

# Activation of Mitochondria and Release of Mitochondrial Apoptogenic Factors by Betulinic Acid\*

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**Different classes of anticancer drugs may trigger apoptosis by acting on different subcellular targets and by activating distinct signaling pathways. Here, we report that betulinic acid (BetA) is a prototype cytotoxic agent that triggers apoptosis by a direct effect on mitochondria. In isolated mitochondria, BetA directly induces loss of transmembrane potential independent of a benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone-inhibitable caspase. This is inhibited by bongkreik acid, an agent that stabilizes the permeability transition pore complex. Mitochondria undergoing BetA-induced permeability transition mediate cleavage of caspase-8 (FLICE/MACH/Mch5) and caspase-3 (CPP32/Yama) in a cell-free system. Soluble factors such as cytochrome *c* or apoptosis-inducing factor released from BetA-treated mitochondria are sufficient for cleavage of caspases and nuclear fragmentation. Addition of cytochrome *c* to cytosolic extracts results in cleavage of caspase-3, but not of caspase-8. However, supernatants of mitochondria, which have undergone permeability transition, and partially purified apoptosis-inducing factor activate both caspase-8 and caspase-3 in cytosolic extracts and suffice to activate recombinant caspase-8. These findings show that induction of mitochondrial permeability transition alone is sufficient to trigger the full apoptosis program and that some cytotoxic drugs such as BetA may induce apoptosis via a direct effect on mitochondria.**

Anticancer agents with different modes of action have been reported to trigger apoptosis in chemosensitive cells (1). Alterations of mitochondrial functions such as permeability transition (PT)<sup>1</sup> have been found to play a major role in the apoptotic

process including cell death induced by chemotherapeutic agents (2–7). Mitochondria undergoing PT release apoptogenic proteins such as cytochrome *c* or apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the cytosol, where they can activate caspases and endonucleases (2, 3, 6, 8–11). However, activated caspases can also induce PT, probably via a direct effect on the PT pore complex (3, 12). These findings suggest that caspases can act upstream and downstream of mitochondria. Mitochondrial function during apoptosis is controlled by the Bcl-2 family of proteins localized to intracellular membranes including the mitochondrial membrane (13). Overexpression of the anti-apoptotic molecules Bcl-2 and Bcl-X<sub>L</sub> has been found to confer resistance to anticancer treatment (14–16). Bcl-2 and Bcl-X<sub>L</sub> may inhibit apoptosis through the capacity to prevent PT and/or to stabilize the barrier function of the outer mitochondrial membrane (5, 6, 13, 17, 18).

Cytotoxic drugs such as doxorubicin can activate apoptosis pathways by inducing ligand/receptor-driven amplifier systems such as the CD95 system (19–26). Upon CD95 ligand/receptor interaction, caspase-8 (FLICE/MACH/Mch5) is cleaved, resulting in activation of a downstream caspase cascade including caspase-3 (CPP32/Yama) (27–35). Induction of the CD95 ligand and up-regulation of CD95 after treatment with cytotoxic drugs such as doxorubicin have been observed in a variety of tumor cells, and blockade of CD95/CD95 ligand interaction by antagonistic antibodies has been found to inhibit drug-induced cell death (19–26).

Betulinic acid (BetA) is a novel anticancer drug with specificity for neuroectodermal tumors (36, 37). We previously found that BetA-induced apoptosis differs from “classical” anticancer agents such as doxorubicin (37). BetA-induced apoptosis is not associated with activation of ligand/receptor systems such as CD95 and does not involve p53. Perturbation of mitochondrial function including loss of mitochondrial permeability transition precedes other key features of apoptosis such as activation of the caspase cascade and nuclear fragmentation (37). This suggests that BetA may have a direct effect on mitochondria. We therefore asked whether BetA would directly activate mitochondria and studied the sequence of the BetA-triggered apoptosis pathway.

