A G-Quadruplex Ligand 3,3′-Diethyloxadicarbocyanine Iodide Induces Mitochondrion-Mediated Apoptosis But Not Decrease of Telomerase Activity in Nasopharyngeal Carcinoma NPC-TW01 Cells

Chung-Pin Li,[1,2] Jen-Hsin Huang,[1] Ai-Chi Chang,[1] Yi-Mei Hung,[1] Chao-Hsiung Lin,[3] Yee Chao,[4] Shou-Dong Lee,[2] Jacqueline Whang-Peng,[1] and Tze-Sing Huang[1,5]

Received June 30, 2003; accepted October 3, 2003

Purpose. The G-quadruplex ligand 3,3′-diethyloxadicarbocyanine iodide (DODC) was reported to enhance the apoptotic potency of pheochromocytoma PC-12 and leukemia HL-60 cells through the inhibition of telomerase activity. In this study, a mitochondrion-mediated apoptotic pathway was demonstrated as another cytotoxic mechanism for DODC action.

Methods. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DNA laddering assays were performed to exhibit the cytotoxicity and apoptosis-inducing activity of DODC. Telomeric repeat amplification protocol (TRAP) assay was used to evaluate the effect of DODC on cellular telomerase. The mitochondrial uptake of probe 3,3′-dihexyloxacarbocyanine iodide was measured by flow cytometry. The mitochondrial proteomes were analyzed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Western blot analyses were adopted to demonstrate the change of the distribution of mitochondrial proteins.

Results. DODC alone was able to induce apoptotic cell death but not decrease of telomerase activity in nasopharyngeal carcinoma NPC-TW01 cells. Instead, we found evidence that DODC significantly affected cellular mitochondria. DODC inhibited the uptake of another mitochondrial probe 3,3′-dihexyloxacarbocyanine iodide. By proteomic comparative analysis, we found that DODC induced the increase of prohibitin level in the mitochondria, indicating the occurrence of mitochondrial perturbation. Moreover, DODC was found to induce the levels of p53 and an 18-kDa truncated Bax on mitochondria, which in turn potentiated the release of cytochrome *c* for activation of caspases.

Conclusions. DODC induces NPC-TW01 cell apoptosis via a mitochondrion-mediated mechanism. This paper demonstrates another cytotoxic mechanism of DODC other than inhibition of telomerase.

KEY WORDS: 3,3′-diethyloxadicarbocyanine iodide; prohibitin; p53; tbax.

INTRODUCTION

Human telomeric DNAs, consisting of tandem d(TTAGGG) repeats, are located at the ends of chromosomes to protect chromosomes from degradation, fusion, and recombination (reviewed in Ref. 1). These G-rich ends can fold up and form the G quadruplex structures (Ref. 2 and references therein), which disadvantage the elongation step catalyzed by telomerase (3). Agents like derivatives of acridine (4) and triazine (5) that stabilize G quadruplexes have recently aroused interest because these compounds can act as potent telomerase inhibitors in cancer cells and can be evaluated as anticancer agents (see Ref. 6 for a review). The fluorescent carbocyanine 3,3′-diethyloxadicarbocyanine iodide (DODC) was used as a mitochondrial dye to probe the microenvironment of cytochrome *c* oxidase (7). Because of the structural characteristics, DODC can interact and form complex with dimeric hairpin G quadruplexes (8). It was shown to cause the decrease of telomerase activity in pheochromocytoma PC-12 cells in a concentration-dependent manner (9). This inhibition renders DODC able to enhance staurosporine, Fe^{2+}, or amyloid β-peptide-induced mitochondrial dysfunction and apoptosis of PC-12 cells (9). Additionally, DODC treatment was also shown to inhibit telomerase activity in leukemia HL-60 cells, and this inhibition sensitizes HL-60 cells to apoptosis induced by adriamycin (10). In this study, we found that treatment of DODC alone was able to induce apoptosis of nasopharyngeal carcinoma (NPC) NPC-TW01 cells. However, DODC did not cause decrease of telomerase activity in NPC-TW01 cells. Instead, DODC induced the perturbation of mitochondrial protein distribution. Our data suggest that DODC induced NPC-TW01 cell apoptosis via a mitochondrion-mediated mechanism.

MATERIALS AND METHODS

Cytotoxicity Assay

Cytotoxicity of DODC (Sigma Chemical Co., St. Louis, MO, USA) was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) assay. Two thousand NPC-TW01 cells were seeded on each well of 96-well dishes and incubated overnight at 37°C in a 5% CO_2 humidified atmosphere. Adherent cells were then exposed to varying concentrations of DODC in 200 μl of medium. After 72 h, 50 μl of 0.25% MTT in phosphate-buffered saline (PBS) was added and incubated for another 4 h. Medium was then removed from each well, and formazan was extracted with 50 μl of dimethyl sulfoxide and quantitated by ELISA reader at 550 nm.

Apoptotic DNA Fragmentation Assay

The apoptotic DNA ladders were isolated from DODC-treated NPC-TW01 cells based on the method previously de-

[1] Cooperative Laboratory, Cancer Research Division, National Health Research Institutes, Taipei, Taiwan, R.O.C.
[2] Division of Gastroenterology, Department of Internal Medicine, Taipei Veterans General Hospital; and Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, R.O.C.
[3] Department of Life Science, National Yang-Ming University, Taipei, Taiwan, R.O.C.
[4] Cancer Center, Taipei Veterans General Hospital, Taipei, Taiwan.
[5] To whom correspondence should be addressed. (e-mail: tshuang@nhri.org.tw)

ABBREVIATIONS: $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DODC, 3,3′-diethyloxadicarbocyanine iodide; IPG, immobilized linear pH gradient; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPC, nasopharyngeal carcinoma; tBax, truncated Bax; TRAP, telomeric repeat amplification protocol.

