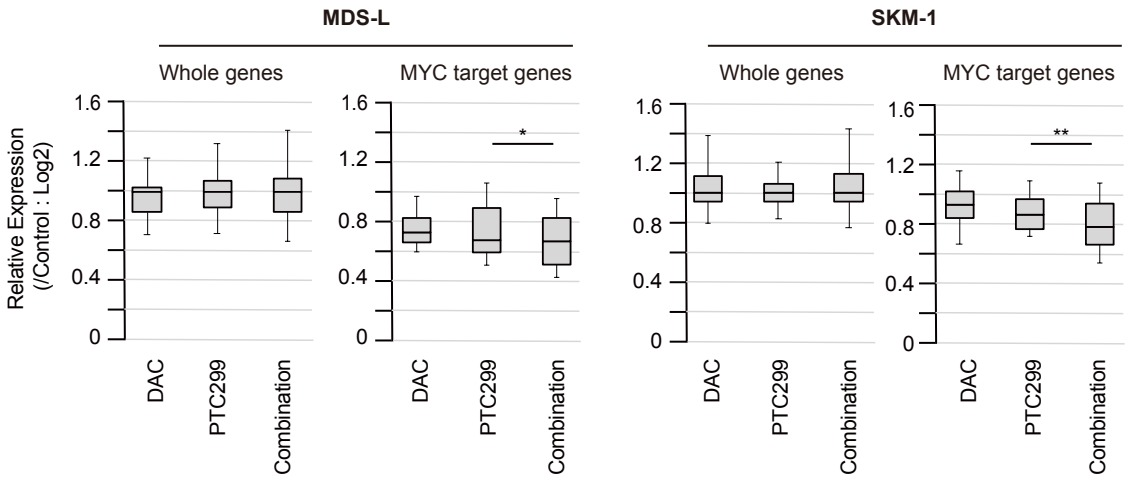
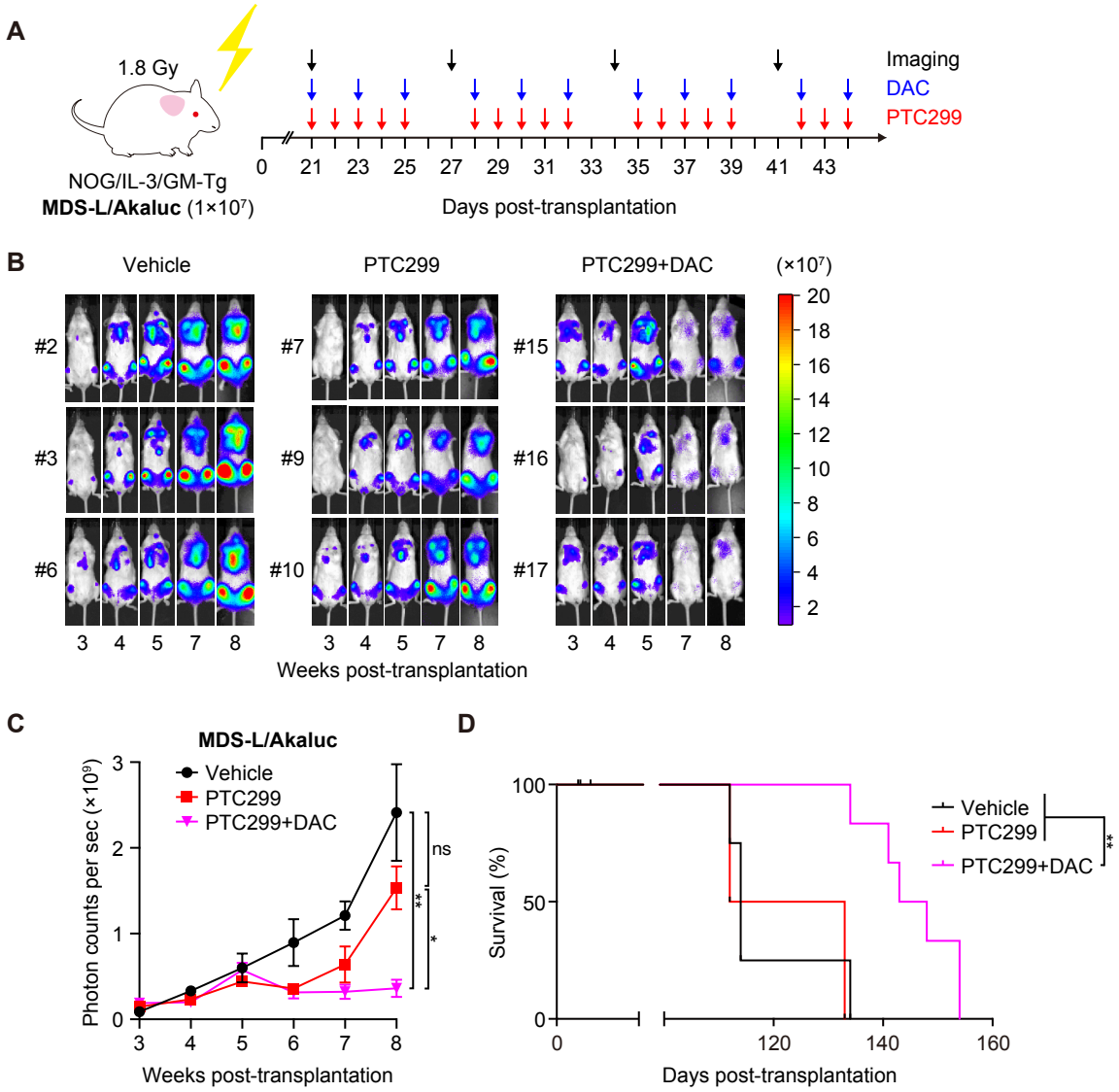


