ELSEVIER

Biochemical Pharmacology 64 (2002) 1079–1090

Biochemical Pharmacology

Effects of FK228, a novel histone deacetylase inhibitor, on human lymphoma U-937 cells *in vitro* and *in vivo*

Yuka Sasakawa*, Yoshinori Naoe, Takeshi Inoue, Tatsuya Sasakawa, Masahiko Matsuo, Toshitaka Manda, Seitaro Mutoh

Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-Ku, Osaka 532-8514, Japan

Received 18 December 2001; accepted 26 February 2002

Abstract

FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] is a novel histone deacetylase inhibitor that shows therapeutic efficacy in Phase I trials of patients with malignant lymphoma. However, its mechanism of action has not been characterized. In this study, we examined the *in vitro* and *in vivo* effects of FK228 on human lymphoma U-937 cells. FK228 very strongly inhibited the growth of U-937 cells with an IC_{50} value of 5.92 nM. In a *scid* mouse lymphoma model, mice treated with FK228 once or twice a week survived longer than control mice, with median survival times of 30.5 (0.56 mg/kg) and 33 days (0.32 mg/kg), respectively (vs. 20 days in control mice). Remarkably, 2 out of 12 mice treated with FK228 (0.56 mg/kg once or twice a week) survived past the observation period of 60 days. The apoptotic population of U-937 cells time-dependently increased to 37.7% after 48 hr of treatment with FK228. In addition, FK228 induced G1 and G2/M arrest and the differentiation of U-937 cells to the $CD11b^+/CD14^+$ phenotype. Expression of $p21^{WAF1/Cip1}$ and gelsolin mRNA increased up to 654- and 152-fold, respectively, after 24 hr of treatment with FK228. FK228 caused histone acetylation in $p21^{WAF1/Cip1}$ promoter regions, including the Sp1-binding sites. In conclusion, (i) FK228 prolonged the survival time of *scid* mice in a lymphoma model, and (ii) the beneficial effects of FK228 on human lymphoma may be exerted through the induction of apoptosis, cell cycle arrest, and differentiation via the modulation of gene expression by histone acetylation.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Lymphoma; U-937; FK228; Histone deacetylase inhibitor

1. Introduction

FK228 [(*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo-[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; FR901228, depsipeptide] was isolated from *Chromobacterium violaceum* Strain WB968. It was discovered by Ueda and coworkers [1] while screening for novel antitumor compounds with the ability to reverse morphological changes in Ha-*ras*-transformed NIH3T3 cells. As shown in Fig. 1, FK228 contains a unique bicyclic peptide with a non-cystine disulfide bridge, and is unrelated to other HDAC inhibitors. FK228 possesses potent antitumor activity against human tumor cell lines and inhibits the growth of human solid tumors implanted in mice [2].

FK228, like TSA, inhibits intracellular HDAC activity, which induces the accumulation of marked amounts of acetylated histone species [3]. It has been proposed that histone acetylation plays a role in transcriptional regulation, probably by relaxing specific segments of DNA and facilitating the binding of transcription factors [4–7]. The acetylation on lysine residues of histone N-terminal tails is controlled by either histone acetyltransferase (HAT) or HDAC. Acetylation leads to a loosening of histone-DNA contacts and the interaction of histones with ATP-dependent chromatin remodeling complexes [7–9].

Studies on the effect of HDAC inhibitors on APL have been performed. APL patients who have a t(15;17) translocation have an abnormal retinoic acid receptor-α (RARα), designated as PML/RARα, resulting from protein fusion; these patients respond well to treatment with ATRA [10].

* Corresponding author. Tel.: +81-6-6390-1154; fax: +81-6-6304-5367.
E-mail address: yuka_sasakawa@po.fujisawa.co.jp (Y. Sasakawa).

Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; ChIP, chromatin immunoprecipitation; CTCL, cutaneous T-cell lymphoma; HDAC, histone deacetylase; PE, phycoerythrin; PTCL, peripheral T-cell lymphoma; q-PCR, quantitative polymerase chain reaction; TSA, trichostatin A; vitamin D_3, 1α,25-dihydroxy-vitamin D_3.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0006-2952(02)01261-3

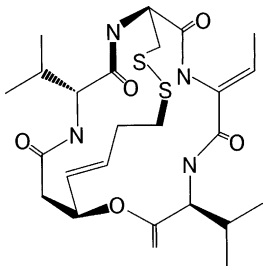

Fig. 1. Chemical structure of FK228.

In contrast, APL patients who have a t(11;17) translocation have a retinoic acid receptor fusion comprised of a promyelocytic leukemia zinc-finger (PLZF) and an RARα (PLZF/RARα); these patients respond poorly to ATRA [10]. HDAC recruited by PLZF-RARα is associated with ATRA resistance, because ATRA cannot inhibit the binding of PLZF to HDAC [11–14]. TSA and ATRA synergistically release the reporter gene harboring the retinoic acid response element (RARE) from transcriptional repression by PLZF-RARα [11–14]. The HDAC inhibitors, TSA or butyrate, alone or in combination with ATRA induce apoptosis, cell cycle arrest, and cell differentiation in APL [15–18]. HDAC inhibitors induce the expression of $p21^{WAF1/Cip1}$ and gelsolin, which may contribute to cell cycle arrest and differentiation, respectively [16,19–22].

Recent Phase I trials demonstrated that FK228 has therapeutic efficacy in the treatment of patients with PTCL or CTCL [23]. Three patients with CTCL had a partial response and 1 patient with PTCL (unspecified) had a complete response. However, the mechanism of this action has not been characterized completely. In this study, we examined both *in vitro* and *in vivo* effects and the mode of action of FK228 using human lymphoma U-937 cells.

2. Materials and methods

2.1. Chemicals

FK228 was prepared at the Fujisawa Pharmaceutical Co., Ltd. The chemical structure of FK228 is shown in Fig. 1. ATRA was purchased from the Sigma Chemical Co. Vitamin D_3 was purchased from Wako Pure Chemical Industries, Ltd. For *in vitro* studies, all drugs were dissolved in ethanol and diluted with each experimental medium. For *in vivo* studies, FK228 was dissolved in and diluted with 10% polyoxyethylated (60 mol) hydrogenated castor oil in saline (HCO60 saline).

2.2. Tumor cells and animals

The following cell lines were used for this study: the histiocytic lymphoma cell line U-937, the chronic myelogenous leukemia cell line K562, and the acute lymphoblastic leukemia cell line CCRF-CEM. These cells were maintained in RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Moregate), 50 units/mL of penicillin, and 50 μg/mL of streptomycin (ICN Biomedicals). The cell numbers were determined with a hemocytometer, and viabilities were assessed by trypan blue dye exclusion. Male *scid* mice (FOX CHASE SCID C.B-17/lcr-*scid*Jcl) were purchased from CLEA Japan Inc.

2.3. Evaluation of in vitro sensitivity

Growth inhibition experiments were carried out in 96-well flat-bottomed microplates (Sumitomo Bakelite Co., Ltd.), and the number of viable cells at the end of incubation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially as described by Mosmann [24]. Briefly, 2×10^4 cells per well in 100 μL culture medium were incubated in the presence or absence of drugs for 72 hr. After the addition of 10 μL of 5 mg/mL of MTT (Sigma) per well, the plates were incubated at 37° under 5% CO_2 for 4 hr. The blue crystals formed in each well were dissolved in 150 μL of 0.04 N HCl in isopropanol. The absorbance was measured at 580 nm using a SPECTRA MAX250 (Wako Pure Chemical Industries). The IC_{50} values were calculated with the converted log concentrations by the least squares method.

2.4. In vivo antitumor activity

We evaluated the antitumor activity of FK228 on lymphomas using a *scid* mouse model and a modification of a previously described method [25–27]. Briefly, male *scid* mice were injected i.p. with 150 mg/kg of cyclophosphamide (Shionogi & Co., Ltd.). All the mice injected with cyclophosphamide alone survived over the observation period (data not shown). Twenty-four hours after cyclophosphamide injection, mice were inoculated i.p. with 1×10^7 U-937 cells in 500 μL PBS. Twenty-four hours after tumor cell inoculation, the mice were treated i.p. with or without FK228 at doses ranging from 0.1 to 1 mg/kg, once or twice a week. Control mice were treated with 10% HCO60 saline twice a week. Six and twelve mice were used for FK228-treated and control groups, respectively. The observation period was 60 days after cell inoculation, and the antitumor effect was evaluated by survival time, which was analyzed for statistical significance by Peto's test.