0724-8741/04/0100-0093/0 © 2004 Plenum Publishing Corporation

scribed (11). The treated cells were lyzed in the buffer consisting of 50 mM Tris-HCl (pH 7.5), 20 mM EDTA, and 1% NP-40. The lysates were further brought to 1% sodium dodecyl sulfate (SDS) and incubated for 1 h with RNase A (final concentration 0.2 μg/μl) at 45°C followed by digestion with proteinase K (final concentration 2.5 μg/μl) at 37°C overnight. The DNA ladders were further extracted from the cell lysates with phenol–chloroform twice and chloroform once and precipitated by ethanol. The DNA samples were resolved on 1% agarose gels, and the pattern of DNA ladders was examined on gels after ethidium bromide staining.

Telomeric Repeat Amplification Protocol

The telomerase activity was assayed according to the protocol previously described (12). Cell lysates were prepared in 200 μl of lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 0.5% CHAPS, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.1 mM benzamidine, and 5 mM β-mercaptoethanol. Then, each telomeric repeat amplification protocol (TRAP) reaction was performed at 30°C for 30 min in a 50-μl reaction mixture containing 20 ng of cell lysate, 20 mM Tris-HCl (pH 8.3), 0.05% Tween-20, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 250 μM dNTPs, Primer Mix (Intergen Co., Purchase, NY, USA), and 2 U of Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA), and followed by 33 cycles of PCR reactions at 94°C for 0.5 min and 59°C for 0.5 min. One-half of each reaction mixture was resolved on a 12.5% polyacrylamide gel, and after staining with SYBR Green (Molecular Probes, Eugene, OR, USA), the image of the telomeric repeat pattern was printed by Alpha-InnoTech IS500 Digital Imaging System (Avery Dennison, CA, USA).

Flow Cytometric Analyses

Flow cytometric determination of cellular DNA content was performed as previously described (13). NPC-TW01 cells were trypsinized and fixed with –20°C prechilled 80% (v/v) ethanol for at least 30 min. After centrifugation, cell pellets were resuspended in 0.5% Triton X-100 and kept on ice for 5 min. The suspensions of permeabilized cells were further treated with 25 μg/ml of propidium iodide plus 0.25% (w/v) of RNase A. Ten minutes later, the DNA content of cell samples was analyzed by the FACStar flow cytometer (BD Biosciences, San Jose, CA, USA). For flow cytometric determination of mitochondrial membrane potential [deltaPsi(m)], the fluorescent dye 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was added to NPC-TW01 cells and incubated at 37°C for 15 min (14). The fluorescence of trypsinized cell samples was analyzed by the FACStar flow cytometer with an argon laser tuned to the 488-nm line for excitation.

Preparation of Mitochondria

The procedure for isolating cellular mitochondria was modified from the method described by Yang *et al.* (15). Adherent cells were scraped and harvested by centrifugation at 600 × *g* for 10 min at 4°C. The cell pellets were washed once with ice-cold PBS and resuspended in five volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose). The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates were centrifuged at 750 × *g* for 10 min at 4°C. The supernatants were subjected to further centrifugation at 10,000 × *g* for 15 min at 4°C. The resultant supernatant fractions were taken as crude cytosolic extracts, and the pellets were successively washed with ice-cold 20 mM Na_2CO_3 (pH 11.0), 0.01% Tween-20, and PBS with each step followed by centrifugation at 16,000 × *g* for 15 min. The final pellets were saved as the mitochondrial fractions. In addition, the crude cytosolic extracts were further centrifuged at 10,000 × *g* for 1 h at 4°C, and the resultant supernants were saved as the cytosolic fractions for further experiments.

Two-Dimensional Gel Electrophoresis and Protein Identification

Immobilized linear pH gradient (IPG) strips, pH 3–10, were rehydrated with cell lysate samples in the dark at room temperature overnight, according to the manufacturer's

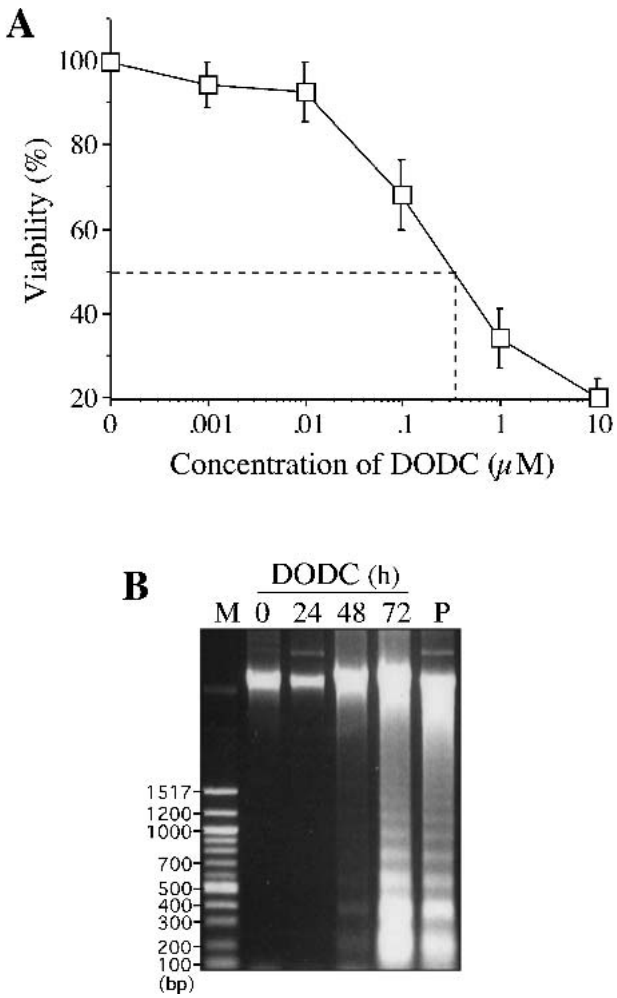

Fig. 1. DODC induces apoptosis in NPC-TW01 cells. (A) The viability curve of NPC-TW01 cells treated 72 h with varying concentrations of DODC. The viability was evaluated by MTT assay as described in the "Materials and Methods" section. The data represent the means of three independent experiments. Each experiment was performed in quintuple. (B) Agarose gel electrophoretic analysis of apoptotic DNA fragmentation from NPC-TW01 cells treated with 0.4 μM of DODC for 24, 48, or 72 h. Lane P, NPC-TW01 cells treated with 18 nM (IC_{50}) of paclitaxel for 72 h. Lane M, molecular weight marker.

