Bcl-2-Associated X Protein Is the Main Mediator of Manumycin A-Induced Apoptosis in Anaplastic Thyroid Cancer Cells

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We previously demonstrated that the combination of paclitaxel and manumycin A, a farnesyltransferase inhibitor, enhanced apoptosis of anaplastic thyroid cancer (ATC) cells. However, the mechanism of the manumycin-induced apoptosis is not fully understood. In this study, we discovered that mitochondrial ultrastructure condensation occurred after treatment with manumycin or manumycin plus paclitaxel. Bongkrekic acid and cyclosporin A, which are known inhibitors of the voltage-dependent anion channel, failed to inhibit cytochrome c release induced by manumycin or manumycin plus paclitaxel, suggesting that mitochondrial permeability transition pores were not involved. We also found that manumycin induced translocation of Bcl-2-associated X protein (Bax), another possible mediator of cytochrome c release, from the cytosol to the mitochondria. Silencing Bax with a specific small interfering RNA blocked manumycin-induced mitochondrial condensation and cytochrome c release, arguing the dependence of manumycin-induced apoptosis on Bax. Using a binary adenoviral system, we found that overexpression of Bax enhanced manumycin-induced apoptosis of ATC cells, and the combination of manumycin and overexpression of Bax increased inhibition of ATC xenograft growth in nude mice. Thus, we concluded that manumycin-induced apoptosis in ATC cells was primarily mediated by Bax and that increasing Bax expression may sensitize ATC cells to manumycin. (J Clin Endocrinol Metab 90: 3583–3591, 2005)

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NAPLASTIC THYROID CANCER (ATC) is one of the most aggressive solid tumors (1). Despite use of the current multimodality approach to treatment of ATC, patients with the disease have a very short survival duration (median, 3–7 months) (2). Thus, development of alternative mechanism-based and targeted therapeutic strategies for ATC is desperately needed. Paclitaxel, an inhibitor of tubulin depolymerization, has been shown to have activity against ATC in vitro (3) and in a clinical trial (4). The farnesyltransferase inhibitor manumycin A (manumycin), a natural product of Streptomyces parvulus, inhibits farnesyltransferase by competing with the farnesyl pyrophosphate substrate (5, 6). We previously demonstrated that manumycin enhanced the antineoplastic activity of paclitaxel against ATC cells in vitro (7) and in a nude mouse xenograft model (7, 8).

The two known major apoptosis pathways are the intrinsic pathway initiated by the mitochondria and the extrinsic pathway initiated by cell surface receptors. Mitochondria-mediated apoptosis is generally recognized to play an important role in cancer therapy (9, 10). Mitochondria are major sites of integration of proapoptotic, e.g. Bcl-2-associated X protein (Bax), and antiapoptotic, e.g. Bcl-2, Bcl-X_L, signals (11). The specific mitochondrial response to an apoptosis-inducing agent may critically depend on the type and intensity of the stimulus, specific mitochondrial function perturbed, and cell type (9).

Manumycin induces apoptosis of ATC cells by eliciting the release of cytochrome c and activation of caspases (12). However, little is known about the mechanisms of cytochrome c release. In the present study, our goal was to characterize the role of mitochondria and molecular mechanisms of cytochrome c release in apoptosis of ATC cells after treatment with manumycin. To do so, using paclitaxel (whose action mechanism is different from that of manumycin) for comparison, we evaluated the effect of manumycin on mitochondrial features known to be associated with apoptosis: the mitochondrial ultrastructure, membrane permeability transition (MPT) pore opening, and Bax translocation to the mitochondria in ATC cells. We also examined the role of Bax in manumycin-induced apoptosis of ATC cells by using molecular techniques to suppress or elevate Bax protein level.

Materials and Methods

Cell culture

The human ATC cell lines KAT-4 and ARO were cultured in RPMI 1640 medium as described previously (7). All of the cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. Adherent cultures were incubated with drugs in the culture medium without changes in the fetal bovine serum concentration because serum withdrawal is a known stimulus for apoptosis.

