Involvement of p21$^{waf1/cip1}$ expression in the cytotoxicity of the potent histone deacetylase inhibitor spiruchostatin B towards susceptible NALM-6 human B cell leukemia cells

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Abstract. Spiruchostatin B (SP-B) is a potent histone deacetylase (HDAC) inhibitor that has potential for the chemotherapy of leukemia. The aim of this study was to study the susceptibility of human leukemia cell lines to SP-B. We found that NALM-6 human B cell leukemia cells are the most susceptible to SP-B. There was a low correlation between the expression of *HDAC1* mRNA and HDI susceptibility of leukemia cells. NALM-6 has higher endogenous *p21$^{waf1/cip1}$* mRNA expression than other leukemia cells. SP-B-induced cytotoxicity was mediated by induction of histone acetylation via inhibition of HDACs, and this effect of SP-B was associated with apoptosis, which was mediated by caspase activation in NALM-6 cells. SP-B time-dependently increased the size of the sub-G1 (apoptotic) peak, and this effect correlated with SP-B induction of cellular apoptotic features such as changes in nuclear morphology. SP-B significantly increased p21$^{waf1/cip1}$ expression prior to induction of apoptosis. In conclusion, NALM-6 cells, which have a higher expression of *p21$^{waf1/cip1}$* mRNA than other leukemia cell lines, were susceptible to SP-B-induced cytotoxicity that resulted in induction of apoptosis. Our findings may be useful when establishing a therapeutic strategy based on SP-B.

Introduction

Since inhibition of histone deacetylase (HDAC) activity reverses the epigenetic silencing frequently observed in cancer, various HDAC inhibitors (HDIs) have been developed for therapeutic purposes (1). Studies of leukemia have provided examples for the functional implications of HDACs in cancer development and progression, as well as their relevance for therapeutic targeting (2). There has been intensive research regarding the clinical use of HDIs (3), and clinical trials involving systemic HDI treatment have been reported (4). Different classes of agents with HDAC inhibitor activity exert different biological effects, possibly because of additional distinct effects on mechanisms of cell regulation.

The chemical structures of typical HDIs that were used for experiments in this study are shown in Fig. 1. Sodium butyrate (NaB) is a short-chain acid that is produced by anaerobic bacterial fermentation of dietary fibers, which has been shown to inhibit HDAC activity (5). Trichostatin A (TSA) is a naturally occurring hydroxamate that is a potent HDI (6), but is considered too toxic for clinical development (7). Romidepsin (FK228), which is a more potent HDI than either of the above chemicals, is a naturally occurring 16-membered bicyclic depsipeptide that was isolated from *Chromobacterium violaceum* in 1994 (8). Preclinical studies with FK228 in leukemia patients demonstrated that it effectively induced apoptosis at concentrations at which HDAC inhibition occurs (9). Subsequently, structurally similar 15-membered bicyclic depsipeptides, spiruchostatins A and B, isolated from a culture broth of *Pseudomonas sp.* by Masuoka *et al* in 2001 (10), were also found to exhibit potent HDAC inhibitory activity (11,12). In a previous study, we compared the activity of these depsipeptide compounds using a panel of 39 human cancer cell lines and found that spiruchostatin B (SP-B) was the most potent inhibitor of HDAC1 and displayed the most potent growth-inhibitory activity (13).

The anti-tumor effects of HDIs have been shown to be mediated by various signaling mechanisms. The induction of p21$^{waf1/cip1}$ and the resulting blockage of cell cycle progression are critical for the anti-tumor activities of all HDIs (14-17). This induction is independent of p53 and correlates with alterations in proteins associated with the p21$^{waf1/cip1}$ promoter, including an increase in the acetylation of histones (18,19).

The aims of this study were to better understand the signaling mechanism of SP-B anti-tumor activity with a view to its clinical application, we examined the susceptibility of human leukemia cell lines to SP-B. Our study suggests that the human B cell leukemia NALM-6 cell line, which has higher

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p21$^{waf1/cip1}$ expression than other leukemia cells, is the most susceptible to SP-B. These data may prove useful with respect to the clinical application of SP-B.

