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## Histone deacetylase inhibitor, FK228, induces apoptosis and suppresses cell proliferation of human glioblastoma cells in vitro and in vivo

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**Abstract** We investigated the effects of FK228 on cell proliferation and apoptosis against human glioblastoma (GM) T98G, U251MG, and U87MG cells. Upon exposure to FK228, cell proliferation was inhibited, and apoptosis detected by the cleavage of CPP32 was induced. FK228 increased the expression levels of p21 (WAF-1) and of pro-apoptotic Bad protein in all GM cells. Furthermore, FK228 treatment also reduced the anti-apoptotic protein Bcl-xL in all GM cells and anti-apoptotic Bcl-2 in U87MG cells, thereby shifting the cellular equilibrium from life to death. An increased accumulation of histone H4 was detected in the p21 (WAF-1) promoter and the structural gene (exon 2) and the Bad structural gene (exon 2 and 3) upon treatment with FK228, as assessed by chromatin immunoprecipitation (ChIP) assay. Thus, the results indicated that an increased expression of p21 (WAF1) and Bad due to FK228 is regulated, at least in part, by the degree of acetylation of the gene-associated histone. We also found that FK228 inhibits cellular invasiveness and decreases MMP-2 activity. In addition, the growth of transplanted human GM m-3 cells into the subcutaneous tissue of hereditary athymic mice was significantly inhibited, and apoptosis was induced with FK228 treatment. The results suggested that FK228 might be useful in the treatment of human GM, although further studies will be needed.

**Keywords** Histone deacetylase inhibitor · FK228 · Glioblastoma · Apoptosis · MMP

### Introduction

Histone deacetylase (HDAC) and histone acetyltransferase (HAT) are enzymes that influence transcription by selectively deacetylating or acetylating the  $\epsilon$ -amino groups of lysines located near the amino-termini of core histone proteins [8, 15]. Various agents, such as sodium butyrate (SB) or trichostatin A (TSA) inhibit HDAC activities. We previously showed that SB treatment inhibited the G1-S transition associated with increased expression of p21 (WAF-1) and reduced the phosphorylation of the retinoblastoma gene (Rb) [5]. Activation of cyclin-dependent kinase (CDK) 4/6 and CDK2 by coupling with timely expressed cyclin D and cyclin E, respectively, is required to phosphorylate pRb and to advance the cell cycle through the G1 to S phase. P21 (WAF-1) forms complexes with cyclin D and CDK 4 or 6 as well as with cyclin E and CDK 2, thereby reducing CDK activities that lead to inhibition of the G1-S transition. In addition, we showed that either SB or TSA induced apoptotic cell death of human glioma cells through an increased expression of the bcl-2-related protein Bad [22]. A new cyclic depsipeptide, FK228 (formerly designated FK901228), exhibited in vitro cytotoxicity in various cancer cell lines [18, 28] and malignant lymphoid cells [12]. An in vivo study using SCID mouse also showed the antitumor effect of FK228 in human acute promyelocytic leukemia [7]. In the present study, we determined whether FK228 suppressed cell proliferation and whether it induced apoptotic cell death in human glioblastoma (GM) cells in vitro and in vivo. We found that FK228 suppressed cell proliferation and induced apoptotic cell death in human GM cells at nanomolar levels. Furthermore, we showed that FK228 induced the accumulation of acetylated histone in chromatin associated with the p21 (WAF-1) and Bad genes. The results suggested that the induction of p21 (WAF1) and Bad by FK228 might be regulated by acetylation of the gene-associated histone. In animal models using transplantable human GM m-3 cells, FK228 significantly inhibited tumor growth in the subcutaneous tissues of hereditary athymic mice. The results suggested that FK228 might be a promising approach for GM therapy.

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## Materials and methods

FK228 (MW 540.71) was kindly provided by Fujisawa Pharmaceutical. FK228 was dissolved in 100% dimethylsulfoamide (DMSO) and stored at  $-80^{\circ}\text{C}$  as a stock solution until use. The primary antibodies used were as follows: anti-acetylated histone H4 (1:1,000 dilution) (rabbit polyclonal, Chemicon, CA, USA), anti-p21 (WAF-1) antibody (1:1,000 dilution) (mouse monoclonal, Ab-1, Calbiochem, MA, USA), anti-Bcl-2 antibody (1:500 dilution) (mouse monoclonal, Dako, Glostrup, Denmark), anti-Bax antibody (1:3,000 dilution) (rabbit polyclonal, N-20, Santa Cruz, CA), anti-Bcl-xL (1:4,000 dilution) (rabbit polyclonal, Transduction Laboratories, KY, USA), anti-Bad antibody (1:4,000 dilution) (mouse monoclonal, Transduction Laboratories), anti-Fas antibody (1:4,000 dilution) (mouse monoclonal, Transduction Laboratories), and anti-CPP32 antibody (1:4,000 dilution) (mouse monoclonal, Transduction Laboratories). Anti-BrdU antibody (Br-3, mouse monoclonal) was purchased from Caltag, CA, USA.

The human GM cells (T98G, U251MG, and U87MG) were obtained from the American Type Culture Collection (ATCC) and maintained in Eagle's MEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The p53 of T98G and U251MG is mutant-type; U87MG is wild-type. Morphological changes with FK228 were observed and photographed under the microscope (Olympus, Tokyo, Japan). To detect nuclear fragmentation, the cells were cultured in Labtec chamber slides (Nunc, Roskilde, Denmark) and treated with FK228 (1 ng/ml) for 24 h. The cells were fixed with 3.7% paraformaldehyde solution and stained with Hoechst 33258 for 10 min at room temperature. The cells were observed and photographed under fluorescent microscopy (Carl Zeiss, Oberkochen, Germany).

