P-PKSR
Preclinical
Pharmacokinetics

PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program

P-PKSR Study 830648 - 3159863

STUDY TITLE:

SCREENING PLASMA PHARMACOKINETICS (SPPK) OF DEFACTINIB IN FEMALE ATHYMIC NUDE MICE AFTER A SINGLE ORAL DOSE

SHORT TITLE:	Defactinib Screening Plasma PK (SPPK)
TEST ARTICLE:	Defactinib (as hydrochloride salt) (VS-6063; PF-04554878)
SECTION:	Nonclinical Pharmacokinetics (Non-GLP)
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REPORT STATUS:	FINAL
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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 pharmacokinetic (PK) profile of the FAK inhibitor defactinib was evaluated in female athymic nude mice (Charles River), approximately 12 weeks in age. Defactinib hydrochloride (SJ000981038-10, MedChemExpress, Cat# HY-12289A, Lot# 180406, purity 98.03%) was suspended in 0.5% sodium carboxymethylcellulose (NaCMC) / 0.1% polysorbate 80 (PS80) in ultrapure water (UP H2O), 5 mg/mL for a 5 mL/kg oral gavage, yielding a 25 mg/kg dose. Three survival blood samples were obtained from each mouse via retro-orbital plexus using 70 µL glass microhematocrit capillary tubes (Fisherbrand, Cat 22362574), and a fourth final sample by cardiac puncture, all using KEDTA as the anticoagulant. Samples were obtained at various times up to 24 hours post-dose, immediately processed to plasma, and stored at -80 °C until analysis. Remaining dosing suspension was submitted at 4 °C for the verification of potency, and chemical and physical stability during the study period.

1.2 Bioanalysis (BA)

Plasma samples were analyzed for defactinib (SJ000981038-2, Medkoo Biosciences, CAT # 205583, LOT # A7T12K13, Purity 98%) using a qualified liquid chromatography – tandem mass spectrometry (LCMS/MS) assay. Plasma calibrators and quality controls were spiked with solutions, corrected for salt content and purity as necessary, prepared in methanol. Plasma samples, 25 µL each, were protein precipitated with 115 µL of 5 ng/mL VS-4718 (MedChemExpress, CAT# HY-13917, LOT# 16765, Purity 98.85%) in acetonitrile-methanol (90:10, v/v) as an 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 autosampler. The LC separation was performed using a Phenomenex Kinetex F5 (2.6 µm, 50 mm x 2.1 mm) column maintained at 50 °C with gradient elution at a flow rate of 0.4 mL/min. The binary mobile phase consisted of water-formic acid (100:0.1 v/v) in reservoir A and acetonitrile-formic acid (100:0.1 v/v) in reservoir B. The initial mobile phase consisted of 5% B for 0.5 min with a linear increase to 95% B in 1.7 min. The column was then rinsed for 1.3 min at 95% B and returned to initial mobile phase conditions in 0.3 mins, then equilibrated the column for another 1.2 min for a total run time of 5.0 min. Under these conditions, the analyte and IS eluted at 2.52 and 2.44 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: defactinib 511.1 → 312.0, VS-4718 502.2 → 471.2. The method qualification and bioanalytical runs all passed acceptance criteria for non-GLP assay performance. A linear model ($1/X_2$ weighting) fits the calibrators across the 0.5 to 250 ng/mL range, with a correlation coefficient (R) of ≥ 0.9972. 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 0.5 ng/mL. Sample dilution integrity was confirmed. No significant matrix effects, ion enhancement or suppression, were detected in blank EDTA plasma harvested from untreated female athymic nude mice or in tumor homogenates. The intra-run precision and accuracy was ≤ 5.11% CV and 93.0% to 104.53%, respectively.

1.3 Pharmacokinetic (PK) Analysis

Plasma concentration-time (Ct) data for defactinib were grouped by 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).

Defactinib fractions unbound in mouse and human plasma (Fu,p,m and Fu,p,h) were determined using a HTD-96B equilibrium dialysis device (12-14K MWCO, HTDialysis LLC, Gales Ferry, CT). Briefly, blank plasma was spiked with compound at a final concentration of 5 µM. The spiked plasma was placed in one side of the membrane on the HTD-96B plate in sextuplicates, dialyzed against equal volume of the 100 mM PBS (pH 7.4), and permitted to equilibrate for 6 hours at 37 °C with 5% CO2. Compound concentrations were assayed on both side of the dialysis membrane using the qualified LC-MS/MS assay, with the fraction unbound calculated as the ratio of concentration in receiver to donor, adjusted for any dilution if necessary [1].

