ORIGINAL ARTICLE

The cytotoxic macrolide FD-891 induces caspase-8-dependent mitochondrial release of cytochrome *c* and subsequent apoptosis in human leukemia Jurkat cells

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The 16-membered macrolide FD-891 exerts cytotoxicity toward several cancer cell lines. In this study, we showed that FD-891 induces apoptosis in various human cancer cell lines. Human leukemia Jurkat cells were highly sensitive to FD-891, exhibiting caspase activation and mitochondrial release of cytochrome *c* into the cytosol at early time points after exposure to FD-891. By contrast, Jurkat cells deficient in caspase-8 were resistant to FD-891-induced apoptosis and manifested little induction of cytochrome *c* release as well as caspase-9 processing. Consistent with these results, the overexpression of the Bcl-2 family member Bcl-x_L or the caspase-8 modulator c-FLIP_L markedly prevented FD-891-induced apoptosis. These results clearly demonstrate that FD-891 triggers caspase-8-dependent mitochondrial release of cytochrome *c* and subsequent apoptosis in Jurkat cells.

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INTRODUCTION

Apoptosis is a physiological process that has an important role in the homeostasis of mature tissues. Caspases are a family of cysteine proteinases that are essential for apoptosis and act by cleaving selected substrates.^{1–3} Initiator caspase-8 is converted into active forms via dimerization and self-processing, and subsequently cleaves various substrates, such as effector caspases and BH3-only protein Bid, to produce their active forms.^{1–3} Truncated Bid is translocated to mitochondria where it initiates the release of pro-apoptotic proteins into the cytosol.^{4,5} Mitochondrial pro-apoptotic proteins, such as cytochrome *c*, are confined to intermembrane space and liberated into the cytosol in response to apoptotic stimuli.^{6,7} Cytochrome *c* collaborates with adaptor protein Apaf-1 to activate initiator caspase-9.^{8–10} Effector caspases-3, -6, and -7 are processed into their active forms by activated caspase-9 and the activated caspases mediate the proteolytic cleavage of cellular proteins required for apoptosis.¹¹

The cytotoxic macrolide FD-891 was originally isolated from the fermentation broth of *Streptomyces graminofaciens* A-8890.^{12,13} The absolute structure of FD-891 was determined in our earlier work (Figure 1a)^{14,15} and its planar structure turned out to be the same as that of BE-45653.¹⁶ The total synthesis of FD-891 was recently reported.^{17–19} It has been shown that FD-891 exerts cytotoxicity

toward several cancer cell lines.^{12,19} Moreover, as regards immunosuppressive activity, FD-891 prevents cytotoxic T lymphocyte (CTL) mediated killing pathway.²⁰ In this study, we investigated the molecular mechanism underlying the cytotoxicity of FD-891. Our present results demonstrate that FD-891 triggers caspase-8-dependent mitochondrial release of cytochrome *c* and subsequent apoptosis in a human leukemia cell line highly sensitive to FD-891.

MATERIALS AND METHODS

Cells

Human lung carcinoma A549 cells, human fibrosarcoma HT-1080 cells and human rhabdomyosarcoma Kym-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan). Caspase-8-deficient Jurkat cell clone (J9.2),²¹ c-FLIP_L-transfected Jurkat cell clone (JFL2),²² and Bcl-x_L-transfected Jurkat cell clone (Jurkat-Bcl-x_L #1)²³ were described previously. Human cancer cell lines were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS, USA), penicillin G (100 U ml⁻¹) (Sigma-Aldrich, St Louis, MO, USA) and streptomycin (100 µg ml⁻¹) (Sigma-Aldrich).

Reagents

FD-891 was purified from the fermentation broth of *S. graminofaciens* A-8890 as previously described.¹² Doxorubicin, etoposide and vincristine were purchased from Sigma-Aldrich.

