

KBH-A42, a histone deacetylase inhibitor, inhibits the growth of doxorubicin-resistant leukemia cells expressing P-glycoprotein

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Abstract. Multidrug resistance mediated by the drug efflux protein, P-glycoprotein (P-gp), is one of the principal mechanisms by which tumor cells escape the cell death induced by chemotherapeutic agents. In our previous study, we demonstrated that KBH-A42 [N-hydroxy-3-(2-oxo-1-(3-phenylpropyl)-1,2,5,6-tetrahydropyridin-3-yl)propanamide], a synthetic histone deacetylase inhibitor, effectively inhibited the growth of several human cancer cell lines. In this study, we attempted to determine whether KBH-A42 was also capable of inhibiting the growth of multidrug-resistant cells. Doxorubicin dose-dependently inhibited the growth of P-gp-negative K562 human leukemia cells, but did not show substantial inhibition on the growth of P-gp-positive K562/ADR cells even at 10 μ M, the highest concentration of KBH-A42 used, which increased the acetylation of histones in these leukemia cells, dose-dependently and effectively inhibited the cell growth, regardless of the presence of P-gp in the cells. KBH-A42 mediated G0/G1 cell cycle arrest, probably as the result of the down-regulation of CDK2, CDK4 and CDK6 and the up-regulation of p21^{WAF1}. When the expression of p21^{WAF1} was ablated by a specific siRNA, the inhibition of cell growth by KBH-A42 was partly reduced in both cell lines. In addition to the cell cycle arrest, KBH-A42

also induced apoptosis in these cells, which was accompanied by the activation of caspases, including caspase-9, caspase-8 and caspase-3. The pan-caspase inhibitor, Z-VAD-fmk, partially blocked the cell death induced by KBH-A42. These results indicate that KBH-A42 induces cell cycle arrest and apoptosis via the up-regulation of p21^{WAF1} and caspase activation, respectively, regardless of the presence of P-gp in the leukemia cells.

Introduction

The acquisition of the multidrug-resistant (MDR) phenotype by tumor cells represents an undesirable process of clinical relevance, which has been associated with poor prognosis and constitutes a major impediment to the success of cancer chemotherapy (1). The most consistent MDR-associated molecular alteration is the overexpression of the transmembrane protein P-glycoprotein (P-gp) (2,3). P-gp is a large (170 kDa) molecule that belongs to the ATP binding cassette (ABC) transporter superfamily, which promotes the export of toxic xenobiotics or prevents their absorption in normal tissues (2). The overexpression of P-gp in human tumor cells appears to be related to prior chemotherapy (4), whereas in cancer cell cultures, the P-gp protein is overexpressed upon the growth of the cells in the continuous presence of sublethal concentrations of selected anti-tumor drugs. Cells grown in culture evidence resistance patterns similar to those observed *in vivo* and when they acquire the drug-resistant phenotype, P-gp accounts for the active drug efflux of several chemically unrelated anti-tumor drugs, thereby contributing to an efficient lowering of the intracellular accumulation of such drugs (5).

Histone deacetylase (HDAC) inhibitors, a promising class of chemotherapeutic agents (6), can activate the transcription of specific genes via the accumulation of acetylated histones in the nucleus, and can subsequently induce a variety of phenotypic changes, including cell cycle arrest, differentiation and apoptosis (7,8). Additionally, SAHA, a representative histone deacetylase inhibitor, induced equivalent death in P-gp-positive cells as compared with P-gp-negative cells (9) and Trichostatin A and SAHA dramatically reduce cell viability and promote apoptosis in different drug-resistant

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cells, although exerting far less profound effects than in their parental drug-sensitive counterparts (10).

Treatment with HDAC inhibitors increases the acetylation of chromatin in the promoter region of the p21^{WAF1} gene, thereby increasing access to a specific protein (Sp)1/Sp3 site that has been correlated with increased gene transcription (11,12), although other modifications to the transcriptional machinery also appear to be required for full transcriptional activation (13). Apoptosis is mediated principally by a family of caspases, the activation of which can occur via two existing pathways: the death receptor pathway and the mitochondrial pathway (14). The death receptor pathway is initiated by ligations of transmembrane death receptors such as the Fas receptor, which activates caspase-8 followed by effector caspase-3. The mitochondrial pathway requires the disruption of the mitochondrial membrane and the consequent release of cytochrome c, which functions in conjunction with Apaf-1 to activate caspase-9, thereby promoting the activation of effector caspase-3 (15).

Our previous study showed that KBH-A42, a synthetic histone deacetylase inhibitor, evidenced potent anti-tumor effects in colon cancer cells (16). However, thus far, there have been no studies assessing the effects of KBH-A42 on P-gp-positive cells. Herein, we have analyzed the alterations caused by KBH-A42 in P-gp-positive and P-gp-negative leukemia cell lines. KBH-A42 profoundly inhibited the growth of P-gp-negative and -positive leukemia cells via cell cycle arrest at the G0/G1 phase, as well as caspase-dependent apoptosis. This finding implies that KBH-A42 has the potential to be a therapeutic candidate for the treatment of MDR cancers.

Materials and methods

Cell culture and reagents. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The human chronic myelogenous leukemia cell line K562 and its doxorubicin-resistant subline K562/ADR were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) and the media were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine and 50 µg/ml of gentamycin (Gibco BRL). The cells were maintained at 37°C in 5% CO₂ humidified air. In order to maintain the multidrug resistance of K562/ADR, 500 nM of doxorubicin was added to the culture medium. The K562/ADR cells were grown for 7 days in the presence of doxorubicin until used for the experiments.

