

Reactivation of Silenced WT1 Transgene by Hypomethylating Agents - Implications for *in vitro* Modeling of Chemoimmunotherapy

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Background: A cell line with transfected Wilms' tumor protein 1 (WT1) is has been used for the preclinical evaluation of novel treatment strategies of WT1 immunotherapy for leukemia due to the lack of appropriate murine leukemia cell line with endogenous WT1. However, silencing of the transgene occurs. Regarding the effects of hypomethylating agents (HMAs) on reactivation of silenced genes, HMAs are considered to be immune enhancers. **Methods:** We treated murine WT1- transfected C1498 (mWT1-C1498) with increasing doses of decitabine (DAC) and azacitidine (AZA) to analyze their effects on transgene reactivation. **Results:** DAC and AZA decreased the number of viable cells in a dose- or time-dependent manner. Quantification of WT1 mRNA level was analyzed by real-time polymerase chain reaction after mWT1-C1498 treated with increasing dose of HMA. DAC treatment for 48 h induced 1.4-, 14.6-, and 15.5-fold increment of WT1 mRNA level, compared to untreated sample, at 0.1, 1, and 10 μ M, respectively. Further increment of WT1 expression in the presence of 1 and 10 μ M DAC was evident at 72 h. AZA treatment also induced up-regulation of mRNA, but not to the same degree as with DAC treatment. The correlation between the incremental increases in WT1 mRNA by DAC was confirmed by Western blot and concomitant down-regulation of WT1 promoter methylation was revealed. **Conclusion:** The *in vitro* data show that HMA can induce reactivation of WT1 transgene and that DAC is more effective, at least in mWT1-C1498 cells, which suggests that the combination of DAC and mWT1-C1498 can be used for the development of the experimental model of HMA-combined WT1 immunotherapy targeting leukemia.

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INTRODUCTION

Although chemotherapy and allogeneic hematopoietic stem cell transplantation (allo-HSCT) are still the mainstays of therapy for leukemia, immunotherapy is one of the alternative promising treatment options. The graft-versus-leukemia effects induced by allo-HSCT or post-transplant donor lymphocyte infusion are the most powerful evidence of immunological eradication of leukemia, showing that allo-HSCT itself is a powerful immunotherapy (1). Immunotherapeutic approaches other than HSCT involve the immunization of patients with cancer antigen with the goal of stimulating antigen-specific cytotoxic T lymphocytes (CTL) or the adoptive transfer of *ex-vivo* generated cancer specific CTL that will recognize and kill the tumor.

Among the various immunotherapeutic targets, Wilms' tumor protein 1 (WT1) was ranked first in a list of 75 cancer antigens in a recent report from National Cancer Institute prioritization project (2,3). Also, WT1 is the most promising potential target of leukemia as it is more abundant in leukemia cells than in normal hematopoietic cells (4,5) and seems to participate in leukemogenesis and may be necessary to maintain the viability of leukemia cells (6). Furthermore, WT1 is apparently immunogenic as shown by spontaneous immune responses in leukemic patients (7,8). A growing body of evi-

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dence supports WT1 as a promising target for hematological malignancies, and various clinical trials have demonstrated the feasibility of this approach (3,9-15).

Regarding the wide applicability of WT1 immunotherapy, preclinical experiments using murine WT1-expressing leukemia cell lines could lead to resolution of many of the critical issues for the development of clinical treatment strategies, of which usefulness is further strengthened by the similarity between murine and human WT1 (16,17). However, none of the murine cell lines with endogenous WT1 expression are available, except the FBL3 cell line used for an earlier animal study (18), but which was substituted for by murine WT1-transfected C1498 (mWT1-C1498) as FBL3 was too aggressive and not appropriate for immunotherapy model.

The transgene system such as mWT1-C1498 provides a convenient way for understanding the gene function and generating preclinical experimental models. However, expression levels of transgene in transfected cells often declines with time (19,20). The molecular basis of this gene silencing is not clearly understood but is thought to be associated with hypermethylation of transgene DNA (21,22), which is a similar way by which tumors escape immune reaction by editing tumor antigens or immune related genes. Although transgene silencing has generally been regarded as one of the major drawbacks for experimental use, we assumed that this phenomenon was amenable to being adopted for the development of an experimental model where the potential role of hypomethylating agents (HMAs) for the reactivation of silenced tumor antigen could be tested as an immune enhancer (23,24).

