PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program

P-PKSR Study 124103-1295433

STUDY TITLE:

ABEMACICLIB IN CD1 NU/NU MICE WITH AND WITHOUT ORTHOTOPIC RHABDOMYOSARCOMA (RMS) XENOGRAFTS: INTER-GROUP QUALIFICATION BY POPULATION PHARMACOKINETIC (PK) ANALYSIS

SHORT TITLE:	**Abemaciclib PopPK CD1 nu RMS OTX**
TEST ARTICLE:	Abemaciclib mesylate
SECTION:	Nonclinical Pharmacokinetics (Non-GLP)
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Quality Statement

This non-GLP study was conducted using sound scientific principles and established techniques in accordance with the relevant guidelines and standard operating procedures (SOPs) of the Preclinical Pharmacokinetic Shared Resource (P-PKSR) and St. Jude Children's Research Hospital (SJCRH), Memphis, TN, USA. This report accurately reflects the data obtained during the course of this study.

These results represent part of an early phase preclinical pharmacology program. This study has been conducted to provide preliminary insights into the pharmacokinetic (PK) properties of the compound(s) in the indicated preclinical model(s). This study and its results are not intended to provide a comprehensive PK evaluation of the compound(s). The applied bioanalytical method was validated/qualified to support this specific study and discovery-style sample analyses.

Substantial study-to-study and inter-animal variability in preclinical PK exists. Such variability depends upon the in vivo scientists' experience, variations in compound purity and formulation, animal strains, sex and age, and other situational fixed effects (i.e. husbandry conditions, chow constituents, presence or absence of disease, concomitant drugs). As such, the actual PK, plasma or tissue compound concentrations, or equivalent dose in other studies or preclinical models may vary significantly from that reported herein.

1.0 METHODS

1.1 In Vivo Pharmacokinetic (PK) Studies

Over a period of approximately two years, from Spring 2016 to 2018, a variety of mouse plasma and rhabdomyosarcoma (RMS) orthotopic xenograft pharmacokinetic (PK) studies were performed by personnel from St. Jude Children's Research Hospital's Developmental Biology and Solid Tumor Program (DBSTP, Stewart, Elizabeth <Elizabeth.Stewart@STJUDE.ORG>) and the Center for In Vivo Imaging and Therapeutics (CIVIT, Amanda <Amanda.May@STJUDE.ORG>; Millican, Krista <Krista.Millican@STJUDE.ORG>).

Abemaciclib mesylate (LY2835219, Abmole, M2112, Lot 2, purity >98%) was suspended in 1% hydroxyethylcellulose (HEC) / 0.25% Tween 80 / ~0.05% antifoam in ultrapure water at a nominal free base concentration of 5 mg/mL for a 10 mL/kg oral gavage, with PK samples obtained under isoflurane for survival procedures and terminally up to 24 hours post-dose. The same compound substance and oral suspension vehicle substitutes from single lots were used in each study (see **Attached File 5.1**). For the CIVIT studies, each formulation was assessed for concentration in remaining dose suspension using a modification of the P-PKSR's LC-MS/MS plasma assay. All these formulations met specification upon testing (within 15% of nominal concentration with <10% CV across all replicates). Mouse total body weight and tumor weight in grams was recorded within 1 day of study, and upon harvest respectively.

The objective of these studies was to compare and contrast the PK results, thus determining whether statistical or practical (> 2-fold) differences existed based upon: different bioanalytical assay (internal qualified P-PKSR vs. external qualified SAI, ltd.), study personnel, or sampling sites or technique. An overview of the individual studies is provided in **Table 1.1**.

