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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 and St. Jude Children's Research Hospital, 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, 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.

PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program (DBSTP)

P-PKSR Study 63659-604048

STUDY TITLE:

SCREENING PLASMA AND TUMOR PHARMACOKINETICS OF PALBOCICLIB IN FEMALE CD1 NU/NU MICE BEARING RHABDOMYOSARCOMA (SJRHB026) ORTHOTOPIC XENOGRAFTS AFTER A SINGLE ORAL ADMINISTRATION

SHORT TITLE:	**Palbociclib Screening PK in SJRHB026 RMS OTX**
TEST ARTICLE:	Palbociclib Hydrochloride
SECTION:	Nonclinical Pharmacokinetics (Non-GLP)
PRINCIPAL INVESTIGATOR(S)	Stewart, Elizabeth <Elizabeth.Stewart@STJUDE.ORG>
SJCRH SRM2 O/R:	63659-604048 Preclinical Pharmacokinetic Shared Resource
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REPORT STATUS:	FINAL
DATE:	2016-04-19

FOR P-PKSR APPROVED USE AND DISTRIBUTION

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Research Associate
Department of Oncology
Developmental Biology and Solid Tumor Program

4/19/16

Date

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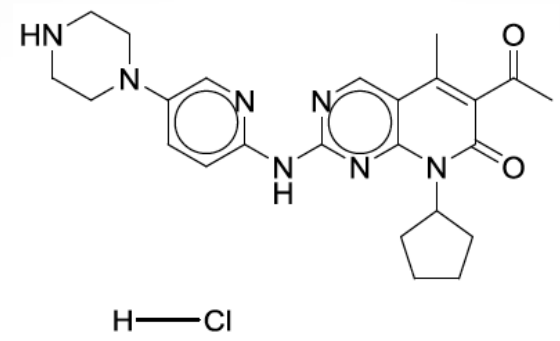
1.0 INTRODUCTION

The CDK4/6 inhibitor palbociclib (Ibrance, Pfizer) is being investigated by the DBSTP (PI Dr. Elizabeth Stewart) for single agent and combination therapies in several pediatric solid tumors. Rhabdomyosarcomas (RHB) may be susceptible to CDK4/6 inhibition, given their mutational profile in RAS and CDKN2A, and the presence of functional pRb protein. As part of the in vivo efficacy evaluation of this compound in murine orthotopic xenograft (OTX) models of pediatric cancer, we have undertaken a discovery-style, screening pharmacokinetic (PK) study of palbociclib, assessing its concentration in plasma and tumors after a single oral dose in OTX-bearing mice. The goals of the PK study are to 1) determine a clinically relevant dosage and exposure for future murine studies, and 2) identify potential blood-tumor barrier issues that could impact compound in vivo efficacy.

2.0 MATERIALS AND METHODS

2.1 Test Article



Compound	Palbociclib Hydrochloride
Molecular Weight	483.08 (447.24 as free base)
SJ REG #	SJ000784215-12
CAS #	827022-32-2
Vendor	MedKoo
Lot #	SSC40317
Exp. Date	NA
Purity	1100% (CBT-HTAC QC) 99.0% (Vendor)

2.2 Formulation

Formulation: Palbociclib HCl in 1% methylcellulose (MC, type 400 cPs) and 1% Tween 80, 1.61 mg/mL free base equivalent final nominal concentration, 10 mL/kg gavage volume, 16.1 mg/kg dosage.

Item	Vendor	Lot #	Exp. Date
MC (400 cPs)	Sigma	MKBK1179V	2017-02
Tween 80	Fisher	111721	2016-05
DDI H_2O	Millipore	NA	NA

The suspension was prepared by William Caufield on 2015-12-01 using a standardized procedure. Briefly, 8.707 mg of palbociclib HCl was weighed and transferred into a 5 mL volumetric flask. The vehicle was then added to the flask slowly with agitation and vortexing. The flask was then indirectly sonicated in a water bath for 30 minutes. The suspension appeared visually homogenous, and was stored in an 8 mL amber glass vial at 4ºC protected from light until use (see **§ 2.4 Dosing**). The study plan called for 17.5 mg/kg palbociclib free base equivalents; however, due to a lack of accounting of the HCl salt component of the compound powder during weighing, a nominal 16.1 mg/kg dose was formulated. The study plan was also incorrect in the vehicle formulation recepie, and was appropriately adjusted for (i.e. 1 mL of Tween80 changed to 0.5 mL in a 50 mL total volume of vehicle).