To test this possibility, the present study represents the first step to investigate the effects of decitabine (DAC) and azacitidine (AZA) on transgene-containing cell lines and silencing WT1 from the viewpoint of cell viability, WT1 expression level, and accompanying changes in methylation status induced by the two HMAs.

MATERIALS AND METHODS

Cell culture and drug treatment

mWT1-C1498 and mock-transfected C1498 (mock-C1498) were kindly provided by Dr. H. Sugiyama (University of Osaka, Japan). Cells were grown in DMEM (Dulbecco's modified Eagle's medium) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO) and antibiotics (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

DAC and AZA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of DAC (40 μM) and AZA (400 μM) were prepared in dimethyl sulfoxide (DMSO) and stored at -70°C. The stock solutions were further diluted in DMSO (0.1~10 μM; <0.1% DMSO final concentration) for cell culture experiments.

Cell viability assay

To evaluate the growth inhibitory effect of HMA on C1498 cells with or without WT1 transgene, an MTS assay using CellTiter 96[®] AQueous One Solution Cell Proliferation (Promega, Madison, WI, USA) was done according to the manufacturer's instruction. Briefly, cells were seeded in 96-well plates at a density of 1×10⁴/100 μl of culture medium for 24 hours to reach exponential phase of growth when various concentrations of HMA or vehicle control were added. Following 72 hours incubation, reagent was added into each well containing the samples and the plate was incubated for 1 hour at 37°C in a humidified, 5% CO₂ atmosphere. Absorbance at 490 nm was recorded using a 96-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The doses that inhibited 50% proliferation (IC₅₀) were analyzed by the median-effect method (CalcuSyn software; Biosoft, Cambridge, UK).

RNA extraction and cDNA synthesis

RNA from the cells was extracted by TRIZOL reagent (Invitrogen) and recovered from the aqueous phase by an isopropyl alcohol precipitation. RNA was re-suspended in diethylpyr-carbonate-treated water and quantitated by spectrophotometry (UV/VIS Spectrophotometer ND-100; Nanodrop, Wilmington, DE, USA) and reverse cDNA transcription was performed on 1 μg of total RNA. cDNA was synthesized in 20 μl of a solution containing random hexameric primers (Promega) with the use of AMV reverse transcriptase (Promega), 5X RT buffer (Promega), 10 mM dNTP mixture (Promega), and RNase inhibitor (Promega). The mixtures were incubated at 20°C for 10 min and subsequently incubated at 42°C for 80 min, and denatured by heating at 95°C for 5 min.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

RT-PCR was performed on 1 μl of cDNA using GoTaq[®] Green Master Mix (Promega). RT-PCR conditions for all genes were as follows: 1 cycle at 95°C for 2 min followed by 35 cycles at 95°C for 10 sec, 60°C for 5 sec, and 72°C for 10

sec. WT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were determined by visualizing DNA bands on SYBR[®] Safe DNA gel stain (Invitrogen) stained 1.5% agarose gels. WT1 mRNA expression levels were quantified by real-time PCR using SYBR Green assay (Bio-Rad, Hercules, CA, USA) with Bio-CFX96 (Bio-Rad). The WT1 primers for RT-PCR or real-time PCR were aWT1 sense (5'-taca-gatcatagccggaagc-3') and WT1 antisense (5'-cctgtgtctcctt-ggtg-3'). GAPDH primer sets used for normalizing the quantity of WT1 were GAPDH sense (5'-ggtgtgaacgattggccgtatt-3') and GAPDH antisense (5'-ggccttgactgtgccgtgaatt-3'). Real-time PCR was performed using CFX96[™] on 1 μ l cDNA in 2X iQTM SYBR Green Supermix (Bio-Rad) under following conditions: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 10 sec, 60°C for 5 sec, and 72°C for 10 sec. Samples were measured in triplicate. The resulting data were analyzed with CFX96 manager software (Bio-Rad). The amount of target gene was normalized to the endogenous level of GAPDH. This was done to obtain the relative threshold cycle (ΔC_t) in relation to the C_t of the control gene to measure the relative expression level ($2^{-\Delta\Delta C_t}$) of the target gene.

Evaluation of WT1 protein level using Western analysis

Mock and mWT1-C1498 cells treated with DAC or vehicle for 48 h and 72 h were lysed by RIPA buffer (Thermo Scientific, Rockford, IL, USA) with protease inhibitor cocktail (Thermo Scientific). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). Membranes were incubated with anti-WT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti- β -actin (Abcam, Cambridge, MA, USA) antibodies. X-ray image of the membrane was developed using a SuperSignal[®] West Pico Chemiluminescent Substrate (Thermo Scientific).