Materials

Manumycin, paclitaxel, staurosporine, cyclosporin A, and bongkrekic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Stock

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Abbreviations: ATC, Anaplastic thyroid cancer; Bax, Bcl-2-associated X protein; DMSO, dimethyl sulfoxide; manumycin, manumycin A; MMP, mitochondrial membrane permeabilization; MOI, multiplicity of infection; MPT, mitochondrial permeability transition; PARP, poly(ADP-ribose) polymerase; RIPA, radioimmunoprecipitation assay; siRNA, small interfering RNA.

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solutions of manumycin, pactaxel, staurosporine, and cyclosporin A were prepared in dimethyl sulfoxide (DMSO). A stock solution of bongkrekic acid was prepared in tissue culture medium. All these stock solutions were then further diluted in tissue culture medium to the desired concentration. The final concentration of DMSO in the final solution was 0.1% vol/vol or less for in vitro treatment.

The antibodies for the antigens were purchased from the following sources: actin (clone AC-15, Sigma), Bax (clone ID3, Calbiochem, EMD, and Merck Biosciences, San Diego, CA), cytochrome c (clone 6H2B4, BD-PharMingen, San Diego, CA), and poly(ADP-ribose) polymerase (PARP) (clone 4C10–5, BD-PharMingen).

Transmission electron microscopy

Cell cultures on coverslips that adhered overnight were treated with the study drugs and then fixed with 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h (13). After fixation, the samples were washed with 1% cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 1 h, and stained en bloc with 1% uranyl acetate. The samples were dehydrated in ethanol and embedded in Spurr’s low-viscosity medium. The samples were polymerized in an oven at 60 C for 2 d.

Ultrathin sections of the samples stained with uranyl acetate and lead citrate were examined under a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Details of the mitochondrial morphology were obtained at a magnification of ≥27,000. The morphology was classified as orthodox, swollen, or condensed. Orthodox mitochondria had closely spaced and regular cristae, swollen mitochondria had closely spaced and regular cristae but were pale with a diluted matrix and larger than orthodox mitochondria, and condensed mitochondria had many tubular cristae with dilated ends but a shrunken matrix with increased staining density (14). The percentage of condensed and swollen mitochondria in each treatment group was determined by manual counting of mitochondria in all three categories on electron micrographs obtained at a magnification of ×6,000.

Preparation of ATC cell extracts and Western blot analysis

Total cell extract. After treatment with the study drugs, floating cells were pelleted by centrifugation. After the adherent cells were freed from the culture vessel with trypsin/EDTA, the floating and adherent cells were combined and washed with ice-cold PBS. Next, the whole-cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer [1× PBS, 1.0% octylphenyl-polyleglycerol glycol oxalyl (Igepal CA-630), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 mg/ml phenylmethylsulfonyl fluoride, and Complete protease inhibitor mix (Roche, Nutley, NJ; one tablet per 50 ml)]. The DNA in the RIPA lysates was sonicated by shear with one 8-sec bursts at medium power.

Extracts of subcellular fractions. After treatment with the study drugs, cytosolic proteins were extracted from the cells by using an ice-cold digitonin buffer [10 mM 1,4-piperazine diethane sulfonic acid (pH 6.8), 0.015% (wt/vol) digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] as described previously (12). To obtain both the mitochondrial and cytosolic fractions after experimental treatment, a mitochondria fractionating kit from Clontech (Palo Alto, CA) was used according to the manufacturer’s protocol, except that the cytosolic fraction underwent an additional ultracentrifugation step at 100,000 × g for 3 h at 4 C.

The protein concentrations of the various protein extracts were determined by using a modified Lowry method (Bio-Rad Laboratories, Hercules, CA), SDS-PAGE and immunoblotting were performed by using standard methods. Equal amounts of proteins were loaded into each lane in the gels. Anticat immunoblotting was performed to ensure equal sample loading.

Transfection of anti-Bax small interfering (si) RNA

An anti-Bax siRNA transfection kit was purchased from Cell Signaling Technology (Beverly, MA). The sequences of the siRNA duplex were 5’-GGUGGCGGAAACUGAAGATGTT-3’ and 5’-UCUGAUCAGUUC-CGGACCTT-3’. Transfection of the synthesized siRNA duplex of either Bax or a scrambled unrelated control (included in the kit) into KAT-4 cells was performed by using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Briefly, cells were grown in a 6-well plate to 40% confluence and then incubated with a mixture of a 200 pm siRNA duplex and 8 μl Oligofectamine per well. After 4 h, fetal bovine serum was added to a final concentration of 10% (vol/vol). A second transfection was performed 24 h later. Experimental drug treatments were carried out 24 h after the beginning of the second transfection.

