Cediranib for Metastatic Alveolar Soft Part Sarcoma

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SUPPLEMENTARY APPENDIX

METHODS AND RESULTS

RNA Isolation from Biopsies

Tumor biopsies were collected in 2 mL Eppendorf tubes filled with RNAlater (Qiagen) following the manufacturer's recommendations. Biopsies were transferred into FastPrep Lysing Matrix D tubes (MP Biomedicals) and 600 µL of Buffer RLT (Qiagen) was added. Samples were homogenized in a FastPrep Instrument (MP Biomedicals) for 20 seconds at a speed of 4.5 m/s followed by centrifugation at 13,000 rpm for 10 minutes at 4ºC. Total RNA from sample lysates was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop) and quality was assessed using an RNA 6000 Nano Kit (Agilent) and Agilent 2100 Bioanalyzer. Total RNA was stored at -80^{o}C until analysis.

Microarray and Statistical Analysis

All RNA samples used for microarray analysis had a high quality score (RIN > 7). RNA (100 ng) isolated from pre- and post-cediranib treatment tumor biopsies from 8 patients (patient 23, 24, 25, 32, 34, 38, 41, and 44) was reverse transcribed and labeled with biotin following the manufacturer's protocol for the GeneChip 3' IVT Express Labeling Kit (Affymetrix). Hybridization, staining, and scanning were performed in quadruplicate using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix), which queries 47,000 transcripts genome-wide using 54,000 probe sets, and the Affymetrix GeneChip Scanner 3000 according to the manufacturer's protocol. The stored images were aligned and analyzed using the GeneChip Operating System software (GCOS; Affymetrix).

Bioconductor version 2.10[1] and R version 2.15.1[2] were used for analysis of the microarray data. Arrays were pre-processed with the RMA algorithm for background correction, quantile normalization, and transcript summarization. Probe sets with low variability were removed from the analysis set. Additionally, if multiple probe sets mapped to the same gene, then the probe set with the largest overall variability was retained for analysis; after screening, there were 10,034 probe sets/genes. A paired, moderated t- test was conducted for each probe set with the LIMMA software package[3] and the P values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate procedure.[4] Expression data were $\log_2$ transformed.

Quantitative Real-Time PCR

Following total RNA isolation, cDNA was prepared from 500 ng total RNA for pre- and post-cediranib tumor biopsy samples using TaqMan High Capacity Reverse Transcription Reagents and the GeneAmp PCR System 9700 (Applied Biosystems) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) reactions using 10 ng cDNA were performed in triplicate using the TaqMan Fast Universal PCR Master Mix and the StepOnePlus Real-Time PCR System (Applied Biosystems) following the manufacturer's protocol. Commercially available TaqMan gene expression assays from Applied Biosystems included ANGPT2, CCL2, CD163, CXCR7, EMILIN2, ESM-1, FLT1 (VEGFR1), FOLH1, KDR (VEGFR2), and TEK (Table S3). TaqMan GAPDH control reagents were used as endogenous controls. Results from qRT-PCR for genes of interest were normalized to GAPDH using the comparative Ct method as described previously.[5] Relative gene expression was expressed as the fold-change between pre- and post-treatment samples and statistical changes in gene expression from vehicle were determined using a paired Students t-test (n=7) with the significance level (α) set at 0.05.

^{18}F-FDG PET/CT Technique and Data Analysis

Patients underwent a whole body ^{18}F- fluorodeoxyglucose positron-emission tomography/computed tomography (^{18}F-FDG PET/CT) scan using a Philips Gemini TF PET/CT camera. The intravenous dose of ^{18}F-FDG administered ranged from 10 to 15 mCi; patients were rested for approximately 60 min before scanning. To standardize imaging conditions and assure that elevated blood glucose levels would not affect ^{18}F-FDG uptake measurements, patients were instructed to fast for at least 6 hours prior to PET/CT examination. Blood glucose levels were normal in peripheral blood at the time of the radiotracer injection for all patients.