Cell proliferation assay. Cells were plated at 9x10³ cells/well in 96-well plates, incubated overnight and treated for 24 h with KBH-A42. Cell proliferation assays were conducted using a Cell Proliferation Kit II (XTT Roche Applied Science Mannheim, Germany) in accordance with the manufacturer's instructions. In brief, the XTT labeling mixture was prepared by mixing 50 volumes of 1 µg/ml sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate with 1 volume of 0.383 µg/ml of N-methyl-dibenzopyrazine methyl sulfate. This XTT labeling mixture was added to the cultures and incubated for 2 h at 37°C. Absorbance was measured at 490 nm with a reference

wavelength at 650 nm. In order to further evaluate the role of caspase on KBH-A42-induced apoptosis, cells were incubated with Z-VAD-fmk (20 µM, R&D system), the pan-caspase inhibitor, for 2 h and were then treated for an additional 24 h with KBH-A42.

Calcein accumulation assay. Calcein-AM has been previously utilized as a functional probe for measurements of P-gp transport activity, and by using the appropriate inhibitors, the functions of P-gp can be effectively differentiated (17). Cells expressing P-gp activity rapidly remove the non-fluorescent probe calcein-AM, thereby resulting in reduced accumulation of the fluorescent dye calcein in the cytoplasmic compartment. In brief, cells were harvested and plated in a volume of 200 µl at 1x10⁵ cells/well in 96-well turbid microtiter plates. Calcein-AM (at a final concentration of 1 µM) was added to the cells with or without KBH-A42 or verapamil, after which the cells were incubated for 0-60 min. Fluorescence with excitation at 485 nm and emission at 530 nm was read immediately on a Victor™ system (PerkinElmer).

Cell cycle analysis. Cell cycle analysis was conducted using a previously described protocol (18). In brief, cells were plated at 1x10⁶ cells/dish in 100-mm dishes, incubated overnight, then treated with the indicated concentrations of KBH-A42. After 24 h, the cells were harvested and washed with PBS. After cell counting with trypan blue staining, 1x10⁶ cells were pelleted and fixed for 1 h in 70% ethanol at 4°C. The cells were then resuspended in 1 ml of Krishan's buffer (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% Triton X-100 and 50 µg/ml propidium iodide, pH 7.4) for 1 h at 4°C. The samples were centrifuged, resuspended in 1 ml of PBS buffer and analyzed via flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The data were collected for 10,000 events. The Modfit LT program (Verity Software House; Maine, ME, USA) was employed for cell cycle modeling.

siRNA transfection. The role of p21^{WAF1} on KBH-A42-induced cell cycle arrest was evaluated using antisense oligonucleotides by Ambion (#AM51324, TX, USA) and Bioneer (#1029367, #1029369 and #1029372, Korea). The oligonucleotide mixture was delivered to K562 and K562/ADR cells using Lipofectamine™ RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. In brief, the oligonucleotide mixture was premixed with RNAiMAX in Opti-MEM medium for 20 min at room temperature. K562 and K562/ADR cells were transfected for 24 h with the prepared complex in Opti-MEM complete growth medium at 37°C. After transfection, the cells were treated for an additional 24 h with KBH-A42.

Apoptosis analysis. Apoptosis analysis was conducted using an Annexin V-FITC Apoptosis Detection Kit II (BD Bioscience, San Jose, CA, USA) in accordance with the manufacturer's instructions. In brief, the cells were plated at 1x10⁶ cells/dish in 100-mm dishes, incubated overnight and treated for 24 h KBH-A42 at the indicated concentrations. The cells were harvested, washed in PBS and combined with a binding buffer containing annexin V-FITC and propidium



After 15 min of incubation in darkness, the cells were via flow cytometry using a FACSCalibur flow cytometer.

Caspase activity assay. The activities of caspases were determined via a Caspase-Glo™ assay (Promega, Madison, WI, USA) conducted in accordance with the manufacturer's instructions. In brief, the cells were plated at 9×10^3 cells/well in 96-well plates, incubated overnight and treated for 24 h with the indicated concentrations of KBH-A42. The culture supernatants were collected on 96-well turbid microtiter plates and 50 μ l of proluminescent caspase-9, caspase-8 and caspase-3/7 substrate were added. After 1 h of incubation at 37°C, luminescence was measured using a Victor Light system (PerkinElmer).

Western immunoblot analysis. Total protein extracts were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations in the lysates were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in accordance with the manufacturer's instructions. The samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer (Tris-buffered saline containing 0.2% Tween-20 and 3% non-fat dried milk) and probed with the indicated primary antibodies. After washing, membranes were probed with horseradish peroxidase-conjugated secondary antibodies. Detection was performed using an enhanced chemiluminescent protein (ECL) detection system (Amersham Biosciences, Little Chalfont, UK). The membranes were subsequently stripped using a Restore™ Western blot stripping buffer (Thermo Scientific, Rockford, USA) and re-probed with other primary antibodies where indicated.

Statistical analysis. The results are expressed as mean \pm SD. A paired t-test was used to compare two groups and one-way ANOVA and Dunnett's t-test were used for multiple comparisons using GraphPad Prism (GraphPad Software, Inc.; San Diego, CA, USA). The criterion for statistical significance was set at $P < 0.05$.

Results

KBH-A42 effectively inhibits growth of both P-gp-positive and P-gp-negative leukemia cells. In order to determine whether KBH-A42 could inhibit the proliferation of P-gp-expressing cells, P-gp-positive (K562/ADR) and P-gp-negative (K562) cells were treated with 0 to 10 μ M of KBH-A42. The GI50 values of KBH-A42 against K562 cells and K562/ADR cells were 1.903 and 2.815 μ M, respectively. We detected little difference in the ability of KBH-A42 to inhibit the growth of both cell lines. However, whereas doxorubicin (ADR) dose-dependently inhibited the growth of K562 cells (GI50 = 1.588 μ M), the growth of K562/ADR cells remained almost stable (Fig. 1A).

The acetylation status of histone H3 was assessed to determine whether KBH-A42 appropriately exerted its HDAC inhibitory activity in K562 and K562/ADR cells. After

the treatment of both cell types for 24 h with 5 μ M of KBH-A42, the whole cell lysates were extracted and subjected to Western immunoblot analysis. The results demonstrate that the acetylation levels of histone H3 were increased in both types of cells by KBH-A42, but the MDR-1 (P-glycoprotein) protein was overexpressed only in the K562/ADR cells (Fig. 1B).

