

Induction of a Ribotoxic Stress Response That Stimulates Stress-Activated Protein Kinases by 13-Deoxytedanolide, an Antitumor Marine Macrolide

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13-Deoxytedanolide is a structurally unique macrolide with strong antitumor activity isolated from a marine sponge. Recently, we showed that 13-deoxytedanolide bound to the large subunit of the yeast ribosome and inhibited polypeptide elongation *in vitro*, but the mechanism by which it exerts antitumor activity is still unknown. Here we show that 13-deoxytedanolide strongly induces plasminogen activator inhibitor 1 (PAI-1) promoter-derived gene expression. 13-Deoxytedanolide, unlike TGF- β , did not cause apparent nuclear translocation of Smad2/3, but it relocalized the temperature-sensitive mutant of mouse p53 (p53Val153) from the cytoplasm to the nucleus at a nonpermissive temperature, suggesting that 13-deoxytedanolide inhibits protein synthesis. Indeed, the drug inhibited *in vivo* protein synthesis at low nanomolar concentrations and strongly activated stress-activated protein kinases such as p38 mitogen-activated protein kinase and Jun NH₂-terminal protein kinase (JNK). Anisomycin, a well-known inducer of ribotoxic stress that activates both p38 and JNK, also activated PAI-1 gene expression, while other protein synthesis inhibitors that do not activate the kinases failed to do so. PAI-1 gene expression by 13-deoxytedanolide and anisomycin was blocked by SB202190, a specific inhibitor of p38, and SP600125, an inhibitor of both p38 and JNK. 13-Deoxytedanolide and anisomycin caused activation of apoptosis signal-regulating kinase 1, MKK3/MKK6, and SEK1/MKK4, the regulatory kinases upstream of p38 and JNK. These results suggest that 13-deoxytedanolide, like anisomycin, triggers a ribotoxic stress response that activates stress-activated protein kinase cascades, thereby inducing PAI-1 gene expression and apoptosis.

Key words: anisomycin; Jun NH₂-terminal protein kinase; p38 MAP kinase; p53; TGF- β

Ribotoxic stress response is a conserved cellular reaction to cytotoxic interference with the function of the large ribosomal (23S or 28S) RNA.¹⁾ In mammalian cells, the ribotoxic stress response involves activation of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38 mitogen-activated protein kinase, and subsequent transcriptional induction of immediate early genes such as c-fos and c-jun. The best-known inducer of the ribotoxic stress response is anisomycin, an inhibitor of the eukaryotic peptidyl transferase reaction.^{2,3)} But, inhibition of protein synthesis *per se* does not stimulate JNK and p38. Pactamycin and emetine, which block translational initiation and ribosomal translocation respectively, have been reported to fail to activate JNK. In addition, cycloheximide and puromycin are very weak inducers.⁴⁾ Efficient kinase activation has been achieved with concentrations of anisomycin that inhibited protein synthesis by less than 50%; hence it was concluded that anisomycin activates protein kinases independently of its ability to inhibit protein synthesis.^{5,6)} On the other hand, ricin A chain and α -sarcin, ribotoxic enzymes that catalyze sequence-specific RNA damage in the 28S rRNA, stimulated the ribotoxic stress response.⁴⁾ UV radiation causes specific damage to the 28S rRNA, thus inducing inhibition of protein synthesis and JNK activation in the presence of active ribosomes.⁷⁾ Anisomycin induces rapid apoptosis in human lymphoid cells in contrast with the delayed apoptosis induced by many other protein synthesis inhibitors that do not activate JNK and p38,^{8,9)} suggesting an important role of these kinases in anisomycin-induced apoptosis in tumor cells. Trichothecene mycotoxins also trigger a ribotoxic stress response and activate JNK and p38. Among trichothecenes that strongly inhibit protein synthesis, induction of apoptosis increases linearly with activation of JNK/p38 kinases,

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while derivatives that inhibit protein synthesis without activating JNK/p38 inhibit the induction of apoptosis by trichothecenes and anisomycin.¹⁰ These kinase pathways in response to stress are thought to be important in controlling cancer.^{11,12} It is therefore possible that the ribosome function of sensing ribotoxic stress might be a good target for cancer treatment.

13-Deoxytendanolide (13-DT) is a macrolide isolated from the marine sponge *Mycale adhaerens* that inhibits the proliferation of cultured tumor cells at low nanomolar concentrations. It has been reported that 13-DT slows the growth rate of P388 tumors implanted in mice with a T/C value of 189% at a dose of 0.125 mg/kg.¹³ Although we have reported that 13-DT binds to the 60S large subunit of the yeast ribosome, thereby inhibiting *in vitro* polypeptide elongation, the mechanism that underlies the antitumor activity is poorly understood.¹⁴ During the course of screening for compounds that increase plasminogen activator inhibitor-1 (PAI-1) gene expression, we found that 13-DT induces PAI-1 gene expression in a low nanomolar concentration range. We show that 13-DT strongly activates both p38 and JNK, and that the activation of these kinase pathways might be responsible for PAI-1 gene activation and apoptosis induction. Comparative studies with anisomycin and other protein synthesis inhibitors suggest that 13-DT is a potent protein synthesis inhibitor that triggers a ribotoxic stress response. Our data indicate that 13-DT is the most potent chemical ribotoxic stress inducer so far identified.

Materials and Methods

Cells, culture conditions, and reagents. TMLEC, a mink lung epithelial cell line (Mv1Lu) transformed with the firefly luciferase reporter gene downstream of the plasminogen activator inhibitor-1 (PAI-1) promoter, was kindly provided by M. Abe. To obtain a control cell line, the CMV enhancer/promoter-driven luciferase reporter vector pBC-Luc was introduced into Mv1Lu cells and the stably transformed cell line MCL-5 was selected with 400 µg/ml Geneticin (G418, Sigma-Aldrich, St. Louis, MO). Clone 6, a rat fibroblast cell line transformed with p53Val135A and activated ras, was kindly provided by S. Khochbin.

TMLEC, MCL-5, clone 6, HeLa, and NIH3T3 cells were cultured in Dulbecco's modified Eagles Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). For luciferase assay, a medium without phenol red was used. Cells were grown at 37 °C in a 5% CO₂ atmosphere, and the temperature was shifted to 32 °C when necessary.