## EXPERIMENTAL PROCEDURES

**Drugs**—BetA (Sigma, Deisenhofen, Germany) was provided as a pure substance and dissolved in dimethyl sulfoxide.

**Cell Culture**—The human neuroblastoma cell line SHEP was kindly provided by M. Schwab (German Cancer Research Center, Heidelberg, Germany); maintained in monolayer culture in 75-cm<sup>2</sup> tissue culture

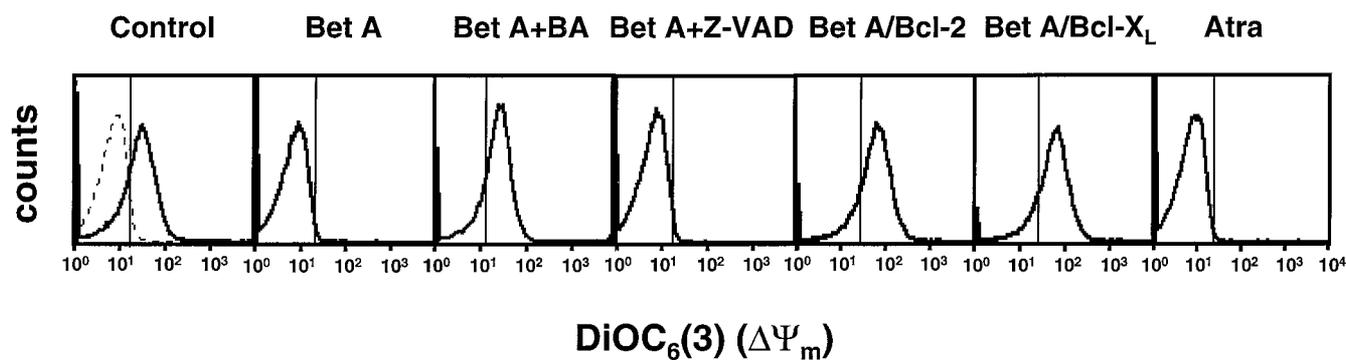
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<sup>1</sup> The abbreviations used are: PT, permeability transition; AIF, apoptosis-inducing factor; BetA, betulinic acid; FACS, fluorescence-activated cell sorting; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; BA, bongkreik acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum

albumin; PARP, poly(ADP-ribose) polymerase; mAb, monoclonal antibody; PIPES, 1,4-piperazinediethanesulfonic acid;  $\Delta\Psi_m$ , mitochondrial transmembrane potential.



**FIG. 1. BetA directly triggers mitochondrial permeability transition.** Mitochondria isolated from SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only were left untreated (*Control*) or were treated with 10  $\mu\text{g/ml}$  BetA for 30 min in the presence or absence of 50  $\mu\text{M}$  BA or 60  $\mu\text{M}$  Z-VAD-fmk, 5 mM atractyloside (*Atra*), a direct mitochondrial activator, was used as a positive control.  $\Delta\Psi_m$  was determined by staining mitochondria with the fluorochrome 3,3'-dihexyloxycarbocyanide iodide (*DiOC<sub>6</sub>(3)*). The *dashed line* in the first histogram indicates the staining profile obtained in the presence of the  $\Delta\Psi_m$ -dissipating agent carbonyl cyanide *m*-chlorophenylhydrazone.

flasks (Falcon, Heidelberg) in RPMI 1640 medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat inactivated fetal calf serum (Conco, Wiesbaden, Germany), 10 mM HEPES, pH 7.4 (Biochrom, Berlin, Germany), 100 units/ml penicillin (Life Technologies, Inc.), 100  $\mu\text{g/ml}$  streptomycin (Life Technologies, Inc.), and 2 mM L-glutamine (Biochrom); and incubated at 37 °C in 95% air and 5% CO<sub>2</sub>. SHEP neuroblastoma cells stably transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a vector control were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 500  $\mu\text{g/ml}$  G418 (Geneticin, Life Technologies, Inc.) (15, 16).

**Determination of Apoptosis**—Cells were incubated for the indicated times with BetA and harvested by trypsinization using 0.05% trypsin and 0.02% EDTA without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Life Technologies, Inc.). Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei as described previously (38), using CELLQuest software (Becton Dickinson, Heidelberg).

**Inhibition of Drug-induced Apoptosis by Benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl Ketone (Z-VAD-fmk) or Bongkreic Acid (BA)**—The broad spectrum tripeptide caspase inhibitor Z-VAD-fmk (Enzyme Systems Products, Dublin, CA) was used at a concentration of 60  $\mu\text{M}$ , and the mitochondrion-specific inhibitor BA was used at a concentration of 50  $\mu\text{M}$  (kindly provided by Dr. Duine, University of Delft, Delft, The Netherlands).

