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Efficacy of the novel tubulin polymerization inhibitor PTC-028 for myelodysplastic syndrome

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Abstract

Monomer tubulin polymerize into microtubules, which are highly dynamic and play a critical role in mitosis. Therefore, microtubule dynamics are an important target for anticancer drugs. The inhibition of tubulin polymerization or depolymerization was previously targeted and exhibited efficacy against solid tumors. The novel small molecule PTC596 directly binds tubulin, inhibits microtubule polymerization, down-regulates MCL-1, and induces p53-independent apoptosis in acute myeloid leukemia cells. We herein investigated the efficacy of PTC-028, a structural analog of PTC596, for myelodysplastic syndrome (MDS). PTC-028 suppressed growth and induced apoptosis in MDS cell lines. The efficacy of PTC028 in primary MDS samples was confirmed using cell proliferation assays. PTC-028 synergized with hypomethylating agents, such as decitabine and azacitidine, to inhibit growth and induce apoptosis in MDS cells. Mechanistically, a treatment with PTC-028 induced G2/M arrest followed by apoptotic cell death. We also assessed the efficacy of PTC-028 in a xenograft mouse model of MDS using the MDS cell line, MDS-L, and the AkaBLI bioluminescence imaging system, which is composed of AkaLumine-HCl and Akaluc. PTC-028 prolonged the survival of mice in xenograft models. The present results suggest a chemotherapeutic strategy for MDS through the disruption of microtubule dynamics in combination with DNA hypomethylating agents.

KEYWORDS

Tubulin polymerization inhibitor, chemotherapy, DNA hypomethylating agents, Myelodysplastic syndrome

Cheng Zhong and Kensuke Kayamori contributed equally to this work.

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1 | INTRODUCTION

Myelodysplastic syndrome (MDS) is a type of hematological malignancy in which blood cells stop differentiation at an immature state. This leads to the formation of dysfunctional blood cells in bone marrow (BM). In most cases, defective blood cells will be destroyed before leaving the BM and impaired hematopoiesis results in fewer blood cells. In some types of MDS, immature blood cells, called myeloblasts, increase in peripheral blood (PB) and BM. MDS patients develop acute myeloid leukemia (AML) as the final stage if myeloblasts exceed the cut-off point of 20%.[1] The DNA hypomethylating agents decitabine and azacitidine are cytidine analogs and first-line drugs for the treatment of MDS. They may be incorporated into DNA and cause covalent binding with DNA methyltransferase, which prevents DNA synthesis and results in cytotoxicity. The inhibition of DNA methylation has extended the overall survival of patients with MDS and AML transformed from MDS.[2] Despite the administration of these chemotherapeutic agents, these diseases generally recur and often become uncontrollable. Therefore, an innovative and more efficacious therapeutic strategy needs to be considered.

Microtubules are highly dynamic and involved in intracellular migration, cell movement, and mitosis. They play an important role in the attachment and segregation of chromosomes in various phases of cell division. Therefore, the targeting of microtubules represents a therapeutic strategy against both solid and hematological cancers.[3] The first microtubule-targeted agent (MTA) approved by the FDA was vincristine, which has been clinically used to treat multiple types of cancers, particularly hematological malignancies. Over the past few decades, additional MTA have been developed and received FDA approval, mostly for applications to cancer therapies. These agents have been classified by their binding sites on tubulin, which influences their roles in the inhibition or stabilization of polymerized microtubules. The novel small molecule PTC596 directly binds tubulin and inhibits microtubule polymerization.[4] PTC596 was originally developed to target BMI1-positive cancer stem cells. BMI1 protein is a component of polycomb repressive complex 1 that maintains the transcriptional repression of target genes via ubiquityl histone H2A.[5-7] PTC596 was recently identified as a direct microtubule polymerization inhibitor in a preclinical study on pancreatic ductal adenocarcinoma and its function as a BMI1 modulator was shown to be a secondary effect.[4] PTC596 induces cytotoxicity in various tumor cell lines and exerts preclinical effects on hematological malignancies, such as AML, mantle cell leukemia, and multiple myeloma.[5-7] Clinical trials on PTC596 are ongoing for diffuse intrinsic pontine glioma, leiomyosarcoma, and ovarian cancer (ClinicalTrials.gov identifiers: NCT03605550, NCT03761095, and NCT03206645). PTC-028, an analog of PTC596, has also been characterized as a BMI1 modulator that has therapeutic potential in ovarian cancer, endometrial cancer, and medulloblastoma.[8-10] However, PTC-028 as well as PTC596 exhibited cytotoxic activity independent of BMI1.[7] These findings suggested that PTC-028 also functions as an inhibitor of tubulin assembly.

In this study, we investigated the efficacy of PTC-028 in MDS and found its role as a microtubule polymerization inhibitor that synergizes with hypomethylating agents to inhibit growth and induce apoptosis in MDS cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and drug treatment

The HL-60, THP-1, and MOLM-13 cell lines were acquired from ATCC. The TF-1 cell line was provided by Toshio Kitamura (The University of Tokyo, Japan). The SKM-1 and SKK-1 cell lines were provided by Dr Hiroshi Matsuoka (Kobe University, Japan). Cells were maintained in RPMI-1640 supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco). SKM-1 cells were cultured in RPMI supplemented with 20% FBS. MDS-L cells[11-15] were cultured in the presence of human IL-3 (10 ng/mL) (BioLegend). TF-1 and SKK-1 were cultured in the presence of 1 and 10 ng/mL of GM-CSF (1 ng/mL) (BioLegend), respectively. Stocks of PTC-028 (synthesized for PTC Therapeutics) and decitabine (Wako) were prepared in DMSO (Sigma-Aldrich) at concentrations of 3 and 10 mM, respectively. In growth assays, cells were seeded on 24-well plates at 1×10^5 cells/mL in triplicate and treated with graded concentrations of PTC-028 and decitabine. Cells were counted using 0.1% trypan blue dye. MTS viability tests were conducted according to the manufacturer's instructions (Promega). Synergism was assessed

by calculating the proportion of cell growth using CompuSyn software (ComboSyn).[16]

Apoptosis and cell cycle assays

Apoptosis and the cell cycle were examined using an Annexin V Apoptosis Kit and a BrdU Flow Kit (BD Pharmingen) according to the manufacturer's instructions. Flow cytometric analyses were performed on BD FACS Celesta (BD Bioscience).

2.2 | RNA sequencing

Total RNA was isolated from MDS-L and SKM-1 cells treated with DMSO or 30 nM of PTC-028 using the RNeasy Mini Kit (Qiagen). After reverse transcription, the libraries for RNA-seq were generated from fragmented DNA with 15 cycles of amplification using a NEB Next Ultra DNA Library Prep Kit (New England BioLabs). After the libraries had been quantified using TapeStation (Agilent), samples were subjected to sequencing with Hiseq2500 (Illumina) and 61 cycles of sequencing reactions were performed. RNA-seq raw reads (fastq files) were mapped to a human genome. Gene level counts for fragments mapping uniquely to the human genome were extracted from BAM files. Gene expression values were then calculated as reads per kilobase of exon units per million mapped reads using cufflinks (version 2.2.1). A gene set enrichment analysis (GSEA) was performed based on curated gene sets from the Broad Institute's molecular signatures database MSigDB.