SB202190 and SP600125 were purchased from Calbiochem (San Diego, CA). Anisomycin was purchased from Sigma-Aldrich. An anti-Smad2/3 monoclonal antibody was purchased from BD Biosciences (Boston, MA). An anti-p53 monoclonal antibody (Ab-1) was purchased from Oncogene Research Products (Boston, MA). Antibodies specific for p38, phospho-

p38, JNK, phospho-JNK, ATF-2, phospho-ATF-2, c-Jun, and phospho-c-Jun II were purchased from Cell Signaling Technology (Beverly, MA). Alexa Fluor 488-conjugated antibodies (from goat) against mouse and rabbit Ig were obtained from Molecular Probes (Eugene, Oregon). An FITC-conjugated and a horseradish peroxidase-linked secondary antibody were purchased from Amersham Pharmacia Biotech UK (Buckinghamshire, England).

Assay for luciferase. TMLEC or MCL-5 cells were plated 8.5×10^5 /ml in 96-well culture plates (Packard BioScience, Meriden, CT) in a medium without phenol red. After 6 h incubation for attachment, cells were incubated with screening samples for 16–18 h, and then luciferase activity was measured using the LucLite substrate (Packard BioScience) and a luminometer. All of the readouts were compared with control, and a number reflecting the relative increase in luciferase activity was calculated for each chemical.

Indirect immunofluorescence microscopy. TMLEC or NIH3T3 cells were grown to 70% confluence in 6-well plates (Falcon Labware, Oxnard, CA) on glass coverslips in a complete medium, and were then serum-starved (0.2% fetal bovine serum) overnight prior to incubation with drugs for 1 h. Clone 6 cells grown on glass coverslips to 70% confluence were incubated with or without drugs for 12 h, or incubated at 32 °C for 12 h. They were then rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. To minimize nonspecific binding, the samples were preincubated with 2% fetal bovine serum in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.1% (vol/vol) Tween 20 for 1 h at room temperature, and then treated with the primary antibody against Smad2/3 (1:50), phospho-ATF-2 (1:50), phospho-c-Jun II (1:50), or p53 (1:50) for 1 h, followed by incubation with the secondary antibody Alexa Fluor 488 (1:600, anti-mouse IgG), Alexa Fluor 488 (1:600, anti-rabbit IgG), or FITC (1:100, anti-mouse IgG) for 45 min. The coverslips were washed, mounted with 1 µg/ml DAPI in Vectashield (Vector Laboratories, Burlingame, CA), and observed under a fluorescent microscope (Axiophoto 2, Carl Zeiss).

Analysis of protein synthesis in vivo. The *in vivo* effects of the active compounds on protein synthesis were determined by measuring the incorporation of [³⁵S]methionine into cellular proteins. The NIH3T3 cells were grown to confluence in 35-mm culture dishes, washed with PBS, transferred to 10% DMEM with [³⁵S]methionine (10 µCi/ml) but without methionine and cystine, and cultured with various concentrations of drugs. After incubation, the cells were quickly washed twice with ice-cold PBS and lysed with ice-cold lysis

buffer containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM DTT, 0.2% Sarkosyl, 1 mM NaF, 1 mM sodium vanadate, 0.5 mM PMSF, and 0.5 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin. Trichloroacetic acid (10%) was added to the lysates, and the acid insoluble fractions were taken for determination of radioactivity.

Western blot analysis. 5×10^5 /ml NIH3T3 cells treated with each compound were lysed with ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM PMSF, 10 mM beta-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, and 10 mM leupeptin. The lysates were centrifuged at 15,000 rpm for 15 min at 4°C, and protein concentrations of the supernatants were determined by the Bradford method. An equal amount of protein was separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blotting, and visualized with an ECL Western blotting detection kit (Amersham Pharmacia Biotech).

In vitro kinase assay. NIH3T3 cells grown to 80% confluence in 10 cm-diameter dishes were incubated with or without drugs. The ability to phosphorylate ATF-2 or c-Jun in the cells treated with the compounds was measured *in vitro* using a p38 kinase or SAPK/JNK assay kit (Cell Signaling Technology, Beverly, MA) according to the manufacture's instructions.

RNA extraction and RT-PCR. TMLEC cells grown to 70% confluence in 10 cm dishes were incubated with or without drugs. Total RNAs were extracted with ISOGEN (Nippon Gene, Tokyo, Japan). Poly(A)⁺ RNA were isolated with a Poly(A)⁺ Isolation Kit (Nippon Gene) according to the manufacture's protocol. RT and PCR reactions were performed using luciferase primers 5'-GAAGAGATACGCCCTGGTTCCTGG-3' and 3'-GTACATCGACTGAAATCCCTGGTAATCCGT-5'.

Measurement of apoptotic cells. Cells (2.0×10^4 /well) were seeded in a 96-well flat-bottomed chimney-well plate (Greiner Bio-One, Frickenhausen, Germany) and incubated for 24 h. After treatment with drugs for an additional 24 h, the cells were fixed with 3.7% paraformaldehyde in PBS for 10 min. Following one washing with PBS, cells were treated with Hoechst-33342 in PBS (0.5 µg/ml). Hoechst-stained DNA was observed using a conventional fluorescence microscope (Olympus) with a DAPI filter.