**Western Blot Analysis**—Cells were lysed for 30 min at 4 °C in PBS with 0.5% Triton X-100 (Serva, Heidelberg) and 1 mM phenylmethylsulfonyl fluoride (Sigma), followed by high speed centrifugation. Membrane proteins were eluted in buffer containing 0.1 M glycine, pH 3.0, and 1.5 M Tris, pH 8.8. Protein concentration was assayed using bicinchoninic acid (Pierce). 40  $\mu\text{g}$  of protein/lane was separated by 12 or 15% SDS-PAGE and electroblotted onto nitrocellulose (Amersham Pharmacia Biotech, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of caspase-3 and caspase-8, PARP, and cytochrome *c* protein was done using mouse anti-caspase-8 mAb C15 (1:5 dilution of hybridoma supernatant) (19), mouse anti-caspase-3 mAb (1:1000; Transduction Laboratories, Lexington, KY), rabbit anti-PARP polyclonal antibody (1:10000; Enzyme Systems Products), or mouse anti-cytochrome *c* mAb (1:5000; Pharmingen, San Diego, CA). Goat anti-mouse IgG or goat anti-rabbit IgG (1:5000; Santa Cruz Biotechnology) followed by ECL (Amersham Pharmacia Biotech) was used for detection.

**Preparation of Mitochondria, Cytosolic Extracts, Nuclei, and Mitochondrial Supernatant**—For isolation of mitochondria, cells ( $3 \times 10^8$ /sample) were washed twice with ice-cold PBS, resuspended in 5 volumes of buffer A (50 mM Tris, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.2% BSA, 10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.6, and 0.4 M sucrose), and allowed to swell on ice for 20 min. Cells were homogenized with 30 strokes of a Teflon homogenizer and centrifuged at 10,000  $\times g$  for 10 min at 4 °C. The resulting pellets were resuspended in buffer B (10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, 0.3 mM mannitol, and 0.1% BSA). Mitochondria were separated by sucrose gradient (lower layer: 1.6 M sucrose, 10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5, and 0.1% BSA; upper layer: 1.2 M sucrose, 10 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5, and 0.1% BSA). Interphases containing mitochondria were washed with buffer B at 18,000  $\times g$  for 10 min at 4 °C, and the resulting mitochondrial pellets were resuspended in buffer B. For preparation of cytosolic

extracts, cells ( $1 \times 10^8$ /sample) were washed twice with ice-cold PBS, resuspended in 1 volume of buffer A, and allowed to swell on ice for 20 min. Cells were homogenized with 30 strokes of a Dounce homogenizer and centrifuged at 15,000  $\times g$  for 15 min at 4 °C. The protein concentration of mitochondria or cytosolic extracts was determined by the Bradford method (Bio-Rad). For isolation of nuclei, cells were washed twice with ice-cold PBS, resuspended in 10 volumes of buffer C (10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu\text{M}$  cytochalasin B), allowed to swell on ice for 20 min, and homogenized using a Teflon homogenizer. Homogenates were layered over 30% sucrose in buffer C and centrifuged at 800  $\times g$  for 10 min. The resulting nuclear pellets were resuspended in buffer C and washed three times. Nuclei were stored at -80 °C in aliquots of 10<sup>8</sup> nuclei/ml until required. An AIF-containing mitochondrial supernatant was prepared as described previously (3, 6).

