PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program

P-PKSR Study 186813 - 1936416

STUDY TITLE:

SCREENING PLASMA AND TUMOR PHARMACOKINETICS (SPTPK) OF AFATINIB IN FEMALE ATHYMIC NUDE MICE AFTER A SINGLE ORAL DOSE

SHORT TITLE:	**Afatinib Screening Plasma Tumor PK (SPTPK)**
TEST ARTICLE:	Afatinib (as dimaleate salt)
SECTION:	Nonclinical Pharmacokinetics (Non-GLP)
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SJCRH SRM2 O/R:	186813-1936416 Preclinical Pharmacokinetic Shared Resource
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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) STUDY

The plasma and tumor pharmacokinetic (PK) profile of afatinib was evaluated in female Athymic Nude mice (Charles River) approximately 12 weeks in age bearing orthotopic rhabdomyosarcoma (RMS) MAST39 tumors. Afatinib dimaleate (SJ000855122, OCHEM, CAT# 140A737, LOT# 150318G1) was suspended in 1% Methylcellulose (type 400 cPs) / 1% Tween 80 in ultrapure water, at 3 mg/mL for a 30 mg/kg free base equivalent dose as a 10 mL/kg oral gavage. Terminal samples were collected over a 16 hour post-dose period by cardiac puncture using a 1 mL syringe, and the blood placed in a Sarstedt Microvette K3EDTA 500 µL tube, and immediately separated to plasma. The carcass was then perfused with PBS, the tumor extracted, rinsed, and placed in a microcentrifuge tube. All samples were immediately stored on dry ice and transferred to -80 °C until analysis.

1.2 Bioanalysis

Tumor samples were weighed in 15 mL Lysing matrix D (MP Biomedicals, Santa Ana, CA), diluted with a 1:5 volume of ultrapure water, and homogenized using a FastPrep-24 system (MP Biomedicals, Santa Ana, CA) for five cycles of 1 min vibration at 6.5 M/S speed, with 5 min in ice bath between each cycle to prevent over-heating. The homogenates were then stored at -80 °C until analysis.

Plasma and tumor samples were analyzed for afatinib (dimaleate salt, SJ000855122, OCHEM, CAT# 140A737, LOT# 150318G1) using a qualified liquid chromatography – tandem mass spectrometry (LC-MS/MS) assay. Plasma and tumor homogenate calibrators and quality controls were spiked with solutions, corrected for salt content and purity, prepared in methanol. Matrix samples, 25 µL each, were protein precipitated with 100 µL of 10 mM ammonium acetate in water-acetonitrile (5:95 v/v, pH 6.8) and 25 µL of 20 ng/mL erlotinib (hydrochloride salt, SJ000312295, LC Laboratories, CAT# E-4007, LOT# BBE-106) in methanol as internal standard (IS). A 5 µL aliquot of the extracted supernatant was injected onto a SCIEX ExionLC high performance liquid chromatography system via a SCIEX ExionLC AC autosampler. The LC separation was performed using a Waters XBridge BEH C18 (2.5 µm, 75 mm x 2.1 mm) XP column maintained at 40 °C with gradient elution at a flow rate of 0.25 mL/min. The binary mobile phase consisted of 10 mM ammonium acetate in water-acetonitrile (90:10 v/v, pH 6.8) in reservoir A and 10 mM ammonium acetate in water-acetonitrile (5:95 v/v, pH 6.8) in reservoir B. The initial mobile phase consisted of 10% B for 0.5 min with a linear increase to 100% B in 2 min. The column was then rinsed for 1 min at 100% B and then equilibrated at the initial conditions for 1.5 min for a total run time of 5 min. Under these conditions, the analyte and IS eluted at 3.45 and 3.76 min, respectively.

Analyte and IS were detected with tandem mass spectrometry using a SCIEX Triple Quad 3500 in the positive ESI mode and the following mass transitions were monitored: afatinib 486.1 -> 371.2, erlotinib 394.3 -> 278.2. The method qualification and bioanalytical runs all passed acceptance criteria for non-GLP assay performance. A linear model ($1/X^2$ weighting) fit the plasma calibrators across the 1 to 250 ng/mL range, with a correlation coefficient (R) of ≥ 0.9961. A quadratic model ($1/X^2$ weighting) fit the tumor homogenate calibrators across the 1 to 250 ng/mL range, with a correlation coefficient (R) of ≥ 0.9959. 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 for both plasma and tumor homogenate. Sample dilution integrity was confirmed. The intra-run precision and accuracy was ≤ 10.13% CV and 88.3% to 111%, respectively.

