[CANCER RESEARCH 62, 4916–4921, September 1, 2002]

FK228 (Depsipeptide) as a Natural Prodrug That Inhibits Class I Histone Deacetylases[1]

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ABSTRACT

FK228 is a histone deacetylase (HDAC) inhibitor, the molecular mechanism of inhibition of which has been unknown. Here we show that reduction of an intramolecular disulfide bond of FK228 greatly enhanced its inhibitory activity and that the disulfide bond was rapidly reduced in cells by cellular reducing activity involving glutathione. Computer modeling suggests that one of the sulfhydryl groups of the reduced form of FK228 (redFK) interacts with the active-site zinc, preventing the access of the substrate. HDAC1 and HDAC2 were more strongly inhibited by redFK than HDAC4 and HDAC6. redFK was less active than FK228 in inhibiting *in vivo* HDAC activity, due to rapid inactivation in medium and serum. Thus, FK228 serves as a stable prodrug to inhibit class I enzymes and is activated by reduction after uptake into the cells. The glutathione-mediated activation also implicates its clinical usefulness for counteracting glutathione-mediated drug resistance in chemotherapy.

INTRODUCTION

Several lines of evidence have suggested that aberrant histone acetylation is linked to tumorigenesis. Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases and HDACs,[3] respectively (1). Histone acetyltransferase enzymes constitute a multienzyme family, and some members of this family were identified as transcriptional coactivators. On the other hand, HDAC enzymes were found to be transcriptional regulators related to yeast Rpd3 (2), Hda1 (3), and Sir2 (4). A number of transcriptional repressors and corepressors were shown to recruit the HDAC complex to the promoter regions. In the case of nuclear hormone receptors, they associate HDAC in the absence of ligand to repress transcription (5). The PML-RARα fusion protein generated by chromosomal translocation in acute promyelocytic leukemia cells strongly represses the gene expression required for myeloid differentiation by recruiting many HDAC molecules by oligomerization. However, because the protein is still able to bind the ligand retinoic acid, pharmacological concentrations of retinoic acid can induce the release of repression and therefore cause reinitiation of differentiation. Thus, most acute promyelocytic leukemia patients can be cured by all-*trans*-retinoic acid treatment. In contrast, another fusion protein, PLZF-RARα, is unable to be activated because PLZF also binds the corepressor-HDAC complex. In this case, the combined treatment with HDAC inhibitors can experimentally overcome the transcriptional repression (6–8).

Cocrystallization of a HDAC-like protein with HDAC inhibitors, such as TSA, demonstrated that these inhibitors mimic the substrate and that chelation of the zinc in the catalytic pocket by the hydroxamic acid group is the main mechanism of inhibition (9). Consistent with the structure of the enzyme-inhibitor complex, most of the HDAC inhibitors consist of cap, spacer, and functional groups. Some of the new HDAC inhibitors were designed based on structural information (10, 11).

FK228 (formerly named FR901228), also known as depsipeptide, is produced by *Chromobacterium violaceum* and shows potent *in vivo* antitumor activity against both human tumor xenografts and murine tumors (12, 13). A Phase I study of FK228 in the Medicine Branch, National Cancer Institute showed that cutaneous T-cell lymphoma patients had a complete or partial response (14). Thus, FK228 is at least a novel and potentially effective agent for patients with T-cell lymphoma. We have previously shown that it strongly inhibits HDAC (15). However, FK228 has no apparent chemical structure that interacts with the HDAC active-site pocket. In this study, we show that FK228 is converted to its active, reduced form (redFK) by cellular reducing activity. redFK possesses a functional sulfhydryl group capable of interacting with the zinc in the active-site pocket. Because FK228 is much more stable than redFK in medium and serum, we propose that FK228 is a natural prodrug, which is activated after incorporation into the cells.

MATERIALS AND METHODS

Preparation of redFK and Dimethyl FK228. To a mixture of FK228 (51.6 mg, 95 μmol), water (40 ml), and acetonitrile (10 ml), DTT (412 mg, 2.66 mmol) was added, and the mixture was left at room temperature overnight. After removal of acetonitrile by evaporation, the mixture was purified by preparative HPLC using a solvent system of 50% acetonitrile in a 0.05% aqueous solution of trifluoroacetic acid. The fractions collected were lyophilized to give redFK (14.8 mg, 28.7%) as a powder. To a mixture of redFK (3.4 mg, 6 μmol), ethanol (2 ml), CH_3I (8.52 mg, 60 μmol), and K_2CO_3 (8.3 mg, 60 μmol) were added and left at room temperature overnight. After removal of ethanol by evaporation, the mixture was purified by preparative HPLC using the same solvent system. The fractions collected were lyophilized to give dimethyl FK228 (1.3 mg, 38%) as a powder. The HPLC analysis was carried out using a YMC-PACK Pro C18 column (4.6 mm $\phi \times$ 150 mm) at a flow rate of 1 ml/min. The retention times were 4.24 min for redFK and 4.64 min for FK228, respectively. The structures and purity of redFK and dimethyl FK228 were confirmed by fast atom bombardment mass spectrometry and ^{1}H nuclear magnetic resonance.

Cells, Strains, and Culture Conditions. 293T, HeLa, and Mv1Lu cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. *Schizosaccharomyces pombe* strains used in this study are JY265 (*h$^-$ leu1*), MN55 (*h$^-$ leu1 gcs1*), and MN101 (*h$^-$ leu1 gsh2*). MN55 and MN101 were kindly provided by Dr. N. Mutoh (Aichi Human Service Center). The yeast cells were grown in a YE plate (0.75% yeast extract, 3% glucose, and 2% agar) and supplemented with 150 mg/liter adenine for 2 days at 30°C.

Received 3/1/02; accepted 7/1/02.

