A Phase 1 Dose Escalation Study of the Pyruvate Kinase Activator Mitapivat (AG-348) in Sickle Cell Disease

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SUPPLEMENTAL METHODS

Mitapivat quantification in plasma

Mitapivat plasma concentration was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) following a simple protein precipitation method. A 20-µL aliquot of the plasma (K_2-EDTA) from study samples was mixed with 200 µL acetonitrile (with 0.1% FA) and the internal standard (mitapivat d8, 200 ng/mL). The mixture was vortexed at 1500 rpm for 2 min and centrifuged at 4000 rpm for 5 min. Subsequently, 20-µL aliquot of supernatant was diluted with 180-µL water (with 0.1% FA). The contents were mixed and 10-µL samples was

injected for analysis by LC-MS/MS. A reversed-phase gradient method utilizing Water (with 0.1% FA, mobile phase A) and Acetonitrile (with 0.1% FA, mobile phase B) was used for chromatographic separation on an Hypersil Gold™ C18 (3 µm, 2.1x 50 mm, ThermoFisher Scientific, Framingham, MA). The detection of eluting analytes was carried on a SCIEX 6500 QTRAP mass spectrometer (SCIEX. Framingham, MA) in positive electrospray ionization mode. The total run time of the assay was 3.5 min and analytical column was kept at 40°C throughout the analysis while sample manager was maintained at 7°C.

ATP and 2,3-DPG quantification in whole blood

Whole blood levels of ATP and 2,3-DPG were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). A 5-µL of whole blood sample was mixed with 10 µL of water containing the internal standards $^{13}C_3$-2,3-diphosphoglycerate (200 µg/mL) and $^{13}C_{10}$,$^{15}N_5$-ATP (200 µg/mL). Samples were then vortex mixed and 40-µL water was added. Subsequently, samples were extracted with 320 µL of methanol, mixed for 10 min and then centrifuged at 4000 rpm for 10 minutes. A 40-µL supernatant was transferred to another plate and 320 µL of acetonitrile was added. The mixture was mixed for 2 minutes, followed by centrifugation at 4000 rpm for 5 minutes. The resulting supernatant was transferred to an injection plate for LC-MS analysis. A 5-µL aliquot of supernatant was injected for analysis by LC-MS/MS. An ion exchange method was used for separation of ATP and 2,3 DPG on a BioBasic AX analytical column (2.1 X 50 mm, 5 µM, Thermo Fisher Scientific, Waltham, MA). Detection was done on a SCIEX 6500 QTRAP mass spectrometer (SCIEX. Framingham, MA) in negative electrospray ionization mode. Calibration curves were established using ATP and 2,3 DPG authentic standards (50 to 2000 µg/mL). The peak area ratios of the analyte relative to the internal standard were used for quantitation.

Sickling assay

When HbS polymerizes upon deoxygenation in erythrocytes, the cell shape is simultaneously distorted,[1] which is readily detected using an optical microscope. Images of SS erythrocytes suspended in a pH 7.3 300 mOsM phosphate buffered saline solution (32 mM Na_2HPO_4, 8 mM KH_2PO_4, 130 mM NaCl, 1 mg/mL BSA, 1 mg/mL dextrose) were collected following the initiation of deoxygenation using a Lionheart FX automated microscope system (BioTek Instruments, Inc.) in a 37°C humidified chamber; nitrogen flow was regulated to reach and maintain 5% oxygen inside the instrument using the Biotek gas controller. Deoxygenation of the hemoglobin inside the erythrocytes required about 30 min. Several metrics were used to determine the time at which a cell sickles, loss of circular shape, loss of more transparent center characteristic of a biconcave disc, and a decrease in area of the cell. The output of the assay is the fraction of cells sickled as a function of time after the initiation of deoxygenation, from which the time required

for 50% of the cells to sickle (t50) is obtained. An increase in t50 corresponds to a decrease in sickling. More details of the assay have been previously reported.[2]

References:

1. Mozzarelli A, Hofrichter J, Eaton WA. Delay time of hemoglobin S polymerization prevents most cells from sickling in vivo. *Science*. 1987;237(4814):500-506.
2. Dunkelberger EB, Metaferia B, Cellmer T, Henry ER. Theoretical Simulation of Red Cell Sickling Upon Deoxygenation Based on the Physical Chemistry of Sickle Hemoglobin Fiber Formation. *J Phys Chem B*. 2018;122(49):11579-11590.

