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MT-21 Is a Synthetic Apoptosis Inducer That Directly Induces Cytochrome *c* Release from Mitochondria[1]

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ABSTRACT

We reported previously that a synthetic compound, MT-21, induced apoptosis by activating c-Jun-NH_2-terminal kinase via the Krs/MST protein, which is activated by caspase-3 cleavage dependent on reactive oxygen species production. Here we examine the activation mechanism of caspase-3, an important cysteine aspartic protease, during MT-21-induced apoptosis. We found that MT-21 activated caspase-3 via caspase-9, but not via caspase-8. In addition, MT-21 induced the release of cytochrome *c* from the mitochondria that is necessary to activate caspase-9, and this release occurred before a change in membrane potential. This initiation process of MT-21-induced apoptosis was suppressed by overexpression of Bcl-2, which is known to prevent cells from undergoing apoptosis in response to a variety of stimuli. Moreover, when we treated mitochondria isolated from the cells with MT-21, the direct release of cytochrome *c* from the mitochondria was observed, whereas this effect was not observed in the mitochondria isolated from cells that overexpressed Bcl-2. Other apoptosis-inducing agents known to induce apoptosis via cytochrome *c* release from the mitochondria failed to release cytochrome *c* directly from isolated mitochondria. These findings indicate that MT-21 is a possible candidate antitumor agent that is able to induce apoptosis via the direct release of cytochrome *c* from the mitochondria.

INTRODUCTION

Apoptosis-inducing compounds are possible candidate antitumor agents. Recent advances have led to a widely accepted model that a conserved family of cysteine proteases (caspases) plays important roles in apoptosis (1). Caspases themselves are present as proenzymes that are readily cleaved and activated during apoptosis, providing the cell with a means to rapidly amplify its apoptotic response (2). In addition, the similar proteolytic cascade was induced, and consequent morphological changes occurred during apoptosis, irrespective of the type of stimuli.

There are various pathways leading to the caspase cascade, depending on the apoptotic stimuli. For example, apoptosis caused by activation of the CD95/Fas receptors is brought about by recruitment of the adaptor protein Fas-associated protein with death domain (3). Fas-associated protein with death domain, in turn, recruits procaspase-8 via a homophilic interaction between the death effector domains of these proteins, and this event is followed by autocatalytic cleavage and activation of the clustered procaspase (4, 5). In contrast, activation of caspase-9 by apoptotic stimuli proceeds via recruitment of the procaspase to a signaling complex containing apoptotic protease-activating factor 1 and cytochrome *c* (6–8), which is believed to be released from the mitochondria as an early event in several models of apoptosis (9). Cytochrome *c* is encoded by a nuclear gene and translated by cytosolic ribosomes as apocytochrome *c* (10). Apocytochrome *c* is subsequently translocated into the mitochondria, where a heme group is attached covalently to form holocytochrome *c* (10). The increase in cytosolic holocytochrome *c* on apoptosis suggests that mitochondria may participate in apoptosis by releasing cytochrome *c*. Based on the finding that both caspase-8 (11) and caspase-9 (6) cleave and activate caspase-3 in cell-free assays of apoptosis, it is widely believed that these initiator caspases lie at the apex of separate apoptotic cascades that converge on activation of downstream effector caspases.

Recently, we found that MT-21, a synthetic compound, induced apoptosis in human promyelocytic leukemia HL-60 cells and activated JNK[3] by activation of a M_r 36,000 kinase termed p36 MBP kinase via caspase-3 activation during MT-21- induced apoptosis (12). In addition, p36 MBP kinase is an active proteolytic product of Krs-1 and Krs-2, which were originally cloned by virtue of their homology to the budding yeast Ste20 kinase (12–15). Furthermore, cytotrienin A, a novel ansamycin antitumor agent that we isolated from *Streptomyces* sp. as an apoptosis inducer (16, 17), also generated the proteolytically activated Krs protein p36 MBP kinase mediated by caspase-3 during apoptosis (15). Recently, two other groups (18, 19) reported that Krs-2 is cleaved by a caspase-3-like activity during apoptosis induced by Fas (CD95/APO-1). In the present study, we reveal that most known antitumor agents indirectly induce cytochrome *c* release from the mitochondria and that MT-21 directly induces cytochrome *c* release and activates the caspase cascade during apoptosis.

MATERIALS AND METHODS

Chemicals. MT compounds were synthesized as described previously (20). Z-IETD-FMK and Z-LEHD-FMK were purchased from Kamiya Biomedical Company (Seattle, WA) and are caspase-8 and caspase-9 inhibitors, respectively. Anti-caspase-3 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-cytochrome *c* antibody was obtained from PharMingen (San Diego, CA), and anti-Bcl-2 antibody was obtained from DAKO (Kyoto, Japan).

Cells. Human promyelocytic leukemia HL-60 and U937 cells were cultured in RPMI 1640 supplemented with 10% FCS at 37°C in a 5% CO_2 humidified atmosphere.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. HL-60 cells were lysed with a solution consisting of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) SDS, and 0.1% (w/v) RNase A and incubated for 60 min at 50°C. The lysate was incubated for an additional 60 min at 50°C with 1 mg/ml proteinase K, and phenol/chloroform-extracted DNA was electrophoresed on a 2% (w/v) agarose gel in 40 mM Tris-acetate (pH 7.5) containing 1 mM EDTA for 90 min at 50 V. After electrophoresis, DNA was visualized by ethidium bromide staining.

Preparation of Cell Lysate. HL-60 cells were lysed with 0.1 ml of lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% (w/v) Triton X-100. The cell lysate was centrifuged at 15,000 $\times$ *g* for 20 min. The supernatant thus obtained was used for immunoblotting with an anti-caspase-3 antibody and for measuring protein kinase activities.

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[3] The abbreviations used are: JNK, c-Jun-NH_2-terminal kinase; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride; TBS, 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl; DCFH-DA, dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; NAS, *N*-acetylserine; CsA, cyclosporin A.

Immunoblotting. Cell lysates containing 20 μg of protein were subjected to SDS-PAGE and separated. The proteins were then transferred to a polyvinylidene difluoride membrane with a semidry blotting apparatus at 2 mA/cm^2 constant current for 1 h. The membrane was first washed with TBS and then blocked with 3% (w/v) BSA in TBS for 1 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with the primary antibody diluted 1:1000 in TBS containing 0.1% (w/v) BSA. After washing with TBS containing 0.1% (w/v) Tween 20 (TTBS), the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in TBS containing 0.1% (w/v) BSA. After washing with TTBS, protein bands on the membrane were visualized by an enhanced chemiluminescence Western blotting detection system (DuPont New England Nuclear, Boston, MA).

