Anticancer Effects of Mesothelin-targeted Immunotoxin Therapy are Regulated by Tyrosine Kinase DDR1

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Mesothelin surface expression

Cell surface expression of mesothelin was measured after knockdown of DDR1 as described previously (1).

Internalization of RIT

RG7787 was labeled with the Alexa Fluor 647 labeling kit (Invitrogen) and purified according to the manufacturer's guidelines. After transfection of cells for 48 hours, media was removed and changed with medium containing RG7787-Alexa647 (1 μg/ml) and cells were incubated at 37^{o}C for 5, 30, 60, and 180 minutes. Surface bound labeled toxin was removed by treatment with ice-cold stripping buffer (0.2M Glycine-HCL/1% BSA, pH 2.5) for 5 minutes, followed by neutralization with 1M Tris-HCL (pH 8). Cells were washed with Dulbecco's phosphate buffered saline (D-PBS) and harvested by trypsin. Cells were washed with FACS buffer (5% FBS, 0.1% sodium azide in D-PBS without calcium and magnesium) 2 times before measuring fluorescence intensity using CellQuest software on FACS Calibur (BD Bioscience). Data were analyzed using FlowJo software (Tree Star, Inc.).

Tumor antigen expression and histology studies

To measure the effect of DDR1 inhibition on mesothelin expression in A431/H9 tumors, mice were given 7rh daily for 3 days and 3 hours after the third treatment mice were anesthetized by $C0_2$ and tumors were harvested. One-half of the tumor was snap frozen and stored at -80 for western blot (WB) analysis. The other half was collected in RNALater solution for RT-PCR analysis.

For target validation of 7rh, tumors were weighed and homogenized in lysis buffer (Cell signaling; 2X buffer with PMSF and protease and phosphatase inhibitor) using a dounce homogenizer, followed by 10 seconds sonication on ice. Lysates were centrifuged at maximum speed for 10 minutes and immunoprecipitated with a goat anti-human DDR1 antibody (R & D). WB analysis was performed using phospho DDR1 antibody (Origene) to confirm inhibition of DDR1 in tumors.

For histological analysis, tumors were harvested after administering four combination treatments. Tumors were fixed in 10% formalin for 24 hours and then transferred to 70% ethanol. Hematoxylin and eosin (H &E) staining was performed by Histoserv Inc. using standard methods. Images of the slides were taken as described above.

Supplementary Reference

1. Alewine C, Xiang L, Yamori T, Niederfellner G, Bosslet K, Pastan I. Efficacy of RG7787, a next-generation mesothelin-targeted immunotoxin, against triple-negative breast and gastric cancers. Mol Cancer Ther 2014;13:2653-61.

Supplementary Figure Legends

Supplementary Figure S1. Effect of DDR1 knockdown on internalization of alexa-647 labeled RG7787. After DDR1 knockdown for 48 hours, cells were incubated with 2 μg/ml RG7787-alexa647 for 10, 35, 60, or 120 minutes at 37°C. After incubation for different time intervals, cell-surface bound RG7787 was stripped using stripping buffer and internalized RG7787 was measured by flow cytometry. Internalization of RG7787-alexa647 is shown in **A**) KB, **B**) HAY, **C**) KLM1, and **D**) A431/H9 cells.

Supplementary Figure S2. Effect of DDR1 knockdown on mesothelin expression. A) After DDR1 knockdown for 48 hours, surface MSLN expression was measured by incubating with anti-MSLN primary antibody followed by secondary antibody with phycoerythrin-conjugate and FACS analysis. B) Cells were harvested and total RNA isolated after 48 hour reverse transfection for DDR1 knockdown, and analyzed by RT-PCR. Fold change in MSLN mRNA expression was normalized to GAPDH expression. C) Total miRNAs were isolated after DDR1 knockdown and fold change in expression of miRNA198 was normalized to U6 expression.

Supplementary Figure S3. KB or A431/H9 cells were transfected with control siRNA or DDR1 siRNAs for 48 hours, total RNA was isolated and RPL mRNA expression was analyzed by qRT-PCR. Average CTs of three biological and three technical replicates are summarized.

Supplementary Figure S4. Effect of collagen I on DDR1 kinase activation in **A**) A431/H9 and **B**) KLM1 is shown. A431/H9 or KLM1 cells were plated on plastic or collagen I coated plates for 24 hours followed by treatment with 25 μg/ml collagen I for 6 hours and harvested for analysis by WB. Blots were probed using anti-phospho DDR1, anti-DDR1 or anti-Actin antibody.

