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CDKN1A Gene Expression in Two Multiple Myeloma Cell Lines With Different P53 Functionality

DENISA HRCKOVA DROZDKOVA[1,2], JAN GURSKY[3], JIRI MINARIK[4,5], IVO ÜBERALL[1], ZDENEK KOLAR[1,4] and KATERINA SMESNY TRTKOVA[1,2]

[1]*Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic;*
[2]*Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic;*
[3]*Department of Biology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic;*
[4]*Department of Hemato-Oncology, University Hospital Olomouc, Olomouc, Czech Republic;*
[5]*Department of Hemato-Oncology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic*

Abstract. *Background/Aim: Multiple myeloma is a highly heterogeneous disease of clonal plasma cells. Histone deacetylase (HDAC) inhibitors are promising anticancer drugs but their precise mechanisms of actions are not well understood. Materials and Methods: Cell-cycle regulation and pro-apoptotic effects of two histone deacetylase inhibitors, suberohydroxamic acid (SAHA) and suberoylanilide hydroxamic acid (SBHA), were analyzed in multiple myeloma cell lines RPMI8226 and U266 with differing TP53 status using gene-expression analysis. Results: Enhanced expression of cyclin-dependent kinase inhibitor 1A (CDKN1A/p21$^{WAF/CIP1}$) detected in the TP53-deleted U266 cell line after SAHA treatment indicates the P53-independent mode of transcriptional activation of CDKN1A gene. In contrast, CDKN1A gene expression was significantly increased by both SBHA and SAHA treatment of TP53-mutated RPMI8226 cells. Conclusion: SAHA appears to be a potentially effective pro-apoptotic and anticancer drug with universal application in the treatment of heterogeneous populations of multiple myeloma cells.*

Correspondence to: Denisa Hrckova Drozdkova, ORCID ID 0000-0002-8952-4454, Laboratory of Molecular Pathology, Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Hnevotinska 3, 775 15 Olomouc, Czech Republic. E-mail: denisa.drozdkova@seznam.cz and Katerina Smesny Trtkova, ORCID ID 0000-0002-6104-8118, Laboratory of Molecular Pathology, Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Hnevotinska 3, 775 15 Olomouc, Czech Republic. E-mail: katerina.smesny@upol.cz

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Multiple myeloma (MM) is a disease of terminally differentiated B-cells known as plasma cells. It is characterized by clonal proliferation of malignant plasma cells with extensive skeletal destruction, infections, anemia, and hypercalcemia (1). MM accounts for approximately 1% of all malignancies, with a mean diagnostic age of 65 years. It is more common in men than in women and twice as common in African Americans (2).

Genomic events occurring during the disease can be divided into primary and secondary events. Primary events are further divided into hyperdiploid (HRD) and non-HRD subtypes, which are defined by a number of recurrent chromosomal translocations. Primary HRDs are usually triple odd numbers involving chromosomes 3, 5, 7, 9, 11, 15, 19, and/or 21 (3, 4). Primary non-HRD events include translocations of immunoglobulin heavy chains, with the most common translocations being t(11;14), t(4;14), t(14;16), as well as del13q, the most common deletion in MM (5). Patients with MM may have HRD, trisomy, translocation, and deletion at the same time (6).

Oncogenic mutations are likely to be more clonal than mutations in tumor-suppressor genes, with the notable exception of *TP53* (7). P53 protein is encoded by the *TP53* gene located on chromosome 17p13.1, while a deletion of the chromosomal region 17p13-del (17p) is associated with poor prognosis in multiple myeloma (8). Once activated, P53 induces either cell-cycle arrest or apoptosis by transactivating its downstream genes, such as cyclin-dependent kinase inhibitor 1A (*CDKN1A*), leading to cell-

cycle arrest, and BCL2 apoptosis regulator (BCL2)-associated X (*BAX),* P53-up-regulated modulator of apoptosis *(PUMA)* and phorbol-12-myristate-13-acetate-induced protein 1 *(NOXA)* genes, which induce apoptosis (9). In MM, *TP53* mutations are uncommon in diagnosis (~8%) (8, 10). An increase in incidence of *TP53* mutations in advanced stages of the disease and a key role in the progression of MM have been suggested (10, 11).

To elucidate the different effects on cell-cycle progression and apoptosis in the myeloma cell lines studied, we used histone deacetylase (HDAC) inhibitors: Suberohydroxamic acid (SAHA) and suberoylanilide hydroxamic acid (SBHA). SBHA is a non-selective HDAC inhibitor, causes cell differentiation, cell-cycle arrest and apoptosis, and has an antiproliferative effect against tumor cell lines (12-14). HDAC inhibitors are known to arrest human tumor cells in the G_1 phase of the cell cycle and activate CDK inhibitors (15, 16).