guidelines (Amersham Biosciences, Piscataway, NJ, USA). Isoelectric focusing was performed using the IPGphor apparatus (Amersham Biosciences) for a total of 17500 Voltage-hour at 20°C. Strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% (w/v) SDS, 64 mM dithiothreitol, 30% (v/v) glycerol, and a trace of bromophenol blue. Equilibrated IPG strips were further transferred onto 16 × 18-cm 12% polyacrylamide gels. Standard SDS-polyacrylamide gel electrophoresis was performed, and the two-dimensional (2D) gels were stained with Coomassie blue. Spots of interest were manually excised from 2D gels. Gel pieces were washed twice in 25 mM ammonium bicarbonate/50% acetonitrile and once in 100% acetonitrile. After drying in a SpeedVac for 10 min, gel pieces were rehydrated with 10 μg/ml of trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8.0. Proteins were proteolyzed for 16–24 h at 37°C. Supernatants were collected, and the peptides in gel pieces were further extracted with 5% trifluoroacetic acid (TFA)/50% acetonitrile. The peptide extracts were vacuum-dried and solubilized in 10 μl of 0.1% TFA. Before the analyses by MALDI-TOF MS, peptide extracts were further desalted by $ZipTip_{C18}$ according to the manufacturer's protocol (Millipore, Bedford, MA, USA). The final peptide extracts were in 5 μl of 0.1% TFA/33% acetonitrile. One microliter for each sample was spotted onto the MALDI target plate and added with α-cyano-4-hydroxycinnamic acid as matrix. MALDI-TOF MS was performed using M@LDI *R*™ reflector time-of-flight mass spectrometer (Micromass, Manchester, UK). All mass spectra were internally calibrated with ACTH peptide and trypsinized alcohol dehydrogenase peaks. Peptide mass mapping was carried out using the MS-Fit program (Protein Prospector, University of California, San Francisco, CA, USA).

Western Blot Analysis

Cell lysates were prepared in lysis buffer consisting of PBS, 0.3% SDS, 0.5% deoxycholate, and 1% NP-40 (16). Forty micrograms of each sample were separated on 12% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes. After blocking with PBST (PBS plus 0.1% Tween-20) plus 5% nonfat milk, the membranes were incubated with indicated primary antibody (in PBST plus 5% powdered nonfat dry milk) at 4°C for 12 h. The anti-PKC-δ antibody was obtained from BD Transduction Laboratories (San Diego, CA, USA), and other antibodies, including anti-Bax, Bcl-2, p53, JNK-2, cytochrome *c*, HSC-70, and so forth, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were then washed three times with PBST buffer and incubated with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. After washing three times with PBST buffer, the protein bands were detected by enhanced chemiluminescence (Amersham Biosciences).

Caspase Activity Assay

NPC-TW01 cells were suspended in buffer consisting of 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. After three freeze-thaw cycles, the cell suspensions were subjected to centrifugation at 15,000 × *g* for 20 min at 4°C. The supernatants were saved as cell lysates and used to determine

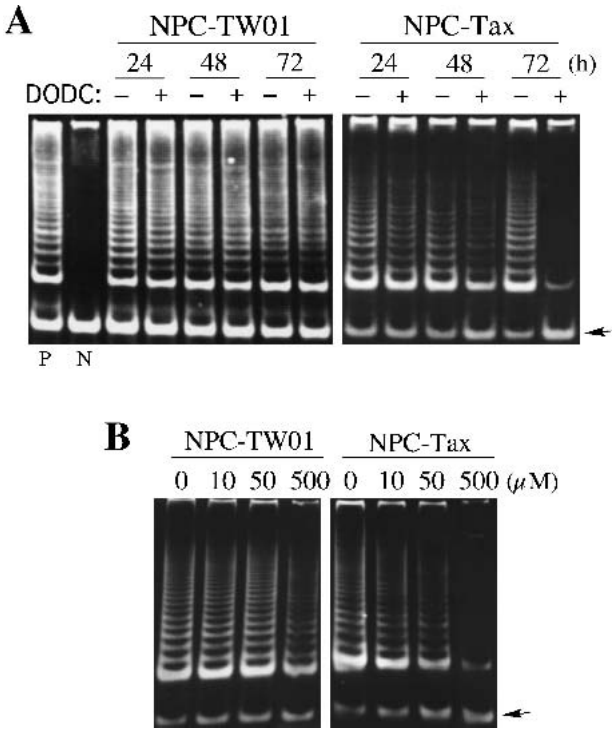

Fig. 2. DODC does not inhibit the telomerase activity of NPC-TW01 cells. (A) Detection of telomerase activity from NPC-TW01 cells treated with or without 0.4 μM of DODC for 24, 48, or 72 h. As a control, another NPC cell line, NPC-Tax, was treated with 0.7 μM (IC_{50}) of DODC for same time periods. The telomerase activity was assayed based on the protocol of the TRAPeze telomerase detection kit (Intergen Co.). The bands indicated by the arrowhead were the 36-bp products used as the internal control, which were produced by including the TSK1 template in each TRAP reaction. Lane P, a positive control using the cell lysate provided in TRAPeze kit. Lane N, a negative control using heat-inactivated cell lysate. (B) Assay of telomerase activity when DODC was present in the TRAP reactions. Various concentrations of DODC were added in the cell lysate from untreated NPC-TW01 or NPC-Tax cells before TRAP reactions. The arrowhead indicates the 36-bp bands of internal control. It was noted that DODC exhibited a significant inhibitory effect on the telomerase activity of NPC-Tax but not NPC-TW01 cell lysate.

protein concentration (Bradford method). Aliquots of cell lysates (80 μg for each) were incubated with 300 μM of Ac-DEVD-*p*NA and Ac-LEHD-*p*NA substrates for determination of caspase-3 and caspase-9 activity, respectively. The reaction was performed at 37°C for the appropriate time,