2.5. Apoptosis analysis

Cells were seeded at 1×10^6 per 25-cm^2 flask (Asahi Technoglass Co., Ltd.) and incubated with or without 5 ng/mL of FK228, at 37° under 5% CO_2 for the indicated time periods. Apoptotic cells were measured with a MEBCYTO Apoptosis Kit (Medical & Biological Laboratories). The relative fluorescent content was measured by flow cytometry (FACScan, Becton Dickinson). Ten thousand events were analyzed for each sample.

2.6. Cell cycle analysis

Unsynchronized cells were seeded at 2×10^6 per 25-cm^2 flask and incubated with or without 5 ng/mL of FK228, at 37° under 5% CO_2 for the indicated time periods. In another test, cells were treated with or without 0.5 ng/mL of FK228 for 48 hr and cultured in drug-free medium for another 24 hr, according to a previously described method [28]. Cell cycle progression was evaluated by propidium iodide (PI) staining, with a modification of a previously described method [29]. Briefly, after fixing with 70% ethanol for more than 30 min, the nuclei were treated with 100 μg/mL of Ribonuclease A (Sigma) and 8 μg/mL of PI (PharMingen) for 30 min in the dark at room temperature. The relative DNA content was measured by immunofluorescence using flow cytometry. Ten thousand events were analyzed for each sample.

2.7. Differentiation analysis

Cells were seeded at 1×10^6 per 25-cm^2 flask and incubated with or without 5 and 0.5 ng/mL of FK228, at 37° under 5% CO_2 for the indicated time periods. ATRA and vitamin D_3 were also used at concentrations of 1 and 0.1 μM, respectively, as described in previous reports [30,31]. The surface antigen expression was measured by immunofluorescence using flow cytometry. PBS plus 0.2% BSA (Sigma) and 0.1% NaN_3 was used as the staining medium. Briefly, the cells were washed twice with staining medium and incubated with anti-human-CD11b PE-labeled antibody (PharMingen) and anti-human-CD14 FITC-labeled antibody (PharMingen), at 4° for 30 min in the dark. After washing twice, cells were resuspended with 500 μL of staining medium; then PE and fluorescein isothiocyanate (FITC) fluorescence were measured by flow cytometry. Ten thousand events were analyzed for each sample.

2.8. Detection of $p21^{WAF1/Cip1}$ and gelsolin mRNA by real-time q-PCR

Cells were seeded at 2×10^6 per 25-cm^2 flask and incubated with or without 5 ng/mL of FK228, at 37° under 5% CO_2 for the indicated time periods. Cells were homogenized with TRIzol reagent (Gibco BRL), and the RNA was extracted according to established methods [32]. The RNA was reverse-transcribed using Taq-Man reverse transcription reagents (PE Biosystems). Primers for $p21^{WAF1/Cip1}$, gelsolin, and β-actin were designed using "primer express" software (PE Biosystems) and ordered from the Funakoshi Co., Ltd. The following sequences were used for this study: 5′-GGC AGA CCA GCA TGA CAG ATT-3′ ($p21^{WAF1/Cip1}$ upstream), 5′-GGA TTA GGG CTT CCT CTT GGA G-3′ ($p21^{WAF1/Cip1}$ downstream), 5′-TGG TGC AGA GAC TCT TCC AGG T-3′ (gelsolin upstream), 5′-TGT TGA AGC TCT CCC AGG ACA-3′ (gelsolin downstream), 5′-CAT TGG CAA TGA GCG GTT C-3′ (β-actin upstream), and 5′-GGT AGT TTC GTG GAT GCC ACA-3′ (β-actin downstream). The expression of $p21^{WAF1/Cip1}$, gelsolin, and β-actin genes was estimated by real-time q-PCR using SYBR green PCR master mix (PE Biosystems). The final expression value was calculated as follows: the expression level of $p21^{WAF1/Cip1}$ or gelsolin mRNA/the expression level of β-actin. Each value at 0 hr was set as 1.

2.9. ChIP assay

The ChIP assay was performed using a modification of a previously described method [22]. Cells were seeded at 4×10^6 per 75-cm^2 flask and incubated overnight at 37° with 5% CO_2. The next day, cells were cultured with 5 ng/mL of FK228 for 2 hr. Formaldehyde was added to the cells to a final concentration of 1%, and they were incubated for 10 min at 37°. The medium was removed, and the cells were resuspended in 1 mL of ice-cold PBS containing the serine protease inhibitor phenylmethylsulfonyl fluoride (0.1 mM). Cells were pelleted, resuspended in 0.5 mL of SDS lysis buffer (1% SDS/10 mM EDTA/50 mM Tris–HCl, pH 8.1), and then incubated on ice for 10 min. Lysates were sonicated with 3 sets of 10-sec pulses. Debris was removed from the samples by centrifugation at 15,000 *g* for 10 min at 4°. An aliquot of the chromatin preparation was set aside and designated as the "input fraction". Supernatants were diluted 5-fold in immunoprecipitation buffer (0.01% SDS/1.1% Triton X-100/1.2 mM EDTA/16.7 mM NaCl/16.7 mM Tris–HCl, pH 8.1), and 80 μL of a 50% protein A Sepharose slurry containing 20 μg of sonicated salmon sperm DNA and 1 mg/mL of BSA in TE buffer (10 mM Tris–HCl/1 mM EDTA, pH 8.0) was added and incubated for 30 min at 4° with rocking. The Sepharose beads were pelleted by centrifugation at 12,000 *g* for 1 min at 4°, and the supernatants were placed in fresh tubes with 5 μg of anti-acetylhistone H3 or anti-acetylhistone H4 antibody (Upstate Biotechnology, Inc.) and incubated overnight at 4°. A protein A Sepharose slurry (60 μL) was added, and the samples were rocked for 1 hr at 4°. The protein A complexes were centrifuged at 12,000 *g* for 1 min at 4° and washed five times for 5 min each. Immune complexes were eluted twice with 250 μL of elution buffer (1% SDS/0.1 M $NaHCO_3$) for 15 min at room temperature. Twenty microliters of 5 M NaCl was added to the combined eluates, and the samples were incubated at 65° for 4 hr. EDTA, Tris–HCl, pH 6.5, and proteinase K were then added to the samples at final concentrations of 10, 40 mM, and 0.04 μg/mL, respectively, and the samples were incubated for 1 hr at 45°. Immunoprecipitated DNA (both immunoprecipitation samples and input) was recovered by phenol/chloroform extraction and ethanol precipitation and then was analyzed by PCR. $p21^{WAF1/Cip1}$-specific primers were used to carry out PCR from DNA isolated from ChIP experiments and input samples. The optimal reaction conditions for PCR were determined for each

primer pair. Cycle parameters were: denaturation at 95° for 1 min and annealing at 60° for 1 min, followed by elongation at 72° for 1 min. PCR products were analyzed by 3% agarose/ethidium bromide gel electrophoresis. The primer pairs used for $p21^{WAF1/Cip1}$ ChIP analysis were: 5′-ACC AAC GCA GGC GAG GGA CT-3′ (P1 upstream), 5′-CCG GCT CCA CAA GGA ACT GA-3′ (P1 downstream), 5′-GGT GTC TAG GTG CTC CAG GT-3′ (P2 upstream), 5′-GCA CTC TCC AGG AGG ACA CA-3′ (P2 downstream), 5′-CGT GGT GGT GGT GAG CTA GA-3′ (P3 upstream), 5′-CTG TCT GCA CCT TCG CTC CT-3′ (P3 downstream), and 5′-AGC TGA GCC GCG ACT GTG AT-3′ (E2 upstream), 5′-CTG AGC GCG GCA CAA GGG TA-3′ (E2 downstream).