Results

13-DT as a potent inducer of p38 kinase and p38 kinase inhibitor-1 gene expression

The transforming growth factor-beta (TGF-beta) signaling cascade has a tumor suppressor function.¹⁵⁾

Smad2 and Smad3, mediators of TGF-beta signaling, are phosphorylated upon ligand binding to the TGF-beta receptor, and together with Smad4, they transfer the TGF-beta signal from the cell surface receptors to nuclear DNA. The key signaling molecules such as TGF-beta receptors and Smads are lost in many tumor-derived cell lines. Therefore, it is plausible that the activation of the downstream signaling pathway by natural or synthetic small-molecule compounds might be a novel therapy against tumors lacking the TGF-beta response. To identify such substances, which stimulate the downstream signaling pathway of TGF-beta, we screened a marine natural product collection using Mv1Lu mink lung epithelial cells stably transfected with a plasminogen activator inhibitor-1 (PAI-1) promoter-luciferase construct (the TMLEC cell line), because PAI-1 is one of the TGF-beta-responsive genes. During the course of screening, we found that 13-DT (Fig. 1A) from a marine sponge caused a remarkable activation of the PAI-1 gene promoter at low nanomolar concentrations. As shown in Fig. 1B, 13-DT greatly stimulated PAI-1 gene expression in a concentration range from 1 to 10 nM, while it did not affect CMV promoter-directed gene expression in MV1Lu cells stably transfected with the CMV promoter-luciferase construct. 13-DT is a cytotoxic macrolide, while a derivative lacking the epoxide-containing side chain is inactive.¹⁶⁾ The inactive derivative did not induce PAI-1 gene expression, suggesting that cytotoxicity and PAI-1-inducing activity are interrelated.

To test whether 13-DT treatment causes nuclear translocation of Smad2/3, we determined the subcellular localization of endogenous Smad2/3 in the presence and absence of 13-DT by immunofluorescent microscopy using an anti-Smad2/3 antibody. As a control experiment, we also examined the effect of 13-DT on the subcellular localization of temperature-sensitive p53 (tsp53), the nuclear translocation of which is induced by protein synthesis inhibition at nonpermissive temperatures but not by TGF-beta signaling activation.^{17,18)} As shown in Fig. 2, no obvious accumulation of Smad2/3 in the nuclei was observed in cells treated with 13-DT (1.7 nM), although TGF-beta (10 ng/ml) induced nuclear accumulation of Smad2/3. A longer incubation with 13-DT also failed to induce nuclear translocation of Smad2/3 (not shown). Surprisingly, however, 13-DT caused a dramatic change in the subcellular localization of tsp53 from the cytoplasm to the nucleus at the nonpermissive temperature of 37°C.

Inhibition of protein synthesis by 13-DT

To test whether 13-DT induces nuclear translocation of tsp53 at nonpermissive temperatures by inhibiting protein synthesis, we examined the effect of 13-DT on the incorporation of labeled methionine into the proteins in cells. As shown in Fig. 3A, a low nanomolar concentration of 13-DT inhibited methionine incorporation, as did anisomycin at much higher concentrations.

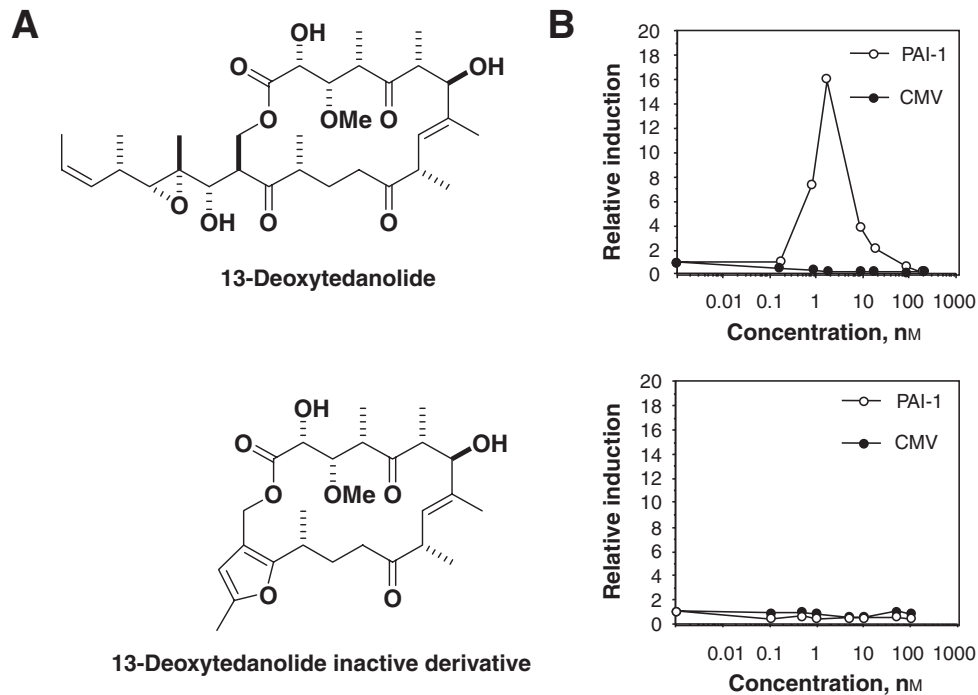


Fig. 1. Activation of PAI-1 Promoter by 13-DT.

A, Chemical structures of 13-DT and its inactive derivative. B, Effect of 13-DT (upper) and its inactive derivative (lower) on PAI-1 and CMV promoters. TMLEC cells for PAI-1 or MCL-5 for the CMV promoter were incubated with various concentrations of drugs for 16h, and then luciferase activity in the cells was measured, as described in "Materials and Methods".

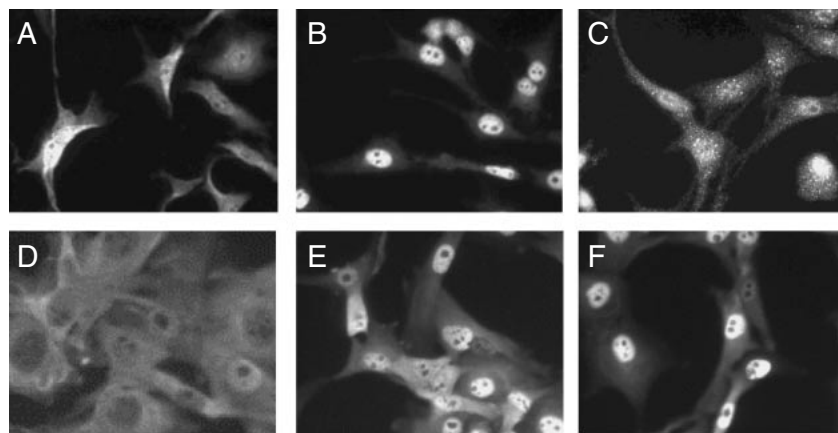


Fig. 2. Effect of 13-DT on Nuclear Translocation of Smad2/3.

