present in a newly crystallized sample, but also for assessing the polymorphic purity of such a sample. Figure 11 shows the experimental XRD pattern of a polymorph of the non-steroidal anti-inflammatory drug tenoxicam and, for comparison, the computed pattern based on the single crystal X-ray data [106]. The level of agreement in peak positions attests to the polymorphic purity of the sample (no extraneous peaks being evident in the experimental pattern) while the discrepancies in corresponding peak intensities indicate some degree of preferred orientation in the sample. Finally, since the sample was a powder derived

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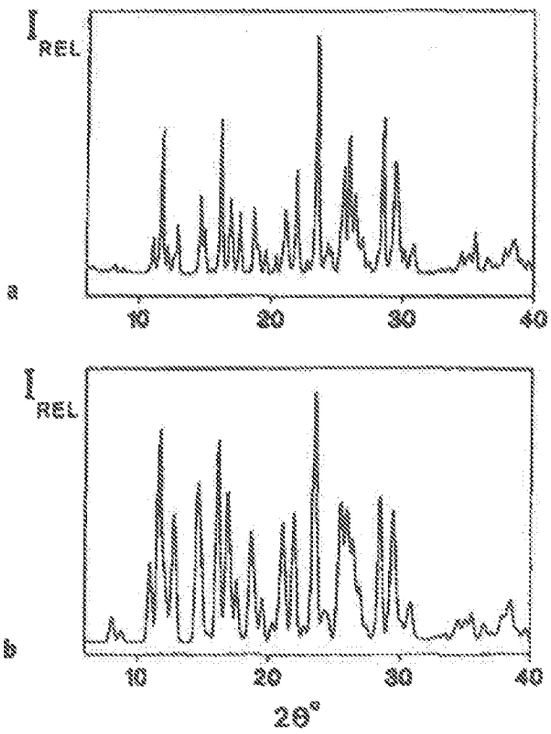



Fig. 11a, b. XRD patterns for a polymorph of tenoxicam: a experimental; b calculated from single crystal data. (Reprinted with permission from [106], copyright 1995, American Chemical Society and American Pharmaceutical Association)

from large single crystals previously identified by X-ray analysis, the overall agreement of the patterns reveals the non-trivial result that grinding single crystals of the polymorph does not effect a phase transformation to another polymorph. In the case of the drug probucol (Fig. 3), the experimental XRD patterns for the two polymorphs were identical as a result of transformation of one polymorph into the other during sample preparation [59].

Neutron and electron diffraction techniques have also been used to some extent to study the polymorphism of organic substances. Owing to its ability to locate hydrogen atoms accurately in crystals, neutron diffraction is the method of choice for distinguishing subtle differences in hydrogen bonding for polymorphic crystal structures. However, the technique is costly and the low intensity of neutron beams demands relatively large single crystals (around 1 $mm^3$ or larger) which are seldom available. Crystallographic studies are therefore usually carried out on polycrystalline samples, yielding powder neutron diffraction patterns analogous to XRD patterns. A fairly recent development is the use of pulsed neutron sources coupled with time-of-flight analysis [100]. As an example of its application to the study of polymorphism, the case of cyclohexanol may be cited [128]. Structural elucidation of three polymorphs (cubic (I), monoclinic (II) and orthorhombic (III)) revealed that I is disordered, that II contains a cyclic dimer of cyclohexanol, and that a polymeric arrangement exists in III.

Because incident electrons interact strongly with crystals, electron diffraction is capable of yielding detailed structural information from very small crystals

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(typically 0.01–0.02 mm). It has been cited as a more reliable method for determining crystal unit cell and space group data than computer-assisted indexing of XRD patterns [100]. The crystal structure of a high-temperature polymorph of chitosan from electron diffraction data has recently been reported [129]. Structural modelling of this complex molecule involved constrained linked-atom least-squares refinement with stereochemical restraints.

Dilatometric analysis is a less widely used technique for characterising polymorphs, but has, for example, been applied to the study of polymorphic transformations occurring in theobroma oil, methyl stearate and chloramphenicol [25]. Substances which contract as they transform from a metastable (less dense) polymorph to a stable (more dense) polymorph can be studied by measuring their specific volume as a function of temperature. A recent study involved the combined use of dilatometry and neutron scattering to characterise the orthorhombic and monoclinic polymorphs of *m*-nitrophenol [130]. Crystals of both polymorphs showed significant anisotropy of expansion and it was possible to reconcile the direction of lowest expansion with that of the hydrogen bonding interactions. Attempts to correlate these results with those obtained from IR and Raman spectra were subsequently reported [131].

Solubility and dissolution rate analyses are of vital importance for polymorphs and pseudopolymorphs of pharmaceutical relevance. For a given drug, metastable polymorphs tend to have higher solubilities and faster dissolution rates than the stable polymorph. When metastable forms are employed in solid dosage forms (tablets, capsules), they generally yield higher and earlier blood serum levels [25]. Thus, for potent drugs with a narrow therapeutic index (e.g. the cardiotonic digoxin), inadvertent use of a metastable polymorph in a tablet could result in patient death from overdose. In vitro dissolution testing is therefore carried out routinely as part of the quality control of manufactured tablets and capsules. It may also be performed directly on powders or single crystals of the drug polymorphs or pseudopolymorphs comprising the active agent. Essentially, this involves placing the sample in the dissolution fluid, agitating it in a reproducible manner at constant temperature (usually 37°C), and measuring the drug concentration in solution as a function of time. Compendial testing methods include the rotating basket, rotating paddle, and flow-through cell techniques. Automated systems employ a peristaltic pump which circulates the dissolution fluid through a UV spectrophotometer flow-cell for continuous monitoring of drug concentration. The advantages and disadvantages of modern analytical techniques available for dissolution testing have been reviewed recently [22].

Polymorphs generally dissolve more rapidly than their hydrates, but there have been reports of drug pseudopolymorphs containing, e.g., ethyl acetate or *n*-pentanol, which display enhanced solubilities, both in vitro and in vivo, when compared with their nonsolvated forms [25]. Figure 12 shows powder dissolution rate curves for two polymorphs of nitrofurantoin. The significance of the curves for this system is that the retarded initial dissolution rate for the $\alpha$-polymorph may render it more favourable for pharmaceutical formulation since there is evidence that adverse side effects may be associated with rapid absorption of the $\beta$-polymorph [61].

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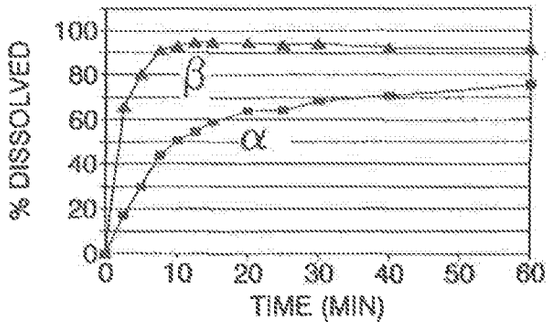



Fig. 12. Powder dissolution rate curves for two nitrofurantoin polymorphs. (Reprinted with permission from [61], copyright 1996, Gordon and Breach Publishers)

Measurements of the equilibrium solubilities of a polymorph as a function of temperature permit evaluation of the enthalpy of dissolution from a van't Hoff plot (i.e. logarithm of solubility vs $T^{-1}$). The point at which such plots intersect for two different polymorphs of the same compound corresponds to the transition temperature for their interconversion.

It should be stressed that the analytical methods surveyed above achieve their optimum effectiveness and lead to deeper insights into polymorphism and pseudopolymorphism when used in combination. A simple illustration is the combined use of dissolution rate analysis and crystal structure analysis of two polymorphs. Used individually, these techniques would produce merely data, whereas the combined approach could lead to an explanation for the differences in dissolution rates based on differences in crystal packing. Some reported studies utilise a wide range of techniques, permitting a comprehensive analysis of a polymorphic system, while others may use only two, but nevertheless effective, complementary techniques (e.g. DSC and XRD). The following examples selected from the recent literature illustrate this point.

Four polymorphic forms (I-IV) of the drug diflunisal (analgesic, anti-inflammatory) were prepared and characterised by DSC, HSM, IR, XRD, and dissolution studies [132]. The thermoanalytical methods revealed that on heating, all polymorphs recrystallized to the stable one (Form I) prior to melting. Only one transition peak in the DSC traces was observed, for the transformation of Form III to Form I. Intermolecular hydrogen bonding features were deduced from IR spectroscopy and this method was also shown to distinguish all the forms. Measurements of the intrinsic dissolution rates indicated (unexpectedly) that Forms II, III and IV had slightly lower values than that of the stable form. Results of this kind are invaluable for ensuring correct formulation of the drug. Some examples of other important drug polymorphic systems for which similar, fairly comprehensive studies have recently been reported include acyclovir [133], meprobamate [134], prasterone [111], ranitidine hydrochloride [135], and chloramphenicol palmitate [136].

Other compounds whose polymorphism and/or pseudopolymorphism have been investigated in recent years, together with the methods used, include pentachloropyridine (DSC, Raman and IR spectroscopy) [137], cyclohexanol (far-IR, adiabatic calorimetry) [138], nifedipine (HSM, IR, DSC) [139], sulfanil-

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amide (DSC, XRD) [140], paraffins $C_{26} - C_{60}$ (DTA, thermobarometric analysis) [141] and tripalmitin (DSC, thermodiffractometry, microcalorimetry) [142]. This list represents a very small selection, merely serving to indicate the variety of techniques employed.

Several recent developments in the areas of structure determination are having a significant impact on the study of organic crystal polymorphism and these are now surveyed, together with examples of applications to specific systems. In the discussion above, it has been maintained that a knowledge of the crystal structure of a polymorph provides a sound basis for interpretation of other analytical data pertaining to that species and is crucial for even a rudimentary understanding of phase transformations at the molecular level. It is also well known that many polymorphs of technological interest cannot readily be obtained as single crystals suitable for X-ray structural elucidation. However, since the advent of the Rietveld, or "whole-pattern", refinement technique [143] which allows crystal structure refinement using X-ray or neutron powder diffraction data, the status of powder XRD has been elevated from that of a mere "fingerprinting" technique to one of formidable power as a structure-solving tool. This power has been significantly enhanced by recent progress in obtaining trial structural models for Rietveld refinement directly from the diffraction intensities. Since the powder pattern is a one-dimensional projection (on $2\theta$) of the three-dimensional single crystal diffraction data, a key problem in this challenging research area is unravelling the intensities of severely, or exactly, overlapping reflections. In a recent study, an algorithm for achieving this was reported [144] and, together with software routines for space group determination and a fast iterative Patterson squaring routine, this combination led to the successful ab initio crystal structure determination of an aluminophosphate-based molecular sieve from X-ray powder data exclusively. It is noteworthy that as many as 65 % of the reflection data used in this analysis suffered from severe overlap. This approach holds great promise for future structure determination of polymorphs which hitherto have been obtained in the form of powders only.

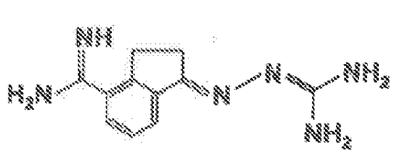
More recently, a method using powder diffraction for structure solution was described in which structural information is not extracted directly from the intensities; instead, trial structures are generated using a Monte Carlo approach [145] commencing with a collection of atoms initially randomly placed in the crystal unit cell. A series of trial structures is then generated by random movement of the atoms, each trial structure being accepted or rejected depending on the level of agreement between the calculated and experimental powder patterns. The Monte Carlo method employed is based on the Metropolis importance sampling algorithm using the crystallographic R-factor as the basis for constructing trial models. The best model is then used in a conventional Rietveld refinement. The structures of two compounds, with eleven and twelve non-hydrogen atoms in the respective asymmetric units, were solved by this procedure using high-resolution powder X-ray intensity data measured with a position-sensitive detector. The novel feature of this method is the use of the Monte Carlo algorithm for generating trial structures.

Crystal structure prediction based on lattice energy minimisation has already been discussed and attention has been drawn to the serious drawbacks of this

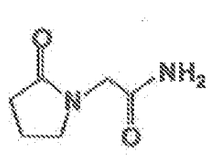
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approach when used on its own. However, used in combination with the Rietveld method, its chances of success are very significantly improved, as recently demonstrated for a polymorphic system [146]. Of two polymorphs, A and B, of a guanidine derivative (1), only form B was available in the form of single crystals and its structure was solved in the conventional manner using X-ray diffraction methods. Polymorph A was available only as a powder and the structural problem was further complicated by molecular features allowing not only for different conformers of the molecule, but also different tautomers. Indexing of 26 lines of an XRD pattern of polymorph A measured on a Guinier camera led to the assignment of the triclinic system. Energy calculations using MOPAC93 were used to search for likely conformers and tautomers suitable for crystal packing calculations. High-precision ab initio quantum mechanical methods were used to confirm the trial models, the four most stable ones being chosen for packing analysis using a corrected Dreiding force-field. Two routes were pursued, one assuming the XRD indexing results, the other ignoring them. In the first case, crystal structures were generated for the space group P1 only, leading to a most stable structure with unit cell parameters very close to those deduced from the indexing. Subsequent optimisation and Rietveld refinement yielded a final structure with an R-factor of around 10%. When the indexing results were ignored, stable conformers were packed in common space groups (e.g. $P\bar{1}$, $P2_1/c$, $P2_12_12_1$). Of the predicted force-field minimised structures, that which gave the best agreement with the experimental XRD pattern turned out to be one in the space group $P\bar{1}$. Following interactive Rietveld refinement, this structure (gratifyingly) converged to the same one as determined assuming the indexing results. A very important feature of this study was the use of an ordinary in-house XRD pattern (as opposed to a high-resolution pattern) for the indexing and Rietveld refinement steps. The authors pointed out that both the ab initio quantum mechanical as well as the packing calculations for this problem consumed enormous amounts of computational resources. They nevertheless predicted that structure determination based on an ordinary powder pattern and packing calculations could evolve into a reliable and inexpensive routine procedure in view of the envisaged drop in computational costs which they state is about an order of magnitude every three years.

The structure of a metastable and shortlived polymorph of the drug piracetam (2) has been solved from XRD data using the AAP method [147]. This species (Form I) was prepared by heating a mixture of Forms II and III and quenching at room temperature. Form I spontaneously transforms into II at 298 K within a few hours of quenching, but it was possible to capture high-resolution XRD data within two hours using a position-sensitive detector. The same (known) molecular conformation as occurs in Forms II and III




1 guanidine derivative




2 piracetam

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was assumed in the crystal packing calculations and the AAP method yielded two distinct minima, only the higher energy structure being correct (consistent with the metastability of Form I) as shown by successful Rietveld refinement. This impressive study supports the contention that the need for single crystals for structure solution is becoming less critical [65] and also demonstrates that even shortlived organic polymorphic species can be structurally characterised.

Another major development which continues to have a significant impact on structure determination of polymorphs has been the advent of synchrotron radiation as a source for diffraction studies [148]. This radiation, which is tuneable and whose intensity exceeds that of conventional X-ray sources by orders of magnitude, is bringing about a revolution in structure determination, not only for polymorphs, but for materials in general. In particular, very small crystals (in the micron range) can now be analysed using data generated using synchrotron radiation and collected with ultra-rapid detection devices. Time-resolved studies are also amenable to study with synchrotron radiation owing to its pulsed time structure and the possibility of recording the X-ray diffraction data from picosecond exposures. An on-line system using in situ X-ray diffraction has recently been developed for examination of the polymorphic forms of pharmaceutical materials [149]. It comprises a temperature-controlled stainless steel in situ cell for X-ray measurements attached to a two-circle diffractometer on a beamline at the Daresbury Synchrotron Radiation Source. The pH of a solution of a drug is varied by titration in a glass solution-saturation vessel and crystallization due to change in the solute solubility yields a crystallized slurry which is circulated through the cell for continuous monitoring of its diffraction pattern.

For the (unnamed) antibiotic investigated, Fig. 13 shows a series of diffraction patterns measured at different temperatures. From these, the transformation between two solvates of the drug at around 309 K is clearly evident. Using the system described, enthalpy release following crystallization can also be measured by monitoring the temperature of the crystallization solution. The authors point out the growing awareness of the importance of understanding crystallization processes for many specialty chemicals in process technology and the increasing popularity of on-line characterization techniques. In situ synchrotron X-ray diffraction offers the advantages of non-invasive characterization of polymorphism and polymorphic changes, and the possibility of studying very small crystalline particles.

The investigation of polymorphism and pseudopolymorphism by means of solid-state NMR spectroscopy is also gaining in popularity owing to recent advances in both instrumentation and computer pulse sequence strategies [27]. Most studies employ $^{13}C$ or $^{15}N$ NMR since $^{1}H$ NMR produces broad, uninformative peaks even after removal of proton-proton dipolar interactions. In the MAS ('magic angle spinning') technique, the sample is spun at high velocity at the critical angle of 54.74° with respect to the applied magnetic field. The resulting averaging of the chemical shift anisotropy reduces spectral broadening considerably, yielding sharp peaks with fine structure from which valuable information on individual atomic chemical environments can be deduced. To

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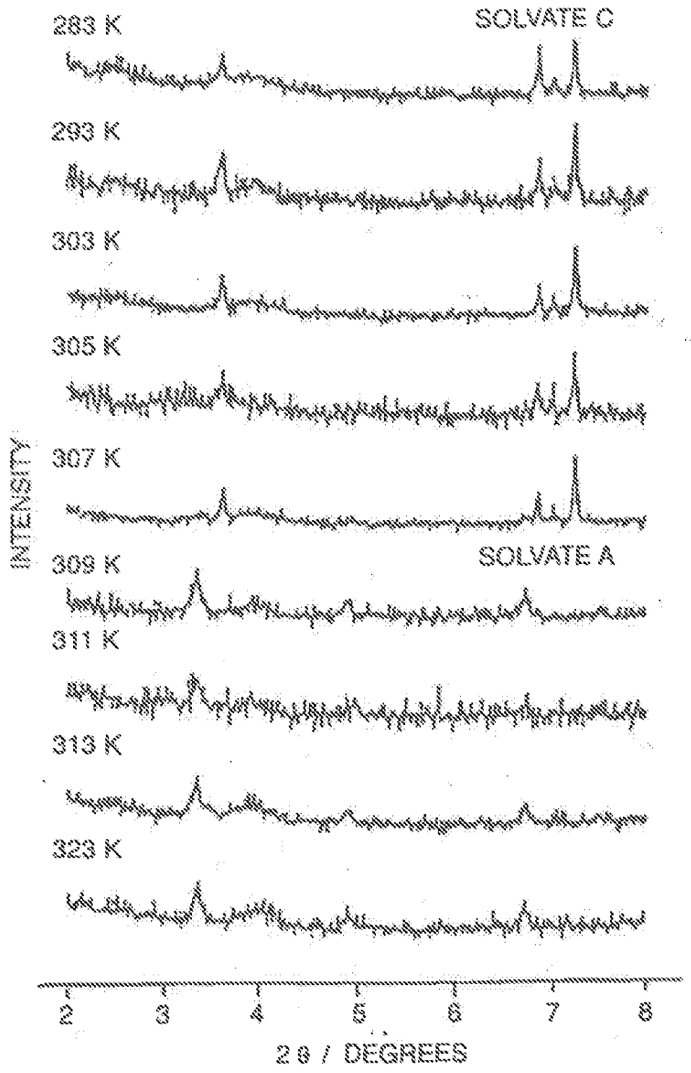



**Fig. 13.** In situ X-ray diffraction patterns of an antibiotic substance for crystallization range 283–323 K. (Reprinted with permission from [149])

accelerate data-acquisition, enhancement of $^{13}C$ magnetisation is achieved by the CP ("cross polarization") strategy and the combined method is referred to as CPMAS NMR spectroscopy. With it, polymorphic conformational differences, crystal packing differences and the effects of hydrogen bonding may be characterised. Varying the sample temperature allows dynamic studies of solid-solid phase transformations. Incorporation of solvents in pseudopolymorphs introduces significant changes in local environmental magnetic effects through, e.g., hydrogen bonding, and such changes are therefore also open to study by the CPMAS technique.

Figure 14 shows $^{15}N$ CPMAS spectra for four polymorphic modifications [150] of the imidazole derivative (3), from which it is clear that $^{15}N$ chemical shifts are good indices for their identification. $^{13}C$ CPMAS has also recently been used to distinguish three polymorphs of complex triacylglycerols [151]. Spectral

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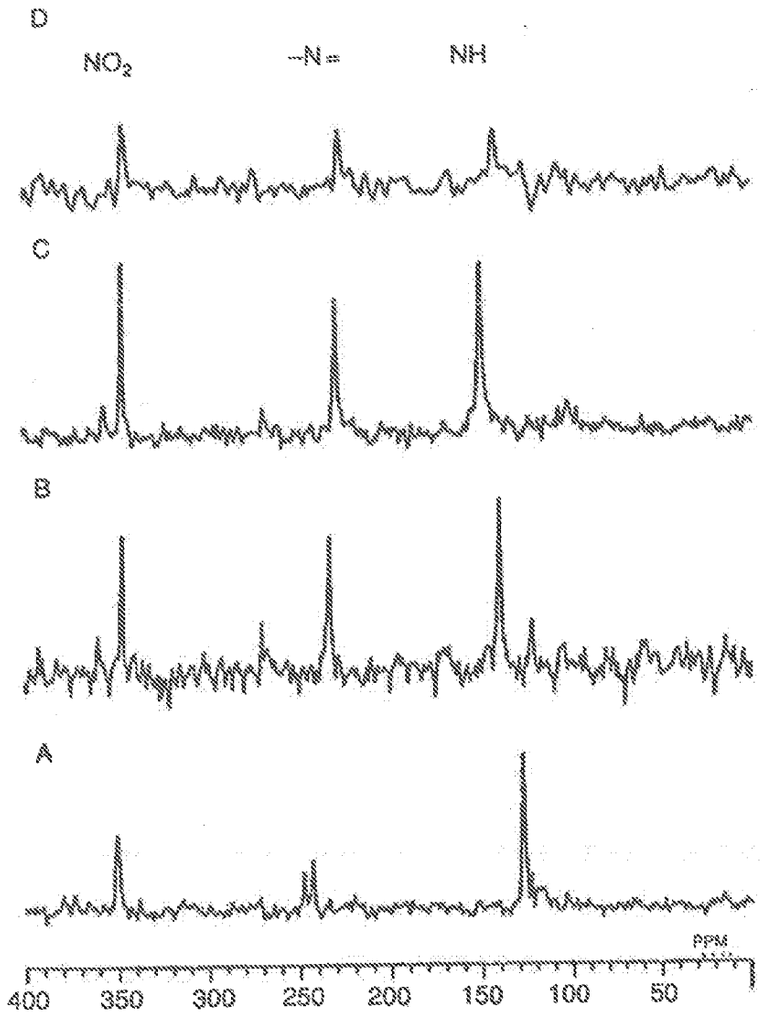
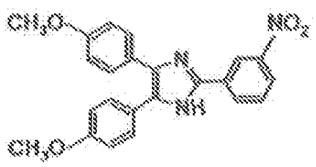


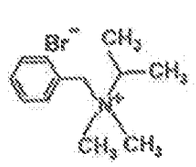

Fig. 14. $^{15}N$ CPMAS spectra for four polymorphs of the imidazole derivative 3. (Reprinted with permission from [150], copyright 1996, Gordon and Breach Publishers)

differences were used to characterise conformations and crystal packing differences in the three forms. Examples of applications of CPMAS to the study of polymorphism and pseudopolymorphism of pure drug substances [27] as well as drug-excipient mixtures [152] have been described.

In a recent study of the molecular motion in the crystal of the salt (4), the results of $^{13}C$ CPMAS NMR and high-resolution powder XRD were combined




3 imidazole derivative




4 salt

Ex. 2001-0447

[153]. An important feature of this study was the determination of the activation energy for rotation of one of the N-methyl groups in the crystal from variable-temperature NMR data. The X-ray crystal structure was solved from high-resolution powder XRD data using direct phasing methods and refined by the Rietveld method. It was possible to reconcile the NMR-derived activation energy with the crystal packing which revealed that one of the N-methyl groups is significantly more hindered than the other. This study illustrates the potential of a combined approach to probing molecular motion in the solid-state. Its application to polymorphic systems is an obvious and desirable extension.

### 3.3
### Polymorphic Systems and Polymorphic Changes – Case Studies

Four selected case studies are cited here to illustrate some of the difficulties encountered in research on organic crystal polymorphism, even when relatively simple molecules are involved. Since space does not permit discussion, précis of these model studies are presented in the hope that the interested reader will consult the original, absorbing accounts for details.

The polymorphism of anthranilic acid has been studied for over a century. Despite this, many properties of crystals of this simple molecule remain obscure and a recent re-examination of the phase transitions was undertaken using X-ray and FT-IR techniques [154]. A special feature of this system is that phase transitions appear to depend subtly on grinding pressure. The main conclusion drawn from the investigation cited is that polymorph I is transformed into polymorph III on heating, not into polymorph II as commonly stated in the literature.

The crystallization behaviour of the high explosive 2,4,6-trinitrotoluene (TNT) has invoked intensive research since the first report on its optical investigation in 1879 [155]. Despite numerous on-going studies, the conditions for crystallizing the monoclinic and orthorhombic forms lack definition. A systematic re-examination using Laue and Weissenberg X-ray methods, DSC, goniometry and computer simulations was undertaken to establish the role of crystallization conditions in determining the nature of polymorphism as well as TNT crystal morphology [156]. Complicating features of this system are extensive twinning in the monoclinic phase and remarkable similarity in the structures of the two polymorphs which results in their XRD patterns being almost indistinguishable. The study established (inter alia) conditions for crystallizing the two forms, finding that their occurrence was determined by growth solvent only and was independent of the rate of crystallization. The orthorhombic polymorph was shown to be metastable relative to the monoclinic polymorph.

Terephthalic acid (TA) is an important industrial chemical used in the production of polyester. Its purification involves multi-stage recrystallization under extreme conditions. The phase characterisation and morphologies of Forms I and II of TA were studied by HSM, thermal analysis, Raman spectroscopy, single crystal Laue X-ray diffraction, lattice energy and morphological calculations [157]. Contrary to earlier reports, this study revealed that Form II (not Form I) is the thermodynamically stable form of TA under ambient conditions. The apparent stability of Form I was shown to be due to twinning in microcrystals

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leading to "collective stabilisation", which prevents spontaneous transformation to Form II.

Structural, thermodynamic, kinetic and mechanistic aspects of phase transformations among three differently coloured forms of dimethyl 3,6-dichloro-2,5-dihydroxyterephthalate have been studied by various methods [108] including simultaneous DSC and optical microscopy using the apparatus shown in Fig. 8. The three forms, (Y) yellow, (LY) light yellow and (W) white, which exhibit extraordinary polymorphic behaviour, are now known to be conformational polymorphs and the transformations among them appear to occur by nucleation and growth of the new phase outside the domains of the old one, not by single-crystal to single-crystal transformation. Some perspective on research on polymorphism can be gained from the concluding remarks of this account which stress the prevailing lack of understanding of the complexity of phase transitions and the formidable experimental difficulties experienced in obtaining reproducible results.

## 4
## Towards Control of Polymorphism

### 4.1
### The Need for Polymorphic Control

In a review sketching the important developments in synthetic organic chemistry during the past 25 years and predicting future directions [158], it was emphasised that the exciting synthetic targets today are no longer molecules to be prepared "for their own sake", but rather, they are systems possessing specific functions or properties. The systems which are being pursued with this goal in mind are largely those whose properties are governed by non-covalent interactions, as evidenced by the rapid growth of the discipline of supramolecular chemistry in recent years. In the realm of solid-state chemistry, the goals of crystal engineering [3] are, analogously, the design and preparation of materials with specific properties (e.g. metallic conductivity, thermochromism, photoactivity, second-harmonic generation), these properties being strictly dictated by the crystalline assembly or supramolecular organization in the solid-state. Preparation of such assemblies has been severely impeded by the lack of an underlying theoretical framework for understanding the subtle interplay of factors determining crystal packing. There is no guarantee, in the first instance, that a given molecule will form a stable crystal which will survive the treatment to which it will be subjected. The occurrence of polymorphism (in which both thermodynamics and kinetics play a role) is an additional complicating factor, since the three-dimensional array possessing the desired property is but one, unique arrangement among many alternative arrays having similar free energies, but lacking that property. As an example, one property of solid materials currently attracting widespread attention is that of non-linear optical (NLO) behaviour, whose effect is second-harmonic generation (SHG). This property has potential for the design of laser devices, optical communications and information processing. SHG depends on both the molecular hyperpolarizability

Ex. 2001-0449

($\beta$) of the compound in question and on the orientation of the molecules in the crystal. Specifically, a non-centrosymmetric packing arrangement is required for a solid to exhibit NLO properties and, while it is possible to alter the value of $\beta$ by synthetic modification, encouraging molecules to adopt a non-centrosymmetric packing arrangement is a formidable challenge.

The question of whether polymorphism is the "nemesis of crystal design" was discussed at length nearly a decade ago [3]. Since then, however, systematic studies in the areas of crystal nucleation, growth and morphology have led to definite proposals for rational stereospecific control of polymorphism as well as some success in its attainment in special cases [36]. Thus, while ab initio crystal structure prediction is still a distant goal [72] and the preparation of specific, desired phases is still elusive, there is at least the possibility of rational intervention during the nucleation or growth stages of crystallization for influencing the outcome, provided that the detailed polymorphic behaviour of the system has been established from systematic study. This again provides strong justification for the application of a wide range of techniques for comprehensive studies of systems exhibiting polymorphism. As implied by case studies described earlier in this report, there are numerous instances in industrial environments where control of both crystal morphology and the polymorphic form of a compound is vital, and here the outlook for success, based on the strategies to be described briefly below, is promising.

### 4.2
### Current Strategies and Prognosis for Crystal Engineering

Practical "control" of the polymorphic outcome of a crystallization process can be interpreted at different levels, ranging from the purely empirical, such as the use of identical crystallization conditions in the hope of obtaining a particular polymorph in a reproducible manner, to a much more ambitious strategy based on, e.g., rational design of additives which could suppress or inhibit nucleation of undesired polymorphs through selective molecular recognition mechanisms, thus allowing unhindered growth of a desired metastable polymorph. Between these extremes, there are different variations, both physical and chemical, that are effective in producing specific polymorphs, as exemplified below. Reports of this kind appear in the recent patent literature, attesting to the technological and commercial importance of the polymorphs concerned. A patent for the preparation of Form 1 of the widely used antiulcerative drug ranitidine hydrochloride describes its crystallization from a mixed solvent in the presence of seed crystals, the preferred process involving in situ reaction of HCl with the free-base [159]. Industrial-scale preparation of the stable polymorphic form of *N*-(4-hydroxyphenyl)retinamide is effected by successive chemical reactions with all-*trans* retinoic acid as the starting material [160]. The desired polymorph is formed by recrystallizing the wet product from 95% EtOH. The $\beta$-form of an organic phosphite, reported to be an effective process stabiliser for polyolefins, has been prepared by heating the compound at 125 °C in the absence of solvent [161].

Desolvation of pseudopolymorphs may be considered as a means of polymorphic control. This was discussed earlier in this report in the context of

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altering the rheological properties of drug polymorphs in particular. However, thermal treatment or vacuum drying has also been applied to more general inclusion compounds containing both natural and synthetic host molecules in order to prepare pure polymorphic forms of the host. Of the seven polymorphic forms of gossypol (5) identified thus far [162], only two were obtained by appropriate recrystallizations from solution, while the remainder resulted from desolvation of various gossypol clathrates. The latter could be divided into isostructural groups and it was found that all members of a given group yielded the same polymorph on desolvation. These unusual and intriguing results imply the existence of a "template" effect in this system. Similar experiments with pseudopolymorphs of indomethacin (6) also yielded a series of isostructural pseudopolymorphs [83], but in this case there was virtually no correlation between a given series and the identity of the polymorph resulting from desolvation. A systematic study of such systems to elucidate the mechanisms of desolvation is desirable if this process is to be exploited in a predictive way for the control of polymorphism.

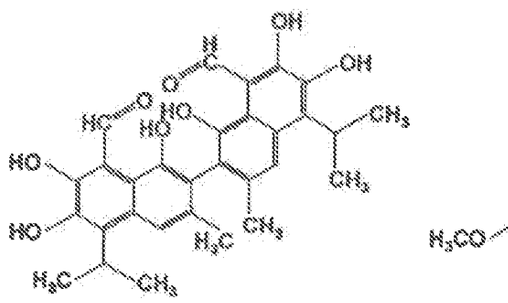
Polymorphic inclusion compounds of a host molecule with the same guest can occur if the host-guest interactions are flexible enough to allow nearly equienergetic, but crystallographically distinct host-guest packing arrangements. Reports of the preparations of such polymorphs are rare [163], but would probably increase in frequency if a variety of preparative conditions were to be employed.

As with most organic compounds, inclusion complexes are usually prepared only once and the chances of detecting polymorphism are correspondingly small. The well known host molecule cholic acid (7) has been shown to form different polymorphic inclusion complexes with the same guest [164]. The polymorph which crystallizes is determined by the absence or presence of a third chemical species. Crystallization of cholic acid from, e.g., acrylonitrile yielded the 1:1 host-guest complex with space group $P2_12_12_1$, while addition of acrylonitrile to a saturated solution of the host in butan-1-ol yielded a 1:1 cholic acid - acrylonitrile complex with space group $P2_1$. X-ray analyses confirmed that the polymorphs contained different packing arrangements as well as different hydrogen bonding networks. The controlling role of the third component in effecting precipitation of a specific polymorph was not clarified in the report but selective adsorption of solvent molecules on the surfaces of growing crystals may be involved.