The aim of our study was to compare the effects of HDAC inhibition on cell-cycle progression, induction of apoptosis, and expression of CDK inhibitors in two MM cell lines, RPMI8226 (17) and U266 (U266B1) (18) with different *TP53* status.

Materials and Methods

Reagents. SAHA, SBHA, 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI), sodium pyruvate, penicillin-streptomycin, L-glutamine, RPMI 1640 medium, ApoScreen^R Annexin-V, CellEvent™ Caspase-3/7 Green, HEPES, propidium iodide (PI), 7-aminoactinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). Taq-Man probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). JNJ–7706621, an inhibitor of CDK1 and CDK2, was purchased from Selleckchem (Houston, TX, USA).

Cell culture and cell conditions. According to Keats Lab (www.keatslab.org), U266 cell line has A161T missense mutation on *TP53* gene, while RPMI8226 cell line has E285K missense mutation on *TP53* gene (19). The U266 cell line has deletions of chromosome 13 and 17p, which involve RB transcriptional co-repressor 1 *(RB1)* and *TP53* genes, respectively. The RPMI8226 cell line has no deletion of neither chromosome 13, nor chromosome 17p (19).

The human MM cell lines RPMI8226 (CCL155™) and U266B1 were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium supplemented with 10% or 15% fetal bovine serum (FBS), respectively, and 1% penicillin-streptomycin antibiotics, 1% L-glutamine, and 100 mM sodium pyruvate at 37˚C in an atmosphere with 5% CO_2.

Both cell lines were seeded on 6-well plates at 5×10^5 cells per well and treated with SAHA (5 μM), SBHA (0.5 μM, 5 μM), and JNJ-7706621 (0.5 μM, 5 μM) for 24 h. Untreated cells cultivated with 10% dimethyl sulfoxide were used as a control sample. All treatments were performed in triplicate.

Assessment of apoptosis by Annexin-V and caspases 3 and 7. Annexin-V staining was carried out to semi-quantify the viable, apoptotic, and necrotic cells. For determination of apoptosis by annexin-V, cells were harvested after 24 h, centrifuged, and kept continuously on ice. Binding buffer (140 mM NaCl, 4 mM KCl, 0.75 mM $MgCl_2$ and 10 mM HEPES-KOH, pH 7.8), annexin V, 7-aminoactinomycin D and $CaCl_2$ were added sequentially. The samples were incubated on ice for 15 min without the presence of light. For determination of apoptosis by caspase 3 and 7 activity, cells were harvested after 24 h, centrifuged, and kept on ice. Binding buffer and 2% FBS was added. Subsequently, CellEvent™ Caspase-3/7 Green Detection Reagent was added at a final concentration of 500 nM. Samples were incubated at room temperature for 40 min without light. Finally, DAPI was added and samples were incubated for 5 min without the presence of light. For both methods, at least 10,000 cells per sample were analyzed. Apoptosis was analyzed by flow cytometry (BD FACSVerse; BD Biosciences, San Jose, CA, USA), and the collected data were processed using BD FACSuite software (BD Biosciences).

Cell-cycle analysis. Flow cytometry was performed for cell-cycle analysis using PI to stain cellular DNA. Cells were treated for 24 h, harvested, and fixed with 96% ice-cold ethanol at −20˚C. After cell washing with ice-cold PBS plus 2% FBS, cells were incubated with 0.2 mg/ml RNAse A and PBS for 30 min. PI (200 μl) was then added and the cell distribution was analyzed by flow cytometry. The results were analyzed using BD FACSuite software (BD Biosciences).

mRNA analysis using quantitative real-time PCR (RT-qPCR). The cells were seeded in 6-well plates at a density of 1×10^5 cells/well, and were harvested after 24 h of SAHA, SBHA, or JNJ-7706621 treatment. Total RNA from both cell lines was isolated with a High Pure RNA Isolation kit (Roche, Basel, Switzerland) and 100 ng of total RNA was converted to mRNA using First Strand cDNA Synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions. The real-time PCR was carried out using Taq-Man probes with Xceed qPCR Probe Mix (Institute of Applied Biotechnologies, Prague, Czech Republic) using Light cycler®480 System (Roche, Basel, Switzerland). The expression of *CDKN2A* (Hs00923894_m1), *CDKN2B* (Hs00793225_m1), and *CDKN1A* (Hs00355782_m1) genes was normalized to expression of the endogenous housekeeping control gene *GAPDH* (Hs01041237_g_1). All probes used were provided by Thermo Fisher Scientific (Waltham, MA, USA). Control (untreated) cells were used as the calibrator (control) for $2^{-\Delta\Delta Ct}$ quantification. The experiments were performed in triplicate with a similar pattern of results.

Statistical analysis. Data are expressed as the mean±SD. The significance for RT-qPCR assay was set at >2-fold and >4-fold changes comparing between the control and treated cells.