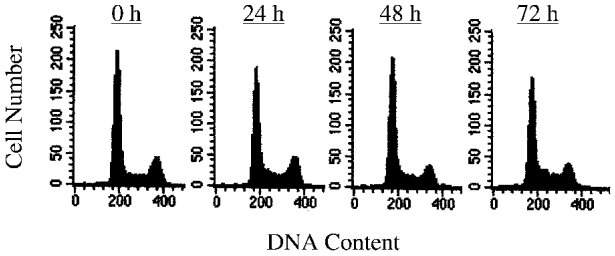

Fig. 3. DODC does not affect the cell-cycle stage distribution of NPC-TW01 cells. NPC-TW01 cells were treated with 0.4 μM of DODC for 24, 48, or 72 h, and adherent cells were collected for flow cytometric determination of DNA content as described in the "Materials and Methods" section.

and the quantity of caspase-cleaved *p*-nitroanilide was measured by a SpectraCount reader (Packard, Meriden, CT, USA) set at 405 nm wavelength.

RESULTS

DODC Induces Apoptosis in NPC-TW01 Cells

DODC is cytotoxic to NPC-TW01 cells. The viability curve of the NPC-TW01 cells treated for 72 h with varying concentrations of DODC was plotted based on MTT assay data (Fig. 1A). The concentration for killing 50% of NPC-TW01 cells (i.e., IC_{50}) was approximate 0.4 μM. When NPC-TW01 cells were treated with 0.4 μM of DODC for different time periods, cellular blebbing morphology and internucleosomal DNA fragmentation were observed at 72 h (Fig. 1B and data not shown), suggesting that DODC was able to induce apoptotic cell death in NPC-TW01 cells.

DODC Does Not Inhibit the Telomerase Activity and the Cell-Cycle Distribution of NPC-TW01 Cells

Since it was reported that DODC could inhibit telomerase activity by stabilizing the G-quartet structure at the telomere, we investigated the possibility of telomerase inhibition by DODC in NPC-TW01 cells. NPC-TW01 cells were treated with 0.4 μM of DODC for 8, 24, 48, or 72 h, and cell lysates were prepared for TRAP telomerase activity assay. The result revealed that no inhibition of telomerase occurred with DODC-treated NPC-TW01 cells (Fig. 2A). In the same figure, NPC-Tax cells (a control NPC cell line) showed decreased telomerase activity after DODC treatment. In addition, cell lysates from proliferating NPC-TW01 and NPC-Tax cells were prepared. The TRAP assay was performed on these cell lysates using increasing concentrations of DODC. The result indicated that the exogenous addition of DODC did not inhibit telomeric repeat elongation in the NPC-TW01 cell lysate until the concentration of DODC reached at 500 μM (Fig. 2B). Taken together, these two pieces of data indicated that DODC was not a potent inhibitor of telomerase activity in NPC-TW01 cell system. We also examined the DNA content of NPC-TW01 cells after DODC treatment of 24, 48, or 72 h. The flow cytometric data suggested that DODC did not significantly affect the distribution of cell-cycle stages in NPC-TW01 cells (Fig. 3).

DODC Induces NPC-TW01 Cell Apoptosis via a Mitochondrion-Mediated Mechanism

DODC can detect the microenvironment of cytochrome *c* oxidase and has been used as a mitochondrial dye. To investigate whether DODC can affect the mitochondrial activity of NPC-TW01 cells, we adapted a flow cytometry method for the quantitative measurement of the uptake level of the fluorescent probe $DiOC_6(3)$. After treated with the IC_{50} of DODC for 2 or 4 h, the NPC-TW01 cells were further incubated with $DiOC_6(3)$ for another 15 min. The flow cytometric analysis indicated that DODC was able to block the $DiOC_6(3)$ uptake level of NPC-TW01 cells (Fig. 4). The same phenomenon could also be observed with NPC-Tax cells (data not shown), suggesting that DODC indeed affected the mitochondria.

Furthermore, we investigated the effects of DODC on mitochondrial proteins. Mitochondrial proteins were isolated from the control and DODC-treated NPC-TW01 cells and were resolved by 2D gel electrophoresis. Protein spots were cut from gels and after in-gel trypsin digestion and peptide extraction were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. After analysis of the protein spots, it was found that the level of prohibitin significantly increased after DODC treatment

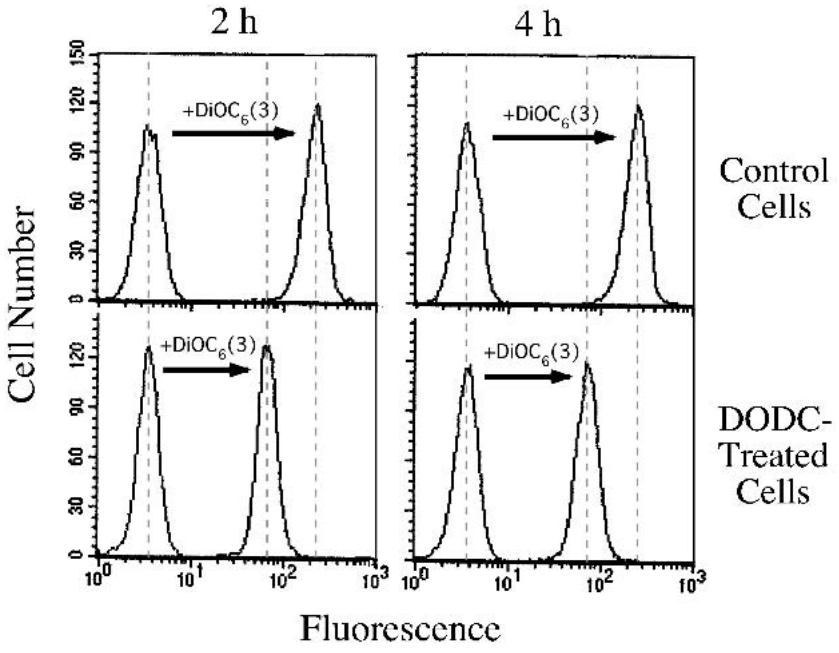

Fig. 4. DODC inhibits the uptake level of the fluorescent probe $DiOC_6(3)$ in NPC-TW01 cells. NPC-TW01 cells were treated with or without 0.4 μM of DODC for 2 or 4 h, and after incubated with $DiOC_6(3)$ for another 15 min, cells were trypsinized and subjected to flow cytometric analysis with an argon laser tuned to the 488-nm line for excitation.