Cytochrome *c* Release from Mitochondria in Drug-treated Cells. Untreated and drug-treated cells were harvested by centrifugation at 1,000 × *g* for 5 min at 4°C. The cells pellets were washed once with ice-cold PBS and resuspended with 5 volumes of 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. The cells were homogenized with a 22-gauge needle, and the homogenates were centrifuged at 750 × *g* for 10 min at 4°C. The supernatants were then centrifuged at 10,000 × *g* for 15 min at 4°C. The pellets were lysed with 0.1 ml of lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% SDS. The lysed solution was used for the identification of mitochondrial cytochrome *c* by immunoblotting. The supernatants were centrifuged at 100,000 × *g* for 15 min at 4°C, and the obtained supernatants were used for identification of cytosolic cytochrome *c* by immunoblotting.

Cytochrome *c* Release from Mitochondria in a Cell-free System. Mitochondria were purified from the cells by differential centrifugation and separation on a sucrose gradient (21). Mitochondria were suspended with 20 μl of CFS [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 mM succinate, 1 mM ADP, and 250 mM sucrose], and MT compounds were added. After a 2-h incubation at 37°C, this suspension was centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatants were used for identification of cytochrome *c* release from mitochondria by immunoblotting.

Changes in Mitochondrial Transmembrane Potential (Δψm). To measure Δψm, the cells were incubated with 10 μg/ml JC-1 (Molecular Probes, Eugene, OR) for 30 min in culture medium at 37°C. The cells were washed once with PBS and analyzed immediately by flow cytometry (Epics Elite; Coulter, Hialeah, FL). At least 50,000 events were collected per sample.

Evaluation of Intracellular H_2O_2. The cells were treated with chemicals in the presence of 5 μM dichlorodihydrofluorescein diacetate (DCFH-DA). The esterified form of DCFH-DA can permeate cell membranes and can then be deacetylated by intracellular esterases. The resulting compound is reactive with H_2O_2 to give a fluorescent compound, dichlorofluorescein (12). The amount of intracellular H_2O_2 was detected by flow cytometry (Epics Elite; Coulter).

Immunofluorescence Microscopy. Immunocytochemical study of cytochrome *c* was performed as described previously (22). Cells treated with 40 μM MT-21 were washed once with PBS and then fixed in PBS containing 3.7% formaldehyde for 5 min at room temperature. Cells were then permeabilized with PBS containing 0.2% Triton X-100 for 5 min and then washed three times with PBS. Incubation with primary antibody (anti-cytochrome *c* at a 1:100 dilution) was carried out for 1 h at room temperature. Excess antibody was removed by washing three times with PBS. This was followed by incubation with an appropriate FITC-conjugated secondary antibody for 1 h at room temperature in an area protected against light. After excess antibody was removed by washing three times with PBS, images were collected by fluorescence microscopy.

Detection of MBP Kinase Activity in SDS-Polyacrylamide Gel. Separation gel containing 10% (w/v) polyacrylamide, 0.1% (w/v) SDS, and 0.5 mg/ml MBP was prepared as described previously (12), and 40 μg of protein of cell lysates were subjected to electrophoresis. After electrophoresis, the gel was incubated for 1 h in 50 mM Tris-HCl (pH 8.0) containing 20% (v/v) isopropyl alcohol to remove SDS and then incubated for 1 h in Buffer A consisting of 50 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol. Subsequently, proteins in the gel were denatured for 1 h in Buffer A containing 6 M guanidine-HCl. The denatured proteins were renatured by incubating the gel for 12 h at 4°C in four changes of Buffer A containing 0.05% (w/v) Tween 20. The entire gel was then subjected to a kinase assay. After a 30-min preincubation at room temperature in Buffer B [40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, and 0.1 mM EGTA], the gel was incubated for 1 h in Buffer B containing 20 μM [γ-^{32}P]ATP (25 μCi) at 30°C. The gel was washed, extensively with a solution consisting of 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate, dried, and subjected to autoradiography at −80°C using Kodak X-OMAT AR5 X-ray film and an intensifying screen.

Determination of JNK Activity. JNK activity in the whole cell extracts (0.1 mg) was measured by incubation with 10 μg of glutathione *S*-transferase-c-Jun(1–79) conjugated to glutathione-agarose beads [prepared according to the manufacturer's instructions (Pharmacia)] for 1 h at 4°C. The agarose beads were collected by quick microcentrifugation and washed five times with HEPES binding buffer [20 mM HEPES (pH 8.0), 20 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, and 0.05% Triton X-100]. The final wash was performed in kinase buffer [20 mM HEPES (pH 8.0), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 2 mM DTT]. The kinase reaction was initiated by resuspending the pelleted beads in 30 μl of kinase buffer containing [γ-^{32}P]ATP (20 μM; 0.5 μCi/reaction) for 10 min at 30°C. The reaction was terminated by the addition of 1 ml of ice-cold HEPES binding buffer. The beads were pelleted, resuspended in SDS sample buffer, and boiled for 5 min. Proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel followed by autoradiography.

RESULTS

Activation of Caspase-9 by MT-21. The active form of caspase-3 consists of two subunits, p20 and p12, and is derived from a precursor protein (p32) by proteolytic processing. Therefore, the activation of caspase-3 by MT-21 is detected as the decrease of the precursor form of caspase-3. To examine whether the activation of caspase-3 induced by MT-21 was caused by caspase-8 or -9, we performed immunoblotting with an anti-caspase-3 antibody using each specific inhibitor against caspase-8 or -9. IETD, a caspase-8-specific inhibitor, inhibited the activation of caspase-3 induced by tumor necrosis factor α, but not the activation induced by MT-21 (Fig. 1*A*). We obtained the same result on the induction of a ladder pattern typical of internucleosomal fragmentation, which is considered to be an early event in apoptosis (Fig. 1*B*). On the other hand, LEHD, a caspase-9-specific inhibitor, inhibited the activation of caspase-3 and DNA fragmentation by MT-21 (Fig. 1, *C* and *D*). These results suggest that MT-21 activates caspase-3 via caspase-9 activation during apoptosis.

Release of Cytochrome *c* from Mitochondria Induced by MT-21. Because caspase-9 is activated by complex formation with apoptotic protease-activating factor 1 and cytochrome *c*, we examined the effect of MT- 21 on the release of cytochrome *c* from mitochondria. Without MT-21 treatment, most of the detectable cytochrome *c* was localized on mitochondria in the cells (Fig. 2*A*). Cytochrome *c* in the cytosol of the cells increased significantly after 3 h of treatment with MT-21 (Fig. 2*B*). The amounts of cytochrome *c* in mitochondria showed a corresponding decrease. In our previous report, MT-21 caused the production of ROS before apoptosis induction, and NAC, a scavenger of H_2O_2, suppressed MT-21-induced apoptosis (12). Therefore, we tested the effect of antioxidants on MT-21-induced release of cytochrome *c* from mitochondria. As shown in Fig. 2*C*, the increase of cytochrome *c* in the cytosol induced by MT-21 was suppressed by NAC but not by NAS, a negative control of NAC. These results indicated that MT-21 induced the release of cytochrome *c* from mitochondria via ROS production during apoptosis.

