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Apoptotic Cytotoxic Effects of a Histone Deacetylase Inhibitor, FK228, on Malignant Lymphoid Cells

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Histone deacetylases are promising targets for cancer treatment. Here we studied the *in vitro* effects of a potent histone deacetylase inhibitor, FK228 (formerly FR901228), on human leukemia/lymphoma cells and cell lines compared with normal hematopoietic cells. In a lymphoma cell line, Raji, a nanomolar concentration of FK228 induced G1 arrest and/or apoptotic cell death, depending on the concentration and exposure time. Growth of lymphoid cell lines including Raji (N=13) was inhibited by 50% (IC_{50}) after 2-day treatment at concentrations of 0.83 to 1.87 ng/ml. Viability of clinical samples from patients with acute lymphoblastic leukemia was decreased by 50% at 0.78±0.46 ng/ml, whereas the IC_{50} values for normal mononuclear cells from peripheral blood and bone marrow were 2.3±0.96 and 7.8±1.0 ng/ml, respectively. The IC_{50} values for normal progenitor cells were 3.1, 4.4 and 7.8 ng/ml for BFU-E, CFU-GM and CFU-Mix, respectively. Expression levels of HDAC-1 and HDAC-3 proteins, which varied among cell lines, but were stable during the treatment with FK228, did not correlate with the sensitivity to FK288. This novel agent might be useful in the treatment of lymphoid malignancies, because the above concentrations are clinically achievable *in vivo* according to a recent clinical study.

Key words: Leukemia — Lymphoma — Apoptosis — Histone deacetylase inhibitor — FK228

Histone acetylation/deacetylation is a key mechanism for regulating transcription.[1, 2] Histone acetyltranferases (HATs) are recruited by transcriptional factors and activate transcription. On the other hand, histone deacetylases (HDACs), recruited by repressors of transcription, are involved in silencing transcription, and their deregulation is associated with some leukemias.[3] HDAC activities are inhibited by various agents such as sodium butyrate (SB),[4] trichostatin A (TSA)[5] and trapoxin (TPX).[6] Recently, TSA was found to bind directly with the catalytic pocket of HDAC-1 in a crystal structure analysis.[7]

HDAC inhibitors (HDACIs) are considered as potential therapeutic agents against malignancies. HDACIs increase or decrease the transcriptional levels of a limited number of genes in addition to causing hyperacetylation of histones,[8, 9] although the mechanisms involved are still under investigation. The treatment of cancer/leukemia cell lines with HDACIs induces cell-cycle arrest, differentiation and apoptosis.[5, 8, 10] TSA can induce differentiation of retinoic acid-resistant acute promyelocytic leukemia cell lines and cells.[11–14] In CCRF-CEM lymphoid cell lines, SB is known to induce apoptosis,[15] although it is not clear whether lymphoblastic cells from patients are sensitive to HDACIs.

A new cyclic depsipeptide, FK228 (formerly FR 901228), originally developed as an anti-cancer agent,[16] shows strong HDACI activity.[17] FK228 exhibited *in vitro* cytotoxicity in various cancer cell lines and chronic lymphocytic leukemia cells.[18, 19] Our preliminary experiment suggested that FK228 had a stronger cytotoxic activity than TSA. Furthermore, its clinical application has been started in a phase I study against solid tumors in the USA.[20] In this study, we investigated the *in vitro* cytotoxic effects of FK228 on malignant lymphoid cells and cell lines in comparison with normal hematopoietic cells.

MATERIALS AND METHODS

Cells Blood samples were obtained from patients with acute lymphoblastic leukemia (ALL) and healthy volunteers with written informed consent. Mononuclear cells (MNC) were separated from peripheral blood or bone marrow (BM) using density gradient centrifugation (Ficoll-Conray). All the samples were cryopreserved until used. After thawing, cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, NY) and incubated at 37°C in a 5% CO_2 incubator. The following cell lines were used for this study: precursor B-cell leukemia cell lines NALM-1, NALM-6, and ALL-1, the B-cell leukemia cell line BALL-1, the Burkitt's lymphoma cell lines Raji and Daudi, the precursor T-cell leukemia

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cell lines MOLT-3 and Jurkat, the T-cell leukemia cell lines CCRF-CEM and RPMI-8402, the adult T-cell leukemia cell lines ATN-1 and MT-1, and lymphoid-myeloid mixed leukemia cell line MR-87.