Evaluation of methylation status of WT1 promoter

Genomic DNA was prepared from mWT1-C1498 cells treated with or without DAC. Bisulfite conversion of genomic DNA was carried out using an EZ Methylation kit (Zymo Research, Orange, CA, USA) as follows: 500 ng of DNA was sodium bisulfite treated, denatured at 98°C for 10 min, bisulfite converted at 64°C for 2.5 hr, and then desulfonated and eluted using a column preparation. Primer sequences of WT1 gene amplification conditions were sense (5'-cgggacgagcagcgcc-3') and antisense (5'-gtcagtcagctcatctcgtcg-3'). Pyrosequencing

was performed using PyroMark[®] CpG assay (Mm_Wt1_05_PM) (QIAGEN, Hilden, Germany) reagents and the PSQ 96MA machine (Biotage, Uppsala, Sweden). CpG site methylation analysis was performed with the Pyro Q-CpG 1.0.9 software (Biotage), and for each CpG site a methylation percentage was calculated. Mock-C1498 cells treated with vehicle were used as negative control for methylation-specific assays. Busifite DNA (QIAGEN) universally methylated for all genes was used as a positive control for methylated alleles (data not shown).

Statistical analysis

The experimental results are presented as mean \pm standard deviation from triplicate samples of three independent experiments. Statistical analysis was performed using Prism version 5 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered to be statistically significant.

RESULTS

Effects of HMA on cell viability

We examined the effects of DAC and AZA on the growth and survival of mock- and mWT1-C1498 cells, which were evaluated with MTS assay after culture with the drugs at a dose of 0, 0.1, 0.5, 1, 5, and 10 μ M for 24 h, 48 h, and 72 h. During 24 h culture, cell viability did not change with increasing concentrations of DAC in both cell lines. Meanwhile, AZA decreased cell viability in a dose-dependent manner. On the long-term culture (48 and 72 h), it was revealed that DAC decreased the number of viable cells in both cell lines in a dose- and time-dependent manner, while AZA decreased cell viability in a dose-dependent manner rather than a time-dependent manner (Fig. 1). When the IC₅₀ of the drugs at 48 and 72 h were compared, AZA showed higher IC₅₀ compared to DAC in mWT1-C1498 cells at both time points, but vulnerability of mock-C1498 to the drugs were different according to exposure time. Generally, mWT1-C1498 cells were likely to be more susceptible to DAC than mock-C1498 cells when treated for 48 and 72 h (Table I). These results indicate that both HMA have a significant effect on cell viability at 48 h or longer treatment at doses $\geq 5 \mu$ M, but that exposure time was more important in DAC treatment.

Effect of HMA on mRNA expression of WT1

Initial screening by RT-PCR indicated the absence and presence of WT1 in mock- and mWT1-C1498, respectively, and