Table 1.1: Summary of Abemaciclib Mouse PK Studies

Study	Remarks
Study 1	Reference, original DBSTP RMS tumor bearing PK performed by Elizabeth Stewart and group, June 2016 (see SRM2 O/R 74806-737948). Plasma (cardiac puncture, CP) and tumor homogenate samples were analyzed using both P-PKSR and SAI, ltd. qualified assays.
Study 2	CIVIT survival plasma only PK, with sampling by retro-orbital bleed (ROB) using Sarstedt 50 uL POCT devices, Dec 2017. When possible, simultaneous ROB and CP upon terminal last sampling obtained. Bioanalysis by P-PKSR, with CP and ROB samples analyzed in duplicate when possible.
Study 3	CIVIT MAST39 tumor bearing PK, Dec 2017. Plasma and tumor homogenate samples analyzed in duplicate by P-PKSR, CP only.
Study 4	CIVIT 2nd survival plasma only PK, with sampling by ROB and terminal CP on last sampling, Jan 2018. Bioanalysis by P-PKSR, with CP and ROB samples analyzed in duplicate when possible.

1.2 Bioanalysis (P-PKSR Assay)

Total plasma and tumor homogenate abemaciclib concentrations were assessed using a sensitive and specific liquid chromatography, tandem mass spectrometry assay. First, tumor samples were diluted with a 5:1 volume of ultrapure water, and homogenized with a bead-based technique [1] on a FastPrep-24 system (MP Biomedicals, Santa Ana, CA). 1.4 mm ceramic spheres (MP Biomedicals, Lysing matrix D, 10 mg per mg of tumor) were added to the microcentrifuge tubes containing samples. The samples were then subjected to three 60 M/S vibratory cycles of 1 min each on the FastPrep-24 system. To prevent

over-heating due to friction, samples were placed on wet ice for 5 min between each cycle. The homogenates were then stored at -80 °C until analysis.

Abemaciclib mesylate (LY2835219, Abmole, M2112, Lot 2, purity >98%) stock solutions corrected for salt content were prepared in acetonitrile and used to spike matrix calibrators and quality controls. Plasma and tumor homogenate samples, 25 µL each, were protein precipitated with 100 µL of 150 ng/mL palbociclib (LC Labs, P-7788, Lot PLH-103, Purity >99%) in acetonitrile as an internal standard. A 2 µL aliquot of the extracted supernatant was injected onto a Shimadzu LC-20ADXR high performance liquid chromatography system via a LEAP CTC PAL autosampler. The LC separation was performed using a Phenomenex Kinetex 2.6 µm EVO C18 (100 Å, 50 x 2.1 mm) maintained at 50 °C with gradient elution at a flow rate of 0.5 mL/min. The binary mobile phase consisted of 20 mM ammonium acetate in H_2O in reservoir A and methanol: acetonitrile: 2-propanol (40:30:30 v/v) in reservoir B. The initial mobile phase was maintained at 25% B for 0.5 minutes followed by a linear increase to 100% B in 2.5 minutes. The column was then rinsed for 2.0 minutes at 100% B and then equilibrated at the initial conditions for two minutes for a total run time of seven minutes. Under these conditions, the analyte and IS eluted at 1.37 and 0.98 minutes, respectively.

Analyte and IS were detected with tandem mass spectrometry using a SCIEX API 5500 Q-TRAP in the positive ESI mode with monitoring of the following mass transitions: abemaciclib 507.28 -> 393.20, palbociclib 448.25 -> 380.10.

The experimental bioanalytical runs were all found to be acceptable for the purpose of a singlicate non-GLP, preclinical PK assessment. A linear model ($1/X^2$ weighting) fit the calibrators across the 5.00 to 500 ng/mL range, with a correlation coefficient (R) of ≥0.9967. The lower limit of quantitation (LLOQ), defined as a peak area signal-to-noise ratio of 5 or greater verses a matrix blank with IS, was 5.00 ng/mL. The intra-run precision and accuracy was < 15.6% CV and 90.7% to 112%, respectively, across the matrices. In experimental runs, multiple preparations of the same sample were often analyzed to better address assay error.

1.3 Bioanalysis (SAI, ltd. Assay)

Tumor homogenate samples were prepared by the P-PKSR using a standardized bead-based technique, and plasma and tumor homogenate experimental samples were then sent to SAI, ltd (Pune, India) in a temperature-controlled shipment. The extraction procedure for plasma samples, tumor homogenate samples and the spiked plasma and tumor homogenate calibration standards were identical: A 15 µL of study sample or spiked calibration standard was added to individual pre-labeled micro-centrifuge tubes followed by 350 µL of internal standard prepared in acetonitrile with 0.1% formic acid (Glipizide, 500 ng/mL) was added except for blank, where 350 µL of 0.1% formic acid in acetonitrile was added. Samples were vortexed for 5 minutes. Samples were centrifuged for 5 minutes at a speed of 4000 rpm at 4 °C. Following centrifugation, 300 µL of clear supernatant was transferred in 96 well plates and analyzed using LC-MS/MS.

