Tetrocin A Inhibits Mitochondrial Functions of Bcl-2 and Suppresses Its Anti-apoptotic Activity

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INTRODUCTION

Bcl-2 is an integral, intracellular membrane protein that prevents cells from undergoing apoptosis in response to a variety of cell death signals. It negatively regulates the activation of Caspase-3, which functions as effector of mammalian cell death pathways. Overexpression of Bcl-2 inhibits the caspase activities and apoptosis. A microbial secondary metabolite, Tetrocin A (TC-A), was identified as an inhibitor of the anti-apoptotic function of Bcl-2. Apoptosis could be induced in cell lines that overexpress Bcl-2 or Bcl-X, when the cells were treated with anti-Fas antibody, tumor necrosis factor α, staurosporine, or Bax, in addition to TC-A. TC-A showed selectivity against the pro-apoptotic Bcl-2 family members, in that cell overexpressing CrmA or dominant-negative FADD could not undergo apoptosis with TC-A treatment. In Bcl-2-overexpressing cell lines, TC-A inhibited mitochondrial functions regulated by Bcl-2, resulting in Fas-triggered mitochondrial transmembrane potential loss and cytochrome c release. Inhibition of the mitochondrial functions of Bcl-2 and, thereby, its anti-apoptotic effect could serve as useful pharmacological targets. Thus, TC-A should serve as an archetype for specific inhibitors of Bcl-2 functions.

MATERIALS AND METHODS

Cell Culture. HeLa/bcl-2 is a stable, bcl-2-expressing cell line derived from HeLa cells transfected with a bcl-2 expression plasmid. The details of its construction have been reported previously (28). HeLa/bcl-X, cell is a stable, bcl-X-expressing cell line derived from HeLa cells transfected with an expression vector containing a cDNA encoding human bcl-X. Bcl-X, overexpressing clones were isolated by selecting G418-resistant clones. 293/bcl-2 or 293/bcl-X cells are transiently transfected 293 cells. All of the cells were grown in DMEM supplemented with 10% fetal bovine serum.

Treatment of Cells with Reagents. Cells were untreated or treated with cell-death-inducing stimuli including, αFas (100 ng/ml) and CHX (0.2 µg/ml), STA (1 µM), or TNF-α (10 ng/ml), and cultured in the presence of TC-A. Cultivation time for TC-A treatment was 6, 24, 8, 6, and 24 h for DEVD-cleavage, PARP-cleavage, DiOC₃(3) staining, the release of cytochrome c, and XTT cell viability assays, respectively.

Viability Assays. Cell viability was determined by XTT assay. Briefly, cells (1.5 x 10⁵) were plated per well, and treated with various reagents, as described above. Cells were then cultured in the presence of various concentrations of TC-A for 24 h and assayed for uptake of XTT as described previously (29).

PARP Cleavage Assay. Cells (2 x 10⁵) were treated by the reagents described above. After 24 h of cultivation, the cells were washed with PBS and lysed in 20 µl of lysis buffer [250 mM NaCl/1.0% NP40/50 mM HEPES-NaOH (pH 7.5)/protease inhibitors/1 mM DTT/1 mM EDTA]. Lysates were clarified by centrifugation, and the protein concentration of the supernatants was determined. Each protein sample (10 µg) was subjected to SDS/PAGE (12.5% acrylamide) and transferred to nitrocellulose filters. The filters were blocked and incubated with anti-PARP (Enzyme Systems Products) or anti-Bcl-2 (Dako) antibodies.

DEVD-4-methyl-coumaryl-7-amide Cleavage Assay. Cells (3 x 10⁵) were treated with reagents as described above. After 6 h of cultivation, the cells were lysed as described previously (28). The DEVD-specific caspase activity was measured by incubating cell extracts (25 µl) with Acetyl-L-Aspartyl-L-Glutamyl-L-Valyl-L-Aspartic Acid α-(4-Methyl-Coumaryl-7-Amide) (50 µM, Peptide Institute) in 50 µl of buffer A (20 mM Pipes (pH 7.2), 100 mM NaCl, 5 mM DTT, 1 mM EDTA, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate). After 120 min, the reaction was stopped by the addition of
100 µl of 0.75 M acetic acid and placed on ice. Fluorescence at wavelength 380 to 460 nm was compared with a standard curve of 7-amino-4-methylcoumarin (AMC, Peptide Institute).