A whole-body, non-diagnostic, low-dose CT scan (140 kV, 80 mA) was performed for attenuation correction purposes and anatomic localization of ^{18}F-FDG uptake. No iodinated contrast material was administered for the CT scan. Immediately after the CT, an emission whole-body PET scan was acquired over the same anatomical regions. The acquisition PET scan duration was 2 to 3 minutes per bed position, depending on patient body weight.

The PET emission scan was corrected using segmented attenuation data from the CT scan. Transverse, sagittal, and coronal PET reconstructions as well as 3D rotating maximum pixel intensity projection images were generated.

Co-registered PET/CT scans were displayed using MIM 4.2 software (MIM Software Inc.). The resulting axial, coronal, and sagittal slices were analyzed by one experienced nuclear medicine physician, who was blinded to histopathologic treatment response and CT size measurements.

Suspicious hypermetabolic foci were analyzed semi-quantitatively using the standardized uptake value (SUV). Volumes of interest were outlined in the tumor sites seen on CT, which showed

abnormal increased ^{18}F-FDG uptake, to obtain a corresponding SUV_{max}, defined as the mean value of the 80% threshold of the maximum pixel value. This approach was used for baseline and follow-up scans. Percentage of SUV change was calculated between baseline and follow-up PET/CT scans. A greater than 25% change in SUV_{max} was considered significant.

Gene Expression in HUVECs and in an ASPS-1 Cell Line

Human umbilical vein endothelial cells (HUVECs) were obtained from and cultured in EBM-2 media supplemented with EGM-2 BulletKit (Lonza). ASPS-1 cells were a kind gift from Dr. David Vistica, DTCD/DTP Screening Technologies Branch, Frederick National Laboratory for Cancer Research, and were cultured in DMEM/F-12 50/50 (Mediatech Inc.) supplemented with 10% FBS. HT29 cells were obtained from the NCI-60 Human Tumor Cell Line Screen (Frederick National Laboratory for Cancer Research) and cultured in RPMI-1640 (Quality Biological) supplemented with 5% FBS.

HUVEC and ASPS-1 cells were seeded into 6 well plates at a density of 2.5 $X10^5$ cells/well, and after 24 h, cediranib and sunitinib were added to a final concentration of 2000 nM. Two, 6, and 24 hours after the addition of the drugs, media/drug was aspirated from the wells, and the cell monolayer was lysed with Qiagen's buffer RLT. Total RNA was isolated from the lysates of three independent treatments using the RNeasy mini kit (Qiagen) following manufacturer's protocols.

Five hundred nanograms of total RNA for each sample was reverse transcribed using the GeneAmp® PCR System 9700 and TaqMan® Reverse Transcription Reagents kit. Quantitative

real time PCR reactions were conducted and measured using the ABI StepOnePlus™ real time PCR system and Fast TaqMan® chemistries using pre-designed ABI TaqMan® assays for the genes of interest. Samples were tested in triplicate wells for the genes of interest and for the endogenous control, GAPDH. Data were analyzed using the comparative Ct method as described in the Perkin Elmer User Bulletin #2 (ABI Prism® 7700 Sequence Detection System, 1997) and expressed as a fold induction of the gene in the drug treated samples compared to the untreated control samples.

Statistical and clustering analysis was performed using Partek software, version 6.5 (Partek Inc), with RMA background correction and quantile normalization. Differentially expressed genes were identified with a 1-way ANOVA model. Genes were selected that were up- or down-regulated more than 1.5 fold and with a $P < 0.01$.

Characterization of selected response genes

Because vascular and inflammatory responses to cediranib were observed, we examined the expression of these genes in ASPS-1 and HUVEC cells 2, 6, and 24 hours post treatment. Gene responses for HUVEC and ASPS-1 cells were most highly modulated 6 hours after treatment. Figure S1 compares the response of selected genes in HUVEC cells and the ASPS-1 cell lines to cediranib (2 μM) and sunitinib (2 μM) after treatment for 6 hours. These data suggest that, although both VEGFR inhibitors modulate some of the genes in a similar manner, there are distinct differences. Notably, ESM-1, the most highly down-regulated cediranib-response gene, was significantly induced by sunitinib, but down regulated by cediranib, in both HUVEC and ASPS-1 cells. We confirmed that ESM-1 was down-regulated in ASPS-1 cells following 3 days

of daily treatment with 150 nM cediranib (data not shown). Moreover, ANGPT1 and FOLH1, two marker genes for cediranib response, were also differentially regulated by the 2 agents in HUVEC cells, although they were not significantly expressed in ASPS-1 cells.

