Histone Deacetylase Inhibitor FK228 Activates Tumor Suppressor Prdx1 with Apoptosis Induction in Esophageal Cancer Cells

Isamu Hoshino,1 Hisahiro Matsubara,1 Naoyuki Hanari,1 Mikito Mori,1 Takanori Nishimori,1 Yasuo Yoneyama,1 Yasunori Akutsu,1 Haruhito Sakata,1 Kazuyuki Matsushita,1 Naohiko Seki,2 and Takenori Ochiai1

Abstract

Purpose: The histone deacetylase inhibitor FK228 shows strong activity as a potent antitumor drug but its precise mechanism is still obscure. The purpose of this study is to reveal the effect of FK228 on gene expression in the cell and to determine the mechanism of the antitumor activity of FK228 for further clinical applications.

Experimental Design and Results: Microarray analysis was applied to verify the gene expression profiles of 4,608 genes after FK228 treatment using human esophageal squamous cell cancer cell lines TTN and TE2. Among them, peroxiredoxin 1 (Prdx1), a member of the peroxiredoxin family of antioxidant enzymes having cell growth suppression activity, as well as p21WAF1, were significantly activated by FK228. In addition, FK228 strongly inhibited the cell growth of TTN and TE2 by the induction of apoptosis. Further, chromatin immunoprecipitation analysis revealed that FK228 induced the accumulation of acetylated histones H3 and H4 in Prdx1 promoter, including the Sp1-binding site. In mouse xenograft models of TTN and TE2 cells, FK228 injection resulted in significant tumor regression as well as activated Prdx1 expression in tumor tissues. Prdx1 suppression by RNA interference hindered the antitumor effect of FK228.

Conclusion: Our results indicate that the antitumor effect of FK228 in esophageal cancer cells is shown at least in part through Prdx1 activation by modulating acetylation of histones in the promoter, resulting in tumor growth inhibition with apoptosis induction.

Esophageal squamous cell carcinoma is a highly malignant disease with poor prognosis, the overall 5-year survival rate being only in a range of 5% to 25% (1, 2). Recently, applications of combined chemotherapy and radiotherapy, or the combination therapy as an adjunct to surgery, have improved the prognosis of esophageal squamous cell carcinoma patients (3, 4). Thus, there is some urgency for the development of new antitumor agents so as to further improve the prognosis of esophageal squamous cell carcinoma patients. In this regard, among the most promising candidates as antitumor agents are histone deacetylase inhibitors (HDACi) such as FK228 (5, 6).

In chromatin, the four inner histones are wrapped inside 146 bp of DNA to make a nucleosome and they are aggregated to form a higher-order structure of chromatin (7). Chromatin structure is closely related to gene transcription (8). At least two mechanisms of chromatin remodeling are now understood: (a) ATP-dependent chromatin remodelers make chromatin remodeling complexes and slide nucleosome core histone along DNA, making DNA more accessible to transcription factors and DNA binding factors to promote transcription (9) and (b) the chromatin structure of nucleosomes is modified by histone acetyltransferases and HDACs (10). Histone acetyltransferases, which acetylate lysine residues of histone proteins, facilitate the access of many transcriptional factors to DNA and consequently activate the expression of the target genes. In contrast, HDACs, which deacetylate lysine residues, repress transcription (11, 12). One of the histone deacetylase inhibitors, FK228 (depsipeptide), isolated from Chromobacterium violaceum, is a new anticancer agent with potent HDAC-inhibiting activities (5, 6). FK228 induces histone acetylation, cell cycle arrest, and differentiation or apoptosis in vitro and in vivo in some human cancer cell lines (13–16). Recently, it was shown that HDACi (e.g., FK228, suberoylanilide hydroxamic acid, and trichostatin A) activate transcription of the cyclin-dependent kinase inhibitor p21WAF1 (15, 17, 18) and induction of p21WAF1 by HDACi requires the Sp-1 binding site and is associated with the accumulation of acetylated histones in the promoter region of the gene (17–20).