A surrogate assay using calcein-AM, a P-gp substrate, for P-gp inhibition was employed to assess whether the P-gp expressed in K562/ADR cells functions correctly. The accumulation of calcein-AM increased rapidly over time in K562 cells in the presence and absence of verapamil, a P-gp inhibitor, or KBH-A42, because the K562 cells did not express P-gp to pump calcein-AM out of the cells. However, in the K562/ADR cells, calcein-AM accumulation increased rapidly with time only in the presence of verapamil. Additionally, the rate of calcein-AM accumulation was substantially retarded when only DMSO, the vehicle used for this experiment, was present. This result can be interpreted to indicate that calcein-AM was being actively eliminated by P-gp (Fig. 2). The addition of KBH-A42, regardless of concentration, did not influence calcein-AM accumulation in the K562/ADR cells, thereby suggesting that KBH-A42 did not compete with calcein-AM to be pumped out of the cells by P-gp and KBH-A42 did not, therefore, appear to function as a P-gp substrate.

KBH-A42 induces a G0/G1 cell cycle arrest by p21^{WAF1} induction. In a previous study, it was demonstrated that KBH-A42 induces a variety of phenotypic changes, including cell cycle arrest and apoptosis (16). Flow cytometric analysis was conducted to determine the cell cycle profiles of K562 and K562/ADR cells that were treated for 24 h with 5 μ M KBH-A42, which induced G0/G1 accumulation in both K562 and K562/ADR cells (Fig. 3). The percentage of G0/G1 was increased as the result of KBH-A42 treatment, from 38.3 to 79.4% in the K562 cells and from 45.1 to 75.2% in the K562/ADR cells (Fig. 3). These results indicate that G0/G1 cell cycle arrest is one of the principal contributors to the inhibition of growth of these leukemia cells.

In order to assess the possible mechanisms by which KBH-A42-induced cell cycle alterations are induced, the effects of KBH-A42 on cell cycle regulatory proteins were evaluated. K562 and K562/ADR cells were incubated for 24 h in the presence and absence of 5 μ M KBH-A42 and subjected to Western immunoblot analysis. As is shown in Fig. 4A, p21^{WAF1} was substantially induced and the expression of CDK2, CDK4 and CDK6 were reduced as the result of KBH-A42 treatment. In order to determine more precisely whether p21^{WAF1} up-regulation was necessary for the KBH-A42-induced arrest of cell growth, siRNA targeting p21^{WAF1} was applied to K562 and K562/ADR cells. The cells transfected with the siRNA were incubated for 24 h in the presence and absence of 5 μ M KBH-A42. The presence of p21^{WAF1} siRNA effected the blockage of the KBH-A42-mediated up-regulation of p21^{WAF1} protein. The survival of the cells treated with KBH-A42 was increased slightly via the suppression of p21^{WAF1} protein expression in K562 (9.7%) and K562/ADR (11.0%) cells (Fig. 4B). These results show that G0/G1 cell cycle arrest by KBH-A42 is a principal regulator in the inhibition

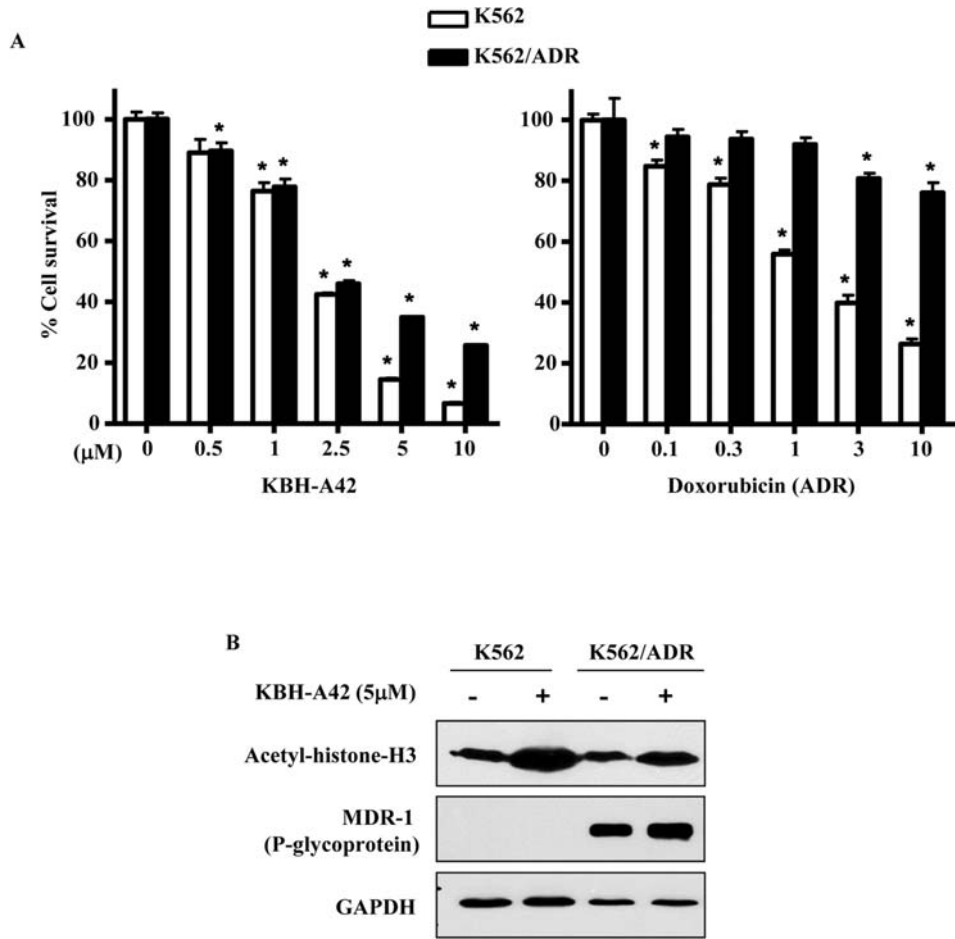


Figure 1. KBH-A42 effectively induces cell growth inhibition in P-gp-positive (K562) and P-gp-negative (K562/ADR) cells. (A) Cells were cultured for 24 h with 0 to 10 µM KBH-A42 or doxorubicin (ADR) and cell proliferation was determined via an XTT assay, as described in the Materials and methods section. Significance was determined using the ANOVA test vs. DMSO-treated controls (*P<0.05). (B) Cells were treated with 5 µM of KBH-A42 for 24 h. Acetylated histone-H3, MDR-1 (P-glycoprotein) and GAPDH in the total cell lysates were determined via Western immunoblot analysis.