High level summary on pharmacokinetics of mitapivat

The pharmacokinetics of mitapivat were evaluated in subjects with SCD for the first time in this study. The exposure metrics, $AUC_{0\text{-last}}$ and C_{max}, of mitapivat generally increased in a dose-proportional manner after the first dose of mitapivat was administered in each of the dosing periods. The exposure of mitapivat after the first 50-mg dose and after 2 weeks of repeated dosing with 50 mg BID were found to be similar. The exposure of mitapivat after 2 weeks of repeated dosing of 100 mg BID was approximately 20% less than that observed after administration of the first 100-mg dose. A decrease in exposure after repeated dosing has previously been observed at doses of 120 mg BID or higher in studies in healthy subjects, at the 100-mg BID dose level in subjects with thalassemia, and at the 300-mg BID dose level in subjects with pyruvate kinase deficiency. This is likely due to the autoinduction effect of mitapivat on CYP3A4. Overall, low to moderate variability in pharmacokinetics of mitapivat was observed between subjects.

STATISTICAL MODELING

Regression Modeling

In the regression equation below, Y_{ij} denotes a given outcome (e.g. hemoglobin) for the i^{th} person at the j^{th} measurement (either Baseline, 5mg dose, 20mg dose, 50mg dose, 100mg dose, taper, or last):

$$\begin{aligned} Y_{ij} = \quad & \alpha_i + \beta_0 + \beta_5 I[Measurement_j = 5mg\ Dose] + \beta_{20} I[Measurement_j = 20mg\ Dose] + \\ & \beta_{50} I[Measurement_j = 50mg\ Dose] + \beta_{100} I[Measurement_j = 100mg\ Dose] + \\ & \beta_{Taper} I[Measurement_j = Taper] + \beta_{Last} I[Measurement_j = Last] + \\ & \beta_{Age}(Age.at.tx_i - \text{average age}) + \beta_{Gen} I[Gender_i = M] + \epsilon_{ij} \end{aligned}$$

In the equation, $I[Measurement_j = 5mg\ Dose]$ denotes an indicator function equal to 1 if measurement j corresponds to obtaining the outcome when dosing was at $5mg$ and 0 otherwise. The other indicator variables are similarly interpreted. The α_i is a mean zero Gaussian distributed, individual-specific random effect allowing for individual variation in the level of hemoglobin, and ϵ_{ij} are independent error terms following a mean zero Gaussian distribution that is independent of the α_i. β_0 represents an overall baseline mean average and the $\beta_5, \beta_{20}, \beta_{50}, \ldots \beta_{Last}$ coefficients measure the change from baseline for the corresponding measurements, e.g. β_{100} is the estimated average change in hemoglobin from baseline to dose 100mg after adjustments for age, gender (denoted by "gender M" in the tables; 0 = female; 1 = male), and random effect α_i.

As a supplementary analysis we include a second regression model that has additional regression coefficient (denoted by "hu" in the tables) corresponding to the effect of hydroxyurea (0 = not on hydroxyurea; 1 = on hydroxyurea). Power for this additional covariate will usually be low because only 4 individuals were not on hydroxyurea. No patients were initiated or uptitrated on hydroxyurea during the study period so hydroxyurea use patterns were constant within each individual.

Below we provide the basic regression model and the extended regression model for each outcome variable.

Hemoglobin

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	8.73	0.51	81	16.95	< 0.0001
Change-Dose.5	0.34	0.22	81	1.56	0.12
Change-Dose.20	0.76	0.22	81	3.53	0.0007
Change-Dose.50	1.19	0.22	81	5.5	< 0.0001
Change-Dose.100	0.92	0.26	81	3.52	0.0007
Change-Taper	0.34	0.22	81	1.52	0.13
Change-Last	0.37	0.22	81	1.7	0.09
age	-0.02	0.03	13	-0.49	0.63
gender M	0.68	0.6	13	1.13	0.28