Materials and methods

Materials and cell culture. SP-B and FK228 were prepared as previously described (13,20). TSA, and all other reagents, unless stated, were of the highest grade available and were supplied by either Sigma (St. Louis, MO, USA) or Wako Pure Chemical Industries, Ltd (Osaka, Japan). Normal human leukocyte cDNA was purchased from OriGene Technologies Inc (Rockville, MD, USA). All cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were routinely cultured using standard methods as described in our previous reports (21).

Cytotoxicity and apoptosis. Cytotoxicity was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, and apoptosis was estimated by nuclear morphological observation or by analysis of qualitative damage to genomic DNA (DNA fragmentation) using a modification of our previously described method (22).

Cell cycle analyses by flow cytometry. Analyses of the cell cycle were performed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with CELLQuest Pro software (BD Biosciences). Data analyses for cell cycle distribution were performed on linear propidium iodide (PI) histograms using the mathematical software ModFit LT 2.0 (Verity Software House; Topsham, ME, USA).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) assay. The mRNA expression level of *p21$^{waf1/cip1}$* (GenBank Accession no. NM_000389.4), *HDAC1* (NM_004964.2), in human leukemia cells was quantified using the real-time polymerase chain reaction (qPCR) with a Light Cycler (Roche, Basel, Switzerland). Briefly, total RNA was extracted from each cell line with the Isogen reagent (Nippon Gene, Tokyo, Japan) and 0.1 μg of total RNA was then reverse transcribed to single-strand cDNA using the RverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Aliquots of the cDNA preparations were subjected to qPCR analysis using SYBR® Premix Ex Taq™ (Takara Bio, Shiga, Japan) to quantify the expression of each target gene and of the internal standard β-actin (GenBank Accession no. NM_001101.3) using Light Cycler. The primer pairs used were from the QuantiTect® Primer Assay (Qiagen, Valencia, CA) or were Takara Perfect Real Time Primers (Takara Bio). The results of all assays were checked with the melting curves to confirm the presence of single PCR products.

Western blotting. Changes in the expression level of apoptosis related proteins or of the p21$^{waf1/cip1}$ protein were detected using Western blotting as described in our previous reports (23). All antibodies used were purchased from Cell signaling Technology, Inc. (Beverly, MA, USA). Protein levels were analyzed by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, Arlington Heights, IL, USA).

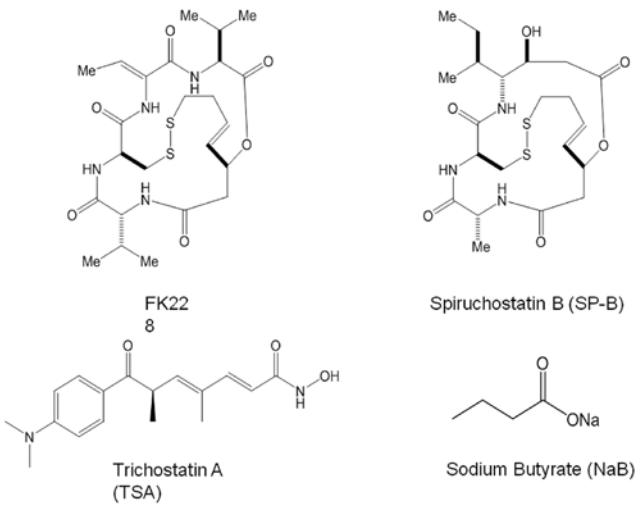

Figure 1. Chemical structures of HDIs.

Statistical analysis. Statistical analysis of the results was performed using a one-way analysis of variance (ANOVA) followed by the Williams' type multiple comparison test or a Bonferroni test among multiple groups. A $p<0.05$ was considered significant.