Supplemental Figure 1. Alterations in expression of whole genes and MYC target genes
Relative expression of all RefSeq genes and MYC target genes in MDS-L and SKM-1 cells treated with PTC299, DAC, and combination (PTC299 and DAC) relative to the DMSO control. RNA-seq data in Figure 3 was used. $p<0.05$, $^{**}p<0.01$ by a one-way ANOVA.



Supplemental Figure 2. PTC299 and decitabine exert synergistic effects in the xenograft MDS model (another cohort) (A) Schematic representation of the xenograft MDS model using NOG IL-3/GM-TG mice. (B) Bioluminescence signals in NOG IL-3/GM-TG mice infused with 1×10^7 MDS-L/Akaluc cells via the tail vein. NOG IL-3/GM-TG mice were irradiated at a dose of 1.8 Gy just before transplantation. Starting on day 21 post-transplantation, recipient mice received vehicle QD orally for 5 consecutive days per week (n=6), 10 mg/kg of PTC299 QD orally for 5 consecutive days per week (n=6), and PTC299 and DAC (n=7) for 5 weeks. DAC was administered in a dose of 0.3 mg/kg intraperitoneally 3 times per week. (C) Quantification of photon counts from MDS-L/Akaluc cells in xenograft MDS mice. Bioluminescence activity in treated mice was detected weekly. Images of bioluminescence signals in representative mice (3 mice each) at different time points during the treatment are shown in the left panel. The quantitative photon count in each group was shown in the right panel. (D) A Kaplan–Meier survival curve. Data are shown as the mean ± SEM. * $p<0.05$, ** $p<0.01$, ns, not significant by a one-way ANOVA.

Supplemental Table 1. Patient Characteristics

	ID-01	ID-02	ID-03	ID-04	ID-05	ID-06	ID-07	ID-08
Sample	BM	BM	PB	BM	BM	PB	BM	BM
Diagnosis	MDS-EB2	MDS-EB1	MDS-EB2	MDS/AML	MDS-EB1	RCMD	MDS/AML	MDS-EB1
Age	60	63	68	62	59	60	49	60
Sex	M	M	F	F	F	F	M	M
Blast (%)	3.0	3.3	5.0	46.8	4.6	11.5	19.8	2.9
Karyotype	Normal	Normal	Complex*	Normal	-7	Normal	t(11;19)	Normal
WBC (/μl)	1000	300	16100	1700	1700	46400	1000	2400
Hb (g/dl)	13.8	6.8	7.0	8.5	9.3	8.2	6.6	9.5
MCV(fl)	101.3	87.1	90.7	84.0	101.1	84.5	88.5	87.8
PLT ($\times 10^4$/μl)	6.5	1.8	8.2	2.9	7.8	2.7	1.6	14.3
R-IPSS	5	4.5	7.5	6	7	7	8	3.5
WT-1mRNA (copy/μgRNA)	89	25534	<50	840	5314	32559	393	4224

*46,XX,del(5)(q?),-7,+8,add(12)(p11.2),-21,+mar1

Prognostic indexes	Total (n=8)
Diagnosis	
RCMD	1
MDS-EB-1	3
MDS-EB-2	2
MDS/AML	2
Age(yrs)	
median (range)	60 (49-68)
Sex	
(F/M)	4 / 4
Karyotype	
Normal	5
-7	1
t(11;19)	1
Complex	1
R-IPSS	6.5