Release of Cytochrome *c* Occurs before the Loss of Δψm. Because some observations have suggested that the change in Δψm is major cause of apoptosis (23–26), alteration in the Δψm was studied using JC-1 dye. JC-1 is a potential-sensitive dye that undergoes a molecular aggregation and shift in fluorescence from green to red at high Δψm. As shown in Fig. 3, valinomycin, which is a K^+ iono-

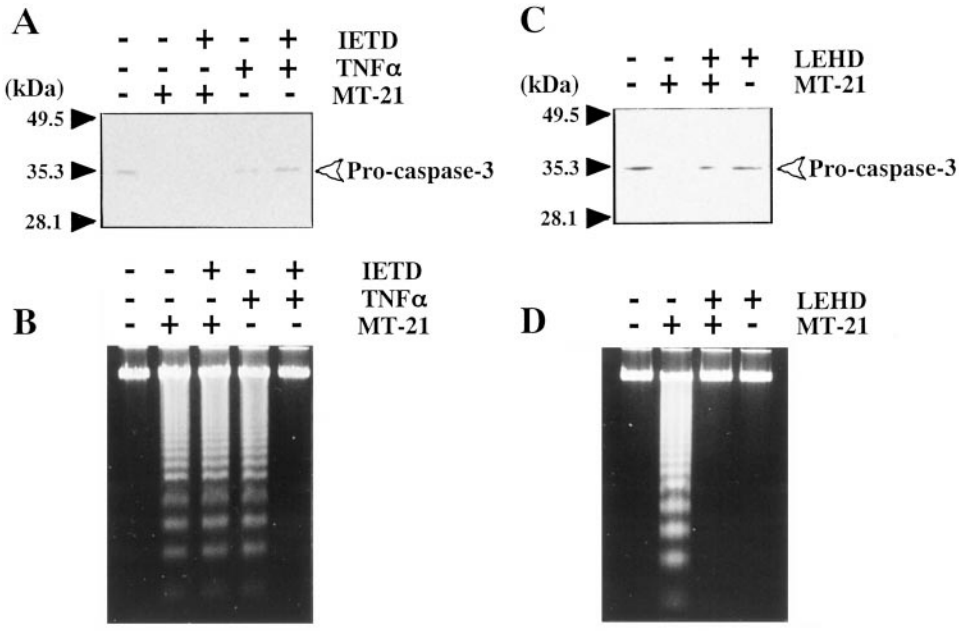


Fig. 1. Activation of caspase-9 but not caspase-8 by MT-21 in HL-60 cells. HL-60 cells were treated with 40 μM MT-21 for 6 h or with 50 ng/ml tumor necrosis factor α for 16 h in the absence or presence of 20 μM Z-IETD-FMK (*A* and *B*) or 40 μM Z-LEHD-FMK (*C* and *D*). Immunoblotting analysis was performed using anti-caspase-3 antibodies (*A* and *C*). Cellular DNA extracted from the cells was analyzed by agarose gel electrophoresis and stained with ethidium bromide (*B* and *D*).

phore, dissipated the $\Delta\psi$m, as confirmed by an increase in the green fluorescence of JC-1 and a decrease in the red fluorescence of JC-1 aggregates. Under the same conditions, we examined the change in $\Delta\psi$m in MT-21-treated cells. For at least 3 h after MT-21 treatment, $\Delta\psi$m was largely retained under the condition where cytochrome *c* was already released (Fig. 3). Further treatment with MT-21 slightly enhanced the loss of $\Delta\psi$m. This is a consequence of cytochrome *c* loss because cytochrome *c* takes part in generating $\Delta\psi$m through the mitochondrial respiratory chain. These observations suggest that the release of mitochondrial cytochrome *c* into the cytosol is not caused by the change in $\Delta\psi$m.

Induction of Permeability Transition by MT-21. CsA is known as the permeability transition pore inhibitor. Therefore, we examined whether MT-21 induced cytochrome *c* release from mitochondria via the induction of permeability transition during apoptosis using CsA. Fig. 4*A* shows that CsA caused dose-dependent inhibition of MT-21-induced cytochrome *c* release from mitochondria and the activation of caspase-9 monitoring the proteolytic digestion of pro-caspase-3. We also examined the effect of CsA on MT-21-induced DNA fragmentation. CsA suppressed DNA fragmentation by MT-21 in a dose-dependent manner (Fig. 4*B*). Moreover, we examined the effect of CsA on ROS production by MT-21. As shown in Fig. 4*C*, CsA could not suppress ROS production by MT-21. These results indicate that MT-21 induces cytochrome *c* release from mitochondria via the induction of permeability transition by ROS production during apoptosis.

Effect of Bcl-2 Overexpression on MT-21-induced Apoptosis. Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria and is known to prevent cell apoptosis in response to a variety of stimuli. To evaluate the influence of Bcl-2 overexpression on MT-21-induced release of cytochrome *c* from mitochondria and on apoptosis, cells transfected with vector alone (vector cells) or with same vector containing a cDNA encoding human Bcl-2 (Bcl-2 cells) were treated with MT-21. An increase in cytosolic cytochrome *c* was induced by MT-21 in vector cells but not in Bcl-2 cells (Fig. 5*A*). Bcl-2 expression in vector and Bcl-2 cells was confirmed by immunoblotting with anti-Bcl-2 antibody (Fig. 5*B*). Moreover, we carried out immunocytochemical studies of cytochrome *c* distribution in MT-21-