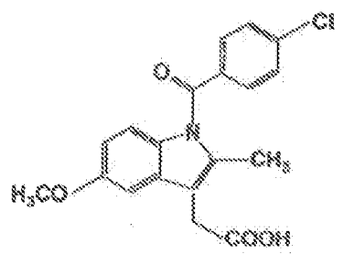
During the last ten years, considerable progress has been made in the control and modification of crystal properties using "tailor-made auxiliaries" [35, 36]. These auxiliaries may act either as promoters of crystal growth (making them useful for the study of nucleation processes) or as inhibitors (for use in the control of, e.g., morphology, enantiomeric resolution and polymorphism). The strategy for effecting kinetic resolution of a conglomerate involves the design of chiral-resolved polymeric inhibitors which will, in solution, bind selectively to only one of the growing enantiomeric crystalline phases, thus preventing its growth and permitting unhindered growth of the other enantiomeric phase. Where simultaneous crystallization of the racemic phase might occur, design of the inhibitor would have to take this into account. It must be emphasised that

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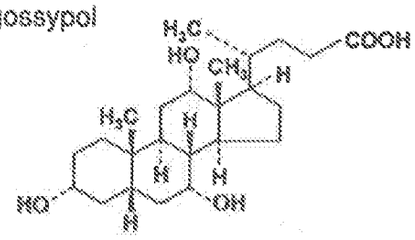
successful design of auxiliaries depends on a detailed knowledge of the packing arrangements, as well as the relationship between packing arrangements and crystal morphology for all species that might appear in the system. An early demonstration of this approach (which was subsequently successfully applied to several other systems) involved resolution of racemic histidine hydrochloride [165]. In the report of this resolution, it was mentioned that this approach was being tested for use in the precipitation of metastable polymorphic crystals by kinetic control.



5 gossypol



6 indomethacin



7 cholic acid

Recent application of these principles to polymorphic control is shown schematically in Fig. 15 for a hypothetical dimorphic system in which one polymorph is centrosymmetric and the other crystallizes in a polar space group [36]. In the former crystal, the molecules are arranged in antiparallel orientation whereas in the latter they are aligned along a common direction. A tailor-made auxiliary which binds to both crystals would do so at the two (indistinguishable) ends of the centrosymmetric crystal but only at one end of the polar crystal. The latter would therefore grow at the expense of the former and polymorphic control will have been achieved.

This concept was successfully applied to *N*-(2-acetamido-4-nitrophenyl)pyrrolidene (PAN) where crystallization of the metastable polymorph of PAN could be induced by addition of a remarkably small amount (0.03%) of the designed inhibitor [36]. Generalisation of this strategy is not straightforward, however, since polar crystals may belong to space groups of relatively high symmetry which require the constituent molecules to adopt a variety of orientations with respect to the polar axis [35]. Strategies which have been explored to engineer non-centrosymmetry into crystals for NLO properties have been listed in a recent paper describing the polymorphism of 1,3-bis(*m*-nitrophenyl)urea [166].

Research on the use of tailor-made surfaces for promotion of nucleation has been pursued in parallel with studies of crystal growth inhibition [35, 36]. The objective here is to gain a deeper understanding of the mechanism of molecular

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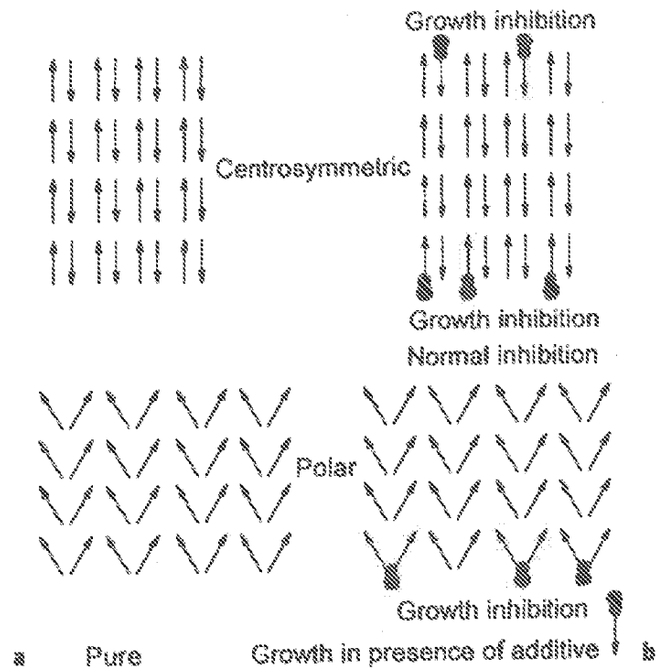



**Fig. 15** a Schematic illustration of the packing arrangement in a polar and a centrosymmetric crystal. b The effect of a designed inhibitor on crystal growth. (Adapted from [36] with permission

clustering associated with the formation of pre-critical nuclei of crystalline phases. The relevance for organic crystal polymorphism is that direct observation of the dynamics of crystal nucleation could elucidate the effects of additives on polymorphic control. Much of this work has been based on the use of Langmuir films to induce nucleation at the air-water interface [36]. To illustrate the level of interpretation which is currently possible from such experiments, a recent study of the self-assembly of crystalline monolayers and multilayers of *n*-alkanes on a water surface is cited [167]. Surface observations were carried out using synchrotron grazing incidence X-ray diffraction and specular X-ray reflectivity. These allowed detailed characterisation of the crystalline films including determination of their space groups. It is noteworthy that these were found to be identical to those of the corresponding three-dimensional crystals, thus demonstrating that the full crystal symmetry already exists even for crystallites whose thickness corresponds to only a few molecular layers.

Related to the above strategy is the concept of engineering solid surfaces for promoting nucleation and epitaxial growth of desired polymorphs. If it is assumed that the pre-nucleation aggregate for a polymorph resembles the mature crystal, it should be possible to design a surface which mimics a particular crystal plane of the desired species, and upon which heterogeneous nucleation will occur and epitaxial polymorphic growth will result. This has been realised in the technique of ledge-directed epitaxy [168] in which the intersection of a terrace plane and a step on a crystal surface (a "ledge") functions as a nucleation site for organic crystals. For polymorphic control, the crucial requirement is that two well-defined, close-packed crystal planes of the polymorph have a dihedral

Ex. 2001-0453

angle which matches that of the substrate ledge site. Successful application of this principle to the selective nucleation and growth of a thermodynamically less favoured polymorph of a complex organic salt on succinic acid single crystals as substrates has recently been reported [169].

The approaches outlined above continue to provide new insights into the fundamental process of crystallization of organic molecules and studies of the type described promise to contribute very significantly in the future to a detailed understanding of the molecular origins of crystal polymorphism and to its effective control. The author believes that the phenomenon of polymorphism will, notwithstanding such progress, retain its aura as a remarkable manifestation of Nature's diversity.

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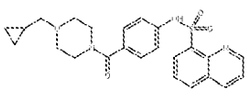
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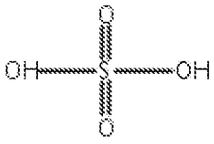
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8-Quinolinesulfonamide, *N*-[4-[[4-(cyclopropylmethyl)-1-piperazinyl]carbonyl]phenyl]-, sulfate, hydrate (2:1:3)
Other Names: AG 348 sulfate hydrate; Mitapivat sulfate; *N*-[4-[4-(Cyclopropylmethyl)piperazine-1-carbonyl]phenyl]quinoline-8-sulfonamide sulfate hydrate (2:1:3)

1260075-17-9
$C_{24}H_{26}N_4O_3S$



7664-93-9
$H_2O_4S$




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# Preclinical pharmacokinetic/pharmacodynamic relationships for AG-348, an investigational small-molecule activator of pyruvate kinase

Yue Chen, Penelope Kosinski, Gavin Hasten, Charles Kung, Hyerytun Kim, Lenny Gang, Jasmina Popovici-Muller, Jeffrey Hixon, Lee Silverman, Scott Biller, Hua Yang

Agios Pharmaceuticals, Cambridge, MA, USA

## BACKGROUND

- Pyruvate kinase (PK) deficiency is a rare, autosomal recessive, inborn error of metabolism caused by a functional deficiency of the R isoform of PK (PK-R).[1]
- PK deficiency is characterized by life-long hemolytic disease and serious long-term comorbidities such as poor growth and development in children, and iron overload and multiorgan dysfunction in adults.[2]
- Current treatments are palliative only, and there are no approved drugs that directly target the underlying functional deficiency.
- PK-deficient red blood cells (RBCs) display metabolic changes related to defective glycolysis, including a buildup of the upstream intermediate, 2,3-diphosphoglycerate (2,3-DPG), and a deficiency in the product, adenosine triphosphate (ATP).
- AG-348 is a first-in-class, oral, small-molecule allosteric activator of PK-R. Treatment with AG-348 in PK-deficient RBCs *ex vivo* increased PK-R enzyme activity and induced metabolic changes consistent with increased glycolytic activity, including reductions in 2,3-DPG levels and increases in ATP levels.
- First-in-human, phase 1 trials of AG-348 in healthy volunteers recently have been completed.
  - Data were presented previously,[3] and in abstract S138 of this conference.

## OBJECTIVE

- We conducted pharmacokinetic/pharmacodynamic (PK/PD) studies to evaluate the effect of AG-348 on RBC metabolism *in vivo* in PK-R wild-type (WT) mice, rats and monkeys.

## METHODS

- Single or multiple (twice daily, BID) doses of AG-348 or vehicle were administered by oral gavage to PK-R WT C57/BL6 mice, Sprague Dawley rats and cynomolgus monkeys.
  - AG-348 dosing regimens are shown in **Table 1**.
- After the first and last doses, blood samples were collected to evaluate plasma drug exposure and PD markers *in vivo* in whole blood, including 2,3-DPG and ATP levels and PK-R activity.
  - PK-R activity was also assessed *in vitro* in RBCs.
- 2,3-DPG and ATP levels were analyzed by liquid chromatography–mass spectrometry
- PK-R activity was determined by assessing reaction rate in RBC lysates isolated from whole blood using a spectrophotometric absorbance assay.

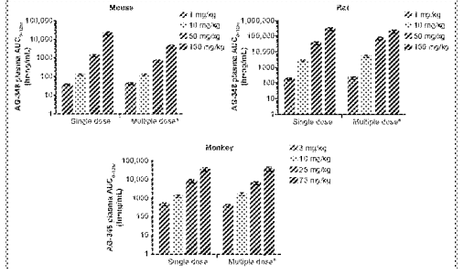
*Table 1. Dosing regimens in mice, rats and monkeys*

| Species | AG-348 dose (BID)[a] | Duration |
|---|---|---|
| Mice | 1 mg/kg, 10 mg/kg, 50 mg/kg, 150 mg/kg | 7 days (13 doses) |
| Rats | 1 mg/kg, 10 mg/kg, 50 mg/kg, 150 mg/kg | 7 days (13 doses) |
| Monkeys | 1 mg/kg, 10 mg/kg, 25 mg/kg, 75 mg/kg | 5 days (10 doses) |

## RESULTS

- In WT mice, rats and monkeys, AG-348 mean plasma exposure was dose dependent (**Figure 1**).
- A single dose of AG-348 resulted in dose- and exposure-related increases in PK-R activity concomitant with a reduction in 2,3-DPG levels, but no significant change in ATP levels (**Figures 2** and **3**).
- After multiple BID doses of AG-348, increase in PK-R activity and decrease in 2,3-DPG was comparable with that observed after a single dose (**Figures 4** and **5**).
- In contrast to the single-dose study, ATP levels increased in an exposure-related manner after multiple doses (**Figure 5**).
- PK-R activity *in vitro* half-maximal activity concentration ($AC_{50}$) values and estimated *in vivo* values were similar across species (**Table 2**).

*Figure 1. AG-348 mean plasma exposure in WT mice, rats and monkeys*




*BID dosing for 7 days (13 doses total). †BID dosing for 5 days (10 doses total)
$AUC_{0-12h}$ = area under the concentration–time curve from 0–12 hr

*Figure 2. Change in PK-R biomarkers in WT mice, rats and monkeys after a single dose of AG-348*

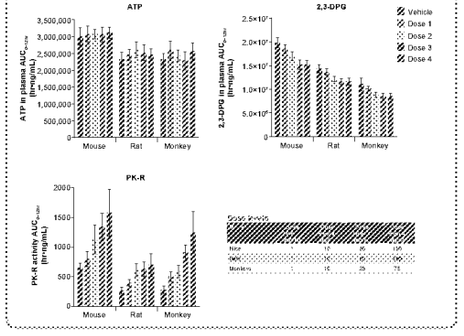



*Figure 3. Correlations of AG-348 plasma exposure and PK-R biomarkers in WT mice, rats and monkeys after a single dose*

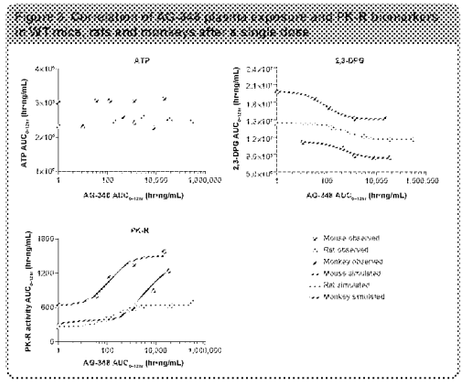



*Figure 4. Change in PK-R biomarkers in WT mice, rats and monkeys after multiple doses of AG-348*

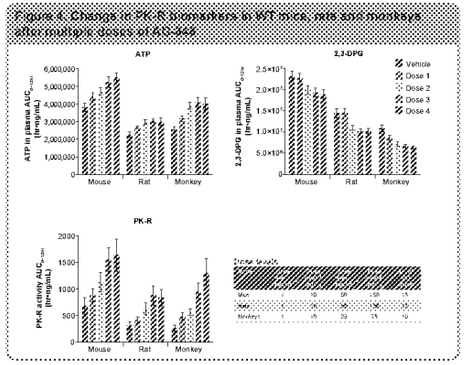



*Figure 5. Correlation of AG-348 plasma exposure and PK-R biomarkers in WT mice, rats and monkeys after multiple BID doses*

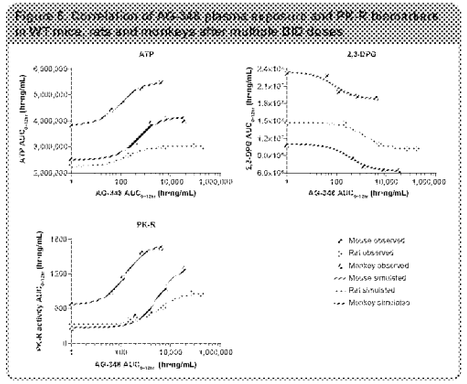



*Table 2. $AC_{50}$ values for PK-R activity in vitro (purified RBCs) and in vivo*

| Species | Biomarker | In vitro PK-R activity RBC $AC_{50}$, nM | In vivo RBC $AC_{50}$, ng/mL | | In vivo plasma $EC_{1.5x}$, ng/mL | |
|---|---|---|---|---|---|---|
| | | | Single dose | Multiple doses | Single dose | Multiple doses |
| Mouse | ATP | ND | NA | 0.58 | NA | 42.6 |
| | 2,3-DPG | ND | 0.55 | 0.60 | 31.5 | 25.5 |
| | PK-R activation | 1.9 | 0.77 | 1.1 | 38.5 | 55.2 |
| Rat | ATP | ND | NA | 0.59 | NA | 33.9 |
| | 2,3-DPG | ND | 2.2 | 4.8 | 24.4 | 48.4 |
| | PK-R activation | 8.3 | 0.81 | 19.7 | 36.0 | 89.9 |
| Monkey | ATP | ND | NA | 5.4 | NA | 65.4 |
| | 2,3-DPG | ND | 5.5 | 4.1 | 34.5 | 72.9 |
| | PK-R activation | 6.8 | 34.6 | 32.4 | 278 | 269 |

*PK-R activity $AC_{50}$ values in human RBCs *in vitro* = 12 nM (male) and 10 nM (female)
$E_{1.5x}$ = maximal PD change against $E_{bl}$. NA = not applicable; ND = not determined

## CONCLUSIONS

- *In vivo* PK/PD studies in WT mice, rats and monkeys found no significant species-related differences in pharmacokinetics after single and multiple oral doses of AG-348, and PK/PD relationships were described by an indirect model across all three species.
- In whole blood samples, robust changes in all three PD markers (2,3-DPG, ATP and PK-R activity) were observed after multiple doses, which correlated with AG-348 plasma exposure.
- Changes in PD markers showed similar trends across species, and there was good correlation of *in vivo* and *in vitro* data following multiple oral doses.
- These animal models effectively predicted changes in 2,3-DPG and ATP levels observed in healthy volunteers enrolled in single and multiple ascending dose clinical studies of AG-348, with the greatest similarity seen between mouse models and humans.
  - Clinical data were presented in poster 4007 at the 56th American Society of Hematology Annual Meeting & Exposition, 6–9 December 2014, San Francisco, CA, USA, and in abstract S138 of this conference.

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### Disclosures

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Ex. 2001-0460

# Abstract Submission

***29. Non-malignant hematopoietic disorders***

EHA-3722

**PRECLINICAL PHARMACOKINETIC/PHARMACODYNAMIC RELATIONSHIPS FOR AG-348, AN INVESTIGATIONAL SMALL-MOLECULE ACTIVATOR OF PYRUVATE KINASE**

Yue Chen* [1], Penelope Kosinski[1], Gavin Histen[1], Charles Kung[1], Hyeryun Kim[1], Lenny Dang[1], Janeta Popovici-Muller[1], Jeffrey Hixon[1], Lee Silverman[1], Scott Biller[1], Hua Yang[1]

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**Please indicate your presentation preference:** No Preference

**Has the submitted material been published in a journal (printed or online)?:** No

**Has the submitted material been presented or submitted to another event?:** No

**Background:** Pyruvate kinase (PK) deficiency is a rare, autosomal recessive, inbom error of metabolism caused by a functional deficiency of the R isoform of PK (PK-R). It is characterized by life-long hemolytic disease and serious long-term comorbidities such as poor growth and development in children, iron overload, and multi-organ dysfunction. Current treatments are palliative only, and there are no approved drugs that directly target the underlying enzyme defect. PK-deficient red blood cells display metabolic changes related to defective glycolysis, including a build-up of upstream intermediate, 2,3-diphosphoglycerate (2,3-DPG) and a deficiency in the product, adenosine triphosphate (ATP). AG-348 is a first-in-class, oral, small molecule allosteric activator of PK-R. AG-348 treatment of PK-deficient red blood cells *ex vivo* increased PK-R enzyme activity and induced metabolic changes consistent with increased glycolytic activity, including reductions in 2,3-DPG levels and increases in ATP levels. First-in-human, phase 1 trials of AG-348 in healthy volunteers have recently been completed.

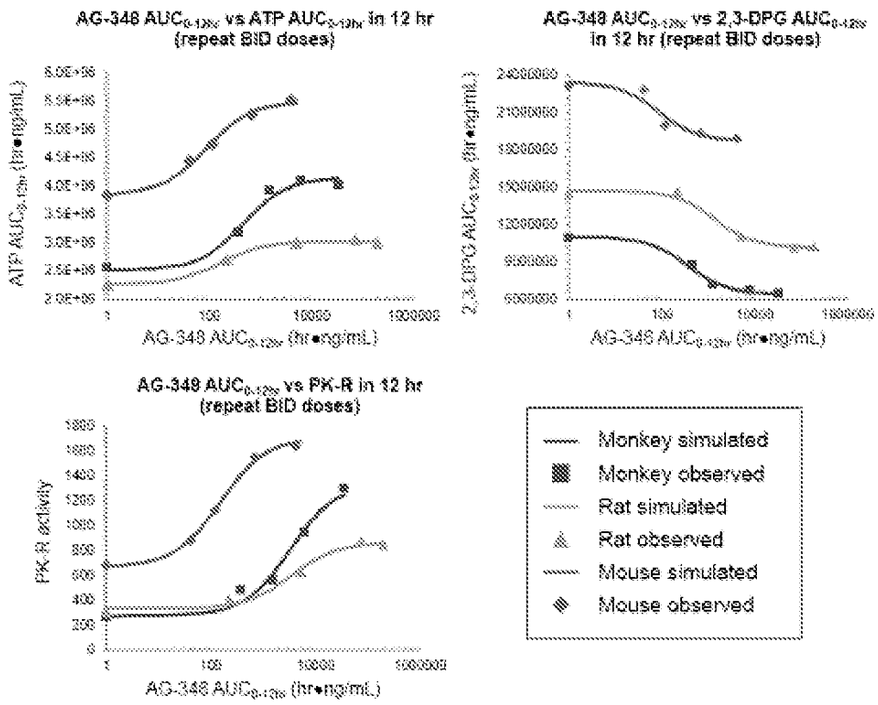
**Aims:** Pharmacokinetic/pharmacodynamic (PK/PD) studies were conducted to evaluate the effect of AG-348 on red blood cell metabolism *in vivo* in PK-R wild-type (WT) mice, rats, and monkeys.

**Methods:** PK-R WT C57/BL6 mice, Sprague Dawley rats, and cynomolgus monkeys were administered single or multiple (twice daily) doses of AG-348 by oral gavage for 3 and 7 days (mice and rats) or 5 days (monkeys). Dose levels tested were 1 mg/kg, 10 mg/kg, 50 mg/kg, and 150 mg/kg in mice and rats, and 3 mg/kg, 10 mg/kg, 25 mg/kg, and 70 mg/kg in monkeys. After the first and last dose, blood samples were collected to evaluate plasma drug exposure and pharmacodynamic (PD) markers in whole blood, including 2,3-DPG and ATP levels, and PK-R activity.

**Results:** In WT mice, rats, and monkeys, a single dose of AG-348 resulted in AG-348 exposure-related increases in PK-R activity concomitant with a reduction in 2,3-DPG levels, but no significant change in ATP levels. After multiple doses of AG-348, the increase in PK-R activity and decrease in 2,3-DPG was comparable to that observed after a single dose. In contrast to the single-dose study, ATP levels increased in an exposure-related manner after multiple doses (**Figure**). *In vitro* $IC_{50}$ values for purified red blood cell PK-R activity correlated well with estimated *in vivo* potencies in mice, rats, and monkeys.

**Image/Pictures:**

**Correlation of AG-348 plasma exposure and ATP, 2,3-DPG and PK-R activity in WT mice, rats, and monkeys following multiple, twice daily (BID) dosing**




**Summary/Conclusion:** *In vivo* PK/PD studies in WT mice, rats, and monkeys found no significant species-related differences in pharmacokinetics following single and multiple oral doses of AG-348 and demonstrated an indirect PK/PD relationship across all three species. In whole blood samples, robust changes in all three PD markers (2,3-DPG, ATP, and PK-R activity) were observed following multiple doses, which correlated with AG-348 plasma exposure. Changes in PD markers showed good cross-species consistency, and there was good correlation of *in vivo* and *in vitro* data. These studies in mice, rats, and monkeys effectively predicted the changes in 2,3-DPG and ATP levels observed in single and multiple ascending dose clinical studies in healthy volunteers.

**Keywords**: Anemia, Animal model, Pharmacokinetic, Red blood cell

# Characterization of metabolic response to AG-348, an allosteric activator of red cell pyruvate kinase, in healthy volunteers and pyruvate kinase deficiency patients

Victor Chubukov, Kendall Johnson, Penelope A Kosinski, Michelle Clasquin, Abhishek Jha, Hyeryun Kim, Thomas P Roddy, Elizabeth Merica, Ann J Barbier, Lenny Dang, Lee Silverman, Charles Kung

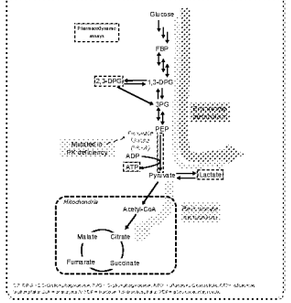
Agios Pharmaceuticals, Inc., Cambridge, MA, USA

## BACKGROUND

PK deficiency

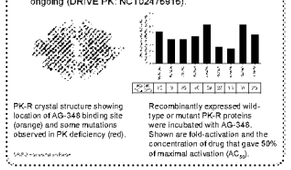
- Pyruvate kinase (PK) deficiency is a glycolytic enzymopathy that causes lifelong chronic hemolytic anemia.
- PK deficiency is caused by abnormalities of the PK red blood cell isoform R (PK-R) due to mutations in the *PKLR* gene.
- Mutations in PK-R typically affect protein stability, catalytic activity, or both, which adversely affects glycolysis and leads to severe energy starvation in red blood cells.

### Figure 1. Glycolytic pathway




### Figure 2. AG-348 is a first-in-class allosteric activator of PK-R and is in clinical development to treat PK deficiency

- Phase 1 studies of AG-348 in healthy volunteers (NCT02108106, NCT02149966) have been completed, and a phase 2 study in patients with PK deficiency is ongoing (DRIVE PK; NCT02476916).

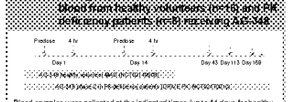


PK-R crystal structure showing location of AG-348 binding site (orange) and some mutations observed in PK deficiency (red).

Recombinantly expressed wild-type or mutant PK-R proteins were incubated with AG-348. Shown are fold-activation and the concentration of drug that gave 50% of maximal activation ($AC_{50}$).

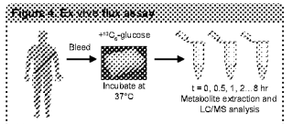
## METHODS

### Figure 3. Metabolic profiling and stable isotope tracing experiments were conducted in blood from healthy volunteers (n=16) and PK deficiency patients (n=8) receiving AG-348




Blood samples were collected at the indicated times (up to 14 days for healthy volunteers and up to 6 months for PK deficiency patients) to assess PK-R activity, levels of ATP/2,3-DPG, and PK-R flux, and for metabolomic profiling. Dose levels evaluated in this study include 120 mg BID and 360 mg BID (healthy volunteers) and 50 mg BID and 300 mg BID (PK deficiency patients).

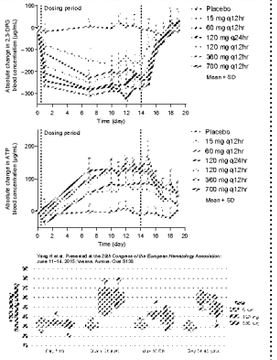
### Figure 4. Ex vivo flux assay




A metabolic tracer, U-$^{13}C$ glucose, was added to blood collected from volunteers and PK deficiency patients treated with AG-348. Blood was incubated *ex vivo* at 37°C, and samples were taken continuously over an 8-hr time course to monitor the propagation of glucose carbon through glycolysis. The labeling patterns of glycolytic intermediates such as 2,3-DPG, as well as the accumulation rates of end-product metabolites such as lactate, were used to characterize blood cell metabolism.

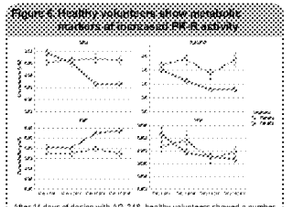
## RESULTS

### Figure 5. AG-348 increases PK-R activity in healthy volunteers



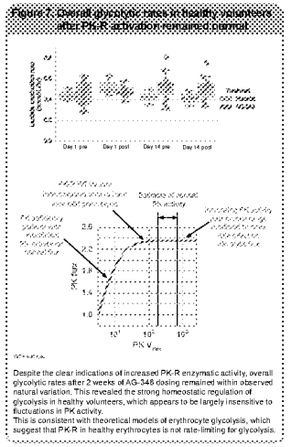

Previously, we had demonstrated that 2,3-DPG levels are decreased and ATP levels are increased in blood samples from healthy volunteers treated with AG-348. PK-R activity was measured in red blood cells from these volunteers and found to be elevated posttreatment.

### Figure 6. Healthy volunteers show metabolic markers of increased PK-R activity



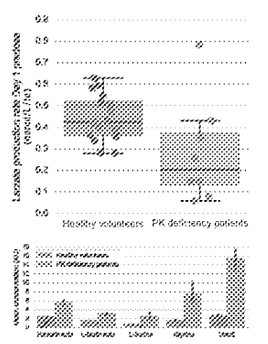
After 14 days of dosing with AG-348, healthy volunteers showed a number of metabolic phenotypes consistent with activation of the wild-type PK-R enzyme. These included decreased concentrations of glycolytic intermediates such as 2,3-DPG and PEP relative to both predosing samples and placebo control volunteers.

### Figure 7. Overall glycolytic rates in healthy volunteers after PK-R activation remained normal



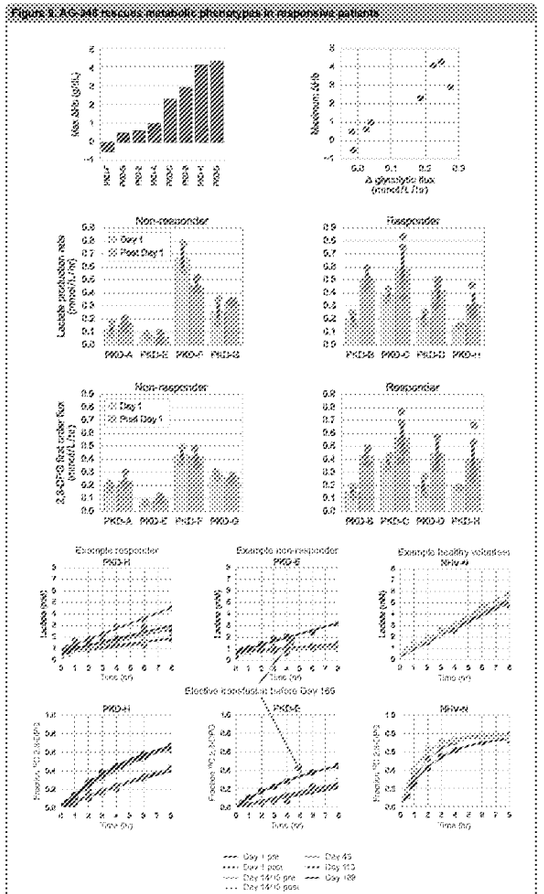
Despite the clear indications of increased PK-R enzymatic activity, overall glycolytic rates after 2 weeks of AG-348 dosing remained within observed natural variation. This revealed the strong homeostatic regulation of glycolysis in healthy volunteers, which appears to be largely insensitive to fluctuations in PK activity.
This is consistent with theoretical models of erythrocyte glycolysis, which suggest that PK-R in healthy erythrocytes is not rate-limiting for glycolysis.

### Figure 8. Metabolic analysis reveals reduced glycolytic flux in PK-deficient blood



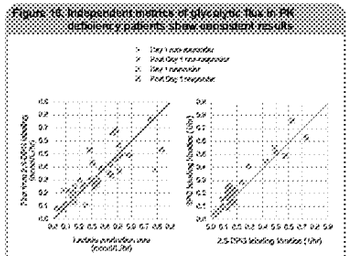

34 PK deficiency patients have enrolled in the DRIVE PK study, and 15 of 32 patients with ≥3 weeks of data had a maximal increase in hemoglobin >1.0 g/dL.
Eight PK deficiency patients have undergone extensive metabolic characterization to determine unique qualities of metabolism in PK deficiency. Compared with healthy volunteers, PK deficiency patient blood cells showed significantly decreased lactate production rates, consistent with a metabolic bottleneck in glycolysis caused by the PK-R mutation. Additionally, PK deficiency patients exhibited a number of other distinct metabolic qualities, such as high concentrations of nucleotides, amino acids, and tricarboxylic acid cycle metabolites.

### Figure 9. AG-348 rescues metabolic phenotypes in responsive patients



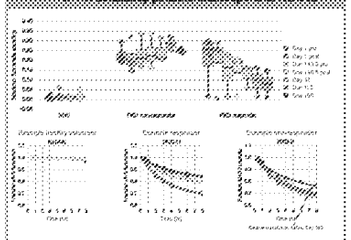

Of the eight PK deficiency patients undergoing extensive metabolic characterization, four had a maximal increase in hemoglobin of >1.0 g/dL. In those patients, we observed a >0.1 mmol/L/hr (>50%) increase in glycolytic rates. Additionally, we observed increased incorporation of $^{13}C$ into glycolytic intermediates such as 2,3-DPG, suggesting an increase in metabolically active erythrocytes. None of the four PK deficiency patients that did not have a hemoglobin increase of >1.0 g/dL showed significant metabolic changes.

### Figure 10. Independent metrics of glycolytic flux in PK deficiency patients show consistent results




Two independent metrics were used to assess glycolytic flux in PK deficiency patients: the total lactate production rate and the rate of labeling in 2,3-DPG. Use of 2,3-DPG first-order kinetics was justified by the fact that it accounts for the vast majority of the pool of glycolytic metabolites, and that metabolites in lower glycolysis showed virtually identical labeling kinetics. The two metrics gave highly consistent results.

### Figure 11. Metabolic markers of immature red cells are reduced in PK deficiency patients that respond to AG-348




An additional observation from the metabolic flux analysis of PK deficiency patient blood was the significant propagation of $^{13}C$ from glucose into tricarboxylic acid cycle metabolites such as citrate, fumarate, and malate. This labeling, which was absent in healthy volunteers, strongly suggested the presence of active mitochondrial metabolism. Since erythrocytes lose their mitochondria during the development process, this metabolism is likely performed by reticulocytes, consistent with the fact that PK deficiency patients tend to have hyperactive bone marrow and increased reticulocyte counts in peripheral blood. Additionally, incomplete labeling of glycolytic intermediates demonstrated the existence of a metabolically inactive cell subpopulation, further suggesting that reticulocytes play an important role in maintaining blood metabolism in PK deficiency patients.

