The histone deacetylase inhibitor FR901228 induces caspase-dependent apoptosis via the mitochondrial pathway in small cell lung cancer cells

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Abstract
Histone deacetylase inhibitors modulate the transcription of target genes and represent a new class of anticancer agents. The histone deacetylase inhibitor FR901228 has been reported to show antiproliferative and apoptotic effects in various malignancies including small cell lung cancer (SCLC) in vitro; however, the underlying mechanism is not fully understood. BCL-2 and BCL-XL are antiapoptotic proteins, of which overexpression has been reported to confer resistance to anticancer agents. High levels of BCL-2 and BCL-XL are frequently expressed in SCLC tumors. The present study was designed to clarify the apoptotic pathway of FR901228 in SCLC cells in vitro. FR901228 induced apoptosis in three SCLC cell lines after 24 hours of treatment. FR901228 activated caspase-9 and caspase-3 but not caspase-8, and the caspase-3 inhibitor Z-DEVD-fmk blocked the cytotoxicity of FR901228. FR901228 down-regulated the expression of bcl-2 and bcl-xL mRNA through de novo protein synthesis and suppressed the expression of BCL-2 and BCL-XL proteins. In addition, the combination of bcl-2 antisense oligonucleotides with FR901228 enhanced FR901228-induced caspase-3 activity and cytotoxicity. These findings suggest that FR901228 induces caspase-dependent apoptosis via the mitochondrial pathway rather than the death receptor pathway. Considering the possible contributions of BCL-2 and BCL-XL to multidrug resistance, FR901228 is a promising agent in the treatment of refractory as well as primary SCLC tumors. [Mol Cancer Ther 2004;3(11):1397–402]

Introduction
Histones comprise the core protein of the nucleosome wrapped in DNA, and acetylation of histones is regulated by histone acetyltransferase and histone deacetylase (HDAC). Inhibition of HDAC modulates the transcription of target genes through the relaxation of DNA conformation (1, 2). FR901228 [((E)-(15S,105S,21R)-7-[((Z)-ethyldiene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,19,22-pentanone; depsipeptide] is a novel HDAC inhibitor, which was originally isolated from the fermentation broth of Chromobacterium violaceum (1, 2). FR901228 has been reported to inhibit HDAC1 and HDAC2 class I enzymes (3), induce cell differentiation, and enhance cell death in several types of cancer cells (2). FR901228 is currently under clinical trial with solid and hematologic malignancies (4, 5). We reported recently that FR901228 effectively induced apoptosis and inhibited the proliferation of small cell lung cancer (SCLC) cells in vitro (6). However, the exact apoptotic pathway of FR901228 remains undetermined.

Apoptosis is mainly mediated by a family of caspases, with two pathways leading to caspase activation: the death receptor pathway and the mitochondrial pathway (7). The death receptor pathway is initiated by ligations of transmembrane death receptors such as the Fas receptor, which activates caspase-8 and subsequently effector caspase-3. The mitochondrial pathway requires disruption of the mitochondrial membrane to release cytochrome c. Cytochrome c functions with Apaf-1 to activate caspase-9, thereby promoting the activation of effector caspase-3. The BCL-2 family of proteins is known to regulate the release of mitochondrial cytochrome c: BCL-2 and BCL-XL prevent the release of cytochrome c, whereas BAX promotes release. BCL-2 and BCL-XL function as antiapoptotic proteins, and high levels of BCL-2 and BCL-XL are frequently expressed in SCLC tumors (8, 9). Several in vitro studies have shown that BCL-2 is critical to the survival of SCLC cells (10, 11) and that overexpression of BCL-2 and BCL-XL confers resistance to anticancer agents in SCLC cells (12–14). The present study was designed to clarify the apoptotic pathway of FR901228 in SCLC cells in vitro. Here, we showed that FR901228 induced caspase-dependent apoptosis through the mitochondrial pathway, accompanied by decreased BCL-2 and BCL-XL expression in SCLC cell lines.
Materials and Methods

Cell Culture
RPMI 1640 and FCS were obtained from Life Technologies (Tokyo, Japan). FR901228 was obtained from the Fujisawa Pharmaceutical Co. (Osaka, Japan). Human SCLC cell lines of H69 and H526 cells were obtained from the American Type Culture Collection (Rockville, MD), and PC-6 cells of a human SCLC cell line were kindly provided by Dr. Akiko Tohgo (Daichi Pharmaceutical Co., Tokyo, Japan). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in RPMI 1640 with 10% FCS.