2.3 Animals

Fifteen (15) female CD1 nu/nu mice (Jax Laboratories), aged 12-16 weeks and weighing from 22.2-32.4 g, bearing orthotopic SJRHB026_X1 tumors in the quadriceps area were used. Mice were permitted standard chow and purified water *ad libitium* during the study, and were housed under SJCRH IACUC-approved animal husbandry conditions.

2.4 Dosing

Animals were dosed with 16.1 mg/kg palbociclib free base equivalents (1.61 mg/mL in 1% MC / 1% Tween 80 at ambient temperature) via a 10 mL/kg oral gavage using a 20 gauge flexible plastic feeding tube (Instech FTP-20-38) attached to a 1 mL syringe. Individual dosages were determined based upon the total body weight of each animal recorded within 24 hours prior to dosing. The calculated volume gavaged in mL was rounded to the nearest hundredth decimal place. Animals were dosed starting at 2015-12-03T09:48:00-06:00 and ending at 2015-12-03T10:30:00-06:00.

2.5 Plasma and Tissue Sample Collection

A serial sacrifice study design was used, whereby each animal provided one sample at one time point upon termination with an IACUC-approved technique. **Table 2.1** below lists the scheduled sampling schema. No individual animals were sampled outside the acceptable window for a nominal scheduled time point, i.e. ± 16.7% of the nominal time relative to dosage. For more information, see **Appendix 6.1. and 6.2.**

Table 2.1 Sample Collection Schedule

Time after dose (hr)	0.167	1	4	8	24
Animal IDs	6026(M1) 6017(M2) 6032(M3)	6018(M4) 6045(M5) 6041(M6)	6040(M7) 6033(M8) 6020(M9)	6042(M10) 6049(M11) 6030(M12)	6036(M13) 6021(M14) 6024(M15)
* Sample time deviation. None reported, 100% sampled at nominal scheduled times					

At each sampling time point, the mouse was anesthetized with 0.3 mL of Avertin (tribromoethanol, 25 mg/mL) by intraperitoneal injection. Then 0.5 – 1 mL of whole blood was collected from an open cardiac puncture using a heparin charged 25 gauge needle attached to a 1 mL syringe and transferred into a plastic microcentrifuge tube (1.5 mL, Fisher cat # 05-408-129) containing ~10 µL of Sodium Heparin anticoagulant (1000 U/mL,Fresenius Kabi 401811I). The blood was immediately centrifuged at ambient temperature for 2 min at 10000 rpm to generate plasma. Each plasma supernatant was transferred into an appropriately labeled microcentrifuge tube, placed on dry ice for remainder study, and transferred to -80ºC until analysis.

After blood collection from each animal, the right ventricle was punctured and the animal was perfused with 10 mL of calcium- and magnesium-free PBS through the left ventricle. The orthotopic xenograft was excised, rinsed with PBS, weighed, and divided into aliquots. The portion submitted for compound bioanalysis was transferred into an appropriately labeled microcentrifuge tube or Falcon tube, placed on dry ice for remainder of study, and transferred to -80ºC until analysis. Total tumor tissue masses ranged from 2.28-7.88 g, with the submitted portions ranging from 0.380-1.10 g.

2.6 Bioanalytical Summary

Matrix samples, 25 µL each, were protein precipitated with 75 µL of 10 ng/mL LEE011 (Cayman Chemical Co., Lot 0467691-2, Purity ≥95%) 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 Gemini C6 Phenyl (3.0 µm, 30 mm x 2.0 mm) column maintained at 60 °C with gradient elution at a flow rate of 0.50

mL/min. The binary mobile phase consisted of water-200 mM ammonium acetate pH 6.0 (90:10 v/v) in reservoir A and acetonitrile-water-200 mM ammonium acetate pH 6.0 (90:10:10 v/v) in reservoir B. The initial mobile phase consisted of 25% B with a linear increase to 65% B in three minutes. The column was then rinsed for two 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 1.36 and 1.04 minutes, respectively.