3. Results

3.1. In vitro sensitivities of human lymphoma U-937 and leukemia cell lines to FK228

We examined the *in vitro* sensitivities of human lymphoma U-937 and leukemia cell lines (K562 and CCRF-CEM) to FK228. FK228 inhibited the growth of U-937, K562, and CCRF-CEM cells with IC_{50} values of 5.92, 8.36, and 6.95 nM, respectively (Table 1). The sensitivity of U-937 cells was almost the same as that of the leukemia cell lines.

3.2. In vivo antitumor effect of FK228 on U-937 lymphoma in a scid mouse model

The *in vivo* antitumor activity of FK228 on lymphomas was evaluated in a *scid* mouse model. Mice inoculated with U-937 cells were unhealthy in appearance and had swollen abdomens. They started to die from 18 days after tumor cell inoculation, and the median survival time (MST) was 20 days (Table 2). Mice treated with FK228 at 0.1–1 mg/kg twice a week survived significantly longer (MST of 25–33 days) than control mice (Table 2 and Fig. 2B). The mice treated with FK228 at 0.1 to 1 mg/kg once a week also survived significantly longer (MST of 22.5–30.5 days) than control mice (Table 2 and Fig. 2A). All the mice treated with FK228 once a week at 0.18 and 0.56 mg/kg or twice a week at 0.32 and 0.56 mg/kg, were still alive on day 24, the time at which 11 out of 12 mice had died in the control group. Of note, 1 out of 6 mice treated once a week with FK228 at 0.56 mg/kg and 1 out of 6 mice treated twice a week with FK228 at 0.56 mg/kg survived past the observation period

Table 1
In vitro sensitivities of human lymphoma U-937 and leukemia cell lines to FK228

Cell line	IC_{50} (nM)
U-937	5.92
K562	8.36
CCRF-CEM	6.95

U-937 cells and leukemia cell lines were cultured with FK228 for 72 hr, and growth inhibitory activity was determined. The experiment was repeated at least three times, and typical result is shown.

Table 2
Effect of FK228 on the survival time of *scid* mice inoculated with lymphoma U-937

Treatment	Dose (mg/kg)	MST (days)	% of Control
Control		20.0	100
FK228 once a week	0.1	22.5	113*
	0.18	27.0	135*
	0.32	28.0	140*
	0.56	30.5	153*
	1	28.0	140*
FK228 twice a week	0.1	25.0	125*
	0.18	26.0	130*
	0.32	33.0	165*
	0.56	27.0	135*
	1	25.5	128*

The observation period was 60 days after tumor cell inoculation, and the antitumor effect was evaluated by survival time. Survival was analyzed for statistical significance by Peto's test. Six and twelve mice were used for FK228-treated and control groups, respectively.

* $P < 0.01$ vs. control.

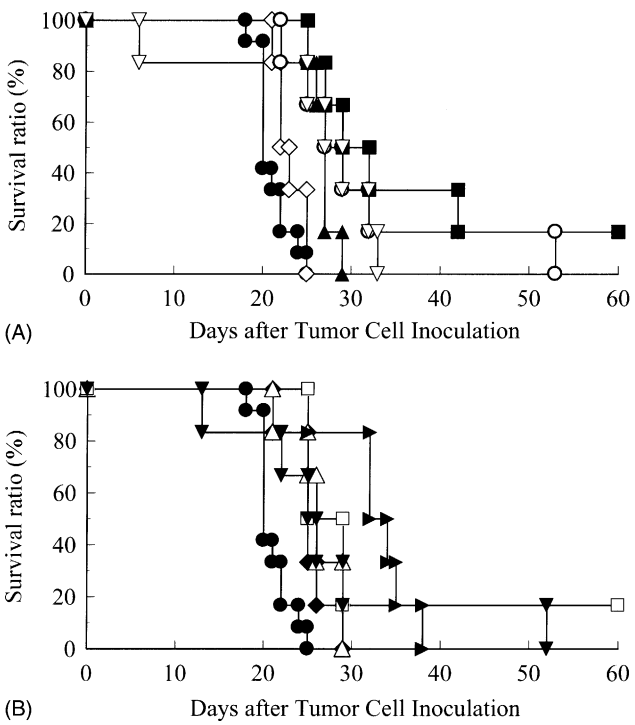

Fig. 2. Prolongation by FK228 of the survival time of *scid* mice inoculated with lymphoma U-937 cells. (A) The mice were treated with FK228 at 0.1 (◇), 0.18 (▲), 0.32 (○), 0.56 (■), and 1 (▽) mg/kg once a week 24 hr after U-937 cell inoculation. (B) The mice were treated with FK228 at 0.1 (◆), 0.18 (Δ), 0.32 (▶), 0.56 (□), and 1 (▼) mg/kg twice a week 24 hr after inoculation with U-937 cells. Control mice were treated with 10% HCO60 saline (●) twice a week. The observation period was for 60 days after tumor cell inoculation. Six and twelve mice were used for FK228-treated and control groups, respectively.

of 60 days. Mice treated with 1 mg/kg of FK228 once or twice a week started to die earlier than control mice, demonstrating toxicity at this higher concentration. Thus, FK228 had antitumor activity on lymphomas as it prolonged the survival time of *scid* mice inoculated with U-937 cells.

3.3. Effect of FK228 on apoptosis of U-937 lymphoma cells

To characterize the antitumor mechanism of FK228 on lymphomas, we examined the effects of FK228 on apop-