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[1] Supported in part by CREST Research Project, Japan Science and Technology Corp., a special grant for Advanced Research on Cancer, and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture Sports, Science and Technology of Japan.

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[3] The abbreviations used are: HDAC, histone deacetylase; TSA, trichostatin A; redFK, reduced form of FK228; MRP, multidrug resistance-associated protein; HPLC, high-performance liquid chromatography; γ-GCS, γ-glutamylcysteine synthetase; TPX, trapoxin; YE, yeast extract.

Enzyme Preparation and Assay. 293T cells ($1–2 \times 10^6$) were grown in a 100-mm dish for 24 h and transiently transfected with 10 μg each of the vector pcDNA3-HD1 for human HDAC1, pME18S-HDAC2 for human HDAC2, pcDNA3.1(+)-HD4 for human HDAC4, or pcDNA-mHDA2/HDAC6 for mouse HDAC, using LipofectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD). To construct a mutant HDAC1 (C151S) expression vector, we introduced a mutation into pcDNA-HDAC1-FLAG using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with synthetic oligonucleotide primers of 5′-GCATCTGGCTTCAGTTACGT-CAATG-3′ and 5′-CGTAGACCGAAGTCAATGCAGTTAC-3′. After successive cultivation in Opti-MEM for 5 h and DMEM for 19 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40. The soluble fraction collected by microcentrifugation was precleared by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnologies, Inc.). After the cleared supernatant had been incubated for 1 h at 4°C with 8 μg/ml of an anti-FLAG M2 antibody (Sigma-Aldrich Inc.) for HDAC1, HDAC2, and HDAC4 or an anti-hemagglutinin antibody (Santa Cruz Biotechnologies, Inc.) for HDAC6, the agarose beads were washed three times with lysis buffer and once with histone deacetylase buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, and a complete protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). The bound proteins were released from the immune complex by incubation for 1 h at 4°C with 100 μg of the FLAG or the hemagglutinin peptide (Sigma-Aldrich Inc.) in histone deacetylase buffer (1.25 ml). The supernatant was collected after centrifugation and diluted to give 1,000–2,000 cpm in the following standard enzyme assay. Bead-bound HDAC1 was used for the drug reversibility test. For the enzyme assay, 10 μl of [^{3}H]acetyl-labeled histones (25,000 cpm/10 μg) were added to 90 μl of the enzyme fraction, and the mixture was incubated at 37°C for 15 min. The enzyme reaction was linear for at least 1 h under these conditions, as described previously (16). The reaction was stopped by the addition of 10 μl of concentrated HCl. The released [3H]acetic acid was extracted with 1 ml of ethylacetate, and 0.9 ml of the solvent layer was taken into 5 ml of aqueous counting scintillant II solution (Amersham Life Science) for determination of radioactivity. DTT or H_2O_2 was added simultaneously with drugs to the enzyme solutions.

Stable Transfection and Luciferase Assay. The human wild-type p21 promoter-luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein. A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4-kb genomic fragment containing the transcription start site into *Hin*dIII and *Sma*I sites of the pGL3-Basic plasmid (Promega Co., Madison, WI). Mv1Lu (mink lung epithelial cell line) cells were transfected with pGW-FL and a phagemid expressing the neomycin/kanamycin resistance gene (pBK-CMV; Stratagene) with LipofectAMINE reagent (Life Technologies, Inc.). After the transfected cells had been selected by 400 μg/ml Geneticin (G418; Life Technologies, Inc.), the colonies formed were isolated. One of the clones was selected and named MFLL-9. MFLL-9 expressed a low level of luciferase, and luciferase activity was enhanced by TSA in a dose-dependent manner.

Western Blot Analysis. *S. pombe* cells were grown to mid-log phase at 30°C. FK228 was added at a concentration of 10 μM, and cells were incubated further at 30°C. Twelve h after drug addition, cells were harvested. Crude cell extracts were prepared by disrupting the cells with glass beads in TNE buffer [10 mM Tris-Cl (pH 7.8), 110 mM NaCl, and 1 mM EDTA]. HeLa cells treated with various concentrations of drugs for 24 h were harvested and lysed with lysis buffer [50 mM Tris-Cl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40], followed by sonication. Samples were analyzed by Western blot analysis using an antihuman $p21^{WAF1/Cip1}$ antibody (Transduction Laboratories, Inc.), a monoclonal anti-acetylated lysine antibody (AL3D5) described previously (10, 11), and an anti-α-tubulin antibody (Sigma).

Computer Modeling. Docking simulation was carried out with the discover module in Insight II. CFF was used as force field. Chain A of 1C3R, the HDAC-like protein structure registered in the Protein Data Bank, was used as an initial model. The positions of residues coordinated to zinc and zinc itself were restrained during simulation.

RESULTS

Enhancement of HDAC-inhibitory Activity by Reduction of FK228. FK228 is a bicyclic depsipeptide that is structurally distinct from other HDAC inhibitors. However, reduction of an internal disulfide bond was supposed to yield two free sulfhydryl groups, one of which is potentially accessible to catalytic residues in the active-site pocket (Fig. 1). Indeed, HPLC analysis showed that FK228 and its reduced form (redFK) were interconvertible by adding DTT and H_2O_2, respectively. We therefore hypothesized that redFK is the active form that inhibits HDAC. To test this possibility, we first examined the effect of DTT on its HDAC1-inhibitory activity. When FK228 was preincubated with 100 μM DTT at a concentration that does not affect HDAC1 activity, the inhibitory potency of FK228 was 36-fold enhanced compared with that in the absence of DTT (Table 1). On the other hand, the addition of 1% H_2O_2 markedly reduced the activity of FK228 ($IC_{50} = 2000 \pm 370$ nM). We next purified redFK from the FK228/DTT mixture, carefully avoiding oxidation by air, and determined its inhibitory potency. The purified redFK was as strong as that in the presence of DTT in inhibiting HDAC1 ($IC_{50} = 1.6 \pm 0.9$ nM). However, the activity of redFK was almost completely abolished by methylation of the two sulfhydryl groups (dimethyl FK228). These results indicate, as expected, that redFK is the active form. The relatively potent inhibition of HDAC1 by FK228 ($IC_{50} = 36 \pm 16$ nM) can be ascribed to some cellular reducing agents such as glutathione in the enzyme sample, resulting in the reduction of a small population of FK228 molecules.