**Transient Transfection.** Transfections were performed by using Lipofectamine (Life Technologies, Inc.) according to the supplier’s protocol. 293 cells were seeded in 24-well dishes and transfected with 0.5 µg of each expression vector (pcDNA3-Bax, pcDNA3-Bcl-2, or pcDNA3-Bcl-XL). Twenty-four h after transfection, the cells were treated with TC-A (2 µM) for 12 h. Cleavage of PARP was assessed as described in “Materials and Methods” above.

HeLa cells were seeded in 6-well dishes and transfected with 1 µg of each expression vector, together with 0.1 µg of green fluorescent protein (GFP) expression vector (pCMX-SA/Y145F, kindly donated by K. Umezono at...
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RESULTS AND DISCUSSION

Discovery of TC-A as an Inhibitor of the Anti-apoptotic Functions of Bcl-2. TC-A was identified by screening a library of natural products—a rich source of chemically unique, biologically active compounds—for its ability to inhibit the anti-apoptotic function(s) of Bcl-2 and, thereby, cause apoptosis in Bcl-2-overexpressing cell lines (Fig. 2A). Untransfected HeLa cells are susceptible to cell death induced by cell-death stimuli such as αFas in the presence of the protein synthesis inhibitor αFas/CHX or STA, both of which cause the cleavage of PARP. HeLa/bcl-2 cells are, however, resistant to apoptosis induced by those same cell-death-inducing stimuli, and no PARP-cleavage was observed (Fig. 2B). Cotreatment of the apoptosis-resistant HeLa/bcl-2 with TC-A and αFas/CHX did activate caspases, as indicated by the cleavage of PARP (Fig. 2C) and tetrapeptide-substrate, Ac-DEVD-MCA (Fig. 2D). Caspase activation resulted in the loss of cell viability, and the inhibition of caspase activation by z-DEVD-fmk prevented cells from dying (Fig. 2, D and E). Cell morphological changes were consistent with the reduced viability of HeLa/bcl-2 cells treated with TC-A and αFas/CHX. That is, cell rounding and detachment from the substrate were observed in HeLa/bcl-2 treated with TC-A and αFas/CHX, resembling that observed in HeLa cells treated with αFas/CHX (Fig. 2F).

The effect of TC-A was dose-dependent. TC-A (2.2 μM) caused complete cleavage of M116,000 PARP, with a significant loss of cell viability. The small fragment of Bcl-2 that appeared with 2.2 μM of TC-A may be the COOH-terminal Bcl-2 cleavage product, because Bcl-2 is reported to be cleaved at Asp34 of the loop domain by caspase-3 (31). TC-A alone did not show any significant signs of apoptosis in HeLa/bcl-2 cells. These results indicate that TC-A seems to abrogate the cell-death block caused by Bcl-2 overexpression, because the apoptotic signal due to Fas ligation led to the death of Fas-resistant HeLa/bcl-2 cells.

Fig. 2G shows the effect of TC-A on nontransfected, parental HeLa cell line. Increased concentrations of TC-A did not affect the time course of PARP-cleavage induced by αFas/CHX treatment. Therefore, without Bcl-2 overexpression, TC-A did not sensitize HeLa cells to αFas/CHX. Furthermore, TC-A did not induce apoptosis in HeLa cells at the concentrations that induced Fas-dependent apoptosis in HeLa/bcl-2 cells (data not shown), thus indicating that TC-A itself had no activity as a cell-death stimulus at those drug concentrations in HeLa cells. Taken together, these data indicate that TC-A does not.