1.3 Pharmacokinetic (PK) Analysis

Afatinib concentration-time (Ct) data were grouped by matrix and nominal time point. Manual imputation of data below the lower limit of quantitation (BLOQ) was as follows: IF at any time point ≥ 2/3rds of the Ct results were above the LLOQ, the BLOQ data were replaced with a value of ½ LLOQ, ELSE the entire time point's data were treated as missing. Summary statistics were calculated and the arithmetic mean Ct

values were subjected to noncompartmental analysis (NCA) using Phoenix WinNonlin 8.1 (Certara USA, Inc., Princeton, NJ). The extravascular model 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 (Kel) was estimated using an unweighted log-linear regression of the terminal phase. The terminal elimination half-life (T1/2) was estimated as 0.693/Kel, and the AUC from time 0 to infinity (AUCinf) was estimated as the AUC to the last time point (AUClast) + Clast (predicted)/Kel. 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 plasma-to-tumor partition coefficient (Kp,inf) was estimated as the ratio of the AUCinf in tissue to AUCinf plasma, whereas Kp,last was similarly estimated using AUClast values.

A clinically relevant dose (CRD) for mice was estimated from unbound plasma PK and exposure. The CRD was defined as the mouse dose achieving a predicted mean steady state unbound plasma AUC (AUCu) similar to humans at the single agent maximum tolerated dose (MTD), recommended Phase II dose (RP2D), or FDA-approved dose. Dose proportional, linear, and time-invariant PK across species was assumed. Human and mouse plasma protein binding were assumed similar when data were not available. This is similar to the clinical relevance approach proposed by Spilker [1] which uses unbound plasma average steady state concentrations. Some latitude in dose rounding was permitted in the CRD recommendation, and an unbound exposure within 2-fold of the clinical target was considered acceptable. Additional considerations influenced the final recommended mouse dose, including mouse dosing regimens prevalent in the literature and the tolerability of the compound in mice.

2.0 RESULTS

The afatinib Ct data demonstrated high variability between mice for plasma and tumor, with coefficients of variation ranging from 7.04% to 72.1%. Most of the variability was seen during the putative absorption phase. One plasma concentration result (M10, PLA, 8 hr) was excluded from statistics and NCA, as it was inconsistent with the overall Ct profile across mice and deemed an outlier. The absorption rate of afatinib was relatively slow, with the Tmax occurring at 4 hours post-dose for both plasma and tumor. After Cmax, plasma and tumor concentrations diminished in a mono-exponential manner. The apparent terminal half-life of afatinib was 1.93 hours for plasma, and 3.75 hours for tumor. The apparent clearance (CL/F) of afatinib was high at 186.67 mL/min/kg for plasma, or approximately 2-fold in excess of murine hepatic blood flow. The apparent terminal volume of distribution (Vz/F) for afatinib in plasma was also high at 31.3 L/kg for plasma. The tumor penetration was high, with a Kp,inf of 5.45, and Kp,last of 5.07. Compared with our previous screening plasma PK study at the same dose (RPT.186809-1936319), the plasma AUCinf in the current study is about 35% higher, and the plasma apparent terminal half-life is shorter by 25%.

In clinical studies, the total plasma AUC of afatinib at steady state was reported as 908 hr-ng/mL with the dose of 40 mg PO QD [2]. The fraction unbound in plasma (Fu,p) for afatinib is similar for humans and mice, and estimated at 0.05 and 0.057 respectively [4]. From this current study's results, the precise CRD for mice calculated by unbound AUCs is afatinib 8.95 mg/kg PO QD. Given our overall afatinib PK findings, doses of 7.5 to 30 mg/kg are clinically relevant by our 2-fold exposure criteria. Afatinib 15 mg/kg PO QD – a commonly used effective dose in mice [3]– is recommended.