Analyte and IS were detected with tandem mass spectrometry using a SCIEX API 5500 Q-TRAP in the positive ESI mode with the following mass were transitions monitored: Palbociclib 448.25 -> 380.10, LEE011 435.26 -> 322.20.

The method qualification and bioanalytical runs all passed P-PKSR's acceptance criteria for non-GLP assay performance. A quadratic model ($1/X^2$ weighting) fit the calibrators across the 1 to 500 ng/mL range, with a correlation coefficient (R) of ≥0.9951. 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 1 ng/mL. The intra-run precision and accuracy was < 14.9% CV and 103% to 114%, respectively.

For more information, please refer the bioanalytical method qualification and run report archived by the P-PKSR. NOTE: Bioanalytical validation, qualification, and/or run reports are marked "COMPANY CONFIDENTIAL," and are not for distribution outside SJCRH as per P-PKSR policy.

2.7 Data and Statistical Analyses

The bioanalytical concentration results were processed by run and matrix using Analyst 1.6.1 software (SCIEX, Framingham, MA) and outputted as standardized tab delimited text (.txt) files with MultiQuant 2.1.1 software (SCIEX, Framingham, MA). These .txt files were subsequently processed using R software [1]. The concentrations for analytes were grouped by matrix (plasma or tumor) and nominal sample time, and arithmetic means (Mean) and standard deviations (SD) were generated. If at any time point, ≥ 2/3rds of the results were below the assay LLOQ (BLOQ), then the entire time point was treated as missing. Otherwise, any data BLOQ were replaced with a value of ½ LLOQ, and the concentration Mean and SD values calculated.

2.8 Pharmacokinetic (PK) Analyses

The palbociclib arithmetic mean concentration-time (Ct) data for each matrix was subjected to noncompartmental pharmacokinetic analysis (NCA) using Phoenix WinNonlin 6.4 (Certara USA, Inc., Princeton, NJ). The extravascular model (Model 202) was applied, and area under the Ct curve (AUC) values were estimated using the "linear up log down" method. The terminal phase was defined as at least three time points at the end of the Ct profile, and the elimination rate constant (Ke) was estimated using an unweighted log-linear regression of the terminal phase. The terminal elimination half-life (T1/2) was estimated as 0.693/Ke, and the AUC from time 0 to infinity (AUCinf) was estimated as the AUC to the last time point (AUClast) + Clast/Ke. Other parameters estimated included observed maximum concentration (Cmax), time of Cmax (Tmax), concentration at the last observed time point (Clast), time of Clast (Tlast), apparent clearance (CL/F = Dose/AUCinf), and apparent terminal volume of distribution (Vz/F). The apparent partition coefficient of compound from the plasma to tumor (Kp,tumor) was estimated as the ratio of the AUCinf, tumor to AUCinf plasma when available.

3.0 RESULTS AND DISCUSSION

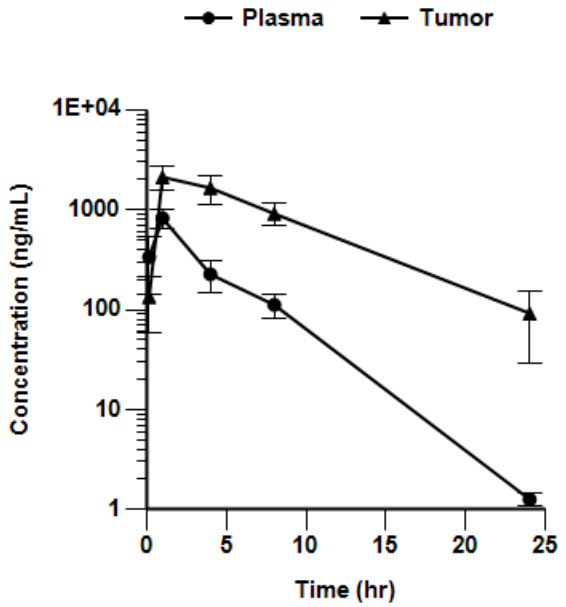
Palbociclib displayed moderate within-study PK variability considering the serial sacrifice design. Concentration coefficients of variation (CV) ranged from 13.5% to 58.4% for plasma, and from 25.3% to 68.5% for tumor. Most variability occurred at the first sampling time point of 10 min, which was during the absorption phase. The apparent plasma clearance (CL/F) estimate of 5.47 L/hr/kg was similar to that derived from previously published literature (4.0 to 5.9 L/hr/kg) [2,3]. The apparent terminal half-life (T1/2)