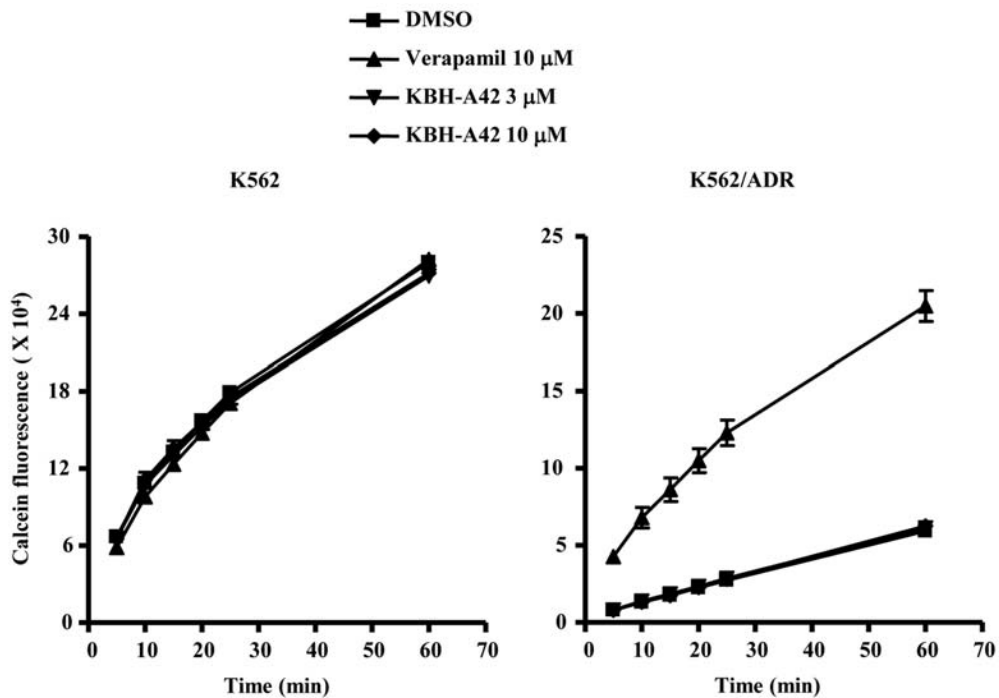


Figure 2. The accumulation of calcein which exhibits fluorescence, as described in the Materials and methods section, in K562 and K562/ADR cells. Cells exposed to 1 µM of calcein-AM with or without KBH-A42 or verapamil for 0-60 min.