TMLEC cells (A) were treated with 10 ng/ml TGF-beta (B) or 1.7 nM of 13-DT (C) for 1 h. As a control, rat fibroblast clone 6 cells transformed with temperature-sensitive p53 (p53Val135) were cultured for 12 h at 37 °C (D) or 32 °C (E), and with 1.7 nM of 13-DT at 37 °C (F). Localization of Smad2/3 and p53 was determined by immunofluorescent microscopy.

Dose response curves indicate that the IC_{50} value of 13-DT was approximately 1 nM, while that of anisomycin was 200 nM (Fig. 3B). These results clearly show that 13-DT is a strong inhibitor of protein synthesis in mammalian cells. This result is consistent with recent observations that 13-DT binds to the yeast 60S large ribosomal subunit.¹⁸⁾ To see whether protein synthesis inhibition by 13-DT is involved in the activity of inducing PAI-1 gene expression, we determined the ability of several protein synthesis inhibitors to activate

the PAI-1 gene promoter. As shown in Fig. 4A, the luciferase reporter assay demonstrated that anisomycin showed potent activity to induce PAI-1 gene expression, whereas neither puromycin nor cycloheximide induced expression, or showed very weak activity, if any, as compared with anisomycin. Anisomycin failed to activate the CMV promoter, as did 13-DT (Fig. 4B). RT-PCR demonstrated that both anisomycin and 13-DT, as well as trichostatin A (TSA), a histone deacetylase inhibitor known as an activator of TGF-beta-responsive

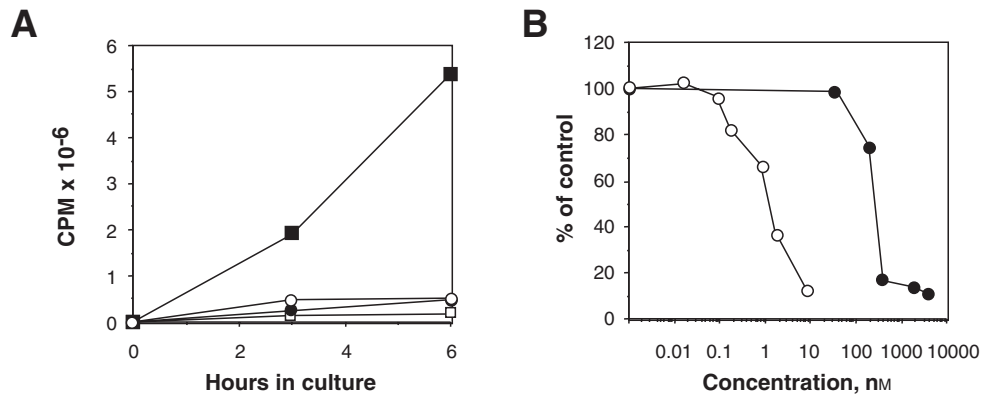


Fig. 3. Inhibition of Protein Synthesis by 13-DT.

A, Incorporation of labeled methionine into the cellular insoluble fractions was determined after incubation with the drug at the indicated times. Control (closed square); 1.7 nM 13-DT (open circle); 3.6 μ M cycloheximide (closed circle); and 3.8 μ M anisomycin (open square). B, Dose response of protein synthesis inhibition by 13-DT (open circle) and anisomycin (closed circle). Cells were treated with drugs for 3 h.

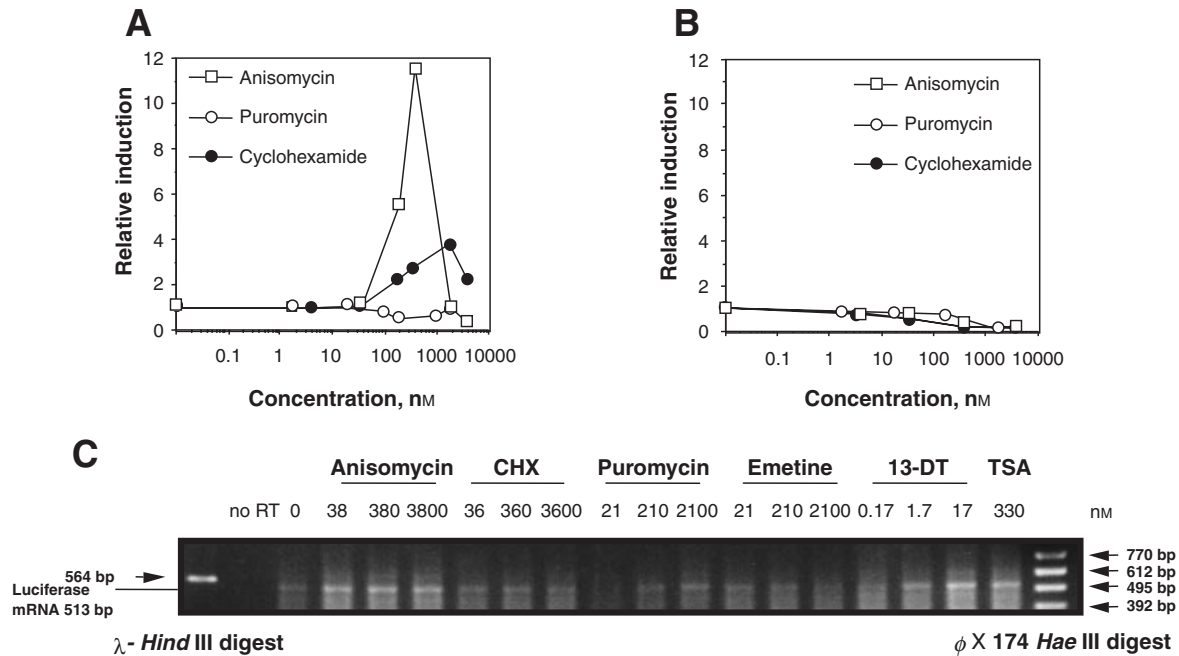


Fig. 4. Effect of Protein Synthesis Inhibitors on PAI-1 Gene Expression.