### Proliferation assay

Cell proliferation was evaluated with the alamarBlue assay (BioSource International, CA, USA). The alamarBlue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system uses an oxidation-reduction (redox) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. In brief, 1000 cells in 90  $\mu\text{l}$  of the culture media were plated in each well of a 96-well plate (Nunc) and cultured in the presence or absence of FK228. Then 10  $\mu\text{l}$  of alamarBlue solution was added to each well at the indicated time. The plate was incubated for 4 h in a humidified atmosphere containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Absorbance was monitored at the wavelengths of 570 nm and 600 nm with a Corona microplate reader MTP-100 (Corona, Tokyo, Japan).

### Immunoblot analysis

Equal amounts of proteins (5  $\mu\text{g}/\text{lane}$ ) were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to the PVDF membranes. The membranes were incubated for 1 h with 1% fat-free milk solution in PBS to quench the nonspecific bindings and were reacted for 1 h at room temperature with the specific antibodies against the apoptosis-related proteins described above. The membranes were washed three times with 50 mM Tris HCl, 150 mM NaCl (pH 7.6) (TBS) and were reacted for 1 h at room temperature with the horseradish peroxidase (HRP)-conjugated secondary antibodies. The HRP reaction was performed with the enhanced chemiluminescence system (Amersham, Buckinghamshire, England) and was visualized with a Lumino image analyzer LAS-1000 (Fuji Film, Tokyo, Japan). The cleavages of CPP32 were also studied with immunoblotting, because CPP32 degrades two subunits consisting of approximately 20 kDa and 10 kDa molecular weight during apoptosis.

### Chromatin immunoprecipitation assay

U251MG cells were cultured in the presence or absence of FK228 (1 ng/ml) for 2 or 16 h. Formaldehyde was then added to the cells to a final concentration of 1%, and the cells were incubated at  $37^{\circ}\text{C}$  for 10 min. The medium was removed, and the cells were suspended in 1 ml of ice-cold PBS containing protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany). Cells were pelleted, resuspended in 0.5 ml of SDS lysis buffer (1% SDS, 2 mM EDTA, 50 mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. Lysates were sonicated with  $15 \times 10\text{-s}$  bursts. Debris was removed from samples by centrifugation for 10 min at  $15,000 \times g$  at  $4^{\circ}\text{C}$ . Supernatants were diluted 5-fold in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl), and 80  $\mu\text{l}$  of a 50% protein A Sepharose slurry containing 20  $\mu\text{g}/\text{ml}$  sonicated salmon sperm DNA and 1 mg/ml BSA in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) were added, and the solutions incubated, rocking for 30 min at  $4^{\circ}\text{C}$ . Beads were pelleted by centrifugation, and supernatants were placed in fresh tubes with 5  $\mu\text{g}$  of anti-acetylated histone H4 antibody and incubated overnight at  $4^{\circ}\text{C}$ . Protein A Sepharose slurry (60  $\mu\text{l}$ ) was added, and samples were rocked for 1 h at  $4^{\circ}\text{C}$ . Protein A complexes were centrifuged and washed 5 times for 5 min each. Immune complexes were eluted twice with 250  $\mu\text{l}$  of elution buffer (1% SDS, 0.1 M  $\text{NaHCO}_3$ ) for 15 min at room temperature. Then 20  $\mu\text{l}$  of 5 M NaCl were added to the combined elutes, and the samples were incubated at  $65^{\circ}\text{C}$  for 4 h. EDTA, Tris-HCl, pH 6.5, and proteinase K were then added to the samples at a final concentration of 10 mM, 40 mM, and 0.04  $\mu\text{g}/\mu\text{l}$ , respectively, and the samples were incubated at  $45^{\circ}\text{C}$  for 1 h. Immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. Actin, p21 (WAF-1) or Bad-specific primers were used to carry out PCR from DNA isolated from ChIP experiments. The optimal reaction conditions for PCR were determined for each primer pair. PCR was performed as follows: denaturation at  $95^{\circ}\text{C}$  for 1 min and annealing at  $60^{\circ}\text{C}$  for 1 min, followed by elongation at  $72^{\circ}\text{C}$  for 1 min. PCR products were analyzed by 2% agarose/ethidium bromide gel electrophoresis. The primer pairs used for p21 (WAF-1) chromatin immunoprecipitation (ChIP) analysis were: 5'-GGTGTCTAGGT-GCTCCAGGT-3' (uP), 5'-GCCTCTCCAGGAGGACACA-3' (dP), 5'-AGCTGAGCCGCGACTGTCTAT-3' (uE), 5'-ATGTTCCAGATCCCAGAGTTTGAG-3' (dE). The primer pairs used for Bad ChIP analysis were: 5'-ATGTTCCAGATCCCAGAGTTTGAG-3' (uE2), 5'-ATGATGGCTGCTGCTGGTTGGCTG-3' (dE2), 5'-GCTGTGGAGATCCGGAGTCGCCAC-3' (uE3), 5'-CATCTCCGGAGCTCGCGGCCATA-3' (dE3). The primers used for actin ChIP analysis were: 5'-GCCAGCTCTCGACTCTGTT-3' and 5'-AGATCGCAACCGCCTGGAAC-3'.