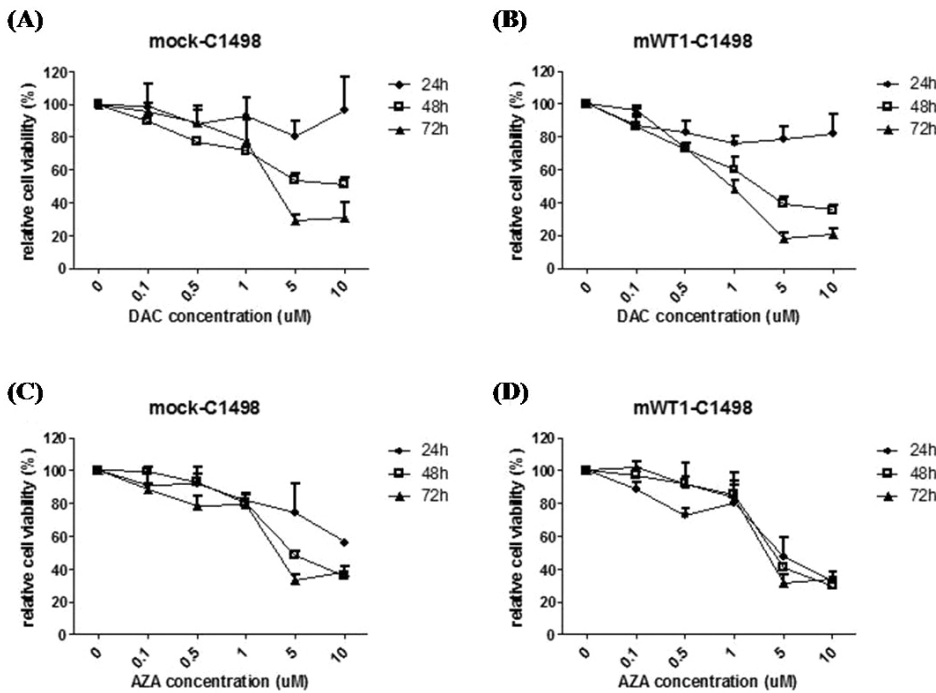


Figure 1. Effects of HMA on cell viability. In all cases, cell viability was measured using MTS assay as described. (A) C1498 cells were cultured with increasing concentrations of HMA for 24 h, 48 h, and 72 h. (B) mWT1-C1498 cells treated with 0.1~10 μ M DAC for 24 h up to 72 h. (C) mock-C1498 cells treated with 0.1~10 μ M AZA for 24 h up to 72 h. (D) mWT1-C1498 cells treated with 0.1~10 μ M AZA for 24 h up to 72 h. Samples were measured in triplicates.

Table I. Measurement of IC₅₀ of Decitabine and Azacitidine in mock- and mWT1-C1498 cell lines

Cell lines	IC ₅₀ (μ M)			
	Decitabine		Azacitidine	
	48 h	72 h	48 h	72 h
mock-C1498	9.07 \pm 4.59	3.57 \pm 2.15	6.13 \pm 3.47	3.86 \pm 1.56
mWT1-C1498	2.83 \pm 1.4	1.41 \pm 0.38	4.44 \pm 1.61	1.89 \pm 1.89

Values are presented as mean \pm SD of three independent experiments.

treatment with DAC could change WT1 mRNA expression level in mWT1-C1498 without non-specific response in the control cell line (Fig. 2A). Quantification of WT1 mRNA level was further analyzed by real-time PCR after mWT1-C1498 cells were treated with 0, 0.1, 1, and 10 μ M DAC and AZA for 48 h and 72 h. DAC treatment for 48 h induced 1.4-, 14.6-, and 15.5-fold increment of WT1 mRNA level compared to untreated sample at 0.1, 1, and 10 μ M, respectively, with the latter change being statistically significant (Fig. 2B). Further increment of WT1 expression level at 1 and 10 μ M was observed with a 72 h treatment with DAC. AZA treatment also induced 1.48-, 3.99-, and 18.94-fold WT1 gene re-expression at 0.1, 1, and 10 μ M, respectively, for 48 h (Fig. 2C). Contrary to DAC, further treatment with AZA up to 72 h re-

sulted in the down-regulation of WT1 mRNA. Collectively, DAC was more potent compared to AZA in inducing re-expression of WT1 transgene at a dose of 1 and 10 μ M, and longer treatment with DAC induced higher expression. On the basis of these results, DAC was selected for the following experiments using mWT1-C1498.

Effect of DAC on protein expression level of WT1

Western blot analysis was performed on mWT1-C1498 cells treated with DAC at various doses (0.1, 1, 10 μ M) for 48 and 72 h. When a total of 100 μ g protein for each lane were loaded, a visible band was not observed in untreated samples or samples treated with 0.1 μ M DAC, but strong bands were observed at higher doses of 1 and 10 μ M, which correlated with