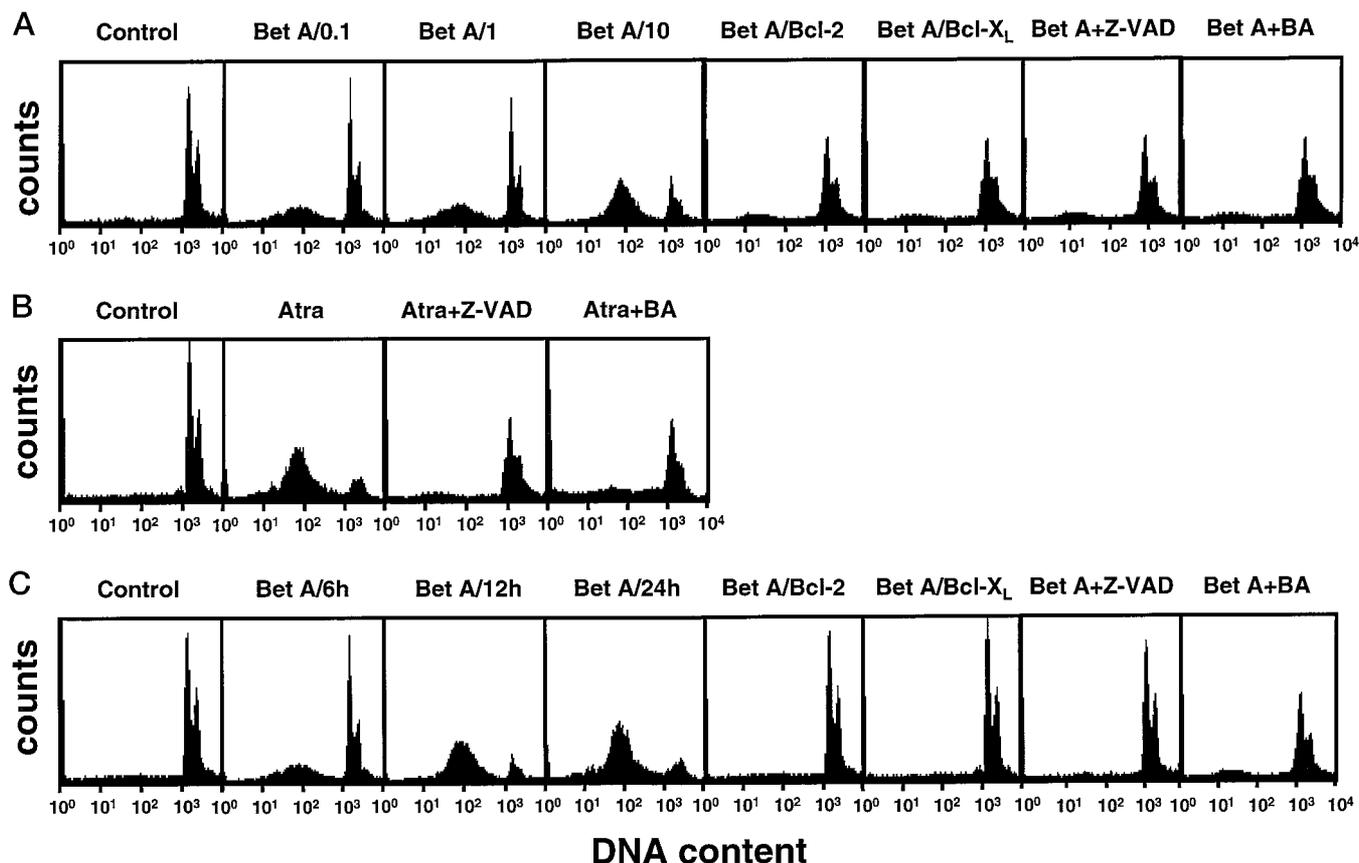
**Cell-free System of Apoptosis**—For determination of nuclear fragmentation, nuclei (10<sup>3</sup>/ $\mu\text{l}$ ) were incubated with mitochondria (1  $\mu\text{g}/\mu\text{l}$ ) in buffer D (10 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 10 mM phosphocreatine, 50  $\mu\text{g/ml}$  creatine kinase, and 10  $\mu\text{M}$  cytochalasin B) for 2 h at 37 °C. Nuclei were stained with propidium iodide (10  $\mu\text{g}/\mu\text{l}$ ) and analyzed by flow cytometry. For determination of caspase activation, cytosolic extracts (2  $\mu\text{g}/\mu\text{l}$ ) were incubated with mitochondria (1  $\mu\text{g}/\mu\text{l}$ ), cytochrome *c* (0.1–100  $\mu\text{M}$ ), or the AIF-containing mitochondrial supernatant (0.5  $\mu\text{g}/\mu\text{l}$ ) in buffer D for 2 h at 37 °C. Partially purified AIF (cytochrome *c*-free; 0.5 mg/ml) was prepared as described previously (3, 6). Proteins were separated by 15% SDS-PAGE, and Western blot analysis was performed as described above. To confirm equal loading of mitochondrial protein, all Western blots were also developed with an antibody directed against a 60-kDa mitochondrial antigen (data not shown).