## CONCLUSIONS

- A >50% increase in glycolytic flux was observed in PK deficiency patients treated with AG-348 who had a hemoglobin increase >1.0 g/dL, but was not observed in patients without such an increase.
- Metabolic markers of immature red cells are reduced in PK deficiency patients that respond to AG-348.
- Strong homeostatic regulation of overall rates of glycolysis was observed in healthy volunteers, even in the presence of activated PK-R.
- These data demonstrate that hemoglobin increases in PK deficiency patients treated with AG-348 are associated with increased red cell glycolysis.

Disclosures
This study was funded by Agios Pharmaceuticals. VC, KJ, PAK, AJ, HK, TPR, AJB, LD, LS, CK: Agios – employment and stockholder; MC, EM: Agios – employment and stockholder at time of study. Editorial assistance was provided by Helen Varley, PhD, CMPP, Excel Scientific Solutions, Horsham, UK, and supported by Agios.

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101. RED CELLS AND ERYTHROPOIESIS, STRUCTURE AND FUNCTION, METABOLISM, AND SURVIVAL, EXCLUDING IRON: POSTER II | DECEMBER 2, 2016

# Characterization of Metabolic Response to AG-348, an Allosteric Activator of Red Cell Pyruvate Kinase, in Healthy Volunteers and Pyruvate Kinase Deficiency Patients

Victor Chubukov, Kendall Johnson, Penelope A Kosinski, Michelle Clasquin, Abhishek Jha, Hyeryun Kim, Thomas P Roddy, Elizabeth Merica, Ann J Barbier, Lenny Dang, Lee Silverman, Charles Kung

Check for updates

*Blood* (2016) 128 (22): 2452.

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## Abstract

Pyruvate kinase (PK) deficiency is a glycolytic enzymopathy that causes lifelong chronic hemolytic anemia. AG-348 is an allosteric activator of the red cell isoform of pyruvate kinase (PK-R) that is in clinical development to treat PK deficiency. Phase 1 studies of AG-348 in healthy volunteers (NCT02108106, NCT02149966) have been completed, and a phase 2 study in patients with PK deficiency is in progress (DRIVE PK, NCT02476916). We have previously reported that in the healthy volunteer studies, AG-348 induced changes in levels of the metabolites adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) that are consistent with PK-R activation. A preliminary analysis of data showed that nine out of 18 DRIVE PK patients achieved a maximal increase in hemoglobin (Hb) levels of >1.0 g/dL.

In this study, we characterize whole blood metabolism of healthy human subjects (from the multiple-ascending dose study NCT02149966) as well as PK-deficient patients (from DRIVE PK), before and after administration of AG-348, with a focus on the flux through the PK-R reaction. Whole blood taken



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isotope tracer, [$^{U-13}C_6$]-glucose. Glycolytic flux through PK-R was estimated by kinetic flux profiling based on the isotope labeling, as well as by the specific lactate production rate, with the two methods giving consistent results.

Blood cells from healthy subjects exhibited classic red blood cell (RBC) metabolism, with the majority of glucose catabolized through glycolysis. AG-348 was shown to significantly increase the maximal PK-R protein activity in these subjects, and also to have metabolic effects consistent with PK-R activation, marked by decreased concentrations of glycolytic intermediates such as 2,3-DPG and phosphoenolpyruvate (PEP). Increases in ATP concentrations were also observed, with the magnitude and kinetics of the increase strongly suggesting enhanced adenosine salvage or synthesis. The overall glycolytic rates, however, did not change significantly after two weeks of AG-348 dosing, revealing the homeostatic regulation of RBC glycolysis in healthy blood.

Five of the first 18 DRIVE PK patients underwent an extensive sampling protocol for metabolic analysis. This analysis revealed a number of distinct metabolic qualities in PK-deficient patients at baseline compared with healthy subjects. These included significantly reduced rates of lactate production and high concentrations of nucleotides, amino acids, and Krebs cycle intermediates. $^{13}C$ labeling was observed in Krebs cycle intermediates, demonstrating significant respiratory metabolism in whole blood cells from PK-deficient patients, while incomplete labeling of glycolytic intermediates suggested the presence of a metabolically inactive cell subpopulation. These observations are most consistent with the hypothesis that PK-deficient whole blood is dominated metabolically by immature erythrocytes that retain residual mitochondrial activity.

With AG-348 treatment, three of the five DRIVE PK patients had increases in Hb of >1.0 g/dL. In those three patients, we observed increased incorporation of $^{13}C$ label into glycolytic intermediates such as 2,3-DPG, suggesting an increase in metabolically active erythrocytes. First order flux estimates based on either 2,3-DPG labeling kinetics or lactate production rates showed a >0.1 mmol/L/hr (>50%) increase in glycolytic flux. Neither of the two DRIVE PK patients that did not have an Hb increase of >1.0 g/dL showed significant metabolic changes.

In conclusion, metabolic profiling and stable isotope tracing experiments in blood from healthy subjects treated with AG-348 revealed strong homeostatic regulation of glycolysis even in the presence of activated PK-R. Analysis of data from a small number (n=5) of PK-deficient patients treated with AG-348 for two weeks showed that the three patients with Hb increases >1.0 g/dL also had increased glycolytic flux. While the small number of patients makes these results preliminary, it


Abstract

activator AG-348 and the resulting hematological response as assessed by increases in Hb levels. Updated analyses including additional patients will be presented as more data are collected in the ongoing study.

Author Notes

## Disclosures

**Chubukov:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Johnson:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Kosinski:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Clasquin:***Agios Pharmaceuticals, Inc.:* Other: former employee and stock holder; *Pfizer, Inc.:* Employment. **Jha:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Kim:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Roddy:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Merica:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Barbier:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Dang:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Silverman:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership. **Kung:***Agios Pharmaceuticals, Inc.:* Employment, Equity Ownership.

**Topics:** erythrocytes, pyruvate kinase, pyruvate kinase deficiency, transcriptional activation, lactates, glucose, isotopes, adenosine, adenosine triphosphate, amino acids

## Author notes

* Asterisk with author names denotes non-ASH members.

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Abstract
Abstract

# Metabolic consequences of Pyruvate Kinase Deficiency (PKD), & their correction with AG-348

CPSA Conference

October 2nd, 2014

## Pyruvate kinase deficiency (PKD) results in hemolytic anemia

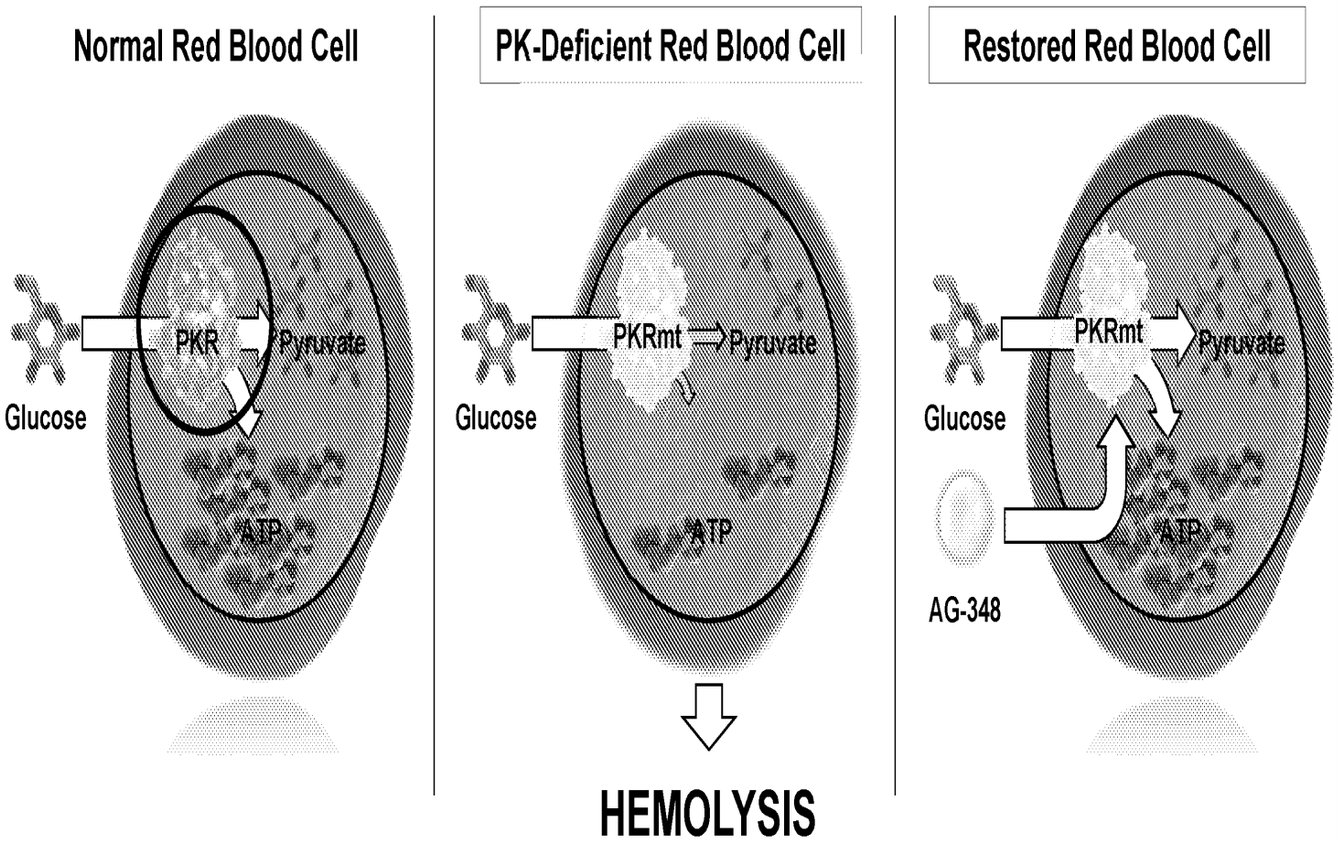
- PKD is a rare, autosomal recessive glycolytic enzymopathy caused by mutations in the red cell isoform of pyruvate kinase (PKR)
- Over 180 mutations have been described, most of which have deleterious effects on protein activity, stability, or expression
- PKD can be a debilitating disease with severe co-morbidities
  - Can be fatal at birth, with necessity for whole body exchange transfusions
  - Adults can be transfusion-dependent
  - Disease can result in significant problems with iron deposition in heart, liver, and other tissues, jaundice, gallstones
- Current standard of care includes blood transfusions, splenectomy, chelation therapy, & interventions for other treatment- & disease-related morbidities
- There are no FDA-approved treatments that target the underlying enzymatic defect



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# Therapeutic Approach to Treating PK Deficiency

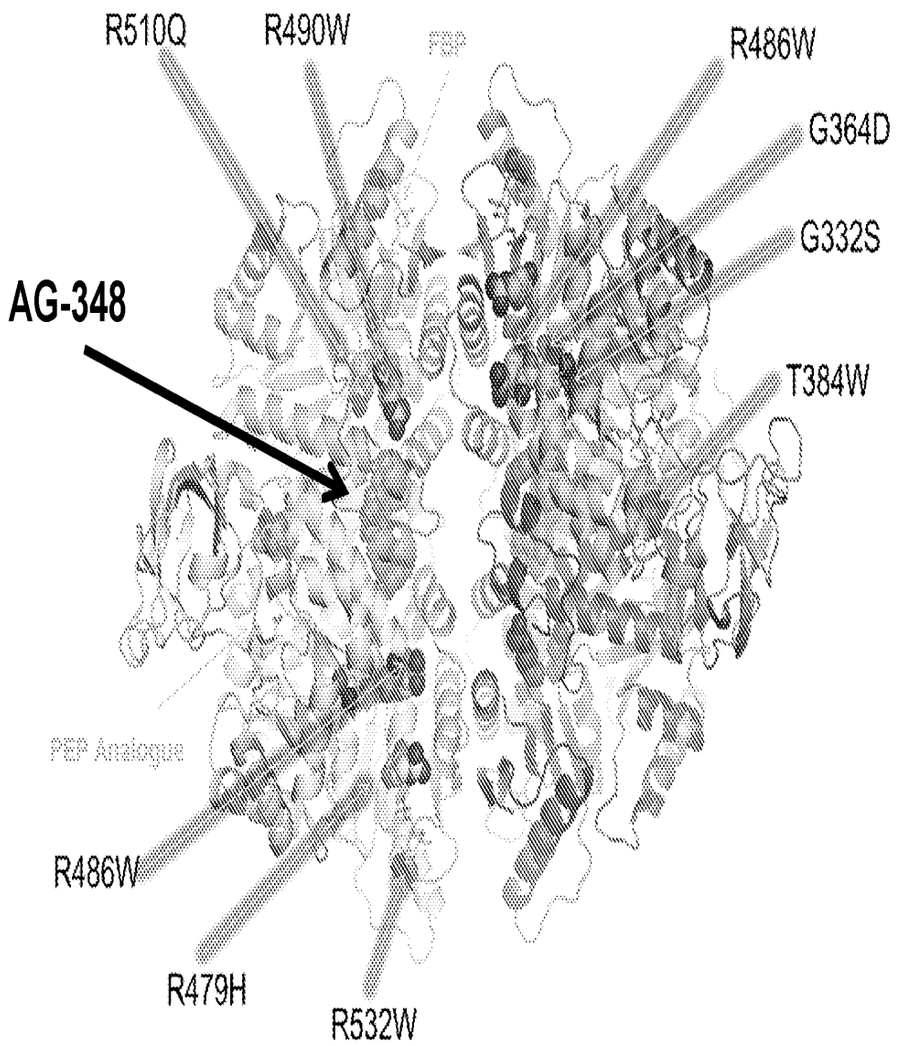
- Glycolysis is the only source of energy (ATP) for red blood cells
- PKR enzyme catalyzes the final step in glycolysis in red blood cells
- In PK Deficiency, low PKR activity leads to low ATP levels and high rate of hemolysis in red blood cells
- Agios has developed AG-348, a small molecule activator that restores activity of mutant PKR



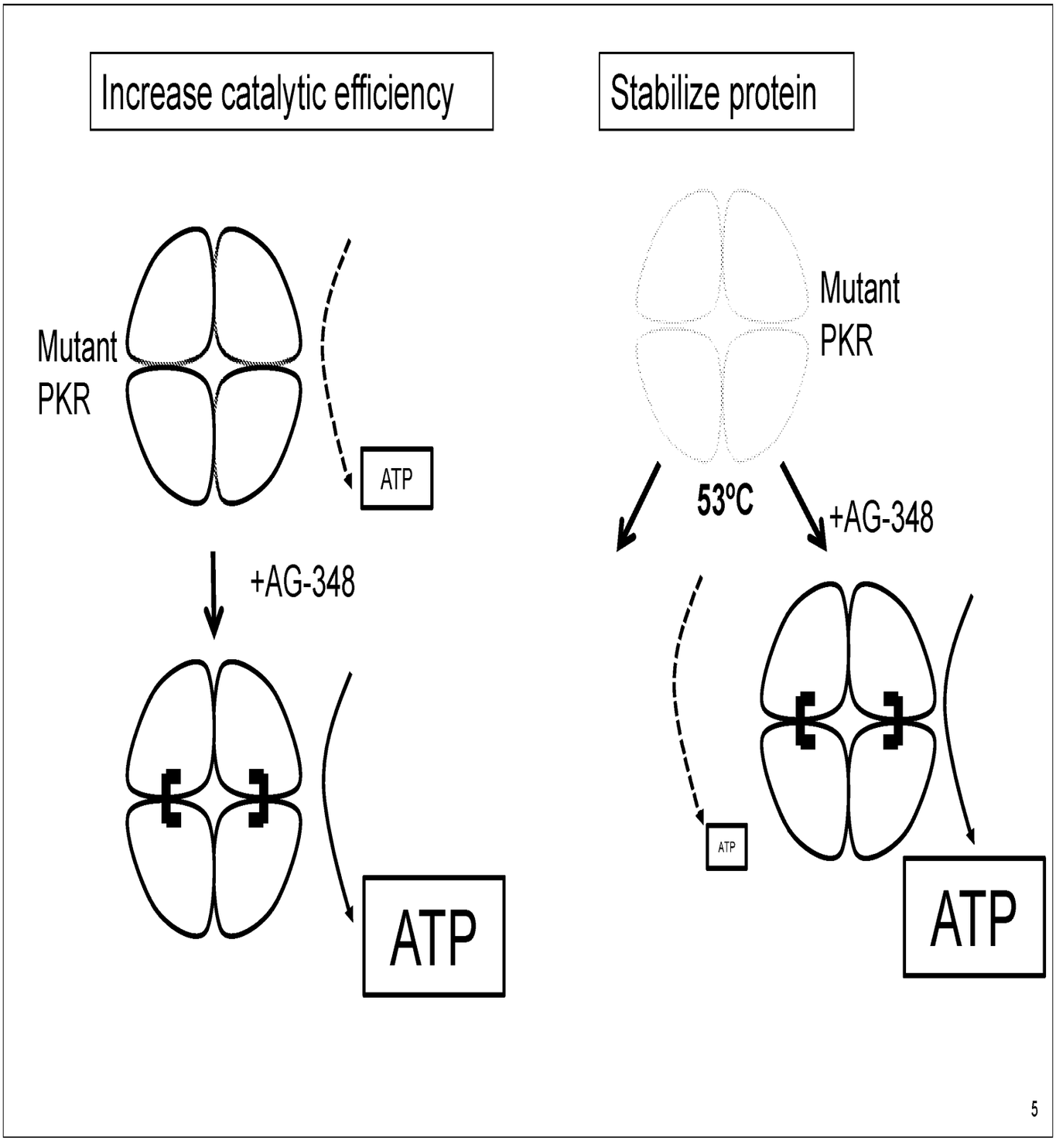



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# AG-348 binds at the PKR dimer-dimer interface

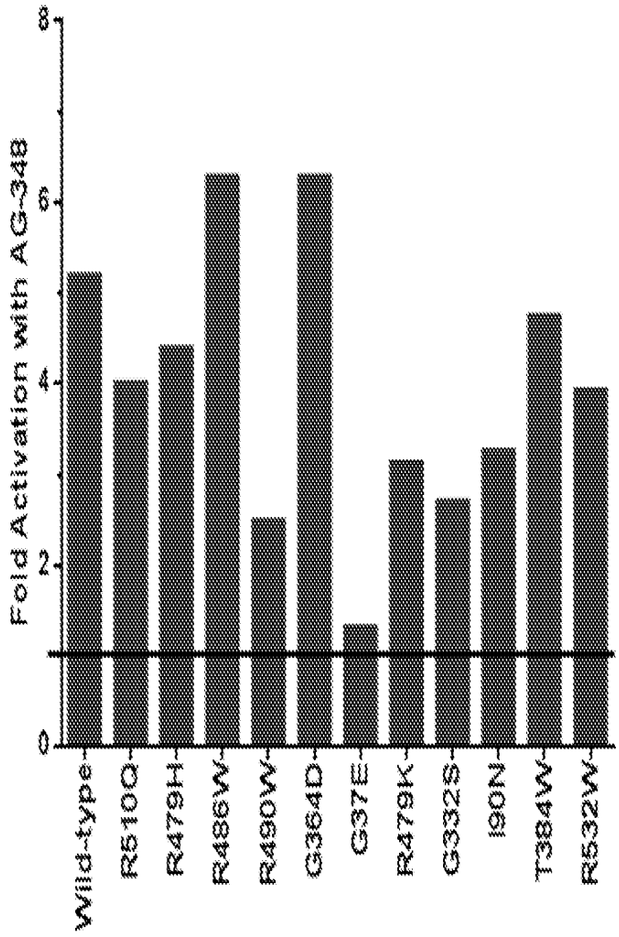


# Two mechanisms for AG-348 Activation

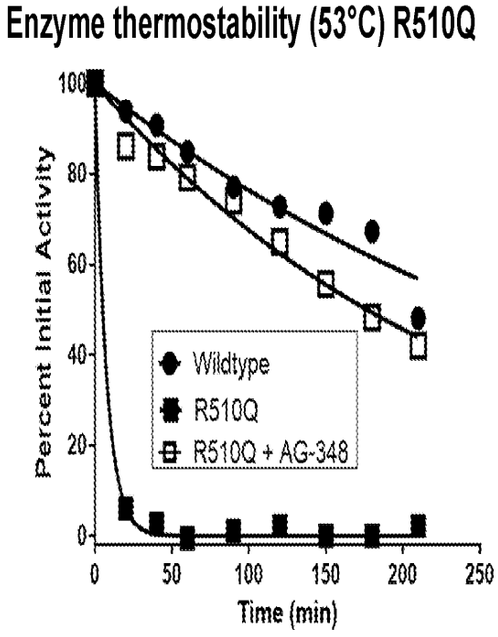


# AG-348 increases catalytic efficiency and improves stability of mutant enzymes in biochemical assays

Increase catalytic efficiency




Stabilize protein



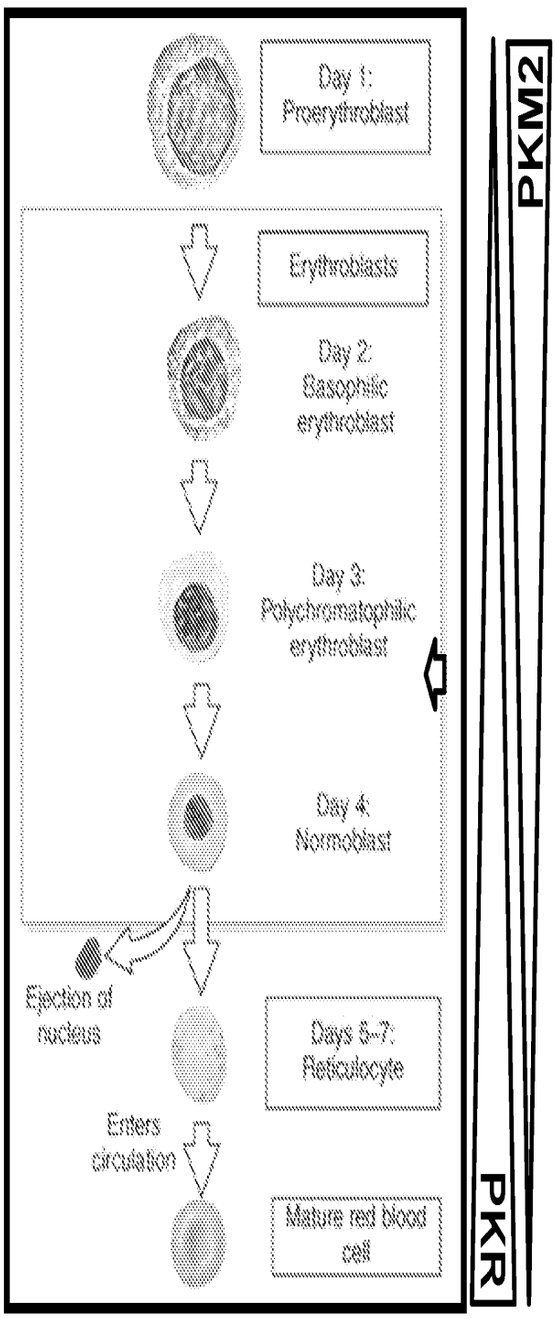
# Key questions

- Are proximal metabolite concentrations altered in either whole blood or RBCs isolated from PKR patients?
  - Can these be reproducibly measured?
- Are the metabolic defects normalized following AG-348 treatment?
  - True across diverse genotypes?
- Can we design clinically translatable pharmacodynamic readouts, quantifying the functional activation of glycolysis?



Ex. 2001-0473

# Erythrocyte maturation and pyruvate kinase




**L gene** → **L isozyme**

**Liver, kidney, intestine**
- allosteric regulation by FBP
- hormonal control

**L gene** → **R isozyme**

**Erythrocytes**
- allosteric regulation by FBP

**M gene** → **M1 isozyme**

**Adult tissues - muscle, brain**
- constitutively active

**M gene** → **M2 isozyme**

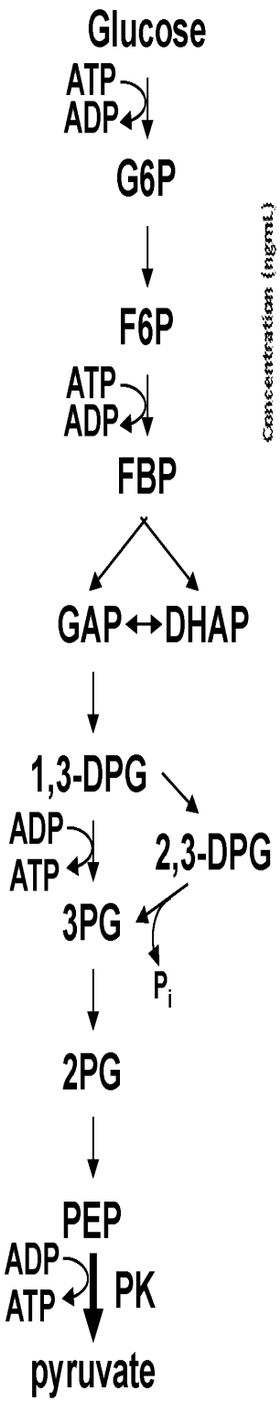
**Fetal tissue/ undifferentiated cells**
- allosteric activation by FBP
- re-expressed in tumors

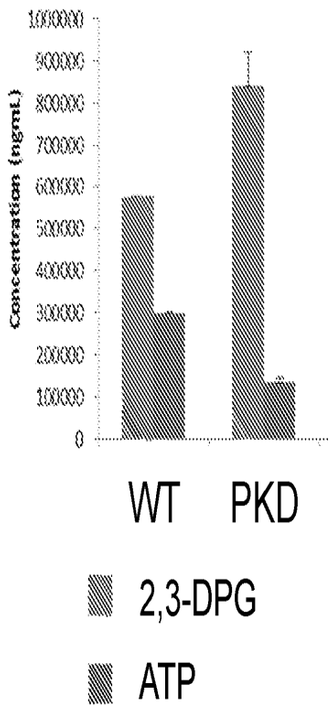
- Blood from each individual PKD patient represents a complex mixture of cells of different type/age/etc.



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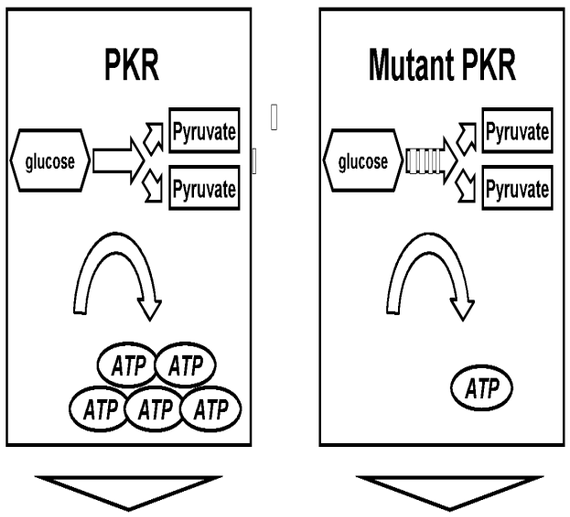
# Proximal metabolites ATP and 2,3-DPG are altered in PKD red cells, potentially leading to chronic red cell hemolysis

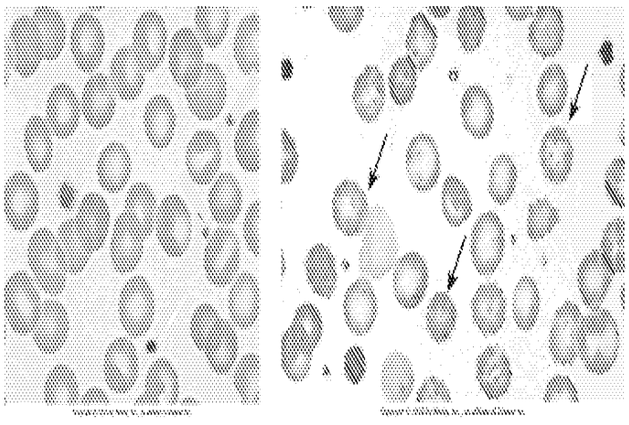



In PK Deficiency:

- 2,3-DPG levels are elevated
- ATP levels reduced



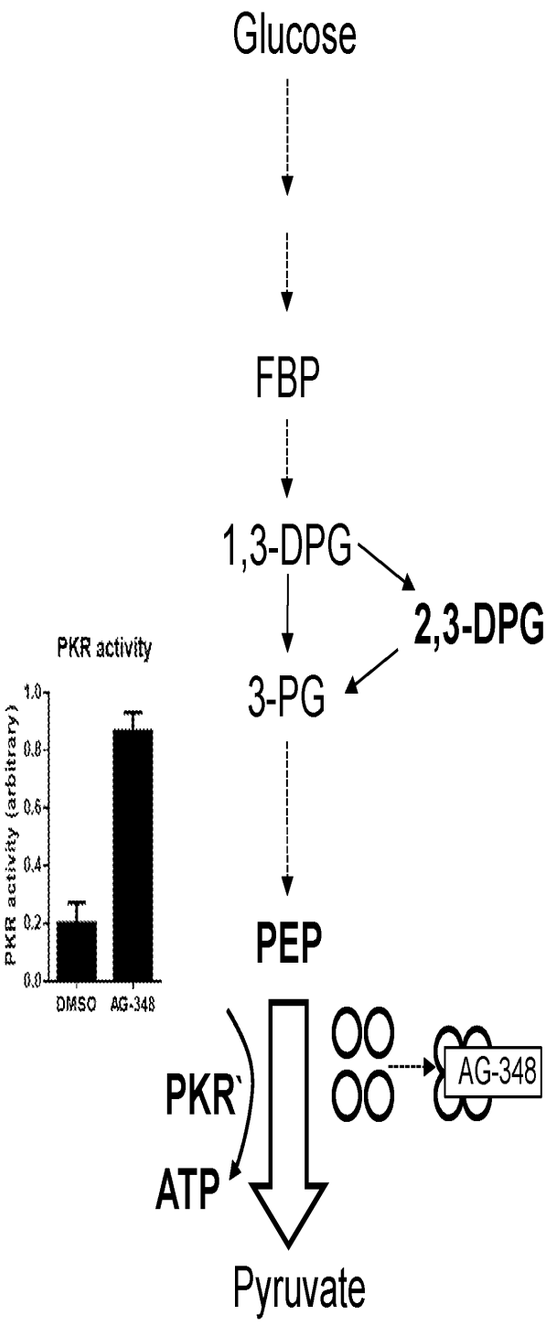



Arrows = features of echinocytosis

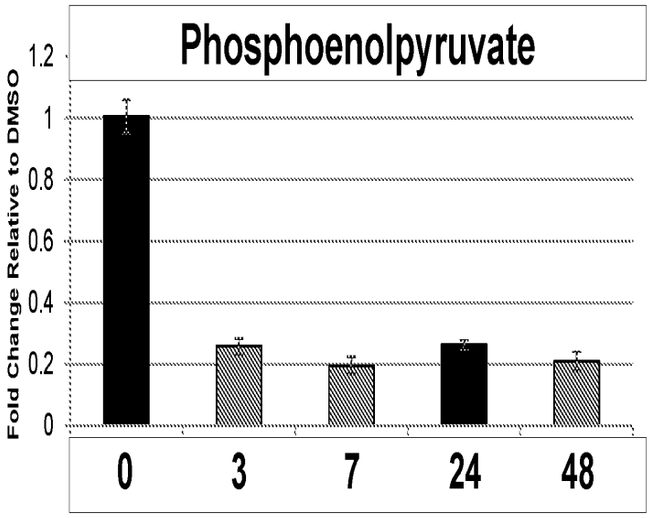


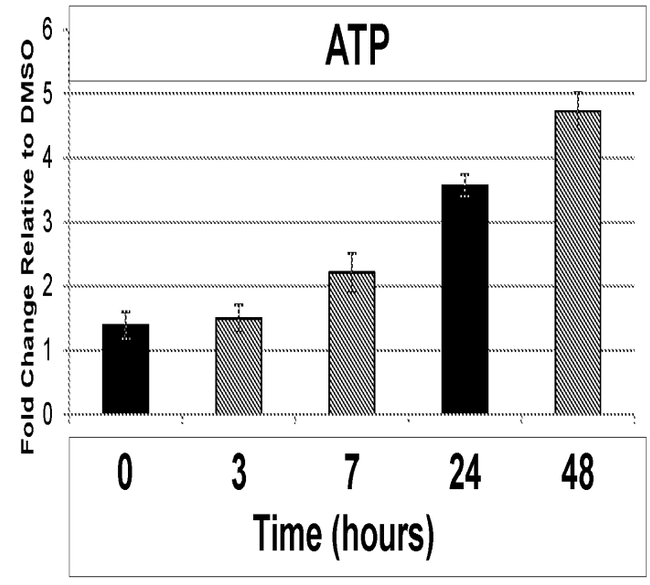
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# AG-348 treatment modulates proximal metabolites ATP and 2,3-DPG in PKD patient red cells