Reagents FK228 was a gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka). A 200 μg/liter solution of FK228 was prepared in dimethyl sulfoxide (DMSO) and stocked at −20°C.

Viability assay MNC adjusted to 1×10^6 cells/ml and cell lines adjusted to 1×10^5 cells/ml were cultured in a 24-well plate. To each well, 1 to 5 ng/ml FK228 was added. As the control for FK228, the same volume of DMSO was added to media. Viable cells were counted by means of the trypan blue dye-exclusion test. The concentration of FK228 required to produce a 50% reduction in the viable cell count was determined as the IC_{50}.

Colony assay Normal MNC from bone marrow (BMMNC) or peripheral blood (PBMNC) at 1×10^6 cells/ml were cultured with FK228 at concentrations from 0.5 to 10 ng/ml for 48 h. After culture, the cells were washed with media three times. Sixty thousand cells were resuspended in 3 ml of MethoCult GF medium (StemCell Technologies Inc., Vancouver, Canada) for progenitor assay. A total of 1 ml of culture medium was added to each of three 35-mm petri dishes. The growth of erythroid (BFU-E), granulocyte-macrophage (CFU-GM) and mixed (CFU-Mix) hematopoietic progenitors was investigated after 14 days of incubation at 37°C with 5% CO_2 in a humidified atmosphere. The data are based on the actual colony counts from the assays (N=3).

DNA histogram To prepare DNA histograms, cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 0.1% Triton X-100 and 50 μg/ml propidium iodide (PI, Sigma Chemical Co., St. Louis, MO). Then, the specimens were quantified by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA). For the detection of apoptotic cells, expression of annexin-V and exclusion of PI were simultaneously detected using two-color flow cytometry. Cells were washed with PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing 2 μl of FITC-labeled anti-annexin V antibody (MBL, Nagoya) and 10 μl of 20 μg/ml PI. After incubation for 10 min at room temperature in a light-protected area, the specimens were quantified on the FACScan.

Immunoblot analysis The expression of HDAC-1 and HDAC-3 proteins was analyzed by immunoblot analysis. The method was described previously. Briefly, whole cell lysates were loaded onto 10% polyacrylamide gel for electrophoresis. The proteins were transferred to 0.2-μm nitrocellulose (Sequi-Blot PVDF Membrane, Bio-Rad Laboratories, Hercules, CA) using an electroblot apparatus (Trans-Blot SD, Bio-Rad Laboratories). After having been blocked in TBS-T (Tris-buffered saline-0.05% Tween; WAKO, Osaka) containing 5% skim milk for 2 h, the membranes were incubated with either goat antihuman HDAC-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or goat antihuman HDAC-3 antibody (Santa Cruz Biotechnology) diluted in TBS-T with 5% skim milk for 24 h at 4°C or for 1 h at room temperature. The blots were incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (Santa Cruz Biotechnology) diluted with 5% skim milk in TBS-T for 45 min at room temperature. A chemiluminescence system (ECL-Plus, Amersham, Buckinghamshire, UK) was used for visualization. Gel loading equivalence was confirmed by reprobing with mouse monoclonal antibody to actin (Boehringer Mannheim Yamanouchi, Tokyo) followed by HRP anti-mouse IgG (DAKO Japan, Tokyo).

RESULTS

Cytotoxic effects of FK228 on Raji cells Treatment with a nanomolar level of FK228 decreased the number of viable Raji cells (Fig. 1). Growth of Raji cells was inhibited by 50% (IC_{50}) after 2-day treatment at 1.47 ng/ml (2.72 nM). To elucidate whether the growth inhibition was caused by growth-arrest or by cell-death, the DNA histogram was studied (Fig. 2). Treatment with 1 ng/ml FK228 caused G1-arrest after 24 h, which corresponded to the flat cell-growth curve in Fig. 1. At 2 ng/ml, FK228 increased the sub-G1 fraction in addition to causing G1-arrest after 24 h. Morphologically, Raji cells treated with 2 ng/ml or more for 2 days showed characteristics of apoptosis, such