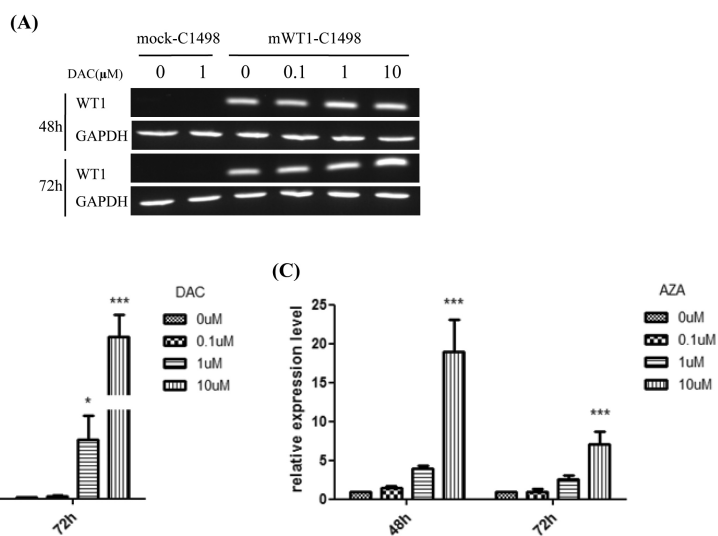


Figure 2. (A) HMA mediates induction of WT1 in C1498 cells in a time- and dose-dependent manner by RT-PCR results. (B, C) Expression levels of WT1 mRNA were determined by real-time PCR in untreated and HMA treated mWT1-C1498 cells at 48 h and 72 h. Shown are the mean mRNA values normalized to GAPDH from at least three experiments. Error bars indicate the standard deviation. Data are expressed as mean \pm SD; * $p < 0.05$, *** $p < 0.001$.

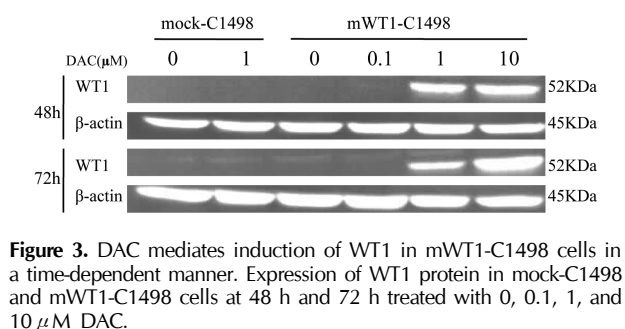


Figure 3. DAC mediates induction of WT1 in mWT1-C1498 cells in a time-dependent manner. Expression of WT1 protein in mock-C1498 and mWT1-C1498 cells at 48 h and 72 h treated with 0, 0.1, 1, and 10 μ M DAC.

the changes in mRNA level by real-time PCR (Fig. 3).

Hypomethylation of CpG islands in mWT1-C1498 accompanied with WT1 gene transcriptional activity

We then assessed whether methylation patterns of CpG islands were accompanied with transcriptional activity of the WT1 gene. mWT1-C1498 cells were treated with DAC at 0.1, 1, and 10 μ M for 48 h and 72 h, and their methylation status was determined by pyrosequencing and compared to vehicle control. After 48 h DAC treatment, mean methylation status of four sites were 94.8%, 93.5%, 78.48%, and 60.75% at 0, 0.1, 1, and 10 μ M, respectively. Differences of methylation status between control and 1 or 10 μ M were statistically significant and correlated with the WT1 up-regulation at the mRNA and protein levels (Fig. 4). Further treatment up to 72 h also showed the decrement of methylation status, but it was not different from those of 48 h.

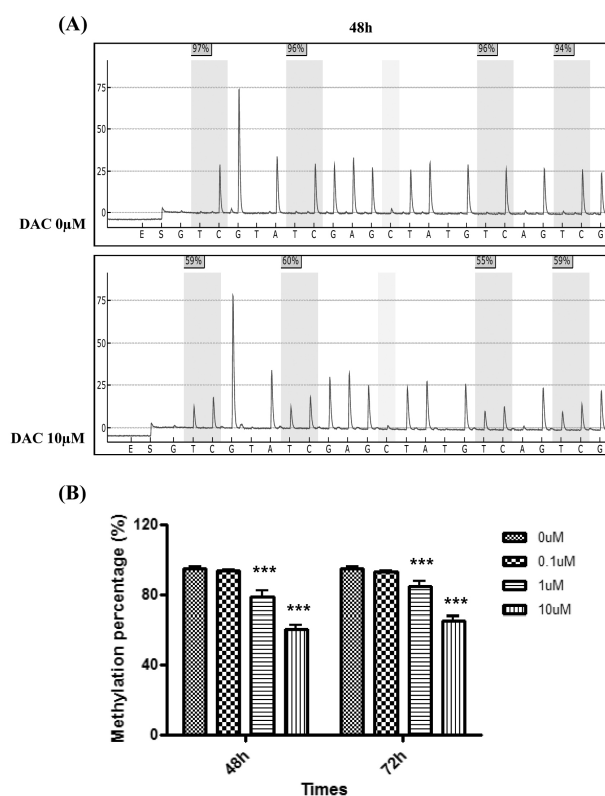


Figure 4. Dose-dependent hypomethylation induction by DAC in mWT1-C1498 cell lines. (A) A representative methylation analysis of four CpG-sites of the WT1 promoter using pyrosequencing. (B) Average percentage for each sample is displayed as bar graphs. Data are expressed as mean \pm SD; *** $p < 0.001$.