Kyoto University, Kyoto, Japan). Expression vector for CrmA, Bcl-2, and Bcl-XL were described previously (30). The expression vector for the dominant negative form of FADD was kindly donated by V. M. Dixit (University of Michigan Medical School, Ann Arbor, MI). Forty-eight h after transfection, cells were treated with TNF-α (10 ng/ml) and cycloheximide (10 μg/ml), together with TC-A (2 μM) for 5 h. Cells were then washed with PBS and fixed with 4% paraformaldehyde/PBS for 10 min. GFP-positive cells were examined with an Olympus fluorescence microscope (model IX70). Apoptotic cells were small, dense, and frequently fragmented, whereas surviving cells were flat and well attached to the dish as described previously (30).

Immunostaining of HeLa/bcl-2 Cells. HeLa/bcl-2 cells were treated with or without 2 μM of TC-A for 12 h, then fixed with 4% paraformaldehyde/PBS for 10 min, and permeabilized in 0.1% Triton X-100/PBS for 10 min at room temperature. The cells were washed 3 times with PBS and blocked with 4% normal goat serum in PBS (blocking buffer) for 10 min. They were incubated with monoclonal, antimouse Bcl-2 antibody (1:200 dilution, DAKO, Denmark) for 1 h at room temperature and washed with PBS three times. The samples were incubated with Rhodamine-labeled antimouse IgG (1:200 dilution, Jackson Laboratory) for 1 h and washed in PBS three times. The samples were mounted with PermaFlour Aqueous Mounting Medium (Immunon) and examined with an Olympus confocal laser scanning microscope (scale bar, 25 μm).

Assessment of Mitochondrial ΔΨm. Changes in the inner membrane transmembrane potential (ΔΨm) were determined by incubating 3 × 105 cells in 40 nm DiOC6(3) for 20 min at 37°C. The cells were assayed using FACScan flow cytometry (Becton Dickinson, Mountain View, CA). In all of the cases, the cells were gated to exclude cellular debris, which physically prevents proper fluorescence-activated cell sorting detection.

Subfractionation. HeLa/bcl-2 cells were treated with PBS, and the pellets were resuspended in ice-cold, buffer B [20 mM HEPES (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors] containing 250 mM sucrose. The cells were homogenized by douncing 80 times in a Dounce homogenizer (Wheaton). Nuclei and unbroken cells were separated by a centrifugation at 10,000 × g for 10 min. The resulting pellet (ppt) and supernatant fractions including cytosolic cytochrome c were subjected to Western analysis as described in “Materials and Methods.”

Fig. 3. Effect of TC-A on Fas-mediated apoptosis in HeLa/bcl-XL cells. PARP cleavage (A, B) and cell viability assay (C) of HeLa/bcl-XL. Cells were cultured in the presence of 100 μM z-DEVD-fmk for 18 h and then treated with αFas/CHX.

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simply sensitize the cells to Fas/CHX but, rather, blocks the anti-apoptosis function of Bcl-2 in HeLa/bcl-2 cells. TC-A also inhibits the anti-apoptotic function of Bcl-X \textsubscript{L}.