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	9.13	0.78	81	11.63	< 0.0001
Change-Dose.5	0.34	0.22	81	1.56	0.12
Change-Dose.20	0.76	0.22	81	3.53	0.0007
Change-Dose.50	1.19	0.22	81	5.5	< 0.0001
Change-Dose.100	0.92	0.26	81	3.53	0.0007
Change-Taper	0.34	0.22	81	1.53	0.13
Change-Last	0.37	0.22	81	1.7	0.09
age	0	0.03	12	-0.14	0.89
gender M	0.63	0.61	12	1.03	0.32
hu	-0.5	0.73	12	-0.69	0.50

LDH

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	348.4	51.21	80	6.8	< 0.0001
Change-Dose.5	-7.81	27.81	80	-0.28	0.78
Change-Dose.20	-39.94	27.81	80	-1.44	0.15
Change-Dose.50	-25.31	27.81	80	-0.91	0.37
Change-Dose.100	-37.89	34.95	80	-1.08	0.28
Change-Taper	20.63	28.35	80	0.73	0.47
Change-Last	30.72	28.34	80	1.08	0.28
age	-5.69	2.96	13	-1.92	0.08
gender M	41.59	57.87	13	0.72	0.49

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	368.8	77.75	80	4.74	< 0.0001
Change-Dose.5	-7.81	27.81	80	-0.28	0.78

Change-Dose.20	-39.94	27.81	80	-1.44	0.15
Change-Dose.50	-25.31	27.81	80	-0.91	0.37
Change-Dose.100	-37.92	34.97	80	-1.08	0.28
Change-Taper	20.71	28.36	80	0.73	0.47
Change-Last	30.78	28.35	80	1.09	0.28
age	-5.17	3.4	12	-1.52	0.15
gender M	39.39	60.18	12	0.65	0.53
hu	-25.41	71.1	12	-0.36	0.73

Total Bilirubin

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	1.82	0.28	81	6.42	< 0.0001
Change-Dose.5	-0.19	0.17	81	-1.17	0.24
Change-Dose.20	-0.56	0.17	81	-3.36	0.001
Change-Dose.50	-0.77	0.17	81	-4.65	< 0.0001
Change-Dose.100	-0.87	0.2	81	-4.36	< 0.0001
Change-Taper	-0.19	0.17	81	-1.14	0.26
Change-Last	0.1	0.17	81	0.62	0.54
age	-0.04	0.02	13	-2.61	0.02
gender M	0.23	0.32	13	0.73	0.48

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	2.11	0.41	81	5.1	< 0.0001
Change-Dose.5	-0.19	0.17	81	-1.17	0.24
Change-Dose.20	-0.56	0.17	81	-3.36	0.001
Change-Dose.50	-0.77	0.17	81	-4.65	< 0.0001
Change-Dose.100	-0.87	0.2	81	-4.35	< 0.0001

Change-Taper	-0.19	0.17	81	-1.12	0.26
Change-Last	0.1	0.17	81	0.62	0.54
age	-0.03	0.02	12	-1.93	0.08
gender M	0.2	0.32	12	0.62	0.55
hu	-0.37	0.38	12	-0.98	0.35

Absolute Reticulocyte Count

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	196.44	39.88	81	4.93	< 0.0001
Change-Dose.5	-20.97	13.95	81	-1.5	0.14
Change-Dose.20	-20.72	13.95	81	-1.49	0.14
Change-Dose.50	-44.99	13.95	81	-3.23	0.002
Change-Dose.100	-34.1	16.82	81	-2.03	0.05
Change-Taper	-13.77	14.22	81	-0.97	0.34
Change-Last	8.1	14.22	81	0.57	0.57
age	-2.73	2.41	13	-1.14	0.28
gender M	-10.75	46.88	13	-0.23	0.82