was slightly shorter compared with literature (2.61 hr vs. approximately 3.4 hr). Palbociclib distributed well into the orthotopic RHB xenograft, achieving concentrations approximately 6–fold higher than the plasma. The T1/2 in the tumor was 1.8-fold longer than the plasma. **Table 3.1** and **Figure 3.1** present the NCA PK parameter estimates and the Mean (SD) Ct profile for the compound, respectively.

Table 3.1 Noncompartmental PK Parameter Estimates

Parameter	Units	Plasma	Tumor
Cmax	ug/L	823	2120
Tmax	hr	1	1
AUClast	hr*ug/L	2940	17300
AUCInf	hr*ug/L	2950	17900
AUC %Extrap	%	0.168	3.55
T1/2	hr	2.61	4.81
CL/F	L/hr/kg	5.47	0.898
Vz/F	L/kg	20.6	6.23
Clast	ug/L	1.26	91.9
Tlast	hr	24	24
Kp,tumor	-	-	6.07

Figure 3.1 Mean (SD) Compound Ct Profile



The common FDA-approved dose for palbociclib is 125 mg PO QD for 21 days followed by 7 days of rest [4]. The following PK and ADME information was obtained from the palbociclib FDA NDA reviews [5,6]. A population PK analysis of palbociclib revealed a mean plasma CL/F of 60.2 L/hr for a typical patient,

resulting in an estimated total plasma AUCtau of 2080 hr-ng/mL at steady state. The reported plasma half-life of palbociclib in patients is 22 to 29 hours. Moreover, after multiple dosing of palbociclib 125 mg PO QD in two clinical studies, the mean total plasma Cmax ranged from 94.9 to 116 ng/mL, and the mean Ctrough was 61 ng/mL. Using standard calculations, the estimated total plasma Cavg,ss in patients is about 85 ng/mL. As there is no appreciable difference in plasma protein binding between mice and humans (Fu,p 0.159 vs 0.147 respectively), total plasma concentrations can be applied to estimate a murine equivalent dose (MED). Assuming dose- and time-linear PK and minimal inter-study PK variability in mice, and based upon the total plasma AUCinf vs the AUCtau in humans, the MED is estimated at 10 mg/kg palbociclib free base equivalents daily, with this formulation under similar study conditions.

4.0 CONCLUSIONS

- Palbociclib demonstrated similar apparent plasma clearance (CL/F) to previously published studies, yet had a shorter terminal half-life (2.61 hr) in this study.
- Palbociclib distributed into the RHB orthotopic xenograft, achieving 6-fold higher concentrations compared with plasma.
- The murine equivalent dose (MED) for palbociclib, based upon total plasma AUCs, is 10 mg/kg free base equivalents daily. This equates to the FDA-approved human dose of 125 mg PO QD.
- Inter-study and inter-animal PK variability is unknown, as are potential PK interaction with planned concomitant therapy (i.e., trametinib). Additional plasma PK studies are recommended.

5.0 REFERENCES

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6. FDA. Drug Approval Package: Ibrance (palbociclib) NDA #207103 Clinical Pharmacology and Biopharmaceutics Reveiw [Internet]. Drugs@FDA. [cited 2016 Mar 14]. Available from: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/207103Orig1s000ClinPharmR.pdf

6.0 RELATED DOCUMENTS

1. BA.VQP.63659-604048_Palbociclib_PLA.pdf – Validation Plan for LC-MS/MS Quantitation of Palbociclib in Mouse Plasma (Tier 3, Qualification)

2. BA.VQP.63659-604048_Palbociclib_TH.pdf – Validation Plan for LC-MS/MS Quantitation of Palbociclib in Tumor Homogenate (Tier 3, Qualification)

3. BA.VQR.63659-604048_Palbociclib_PLA.pdf – Validation Report for for LC-MS/MS Quantitation of Palbociclib in Mouse Plasma (Tier 3, Qualification)