Supplemental Table 2. Raw Data of MTS Assay in Figure 2C

MDS-L

Plated cell 10,000/well

PTC299	DAC 0 nM			DAC 12.5 nM			DAC 25 nM		
0	96.3%	101.9%	101.8%	49.1%	48.3%	49.9%	36.1%	34.2%	35.3%
30	46.5%	49.0%	51.7%	28.8%	28.5%	28.6%	25.6%	25.6%	25.1%
60	36.1%	41.0%	39.8%	27.5%	23.4%	24.1%	22.1%	22.1%	20.7%
90	26.8%	32.3%	34.3%	21.0%	20.6%	20.5%	18.5%	19.0%	19.9%

SKM-1

Plated cell 1,000/well

PTC299	DAC 0 nM			DAC 50 nM			DAC 100 nM		
0	90.2%	101.3%	108.5%	30.9%	33.1%	37.0%	27.0%	23.0%	19.4%
60	54.1%	53.2%	55.3%	12.6%	11.7%	12.2%	9.9%	9.6%	9.9%
120	48.8%	44.3%	48.1%	13.6%	16.6%	15.0%	11.0%	9.6%	12.7%
240	45.2%	43.6%	43.8%	14.1%	10.6%	18.0%	8.9%	9.8%	11.3%

MDS-L

Plated cell 10,000/well

PTC299	AZA 0 nM			AZA 50 nM			AZA 100 nM		
0	91.8%	106.6%	101.6%	60.0%	76.7%	79.0%	57.3%	73.9%	82.3%
30	70.0%	72.6%	71.9%	52.6%	51.0%	53.6%	51.5%	51.9%	52.4%
60	63.7%	58.6%	73.5%	48.9%	45.7%	56.4%	45.9%	42.2%	54.3%
90	61.7%	60.7%	57.5%	46.8%	45.2%	44.3%	44.0%	45.9%	41.1%

MDS-L

Plated cell 10,000/well

BRQ	DAC 0 nM			DAC 5 nM			DAC 20 nM		
0	107.9%	95.5%	96.6%	64.3%	72.2%	78.3%	66.0%	66.1%	68.3%
10	93.2%	92.2%	97.1%	73.6%	68.3%	69.6%	62.8%	62.9%	67.3%
20	85.3%	89.4%	87.8%	68.0%	62.0%	60.9%	63.1%	69.3%	63.1%
40	76.0%	68.9%	77.6%	59.6%	60.9%	63.4%	57.3%	34.3%	55.6%

Supplemental Methods

Measurement of decitabine incorporation by LC-MS/MS

DNA isolation

MDS-L cells (1×10^7 cells) were pre-treated with or without 30 nM of PTC299 for 24 hours, and then treated with 0, 100, and 200 nM of decitabine for 24 hours at 37 °C in a humidified 10% CO_2 incubator. After being treated, cells were washed twice with PBS. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). After extractions, DNA was reconstituted in 5 mM BIS-TRIS buffer (pH 7.0) to a concentration of 0.5 mg/mL. DNA hydrolysis was performed following the published protocol.[1] DNA was denatured to ss-DNA in 95°C heat block for 30 min followed by a one-step tetra-enzyme digestion. The tetra-enzyme cocktail was prepared by mixing the enzyme solutions as follows: 10.0 µL of deoxyribonuclease I type II (DNase I) (20,000 U/mL) (Sigma-Aldrich), 15.0 µL of nuclease P1 (NP1) (200 U/mL) (Sigma-Aldrich), 40.0 µL of nuclease phosphodiesterase I (PDE I) (100 U/mL) (Worthington Biochemical), and 0.35 µL of bovine alkaline phosphatase (ALP) (67,500 U/mL) (Sigma-Aldrich). Four microliters of the tetra-enzyme cocktail was added to 50.0 µL of DNA and mixed well, and the mixture was then kept at 37 °C overnight to secure the complete digestion of DNA to mononucleosides. After the enzymatic digestion, 20 µL of 2'-deoxycytidine $_{15}N^3$ (Aptochem) (500 ng/mL, an internal standard) was added to the digest, and mixed well. Each digest was deproteinized by adding 160 µL of acetonitrile and centrifuged at 9,100 × *g* for 5 minutes. The supernatant was used for analytical sample. When analyte concentration was exceeded the upper limit of quantification, the supernatant was diluted 100-fold with 90% acetonitrile containing internal standard (IS) at the same concentration as analytical samples.