2.3 | Deposition of data

RNA sequence data were deposited in the DNA Data Bank of Japan (DDBJ) (accession number DRA10205).

2.4 | AkaBLI system

In vivo bioluminescence imaging (BLI) is a non–invasive method for measuring light output produced by the enzyme-catalyzed oxidation reaction of a substrate. The AkaBLI system, composed of AkaLumine-HCl and Akaluc, provides a light source of sufficient strength to penetrate body walls, even in deep tissue areas.[17] pcDNA3/Venus-Akaluc was obtained from the RIKEN BioResource Research Center (Catalog No: RDB15781). The nucleotide sequence of the synthetic construct Akaluc gene for the firefly luciferase mutant protein Akaluc is available under DDBJ accession No: LC320664. Regarding the generation of the CS-CDF-UbC-mScarlet-P2A-Akaluc-PRE lentiviral vector, mScarlet (synthesized by FASMAC) and Akaluc CDS were cloned into the CS-CDF-UbCG-PRE lentiviral vector (Catalog No: RDB08363, a gift from Dr H. Miyoshi) downstream of the ubiquitin C promoter (UbC) using the AgeI and XhoI restriction sites, replacing the existing GFP CDS. Cloning primers were designed to include an in-frame addition of the GSG-P2A self-cleaving peptide sequence between the mScarlet and Akaluc sequences.

2.5 | Lentiviral production and transduction

A recombinant mScarlet-P2A-Akaluc lentivirus (LV) was generated by the transient co–transfection of HEK293T cells with CS-CDF-UbC-mScarlet-2A-Akaluc-PRE and the helper plasmids pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) using the polyethylenimine method. Culture supernatants were collected after 96 hours and filtered (22 μm), followed by the concentration of LV particles by centrifugation at 40 000 *g* for 4 hours.

2.6 | Xenograft studies

All studies involving animals were performed in accordance with the institutional guidelines for the use of laboratory animals and approved by the Review Board for Animal Experiments of the University of Tokyo (approval ID PA18-42). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug} Tg (SRa-IL3, CSF2)/Jic (NOG IL-3/GM-Tg) mice expressing human IL-3 and GM-CSF were purchased from the Central Institute for Experimental Animals (Kawasaki Japan).[18] MDS-L/Akaluc cells (1×10^7 cells) were inoculated into female NOG IL-3/GM-TG mice irradiated at a dose of 1.8 Gy. Three weeks after tumor inoculation, mice were randomly divided into three groups (6 mice per group) and treated with the indicated compounds. Just before the imaging analysis, 100 μL of 5 mM AkaLumine-HCl (Wako) was injected intraperitoneally into mice, and mice under isoflurane anesthesia were imaged within 5–10 minutes of the injection. The following conditions were used for image acquisition: open for total bioluminescence, exposure time = 60 seconds, binning = 4-8, field of view = 25 × 25 cm, and f/stop = 1. In vivo photon counting was conducted with an IVIS system using Living Image 2.5 software (Xenogen). Mice were monitored until they became moribund, at which time they were killed.

2.7 | Primary myelodysplastic syndrome samples

Freshly isolated primary MDS cells were obtained from the BM aspirates of 1 patient with MDS and 1 with MDS/AML. All patients provided written informed consent according to institutional guidelines. The present study was approved by the Institutional Review Board at the University of Tokyo and Chiba University (approval #30-47-B1002 and #844, respectively). BM-MNC were isolated using LymphoPrep (Cosmo Bio) and CD34$^+$ cells were obtained from BM-MNC using a CD34 MicroBead Kit (Miltenyi Biotec). CD34$^+$ cells were seeded into culture flasks in RPMI medium supplemented with 1% penicillin/streptomycin, 20% FBS, and 10 ng/mL of SCF, TPO, IL-3, GM-CSF, and FLT3 ligand (BioLegend). The effects of PTC-028 and decitabine in combination were evaluated using the MTS viability test (Promega).

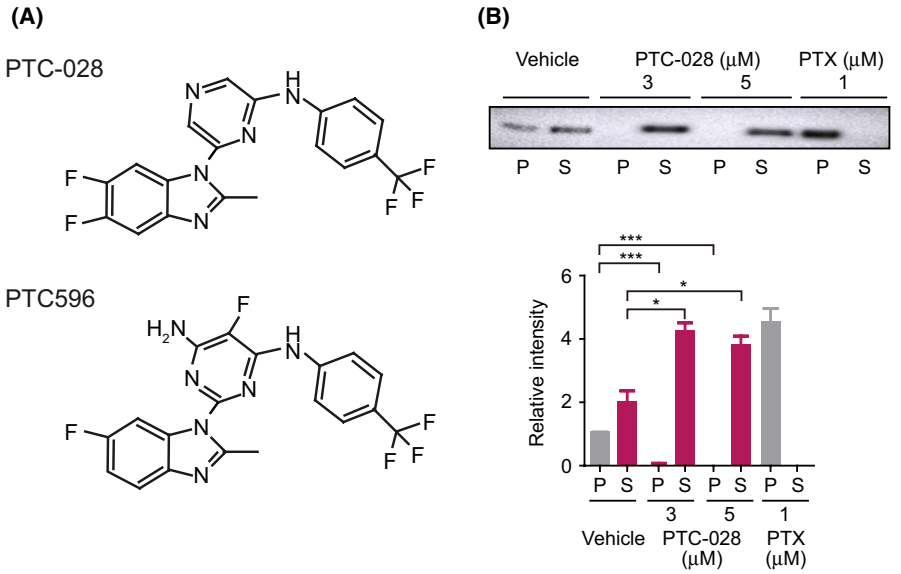


FIGURE 1 PTC-028 disrupts tubulin integrity in myelodysplastic syndrome (MDS) cells. (A) Chemical structure of PTC-028 and PTC596. (B) Distribution of tubulin in polymerized (P) vs soluble (S) fractions analyzed by immunoblotting in PTC-028-treated MDS-L cells. MDS-L cells were treated with 3 and 5 μM of PTC-028 and 1 μM paclitaxel for 4 h. The fractions containing soluble and polymerized tubulin were collected and separated by SDS-PAGE. The α-tubulin antibody was used to detect tubulin by western blotting. Band intensity was calculated using Image lab (Bio-Rad) and is shown as means ± SD (n = 3). $^{*}P < 0.05$, $^{***}P < 0.001$ by Student's *t*-test

2.8 | Immunoblot analysis of tubulin in myelodysplastic syndrome cells

MDS-L cells were cultured in the presence of PTC-028 for 4 hours. Cells were washed with PBS, permeabilized with 200 μL of pre-warmed buffer (80 mM PIPES-KOH [pH 6.8], 1 mM $MgCl_2$, 1 mM EGTA, 0.2% Triton X-100, 10% glycerol, and 1 × Protease inhibitor), and incubated at 30°C for 5 minutes. Supernatants containing the soluble fraction of microtubules were separated after centrifugation, mixed with 4 × Laemmli gel sample buffer, and boiled for 3 minute. To collect the insoluble polymerized tubulin fraction, 250 μL of 1 × Laemmli gel sample buffer was added to the pellet, followed by boiling for 3 minutes. Microtubules were detected by western blotting and probed with mouse anti–human α-tubulin antibodies.