Supplementary Figure S5. Nilotinib enhances cytotoxicity of RG7787. **A**) A431/H9 and **B**) KB cells were treated with different concentrations of Nilotinib for 3 hours and followed by treatment with varying concentrations of RG7787 for 72 hours and assayed by cell titer glow ATP assay.

Supplementary Figure S6. Combination of 7rh enhances cytotoxicity of native PE and HB21. **A**) KB, **B**) A431/H9, **C**) SUM149 and **D**) HAY cells were treated with 7rh for 3 hours followed by different concentrations of PE for 72 hours and assayed by cell titer glow ATP assay. **E, F**) Activity of HB21 was enhanced by combination with 7rh in SUM149 and HAY cells, respectively.

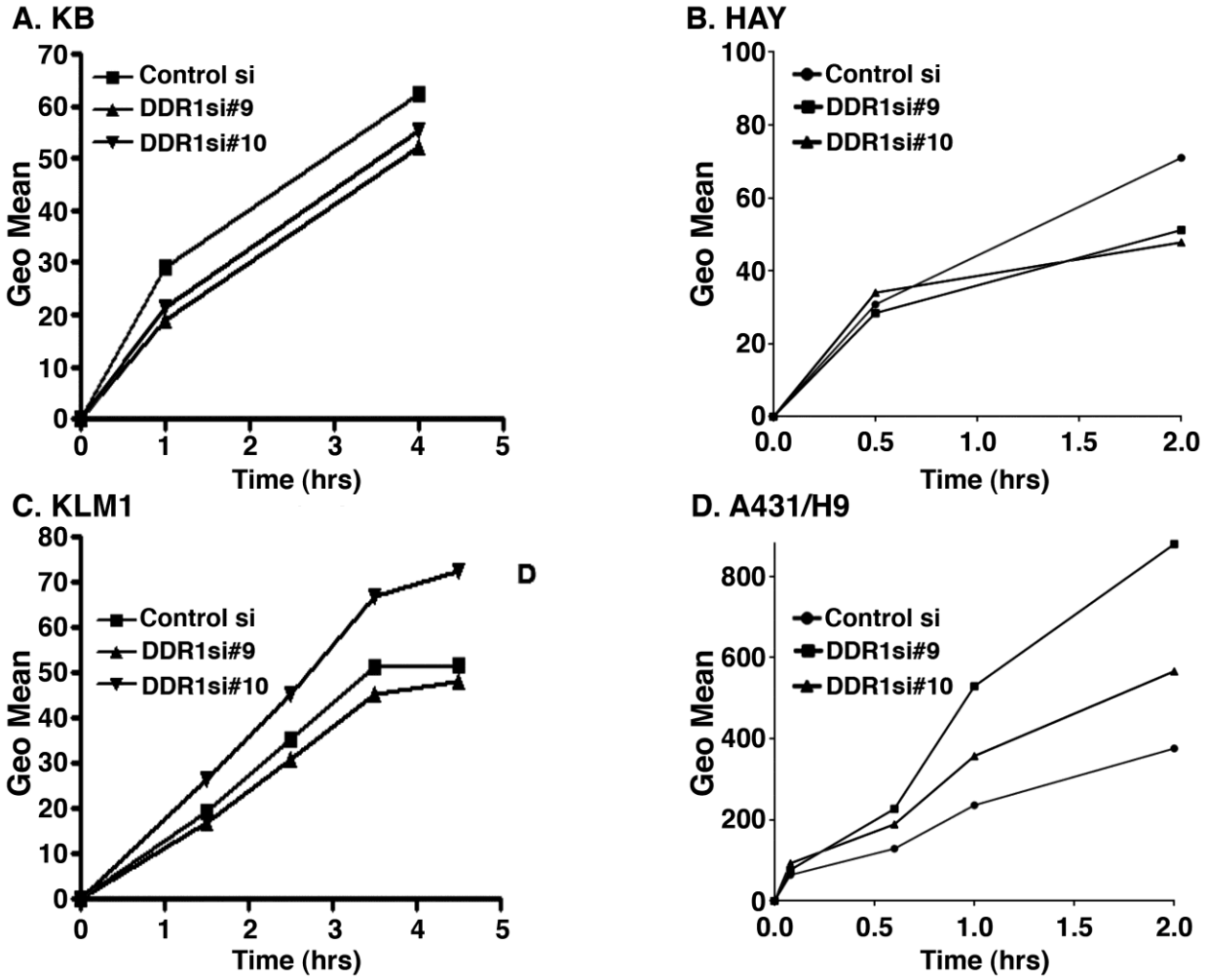
Supplementary Figure S7. 7rh inhibits DDR1 phosphorylation in A431/H9 tumors. **A**) Mice with A431/H9 xenografts were treated with vehicle, 10 or 20mg/kg 7rh daily by oral gavage for 3 days and tumors were harvested. Tumor lysate from 2-3 mice per group was