Results

Susceptibility of human leukemia cell lines to HDIs. We first examined the susceptibility of human leukemia cell lines to the cytotoxicity of typical HDIs; SP-B, TSA, or NaB, by incubation of the cells with HDIs for 24 h. Susceptibility was estimated by analysis of cell growth using the MTT assay, in which the HDI concentration that induced 50% cell growth inhibition (IC_{50}) was calculated using GraphPad Prism software version 4.0 (Table I). SP-B displayed the most potent cytotoxicity towards each leukemia cell line, showing cytotoxicity at nanomolar (nM) concentrations. Compared to SP-B, TSA showed moderate cytotoxicity at micromolar (μM) concentrations towards all leukemia cell lines except for NALM-6 cells. All cell lines were considerably less susceptible to NaB. Thus, SP-B has potent cytotoxic effects on leukemia cells. In addition, NALM-6 cells were the most susceptible of all of the leukemia cells tested to the cytotoxic activity of all of the HDIs. To determine the factor(s) that makes human leukemia cells susceptible to HDIs, we carried out qPCR to assess the mRNA levels of *HDAC1* and *p21$^{waf1/cip1}$* in the leukemic cells compared to normal human leukocytes (Fig. 2). Compared to control, the mRNA expression of *HDAC1* was higher in MOLT-4 (2.0-fold higher) and K562 (2.3-fold higher) cells. The mRNA expression of *p21$^{waf1/cip1}$* was extremely high in NALM-6 (3.8-fold higher than control), but its expression in the other leukemia cells was lower than in normal leukocytes.

SP-B-induced cytotoxicity and apoptosis in NALM-6 cells. Since NALM-6 was the most susceptible cell line to SP-B, as shown in Table I, we used this cell line to confirm that SP-B-induced cytotoxicity depends on apoptosis and HDAC inhibition. We first determined the best conditions for SP-B treatment of NALM-6 cells for induction of cytotoxicity.

Table I. HDAC inhibitor-induced cytotoxicity in human leukemia cell lines.[a]

Cell line	Characteristic	SP-B (nM)	TSA (μM)	NaB (mM)
NALM-6	B cell leukemia	5.168 (2.981-8.960)	0.085 (0.050-0.144)	1.232 (0.837-1.811)
U937	Diffuse histiocytic lymphoma	27.47 (19.050-39.600)	2.840 (1.669-4.833)	11.19 (8.164-15.350)
MOLT-4	T cell leukemia	29.87 (16.130-55.310)	1.405 (0.680-2.902)	7.947 (5.815-10.860)
HL-60	Promyelocytic	64.22 (40.960-100.700)	1.840 (1.063-3.186)	10.03 (7.351-13.680)
K562	Erythroleukemia	536.4 (319.100-901.800)	35.79 (2.135-59.998)	>10

[a]Cytotoxicity was assessed using the MTT assay following incubation of the cells with the indicated HDIs for 24 h. Data were calculated relative to each control group and are expressed as the concentration which induces 50% cell growth inhibition (IC_{50}). The range of values obtained is shown in brackets under each result.