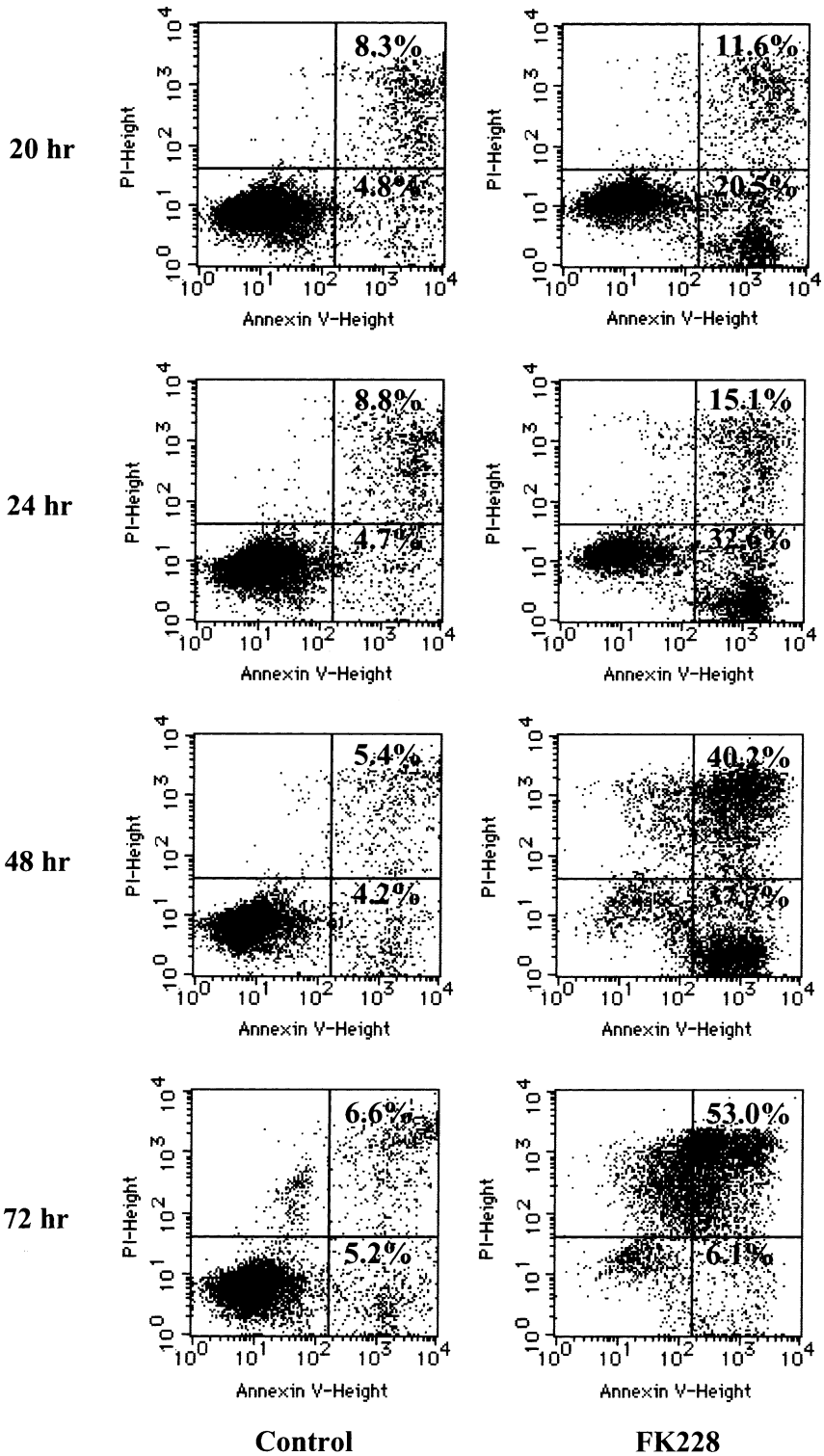

Fig. 3. Induction of apoptosis in U-937 cells treated with FK228. Apoptosis in U-937 cells was detected by the annexin V/PI assay after treatment with or without 5 ng/mL of FK228 for 20, 24, 48, and 72 hr. Cells that were both annexin V-positive and PI-negative were in the early stage of apoptosis, whereas cells that were annexin V-positive and PI-positive were in the late stages of apoptosis or necrosis.

tosis, cell cycle arrest, and differentiation of U-937 cells. In this test, FK228 was used at a concentration of 5 ng/mL, based on the IC_{50} values of the *in vitro* sensitivity tests. Up until 16 hr, there was no difference between FK228-treated and control cells (data not shown). Control cells that were both annexin V-positive and PI-negative corresponded to a group in the early phase of apoptosis and represented 4.8% of the total population at 20 hr; this percentage did not change up to 72 hr (Fig. 3). With FK228 treatment, the proportion of apoptotic cells at 20 hr was 20.5% and this percentage increased in a time-dependent manner up until 48 hr. The annexin V-positive and PI-positive population, which corresponds to cells in the late phases of apoptosis or necrosis, was not changed from 20 to 72 hr in the controls. This FK228-treated cell population increased in a time-dependent manner up until 72 hr at which time it comprised 53.0% of the cells. Thus, the growth inhibitory effect of FK228 on these lymphoma cells may, in part, be through the induction of apoptosis.

3.4. Effect of FK228 on the U-937 cell cycle

To evaluate the effect of FK228 on the U-937 cell cycle, DNA histograms were studied. Up until 10 hr, there was

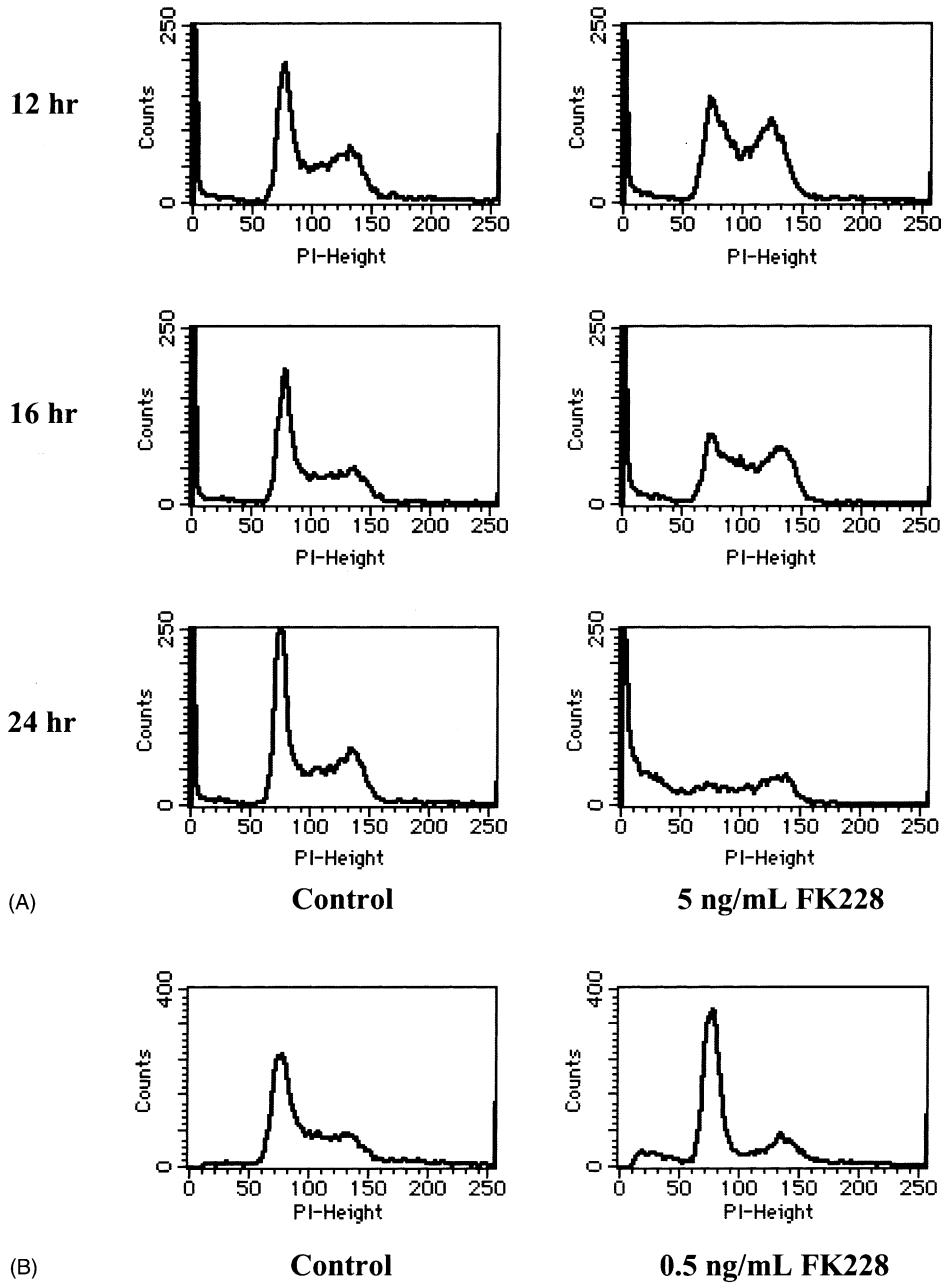

Fig. 4. DNA histogram of U-937 cells treated with FK228. (A) Cells were treated with or without 5 ng/mL of FK228 for 12, 16, and 24 hr. (B) Cells were treated with or without 0.5 ng/mL of FK228 for 48 hr and cultured in drug-free medium for another 24 hr.