Purified red cells treated in AGAM buffer for indicated time with AG-348



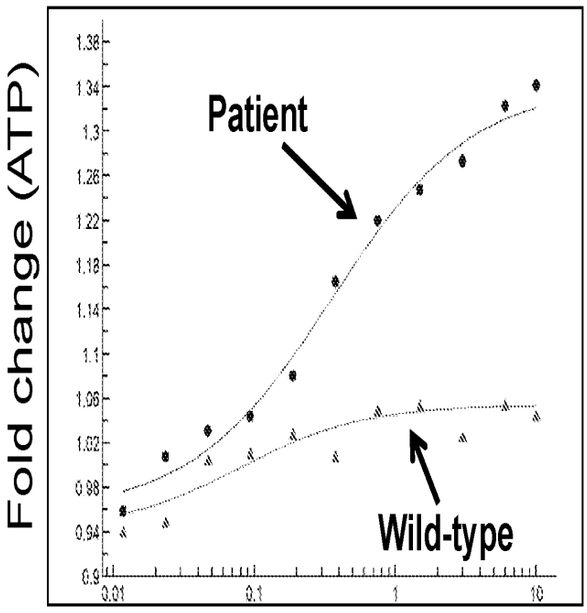

*Patient sample #3: R510Q/G511R*

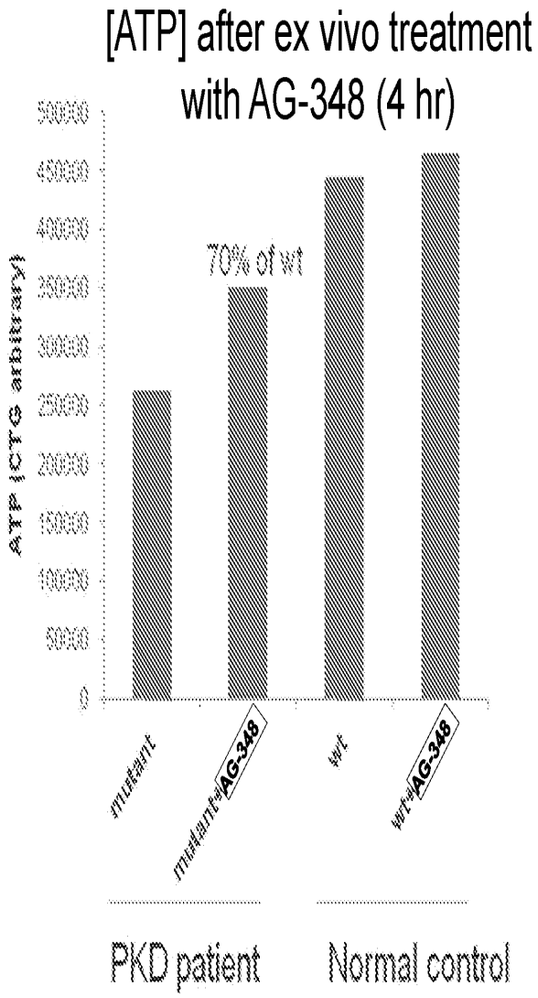


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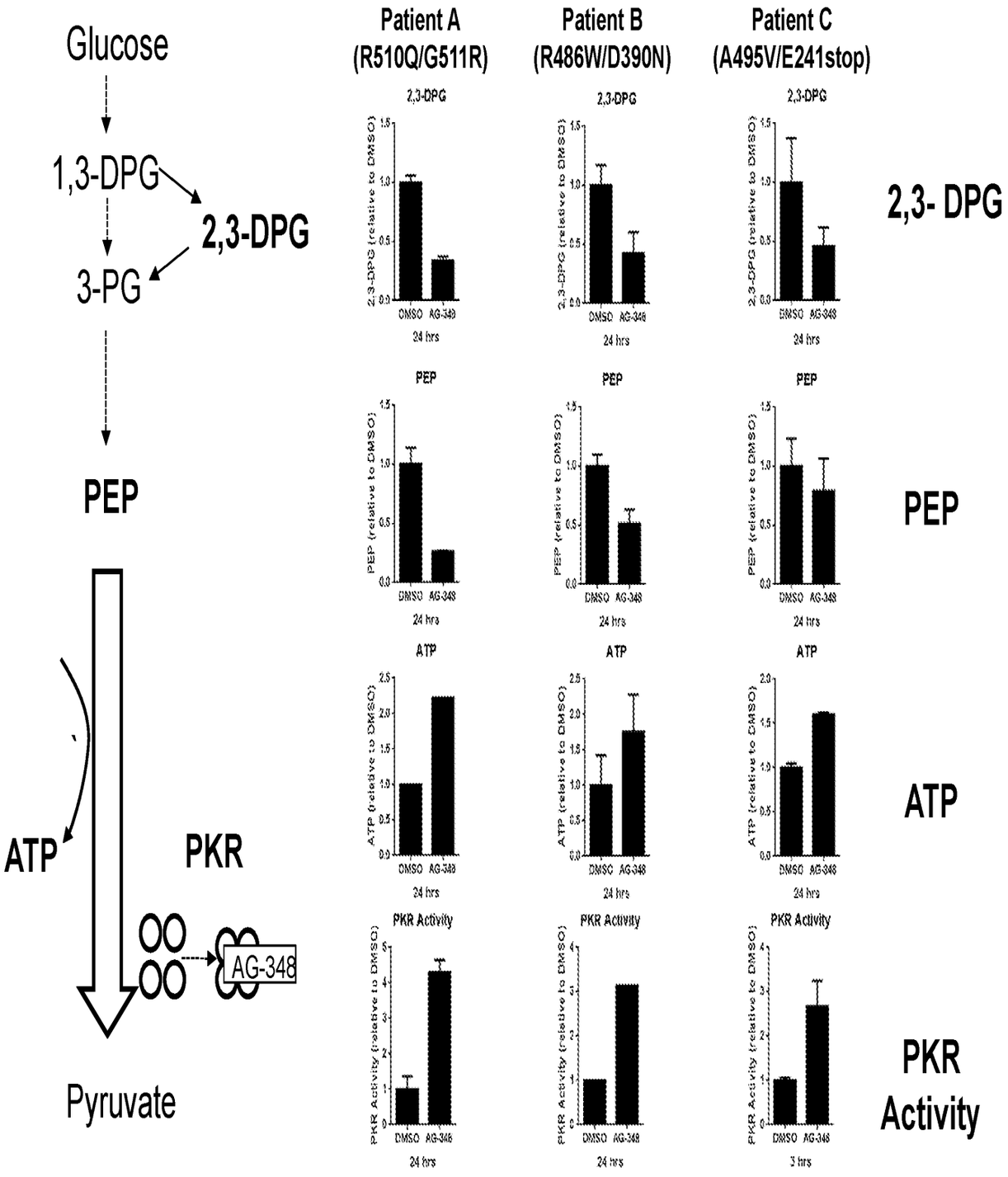
## AG-348 increases ATP levels in a dose-dependent fashion in PKD patient red cells (R510Q/G511R)

- Purified red cells were treated with AG-348 for 24 hrs with AG-348



# Across three genotypes, AG-348 activates mutant PKR enzyme and induces metabolic changes consistent with increased glycolysis in PKD patient red cells

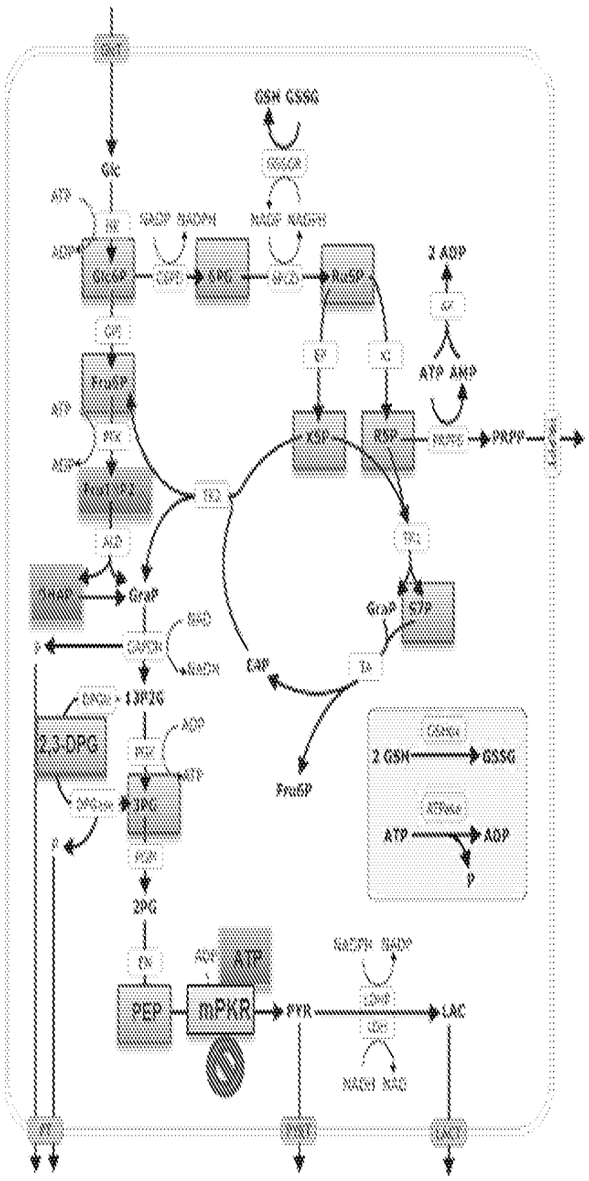





Ex. 2001-0478

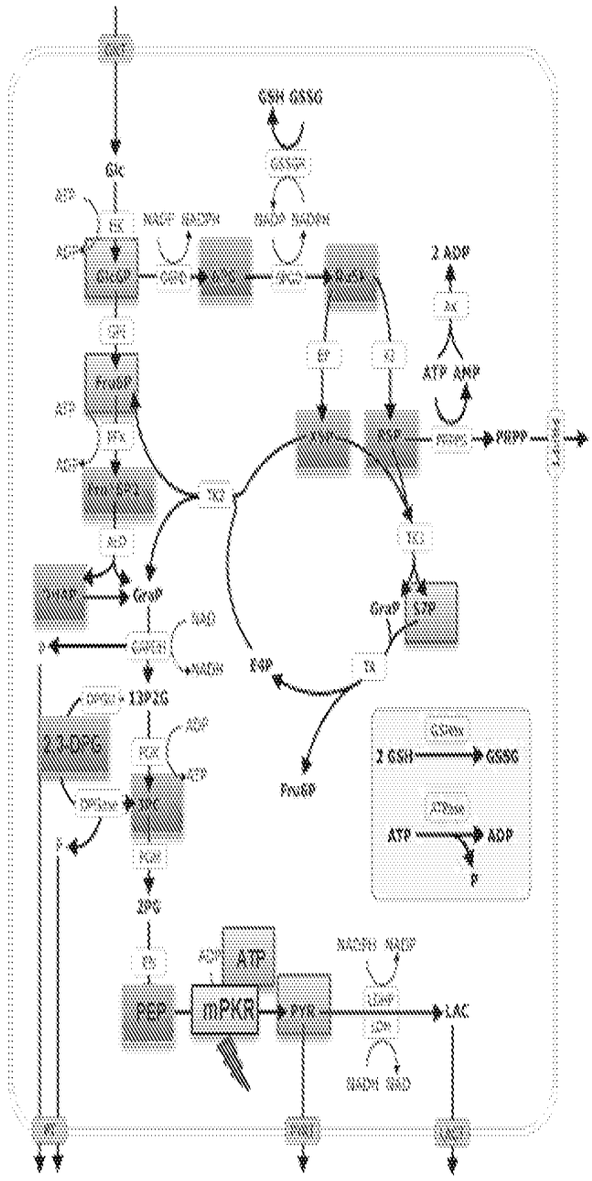
# Isolated RBCs from PKD patient show broader metabolic defects, corrected by ex vivo treatment with AG-348

**Patient A vs WT**




| Up in patient | Down in patient |
|---|---|

**Patient A + AG-348**




| Up vs. control | Down vs. control |
|---|---|



Ex. 2001-0479

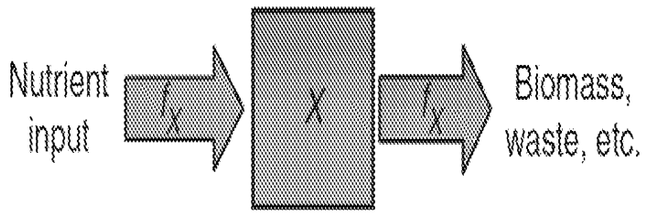
## Key questions

- Are proximal metabolite concentrations altered in either whole blood or RBCs isolated from PKR patients?
  - Can these be reproducibly measured?
- Are the metabolic defects normalized following AG-348 treatment?
  - True across diverse genotypes?
- Can we design clinically translatable pharmacodynamic readouts, quantifying the functional defect of glycolysis in PKD patients, & subsequent activation by AG-348?



Ex. 2001-0480

# Kinetic flux profiling (KFP) can be applied to quantify glycolytic flux




With $X^T$ at steady state,

$dX^U/dt = -f_X(X^U/X^T)$

The analytical solution is

$X^U/X^T = \exp(-f_X t/X^T)$

Setting $k_X = f_X/X^T$, we get

$X^U/X^T = \exp(-k_X t)$

Labeled form

Unlabeled form

Basic KFP procedure:

- Rapid switch to labeled nutrient followed by quenching at various time points

Adaptation for clinical use:

- Spike U-$^{13}C_6$-glucose into whole blood ex vivo, ~50/50 unlabeled/labeled

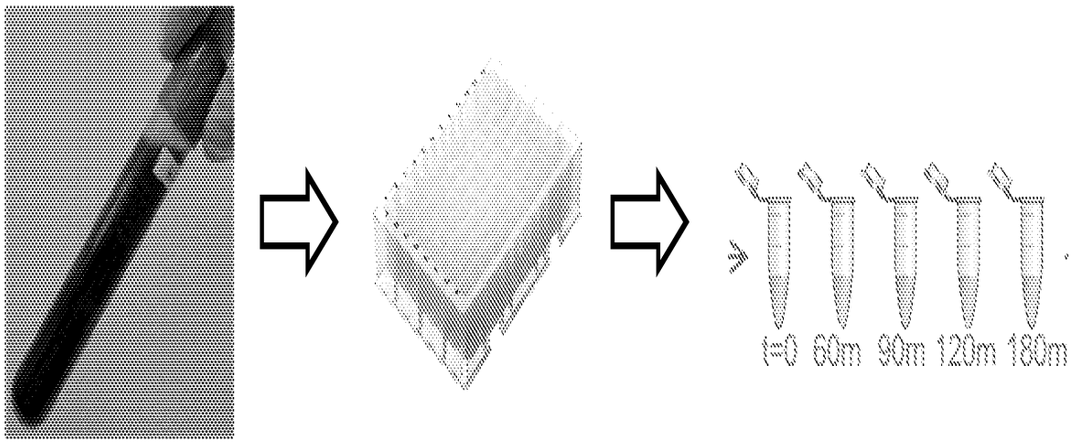
*Note: Requires metabolite concentrations ($X^T$) to be at constant*

*Juan, Nat. Chem. Bio, (2006)*



Ex. 2001-0481

# *Ex vivo* Kinetic Flux Profiling strategy designed as clinically translatable pharmacodynamic readout




Collect whole blood in anticoagulant tubes

Immediately transfer to plate with pre-aliquoted $^{13}C$ glucose. Incubate at 37 degrees.

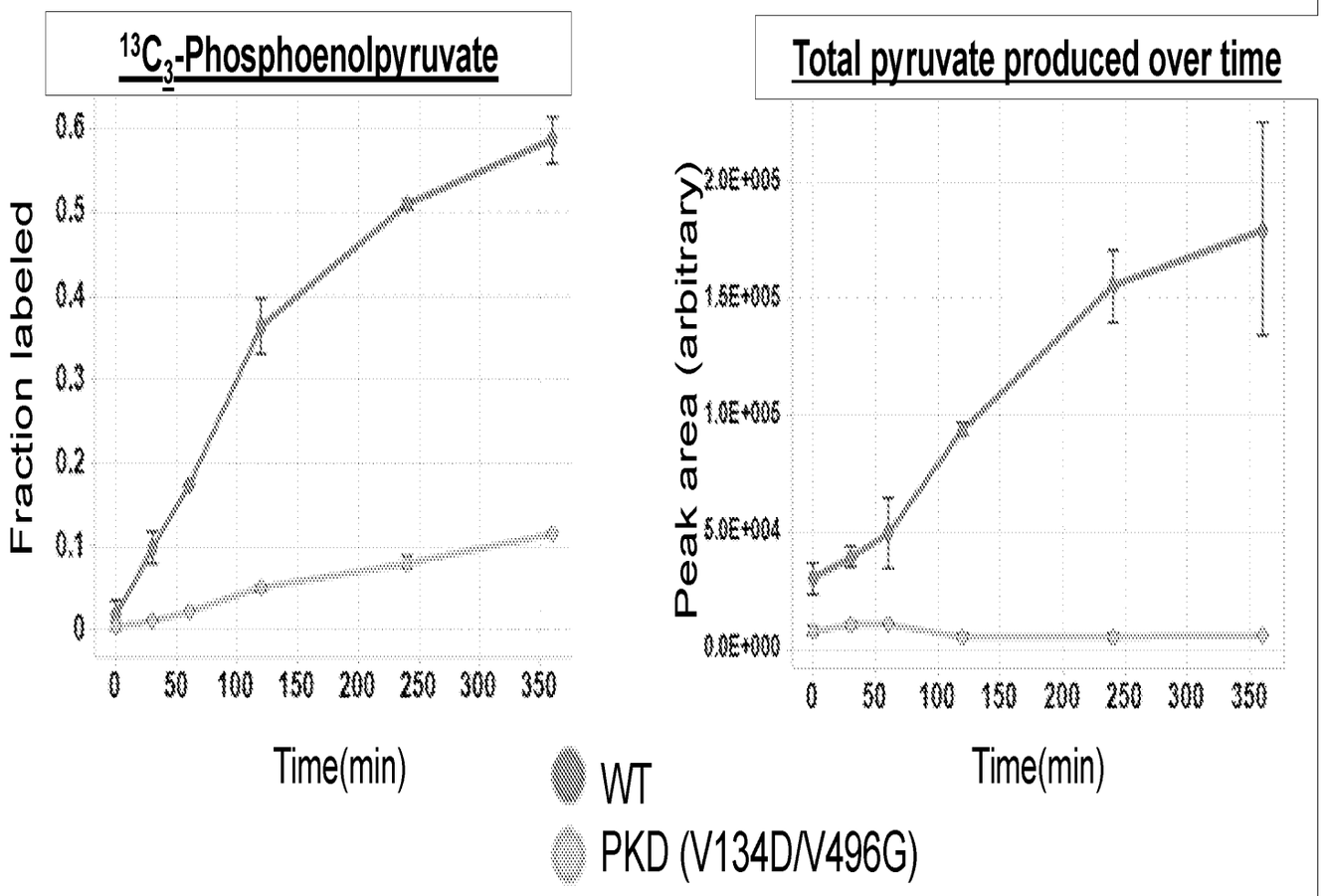
Remove aliquots at specified timepoints and snap freeze (e.g. 30, 60, 120, 180, 240 min etc.)

LC / QExactive MS applied to quantify metabolite pool sizes, & trace label throughout time course



Ex. 2001-0482

## Modeling of kinetic flux profiling data leads to quantitative measures of diminished glycolytic rate in PKD patient




- PKD blood shows dramatic decrease of % labeling into glycolytic intermediates.
- Pyruvate is actively made and accumulates in WT blood, but not in PKD blood.
- First Agios measurement of patient flux; very little literature precedent



Ex. 2001-0483

# Summary

- AG-348 is a potent, orally available, allosteric activator of mutant PKR (mPKR) enzymes
- In biochemical experiments, 10/11 of tested mutant enzymes associated with PKD can be activated >2-fold
- AG-348 can activate mPKR enzymes in PKD red cells, and induces metabolic changes consistent with increased glycolysis
  - Increase [ATP], decrease [2,3-DPG]
- Adapted kinetic flux profiling protocols are being used to quantify glycolytic flux in ongoing clinical trials
- Using this method, reduced glycolytic flux is observed in PKD patients



Ex. 2001-0484

# Acknowledgements


- Colleagues at Agios Pharmaceuticals
- PKD patient blood donors

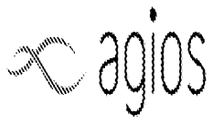



Ex. 2001-0485

# Citations



Small Molecule Activation of Pyruvate Kinase Normalizes Metabolic Activity in Red Cells From Patients With Pyruvate Kinase Deficiency-associated Hemolytic Anemia



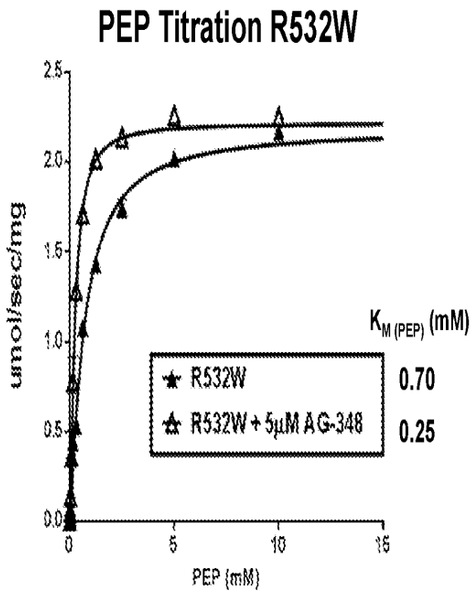

Charles Kung[1*], Jeff Hixon[1], Penelope Kosinski[1], Gavin Histen[1], Collin Hill[1], Yaguang Si[1], Andrew Kernytsky[1], Yue Chen[1], Byron DeLaBarre[1], Michelle Clasquin[1], Helen Ho[1], Furha Cossor[2], Ellis J Neufeld[3], Frank Salituro[1], Janeta Popovici-Muller[1], Sam Agresta[1], Lee Silverman[1], and Lenny Dang[1]

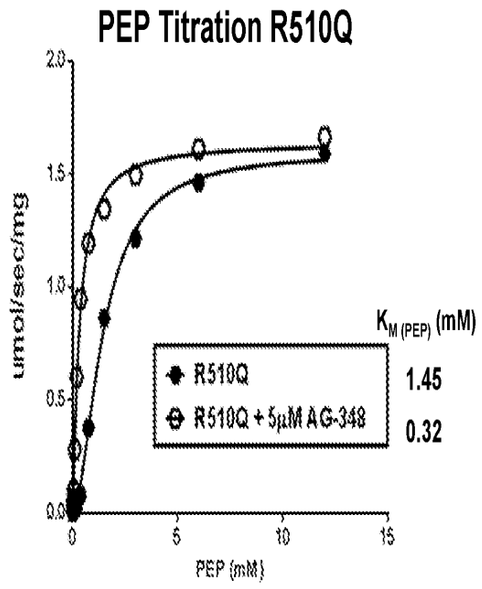
[1]Agios Pharmaceuticals, 38 Sidney St., Cambridge, MA 02139, [2]Tufts Medical Center, 800 Washington St., Boston, MA 02111, [3]Boston Children's Hospital, 300 Longwood Ave, Boston, MA 02115 *[illegible]

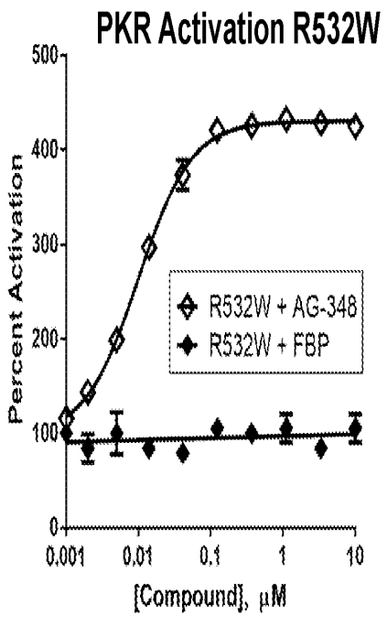
- Yuan, J., Fowler, W.U., Kimball, E., Lu, W. & Rabinowitz, J.D. Kinetic flux profiling of nitrogen assimilation in Escherichia coli. *Nat. Chem. Biol.*, **2**, 529–530 (2006).

# Back up

# AG-348 increases catalytic efficiency and stability of PKD-associated mutants






Ex. 2001-0488



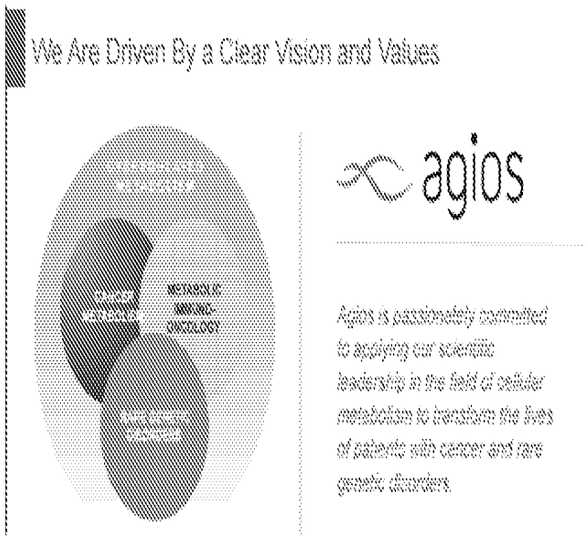
# Using metabolism to support drug discovery: from target identification to biomarker validation

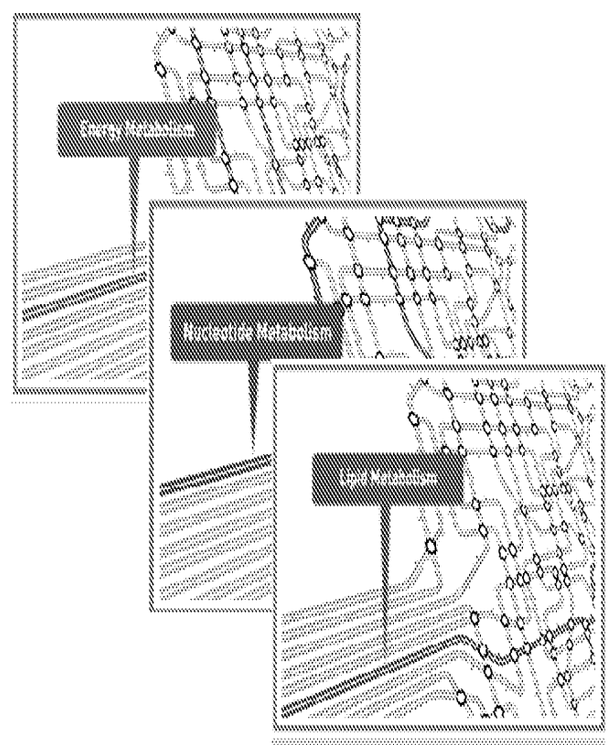
Feb. 27 2017

--Cell metabolism team--
David Pirman, PhD

# Agios Pharmaceuticals

- Small-ish biotech company (300+ employees)
- Founded by cancer metabolism experts: Tak Mak, Lew Cantley and Craig Thompson
- Focuses primarily on metabolic disease research
  - Cancer
  - Rare Genetic Diseases
  - Metabolic Immuno-oncology (*new)
- Cell metabolism group
  - Fall in between DMPK and Biology
  - Supports all research functions
    - Target discovery and validation
    - Biomarker discovery
    - Clinical metabolic characterization



# What do we do?

## Drug discovery timeline

Target ID/Validation → Hit Finding/Validation → Cell model/animal model → Lead op SAR → Clinical trials!

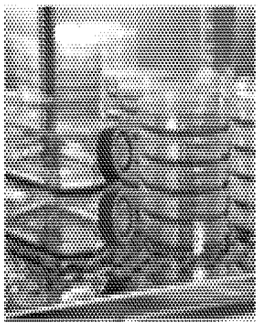
OGDH for Cancer

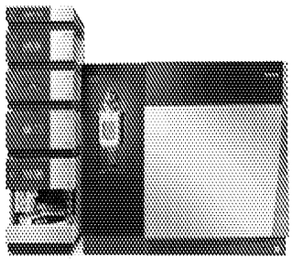
Pyruvate Kinase activator for PK-Deficiency patients

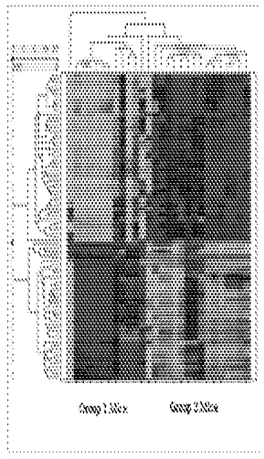
CELL METABOLISM



# Biology and metabolism: a symbiotic relationship







## Biology

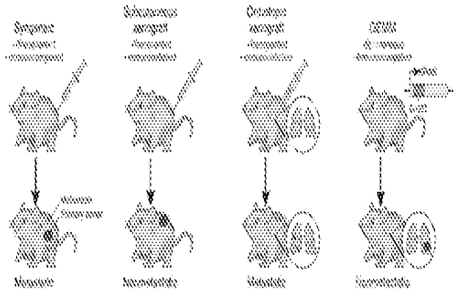
- *In vitro, in vivo*, patient samples
- Model development
- Target validation

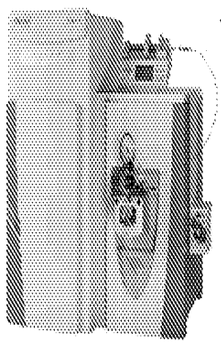
## Analytical

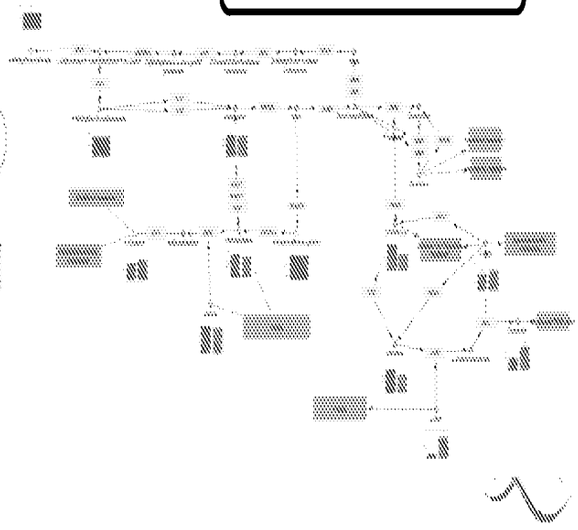
- Multi-omics platforms
- Metabolism, lipidomics, proteomics
- Targeted assays
- Biomarker identification

## Informatics

- Automated data analysis
- Metabolomics (targeted and untargeted)
- Flux Analysis
- Pathway analysis

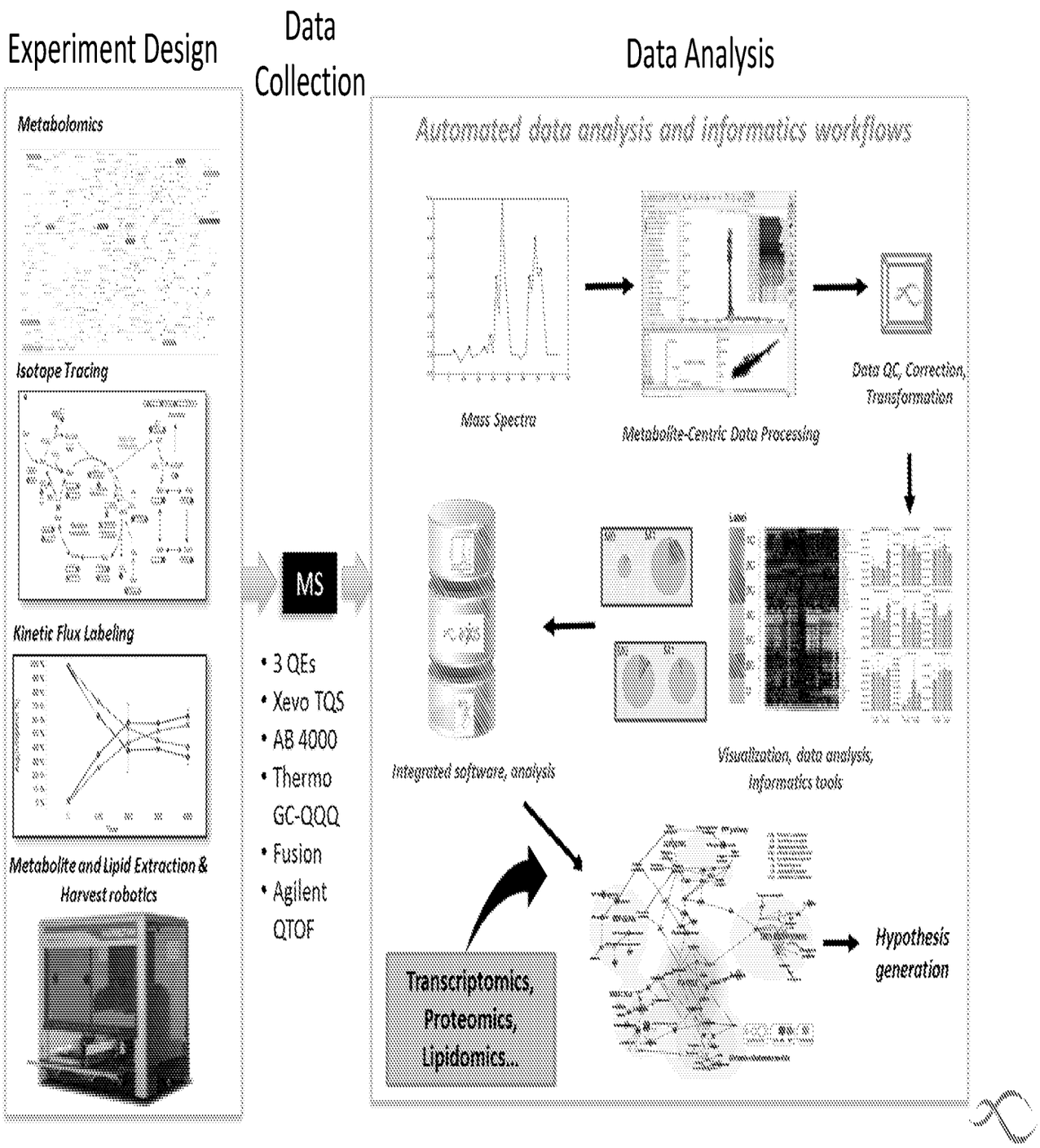






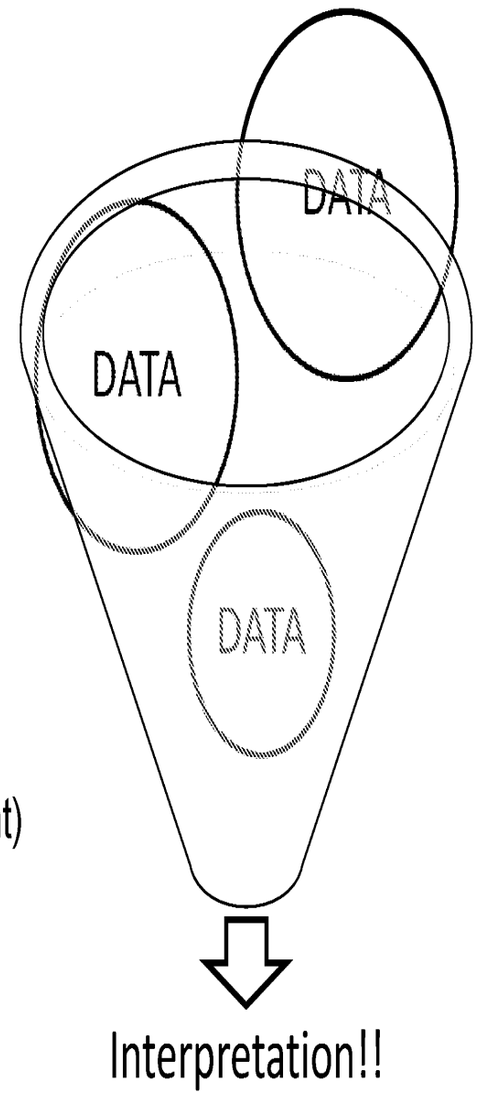
# Agios Metabolism Platform: Tools and Technology

*Automation and Mass Informatics Workflows (scale reflects effort)*



# Informatics analysis—huge bottleneck in metabolomics

- Metabolomics-Automated workflows
  - Peak library matching (m/z and RTs)
  - Peak annotation
  - Maven Software continuously optimized
  - Statistical characterization
- Combi-Omics
  - Metabolism-transcript (automated)
  - Protein-transcript (development)
  - Metabolism-protein-transcript (development)
- Flux analysis
  - Rate of specific metabolite production and consumption
  - Typically utilizing stable-labeled isotopes



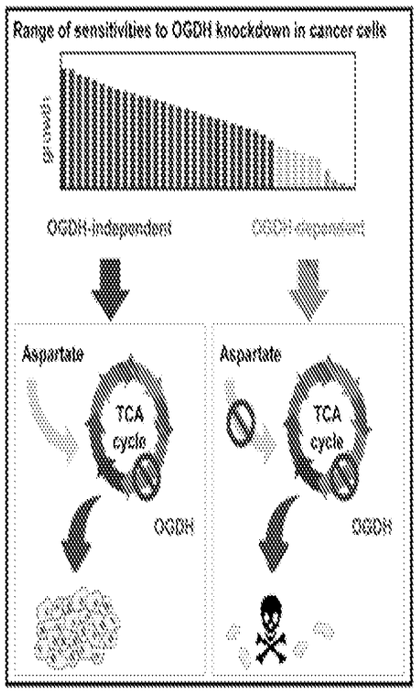
# Metabolic studies of cancer targets

Article

Cell Reports

## Differential Aspartate Usage Identifies a Subset of Cancer Cells Particularly Dependent on OGDH

Graphical Abstract




Authors

Eric L. Allen, Danielle B. Ulanet, David Pirman, ..., Marion Dorsch, Shengfang Jin, Gromoslaw A. Smolen

Correspondence

gromoslaw.smolen@agios.com

In Brief

Allen et al. show that knockdown of alpha-ketoglutarate dehydrogenase E1 subunit, OGDH, results in profound growth defects in a subset of cancer cell lines in vitro and in vivo. Differential aspartate utilization, via malate-aspartate shuttle, serves as a marker of OGDH dependence and suggests broader utility of nutrient utilization screens to identify cancer targets.