### In vitro invasion assay and analysis of MMP activity

Some 100,000 cells of U251MG (400  $\mu\text{l}$ ) were plated in the upper compartment of a Transwell chamber (Iwaki, Tokyo, Japan) coated with 100  $\mu\text{l}$  of matrigel (0.5 mg/ml) in triplicate and cultured in the absence or presence of FK228 (0.1 or 0.5 ng/ml). Its lower compartment was filled with Eagle's MEM containing 10% fetal bovine serum. After 48 h, the cells in the compartment that had moved through 12  $\mu\text{m}$  pores in the well-polycarbonate membrane were stained with 0.4% trypan blue. The number of stained cells in 10 fields was counted under light microscopy (original magnification  $\times 200$ ). Analysis of MMP-2 and MMP-9 activity was performed using SDS-PAGE gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v) as described [14]. Cells were grown in 100- $\text{cm}^2$  tissue-culture plates in Eagle's MEM containing 10% FBS until they reached sub-confluence, washed with serum-free Eagle's MEM, and replaced with 10 ml of Eagle's MEM. The conditioned media were collected after 48 h and centrifuged to remove cellular debris. Equal amounts (10  $\mu\text{g}/\text{lane}$ ) of protein were mixed with Laemmli sample buffer (minus reductant) prior to electrophoresis. Gels were run at a constant current, washed twice for 30 min in 10 mM Tris-HCl (pH 8.0) and 2.5% Triton X-100, and then

washed for 30 min with 10 mM Tris-HCl (pH 8.0). Gels were incubated for 24 h at 37°C in 50 mM Tris-HCl (pH 8.0), 0.5 mM CaCl<sub>2</sub>, 10<sup>-6</sup> M ZnCl<sub>2</sub>, and then the gels were stained with Coomassie Brilliant Blue R-250 for 10 min. The gels were de-stained with a solution of 5% acetic acid and 10% methanol.

#### Heterotransplantation of human glioblastoma m-3 cells into subcutaneous tissue of hereditary athymic nude mice

For inoculation into subcutaneous tissue, 1x10<sup>6</sup> m-3 cells [24] which were produced by the transfection of mutant EGFR to U87MG cells were resuspended in 100 µl of Eagle's MEM and inoculated in the subcutaneous tissue of the flanks of 5-week-old nude mice (female). Tumor volume was determined from the length of the major and minor axes of the tumor. Tumor volume (mg) = (length of the major axis in mm) x (length of the minor axis in mm)<sup>2</sup>/2. At 8 and 12 days after transplantation, the tumors were treated with FK228 (0.5 µg/g body weight) by intraperitoneal or peritumoral injection. As a control, 100 µl of saline containing 0.1% DMSO was injected intraperitoneally into each mouse. The tumor tissues that grew in the flank of nude mice were resected and were kept at -80°C until use. The tissues (approximately 100 mg) were cut with scissors, and 200 µl of TBS containing 1% SDS were added. The tissues were sonicated for 5 min on ice. The samples were heated at 95°C for 5 min and were centrifuged at 12,000xg for 10 min. The supernatant was mixed with 2x sample buffer and was heated at 95°C for 5 min. Immunoblot analysis was done as described above.

#### Microscopical observation and BrdU labeling

The tumor-laden mice treated with FK228 or control saline were injected with 100 µl of 2 mM 5'-bromo-2-deoxyuridine (BrdU) 2 h before killing. The tumor tissues were fixed with 70% ethanol or 10% buffered formalin and embedded in paraffin, then cut into 5-µm-thick sections on glass slides. The deparaffinized tissues were treated with 4 N HCl for 20 min and incubated for 30 min in 10% normal goat serum to quench nonspecific binding. The tissues were reacted with 1:3000 diluted solution of anti-BrdU antibody

(Br-3) in TBS containing 0.05% Tween 20 for 1 h at room temperature. The slides were washed three times with TBS containing 0.05% Tween 20 and were incubated with 1:200 diluted solution of biotin-conjugated anti-mouse IgG. The slides were washed with TBS and then reacted with peroxidase-conjugated avidin-biotin complex (Dako) for 30 min. Peroxidase reaction was performed in 0.4 mg/ml diaminobenzidine tetrachloride (DAB) containing 0.03% H<sub>2</sub>O<sub>2</sub>. The nuclear staining was done by 1% hematoxylin solution. The tumor tissues were also stained with hematoxylin and eosin and were observed under light microscopy.

#### Tunel assay

The transplanted tumor tissues resected from nude mice were fixed with 10% buffered formalin and embedded in paraffin, then cut into 5-µm-thick sections on glass slides. The tissues were submitted to Tunel assay using the In situ Apoptosis Detection Kit (TaKaRa, Osaka, Japan) according to the manufacturer's protocols. The slides were treated for 15 min with proteinase K (20 µg/ml) and washed with TBS. To quench endogenous peroxidase activity, the slides were treated for 30 min with methanol containing 3% H<sub>2</sub>O<sub>2</sub>. The reaction solution consisting of TdT enzyme and FITC-conjugated dUTP was applied on the slides, which were incubated for 90 min at 37°C in a humidified box. After washing with TBS, the slides were incubated for 30 min at 37°C with HRP conjugate anti-FITC antibody. Peroxidase reaction was performed with DAB containing 0.03% H<sub>2</sub>O<sub>2</sub>. The nuclear staining was done with 1% hematoxylin solution.