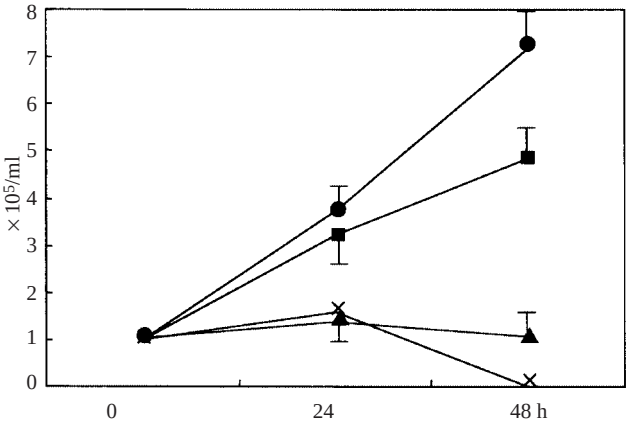

Fig. 1. Anti-proliferative effects of FK228 on Raji cells. Raji cells seeded at 1×10^5 cells/ml were incubated with media alone (●) or containing FK228 (1 ng/ml, ■; 2 ng/ml, ▲; or 5 ng/ml, ×) for 48 h. At intervals of 24 h, the number of viable cells was determined by trypan blue dye-exclusion test. Each value represents a mean of triplicates and error bars represent standard deviation.

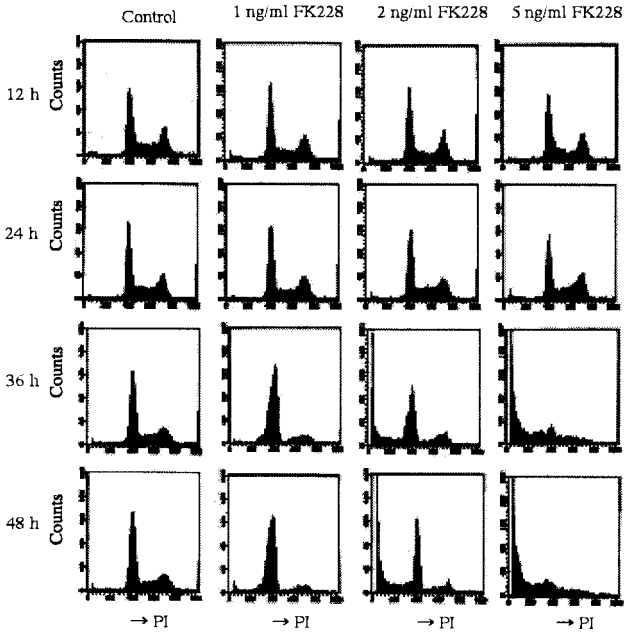

Fig. 2. DNA histogram of Raji cells treated with FK228. Treatment with 1 ng/ml FK228 for more than 24 h caused G1-arrest. Treatment with 2 ng/ml FK228 for more than 24 h increased both G1 and sub-G1 fractions. Five ng/ml FK228 increased the sub-G1 fraction without accumulation at G1.

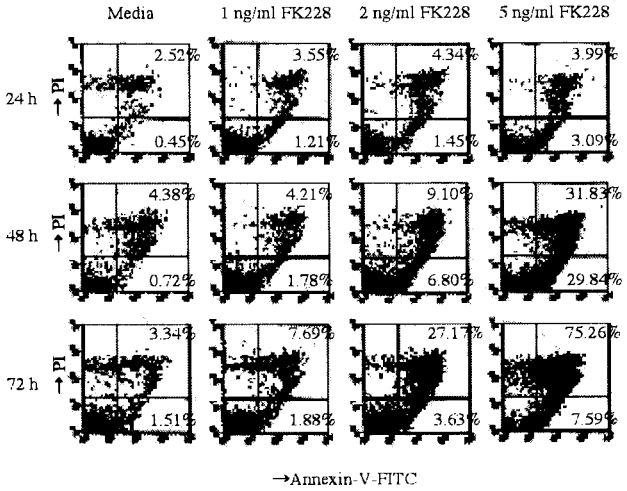

Fig. 3. Detection of apoptosis in Raji cells by annexin-V/PI assay. Cells in an annexin-V-positive and PI-negative fraction are observed during the early stage of apoptosis. Cells in an annexin-V-positive and PI-positive fraction are in the late stage of apoptosis or are necrotic.

as cell shrinkage, membrane blebbing, cellular fragmentation, and formation of condensed apoptotic bodies (data not shown). To study further whether FK228 induced apoptosis in Raji cells, we examined the expression of annexin-V and exclusion of PI using two-color flow cytometry (Fig. 3). Treatment with 1 ng/ml FK228 had little effect on the annexin-V- and PI-positivities. Upon treatment of the cells with 2 or 5 ng/ml FK228, the annexin-V-positive and PI-negative population, which corresponded to cells in an early phase of apoptosis, increased at 48 h. At 72 h, the annexin-V-positive and PI-positive population, which corresponded to a late phase of apoptosis or necrosis, increased. Thus, FK228 caused growth-arrest and/or apoptosis, depending on concentration.

