Supplemental Materials

Characterization of a novel PERK kinase inhibitor with anti-tumor and anti-angiogenic activity

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Materials and Methods

Immunohistochemistry

Mice bearing BxPC3 tumor xenografts were treated with 50 or 150 mg/kg GSK2656157 twice daily for 3 weeks. Four hours following the last dose, tumors were harvested and fixed in 10% neutral buffered formalin for 24 h then paraffin embedded. Sections were cut (6 µM) and immunohistochemical analysis was carried using the Ventana Discovery XT system (Ventana/Roche Tuscon, Az). Briefly, sections were deparaffinized, hydrated and loaded on the Discovery XT. Antigen retrieval was performed using Tris based (EDTA) buffer solution, CCL (Ventana) at 95-100°C for 20-40 min. Endogenous peroxidases were quenched using the Inhibitor-D 3% H_2O_2 reagent (Ventana) for 4 minutes at 37°C. Primary antibodies were diluted in antibody diluent (DAKO Cytomation) according to manufacturers' instructions. Primary antibodies included rat anti-mouse pan-endothelial cell antigen (MECA-32, BD Biosciences, San Jose, CA) and anti-von Willerbrand factor (Dako, Carpinteria, CA). Antibodies were applied to the sections for 2 h then detected using a mouse adsorbed Rabbit anti-Rat linker (Vector Labs) in combination with OmniMap HRP anti-Rb (Ventana) and the ChromaMap DAB detection kit (Ventana). Tissues were counterstained with Harris' hematoxylin (Lerner Laboratories, Pittsburg, PA), dehydrated, cleared, and coverslipped. Images were acquired with an Axio Imager D2 microscope (Zeiss) equipped with an Axiocam HRc digital camera (Zeiss). Image analysis was carried out using the MetaMorph Imaging program (Molecular Devices).

Magnetic Resonance Imaging (MRI)

Twelve week old female nude mice bearing subcutaneous BxPC3 tumor xenografts were treated with vehicle (n=6) or 150 mg/kg GSK2656157 (n=12) (PO, BID for 14 days). MRI was performed pre-treatment (day 0), day 7, and day 14 post-treatment using a 9.4T/30 cm Bruker system (Bruker Biospin GmbH, Germany). Mice were anesthetized with continuously inhaled isoflurane (1.5–2%) and a constant body temperature of 37°C was maintained using a circulating-water heating system. Respiration was monitored using a respiratory sensor (SA Instruments, Inc, Stony Brook, NY) placed on the abdomen of the animal.

Following a scout image, axial T2-weighted images were acquired using a fast spin-echo sequence with TR/TE=5000/12 ms, Rare factor = 8, Field of View (FOV =3.2 x 3.2 cm), Matrix = 128 x 128, slice thickness = 2 mm, and number of average = 4. A saturation recovery sequence with nine repetition times (TR = 0.2, 0.4, 0.8, 1.2, 2, 4, 6, 8, 12 sec) was used to acquire a T1 map before contrast administration to estimate the intrinsic T1 relaxation rate in tumor. Dynamic Contrast Enhanced (DCE) images were acquired using a 2D-FLASH sequence before (17 frames) and after (33 frames) Gd-DTPA injection (0.3 mmol/kg) with the following parameters: TR/TE = 64.85/2.62 ms, Flip Angle = 58 deg, and a temporal resolution of 7 sec (for each frame).

The Arterial Input Function (AIF) was obtained by analyzing blood samples in vitro to determine the concentration of Gd-DTPA in the blood of the mice (Benjaminsen et al 2004 #27). Image processing and data analysis were performed on a voxel-by-voxel basis by external observer (Mango Solutions, London, UK). All images were stored in ANALYZE format and were converted to NIfTI for data processing. Two axial slices of each DCE acquisition were analyzed; regions of interest (ROIs) that cover the tumor were defined and the T1 relaxation rate was calculated from the T1 map. The signal intensities from the dynamic acquisition (DCE) were converted into gadolinium contrast-agent concentration to calculate the K^{trans} value for each pixel. The median K^{trans} values (in min^{-1}) from each imaging session, across all subjects, were determined and the K^{trans} values at baseline, Day 7 and Day 14, were calculated (Yankeelov et al 2007 #28).