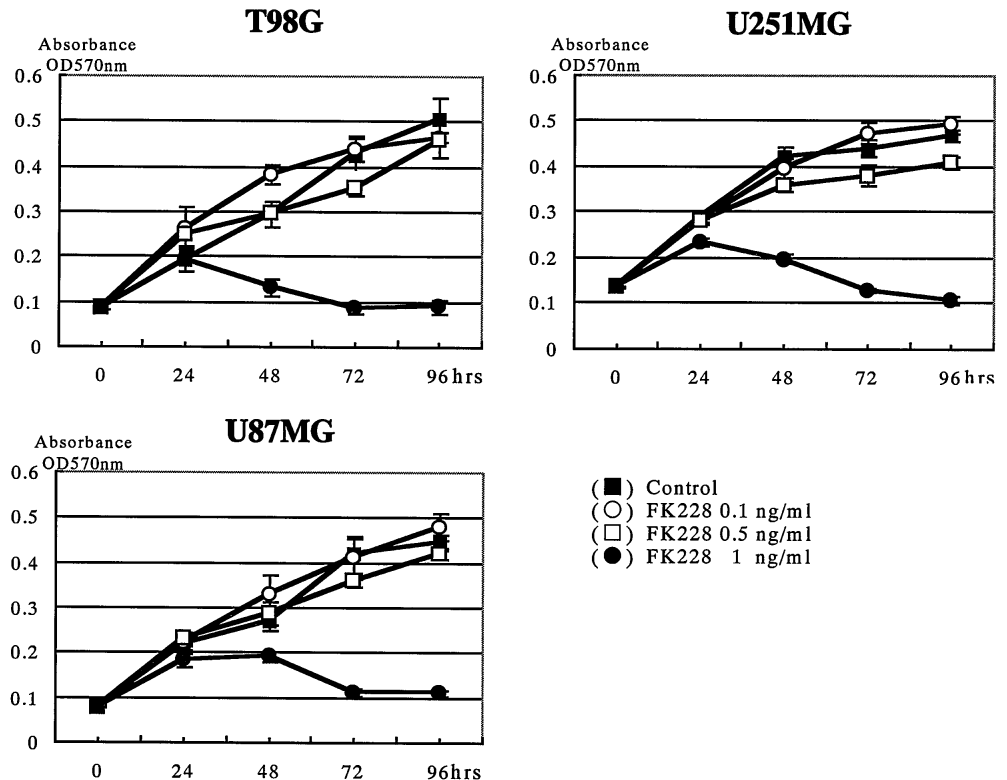
## Results

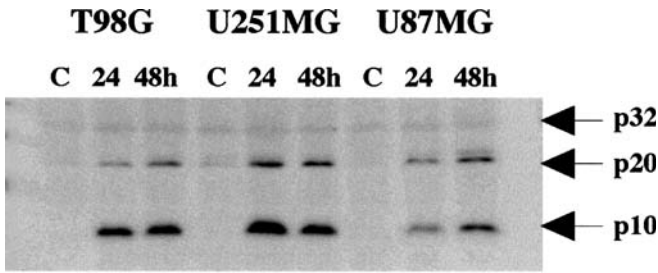
### FK228 inhibits proliferation of human glioma cells in vitro

To determine whether the HDAC inhibitor, FK228, could suppress the in vitro growth of human GM cells, we first

**Fig. 1** Growth suppression of human glioblastoma (GM) cells by treatment with FK228.

T98G, U251MG, and U87MG cells were cultured in the absence or presence of FK228 (0.1, 0.5, and 1 ng/ml) for 96 h, and the cell growth was assessed by the alamarBlue assay. The results represent the mean±SD of three different wells. The experiment was independently repeated twice





**Fig. 2** Apoptosis induction upon treatment with FK228 in human GM cells in vitro. T98G, U251MG, and U87MG cells were treated with FK228 (1 ng/ml) for 24 and 48 h, then the cleavages of CPP32 were analyzed. Treatment with FK228 increased the cleavage products (p20 and p10, *solid arrows*) of CPP32 in all human GM cells, indicating that they may induce apoptosis

tested the effects of FK228 on proliferation by using the alamarBlue assay. Treatment with FK228 (1 ng/ml) significantly reduced the proliferation rates of human glioma cells, although a lower concentration of FK228 (0.5 or 0.1 ng/ml) was not effective against the cell proliferation of human glioma cells as shown in Fig. 1.

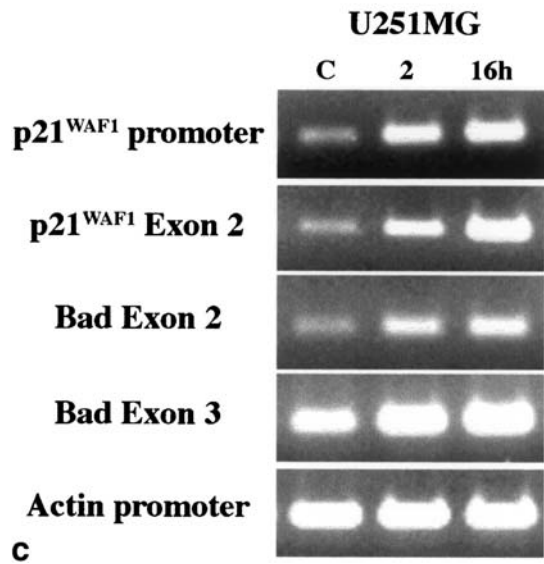
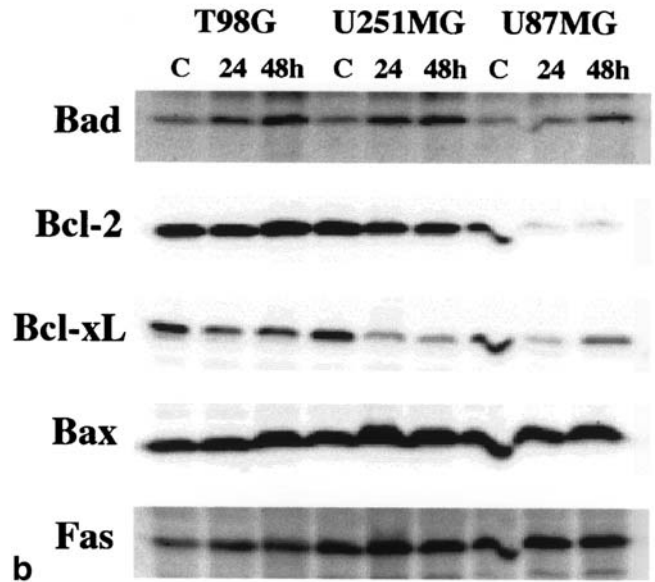
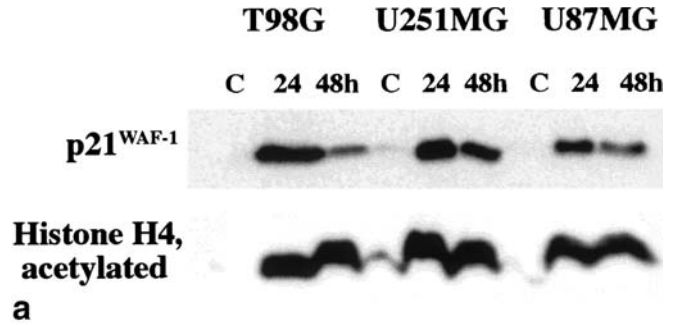
**Apoptosis induction by FK228 in vitro**

T98G, U251MG, and U87MG cells treated with FK228 showed the formation of long processes and also became round like apoptotic cells (data not shown). The cells were cultured in the presence or absence of FK228 (1 ng/ml) for 24 h and were stained with Hoechst 33258. The cells treated with FK228 showed nuclear fragmentation, suggesting apoptosis (data not shown). To further confirm the induction of apoptosis with FK228, the cleavages of CPP32 were studied. As shown in Fig. 2, treatment with FK228 increased the cleavage products (p20 and p10) of CPP32 in all human GM cells, indicating that it may induce apoptosis in human GM cells in vitro.