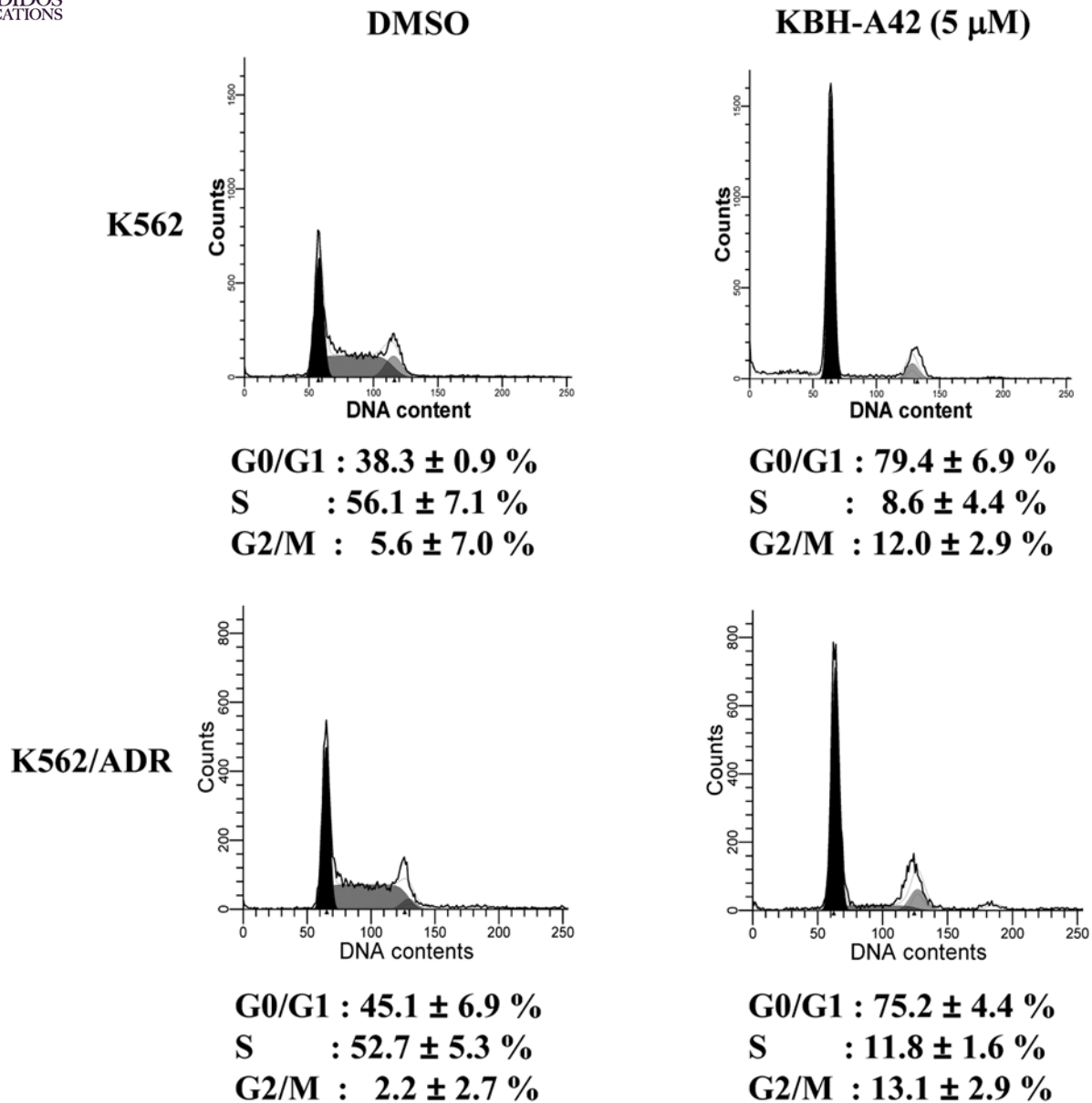


Figure 3. Effects of KBH-A42 on cell cycle progression. The cells were treated for 24 h with 5 μ M KBH-A42. Cell cycle distribution was assessed via flow cytometry. The results are presented as the mean \pm SD of triplicate determinations.

of the growth of these leukemia cells and p21^{WAF1} appears to contribute partially to the cell cycle arrest. However, it appears that this is not sufficient to explain the inhibition of cell growth induced by KBH-A42 only in conjunction with these cell cycle-related events. There remains the possibility that KBH-A42 may regulate other cellular activities, including apoptosis, to inhibit the growth of these leukemia cells.

KBH-A42 induces apoptosis by caspase activation. Previous studies have demonstrated that P-gp-positive cell lines over-expressing P-gp were less sensitive to multiple forms of caspase-dependent cell death, including the cell death mediated by cytotoxic drugs (19). As KBH-A42 was demonstrated in a previous study to induce the activation of caspases (16), we attempted to determine whether caspase activation in P-gp-expressing cells would also be affected by KBH-A42. K562

and K562/ADR cells were treated for 24 h with 5 μ M KBH-A42, harvested, stained with PI and annexin V-FITC and analyzed via flow cytometry. The percentage of apoptosis induced in K562 and K562/ADR by KBH-A42 was 17.2 and 8.6%, respectively (Fig. 5). We also attempted to determine whether KBH-A42 activated caspases, key enzymes involved in the apoptotic signaling cascade. KBH-A42 activated caspase-9, caspase-8, and caspase-3/7 enzyme activity (Fig. 6A) and enhanced the protein levels of cleaved caspase-9, caspase-8, and caspase-3/7, both of which were active forms of caspases in the cells (Fig. 6B). In order to determine whether KBH-A42-induced caspase activation could be inhibited by caspase inhibitor treatment, the cells were pretreated for 2 h with pan-caspase inhibitor (Z-VAD-fmk) and then cultured for 24 h with 5 μ M KBH-A42. As expected, Z-VAD-fmk increased the survival durations of

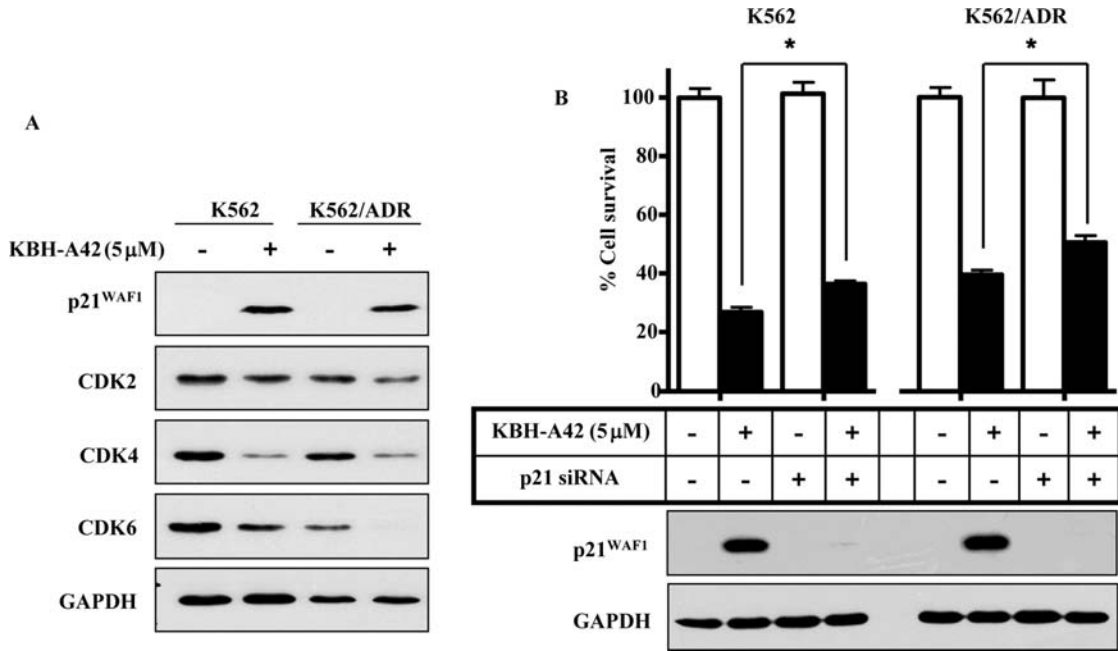


Figure 4. Effects of KBH-A42 on the expression of cell cycle regulators. (A) Cells were treated for 24 h with the indicated concentrations of KBH-A42. Expression levels of p21^{WAF1}, CDK2, CDK4, CDK6 and GAPDH in total cell lysates were determined via Western immunoblot analysis. (B) Cells were transfected with non-targeting or double-stranded p21^{WAF1} siRNA oligonucleotides, plated at a density of 6,000 cells per well in 96-well plates and incubated for 24 h in the absence or presence of 5 μM KBH-A42. To confirm p21^{WAF1} knockdown, cells were plated (60-mm plates) at a density of 400,000 per plate and transfected, the cell lysates were probed with an anti-p21^{WAF1} antibody, and cell proliferation was evaluated as described in the Materials and methods section. Significance was determined via an ANOVA test vs. DMSO-treated controls (*P<0.05).