(Fig. 5A). This result was confirmed by Western blot analysis as well (Fig. 5B). Other protein spots identified, including ATP synthase β-chain, GRP75, GRP78, HSC-70, VDAC-2, and annexin I, were not significantly changed by DODC treatment (Fig. 5 and data not shown).

Because elevation of prohibitin levels is thought to be an indicator of mitochondrial destabilization during apoptosis-related events (17), we investigated the effect of DODC treatment on cytochrome *c* release from mitochondria to cytosol. As shown in Fig. 6A, DODC was able to induce leakage of cytochrome *c* out of mitochondria. Considering that the released cytochrome *c* can cause activation of caspase-9 and in turn activation of more downstream executor caspases such as caspase-3 (15), we next examined whether DODC treatment would furthermore induce the caspase activation cascade. NPC-TW01 cells were treated with 0.4 μM of DODC for 8, 16, 24, 36, 48, 60, or 72 h, and the cell lysates were prepared for caspase activity assay. The result showed that caspase-9 activity was slightly induced by DODC treatment (Fig. 6B). The low-fold induction of caspase-9 activity is likely due to the low abundance of caspase-9 in NPC-TW01 cells. In addition, the activity of caspase-3 was significantly induced by DODC treatment. The maximal induction of caspase-3 activity occurred at 60 h (Fig. 6C).

Several proteins, such as p53, SAPK/JNK, PKC-δ, and so forth, have been found to translocate to mitochondria upon the induction of apoptosis (18–20), though the roles of some of them remain unclear. Therefore, we investigated the mitochondrial levels of p53, JNK-2, PKC-δ, Bax, Bcl-2, Bad, Bcl-X_L, HSP-60, and HSP-27 in the NPC-TW01 cells with or without 4-h DODC treatment. We found that the levels of p53 and truncated Bax (tBax) were increased in the mitochondria of DODC-treated NPC-TW01 cells (Fig. 7). The levels of other proteins, including JNK-2, PKC-δ, Bcl-2, Bad, Bcl-X_L, HSP-60, and HSP-27, were not increased or changed by DODC treatment.

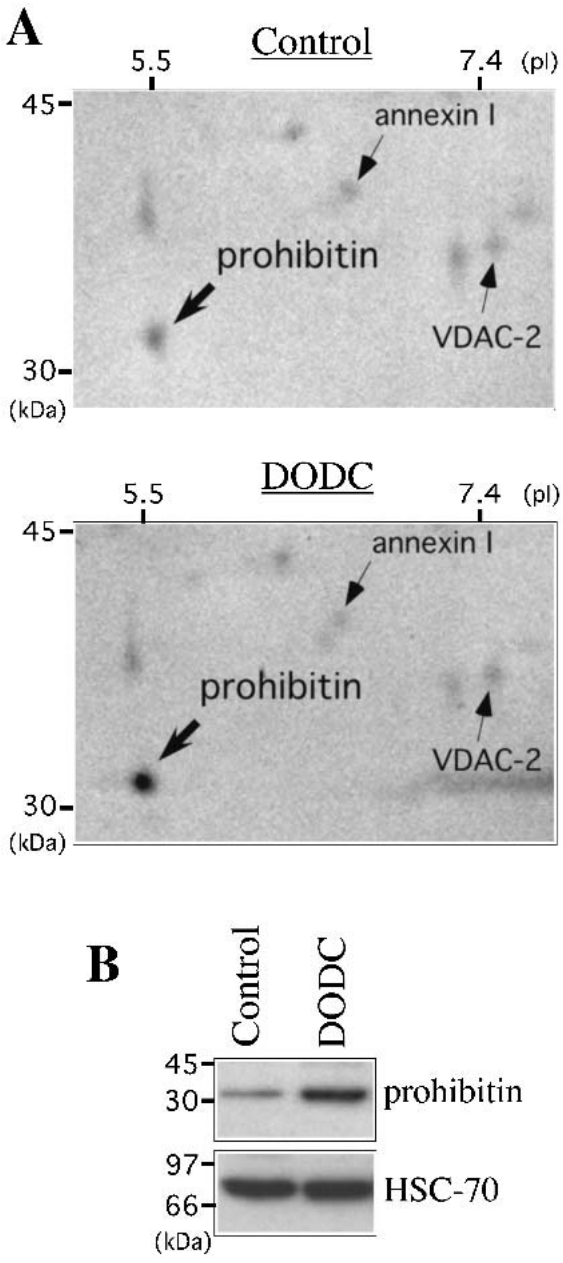

Fig. 5. DODC induces increase of prohibitin in the mitochondria of NPC-TW01 cells. (A) Two-dimensional gel electrophoretic patterns of control vs. DODC-treated NPC-TW01 cells. The mitochondrial proteins were isolated from NPC-TW01 cells treated with or without 0.4 μM of DODC for 4 h, and 40 μg of each sample were moreover resolved in pH 3–10 IPG strips and 12% SDS-polyacrylamide gels as described in the "Materials and Methods" section. After 2D gels were stained with Coomassie blue, the protein spots of interest were identified by MALDI-TOF mass spectrometry. (B) Western blot analyses of the prohibitin and HSC-70 protein levels in the mitochondria of NPC-TW01 cells treated with or without 0.4 μM of DODC for 4 h.