In this study, microarray analysis revealed that FK228 activated peroxiredoxin 1 (Prdx1) gene expression, Prdx1 being a member of the peroxiredoxin family of antioxidant enzymes (21–25) in both TE2 and T.Tn cell lines. The Sp-1 binding site

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of the Prdx1 promoter was acetylated by FK228. Taken together, we propose a novel antitumor mechanism of FK228 that induces cell growth arrest and/or apoptosis induction augmented by Prdx1 activation with promoter acetylation in human esophageal cancer cell lines and suggest its applicability to esophageal cancer treatment.

Materials and Methods

Cell culture and chemicals. The human esophageal cell lines were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% FCS. T.Tn cells were from Japanese Cancer Research Resources Bank. TE2 cells were kindly provided by Dr. T. Nishihira

Table 1. TUNEL-positive cells for apoptotic index

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<th></th>
<th>TTN</th>
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<td>0 h</td>
<td>1.50 ± 0.30</td>
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<tr>
<td>24 h</td>
<td>3.17 ± 0.45</td>
<td>6.20 ± 0.30</td>
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<tr>
<td>72 h</td>
<td>3.23 ± 0.35</td>
<td>39.4 ± 1.88</td>
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Table 1. TUNEL-positive cells for apoptotic index

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<tr>
<td>0 h</td>
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<td>24 h</td>
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<td>5.60 ± 0.17</td>
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<tr>
<td>72 h</td>
<td>3.20 ± 0.36</td>
<td>50.9 ± 2.16</td>
<td>&lt;0.0001</td>
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NOTE: The apoptotic index was defined as the percentage of positive cells in 1,000 cells from three arbitrary microscopic fields. Data are expressed as mean ± SD. Statistical significance was evaluated by unpaired Student’s t test.
FK228 was dissolved in 100% ethanol and diluted with each experimental medium. In in vitro studies, FK228 was dissolved and diluted with 10% polyoxymethylated (60 mol) hydrogenated castor oil (HCO60) in saline.

**Cytotoxicity assay.** Cell growth was determined with Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan). FK228 was provided by Fujisawa Pharmaceutical Company (Tokyo, Japan). The apoptotic index was defined as the percentage of the sub-G1 population in the cell cycle profile.

Apoptosis was also detected by the analysis of DNA fragmentation for those genes that showed 2-fold changes for at least one time point.

**Cell cycle analysis and terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.** For cell cycle analysis, cells were incubated with propidium iodine in a reaction solution containing 1 mg/mL RNase A. Fluorescence emitted from the propidium iodine-DNA complex was quantified using FACScan (Becton Dickinson Dickinson Medical Systems, Sharon, MA). Apoptosis was determined by the analysis of DNA fragmentation using terminal deoxynucleotidyl transferase–mediated nick end labeling assay (TUNEL) with an in situ Apoptosis Detection kit (Takara, Tokyo, Japan). The apoptotic index was defined as the percentage of positive cells in 1,000 cells from three arbitrary microscopic fields. Western blot analysis. Whole cell pellets were washed thrice in PBS, resuspended in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 5% NP40, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 50 μmol/L leupeptin, 50 μmol/L antipain, 50 μmol/L pepstatin, 50 μmol/L N-acetyl-leucyl-leucyl-norleucinal]. Protein extracts (25 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in PBS containing 5% nonfat dried milk and 0.1% Tween 20. Anti-actin antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Prdx1 polyclonal antibody (1:500; Alexis Biochemicals, Lausen, Switzerland), and anti-β-actin antibody (1:200; Cruz Biotechnology, Santa Cruz, CA) were used. After incubation with a primary antibody for 3 hours at room temperature, the membranes were incubated for 2 hours in the second antibody in PBS and visualized using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

**Messenger RNA preparation and cDNA microarray analysis.** Cells were seeded into a 225 cm² flask and incubated for 48 hours, then treated with or without an IC₅₀ concentration of FK228 and harvested at 0, 6, 12, 24, and 48 hours. Cells were washed with PBS and processed for RNA extraction with RNeasy kit (Qiagen, Inc., Chatsworth, CA). Twenty micrograms of total RNA from cells cultured with FK228 were compared with 20 μg of total RNA from cells cultured without FK228 by using a cDNA microarray consisting of 4,608 distinct cDNA clones generated as described previously (26).