A 5 µL aliquot of the extracted supernatant was injected onto a Waters Aquity UPLC system. The UPLC separation was performed using a Waters XSelect CSH,C18, 2.1 X 50 mm, 2.5µm column, maintained at 45 °C with gradient elution at a flow rate of 0.5 mL/min. The binary mobile phase consisted of 0.1% formic acid in acetonitrile in reservoir A and 10 mM ammonium formate in reservoir B. The initial mobile phase was maintained at 90% B for 0.3 minutes followed by a linear increase to 90% A in 0.2 minutes, and maintained for 0.7 minutes. The column was then rinsed for 0.2 minutes at 90% B and then equilibrated at the initial conditions for 0.4 minutes for a total run time of 1.8 minutes. Under these conditions, the analyte and IS (glipizide) eluted at 0.64 and 0.69 minutes, respectively.

Analyte and IS were detected with tandem mass spectrometry using a SCIEX API 4000 in the positive ESI mode with monitoring of the following mass transitions: abemaciclib 507.4 -> 393.3, glipizide 446.3 -> 347.0.

The experimental bioanalytical runs were all found to be acceptable for the purpose of a singlicate non-GLP, preclinical PK assessment. A linear model ($1/X^2$ weighting) fit the calibrators across the 8.18 to 508.33 ng/mL range, with a correlation coefficient (R) of ≥0.9985. The lower limit of quantitation (LLOQ), defined as a peak area signal-to-noise ratio of 5 or greater verses a matrix blank with IS, was 8.18 ng/mL. The intra-run precision and accuracy, derived from the quality control samples, was ≤ 8.61% CV and 86.2% to 108%, respectively across the matrices.

1.4 Pharmacokinetic (PK) Analysis

Abemaciclib plasma and tumor homogenate abemaciclib concentration-time (Ct) were grouped by study, individual mouse, and matrix and analyzed using nonlinear mixed effect (NLME) modeling implemented in Monolix 2019R1 (Lixoft SAS, Antony, France). Parameters and the Fisher Information Matrix (FIM) were estimated using the stochastic approximation expectation maximization (SAEM) algorithm, and the final log-likelihood estimated with importance sampling.

A variety of models were fit to the Ct data, parameterized using apparent clearances or rate constants, volumes of distribution, and absorption rates as needed. The tumor compartment was modeled using an apparent flow rate (Q2) and a partition coefficient (Kp), with the volume fixed to the observed volume upon harvest, or the average if observation was not reported/missing. These models were assessed for goodness of fit using the -2 log likelihood (-2LL) value, Akaike and Bayesian Information Criterion (AIC, BIC), visual predictive checks, plots of model individual and population predicted vs. observed data, residual plots, and the standard errors of parameter estimates. A log-normal inter-individual and inter-occasion distribution was assumed on selected supported parameters, with only diagonal elements of parameter covariance matrices estimated. Additive and/or proportional error models were tested and implemented as supported. Beal's M3 method was used to handle any data that were below the LLOQ [2].

Conditions, including assay applied, specific study number, and the blood sampling site (ROB vs CP) were tested as categorical covariates on supported PK parameters, primarily the apparent volume of distribution (V1). A covariate effect was considered statistically significant if its addition reduced the -2LL by at least 3.84 units ($P < 0.05$, based on the χ^2 test for the difference in the -2LL between two hierarchical models that differ by 1 degree of freedom). Additionally, Wald test P values were outputted for each covariate effect by the software.