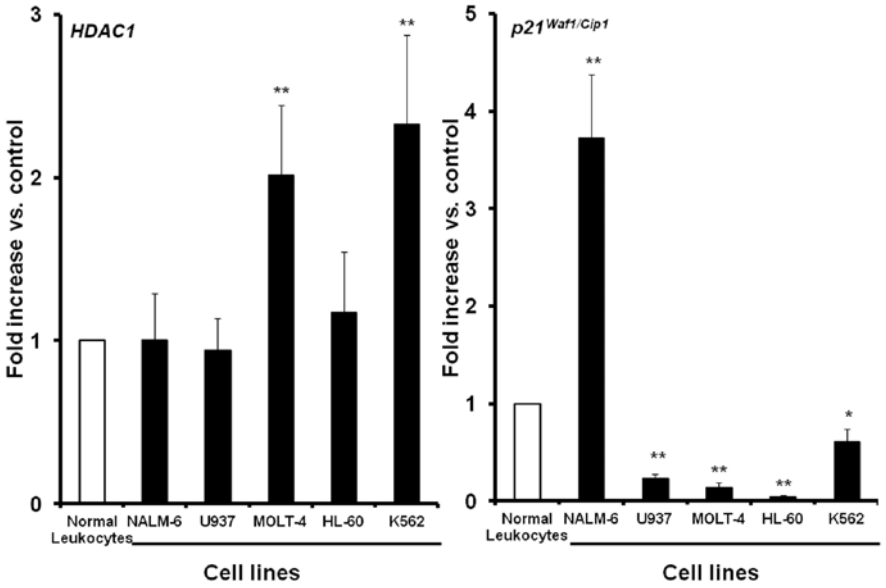

Figure 2. Comparison of *HDAC1* or *p21*$^{waf1/cip1}$ mRNA expression in human leukemia cell lines. mRNA expression was analyzed using qPCR and data are expressed as fold change in the mRNA level relative to normal human leukocytes. *p<0.05, **p<0.01 compared to expression in normal human leukocytes.

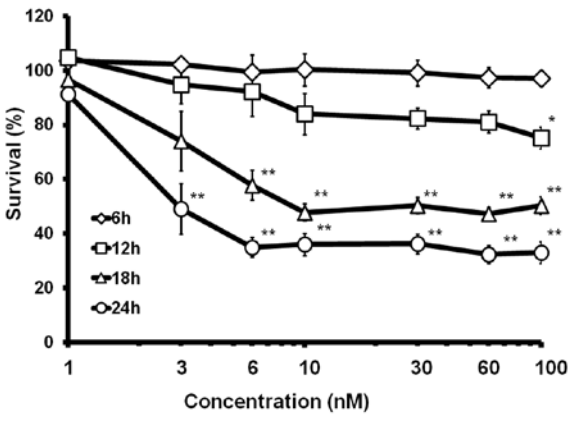

Figure 3. SP-B-induced cytotoxicity towards NALM-6 cells. NALM-6 cells were incubated with the indicated concentrations of SP-B for the indicated times. Cytotoxicity was assessed using the MTT assay, survival (%) was calculated relative to the control (SP-B vehicle). The results are means ± SEM of three individual studies. *p<0.05, **p<0.01 compared with the control group under the indicated culture condition.

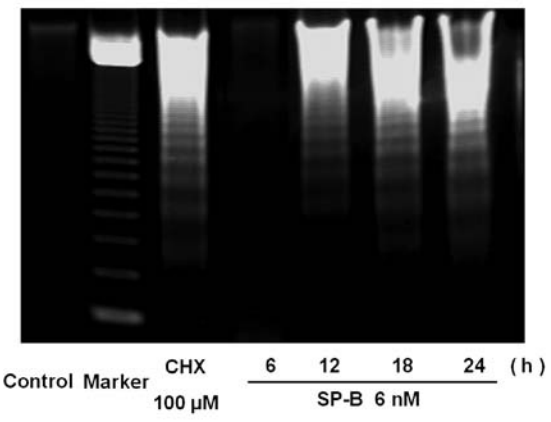

Figure 4. SP-B induces DNA damage (DNA fragmentation). SP-B-induced damage to genomic DNA was qualitatively estimated by agarose gel electrophoresis following incubation of NALM-6 cells with 6 nM SP-B for the indicated times. Cycloheximide (CHX) was used as a positive control and no SP-B vehicle as a negative control. DNA samples were electrophoresed on 1.4% agarose gel containing ethidium bromide, visualized under UV light, and photographed.