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	290.89	50.89	81	5.72	< 0.0001
Change-Dose.5	-20.97	13.95	81	-1.5	0.14
Change-Dose.20	-20.72	13.95	81	-1.49	0.14
Change-Dose.50	-44.99	13.95	81	-3.23	0.002
Change-Dose.100	-33.62	16.81	81	-2	0.05
Change-Taper	-13.6	14.22	81	-0.96	0.34
Change-Last	7.94	14.22	81	0.56	0.58
age	-0.32	2.25	12	-0.14	0.89

gender M	-21.23	39.81	12	-0.53	0.60
hu	-117.35	47.05	12	-2.49	0.03

AST

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	33.88	5.94	81	5.71	< 0.0001
Change-Dose.5	-3.31	3.43	81	-0.97	0.34
Change-Dose.20	-3.37	3.43	81	-0.98	0.33
Change-Dose.50	-2	3.43	81	-0.58	0.56
Change-Dose.100	-3.54	4.13	81	-0.86	0.39
Change-Taper	-2.49	3.49	81	-0.71	0.48
Change-Last	3.02	3.49	81	0.86	0.39
age	-0.17	0.34	13	-0.49	0.63
gender M	3.07	6.64	13	0.46	0.65

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	42.81	8.33	81	5.14	< 0.0001
Change-Dose.5	-3.31	3.43	81	-0.97	0.34
Change-Dose.20	-3.37	3.43	81	-0.98	0.33
Change-Dose.50	-2	3.43	81	-0.58	0.56
Change-Dose.100	-3.42	4.13	81	-0.83	0.41
Change-Taper	-2.42	3.5	81	-0.69	0.49
Change-Last	3	3.49	81	0.86	0.39
age	0.06	0.36	12	0.18	0.86
gender M	2.12	6.39	12	0.33	0.75
hu	-11.13	7.56	12	-1.47	0.17

MCV

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	103.32	7.42	79	13.93	< 0.0001
Change-Dose.5	-0.5	0.8	79	-0.62	0.54
Change-Dose.20	0.52	0.8	79	0.64	0.52
Change-Dose.50	0.42	0.8	79	0.52	0.60
Change-Dose.100	1.98	1.01	79	1.95	0.05
Change-Taper	-0.25	0.84	79	-0.3	0.77
Change-Last	-0.64	0.82	79	-0.78	0.44
age	0.61	0.46	13	1.34	0.20
gender M	-1.82	8.93	13	-0.2	0.84

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	79.02	7.24	79	10.92	< 0.0001
Change-Dose.5	-0.5	0.8	79	-0.62	0.54
Change-Dose.20	0.52	0.8	79	0.64	0.52
Change-Dose.50	0.42	0.8	79	0.52	0.60
Change-Dose.100	1.97	1.01	79	1.94	0.06
Change-Taper	-0.26	0.84	79	-0.31	0.76
Change-Last	-0.63	0.82	79	-0.77	0.44
age	0	0.32	12	-0.02	0.99
gender M	0.88	5.74	12	0.15	0.88
hu	30.18	6.78	12	4.45	0.0008

Hb F Percentage

Basic Model

	Value	Std.Error	DF	t-value	p-value
Baseline	20.39	4.26	75	4.79	< 0.0001
Change-Dose.5	-0.42	0.49	75	-0.86	0.39
Change-Dose.20	-1.02	0.49	75	-2.1	0.04
Change-Dose.50	-1.31	0.49	75	-2.68	0.009
Change-Dose.100	-0.34	0.56	75	-0.6	0.55
Change-Taper	0.19	0.47	75	0.41	0.68
Change-Last	0.81	0.47	75	1.71	0.09
age	0.52	0.26	13	1.97	0.07
gender M	-2.33	5.13	13	-0.45	0.66

Extended Model

	Value	Std.Error	DF	t-value	p-value
Baseline	9.68	5.38	75	1.8	0.08
Change-Dose.5	-0.42	0.49	75	-0.87	0.39
Change-Dose.20	-1.02	0.49	75	-2.1	0.04
Change-Dose.50	-1.31	0.49	75	-2.69	0.009
Change-Dose.100	-0.34	0.56	75	-0.61	0.54
Change-Taper	0.19	0.47	75	0.4	0.69
Change-Last	0.81	0.47	75	1.71	0.09
age	0.25	0.24	12	1.02	0.33
gender M	-1.14	4.27	12	-0.27	0.79
hu	13.31	5.04	12	2.64	0.02

2,3-DPG

Basic Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	-3.74	3.65	66	-1.02	0.31
% Change-Dose.20	-16.08	3.65	66	-4.4	< 0.0001