2.0 RESULTS

The abemaciclib plasma data was well-described with a 1-compartment, first-order absorption model (ka), parameterized with apparent volume of distribution (V1, L/kg) and a linear elimination rate (k, 1/hr). The plasma was used as a forcing function to drive the RMS orthotopic xenograft tumor PK, parameterized with a highly variable apparent flow (Q2) and a partition coefficient Kp. Apparent plasma clearance was high (70.7 mL/min/kg), approaching murine hepatic blood flow (90 mL/min/kg), and a high apparent volume of distribution was noted. The oral bioavailability of abemacicilb was not assessed in this study; however, it has been reported as ~50% previously in rats. A moderate terminal half-life of ca. 4 hours for plasma and tumor was observed, with tumor concentrations paralleling the plasma. Tumor penetration was high, with a Kp value of 5.15. Inter-individual variability in parameters was moderate-to-high at 31.6-126% CV, while intra-individual or inter-occasion variability was low at 10%. The proportional residual error, which represents the model adequacy and a measure of assay random variability, was also very low at ~4.5%.

The effect of assay, P-PKSR vs SAI, upon V1 was not significant. The effect of sampling site was statistically significant in the model, with ROBs yielding roughly 14% lower concentration results vs. paired CPs. Plasma exposures between Study 1 and Studies 2 and 4 did not differ significantly. There was a statistically significant effect of Study 3 upon V1, which resulted in a ca. 30% lower plasma exposure. The final parameter estimates from the model are presented in **Table 4.1**. The raw Ct data,

with time in hours and concentration in ug/L, are included in the Monolix data file format as **Attached File 5.2**.

In conclusion, there were no practical differences in the plasma or tumor PK of abemaciclib between the reference DBSTP study and the 3 repeated CIVIT studies, nor were there any significant differences in the performance of the P-PKSR or SAI assays. While the CIVIT tumor bearing study demonstrated a slightly lower exposure (-30%) it was on par with the observed inter-individual variability (31.6-33.4% CV), and was well within the 2-fold "rule of thumb" threshold. For all intents it did not practically differ. For an unknown reason, samples obtained by retro-orbital bleed with the Sarstedt 50 uL POCT devices were 14% lower than paired samples via cardiac puncture. These findings show consistent, reproducible results for abemaciclib plasma and RMS OTX tumor PK across two in vivo groups, and as analyzed by two separate qualified LC-MS/MS assays.

3.0 REFERENCES

1. Liang X, Ubhayakar S, Liederer BM, Dean B, Ran-Ran Qin A, Shahidi-Latham S, Deng Y. Evaluation of homogenization techniques for the preparation of mouse tissue samples to support drug discovery. Bioanalysis. 2011 Sep;3(17):1923–33.

2. Beal SL. Ways to fit a PK model with some data below the quantification limit. J Pharmacokinet Pharmacodyn. 2001 Oct;28(5):481–504.

