PRECLINICAL PHARMACOKINETIC REPORT

Developmental Biology and Solid Tumor Program

P-PKSR Study 821573 - 3094397

STUDY TITLE:

SCREENING PLASMA AND TUMOR PHARMACOKINETICS OF VS-6766 IN ATHYMIC NUDE MICE BEARING MAST 39 XENOGRAFT TUMORS AFTER A SINGLE ORAL DOSE

SHORT TITLE:	VS-6766 Screening Plasma and Tumor PK (SPTPK)
TEST ARTICLE:	VS-6766 (free base), avutometinib, CH5126766, RO5126766
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) Study

The plasma and tumor pharmacokinetic (PK) profiles of VS-6766 were evaluated in normal female Athymic nude mice (Charles River), approximately 8 to 12 weeks in age. The test article VS-6766 (SJ000836349, MedChemExpress, CAT# HY-18652, LOT# 241116) was suspended in 5% DMSO / 10% hydroxypropyl cyclodextrin in sterile water, at 0.03 mg/mL for a 0.3 mg/kg free base equivalent dose as a 10 mL/kg oral gavage. One survival blood sample was obtained from each mouse via cardiac chamber puncture with 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.

1.2 Bioanalysis

MAST39 tumor samples were weighed in 2.0 mL Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA), diluted with a 1:5 volume of 0.1 M phosphate buffered saline (pH 7.4), and homogenized using a FastPrep-24 system (MP Biomedicals, Santa Ana, CA) for three 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 for 24 hours until analysis.

Plasma and tumor homogenate samples were analyzed for VS-6766 (SJ000836349, MedChemExpress, CAT# HY-18652, LOT# 241116) using a qualified liquid chromatography – tandem mass spectrometry (LC-MS/MS) assay. Plasma calibrators and quality controls were spiked with solutions, corrected for salt content and purity as necessary, prepared in dimethyl sulfoxide. Plasma samples, 25 µL each, were protein precipitated with 75 µL of 6 ng/mL Encorafenib (MedChemExpress, CAT# HY-15605, LOT# 255518) in methanol as an internal standard (IS). A 4 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 C18 (2.6 µm, 50 mm x 2.1 mm) column maintained at 50 °C with gradient elution at a flow rate of 0.60 mL/min. The binary mobile phase consisted of water-acetonitrile-0.2 M ammonium acetate, pH 6.0 (90:10:10 v/v) in reservoir A and methanol-water-0.2 M ammonium acetate, pH 6.0 (90:10:10 v/v) in reservoir B. The initial mobile phase consisted of 35% B and is followed by a linear increase to 100% B in 1.5 min. The column was then rinsed for 1.5 min at 100% B and then equilibrated at the initial conditions for 2.0 min for a total run time of 5 min. Under these conditions, the analyte and IS eluted at 0.96 and 1.46 min, respectively.

Analyte and IS were detected with tandem mass spectrometry using a SCIEX QTRAP 5500 in the positive ESI mode and the following mass transitions were monitored: VS-6766 472.1 → 441.1, encorafenib 540.1 → 508.1. The method qualification and bioanalytical runs all passed acceptance criteria for non-GLP assay performance. A linear model ($1/X^2$ weighting) fit the calibrators across the 1 to 500 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. Sample dilution integrity was confirmed. The intra-run precision and accuracy was ≤ 9.76% CV and 87.0% to 115%, respectively.

1.3 Pharmacokinetic (PK) Analysis

Plasma concentration-time (Ct) data for VS-6766 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).

VS-6766 fractions unbound in mouse plasma (Fu,p,m) and human plasma (Fu,p,h) were determined using equilibrium dialysis device HTD-96B (12-14K MWCO, HTDialysis LLC, Gales Ferry, CT). Briefly, blank mouse and human plasma were spiked with VS-6766 DMSO stock, achieving back calculated final concentrations 5 µM. The spiked plasma was placed in one side of the membrane on the HTD-96B plate in sextuplicate and 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. Compounds 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 [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

The VS-6766 plasma Ct data demonstrated low variability between and within mice, with coefficients of variation ranging from 4.46% to 28.2%. Most of the variability was seen during the later time points. The absorption rate of VS-6766 was rapid, with the Tmax occurring at 0.5 hours post-dose. After Cmax, plasma concentrations diminished in a mono-exponential manner with no BLOQ observations. The apparent plasma terminal half-life of VS-6766 was 3.51 hours. The apparent plasma clearance (CL/F) of VS-6766 was low at 1.49 mL/min/kg, or approximately 1.66% of murine hepatic blood flow. The apparent plasma terminal volume of distribution (Vz/F) for VS-6766 was low at 0.453 L/kg, below total body water for a mouse. The oral bioavailability of VS-6766 was unknown in the current study but has been reported to be 93% [3].