**Table 2.** List of 11 genes up-regulated (>2-fold changes at least at one time point) in both TTn and TE2 cells

<table>
<thead>
<tr>
<th>Genbank accession no.</th>
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<th>Description</th>
<th>Function</th>
<th>Maximum fold change</th>
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<tr>
<td>X67951</td>
<td>Prdx1</td>
<td>Peroxidoxine I</td>
<td>Metabolism/enzyme</td>
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<tr>
<td>U030106</td>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>Cell cycle</td>
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<tr>
<td>M61733</td>
<td>EPB41</td>
<td>Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)</td>
<td>Cytoskeleton</td>
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<td>M59828</td>
<td>HSPA1A</td>
<td>Heat shock 70 kDa protein 1A</td>
<td>Transcription factor</td>
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<td>AK000715</td>
<td>P3TF</td>
<td>Polymerase I and transcript release factor</td>
<td>Translation factor</td>
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<td>X62320</td>
<td>GRN</td>
<td>Granulin</td>
<td>Translation factor</td>
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<td>AL132826</td>
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<td>Fibronectin leucine-rich transmembrane protein 3</td>
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<td>Ribophorin II</td>
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**Table 3.** List of four genes down-regulated (>2-fold changes at least at one time point) in both TTn and TE2 cells

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<th>Genbank accession no.</th>
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<th>Function</th>
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<td>Laminin receptor 1 (ribosomal protein SA, 67 kDa)</td>
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<td>HNRPA1</td>
<td>Heterogeneous nuclear ribonucleoprotein A1</td>
<td>Translation factor</td>
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**Real-time quantitative reverse transcription-PCR.** The expression changes of p21WAF1 and Prdx1 genes were examined by real-time quantitative PCR using the LightCycler technique (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA templates for real-time PCR were synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase and an oligo-dT primer. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene served as internal control. The PCR reaction mixture consisted of DNA Master SYBR Green 1 mix (LightCycler-FastStart DNA Master SYBR Green 1 kit, Roche Diagnostics), containing Taq DNA polymerase, deoxyribonucleotide triphosphate, 3 mmol/L MgCl2, and SYBR green dye), 0.5 mmol/L of each primer, and cDNA. The PCR processes were as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 10 seconds, and elongation at 72°C for 8 to 18 seconds. The Fit Points method provided by the LightCycler software was used to estimate the concentration of each sample. Primers were chosen using Primer3 (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The following primer sequences for each mRNA/expression level of GAPDH. Experiments were done in triplicate and data were presented as mean ± SD.