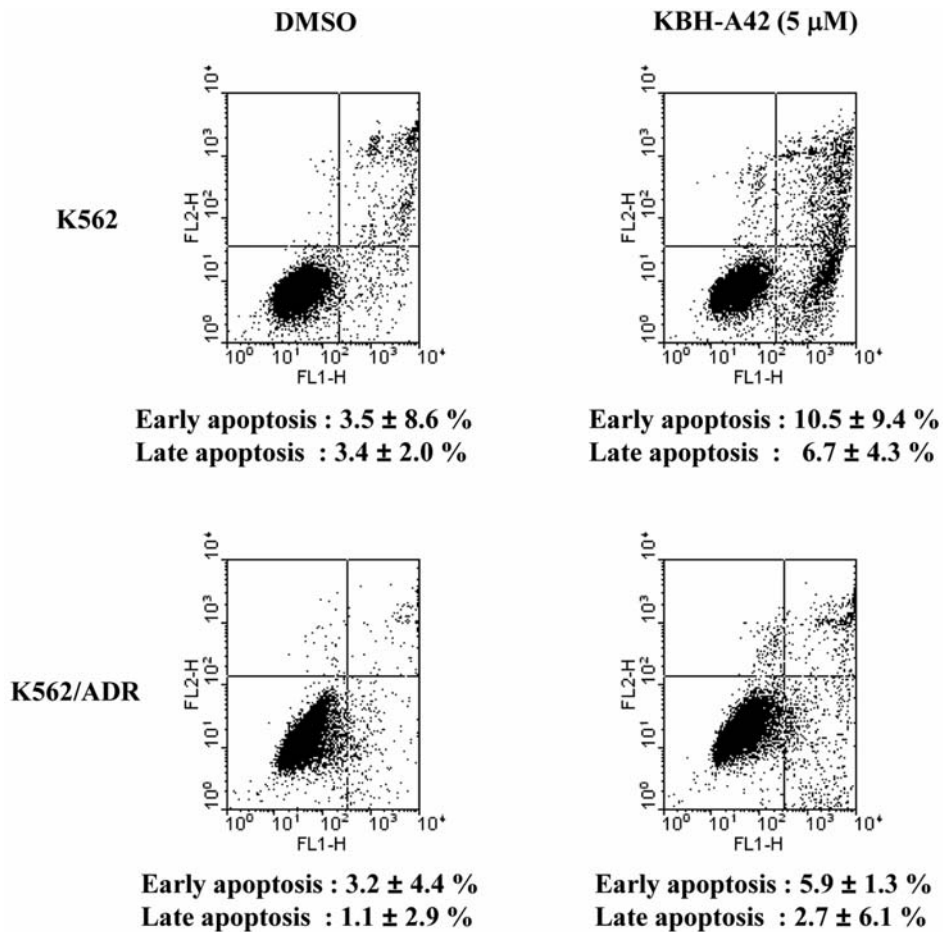


Figure 5. Induction of apoptosis by KBH-A42. Cells were stained with propidium iodide and a FITC-conjugated antibody directed against annexin V, then analyzed via flow cytometry as described in the Materials and methods section. The results are presented as the mean ± SD of triplicate determinations.