Measurement of Cell Viability
Cell viability was determined by the WST-1 assay (Boehringer Mannheim, Mannheim, Germany) as described previously (6). Cells were incubated with FR901228 (1–10 nmol/L). Clinical trials with FR901228 showed that the serum levels reached 500 ng/mL (920 nmol/L) and that the free FR901228 concentration exceeded 50 ng/mL (92 nmol/L; ref. 5). Accordingly, the concentrations used in the present study are clinically achievable.

Analysis of Apoptosis
Apoptosis was determined by both the sub-G₁ population in the cell cycle profile and the fragmented DNA. For the cell cycle experiment, cells were incubated with FR901228 (10 nmol/L) for 48 hours. Cells were then mixed with propidium iodide (50 µg/mL), and fluorescence was quantified using a flow cytometer (Becton Dickinson Medical Systems, Sharon, MA). DNA fragmentation was determined by the Apoptosis Ladder Detection Kit (Wako Co., Osaka, Japan). Briefly, cells (1 × 10⁵) were lysed in a solution containing RNase and protein digestion enzymes and incubated at 50°C for 30 minutes. DNA was extracted by isopropl alcohol, and ethanol precipitation was performed. The extracts were electrophoresed on 1.5% agarose gels and stained with SYBER Green I (Molecular Probes, Inc., Eugene, OR). DNA ladders (126 bp) were used as control.

Measurement of Caspase Activity
A colorimetric reaction assay kit (R&D Systems, Minneapolis, MN) was used to determine the enzymatic activity of caspases-3, caspase-8, and caspase-9 according to the manufacturer’s instructions. Briefly, the supernatant of the cell lysate was incubated with the caspase-specific substrate conjugated to the color reporter molecule, and the substrate cleavage was measured at 405 nm using a microplate reader. To further evaluate the role of caspase-3 on FR901228-induced apoptosis, cells were incubated with Z-DEVD-fmk (2 µmol/L, VioVision, Mountain View, CA), the caspase-3 specific inhibitor, for 2 hours and were then treated with FR901228 (5 nmol/L) for 24 hours.

Reverse Transcription-PCR
The target sequence for each mRNA was separately amplified with gene-specific primers according to the protocol provided by the manufacturer (Thermoscript RT-PCR System; Life Technologies, Inc., Carlsbad, CA). The PCR primer sets were as follows (15): 5' - GGTGGCACCCTGTTGTCACCT-3' and 5' - CCCTCACTTTGTCGCCCAAGATA GG-3' for bcl-xL, and 5' - CTGACATTTTCTGACGGCCAGC-3' and 5' - TCAAGCCCATCCTTCCAGA-3' for bax. The reaction conditions were 32 cycles at 94°C for 40 seconds, 60°C for 40 seconds, and 72°C for 60 seconds. The amplified products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Western Blot Analysis
Whole cell proteins were electrophoresed on 12.5% polyacrylamide gel and electrotransferred to nitrocellular membranes. The membranes were incubated with either mouse anti-BCL-2 antibody (Oncogene Research Products, Boston, MA) or mouse anti-BCL-XL antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, membranes were incubated with peroxidase-conjugated goat anti-mouse secondary antibody (Amersham Biosciences, Buckinghamshire, United Kingdom). The immunocomplexes were visualized using the Enhanced Chemiluminescence Plus Western detection system (Amersham Biosciences).

Treatment with bcl-2 Antisense Oligonucleotides
The role of BCL-2 on FR901228-induced apoptosis was evaluated using antisense oligonucleotides. The phosphorothioate oligonucleotides were provided by Amersham Pharmacia Biotech (Tokyo, Japan). The sequences were as follows: 5' - TCTCCCAGGTCCGCAAT-3' and control reverse sense oligonucleotides (5' - TACGGTGCGGCCACCT-3'). The sequence of bcl-2 antisense is complementary to the first six codons of the bcl-2 mRNA and is widely known as G3139 in clinical trials (16). The oligonucleotides were delivered to H69 cells in the form of complexes with the cationic liposome, DMRIE-C reagent (Life Technologies, Tokyo, Japan). The oligonucleotides were premixed with DMRIE-C in Opti-MEM medium (Life Technologies, Tokyo, Japan) for 45 minutes at room temperature. Subconfluent H69 cells were transfected with the prepared complex in Opti-MEM medium for 4 hours at 37°C. Then, cells were incubated with the addition of 10% FCS. Twenty-four hours after the start of transfection, cells were treated with 5 nmol/L FR901228 for an additional 24 hours.