Adenoviral overexpression of Bax

A binary adenoviral vector system for expressing Bax (Ad/PKG/GV16 plus Ad/GT-Bax or Ad/GT-LacZ) was used in this investigation as described previously (15). The recombinant adenoviruses containing human Bax gene cDNA (Ad/GT-Bax) or Escherichia coli β-galactosidase gene (Ad/GT-LacZ) were regulated by the GT3 minimal synthetic promoter; the GV16 transactivating protein for the GT promoter was under the control of the PCK promoter (Ad/PKG-GV16) in this helper virus. The total multiplicity of infection (MOI) in the experiments was 1001, in which one third of the viral particles were Ad/PKG/GV16 and two thirds were Ad/GT-LacZ or Ad/GT-Bax. This MOI was determined based on preliminary optimization experiments with KAT-4 and ARO cells, in which more than 95% of the cells became positive for LacZ without cytotoxicity after infection at an MOI of 100:1 (data not shown). ATC cells were seeded in 100-mm plate at 1 × 105 cells/dish or 3000 cells/well in 96-well plates. The next morning, infection was performed for 3 h by using 1 ml of infection medium (serum-free medium plus adenoviruses) per 100-mm plate or 20 μl of infection medium per well in a 96-well plate in a 5% CO2 incubator at 37 C followed by replacement of the normal volume of fresh complete medium and incubation overnight. The cells were then ready for drug treatments.

Sulforhodamine B protein biomass assay

After drug treatments, the cells in the 96-well plates were fixed with cold 50% trichloroacetic acid at 50 μl/well for 1 h at 4 C. After fixation, the plates were washed several times with tap water and air-dried. Trichloroacetic acid-fixed cells were stained for 20 min with sulforhodamine B [0.4% (wt/vol) in 1% acetic acid]. The unbound dye was washed out with 1% acetic acid after staining. Two hundred microliters of 10 mM Tris base (pH 10.5) were then added to each well of the dried plate. Absorbance was measured by using a Tecan SpectraFluor Plus plate reader (Tecan Inc., Mannedorf, Switzerland) at 570 nm.

Nude mouse xenograft model

One million KAT-4 cells suspended in RPMI 1640 medium were injected sc into a flank of each 5-wk-old nude mouse (nu/nu BALB-c). Tumor volumes were calculated by the formula a2 × b × 0.4, where a is the smallest diameter and b is the diameter perpendicular to a (16). After the tumors reached at least 50 mm3, the mice were randomly assigned to experimental or control groups. The animals were cared for under specific pathogen-free conditions in approved facilities. The body weight, feeding behavior, and avoidance behavior of each animal were monitored every Monday, Wednesday, and Friday as indicators of general health. Animals with any of the following were killed: more than 10% body weight loss, motor retardation as defined by loss of avoidance behavior, inability to obtain food or water, ruffled hair, or largest tumor diameter exceeding 15 mm. When the animals were killed, tumor xenografts were dissected and weighed. Manumycin was dissolved in DMSO before dilution in RPMI 1640 culture medium. Manumycin was injected ip at 12 mg/kg body weight on d 1 and 4 of each 7-d cycle. Placebo injections consisted of RPMI 1640 with DMSO 3% (vol/vol) administered in the same way. The adenoviral vectors used in this study were the binary vector system for expressing Bax [Ad/PKG/GV16 plus Ad/GT-Bax or Ad/GT-LacZ (for control)] as described previously (15). When tumors had reached a diameter of about 5 mm, each mouse was given a weekly intratumoral injection of 100 μl of 9 × 1010 total viral particles of Ad/GT-Bax or Ad/GT-LacZ mixed at a 2:1 ratio with Ad/PKG-GV16.
Results