3.0 REFERENCES

1. Spilker ME, Chen X, Visswanathan R, Vage C, Yamazaki S, Li G, Lucas J, Bradshaw-Pierce EL, Vicini P. Found in Translation: Maximizing the Clinical Relevance of Nonclinical Oncology Studies. Clin Cancer Res. 2017 Feb 15;23(4):1080–90.

2. Wind S, Schnell D, Ebner T, Freiwald M, Stopfer P. Clinical Pharmacokinetics and Pharmacodynamics of Afatinib. Clin Pharmacokinet. 2017 Mar 1;56(3):235–50.

3. Zhang S, Zhu L, Jiang Y, Zhang J, Xu R, Xu Y, Xia B, Ma S. Efficacy of afatinib, an irreversible ErbB family blocker, in the treatment of intracerebral metastases of non-small cell lung cancer in mice. Acta Pharmacol Sin. 2017 Feb;38(2):233–40.

4.0 TABLES, LISTINGS, AND FIGURES (TLFS)

Figure 4.1: Mean (SD) Ct Profiles by Analyte and Group

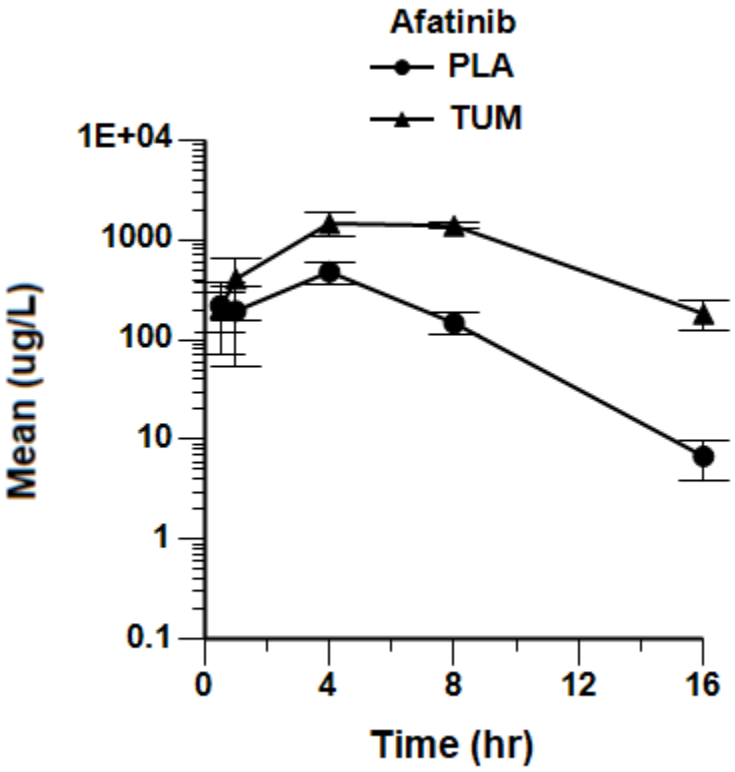

Table 4.1: NCA Parameter Estimates by Analyte and Group

		Analyte	
		Afatinib	
		Group	
		PLA	TUM
Parameter	Unit	Value	
AUCinf	hr*ug/L	2670	14600
AUClast	hr*ug/L	2650	13400
CL/F	L/hr/kg	11.2	2.06
Clast	ug/L	6.81	183
Cmax	ug/L	478	1460
Kel	1/hr	0.359	0.185
Kp_inf			5.45
Kp_last			5.07
T1/2	hr	1.93	3.75
Tlast	hr	16.0	16.0

		Analyte	
		Afatinib	
		Group	
		PLA	TUM
Parameter	Unit	Value	
Tmax	hr	4.00	4.00
Vz/F	L/kg	31.3	11.1