**Determination of Mitochondrial Membrane Potential**—Mitochondria ( $5 \times 10^6$ /ml) were treated with 10  $\mu\text{g/ml}$  BetA for 30 min, incubated with 3,3'-dihexyloxycarbocyanide iodide (40 nM; Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C, and analyzed on a flow cytometer (FACS Vantage, Becton Dickinson). As a control, cells were treated with the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (200  $\mu\text{M}$ ; Sigma).

**In Vitro Translation and in Vitro Cleavage Assay**—*In vitro* translation and *in vitro* cleavage assay of caspase-8 was performed as described previously (35).

## RESULTS

**BetA Triggers Mitochondrial PT in Isolated Mitochondria**—BetA induces apoptosis in SHEP neuroblastoma cells, where it provokes a dissipation of the mitochondrial transmembrane potential prior to activation of nucleases and caspases (37). All these phenomena are blocked by overexpression of Bcl-2 or Bcl-X<sub>L</sub> (37), which have been found to block apoptosis at the mitochondrial level (2, 6). We therefore asked whether BetA has a direct effect on mitochondria. Isolated mitochondria were incubated with BetA and stained with the dye 3,3'-dihexyloxycarbocyanide iodide to assess the mitochondrial membrane potential. Mitochondria isolated from wild-type SHEP cells or from vector only-transfected cells underwent a loss of  $\Delta\Psi_m$



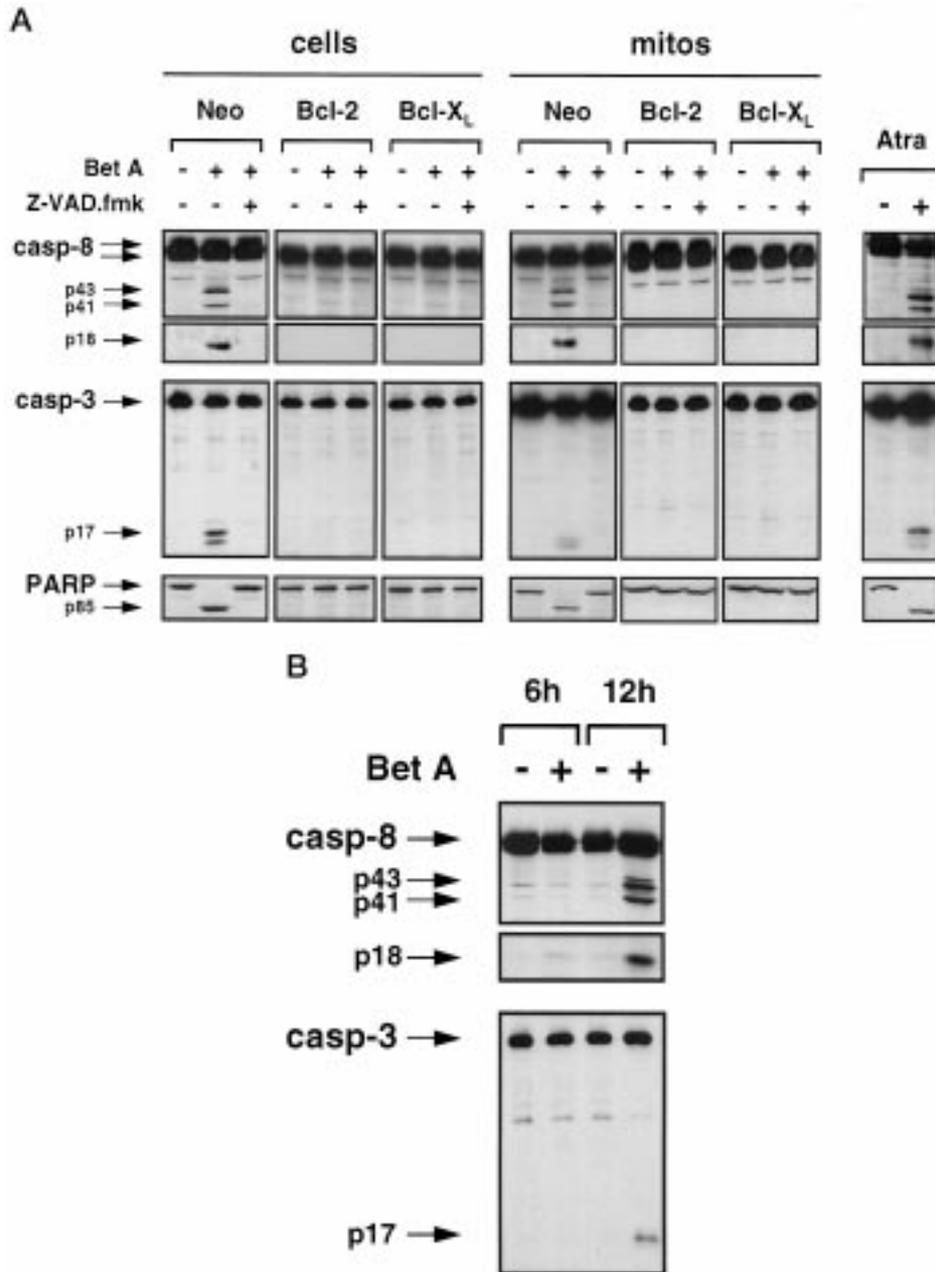
**FIG. 2. BetA-induced mitochondrial permeability transition triggers nuclear fragmentation.** A and B, mitochondria isolated from SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only were incubated for 6 h with nuclei and 0.1–10  $\mu\text{g/ml}$  BetA (A) or 5 mM atractyloside (Atra; B) in the presence or absence of 50  $\mu\text{M}$  BA or 60  $\mu\text{M}$  Z-VAD-fmk. Nuclei incubated either with mitochondria from vector-only cells or with BetA were used as a control. Nuclear apoptosis was determined by FACS analysis of propidium iodide-stained DNA content. C, SHEP cells transfected with *Bcl-2*, *Bcl-X<sub>L</sub>*, or a neomycin resistance vector only were treated with 10  $\mu\text{g/ml}$  BetA or 5 mM atractyloside for 6–24 h. Mitochondria were isolated and incubated with nuclei in the presence or absence of 50  $\mu\text{M}$  BA or 60  $\mu\text{M}$  Z-VAD-fmk. Nuclei incubated either with mitochondria from vector-only cells or with BetA were used as a control. Nuclear apoptosis was determined by FACS analysis of propidium iodide-stained DNA content.