**Chromatin immunoprecipitation assay.** The chromatin immunoprecipitation assay was done by chromatin immunoprecipitation assay kit (Upstate Biotechnology). Briefly, cells were seeded at 2 × 10⁶ per 10 cm dish and incubated for 48 hours at 37°C under 5% CO2. The cells were then cultured with 2.0 ng/mL of FK228 for 2 hours at 37°C under 5% CO2. Formaldehyde was added to the cells to a final concentration of 1% and they were further incubated for 10 minutes at 37°C. The harvested cells were precleared with salmon sperm DNA/protein A agarose and subsequently incubated with either 5 μL of anti-acetyl-histone H3 or anti-acetyl-histone H4 antibody at 4°C overnight. Chromatin samples were immunoprecipitated using salmon sperm DNA/protein A. Samples were washed once with low salt immune complex wash buffer, once with high salt immune complex wash buffer, once with LiCl immune complex wash buffer, and twice with TE buffer. Immune complexes were eluted and subsequently reverse cross-linked and purified by phenol/chloroform extraction and ethanol precipitation. The supernatant of an immunoprecipitation reaction was done in the absence of any antibody was purified and used as control to show total input DNA. PCR analysis by Prdx1-specific primers was carried out using DNA from chromatin immunoprecipitation experiments and input samples. The PCR processes were as follows: initial denaturation at 95°C for 1 minute followed by 30 cycles of denaturation at 95°C for 30 seconds and annealing and elongation at 68°C for 1.5 minutes. PCR products were run on 2% agarose/ethidium bromide gel. The following primer sequences for Prdx1 were used: P1, 5'-CCCAAGAGGCTCTAGGAGGT-3' and 5'-TGGTGAACAC-GGTGCTGTGCT-3'; P2, 5'-CTGAAATCAGGCTCCAAAGATG-3' and 5'-ACTACGAGTCGTCCTCAAT-3'; P3, 5'-CTCACCTCAAGCTCAGGCAT-3' and 5'-ACTCACACTGCTTCAC-3'; and P4, 5'-ATCACAGTGGACATGCAGA-3' and 5'-ACTCAAGTGGACATGCAGA-3'.

**Small interfering RNA transfections.** The anti-Prdx1 small interfering RNA (siRNA) sequences and the control siRNA sequence used were as follows: Prdx1-si-1, CAGATGTCAGTTTAAAGATA; Prdx1-si-2, CAGCC-GATGGTCAGTTTAAAGATA; control, AATTCTCCGAACGTGTCACGT. A total of 10,000 cells per well were seeded in a 96-well plate 1 day before transfection. Transfection was done with 0.25 μg siRNA and 2 μL transmembrane per well using a transmembrane transfection kit (Qiagen). At 48 hours after transfection, cells were treated in the presence or absence of FK228 for 72 hours. Cell growth was determined by the Cell Counting Kit-8 (Dojindo) as described above. In parallel, RNA from ~ 20,000 cells was isolated using the RNeasy kit (Qiagen) 48 hours after transfection. The relative amount of mRNA was determined by reverse-transcription-PCR and real-time quantitative PCR as described above. All experiments were done in triplicate and data were presented as mean ± SD.

**In vivo animal model.** T.Tn (2.5 × 10⁶ cells) and TE2 (2.5 × 10⁶ cells) were injected into the backs of BALB/c nu/nu mice. When the
estimated tumor weight reached ~200 mg, animals were divided into four groups and treated i.v. thrice at 4-day intervals with 0, 1.0, 2.0, or 3.0 mg/kg, respectively. Tumor weight was calculated from the following formula: tumor weight (mg) = length /C2 width /2.

Xenograft model of real-time quantitative PCR for Prdx1 gene. T.Tn (2.5 × 10⁶ cells) and TE2 (2.5 × 10⁶ cells) were injected into the backs of BALB/c nu/nu mice. When the estimated tumor weight reached ~200 mg, animals were divided into five groups, treated i.v. once with 3.0 mg/kg of FK228, and RNA was extracted from solid tumors at 0, 6, 12, 24, and 48 hours postinjection by RNeasy Mini kit (Qiagen). RNA was reverse-transcribed and PCR analysis for Prdx1 and GAPDH was done as described above.

**Results**

**FK228 showed antiproliferative activity by inducing cell arrest and apoptosis in human esophageal cancer cell lines.** FK228 has apparent antitumor efficacy in several human cancer cell lines (5, 6, 13–16). In this study, we assessed the antitumor activity of FK228 in human esophageal cancer cell lines T.Tn and TE2 and the sensitivities of these cells were evaluated with IC₅₀ values of 0.373 and 0.781 ng/mL, respectively (Fig. 1A). In both T.Tn and TE2 cell lines, the percentage of G₂-M cells was increased at 24 hours with IC₅₀ FK228 treatment, and sub-G₁ cells were increased in a time-dependent manner (Fig. 1B). For TUNEL assay, T.Tn and TE2 cells were cultured with IC₅₀ concentrations of FK228 for 24 and 72 hours, respectively. In both T.Tn and TE2, TUNEL-positive cells increased in a time-dependent manner (Fig. 1C). TUNEL index also showed that FK228 increased sub-G₁ fractions, representing the induction of apoptosis (Table 1).