4.0 TABLES, LISTINGS, AND FIGURES (TLFS)

Figure 4.1: Abemaciclib Population Mean (90% PI) Ct Profile: Plasma

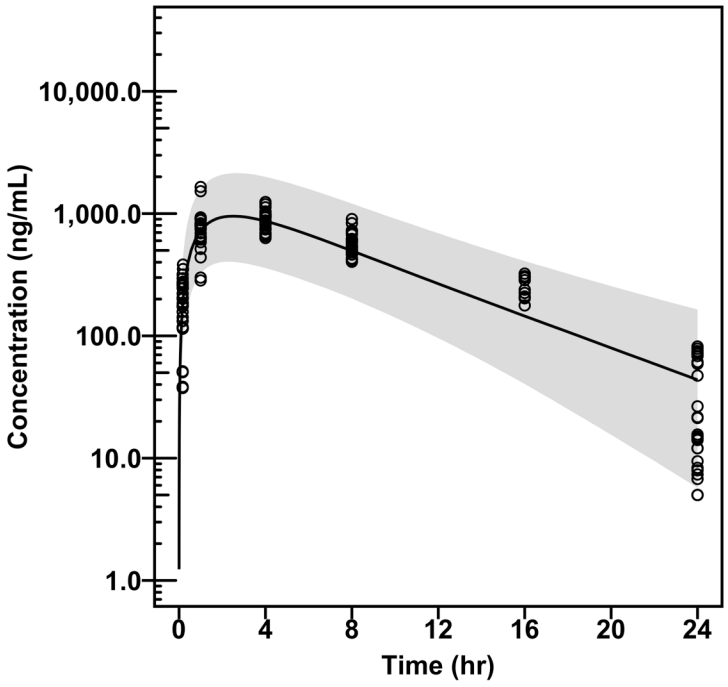

Figure 4.2: Abemaciclib Population Mean (90% PI) Ct Profile: Tumor

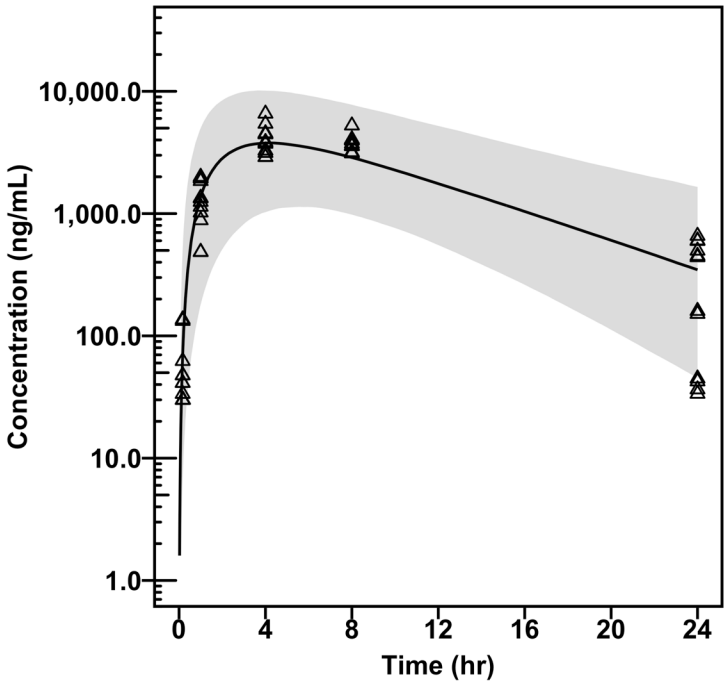

Table 4.1: Abemaciclib Population PK Parameter Estimates

	VALUE	STOCH. APPROX.	
		S.E.	R.S.E.(%)
Fixed Effects			
ka_pop (hr^{-1})	0.153	2.19e-5	0.0144
k_pop (hr^{-1})	0.814	7.28e-6	0.000895
V1_pop (L/kg)	5.32	0.59	11.1
beta_V1_ASSAY_SAI	0.0915	0.0453	49.5
beta_V1_STUDY_2	0.138	0.172	**125**
beta_V1_STUDY_3	0.35	0.163	46.5
beta_V1_STUDY_4	0.212	0.187	**88.6**
beta_V1_WBSITE_ROB	0.13	0.0372	28.5
Q2_pop (L/hr/kg)	0.405	6.65e-5	0.0164
Kp_pop	5.15	0.0374	0.727
Standard Deviation of the Random Effects			
omega_ka	0.327	0.052	15.9
omega_k	0.334	0.0707	21.1
omega_V1	0.316	0.0618	19.5
omega_Q2	1.26	0.252	19.9
omega_Kp	0.258	0.0679	26.3
gamma_V1	0.108	0.00851	7.91

	VALUE	STOCH. APPROX.	
		S.E.	R.S.E.(%)
Error Model Parameters			
b1	0.049	0.00815	16.6
b2	0.0411	0.00937	22.8

5.0 ATTACHED FILES

Attached File 5.1 CofAs and Source Quality Docs.zip – *Information regarding compound and reagent source documentation and quality testing analysis*

Attached File 5.2 abemaciclib_dat.csv – *Complete listing of raw Ct data used in abemeciclib population model in Monolix format. Dose in ug/kg, concentration in ug/L, and time in hr. YTYPE = 1 for plasma, 2 for tumor.*

Attached File 5.3 CIVIT DBSTP PK QUAL Abemaciclib MAST39 2018.pdf – *Overview of abemaciclib PK and BA qualification in supplementary materials format, as originally uploaded to SRM2.*

Attached File 5.4 abemaciclib study information.zip – *In vivo study plans and data collection forms, by study number.*