Tetrocarcin 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, Tetrocarcin 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-XL 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 cells 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.

ABSTRACT

Bcl-2 is an integral, intracellular membrane protein that prevents apoptosis or programmed cell death induced by diverse stimuli in many cell types (1–3). Both biochemical and genetic evidence indicate that Bcl-2 prevents apoptosis at the point of activation of CED-3 family proteases, such as Caspase-3 (CPP32; 4–6). Recent evidence has shown that the mitochondria play a crucial role in apoptosis by releasing apoptogenic factors, such as cytochrome c and apoptosis-inducing factor, from the intermembrane space into the cytoplasm (7). Cytochrome c release activates caspases through its effects on a protein called Apaf-1 (8, 9). Anti-apoptotic Bcl-2 and Bcl-XL inhibit the apoptosis-associated release of both cytochrome c and apoptosis-inducing factor, although the mechanism of these actions has remained elusive.

Small molecule inhibitors of the anti-apoptotic functions of Bcl-2, which we would like to refer to as Bcl-2 antagonists, would be useful reagents for understanding Bcl-2 function. In addition to aiding in mechanistic studies, they could also be useful in the treatment of those diseases in which Bcl-2 plays a role. Elevated expression of Bcl-2 is frequently found in human cancers. Consequently, those cancer cells resist apoptosis (10). In many cases, cancers, such as follicular lymphoma and hormone-refractory prostate cancer, that overexpress Bcl-2 are also resistant to chemotherapeutic agents. Such cancers have been associated with an unfavorable prognosis (11–17). Thus, Bcl-2 antagonists have the potential of treating those human malignancies composed of Bcl-2-overexpressing cells. Several approaches have been pursued to inhibit the function of Bcl-2 in human cancers. Most of them are oligonucleotide-based, such as the use of Bcl-2 antisense RNA or hammerhead ribozyme against bcl-2 (18–21). However, small molecule Bcl-2 antagonists have yet to be reported.

We describe here a natural product produced by Actinomycete, TC-A (Fig. 1), which inhibits the anti-apoptotic functions of the Bcl-2 family. TC-A was originally discovered as an antibiotic active against Gram-positive bacteria (22–27). It also showed antitumor activity in murine experimental tumor models, such as Sarcoma 180, P388 leukemia, and B16 melanoma, although the mechanism of its antitumor activity is unknown. The present study shows that TC-A inhibits the mitochondrial functions of Bcl-2 and, thereby, suppresses its anti-apoptotic function in cells overexpressing Bcl-2. These results pharmacologically indicate that the mitochondrial functions regulated by Bcl-2 are crucial for the death-suppressor activity of Bcl-2 family proteins.

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-XL cell is a stable, bcl-XL-expressing cell line derived from HeLa cells transfected with an expression vector containing a cDNA encoding human bcl-XL. Bcl-XL overexpressing clones were isolated by selecting G418-resistant clones. 293/bcl-2 or 293/bcl-XL 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-cleave, PARP-cleave, DiOC6(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 × 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 × 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-amine Cleavage Assay. Cells (3 × 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 Lipo-fectamine (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/H/Y145F, kindly donated by K. Umezono at Fig. 1. Structure of TC-A

![Fig. 1. Structure of TC-A](image)

Fig. 2. Effect of TC-A on Fas-mediated apoptosis in HeLa/bcl-2 cells. A, Western analysis of the expression level of Bcl-2 and Bcl-XL. Erk2 was used as the control for equal protein loading. B, time course of PARP cleavage in HeLa and HeLa/bcl-2. HeLa and HeLa/bcl-2 cells were treated with indicated reagents and at the indicated times; cleavage of PARP was analyzed as described in “Materials and Methods.” C-F, PARP cleavage (C), DEVD cleavage (D), cell viability (E), and cell morphology (F) of HeLa/bcl-2 treated with TC-A. Cells were treated with indicated reagents and analyzed as described in “Materials and Methods” [C, +, the COOH-terminal Bcl-2 cleavage product (8)]. G, time course of PARP cleavage induced by oFas/CHX in HeLa cells in the presence of various concentrations of TC-A.

![Fig. 2. Effect of TC-A on Fas-mediated apoptosis in HeLa/bcl-2 cells.](image)
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 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 M₄₁₁,000 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
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-XL.

We next tested whether TC-A inhibited the anti-apoptotic function(s) of another Bcl-2 family protein, Bcl-XL, using HeLa cells stably overexpressing Bcl-XL (HeLa/bcl-XL cells; Fig. 2A). When these cells were treated with Fas/CHX, no PARP cleavage was observed, indicating that the HeLa/bcl-XL cell line was also resistant to Fas-induced apoptosis (Fig. 3A). The addition of TC-A caused a Fas-dependent apoptosis in HeLa/bcl-XL 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 αFas/CHX-treated HeLa/bcl-2 cells, marked cleavage of the Bcl-XL protein occurred, with a dose dependency similar to that observed for PARP cleavage and reduced cell viability. HeLa/bcl-XL cells treated with the caspase inhibitor z-DEVD-fmk blocked the cleavage of Bcl-XL, which indicated that DEVD-cleaving caspases were responsible for the proteolysis of Bcl-XL (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 αFas-treated HeLa/bcl-XL 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-XL was reported in cells induced to undergo apoptotic death after Sindbis virus infection or interleukin 3 withdrawal. Furthermore, the COOH-terminal fragment of Bcl-XL was shown to potently induce apoptosis (34). These results suggest that the inhibition of the anti-apoptotic functions of Bcl-XL by TC-A results in the activation of a subset of caspases that are sensitive to Bcl-XL, which in turn cleaves the Bcl-XL.

**TC-A INHIBITS MITOCHONDRIAL FUNCTIONS OF BCL-2**

**Fig. 4.** The effect of TC-A on TNF-α- 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-α and 10 μg/ml CHX (A) or 1 μM 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 μM 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-α and cycloheximide (10 μg/ml) together with TC-A (2 μM) for 5 h. Apoptotic cells were counted as described in “Materials and Methods.”
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-XL or common effector machinery that can be antagonized by Bcl-2/Bcl-XL.

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-XL 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-XL. 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.” 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.

Fig. 7. The effect of TC-A on the localization of Bcl-2 in HeLa cells. HeLa/bcl-2 cells were treated with DMSO (a) or 2 μM TC-A (b) for 12 h and were then fixed and stained as described in “Materials and Methods.”

Fig. 8. The effect of TC-A on Fas-induced ΔΨm loss and cytochrome c release. A, DiOC6(3) staining of HeLa or HeLa/bcl-2 untreated or treated with αFas/CHX in the presence of various concentrations of TC-A. B, Western blot analysis of the release of cytochrome c in HeLa and HeLa/bcl-2 cells. Cells were treated with reagents as indicated and were fractionated and analyzed as described in “Materials and Methods.”
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 resulted 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 affects the ΔΨm, which suggests that TC-A itself has some effect on the function of the mitochondrion. 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 cells 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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