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 [2] 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

Defactinib plasma Ct data showed high variability in our mice, with coefficients of variation (CVs) of up to 112%. For the terminal time points, there was notable consistency between the retro-orbital and cardiac puncture results (R = 0.999), with one exception of a retro-orbital high-end outlier (M3, 16-hr). For consistency, only the retro-orbital data was used in PK analyses. Generally, plasma volumes from the retro-orbital bleeds were less than the required 25 µL; therefore, many samples required dilution with blank mouse plasma to bring up to minimal volume for analysis. As sample dilution integrity was verified in the method qualification, this should not have negatively impacted the results.

The absorption rate of defactinib was rapid with the Tmax occurring at 0.25 hours post-dose. After Cmax, plasma concentrations diminished in a nearly bi-exponential manner, with observations at 16-hr and 24-hr time points being BLOQ. Rising concentrations were noted at the 8-hr time point, which precluded an accurate terminal phase regression. As such, terminal phase parameters could not be estimated (T1/2, CL/F, Vz/F). The oral bioavailability (F) was not assessed in this study and remains unknown in mice. The formulation met specification (4.98 ± 0.192 mg/mL with %CV ≤ 3.85) and was stable for at least 8 days when stored at 4 °C and protected from light. Defactinib plasma protein binding was higher in human plasma by 1.6-fold. Fraction unbound values were 0.0437 ± 0.00178 vs 0.0706 ± 0.00149 for humans and mice, respectively.

Currently, the RP2D for defactinib in combination with the RAF-MEK clamp avutometinib in adults is 200 mg PO BID for 3 of 4 weeks [3,4], yielding a mean plasma AUCtau of 2150 to 6240 hr-ug/L at steady state [5,6]. Notably, defactinib plasma exposures in patients are variable and fail to increase with doses of 300 mg and beyond, most likely due to solubility-limited or saturable absorption. Given our current PK results and plasma protein binding, a CRD for mice would be estimated as 120 to 350 mg/kg, which is orders of magnitude higher than the doses reported in mice to date [7–9]. Such doses are also inconsistent with standard weight-based allometry, which assumes similar bioavailability, protein binding, and metabolism between species. Paradis et al. noted that an alternate FAK inhibitor VS-4718 has better PK properties than defactinib in mice, but did not elaborate on how so or why [10]. It's unclear why the PK

of defactinib is unfavorable in mice, but it could be due to poor solubility or an inadequate formulation, saturable absorption, or high first-pass extraction in gut and liver. It is recommended that an alternate published formulation be trialed (5-10% DMSO / 5% Gelucire 44/14 / 90% UP H2O) [8,11], and plasma PK reassessed.

3.0 REFERENCES

1. Romer J, Bickel MH. A method to estimate binding constants at variable protein concentrations. J Pharm Pharmacol. 1979;31(1):7–11.
2. 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.
3. Banerjee SN, Ring KL, Van Nieuwenhuysen E, Fabbro M, Aghajanian C, Oaknin A, Colombo N, Santin A, Clamp AR, Moore KN, Rose PG, O'Malley DM, Chon HS, Salinas EA, Prendergast EN, Lustgarten S, Rodrigues M, Gennigens C, Monk BJ, Grisham RN. Initial efficacy and safety results from ENGOT-ov60/GOG-3052/RAMP 201: A phase 2 study of avutometinib (VS-6766) ± defactinib in recurrent low-grade serous ovarian cancer (LGSOC). J Clin Oncol. 2023 Jun;41(16_suppl):5515–5515.
4. Reuss JE, Gandhi SG, Spigel DR, Janne PA, Paz-Ares LG, Gadgeel SM, Patel JD, Passiglia F, Spira AI, Edelman MJ, Blumenschein GR, Shergill A, Burns TF, Bhambhani V, Patrick G, Pachter JA, Denis LJ, Camidge DR. RAMP 202: A phase 2 study of avutometinib (VS-6766) ± defactinib, in patients with advanced KRAS G12V mutant non–small cell lung cancer (NSCLC). J Clin Oncol. 2023 Jun;41(16_suppl):9100–9100.
5. Shimizu T, Fukuoka K, Takeda M, Iwasa T, Yoshida T, Horobin J, Keegan M, Vaickus L, Chavan A, Padval M, Nakagawa K. A first-in-Asian phase 1 study to evaluate safety, pharmacokinetics and clinical activity of VS-6063, a focal adhesion kinase (FAK) inhibitor in Japanese patients with advanced solid tumors. Cancer Chemother Pharmacol. 2016 May 1;77(5):997–1003.
6. Jones SF, Siu LL, Bendell JC, Cleary JM, Razak ARA, Infante JR, Pandya SS, Bedard PL, Pierce KJ, Houk B, Roberts WG, Shreeve SM, Shapiro GI. A phase I study of VS-6063, a second-generation focal adhesion kinase inhibitor, in patients with advanced solid tumors. Invest New Drugs. 2015 Oct 1;33(5):1100–7.
7. Kang Y, Hu W, Ivan C, Dalton HJ, Miyake T, Pecot CV, Zand B, Liu T, Huang J, Jennings NB, Rupaimoole R, Taylor M, Pradeep S, Wu SY, Lu C, Wen Y, Huang J, Liu J, Sood AK. Role of Focal Adhesion Kinase in Regulating YB–1–Mediated Paclitaxel Resistance in Ovarian Cancer. JNCI J Natl Cancer Inst. 2013 Oct 2;105(19):1485–95.
8. Lin HM, Lee BY, Castillo L, Spielman C, Grogan J, Yeung NK, Kench JG, Stricker PD, Haynes AM, Centenera MM, Butler LM, Shreeve SM, Horvath LG, Daly RJ. Effect of FAK inhibitor VS-6063 (defactinib) on docetaxel efficacy in prostate cancer. The Prostate. 2018;78(4):308–17.
9. Hallur G, Tamizharasan N, Sulochana SP, Saini NK, Zainuddin M, Mullangi R. LC-ESI–MS/MS determination of defactinib, a novel FAK inhibitor in mice plasma and its application to a pharmacokinetic study in mice. J Pharm Biomed Anal. 2018 Feb 5;149:358–64.
10. Paradis JS, Acosta M, Saddawi-Konefka R, Kishore A, Lubrano S, Gomes F, Arang N, Tiago M, Coma S, Wu X, Ford K, Day CP, Merlino G, Mali P, Pachter JA, Sato T, Aplin AE, Gutkind JS. Synthetic Lethal Screens Reveal Cotargeting FAK and MEK as a Multimodal Precision Therapy for GNAQ-Driven Uveal Melanoma. Clin Cancer Res. 2021 Jun 1;27(11):3190–200.