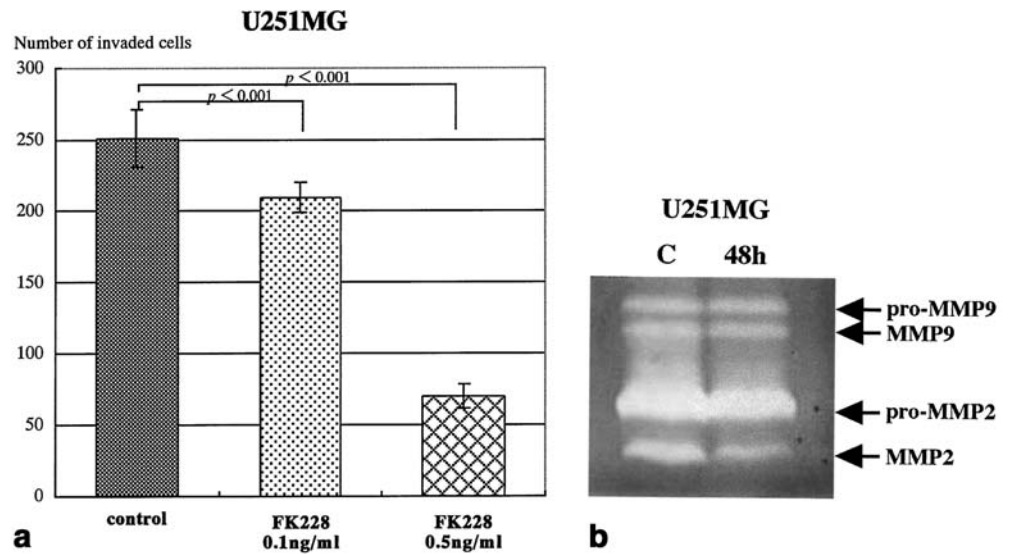
**Fig. 3 a** Increased expression of p21 (WAF-1) with FK228 treatment in vitro. T98G, U251MG, and U87MG cells were cultured in the absence or presence of FK228 (1 ng/ml) for 24 and 48 h. FK228 increased the expression of p21 (WAF-1), though the extent of p21 (WAF-1) expression was different in the cell types because of exposure times (*upper columns*). FK228 increased the expression levels of acetylated histone H4 (*lower columns*). **b** Expression of apoptosis-related proteins upon FK228 treatment. T98G, U251MG, and U87MG cells were cultured with or without FK228 (1 ng/ml) for 24 and 48 h. Treatment with FK228 increased the expression of Bad in all human GM cells examined. In addition, FK228 also reduced the anti-apoptotic protein Bcl-xL in all human GM cells examined. The expression level of anti-apoptotic protein Bcl-2 in U87MG cells was significantly reduced with FK228 treatment. The expression of Bax and Fas did not change with FK228 treatment. **c** FK228 induces accumulation of acetylated histone H4 in chromatin associated with the p21 (WAF-1) and Bad genes. U251MG cells were treated with FK228 (1 ng/ml) for 2 or 16 h. It is demonstrated that p21 (WAF1) and Bad DNA of U251MG cells treated with FK228 were associated with highly acetylated histone, compared with cells in the absence of FK228. The level of histone acetylation did not affect the chromatin associated with the actin promoter

FK228 increases the expression levels of p21 (WAF-1) in vitro

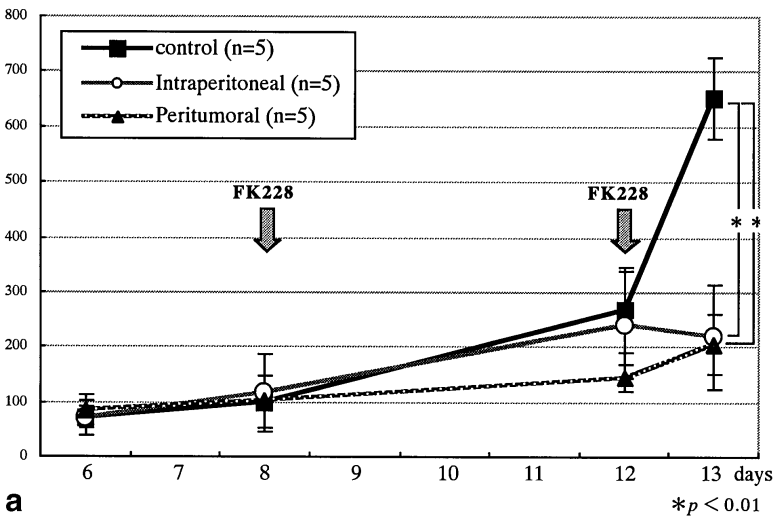
We previously reported that sodium butyrate treatment resulted in an increase of expression levels of p21 (WAF-1)