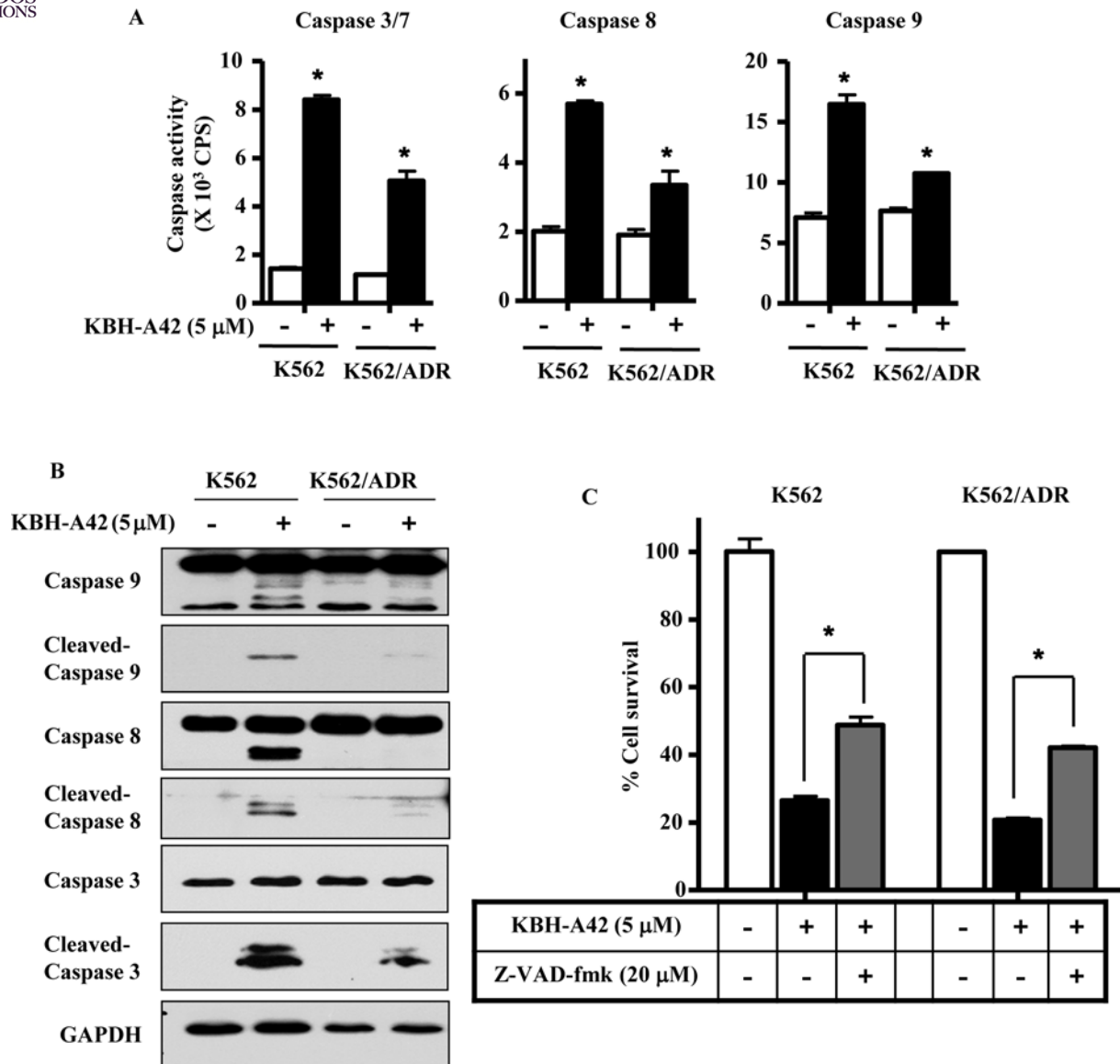


Figure 6. Effects of KBH-A42 induced caspase activation on apoptosis. Cells were treated for 24 h with 5 μM KBH-A42. (A) Culture supernatants were collected, and the activity of caspase-9, caspase-8, and caspase-3/7 was determined as described in the Materials and methods section. (B) Levels of caspase-9, cleaved caspase-9, caspase-8, cleaved caspase-8, caspase-3, cleaved caspase-3 and GAPDH in the total cell lysates were determined via Western immunoblot analysis. (C) The effects of pan-caspase inhibitor on KBH-A42-induced apoptosis in K562 and K562/ADR cells were determined via an XTT assay. The results are expressed as the mean ± SD of triplicate determinations. Significance was determined using the ANOVA test vs. DMSO-treated controls (*P<0.05).

K562 and K562/ADR cells treated with KBH-A42 by 22.3 and 21.3%, respectively (Fig. 6C), thereby suggesting that apoptosis induced via the activation of a variety of caspases contributes to the inhibition of growth induced by KBH-A42 in these leukemia cells.

Discussion

The pleiotropic nature of the MDR phenomenon comprises the alteration of multiple genetic events (20), providing an excellent cellular situation to study the activation or repression of endogenous genes, the transcription of which involves the acetylation/deacetylation of histones. In this regard, a question arises as to what degree the principal characteristics of the drug-resistant tumor cells are affected by drugs that could

efficiently overcome the MDR phenotype by altering the acetylated/deacetylated status of the cells.

The P-gp responsible for MDR has been shown to protect human tumor cells against multiple forms of caspase activation (19). It has been established that chemotherapeutic drugs of diverse structure and specificity can induce the death of target cells via the activation of physiologic apoptotic pathways and drugs, such as doxorubicin, require caspase activation for full cytotoxic function (21). Therefore, we have hypothesized that drugs which are not P-gp substrates and can overcome the protective mechanisms of MDR tumor cells against chemotherapeutic drugs should be capable of inducing death in P-gp-positive MDR tumor cells.

In the current study, it was shown that KBH-A42, a synthetic HDAC inhibitor, could function similarly to induce growth arrest and histone acetylation in both K562 and K562/ADR cells. Additionally, a calcein-AM accumulation assay revealed that KBH-A42 did not appear to be a P-gp substrate. Therefore, it seems that KBH-A42 may be a candidate factor that escapes the pumping-out action of P-gp and functions as an HDAC inhibitor. Castro-Galache *et al* previously demonstrated that TSA and SAHA, both of which are representative HDAC inhibitors, promoted the endogenous down-regulation of P-gp (10). On the other hand, some studies have reported no change in P-gp expression in human CEM cells as the result of SAHA (22) or an increase in the steady-state level of MDR1 mRNA in human colon carcinoma SW620 cells upon treatment with TSA (23). In the case of our study, KBH-A42 slightly induced P-gp expression in K562/ADR cells. Although the basis for this discrepancy is unknown, the regulation of P-gp expression by KBH-A42 may be the result of the transcriptional control of the endogenous P-gp promoter.

We also demonstrated that KBH-A42 induced G0/G1 cell cycle arrest in K562 and K562/ADR cells, which was probably the result of the down-regulation of CDK2, CDK4 and CDK6 expression. Huang *et al* (11) demonstrated that SAHA induced the up-regulation of the cyclin-dependent kinase inhibitor p21^{WAF1} by altering the association of proteins on the p21^{WAF1} promoter, thereby indicating that p21^{WAF1} is the direct target of an HDAC inhibitor (13,24). In K562 and K562/ADR cells, we noted a marked up-regulation of p21^{WAF1} by KBH-A42. When p21^{WAF1} induction was ablated using siRNA targeting p21^{WAF1}, the KBH-A42-mediated growth inhibition was partly reduced in both cell lines. These observations indicate that the mechanism of KBH-A42-induced growth arrest is partly dependent on the up-regulation of p21^{WAF1}. Further studies will be necessary to determine what types of cell cycle-related molecules, in addition to p21^{WAF1}, are involved in the inhibition of the cell cycles of K562 and K562/ADR cells by KBH-A42. These results indicate that the growth inhibition of these leukemia cells by KBH-A42 is, at least in part, mediated by the blockage of cell cycle progression.