DISCUSSION

Recent advances in immunotherapy have opened the possibilities of further eradication of post-chemotherapy residual leukemia by targeting leukemia-associated antigens of WT1 (25). One strategy to enhance anti-tumor immune reaction is to combine chemotherapy with immunotherapy, so called chemoimmunotherapy (26), which clearly demonstrates that chemotherapeutic agents can synergize with and enhance T cell response in pre-clinical and clinical studies (27,28). These approaches seem to be helpful in overcoming immune escape mechanism of leukemia with which the phenotypic changes of the leukemic cells characterized by the down-regulation or disappearance of tumor antigen, human leukocyte antigen (HLA), or co-stimulatory molecules are partly involved. In line with this theoretical base, HMA are also considered appropriate candidate agents for chemoimmunotherapy as they augment CTL response by inducing tumor antigens or HLA (29,30). As seen in solid tumors, induction of tumor antigens by HMA was also observed in hematological malignancies (31).

Two HMAs, DAC and AZA, belong to a class of cytosine analogues that inhibit DNA methylation and have shown clinical efficacy in myelodysplastic syndrome and acute myelogenous leukemia. In addition to their antineoplastic activity, recent evidence suggested that HMA can modulate important immune functions via epigenetic modifications (32,33), making it an attractive candidate for pharmacologic manipulation of the immunotherapy. Although epigenetic modulation of immune function has been widely studied, measuring anti-WT1 immune responses in leukemia in association with HMA has never been studied because of the lack of appropriate murine leukemia cell lines and available animal models. WT1 expressing solid tumor cell lines such as TRMP-C were alternatively used for preclinical animal experiments for the evaluation of anti-WT1 immunology in patients with leukemia (34).

In pursuing a new experimental model, we performed *in vitro* experiments and revealed the up-regulation of WT1 transgene expression by treating mWT1-C1498 with HMA, which was related with the hypomethylation of transgene. We first evaluated the cytotoxicity of DAC and AZA on cell viability. With 24 h culture, DAC was minimally toxic to mock- and mWT1-C1498 cells. Meanwhile, AZA showed higher toxicity, especially at doses $\geq 5 \mu\text{M}$. However, longer incubation showed a trend of higher toxicity of DAC, especially in mWT1-C1498 cells when comparing IC_{50} of two

drugs at two time points. There was no differences in IC_{50} between the two cell lines in AZA treatment, but mWT1-C1498 cells were more vulnerable to DAC. When decreased cell growth by DAC was assessed in association with apoptosis, the drug induced apoptosis in dose-dependent and time-dependent manners, similar to the patterns of cell viability. Next, we analyzed the expression level of transgene. A lower dose of DAC or AZA ($0.1 \mu\text{M}$) did not affect the mRNA level of WT1, but higher doses of the drugs induced up-regulation of the gene level. Significant increment was observed with DAC at 1.0 and $10 \mu\text{M}$, but only at $10 \mu\text{M}$ for AZA. At these two dose levels, relative increment of mRNA was prominent in DAC treatment in the comparison with AZA, whether incubation time was 48 h or 72 h, showing higher efficiency of transgene reactivation of DAC. Of course, this result should not be translated to indicate that DAC is superior to AZA in up-regulating silenced tumor antigens. Rather, differences in WT1 transgene reactivation in our study might be explained by the observation by Hollenbach et al. who suggested that the majority of genes regulated by AZA and DAC are drug-specific as they show distinctly different effects in their actions on cell viability, protein synthesis, cell cycle, and gene expression (35). We also observed that up-regulation of WT1 transgene was accompanied by concomitant down-regulation of methylation status, suggesting that transgene expression could be regulated by the epigenetic modifications marking on the promoter (36). Regarding histone decetylation (HDAC) in addition to DNA methylation is the major epigenetic changes associated with gene suppression (37), further studies to combine HMA with HDAC inhibitor could be pursued to modulate transgene silencing.

Our results suggest that treatment of mWT1-C1498 cells with DAC can efficiently reactivate the silenced WT1 transgene by induction of DNA hypomethylation of the promoter region, which suggests the possibility that DAC could enhance immune reaction against silenced WT1 transgene in mWT1-C1498 cells. Further studies are needed to develop an animal model of epigenetically modulated immunotherapy, where novel treatment strategies of chemoimmunotherapy targeting WT1 can be practically investigated.

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CONFLICTS OF INTEREST

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