within 30 min of treatment with BetA (Fig. 1). BetA-induced  $\Delta\Psi_m$  dissipation was inhibited by BA, a ligand of the adenine nucleotide translocator, which inhibits PT (2, 3), and BetA had no effect on mitochondria isolated from cells that had been transfected with *bcl-2* or *bcl-X<sub>L</sub>* (Fig. 1), two endogenous inhibitors of PT (2, 6). However, the caspase inhibitor Z-VAD-fmk did not interfere with the BetA-induced  $\Delta\Psi_m$  loss (Fig. 1). Thus, BetA can directly trigger mitochondrial permeability transition without involvement of a Z-VAD-fmk-inhibitable caspase.

**BetA-induced Mitochondrial PT Induces Apoptosis**—To see whether BetA-induced mitochondrial perturbations would cause apoptosis, we assessed nuclear fragmentation following co-incubation of isolated nuclei with isolated mitochondria in the presence of BetA. In this experimental setup, the combination of mitochondria from Neo control cells plus nuclei and BetA resulted in nuclear DNA fragmentation (Fig. 2A). Removal of mitochondria from this mixture abolished the effect of BetA, indicating that mitochondria were required for BetA-induced nuclear apoptosis in this cell-free system. Mitochondria without addition of BetA had no effect on nuclei. No DNA fragmentation was observed using a combination of mitochondria plus nuclei to which apoptogenic doses of standard cytotoxic drugs such as doxorubicin, *cis*-platin, or etoposide were added (data not shown). In contrast, atractyloside, which specifically triggers mitochondrial PT by binding to the adenine nucleotide translocator at the inner mitochondrial membrane (2), had the same effect as BetA (Fig. 2B). Fragmentation of nuclei induced by BetA was inhibited by Z-VAD-fmk or BA or

when mitochondria were obtained from cells overexpressing *Bcl-2* or *Bcl-X<sub>L</sub>* (Fig. 2A). Nuclear fragmentation could also be induced by mitochondria isolated from cells pretreated with BetA (Fig. 2C). This effect was again blocked by Z-VAD-fmk or BA or by overexpression of *Bcl-2* or *Bcl-X<sub>L</sub>* (Fig. 2C). These findings indicate that BetA has a direct and specific effect on mitochondria, leading to fragmentation of nuclei and apoptotic DNA degradation.