References

1. Benjaminsen IC, Graff BA, Brurberg KG, Rofstad EK. Assessment of tumor blood perfusion by high-resolution dynamic contrast-enhanced MRI: A preclinical study of human melanoma xenografts. Magnetic Resonance in Med. 2004; 52: 269-76.
2. Yankeelov T E, Cron GO, Addison CL, Wallace JC, Wilkins RC, Pappas BA, et al. Comparison of a Reference Region Model With Direct Measurement of an AIF in the Analysis of DCE-MRI Data. Magnetic Resonance in Med. 2007; 57:353-61.

Supplementary Figure 1: Dose-dependent pharmacodynamic effect of GSK2656157 on PERK in mouse pancreas

PK-PD correlation of phospho-PERK and drug concentration in blood and pancreas from mice treated with a single oral dose of GSK2656157 and pancreas and blood were collected 4 h later. Bars represent densitometric analysis of phospho-PERK, normalized to vehicle treated control (mean ± SD).

Supplementary Figure 2. Specificity of phospho-PERK antibody in mouse cells

Murine LL/2 cells were pre-treated with different concentrations of GSK2656157 for 1 h, followed by thapsigargin for an additional 1 h. Cell lysates were analyzed by Western blot for various antigens using antibodies for actin (Sigma), phospho-PERK Thr980, total PERK, phospho-eIF2α Ser51 and total eIF2α (Cell Signaling Technologies).

Supplementary Figure 3. Effect of PERK inhibitor on RNA expression in tumor xenografts

Mice bearing BxPC3, HPAC or RPMI-8226 tumor xenograft were treated twice daily with vehicle or GSK2656157 at 150 mg/kg for 2.5 days. On day 3, tumors were harvested after the single dose and RNA expression was analyzed using RT-PCR. Data represent RNA expression of each gene normalized to actin expression (mean +/- SD, n=4 mice/group).

Supplementary Table 1: Kinase selectivity profiling. GSK2656157 was tested at 10 μM in duplicate against a panel of 300 kinases at Reaction Biology Corp. (Malvern, PA). Reactions were carried out at 10 μM ATP. Data represent percent inhibition with GSK2656157 relative to DMSO control.

	Percent Inhibition with 10 μM GSK2656157
Kinase	**Average**
NEK1	96.76
KHS/MAP4K5	96.28
MEKK2	94.29
c-MER	92.85
MLK2/MAP3K10	91.20
MSK1/RPS6KA5	86.83
BRK	85.23
ACK1	84.62
TRKA	84.20
MEKK3	83.92
Aurora B	83.15
MLK3/MAP3K11	83.08
AXL	82.82
MLCK2/MYLK2	82.79
MLK1/MAP3K9	81.72
LCK	80.75
WNK3	80.09
IRAK1	78.80
NEK7	78.71
CK1epsilon	78.53
MST1/STK4	76.61
YES/YES1	74.48
LOK/STK10	74.16
ABL1	72.30
GCK/MAP4K2	70.66
NEK11	70.44
c-Kit	69.84
IRR/INSRR	68.96
TRKC	68.40
FMS	66.06
ABL2/ARG	63.04
TIE2/TEK	62.63
TRKB	62.36
WNK2	61.08

BMX/ETK	60.88
c-MET	57.92
Aurora C	55.28
FGFR2	54.30
IR	54.25
NEK4	52.14
FGFR1	51.99
TYRO3/SKY	51.65
TAOK1	50.97
IKKe/IKBKE	50.80
EPHB3	49.42
EPHA6	49.31
ROCK2	49.30
PASK	48.13
LRRK2	47.52
CSK	44.68
RSK1	44.08
LYN	44.00
FLT3	43.47
ZIPK/DAPK3	43.29
MST2/STK3	42.30
TAK1	41.51
HCK	41.35
TAOK2/TAO1	40.26
c-Src	38.73
SLK/STK2	38.47
STK39/STLK3	37.00
ULK1	36.63
FLT4/VEGFR3	36.17
FGR	35.14
MNK2	34.64
TXK	34.35
FGFR3	33.56
Haspin	32.36
FER	31.48
Aurora A	30.59
TGFBR2	30.37
MSK2/RPS6KA4	30.06
BTK	29.92
BLK	29.76
ARAF	29.34
MNK1	29.29
FGFR4	28.83
CK1g2	28.07
PDGFRb	28.02