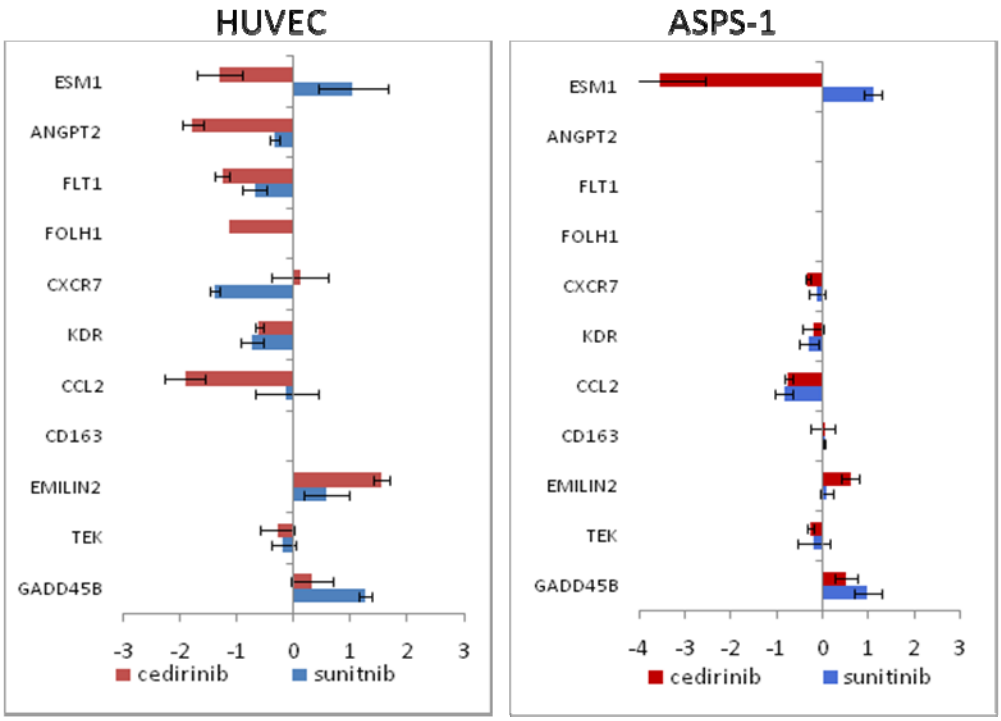

Figure S1: Changes in gene expression measured following 6-hour treatment with 2 µM cediranib or 2 µM sunitinib in HUVEC cells and the ASPS-1 cell line. Error bars represent the average of 3 separate experiments (± SD).

Table S1. Top 100 Up- and Down-Regulated Probe Sets Detected in 8 Patients Ranked by Adjusted P Value