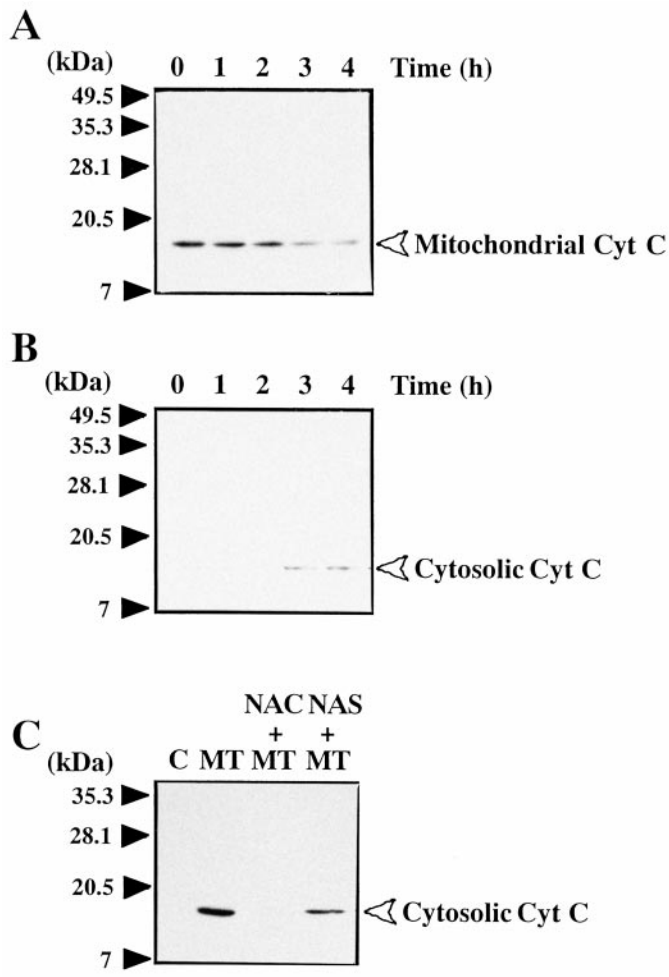


Fig. 2. Release of cytochrome *c* from mitochondria by MT-21. HL-60 cells were treated with 40 μM MT-21 for the indicated time periods. After preparation of the cytosolic or mitochondrial fraction, immunoblotting analysis was performed using anti-cytochrome *c* antibodies (*A* and *B*). *C*, HL-60 cells were pretreated with 3 mM NAC and 3 mM NAS and then treated with 40 μM MT-21 for 6 h. After preparation of the cytosolic fraction, immunoblotting analysis was performed as described in *A*.

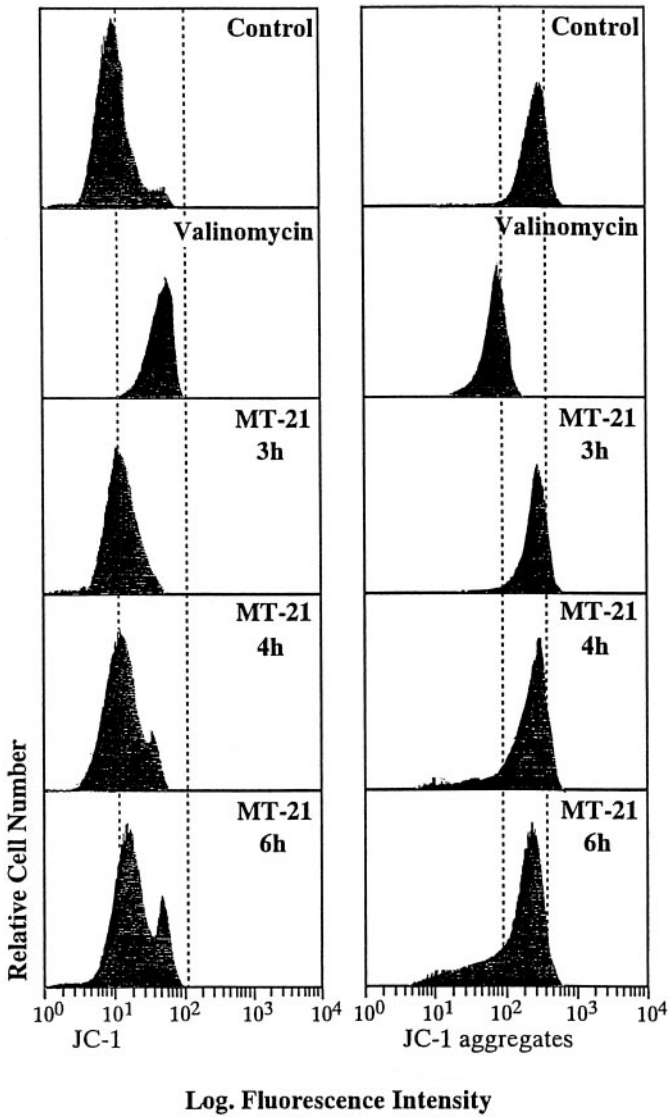


Fig. 3. Changes in $\Delta\psi m$ in MT-21-treated HL-60 cells. Cells were untreated or treated with 40 μM MT-21. At various time points, the cells were harvested and stained with JC-1. As a control for the specificity of JC-1 staining, the cells were treated with 100 nM valinomycin for 30 min. JC-1 monomer (*left*) and JC-1 aggregates (*right*) were measured by flow cytometry.

treated vector and Bcl-2 cells. The immunostaining of untreated vector cells and Bcl-2 cells demonstrated a punctate pattern for cytochrome *c* consistent with mitochondrial localization (Fig. 5*C*, *Control*). On induction of apoptosis by MT-21 in vector cells, cytochrome *c* lost punctate distribution and diffused throughout the cells (Fig. 5*C*, *Vector/MT-21*). On the other hand, in Bcl-2 cells, MT-21 treatment failed to diffuse cytochrome *c* distribution, resulting in the protection of cells from apoptosis (Fig. 5*C*, *Bcl-2/MT-21*). The induction of DNA fragmentation by MT-21 was also suppressed in Bcl-2 cells (Fig. 5*D*). These results indicate that Bcl-2 inhibits MT-21-induced apoptosis through the suppression of cytochrome *c* release from the mitochondria.

Release of Cytochrome *c* from Mitochondria by MT-21 Using a Cell-free System. We examined whether MT-21 released cytochrome *c* from mitochondria directly using a cell-free system. After preparation of the mitochondria fraction from the cells, various concentrations of MT-21 were added directly to the mitochondria. As shown in Fig. 6*A*, MT-21 directly released cytochrome *c* from isolated mitochondria, and this effect was partially suppressed by NAC (Fig. 6*B*). NAS, the negative control of NAC, had no effect on MT-21-induced cytochrome c release (data not shown). CsA also inhibited MT-21-induced cytochrome *c* release from isolated mitochondria (Fig. 6*B*). Benzoyloxycabonyl-Asp-CH_2O(CO)-2,6-dichlorobenzene (Z-Asp), a synthetic inhibitor of caspases (Peptide Institute, Inc., Osaka, Japan), inhibited MT-21-induced apoptosis as described previously (12). As shown in Fig. 6*B*, Z-Asp did not inhibit MT-21-induced cytochrome *c* release from isolated mitochondria. We also examined the effect of MT-21 on mitochondria isolated from Bcl-2 cells. Cytochrome *c* release from mitochondria by MT-21 was detected on mitochondria isolated from vector cells, but cytochrome *c* release was suppressed on mitochondria isolated from Bcl-2 cells (Fig. 6*C*). Recently, it was reported that precursors of caspase-2, -3, and -9 located on mitochondria are released from the mitochondria to the cytosol during the apoptotic process (27). Next, we examined whether MT-21 released caspase-3 from mitochondria. As shown in Fig. 6*C*, the caspase-3 precursor was released directly from mitochondria by MT-21, and this effect was barely detected on mitochondria isolated from Bcl-2 cells. The expression of Bcl-2 in mitochondria isolated from Bcl-2 cells was confirmed by immunoblotting (Fig. 6*C*). These observations indicated that MT-21 acted directly on mitochondria and induced the release of cytochrome *c* and caspase-3 from mitochondria during apoptosis.