Table 4.2: Full Summary Statistics of Ct Data by Analyte and Group

		Analyte	
		Afatinib	
		Group	
		PLA	TUM
Time (hr)		Concentration (ug/L)	
0.500	N	3	3
	Mean	219	207
	SD	149	86.4
	Min	78.6	125
	Median	204	199
	Max	375	297
	CV%	67.8	41.8
	Geometric Mean	182	194
	CV% Geometric Mean	92.8	45.6
1.00	N	3	3
	Mean	194	406
	SD	140	251
	Min	47.1	136
	Median	209	450
	Max	325	632
	CV%	72.1	61.7
	Geometric Mean	147	339
	CV% Geometric Mean	134	95.6
4.00	N	3	3
	Mean	478	1460
	SD	114	377
	Min	354	1160
	Median	503	1340
	Max	577	1880
	CV%	23.8	25.8
	Geometric Mean	468	1430
	CV% Geometric Mean	25.6	25.3
8.00	N	2	3
	Mean	147	1390
	SD	36.6	97.7

		Analyte	
		Afatinib	
		Group	
		PLA	TUM
Time (hr)		Concentration (ug/L)	
	Min	121	1290
	Median	147	1380
	Max	173	1490
	CV%	24.9	7.04
	Geometric Mean	145	1380
	CV% Geometric Mean	25.6	7.05
16.0	N	3	3
	Mean	6.81	183
	SD	2.91	61.0
	Min	3.51	113
	Median	7.87	207
	Max	9.05	228
	CV%	42.8	33.4
	Geometric Mean	6.30	175
	CV% Geometric Mean	54.6	39.3

Table 4.3: Ct Data Listings by Subject, Analyte, Group, and Time

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M1	Afatinib	PLA	0.500	204.40
M1	Afatinib	TUM	0.500	198.55
M2	Afatinib	PLA	0.500	78.60
M2	Afatinib	TUM	0.500	124.64
M3	Afatinib	PLA	0.500	375.15
M3	Afatinib	TUM	0.500	296.94
M4	Afatinib	PLA	1.00	325.36
M4	Afatinib	TUM	1.00	632.06
M5	Afatinib	PLA	1.00	47.11
M5	Afatinib	TUM	1.00	136.30
M6	Afatinib	PLA	1.00	208.59
M6	Afatinib	TUM	1.00	450.33
M7	Afatinib	PLA	4.00	576.96
M7	Afatinib	TUM	4.00	1337.80
M8	Afatinib	PLA	4.00	353.87
M8	Afatinib	TUM	4.00	1158.00
M9	Afatinib	PLA	4.00	503.26
M9	Afatinib	TUM	4.00	1882.40
M10	Afatinib	PLA	8.00	*3702.40

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M10	Afatinib	TUM	8.00	1383.10
M11	Afatinib	PLA	8.00	173.01
M11	Afatinib	TUM	8.00	1291.50
M12	Afatinib	PLA	8.00	121.18
M12	Afatinib	TUM	8.00	1486.70
M13	Afatinib	PLA	16.0	9.05
M13	Afatinib	TUM	16.0	227.73
M14	Afatinib	PLA	16.0	7.87
M14	Afatinib	TUM	16.0	206.90
M15	Afatinib	PLA	16.0	3.51
M15	Afatinib	TUM	16.0	113.13

* Result excluded from statistics and NCA

Table 4.4: Ct Summary (Mean, SD, N) by Analyte and Group

Analyte	Group	Time (hr)	Mean (ug/L)	SD (ug/L)	N
Afatinib	PLA	0.500	219	149	3
Afatinib	PLA	1.00	194	140	3
Afatinib	PLA	4.00	478	114	3
Afatinib	PLA	8.00	147	36.6	2
Afatinib	PLA	16.0	6.81	2.91	3
Afatinib	TUM	0.500	207	86.4	3
Afatinib	TUM	1.00	406	251	3
Afatinib	TUM	4.00	1460	377	3
Afatinib	TUM	8.00	1390	97.7	3
Afatinib	TUM	16.0	183	61.0	3

5.0 ATTACHED FILES

Attached File 5.1 Afatinib Screening Plasma Tumor PK V2.0.docx – *Final in vivo study plan as executed*

Attached File 5.2 Afatinib Screening Plasma Tumor PK TLFs.docx – *Report TLFs as a Word document for manipulation, plotting, and further presentation*

Attached File 5.3 Copy of 186813-1936416_AFA_SPTPK_2020-10-26.xlsx – *Digital data collection form*

Attached File 5.4 Afatinib TB PK Study Sheet.jpg – *Photocopy of original paper data collection form*