Manumycin-induced ultrastructural changes in the mitochondria of KAT-4 cells

To evaluate morphologic changes in the mitochondrial ultrastructure of cells undergoing manumycin-induced apoptosis, we observed the mitochondria by using transmission electron microscopy. In KAT-4 cells, manumycin induced condensation of the mitochondria (Fig. 1A). The mitochondria in the manumycin-treated KAT-4 cells had a densely stained matrix and lightly stained cristae that appeared to be tubular and distended at their ends. Although we did not perform tomographic reconstruction to prove that the cristae were tubular, the two-dimensional appearance of the cristae was similar to that of the class II mitochondria observed by Scorrano et al. (17). However, the condensed mitochondria in the manumycin-treated KAT-4 cells differed from that of the class II mitochondria in that the matrix of the condensed mitochondria was densely stained and the cristae were closely packed. In contrast with manumycin, paclitaxel caused swelling in some of the mitochondria of the KAT-4 cells. About one third of the mitochondria in paclitaxel-treated KAT-4 cells were identified as swollen according to a lightly stained matrix and widely spaced cristae. The combination of manumycin and paclitaxel enhanced apoptosis as compared with that seen with the use of either drug alone, and significant numbers of apoptotic cells and apoptotic bodies were observed in cells treated with this combination.

Among the surviving and early apoptotic cells, the morphology of the mitochondria of cells treated with manumycin plus paclitaxel was similar to that of the mitochondria of cells treated with manumycin alone (condensed). Typical examples of the three morphologic classes of mitochondria (orthodox, swollen, and condensed) are shown in Fig. 1B. The percentages of orthodox, swollen, and condensed mitochondria in the cells after 2 h of treatment are shown in Fig. 1B (stacked bar chart). Manumycin and manumycin plus paclitaxel caused condensation in more than 95% of the mitochondria in the KAT-4 cells. In sharp contrast to manumycin and manumycin plus paclitaxel, paclitaxel alone caused swelling in about one third of the mitochondria. The distribution of the three classes of mitochondria in the KAT-4 cells after 6 h of treatment was similar to that after 2 h of treatment (data not shown).

Cytochrome c release induced by manumycin in an MPT-independent manner

An important link between mitochondria and apoptosis is cytochrome c release from the mitochondria to the cytosol. In ATC cells, cytochrome c release is a crucial event for activation of the caspase cascade after treatment with manumycin or manumycin plus paclitaxel (12). Components of the MPT pores, which are regulators that control cytochrome c release, include the adenine nucleotide translocator and cyclophilin D. Both bongkrekic acid, an inhibitor of the adenine nucleotide translocator, and cyclosporin A, an inhibitor of cyclophilin D, have been shown to inhibit cytochrome c release from the mitochondria through the MPT pores (18). Because mitochondrial swelling is associated with MPT pore opening and condensed mitochondria has been observed in KAT-4 cells, we predicted that MPT pore opening might not be the major pathway involved in manumycin-induced cytochrome c release in KAT-4 cells.

In KAT-4 cells, release of cytochrome c into the cytosol caused by 6 h of treatment of manumycin or manumycin plus paclitaxel was not inhibited by bongkrekic acid (50 μM; Fig. 2A) or cyclosporin A (10 μM; Fig. 2B) in the present study. As a control experiment to demonstrate the effectiveness of bongkrekic acid and cyclosporin A in blocking cytochrome c release at the concentrations used, HeLa cells (19) were treated with 2.5 μM staurosporine for 6 h in the presence or absence of 50 μM bongkrekic acid or 10 μM cyclosporin A. Bongkrekic acid and cyclosporin A inhibited cytochrome c release from staurosporine-treated HeLa cells (Fig. 2C). These results suggested that cytochrome c release induced by
expression Bcl-2 and Bcl-XL had not changed detectably in much lower than that in Jurkat cells. We also found that the Bcl-2 in six ATC cell lines, including ARO and KAT-4, was (22). Our own unpublished data confirmed that the level of induction at the mitochondrial level. Particularly, we focused on the Bcl-2 family of proteins. Unlike normal thyroid epithelium, differentiated thyroid carcinomas frequently have expression of Bcl-X (an antiapoptotic protein) and Bax (a proapoptotic protein) (20), whereas ATC often has expression of Bax only (21). Very low levels of expression of Bcl-2, another antiapoptotic protein, in ATC have been noted (21, 22). Our own unpublished data confirmed that the level of Bcl-2 in six ATC cell lines, including ARO and KAT-4, was much lower than that in Jurkat cells. We also found that the expression Bcl-2 and Bcl-X had not changed detectably in cells undergoing apoptosis induced by manumycin alone or manumycin plus paclitaxel (data not shown). Therefore, we focused on Bax. 