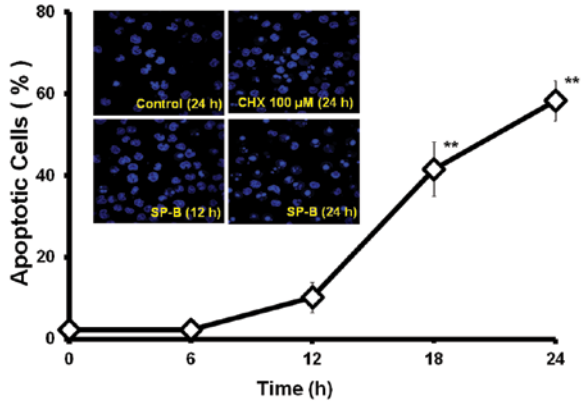

Figure 5. SP-B induces changes in nuclear morphology. NALM-6 cells were treated with 6 nM SP-B or with 100 mM cycloheximide (CHX, positive control) or with a negative control (SP-B vehicle) for the indicated times. Cell nuclei were then stained with H33342. Apoptotic nuclei, which were characterized by chromatin condensation followed by partition into multiple bodies (magnification, x400), were counted and the percentage of apoptotic cells was calculated. The results are means ± SEM of three individual studies. $^{**}p<0.01$ compared with control group.

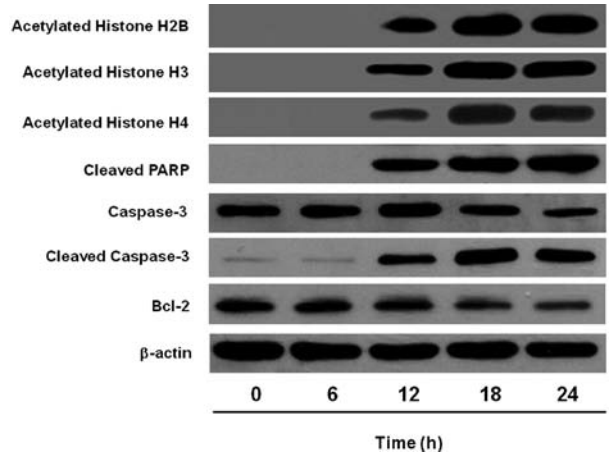

Figure 6. Western blotting analysis of SP-B-induced changes in the level of acetylated histones and apoptosis-related proteins. Cells were incubated with 6 nM SP-B for the indicated times and expression of the indicated proteins was then analyzed by Western blotting using expression of β-actin as a loading control. Experiments shown are representative of a minimum of three separate experiments.

SP-B had significant cytotoxic effects on NALM-6 cells at concentrations greater than or equal to 6 nM, and following incubation for 18 h (Fig. 3). Interestingly, there was little change in the cytotoxic effects of SP-B at concentrations between 6 and 100 nM, when incubated with cells for 24 h. Based on these data we used SP-B at a concentration of 6 nM for the rest of the study. We next analyzed the effect of SP-B on apoptosis. Apoptosis is characterized by oligonucleosomal DNA fragmentation, qualitative damage to genomic DNA and nuclear morphological changes such as chromatin condensation. Since treatment with high doses of cycloheximide (CHX) is known to induce apoptosis in human B leukocytes (24), we used 100 μM CHX as positive control for induction of apop-