**FK228 activates Prdx1 and p21⁰⁵⁶¹ expression as detected by microarray analysis.** To determine the target genes of FK228 in esophageal cancer cell lines, we did cDNA microarray analysis. Two-fold or greater alterations in gene expression, either increasing or decreasing, were considered significant. Ninety-three genes were identified in T.Tn and 65 in TE2. Among them, 11 genes were up-regulated (Table 2) and four were down-regulated (Table 3) in both cell lines, respectively. Especially, the expression levels of p21⁰⁵⁶¹ and Prdx1 genes were remarkably increased. To verify whether p21⁰⁵⁶¹ and Prdx1...
expression levels were activated at transcriptional levels, we did quantitative real-time reverse transcription-PCR analysis of these mRNAs and showed that their expressions were activated at both transcriptional (Fig. 2A) and protein levels (Fig. 2B).

**FK228 acetylated histones in the Prdx1 gene promoter.** FK228 treatment increased acetylated histones in several human cancer cell lines (27). First, to determine whether FK228 can induces histone acetylation in human esophageal cancer cells, T.Tn and TE2 cells were exposed to FK228 at various concentrations for 2 hours. As expected, FK228 induced histones H3 and H4 acetylation in a dose-dependent manner, especially apparent at doses higher than 1.0 ng/mL (Fig. 3). Second, we examined whether FK228 acetylates histones H3 and/or H4 in the promoter of *Prdx1* gene by using chromatin immunoprecipitation assay in T.Tn and TE2 cells. As shown in Fig. 4A, primer sets encompassing the *Prdx1* promoter and the coding gene were used to assess the extent of histone acetylation; the acetylation of histone H3 of *Prdx1* promoter and exon 6 (the last exon of *Prdx1*) was increased in both cell lines (Fig. 4B and C). In TE2 cells, histone H4 in P3 was acetylated, whereas in T.Tn cells histone H4 in P1 and P2 regions was acetylated; however, histone H4 in exon 6 was acetylated in both cell lines (Fig. 4B and C). These results indicate that the regions of histone acetylation differ among esophageal cancer cell lines.

**FK228 did not induce accumulation of acetylated histones in chromatin associated with β-actin genes.** To determine whether this effect was selective for a limited number of genes, we examined the level of histone H3 or H4 acetylation in β-actin. The protein expression level of β-actin was not changed by incubation with FK228 (Fig. 5A) and there was no change in the levels of histone H3 or H4 acetylation after culture with FK228 (Fig. 5B). Taken together, these results suggest that the FK228-induced changes in histone acetylation are localized to specific areas of chromatin.

**Silencing Prdx1 gene expression reduced chemosensitization by FK228.** If FK228 induces antiproliferative effects by activating the *Prdx1* gene, *Prdx1* suppression by RNA interference should reduce the antitumor activity of FK228. To examine this hypothesis, *Prdx1*-specific siRNA was transfected into T.Tn and TE2 cells (Fig. 6A and B). Expectedly, the reduction in *Prdx1* expression significantly down-regulated the chemosensitization by FK228 in both cell lines (Fig. 6C).