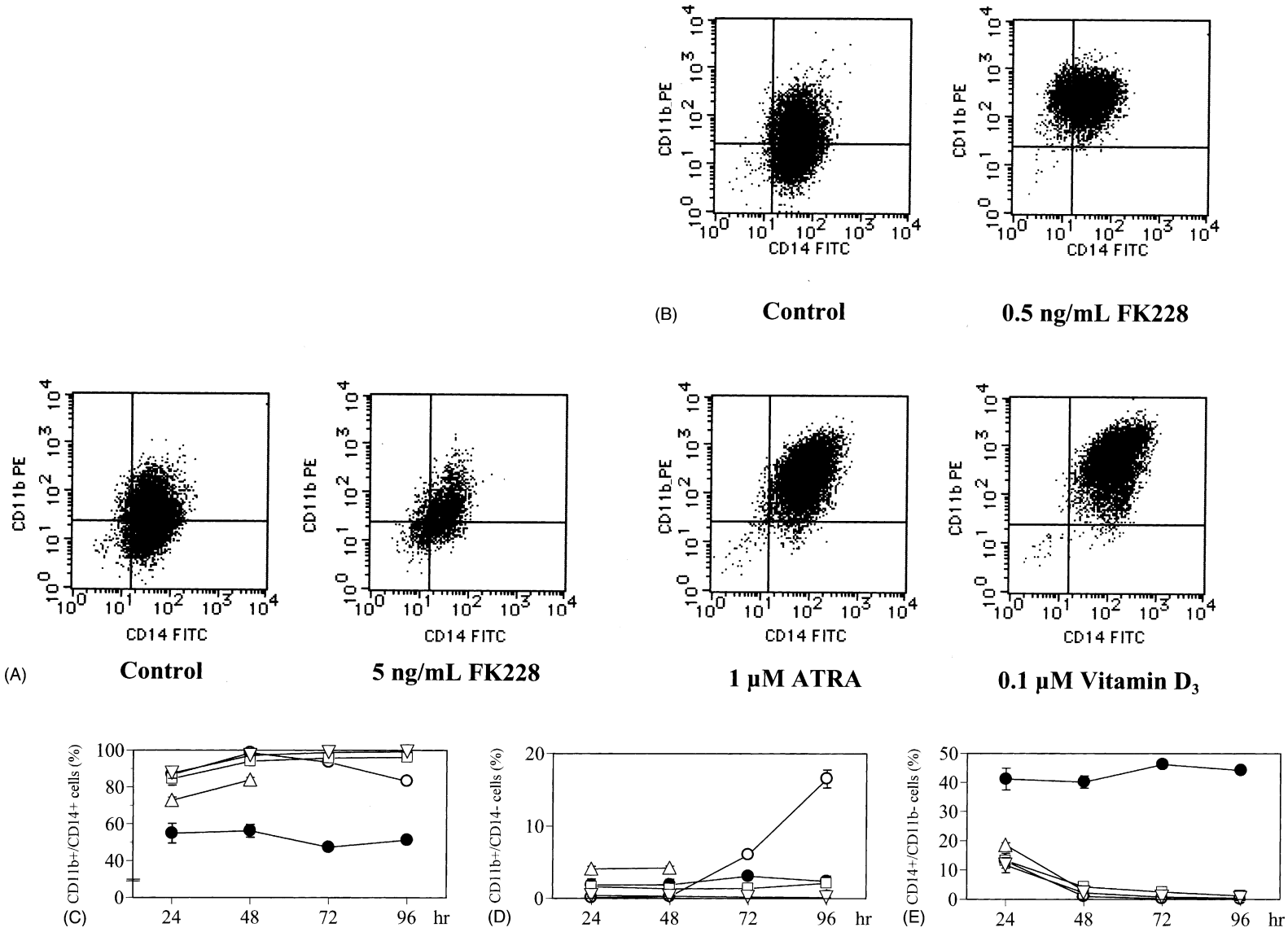

Fig. 5. Expression of CD11b and CD14 antigens in U-937 cells treated with FK228, ATRA, or vitamin D_3. Expression of CD11b and CD14 in U-937 cells was detected by flow cytometry analysis after treatment with or without (A) 5 ng/mL of FK228 for 24 hr, and (B) 0.5 ng/mL of FK228, 1 μM ATRA, or 0.1 μM vitamin D_3 for 96 hr. The percentages of $CD11b^+/CD14^+$ cells (C), $CD11b^+/CD14^-$ cells (D), and $CD14^+/CD11b^-$ cells (E) after treatment with 5 ng/mL of FK228 (Δ) for 24–48 hr, or 0.5 ng/mL of FK228 (○), 1 μM ATRA (□), or 0.1 μM vitamin D_3 (▽) for 24–96 hr are shown as means ± SEM of 3 independent experiments. Control are shown as (●).

no difference between FK228-treated and control cells (data not shown). G2/M arrest was caused by treatment with 5 ng/mL of FK228 for 12 hr (Fig. 4A). Reduction of the G1 fraction started at 12 hr, and the sub-G1 fraction increased at 24 hr in cells treated with 5 ng/mL of FK228. When U-937 cells were treated with 0.5 ng/mL of FK228 for 48 hr and then cultured in drug-free medium for another 24 hr, G1 arrest was observed (Fig. 4B). This result confirmed that we were analyzing the cycle of viable cells since most of the dead cells were removed at the drug-washout step (data not shown). These results indicate that FK228 induced G1 and G2/M cell cycle arrest in U-937 cells.

3.5. Effects of FK228, ATRA, and vitamin D_3 on the differentiation of U-937 cells

To investigate the effect of FK228 on the differentiation of U-937 cells, the expression of CD11b and CD14 was studied. ATRA and vitamin D_3 were used as positive controls at concentrations of 1 and 0.1 μM, respectively. In control cells, the relative proportions of the three populations did not change from 24 to 96 hr (Fig. 5C–E). In the cells treated with 5 ng/mL of FK228, the $CD11b^+/CD14^+$ population increased to 72.8% at 24 hr, but the total number of stained cells was decreased, because 5 ng/mL of FK228-induced apoptosis from 20 hr (Figs. 3 and 5A). To examine the effect of FK228 on differentiation more clearly, U-937 cells were treated with 0.5 ng/mL of FK228. Similarly, the $CD11b^+/CD14^+$ population increased to 86.8% at 24 hr in the cells treated with 0.5 ng/mL of FK228 (Fig. 5C). ATRA at 1 μM and vitamin D_3 at 0.1 μM also induced the differentiation of U-937 cells (to $CD11b^+/CD14^+$-expressing cells) from 24 hr, with the $CD11b^+/CD14^+$-expressing population being 84.6 and 87.6%, respectively, of the total. Interestingly, after 96 hr of treatment with 0.5 ng/mL of FK228, the $CD11b^+/CD14^+$ cell population was reduced while the $CD11b^+/CD14^-$ population was increased (Fig. 5B–D). In contrast, ATRA and vitamin D_3 only induced an increase in cells expressing $CD11b^+/CD14^+$ (Fig. 5B and C). The viability of cells treated with 0.5 ng/mL of FK228, 1 μM ATRA, or 0.1 μM vitamin D_3 was the same as control cells from 24 to 96 hr (data not shown). However, these agents caused growth inhibition starting at 72 hr; by 96 hr inhibition reached 62.1, 85.1, and 54.6%, respectively, of the total cell population (data not shown). Thus, FK228 induced the differentiation of U-937 cells, which may be related to its growth inhibitory effect.

3.6. Changes in the expression of $p21^{WAF1/Cip1}$ and gelsolin mRNA by FK228

We examined the effect of FK228 on the expression of $p21^{WAF1/Cip1}$ and gelsolin mRNA in U-937 cells. To measure the exact level of $p21^{WAF1/Cip1}$ and gelsolin gene expression, we used the real-time q-PCR method. FK228 induced the expression of $p21^{WAF1/Cip1}$ mRNA 9-fold at 1 hr and time-dependently increased it by 654-fold at 24 hr (Fig. 6A). Similarly, FK228 increased the expression of gelsolin mRNA 2-fold at 1 hr and increased it by 152-fold at 24 hr (Fig. 6B). The onset of $p21^{WAF1/Cip1}$ and gelsolin gene expression preceded the onset of apoptosis, cell cycle arrest, and differentiation.

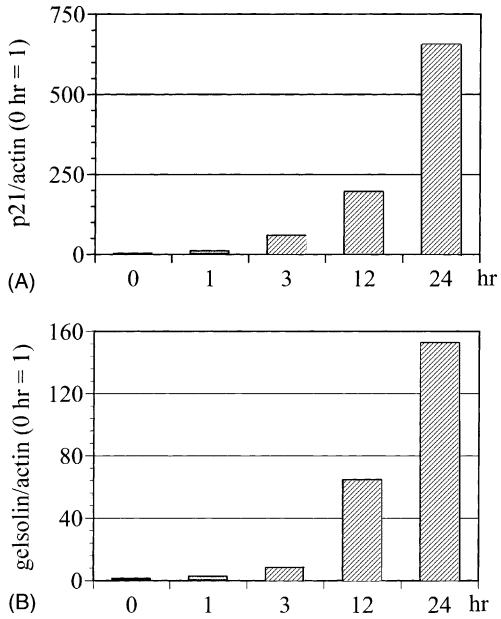

Fig. 6. Changes in the expression of $p21^{WAF1/Cip1}$ and gelsolin mRNA in U-937 cells induced by FK228. The quantitative changes in the expression of $p21^{WAF1/Cip1}$ (A) and gelsolin (B) mRNA by U-937 cells were detected by real-time q-PCR after treatment with or without 5 ng/mL of FK228 for 1–24 hr. Each experiment was repeated at least three times, and typical results are shown.