11. Kessler BE, Sharma V, Zhou Q, Jing X, Pike LA, Kerege AA, Sams SB, Schweppe RE. FAK Expression, Not Kinase Activity, is a Key Mediator of Thyroid Tumorigenesis and Pro-tumorigenic Processes. Mol Cancer Res MCR. 2016 Sep;14(9):869–82.

4.0 TABLES, LISTINGS, AND FIGURES (TLFS)

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

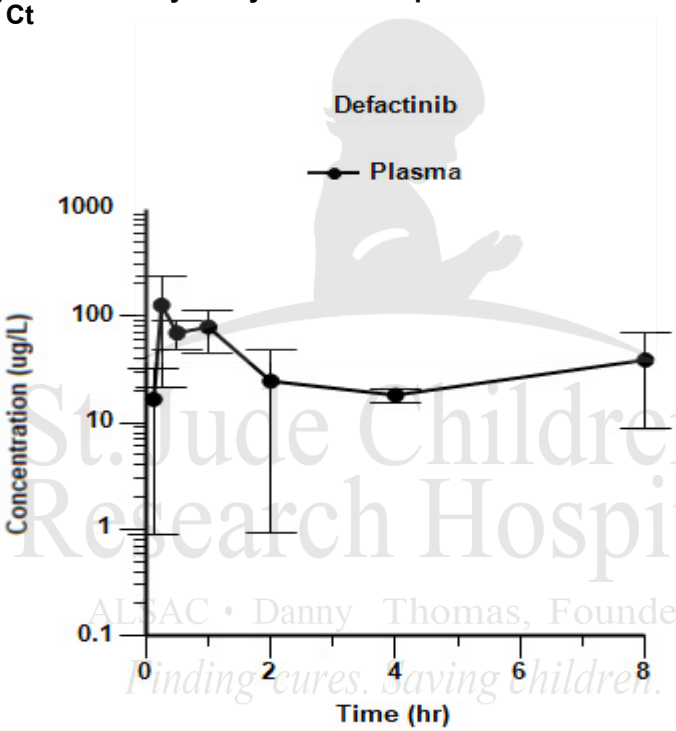

Table 4.1: NCA Parameter Estimates by Analyte and Group

		Analyte
		Defactinib
		Group
		Plasma
Parameter	Units	Estimate
Cmax	ug/L	126
Tmax	hr	0.250
AUClast	hr*ug/L	273
AUCinf	hr*ug/L	
Kel	1/hr	
T1/2	hr	
CL/F	L/hr/kg	
Vz/F	L/kg	
Clast	ug/L	38.7
Tlast	hr	8.00

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

Defactinib Screening Plasma PK (SPPK)