Statistical Analysis
Data are means ± SD, and differences were evaluated by Dunnet's test and Spearman correlation analysis. Data were analyzed with the StatView software program (version 5.0; SAS Institute Inc., Cary, NC), and two-tailed P < 0.05 was considered significant.

Results

Apoptotic Effects of FR901228 in SCLC Cells
The growth of H69, H526, and PC-6 cells was inhibited by FR901228 (1–10 nmol/L) for 48 hours in a dose-dependent manner and the IC₅₀ ranged from 1 to 5 nmol/L (Table 1). These concentrations of FR901228 were clinically achievable in recent phase I trials (5) as reported previously. Cell cycle analysis revealed that sub-G₁ populations showing hypoploid DNA increased with...
treatment with FR901228 (10 nmol/L) for 48 hours (Fig. 1A). In a time course experiment, DNA fragmentation was observed in H526 cells treated with FR901228 for only 24 hours (Fig. 1B).

We examined whether FR901228-induced apoptosis was caspase dependent. The activity of caspase-3 as an effector of apoptosis increased after 24 hours of treatment with FR901228 in a dose-dependent manner (data not shown). In a time course experiment, the activation of caspase-3 became evident 12 hours after treatment with FR901228 (Fig. 2).

The caspase-3 inhibitor Z-DEVD-fmk blocked FR901228-induced caspase-3 activation (Fig. 3A) and cytotoxicity (Fig. 3B). Next, we investigated whether the activation of caspase-3 occurred via the death receptor pathway or the mitochondrial pathway. FR901228 activated caspase-9, which represents the mitochondrial pathway, in comparison with caspase-8, which represents the death receptor pathway (Fig. 4). FR901228 mainly induced caspase-dependent apoptosis via the mitochondrial pathway.

Effects of FR901228 on the BCL-2 Family in SCLC Cells

The effects of FR901228 on the BCL-2 family were investigated because BCL-2 overexpression in SCLC regulates the mitochondrial apoptotic pathway. Treatment with FR901228 for 24 hours suppressed the expression of bcl-2 and bcl-xL mRNA but not totally reduced it (Fig. 5). In contrast, treatment with FR901228 did not modify bax mRNA (Fig. 5). Western blot analysis revealed that FR901228 subsequently decreased the levels of BCL-2 and BCL-XL proteins for 48 hours (Fig. 6).

Furthermore, we examined whether the inhibition of bcl-2 mRNA was necessary for de novo protein synthesis. FR901228 suppressed the expression of bcl-2 mRNA as early as 6 hours after treatment. The protein synthesis inhibitor cycloheximide blocked the decrease in bcl-2 mRNA induced by FR901228 (Fig. 7). Early inhibition of bcl-2 mRNA required newly synthesized proteins, unlike a consequence of apoptosis, which began 24 hours after treatment.

To clarify the role of the BCL-2 protein on cell survival in SCLC cells, we treated H69 cells with bcl-2 antisense oligonucleotides. In this experiment, we used the bcl-2 antisense sequence G3139, which is currently under clinical trial (16). Treatment with bcl-2 antisense oligonucleotides (200 nmol/L) inhibited the levels of BCL-2 protein to 53% of untreated cells for 48 hours, whereas treatment with control oligonucleotides did not reduce the levels (Fig. 8A).

Table 1. Antiproliferative effects of FR901228 in SCLC cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC_{50} of FR901228 (nmol/L), mean ± SD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>24-h exposure</td>
</tr>
<tr>
<td>H69</td>
<td>4.76 ± 0.77</td>
</tr>
<tr>
<td>H526</td>
<td>4.00 ± 0.95</td>
</tr>
<tr>
<td>PC-6</td>
<td>2.76 ± 0.24</td>
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Figure 2. Time course of caspase-3 activity after treatment with FR901228 (10 nmol/L). The activity of caspase-3 is expressed as the value relative to untreated cells. Open columns, H69 cells; filled columns, H526 cells. Columns, mean; bars, SD.