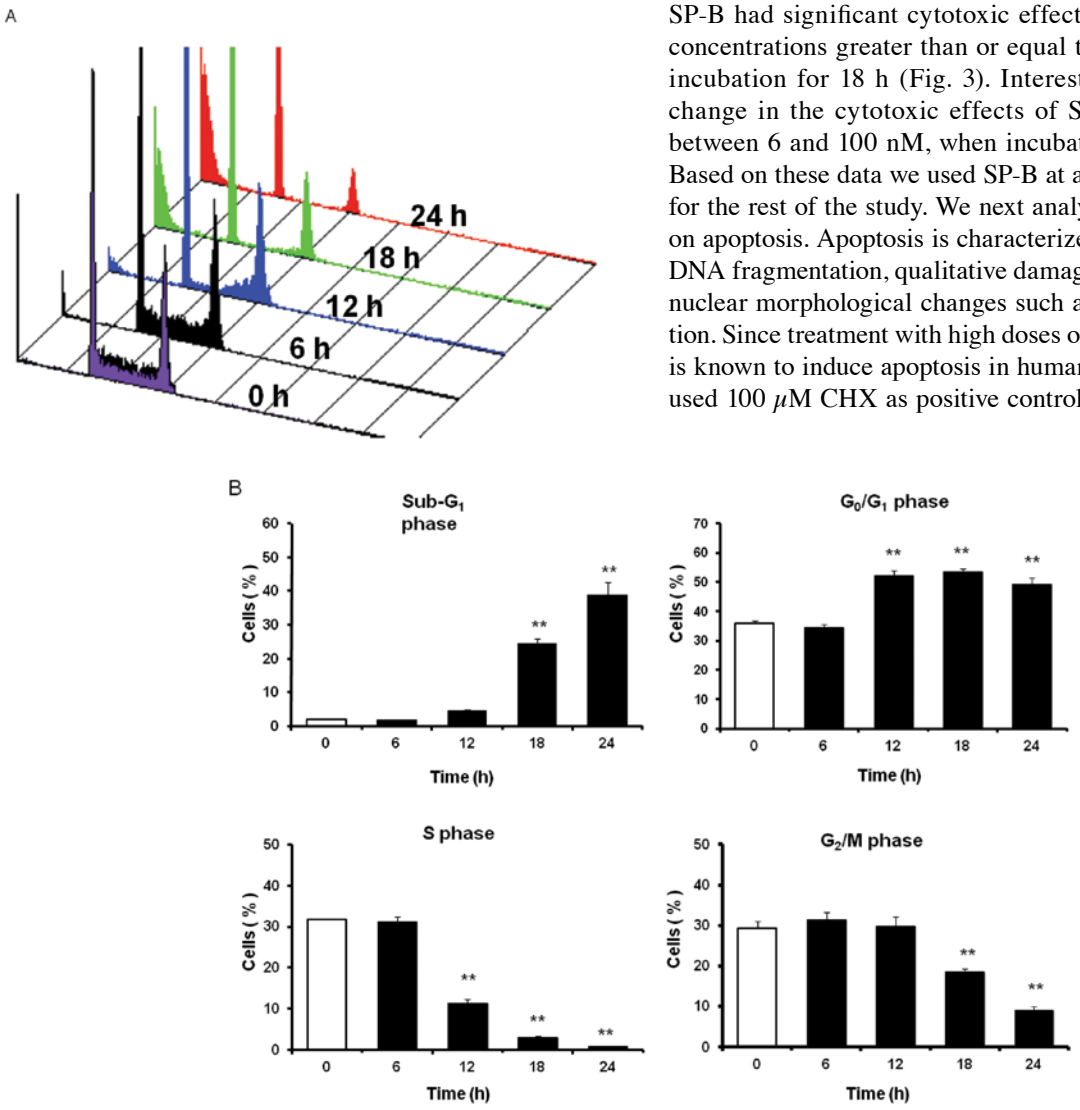

Figure 7. Effects of SP-B on the cell cycle. (A) Representative cell cycle profiles obtained using FACS of NALM-6 cells treated for the indicated times with 6 nM SP-B. (B) The percentage of cells at the indicated stage of the cell cycle in response to SP-B was assessed in NALM-6. The results are means ± SEM of three individual studies. $^{**}p<0.01$ compared with control group.