We next tested whether TC-A inhibited the anti-apoptotic function of another Bcl-2 family protein, Bcl-X \textsubscript{L}, using HeLa cells stably over-expressing Bcl-X \textsubscript{L} (HeLa/bcl-X \textsubscript{L} cells; Fig. 2A). When these cells were treated with Fas/CHX, no PARP cleavage was observed, indicating that the HeLa/bcl-X \textsubscript{L} cell line was also resistant to Fas-induced apoptosis (Fig. 3A). The addition of TC-A caused Fas-dependent apoptosis in HeLa/bcl-X \textsubscript{L} cells, as indicated by PARP cleavage, and an accompanying decrease in cell viability (Fig. 3, A and C). In contrast to the marginal effect of TC-A on the cleavage of Bcl-2 protein in Fas/CHX-treated HeLa/bcl-2 cells, marked cleavage of the Bcl-X \textsubscript{L} protein occurred, with a dose dependency similar to that observed for PARP cleavage and reduced cell viability. HeLa/bcl-X \textsubscript{L} cells treated with the caspase inhibitor z-DEVD-fmk blocked the cleavage of Bcl-X \textsubscript{L}, which indicated that DEVD-cleaving caspases were responsible for the proteolysis of Bcl-X \textsubscript{L} (Fig. 3B). The addition of caspase inhibitors also significantly suppressed the other activities of TC-A, for example PARP cleavage and cell viability loss in Fas-treated HeLa/bcl-X \textsubscript{L} cells. Thus, the action of TC-A is abrogated by the inhibition of DEVD-cleaving caspase activity, which indicates that TC-A inhibits an event upstream of the activation of z-DEVD-fmk-inhibitable caspase(s). This hypothesis is consistent with the fact that anti-apoptotic Bcl-2 family members act upstream of caspase-3 (32, 33). Similar proteolysis of Bcl-X \textsubscript{L} was reported in cells induced to undergo apoptotic death after Sindbis virus infection or interleukin 3 withdrawal. Furthermore, the COOH-terminal fragment of Bcl-X \textsubscript{L} was shown to potently induce apoptosis (34). These results suggest that the inhibition of the anti-apoptotic functions of Bcl-X \textsubscript{L} by TC-A results in the activation of a subset of caspases that are sensitive to Bcl-X \textsubscript{L}, which in turn cleaves the Bcl-X \textsubscript{L}.

Fig. 4. The effect of TC-A on TNF-\textalpha- or STA-mediated apoptosis in HeLa/bcl-2 cells. DEVD cleavage (A, B, left), PARP cleavage (A, B, middle) and cell viability assay (A, B, right) of HeLa/bcl-2. Cells were treated with 10 ng/ml TNF-\textalpha and 10 \mu g/ml CHX (A) or 1 \mu M STA (B) and were cultured in the presence of TC-A. *, the COOH-terminal Bcl-2 cleavage product.

Fig. 5. The effect of TC-A on the anti-apoptotic function of the Bcl-2 family in the Bax-mediated apoptosis. PARP cleavage in transiently transfected 293 cells. Cell were transfected with expression vectors as indicated. Twenty-four h after transfection, cells were treated with 2 \mu M TC-A for an additional 12 h and were analyzed for PARP-cleavage as described in “Materials and Methods.”

Fig. 6. The effect of TC-A on the anti-apoptotic function of CrmA and dominant negative form of FADD. Transfected expression vectors are indicated. Forty-eight h after transfection, cells were treated with TNF-\textalpha and cycloheximide (10 \mu g/ml) together with TC-A (2 \mu M) for 5 h. Apoptotic cells were counted as described in “Materials and Methods.”