Rank	Affymetrix Probe Set	Gene Symbol	Log_2 FC*	Avg Exp	t	P value	Adjusted P value
1	204726_at	CDH13	-1.28	7.25	-9.81	< 0.001	0.02
2	211148_s_at	ANGPT2	-2.14	6.78	-8.92	< 0.001	0.02
3	222922_at	KCNE3	-2.04	4.95	-8.40	< 0.001	0.03
4	212909_at	LYPD1	-1.14	5.66	-7.96	< 0.001	0.03
5	226814_at	ADAMTS9	-1.17	6.93	-7.90	< 0.001	0.03
6	221489_s_at	SPRY4	-0.98	7.13	-7.79	< 0.001	0.03
7	205860_x_at	FOLH1	-2.12	7.23	-7.33	< 0.001	0.03
8	205352_at	SERPINI1	-1.65	7.20	-7.26	< 0.001	0.03
9	229546_at	LOC653602	-1.45	4.24	-7.20	< 0.001	0.03
10	218888_s_at	NETO2	-1.46	6.77	-7.18	< 0.001	0.03
11	228489_at	TM4SF18	-1.47	6.69	-6.82	< 0.001	0.04
12	242447_at	C3orf70	-1.11	7.00	-6.82	< 0.001	0.04
13	225911_at	NPNT	-1.35	6.38	-6.62	< 0.001	0.04
14	205547_s_at	TAGLN	1.18	8.66	6.58	< 0.001	0.04
15	211303_x_at	FOLH1B	-1.71	5.88	-6.53	< 0.001	0.04
16	204914_s_at	SOX11	-1.51	5.41	-6.52	< 0.001	0.04
17	1555561_a_at	UGGT2	-0.67	4.89	-6.48	< 0.001	0.04
18	231513_at	KCNJ2	-1.58	6.34	-6.44	< 0.001	0.04
19	210815_s_at	CALCRL	-1.51	6.44	-6.44	< 0.001	0.04
20	35666_at	SEMA3F	-1.05	8.25	-6.43	< 0.001	0.04
21	219935_at	ADAMTS5	-1.45	8.46	-6.41	< 0.001	0.04
22	215177_s_at	ITGA6	-1.04	7.43	-6.38	< 0.001	0.04
23	212977_at	CXCR7	-1.51	8.86	-6.37	< 0.001	0.04
24	214228_x_at	TNFRSF4	-1.07	6.45	-6.32	< 0.001	0.04
25	217826_s_at	UBE2J1	-0.65	8.59	-6.24	< 0.001	0.04
26	219522_at	FJX1	-0.72	7.26	-6.16	< 0.001	0.04
27	235666_at	ITGA8	-1.67	5.65	-6.03	< 0.001	0.05
28	242943_at	ST8SIA4	-0.81	6.41	-6.03	< 0.001	0.05
29	226498_at	FLT1	-1.90	8.67	-6.01	< 0.001	0.05
30	240317_at	PCDHB4	-0.64	5.76	-5.83	< 0.001	0.06
31	239952_at	ZEB1	-1.21	5.53	-5.78	< 0.001	0.06
32	214022_s_at	IFITM1	0.77	10.24	5.77	< 0.001	0.06
33	202669_s_at	EFNB2	-1.02	6.48	-5.76	< 0.001	0.06
34	205150_s_at	TRIL	-1.30	6.19	-5.74	< 0.001	0.06
35	232013_at	C9orf102	-0.65	5.20	-5.69	< 0.001	0.06
36	230935_at	LOC100506798	-0.82	5.03	-5.66	< 0.001	0.06
37	219213_at	JAM2	0.75	6.53	5.56	< 0.001	0.07
38	222562_s_at	TNKS2	-0.69	7.14	-5.55	< 0.001	0.07