immunoprecipitated with DDR1 antibody and analyzed by WB to measure phosphorylation of Y513 residue of DDR1. **B**) shows densitometric ratio of phosphor-DDR1 to total DDR1.

Supplementary Table S1. siRNA and primer sequences used in this study

Name	**Purpose**	**Sequence**
Non-target Control	siRNA	Cat No. 4390846
DDR1 #9	siRNA	5’-GGGACACCCUUUGCUGGUA
DDR1 # 10	siRNA	5’-GAAUGUCGCUUCCGGCGUG
RPL38 #1	siRNA	5’-AGGACAACGUGAAGUUUAAttt
RPL38 #2	siRNA	5’-CUUUACACCCUGGUCAUCAtt
RPL24 #1	siRNA	5’-GAGAUUUACUGAAGCAGGAtt
RPL24#2	siRNA	5’-CCUAUGUGCCCUGUCUAAAtt
RPL22#1	siRNA	5’-AGAAGAAUAAUCUACGUGAtt
RPL22#2	siRNA	5’-GAGAGUUACGAAUUACGUUtt
RPL10A#1	siRNA	5’-GUCCGGGCCUUAUAUAUCAtt
RPL10A#2	siRNA	5’-UGAAGAAGGUGUUAUGUCUtt
Mesothelin	siRNA	5’-CGGGTCCGAATACTTCGTGAA
Mesothelin	primer	F: 5’-TCCTGTTCCTGCTCTTCAGCC R: 5’- ACACGGGAAGCCAAGGAGTTG
GAPDH	primer	F: 5’-ATCAGCAATGCCTCCTGCAC R: 5’-TGATGGCATGGACTGTGGTC
B-actin	primer	F: 5’-GCCAGCCAGGTCCAGAC R: 5’-AGGCCAACCGCGAGAAGAT
miRNA 198	primer	F: 5’-GTCCAGAGGGGAGATAG R: 5’-GAACATGTCTGCGTACTC
U6	Primer	F: 5’-CTCGCTTCGGCAGCACAT R: 5’-TTTGCGTGTCATCCTTGCG

Figure S1: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DDR1si#10
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Time (hrs)
B. HAY
Control si
DDR1si#9
DDR1si#10
Geo Mean
Time (hrs)
C. KLM1
Control si
DDR1si#9
DDR1si#10
Geo Mean
Time (hrs)
D
D. A431/H9
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Geo Mean
Time (hrs)

Figure S2:

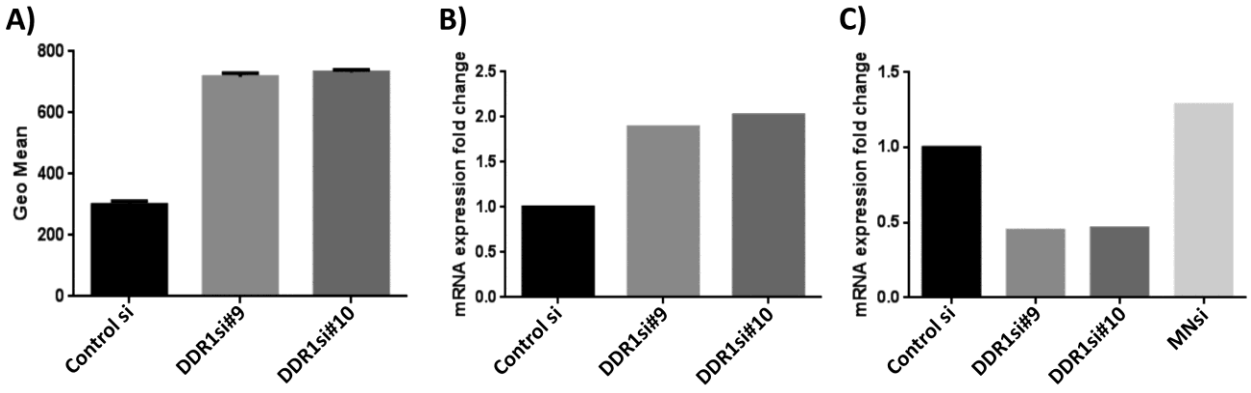


Figure S3:

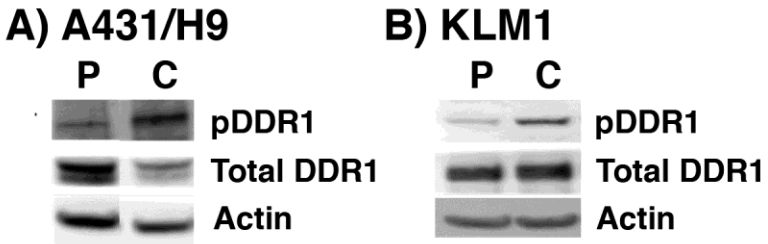
KB

	RPL22	RPL24	RPL38	RPL10A	GAPDH
Control si	22	14	15	13	16
DDR1 si #9	26	15	16	16	16
DDR1 si #10	27	14	15	14	16

A431/H9

	RPL22	RPL24	RPL38	RPL10A	GAPDH
Control si	25	16	17	17	15
DDR1 si #9	27	16	17	17	16
DDR1 si #10	30	15	17	17	15

Figure S4:



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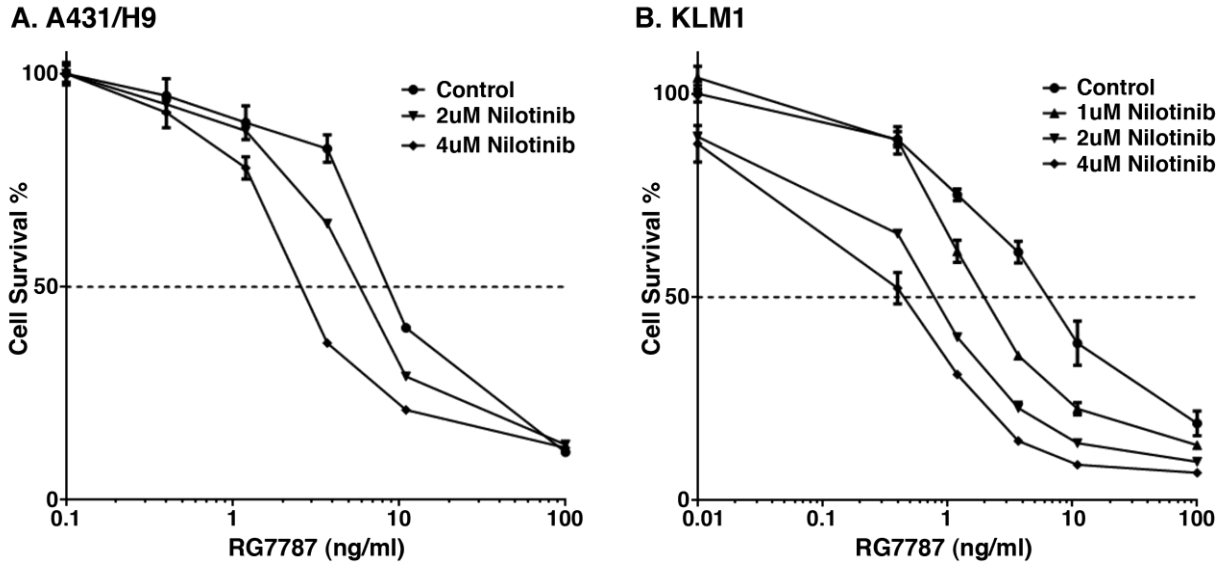