DISCUSSION

DODC is a positively charged, membrane-permeable dye that has been used as a probe for the mitochondrial localization (7). In this study, DODC was found to induce apoptosis in NPC-TW01 cells. Owing to its structural characteristics, DODC was thought to be an inhibitor of telomerase by forming the G-quartet structure with telomeric TTAGGG repeats (8–10). However, we did not detect any significant inhibitory effect of DODC on telomerase activity either by using DODC to treat NPC-TW01 cells or by exogenously adding DODC to TRAP reaction mixtures. DODC was recently reported to induce the decrease of telomerase activity in pheochromocytoma PC-12 cells (9) and leukemia HL-60 cells (10). Besides NPC-TW01 cells, we also investigated the effect of DODC on another NPC cell line, NPC-Tax, and a gastric carcinoma cell line, SC-M1. Interestingly, DODC exhibited its telomerase inhibitory activity in NPC-Tax cells but not SC-M1 cells (Fig. 2 and data not shown). It is intriguing to study why distinct cell contents render cellular telomerase differentially responsive to DODC. We detected a higher level of TTAGGG repeats-binding protein TRF-2 expressed by NPC-TW01 cells rather than NPC-Tax cells (data not shown). Whether the elevated TRF-2 affected the interaction of DODC with telomeric repeats is still unknown. However, NPC-TW01 cells are useful for studying alternative mechanisms of action of DODC besides telomerase inhibitory activity.

Considering that DODC is a mitochondrial dye, we investigated whether DODC induced apoptosis through its action on mitochondria. Mitochondrial membrane potential

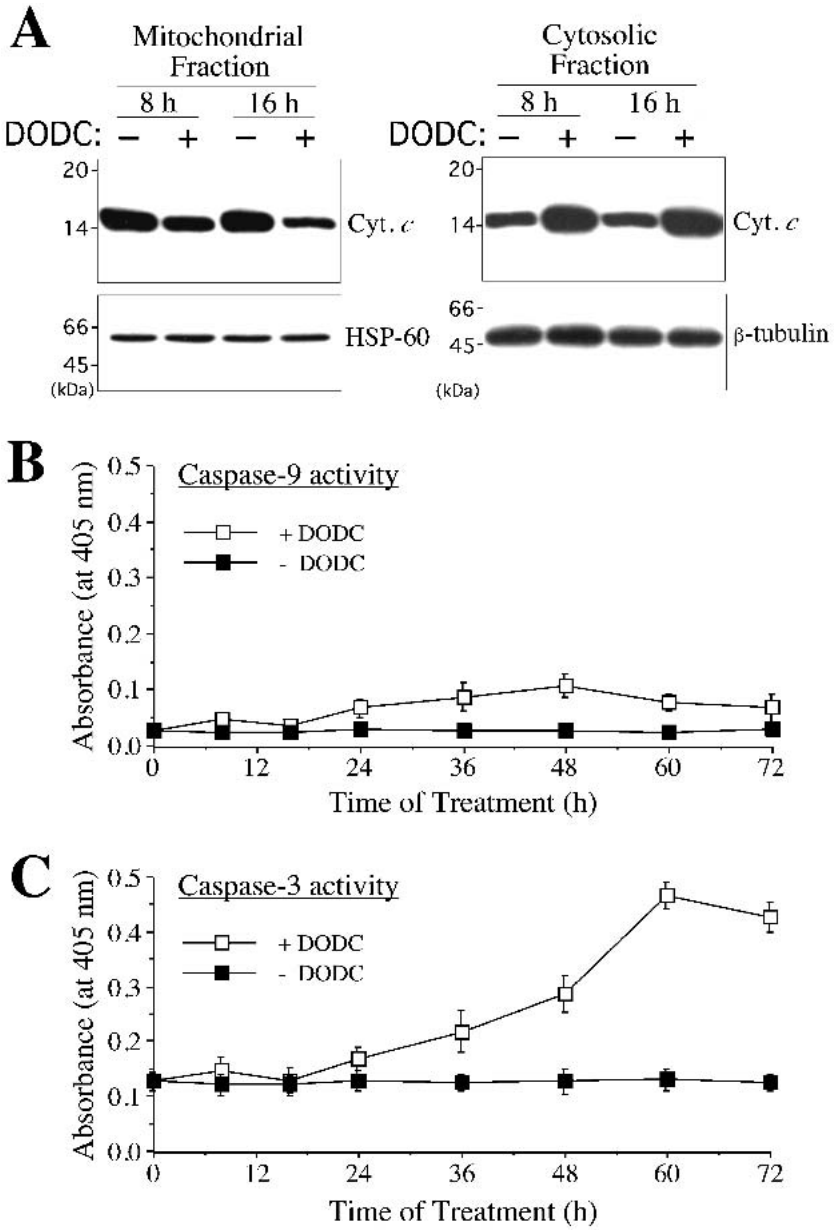

Fig. 6. DODC induces the cytochrome *c* release and the activation of caspases in NPC-TW01 cells. (A) Western blot analyses of the cytochrome *c* levels in the mitochondrial and cytosolic fractions. NPC-TW01 cells were treated with or without 0.4 μM of DODC for 8 or 16 h and were subjected to subcellular fractionation and Western blot analyses. (B) and (C) Activity curves of caspase-9 and -3 of DODC-treated NPC-TW01 cells. NPC-TW01 cells were treated with or without 0.4 μM of DODC for 8, 16, 24, 36, 48, 60, or 72 h, and the cell lysates were subjected to caspase activity assay described in the "Materials and Methods" section. The data represent the mean ± SD of three independent experiments.

[deltaPsi(m)] is an important indicator of mitochondrial function and dysfunction. Early on in apoptosis, there is always a reduction of deltaPsi(m). Because DODC induced apoptosis in NPC-TW01 cells, we wondered if DODC would induce the reduction of deltaPsi(m). We used a lipophilic, cationic, fluorescent probe $DiOC_6(3)$ and a flow cytometry method for the quantitative measurement of deltaPsi(m). The result indicated that DODC was able to inhibit the $DiOC_6(3)$ staining level, possibly because of induction of the deltaPsi(m) reduction. In addition, we performed a mitochondrial proteomic comparative analysis of NPC-TW01 vs. DODC-treated NPC-TW01 cells and found that the level of prohibitin was increased in DODC-treated cells. Prohibitin was originally characterized as a tumor suppressor because of its association with antiproliferation activity (21). Its physiological function was then proposed to act as a chaperone/holdase (22). When there was an imbalance in the levels of mitochondrial translated proteins, the prohibitin amount was increased to ensure/assist correct protein folding/assembly (22). Moreover, induction of prohibitin was suggested as an indicator for mitochondrial destabilization during apoptosis-related events (17). Our data, demonstrating mitochondrial prohibitin levels by DODC treatment, support the notion that the cell-killing effect of DODC is attributable to mitochondrial perturbation.