Release of Cytochrome *c* from Mitochondria by Other MT Compounds. MT-21 is a compound that has a γ-lactam ring and an alkyl chain (n-C_8H_{17}) at the N-1 position. To determine what is necessary to release cytochrome *c* and caspases from mitochondria on a structure characteristic of MT-21, we synthesized a series of derivatives with alkyl chains of different lengths at the N-1 or C-5 position in a γ-lactam ring (Fig. 7*A*). When we examined apoptosis induction by these compounds in HL-60 cells, only the compound (MT-21) that has an alkyl chain (C_8) at the N-1 position induced cytochrome *c* release from mitochondria, activation of proteins (caspases, Krs proteins, and JNKs), and apoptosis induction (Fig. 7, *B—E*). These results suggest that an alkyl chain (n-C_8H_{17}) at the N-1 position but not at the C-5 position in a γ-lactam ring was necessary to release cytochrome *c* from mitochondria and could induce apoptosis by acting directly on mitochondria.

Release of Cytochrome *c* from Mitochondria by Apoptosis-inducing Agents. We examined the effect of other apoptosis-inducing agents including MT-21, cytotrienin A, camptothecin, etoposide, paclitaxel, and staurosporine on the release of cytochrome *c* from mitochondria in HL-60 cells. As shown in Fig. 8*A*, cytochrome *c* release into the cytosol was detected on treatment with all of the apoptosis-inducing agents tested here. In addition, we confirmed that treatment with the same concentration of these apoptosis-inducing agents induced DNA fragmentation (Fig. 8*C*). However, with the exception of MT-21, treatment with these agents did not induce the direct release of cytochrome *c* from cell-free mitochondria (Fig. 8*B*). These results suggest that cytochrome *c* release from the mitochondria may be an event common to the apoptotic program induced by antitumor agents, despite the differences in their modes of action, and MT-21 alone is able to release cytochrome *c* from mitochondria by direct action.

DISCUSSION

It is well known that most antitumor agents induce apoptosis in cancer cells. Triggering of apoptosis by antitumor agents involves simultaneous or subsequent activation of death receptor systems, perturbation of mitochondrial function, and proteolytic processing of caspases, the death effector molecules of apoptosis. Thus, the cell death pathway may be entered at multiple sites, and most drugs may

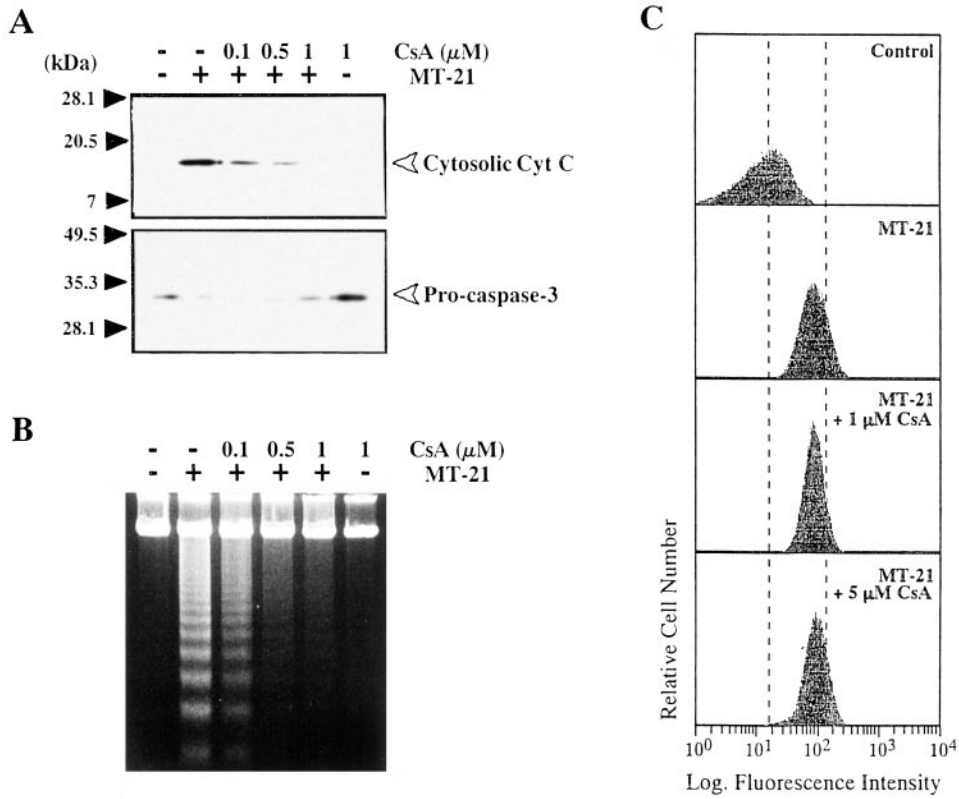


Fig. 4. Inhibition of MT-21-induced apoptosis by CsA. HL-60 cells were treated with 40 μM MT-21 for 6 h in the absence or presence of CsA. Immunoblotting analysis was performed using anti-cytochrome *c* or anti-caspase-3 antibodies (*A*). Cellular DNA extracted from the cells was analyzed by agarose gel electrophoresis and stained with ethidium bromide (*B*). HL-60 cells were pretreated with or without 1 or 5 μM CsA and then treated with 40 μM MT-21. The amount of intracellular H_2O_2 was measured by flow cytometry (*C*).

hit various targets, although the precise molecular mechanisms have not been characterized in detail.

The caspase cascade is activated by various stimuli including serum withdrawal, activation of Fas, ionizing radiation, and chemical compounds (28–33). Fas activation is known to activate the caspase cascade by interacting directly with a signaling complex on the cell membrane (4, 34). However, little is known about the non-receptor signal transduction pathways that operate within the cytoplasm to activate caspases. Only one apoptotic pathway in mammalian cells is shunted through the mitochondria and involves the export of cytochrome *c* to the cytoplasm (7). In the present study, we found that MT-21 activated caspase-9 via cytochrome *c* release, which is involved mainly in non-receptor-mediated signaling, but did not activate caspase-8, which interacts directly with a signaling complex on the cell membrane (Fig. 1).