**BetA-induced Cleavage of Caspases Depends on Mitochondrial PT**—We then investigated cleavage of caspases in cytoplasmic extracts co-incubated either with mitochondria isolated from BetA-treated cells or with mitochondria isolated from untreated cells in the presence of BetA. Incubation of cytoplasmic extracts with mitochondria isolated from BetA-treated cells resulted in processing of caspase-8, caspase-3, and the prototype substrate PARP (Fig. 3A, *cells*). Cleavage of caspases was blocked in the presence of Z-VAD-fmk or when mitochondria from *Bcl-2*- or *Bcl-X<sub>L</sub>*-overexpressing cells were used (Fig. 3A). Similarly, cleavage of caspases was observed when BetA-treated mitochondria were used (Fig. 3A, *mitos*). Treatment of isolated mitochondria with atractyloside also led to activation of caspases (Fig. 3A). Moreover, mitochondria isolated from BetA-treated cells induced cleavage of caspase-8, caspase-3, and PARP in a time-dependent manner, which was first detectable after treatment with BetA for 12 h (Fig. 3B). To see whether BetA could directly induce cleavage of caspases, an *in vitro* cleavage assay was performed. Following incubation of *in vitro* translated, radiolabeled caspase-8 or caspase-3 with

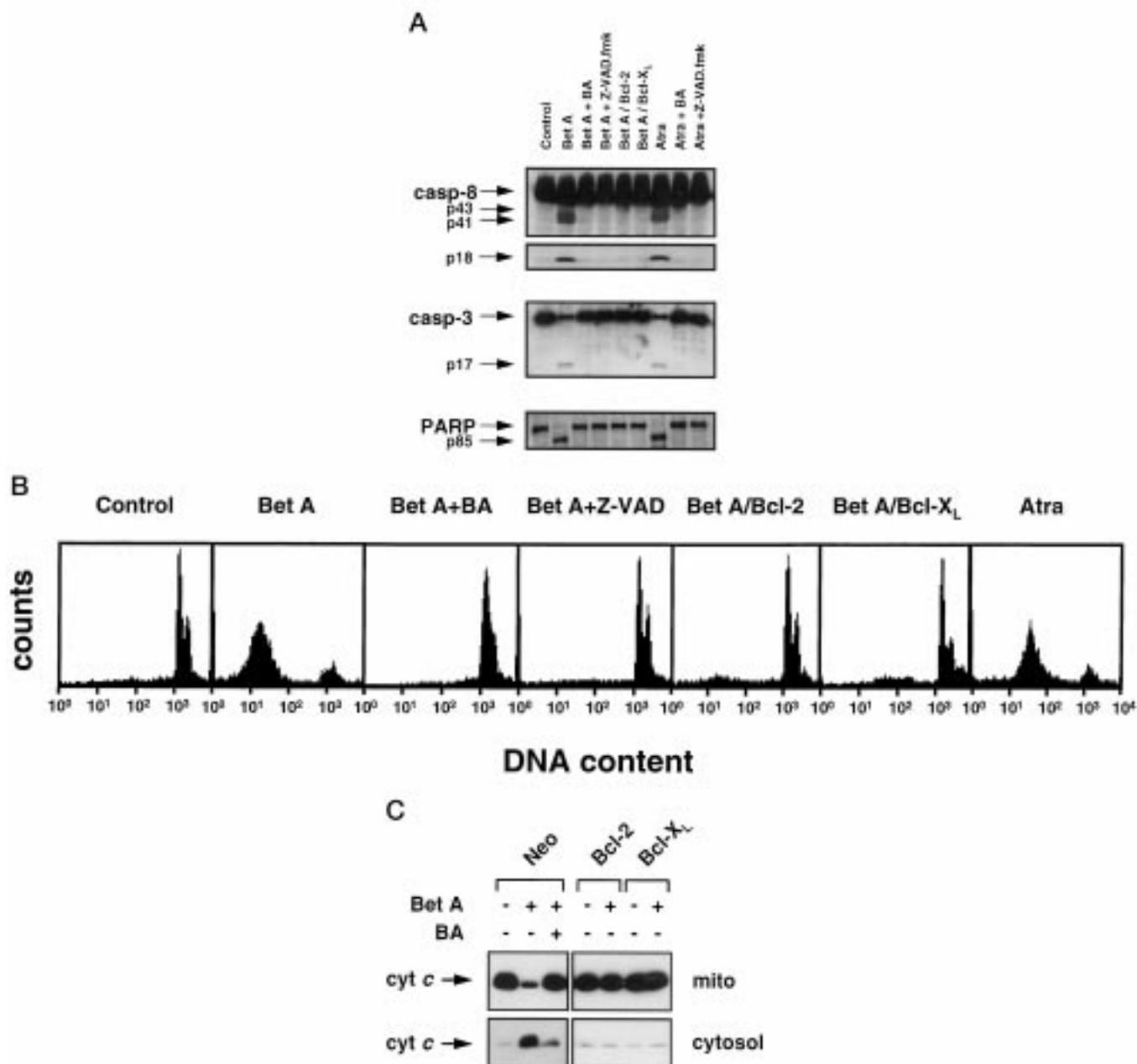


**FIG. 3. BetA-induced cleavage of caspases depends on mitochondrial permeability transition.** *A*, caspases are cleaved by mitochondria undergoing PT. SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only were treated with 10  $\mu\text{g/ml}$  BetA for 16 h. Mitochondria were isolated and incubated with cytosolic extracts for 6 h in the presence or absence of 60  $\mu\text{M}$  Z-VAD-fmk (*left panels, cells*). Alternatively, mitochondria isolated from untreated cells were incubated with 10  $\mu\text{g/ml}$  BetA or 5 mM atractyloside (*Atra*) together with cytosolic extracts for 6 h in the presence or absence of 60  $\mu\text{M}$  Z-VAD-fmk (*right panels, mitos and Atra*). Cytosolic extracts incubated