4. BA.VQR.63659-604048_Palbociclib_TH.pdf – Validation Report for for LC-MS/MS Quantitation of Palbociclib in Tumor Homogenate (Tier 3, Qualification)

5. BA.BTM.63659-604048_Palbociclib_PLA_TH.01.pdf – Bioanalytical Test Method for LC-MS/MS Quantitation of Palbociclib in Mouse Plasma and Tumor Homogenate

7.0 APPENDICES

Appendix 7.1 Palbociclib Prelim PK.docx

Murine Pharmacokinetics (PK) of PALBOCICLIB

Client Investigators: Dr. M. Dyer

Date: TBD

Title: Preliminary plasma and RMS tumor PK of oral Palbociclib

Animals: Female CD1 nu mice bearing RMS xenografts. Aged [WKS] weeks at study execution.

Dosages: 17.5 mg/kg PALBOCICLIB by oral gavage, single dose

Formulation: PALBOCICLIB free base equivalents in 1% methylcellulose (Type 400 cPs) and 1% Tween80 (1.75 mg/mL final concentration)

Design: A total of 15 mice will be dosed, each mouse will be sacrificed at the relative time point

Group	Mouse #s	Mouse Ear Tag IDs	Sample Times
1	1-3		0.167 hr
2	4-6		1 hr
3	7-9		4 hr
4	10-12		8 hr
5	13-15		24 hr

Time	0.167 hr	1 hr	4 hr	8 hr	24 hr
Groups	1	2	3	4	5
Mouse #s	1-3	4-6	7-9	10-12	13-15
Planned Sample Time	Day 1 4:00 PM	Day 2 9:00 AM	Day 2 12:00 PM	Day 2 4:00 PM	Day 2 4:00 PM

Summary:

Materials:

- For plasma, two (2) sets of 15 screw-top microcentrifuge tubes, pre-labeled with PALBOCICLIB, group #, mouse #, and nominal time point in hrs. Set #1 is for whole blood collection and spin down, and Set #2 is for plasma supernatant and freezing
- Two (2) additional sets of 15 screw-top microcentrifuge tubes (one for RMS tumor and one for whole brain), prelabled with PALBOCICLIB, group #, mouse #, and nominal time point in hrs
- Methylcellulose (Type 400 cPs), Tween80, ultrapure water, PBS for flushing mice
- ~10 mg of PALBOCICLIB free base equivalents
- Mouse gavage needle and 1 mL syringes for PO administration
- Heparin 1000 u/mL in a tube, needles and TB/insulin syringes for cardiac punctures
- Centrifuge (10000g) w/ microcentrifuge rotor (4°C preferred, but room temp. will suffice)
- Container of wet ice
- Styrofoam cooler with labeled cardboard vial box and **dry** ice

Procedure:

1. The day before the study, sort mice into groups, 3 mice per cage with 5 cages and perform weighing. Tattoo tails for identification, or refer to mouse ear tag numbers. Label cages with group number, mouse numbers, and nominal time points. Each tattooed stripe represents the number of mouse in the cage's sequence. For example, the mouse with 1 stripe in Cage/Group 2 would be mouse #4 and the mouse with 2 stripes would be mouse #5, and so forth. Weigh each mouse, record weight in grams on the Study Worksheet, and calculate planned doses in mL.

2. The day before the study, compound the oral vehicle [1% w/v methylcellulose (type 400 cPs) and 1% v/v Tween80 in sterile water for injection or ultrapure water]

Murine Pharmacokinetics (PK) of PALBOCICLIB

Client Investigators: Dr. M. Dyer

a. Prepare 1% (w/v) MC solution, 49 mL
    i. Heat 17 mL of SWI or UP water to 80°C.
    ii. Add 500 mg of MC to the hot water with ample agitation and mixing
    iii. Agitate the mixture until the particles are thoroughly wetted and evenly dispersed.
    iv. Add ~32 mL of cold UP water (QS to 50 mL) under continued agitation. Solution should be cooled to 0-5°C for 20-40 min. under continued agitation.
    v. Agitation should continue for at least 30 min. after proper temperature is reached.
b. To the 49 mL of the 1% MC solution, add 1 mL of Tween80 by pipette. Shake or mix gently until the Tween80 is dispersed, and avoid bubbling.