The results of the plasma protein binding study for VS-6766 were: Fu,p,m = 0.0307 ± 0.00526; Fu,p,h = 0.0346 ± 0.00288. The plasma protein binding appeared similar between mice and humans, with only a 14% difference.

MAST39 tumor samples were acquired at each of the time points. All of the tumor concentrations were above the LLOQ of 6 ng/mL, and concentrations ranged from 9.08 to 180 ng/mL. The appearance rate of VS-6766 from plasma into the MAST39 tumors was moderate, with the Tmax occurring at 1.0 hours post-dose. The retention was moderate-to-high with a T1/2 of 5.27 hours. The extent of tumor penetration was rather modest, with a Kp_inf value of 0.515.

The PK profile of VS-6766 in the current study compares well with the limited previous findings in mice. Iikura reported a plasma clearance of 1.1 mL/min/kg in mice and a bioavailability fraction of 0.93 [3]. This would yield an estimated apparent oral clearance of 1.2 mL/min/kg, which compares well with our value of 1.49 mL/min/kg, being within 26%.

In clinical studies, the total plasma AUC of VS-6766 at steady state on Day 15 was 6017.4 hr-ng/mL at one of the regimen-dependent MTDs of 2.25 mg PO QD [4]. Given the similar plasma free fractions for VS-6766 between mice and humans, a CRD estimate using total AUCs is appropriate. Therefore, a CRD

for mice would be VS-6766 0.5 mg/kg PO QD. Doses may vary depending on frequency of administration (i.e. BIW, 4d on / 3d off, etc).

3.0 REFERENCES

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4.0 TABLES, LISTINGS, AND FIGURES (TLFS)

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

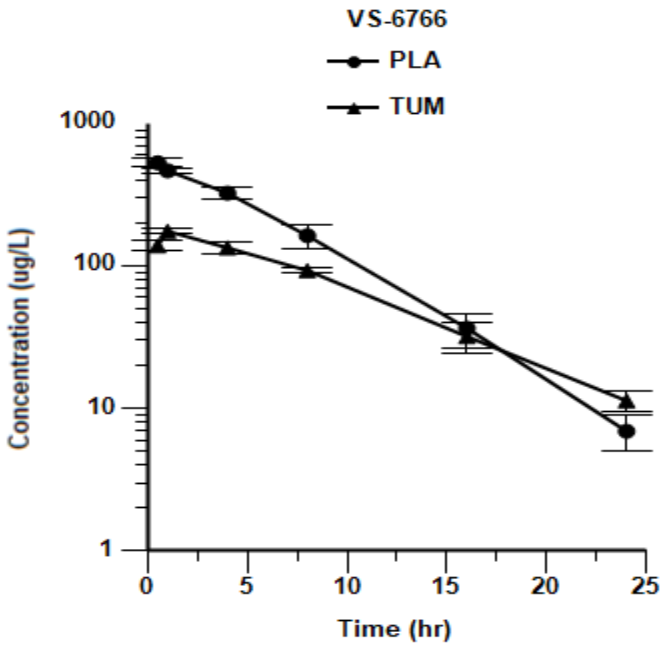

Table 4.1: NCA Parameter Estimates by Analyte and Group

		Analyte	
		VS-6766	
		Group	
		PLA	TUM
Parameter	Unit	Value	
Cmax	ug/L	530	175
Tmax	hr	0.500	1.00
AUClast	hr*ug/L	3310	1640
AUCinf	hr*ug/L	3350	1720
Kel	1/hr	0.198	0.132
T1/2	hr	3.51	5.27
CL/F	L/hr/kg	0.0896	0.174
Vz/F	L/kg	0.453	1.32
Clast	ug/L	6.91	11.3
Tlast	hr	24.0	24.0
Kp_last			0.495
Kp_inf			0.515

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

VS-6766 Screening Plasma and Tumor PK (SPTPK)