Results

Apoptosis in U266 and RPMI8226 cell lines. In both tested cell lines, treatment with 5 μM JNJ-7706621 led to a similar proportion (50-60%) of viable cells. Viable U266 cells decreased after treatment with 5 μM SAHA (to 65.8% by annexin-V staining, and 69.2% by caspase-3 and -7 assay) and 5 μM JNJ-7706621 (to 55.7% by annexin-V staining, and 60.2% by caspase-3 and -7 assay) in comparison to

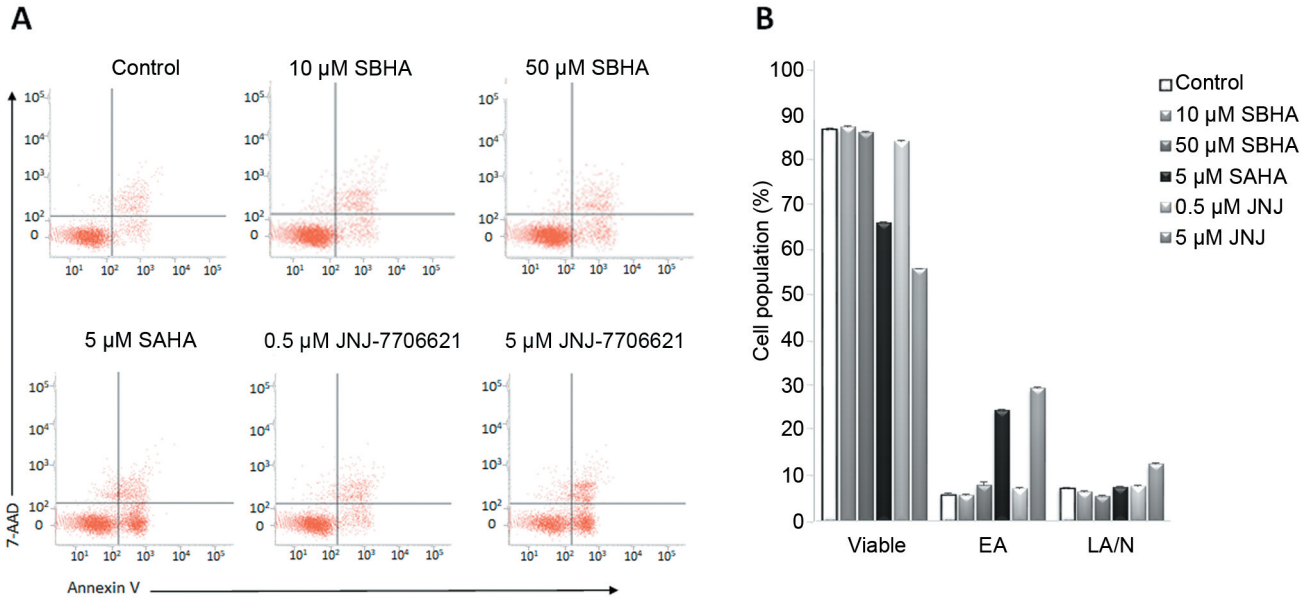

Figure 1. *Flow cytometric analysis using annexin-V/7-aminoactinomycin D (7-AAD) with representative histograms (A) and cell-viability quantification (B) after 24 h of treatment of U266 cell line with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD.*