2.9 | Statistical analysis

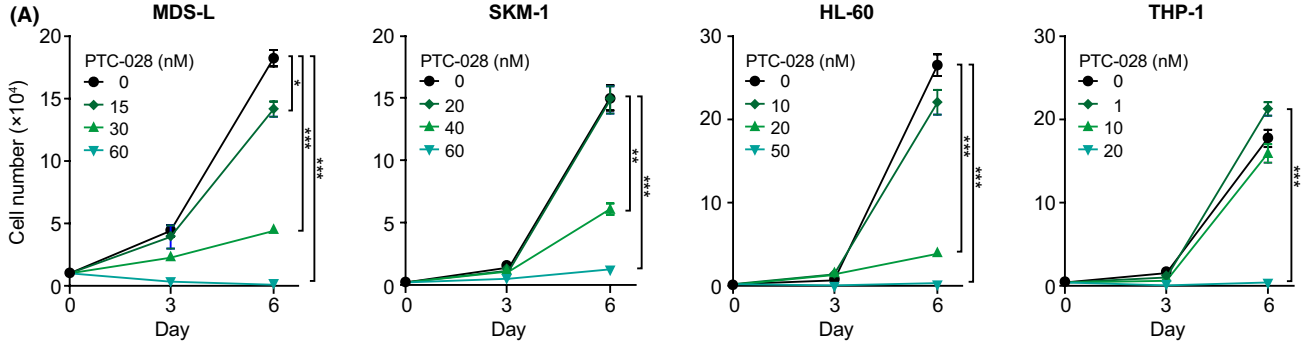
Data are shown as the mean ± SD or SEM. In statistical analyses, *P*-values were derived using unpaired Student's *t*-tests for any studies with only two groups. Otherwise, comparisons of groups were performed on log-transformed data using a one-way ANOVA test. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. All analyses were conducted using GraphPad Prism Software.

3 | RESULTS

3.1 | PTC-028 disrupts tubulin integrity in myelodysplastic syndrome cells

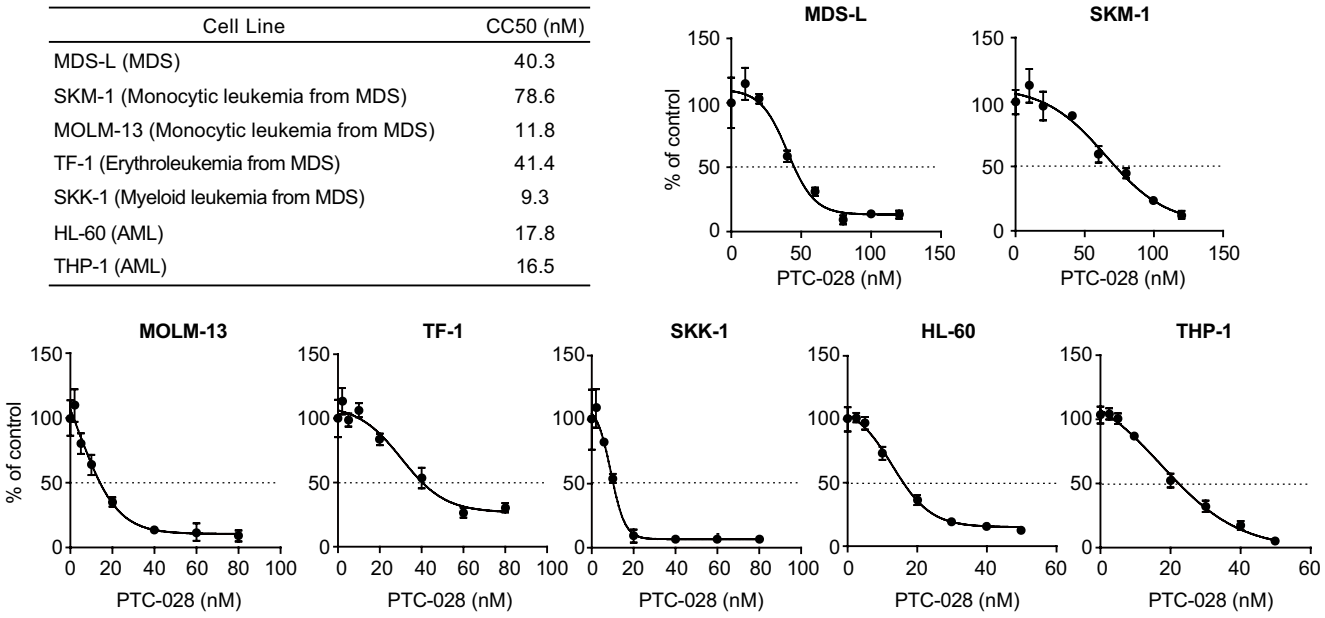
PTC-028 is a novel derivative of PTC596, which was recently shown to directly inhibit microtubule polymerization in pancreatic ductal adenocarcinoma cells (Figure 1A).[4] We first investigated the effects of PTC-028 on the levels of soluble (unpolymerized) versus polymerized tubulin in MDS-L cells. Cells were treated with PTC-028 (3 and 5 μM) and paclitaxel (1 μM) for 4 hours, and cell lysates were then separated into soluble and polymerized fractions by centrifugation. The visualization of

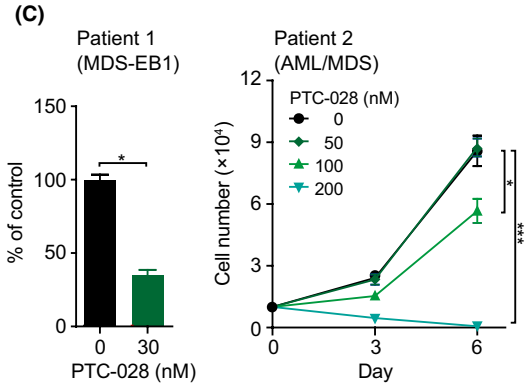
FIGURE 2 PTC-028 suppresses proliferation and induces apoptosis in myelodysplastic syndrome (MDS) cell lines. (A) Growth of MDS-L and SKM-1 MDS cells and HL-60 and THP-1 acute myeloid leukemia (AML) cells treated with the indicated concentrations of PTC-028. The numbers of viable cells on days 3 and 6. Data are shown as means ± SD (n = 3). (B) CC_{50} of MDS and AML cell lines. Cells lines were treated with the indicated concentrations of PTC-028 for 3 d in triplicate (left panels). CC_{50} was defined as the concentration required to reduce cell viability by 50% and is presented in the right panel. Cell viability was assessed by MTS assays. (C) Growth of primary MDS cells treated with PTC-028. $CD34^{+}$ MDS cells were cultured in the presence of SCF, TPO, IL-3, GM-CSF, and FLT3 ligand in the presence of the indicated doses of PTC-028. Cell growth was examined by MTS assays after 48 h in culture (left panel) and viable cells were counted on days 3 and 6 (right panel). Data are shown as means ± SD (n = 3). (D) Caspase-Glo 3/7 values 3 d after the treatment with PTC028. MDS cells were treated with PTC-028 at the indicated doses in triplicate. An equal volume of Caspase-Glo 3/7 agent was added to samples before recording luminescence. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test