We next compared the activity of FK228 and related compounds against several different enzymes. We chose HDAC1 and HDAC2 as class I enzymes and HDAC4 and HDAC6 as class II deacetylases (Table 1). TSA inhibited all of the deacetylases tested to a similar extent. On the other hand, redFK strongly inhibited HDAC1 and HDAC2 at low nanomolar concentrations but was relatively weak in inhibiting HDAC4 and HDAC6. In particular, HDAC6 was almost

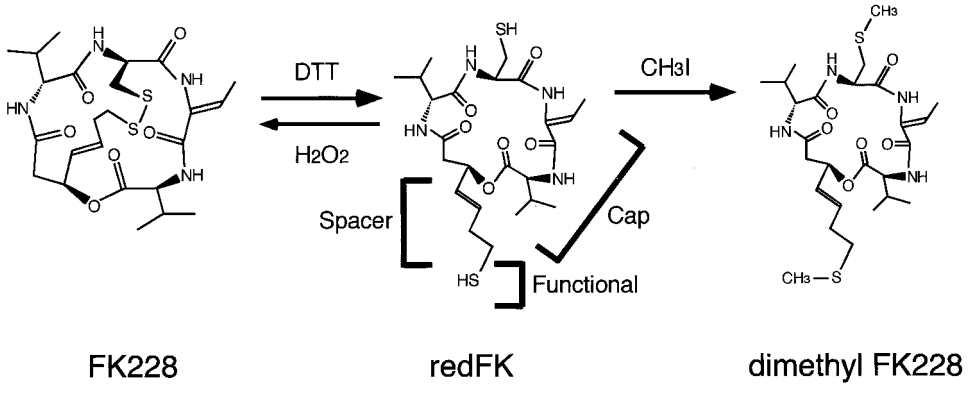

Fig. 1. Scheme for chemical modification of FK228 and working hypothesis of its activation.

Table 1 *Effects of FK228 and its derivatives on HDAC enzymes*

HDAC-inhibitory activity was evaluated by using HDACs prepared from 293T cells, and the (IC_{50}s) were determined as the means ± SD of the concentrations calculated from at least three independent dose-response curves. Each data point of the dose-response curves was the mean of duplicate assays. NT, not tested.

	IC_{50} ± SD (nM)				
	Class I		Class II		HDAC1 (C151S)
Compound	HDAC1	HDAC2	HDAC4	HDAC6	
FK228	36 ± 16	47 ± 18	510 ± 340	14,000 ± 3,100	280 ± 75
FK228 + DTT	1.0 ± 0.1	NT	NT	NT	NT
FK228 + H_2O_2	2,000 ± 370	NT	NT	NT	NT
redFK	1.6 ± 0.9	3.9 ± 2.7	25 ± 7.3	790 ± 110	57 ± 12
redFK + DTT	1.1 ± 0.7	NT	NT	NT	NT
Dimethyl FK228	6,300 ± 1,900	3,300 ± 1,000	11,0000 ± 53,000	>100,000	>100,000
TSA	19 ± 0.9	57 ± 21	50 ± 18	14 ± 4.3	12 ± 2.2

insensitive to redFK. FK228 was 17–23 times weaker than redFK in inhibiting each enzyme, and dimethyl FK228 showed no inhibitory activity against all of the enzymes.

FK228 Is Reduced in Cell-free Extract. If redFK should be the active form that inhibits HDAC, then FK228 was supposedly converted to redFK in cells. We examined the conversion with the cell-free extract of 293T cells by HPLC analysis. During incubation with the 20,000 × *g* supernatant of cell lysate, the amount of FK228 was rapidly decreased to 20% of the FK228 input, whereas redFK increased to reach a peak at 1 h, indicating that FK228 can be reduced by the cellular reducing activity (Fig. 2*A*). The content of redFK was then decreased to an almost undetectable level by 4 h, probably due to the instability of redFK (see below).

Cellular Glutathione Enhances Reduction of FK228. A major cellular reducing agent is glutathione, which is biosynthesized from L-glutamate, L-cysteine, and glycine by two enzymes, γ-GCS and glutathione synthetase. If FK228 is indeed reduced by glutathione in cells, then cells with a low level of glutathione are expected to be resistant to FK228. We tested this possibility by using mutants of the fission yeast *S. pombe*, in which the glutathione levels are markedly reduced (Ref. 17; Fig. 2*B*). The minimal inhibitory concentration of FK228 against wild-type *S. pombe* was 10 μM. However, the *gcs1* cells, in which γ-GCS is mutated, were almost completely resistant to 10 μM FK228, and the *gsh2* cells that were defective in glutathione synthetase also showed partial resistance. Western blot analysis using an anti-acetylated lysine antibody showed that histone H4 in the wild-type cells treated with FK228 was highly acetylated, whereas the acetylation levels in the mutant cells were suppressed, which coincided well with the growth of these cells in the presence of FK228 (Fig. 2*C*).