Bax, which is a proapoptotic Bcl-2 family member, has been reported to trigger apoptosis by translocation from the mitochondrial level. Particularly, we focused on other regulators of apoptosis functioning at the mitochondrial level. Additionally, we examined the impact of Bax silencing on manumycin-induced cytochrome c release. After completion of the second transfection, KAT-4 cells were exposed to manumycin (5 μM) for another 2 h. Anti-Bax siRNA alone (Fig. 4A, left lower panel) and control siRNA alone (Fig. 4A, left upper panel) did not change mitochondrial morphology. Manumycin induced condensation of the mitochondria in control siRNA-treated cells (Fig. 4A, right upper panel). However, manumycin failed to induce mitochondrial condensation in Bax-suppressed cells (Fig. 4A, right lower panel). Therefore, Bax appeared to be required for the manumycin-induced mitochondrial condensation.

We then examined the impact of Bax silencing on manumycin-induced cytochrome c release. After completion of the second transfection, KAT-4 cells were exposed to manumycin (54 μM) for 3 h; cytochrome c release into the cytosol was then evaluated by digitonin extraction as described previously (12). Manumycin induced cytochrome c release into the cytosol, which was blocked to a very large degree by suppressing the expression of Bax with the anti-Bax siRNA duplex (Fig. 4B, digitonin extracts). The percentage of surviving (trypan blue-excluding) cells after 24 h of manumycin treatment was significantly lower (one-way ANOVA, post hoc group-to-group comparisons, and the Tukey test; P < 0.05) in siRNA control cells than cells transfected with anti-Bax siRNA duplex (Fig. 4C). Hence, suppression of the Bax pro-
protein level by siRNA inhibited manumycin-induced cytochrome c release and cell death.

Enhancement of manumycin-induced cytochrome c release by adenovirus-mediated Bax overexpression

To further evaluate the role of Bax in manumycin-induced apoptosis of ATC cells, the effect of raising the level of Bax protein expression on manumycin-induced cytochrome c release and cell death was examined. The tool used to over-express Bax in ATC cells was a binary adenoviral vector system, the first component of which is an adenoviral vector containing a human Bax cDNA (Ad/GT-Bax) driven by a synthetic promoter consisting of five Gal4-binding sites and a TATA box (GT). This structure alone expresses Bax protein at a minimal background level in transfected cells, thus preventing apoptosis of virus-packaging 293 cells. The second component of this system is another adenoviral vector (Ad/PGK/GV16) that produces a powerful transcription factor (Gal4-VP16 fusion protein) under the control of the constitutively active PGK promoter. Cotransfection of these two adenoviruses produced Bax expression at a high level (15, 24). Using the modest adenoviral concentration MOI of 100:1, Bax was dramatically expressed in both ARO and KAT-4 cells for more than 48 h (data not shown). No change in Bax expression was observed in cells coinfected with Ad/PGK/GV16 plus the Ad/GT-LacZ (the control adenovirus in which the Bax cDNA was replaced with the LacZ cDNA) binary system. Sixteen hours after infection with the binary system, cells were exposed to manumycin at 54 μM for 2 h. KAT-4 cells infected with Ad/PGK/GV16 plus Ad/GT-Bax (Adeno-Bax) had the same orthodox mitochondrial morphology as in the cells infected with Ad/PGK/GV16 plus Ad/GT-LacZ (Adeno-LacZ) under the same conditions (data not shown). Therefore, Bax overexpression is not sufficient to trigger mitochondrial morphological alteration in KAT-4 cells under this experimental condition. Sixteen hours after infection with the binary system, cells were exposed to manumycin at 54 μM for another 18 h. As shown in Fig. 5A, whole-cell lysate, KAT-4 cells infected with Adeno-Bax had a markedly increased level of Bax expression in whole cell lysates as compared with cells infected with Adeno-LacZ under the same conditions. In the absence of manumycin, forced overexpression of Bax caused a low level of specific cleavage of PARP (Fig. 5A, whole-cell lysate, cleavage band indicated by the arrow on the right).