		Analyte	
		VS-6766	
		Group	
		PLA	TUM
Time (hr)		Concentration (ug/L)	
0.500	N	3	3
	Mean	530	139
	SD	39.3	12.3
	Min	485	127
	Median	553	138
	Max	553	151
	CV%	7.42	8.86
	Geometric Mean	529	138
	CV% Geometric Mean	7.60	8.89
1.00	N	3	3
	Mean	466	175
	SD	20.8	6.37
	Min	453	168
	Median	455	178
	Max	490	180
	CV%	4.46	3.63
	Geometric Mean	465	175
	CV% Geometric Mean	4.41	3.67
4.00	N	3	3
	Mean	325	134
	SD	28.5	12.1
	Min	293	125
	Median	333	129
	Max	349	148
	CV%	8.79	9.05
	Geometric Mean	324	134
	CV% Geometric Mean	8.98	8.88
8.00	N	3	3
	Mean	163	92.9
	SD	30.5	3.76
	Min	146	88.7
	Median	146	94.0
	Max	198	96.0
	CV%	18.7	4.05
	Geometric Mean	161	92.8
	CV% Geometric Mean	18.0	4.09
16.0	N	3	3
	Mean	36.5	31.9
	SD	9.85	7.56
	Min	26.3	24.8
	Median	37.4	31.1

		Analyte	
		VS-6766	
		Group	
		PLA	TUM
Time (hr)		Concentration (ug/L)	
	Max	45.9	39.9
	CV%	27.0	23.7
	Geometric Mean	35.6	31.3
	CV% Geometric Mean	28.8	24.1
24.0	N	3	3
	Mean	6.91	11.3
	SD	1.95	1.93
	Min	4.75	9.08
	Median	7.48	12.3
	Max	8.51	12.5
	CV%	28.2	17.1
	Geometric Mean	6.71	11.2
	CV% Geometric Mean	31.4	18.2

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

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M1	VS-6766	PLA	0.500	552.78
M1	VS-6766	TUM	0.500	151.18
M2	VS-6766	PLA	0.500	553.12
M2	VS-6766	TUM	0.500	126.60
M3	VS-6766	PLA	0.500	484.83
M3	VS-6766	TUM	0.500	138.46
M4	VS-6766	PLA	1.00	452.60
M4	VS-6766	TUM	1.00	168.06
M5	VS-6766	PLA	1.00	489.69
M5	VS-6766	TUM	1.00	177.89
M6	VS-6766	PLA	1.00	454.99
M6	VS-6766	TUM	1.00	180.00
M7	VS-6766	PLA	4.00	332.63
M7	VS-6766	TUM	4.00	147.98
M8	VS-6766	PLA	4.00	348.60
M8	VS-6766	TUM	4.00	128.96
M9	VS-6766	PLA	4.00	293.14
M9	VS-6766	TUM	4.00	125.41
M10	VS-6766	PLA	8.00	198.46
M10	VS-6766	TUM	8.00	94.00
M11	VS-6766	PLA	8.00	145.57
M11	VS-6766	TUM	8.00	88.70

Subject	Analyte	Group	Time (hr)	Concentration (ug/L)
M12	VS-6766	PLA	8.00	145.65
M12	VS-6766	TUM	8.00	95.96
M13	VS-6766	PLA	16.0	37.39
M13	VS-6766	TUM	16.0	31.15
M14	VS-6766	PLA	16.0	26.28
M14	VS-6766	TUM	16.0	24.79
M15	VS-6766	PLA	16.0	45.92
M15	VS-6766	TUM	16.0	39.85
M16	VS-6766	PLA	24.0	7.48
M16	VS-6766	TUM	24.0	12.29
M17	VS-6766	PLA	24.0	4.75
M17	VS-6766	TUM	24.0	9.08
M18	VS-6766	PLA	24.0	8.51
M18	VS-6766	TUM	24.0	12.54

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

Analyte	Group	Time (hr)	Mean (ug/L)	SD (ug/L)	N
VS-6766	PLA	0.500	530	39.3	3
VS-6766	PLA	1.00	466	20.8	3
VS-6766	PLA	4.00	325	28.5	3
VS-6766	PLA	8.00	163	30.5	3
VS-6766	PLA	16.0	36.5	9.85	3
VS-6766	PLA	24.0	6.91	1.95	3
VS-6766	TUM	0.500	139	12.3	3
VS-6766	TUM	1.00	175	6.37	3
VS-6766	TUM	4.00	134	12.1	3
VS-6766	TUM	8.00	92.9	3.76	3
VS-6766	TUM	16.0	31.9	7.56	3
VS-6766	TUM	24.0	11.3	1.93	3

5.0 ATTACHED FILES

Attached File 5.1 VS-6766 Screening Plasma and Tumor PK V1.0.docx – *Final in vivo study plan as executed*

Attached File 5.2 821573_VS_6766_SPTPK_20240524_TLFs.docx – *Report TLFs as a Word document for manipulation, plotting, and further presentation*

Attached File 5.3 VS-6766 PK Sheet.pdf - *data collection form for the PK study from in vivo scientists.*

Attached File 5.4 Avutometinib-COA-241116-MedChemExpress.pdf - *Certificate of analysis for the VS-6766 lot used in vivo and for bioanalysis.*