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Antibodies

Antibodies to caspase-3 (H-277; Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-6 (3E8; Medical & Biological Laboratories (MBL), Nagoya, Japan), caspase-7 (11E4; Sigma-Aldrich), caspase-8 (5F7; MBL), caspase-9 (5B4; MBL), cytochrome *c* (7H8.2C12; BD Biosciences, Franklin Lakes, NJ, USA), and PARP (C-2-10; Sigma-Aldrich) were commercially obtained.

Assay for cell viability

Cells were pulsed with 500 $\mu\text{g ml}^{-1}$ of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for 4 h. MTT formazan was solubilized with 10%

sodium dodecyl sulfate (SDS) overnight. Absorbance at 595 nm was measured with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Cell viability (%) was calculated as [(experimental absorbance–background absorbance)/(control absorbance–background absorbance)] $\times$ 100.

Assay for apoptosis

Cells were fixed with PBS containing 4% paraformaldehyde at 4 °C overnight, and then stained with 300 μM Hoechst 33342 (Calbiochem, San Diego, CA, USA). Nuclear morphology was observed under a fluorescence light microscope (Axiovert 200M; Carl Zeiss, Jena, Germany). Apoptotic cells (%) were calculated as (condensed nuclei/total nuclei) $\times$ 100.

Preparation of cell lysates

Cells were washed with PBS and lysed in Triton X-100 lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM DTT, 2 mM sodium orthovanadate, and the protease inhibitor mixture Complete (Roche Diagnostics,

Figure 1 FD-891 inhibits cell proliferation. (a) Structure of FD-891. (b) and (c) Jurkat cells were incubated with various concentrations of FD-891 for 72 h (b). Jurkat cells were incubated with FD-891 (1 μM) for the indicated times (c). Cell viability (%) was measured by MTT assay. Data points represent means $\pm$ s.d. of triplicate cultures.

Table 1 Effect of FD-891 on proliferation of human cancer cell lines

Cell line	Source	IC ₅₀ (nM)
A549	Lung carcinoma	310 $\pm$ 70
HeLa	Cervix adenocarcinoma	140 $\pm$ 70
HL-60	Acute promyelocytic leukemia	35 $\pm$ 29
HT-1080	Fibrosarcoma	130 $\pm$ 70
Jurkat	Acute T cell leukemia	32 $\pm$ 24
K-562	chronic myelogenous leukemia	1000 $\pm$ 710
Kym-1	Rhabdosarcoma	250 $\pm$ 80
MCF7	Breast adenocarcinoma	220 $\pm$ 100
Raji	Burkitt's lymphoma	170 $\pm$ 140
THP-1	Acute monocytic lymphoma	30 $\pm$ 20
U-937	Histiocytic lymphoma	41 $\pm$ 34

Human cancer cell lines were incubated with serial dilution of FD-891 for 3 days. Cell viability (%) was measured by MTT assay. The IC₅₀ values (mean $\pm$ s.d.) were calculated from three to four independent experiments.

Figure 2 FD-891 induces apoptosis. (a) and (b) Jurkat cells were incubated with various concentrations of FD-891 for 24 h (a). Jurkat cells were incubated with FD-891 (1 μM) for the indicated times (b). Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent means $\pm$ s.d. of triplicate determinations. (c) Human cancer cell lines were incubated with various concentrations of FD-891 for 24 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. The results are expressed as means $\pm$ s.d. of three independent experiments.

Mannheim, Germany). Postnuclear lysates were collected as supernatants by centrifugation (10 000 g, 5 min). Alternatively, cells were washed with PBS and lysed with digitonin lysis buffer (10 mM Hepes-KOH (pH 7.2), 100 μ M digitonin, 250 mM sucrose, 1 mM DTT, 5 mM EGTA, 2 mM $MgCl_2$, 50 mM NaCl and the protease inhibitor mixture Complete) on ice for 15 min. After centrifugation (10 000 g, 5 min), cell lysates containing the cytosol were recovered as supernatants. Pellets were washed once with digitonin lysis buffer and treated with Triton X-100 lysis buffer on ice for 15 min. After centrifugation (10 000 g, 5 min), cell lysates containing the mitochondria were collected as supernatants (mitochondrial fraction).