The manumycin-induced specific cleavage of PARP was further increased by overexpression of Bax when being detected at 18 h after manumycin treatment. Cytosolic cytochrome c in the digitonin extracts was not detectable in the KAT-4 cells infected with Adeno-LacZ (Fig. 5A, digitonin extract). In comparison, a very low level of cytochrome c was detected in KAT-4 cells infected with Adeno-Bax. Manumycin induced release of cytochrome c, which was further increased by overexpression of Bax. Under the same experimental conditions, similar Bax expression, specific cleavage of PARP, and cytochrome c release were observed in ARO cells (data not shown). Therefore, adenovirus-mediated overexpression of the Bax protein increased manumycin-induced apoptosis as evidenced by release of cytochrome c and specific cleavage of PARP.

The percentage of surviving (trypan blue-excluding) cells after 18 h manumycin treatment was significantly lower (one-way ANOVA, post hoc group-to-group comparison, and the Tukey test; P < 0.05) in cells infected with Adeno-Bax than in cells infected with Adeno-LacZ (Fig. 5B). Hence, overexpression of the Bax protein with the use of adenoviruses increased manumycin-induced cell death. Measurement of the protein biomass with the sulforhodamine B dye-binding assay revealed similar results. KAT-4 cells were treated for 48 h after adenoviral infection. Overexpression of Bax (Adeno-Bax) caused a shift of the manumycin dose-response curve to the left, whereas overexpression of LacZ
Increased inhibition of KAT-4 xenograft growth by the combination of manumycin and adenovirus-mediated Bax overexpression

The nude mouse sc xenograft model was used to evaluate whether adenoviral expression of Bax could increase the antineoplastic effect of manumycin against ATC in vivo. Thirty-two nude mice xenografted with KAT-4 cells were divided into four groups of eight animals: manumycin + Adeno-Bax, placebo + Adeno-Bax, manumycin + Adeno-LacZ, and placebo + Adeno-LacZ. The doses and administration schedules were as described above in Materials and Methods.

Growth curve analysis of estimated tumor volumes. The growth data were transformed by the logarithm of the ratio of estimated tumor volume to the estimated tumor volume when drug treatments began (Fig. 6A). These transformed data

**FIG. 5.** Enhancement of manumycin-induced apoptosis and sensitization of KAT-4 cells to the cytotoxicity of manumycin by overexpression of Bax. KAT-4 cells were infected with an adenovirus expressing Bax or LacZ (control). A, Anti-Bax, anti-PARP, anti-cytochrome c, and antiactin immunoblots. The + signs in the table at the top indicate manumycin at 54 μM and adenoviruses used. The sources of proteins (RIPA whole-cell lysate in cells obtained at 18 h after manumycin exposure or digitonin extracts of cytosolic protein in cells obtained at 3 h after manumycin exposure) are indicated at left. The arrow indicates the cleavage product of PARP specific for apoptosis. B, Cells harvested after 18 h of manumycin treatment were assayed for cell survival by trypan blue staining. Bar chart shows the percentage of live cells for the treatments indicated by the table below the horizontal axis. The error bars indicate the 95% confidence intervals. C, A sulfurhodamine B assay was performed with KAT-4 cells treated with manumycin for 48 h 1 d after adenovirus infection. The dose-response curves for the protein biomass (relative to the control) of KAT-4 cells infected with adenovirus overexpressing Bax (Adeno-Bax), adenovirus overexpressing LacZ (Adeno-LacZ), or no adenovirus were plotted against the concentration of manumycin in a logarithmic scale. *, P < 0.05 for differences in the means of the Adeno-Bax group from the other groups at the same manumycin concentration.