Possible Interaction with Zinc in the Active-site Pocket. Crystallographic studies using the HDAC-like protein-TSA complex have shown that TSA inserts its long aliphatic chain into the tube-like pocket and inhibits enzyme activity by interacting with the zinc and active-site residues through its hydroxamic acid at one end of the aliphatic chain (9). Because redFK has a 4-carbon-long chain between one of the sulfhydryl groups and the cyclic depsipeptide core, this sulfhydryl group is probably accessible to the residues in the active-site pocket. There is only one conserved cysteine residue (Cys-151 for HDAC1) in the pocket of all of the HDAC enzymes identified thus far (Fig. 3*A*). It therefore seemed possible that redFK interacts with the cysteine residue to form a covalent disulfide bond between redFK and the enzyme. To test this possibility, we introduced a single amino acid exchange from Cys-151 to Ser in HDAC1 (C151S), and we examined the drug sensitivity of this mutant enzyme. The C151S mutant was still sensitive to redFK, although the IC_{50} was higher than that for wild-type enzyme (Table 1). We next asked whether redFK irreversibly inhibited HDAC1, as did TPX (18, 19). The affinity-purified, bead-conjugated HDAC1 was incubated with TSA (100 nM), TPX B (100 nM), and redFK (100 nM) for 60 min, and then the residual enzyme activity was determined in the presence or absence of the drug (Fig. 3*B*). The activity of redFK-treated HDAC1 was recovered to a level similar to that of TSA-treated HDAC1 after removal of the drugs, in contrast to the irreversible inhibition by TPX. These results rule out the possibility that redFK is covalently bound to the conserved cysteine residue.

We further carried out a docking simulation using X-ray crystallo-

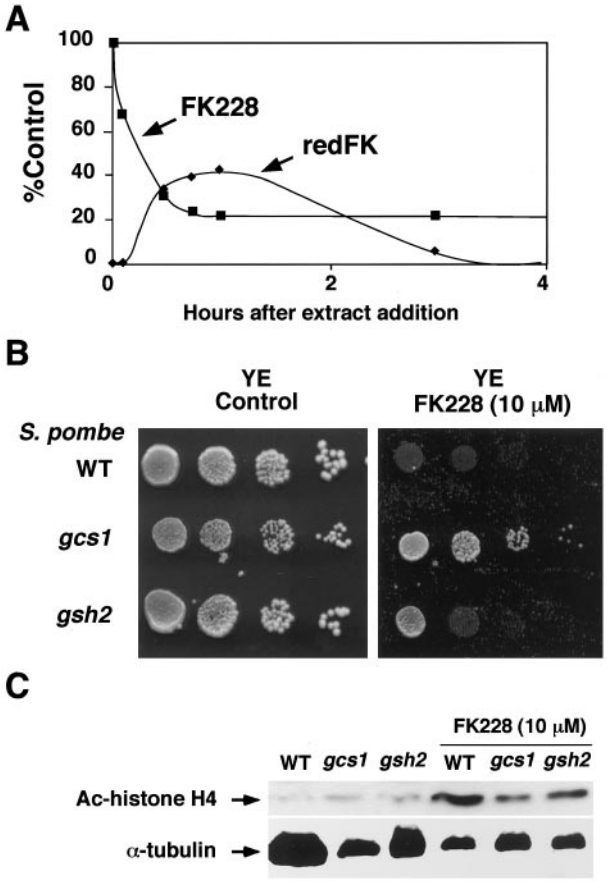

Fig. 2. Reduction of FK228 in mammalian cell lysate and fission yeast cells. *A*, conversion from FK228 to redFK in cell lysate. FK228 was incubated with the cytoplasmic fraction of 293T cells for the indicated times and extracted with ethylacetate from the mixture. The quantities of FK228 and redFK in the extracts were determined by HPLC. *B*, resistance to FK228 of yeast mutants defective in glutathione synthesis. Total cell numbers of 10^7, 10^6, 10^5, and 10^4 of wild-type *S. pombe* and two glutathione synthesis mutants were spotted on YE plates with (*right panel*) or without (*left panel*) 10 μM FK228. *C*, decreased histone acetylation in glutathione synthesis mutants. The amounts of acetylated histone H4 in the wild-type and mutant cells that had been treated with 10 μM FK228 for 12 h were determined by Western blotting using anti-acetylated lysine and anti-α-tubulin antibodies. α-Tubulin was used as an internal control.