Washout experiment of FK228 To examine further the relationship between exposure to FK228 and cytotoxicity, a washout experiment was performed on Raji cells. FK228 was added to cultures at various concentrations for 1, 3, 6, 12 and 48 h. After 48-h culture in total, viable cells were counted. With increasing exposure time up to 24 h, the cytotoxic activity increased (Fig. 4). In treatment for 12 h or less, however, the products of IC_{50} at a particular exposure time and the exposure time were almost constant. For example, the IC_{50} was 5 ng/ml for 12-h treatment, while it

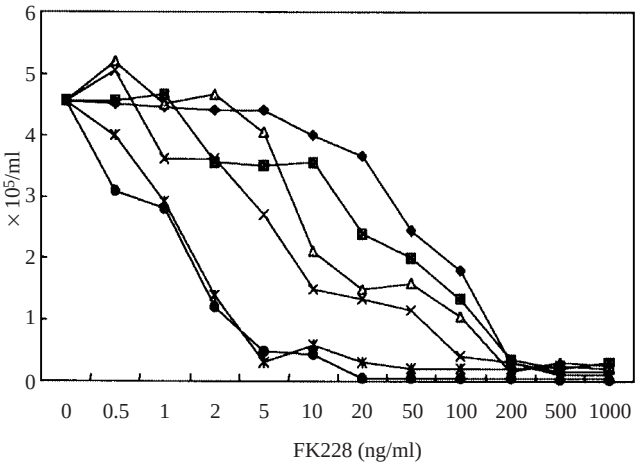

Fig. 4. Wash-out experiment of FK228. Raji cells at 0.1×10^6/ml were treated with FK228 as indicated, then washed 3 times, and cultured again without FK228. After incubation for a total of 48 h, the number of viable cells was determined by trypan-blue dye exclusion test. ◆ 1 h, ■ 3 h, △ 6 h, × 12 h, ✱ 24 h, ● 48 h.

was 20 ng/ml for 3-h treatment. Accordingly, the calculated area under the curve (AUC) for 50% cytotoxicity was 60 ng·h/ml. These findings suggest that the cytotoxic effects of FK228 depend equally on time and concentration at high concentrations.

Table I. IC_{50} Values[a)] of FK228 for Lymphoid Cell Lines

Cell line	(ng/ml)
Raji	1.43
Daudi	1.22
NALM-1	1.19
NALM-6	1.34
ALL-1	1.63
BALL-1	1.86
CCRF-CEM	1.78
MOLT-3	1.87
Jurkat	1.49
RPMI-8402	0.83
ATN-1	0.95
MT-1	1.42
MR-87	1.10

a) Concentrations which inhibited cell growth by 50% after 2-day treatment.

Table II. IC_{50} Values[a)] of FK228 in ALL Cells from Patients, Normal PBMNC and BMMNC

Cells	(ng/ml)
ALL cells (n=6)	0.78±0.46
Normal PBMNC (n=3)	2.28±0.96
Normal BMMNC (n=3)	7.78±1.03

a) Concentrations which reduced cell viability by 50% after 2-day treatment.

Effects of FK228 on other cell lines, clinically isolated samples of ALL, and normal PBMNC and BMMNC The IC_{50} values for lymphoid cell lines after 48-h treatment are presented in Table I. The mean was 1.39 ng/ml, ranging from 0.83 to 1.87 ng/ml. Leukemia cells from six patients with ALL, normal PBMNC from three volunteers, and normal BMMNC from three volunteers were also treated with various concentrations of FK228 for 48 h. The concentrations that reduce viability to 50% of the cultured control (IC_{50}) are shown in Table II. IC_{50} values for ALL samples were comparable to those for the cell lines but lower than those for normal PBMNC or BMMNC. We assessed whether FK228 suppressed the growth of hematopoietic progenitor cells. BMMNC isolated from three normal volunteers were exposed to various concentrations of FK228 for 48 h, washed with PBS, and cultured for colony formation. The effects of FK228 on BM progenitors are shown in Fig. 5. At 2 ng/ml, FK228 did not affect the colony formation of CFU-GM and CFU-Mix, whereas BFU-E colonies were approximately 40% suppressed. The IC_{50} values for BFU-E, CFU-GM and CFU-Mix were 3.1, 4.4 and 7.8 ng/ml, respectively. These