In addition, we reported previously that ROS were involved in MT-21-induced apoptosis in HL-60 cells because caspase cascade activation and apoptosis were diminished by NAC (12). In this study, we showed that ROS were also involved in cytochrome *c* release from mitochondria during MT-21-induced apoptosis because they were inhibited by NAC (Fig. 2*C*). Recently, the involvement of the mitochondrial permeability transition pore in apoptosis has been shown. The pore consists of a complex of the mitochondrial porin channel (voltage-dependent anion channel, VDAC), the adenine nucleotide

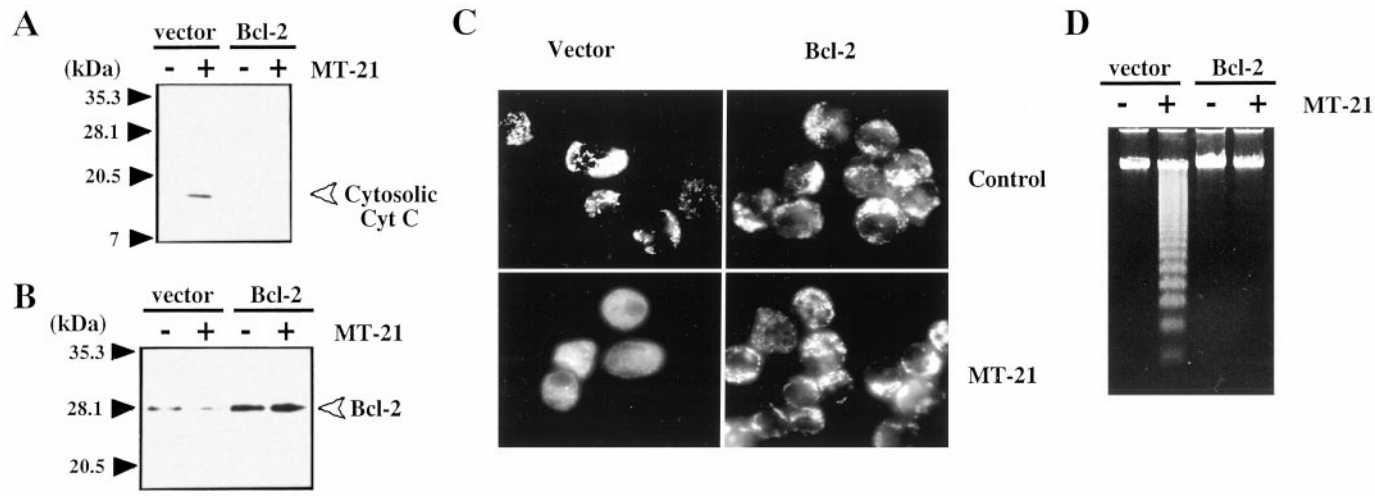


Fig. 5. Inhibition of MT-21-induced cytochrome *c* release from mitochondria and apoptosis by overexpression of Bcl-2. Vector and Bcl-2 cells were treated with 40 μM MT-21 for 8 h. Immunoblotting analysis was performed using anti-cytochrome *c* (*A*) or anti-Bcl-2 antibodies (*B*). C, confocal microscopy of control and MT-21 treatment on vector and Bcl-2 cells labeled with an anti-cytochrome *c* antibody. *D*, cellular DNA extracted from the cells was analyzed by agarose gel electrophoresis and stained with ethidium bromide.

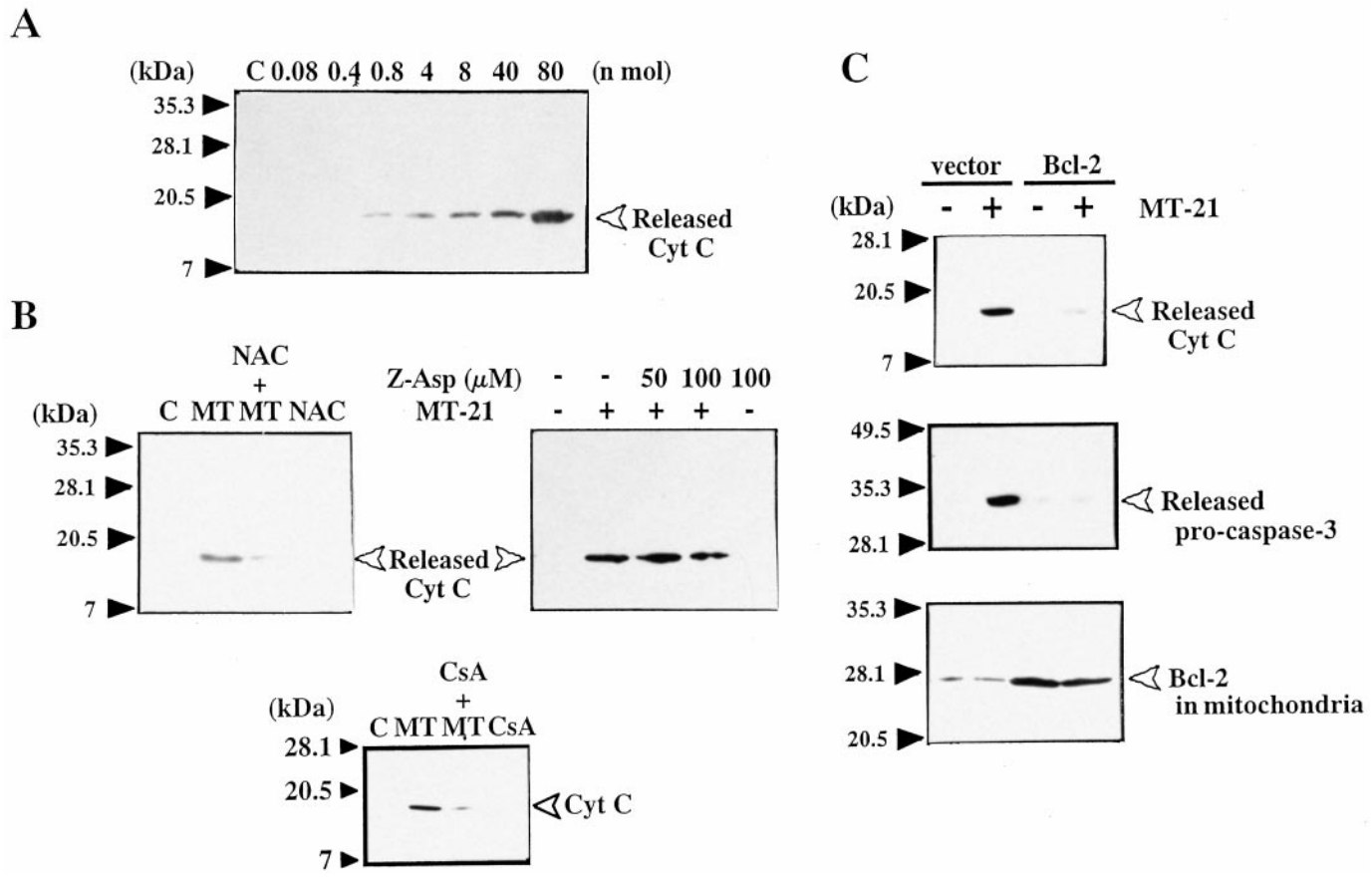


Fig. 6. Release of cytochrome *c* from mitochondria by MT-21 using a cell-free system. *A*, mitochondria isolated from HL-60 cells were added to the indicated concentration of MT-21 at 37°C for 2 h. Using the supernatants obtained by centrifugation, immunoblotting analysis was performed using anti-cytochrome *c* antibodies. *B*, mitochondria isolated from HL-60 cells were pretreated with 3 mM NAC or with the indicated concentration of Z-Asp and then added to 80 nmol of MT-21. After incubation at 37°C for 2 h, immunoblotting analysis was performed as described in *A*. *C*, mitochondria isolated from vector or Bcl-2 cells were added to 80 nmol of MT-21 at 37°C for 2 h. Using the supernatants obtained by centrifugation, immunoblotting analysis was performed with anti-cytochrome *c*, anti-caspase-3, or anti-Bcl-2 antibodies.