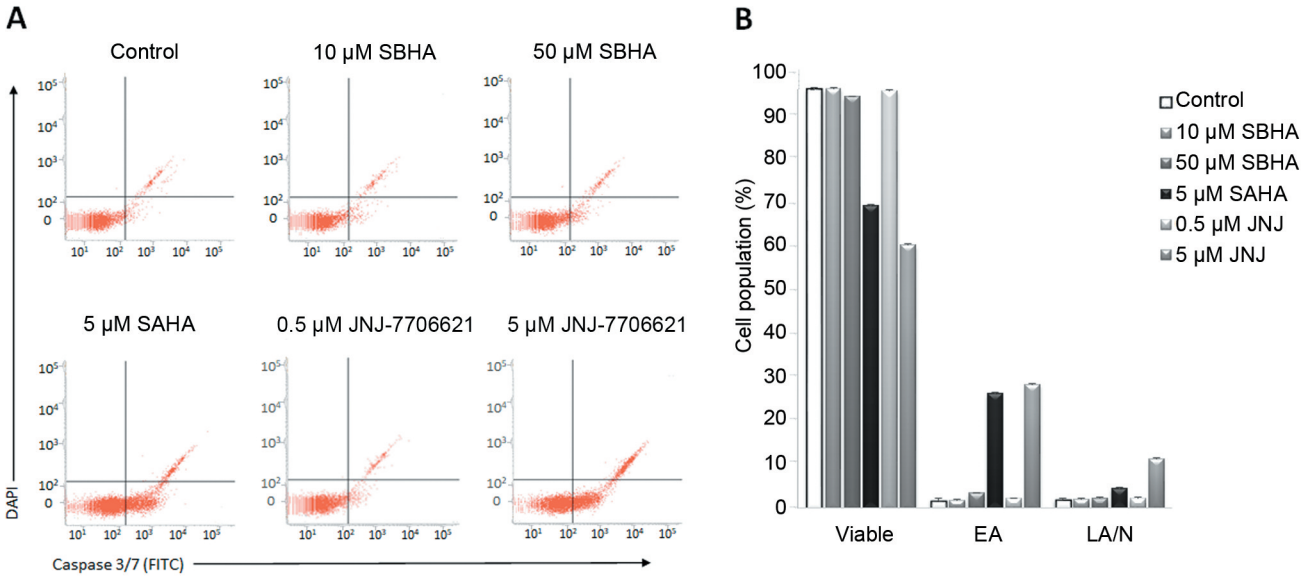

Figure 2. *Determination of apoptosis by caspase-3 and -7 activities with representative histograms (A) and cell viability quantification (B) after 24 h of after 24 h of treatment of U266 cell line with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD. DAPI: 4',6-Diamidino-2-phenylindole.*

untreated cells (86.5% by annexin-V staining, and 95.8% by caspase-3 and -7 assay), which corresponds to the increased number of apoptotic cells in the early and late phases of these treatments (Figures 1 and 2). The RPMI8226 cell line, as shown in Figures 3 and 4, the proportion of viable cells was reduced with 50 μM SBHA and 5 μM JNJ-7706621 treatments, suggesting that apoptosis induced in RPMI8226 cells was dose-dependent.

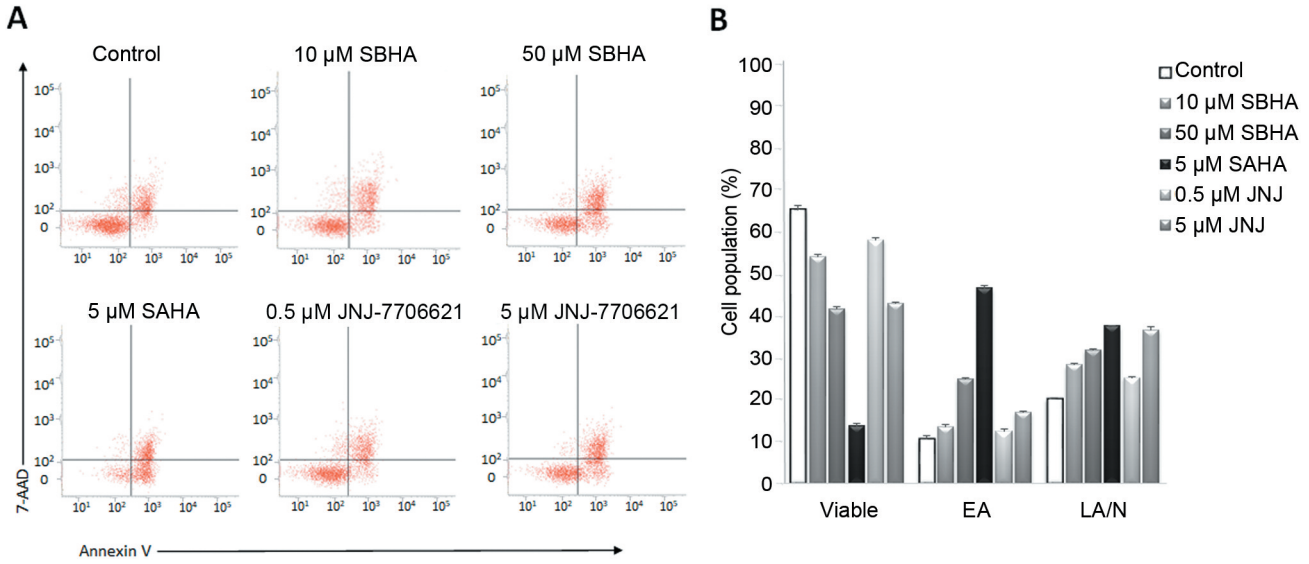

Figure 3. *Flow cytometric analysis using annexin-V/7-aminoactinomycin D (7-AAD) with representative histograms (A) and cell-viability quantification (B) after 24 h of treatment of RPMI822 cell line with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD.*