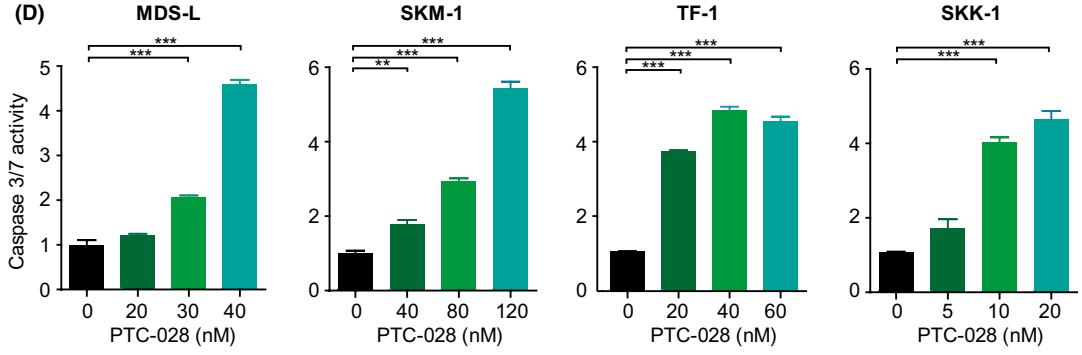


(B)

Cell Line	CC50 (nM)
MDS-L (MDS)	40.3
SKM-1 (Monocytic leukemia from MDS)	78.6
MOLM-13 (Monocytic leukemia from MDS)	11.8
TF-1 (Erythroleukemia from MDS)	41.4
SKK-1 (Myeloid leukemia from MDS)	9.3
HL-60 (AML)	17.8
THP-1 (AML)	16.5







tubulin fractions by western blotting demonstrated that the PTC-028 treatment for 4 hours resulted in the near-complete loss of polymerized microtubules (Figure 1B). In contrast, polymerized microtubules increased in cells treated with paclitaxel, which stabilizes microtubules against depolymerization (Figure 1B).[19] These results indicate that PTC-028 also acts as a microtubule polymerization inhibitor.

3.2 | PTC-028 inhibits cell growth and induces apoptosis in myelodysplastic syndrome cell lines

A previous study reported that PTC596 suppressed cell proliferation and induced apoptosis in AML cell lines.[5] Because MDS is regarded as a pre-leukemic stage, we examined the effects of PTC-028 on MDS cells. MDS-L is a subline derived from the human MDS cell line MDS92, established from the BM of an MDS patient.[11-13] This cell line has complex karyotypic abnormalities, including del(5q) [der(5)(5;19)], monosomy 7, and somatic mutations in *NRAS* and *TP53*, and a *HIST1H3C* mutation (histone H3 K27M).[14] SKM-1 is a cell line derived from a patient with MDS/AML, which has no chromosomal abnormalities but has point mutations in *NRAS* and *KRAS*.[20] We also used the MDS/AML cell lines, TF-1,[21] MOLM-13,[22] and SKK-1,[23] and AML cell lines, HL-60 and THP-1. PTC-028 induced the dose-dependent inhibition of cell proliferation on both MDS and AML cells (Figure 2A). Cell proliferation detected by MTS assays revealed that MDS cells were sensitive to PTC-028, as demonstrated by the low concentrations of PTC-028 needed to inhibit cell viability by 50% (cytotoxic concentration; CC_{50}); however, MDS-L, SKM-1, and TF-1 were less sensitive than others (Figure 2B). We isolated $CD34^+$ cells from primary MDS BM samples and investigated the efficacy of PTC-028 on primary MDS cells. Patient characteristics are shown in Table S1. Cell growth at 48 hours in culture was examined by MTS assays and the counting of viable cells. The growth inhibitory effects of PTC-028 were confirmed in primary MDS cells (Figure 2C). We then performed apoptosis assays. PTC-028 induced caspase 3/7 activities in MDS cells and increased the proportion of annexin V-positive cells (Figure 2D, Figure S1), suggesting the induction of apoptotic cell death by PTC-028.

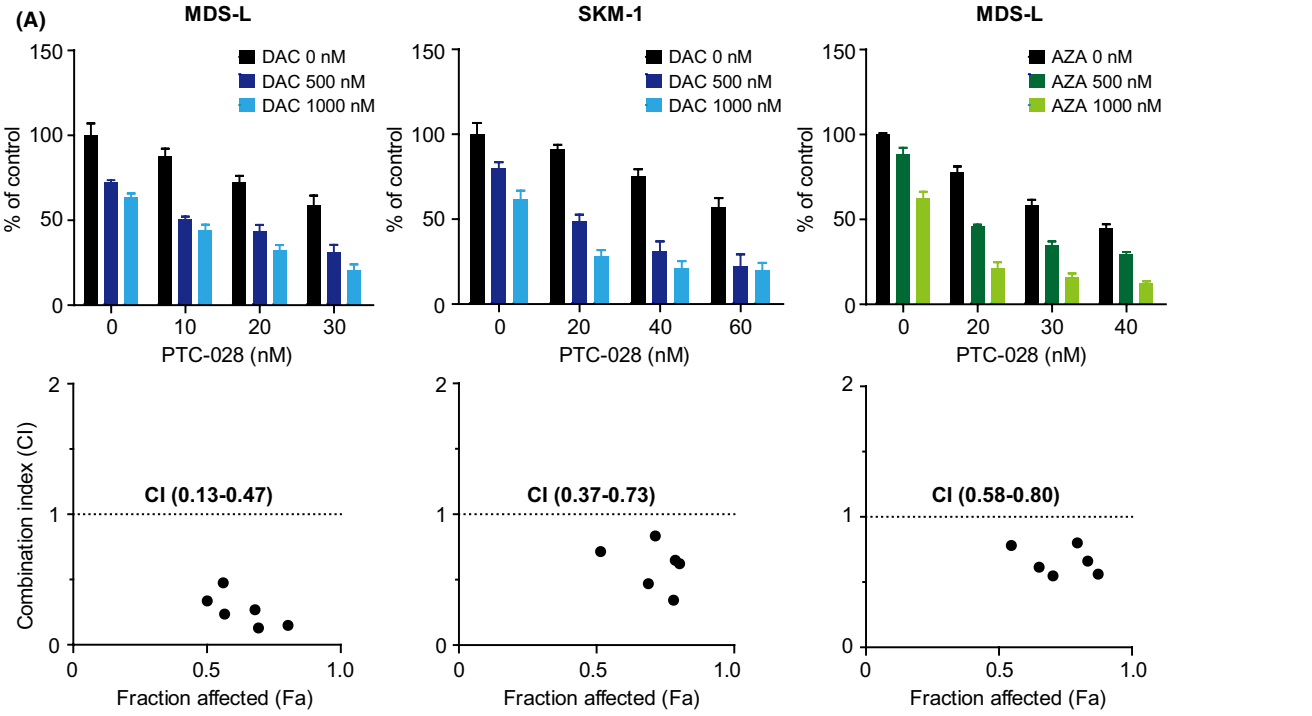
3.3 | Combination of PTC-028 and DNA hypomethylating agents exerts synergistic effects