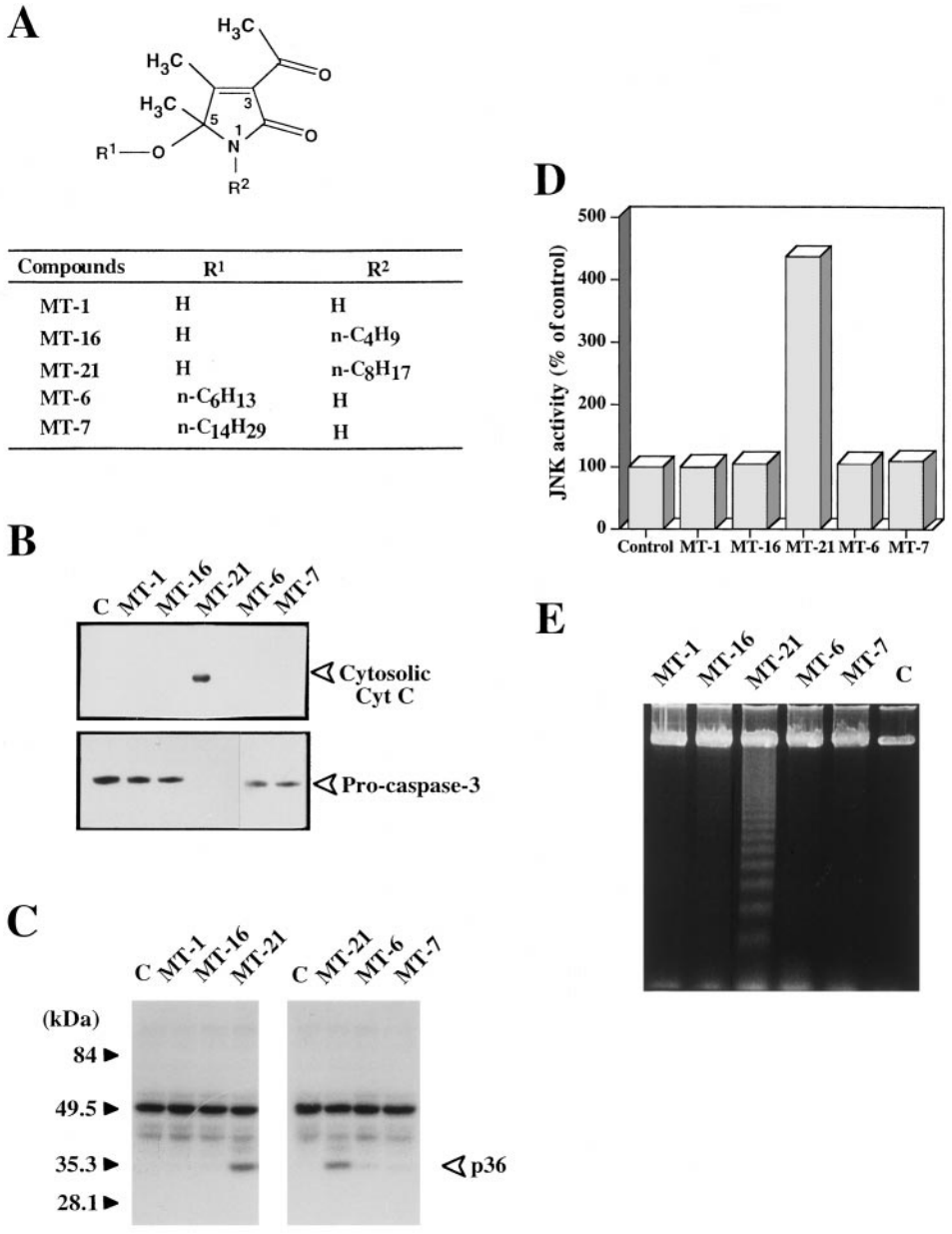
translocase, and cyclophilin D at contact sites between the mitochondrial outer and inner membranes. MT-21 caused little change in $\Delta\psi$m during apoptosis (Fig. 3). Therefore, it is possible for MT-21 to act directly on mitochondria and induce cytochrome *c* release from mitochondria before the change in $\Delta\psi$ m; *i.e.*, it seems that the target of MT-21 is not VDAC.

Using CsA, which inhibits the pore formation, we found that MT-21 released cytochrome *c* from mitochondria via the mitochondrial permeability transition pore during apoptosis (Fig. 4). It has been reported previously that atractyloside, which is known to be an inhibitor of adenine nucleotide translocase, induces the permeability transition (35). We compared atractyloside with MT-21 side by side on cytochrome *c*-releasing and apoptosis-inducing ability in HL-60 cells. MT-21 induced cytochrome *c* release and apoptosis more rapidly and at a lower concentration than did atractyloside (data not shown).

The initiation of the apoptotic cascades is strictly regulated because it is most important for cell survival. Bcl-2, a gene product of proto-oncogene bcl-2, is a widely studied negative regulator of apoptosis (36, 37). Bcl-2 is located mainly on the membrane of mitochondria and prevents the cells from apoptosing in response to a variety of stimuli (37). Previously, it was reported that an overexpression of Bcl-2 blocked cytochrome *c* release from mitochondria (9, 38). In fact, MT-21-induced cytochrome *c* release from mitochondria was inhibited by overexpression of Bcl-2 (Fig. 5). Recently, it was reported that precursors of caspase-2, -3, and -9 localized in both the cytosol and mitochondria are released from mitochondria during the apoptotic process (27). Using a cell-free system, MT-21 also induced the release of caspase-3 from mitochondria in the same way as cytochrome *c*, and this effect was not detected on mitochondria isolated from the cells overexpressing Bcl-2. Thus, the direct release of cytochrome *c* or caspases from mitochondria by MT-21 was regulated by Bcl-2. It is therefore acknowledged that the release of cytochrome *c* or caspases from mitochondria by MT-21 was not a leak caused by the damage against mitochondria, and we suspect that MT-21 activates the mechanism releasing mitochondrial proteins from mitochondria.

In this study, we showed that ROS were involved in the release of cytochrome *c* from mitochondria during MT-21-induced apoptosis because MT-21-induced apoptosis is inhibited by NAC. However, MT-21-induced release of cytochrome *c* in mitochondria isolated from the cells was slightly inhibited by NAC (Fig. 6*B*). Considering these results, we suspect that MT-21 induces ROS production in both cytosol and mitochondria. Because the mechanism by which ROS production is able to activate the caspase cascade during MT-21-induced apoptosis has not yet been clarified, the elucidation of this mechanism will be necessary in the future.