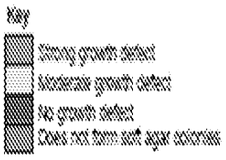
Highlights

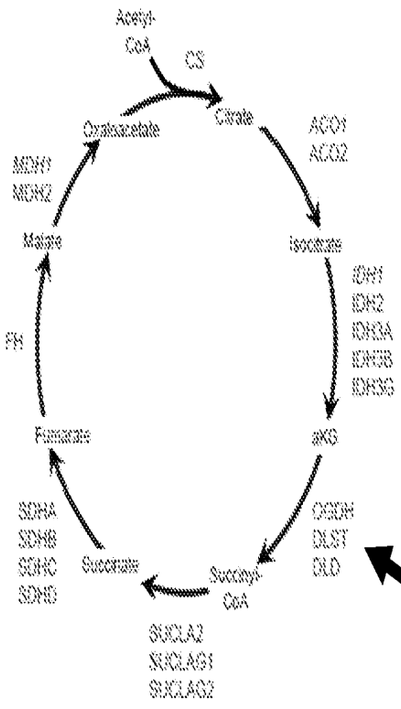
- Cancer cells display a broad range of sensitivities to OGDH knockdown
- Predictive 3D in vitro assays allow for in vivo translation of metabolic vulnerabilities
- OGDH-dependent cell lines display low levels of malate-aspartate shuttle activity

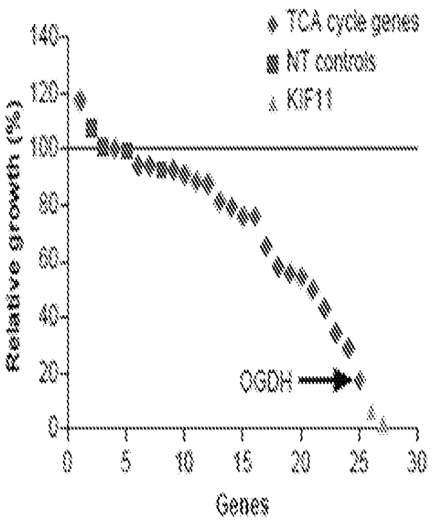
Allen et al. Cell Reports 2016;17:876–890

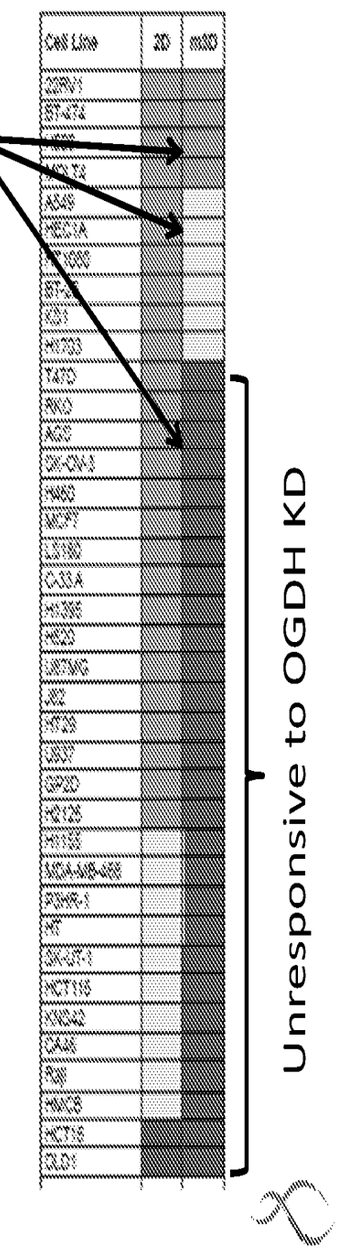
# Metabolic characterization of differential responses to a novel cancer target--OGDH

- siRNA screen targeting TCA genes→identified OGDH as significant hit causing growth defects
- Tested OGDH KD in broader panel of cells (dependence varied)
- 2D→3D growth assay disconnect



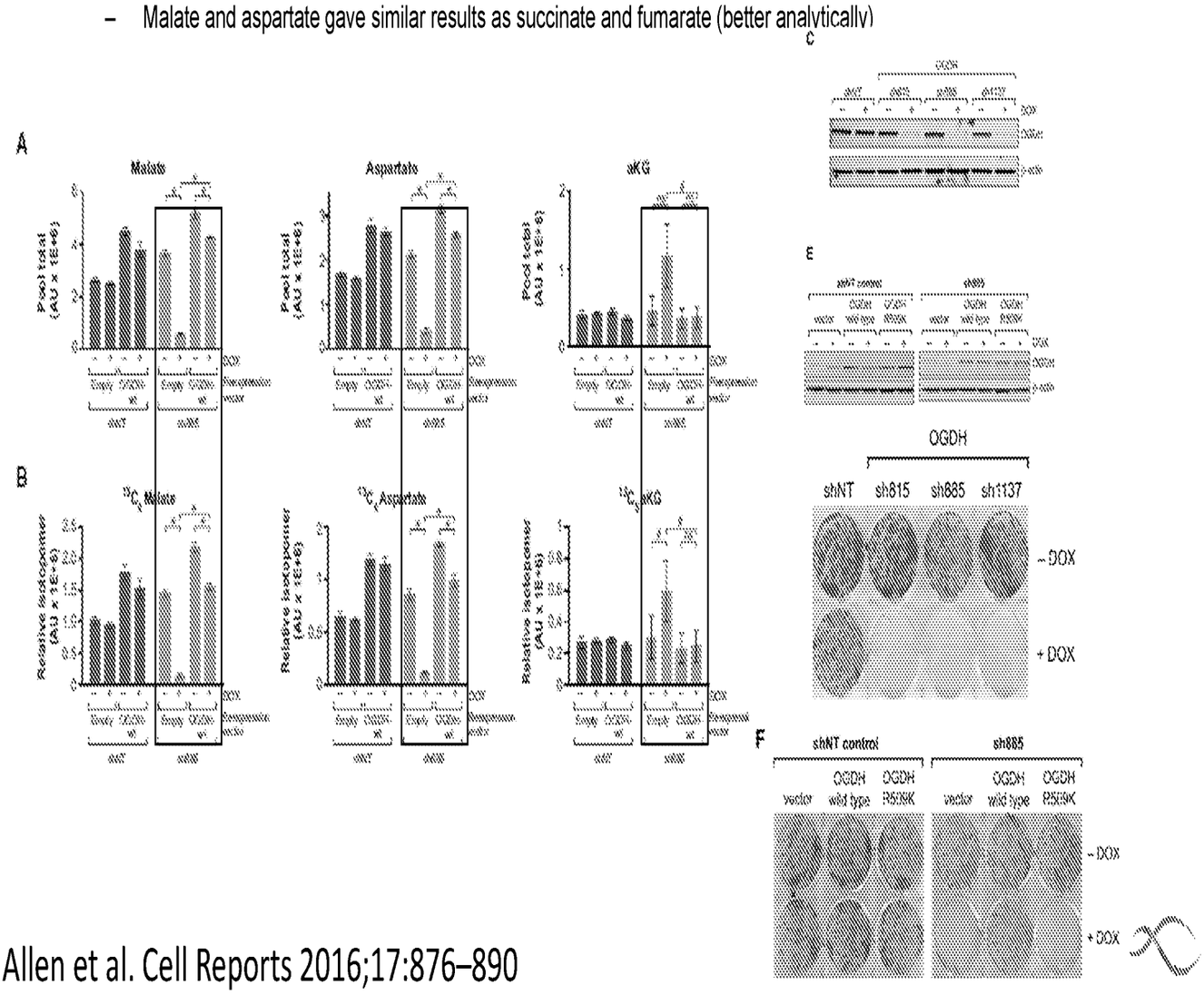




Allen et al. Cell Reports 2016;17:876–890

# Target validation assays to support OGDH KD

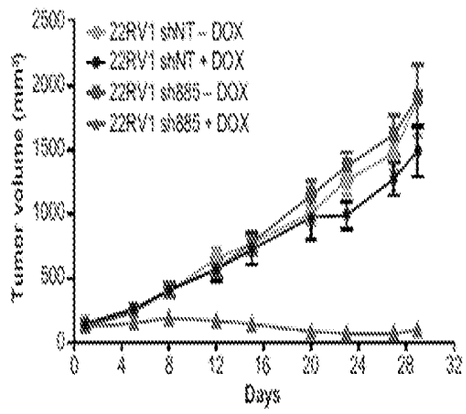
- Ensure siRNA tools were on target to support cell growth target dependence
  - Characterize metabolic phenotype upon OGDH KD
  - Reverse metabolic phenotype with OGDH re-expression (rescue experiment)
- Ultimately utilized labeling into downstream metabolites of OGDH as indication of KD
  - Malate and aspartate gave similar results as succinate and fumarate (better analytically)



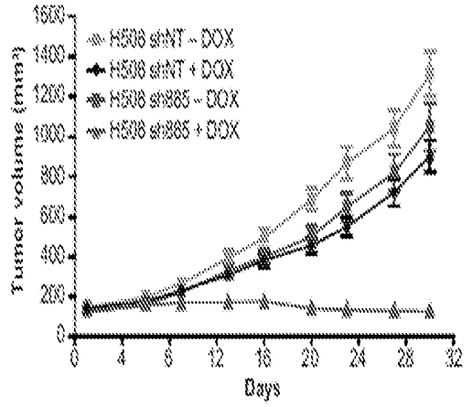

Allen et al. Cell Reports 2016;17:876–890

# OGDH KD in sensitive cells translates to in vivo tumor model: HOW/WHY?

A



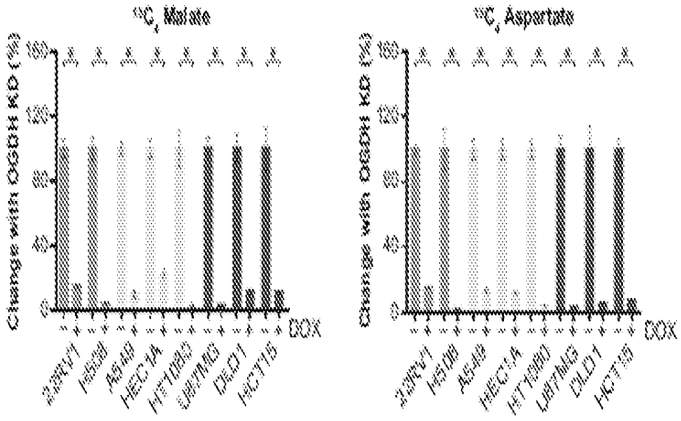

B




C

| | 2D | m3D | % TGI *in vivo* | p-values |
|---|---|---|---|---|
| 22RV1 | | | 104 / 91 | 1.23e-9 / 5.40e-7 |
| H508 | | | 99 / 96 | 9.04e-10 / 3.03e-12 |
| HEC1A | | | 47 / 41 | 1.58e-5 / 1.13e-2 |
| A549 | | | 40 / 49 | 4.16e-2 / 1.72e-4 |
| HT1080 | | | 54 / 61 | 5.08e-4 / 6.00e-6 |
| U87MG | | | 0 | NS |

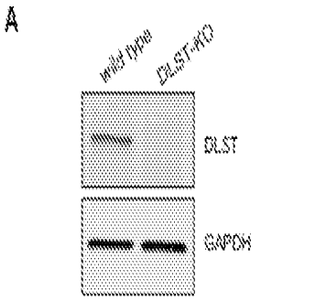
E

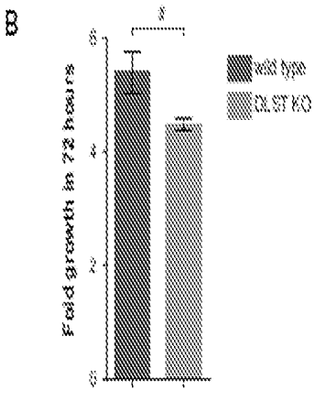


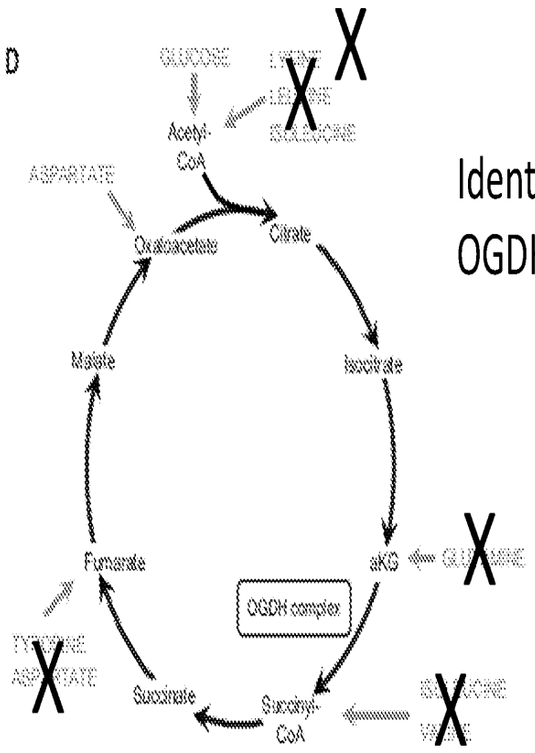

- Remarkable TGI (99%) in sensitive cell lines
- Independent of OGDH KD effects (uniform across cells lines tested)
- Biomarkers that predict OGDH dependence?

Allen et al. Cell Reports 2016;17:876–890

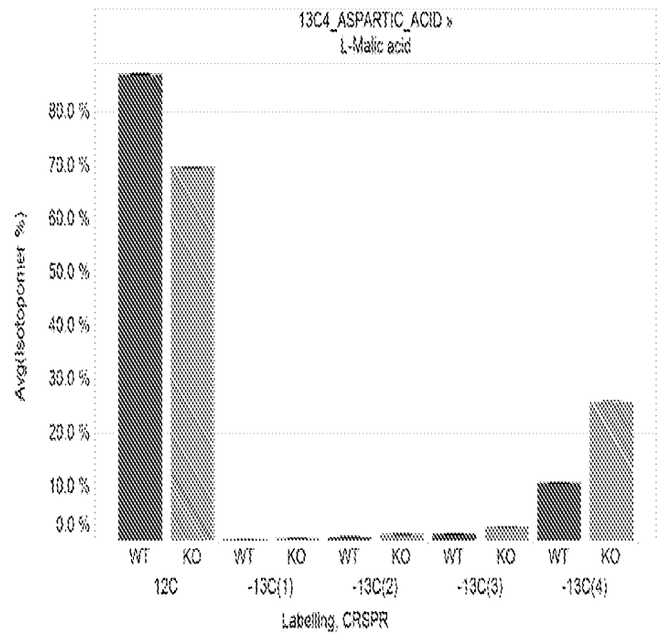
# Anaplerotic to TCA in OGDH independent cell lines—All metabolites were labeled via $^{13}C$



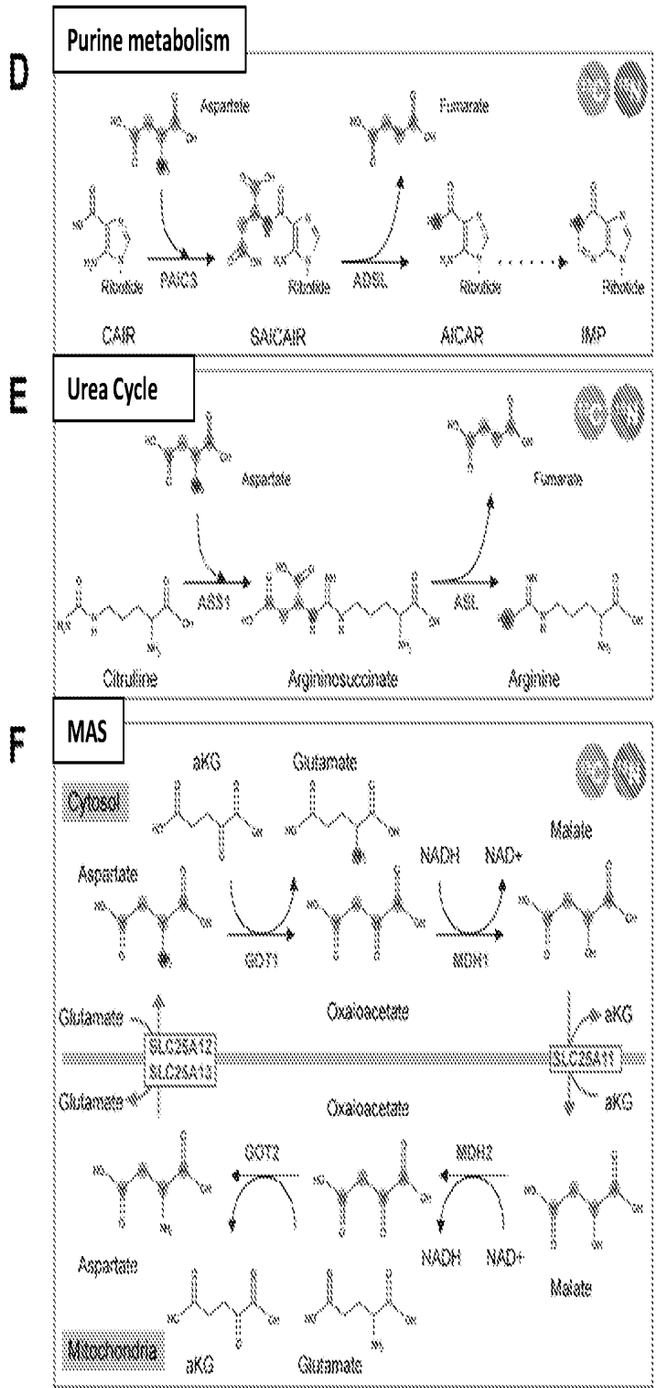



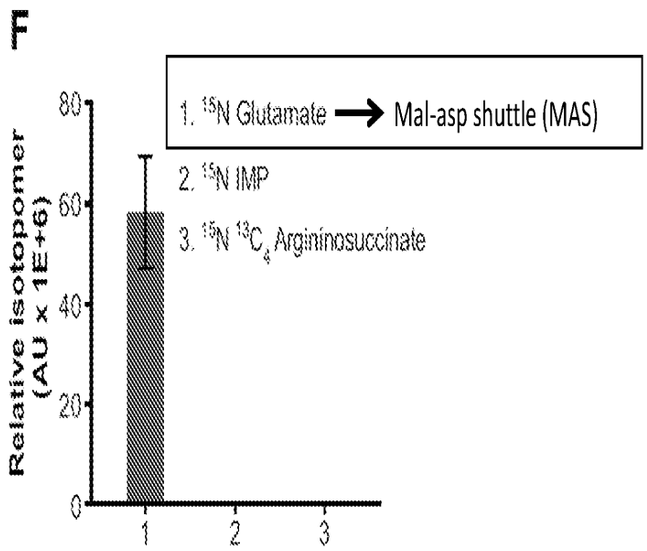



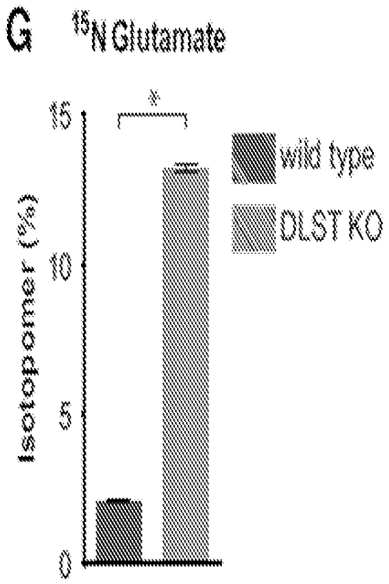

Identify differences between OGDH complex KO versus WT



# Multiple way for Aspartate to support anaplerosis



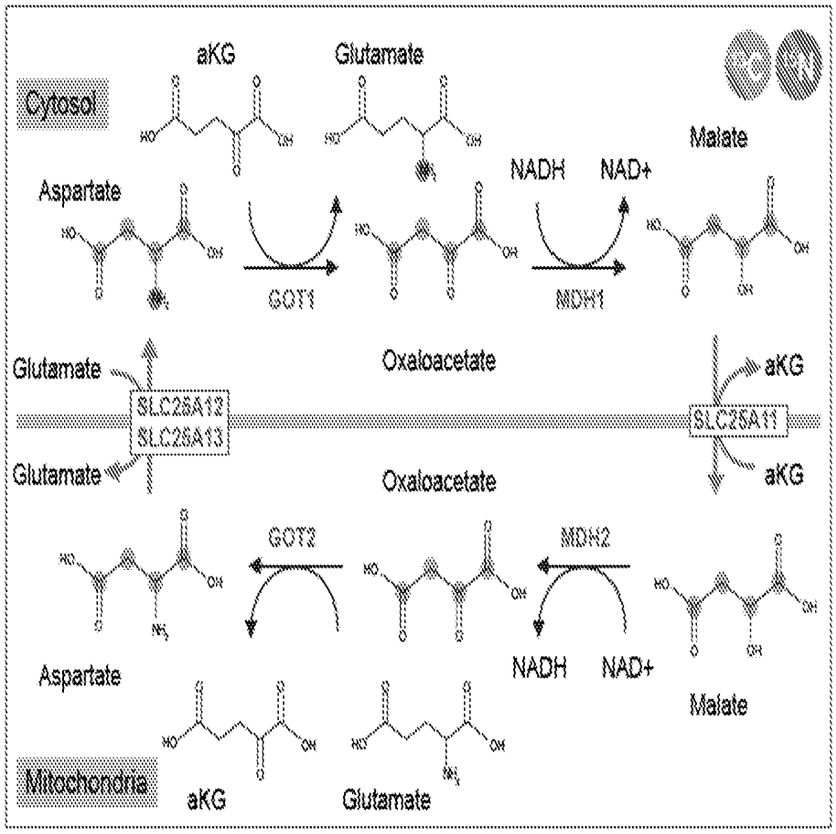



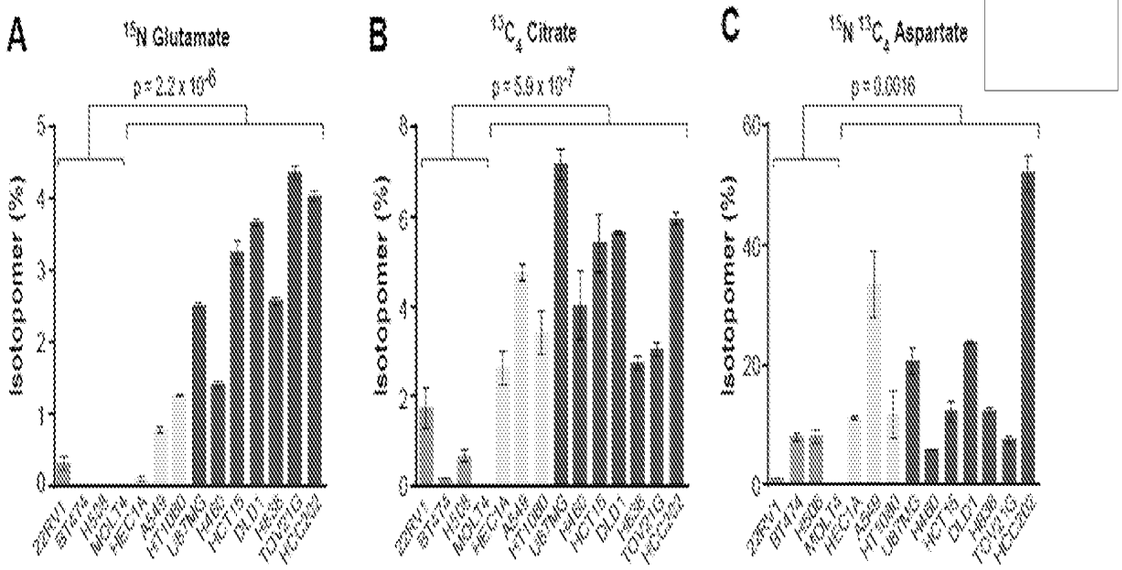




Allen et al. Cell Reports 2016;17:876–890

# Malate-aspartate shuttle activity predicts dependence on OGDH

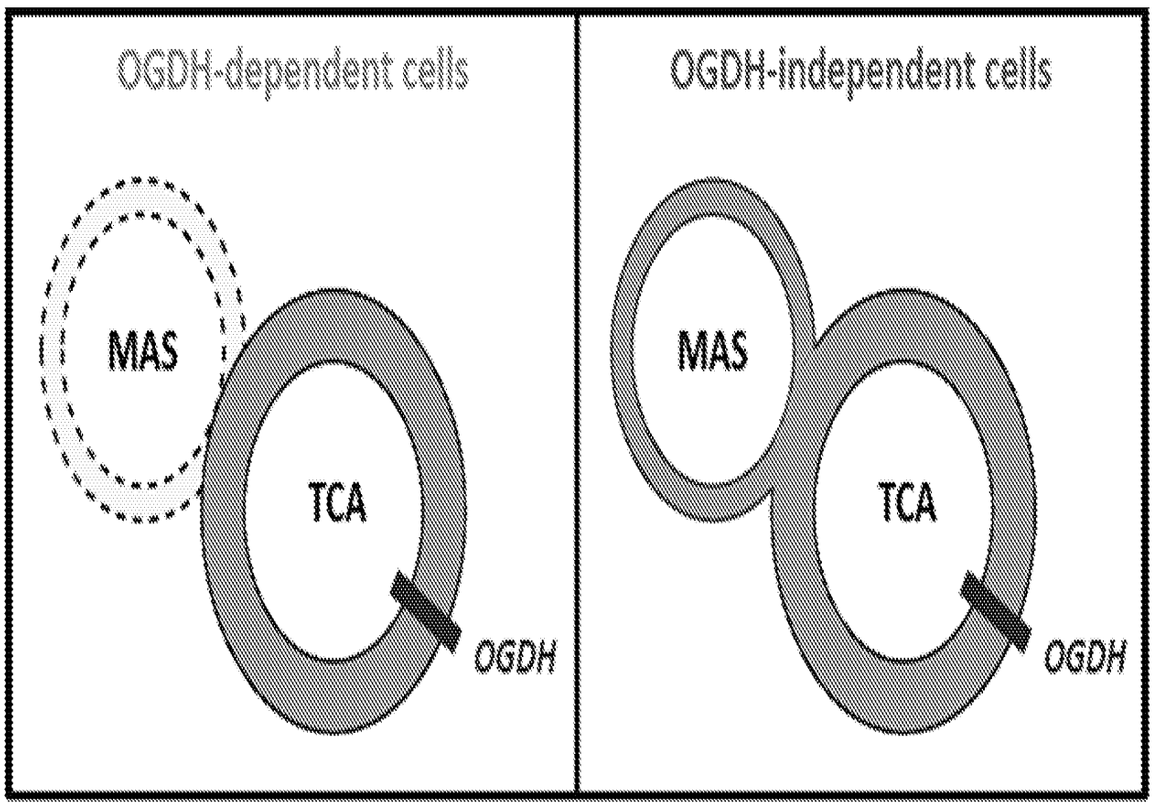
- Added $^{13}C_4$ $^{15}N$ aspartate to track mal-asp shuttle activity in cell line panel
- Confirmed differences between OGDH dependent vs independent cells



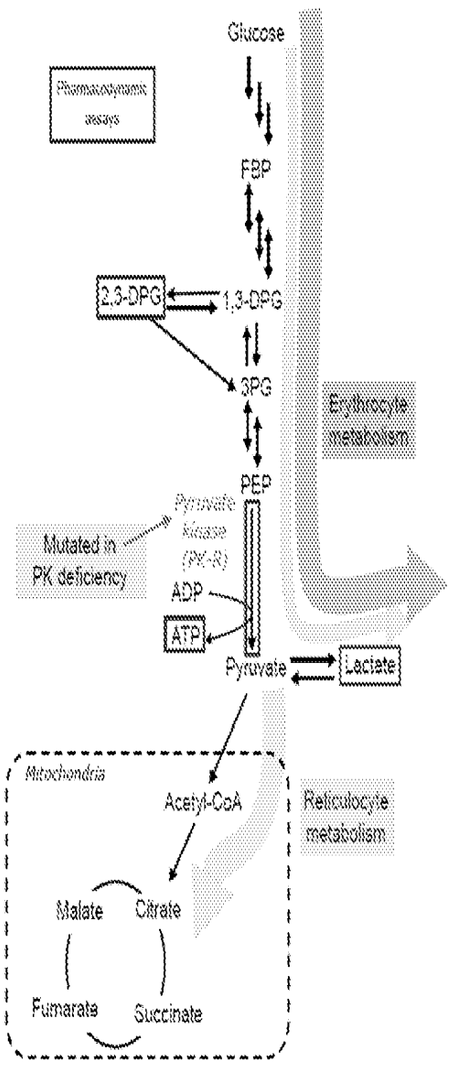

Allen et al. Cell Reports 2016;17:876–890

# Identified malate-aspartate shuttle differences—predicts OGDH sensitivity




Allen et al. Cell Reports 2016;17:876–890

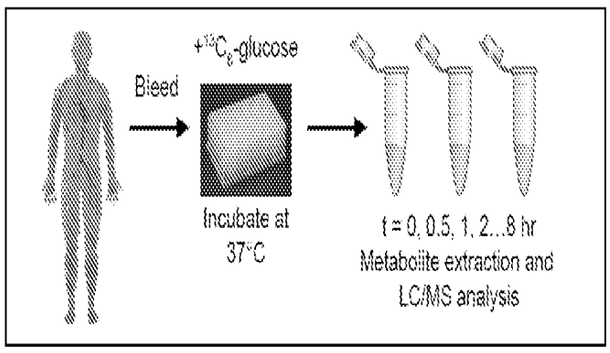
# In vivo Glycolytic flux measured in PK deficiency patients to support AG-348 clinical trial




- PK deficiency patients have destabilizing or inactivating mutations in PKR gene
- Leads to deficiency in ATP production and high rate of hemolysis of red blood cells
- AG-348, a direct activator of mutant PK-R, has completed Phase I in normal healthy volunteers
- Phase II study (DRIVE PK) underway

Chubukov et al. Presented at the 58th American Society of Hematology Annual Meeting and Exposition; 3-6 December 2016; San Diego, CA, USA
Elucidata for data Analysis

# Assay design to measure RBC glycolytic flux—Ex vivo $^{13}C_6$ glucose labeling

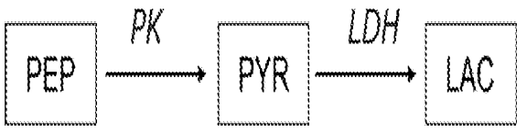



How do we measure flux through PK-R?