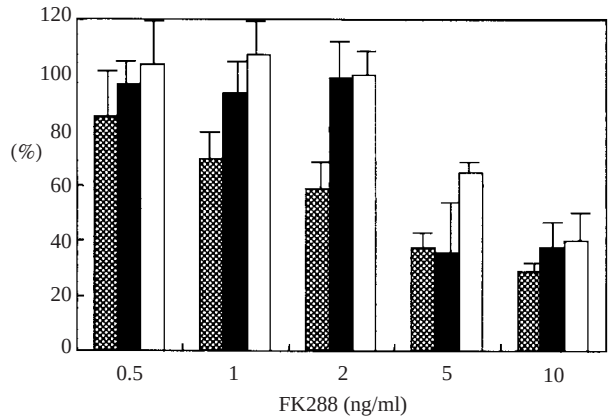

Fig. 5. Effects of FK228 on hematopoietic progenitors. Normal BMMNC were treated with FK228 for 48 h, washed three times, and assayed for colony formation. The effect was evaluated in comparison with the number of hematopoietic progenitors in control media. Each column represents a mean of triplicates and error bars represent standard deviation. ▨ BFU-E, ■ CFU-GM, □ CFU-Mix.

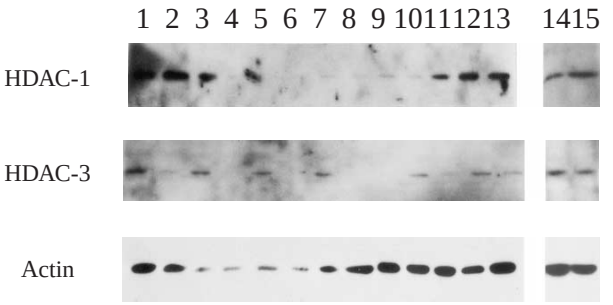

Fig. 6. Immunoblot analysis of HDAC-1 and -3 in cell lines and normal BMMNC. Amounts of proteins loaded were assessed using anti-actin monoclonal antibody. Lane 1, Raji; 2, Daudi; 3, NALM-1; 4, NALM-6; 5, ALL-1; 6, BALL-1; 7, CCRF-CEM; 8, MOLT-3; 9, Jurkat; 10, RPMI-8402; 11, ATN-1; 12, MT-1; 13, MR-87; 14 and 15, normal BMMNC.

results suggest that leukemia cells from patients were more sensitive than BMMNC and hematopoietic progenitor cells.

HDAC-1 and HDAC-3 expression and FK228 To examine the basis of the different sensitivities to FK228 between malignant and normal cells, we compared the expression levels of HDACs in cell lines and BMMNC (Fig. 6). Since anti-HDAC-1 and -HDAC-3 antibodies were available, expression levels were analyzed by immunoblotting. The levels differed among the cell lines, but were equivalent in BMMNC. There was no correlation

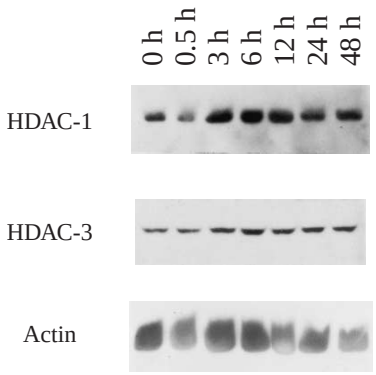

Fig. 7. Expression of HDAC-1 and HDAC-3 in Raji cells treated with FK228. Raji cells were treated with 10 ng/ml FK228, and expression of HDAC-1 and -3 was analyzed by immunoblotting.

between the expression levels of HDACs and IC_{50} levels of FK228. Then, we examined the time-course of HDAC expression in Raji cells treated with 10 ng/ml FK228. Fig. 7 reveals that there was no change in the HDAC-1 and HDAC-3 levels during FK228 treatment.