MT-21 is a compound which has a γ-lactam ring and an alkyl chain (n-C_8H_{17}) at the N-1 position. To determine what is necessary to release the cytochrome *c* and caspases from mitochondria on a structure characteristic of MT-21, we synthesized a series of derivatives with alkyl chains of different lengths at the N-1 position in a γ-lactam ring. When we examined the apoptosis induction by these compounds in HL-60 cells, only MT-21, which has an alkyl chain (C_8) at the N-1 position, induced cytochrome *c* release from mitochondria, the acti-



Compounds	R^1	R^2
MT-1	H	H
MT-16	H	n-C_4H_9
MT-21	H	n-C_8H_{17}
MT-6	n-C_6H_{13}	H
MT-7	n-$C_{14}H_{29}$	H

Fig. 7. Induction of apoptosis by MT-compounds. *A*, structure of our synthetic compounds. *B*, HL-60 cells were treated with 40 μM MT compounds for 6 h, and immunoblotting analysis was performed using anti-cytochrome *c* or anti-caspase-3 antibodies. When HL-60 cells were treated with 40 μM MT compounds for 6 h, MBP kinase activity (*C*), JNK activity (*D*), and DNA fragmentation (*E*) were analyzed as described in "Materials and Methods," respectively.

vation of proteins (caspases, Krs proteins, and JNKs), and apoptosis induction (Fig. 7). Moreover, compounds with an alkyl chain at the C-5 position failed to induce apoptosis (Fig. 7). These results suggest that an alkyl chain (*n*-C_8H_{17}) at the N-1 position but not the C-3 position in a γ-lactam ring was necessary to release cytochrome *c* from mitochondria and could induce apoptosis by acting directly on mitochondria.

Recently, in response to the activation of cell surface death receptors such as Fas, it was shown that a Bcl-2-interacting protein, BID, induced the release of cytochrome *c* from mitochondria by a caspase-dependent mechanism (39–42). BID is cleavaged by caspase-8, and the cleavaged form of BID translocates to the mitochondrial membrane, where it is a potent inducer of cytochrome *c* release. Because MT-21 caused no activation of caspase-8, BID was considered to have no impact on MT-21-induced apoptosis. On the other hand, it was reported that Ca^{2+} and Bax released cytochrome *c* from mitochondria by a caspase-independent mechanism (43, 44). However, HL-60 cells already express a constant level of Bax, and MT-21 caused no change in the amount of Bax (data not shown). MT-21 released cytochrome *c* from mitochondria by a Bax-independent mechanism. Moreover, apoptosis-inducing agents that are able to induce cytochrome *c* release from mitochondria such as camptothecin, etoposide, paclitaxel, staurosporine, and cytotrienin A could not directly release cytochrome *c* from the cell-free mitochondria (Fig. 8). Han *et al.* reported that staurosporine as well as Fas induced cytochrome *c* release from mitochondria via BID (45). Recently, several groups have reported anticancer agents that exert a direct effect on mitochondria, such as betulinic acid, lonidamine, CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid), arsenite, and the peptide motif KLAKKLAKKLAKKLAK (46–49). However, all of these agents induce apoptosis by provoking a disruption of the mitochondrial transmembrane potential ($\Delta\psi$m). MT-21 provoked no change in $\Delta\psi$m. These results suggest that MT-21 is the apoptosis-inducing compound that is able to induce the direct release of cytochrome *c* from mitochondria both *in situ* and *in vitro*, and this is unprecedented because MT-21 has no effect on $\Delta\psi$m.

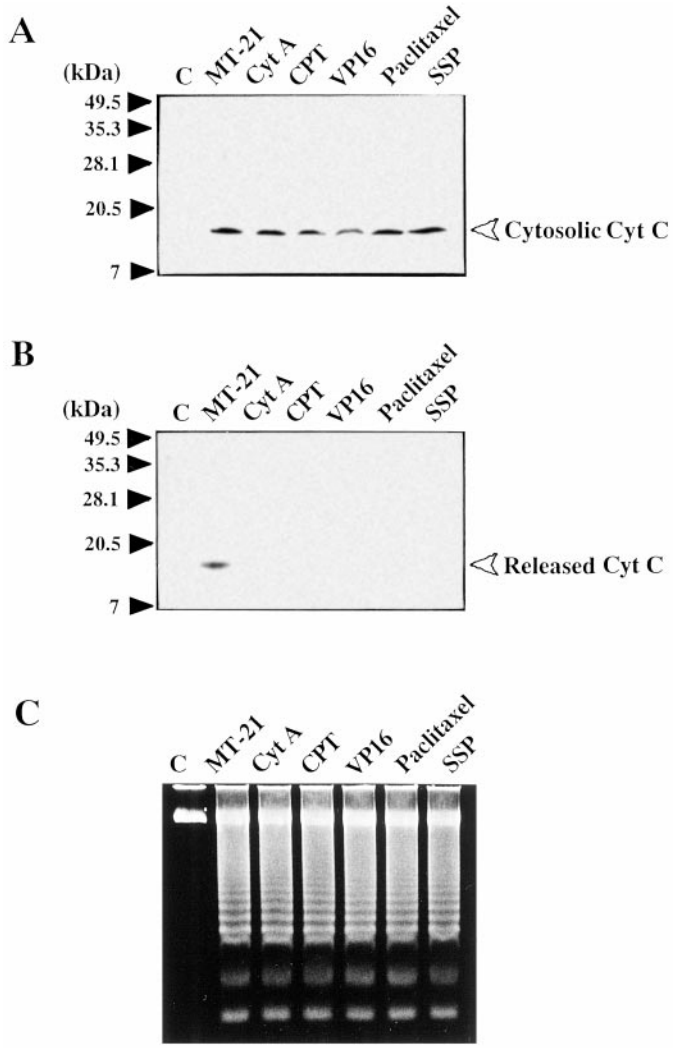


Fig. 8. Release of cytochrome *c* from mitochondria by apoptosis-inducing agents. *A*, HL-60 cells were treated with 40 μM MT-21 (4 h), 300 ng/ml cytotrienin A (2 h), 1 μM camptothecin (6 h), 10 μM etoposide (5 h), 0.1 μM paclitaxel (24 h), and 3 μM staurosporine (6 h), and immunoblotting analysis was performed using anti-cytochrome *c* antibodies. *B*, mitochondria isolated from HL-60 cells were treated with 80 nmol of MT-21, 600 ng of cytotrienin A, 2 nmol of camptothecin, 20 nmol of etoposide, 0.2 nmol of paclitaxel, and 6 nmol of staurosporine. After incubation at 37°C for 2 h, the supernatants obtained by centrifugation were used to perform immunoblotting analysis with anti-cytochrome *c* antibodies. *C*, HL-60 cells were treated with 40 μM MT-21 (6 h), 300 ng/ml cytotrienin A (3 h), 1 μM camptothecin (8 h), 10 μM etoposide (6 h), 0.1 μM paclitaxel (30 h), and 3 μM staurosporine (8 h). Cellular DNA extracted from the cells was analyzed by agarose gel electrophoresis and stained with ethidium bromide.

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