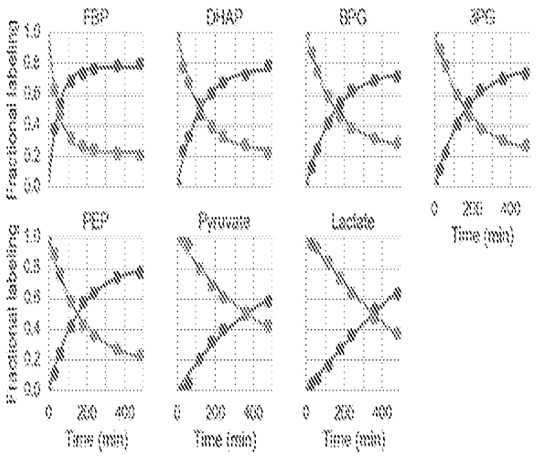
Assuming

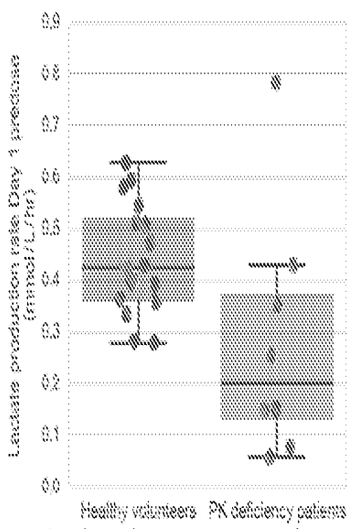
- no significant pyruvate accumulation
- no significant diversion of pyruvate to other pathways

The rate of lactate production should equal PK-R flux.

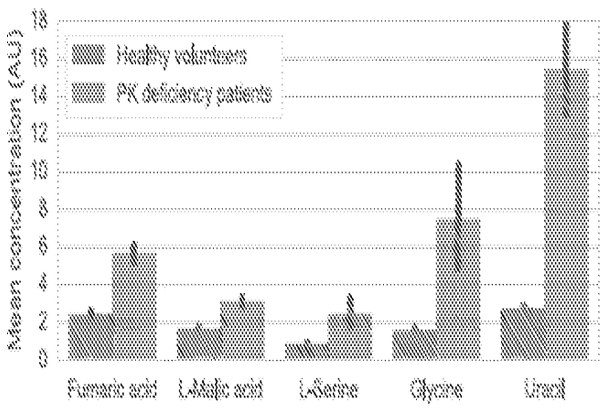



$F_{in} = F_{out}$



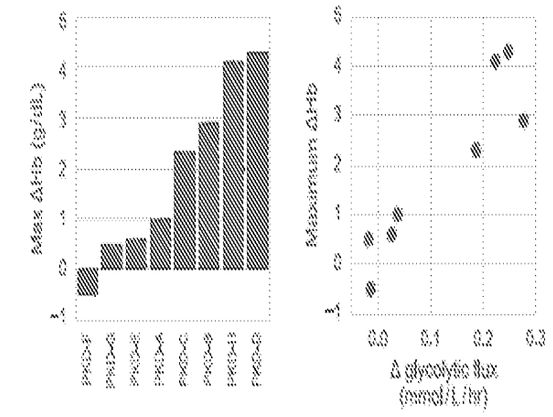

Indicative of mitochondrial metabolism from immature RBC (reticulocytes)

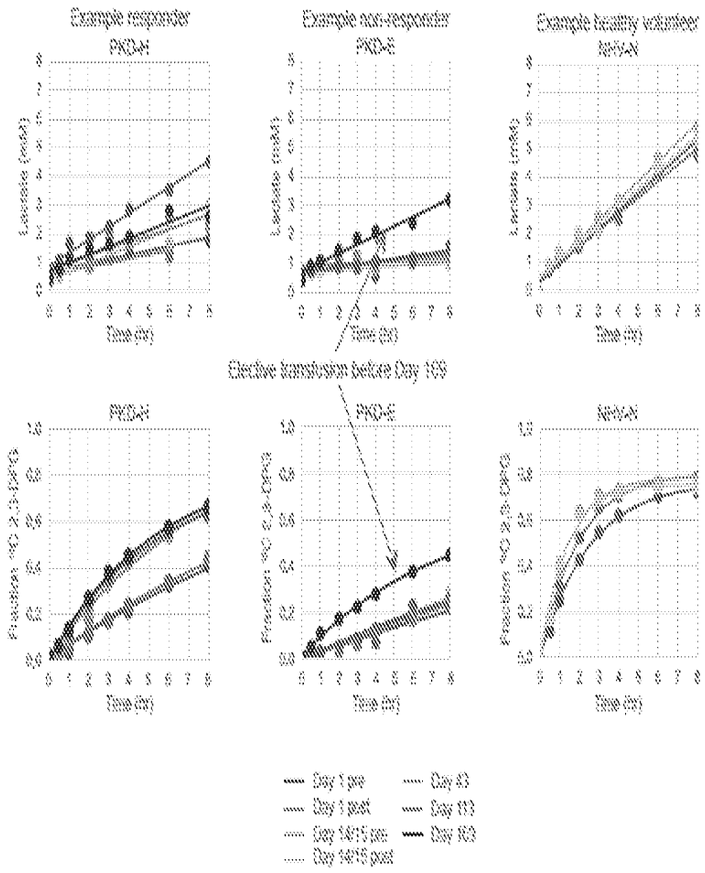


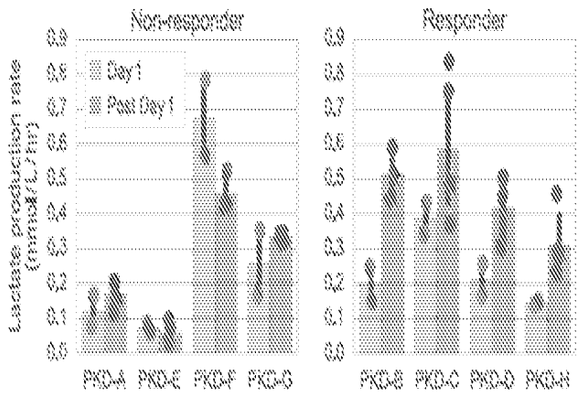

Chubukov et al. Presented at the 58th American Society of Hematology Annual Meeting and Exposition; 3-6 December 2016; San Diego, CA, USA

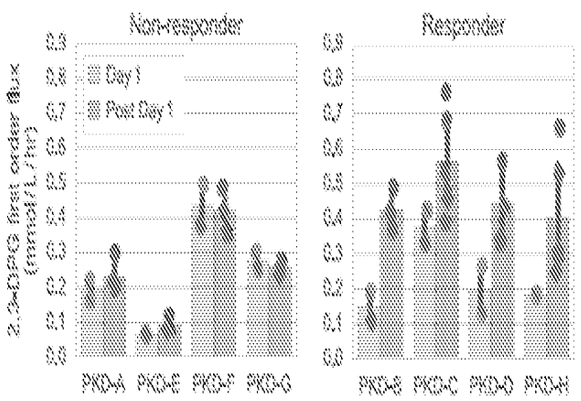
Elucidata for data analysis

# Increase in glycolytic flux correlates with increased Hb after treatment in responding patients



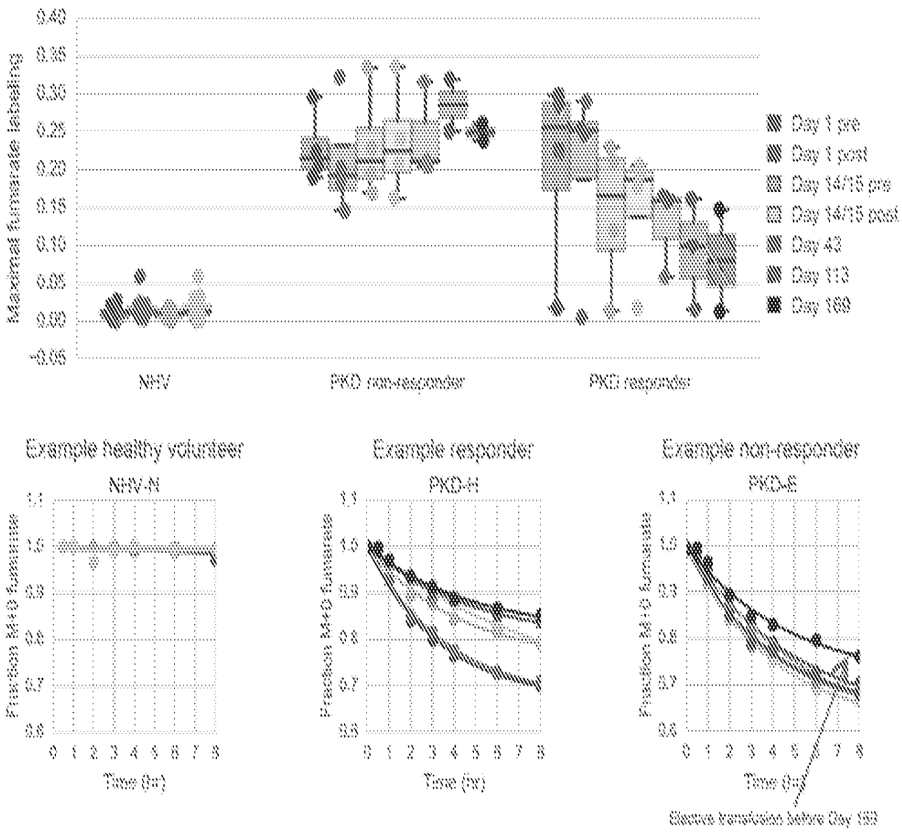




NHV = normal healthy volunteer; PDK-A to H = individualized PK deficiency patients

Of the eight PK deficiency patients undergoing extensive metabolic characterization, four had a maximal increase in hemoglobin of >1.0 g/dL. In those patients, we observed a >0.1 mmol/L/hr (>50%) increase in glycolytic rates. Additionally, we observed increased incorporation of $^{13}C$ into glycolytic intermediates such as 2,3-DPG, suggesting an increase in metabolically active erythrocytes. None of the four PK deficiency patients that did not have a hemoglobin increase of >1.0 g/dL showed significant metabolic changes.

Chubukov et al. Presented at the 58th American Society of Hematology Annual Meeting and Exposition; 3-6 December 2016; San Diego, CA, USA

# Decrease in mitochondrial metabolic markers in whole blood flux measurements in responding patients




- PK deficiency patients have higher levels of immature RBC (contain mitochondria)
- Decrease with treatment to improve RBC lifespan (increases in Hb and ATP)

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Elucidata for data analysis

# Conclusions

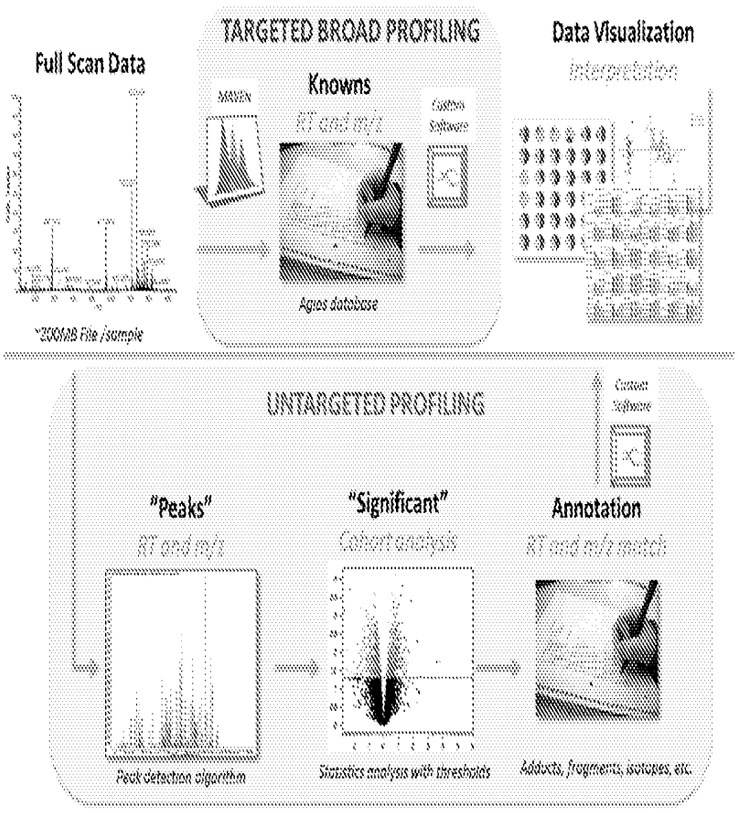
LCMS-based metabolomics is significantly improving our understanding of disease metabolism and metabolic targets

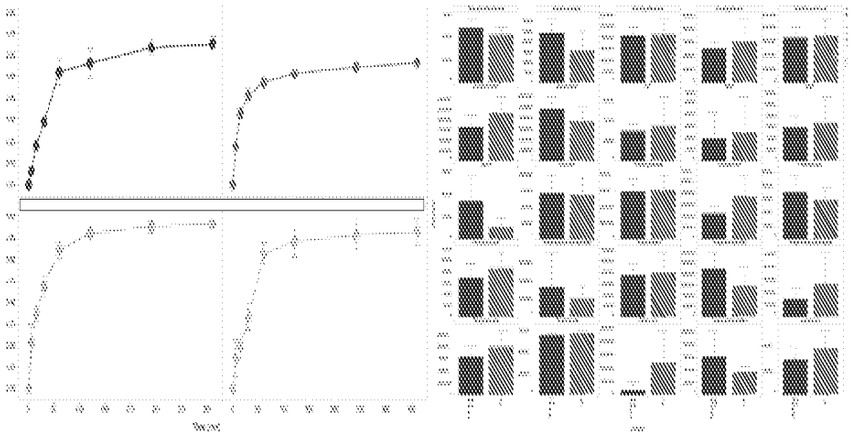
- Target validation and metabolic characterization
- Identify inherent cell metabolism differences to infer target dependency
- Metabolic characterization of disease
- Better understanding of metabolism in response to treatment—PD to efficacy correlations



# LC-HRMS based metabolomics platforms

- Three dedicated Qexactive for metabolomics and stable isotope labeling
- Full scan MS collection
  - Targeted list against internal library
  - Unbiased metabolite detection (metabolomics)
- Both methods combined with HRMS ~500 metabolites (+stable isotopes) detected
- Data analysis tools significantly reduce time for interpretation

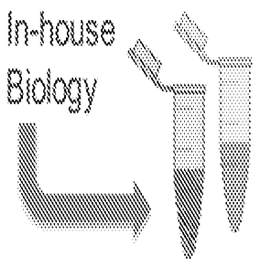


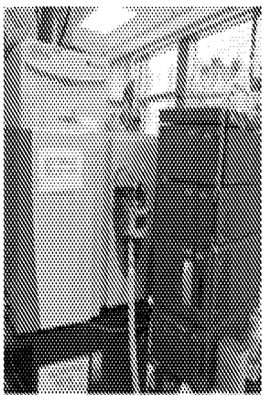
# LC-MS/MS based Lipidomics

Analytical platform

In-house Biology



Extract Lipids



HRMS+MS/MS (post-run)

LipidSearch

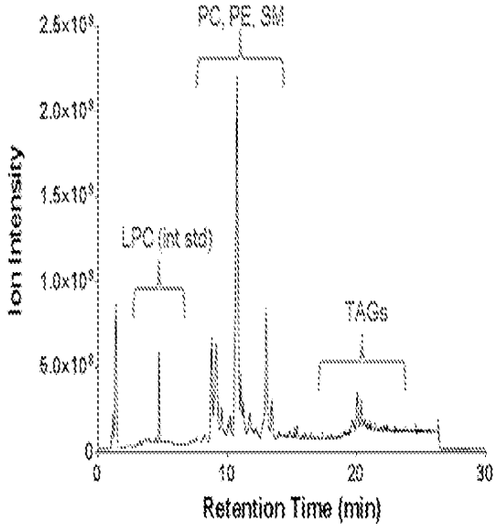
Lipids identify via MS/MS and annotated

Waters iClass Aquity w/ Thermo QExactive

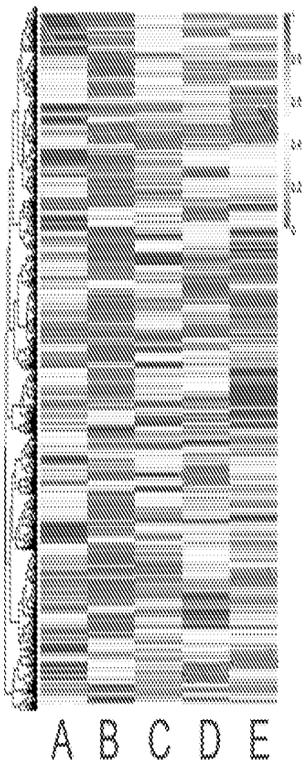
## Observed >800 lipids from biological samples

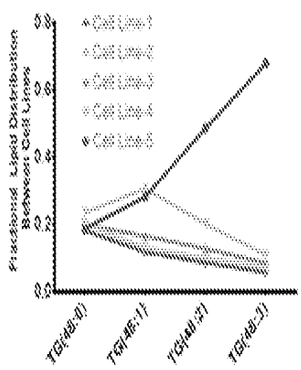
- Phosphoglycerolipids
- Glycerolipids
- Sphingolipids
- Cholesterol esters
- Free fatty acids

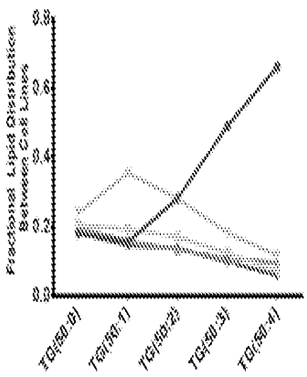
*Limited coverage on sterols, prenols, polyketides*




## Comprehensive data analysis/interpretation to identify lipid metabolic perturbations



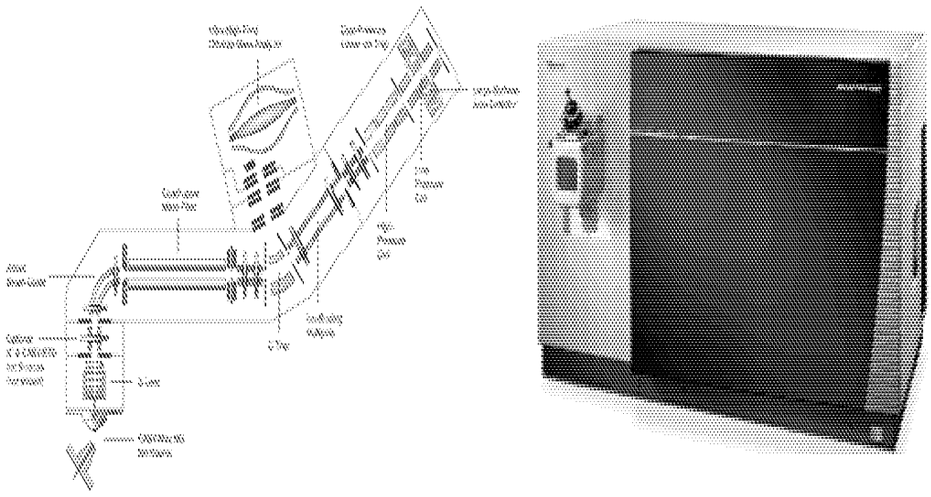



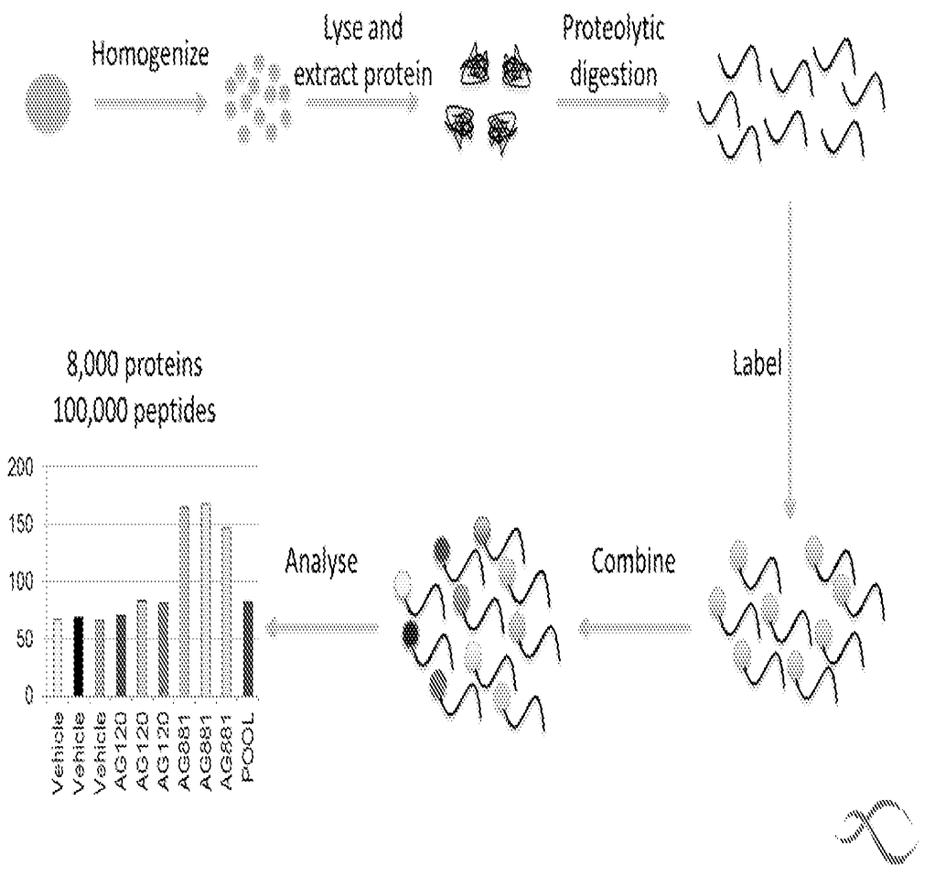




Agios Pharmaceuticals – CONFIDENTIAL

# Tandem Mass Tagged (TMT) Relative Quantitative Proteomics

- Thermo Orbitrap Fusion for TMT proteomics
- Compare protein levels across 10 samples in single analysis
- Typically >7000 proteins detected
- Average of >10 peptides/protein (varies)
- 48 hr analysis time





# ACTIVATE: An ongoing, phase 3, randomized, double-blind, placebo-controlled study with mitapivat (AG-348) in adults with pyruvate kinase deficiency who are not regularly transfused

Andreas Glenthøj[1], Malia P Judge[2], Lei Hua[2], Chris Mix[2], Eduard J van Beers[3]

[1]Danish Center for Hemoglobinopathies, Herlev and Gentofte Hospital, Herlev, Denmark; [2]Agios Pharmaceuticals, Inc., Cambridge, MA, USA; [3]Van Creveldkliniek, University Medical Center Utrecht, University of Utrecht, Utrecht, The Netherlands

## BACKGROUND

### PK deficiency: disease overview and current treatment options

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

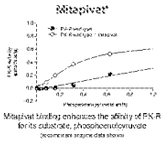
- Underrecognized hereditary disease
- Heterogeneous disease with variable severity among all ages
- Caused by mutations in the *PKLR* gene coding for red cell PK (PK-R), resulting in defective glycolysis and decreased red blood cell lifespan
- Lifelong hemolytic anemia
- Iron overload, jaundice, and 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**[2]



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

**Mitapivat**[4]




Mitapivat binding enhances the affinity of PK-R for its substrate, phosphoenolpyruvate
Sequential enzyme data shown

**PK-R tetramer**[4]



Active PK-R is a tetramer; mutations typically decrease the enzyme activity

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

### Pyruvate kinase deficiency

- Pyruvate kinase (PK) deficiency is a rare, congenital, hemolytic anemia (**Figure 1**).
- Mitapivat sulfate (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.[3,5]

### Mitapivat in PK deficiency

*DRIVE PK study (phase 2)*

- Phase 2, open-label, dose-ranging study (NCT02476916) in 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)[6]
  - Primary endpoint: safety and side-effect profile of mitapivat
  - Secondary endpoints: Hb, markers of hemolysis, pharmacokinetics, and pharmacodynamics
  - Patients 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.

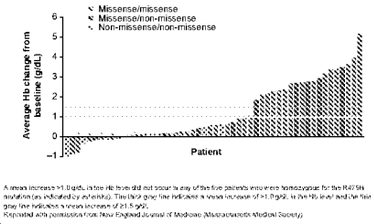
*Cumulative safety summary*

- Mitapivat was generally well tolerated during the core study and an ongoing extension as of data cutoff (August 31, 2018; median duration of 16.4 months [range, 3–35]).[6]
  - 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, hyperglyceridemia, left renal-cell carcinoma, and pleural effusion).
  - There were 18 serious AEs in 15 patients.

*Efficacy (core study)*

- 26 of 52 patients (50%) had an Hb increase from baseline of >1.0 g/dL (**Figure 2**).
  - 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 an Hb increase from baseline of >1.0 g/dL had at least one missense mutation.

### Hb response and *PKLR* genotype




A mean increase in Hb g/dL (blue line) Hb level did not occur in any of the five patients who were homozygous for the R479H mutation (as indicated by asterisks). The third gray line indicates a mean increase of +1.0 g/dL in the Hb level and the fifth gray line indicates a mean increase of +1.5 g/dL.
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## ACTIVATE STUDY (PHASE 3)

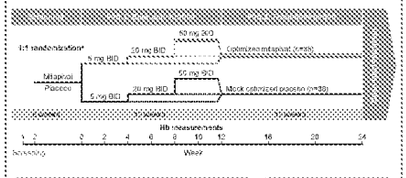
### Summary

- ACTIVATE is an ongoing, phase 3, multicenter, randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of mitapivat in adult patients with PK deficiency who are not regularly transfused (NCT03548220; **Figure 3**).

### Current status

- This phase 3 study in adult patients with PK deficiency is enrolling patients at sites in North America, South America, Europe, and Asia (**Figure 4** and **Table 1**).

### ACTIVATE study design




**Key inclusion criteria**

- ≥18 years of age
- Documented presence of at least two mutated alleles in *PKLR*, at least 1 of which is a missense mutation (based on genotype–PK response correlations in DRIVE PK; see **Figure 2**)
- Hb concentration ≤10.0 g/dL, documented at each of 2 consecutive visits in the 12-month period up to the first day of study treatment, and no transfusions in the 3 months prior to the first dose
- Adequate organ function

**Key exclusion criteria**

- Pathogenic 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

*Defined by average of screening Hb values (<8.5 g/dL or ≥8.5 g/dL) and mutation category (missense/missense vs missense/non-missense)

### Part 1: Individualized dose optimization period

- Eligible patients are randomized 1:1 to mitapivat or matching placebo.
- Maximal Hb increases are sought within an acceptable safety profile.
- Following safety assessment the dose can be increased from 5 to 20 mg BID and, subsequently, from 20 to 50 mg BID to improve Hb response.

### Part 2: Fixed-dose period

- Patients receive mitapivat or matching placebo at their optimized dose (as prescribed at Week 12 visit), with no planned adjustment for the subsequent 12 weeks.
- Dose can be reduced or suspended for safety reasons throughout the study.

### Extension study

- Patients completing the study may have the opportunity to enroll in an ongoing open-label extension study (AG348-C-011; EudraCT 2018-003459-39 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 or its companion study, ACTIVATE-T.

### Key endpoints

*Primary*

- Hb response, defined as a ≥1.5 g/dL (0.93 mmol/L) increase from baseline in Hb concentration that is sustained at two or more scheduled assessments at Weeks 16, 20, and 24 during the fixed-dose period

*Key secondary*

- Average change from baseline in Hb concentration at Weeks 16, 20, and 24.

*Other secondary*

- Maximal Hb concentration increase from baseline.
- Time to achieve an increase from baseline in Hb concentration of ≥1.5 g/dL (0.93 mmol/L).
- Average change from baseline at Weeks 16, 20, and 24 in markers of hemolysis: bilirubin, lactate dehydrogenase, and haptoglobin levels.
- Average change from baseline at Weeks 16, 20, and 24 in markers of hematopoietic activity: reticulocyte percentages.
- Change from baseline in patient-reported, health-related, quality-of-life scores.
- The type, incidence, severity, and relationship to study treatment of AEs and serious AEs, and number of discontinuations due to AEs.
- Plasma concentrations over time and pharmacokinetic parameters of mitapivat.
- Exposure-response relationship between safety parameters and mitapivat concentration and relevant mitapivat pharmacokinetic parameters.

### Statistics

- Assuming a response rate of 35% in the active arm and 5% in the placebo arm, 76 patients (38 per arm) are needed to have 90% power to detect a treatment effect in Hb response rate, on the basis of a two-sided Fisher's exact test at the 0.05 significance level.
- Patients' Hb response status will be analyzed using a logistic regression model that will include Hb response (yes vs no) as a dependent variable and treatment as an independent variable, adjusting for stratification factors, including screening Hb concentrations (<8.5 vs ≥8.5 g/dL) and *PKLR* mutation category (missense/missense vs missense/non-missense).

### Study sites



Table 1. Planned and active sites as of October 2019

| Country | Participating Sites |
|---|---|
| Brazil | Universidade de Campinas, São Paulo; Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo |
| Canada | Toronto General Hospital, ON; Hamilton Health Sciences/McMaster University, Hamilton, ON |
| Czech Republic | Ústav Hematologie a Krevní Transfuze, Prague |
| Denmark | Herlev and Gentofte University Hospital |
| France | AP-HP – Hôpital Henri Mondor, Créteil; CHU Amiens – Hôpital Sud; AP-HM – Hôpital de la Timone, Marseille; CHU de Bordeaux – Hôpital Saint André; IUCT – Institute Universitaire du Cancer de Toulouse |
| Germany | Universitätsklinikum Würzburg; Charité Universitätsmedizin, Berlin |
| Italy | Ospedali Galliera di Genova; A.O.U. Seconda Università Degli Studi di Napoli; IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano |
| Japan | Osaka City Hospital Organization; Kansai Medical University, Osaka; Tohoku University Hospital, Sendai; Mie University Hospital, Tsu |
| South Korea | Yeungnam University Hospital, Gyeongsan |
| Netherlands | University Medical Centre Utrecht |
| Spain | Hospital Universitario La Paz, Madrid; Hospital Universitario Vall d'Hebron, Barcelona; Hospital de la Santa Creu i Sant Pau, Barcelona; Hospital Universitario Virgen de la Arrixaca, Murcia |
| Switzerland | Centre Hospitalier Universitaire Vaudois, Lausanne |
| Thailand | Faculty of Medicine Siriraj Hospital – Mahidol University, Bangkok |
| Turkey | Hacettepe Universitesi, Ankara |
| UK | Imperial College Healthcare NHS Trust, London; University College London Hospitals NHS Foundation Trust, Addenbrooke's Hospital, Cambridge; Manchester Royal Infirmary; Royal Liverpool University Hospital |
| US | California State University, East Bay, Hayward, CA; Massachusetts General Hospital, Boston, MA; Duke University, Durham, NC; Children's Healthcare of Atlanta / Emory University, Atlanta, GA; Primary Children's Hospital / University of Utah, Salt Lake City, UT; Wayne State University School of Medicine / Children's Hospital of Michigan, Detroit, MI; Indiana Hemophilia and Thrombosis Center, Indianapolis, IN; Boston Children's Hospital, Boston, MA; University of Arkansas for Medical Sciences, Little Rock, AR; Houston Methodist Research Institute, Houston, TX; University of Washington, Seattle, WA; Cincinnati Children's Hospital Medical Center, Cincinnati, OH; Phoenix Children's Hospital, Phoenix, AZ |

**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. AG: no conflict of interest to disclose. MPJ, LH, and CM: Agios – employment and stockholder. EJvB: Agios – research funding and advisory board member. Editorial assistance was provided by Mark Porter, Excel Medical Affairs, Fairfield, CT, USA, and supported by Agios. **References** **1.** Grace RF et al. *Am J Hematol* 2015;90:825-36. **2.** Pissey MJ et al. *Blood Cells Mol Dis* 2007;39:159-64. **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;8:246-59. **6.** Grace RF et al. *N Engl J Med* 2019;381:933-44.