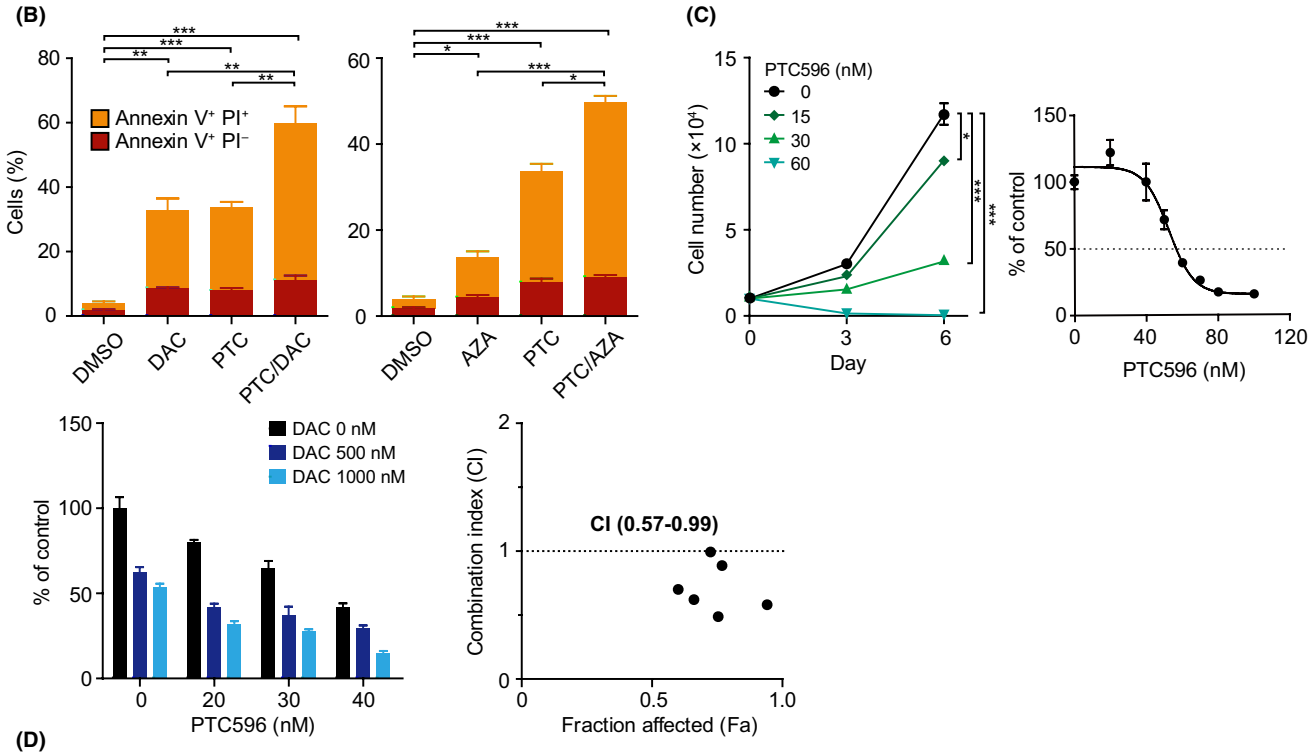
To enhance the therapeutic benefits of PTC-028 on MDS cells, we investigated synergism between PTC-028 and DNA hypomethylating agents. We treated MDS-L and SKM-1 cells with increasing concentrations of PTC-028 in combination with DNA hypomethylating agents, first-line therapeutic agents in the treatment of MDS. After 3 days of culture, cell growth was analyzed by MTS assay. We then calculated the combination index (CI) that indicates the effect quantitatively: additive (CI = 1), synergistic (CI < 1), and antagonistic (CI > 1) effects in drug combinations.[16] PTC-028 and decitabine exerted synergistic cytotoxic effects at most concentrations tested, showing a very low CI, although the effect was rather additive at some concentrations (Figure 3A). Azacitidine was also effective in combination with PTC-028 (Figure 3A). Apoptosis assays using annexin V revealed that PTC-028 and DNA hypomethylating agents both significantly induced apoptosis in MDS-L cells as single agents as well as in combination therapy (Figure 3B). We then investigated the effects of PTC596 on MDS cells. PTC596 inhibited the growth of MDS-L cells and showed moderate synergism when combined with decitabine (Figure 3C,D). The CC_{50} values of PTC596 in MDS and AML cells were moderately higher than those of PTC-028 (Figure 3E), indicating that PTC-028 exerts a better cytotoxic effect than PTC596. We next tested the efficacy of clinically used agents targeting microtubules such as vincristine and paclitaxel. The CC50 value of PTC-028 was lower than those of vincristine and paclitaxel, indicating that PTC-028 is more effective on MDS cells than vincristine and paclitaxel.

3.4 | PTC-028 induces mitotic arrest and changes gene expression in myelodysplastic syndrome cells

To clarify the mechanisms by which PTC-028 plays a role in the cell cycle, we performed BrdU cell cycle assays. The results obtained clearly showed that PTC-028 induced the accumulation of cells at the G2/M phase (Figure 4A). The disruption of tubulin assembly was previously shown to induce cell cycle arrest at the G2/M phase in cancer cells.[24-26]

FIGURE 3 Efficacy of combination therapy using PTC-028 and DNA hypomethylating agents. (A) MTS assays showing the viability of MDS-L and SKM-1 cells treated with the indicated doses of PTC-028 and DAC or azacitidine (AZA) relative to the untreated control. Data are shown as means ± SD (n = 3). Fa-CI plots are shown in the lower panel of each graph. CI, combination index. Fa (fraction affected) indicates the fraction of cells affected by the drug. (B) Apoptosis induced by PTC028 and/or DNA hypomethylating agents. MDS-L cells were treated with PTC-028 and/or DNA hypomethylating agents (DAC or AZA) for 72 h, stained with annexin V and propidium iodide, and then analyzed by flow cytometry. Results are shown as means ± SD (n = 3). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. (C) Growth inhibition of MDS-L cells by PTC596. Growth curve of MDS-L cells treated with the indicated concentrations of PTC596 (left panel) and CC_{50} of PTC596 in MDS-L cells (right panel). Cells were treated with the indicated concentrations of PTC596 for 3 d in triplicate to evaluate CC_{50}. (D) MTS assays showing the viability of MDS-L treated with the indicated doses of PTC596 and DAC relative to the untreated control (left panel) and a Fa-CI plot (right panel). (E) CC_{50} of microtubule-destabilizing agents in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cells. Cell lines were treated with the indicated agents for 3 d in triplicate. Data are shown as means ± SD (n = 3)

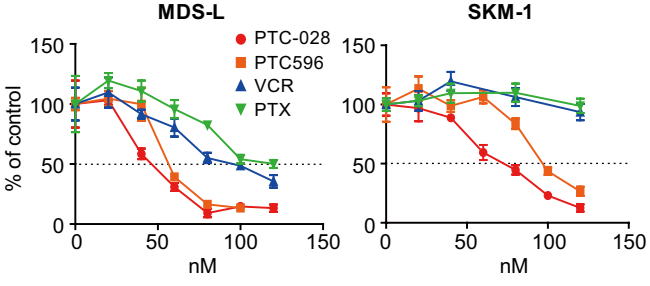




(D)