A and B, Effects of protein synthesis inhibitors on the PAI-1 promoter (A) and the CMV promoter (B) were determined by luciferase reporter gene assay. TMLEC cells (A) and MCL-5 cells (B) were treated with anisomycin (open square), puromycin (open circle), and cycloheximide (closed circle) at the indicated concentrations for 16 h. C, Effects of various drugs on the PAI-1 promoter were determined by analyzing the amount of mRNA by RT-PCR. Cells were treated with each drug for 18 h.

genes,¹⁹⁾ increased the transcription from the PAI-1 gene promoter. On the other hand, cycloheximide, puromycin, and emetine did not cause apparent increases in the amount of the transcript (Fig. 4C). These results suggest that the PAI-1 promoter is responsive to a subset of protein synthesis inhibitors such as anisomycin and 13-DT.

Activation of JNK and p38 by 13-DT

In addition to Smads, stress-activated MAP kinase pathways, including p38²⁰⁻²⁴⁾ and JNK,^{25,26)} have been

shown also to contribute to the TGF-beta-induced gene expression. Hence we tested whether 13-DT induces activation of p38 and JNK by immunoblotting using phosphorylation-specific antibodies against p38 and JNK. As shown in Fig. 5A, 13-DT increased the amount of phosphorylated p38 and JNK in a dose-dependent manner. ATF-2 and c-Jun are well-characterized substrates for p38 kinase and JNK respectively. Western blotting using antibodies specific for phosphorylated ATF-2 and phosphorylated c-Jun demonstrated that both anisomycin and 13-DT greatly enhanced cellular activ-

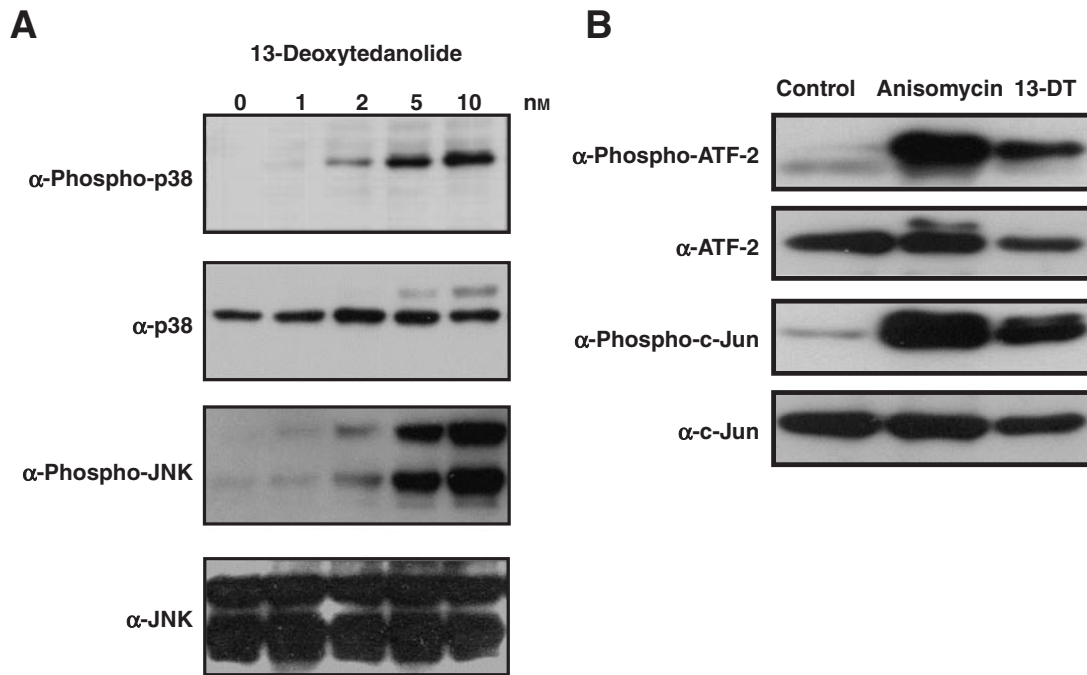


Fig. 5. Activation of p38 MAPK and JNK by 13-DT.

A, The amounts of phosphorylated p38 and phosphorylated JNK in NIH3T3 cells treated with various concentrations of 13-DT for 30 min were determined by Western blot analysis. The total cellular levels of p38 and JNK in treated cells were also determined. B, The *in vitro* p38 kinase and JNK activity in cell lysates prepared from cells treated with anisomycin (500 nM) or 13-DT (5 nM) for 30 min were determined using ATF-2 and c-Jun respectively as the substrates.

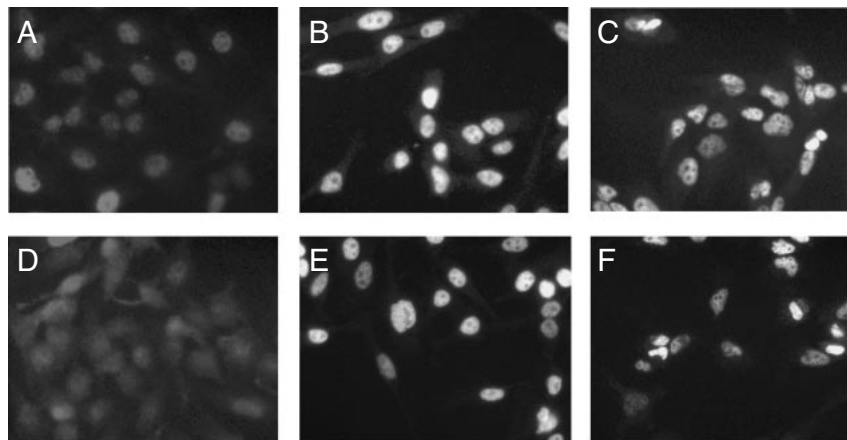


Fig. 6. Phosphorylation of ATF-2 and c-Jun in 13-DT-Treated Cells.

Phosphorylation of ATF-2 (A–C) and c-Jun (D–F) was visualized by immunofluorescent staining of NIH3T3 cells treated with various drugs for 1 h. A and D, control; B and E, 380 nM anisomycin; C and F, 1.7 nM 13-DT.

ity phosphorylating ATF-2 and c-Jun (Fig. 5B). Immunofluorescent microscopy showed that activated, phosphorylated ATF-2 and c-Jun accumulated in the nuclei of cells treated with 13-DT or anisomycin (Fig. 6). These results clearly show that 13-DT activates the stress-activated kinase pathways.