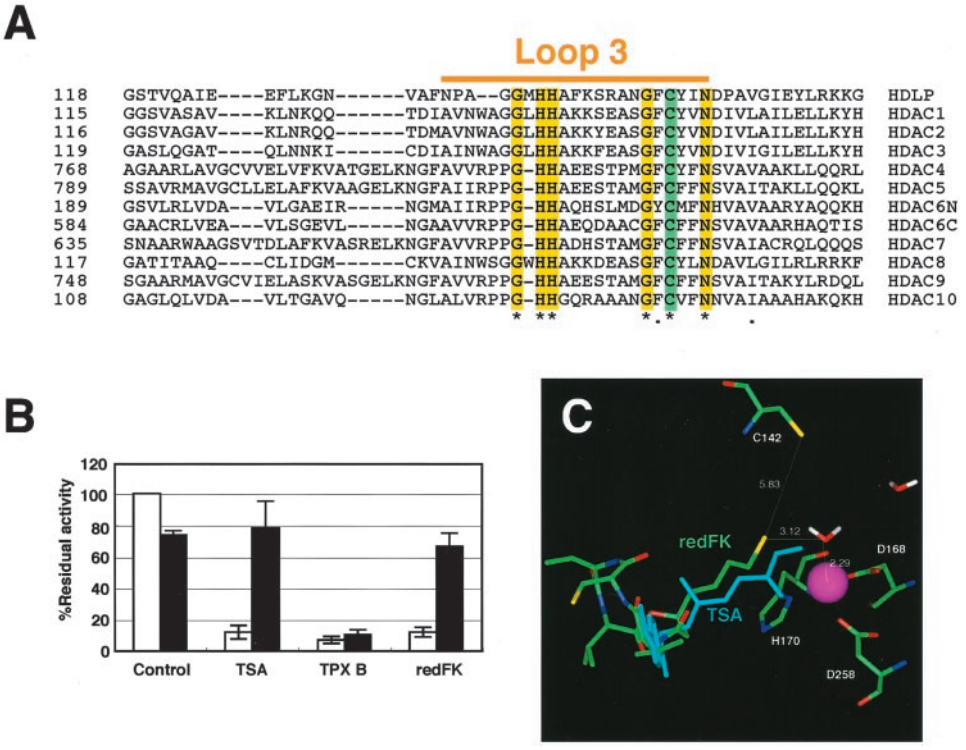

Fig. 3. Possible interaction of redFK with the zinc in the active-site pocket. *A*, amino acid sequence alignment of HDAC enzymes. Residues identical among enzymes are highlighted in *yellow*, and the only cysteine conserved in all enzymes is shown in *green*. Loops that form the active-site pocket are indicated by *orange bars*. *B*, reversibility of enzyme inhibition by redFK. Bead-conjugated, affinity-purified HDAC1 was pretreated with 1% ethanol (control), 100 nM TSA, 100 nM TPX B, and 100 nM redFK for 20 min. The treated enzyme preparations were washed with drug-free buffer, and the residual enzyme activity was determined. The *open* and *filled bars* represent before and after removal of drugs, respectively. Data are represented as the means ± SD of triplicate assays. *C*, computer-aided molecular docking of redFK to HDAC-like protein. The sulfur atom of the redFK longer chain is located 5.83 Å apart from the sulfur atom of Cys-142, the conserved cysteine residue corresponding to HDAC1 Cys-151, and 3.12 Å from a water molecule bound to the zinc.

graphic data of the HDAC-like protein. The position of the sulfur atom of redFK is 5.83 Å apart from the conserved cysteine residue (Cys-142) corresponding to Cys-151 in HDAC1 (Fig. 3*C*). On the other hand, the sulfur atom is located at a position allowing interaction with the active center zinc via a water molecule. These results strongly suggest that redFK reversibly interacts with the zinc, thereby reversibly inhibiting the enzyme activity in a manner similar to TSA.

FK228 Is More Stable than redFK. To examine *in vivo* HDAC inhibition by redFK, we treated HeLa cells with various concentrations of FK228, redFK, and TSA, and the effects on histone acetylation and $p21^{WAF1}$ induction were analyzed by Western blot analysis. Both FK228 and redFK increased histone acetylation and p21 expression (Fig. 4*A*), as did other HDAC inhibitors (20–22). However, FK228 induced acetylation and p21 expression more strongly than redFK, suggesting that the *in vivo* HDAC-inhibitory activity of redFK is weaker than that of FK228, despite its potent *in vitro* inhibition. To quantify the inhibitory activity of these compounds, we constructed a reporter system in which Mv1Lu cells were stably transfected with pGW-FL containing the 2.4-kb p21 promoter and the luciferase gene. The cell line MFLL-9 expresses luciferase activity in a TSA dose-dependent manner (Fig. 4*B*). Using this cell line, we determined the concentrations for half-maximum induction to be 3.0 nM for FK228 and 11 nM for redFK, according to the dose-response curves of FK228 and redFK (Fig. 4*B*). Because HPLC analysis showed that redFK rapidly disappeared during incubation with cell lysate (see Fig. 2*A*), we postulated that the weaker *in vivo* activity of redFK is due to its instability. To test this possibility, we compared the residual activity to induce p21 expression between FK228 and redFK after incubation of the drugs with growth medium or serum for various lengths of time (Fig. 4, *C* and *D*). The half-lives of FK228 and redFK were >12 h and 0.54 h, respectively, in growth medium and 4.7 h and <0.3 h in serum. These results clearly show that FK228 is much more stable than redFK in medium and blood. The functional sulfhydryl group exposed to the medium may be chemically modified or sequestered by some serum proteins.

DISCUSSION

Although FK228 is one of the HDAC inhibitors for which clinical efficacy of antitumor activity has been studied extensively, the molecular details behind the inhibition of HDAC have not been elucidated. The present study shows that the reduced FK228 is the active form. redFK has two sulfhydryl groups, one of which was suggested by computer modeling to interact with the active-site zinc via a water molecule bound to the zinc. The HDAC active-site structure has features of both metalloproteases and serine proteases, and the proposed catalytic mechanism for deacetylation is analogous to that of zinc proteases such as thermolysin and matrix metalloproteinases (9). The zinc protease inhibitors have a zinc-chelating group such as a hydroxamate, sulfhydryl, carboxylate, or phosphinic group (23–25). The hydroxamic acid of these inhibitors coordinates the zinc through its carbonyl and hydroxyl groups, resulting in the formation of a pentacoordinated zinc (26). Crystallographic studies have also shown that TSA binds the zinc and active-site residues through its hydroxamic acid in a manner quite similar to that of the zinc protease-hydroxamate inhibitor complex (27, 28). On the other hand, the sulfhydryl group of the protease inhibitors ligands the zinc with the sulfur, presumably in the anionic form, giving rise to a nearly exact tetrahedral coordination (29, 30). The positions of residues coordinated to the zinc were restrained, and disarrangement of the metal site was not allowed in our simulation. Therefore, it may be possible that the sulfur of redFK also tetrahedrally coordinates to the zinc displacing the water molecule even in HDAC. Because the inhibitory effect of redFK was reversible, and the cysteine mutant enzyme (C151S) was also inhibited by redFK, it is unlikely that the sulfur covalently binds to the enzyme at the cysteine residue in the pocket. However, it is still possible that the cysteine residue has a role in modulating the affinity to redFK because a concentration of redFK but not TSA higher than that for wild-type enzyme was required for mutant enzyme inhibition. The crystal structure of the HDAC-redFK complex is