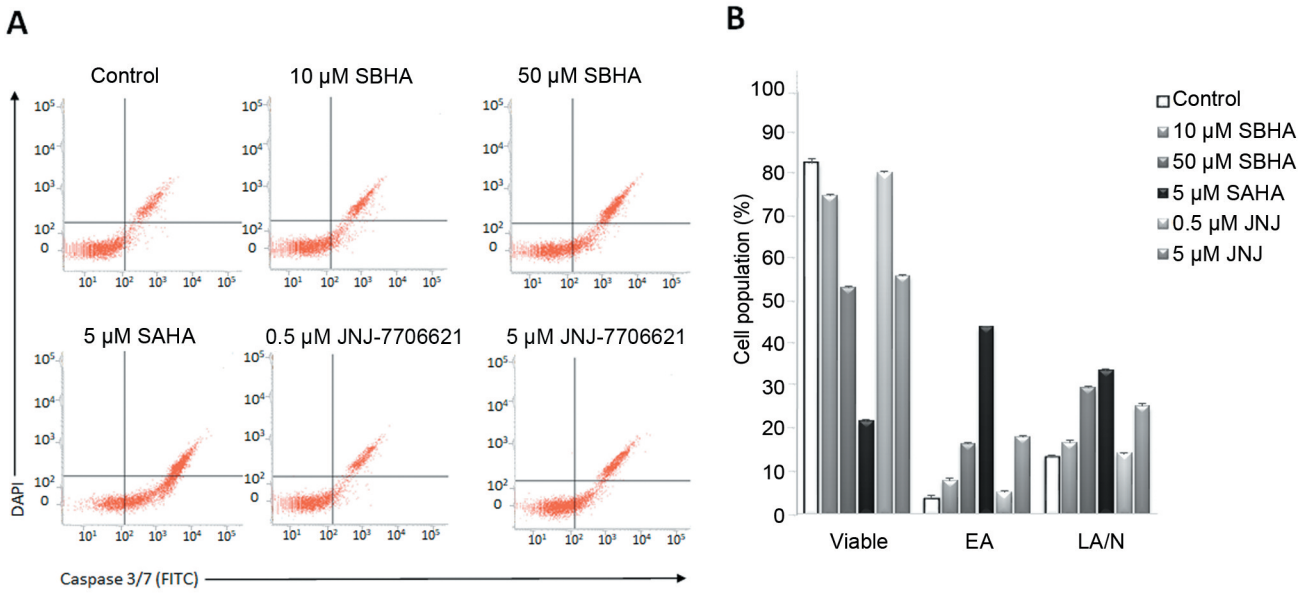

Figure 4. *Determination of apoptosis by caspase-3 and -7 activities with representative histograms (A) and cell viability quantification (B) after 24 h of treatment of RPMI822 cell line with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD. DAPI: 4',6-Diamidino-2-phenylindole.*

Cell-cycle distribution profile in U266 and RPMI8226 cell lines. The effects on cell-cycle progression of the cyclin-dependent kinase inhibitor JNJ-7706621 in comparison with histone deacetylase inhibitor SBHA and pro-apoptotic SAHA were investigated. As illustrated in Figure 5, U266 cells treated with 5 μM JNJ-7706621 and 5 μM SAHA terminated in the cell cycle predominantly at the G_1 checkpoint. In the case of treatment with SBHA, the proportion of G_1 cells

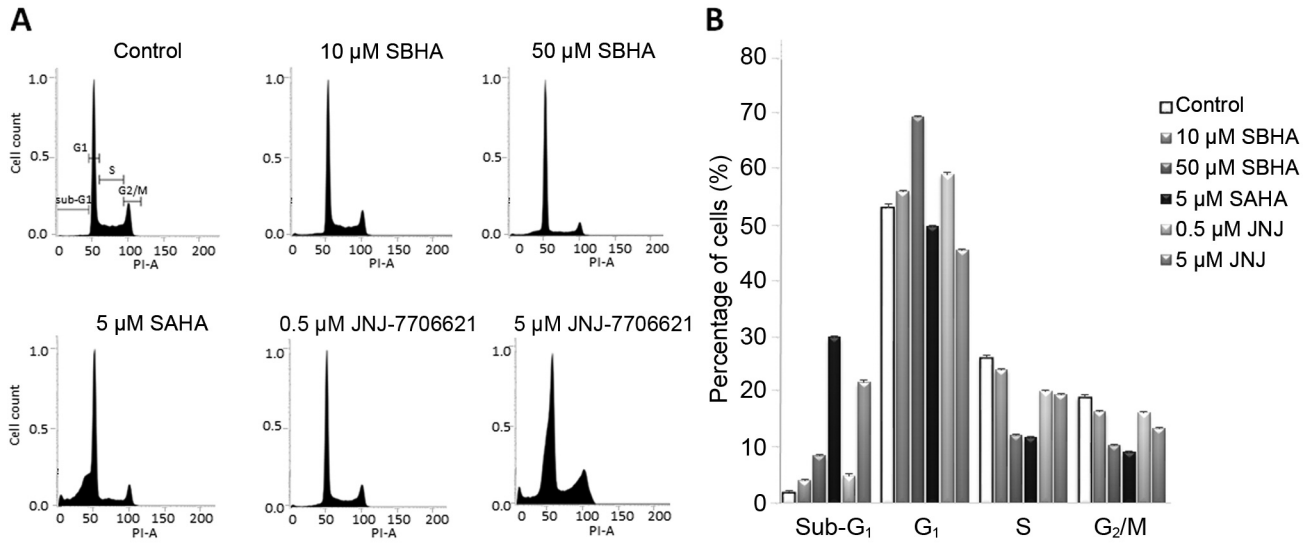

Figure 5. *Cell-cycle analysis (A) and quantification (B) of U266 cells after 24 h of treatment with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD.*