LC-MS/MS system

The LC-MS/MS instrumentation used herein consisted of Nexra UFLC system (Shimadzu, Kyoto, Japan) for analyte separation and LCMS-8060 tandem mass spectrometer (Shimadzu, Kyoto, Japan) for quantitation. The system was controlled by LabSolutions software (version 5.91) for its operation, data acquisition, and processing. The UFLC system included a system controller (CBM-20A), two binary pumps (LC-30AD), a temperature-controlled autosampler (SIL-30AC), and a column oven (CTO-20AC).

Quantitation of decitabine, dG, dC, and 5mdC

LC separation was performed on an Atlantis Silica HILIC Column (50 × 2.1 mm, 3 μm) (Waters Corporation, Milford, MA, USA) using the mobile phases consisting of 10 mM ammonium formate aqueous solution-containing 0.1% formic acid (A), and acetonitrile (B) at the flow rate of 0.300 mL/min. The gradient program of the mobile phase B was as follows :0.0 min 98%, 1.0 min 98%, 6.0 min 85%, 6.1 min 50%, 9.0 min 50%, 9.1 min 98%, and 11.0 min 98%. In each analysis, 1.0 μL of the sample was injected into the system by the autosampler set at 4°C. The mass spectrometer system was equipped with an electrospray ionization (ESI) mode and the analyses were performed under positive ionization mode. Quantitation analyses were performed by a multiple reaction monitoring (MRM) mode, in which the transitions of the precursor ion to the product ion were monitored: m/z 229 → m/z 113 for decitabine, m/z 228 → m/z 112 for 2′-deoxycytidine (dC), m/z 242 → m/z 126 for 5-methyl-2′-deoxycytidine (5mdC), m/z 268 → m/z 152 for 2′-deoxyguanosine (dG), and m/z 231 → m/z 115 for IS. For dC, 5mdC, and dG, the quantitation plot of the analyte/IS peak area ratio versus analyte concentration was constructed, and least squares linear regression was applied to the data. On the other hand, quantitation of decitabine was carried out using an absolute calibration curve of decitabine peak area versus its concentration, since IS ionization was suppressed by dC having higher concentration. The concentrations of

decitabine, dC, dG, and 5mdC in each unknown sample were back calculated by calibration equations using the analyte/IS peak area ratio or the analyte peak area. The LC-MS/MS analysis after enzyme digestion was conducted by Shimadzu Techno-Research, Inc (Kyoto, Japan) referring to the published protocol.[1] The accurate mass concentration of DNA was assessed by the measured molar concentration of dG using the following equation:

[DNA] (mg/L) = [dG] × 618 (g/mol)/0.41

where [dG] is the measured concentration of dG in mM, 618 (g/mol) is the molar mass of the G/C pair, and 0.41 is the percentage of the G/C pair in human DNA.[2] The amount of DNA incorporated decitabine was expressed as fmol of decitabine per μg of DNA.

Quantitative RT-PCR

Total RNA was isolated using an RNA Plus Micro kit (Qiagen) and cDNA was made using the ReverTra Ace qPCR RT Master Mix (TOYOBO) according to manufacturer's instructions. Real-time quantitative PCR was performed using TB Green™ Premix Ex Taq™ GC (Perfect Real Time) (Takara) and FastStart Universal Probe Master (Roche Applied Science) with the indicated combinations of the Universal Probe Library (Roche Applied Science) on a StepOnePlus Real-Time PCR System (Applied Biosystems).

Taqman Assay: *c-MYC*-Forward primer: 5'-GCTGCTTAGACGCTGGATTT-3'; *c-MYC*-Reverse primer: 5'-TAACGTTGAGGGGCATCG-3', Probe #66. *GAPDH*-Forward primer: 5'-CTGACTTCAACAGCGACACC-3'; *GAPDH*-Reverse primer: 5'-TAGCCAAATTCGTTGTCATACC-3', Probe # 25.

SYBR Green Assay: *c-MYC*-Forward primer: 5'-CCGCTTCTCTGAAAGGCTCT-3'; *c-MYC*-Reverse primer: 5'- GACCGCAACGTAGGAGGG-3'. *GAPDH*-Forward primer: 5'-CTGACTTCAACAGCGACACC-3'; *GAPDH*-Reverse primer: 5'-TAGCCAAATTCGTTGTCATACC-3'.

Immunoblotting

Cell extract was prepared by lysing cells in SDS sample buffer followed by incubation with benzonase (70746, Millipore) on ice for 10 min. Samples were boiled at 95 °C for 5 min, loaded to SDS–PAGE, and electrotransferred to nitrocellulose membranes (162-0112, Bio-Rad). Immunoblot analysis was performed with the following antibodies: anti-MYC (D84C12, Cell Signaling) and anti-Vinculin (13901, Cell Signaling).

References

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