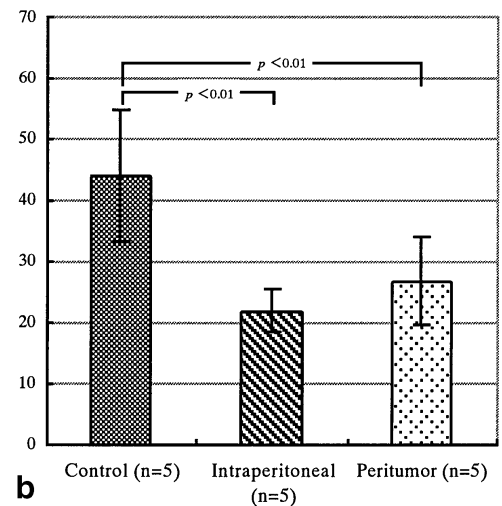
**Fig. 4a,b** FK228 inhibits cellular invasiveness and decreases MMP-2 activity. **a** The number of cells that infiltrated through the pores decreased significantly in the presence of FK228 at a concentration of 0.1 ng/ml ( $p < 0.001$ ) or 0.5 ng/ml ( $p < 0.001$ ). **b** Gelatin zymographic analysis of MMP-2 and MMP-9 in cells treated with 0.5 ng/ml of FK228 for 48 h or untreated cells. Gelatinolytic activity of MMP-2 was reduced in FK228-treated cells



Tumor weight (mg)



BrdU positive cells (%)



**Fig. 5 a** Growth suppression of transplanted m-3 glioma cells by FK228. The tumor growth was significantly suppressed with treatment by FK228. **b** Decrease in BrdU-labeled cells with treatment by FK228. BrdU labeling index of subcutaneous tumors treated with FK228 was significantly reduced ( $p < 0.01$ )

Changes in the expression of apoptosis-related proteins by FK228 in vitro

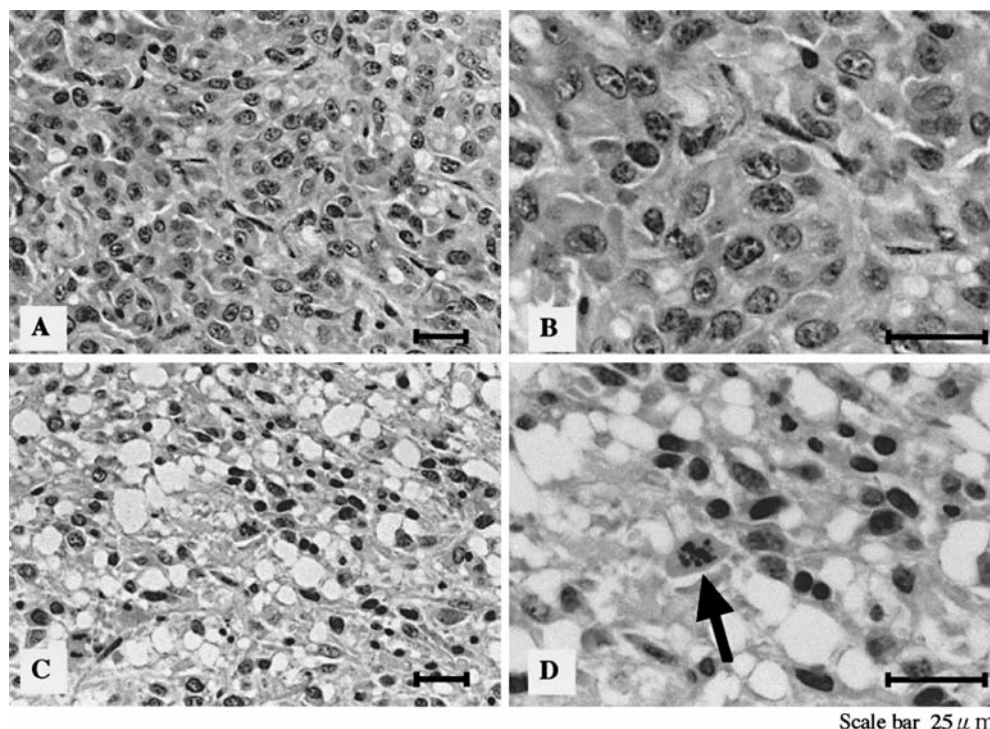
To further determine the underlying mechanisms of FK228-mediated apoptosis, we analyzed the expression levels of apoptosis-related proteins in human GM cells treated with or without 1 ng/ml FK228 for 24 and 48 h. As shown in Fig. 3b, treatment with FK228 increased the expression of Bad and decreased anti-apoptotic protein Bcl-xL in all human GM cells examined. The expression level of the anti-apoptotic protein Bcl-2 in U87MG cells was significantly inhibited by FK228 treatment. The expression of Bax and Fas was not changed in the presence of FK228.

in both U87MG and U251MG cells, and inhibited the G1-S transition of the cell cycle [5]. FK228 treatment also increased the expression levels of p21 (WAF-1) as shown in Fig. 3a. The increased expression levels of p21 (WAF-1) due to FK228 were closely associated with the expression levels of acetylated histone H4 protein. The result suggested that histone acetylation mediated by FK228 may relate to p21 (WAF-1) expression. One mechanism of growth suppression by FK228 may be due to an increase of p21 (WAF-1), since p21 (WAF-1) inhibits the phosphorylation of pRb protein and arrests proliferation in the G1 phase of the cell cycle. Another mechanism of growth suppression may be due to apoptosis induction by FK228.

FK228 induces accumulation of acetylated histone in chromatin associated with the p21 (WAF-1) and Bad genes

It is evident that p21 (WAF1) and Bad DNA in U251MG cells treated with FK228 were associated with highly

**Fig. 6a–d** Morphological changes of subcutaneous tumor tissues by treatment with FK228. **a,b** Control; **c,d** histological changes of subcutaneous tumor tissues with FK228 treatment by the intraperitoneal route. Bar 25  $\mu$ m. Reduced cell density, microcystic change of tumor tissues, and an increased infiltration of inflammatory cells such as lymphocytes and neutrophils were observed at the lower magnification (**a,c**). At the higher magnification, the nuclei of tumor cells treated with FK228 (**d**) become smaller than those of the control (**b**). Nuclear fragmentation (arrow) is seen in FK228-treated tumor (**d**)



acetylated histone, compared with the same region isolated from cells cultured without FK228 (Fig. 3c). The level of histone acetylation did not affect chromatin associated with the actin promoter.