CC50 (nM)

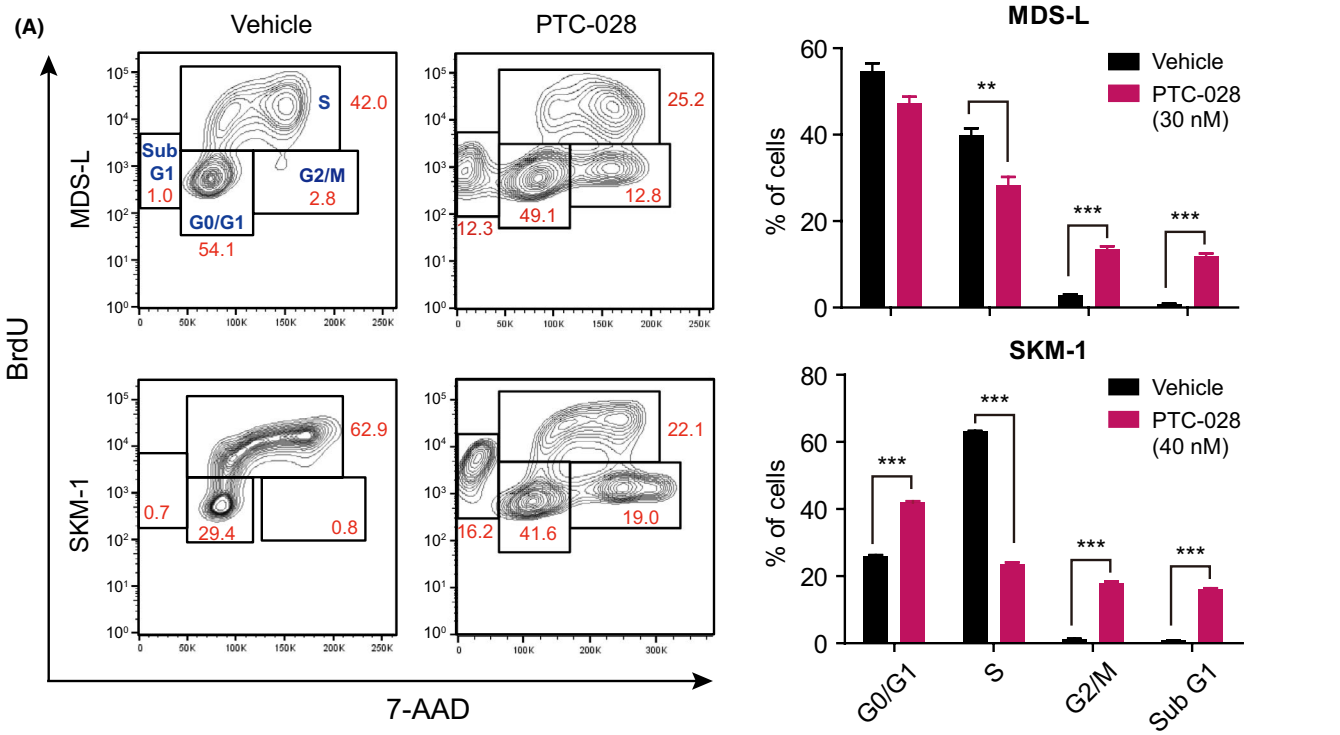
Cell Line	PTC-028	PTC596	VCR	PTX
MDS-L (MDS)	38.5±7.6	52.3±9.8	93.7±10.6	>120.0
SKM-1 (MDS/AML)	80.8±9.5	91.0±8.3	>120.0	>120.0
HL-60 (AML)	17.8±4.6	18.4±5.9	>40.0	38.3±6.2
THP-1 (AML)	16.5±3.4	19.3±3.2	40.1±6.4	>50.0



We next investigated the effects of PTC-028 on the transcriptome; we performed an RNA-seq analysis of MDS-L and SKM-1 cells treated with PTC-028. In total, 163 and 404 genes and 39 and 82 genes were upregulated (≥1.5-fold) and downregulated (≤0.6-fold) in MDS-L and SKM-1 cells, respectively, upon the PTC-028 treatment. GSEA of RNA-seq data revealed that MYC and E2F target gene sets were negatively enriched with significance in PTC-028-treated MDS-L and SKM-1 cells (Figure 4B, Table S2). In contrast, apoptosis gene sets were positively enriched in PTC-028-treated cells (Figure 4B, Table S2). These results were consistent with growth inhibition and enhanced apoptotic cell death by PTC-028. Previous studies reported the activation of NF-κB and inflammatory signaling pathway by microtubule inhibitors.[27,28] PTC-028 also activated gene sets of similar pathways: TNFα signaling by NFκB and inflammatory response (Figure 4C).

3.5 | PTC-028 exerts therapeutic efficacy in the xenograft myelodysplastic syndrome model

We assessed the efficacy of PTC-028 in a xenograft mouse model of MDS. To precisely monitor the tumor burden in mice, we took



(B)

PTC-028 DOWN — PTC-028 UP

	NES NOM p-val/FDR q-val	
	MDS-L	SKM-1
Hallmark_TNFA_Signaling_via_NFKB	1.88 0.00/0.00	2.13 0.00/0.00
Hallmark_Inflammatory_Response	1.63 0.00/0.01	1.83 0.00/0.00
Hallmark_Apoptosis	1.68 0.00/0.00	1.80 0.00/0.00
Hallmark_MYC_Targets_V1	1.86 0.00/0.00	−1.94 0.00/0.00
Hallmark_E2F_Target	−1.67 0.00/0.00	−1.53 0.00/0.02

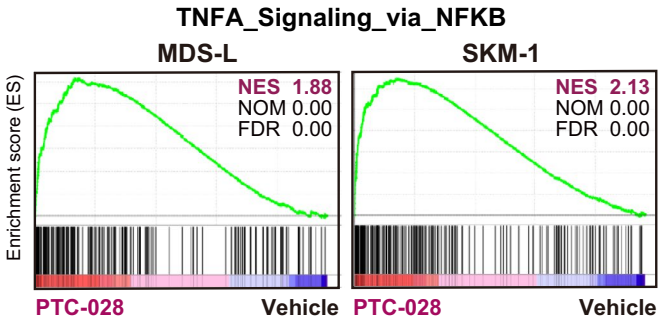


FIGURE 4 Cell cycle arrest and changes of transcriptome induced by PTC-028. (A) Cell cycle arrest induced by PTC-028. MDS-L and SKM-1 were exposed to PTC-028 for 72 h at 40 and 80 nM, respectively. BrdU was added to the culture 4 h before the analysis. Representative contour plots of BrdU incorporation (*y*-axis) versus DNA content assessed by 7-AAD staining (*x*-axis) are shown in the left panels. The proportion of cells at the indicated phase of the cell cycle is shown as means ± SD (n = 3) in the right panels. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. (B) Summary of the gene set enrichment in MDS-L and SKM-1 cells treated with PTC-028 relative to non–treated cells in gene set enrichment analysis (GSEA) using RNA-seq data. MDS-L and SKM-1 cells were cultured in the presence of PTC-028 (MDS-L 30nM; SKM-1 40 nM) for 72 h. Representative GSEA plots are shown in the right panels. Normalized enrichment scores (NES), nominal *P*-values (NOM), and false discovery rates (FDR) are indicated