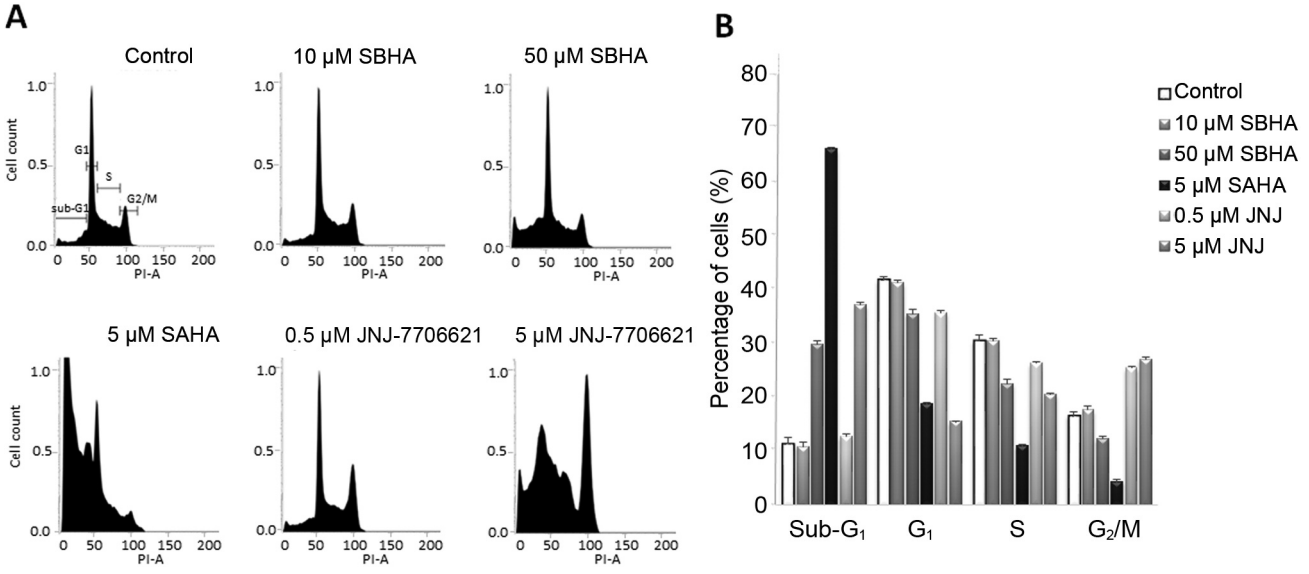

Figure 6. *Cell-cycle analysis (A) and quantification (B) of RPMI8226 cells cells after 24 h of treatment with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD.*

increased gradually in a dose-dependent manner as compared to untreated U266 cells. Treatment with SAHA and 5 μM JNJ-7706621 resulted in cell-death induction, as indicated by the sub-G_1 fraction (29.7% and 21.6%, respectively); however, the cell-cycle stage distribution was basically preserved.

As shown in Figure 6, RPMI8226 cells were more susceptible to induction of cell death than the U266 cell line, as treatment with both 50 μM SBHA and 5 μM JNJ-7706621 increased the percentage of sub-G_1 cells (29.8% and 37.0%, respectively) compared to untreated cells (11.4%). Even more efficient induction of the sub-G_1 fraction was observed after 5 μM SAHA treatment, achieving up to 65%, accompanied by changes in the cell-cycle distribution. The massive increase in the sub-G_1 fraction in JNJ-7706621-