PAI-1 gene expression by ribotoxic stress-induced kinase activation

Anisomycin is known to bind to the 60S large

ribosome subunit, and induces a ribotoxic stress response. Ribotoxic stress response is a conserved cellular reaction to cytotoxic interference with the function of the large ribosomal RNA,¹⁾ which involves activation of stress-activated MAP kinase pathways including JNK and p38, and subsequent transcriptional induction of immediate early genes such as c-fos and c-jun in mammalian cells. The above results raise the possibility that the PAI-1 gene is a target not only of the TGF-beta signaling pathway but also of the ribotoxic stress

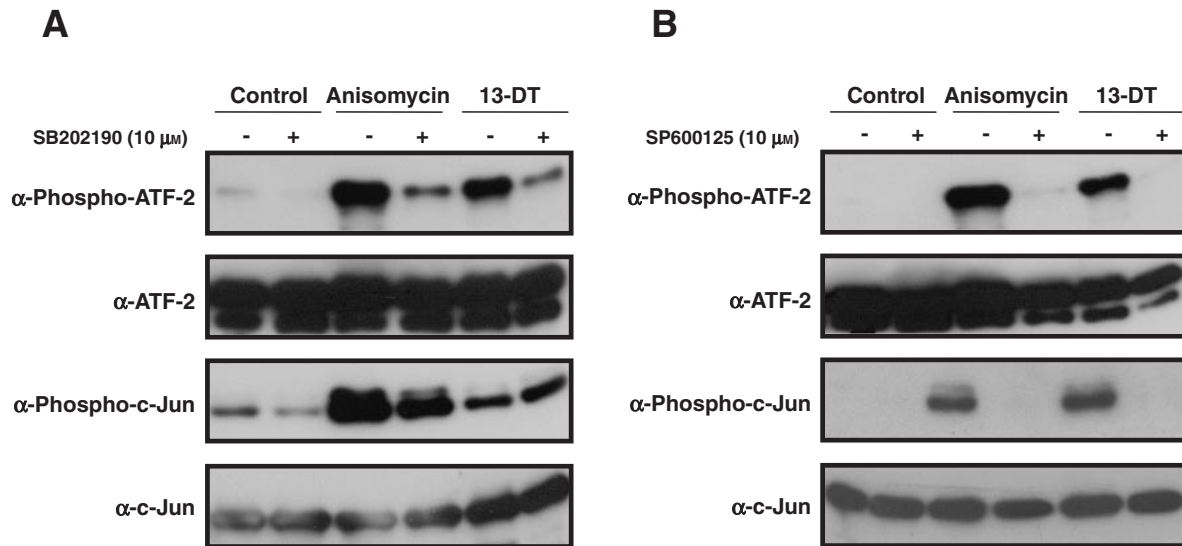


Fig. 7. Effect of Stress-Activated Protein Kinase Inhibitors on 13-DT-Induced Phosphorylation of ATF-2 and c-Jun.

SB202190 (A) or SP600125 (B) was added 30 min before treatment with anisomycin (500 nM) or 13-DT (2 nM) for 30 min. After *in vitro* kinase reaction, the amounts of phosphorylated or unphosphorylated proteins were determined by Western blotting.

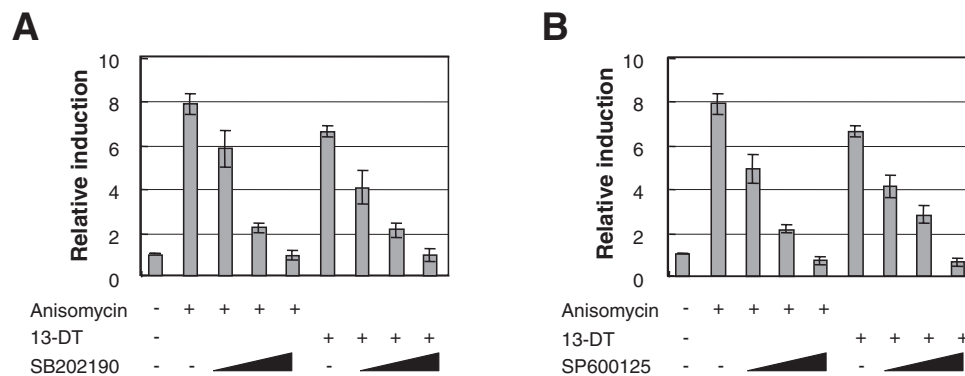


Fig. 8. p38 and/or JNK-Dependent Activation of PAI-1 Gene Expression by 13-DT.

The levels of PAI-1 promoter activation by anisomycin (500 nM) and 13-DT (2 nM) in TMLEC cells were determined by luciferase reporter assay in the presence or absence of various concentrations (0.1, 1, 10 μ M) of SB202190 (A) and SP600125 (B). Cells were treated with the drugs for 16 h.

response pathway. To test whether the 13-DT or anisomycin-induced activation of the kinases is required for PAI-1 gene activation, we analyzed the effect of the inhibitors of p38 and JNK. Pretreatment with SB202190, a specific inhibitor for p38 kinase, greatly reduced the drug-induced phosphorylation of ATF-2, but not of c-Jun (Fig. 7A). In addition, SP600125, an inhibitor for JNK, inhibited ATF-2 phosphorylation by p38 as well as c-Jun phosphorylation by JNK under our experimental conditions (Fig. 7B). Next we examined whether these kinase inhibitors affect PAI-1 gene expression induced by anisomycin or 13-DT. Both SB202190 (Fig. 8A) and SP600125 (Fig. 8B) reduced the PAI-1 promoter activity induced by anisomycin or 13-DT in a dose-dependent manner. These results strongly suggest that 13-DT induces PAI-1 gene expression *via* the stress-activated MAPK pathways but not *via* the Smad pathway. Since

both anisomycin and 13-DT can bind to yeast ribosomes,¹⁸⁾ it is likely that ribotoxic stress induced by anisomycin or 13-DT leads to activation of the PAI-1 promoter.