**FIG. 6.** Effect of the combination of manumycin and intratumoral injection of adenovirus overexpressing Bax on anaplastic thyroid carcinoma xenograft growth. A, The mean logarithm of the estimated tumor volume relative to tumor volume when treatment began was plotted against the number of days after treatment initiation. The error bars represented the SEs. The treatment groups were as labeled by the key to the symbols. B, The mean xenograft weight was plotted against treatment groups labeled on the horizontal axis by the table. The + sign indicated the presence of the labeled agent. The error bars represent the 95% confidence intervals.
thus have no intercepts. These repeated measures were analyzed using linear mixed models (with the help of software SPSS for Windows, version 12.0, SPSS, Inc., Chicago, IL) with the animals grouped by the four different treatments. The diagonal type of repeated covariance was used; estimation was performed using the restricted maximum likelihood method; and main effects for treatments were compared with the manumycin plus Adeno-Bax group as the reference category. Solution for the fixed effects of treatment groups showed that the growth curves of the manumycin plus Adeno-Bax group were significantly different from those of the placebo plus Adeno-LacZ (P < 0.001), the manumycin plus Adeno-LacZ (P < 0.001), and the placebo plus Ad Bax groups (P < 0.001). Therefore, the combination of manumycin and Bax overexpression inhibited KAT-4 tumor growth more than the other three groups.

**Xenograft weights.** The plots of the xenograft weights in each of the four treatment groups for KAT-4 cells are shown in Fig. 6B. One-way ANOVA with post hoc pairwise comparison (Tukey test) showed that the weights of KAT-4 xenografts in the manumycin + Adeno-Bax group were significantly different from the manumycin + Adeno-LacZ (P = 0.005), placebo + Adeno-Bax (P = 0.012), and placebo + Adeno-LacZ groups (P < 0.001). The power of the performed test with α = 0.05 was 0.978. Therefore, considering the growth curve data and the xenograft weight data together, overexpression of Bax increased the antineoplastic activity of manumycin against KAT-4 cells in the nude mouse model.

**Discussion**

In this study, we discovered that the farnesyltransferase inhibitor manumycin induced ultrastructural changes in the mitochondria in ATC cells that were not consistent with MPT pore opening. Taking these findings together with the lack of inhibitory effects of bongkrekic acid and cyclosporin A, which are known inhibitors of the voltage-dependent anion channel, we concluded that MPT pores were not involved in manumycin-induced apoptosis of ATC cells. However, several lines of evidence supported a major role for Bax in manumycin-induced apoptosis of ATC cells: 1) manumycin induced translocation of Bax from the cytosol to the mitochondria; 2) siRNA-mediated specific inhibition of Bax expression blocked mitochondria morphologic change, cytochrome c release, and apoptosis induced by manumycin; and 3) adenovirus-mediated overexpression of Bax increased manumycin-induced apoptosis and decreased cell survival in vitro. Moreover, in vivo experiments showed that adenovirus-mediated Bax overexpression increased the antitumor activity of manumycin on ATC xenograft growth. Therefore, manumycin-induced apoptosis in ATC cells was primarily mediated via Bax. Collectively, these results shed new light on the molecular basis for manumycin-induced apoptosis and suggest a novel method of sensitizing ATC cells to manumycin.

We documented mitochondrial condensation in ATC cells after treatment with manumycin (Fig. 1A). In response to administration of chemotherapeutic agents, mitochondria may exhibit swelling and condensation over the course of apoptosis. Of these two different changes in mitochondrial ultrastructure during apoptosis, mitochondrial swelling is better understood. About one third of the mitochondria in ATC cells treated with paclitaxel alone showed swelling. Mitochondrial swelling involves an osmotic process featuring net solute and water diffusion toward the matrix. There are two potential mechanisms of mitochondrial swelling (9). First, high-energy swelling is a product of electrochemical uptake of monovalent cations, K+ in particular. The inner membrane permeability remains low, and the swollen mitochondria retain a high degree of membrane polarization and high level of coupling to generate ATP. Second, in low-energy swelling, passive diffusion and osmosis occur after an increase in permeability. This type of swelling can be caused by MPT pore opening (25) and occur in deenergized mitochondria. Swelling due to MPT pore opening is followed by collapse of the membrane potential and release of cytochrome c from mitochondria into the cytosol (26). Also, Ali et al. (27) reported that manumycin caused swollen mitochondria in trypanosomes and one mammalian cell line.