Next, we assessed the role of apoptosis in the inhibition of cell growth by KBH-A-2. Flow cytometric analysis demonstrated that KBH-A42 induced apoptosis in both K562 and K562/ADR cells and this was probably initiated by the activation of caspase-9, caspase-8 and caspase-3. Treatment with pan-caspase inhibitor (Z-VAD-fmk) coupled with KBH-A42 also partially reduced the KBH-A42-induced inhibition of cell growth. This result indicates that caspase activation contributes to apoptosis, furthering the inhibition of growth of these leukemia cells.

In summary, the results presented herein demonstrated that KBH-A42 effectively inhibits the growth of P-gp-positive K562/ADR cells, as well as P-gp-negative K562 cells, via the induction of cell cycle arrest at the G0/G1 phase and caspase-mediated apoptosis. Collectively, these findings imply that KBH-A42 may prove to be a promising agent for use in MDR cancer therapy.

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References

1. Tan B, Piwnicka-Worms D and Ratner L: Multidrug resistance transporters and modulation. *Curr Opin Oncol* 12: 450-458, 2000.
2. Gottesman MM and Pastan I: Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 62: 385-427, 1993.
3. Nielsen D and Skovsgaard T: P-glycoprotein as multidrug transporter: a critical review of current multidrug resistant cell lines. *Biochim Biophys Acta* 1139: 169-183, 1992.
4. Grogan TM, Spier CM, Salmon SE, Matzner M, Rybski J, Weinstein RS, Scheper RJ and Dalton WS: P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. *Blood* 81: 490-495, 1993.
5. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM: Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* 39: 361-398, 1999.
6. Carew JS, Giles FJ and Nawrocki ST: Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett* 269: 7-17, 2008.
7. Han JW, Ahn SH, Park SH, Wang SY, Bae GU, Seo DW, Kwon HK, Hong S, Lee HY, Lee YW and Lee HW: Apicidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of p21^{WAF1/Cip1} and gelsolin. *Cancer Res* 60: 6068-6074, 2000.
8. Kwon SH, Ahn SH, Kim YK, Bae GU, Yoon JW, Hong S, Lee HY, Lee YW, Lee HW and Han JW: Apicidin, a histone deacetylase inhibitor, induces apoptosis and Fas/Fas ligand expression in human acute promyelocytic leukemia cells. *J Biol Chem* 277: 2073-2080, 2002.
9. Ruefli AA, Bernhard D, Tainton KM, Kofler R, Smyth MJ and Johnstone RW: Suberoylanilide hydroxamic acid (SAHA) overcomes multidrug resistance and induces cell death in P-glycoprotein-expressing cells. *Int J Cancer* 99: 292-298, 2002.
10. Castro-Galache MD, Ferragut JA, Barbera VM, Martin-Orozco E, Gonzalez-Ros JM, Garcia-Morales P and Saceda M: Susceptibility of multidrug resistance tumor cells to apoptosis induction by histone deacetylase inhibitors. *Int J Cancer* 104: 579-586, 2003.
11. Huang L, Sowa Y, Sakai T and Pardee AB: Activation of the p21^{WAF1/CIP1} promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) through the Sp1 sites. *Oncogene* 19: 5712-5719, 2000.
12. Richon VM, Sandhoff TW, Rifkind RA and Marks PA: Histone deacetylase inhibitor selectively induces p21^{WAF1} expression and gene-associated histone acetylation. *Proc Natl Acad Sci USA* 97: 10014-10019, 2000.
13. Gui CY, Ngo L, Xu WS, Richon VM and Marks PA: Histone deacetylase (HDAC) inhibitor activation of p21^{WAF1} involves changes in promoter-associated proteins, including HDAC1. *Proc Natl Acad Sci USA* 101: 1241-1246, 2004.
14. Johnstone RW, Ruefli AA and Lowe SW: Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108: 153-164, 2002.
15. Doi S, Soda H, Oka M, Tsurutani J, Kitazaki T, Nakamura Y, Fukuda M, Yamada Y, Kamihira S and Kohno S: The histone deacetylase inhibitor FR901228 induces caspase-dependent apoptosis via the mitochondrial pathway in small cell lung cancer cells. *Mol Cancer Ther* 3: 1397-1402, 2004.
16. Kang MR, Kang JS, Han SB, Kim JH, Kim DM, Lee K, Lee CW, Lee KH, Lee CH, Han G, Kim HM and Park SK: A novel delta-lactam-based histone deacetylase inhibitor, KBH-A42, induces cell cycle arrest and apoptosis in colon cancer cells. *Biochem Pharmacol* 78: 486-494, 2009.
17. Shiraki N, Hamada A, Yasuda K, Fujii J, Arimori K and Nakano M: Inhibitory effect of human immunodeficiency virus protease inhibitors on multidrug resistance transporter P-glycoproteins. *Biol Pharm Bull* 23: 1528-1531, 2000.



SPANDIDOS PUBLICATIONS
n A: Rapid flow cytofluorometric analysis of mammalian
scle by propidium iodide staining. *J Cell Biol* 66: 188-193,

- 1713.
19. Smyth MJ, Krasovskis E, Sutton VR and Johnstone RW: The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proc Natl Acad Sci USA* 95: 7024-7029, 1998.
20. Simon SM and Schindler M: Cell biological mechanisms of multidrug resistance in tumors. *Proc Natl Acad Sci USA* 91: 3497-3504, 1994.
21. Johnstone RW, Cretney E and Smyth MJ: P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood* 93: 1075-1085, 1999.
22. Ruefli AA, Ausserlechner MJ, Bernhard D, Sutton VR, Tainton KM, Kofler R, Smyth MJ and Johnstone RW: The histone deacetylase inhibitor and chemotherapeutic agent suberoylanilide hydroxamic acid (SAHA) induces a cell-death pathway characterized by cleavage of Bid and production of reactive oxygen species. *Proc Natl Acad Sci USA* 98: 10833-10838, 2001.
23. Jin S and Scotto KW: Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF- κ B. *Mol Cell Biol* 18: 4377-4384, 1998.
24. Sakajiri S, Kumagai T, Kawamata N, Saitoh T, Said JW and Koeffler HP: Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. *Exp Hematol* 33: 53-61, 2005.