#### FK228 inhibits cellular invasiveness and decreases MMP-2 activity

We determined whether FK228 could also affect the invasiveness of GM cells. U251MG cells were placed in the upper compartment of the Transwell chamber coated with matrigel. The number of cells that infiltrated through the pores decreased significantly in the presence of 0.1 or 0.5 ng/ml FK228, as shown in Fig. 4a. Since cellular invasiveness is thought to be dependent on the production of MMPs, we studied the gelatinolytic activity of MMP-2 and MMP-9, enzymes responsible for extracellular-matrix degradation. After incubation in serum-free medium for 48 h, conditioned media were subjected to gelatin zymography. Figure 4b shows that the gelatinolytic activity of MMP-2 was reduced in FK228-treated cells.

#### Growth suppression of transplanted m-3 glioma cells into hereditary athymic mice with FK228

To estimate the effect of FK228 on the growth of transplanted m-3 glioma cells, tumor volumes were measured by the formula described in Materials and methods in the presence of FK228 (Fig. 5a). The tumor growth was significantly suppressed with treatment by FK228 (arrows). BrdU labeling index of subcutaneous tumors treated with

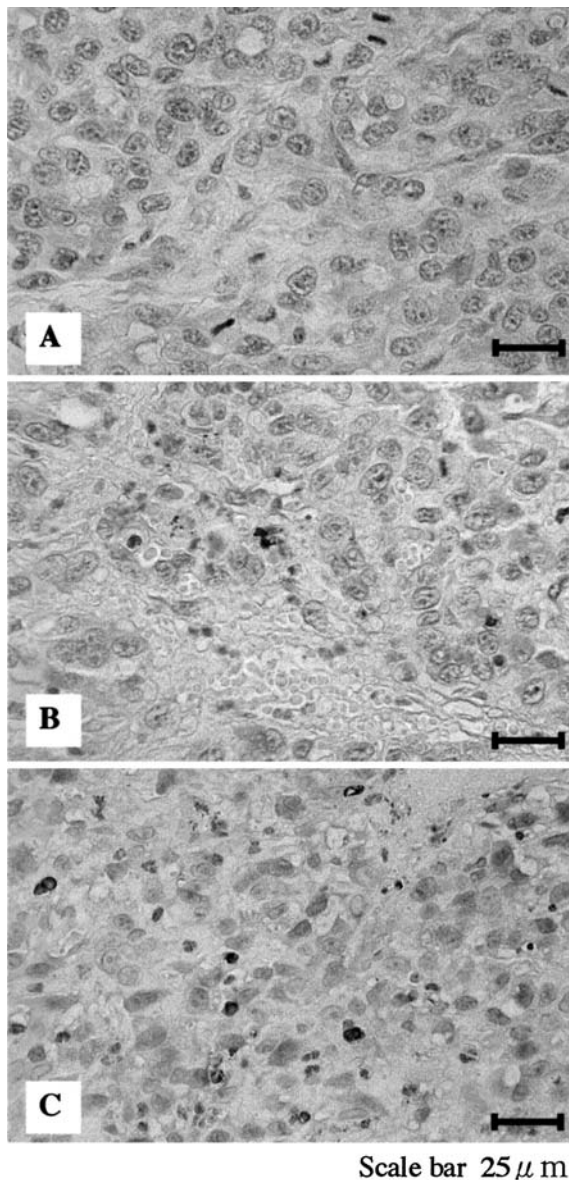
FK228 was significantly ( $p < 0.01$ ) reduced with both intraperitoneal and peritumoral injection of FK228 (Fig. 5b). Immunoblot analysis revealed that FK228 enhanced the expression of p21 (WAF-1) and Bad proteins in the transplanted tumor tissue (data not shown).

#### Morphological changes in subcutaneous tumor tissues by treatment with FK228

Histologically, FK228 treatment reduced the cell density and microcystic change of tumor tissues, while it increased the infiltration of inflammatory cells such as lymphocytes and neutrophils (Fig. 6c). The nuclei of tumor cells treated with FK228 (Fig. 6d) become smaller than those of the control (Fig. 6b) along with nuclear fragmentation (arrow) (Fig. 6d), suggesting that FK228 induced apoptosis in the transplanted tumor cells. The detection of apoptotic cells was evaluated by the TUNEL assay. Scattered TUNEL-positive cells were observed in FK228-treated tumors, compared with control tumors (Fig. 7).

## Discussion

FK228 was originally isolated from *Chromobacterium violaceum* as a novel antitumor agent [26]. An in vitro study revealed that FK228 is a strong and reversible inhibitor of HDAC [13]. We have previously studied the antitumor effects of HDAC inhibitors such as sodium butyrate (SB) or trichostatin A (TSA) [5, 22], and SB treatment inhibited the G1-S transition associated with increased expression of p21 (WAF-1) and reduced pRb phosphorylation [5].