Western blotting

Protein samples (30 μ g per lane) were separated by SDS-PAGE and transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The transferred membranes were stained with Ponceau S and checked for equal loading of proteins before antibody reaction. The membranes were incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), followed by analysis using ECL western blotting detection reagents (GE Healthcare).

RESULTS

FD-891 inhibits proliferation of human cancer cell lines

It has been shown that FD-891 exerts cytotoxicity toward several cancer cell lines.^{12,19} To investigate the inhibition specificity of FD-891

on cancer cell proliferation, various human cancer cell lines were incubated in the presence of FD-891 for 72 h and cell viability was measured by the MTT assay. Although FD-891 prevented cell proliferation at IC_{50} values of 130 to 1000 nM in most of the cancer cell lines, four leukemia cell lines (HL-60, Jurkat, THP-1 and U-937) were highly sensitive to FD-891 at IC_{50} values of approximately 30 to 40 nM (Table 1). In Jurkat cells, FD-891 decreased cell viability by more than 80% at concentrations higher than 100 nM when incubated for 72 h (Figure 1b). Kinetic studies revealed that FD-891 gradually reduced cell viability during incubation for 24 to 72 h (Figure 1c).

FD-891 induces apoptosis of human cancer cell lines

To investigate whether FD-891 is able to induce apoptosis, Jurkat cells were incubated with various concentrations of FD-891 for 24 h and nuclear morphological changes were analyzed by fluorescence microscopy after Hoechst 33342 staining. FD-891 at concentrations higher than 100 nM was found to induce nuclear condensation and fragmentation characteristic of apoptosis within 24 h (Figure 2a). Kinetic studies revealed that FD-891 initiated apoptosis within 6 h and that the number of apoptotic cells steadily increased during 24 h incubation (Figure 2b). Various human cancer cell lines were also found to undergo apoptosis upon treatment with FD-891. FD-891 was able to induce apoptosis very strongly in two leukemia cell lines (that is, Jurkat and HL-60), whereas it induced moderate-to-weak apoptosis in

Figure 3 FD-891 induces activation of effector caspases. (a–c) Jurkat cells were incubated with FD-891 (1 μ M) for the indicated times. The expression of PARP (a), caspases-3, -6, -7 (b), and caspase-9 (c) was analyzed by western blotting. (d) Jurkat cells were incubated with various concentrations of doxorubicin, etoposide, vincristine, and FD-891 for 24 h. Cell viability (%) was measured by MTT assay. The results are means $\pm$ s.d. of triplicate cultures. (e) Jurkat cells were incubated with various concentrations of doxorubicin, etoposide, vincristine and FD-891 for 24 h. The expression of caspases-3, -6 and -7 was analyzed by western blotting.

the other cancer cell lines (Figure 2c). Therefore, the results suggest that the induction of apoptosis is correlated with the inhibitory effect of FD-891 on cell proliferation at least in Jurkat and HL-60 cells. However, non-apoptotic cells harboring two nuclei apparently accumulated in FD-891-treated cells (unpublished observation). Therefore, as previously reported,¹⁹ it seems that FD-891 induces cell cycle arrest at the G₂/M phase, and this biological effect may also contribute to its inhibitory effect on cell proliferation. In this study, the molecular mechanism underlying FD-891-induced apoptosis in Jurkat cells was further addressed.