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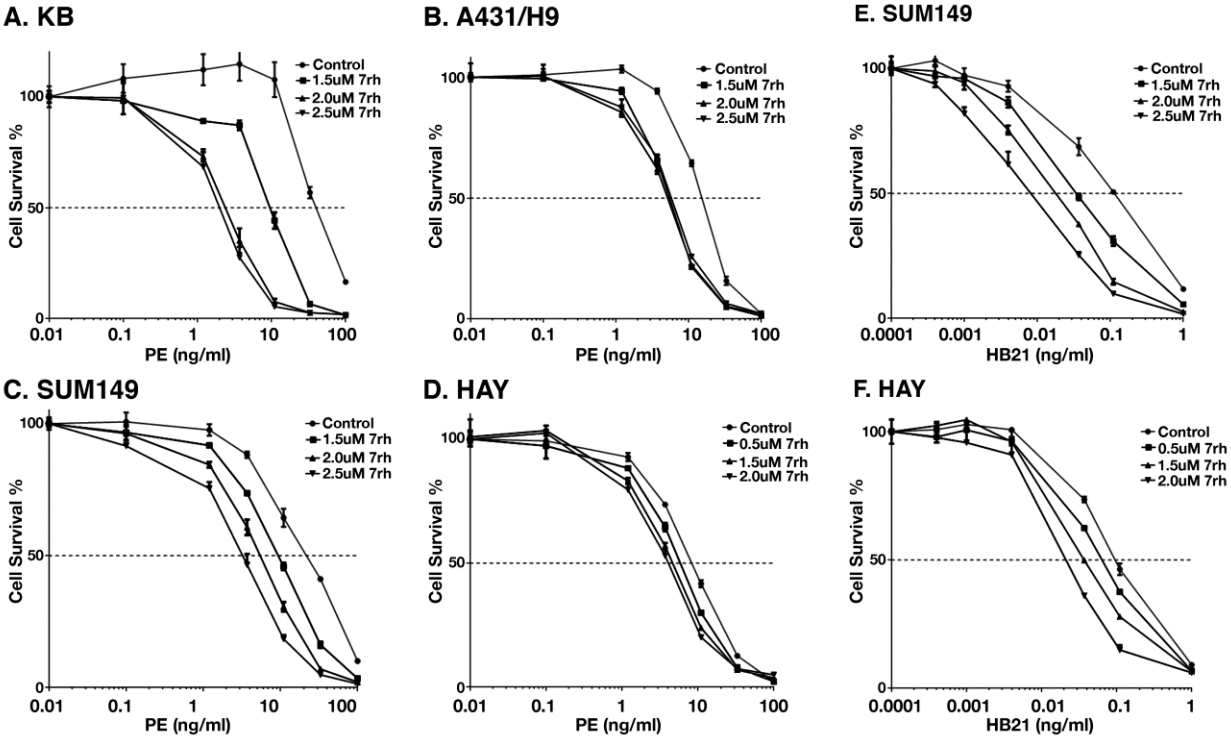

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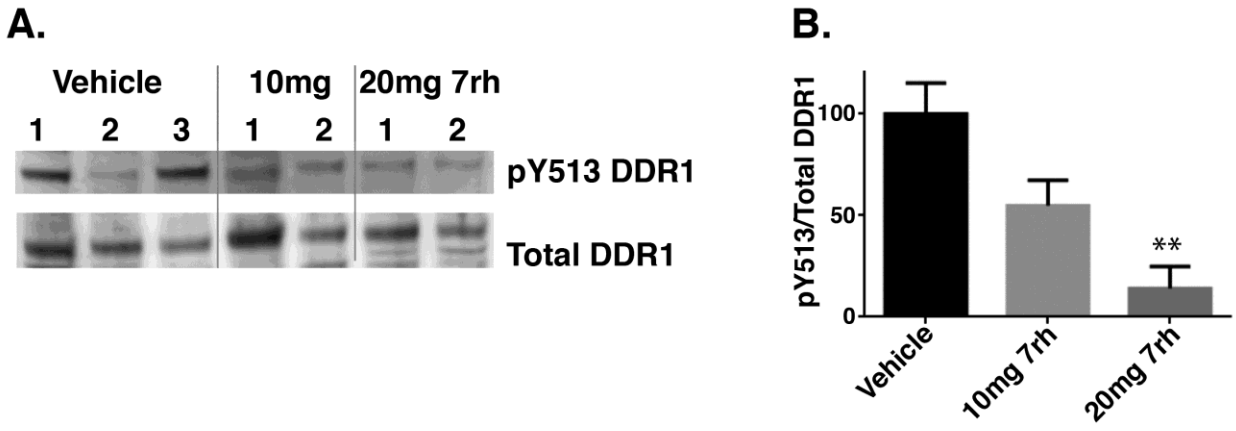
A.
Vehicle
10mg
20mg 7rh
1
2
3
1
2
1
2
pY513 DDR1
Total DDR1
B.
pY513/Total DDR1
100
50
0
**
Vehicle
10mg 7rh
20mg 7rh