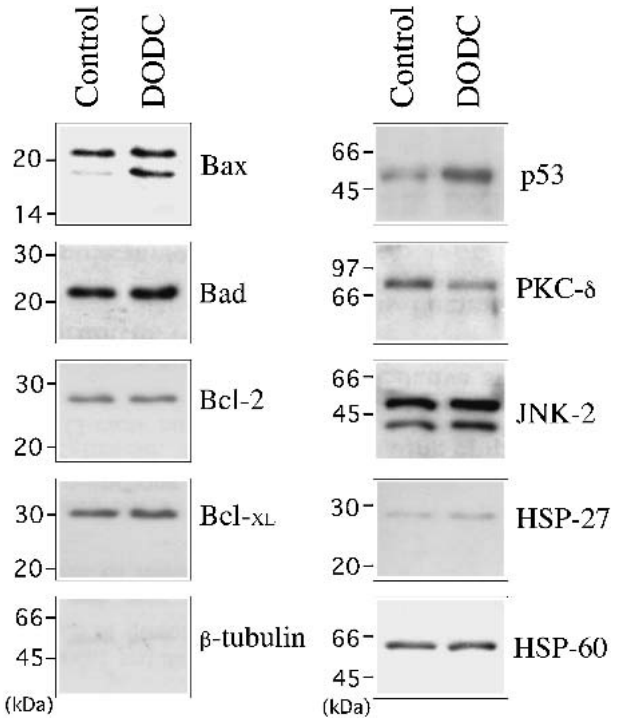

Fig. 7. DODC induces increase of p53 and tBax in the mitochondria of NPC-TW01 cells. NPC-TW01 cells were treated with or without 0.4 μM of DODC for 4 h and were subjected to Western blot analyses of the levels of Bax, Bad, Bcl-2, Bcl-X_L, β-tubulin, p53, PKC-δ, JNK-2, HSP-27, and HSp-60 in the mitochondrial fraction.

The mitochondrial perturbation and thus the reduction of deltaPsi(m) would further result in the change of the distribution of mitochondrial proteins. We found that DODC induced the release of cytochrome *c* from mitochondria to cytosol. In addition, our data revealed that DODC treatment enhanced the mitochondrial levels of p53 and tBax. Bax is well-known to play an important role in enhancing apoptosis. Upon the apoptotic stimuli, Bax migrates from cytosol to mitochondria and associates with voltage-dependent anion channel to form a bigger channel, which facilitates the release of cytochrome *c* from mitochondria (23). The 18-kDa tBax can be produced in some interferon-α and chemotherapeutic agent-induced apoptotic cell systems (24,25). This truncated Bax has been reported to be more apoptotic than its wild-type form (26). On the other hand, p53 has also been reported to translocate to mitochondria upon the induction of apoptosis (18). The p53 tumor suppressor can mediate apoptosis by transcriptional activation of target genes like Bax, Apaf-1, Fas, Puma, and so forth. Accumulating evidence also indicates that p53 can induce apoptosis through a transcription-independent pathway. At the onset of p53-dependent apoptosis, a fraction of p53 localizes to mitochondria, which occurs immediately early and precedes change of deltaPsi(m), cytochrome *c* release, and caspase-3 activation (18). Moreover, it has recently been demonstrated that mitochondrial p53 can form complexes with Bcl-2 and Bcl-X_L and thereby indirectly activate Bax and Bak for the release of cytochrome *c* (27). In cytosol, the released cytochrome *c* together with Apaf-1 causes the activation of caspase-9 and in turn the activation of more downstream executor caspases, such as caspase-3 (reviewed in Ref. 28). Caspases are a family of cysteine proteases that can selectively cleave target proteins critical to cellular longevity.

Regardless of other research reports, induction of mitochondrial localization of JNK-2, PKC-δ, Bad, and so forth, was not observed in our experimental system. In contrast, the mitochondrial level of PKC-δ was even decreased after DODC treatment (Fig. 7). Actually, various apoptotic stimuli induce distinct patterns of cellular localization of PKC-δ (29). For example, PKC-δ translocates to mitochondria in response to UV radiation, oxidative stress, and phorbol ester TPA whereas PKC-δ translocates to nuclei in response to etoposide, cytosine arabinoside, γ-irradiation, and Fas ligation (29). Whether DODC can induce translocation of PKC-δ to nuclei or other organelles and what role is played by PKC-δ therein remain to be investigated.

In conclusion, DODC-induced NPC-TW01 cell apoptosis via a mitochondrion-mediated mechanism with involvement of prohibitin, tBax, p53, and cytochrome *c*. DODC was reported to increase the apoptotic potency of cancer cells through the inhibition of telomerase, but the susceptibility of cellular telomerase to DODC seems to vary in different cell lines. In this study, NPC-TW01 cells provide a system to demonstrate another cytotoxic mechanism of DODC.