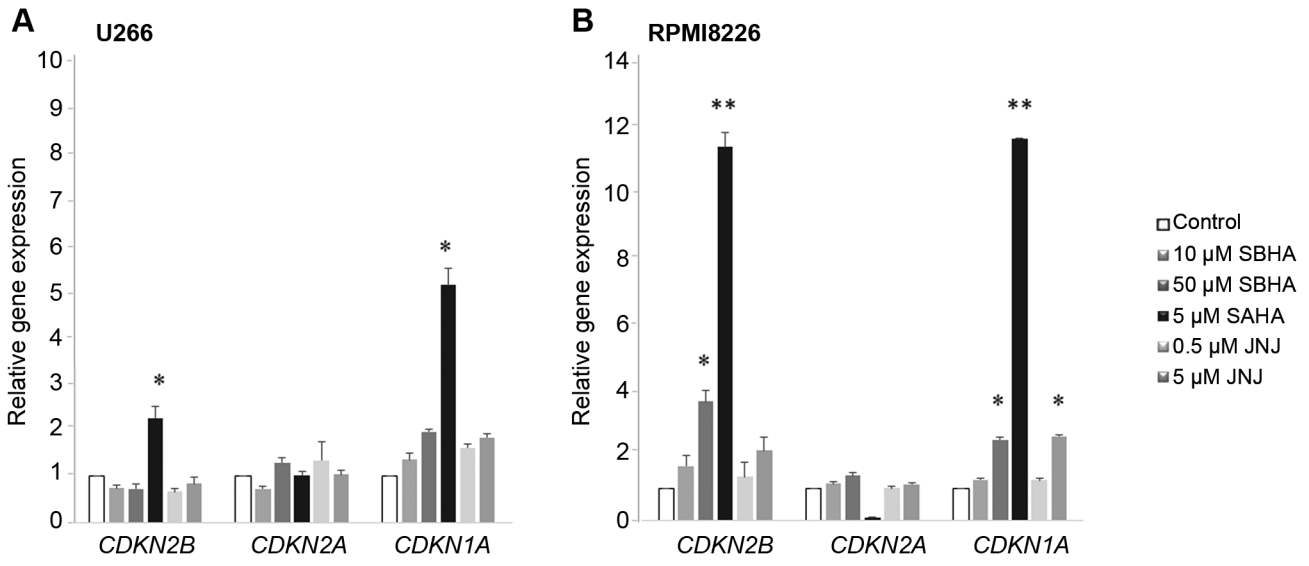

Figure 7. *Analyses of expression of CDKN2B, CDKN2A, and CDKN1A genes in U266 (A) and RPMI8226 (B) cells after 24 h treatment with cells after 24 h of treatment with 10 μM and 50 μM suberoylanilide hydroxamic acid (SBHA), 5 μM suberohydroxamic acid (SAHA), and 0.5 μM and 5 μM pan-cyclin-dependent kinase inhibitor JNJ-7706621. All data are expressed as the mean±SD. *>2-Fold and **>4-fold changes comparing between the control (untreated) and treated cells.*

treated RPMI8226 cells is in contrast with the G_1 accumulation observed in U266 cells, indicating differences in cell-cycle regulation between these cell lines. Thus, an increase in the sub-G_1 phase induced by 5 μM SAHA and 5 μM JNJ-7706621 confirmed their pro-apoptosis effects in both tested cell lines. Like in U266 cells, 50 μM SBHA treatment of RPMI8226 cells induced cell death, indicating its pro-apoptotic effect. Treatment of cells with 10 μM SBHA and 0.5 μM JNJ-7706621 had only a limited impact on cell-cycle progression in both cell lines studied.

CDK gene expression after HDAC treatment. To understand the mechanism of action of the HDAC and CDK inhibitors on genes affecting the course of the MM cell-cycle progression, we performed mRNA quantification of *CDKN2B, CDKN2A*, and *CDKN1A*. After 24 h of treatment of RPMI8226 cells, only 50 μM SBHA, 5 μM SAHA, and 5 μM JNJ-7706621, increased the expression level of *CDKN2B* and *CDKN1A* genes increased. In contrast, only treatment with 5 μM SAHA significantly increased *CDKN2B* and *CDKN1A* gene expression in both cell lines (Figure 7).

Discussion

The disruption of normal mitosis by HDAC inhibitors is a significant contributor to their anticancer effects, although the mechanism of HDAC inhibitor-induced apoptosis is not completely understood. The induction of apoptosis may be due to two major pathways, the extrinsic (death-receptor pathway) and the intrinsic (mitochondrial pathway), although all HDAC inhibitors activate either one or both of these cell death pathways (15, 20). The death-receptor pathway is activated when a ligand such as FAS or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) bind to their death receptor with followed activation of caspase-8 (15). Many studies have suggested that HDAC inhibitor induce apoptosis only *via* the mitochondrial pathway (20-22). The mitochondrial pathway may be invoked due to the ability of HDAC inhibitors to induce pro-apoptotic genes, thus shifting the balance toward cell death (15). Consistent with this idea, overexpression of BCL2 family proteins BCL2 and BCL-xL blocks HDAC inhibitor-mediated apoptosis (20, 22). BCL2 and its relatives act to restrain the cell death mediators BAX and BCL2 antagonist/killer 1 (BAK1), thereby maintaining cellular viability. Additionally, the actions of these proteins are countered by a subclass of the BCL2 family, the BH3-only proteins such as BCL2-modifying factor (BMF), BCL2-like 11 (apoptosis facilitator) (BIM), and BH3 interacting domain death agonist (BID) (23).

In this study, the potential cell-cycle regulating effect *via* apoptotic pathways using two HDAC inhibitors, SAHA and SBHA, towards U266 and RPMI8226 myeloma cell lines was assessed. We found that the SAHA-induced cell-cycle regulating effect involving a reduced number of viable cells was common to both tested MM cell lines, while pro-apoptotic and proliferative effects of SBHA sensitized RPMI8226 cells only.