EPHA7	26.96
ERBB2/HER2	26.81
TAOK3/JIK	26.76
DDR2	25.41
PLK2	24.47
KDR/VEGFR2	23.86
RET	23.63
ARK5/NUAK1	23.25
CK1d	22.72
RON/MST1R	22.61
CAMK1a	22.47
MYO3b	22.26
BRAF	22.23
RIPK2	21.34
WEE1	20.70
LIMK1	20.57
CAMK2d	19.61
TYK1/LTK	19.37
FRK/PTK5	19.34
PKCnu/PRKD3	18.47
CAMK2g	17.69
CAMK1d	17.62
ULK3	17.48
SNARK/NUAK2	17.42
EPHA2	16.77
RSK2	16.74
STK16	16.10
OSR1/OXSR1	16.10
CHK2	15.99
PKCd	15.65
CAMK1b	15.56
EPHB4	15.54
SGK2	15.49
PAK2	15.35
ULK2	15.34
CK1g1	15.17
PAK1	15.05
MEK1	14.54
ZAK/MLTK	14.41
TYK2	14.32
PKCeta	14.28
ROS/ROS1	14.25
CHK1	13.81
PKAcg	13.71
LYN B	13.70

STK33	13.56
ROCK1	13.52
PKD2/PRKD2	13.37
FES/FPS	13.25
LKB1	12.89
EPHA3	12.35
CDK2/cyclin E	11.98
JNK1	11.70
CDK1/cyclin B	11.57
PRKX	11.39
NEK6	11.01
CAMKK1	11.00
p70S6K/RPS6KB1	10.77
CLK4	10.64
FYN	10.63
JAK2	10.59
HIPK4	10.27
MUSK	10.26
PBK/TOPK	10.06
EPHA4	10.03
HIPK3	9.93
IGF1R	9.84
CDK9/cyclin K	9.63
PKCg	9.60
DAPK1	9.43
NEK3	8.93
PDGFRa	8.86
JNK2	8.65
MSSK1/STK23	8.57
SIK2	8.45
NEK9	8.10
CAMKK2	7.99
EPHB1	7.92
CDK5/p25	7.66
MEK2	7.62
PLK3	7.61
PKCepsilon	7.11
PKN2/PRK2	7.05
SGK1	6.91
MLCK/MYLK	6.84
GSK3a	6.71
COT1/MAP3K8	6.62
PKCtheta	6.44
BRSK2	6.32
GRK4	6.16

PYK2	6.10
STK25/YSK1	6.07
PKCiota	5.97
EPHA1	5.89
ERBB4/HER4	5.88
MAPKAPK5/PRAK	5.81
VRK1	5.79
PKCmu/PRKD1	5.73
DMPK	5.68
GRK2	5.65
CAMK1g	5.49
RAF1	5.37
DYRK1/DYRK1A	4.95
CK2a2	4.80
DYRK1B	4.63
MARK4	4.49
GRK6	4.42
DCAMKL2	4.35
TLK2	4.26
TTK	4.23
MINK/MINK1	4.23
CAMK2b	4.21
PHkg1	4.05
DYRK4	4.03
ALK2/ACVR1	3.75
P38d/MAPK13	3.65
HIPK2	3.56
PIM3	3.41
CDK5/p35	3.25
TSSK2	3.22
AKT3	2.91
ZAP70	2.67
ALK	2.65
NEK2	2.56
CK1a1	2.53
PIM1	2.43
CDK9/cyclin T1	2.41
ALK4/ACVR1B	2.24
NLK	2.02
STK38/NDR1	1.95
HGK/MAP4K4	1.82
PKG1a	1.79
PKN1/PRK1	1.72
GRK3	1.65
MARK1	1.51

P38a/MAPK14	1.50
STK22D/TSSK1	1.46
BRSK1	1.46
IKKb/IKBKB	1.45
PAK5	1.33
ALK5/TGFBR1	1.06
CDK6/cyclin D1	1.00
ALK1/ACVRL1	0.94
CDK4/cyclin D3	0.78
CDK1/cyclin A	0.69
FLT1/VEGFR1	0.60
PKG2/PRKG2	0.47
PAK3	0.38
MAPKAPK2	0.30
DAPK2	0.18
MRCKb/CDC42BPB	0.17
PKA	-0.04
PHkg2	-0.16
PAK4	-0.18
CLK3	-0.31
P38b/MAPK11	-0.33
EGFR	-0.39
SGK3/SGKL	-0.45
RSK3	-0.48
EPHB2	-0.51
PAK6	-0.67
CLK1	-0.74
PLK1	-0.81
AKT1	-0.93
DYRK2	-0.95
MARK3	-0.96
TBK1	-0.97
CK1g3	-1.01
GRK7	-1.20
AKT2	-1.23
GSK3b	-1.41
PIM2	-1.76
HIPK1	-1.93
DRAK1/STK17A	-2.52
EPHA8	-2.55
MRCKa/CDC42BPA	-2.63
CAMK4	-2.83
PKCb1	-2.92
CLK2	-3.27
CAMK2a	-3.28