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

Presented at the European Hematology Association Scientific Working Group (EHA-SWG) Scientific Meeting on Red Cell and Iron Metabolism Defects, November 7–8, 2019, Madrid, Spain

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Ex. 2001-0511

# Effects of AG-348, a Pyruvate Kinase Activator, on Anemia and Hemolysis 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 Galacteros[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]Haemaprotein Haegetic, Imperial College Healthcare NHS Trust, London, United Kingdom; [3]Hôpital Saint Vincent de Paul, Lille, France; [4]Central Pennsylvania Clinic, Bellefonte, 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]MEC 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

# PK deficiency and the role of AG-348: an allosteric activator of pyruvate kinase R

**Description**

- Presents at any time of life, as early as the neonatal period with severe hemolytic anemia
- Estimated prevalence ranges from ~1:20K to ~1:485K[1–4]

**Etiology**

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

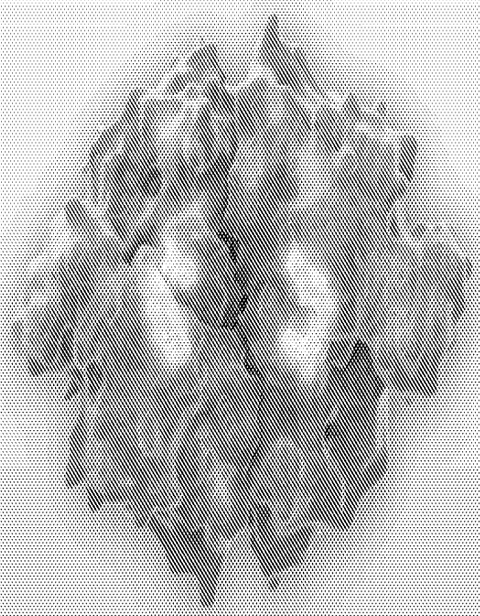
**Disease Burden**

- 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

**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**

Please see Posters 1263, 1264 and 2452 at the current meeting for more information on PK-R activators

Grace R et al. *Am J Hematol* 2015;90(9):825-30. [2] Mohrenweiser HW. *PNAS* 1981;78(8):5046-50. [3] Carey PJ et al. *Blood* 2010;46(12):2065-8. [4] Bainer E & Galeon T. *Blood* 2003;95(11):3585-8. [5] Valentine et al. *Hum Hered* 1992;42(6):179-83.



Ex. 2001-0513

# Study design

DRIVEPK

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



Ex. 2001-0514

# 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 |
|---|---|---|---|
| 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 |

[a]Not reported in 2 patients, 2 patients were Asian; AE = adverse event; SD = standard deviation



Ex. 2001-0515

# 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; 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=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 |
| 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 anemia, not a serious AE. [b]Grade 3 withdrawal hemolysis and anemia 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 = hemoglobin; DXA = Dual energy X-ray absorptiometry



Ex. 2001-0516

# Effect of AG-348 on hormones

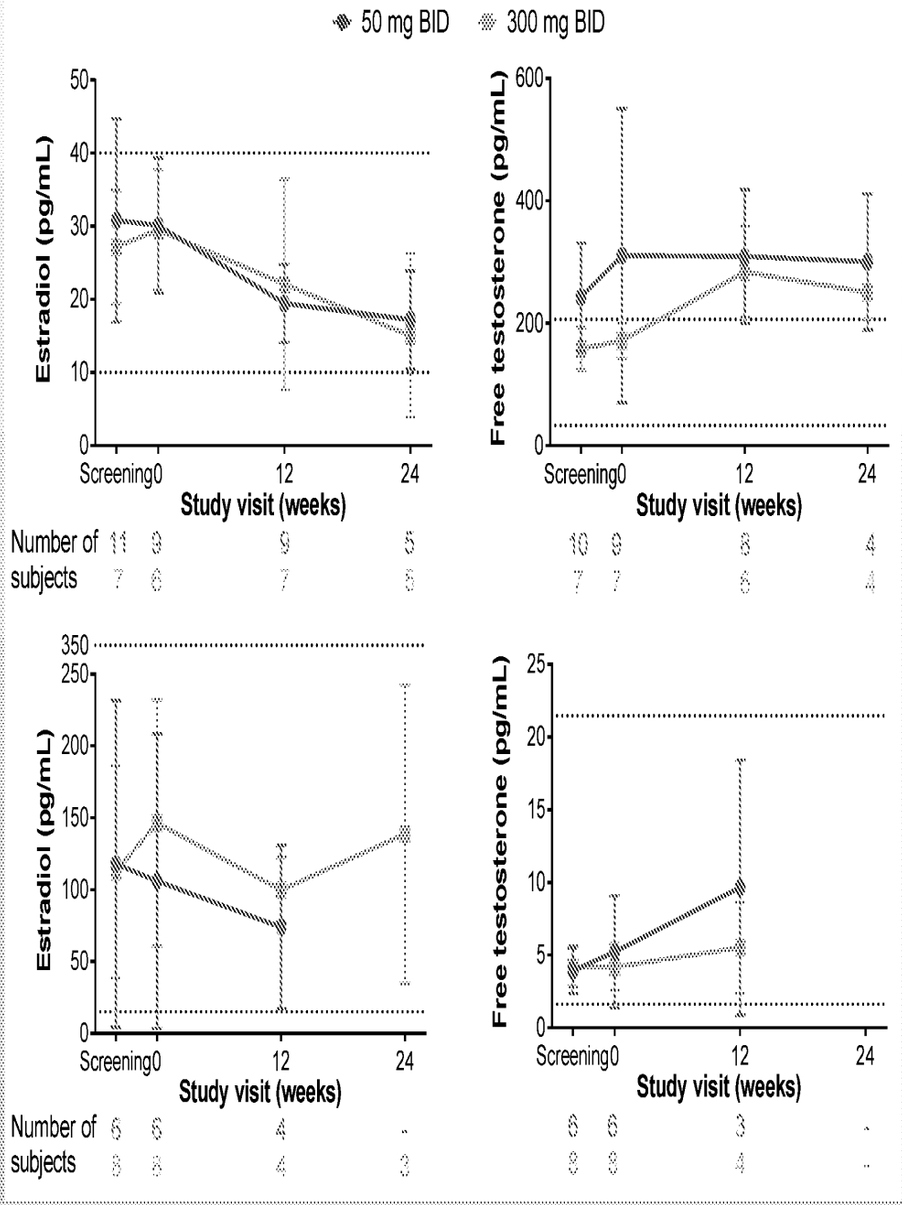
- Preliminary findings are consistent with aromatase inhibition by AG-348

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

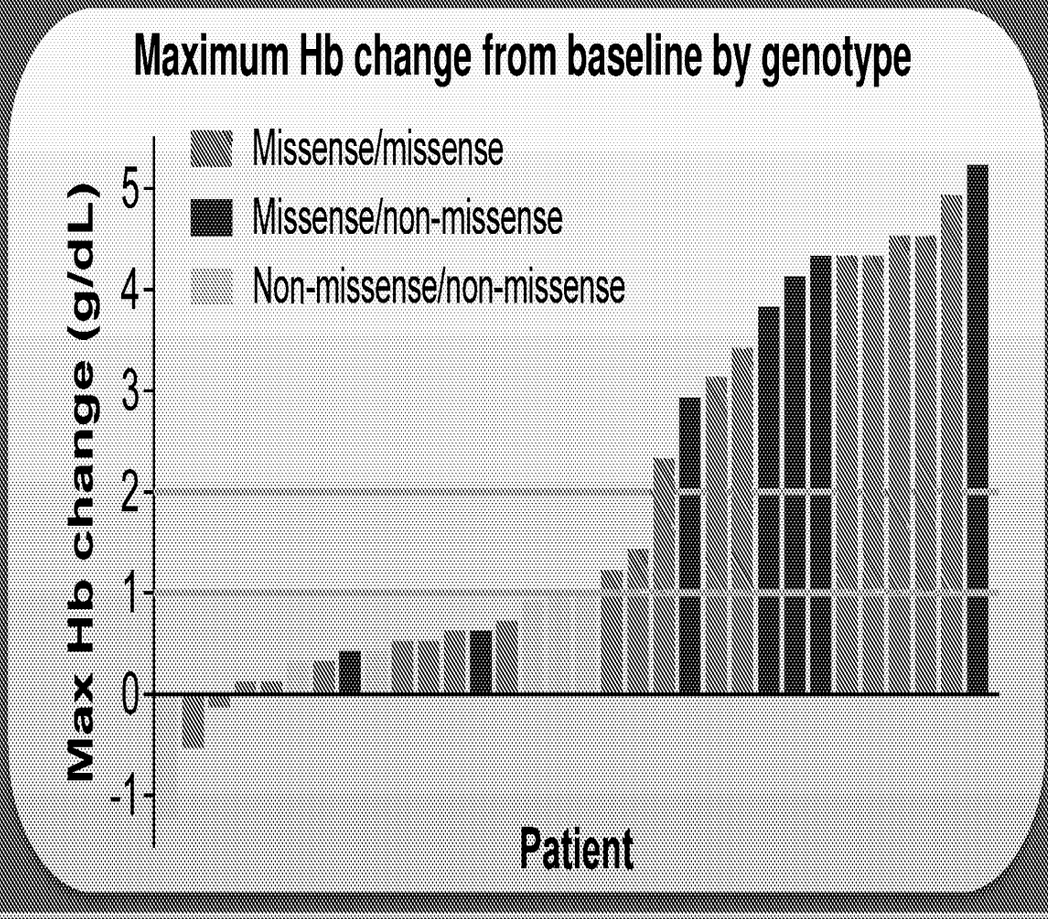


Ex. 2001-0517

# Clinical Activity Results

# Maximum 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 4 doses
  - Robust Hb responses led to dose decreases with maintained Hb



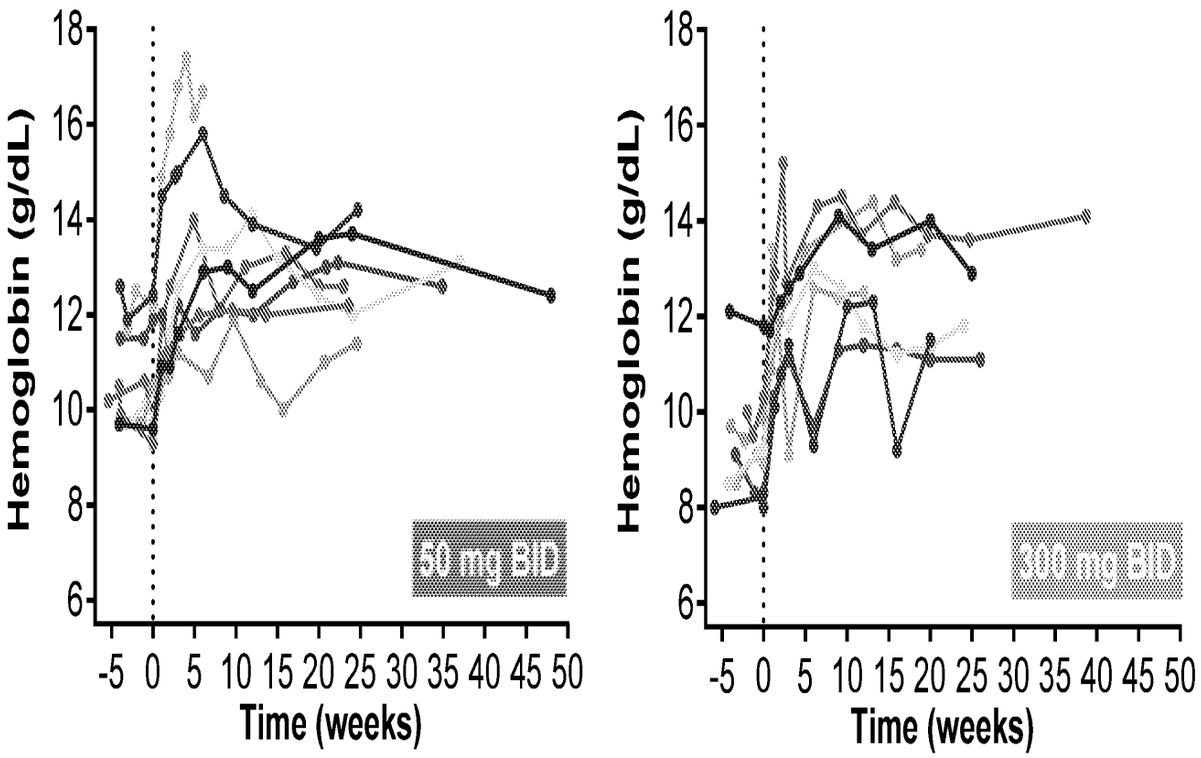

Evaluable analysis set; ≥3 weeks of data



Ex. 2001-0519

# 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]



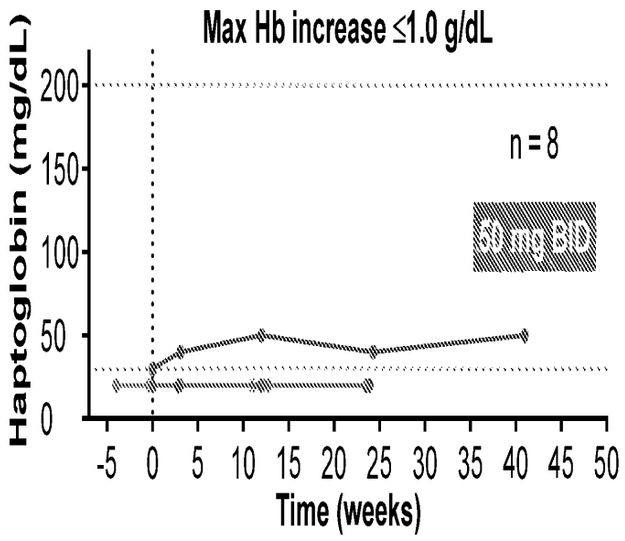

Responders n=15, each line represents an individual patient

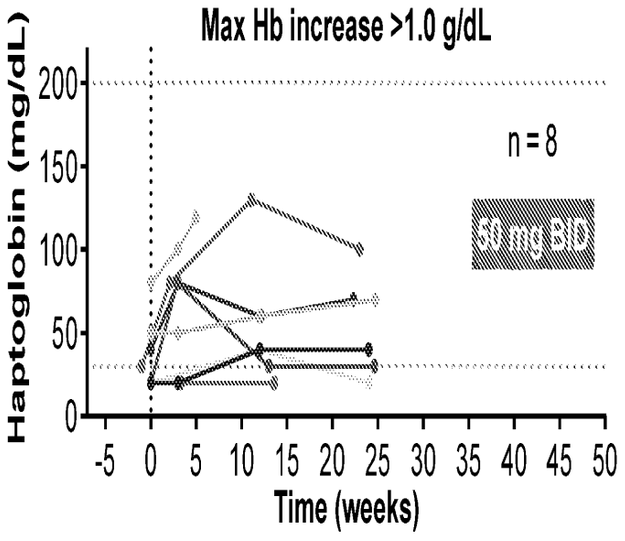
[a]Other dose reductions due to: AEs (n=3), self-reduction due to fatigue (n=1), taper prior to discontinuation (n=1)

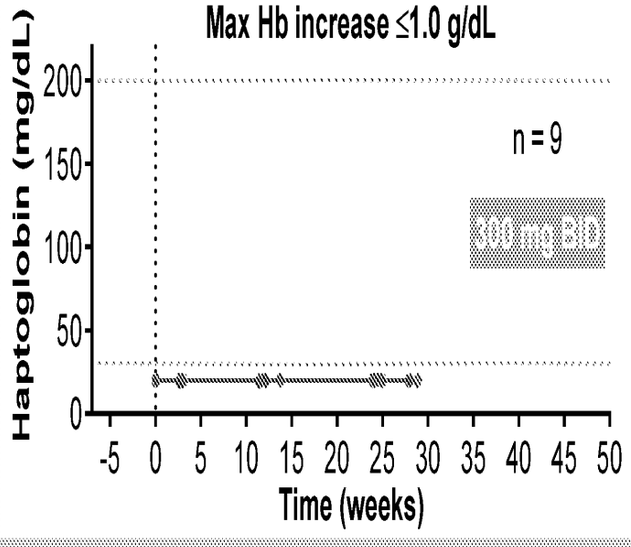


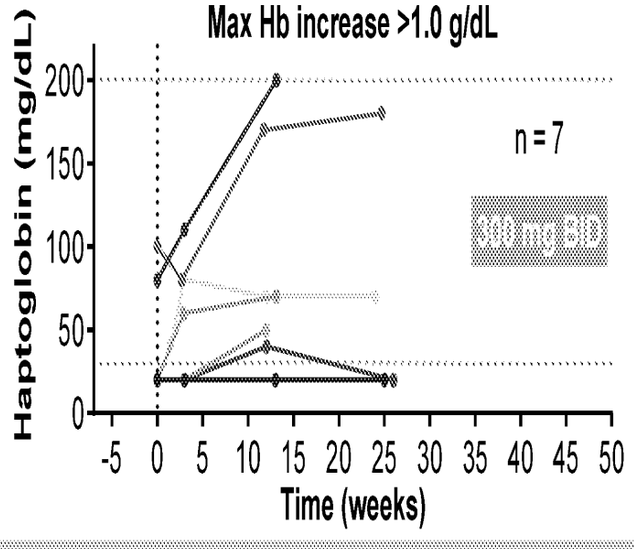
Ex. 2001-0520

# Haptoglobin levels increase in responders, indicating decreased hemolysis




Normal reference low and high limits shown as horizontal dotted lines



Ex. 2001-0521

# Pharmacodynamic Results



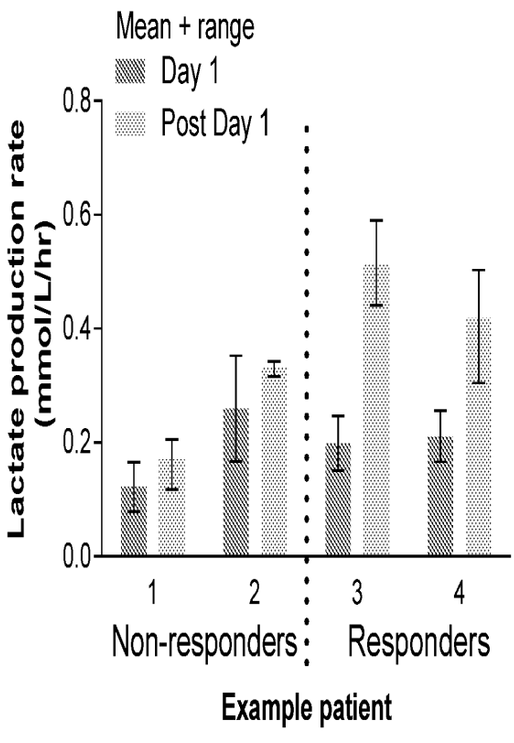
Ex. 2001-0522

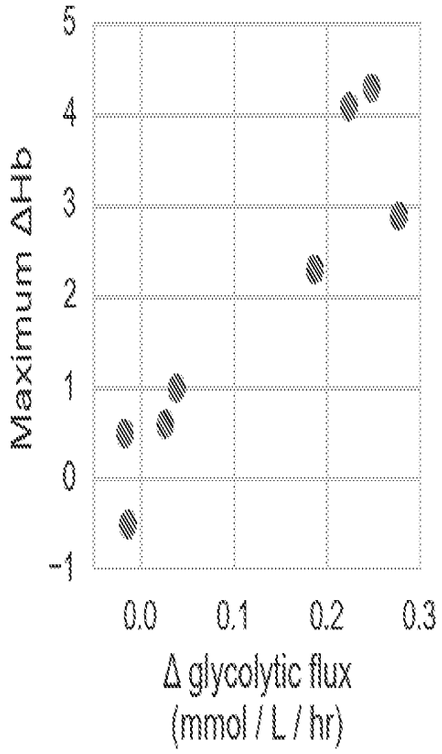
# Patients with Hb increases also had increased rate of metabolism in PK-R pathway in peripheral blood

- 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



Ex. 2001-0523

# DRIVE-PK conclusions

- 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
- These data highlight the potential of PK-R activators as the first disease-altering treatment for patients with PK deficiency



Ex. 2001-0524

# Acknowledgments


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- 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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- This clinical study was funded by Agios Pharmaceuticals

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




Ex. 2001-0525

# Effects of AG-348, a Pyruvate Kinase Activator, on Anemia and Hemolysis in Patients with Pyruvate Kinase Deficiency: Data from the DRIVE PK Study

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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 wild-type and a range of mutated PK-R enzymes *in vitro*, and increases PK-R activity and restores adenosine triphosphate (ATP) levels in red blood cells from patients with PK deficiency *ex vivo*.

AIMS

To describe data from the ongoing DRIVE PK study (NCT02476916), an open-label, dose-ranging trial of AG-348 in transfusion-independent adults with PK deficiency. Early data from this study have been reported (Grace et al. EHA 2016, S466).

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. Sex hormone levels and iron status are evaluated at Baseline, Week 12 and End of Study.

RESULTS

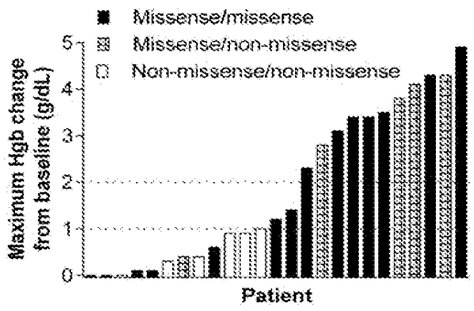
As of the data cutoff date of June 20, 2016, 25 patients have been enrolled and treated for ≥3 weeks.

Adverse events (AEs) were mild to moderate. The most frequent AEs were nausea (n=11/25), headache, and insomnia (each n=8/25). One serious AE (Grade 2 osteoporosis) has been reported. Grade 3 AEs were hypertension (n=1), hypertriglyceridemia (n=1), insomnia (n=2), and anemia (n=1). One patient was discontinued from treatment because anemia worsened. There were no Grade 4 AEs and no deaths. Three patients had dose reductions because of headache, nausea, or insomnia. Serum levels of sex steroids were measured at baseline, 12 and 24 weeks; increases in free and total testosterone, and decreases in estradiol and estrone, indicate aromatase inhibition by AG-348, consistent with previously reported results.

In 25 patients with ≥3 weeks on treatment, 13 patients (52%) showed a hemoglobin (Hgb) increase >1.0 g/dL (range: 1.2–4.9 g/dL; median: 3.40 g/dL). Three patients had dose reductions because of Hgb values exceeding the protocol-mandated maximum. Hgb responses were typically observed within 2 weeks of treatment and were stable with continued treatment. Two patients had dose increases because of insufficient response to 50 mg.

Maximal change in Hgb from baseline according to genotype is shown in Figure 1. Of the 25 enrolled patients with ≥3 weeks on treatment, 6 (24%) had two non-missense mutations and none of these had an Hgb response >1.0 g/dL. Of the 19 patients with at least one missense mutation, 13 (68%) had an Hgb increase exceeding 1.0 g/dL.

Figure 1. Maximum hemoglobin (Hgb) increase by genotype




CONCLUSION

AG-348 is a novel, first-in-class, PK-R activator in clinical testing as a disease-altering therapy to improve anemia in patients with PK deficiency. The ongoing DRIVE PK study has now enrolled over 25 patients, and data from additional patients will be available at the time of presentation. Daily dosing with AG-348 is well tolerated and has demonstrated clinically relevant durable increases in Hgb in the majority of the patients with at least 1 missense mutation. Out of the 19 patients with at least one missense mutation, 13 had an Hgb increase of >1.0 g/dL. These data highlight the potential of AG-348 as the first disease-altering treatment for patients with PK deficiency.



Ex. 2001-0526

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

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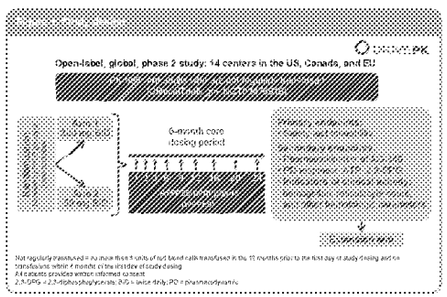
## BACKGROUND

- Pyruvate kinase (PK) deficiency is an under-recognized hereditary disease caused by mutations in the *PKLR* gene, which results in lifelong hemolytic anemia.[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




- Enrollment 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 analyzed 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 hemoglobin (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

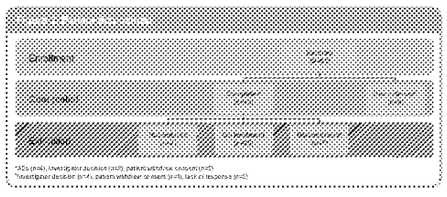



Table 1. Demographic characteristics

| Characteristic | 50 mg BID (n=18) | 300 mg BID (n=34) | Total (N=52) |
|---|---|---|---|
| Male, n (%) | 13 (68.7) | 14 (38.0) | 32 (61.5) |
| Age at randomization, median (range), years | 29 (18–58) | 40 (21–61) | 34 (18–61) |
| White*, n (%) | 22 (51.5) | 21 (54.0) | 43 (52.7) |
| Hb baseline, median (range), g/dL | 9.6 (6.9–12.5) | 8.6 (6.5–12.0) | 8.9 (6.5–12.5) |
| Splenectomy, n (%) | 23 (55.2) | 20 (50.0) | 43 (52.7) |
| Cholecystectomy, n (%) | 19 (70.4) | 19 (79.0) | 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 enrollment, n (%) | 14 (51.9) | 11 (44.0) | 25 (48.1) |
| Duration of AG-348 treatment, median (range), weeks | 34.6 (13.0–92.4) | 30.7 (12.9–86.4) | 37.5 (12.9–92.4) |

*Other races not reported (n=3), Asian (n=2), 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):
  - Hemolytic anemia, hypertriglyceridemia, pharyngitis/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.

Table 2. Most common AEs regardless of causality or grade (occurring in ≥15% of patients)

| | 50 mg BID (n=18) | | 300 mg BID (n=34) | | Total (N=52) | |
|---|---|---|---|---|---|---|
| | All grades n (%) | Grade ≥3 n (%) | All grades n (%) | Grade ≥3 n (%) | All grades n (%) | Grade ≥3 n (%) |
| Patients experiencing ≥1 AE, n (%) | 25 (86.2) | 8 (28.6) | 26 (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 | 5 (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 | 3 (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), myocardial infarction (n=1), pharyngitis (n=1), hypertriglyceridemia (n=1), hemolytic anemia (n=2), hemolysis (n=1), protein calculi in renal pelvis (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.

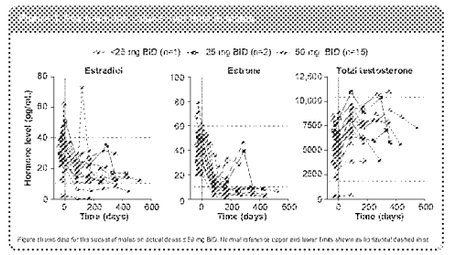
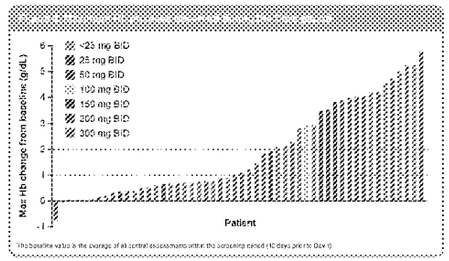



Figure shows data for the subset of males on active dose ≤50 mg BID. Normal reference upper and lower limits shown as horizontal dashed lines

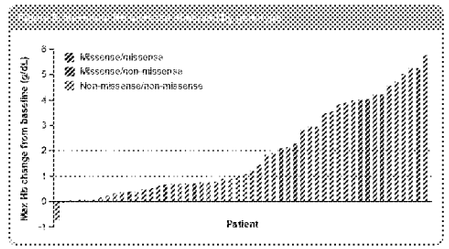
### Clinical activity

- 28 of 52 (53.8%) 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).

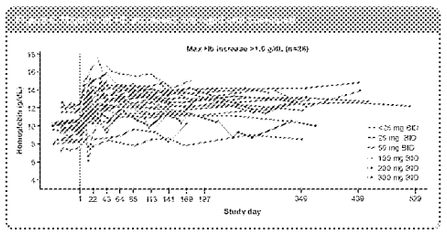



The baseline value is the average of all central assessments within the screening period (42 days prior to Day 0)

- 25 of 42 (59.5%) patients who had ≥1 missense mutation had an Hb increase >1.0 g/dL.



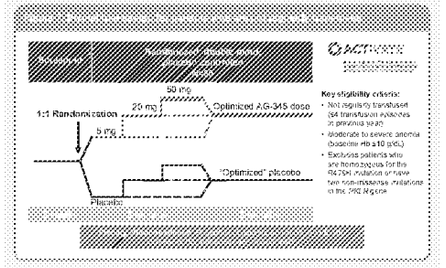

- Median time to the first observation of an Hb increase >1.0 g/dL above baseline was 10 days (range 7–187 days).
  - Median baseline Hb in patients who experienced a maximum Hb increase of >1.0 g/dL was 9.7 g/dL (range 7.3–12.3 g/dL) versus 8.6 g/dL (range 6.5–10.1 g/dL) in patients who did not.
- In 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.
  - 28 of 52 (59%) 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 starting in the first half of 2018:

ACTIVATE N=80 ACTIVATE-T N=20




### Acknowledgements

We would like to thank the patients who agreed to participate in this study, and Dr Kitty Swisher and David Nathan for helpful discussions.

### Disclosures

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RFG: Agios – advisory committee, research funding; DML: Agios – consultancy, speaker bureau; chair – UU PK; Pharvaris; defunct; FG: Agios – consultancy; CR: Agios – advisory committee; WB: Agios – honoraria, research funding; Alexion – honoraria; Novartis – honoraria; DHM: Agios – consultancy; KK: Novartis, Pfizer, GlaxoSmithKline, CSL Behring – consultancy, advisory committee; Shire – consultancy, advisory committee; SS: Agios – study investigator; ARIAD, Astellas – consultancy; JLK: Agios – consultancy, honoraria, research funding; Celgene, Novartis – consultancy; BB: Bluebird bio – employment, stock ownership; CX, VI, HY, PAT, LH, AB, DC: Agios – employment, stock ownership; EvB: Agios – consultancy; CR, RU, RSS, LH: Agios – employment; Roche/dRe, VP: Agios – employment; Shire/Baxter, Novartis – stockholder; RU: Agios – employment, stockholder at time of study; WB: Agios – advisory committee; CR, DHM – no relevant financial relationships to disclose.

Editorial assistance was provided by Helen Yorke, PhD, Excel Scientific Solutions, Horsham, UK, and supported by Agios.

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Scan code to access PDF file of this poster or visit http://bit.ly/2vUIEc1

Presented at the 59th American Society of Hematology (ASH) Annual Meeting, December 9–12, 2017, Atlanta, GA, USA

Ex. 2001-0527

# Results Update from the DRIVE PK Study: Effects of AG-348, a Pyruvate Kinase Activator, in Patients with Pyruvate Kinase Deficiency

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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). Current treatments for PK deficiency are supportive only. 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 in red blood cells from patients with PK deficiency *ex vivo* (Kung C et al. 2017 *Blood* in press).

AIM: To report updated data from the ongoing DRIVE PK study (ClinicalTrials.gov NCT02476916), an open-label, dose-ranging trial of AG-348 in nontransfusion-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; Core Period completers may enter a 2-year Extension Period. Dose modifications were allowed. For this study, nontransfusion-dependence is defined as ≤3 units of red blood cells transfused in the 12 months preceding the first dose and no transfusions in the 4 months preceding the first dose. Patients are followed up at Weeks 1, 2, 3, 6, 9, 12, 16, 20, and 24 in the Core Period, and subsequently every 3 months in the Extension Period. Safety and tolerability (primary endpoint), pharmacokinetics, pharmacodynamics, and hematologic parameters including hemoglobin (Hb) are assessed. Here, we report safety and efficacy data based on the data cutoff date of March 27, 2017; updated data from the Core and Extension Periods and complete data from the Core Period will be available at the meeting. The rate of glycolytic metabolism in peripheral blood samples was measured in a subset of patients before and after treatment to assess PK-R pathway activation.

RESULTS: Enrollment was completed in November 2016 (n=52; median baseline age 34 years; 62% men; 81% white). As of March 27, 2017, 29 patients had completed the Core Period, 15 were ongoing in the Core Period, and 8 had discontinued the Core Period owing to adverse events (AEs) (n=3), investigator decision (n=2), or consent withdrawal (n=3). Of the 29 patients completing the Core Period, 24 entered the Extension Period: 21 were ongoing and 3 had discontinued the Extension Period owing to investigator decision (n=2) or consent withdrawal (n=1). Median (range) treatment duration was 24.9 (12.9–76.9) weeks. As a result of protocol-specified dose modifications, subjects received doses in the range between 5 mg once daily and 300 mg BID.



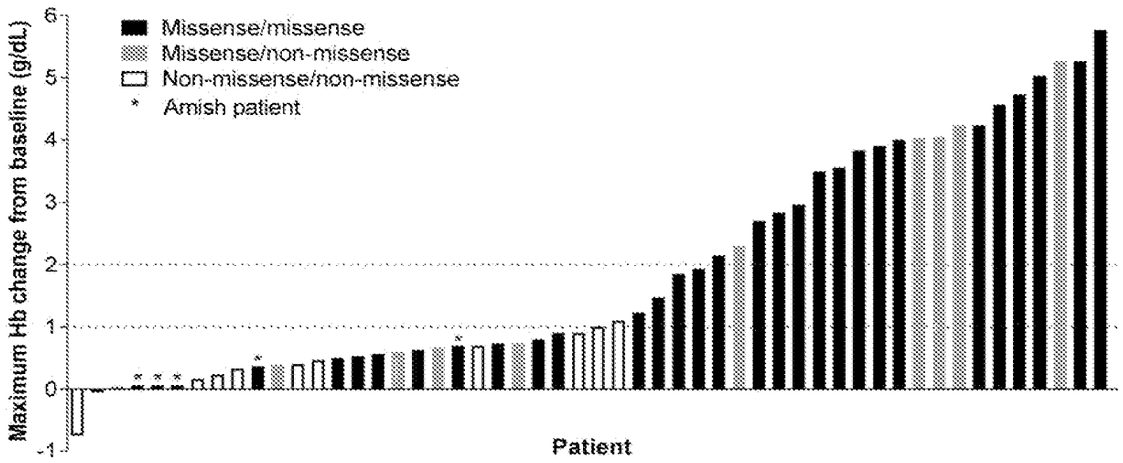
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AG-348 had an acceptable safety profile. The most common AEs (occurring in >10 patients) were headache (44%, n=23), insomnia (39%, n=20), and nausea (37%, n=19). Testosterone levels increased because of known aromatase inhibition; however, most values remained within the normal range. Six drug-related serious AEs occurred in 5 patients (withdrawal hemolysis followed by anemia; anemia; osteoporosis; hypertriglyceridemia; pharyngitis).