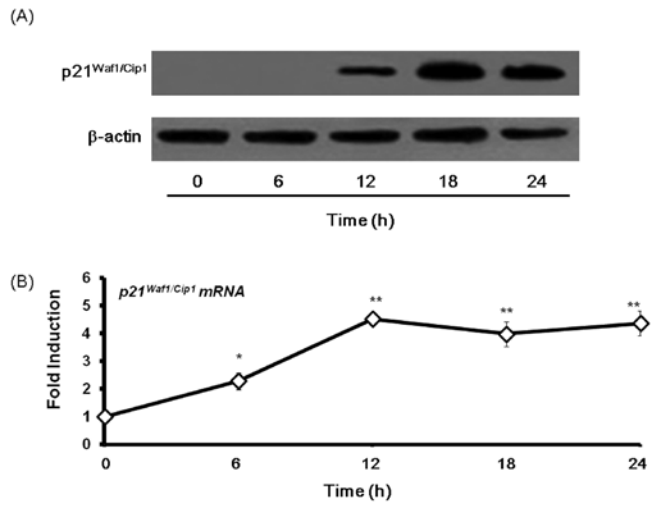

Figure 8. SP-B induces p21$^{waf1/cip1}$ protein and mRNA expression. NALM-6 cells were incubated with SP-B for the indicated times. (A) p21$^{waf1/cip1}$ protein expression was analyzed by Western blotting using β-actin as a loading control. The experiment shown is representative of two separate experiments. (B) *p21$^{waf1/cip1}$* mRNA expression was analyzed using qPCR. The results are means ± SEM of three individual studies. *p<0.05, **p<0.01 compared with control.

tosis in this study. SP-B at a concentration of 6 nM induced DNA fragmentation (Fig. 4) and nuclear morphological change (Fig. 5) following treatment of the cells for more than 12 h and 18 h, respectively. Thus, SP-B induces cellular changes that are characteristic of apoptosis. To confirm the mechanisms by which SP-B induces apoptosis and HDAC inhibition we analyzed the expression of apoptosis related proteins and acetylated histone proteins respectively using Western blotting (Fig. 6). There were no change in these proteins following incubation for 6 h with SP-B, but the expression of acetylated histones H2B, H3 and H4, of cleaved PARP and of cleaved caspase-3, as well as the degradation of total caspase-3 and Bcl-2 proteins were increased following incubation for 12 h or longer. Hence, HDAC inhibition by SP-B was associated with induction of apoptosis and acetylation of histones, which in turn led to cytotoxic effects.

SP-B induces cell cycle arrest and up-regulates p21$^{waf1/cip1}$ expression. We next examined the effect of SP-B on cell cycle progression in NALM-6 cells by FACS analysis of cells stained with PI (Fig. 7A and B). SP-B time-dependently increased the size of the sub-G_1 (apoptotic) peak, and this effect correlated with SP-B induction of cellular apoptotic features such as changes in nuclear morphology (see Fig. 5). Following 12 h incubation with SP-B, G_0/G_1 arrest was accompanied by a marked decrease in the number of cells in the S phase and, at later times of incubation by a decrease in the number of cells in the G_2/M phase. Since induction of p21$^{waf1/cip1}$ results in a blockage of cell cycle progression, and since p21$^{waf1/cip1}$ is also a well-established and key gene expression target of HDIs (14-17), we hypothesized that these affects of SP-B on the cell cycle were dependent on SP-B induction of the transcription factor p21$^{waf1/cip1}$. Indeed, the protein and mRNA levels of p21waf1/cip1 clearly increased in a time-dependent manner following incubation with SP-B (Fig. 8A and B, respectively).

Discussion

At least 80 clinical trials are currently underway in which the anti-tumor activities of at least 11 different HDAC inhibitory agents are being tested on cancers including both hematological and solid malignancies (25). It is therefore desirable to develop useful and highly potent HDIs. In the present study, SP-B was the most potent HDI in terms of induction of cytotoxic effects against several typical human leukemia cell lines (Table I). It is commonly assumed that powerful cytotoxic reagents affect normal cells leading to side effects. However, in our experimental *in vitro* study SP-B did not have significant cytotoxic effects towards normal leukocytes that were supplied by healthy volunteers (data not shown).

Several tumors, including hematological malignancies, are known to have higher HDAC expression than their normal counterparts (26, 27). In addition, clinical studies suggest that the level of *HDAC1* mRNA could potentially serve as a marker for patients with gastric (28), lung (29), or breast (30) cancers. We postulated that the cytotoxic effects of HDIs might be significantly higher on leukemia cell lines that have higher expression of *HDAC1* mRNA. However, there was only a modest correlation between *HDAC1* mRNA expression and SP-B cytotoxicity (Fig. 2). Unexpectedly, K562, which shows a low susceptibility to SP-B, displayed the highest expression of *HDAC1* mRNA of the leukemia cells tested. These data could imply that there is a low correlation between the expression of *HDAC1* mRNA and the HDI susceptibility of leukemia cells.