In contrast with swelling, little is understood about the mechanism of mitochondrial condensation. The appearance of condensed mitochondria may indicate an active mitochondrial condensation. The involvement of MPT-dependent cytochrome c from intramitochondrial cristae with morphologic changes in the cristae. Using purified mitochondria, they found that truncated Bid (BH3 domain only) remodelled mitochondrial cristae into tubular structures. The significance of mitochondria condensation in manumycin-treated KAT-4 cells remains to be determined. However, the mitochondrial ultrastructural changes are consistent with the observation of MPT-independent cytochrome c release induced by manumycin.

Staurosporine, a relatively nonspecific protein kinase inhibitor, is often used as a general method for inducing apoptosis (32). The involvement of MPT-dependent cytochrome c release induced by staurosporine was well characterized in HeLa cells (19). Our control experiment shown in Fig. 2C confirmed the effectiveness of bongkrekic acid and cyclosporin A in blocking MPT-mediated cytochrome c release at the concentrations used, thus allowing us to conclude that in KAT-4 cells, release of cytochrome c into the cytosol caused by 6 h of treatment of manumycin or manumycin plus paclitaxel was not MPT mediated.

Except MPT, Bcl-2 family members are major factors that govern cytochrome c efflux from mitochondria. As described above, we examined the role of Bax in the present study. Bax is one of the well-known proapoptotic genes, overexpression of which leads to apoptosis in a wide variety of cells with or without additional stimuli (33). Mutations of Bax at codon 169 have been found to decrease its proapoptotic activity and play an important role in carcinogenesis in the stomach, colon, rectum, endometrium, and hematologic tissues in hu-
mans (34). Bax does not have a major role in regulating the Ca2+ -induced mitochondrial permeability transition (33) or induce cytochrome c release by acting on the voltage-dependent anion channel (36). Thus, a role for Bax in manumycin-induced apoptosis in ATC cells would be consistent with our present findings. We also found that Bax underwent translocation from the cytosol to the mitochondria in ATC cells after manumycin treatment. Some have suggested that the subcellular relocation of Bax protein is important in apoptosis (37, 38). Bax is generally sequestered in the cytosol and moved from the cytosol into mitochondria with homooligomerization during apoptosis. Bax translocation into mitochondria targets the mitochondrial intermembrane contact sites and releases cytochrome c (38). One question remains, however: how does Bax translocate into mitochondria during apoptosis? Some authors recently reported that activation of p38 MAPK is required for Bax translocation to mitochondria, cytochrome c release, and apoptosis in human cells (39, 40). Our preliminary data showed that p38 MAPK was activated and that blockage of p38 MAPK by its specific inhibitor diminished apoptosis in ATC cells in response to manumycin. Whereas whether translocation of Bax in manumycin-treated KAT-4 cells is mediated through p38 MAPK activation remains to be investigated.

In ATC cells, we proved the importance of Bax in manumycin-induced apoptosis in experiments that perturbed the level of Bax protein expression up or down with molecular techniques. The anti-Bax siRNA duplex, which specifically inhibited Bax expression, blocked manumycin-induced mitochondrial condensation, cytochrome c release, and cell death (Fig. 5). These results suggested that Bax was required for the mitochondrial ultrastructural change and apoptosis. We also examined whether overexpression of Bax by adenovirus delivery could potentiate the effect of manumycin. Ectopic expression of Bax in ATC cells enhanced cytochrome c release, increased specific cleavage of PARP, and further decreased cell survival and the protein biomass after manumycin treatment.

Increasing evidence has revealed that overexpression of Bax enhances chemotherapy and radiotherapy for several different types of cancer and improves treatment outcome in different experimental systems (41). For instance, Bax overexpression has been found to sensitize human ovarian tumor cells to paclitaxel, vincristine, and doxorubicin (42). Hence, Bax appears to be a good candidate for cancer gene therapy because it not only may kill cancer cells directly but also may increase the sensitivity of other cancer treatments (15, 43–45). Our findings that adenovirus-mediated Bax overexpression sensitized ATC cells to manumycin in vitro and that intratumoral injection of adenovirus overexpressing Bax increased the antitumor activity of manumycin in a nude mouse xenograft model warrant further translational studies to further evaluate Bax gene therapy for ATC in combination with manumycin.

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