% Change-Dose.50	-23.49	3.65	66	-6.43	< 0.0001
% Change-Dose.100	-24.13	4.12	66	-5.86	< 0.0001
% Change-Taper	1.97	3.68	66	0.53	0.59
% Change-Last	9.11	3.68	66	2.48	0.02
age	-0.18	0.2	14	-0.89	0.39
gender M	-10.34	3.9	14	-2.65	0.02

Extended Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	-8.88	5.04	66	-1.76	0.08
% Change-Dose.20	-21.22	5.04	66	-4.21	< 0.0001
% Change-Dose.50	-28.63	5.04	66	-5.68	< 0.0001
% Change-Dose.100	-29.38	5.44	66	-5.4	< 0.0001
% Change-Taper	-3.25	5.1	66	-0.64	0.53
% Change-Last	3.98	5.05	66	0.79	0.43
age	-0.31	0.21	13	-1.45	0.17
gender M	-9.81	3.77	13	-2.6	0.02
hu	6.43	4.48	13	1.43	0.18

ATP

Basic Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	13.68	6.29	66	2.17	0.03
% Change-Dose.20	26.95	6.29	66	4.29	< 0.0001
% Change-Dose.50	33.43	6.29	66	5.32	< 0.0001
% Change-Dose.100	39.84	6.74	66	5.91	< 0.0001
% Change-Taper	15.51	6.31	66	2.46	0.02
% Change-Last	12.03	6.31	66	1.91	0.06
age	-0.18	0.37	14	-0.5	0.62

gender M	-8.72	7.13	14	-1.22	0.24

Extended Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	-5.89	6.11	66	-0.96	0.34
% Change-Dose.20	7.38	6.11	66	1.21	0.23
% Change-Dose.50	13.86	6.11	66	2.27	0.03
% Change-Dose.100	20.03	6.63	66	3.02	0.004
% Change-Taper	-4.14	6.19	66	-0.67	0.51
% Change-Last	-7.43	6.13	66	-1.21	0.23
age	-0.68	0.26	13	-2.67	0.02
gender M	-6.53	4.55	13	-1.44	0.17
hu	24.3	5.41	13	4.49	0.0006

p50

Basic Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	0.5	2.88	30	0.17	0.86
% Change-Dose.20	-2.09	3.01	30	-0.7	0.49
% Change-Dose.50	-3.84	3.19	30	-1.21	0.24
% Change-Dose.100	-4.88	3.9	30	-1.25	0.22
% Change-Taper	7.97	3.1	30	2.57	0.02
% Change-Last	10.79	3.39	30	3.18	0.003
age	0.43	0.2	10	2.13	0.06
gender M	-8.91	3.38	10	-2.64	0.02

Extended Model

	Value	Std.Error	DF	t-value	p-value

% Change-Dose.5	-4.04	5	30	-0.81	0.43
% Change-Dose.20	-6.56	5.03	30	-1.3	0.20
% Change-Dose.50	-8.04	4.97	30	-1.62	0.12
% Change-Dose.100	-9.31	5.62	30	-1.66	0.11
% Change-Taper	3.59	5.04	30	0.71	0.48
% Change-Last	6.41	5.22	30	1.23	0.23
age	0.27	0.25	9	1.08	0.31
gender M	-8.35	3.46	9	-2.41	0.04
hu	5.59	5.02	9	1.11	0.29

t50

Basic Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	-0.46	9.68	54	-0.05	0.96
% Change-Dose.20	10.19	9.75	54	1.04	0.30
% Change-Dose.50	7.11	9.84	54	0.72	0.47
% Change-Dose.100	13.98	12.44	54	1.12	0.27
% Change-Taper	-11.38	9.76	54	-1.17	0.25
% Change-Last	1.67	10.13	54	0.16	0.87
age	-0.29	0.53	13	-0.55	0.59
gender M	12.13	10.21	13	1.19	0.26

Extended Model

	Value	Std.Error	DF	t-value	p-value
% Change-Dose.5	21.3	10.95	54	1.94	0.06
% Change-Dose.20	31.88	10.99	54	2.9	0.005
% Change-Dose.50	28.84	11.05	54	2.61	0.01
% Change-Dose.100	36.07	13.59	54	2.65	0.01
% Change-Taper	10.88	11.19	54	0.97	0.34

% Change-Last	22.73	11.2	54	2.03	0.05
age	0.26	0.45	12	0.57	0.58
gender M	9.9	7.98	12	1.24	0.24
hu	-27.51	9.24	12	-2.98	0.01

Footnote:

Please note that absolute baseline values are not provided for the 2,3-DPG, ATP, t50, and p50 results where the statistical models looked at percent change as opposed to raw change.