TC-A also inhibits the Anti-apoptotic Function of Bcl-X \textsubscript{L}. We next tested whether TC-A inhibited the anti-apoptotic function(s) of another Bcl-2 family protein, Bcl-X \textsubscript{L}, using HeLa cells stably over-expressing Bcl-X \textsubscript{L} (HeLa/bcl-X \textsubscript{L} cells; Fig. 2A). When these cells were treated with Fas/CHX, no PARP-cleavage was observed, indicating that the HeLa/bcl-X \textsubscript{L} cell line was also resistant to Fas-induced apoptosis (Fig. 3A). The addition of TC-A caused a Fas-dependent apoptosis in HeLa/bcl-X \textsubscript{L} cells, as indicated by PARP-cleavage, and an accompanying decrease in cell viability (Fig. 3, A and C). In contrast to the marginal effect of TC-A on the cleavage of Bcl-2 protein in Fas/CHX-treated HeLa/bcl-2 cells, marked cleavage of the Bcl-X \textsubscript{L} protein occurred, with a dose dependency similar to that observed for PARP cleavage and reduced cell viability. HeLa/bcl-X \textsubscript{L} cells treated with the caspase inhibitor z-DEVD-fmk blocked the
TC-A Suppresses the Death-suppressor Activity of Bcl-2 in Apoptosis Induced by a Variety of Cell-Death Stimuli. To determine whether the effect of TC-A was restricted to Fas-induced apoptosis, we tested other apoptosis-inducing agents (Fig. 4). HeLa cells are susceptible to cell death by TNF-α/CHX or STA. HeLa/bcl-2 cells are resistant to apoptosis induced by those same cell-death stimuli (28, 35) but could be made sensitive by TC-A treatment. With increasing concentrations of TC-A, the Ac-DEVD-MCA- and PARP-cleaving activities also increased, with a concomitant decrease in cell viability. Similar results were obtained for STA-induced apoptosis in Rat1 cells stably transfected with bcl-2 (data not shown). Compared with the data obtained for Fas-induced apoptosis, TC-A was almost equally effective when TNF-α/CHX or STA were used as cell-death stimuli in HeLa/bcl-2 cells. That is, the concentrations of TC-A that induced the complete cleavage of 116K PARP were 2.2–3.3 μM, using all three of the different apoptotic stimuli. These results suggest that TC-A inhibits a point at which independent signaling pathways to apoptosis converge, most likely Bcl-2/Bcl-X₁, or common effector machinery that can be antagonized by Bcl-2/Bcl-X₁.

The effect of TC-A on the anti-apoptotic function of Bcl-2 was further tested by an experimental system in which the overexpression of Bax, a pro-apoptotic homologue of Bcl-2, induced apoptosis in 293 cells (Fig. 5). The transient expression of Bax induced the activation of caspases in 293 cells, as indicated by PARP-cleavage. Coexpression of Bcl-2 or Bcl-X₁ suppressed Bax-induced apoptosis. The addition of TC-A to the Bax/Bcl-2 coexpressing cells at 24 h after transfection restored the PARP-cleavage induced by Bax, which indicated that TC-A inhibited the anti-apoptotic function of the Bcl-2 family in Bax-mediated apoptosis.

TC-A Preferentially Inhibits the Anti-apoptotic Function of the Bcl-2 Family in Cell-Death Signaling Pathways. To gain further evidence supporting the theory that TC-A acts on Bcl-2 family members in the cell-death signaling pathway and thereby inhibits their anti-apoptotic functions, the effect(s) of TC-A on cell death suppressors other than the Bcl-2 family was studied. The cowpox virus CrmA is a viral serpin protein that can inhibit caspase family proteases (36). FADD mediates cell death by Fas and TNF-α by recruiting caspase-8 to their receptors. A dominant negative mutant of FADD (FADD-DN) lacks the 80 NH₂-terminal amino acids, which contains the death effector domain but retains the death domain. Overexpression of FADD-DN inhibits cell death initiated by Fas and TNF-α (37). HeLa cells were transiently transfected with expression vectors encoding CrmA, FADD-DN, Bcl-2, or Bcl-X₁. The transfected cells were treated with TNF-α/CHX together with TC-A, and the apoptotic cells were measured as described in “Materials and Methods. Similar to the results obtained with the HeLa cells stably transfected with bcl-2 or bcl-X₁, the anti-apoptotic functions of Bcl-2 or Bcl-X₁ were inhibited by TC-A. In contrast to this, TC-A did not show any effects on the anti-apoptotic function(s) of CrmA and FADD-DN, as shown in Fig. 6. Overexpression of CrmA inhibited apoptosis induced by TNFα, with 24% of the cells surviving, but the ratio of surviving cells remain unchanged in the presence of TC-A. HeLa cells transfected with FADD-DN were almost completely resistant to TNF-α/CHX. Treat-
ment of these cells with TC-A did not affect the anti-apoptotic function of FADD-DN. Taken together, these results confirmed that TC-A does not generally inhibit anti-apoptotic effectors but preferentially inhibits the anti-apoptotic function of the Bcl-2 family in the cell-death-signaling pathways of HeLa cells.