FD-891 induces activation of caspases

Poly(ADP-ribose)polymerase (PARP) is a nuclear protein that is processed by effector caspases during apoptosis.²⁴ When Jurkat cells were incubated with FD-891, full-length PARP (116 kDa) was processed into the cleaved form (85 kDa) during 6 to 24 h incubation (Figure 3a). Likewise, caspase-3, caspase-6 and caspase-7 were processed into their active forms in FD-891-treated cells with similar kinetics (Figure 3b). Initiator caspase-9 was also processed into p35 and p37 fragments at earlier time points than other effector caspases when Jurkat cells were exposed to FD-891 (Figure 3c). We previously reported that chemotherapeutic agents, such as doxorubicin, etoposide and vincristine, induce apoptosis in Jurkat cells.²⁵ Similar to FD-891, doxorubicin, etoposide and vincristine were able to induce the processing of caspase-3, caspase-6 and caspase-7 into their active forms (Figure 3e). However, at the concentrations that reduced cell viability to approximately 50% within 24 h, these chemotherapeutic drugs were able to induce the activation of effector caspases more effectively than FD-891 (Figures 3d and e). Thus, the results suggest that FD-891 may induce caspase activation in a manner distinct from doxorubicin, etoposide and vincristine.

FD-891 induces mitochondrial release of cytochrome *c*

Mitochondria has an essential role in the induction of apoptosis by releasing pro-apoptotic proteins, such as cytochrome *c*, into the cytosol.^{6,7} Upon treatment with FD-891, cytochrome *c* was released from the mitochondria into the cytosol in Jurkat cells (Figure 4a). The Bcl-2 family of proteins regulates cytochrome *c* release and subsequent induction of apoptosis.^{6,7} The overexpression of Bcl-x_L markedly inhibited apoptosis induced by FD-891 (Figure 4b). These results suggest that the mitochondrial release of cytochrome *c* is required for FD-891-induced apoptosis.

Caspase-8 is required for mitochondrial release of cytochrome *c* during FD-891-induced apoptosis

Caspase-8 is an initiator caspase that undergoes self-processing into its active forms in response to various stimuli.^{1–3} In Jurkat cells, caspase-8 was processed into p43/p41 fragments during FD-891-induced apoptosis (Figure 5a). However, in contrast to Fas ligand (FasL) stimulation, p18 large fragments were barely detectable in FD-891-treated cells (Figure 5a). To address if caspase-8 is required for FD-891-induced apoptosis, Jurkat cells deficient in caspase-8 were compared with wild-type cells for the induction of apoptosis upon exposure to FD-891. Caspase-8-deficient cells manifested resistance to FD-891-induced apoptosis (Figure 5b). The overexpression of the caspase-8 modulator c-FLIP_L also conferred resistance to FD-891-induced apoptosis in Jurkat cells (Figure 5c), supporting the notion that caspase-8 is required for FD-891-induced apoptosis. In caspase-8-deficient cells, FD-891 barely induced the release of cytochrome *c* into the cytosol (Figure 5d). Consistently, caspase-9 processing was also inhibited in caspase-8-deficient cells treated with FD-891 (Figure 5e).

Figure 4 FD-891 induces mitochondrial release of cytochrome *c*. (a) Jurkat cells were incubated with FD-891 (1 μ M) for 12 h. Cell lysates containing the cytosol and the mitochondria were prepared. The expression of cytochrome *c* was analyzed by western blotting. (b) Wild-type Jurkat cells (black bars) and Bcl-x_L-transfected Jurkat cell clone (gray bars) were incubated with FD-891 (1 μ M) for the indicated times. Apoptotic cells (%) were measured by Hoechst 33342 staining. The results are expressed as means $\pm$ s.d. of triplicate determinations.

Together, the results demonstrate that caspase-8 is required for the release of cytochrome *c* to the cytosol and the subsequent activation of caspase-9.

DISCUSSION

FD-891 was found to induce apoptosis in several human cancer cell lines. In Jurkat cells that were highly sensitive to FD-891, the activation of caspases as well as the mitochondrial release of cytochrome *c* to the cytosol was induced at early time points after treatment with FD-891. Unlike wild-type Jurkat cells, FD-891 barely induced the activation of caspase-9 and the mitochondrial release of cytochrome *c* in caspase-8-deficient Jurkat cells. The overexpression of Bcl-x_L or c-FLIP_L rendered Jurkat cells resistant to FD-891-induced apoptosis. Thus, our present results clearly demonstrate that FD-891 induces caspase-8-dependent mitochondrial release of cytochrome *c* to the cytosol and subsequent apoptosis in Jurkat cells.