13-DT activates upstream kinases for p38 and JNK as a ribotoxic stress inducer

Next we tested whether the known members upstream of p38 and JNK are activated by 13-DT by Western blot analysis using phosphorylated form-specific antibodies (Fig. 9). Both MKK3/6 and SEK1/MKK4, which phosphorylate p38 and JNK respectively, were phosphorylated upon 13-DT treatment in dose- and time-dependent manners, indicating that these upstream kinases are activated. Importantly, phosphorylation of these MAPKs occurred transiently, suggesting that the signal generated from the damaged ribosome to induce

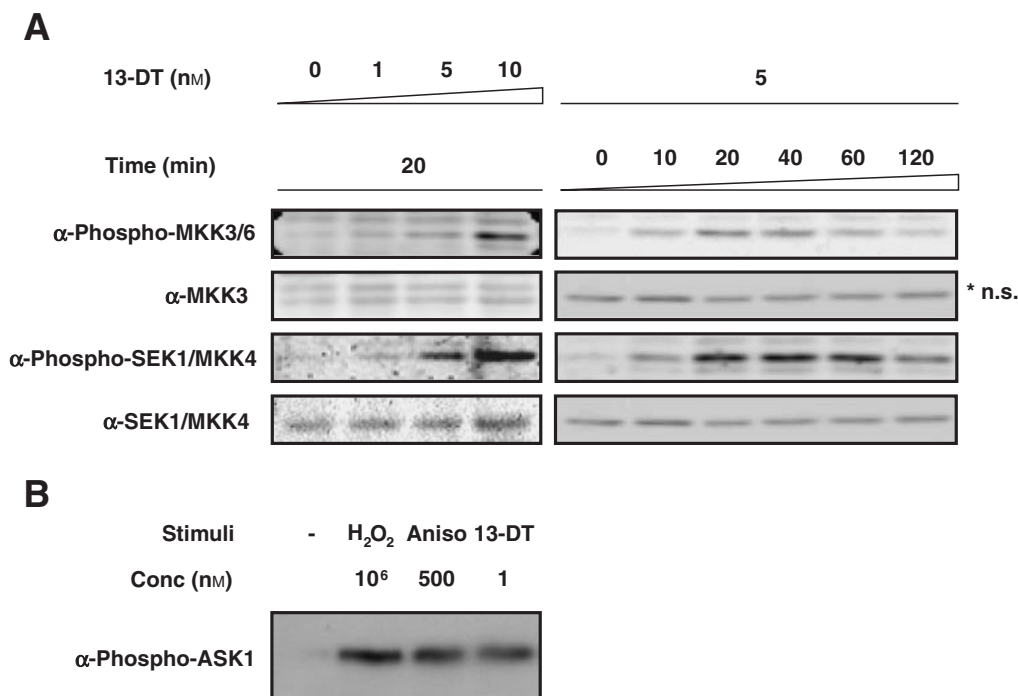


Fig. 9. Activation of MKK3/6, SEK1/MKK4, and ASK1 by 13-DT.

The levels of phosphorylation of the kinases were determined by Western blot analysis using their phosphorylated form-specific antibodies. A, Effects of 13-DT on phosphorylation of MKK3/6 and SEK1/MKK4, members of the MAP kinase family upstream of p38 and JNK respectively. B, Effect of 13-DT on ASK1. NIH3T3 cells were treated with drugs for 10 min and then the levels of phosphorylated ASK1 were determined.

the ribotoxic stress response is not sustained throughout the translation inhibition. Furthermore, apoptosis signal-regulating kinase 1 (ASK1)²⁷⁾ was also activated by 13-DT, like the oxidative stress inducer hydrogen peroxide. Since anisomycin treatment also induced phosphorylation of ASK1, it appears likely that ribotoxic stress uses ASK1 to activate downstream kinases such as MKK3/6 and SEK1/MKK4.

Effect of 13-DT on apoptosis induction

ASK1 is a member of the mitogen-activated protein (MAP) kinase kinase kinase family, which plays a pivotal role in the signaling pathway in cytokine- and stress-induced apoptosis.²⁸⁾ Hence we examined whether 13-DT induces apoptosis in cultured cells (Fig. 10). Changes in the nuclear morphology typical of apoptosis were observed in both NIH3T3 and HeLa cells after a 13-DT challenge. In particular, more than 50% of HeLa cells underwent apoptosis within 24 h during the 13-DT treatment. On the other hand, the effect of cycloheximide was less drastic than that of 13-DT. This is probably due to the weak activity of cycloheximide in inducing the ribotoxic stress response and p38 and JNK activation.⁴⁾

Discussion

To obtain a small molecule activator of the TGF-beta signaling pathway, we screened for compounds that

activate expression of the PAI-1 gene, one of the well-characterized TGF-beta responsive genes. Although 13-DT obtained as a result of this screening induced PAI-1 gene expression at low nanomolar concentrations, it did not stimulate the TGF-beta signaling pathway involving Smads, but rather inhibited eukaryotic protein synthesis. We found that 13-DT strongly activated p38 and JNK, like anisomycin, a potent inducer of ribotoxic stress. Recently, it was also found that 13-DT specifically bound to the 60S large subunit of the eukaryotic ribosome.¹⁸⁾ Hence we concluded that 13-DT induces a ribotoxic stress response by interacting with the large subunit of the ribosome.