DYRK3	-3.71
CDK6/cyclin D3	-4.01
ASK1/MAP3K5	-4.09
CDK3/cyclin E	-4.42
ERK2/MAPK1	-4.70
RSK4	-4.70
MKK6	-4.99
MARK2/PAR-1Ba	-5.04
SYK	-5.10
SRPK1	-5.11
CDK7/cyclin H	-5.35
PDK1/PDPK1	-5.51
SRPK2	-6.09
JAK1	-6.10
PKG1b	-6.51
JNK3	-6.66
EPHA5	-6.67
GRK5	-6.89
P38g	-7.08
PKCzeta	-7.32
CDK2/cyclin A	-7.66
FAK/PTK2	-7.71
JAK3	-7.80
p70S6Kb/RPS6KB2	-10.51
NIK/MAP3K14	-11.67
MAPKAPK3	-11.96
TEC	-13.49
CK2a	-13.77
ITK	-15.45
MELK	-16.68
ERK1	-16.92
CDK4/cyclin D1	-17.02
PKCb2	-17.60
IKKa/CHUK	-18.47
SRMS	-20.54
PKCa	-23.59
CTK/MATK	-26.13
RIPK5	-28.44
IRAK4	-29.36
MST3/STK24	-33.49
MST4	-48.43

Supplementary Table 2: Gene list for Human UPR array from SABiosciences (PAHS-089A)

Refseq	Symbol		Refseq	Symbol
NM_001144	AMFR		NM_013247	HTRA2
NM_006010	ARMET		NM_153692	HTRA4
NM_001675	ATF4		NM_005542	INSIG1
NM_007348	ATF6		NM_016133	INSIG2
NM_004381	ATF6B		NM_002753	MAPK10
NM_004993	ATXN3		NM_002750	MAPK8
NM_004324	BAX		NM_002752	MAPK9
NM_004343	CALR		NM_003791	MBTPS1
NM_001746	CANX		NM_015884	MBTPS2
NM_006430	CCT4		NM_017921	NPLOC4
NM_006429	CCT7		NM_006184	NUCB1
NM_005194	CEBPB		NM_006812	OS9
NM_006368	CREB3		NM_005313	PDIA3
NM_032607	CREB3L3		NM_012394	PFDN2
NM_004083	DDIT3		NM_002624	PFDN5
NM_024295	DERL1		NM_021130	PPIA
NM_016041	DERL2		NM_014330	PPP1R15A
NM_006736	DNAJB2		NM_002743	PRKCSH
NM_012328	DNAJB9		NM_007218	RNF139
NM_018981	DNAJC10		NM_006913	RNF5
NM_006260	DNAJC3		NM_002950	RPN1
NM_005528	DNAJC4		NM_012235	SCAP
NM_014674	EDEM1		NM_003262	SEC62
NM_025191	EDEM3		NM_007214	SEC63
NM_032025	eIF2A		NM_005065	SEL1L
NM_004836	EIF2AK3		NM_203472	SELS
NM_001433	ERN1		NM_014445	SERP1
NM_033266	ERN2		NM_022464	SIL1
NM_014584	ERO1L		NM_004176	SREBF1
NM_019891	ERO1LB		NM_004599	SREBF2
NM_015051	ERP44		NM_172230	SYVN1
NM_018438	FBXO6		NM_030752	TCP1
NM_198334	GANAB		NM_000113	TOR1A
NM_198141	GANC		NM_182688	UBE2G2
NM_014685	HERPUD1		NM_194458	UBE2J2
NM_005346	HSPA1B		NM_014607	UBXN4
NM_005527	HSPA1L		NM_005659	UFD1L
NM_021979	HSPA2		NM_020120	UGCGL1
NM_002154	HSPA4		NM_020121	UGCGL2
NM_014278	HSPA4L		NM_005151	USP14
NM_005347	HSPA5		NM_007126	VCP
NM_006644	HSPH1		NM_005080	XBP1