**Effect of FK228 on the expression of Prdx1 mRNA in T.Tn and TE2 xenografts.** To establish a basis for future clinical applications of FK228, we examined the antitumor activity of FK228 using transplanted human esophageal cancer cell lines in a mouse model. As shown in Fig. 7A, 50 days after the first administration, the growth of TE2 tumors was significantly suppressed compared with the control group. On the other hand, in the case of T.Tn cells, although tumor growth inhibition was observed to some extent, there was no significant difference in tumor size between any of the treated groups. In all cases, there was no effect on the weight of the mice compared with controls. Furthermore, we examined the expression of *Prdx1* mRNA in T.Tn and TE2 xenografts by real-time PCR analysis after the administration of FK228. Even in the xenograft models, the expression of *Prdx1* had its peak at 12 hours postadministration (Fig. 7B), after which the induction levels decreased, reaching basal expression levels at 48 hours.

**Discussion**

We showed for the first time that FK228 induces both apoptosis and cell cycle arrest, resulting in antiproliferative effect on human esophageal cancer cell lines T.Tn and TE2. Further, FK228 activated *Prdx1* gene expression by acetylating histones H3 and H4 of its promoter.

Acetylation and deacetylation of histones play a major role in the regulation of gene transcription and in the modulation of chromatin structure (10). The activity of HDACIs plays a key role in transcriptional modulation for the therapeutic approach that is described as “transcription therapy” (28). However, the molecular events that correlate with growth inhibition by HDACIs are not fully understood and they may differ in each combination of reagent and tumor cells.
In this study, we identified several genes that were significantly up-regulated by FK228, one of the HDACIs, in both T.Tn and TE2 cells. Cyclin-dependent kinase inhibitor \( p21^{WAF1} \) was clearly up-regulated and it has been reported to play an important role in growth arrest, both in G1 and G2-M cell cycle arrest (29). Recent studies also reported that HDACIs exert cell cycle arrest by inducing \( p21^{WAF1} \) in some other cell lines (15, 17, 18). HDACIs, such as trichostatin A, butyrate, suberoylanilide hydroxamic acid, and FK228, induce the acetylation of histones H3 and H4 in the promoter region including the Sp1-binding sites, also called the GC-box, which are important for the expression of the human \( p21^{WAF1} \) gene (17–20). Further experiments are required for revealing the mechanism underlying how \( p21^{WAF1} \) and \( Prdx1 \) up-regulation are directly or indirectly related to apoptosis induction.

In addition, FK228 seems to activate \( Prdx1 \) genes at both transcriptional and protein levels. Peroxiredoxines are a novel family of peroxidases that can reduce \( \text{H}_2\text{O}_2 \) using an electron donor from thioredoxin and/or glutathione (21). Peroxiredoxine has been identified and divided into six groups in mammals (30). \( Prdx1 \) is a highly conserved protein that is up-regulated in ras oncogene–transformed primary mammary epithelial cells (31) and is highly expressed during proliferation (32). Recently, \( Prdx1 \) was reported to inhibit c-Abl kinase activity by interacting with its SH3 domain (22) and to relate to certain c-Myc–dependent functions by interacting with its Myc box II domain (23). Thus, \( Prdx1 \) is a potent tumor suppressor gene. It was also reported that lower expression of \( Prdx1 \) in the tumor correlates to larger tumor size, lymph node metastasis, and clinically advanced stages (24), and that \( Prdx1 \) knockout mice generate malignancies in intestines, lymphomas, and sarcomas (25).

Thus, what is the mechanism of \( Prdx1 \) activation by FK228? One possibility may be that FK228 acetylates histones H3 and/or H4 of promoter region, including the Sp1-binding sites, also called the GC-box, which are important for the expression of the human \( p21^{WAF1} \) gene (17–20). Further experiments are required for revealing the mechanism underlying how \( p21^{WAF1} \) and \( Prdx1 \) up-regulation are directly or indirectly related to apoptosis induction.

In summary, FK228 induces growth inhibition and apoptosis in human esophageal squamous cancer cells, presumably by activating the \( Prdx1 \) gene with histones H3 and H4 acetylation of its promoter. Our findings will provide some clue that may be helpful for the future clinical application of HDACIs in the treatment of esophageal cancer.

References


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