		Analyte
		Defactinib
		Group
		Plasma
Time (hr)		Concentration (ug/L)
0.125	N	3
	Mean	16.6
	SD	15.7
	Min	0.605
	Median	17.2
	Max	31.9
	CV%	94.6
	Geometric Mean	6.92
	CV% Geometric Mean	969
0.250	N	3
	Mean	126
	SD	105
	Min	48.9
	Median	84.5
	Max	246
	CV%	83.1
	Geometric Mean	100
	CV% Geometric Mean	98.2
0.500	N	3
	Mean	69.3
	SD	20.7
	Min	50.2
	Median	66.6
	Max	91.3
	CV%	29.8
	Geometric Mean	67.3
	CV% Geometric Mean	30.6
1.00	N	3
	Mean	78.9
	SD	34.4
	Min	58.8
	Median	59.4
	Max	119
	CV%	43.6
	Geometric Mean	74.5
	CV% Geometric Mean	41.9
2.00	N	3
	Mean	24.5
	SD	23.6
	Min	4.62

Defactinib Screening Plasma PK (SPPK)

		Analyte
		Defactinib
		Group
		Plasma
Time (hr)		Concentration (ug/L)
	Median	18.4
	Max	50.6
	CV%	96.2
	Geometric Mean	16.3
	CV% Geometric Mean	180
4.00	N	3
	Mean	18.1
	SD	2.58
	Min	16.4
	Median	16.9
	Max	21.1
	CV%	14.2
	Geometric Mean	18.0
	CV% Geometric Mean	13.8
8.00	N	3
	Mean	38.7
	SD	29.9
	Min	7.12
	Median	42.5
	Max	66.6
	CV%	77.3
	Geometric Mean	27.2
	CV% Geometric Mean	175
16.0	N	
	Mean	
	SD	
	Min	
	Median	
	Max	
	CV%	
	Geometric Mean	
	CV% Geometric Mean	
24.0	N	
	Mean	
	SD	
	Min	
	Median	
	Max	
	CV%	
	Geometric Mean	

Defactinib Screening Plasma PK (SPPK)

		Analyte
		Defactinib
		Group
		Plasma
Time (hr)		Concentration (ug/L)
	CV% Mean	
	Geometric	

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

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M1	Defactinib	Plasma	0.125	0.60
M1	Defactinib	Plasma	1.00	118.60
M1	Defactinib	Plasma	16.0	
M2	Defactinib	Plasma	0.125	31.93
M2	Defactinib	Plasma	1.00	59.36
M2	Defactinib	Plasma	16.0	
M3	Defactinib	Plasma	0.125	17.17
M3	Defactinib	Plasma	1.00	58.76
M3	Defactinib	Plasma	16.0	
M4	Defactinib	Plasma	0.250	48.87
M4	Defactinib	Plasma	2.00	4.62
M4	Defactinib	Plasma	24.0	
M5	Defactinib	Plasma	0.250	84.46
M5	Defactinib	Plasma	2.00	18.37
M5	Defactinib	Plasma	24.0	
M6	Defactinib	Plasma	0.250	245.90
M6	Defactinib	Plasma	2.00	50.57
M6	Defactinib	Plasma	24.0	
M7	Defactinib	Plasma	0.500	66.61
M7	Defactinib	Plasma	4.00	21.09
M7	Defactinib	Plasma	8.00	7.12
M8	Defactinib	Plasma	0.500	91.26
M8	Defactinib	Plasma	4.00	16.88
M8	Defactinib	Plasma	8.00	42.46
M9	Defactinib	Plasma	0.500	50.16
M9	Defactinib	Plasma	4.00	16.42
M9	Defactinib	Plasma	8.00	66.62

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

Analyte	Group	Time (hr)	Mean (ug/L)	SD (ug/L)	N
Defactinib	Plasma	0.125	16.6	15.7	3

Analyte	Group	Time (hr)	Mean (ug/L)	SD (ug/L)	N
Defactinib	Plasma	0.250	126	105	3
Defactinib	Plasma	0.500	69.3	20.7	3
Defactinib	Plasma	1.00	78.9	34.4	3
Defactinib	Plasma	2.00	24.5	23.6	3
Defactinib	Plasma	4.00	18.1	2.58	3
Defactinib	Plasma	8.00	38.7	29.9	3
Defactinib	Plasma	16.0			0
Defactinib	Plasma	24.0			0

5.0 ATTACHED FILES

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

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

Attached File 5.3 Defactinib_PlasmaStudySheet.jpg – *Handwritten data collection form for defactinib in vivo from client*

Attached File 5.4 Copy of eDCF_830648_DEF_SPPK_2024-07-25.xlsx – *Digital data collection form for defactinib in vivo from client*