3.7. FK228 acetylation of histone associated with the $p21^{WAF1/Cip1}$ gene promoter

To confirm the hypothesis that the modulation of gene expression by FK228 was induced by the acetylation of histone associated with target gene promoter domains, acetylation of histone H3 and H4 associated with the $p21^{WAF1/Cip1}$ promoter was examined by the ChIP assay. U-937 cells were treated with or without 5 ng/mL of FK228 for 2 hr, and the chromatin extracts were immunoprecipitated with antibodies to acetylated histone H3 or H4. The isolated DNA was amplified by using primers to the $p21^{WAF1/Cip1}$ promoter and exon 2 regions, as shown in Fig. 7A. After treatment with FK228, the acetylation of histone H3, specific for the P1 and P2 region of the $p21^{WAF1/Cip1}$ promoter, was increased (Fig. 7B). The acetylation of histone H4 by FK228 was observed in all regions of the $p21^{WAF1/Cip1}$ promoter and exon 2 (Fig. 7B). These findings suggest that FK228 causes histone acetylation of the $p21^{WAF1/Cip1}$ promoter regions and that it contributes to the modulation of gene expression.

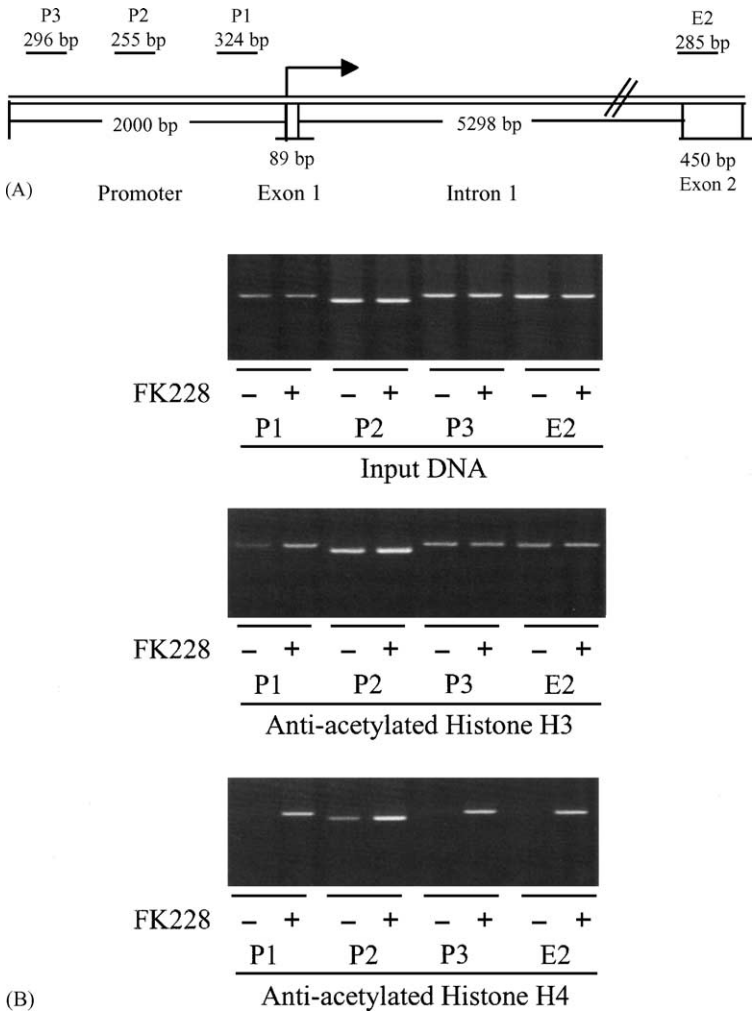

Fig. 7. FK228 induction of the acetylation of the histone associated with the *p21*$^{WAF1/Cip1}$ gene promoter. (A) Position of the primers in the *p21*$^{WAF1/Cip1}$ gene. (B) Treatment of U-937 cells with or without 5 ng/mL of FK228 for 2 hr and immunoprecipitation of chromatin extracts with input DNA or antibodies to acetylated histone H3 or H4.

4. Discussion

In this study, we examined the *in vitro* and *in vivo* effects and the mode of action of FK228 on human lymphoma-derived U-937 cells and on lymphomas raised in *scid* mice. FK228 strongly inhibited the growth of U-937 cells *in vitro* and prolonged the survival time of *scid* mice inoculated with these cells (Tables 1 and 2 and Fig. 2). Our results demonstrated that FK228 has significant curative activity in this model. This is the first report demonstrating that an HDAC inhibitor alone can prolong the survival time of *scid* mice in a lymphoma model. Recent Phase I trials showed that FK228 has therapeutic efficacy in the treatment of patients with PTCL or CTCL [23]. These results suggest that FK228 may also be effective in prolonging the survival time of patients with lymphomas as well as leukemias.

As shown in Fig. 3, 5 ng/mL of FK228-induced apoptosis of U-937 cells at 20 hr. In the present study, FK228 increased the expression of p21$^{WAF1/Cip1}$ and gelsolin mRNA at 1 hr post-treatment. Additionally, we showed that the onset of *p21*$^{WAF1/Cip1}$ and gelsolin gene expression preceded the onset of apoptosis, cell cycle arrest, and differentiation of U-937 cells (Fig. 6). HDAC inhibitors have been demonstrated to induce apoptosis in certain cell lines through a process that requires protein synthesis and leads to the induction of caspase-3 protease activity [20]. One FK228-induced gene that has been linked to apoptosis is gelsolin, a substrate for caspase-3 [20]. The mechanism of the FK228-induced apoptosis of U-937 cells has not been characterized fully but may involve the modulation of gene expression by FK228.

Continuous exposure to 5 ng/mL of FK228 for 12 hr caused G2/M arrest, while cells exposed to 0.5 ng/mL of FK228 for 48 hr and then cultured in drug-free medium for another 24 hr were arrested in G1 phase (Fig. 4). FK228 induced the expression of p21$^{WAF1/Cip1}$ mRNA (Fig. 6A). There is a report that p21$^{WAF1/Cip1}$ has effects on both G1 and G2/M cell cycle arrest [33]. Similar to our results for FK228, suberoylanilide hydroxamic acid (SAHA) induced

both G1 and G2/M cell cycle arrest [34]. The suggested mechanism was that SAHA mediated cell cycle arrest by inducing $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, and by hypophosphorylating retinoblastoma protein [34]. It has also been reported that other HDAC inhibitors (TSA, butyrate, and MS-27-275) induce the expression of $p21^{WAF1/Cip1}$, which may contribute to cell cycle arrest [16,19,22]. The mechanism of U-937 cell cycle arrest by FK228 has not been characterized clearly, but induction of $p21^{WAF1/Cip1}$ mRNA by FK228 may be involved. Furthermore, $p21^{WAF1/Cip1}$ has also been observed to be involved in cell differentiation and senescence [35–37], and may play a role in FK228-mediated reversal of transformed cell phenotypes [1].