Figure 3. The caspase-3 inhibitor Z-DEVD-fmk blocked the activation of caspase-3 and the decrease in viability induced by FR901228. Cells were pretreated with Z-DEVD-fmk (2 μmol/L) and then incubated with FR901228 (5 nmol/L) for 24 hours. A, activity of caspase-3 is expressed as the value relative to untreated control cells. B, number of viable cells is expressed as the value relative to untreated control cells. Columns, mean; bars, SD.

Figure 1. A, cell cycle profile. Percentage of cells with hypoploid DNA (sub-G1 fraction). Cells were treated with FR901228 (10 nmol/L) for 48 hours. B, time course of DNA fragmentation. H526 cells were treated with FR901228 for up to 48 hours (10 nmol/L).
The 48-hour treatment with bcl-2 antisense oligonucleotides increased caspase-3 activity (data not shown) and decreased viability in a dose-dependent manner ($P < 0.01$; Fig. 8B). In addition, the combination of bcl-2 antisense oligonucleotides with FR901228 enhanced caspase-3 activity ($P < 0.01$; Fig. 8C) and the cytotoxicity induced by FR901228 ($P < 0.01$; Fig. 8D).

**Discussion**

SCLC usually responds to initial chemotherapy; however, it relapses and shows resistance to various anticancer drugs. Thus, the limitations of conventional therapy require the introduction of novel targeted therapies. HDAC inhibitors modulate the transcription of target genes and represent a new class of anticancer agents. We reported recently that the novel HDAC inhibitor FR901228 effectively inhibited cell growth in SCLC cells, accompanied by decreased telomerase activity (6). In a present study, FR901228 induced caspase-dependent apoptosis occurred via the mitochondrial pathway rather than the death receptor pathway in SCLC cells. In addition, FR901228 down-regulated the expression of BCL-2 and BCL-XL proteins, which probably contributed to the potentiation of apoptosis.

FR901228 efficiently activated caspase-9 and effector caspase-3 but not caspase-8 in SCLC cells, and the caspase-3 inhibitor prevented the cytotoxic effects of FR901228. These findings suggest that FR901228 triggers caspase-dependent apoptosis mainly via the mitochondrial pathway, which is different from the results of recent studies (17–19). In osteosarcoma and chronic lymphocytic leukemia cells, FR901228-induced apoptosis is caspase dependent and is selectively involved in the death receptor pathway initiating caspase-8 activation (17, 18). In acute lymphocytic leukemia cells, FR901228 induces apoptosis via activation of the mitochondrial pathway, which is not caspase dependent (19). However, SCLC cells have a frequent loss of Fas receptor and caspase-8 protein due to promoter DNA methylation and are highly resistant to the death receptor pathway (20, 21). In addition, the translocation of apoptosis-inducing factor into the nucleus, which represents a caspase-independent pathway, is less pronounced in SCLC cells than in non-SCLC cells after treatment with staurosporine (22). Thus, the apoptotic pathway of FR901228 may vary according to the type of malignancy with different apoptosis-activating cascades.

**Figure 4.** The activities of caspase-3, caspase-8, and caspase-9 are expressed as values relative to untreated control cells. Open columns, untreated control cells; filled columns, cells treated with FR901228 (10 nmol/L) for 24 hours. Columns, mean; bars, SD.

**Figure 5.** Reverse transcription-PCR analysis for bcl-2, bcl-xl, and bax mRNA. Cells were treated with FR901228 (5–10 nmol/L) for 24 hours.