**Fig. 7a-c** TUNEL assay of transplanted tumor tissues with FK228 treatment. FK228 significantly increased the TUNEL-positive cells with both intraperitoneal ( $2.76 \pm 0.011\%$ ,  $p < 0.05$ ) (b) and peritoneal ( $5.03 \pm 0.011\%$ ,  $p < 0.01$ ) (c) injection of FK228, compared with control tumor ( $0.27 \pm 0.003\%$ ) (a)

Furthermore, we have reported that these HDAC inhibitors induced apoptosis through an increase of the bcl-2-related protein Bad [22]. In this paper, we studied the antitumor effects of FK228 for human GM cells in vitro and in vivo. As shown in Fig. 3, treatment with FK228 increased the expression of p21 (WAF-1) and Bad proteins, which were closely related to histone acetylation as confirmed by the ChIP assay (Fig. 3c). In T24 bladder carcinoma cells, suberoylanilide hydroxamic acid (SAHA), possessing the function of a HDAC inhibitor, induced up to a 9-fold increase in the rate of transcription of the p21 (WAF-1) gene and caused an accumulation of acetylated histone H3 and H4 of the p21 (WAF-1) gene [19]. Although the molecu-

lar mechanisms of how FK228 inhibits HDAC activities remain to be clarified, our results suggest that FK228 action might possess a similar biological function to SB, TSA, or SAHA.

The treatment with FK228 increased the expression of Bad in all human GM cells examined. In addition, FK228 also reduced the anti-apoptotic protein Bcl-xL in all GM cells. The expression level of anti-apoptotic protein Bcl-2 in U87MG cells was significantly reduced with FK228 treatment. The Bcl-2 family of proteins, consisting of anti- and pro-apoptotic molecules, constitutes a critical intracellular decision point within a common cell death pathway [2]. The ratio of antagonist [Bcl-2, Bcl-xL, myeloid cell leukemia sequence 1 (Mcl-1), and BCL2-related protein A1 (A1)] to agonist [Bcl-2 associated X protein (Bax), Bcl-2 antagonist/killer (Bak), Bcl-xS, and Bad] dictates whether a cell will respond to a proximal apoptotic stimulus [2, 16]. It has been reported that SB induces apoptosis accompanied by upregulation of Bak in human bladder cancer cells (KoTCC/C) [11], by upregulation of Bak and downregulation of Bcl-2 in human renal cell carcinoma (ACHN) cells [4], and by upregulation of Bak in human colon cancer Caco-2 cells [20]. Tsai et al. [24] showed that Bcl-xS expression was elevated by SB treatment in human gastric cancer TMK-1 cells.

Compared with SB or TSA, there is little information about what FK228 modulates during the cell cycle arrest or the induction of apoptosis. Sandor et al. [20] showed that FK228 causes cyclin D1 downregulation and a p53-independent p21 (WAF1) induction, leading to inhibition of cyclin-dependent kinase and hypophosphorylation of Rb, resulting in growth arrest in the early G1 phase. These data basically agree with our results as shown in Fig. 3. However, Fecteau et al. [3] found that FK228 induced p21 (WAF-1) in nontransformed 10T1/2 cells, but not in *ras*-transformed counterpart, and apoptosis induction of *ras*-transformed 10T1/2 by FK228 correlated with suppression of the extracellular signal-regulated kinase (ERK) pathway through reduction of Raf expression and deactivation of MAP kinase (MEK) and ERK, inhibition of the phosphoinositide-3-kinase pathway indexed by suppression of Akt activity, suppression of p38 activity, and activation of caspase-3. Yu et al. [28] also showed that FK228 treatment led to altered expression of cyclin A, cyclin E, and p21 (WAF-1), and to reduced expression of mutant p53, but not wild-type. Furthermore, FK228-treated cells were depleted of ErbB1, ErbB2, and Raf-1 and exhibited lower ERK1/2 activity. These recent results suggest that FK228 also inhibited various signal transduction pathways through the acetylation of HSP90 [28], for instance, as well as increased the expression of p21 (WAF-1) and modulated the apoptosis-related proteins as shown in Fig. 3. The functional significance of acetylation of nonhistone proteins and the precise mechanisms whereby HDAC inhibitors induce tumor cell growth arrest, differentiation, and/or apoptosis are currently the focus of intensive research [27].

We also showed suppression of cellular invasiveness of U251MG cells on permissive substrates by FK228. Since matrix metalloproteinases (MMPs) have been shown to

play a potent role in helping cancer cells invade through the extracellular matrix and from metastatic lesions, we studied the gelatinolytic activity of MMP-2 and MMP-9, enzymes responsible for extracellular-matrix degradation. Gelatin zymography (Fig. 4) shows that the gelatinolytic activity of MMP-2 was reduced in FK228-treated cells. Recently, Liu et al. [10] reported that TSA increased RECK, a membrane-anchored glycoprotein, which negatively regulated MMPs. The decrease of MMP-2 activity with FK228 treatment may also occur by such a molecular mechanism.

Kosugi et al. [7] have reported that the growth of subcutaneously transplanted acute promyelocytic leukemia cell NB4 into NOD/Shi-scid mice was significantly inhibited by the combined treatment of all-*trans* retinoic acid and FK228. In our experiment using human GM cell m-3, treatment with FK228 alone significantly reduced the tumor growth, as shown Fig. 5a. Both intraperitoneal and peritumoral injection of FK228 significantly inhibited tumor growth (Fig. 5a) and the BrdU labeling index of transplanted human GM cells (Fig. 5b).

Recently, we reported that SB or TSA reduced the secretion of vascular endothelial growth factor (VEGF) into conditioned media of cultured GM cells [23]. It has been also reported that FK228 inhibited the neovascularization of chick embryo and of adult mice in the Matrigel plug assay [9]. This was accompanied by the suppression of angiogenic-stimulating factors such as VEGF or kinase insert domain receptor, along with the induction of angiogenic-inhibiting factors such as von Hippel Lindau and neurofibromin 2 [9].

In conclusion, we showed here that FK228 significantly inhibited the proliferation of human GM cells and induced apoptosis at an excess of 100× lower concentration (1 ng/ml) than SB (200 ng/ml) and TSA (100 ng/ml). Since several tumors are known to have higher HDAC activities than normal cells [1, 6], which probably plays a major role in carcinogenesis and progression of tumors, a new HDAC inhibitor with less cellular toxicity, such as FK228, could possibly be useful as an intractable glioma or cancer therapeutic agent. Further experimental studies into the cellular and molecular biological functions of FK228 should be done in the near future.

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