**TC-A Does Not Affect the Subcellular Localization of Bcl-2.** The proper localization of Bcl-2 in intracellular membranes is required for its death-suppressor activity. Bcl-2 mutants lacking the COOH-terminal, membrane-anchoring tail are localized mainly in the cytosolic fraction. These mutants exhibit a greatly reduced anti-apoptotic activity when compared with wild-type Bcl-2 (38, 39). Therefore, the inhibition of the subcellular localization of Bcl-2 could lead to the inhibition of the anti-apoptotic function of Bcl-2. To test whether TC-A affected the subcellular localization of Bcl-2, HeLa/bcl-2 cells treated with TC-A were incubated with anti-Bcl-2 antibody and processed for immunofluorescence. In the absence of TC-A, Bcl-2 labeled with anti-bcl-2 antibody showed granular and perinuclear staining (Fig. 7A). This staining pattern was not affected by treating the cells with TC-A (Fig. 7B). Thus, TC-A does not affect the localization of Bcl-2 in HeLa/bcl-2 cells.

**TC-A Inhibits Mitochondrial Functions Regulated by Bcl-2.** One of the most extensively studied functions of Bcl-2 in the intracellular membranes is its role in the mitochondrial membrane. Bcl-2 family proteins have been implicated in the regulation of mitochondrial pathophysiology, such as the electrochemical gradient (ΔΨm) across the inner mitochondrial membrane (40) and the release of cytochrome c from mitochondria. We tested the effect of TC-A on these two important aspects of mitochondrial functions during the apoptosis. Treatment of HeLa cells with a combination of αFas/CHX resulted in a release of mitochondrial cytochrome c in HeLa cells. An αFas/CHX treatment also result in a rapid reduction in the mitochondrial transmembrane potential ΔΨm, as assessed by the cationic lipophilic dye DiOC6(3) (Fig. 8). Overexpression of Bcl-2 inhibits the Fas-triggered mitochondrial release of cytochrome c and the reduction of ΔΨm (Fig. 8), in agreement with previous observations (41–46). We assessed the effect of TC-A on those same mitochondrial functions regulated by Bcl-2. In the absence of αFas/CHX, TC-A alone slightly affect the ΔΨm, which suggests that TC-A itself has some effect on the function of the mitochondrial. The precise mechanism of the mitochondrial effects of TC-A is unknown at the present stage but will be studied in our laboratory. In addition to the TC-A effect observed in the absence of αFas/CHX, TC-A clearly inhibited the protective effects of Bcl-2 in HeLa/bcl-2 treated with αFas/CHX. TC-A induced the collapse of ΔΨm in Fas-treated HeLa/bcl-2 cells in a dose-dependent manner (Fig. 8A). Increased concentrations of TC-A caused increases in the cells of cytosolic cytochrome c that were accompanied by cleavage of PARP (Fig. 8B). Taken together, these data indicate that TC-A inhibits the anti-apoptotic functions of Bcl-2 by suppressing those mitochondrial functions regulated by Bcl-2.

In conclusion, TC-A preferentially suppresses the anti-apoptotic functions of the Bcl-2 family in apoptosis triggered by a variety of death stimuli, including Fas, TNFα, STA, and Bax. This could be due to the inhibition of the mitochondrial function(s) of Bcl-2 by TC-A. Thus, the present study pharmacologically demonstrates that the mitochondrial functions regulated by Bcl-2 are crucial for the anti-apoptotic function(s) of the Bcl-2 family. Although additional studies are needed for the elucidation of a precise mechanism(s) for the TC-A-mediated inhibition of Bcl-2 anti-apoptotic function, TC-A is the first Bcl-2 antagonist identified and will be quite useful for pursuing the molecular mechanism of action of Bcl-2. TC-A could also be of therapeutic utility in the treatment of diseases that are associated with the overexpression of Bcl-2 family members.
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