**Figure 6.** Western blot of BCL-2 and BCL-XL proteins. Cells were treated with FR901228 (5–10 nmol/L) for 48 hours.

**Figure 7.** Cycloheximide of a protein synthesis inhibitor blocked the suppression of bcl-2 mRNA induced by FR901228. PC-6 cells were pretreated with cycloheximide (0.25 µg/mL) and then incubated with FR901228 (10 nmol/L) for 6 hours.
FR901228 suppressed the expression of bcl-2 and bcl-xL mRNA and the proteins in SCLC cells. Several investigators have reported similar findings in other malignancies (23, 24). The HDAC inhibitors of trichostatin A and sodium butyrate down-regulate BCL-2 in hepatoma cell lines and BCL-XL in mesothelioma cell lines (23, 24). The ectopic overexpression of BCL-2 or BCL-XL in malignant cell lines blocks apoptosis by FR901228 and another HDAC inhibitor, suberoylanilide hydroxamic acid (19, 25–27). Almost all SCLC cells expressed both BCL-2 and BCL-XL (8, 9). Previous studies as well as our study using antisense strategy showed that BCL-2 is more critical to the survival of SCLC cells (10, 11). In addition, SCLC cells overexpressing BCL-2 or BCL-XL show resistance to various anticancer agents in vitro (12–14). Although the functional differences between BCL-2 and BCL-XL are not well known, some reports have suggested that BCL-2 and BCL-XL can function reciprocally (28, 29). Accordingly, simultaneous inhibition of BCL-2 and BCL-XL by FR901228 may be more useful in the treatment of SCLC.

In the present study, the apoptotic signals preceding the mitochondrial pathway remain unknown. Considering the long half-life of the BCL-2 protein (10–20 hours; ref. 30), decreased BCL-2 and BCL-XL do not seem to be direct triggers of rapid apoptosis. The tumor suppressor p53 is known to regulate apoptosis upstream of the mitochondria (7); however, the role of p53 in apoptosis induced by HDAC inhibitors is controversial (25, 31). FR901228 induced apoptosis in SCLC cells, irrespective of p53 status: wild-type p53 in H69 cells and mutant p53 in H526 cells (6). At least, p53 is unlikely to confer FR901228-induced apoptosis in SCLC cells. It has been reported that the cytotoxic effects of HDAC inhibitors are closely correlated with aberrant mitosis (27, 32, 33). Our previous study also revealed that FR901228 arrested cells at the M phase, concomitant with apoptosis (6). Several investigators have reported that cells with intact G1 or G2 checkpoint mechanisms are resistant to HDAC inhibitors (19, 27). SCLC has a frequent loss of retinoblastoma and 14-3-3 proteins, leading to G1 and G2 checkpoint defects (34). In this respect, SCLC might be a good target tumor for treatment with HDAC inhibitors.

It remains unclear whether the cytotoxic effects of FR901228 are directly mediated through acetylation of DNA histone. FR901228 may have unknown mechanisms of antineoplastic action as a natural product. FR901228 acetylates heat shock protein 90, which promotes the degradation of the client oncoproteins (35). In addition, FR901228 induces formation of aberrant spindles without chromosome attachment (36). The nontranscriptional effects of FR901228 may be partly involved in the cytotoxicity.

A phase I trial of FR901228 was conducted at the National Cancer Institute, and FR901228 was shown to be well tolerated. The major observed toxicities included fatigue, nausea, vomiting, neutropenia, thrombocytopenia, and ST-T wave changes on electrocardiograms without clinical significance (5). In the phase I trial, responses were observed in patients with T-cell lymphoma (4), and a phase II trial has been done in these patients. FR901228 is administered using a 4-hour i.v. infusion on days 1, 8, and 15 of a 28-day cycle. In a recent report, FR901228 has particular activity in cutaneous and peripheral T-cell lymphomas; 7 of 14 patients with cutaneous T-cell lymphoma achieved objective responses for an overall response rate of 50%, and 4 of 17 patients with peripheral T-cell lymphoma achieved a partial response for an overall response rate of 24% (37).

In contrast, there are no available data to evaluate the clinical activity of FR901228 for SCLC. Multidrug resistance is a major obstacle in chemotherapy for SCLC. In a previous study, FR901228 overcame etoposide and irinotecan resistance mediated by MRP1/ABCC1 and BCRP/ABCG2 drug efflux pumps, respectively (6). Furthermore, the present study showed that FR901228 suppressed the expression of BCL-2 and BCL-XL, which possibly confer multidrug resistance. Taken together, these findings suggest that FR901228 may be clinically promising for treating refractory as well as primary SCLC tumors. The present study supports future clinical trials of FR901228 as a single agent or in combination with other therapies for SCLC.
References


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