Rank	Affymetrix Probe Set	Gene Symbol	Log_2 FC*	Avg Exp	t	P value	Adjusted P value
39	206574_s_at	PTP4A3	-1.00	7.28	-5.55	< 0.001	0.07
40	226899_at	UNC5B	-0.81	8.12	-5.52	< 0.001	0.07
41	226499_at	NRARP	-0.78	7.95	-5.47	< 0.001	0.07
42	207677_s_at	NCF4	0.45	6.54	5.43	< 0.001	0.07
43	222911_s_at	CXorf36	-1.19	6.02	-5.43	< 0.001	0.07
44	1566968_at	SPRY4-IT1	-0.83	6.11	-5.42	< 0.001	0.07
45	238066_at	RBP7	-1.05	7.49	-5.42	< 0.001	0.07
46	1555725_a_at	RGS5	-1.67	9.33	-5.42	< 0.001	0.07
47	208394_x_at	ESM1	-2.57	6.60	-5.38	< 0.001	0.07
48	204639_at	ADA	-0.51	7.24	-5.38	< 0.001	0.07
49	221031_s_at	APOLD1	-1.24	7.81	-5.31	< 0.001	0.07
50	203934_at	KDR	-1.20	8.13	-5.26	< 0.001	0.08
51	1552417_a_at	NEDD1	-0.73	5.96	-5.26	< 0.001	0.08
52	206211_at	SELE	1.57	4.14	5.25	< 0.001	0.08
53	205786_s_at	ITGAM	0.56	7.47	5.22	< 0.001	0.08
54	205902_at	KCNN3	-0.94	5.38	-5.18	< 0.001	0.08
55	223525_at	DLL4	-0.78	6.26	-5.16	< 0.001	0.08
56	221011_s_at	LBH	-1.05	8.59	-5.15	< 0.001	0.08
57	226420_at	MECOM	-0.97	7.50	-5.15	< 0.001	0.08
58	206236_at	GPR4	-0.80	6.77	-5.13	< 0.001	0.08
59	1558093_s_at	MATR3	-0.58	7.22	-5.11	< 0.001	0.08
60	218876_at	TPPP3	-0.72	6.45	-5.08	< 0.001	0.08
61	226576_at	ARHGAP26	-0.75	6.35	-5.08	< 0.001	0.08
62	226829_at	AFAP1L2	-0.90	5.06	-5.06	< 0.001	0.08
63	1558404_at	LOC644242	-0.97	4.72	-5.05	< 0.001	0.08
64	219825_at	CYP26B1	1.13	5.78	5.02	< 0.001	0.08
65	219134_at	ELTD1	-0.85	8.84	-5.01	< 0.001	0.08
66	204736_s_at	CSPG4	-0.73	5.88	-5.01	< 0.001	0.08
67	205258_at	INHBB	-0.90	7.65	-4.98	< 0.001	0.09
68	216598_s_at	CCL2	1.59	8.27	4.97	< 0.001	0.09
69	203100_s_at	CDYL	-0.54	7.03	-4.96	< 0.001	0.09
70	205247_at	NOTCH4	-0.69	7.19	-4.95	< 0.001	0.09
71	203130_s_at	KIF5C	-0.62	5.77	-4.94	< 0.001	0.09
72	229459_at	FAM19A5	-1.09	3.96	-4.92	< 0.001	0.09
73	200795_at	SPARCL1	-0.83	10.99	-4.89	< 0.001	0.09
74	223316_at	CCDC3	0.71	7.30	4.89	< 0.001	0.09
75	234993_at	ABHD13	-0.52	5.35	-4.87	< 0.001	0.09
76	213714_at	CACNB2	-1.02	5.41	-4.86	< 0.001	0.09
77	211665_s_at	SOS2	-0.53	5.70	-4.85	< 0.001	0.09
78	212486_s_at	FYN	-0.55	7.08	-4.85	< 0.001	0.09
79	209291_at	ID4	0.82	6.38	4.84	< 0.001	0.09
80	1556364_at	ADAMTS9-AS2	0.59	4.62	4.83	< 0.001	0.09
81	205119_s_at	FPR1	0.72	7.43	4.82	< 0.001	0.09
82	229768_at	OR51E1	-0.81	6.01	-4.81	< 0.001	0.09

Rank	Affymetrix Probe Set	Gene Symbol	Log_2 FC*	Avg Exp	t	P value	Adjusted P value
83	219315_s_at	TMEM204	-0.74	7.99	-4.79	< 0.001	0.09
84	232080_at	HECW2	-1.16	7.35	-4.79	< 0.001	0.09
85	212951_at	GPR116	-0.55	7.96	-4.78	< 0.001	0.09
86	209582_s_at	CD200	-0.96	5.88	-4.77	< 0.001	0.09
87	226122_at	PLEKHG1	-0.56	6.91	-4.77	< 0.001	0.09
88	235484_at	PTAR1	-0.54	6.37	-4.76	< 0.001	0.09
89	204273_at	EDNRB	-1.17	8.60	-4.74	< 0.001	0.09
90	205801_s_at	RASGRP3	-0.59	7.67	-4.71	< 0.001	0.10
91	212558_at	SPRY1	-0.73	8.07	-4.70	< 0.001	0.10
92	1557984_s_at	RPAP3	-0.60	5.45	-4.68	< 0.001	0.10
93	234975_at	GSPT1	-0.80	5.13	-4.62	< 0.001	0.11
94	227140_at	INHBA	1.47	7.56	4.61	0.001	0.11
95	209055_s_at	CDC5L	-0.46	7.78	-4.61	0.001	0.11
96	238907_at	ZNF780A	-0.60	4.27	-4.59	0.001	0.11
97	231789_at	PCDHB15	-0.70	4.62	-4.59	0.001	0.11
98	234304_s_at	IPO11	-0.56	7.22	-4.54	0.001	0.11
99	224374_s_at	EMILIN2	0.75	6.76	4.54	0.001	0.11
100	207968_s_at	MEF2C	-0.67	5.08	-4.53	0.001	0.11