Supplemental Table 1: Probability of detecting at least one adverse event (AE) as a function of sample size and true underlying AE rate.

Sample Size	True Underlying AE Rate		
	15%	**10%**	**5%**
15	91%	79%	54%
20	96%	88%	64%
25	98%	93%	72%

This study was designed to assess the safety and activity of escalating multiple doses of mitapivat in subjects with stable SCD-HbSS. Approximately 20 - 25 patients will be enrolled to achieve 15 patients who have completed all specified dose levels. Specifically, up to 10 subjects will complete the 3 dose levels, and at least 5 subjects will complete the 4 dose levels. The sample size is primarily driven by feasibility considerations and the ability to detect adverse events.

Supplemental Figure 1 - Design of Mitapivat Dose Escalation Study in SCD.

This is a non-randomized, open-label dose escalation study of mitapivat in 17 adults (age ≥ 18 years) with stable Hb SS disease, without a blood transfusion or changes in the dose of hydroxyurea or L-glutamine within 90 days. Following screening, subjects were dose escalated

every 2 weeks from 5mg twice daily up to 50mg twice daily in the original protocol, and up to 100mg twice daily in the amended protocol, followed by a drug taper and a follow-up safety visit 4 weeks after last dose of drug. Subsequent 10 subjects were escalated to 100 mg dose level. The arrows represent the timepoints at which blood samples were collected, and the blue arrows show the time points presented in the efficacy analysis. The primary endpoint was safety and tolerability, in conjunction with changes in hemoglobin level and hemolytic markers. Secondary endpoints included pharmacokinetic and pharmacodynamic parameters, including 2,3-DPG and ATP levels, and also data on p50, a measure of oxygen affinity, and t50, a measure of sickling kinetics.

ATP, adenosine triphosphate; BID, twice daily; DPG, diphosphoglycerate; Hb, hemoglobin.

APPENDIX.

Eligibility Assessment.

All patients with sickle cell anemia (HbSS) will be considered for enrollment.

Inclusion Criteria

- For enrollment, subjects must meet all of the following criteria during the screening period:
- Have provided signed written informed consent prior to performing any study procedure, including screening procedures.
- Age between 18-70 years
- Unequivocal diagnosis of HbSS confirmed by hemoglobin electrophoresis performed on patients at least 90 days after a blood transfusion if previously transfused, or DNA genotyping
- No transfusion in the 90 days prior to the first dose of study drug, or absence of HbA on hemoglobin analysis (by high-performance liquid chromatography; HPLC)
- Have adequate organ function, as defined by:
  a. Serum aspartate aminotransferase (AST) ≤2.5 × Upper Limit of Normal (ULN) (unless the increased AST is assessed by the Investigator as due to hemolysis and/or hepatic iron deposition) and alanine aminotransferase (ALT) ≤2.5 × ULN (unless the increased ALT is assessed by the Investigator as due to hepatic iron deposition).
  b. Serum creatinine ≤1.25 × ULN. If serum creatinine is >1.25 × ULN, then glomerular filtration rate (based on creatinine) must be ≥60 mL/min.
  c. Absolute neutrophil count ≥1.0 × 10^9/L.
  d. Hemoglobin ≥ 7 g/dL

e. Platelet count ≥100 × 10^9/L.
f. Activated partial thromboplastin time and international normalized ratio ≤1.5 × ULN, unless the subject is receiving therapeutic anticoagulants.

- For women of reproductive potential, have a negative serum or urine pregnancy test during the screening period. Women of reproductive potential are defined as sexually mature women who have not undergone a hysterectomy, bilateral oophorectomy, or tubal occlusion; or who have not been naturally postmenopausal (i.e., who have not menstruated at all for at least the preceding 12 months prior to signing informed consent and have an elevated follicle-stimulating hormone level indicative of menopause during the screening period).
- For women of reproductive potential as well as men and their partners who are women of reproductive potential, be abstinent as part of their usual lifestyle, or agree to use 2 effective forms of contraception from the time of giving informed consent, during the study, and for 28 days for women and 90 days for men following the last dose of study treatment. An effective form of contraception is defined as hormonal oral contraceptives, injectables, patches, and barrier methods.
- Be willing to comply with all study procedures for the duration of the study.