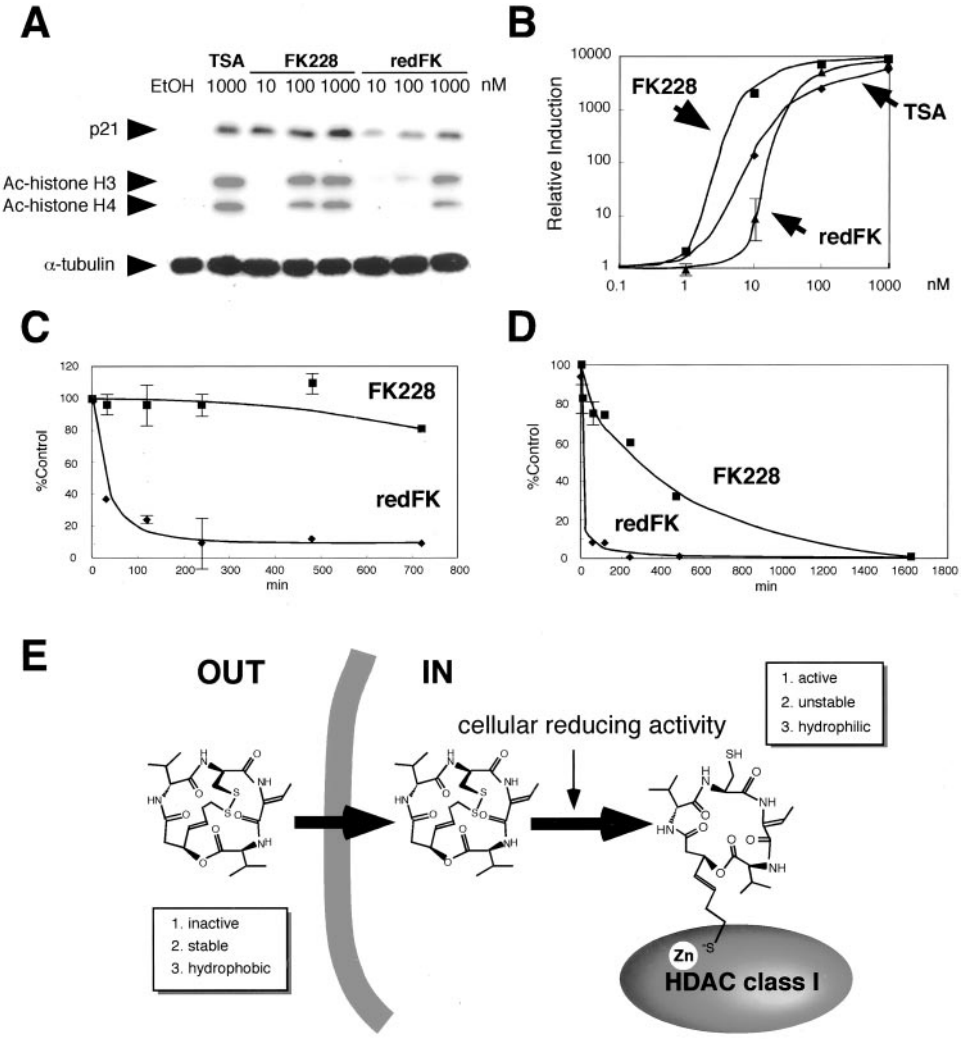

Fig. 4. *In vivo* activity to inhibit HDAC. *A*, accumulation of acetylated histones and p21 in cells. HeLa cells were treated with various concentrations of TSA, FK228, and redFK for 24 h, and the amounts of p21 (*top row*) and acetylated histone (*middle rows*) and α-tubulin (*bottom row*) were determined by Western blotting. *B*, comparison of the *in vivo* activity to induce *p21* gene expression. MFLL-9 cells containing a stable p21 promoter/luciferase construct were treated with various concentrations of TSA, FK228, and redFK for 24 h, and the luciferase activity induced was measured. *C*, stability in medium. After incubation of the drugs with growth medium supplemented with 10% fetal bovine serum for the indicated times, the p21-inducing activity was determined using MFLL-9 cells. *D*, stability in serum. After incubation with fetal bovine serum, the residual p21-inducing activity was determined as described in *C*. Data in *B*−*D* are represented as the means ± SD of triplicate assays. *E*, a model for inhibition of cellular HDAC by FK228.

apparently needed for better understanding of the detailed mechanism of inhibition.

The marked resistance to FK228 of the fission yeast glutathione biosynthesis mutants suggests that cellular glutathione is the important determinant of FK228 sensitivity. Our data suggest that the increased glutathione level leads to the increased FK228 sensitivity. This is in contrast to the multidrug resistance involving the increased glutathione. Multidrug resistance is frequently associated with overexpression of P-glycoprotein and/or MRP1, both of which are members of the ATP-binding cassette superfamily of transporters (31). P-glycoprotein and MRP1 function as ATP-dependent efflux pumps that extrude cytotoxic drugs from tumor cells. Glutathione serves as a cofactor in MRP1-mediated drug transport. The drugs complexed with glutathione after nucleophilic attack of the glutathione thiolate anion are transported out of the cells by MRP1. The genes encoding both MRP1 and the catalytic subunit of γ-GCS are coordinately regulated in cultured cancer cell lines as well as colorectal cancer tissues from colon cancer patients. Glutathione is also involved in the inactivation of anticancer drugs. For instance, conjugation of cisplatin with glutathione inhibits the conversion of monoadducts to cross-links, thereby reducing the cytotoxic potential of the adducts (32). Indeed, depletion of glutathione by buthionine sulfoximine, an inhibitor of γ-GCS, restores the *in vitro* and *in vivo* sensitivity of drug-resistant tumors to anticancer drugs such as cisplatin (33–36). These drug-resistant cells with high glutathione concentrations are likely to be highly sensitive to FK228, due to the higher rate of FK228-redFK conversion. It is therefore conceivable that FK228 is particularly effective on tumor cells with glutathione-mediated drug resistance. The combined use of FK228 with antitumor drugs that are inactivated by glutathione may also be promising.