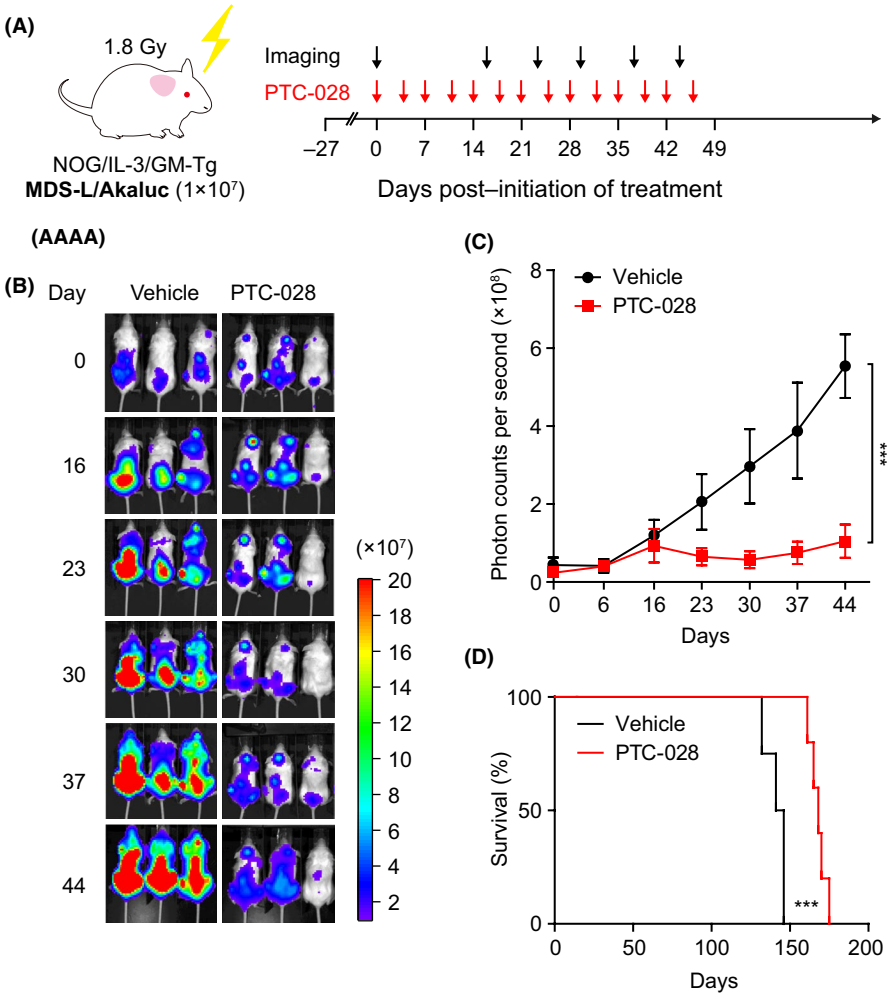
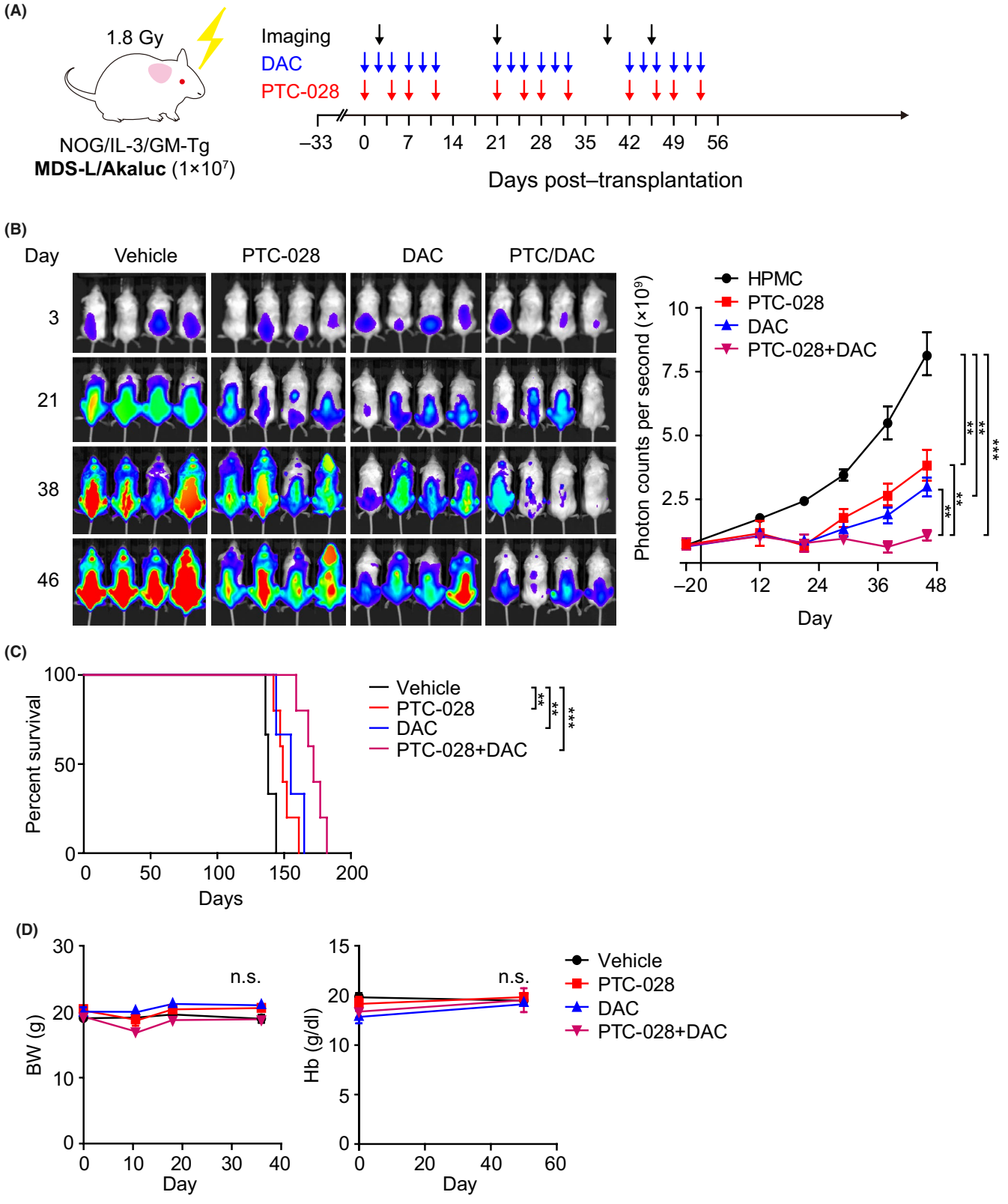