It has been reported that p21$^{waf1/cip1}$ plays a pivotal role in the anti-tumor activities of all HDIs (14-17). Indeed, the mRNA expression of *p21$^{waf1/cip1}$* was markedly increased in NALM-6 cells, which was the cell line that was most susceptible to HDIs in this study. Our data suggest that higher p21waf1/cip1 mRNA expression contributes to susceptibility to HDIs. However, it is unlikely that susceptibility to HDIs including SP-B is dependent solely on *p21$^{waf1/cip1}$* mRNA expression. This is because *p21$^{waf1/cip1}$* mRNA expression was only increased in NALM-6 cells and not in the other HDI-susceptible cell lines, in which expression of *p21$^{waf1/cip1}$* mRNA was even lower than that of normal leukocytes. Therefore it appears that high *p21$^{waf1/cip1}$* mRNA expression is unique to NALM-6.

HDI-induced cytotoxicity has been shown to involve induction of apoptosis that is mediated by caspase activation and Bcl-2 inhibition (31). Our data showed SP-B cytotoxicity following incubation with concentrations of SP-B ≥6 nM for 18 h (Fig. 3). Prior to this time point, after 12 h incubation, SP-B induced apoptosis, as indicated by DNA fragmentation (Fig. 4) and increased changes in nuclear morphology (Fig. 5). We showed that SP-B-induced apoptosis is mediated by caspase activation, since it was accompanied by the cleavage of PARP, which is a major substrate of executioner caspases including caspase-3. Both histone acetylation and changes in the level of apoptosis-related proteins were simultaneously detected following incubation with SP-B for 12 h (Fig. 6). These results support the hypothesis that SP-B-induced cytotoxicity is mediated by induction of histone acetylation via inhibition of HDAC, and that this effect of SP-B is associated with apoptosis, which was mediated by caspase activation. Notably, induction of apoptosis following incubation with SP-B was not observed at incubation times of <12 h in this study. The kinetics of histone acetylation induced

by HDIs have been previously reported, and it was shown that the histone acetylation induced by spiruchostatin A and FK228 is delayed compared with HDIs such as TSA that have a different chemical structure (11). Since SP-B has structural similarity with the former compounds, longer times of incubation were expected for the effect of SP-B on histone acetylation to become evident in NALM-6. As shown in Fig. 7, the change in cell cycle distribution induced by SP-B was primarily a decrease in S phase and a shift to the G_0/G_1 phase, followed by an increased in the sub-G_1 phase that was accompanied by a later decrease in the G_2/M phase. It is well established that *p21$^{waf1/cip1}$* is a negative regulator of the cell cycle, regulates gene transcription, and modulates apoptosis (32). Induction of p21$^{waf1/cip1}$ is a commonly used marker of HDAC inhibition (33). The induction of p21$^{waf1/cip1}$ by SP-B that we observed supports previous data that p21$^{waf1/cip1}$ is involved in events involving HDAC inhibition. Thus, after 6 h incubation with SP-B, there was little change in cell cycle distribution or in apoptotic features, and only a small increase in *p21$^{waf1/cip1}$* mRNA expression (Fig. 8A). However, after 12 h incubation with SP-B, there was a significant increase in both the mRNA and protein expression of p21$^{waf1/cip1}$ (Fig. 8), which was accompanied by SP-B induction of apoptosis and cell cycle arrest (Figs. 4-7).

In conclusion, NALM-6 cells, which have higher expression of *p21$^{waf1/cip1}$* mRNA than other leukemia cell lines, were susceptible to SP-B-induced cytotoxicity that resulted in induction of apoptosis. Expression of *p21$^{waf1/cip1}$* mRNA has prognostic significance in acute lymphoblastic leukemia (34). Interactions between therapy and biomarkers, as well as the emergence of p21$^{waf1/cip1}$ as a prognostic indicator, provide clues as to the underlying biology of leukemia. Our findings may be useful when establishing a therapeutic strategy for SP-B.

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