Because each member of the HDAC family is a component of a distinct physical complex playing a distinct role in gene expression, it is likely that inhibition of a specific enzyme leads to changes in transcription of a specific subset of genes (37). Indeed, FK228 has been shown to modulate both positively and negatively the expression of genes involved in oncogenic signal transduction (38, 39), cell cycle (39, 40), differentiation (41), and angiogenesis (42). However, little is known about the target enzyme specificity of HDAC inhibitors. It is also unknown which HDAC enzyme contributes to tumorigenesis. It was shown that HDAC6 was resistant to TPX, but its inhibitory potency against HDAC4 was as strong as that against HDAC1 and HDAC2 (10). We showed that redFK strongly inhibited HDAC1 and HDAC2 class I enzymes, whereas it was weak in inhibiting HDAC4 and HDAC6 class II enzymes. Given the *in vivo* efficacy of FK228 as an antitumor agent, HDAC1 and HDAC2 may be more importantly involved in tumorigenesis than HDAC4 and HDAC6.

FK228 was fairly stable in medium or serum, whereas redFK was unstable. Thus, the *in vivo*-administered FK228 may be circulated in its stable and inactive form, but after uptake into the tumor cells, it becomes active and reactive by the intracellular reducing activity (Fig.

4*E*). It is also conceivable that FK228 can penetrate the cell membrane better than redFK, due to its more hydrophobic nature. Therefore, FK228 serves as a stable prodrug for strong *in vivo* HDAC inhibition. This finding may explain why FK228 is more effective than other classical HDAC inhibitors such as TSA, TPX, and butyrate in *in vivo* models. These unique properties of FK228 provide a new basis for further development of HDAC inhibitors as antitumor agents.

ACKNOWLEDGMENTS

We thank Drs. E. Seto, E. Verdin, B. Vogelstein, and N. Mutoh for supplying plasmids and yeast strains. We also thank Dr. H. Watanabe for helpful discussion.