*Data presented as the mean log fold-change of microarray results from samples collected from patients 23, 24, 25, 32, 34, 38, 40 and 41. Abbreviations and definitions: Log_2 FC, log_2 fold-change from pre- to post-treatment; Avg Exp, average log_2 expression for pre- and post-treatment; t-test, statistic for the null hypothesis that the expression level didn't change from pre- to post-treatment; P value, unadjusted P value from the moderated paired t test; Adjusted P value, the Benjamini-Hochberg adjusted P value for false discovery rate.

Table S2. Microarray Data for Probe Sets of Genes Used for Further qRT-PCR Analysis

Affymetrix Probe Set	Gene Symbol	Log_2 FC*	Avg Exp	t	P value	Adjusted P value
211148_s_at	ANGPT2	-2.14	6.78	-8.92	< 0.001	0.025
205860_x_at	FOLH1	-2.12	7.23	-7.33	< 0.001	0.033
212977_at	CXCR7	-1.51	8.86	-6.37	< 0.001	0.038
226498_at	FLT1	-1.90	8.67	-6.01	< 0.001	0.048
208394_x_at	ESM1	-2.57	6.60	-5.38	< 0.001	0.069
203934_at	KDR	-1.20	8.13	-5.26	< 0.001	0.076
216598_s_at	CCL2	1.59	8.27	4.97	< 0.001	0.086
224374_s_at	EMILIN2	0.75	6.76	4.54	0.001	0.111
206702_at	TEK	0.54	6.89	2.87	0.017	0.244
216233_at	CD163	0.48	3.92	2.26	0.048	0.306

*Data presented as the mean log fold-change of microarray results from samples collected from patients 23, 24, 25, 32, 34, 38, 40, and 41. Abbreviations and definitions: Log_2 FC, log_2 fold-change from pre- to post-treatment; Avg Exp, average log_2 expression for pre- and post-treatment; t-test, statistic for the null hypothesis that the expression level didn't change from pre- to post-treatment; P value, unadjusted P value from the moderated paired t test; Adjusted P value, the Benjamini-Hochberg adjusted P value for false discovery rate.

Table S3. Genes Selected for Further Investigation by qRT-PCR

Gene symbol	Gene name	TaqMan probe	P value*
ANGPT2	Angiopoietin 2	Hs01048042_m1*	< 0.001
CCL2	Chemokine (C-C motif) ligand 2	Hs00234140_m1*	< 0.001
CD163	CD163 moleculre	Hs00174705_m1*	0.048
CXCR7	Chemokine (C-X-C motif) receptor 7	Hs00604567_m1*	< 0.001
EMILIN2	Elastin microfibril interfacer 2	Hs00230757_m1	0.001
ESM-1	Endothelial cell-specific molecule 1	Hs00199831_m1*	< 0.001
FLT1 (VEGFR1)	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Hs01052961_m1*	< 0.001
FOLH1	Folate hydrolase (prostate-specific membrane antigen) 1	Hs00379515_m1	< 0.001
KDR (VEGFR2)	Kinase insert domain receptor (a type III receptor tyrosine kinase)	Hs00911700_m1*	< 0.001
TEK	TEK tyrosine kinase, endothelial	Hs00945146_m1*	0.017

*Unadjusted P value from the moderated paired t test (n=5) with significance set at 0.05.

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