The mitogen-activated protein (MAP) kinase cascade is evolutionarily conserved in all eukaryotic cells.²⁹⁾ JNK, p38 kinase, and extracellular signal-regulated kinase (ERK) are well-characterized subgroups of a large MAP kinase family. These kinase pathways are structurally similar, but functionally distinct. While ERK is rapidly activated by a variety of cell growth and differentiation stimuli and plays a central role in mitogenic signaling, p38 and JNK are primarily activated by various environmental stresses, including toxins, physical stresses, and inflammatory cytokines.²⁹⁾ TGF-beta also activates p38 and JNK, which are involved in the activation of TGF-beta-responsive genes such as PAI-1.^{20,21,23-26)} A specific p38 kinase inhibitor has been shown to inhibit activation of TGF-beta-responsive genes, including PAI-1.^{23,30)} The PAI-1 gene

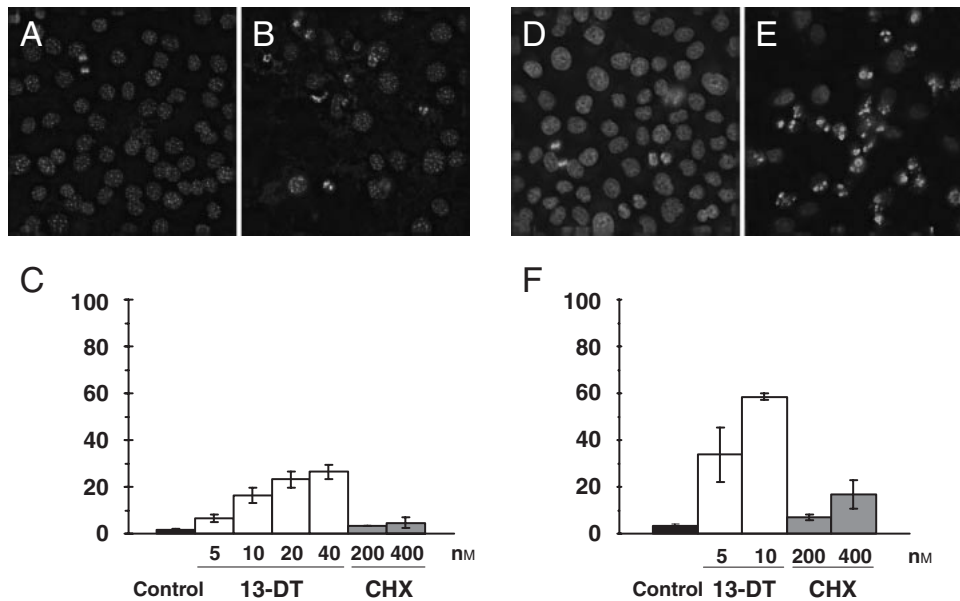


Fig. 10. Effects on Apoptosis in NIH3T3 (A–C) or HeLa (D–F) Cells.

A, B, D, and E, Changes in the morphology of nuclei upon treatment with (B, E) or without (A, D) 13-DT. NIH3T3 and HeLa cells were treated with 40 and 10 nM of 13-DT respectively. Cells were stained with Hoechst-33342 after 24 h of treatment with the drug. C and F, The level of apoptosis induction by treatment with 13-DT or cycloheximide for 24 h was determined by counting apoptotic cells under microscopic observation. Results are expressed as the mean \pm SE of two independent experiments, which were carried out in triplicate assays.

promoter has been shown to be activated by a variety of stresses in addition to TGF- β .^{31–34} In this study, we showed that ribotoxic stress inducers such as anisomycin strongly stimulate transcription from the PAI-1 promoter. On the other hand, other protein synthesis inhibitors that do not cause the ribotoxic stress response failed to induce PAI-1 promoter activation (Fig. 4). These results strongly suggest that the stress-activated MAP kinase pathways are important in the induction of PAI-1 gene expression by ribotoxic stress inducers. Indeed, we showed that inhibition of p38 by the p38 kinase inhibitor SB202190 or the JNK inhibitor SP600125 greatly reduced PAI-1 gene activation by 13-DT or anisomycin (Fig. 8), suggesting that these kinase pathways play a critical role in ribotoxic stress-induced activation of the PAI-1 promoter. Because SB202190, which specifically inhibited p38 kinase activity (Fig. 7A), sufficiently reduced PAI-1 promoter activity (Fig. 8), p38 kinase appeared to be responsible for the 13-DT-induced PAI-1 promoter activation. It is therefore possible that the ribotoxic stress response shares some of TGF- β -responsive genes as the targets, and that ribotoxic stress inducers can partially overcome the defect in TGF- β signaling in tumor cells by activating some of the TGF- β -target genes.

The activity of 13-DT in inducing p38 and JNK activation through the ribotoxic stress response might have relevance to its strong cytotoxic and antitumor activity. Specific inhibitors of the p38 and JNK pathways, or dominant-negative mutants of the kinases, suppress various types of stress-induced apoptosis. Studies on fibroblasts with targeted disruptions of all

the JNK genes established an essential role for JNK in UV-induced and other stress-induced apoptosis.³⁵ These kinase pathways in response to stress are thought to be important in controlling cancer, since many primary tumors as well as transformed cells display higher sensitivity than normal cells to chemotherapeutic drugs due to the potentiation of stress-activated kinase pathways.^{11,12} A number of cytotoxic compounds that inhibit eukaryotic protein synthesis are currently being studied clinically or preclinically as potential anticancer drugs,^{36–38} and the translation machinery has been proposed as a target for cancer therapy.^{39,40} But, the mechanism by which some but not all of the protein synthesis inhibitors can exert antitumor activity is unclear. This study indicated that 13-DT, an antitumor marine natural product, is a eukaryotic protein synthesis inhibitor that strongly activates the stress-activated kinase pathway. This activity might account at least in part for the strong cytotoxic activity and antitumor activity of 13-DT, since 13-DT induced apoptosis in cultured cells more strongly than cycloheximide (Fig. 10).

Among the ribotoxic stress inducers, 13-DT has unique properties. It is the first macrolide compound shown to bind to eukaryotic ribosomes, the 13-DT binding site on the ribosome is different from that of anisomycin,¹⁸ and it might be the strongest compound effective at low nanomolar concentrations. Thus 13-DT should serve as a useful tool for analyzing the mechanism by which the response to ribotoxic stress is generated in cells. Compared with this clear demonstration of the activation of the stress-activated MAP

kinase pathways by ribotoxic stress, the players upstream of the MAP kinases are poorly understood. In the present study, we showed that ASK1, SEK1/MKK4, and MKK3/6 are activated as upstream kinases. Although detailed studies using 13-DT have been hampered because only minute quantities can be obtained from the natural source, the recent establishment of the method for total synthesis^{41,42} should allow extensive studies using 13-DT not only for its preclinical or clinical efficacy but also to understand the molecular mechanism of the ribotoxic stress response and subsequent apoptosis induction.

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