REFERENCES

1. Wolffe, A. P., Wong, J., and Pruss, D. Activators and repressors: making use of chromatin to regulate transcription. Genes Cells, *2:* 291–302, 1997.
2. Taunton, J., Hassig, C. A., and Schreiber, S. L. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science (Wash. DC), *272:* 408–411, 1996.
3. Rundlett, S. E., Carmen, A. A., Kobayashi, R., Bavykin, S., Turner, B. M., and Grunstein, M. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA, *93:* 14503–14508, 1996.
4. Imai, S., Armstrong, C. M., Kaeberlein, M., and Guarente, L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature (Lond.), *403:* 795–800, 2000.
5. Torchia, J., Glass, C., and Rosenfeld, M. G. Co-activators and co-repressors in the integration of transcriptional responses. Curr. Opin. Cell Biol., *10:* 373–383, 1998.
6. Grignani, F., De, M. S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F. F., Zamir, I., Seiser, C., Grignani, F., Lazar, M. A., Minucci, S., and Pelicci, P. G. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature (Lond.), *391:* 815–818, 1998.
7. Lin, R. J., Nagy, L., Inoue, S., Shao, W., Miller, W. J., and Evans, R. M. Role of the histone deacetylase complex in acute promyelocytic leukaemia. Nature (Lond.), *391:* 811–814, 1998.
8. He, L. Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A., and Pandolfi, P. P. Distinct interactions of PML-RARα and PLZF-RARα with co-repressors determine differential responses to RA in APL. Nat. Genet., *18:* 126–135, 1998.
9. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature (Lond.), *401:* 188–193, 1999.
10. Furumai, R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc. Natl. Acad. Sci. USA, *98:* 87–92, 2001.
11. Komatsu, Y., Tomizaki, K-y., Tsukamoto, M., Kato, T., Nishino, N., Sato, S., Yamori, T., Tsuruo, T., Furumai, R., Yoshida, M., Horinouchi, S., and Hayashi, H. Cyclic hydroxamic-acid-containing peptide 31, a potent synthetic histone deacetylase inhibitor with antitumor activity. Cancer Res., *61:* 4459–4466, 2001.
12. Ueda, M., Nakajima, H., Hori, Y., Fujita, T., Nishimura, M., Goto, T., and Okuhara, M. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J. Antibiot. (Tokyo), *47:* 301–310, 1994.
13. Ueda, H., Manda, T., Matsumoto, S., Mukumoto, S., Nishigaki, F., Kawamura, I., and Shimomura, K. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. III. Antitumor activities on experimental tumors in mice. J. Antibiot. (Tokyo), *47:* 315–323, 1994.
14. Piekarz, R. L., Robey, R., Sandor, V., Bakke, S., Wilson, W. H., Dahmoush, L., Kingma, D. M., Turner, M. L., Altemus, R., and Bates, S. E. Inhibitor of histone deacetylation, depsipeptide (FR901228), in the treatment of peripheral and cutaneous T-cell lymphoma: a case report. Blood, *98:* 2865–2868, 2001.
15. Nakajima, H., Kim, Y. B., Terano, H., Yoshida, M., and Horinouchi, S. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp. Cell Res., *241:* 126–133, 1998.
16. Yoshida, M., Kijima, M., Akita, M., and Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J. Biol. Chem., *265:* 17174–17179, 1990.
17. Mutoh, N., and Hayashi, Y. Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small cadmium-binding peptides. Biochem. Biophys. Res. Commun., *151:* 32–39, 1988.
18. Kijima, M., Yoshida, M., Sugita, K., Horinouchi, S., and Beppu, T. Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J. Biol. Chem., *268:* 22429–22435, 1993.
19. Yoshida, M., Horinouchi, S., and Beppu, T. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays, *17:* 423–430, 1995.
20. Archer, S. Y., Meng, S., Shei, A., and Hodin, R. A. p21^{WAF1} is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc. Natl. Acad. Sci. USA, *95:* 6791–6796, 1998.
21. Vaziri, C., Stice, L., and Faller, D. V. Butyrate-induced G_1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. Cell Growth Differ., *9:* 465–474, 1998.
22. Kim, Y. B., Lee, K-H., Sugita, K., Yoshida, M., and Horinouchi, S. Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. Oncogene, *18:* 2461–2470, 1999.
23. Ondetti, M. A., Rubin, B., and Cushman, D. W. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. Science (Wash. DC), *196:* 441–444, 1977.
24. Nishino, N., and Powers, J. C. Design of potent reversible inhibitors for thermolysin. Peptides containing zinc coordinating ligands and their use in affinity chromatography. Biochemistry, *18:* 4340–4347, 1979.
25. Kam, C. M., Nishino, N., and Powers, J. C. Inhibition of thermolysin and carboxypeptidase A by phosphoramidates. Biochemistry, *18:* 3032–3038, 1979.
26. Holmes, M. A., and Matthews, B. W. Binding of hydroxamic acid inhibitors to crystalline thermolysin suggests a pentacoordinate zinc intermediate in catalysis. Biochemistry, *20:* 6912–6920, 1981.
27. Bode, W., Reinemer, P., Huber, R., Kleine, T., Schnierer, S., and Tschesche, H. The X-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity. EMBO J., *13:* 1263–1269, 1994.
28. Borkakoti, N., Winkler, F. K., Williams, D. H., D'Arcy, A., Broadhurst, M. J., Brown, P. A., Johnson, W. H., and Murray, E. J. Structure of the catalytic domain of human fibroblast collagenase complexed with an inhibitor. Nat. Struct. Biol., *1:* 106–110, 1994.
29. Monzingo, A. F., and Matthews, B. W. Structure of a mercaptan-thermolysin complex illustrates mode of inhibition of zinc proteases by substrate-analogue mercaptans. Biochemistry, *21:* 3390–3394, 1982.
30. Grams, F., Reinemer, P., Powers, J. C., Kleine, T., Pieper, M., Tschesche, H., Huber, R., and Bode, W. X-ray structures of human neutrophil collagenase complexed with peptide hydroxamate and peptide thiol inhibitors. Implications for substrate binding and rational drug design. Eur. J. Biochem., *228:* 830–841, 1995.
31. Ishikawa, T., Kuo, M. T., Furuta, K., and Suzuki, M. The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design. Clin. Chem. Lab. Med., *38:* 893–897, 2000.
32. Kartalou, M., and Essigmann, J. M. Mechanisms of resistance to cisplatin. Mutat. Res., *478:* 23–43, 2001.
33. Meijer, C., Mulder, N. H., Timmer-Bosscha, H., Sluiter, W. J., Meersma, G. J., and de Vries, E. G. Relationship of cellular glutathione to the cytotoxicity and resistance of seven platinum compounds. Cancer Res., *52:* 6885–6889, 1992.
34. Xu, B. H., and Singh, S. V. Effect of buthionine sulfoximine and ethacrynic acid on cytotoxic activity of mitomycin C analogues BMY 25282 and BMY 25067. Cancer Res., *52:* 6666–6670, 1992.
35. Dai, J., Weinberg, R. S., Waxman, S., and Jing, Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood, *93:* 268–277, 1999.
36. Benderra, Z., Trussardi, A., Morjani, H., Villa, A. M., Doglia, S. M., and Manfait, M. Regulation of cellular glutathione modulates nuclear accumulation of daunorubicin in human MCF7 cells overexpressing multidrug resistance associated protein. Eur. J. Cancer, *36:* 428–434, 2000.
37. Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berny, D. Functional significance of histone deacetylase diversity. Curr. Opin. Genet. Dev., *11:* 162–166, 2001.
38. Ueda, H., Nakajima, H., Hori, Y., Goto, T., and 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., *58:* 1579–1583, 1994.
39. Yu, X., Guo, Z. S., Marcu, M. G., Neckers, L., Nguyen, D. M., Chen, G. A., and Schrump, D. S. Modulation of p53, ErbB1, ErbB2, and Raf-1 expression in lung cancer cells by depsipeptide FR901228. J. Natl. Cancer. Inst. (Bethesda), *94:* 504–513, 2002.
40. Sandor, V., Senderowicz, A., Mertins, S., Sackett, D., Sausville, E., Blagosklonny, M. V., and Bates, S. E. p21-dependent G_1 arrest with downregulation of cyclin D1 and upregulation of cyclin E by the histone deacetylase inhibitor FR901228. Br. J. Cancer, *83:* 817–825, 2000.
41. Kitazono, M., Robey, R., Zhan, Z., Sarlis, N. J., Skarulis, M. C., Aikou, T., Bates, S., and Fojo, T. Low concentrations of the histone deacetylase inhibitor, depsipeptide (FR901228), increase expression of the Na(+)/I(−) symporter and iodine accumulation in poorly differentiated thyroid carcinoma cells. J. Clin. Endocrinol. Metab., *86:* 3430–3435, 2001.
42. Kwon, H. J., Kim, M. S., Kim, M. J., Nakajima, H., and Kim, K. W. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int. J. Cancer, *97:* 290–296, 2002.