FIGURE 5 Efficacy of PTC-028 as a single agent in the xenograft myelodysplastic syndrome (MDS) model. (A) Schematic representation of the xenograft MDS model using NOG IL-3/GM-TG mice. NOG mice irradiated at a dose of 1.8 Gy were infused with 1×10^7 MDS-L/Akaluc cells via the tail vein. From day 27 post–transplantation, recipient mice (n = 5 in each group) received vehicle and 12.5 mg/kg PTC-028 orally twice a week for 7 wk. (B) The engraftment of MDS-L/Akaluc cells was confirmed by bioluminescence imaging. Images of bioluminescence signals in representative mice (3 mice each) are shown at different time points during the treatment. (C) Quantification of photon counts from MDS-L/Akaluc cells in xenograft MDS mice. Bioluminescence signals taken by a photon-counting analyzer. Data are shown as means ± SD; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. (D) Kaplan–Meier survival of mice. Survival was evaluated from the first day of the treatment to death. The significance of differences between the PTC-028-treated and vehicle-treated groups was assessed using a log-rank test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

advantage of the AkaBLI system, composed of AkaLumine-HCl and Akaluc, which improved performance by a factor of 100 to 1000 over the conventional bioluminescence imaging system composed of D-luciferin and firefly luciferase.[17] MDS-L cells were transduced with the Akaluc gene using a recombinant mScarlet-P2A-Akaluc lentivirus and selected by mScarlet expression as a marker. MDS-L/Akaluc cells (1×10^7) were intravenously inoculated into NOG mice (NOG IL-3/GM TG)[18] irradiated at a dose of 1.8 Gy. The tumor burden was monitored by bioluminescence signals in in vivo imaging assays. MDS-L cells, which retain MDS-like features, expanded very slowly in NOG IL-3/GM-TG mice and induced lethal disease after a long latency. Recipient mice were treated with PTC-028 for 7 weeks (Figure 5A). PTC-028 significantly inhibited the growth of MDS-L cells and prolonged the overall survival of recipient mice (Figure 5B-D). Furthermore, the combination therapy of PTC-028 and decitabine (DAC) significantly reduced the tumor burden compared to PTC-028 or DAC alone (Figure 6A,B) and significantly prolonged the overall survival of recipient mice (Figure 6C). Mice that received combination therapy showed moderate weight loss 11 days after the initiation of the treatment but subsequently recovered (Figure 6D). Hemoglobin levels in the PB of mice did not significantly change during combination therapy (Figure 6D).

4 | DISCUSSION

In the present study, we demonstrated the activities of a novel MTA PTC-028 alone and in combination with decitabine in MDS both in vitro and in vivo. Although PTC-028 has been characterized



as a BMI-1 inhibitor,[8-10] we confirmed that PTC-028 inhibited tubulin polymerization in MDS cells. PTC-028-induced G2/M cell cycle arrest in MDS cells may be attributed to the inhibitory effects of PTC-028 against microtubule polymerization. BMI-1 plays an essential role in MDS cell progression, survival, and drug resistance.[29,30] PTC-028 has been shown to decrease BMI-1 protein and H2A mono–ubiquitination levels in medulloblastoma and ovarian cancer cell lines.[8,10] The underlying pharmacological mechanism involved PTC-028 inducing a reduction in BMI-1 via phosphorylation at the protein level.[10] However, BMI-1 is known to be

FIGURE 6 Combination therapy of PTC-028 and decitabine exerts a synergistic effect in the xenograft myelodysplastic syndrome (MDS) model. (A) Schematic representation of the xenograft MDS model using NOG IL-3/GM-TG mice. NOG mice irradiated at a dose of 1.8 Gy were infused with 1×10^7 MDS-L/Akaluc cells via the tail vein. On day 33 post–transplantation, recipient mice (n = 5 in each group) received vehicle and 6.25 mg/kg PTC-028 orally twice a week. DAC was administered at a dose of 0.3 mg/kg intraperitoneally three times per week. (B) The engraftment of MDS-L/Akaluc cells was confirmed by bioluminescence imaging. Images of bioluminescence signals in representative mice (4 mice each) are shown at different time points during the treatment. Quantification of photon counts from MDS-L/Akaluc cells in xenograft MDS mice. Bioluminescence signals were taken by a photon-counting analyzer. Data are shown as means ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. (C) Kaplan–Meier survival of mice. Survival was evaluated from the first day of the treatment to death. $^{**}P < 0.01$, $^{***}P < 0.001$ by the log-rank test. (D) Body weight (BW) and hemoglobin (Hb) levels of mice. Data are shown as means ± SD. n.s., not significant by Student's *t*-test

hyperphosphorylated and dissociates from chromatin during mitosis.[31] Because PTC-028 induces mitotic cell arrest, these findings suggest that BMI-1 degradation mediated by PTC-028 is a secondary response of mitotic arrest.

PTC-028 significantly induced cytotoxicity in MDS cell lines, primary MDS cells, and MDS cells in a xenograft model. These results demonstrate the preclinical efficacy of PTC-028 as a treatment for MDS. As a therapeutic strategy for MDS, DNA hypomethylating agents are the first choice to treat this heterogeneous disease. A previous study reported that decitabine induced DNA damage, followed by G2/M arrest and caspase-mediated apoptosis. We found that the combination treatment of PTC-028 and decitabine or azacitidine exhibited synergistic cytotoxicity in MDS cells. Our in vivo results obtained using the xenograft model demonstrated that this combination is promising, with a definitive synergy and acceptable tolerability. Therefore, we demonstrated that PTC-028 combined with DNA hypomethylating agents is an effective strategy for MDS therapy.

Similar to other microtubule-destabilizing agents, PTC-028 induces apoptosis in MDS cells. Because microtubules and their dynamic behavior are essential for mitosis, apoptosis induced by microtubule-destabilizing agents is often attributed to the dissolution of mitotic spindles and mitotic arrest. However, the effects of microtubule-destabilizing agents on the microtubule network extend beyond the ability to halt cells in mitosis and include the induction of apoptosis at all phases of the cell cycle, even in non–cycling cells.[3] Acute cell cycle-independent apoptosis in response to microtubule-destabilizing agents has been reported in hematopoietic cells and involves several pathways that dysregulate Bcl-2 family proteins.[3] Further studies are needed to establish whether PTC-028 exerts similar effects to other drugs.

The GSEA analysis showed an upregulated inflammatory response and apoptosis after the treatment with PTC-028. The relationship between inflammation and cell death has been reported in previous studies.[32,33] The release of intracellular material from dead cells triggers inflammatory reactions, including the activation of the NF-κB pathway.[33] However, another mechanism shows that the disruption of microtubule dynamics induces the nuclear translocation of NF-κB via IκB degradation.[34,35] Collectively, these findings explain the enrichment of gene sets targeted by inflammatory responses and the NF-κB signaling pathway. Because the inflammatory cytokines produced through the activation of NF-κB signaling recruit immune cells, which trigger anticancer immunity, MDS cells may also be eliminated by immune activation via PTC-028-induced inflammatory responses.

In conclusion, the present results demonstrate that the inhibition of microtubule polymerization alone and in combination with DNA hypomethylating agents has potential as a novel therapeutic strategy for MDS. The present study provides a preclinical framework for the clinical evaluation of this promising therapeutic approach to improve outcomes in MDS patients.

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DISCLOSURE

WL, JS, and MW are employed by PTC Therapeutics and have received salary compensation for their time and effort and also hold or held financial interests in the company. The remaining authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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