Exclusion Criteria

Subjects who meet any of the following criteria during screening will not receive AG348 and will not be counted toward the final enrollment count for statistical purposes:

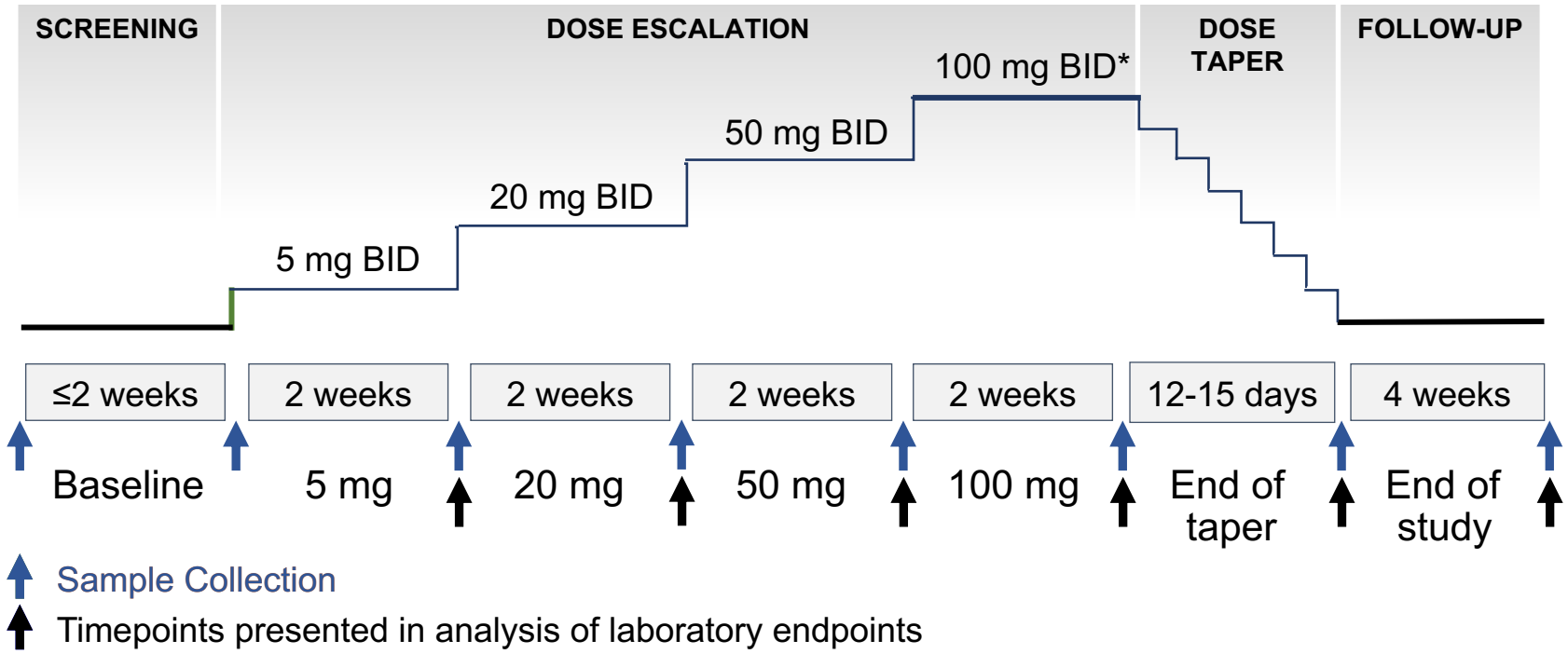
- Documented pyruvate kinase deficiency
- Have a significant medical condition that confers an unacceptable risk to participating in the study, and/or that could confound the interpretation of the study data. Such significant medical conditions include, but are not limited to the following:
  a. Poorly controlled hypertension (defined as systolic blood pressure [BP]
  b. >150 mmHg or diastolic BP >90 mmHg) refractory to medical management.
  c. History of recent (within 6 months prior to signing informed consent) congestive heart failure; myocardial infarction or unstable angina pectoris; hemorrhagic, embolic, or thrombotic stroke; deep venous thrombosis; or pulmonary or arterial embolism.
  d. Cardiac dysrhythmias judged as clinically significant by the Investigator.
  e. Heart-rate corrected QT interval-Fredericia's method (QTcF) >480 msec with

the exception of subjects with right or left bundle branch block.