3. The day before or the morning of the study, formulate PALBOCICLIB in vehicle as a suspension for oral gavage (1.75 mg/mL, 0.25 mL for a 25 g mouse = 17.5 mg/kg)
    a. To 8.75 mg of free base equivalents of PALBOCICLIB, add vehicle QS to 5 mL. Pipette and gently shake/stir to wet the PALBOCICLIB.
    b. Vortex and/or sonicate for up to 30 min. to ensure a homogenous suspension. Store at 4°C. Immediately prior to administration, vortex and check for visual homogeneity.

4. Execute in vivo study according to the Study Worksheet
    a. NOTE: All actual times for dosing and samples should be referenced to the same study clock.
    b. Dose mice by PO gavage; record the actual dose volume administered in mL and the actual times of administration.
    c. At each terminal time point, collect the blood sample by the indicated means and record the actual sample time (from the start of the collection), and make notes of any issues.
        i. R: Retro-orbital bleed – Anesthetize the mouse per ACUC protocol. If necessary, heparinize the Pasture pipette by allowing a small volume to travel up column by capillary motion, then flick excess out. Proceed to collect 500-1000 uL of whole blood from the retro-orbital plexus. Place blood into appropriate pre-labeled tube from Set #1. All samples should be processed to plasma ASAP, but if necessary, put on wet ice until centrifugation.
        ii. T: Terminal cardiac puncture – Anesthetize the mouse per IACUC protocol. Heparinize the needle and syringe by drawing residual volume of heparin into syringe. Proceed to collect 500-1000 uL of whole blood from aorta. Place blood into appropriate pre-labeled tube from Set #1. All samples should be processed to plasma ASAP, but if necessary, put on wet ice until centrifugation.
    d. Centrifuge the whole blood samples at 10000g for 2 min. to generate plasma.
    e. Remove plasma supernatant, place in appropriate pre-labeled tube from Set #2; place in vial box in cooler on dry ice and transfer to -80°C as soon as possible.
    f. Perfuse animal with PBS or equivalent to flush blood from vasculature.
    g. Extract RMS tumor and rinse with PBS, place in microcentrifuge tube in cooler on dry ice and transfer to -80°C as soon as possible.

Palbociclib Screening PK in SJRHB026 RMS OTX

Appendix 7.2 Palbociclib PK Study Sheet.xlsx

				Palbociclib	PK Study			
ET#	Time point	Mouse #	Mouse weight	Mouse dose	Dose Time	Harvest Date/Time	Total tumor (g)	Tumor wt in tube (g)
6026	10 min	1	22.2	0.22	9:48AM	9:58AM	3.95	0.86
6017	10 min	2	25.6	0.26	9:59AM	10:09AM	6.08	0.51
6032	10 min	3	27.8	0.28	10:07AM	10:17AM	7.88	0.75
6018	1 hour	4	24.53	0.25	10:15AM	11:15AM	5.26	0.5
6045	1 hour	5	29.27	0.29	10:22AM	11:22AM	5.57	1.1
6041	1 hour	6	27.68	0.28	10:29AM	11:29AM	4.26	0.8
6040	4 hour	7	27.37	0.27	10:18AM	2:18PM	5.28	0.47
6033	4 hour	8	29.68	0.3	10:24AM	2:24PM	3.46	0.48
6020	4 hour	9	26.03	0.26	10:30AM	2:30PM	2.47	0.44
6042	8 hour	10	32.4	0.32	8:57AM	4:57PM	2.83	0.43
6049	8 hour	11	29.12	0.29	8:58AM	4:58PM	5.1	0.69
6030	8 hour	12	25.56	0.26	9:00AM	5:00PM	2.57	0.4
6036	24 hour	13	26.54	0.27	10:20AM	10:20AM (12/4/15)	2.78	0.38
6021	24 hour	14	25.21	0.25	10:23AM	10:23AM (12/4/15)	2.28	0.59
6024	24 hour	15	28.33	0.28	10:25AM	10:25AM (12/4/15)	2.86	0.6

Date: 12/3/15 - 12/4/15
CD-1 Nude Female
RMS: SJRHB026_X1 tumor bearing mice
Drug formulated by William Caufield
Study performed by Beth Stewart, Rosa Nguyen, Victoria Honnell, Monica Ocarz