REFERENCES

1. C. W. Greider. Telomere length regulation. *Annu. Rev. Biochem.* **65**:337–365 (1996).
2. G. N. Parkinson, M. P. H. Lee, and S. Neidle. Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature* **417**: 876–880 (2002).
3. A. M. Zahler, J. R. Williamson, T. R. Cech, and D. M. Prescott. Inhibition of telomerase by G-quartet DNA structures. *Nature* **350**:718–720 (1991).
4. R. J. Harrison, S. M. Gowan, L. R. Kelland, and S. Neidle. Human telomerase inhibition by substituted acridine derivatives. *Bioorg. Med. Chem. Lett.* **9**:2463–2468 (1999).
5. J. F. Riou, L. Guittat, P. Mailliet, A. Laoui, E. Renou, O. Petitgenet, F. Megnin-Chanet, C. Helene, and J. L. Mergny. Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands. *Proc. Natl. Acad. Sci. U. S. A.* **99**:2672–2677 (2002).
6. J. L. Mergny, J. F. Riou, P. Mailliet, M-P. Teulade-Fichou, and E. Gilson. Survey and summary. Natural and pharmacological regulation of telomerase. *Nucleic Acids Res.* **30**:839–865 (2002).
7. T. K. Das and S. Mazumdar. Effect of adriamycin on the boundary lipid structure of cytochrome *c* oxidase: pico-second time-resolved fluorescence depolarization studies. *Biophys. Chem.* **86**: 15–28 (2000).
8. Q. Chen, I. D. Kuntz, and R. H. Shafer. Spectroscopic recognition of guanine dimeric hairpin quadruplexes by a carbocyanine dye. *Proc. Natl. Acad. Sci. U. S. A.* **93**:2635–2639 (1996).
9. W. Fu, J. G. Begley, M. W. Killen, and M. P. Mattson. Anti-apoptotic role of telomerase in pheochromocytoma cells. *J. Biol. Chem.* **274**:7264–7271 (1999).
10. J. M. Kraveka, B. Ogretmen, J. C. Barredo, and A. R. Safa. Association of telomerase activity with drug resistance in HL-60 and its multidrug resistant derivatives HL-60/adr and HL-60/vcr cells. *Proc. Am. Assoc. Cancer Res.* **41**:2864 (2000).
11. T-S. Huang, C-H. Shu, Y. Chao, and L-T. Chen. Evaluation of GL331 in combination with paclitaxel: GL331's interference with paclitaxel-induced cell cycle perturbation and apoptosis. *Anti-Cancer Drug.* **12**:259–266 (2001).
12. C-C. Lee and T-S. Huang. A novel topoisomerase II poison GL331 preferentially induces DNA cleavage at (C/G)T sites and can cause telomere DNA damage. *Pharm. Res.* **18**:846–851 (2001).
13. S. Lin, H-C. Huang, L-L. Chen, C-C. Lee, and T-S. Huang. GL331 induces down-regulation of cyclin D1 expression via en-

hanced proteolysis and repressed transcription. *Mol. Pharmacol.* **60**:768–775 (2001).
14. T. Hishita, S. Tada-Oikawa, K. Tohyama, Y. Miura, T. Nishihara, Y. Tohyama, Y. Yoshida, T. Uchiyama, and S. Kawanishi. Caspase-3 activation by lysosomal enzymes in cytochome *c*-independent apoptosis in myelodysplastic syndrome-derived cell line P39. *Cancer Res.* **61**:2878–2884 (2001).
15. J. Yang, X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. Peng, D. P. Jones, and X. Wang. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* **275**:1129–1132 (1997).
16. T-S. Huang, C-C. Lee, A-C. Chang, S. Lin, C-C. Chao, Y-S. Jou, Y-W. Chu, C-W. Wu, and J. Whang-Peng. Shortening of microsatellite deoxy(CA) repeats involved in GL331-induced downregulation of matrix metalloproteinase-9 gene expression. *Biochem. Biophys. Res. Commun.* **300**:901–907 (2003).
17. W. E. Thompson, A. Branch, J. A. Whittaker, D. Lyn, M. Zilberstein, K. E. Mayo, and K. Thomas. Characterization of prohibitin in a newly established rat ovarian granulosa cell line. *Endocrinology* **142**:4076–4085 (2001).
18. N. D. Marchenko, A. Zaika, and U. M. Moll. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J. Biol. Chem.* **275**:16202–16212 (2000).
19. S. Kharbanda, S. Saxena, K. Yoshida, P. Pandey, M. Kaneki, Q. Wang, K. Cheng, Y. N. Chen, A. Campbell, T. Sudha, Z. M. Yuan, J. Narula, R. Weichselbaum, C. Nalin, and D. Kufe. Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. *J. Biol. Chem.* **275**:322–327 (2000).
20. P. K. Majumder, P. Pandey, X. Sun, K. Cheng, R. Datta, S. Saxena, S. Kharbanda, and D. Kufe. Mitochondrial translocation of protein kinase C delta in phorbol ester-induced cytochrome *c* release and apoptosis. *J. Biol. Chem.* **275**:21793–21796 (2000).
21. J. K. McClung, D. B. Danner, D. A. Stewart, J. R. Smith, E. L. Schneider, C. K. Lumpkin, R. T. Dell'Orco, and M. J. Nuell. Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. *Biochem. Biophys. Res. Commun.* **164**:1316–1322 (1989).
22. L. G. J. Nijtmans, L. de Jong, M. Artal Sanz, P. J. Coates, J. A. Berden, J. Willem Back, A. O. Muijsers, H. van der Spek, and L. A. Grivell. Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J.* **19**:2444–2451 (2000).
23. S. Shimizu, T. Ide, T. Yanagida, and Y. Tsujimoto. Electrophysiological study of a novel large pore formed by Bax and the voltage-dependent anion channel that is permeable to cytochrome *c*. *J. Biol. Chem.* **275**:12321–12325 (2000).
24. N. Yanase, E. Takada, I. Yoshihama, H. Ikegami, and J. Mizuguchi. Participation of Bax-alpha in IFN-alpha-mediated apoptosis in Daudi B lymphoma cells. *J. Interferon Cytokine Res.* **18**: 855–861 (1998).
25. D. E. Wood, A. Thomas, L. A. Devi, Y. Berman, R. C. Beavis, J. C. Reed, and E. W. Newcomb. Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* **17**:1069–1078 (1998).
26. H. Toyota, N. Yanase, T. Yoshimoto, M. Moriyama, T. Sudo, and J. Mizuguchi. Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. *Cancer Lett.* **189**:221–230 (2003).
27. M. Mihara, S. Erster, A. Zaika, O. Petrenko, T. Chittenden, P. Pancoska, and U. M. Moll. p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* **11**:577–590 (2003).
28. X. Wang. The expanding role of mitochondria in apoptosis. *Genes Dev.* **15**:2922–2933 (2001).
29. C. Brodie and P. M. Blumberg. Regulation of cell apoptosis by protein kinase C δ. *Apoptosis* **8**:19–27 (2003).