f. Clinically symptomatic cholelithiasis or cholecystitis. Prior cholecystectomy is not exclusionary. Subjects with symptomatic cholelithiasis or cholecystitis may be rescreened once the disorder has been treated and clinical symptoms have resolved.
g. History of drug-induced cholestatic hepatitis.
h. Iron overload sufficiently severe to result in a clinical diagnosis by the Investigator of cardiac (e.g., clinically significant impaired left ventricular ejection fraction), hepatic (e.g., fibrosis, cirrhosis), or pancreatic (e.g., diabetes) dysfunction.
i. Have a diagnosis of any other congenital or acquired blood disorder, or any other hemolytic process as defined by a positive direct antiglobulin test (DAT), except mild allo-immunization as a consequence of transfusion therapy.
j. Positive test for hepatitis B surface antigen or hepatitis C virus (HCV) antibody (Ab) with signs of active hepatitis B or C virus infection. If the subject is positive for HCV Ab, a reverse transcriptase-polymerase chain reaction test will be conducted. Subjects with hepatitis C may be rescreened after receiving appropriate hepatitis C treatment.
k. Positive test for human immunodeficiency virus 1 or 2 Ab.
l. Active infection requiring any use of systemic antimicrobial agents (parenteral or oral) or Grade ≥3 in severity (per National Cancer Institute Common Terminology Criteria for Adverse Events) within 2 months prior to signing informed consent.
m. Diabetes mellitus judged to be under poor control by the Investigator or requiring >3 antidiabetic agents, including insulin (all insulins are considered 1 agent); use of insulin per se is not exclusionary.
n. History of any primary malignancy, with the exception of: curatively treated nonmelanomatous skin cancer; curatively treated cervical or breast carcinoma in situ; or other primary tumor treated with curative intent, no known active disease present, and no treatment administered during the last 3 years.
o. Unstable extramedullary hematopoiesis that could pose a risk of imminent neurologic compromise.
p. Current or recent history of psychiatric disorder that, in the opinion of the Investigator or Medical Monitor, could compromise the ability of the subject to cooperate with study visits and procedures.
q. Are currently enrolled in another therapeutic clinical trial involving ongoing therapy with any investigational or marketed product or placebo. Sickle cell

anemia subjects on hydroxyurea or L-glutamine will also be considered, provided that they have been on an unchanged dose of hydroxyurea or L-Glutamine for three months prior to enrollment.

r. Have exposure to any investigational drug, device, or invasive procedure within 3 months prior to the first dose of study treatment. All non-investigational invasive procedures within 3 months of starting study treatment may be considered as a potential exclusion criteria per the PI's discretion.
s. Have had any prior treatment with a pyruvate kinase activator.
t. Have received crizanlizumab or voxelotor in the 12 weeks prior to signing consent.
u. Have a prior bone marrow or stem cell transplant.
v. Are currently pregnant or breastfeeding.
w. Are currently receiving medications that are strong inhibitors of cytochrome P450 (CYP)3A4 or strong inducers of CYP3A4 that have not been stopped for a duration of at least 5 days or a timeframe equivalent to 5 half-lives (whichever is longer) prior to the first dose of AG-348.
x. Are currently receiving hematopoietic stimulating agents (e.g., erythropoietins, granulocyte colony stimulating factors, thrombopoietins) that have not been stopped for a duration of at least 28 days prior to the first dose of study treatment.
y. Have a history of allergy to sulfonamides if characterized by acute hemolytic anemia, drug-induced liver injury, anaphylaxis, rash of erythema multiforme type or Stevens-Johnson syndrome, cholestatic hepatitis, or other serious clinical manifestation
z. Have a history of allergy to AG-348 or its excipients (microcrystalline cellulose, croscarmellose sodium, sodium stearyl fumarate, and mannitol).



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