FK228 at 5 and 0.5 ng/mL induced the differentiation of U-937 cells, to $CD11b^+/CD14^+$-expressing cells, after 24 hr (Fig. 5A and C). Interestingly, after treatment with 0.5 ng/mL of FK228 for 96 hr, the number of $CD11b^+/CD14^+$ cells was reduced while $CD11b^+/CD14^-$ cell numbers increased (Fig. 5B–D). In contrast, ATRA and vitamin D_3 only induced an increase of the $CD11b^+/CD14^+$ cell population (Fig. 5B and C). CD11b (β_2-integrin) is a granulocyte/monocyte differentiation marker, and CD14 is a monocyte-associated antigen. $CD11b^+/CD14^+$ cells and $CD11b^+/CD14^-$ cells correspond to monocytes and granulocytes, respectively [38–40]. The present results for ATRA and vitamin D_3 are consistent with a previous report, in which ATRA and vitamin D_3 induced distinct monocytic differentiation pathways in U-937 cells [41]. Differentiation induced by ATRA resulted in increased retinoic acid receptor-β (RARβ)/retinoid X receptor (RXR) DNA binding, the activation of retinoic acid responsive element (RARE)-dependent transcription, and the increased expression of RARβ by vitamin D_3. This caused the formation of vitamin D_3 receptor/RXR DNA-binding complexes on both vitamin D_3 responsive elements and RAREs [41]. Our results demonstrate that FK228 may not only mediate the differentiation of U-937 cells to monocytes but also to granulocytes. FK228 induced the expression of gelsolin mRNA (Fig. 6B). It has been reported that HDAC inhibitors, such as TSA and MS-27-275, induce the expression of gelsolin, which may contribute to differentiation [20,21]. The mechanism of FK228-induced U-937 cell differentiation has not been characterized, but the induction of gelsolin mRNA by FK228 may have a role. It has been suggested that the differentiation of tumor cells brings with it some antitumor effects, such as the terminal differentiation of primary blast cells, growth inhibition, and the up-regulation of cytotoxic T lymphocyte activity by the expression of surface makers on tumor cells [42–44]. FK228 may exert such favorable effects by inducing differentiation.

In the ChIP assay, treatment of U-937 cells with 5 ng/mL of FK228 for 2 hr specifically caused the acetylation of histone H3 in the P1 and P2 regions of the $p21^{WAF1/Cip1}$ promoter and the acetylation of histone H4 in all regions of the $p21^{WAF1/Cip1}$ promoter and exon 2 (Fig. 7B). The P1 region includes the Sp1-binding sites, also called the GC-box, which are important for the expression of the human $p21^{WAF1/Cip1}$ gene induced by HDAC inhibitors, such as TSA, butyrate, and SAHA [45–47]. Transcription modulators of Sp1, Sp3, and ZBP-89/BFCOL1 bind to the Sp1-binding sites of the $p21^{WAF1/Cip1}$ promoter and play an important role in $p21^{WAF1/Cip1}$ gene expression induced by HDAC inhibitors [48–52]. These results suggest that FK228 causes acetylation of histone H3 and H4 in Sp1-binding sites, which induces the expression of $p21^{WAF1/Cip1}$. It has been reported that an HDAC inhibitor, trapoxin (TPX), increased the acetylation of histone H3 in both the −179 to +44 and −345 to +44 regions of the $p21^{WAF1/Cip1}$ promoter, which overlap with the P1 region, but did not cause the acetylation of histone H4 in these regions in MDA-MB-435 cells, a human ductal breast carcinoma cell line [53]. SAHA caused the acetylation of histone H3 and H4 specifically in the P2 and P3 regions, but the acetylation of histone H4 in the P1 region was increased only 1.1-fold in T24 cells, a human bladder carcinoma cell line [22]. It is surprising that the acetylation pattern is different for FK228, TPX, and SAHA treatments, which may be due to differences in the cell types used or inhibition patterns of HDAC subtypes (HDAC 1–9) [54]. In any case, our results strongly suggest that the acetylation of histone H3 or H4 in the $p21^{WAF1/Cip1}$ promoter region by HDAC inhibitors induces the expression of the $p21^{WAF1/Cip1}$ gene.

Most patients with malignant lymphoma have the non-Hodgkin's type and usually receive treatment with a doxorubicin-containing regimen, such as cyclophosphamide, doxorubicin, vincristine, and prednisone [55,56]. However, the cure rate by chemotherapy alone is only approximately 50% [56]. Although new drugs for malignant lymphoma such as interferon-α have been developed and used in combination with conventional chemotherapy regimens, their efficacy has not been established [57–59]. HDAC inhibitors show promise as potential candidate drugs for use in the treatment of lymphomas.

HDAC inhibitors are also expected to have a beneficial effect on APL [15–18]. There are characteristic chromosomal translocations in APL that code for transforming fusion proteins capable of recruiting HDAC. APL cells with PLZF/RARα, an abnormal retinoic acid receptor formed by protein fusion, respond poorly to treatment with ATRA [10–14]. ATRA treatment in combination with HDAC inhibitors can overcome ATRA resistance by inducing APL cell apoptosis, cycle arrest, and differentiation [15–18]. The apoptotic effect induced by HDAC inhibitors can prevent APL cells from returning to the malignant state.

In conclusion, (i) FK228 can prolong the survival time of *scid* mice in a lymphoma model, and (ii) the beneficial effect of FK228 on human lymphoma may be brought on through the induction of apoptosis, cell cycle arrest, and differentiation driven by the modulation of gene expression by histone acetylation.

References

[1] Ueda H, Nakajima H, Hori Y, Fujita T, Nishimura M, Goto T, Okuhara M. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chomobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J Antibiot (Tokyo) 1994;47:301–10.

[2] Ueda H, Manda T, Matsumoto S, Mukumoto S, Nishigaki F, Kawamura I, Shimomura K. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chomobacterium violaceum* No. 968. III. Antitumor activities on experimental tumors in mice. J Antibiot (Tokyo) 1994;47:315–23.

[3] Nakajima H, Kim YB, Terano H, Yoshida M, Horinouchi S. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res 1998;241:126–33.

[4] Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349–52.

[5] Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996;87:953–9.

[6] Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. Nature 1996;384:641–3.

[7] Lee DY, Hayes JJ, Pruss D, Wolffe AP. A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell 1993;72:73–84.

[8] Kingston RE, Bunker CA, Imbalzano AN. Repression and activation by multiprotein complexes that alter chromatin structure. Genes Dev 1996;10:905–20.

[9] Ura K, Kurumizaka H, Dimitrov S, Almouzni G, Wolffe AP. Histone acetylation: influence on transcription, nucleosome mobility and positioning, and linker histone-dependent transcriptional repression. EMBO J 1997;16:2096–107.

[10] Guidez F, Huang W, Tong J-H, Dubois C, Balitrand N, Waxman S, Michaux JL, Martiat P, Degos L, Chen Z, Chomienne C. Poor response to all-*trans* retinoic acid therapy in a t(11;17) PLZF/RARα patient. Leukemia 1994;8:312–7.

[11] David G, Alland L, Hong SH, Wong CW, DePinho RA, Dejean A. Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene 1998;16:2549–56.

[12] Lin RJ, Nagy L, Inoue S, Shao W, Miller Jr WH, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature 1998;391:811–4.

[13] Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, Fanelli M, Ruthardt M, Ferrara FF, Zamir I, Seiser C, Grignani F, Lazar MA, Minucci S, Pelicci PG. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature 1998;391:815–8.

[14] He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A, Pandolfi PP. Distinct interactions of PML–RARα and PLZF-RARα with co-repressors determine differential responses to RA in APL. Nat Genet 1998;18:126–35.

[15] Kitamura K, Hoshi S, Koike M, Kiyoi H, Saito H, Naoe T. Histone deacetylase inhibitor but not arsenic trioxide differentiates acute promyelocytic leukaemia cells with t(11;17) in combination with all-*trans* retinoic acid. Br J Haematol 2000;108:696–702.

[16] Kosugi H, Towatari M, Hatano S, Kitamura K, Kiyoi H, Kinoshita T, Tanimoto M, Murate T, Kawashima K, Saito H, Naoe T. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. Leukemia 1999;13:1316–24.

[17] Calabresse C, Venturini L, Ronco G, Villa P, Chomienne C, Belpomme D. Butyric acid and its monosaccharide ester induce apoptosis in the HL-60 cell line. Biochem Biophys Res Commun 1993;195:31–8.

[18] Yen A, Brown D, Fishbaugh J. Control of HL-60 monocytic differentiation. Different pathways and uncoupled expression of differentiation markers. Exp Cell Res 1987;168:247–54.

[19] Higgins PJ, Ryan MP. p52(PAI-1) and actin expression in butyrate-induced flat revertants of v-*ras*-transformed rat kidney cells. Biochem J 1991;279:883–90.

[20] Hoshikawa Y, Kwon HJ, Yoshida M, Horinouchi S, Beppu T. Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. Exp Cell Res 1994;214:189–97.