DISCUSSION

In this study, we observed that FK228 induced G1 arrest and/or apoptosis in a concentration-dependent manner at nanomolar levels. At higher concentrations, the cytotoxic effects depended equally on time and concentration. Furthermore, bone marrow progenitor cells were less susceptible than clinically isolated ALL cells. However, the HDAC expression levels did not explain the different sensitivities to FK228.

FK228 was originally isolated from *Chromobacterium violaceum* as a novel anti-tumor agent,[16] which induced morphological reversion of transformed NIH/3T3 cells. An *in vitro* study revealed that FK228 is a strong and irreversible inhibitor of HDAC.[17] Treatment with less than 10 n*M* FK228 caused hyperacetylation of histones and inhibited tumor cells by causing cell cycle arrest at the G1 and G2/M phases. These effects are similar to those of TSA. Notably, FK228 is 100 times more potent than TSA in terms of apoptotic effects on leukemia cells (paper submitted), although IC_{50} of FK228 was half that of TSA for inhibiting HDAC activity *in vitro*. Pharmacokinetics in phase I trial patients treated with FK228 demonstrated that effective concentrations are clinically achievable *in vivo*.[20]

HDAC itself is a component of the complex mediating transcription repression and chromatin remodeling.[1, 2, 21] Many reports have shown that transcription factors associated with gene silencing recruit co-repressor/HDAC complex, and that subsequent deacetylation of chromatin inhibits transcription. However, global hyperacetylation evoked by HDACIs does not induce a general increase of gene transcription. A limited number of genes, including $p21^{waf1}$ and gelsolin, are reportedly activated by treatment with HDACIs.[8, 9] However, it remains to be elucidated why such genes are specifically induced by treatment with HDACIs. First, the treatment with HDACIs acetylates proteins other than histone. Some transcriptional factors such as p53 might be acetylated and then functionally activated.[22] Second, a specific regional chromatin might be preferentially acetylated by treatment with HDACI. A specific sequence within the promoter region is necessary for the transcriptional activation.[23] Cell-cycle arrest might be thus associated with the induction of $p21^{waf1}$. The next question is the mechanism of apoptosis induced by HDACIs. The apoptosis seems to require new protein synthesis.[10] Hyperacetylation of histone and change of chromatin structure may trigger the apoptotic signals. Although induction of Fas/Fas ligand expression was reported in a neuroblastoma cell line treated with hybrid polar HDACI,[24] FK228 did not induce it in Raji cells (data not shown).

Importantly, we observed that IC_{50} values of FK228 in leukemia cells from patients were lower than those in normal BMMNC and hematopoietic progenitor cells. After the treatment with FK228, the gross acetylation states were not significantly different between leukemia cells and normal hematopoietic cells (data not shown). The question then arises, why do leukemia cells show increased sensitivity to hyper-acetylation of histones? First, the different sensitivities might be caused by the imbalance between acetylase and deacetylase in malignant cells, because leukemia is frequently accompanied by deregulation of transcription.[25] Second, our preliminary data indicated that FK228 is sensitive to P-glycoprotein (data not shown). Accordingly, FK228 might be less toxic to normal progenitor cells, which physiologically express a higher level of P-glycoprotein than leukemia cells.[26] Third, the expression and activity of HATs and HDACs may be altered in leukemia cells. In this study, we examined the expression of HDAC-1 and HDAC-3 by immunoblot analysis. The expression levels significantly differed among samples, although the implications of this are unclear. However, there was no apparent relationship between the expression levels and the sensitivities to FK228. Since the sensitivity to MS-275, another HDACI, was reportedly correlated with the response of $p21^{waf1}$ induction, it should be studied in normal hematopoietic cells.

It is also unknown how FK228 inhibits HDAC activities. While TSA and suberoylanilide hydroxamic acid (SAHA) have been shown to bind directly with the catalytic pocket of HDAC-1,[7] the molecular mechanism of

FK228 action remains unknown. Thus we checked whether FK228 down-regulates the expression level of HDACs. During the treatment with FK228, however, there was no change in the expression levels of HDAC-1 and -3.

Finally, this study suggests that lymphoid leukemia/lymphoma might be a promising target for HDACI therapy. Further investigation is needed to establish the mechanism by which HDACI induces apoptosis.

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