It has been shown that FD-891 inhibits cell proliferation in cancer cell lines.^{12,19} Consistent with these findings, we have shown that FD-891 is able to inhibit cell proliferation in many human cancer cell lines at IC₅₀ values of 0.03 to 1 μ M. Human leukemia cell lines, such as Jurkat cells and HeLa cells, were highly sensitive to FD-891 and underwent rapid apoptosis upon treatment with FD-891. However, in some cancer cell lines, FD-891 barely induced apoptosis even at 10 μ M, suggesting that the FD-891-induced apoptosis is cell type-specific. It has been shown that FD-891 induces accumulation of cancer cells at the G₂/M phase during the cell cycle.¹⁹ Therefore, it seems likely that FD-891 predominantly induces cell cycle arrest, thereby inhibiting cell proliferation in cancer cell lines refractory to apoptosis.

Chemotherapeutic drugs, such as doxorubicin, etoposide and vincristine, are known to induce caspase-dependent apoptosis in various types of cancer cells. We previously showed that the over-

Figure 5 Caspase-8 is required for cytochrome *c* release and caspase-9 activation during FD-891-induced apoptosis. **(a)** Jurkat cells were incubated with or without FD-891 (1 μM) or FasL (200 ng ml⁻¹) for the indicated times. The expression of caspase-8 was analyzed by western blotting. **(b)** Wild-type Jurkat cells (black bars) and caspase-8-deficient Jurkat cells (gray bars) were incubated with various concentrations of FD-891 for 24 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. The results are means ± s.d. of triplicate determinations. **(c)** Wild-type Jurkat cells (black bars) and c-FLIP_L-transfected Jurkat cell clone JFL2 (gray bars) were incubated with FD-891 (1 μM) for the indicated times. Apoptotic cells (%) were measured by Hoechst 33342 staining. The results are means ± s.d. of triplicate determinations. **(d)** Wild-type Jurkat cells and caspase-8-deficient Jurkat cells were incubated with FD-891 (1 μM) for the indicated times. Cell lysates containing the cytosol and the mitochondria were prepared. The expression of cytochrome *c* was analyzed by western blotting. **(e)** Wild-type Jurkat cells and caspase-8-deficient Jurkat cells were incubated with FD-891 (1 μM) for the indicated times. The expression of caspase-9 was analyzed by western blotting.

expression of c-FLIP_L does not prevent apoptosis induced by doxorubicin, etoposide and vincristine in Jurkat cells,²⁵ indicating that caspase-8 is dispensable for apoptosis induced by these chemotherapeutic drugs. However, it seems that caspase-8 activation is required

for FD-891-induced apoptosis, as c-FLIP_L overexpression or caspase-8 deficiency markedly prevented FD-891-induced apoptosis. Therefore, as the main apoptosis-signaling pathway, FD-891 is likely to induce the mitochondrial release of cytochrome *c* as well as caspase-9 processing through caspase-8 activation.

In addition to the cytotoxicity toward cancer cells, we have previously shown that FD-891 prevents the interaction between CTLs and target cells, thereby inhibiting CTL-mediated killing pathways.²⁰ Structurally related 16- and 18-membered macrolides (bafilomycin A₁ and concanamycin A, respectively), known as inhibitors of vacuolar-type H⁺-ATPase, manifested inhibitory profiles different from FD-891 in the CTL-mediated killing pathway,²⁶ in agreement with the fact that FD-891 does not affect vacuolar acidification.²⁰ The mechanism of action of FD-891 is also distinct from that of known chemotherapeutic drugs (doxorubicin, etoposide and vincristine). To understand anti-cancer and immunosuppressive activities at the molecular level, the molecular target(s) of FD-891 should be identified.

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