[21] Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T, Nakanishi O. A synthetic inhibitor of histone deacetylase, MS-27-275, with marked *in vivo* antitumor activity against human tumors. Proc Natl Acad Sci USA 1999;96:4592–7.

[22] Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 2000;97:10014–9.

[23] Piekarz RL, Robey R, Sandor V, Bakke S, Wilson WH, Dahmoush L, Kingma DM, Turner ML, Altemus R, Bates SE. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. Blood 2001;98:2865–8.

[24] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[25] Paine-Murrieta GD, Taylor CW, Curtis RA, Lopez MH, Dorr RT, Johnson CS, Funk CY, Thompson F, Hersh EM. Human tumor models in the severe combined immune deficient (*scid*) mouse. Cancer Chemother Pharmacol 1997;40:209–14.

[26] Honma Y, Matsuo Y, Hayashi Y, Omura S. Treatment of Philadelphia-chromosome-positive human leukemia in SCID mouse model with herbimycin A, bcr-abl tyrosine kinase activity inhibitor. Int J Cancer 1995;60:685–8.

[27] Kawata A, Yoshida M, Okazaki M, Yokota S, Barcos M, Seon BK. Establishment of new SCID and nude mouse models of human B leukemia/lymphoma and effective therapy of the tumors with immunotoxin and monoclonal antibody: marked difference between the SCID and nude mouse models in the antitumor efficacy of monoclonal antibody. Cancer Res 1994;54:2688–94.

[28] Ueda H, Nakajima H, Hori Y, Goto T, Okuhara M. Action of FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968, on Ha-*ras* transformed NIH3T3 cells. Biosci Biotechnol Biochem 1994;58:1579–83.

[29] Rajgolikar G, Chan KK, Wang HC. Effects of a novel antitumor depsipeptide, FR901228, on human breast cancer cells. Breast Cancer Res Treat 1998;51:29–38.

[30] Dimberg A, Nilsson K, Oberg F. Phosphorylation-deficient Stat1 inhibits retinoic acid-induced differentiation and cell cycle arrest in U-937 monoblasts. Blood 2000;96:2870–8.

[31] James SY, Williams MA, Kelsey SM, Newland AC, Colston KW. The role of vitamin D derivatives and retinoids in the differentiation of human leukaemia cells. Biochem Pharmacol 1997;54:625–34.

[32] Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, Nakanishi S. Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. J Biol Chem 1993;268:2836–43.

[33] Niculescu III AB, Chen X, Smeets M, Hengst L, Prives C, Reed SI. Effects of $p21^{Cip1/Waf1}$ at both the G_1/S and the G_2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 1998;18:629–43.

[34] Huang L, Pardee AB. Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. Mol Med 2000;6:849–66.

[35] Futamura M, Monden Y, Okabe T, Fujita-Yoshigaki J, Yokoyama S, Nishimura S. Trichostatin A inhibits both *ras*-induced neurite

outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. Oncogene 1995;10:1119–23.

[36] Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledge SJ. p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science 1995;267: 1024–7.

[37] Zhang W, Grasso L, McClain CD, Gambel AM, Cha Y, Travali S, Deisseroth AB, Mercer WE. p53-independent induction of WAF1/ CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. Cancer Res 1995;55:668–74.

[38] Carlos TM, Harlan JM. Membrane proteins involved in phagocyte adherence to endothelium. Immunol Rev 1990;114:5–28.

[39] Perussia B, Lebman D, Ip SH, Rovera G, Trinchieri G. Terminal differentiation surface antigens of myelomonocytic cells are expressed in human promyelocytic leukemia cells (HL60) treated with chemical inducers. Blood 1981;58:836–43.

[40] Dimitriu-Bona A, Burmester GR, Waters SJ, Winchester RJ. Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 1983;130:145–52.

[41] Botling J, Öberg F, Törmä H, Tuohimaa P, Bläuer M, Nilsson K. Vitamin D3- and retinoic acid-induced monocytic differentiation: interactions between the endogenous vitamin D3 receptor, retinoic acid receptors, and retinoid X receptors in U-937 cells. Cell Growth Differ 1996;7:1239–49.

[42] Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, Mancini M, Pelicci PG, Lo Coco F, Nervi C. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res 2001;61:2–7.

[43] Witt O, Sand K, Pekrun A. Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathways. Blood 2000;95:2391–6.

[44] Passmore JS, Lukey PT, Ress SR. The human macrophage cell line U937 as an *in vitro* model for selective evaluation of mycobacterial antigen-specific cytotoxic T-cell function. Immunology 2001;102: 146–56.

[45] Sowa Y, Orita T, Minamikawa S, Nakano K, Mizuno T, Nomura H, Sakai T. Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. Biochem Biophys Res Commun 1997;241:142–50.

[46] Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, Fujita T, Ohtani-Fujita N, Matsukawa Y, Tokino T, Yamagishi H, Oka T, Nomura H, Sakai T. Butyrate activates the *WAF1/Cip1* gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 1997;272:22199–206.

[47] Huang L, Sowa Y, Sakai T, 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 2000;19:5712–9.

[48] Bai L, Merchant JL. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of $p21^{waf1}$ transcription in human cells. J Biol Chem 2000;275:30725–33.

[49] Doetzlhofer A, Rotheneder H, Lagger G, Koranda M, Kurtev V, Brosch G, Wintersberger E, Seiser C. Histone deacetylase 1 can repress transcription by binding to Sp1. Mol Cell Biol 1999;19: 5504–11.

[50] Xiao H, Hasegawa T, Isobe K. p300 collaborates with Sp1 and Sp3 in $p21^{waf1/cip1}$ promoter activation induced by histone deacetylase inhibitor. J Biol Chem 2000;275:1371–6.

[51] Sowa Y, Orita T, Minamikawa-Hiranabe S, Mizuno T, Nomura H, Sakai T. Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. Cancer Res 1999;59:4266–70.

[52] Hasegawa T, Xiao H, Isobe K. Cloning of a GADD34-like gene that interacts with the zinc-finger transcription factor which binds to the $p21^{WAF}$ promoter. Biochem Biophys Res Commun 1999;256:249–54.

[53] Sambucetti LC, Fischer DD, Zabludoff S, Kwon PO, Chamberlin H, Trogani N, Xu H, Cohen D. Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. J Biol Chem 1999;274:34940–7.

[54] Zhou X, Marks PA, Rifkind RA, Richon VM. Cloning and characterization of a histone deacetylase, HDAC9. Proc Natl Acad Sci USA 2001;98:10572–7.

[55] Miller TP, Dahlberg S, Cassady JR, Adelstein DJ, Spier CM, Grogan TM, LeBlanc M, Carlin S, Chase E, Fisher RI. Chemotherapy alone compared with chemotherapy plus radiotherapy for localized intermediate- and high-grade non-Hodgkin's lymphoma. N Engl J Med 1998;339:21–6.

[56] Fisher RI, Gaynor ER, Dahlberg S, Oken MM, Grogan TM, Mize EM, Glick JH, Coltman Jr CA, Miller TP. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med 1993;328:1002–6.

[57] Smalley RV, Andersen JW, Hawkins MJ, Bhide V, O'Connell MJ, Oken MM, Borden EC. Interferon alfa combined with cytotoxic chemotherapy for patients with non-Hodgkin's lymphoma. N Engl J Med 1992;327:1336–41.

[58] Solal-Celigny P, Lepage E, Brousse N, Reyes F, Haioun C, Leporrier M, Peuchmaur M, Bosly A, Parlier Y, Brice P, Coiffier B. Gisselbrecht C for the groupe d'etude des lymphomes de l'Adulte. Recombinant interferon alfa-2b combined with a regimen containing doxorubicin in patients with advanced follicular lymphoma. N Engl J Med 1993; 329:1608–14.

[59] Fisher RI, Dana BW, LeBlanc M, Kjeldsberg C, Forman JD, Unger JM, Balcerzak SP, Gaynor ER, Roy V, Miller T. Interferon alfa consolidation after intensive chemotherapy does not prolong the progression-free survival of patients with low-grade non-Hodgkin's lymphoma: results of the Southwest Oncology Group Randomized Phase III Study 8809. J Clin Oncol 2000;18:2010–6.