Of 52 patients, 25 (48%) experienced a maximum Hb increase >1.0 g/dL (mean maximum increase 3.5 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 BID. Some genotype–Hb response correlations were observed (Figure), in which those with at least 1 missense mutation were more likely to have an Hb increase. Hemolysis markers (reticulocytes, indirect bilirubin, haptoglobin) improved in patients who experienced a maximum Hb increase >1.0 g/dL. In a subset analysis (n=8), a positive correlation was observed between increased Hb and activation of the PK-R pathway, as measured *ex vivo* via incorporation of labelled glucose into lactate, the end product of the PK reaction.

CONCLUSIONS: AG-348 is a novel, first-in-class PK-R activator in clinical testing for PK deficiency. The ongoing DRIVE PK study has completed enrollment, and final Core Period data will be available at the time of presentation. Chronic daily dosing with AG-348 has been generally well tolerated and has resulted in clinically relevant, durable increases in Hb and reductions in markers of hemolysis across a range of doses. These data support the potential of AG-348 as the first disease-altering therapy for PK deficiency.

Figure. Maximum hemoglobin (Hb) change from baseline by genotype






Ex. 2001-0529

# Long-term safety and efficacy of mitapivat (AG-348), a pyruvate kinase activator, in patients with pyruvate kinase deficiency: The DRIVE PK study

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## 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 PK-R, the red blood cell–specific PK enzyme, which results in lifelong hemolytic anemia.[1,2]
- Acute and chronic complications of supportive care (e.g. transfusions, splenectomy, or iron chelation) can additionally burden patients with PK deficiency.
- Mitapivat sulfate (mitapivat, AG-348)—an oral, small-molecule, allosteric activator of both wild type and mutant PK-R[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]

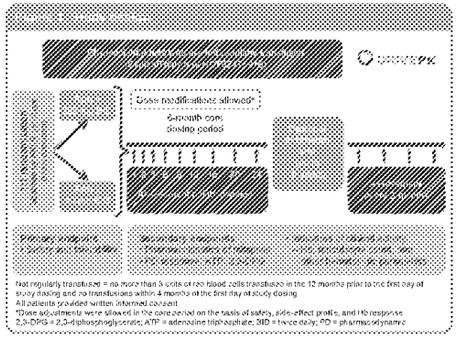
## OBJECTIVE

- 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**).

**Figure 1. Study design**

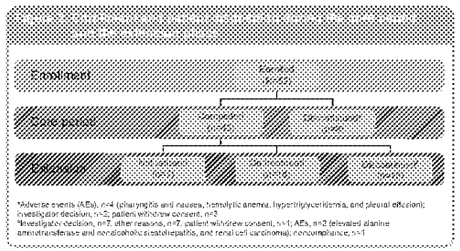



- Protocol amendments during the extension period required that:
  - Patients who did not have an increase from baseline Hb of ≥1.0 g/dL for ≥3 of prior 4 measurements withdraw from the study.
  - Patients treated with mitapivat doses >25 mg BID undergo a dose taper and continue on a dose that maintained their Hb level at no lower than 1.0 g/dL below their pre-taper 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 March 27, 2019 (**Figure 2**).

**Figure 2. Patient disposition**




## RESULTS

### Treatment

- Of the 18 patients continuing in the extension period, 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.

### 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.

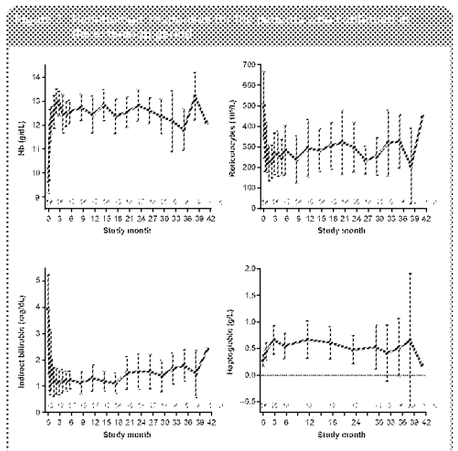
**Table 1. Demographic characteristics of all patients and those who continued in the extension period**

| Characteristic | Total cohort (n=52) | Core period (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) | 9.7 (7.8–12.0) |
| 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) |

### Efficacy

- Improvements in hemoglobin 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**).

**Figure 3. Hb, reticulocytes, indirect bilirubin, and haptoglobin over time**




Data are presented as mean ± 95% CI

### 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 (%) | Continued (n=18) | |
|---|---|---|
| | 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) |
| Hypertriglyceridemia | 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) |
| Dysmenorrhoea | 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 21.0
[b]AEs occurring in >15% of the 18 continuing patients in either the core or extension periods

## CONCLUSIONS

- Mitapivat is a novel, first-in-class, PK-R 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 N=76

ACTIVATE-T N=20–40

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

*Disclosures*
This study was funded by Agios Pharmaceuticals, Inc.

RFG: Agios – consultant, advisory committee member, research funding; Novartis – research funding. DML: Agios; Novartis, – advisory committee member; Cerus – data safety monitoring board member. FG: Addmedica – advisory committee member. WB: Agios; Bioverativ, Incyte – consultant, advisory board member; Apellis – consultant; Alexion – consultant, research funding, speakers bureau member; Novartis – research funding, speakers bureau member. EJvB: Agios – advisory committee member, research funding. FMK: no conflict of interest to disclose. KG: Agios – study investigator, research funding. SS, AMK, JLK, LH, PFH, CX: Agios – employment and stockholder. GG: Agios – consultant; Apopharma, Novartis, Taruna – research funding; Bluebird Bio – consultant, research funding. LH and CM: Agios – employment and stockholder. PFH: Agios – employment and stockholder; Bristol-Myers Squibb, Infinity Pharma, Jazz – stockholder. GG: Agios – consultant, advisory committee member, research funding.

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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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# Long-Term Safety and Efficacy of Mitapivat (AG-348), a Pyruvate Kinase Activator, in Patients with Pyruvate Kinase Deficiency: The DRIVE PK Study

Rachael F. Grace, MD, D. Mark Layton, MB BS, FRCP, FRCPCH, Frédéric Galactéros, MD PhD, Wilma Barcellini, MD, Eduard J. van Beers, MD PhD, Hassan M. Yaish, MD, Yaddanapudi Ravindranath, MBBS, Kevin H. M. Kuo, MDMSc,FRCPC, Sujit Sheth, MD, Janet L. Kwiatkowski, MD MSCE, Lei Hua, PhD, Peter F. Hawkins, PhD, Chris Mix, MD, Bertil Glader, MD PhD

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*Blood* (2019) 134 (Supplement_1): 3512.

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**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). Current treatments for PK deficiency are supportive only. Mitapivat (AG-348) is an oral, small-molecule, allosteric PK-R activator in clinical trials for PK deficiency. We previously described results from DRIVE PK, a phase 2, randomized, open-label, dose-ranging study in adults with PK deficiency (N=52) treated with mitapivat for a median of 6 months.

**Aim:** To report long-term safety and efficacy of mitapivat in patients who continue treatment in the ongoing Extension period of the DRIVE PK study (ClinicalTrials.gov NCT02476916).

**Methods:** Patients were eligible to participate if ≥18 years of age with a confirmed diagnosis of PK



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deficiency (enzyme and molecular testing); baseline hemoglobin (Hb) levels ≤12.0 g/dL (males) or ≤11.0 g/dL (females); and if they had not received more than 3 units of red blood cells in the prior 12 months, with no transfusions in the prior 4 months. Patients were initially randomized 1:1 to receive mitapivat 50 mg twice daily (BID) or 300 mg BID for a 6-month Core period. Dose adjustment was allowed during the Core period based on safety and efficacy. Patients experiencing clinical benefit without concerning safety issues related to mitapivat (investigator discretion) could opt to enter the Extension period, with follow-up visits every 3 months. Safety (adverse events [AEs]) and efficacy (hematologic parameters including Hb) were assessed. Protocol amendments during the Extension period required that (1) patients who did not have an increase from baseline Hb of ≥1.0 g/dL for ≥3 of the prior 4 measurements withdraw from the study, and (2) patients treated with mitapivat doses >25 mg BID undergo a dose taper and continue on the dose that maintained their Hb level no lower than 1.0 g/dL below their pre-taper Hb level.

**Results:** Fifty-two patients enrolled in this study and were treated in the 24-week Core period; 43 (83%) patients completed the Core period and 36 (69%) entered the Extension period. Eighteen patients discontinued from the Extension period: investigator decision (n=8), AEs (n=1), consent withdrawal (n=1), noncompliance (n=1), or other (n=7). Thus, 18 patients, all of whom received ≥29 months of treatment with mitapivat (median 35.6, range 28.7-41.9) have continued treatment. Ten of these 18 patients were male, 11 had a prior splenectomy, and 5 had a history of iron chelation. Median age was 33.5 (range 19-61) years; mean baseline Hb was 9.7 (range 7.9-12.0) g/dL. All patients had ≥1 missense *PKLR* mutation. The doses (post-taper) at which treatment was continued were (BID): ≤25 mg (n=12), 50 mg (n=5), and 200 mg (n=1).

Improvements in Hb levels and markers of hemolysis (reticulocytes, indirect bilirubin, haptoglobin) were sustained (Figure). Among the 18 patients, headache was the most commonly reported AE during both the Extension (n=7, 38.9%) and Core (n=10, 55.6%) periods. Reports of insomnia and fatigue during the Extension period (n=5, 27.8% each) were the same as or similar to those during the Core period. There were fewer reports of nausea (2 vs 6) and hot flush (0 vs 5) in the Extension period. Nasopharyngitis was reported in 5 patients in the Extension period vs 1 patient in the Core period. These data are consistent with the AE profile for the 52 patients treated overall in the Core period, in that headache (44%), insomnia (40%), and nausea (38%) were the most commonly reported AEs and were transient (generally resolved within 7 days without intervention).

**Conclusion:** Chronic daily dosing with mitapivat for a median of 3 years was well tolerated, with no new safety signals reported. Increased Hb levels and improvements in hemolysis markers were



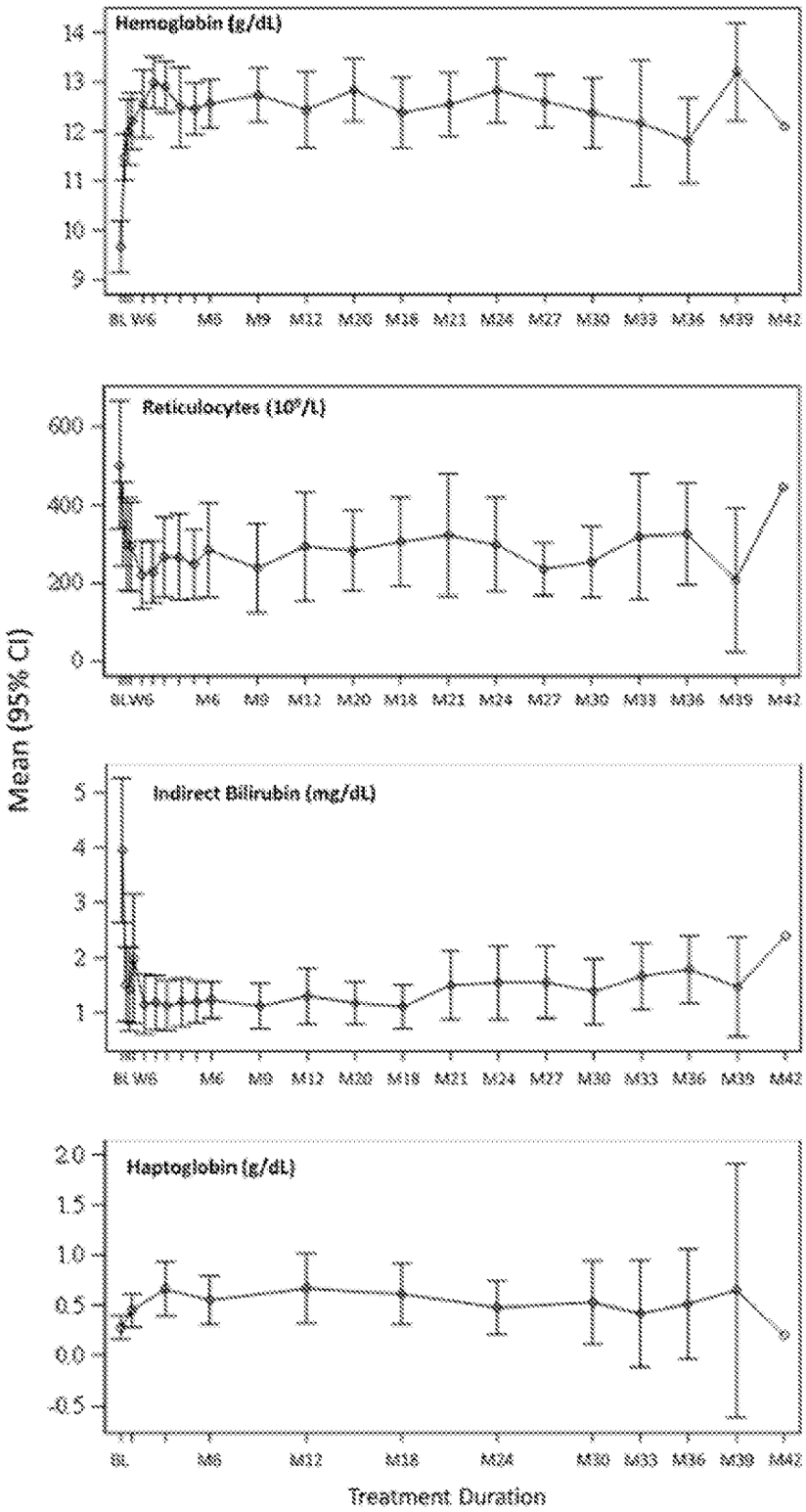
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sustained at the optimized individual doses. These long-term data support the potential of mitapivat as the first disease-altering therapy for PK deficiency. Two phase 3 trials are underway to further study the effect of mitapivat in patients with PK deficiency.



Ex. 2001-0533

**Figure:** Improvements in Hb levels and markers of hemolysis (reticulocytes, indirect bilirubin, haptoglobin) were sustained






Ex. 2001-0534

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**Topics:** intrinsic drive, pyruvate kinase, pyruvate kinase deficiency, transcriptional activation, enzymes, headache, nausea, adverse event, anemia, hemolytic, congenital, blood transfusion

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Ex. 2001-0535

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

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*Presented at the British Blood Transfusion Society Red Cell Special Interest Group, 13 September 2017*

Ex. 2001-0536

# Disclosures

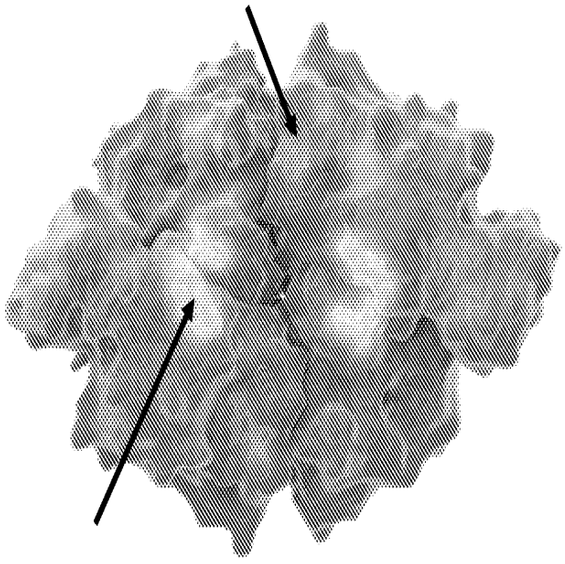
- This study was funded by Agios Pharmaceuticals Inc.
- Rachael F Grace: Agios – study co-principal investigator and scientific advisor
- D Mark Layton: Agios – study investigator
- Frédéric Galactéros – study investigator
- Christian Rose: Agios – study investigator
- Wilma Barcellini: Agios – study 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. 2001-0537

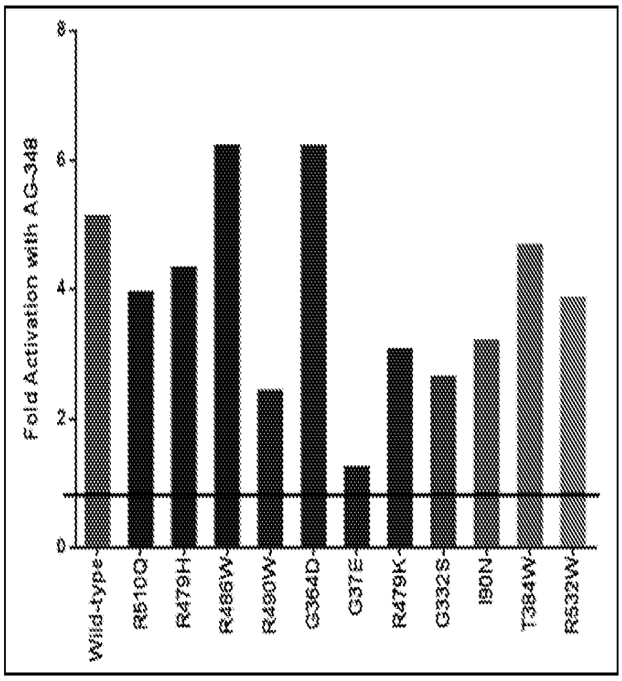
# AG-348: allosteric activator of wild-type and mutant PK-R

**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**

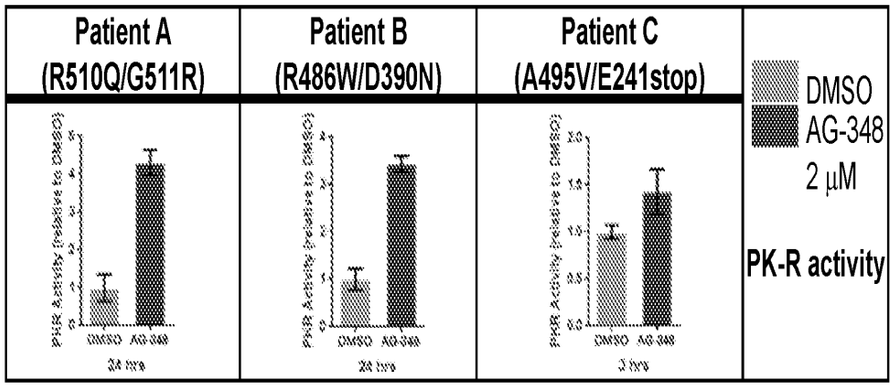
**AG-348 potently activates recombinant PK-R wild type and nearly all mutant enzymes tested *in vitro***



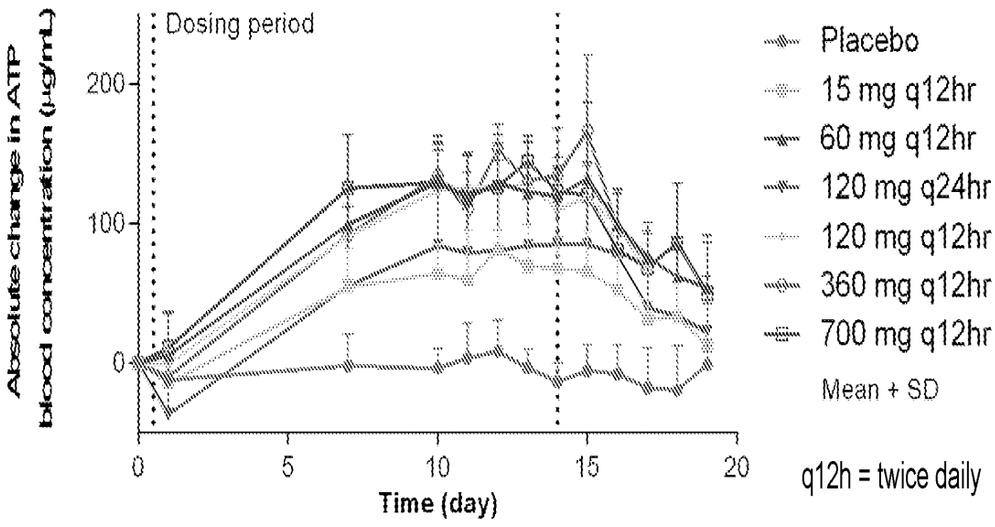

Kung C et al. 55th ASH Annual meeting 2013, Abstract 2180; Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010

# AG-348 activates the glycolytic pathway

PK deficient patient red blood cell samples




ATP levels in healthy volunteers given AG-348 for 14 days




Kung C et al. 56th ASH Annual meeting 2014, Abstract 4010; Yang et al. 20th EHA Congress, 2015, Abstract S138

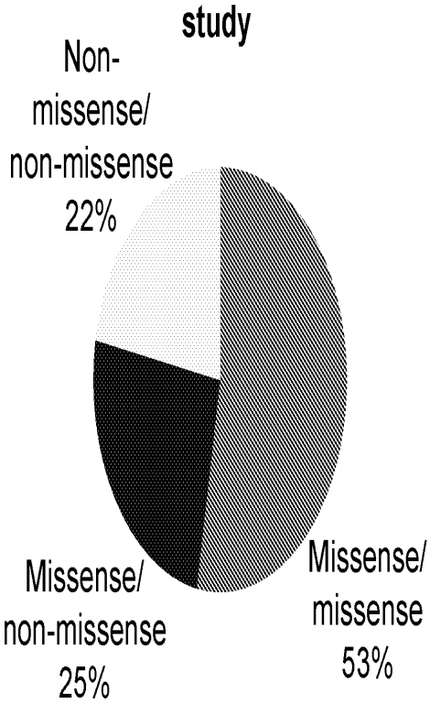


Ex. 2001-0539

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

| | |
|---|---|
| **Description** | • Presents in childhood with severe haemolytic anaemia |
| **Aetiology** | • Caused by mutations in the *PK-LR* gene coding for erythrocyte pyruvate kinase (PK-R) |
| **Disease burden** | • Lifelong haemolytic anaemia<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**



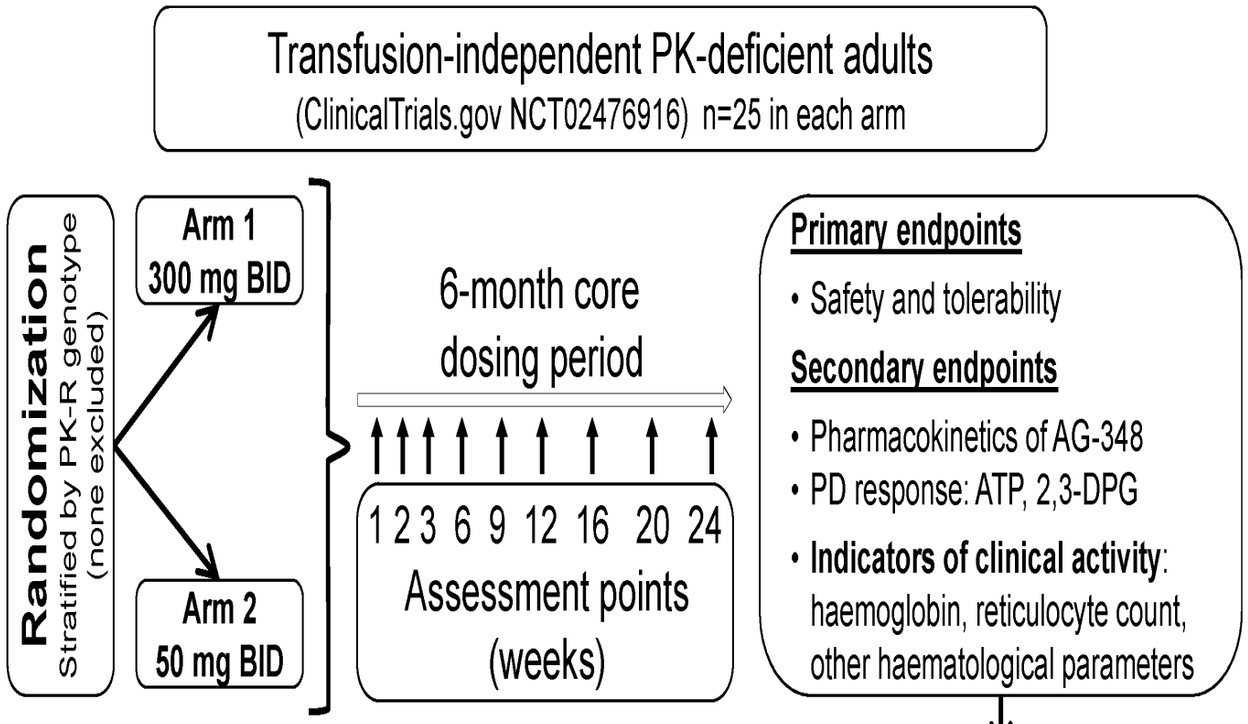

Source: Grace R et al. *Am J Hematol* 2015;90(9):825-30; Bianchi P et al. 57th ASH Annual Meeting 2015, Abstract 3337



Ex. 2001-0540

# 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 diphosphoglycerate; BID = twice daily; PD = pharmacodynamic



Ex. 2001-0541

# 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) |
| Haemoglobin (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. 2001-0542

# 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, anaemia
- 13 serious AEs in 10 patients
  - Six drug-related SAEs in 5 patients: Withdrawal haemolysis followed by anaemia; anaemia; osteoporosis; hypertriglyceridaemia; pharyngitis
    - Grade 4 hypertriglyceridaemia 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. 2001-0543

# 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. 2001-0544

# 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

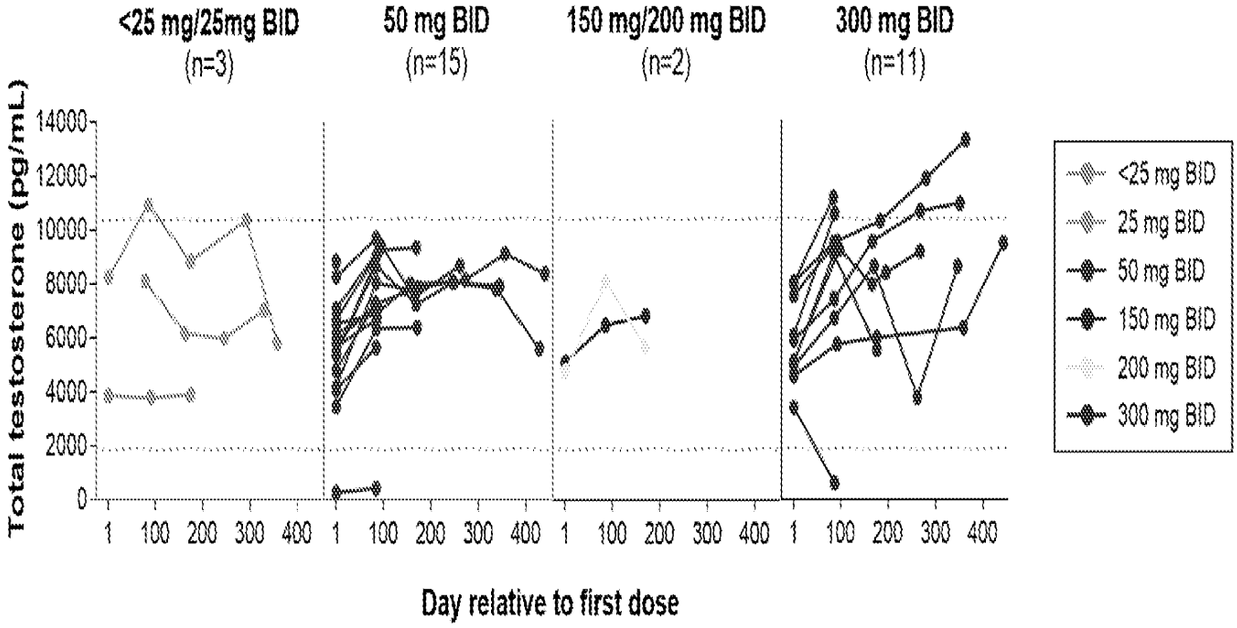
QD = once daily



Ex. 2001-0545

# 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

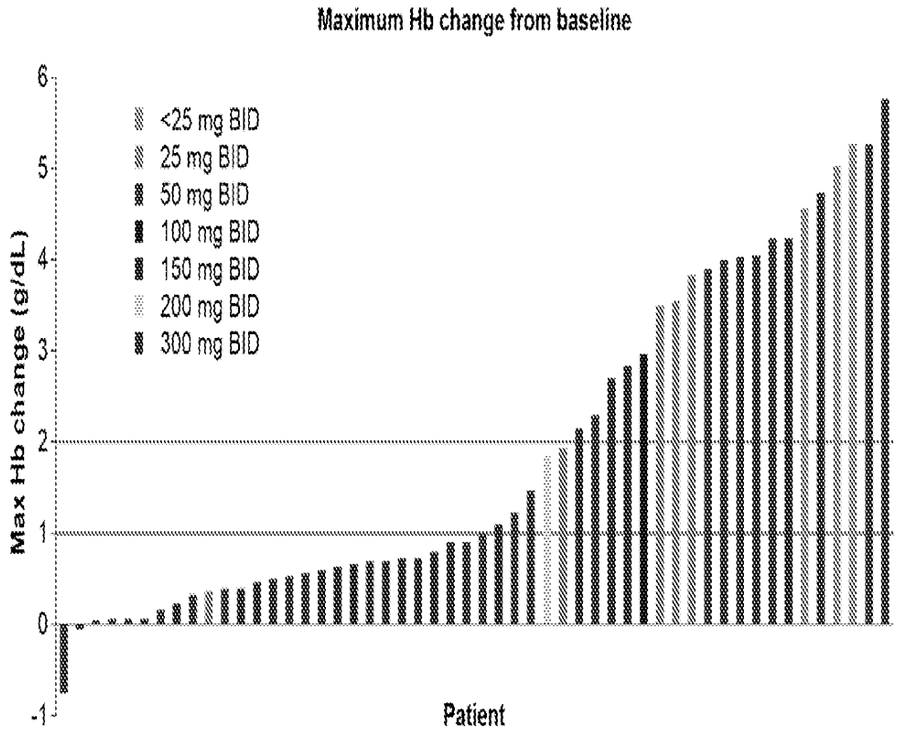


Ex. 2001-0546

# 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)



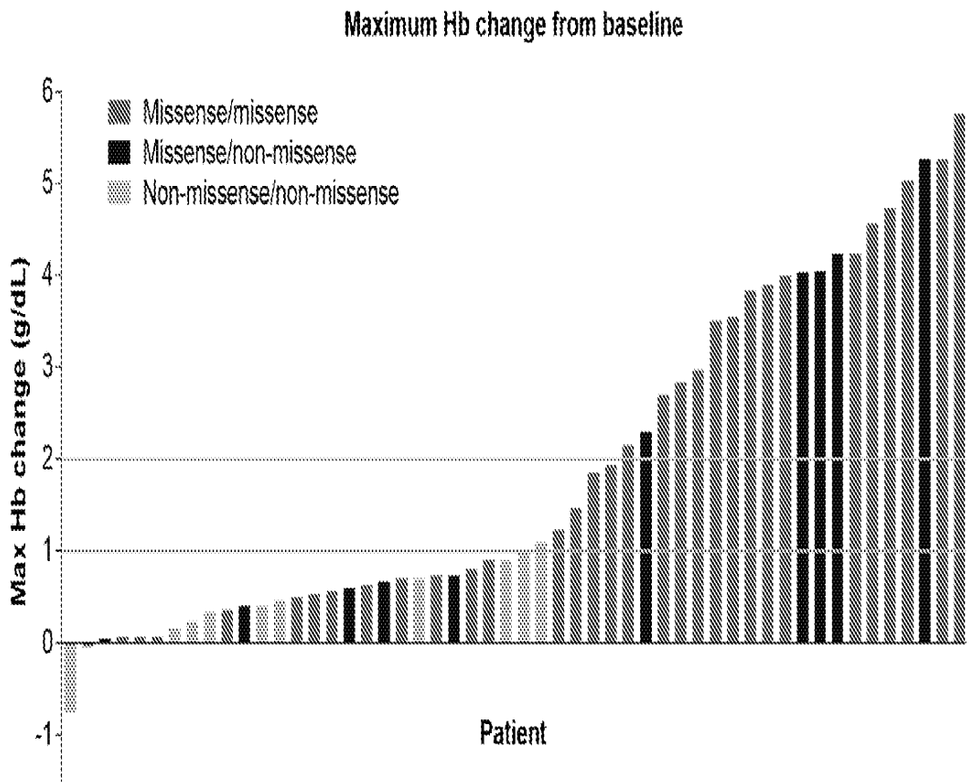

The baseline value is the average of all central assessments within the screening period (42 days prior to Day 1)



Ex. 2001-0548

# 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. 2001-0549