P53-mediated apoptosis has been reported upon SAHA treatment (22, 24). However, Sonnemann *et al*. described a

SAHA stimulative effect in *TP53*-wild type and null cells (25). SAHA, under the name of vorinostat, was originally approved by the US Food and Drug Administration for treatment of cutaneous T-cell lymphoma; because of its low toxicity, SAHA is currently being evaluated in clinical trials for the treatment of cancer (NCT00109109) (26). SAHA initiates cell death by inducing mitochondria-mediated pathway characterized by cytochrome *c* release and reactive oxygen species (ROS) stimulation. The mitochondrial disruption is achieved by means of cleavage of the BH3-only protein BID (22, 27). Caspase-9 is critical for SAHA-induced apoptosis and activation of caspase-2, caspase-8, and caspase-7 depends on caspase-9 (24). Furthermore, SAHA-induced cell death does not require activation of the death-receptor pathway nor the expression of functional P53 (22).

Similarly to SAHA, SBHA-induced apoptosis is dependent on induction of mitochondrial membrane permeability. In MM cell lines (21, 28) is SBHA described as being able to enhance TRAIL-induced cell death by the up-regulation of the pro-apoptotic proteins BIM, BID, BAK and BAX, while at the same time down-regulating the anti-apoptotic proteins BCL-xL, induced myeloid leukemia cell differentiation protein MCL1, and X-linked inhibitor of apoptosis (XIAP) (29). The mechanism by which SBHA regulates such a diverse array of genes involved in apoptosis is not clear. Likely, SBHA targets HDACs associated with transcriptional factors that are involved in the regulation of apoptosis such as TP53 or c-MYC. Apoptosis induced by SBHA occurred relatively late (24-48 h) after addition of the drug (21), and this property of SBHA may affect potential later onset of apoptosis in RPMI8226 cells.

However, the prominent difference between U266 and RPMI8226 cells in their *RB1* and *TP53* status may explain, in part, the lower sensitivity of U266 cells to SBHA-induced cell death in comparison to the RPMI8226 cells. On the other hand, a similar impact of SAHA treatment on apoptosis and cell-cycle course in both tested cell lines might be related to its P53-independent mechanism of action.

HDACs are chromatin-modifying enzymes, which facilitate closed chromatin and hence transcriptional repression. Several studies have shown that HDAC inhibitors, including SAHA, activate the expression of *CDKN1A* through an increase of the acetylation of histones H3 and H4 around the *CDKN1A* promoter and this effect is most commonly associated with an increase in *CDKN1A* due to its P53-independent induction (30). Furthermore, the Sp1 sites on the *CDKN1A* promoter release the repressor HDAC1 from its binding (31). Thus, *CDKN1A* expression is regulated in a P53-dependent and P53-independent manner. In our study, an expression analysis of three cell-cycle regulators *CDKN2B*, *CDKN2A*, and *CDKN1A* was performed. The enhanced level of *CDKN1A* detected in the *TP53*-deleted U266 cell line only after SAHA treatment indicates the P53-independent transcriptional activation of *CDKN1A* gene in U266 cells. On the contrary, *CDKN1A* gene expression was significantly increased under both SBHA and SAHA treatments of *TP53*-mutated RPMI8226 cells. Therefore, SBHA apparently affects gene transcriptional activation in a P53-dependent way, whereas SAHA seems to be a universal drug, inducing apoptosis in both P53-dependent and -independent manners.

Ingersoll *et al*. examined interleukin-6 (IL6) expression in both U266 and RPMI8226 cell lines and found that U266 expresses *IL6* mRNA, while RPMI8226 does not (32). IL6 is known to play a significant role in the pathophysiology of MM. Its impact on apoptosis has not been closely investigated and numerous factors are implicated in both the pro-apoptotic and anti-apoptotic effects of IL6 (33). Importantly, IL6 is also involved in epigenetic modulation of cell-cycle proteins (32, 33). For example, despite the activation of DNA methyltransferases, IL6 is involved in inhibiting P53 as a 'genome guard', thereby removing cells from cell-cycle control mechanisms and their uncontrolled division (34). The U266 cell line is characterized by overexpression of IL6, and thus may be less sensitive to cell-cycle inhibitory drugs.

In conclusion, the overall results show that SAHA is more potent against RPMI8226 cells than U266 cells, suggesting that it would potentially be a more effective pro-apoptotic and anticancer drug for treatment of heterogeneous MM disease.

Conflicts of Interest

The Authors declare that they have no competitive interests.

Authors' Contributions

DHD designed the study, examined the data, performed the experiments and meta-analyses and wrote the article. JG examined the data, contributed to the Discussion, and checked the article. JM contributed to the discussion and checked the article. IU and ZK contributed to the discussion and reviewed the article. KST designed the study, performed the meta-analysis, contributed to the discussion, and wrote the article.

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