with mitochondria isolated from untreated cells or with untreated mitochondria were used as a control. Proteins (40  $\mu\text{g/lane}$ ) isolated from cell lysates were separated by 15% SDS-PAGE. Immunodetection of caspase-3 (*casp-3*), caspase-8, and PARP protein was performed by mouse anti-caspase-3 mAb, mouse anti-caspase-8 mAb, rabbit anti-PARP polyclonal antibody, and ECL. *B*, kinetics of BetA-induced cleavage of caspases in a cell-free system. SHEP cells were treated with 10  $\mu\text{g/ml}$  BetA for the indicated times. Mitochondria were isolated and incubated with cytosolic extracts for 6 h. Western blot analysis was performed as described for *A*.

BetA, no cleavage products were detected (data not shown), indicating that BetA does not directly cleave caspase-8 or caspase-3. These findings suggest that BetA-induced caspase activation is mediated by mitochondrial PT.

**BetA Causes Release of Apoptogenic Factors from Isolated Mitochondria**—Upon mitochondrial permeability transition, apoptogenic proteins such as cytochrome *c* and AIF are released from the mitochondrial interspace into the cytosol, where they can activate caspases and endonucleases (2, 3). To test whether BetA triggered the mitochondrial release of soluble factor(s) that mediated activation of caspases and nuclear

fragmentation, we treated mitochondria with BetA or atractyloside and analyzed the mitochondrial supernatants for the capacity to induce cleavage of caspases or nuclear fragmentation. When supernatants from BetA-treated mitochondria were added to cytosolic extracts, caspase-8, caspase-3, and PARP were cleaved (Fig. 4A). Processing of caspases was inhibited by BA or Z-VAD-fmk or in mitochondria from Bcl-2- or Bcl-X<sub>L</sub>-overexpressing cells (Fig. 4A). In addition, supernatants from BetA-treated mitochondria induced DNA fragmentation, and this effect was also blocked in the presence of BA or Z-VAD-fmk or by overexpression of Bcl-2 or Bcl-X<sub>L</sub> (Fig. 4B). Similarly,

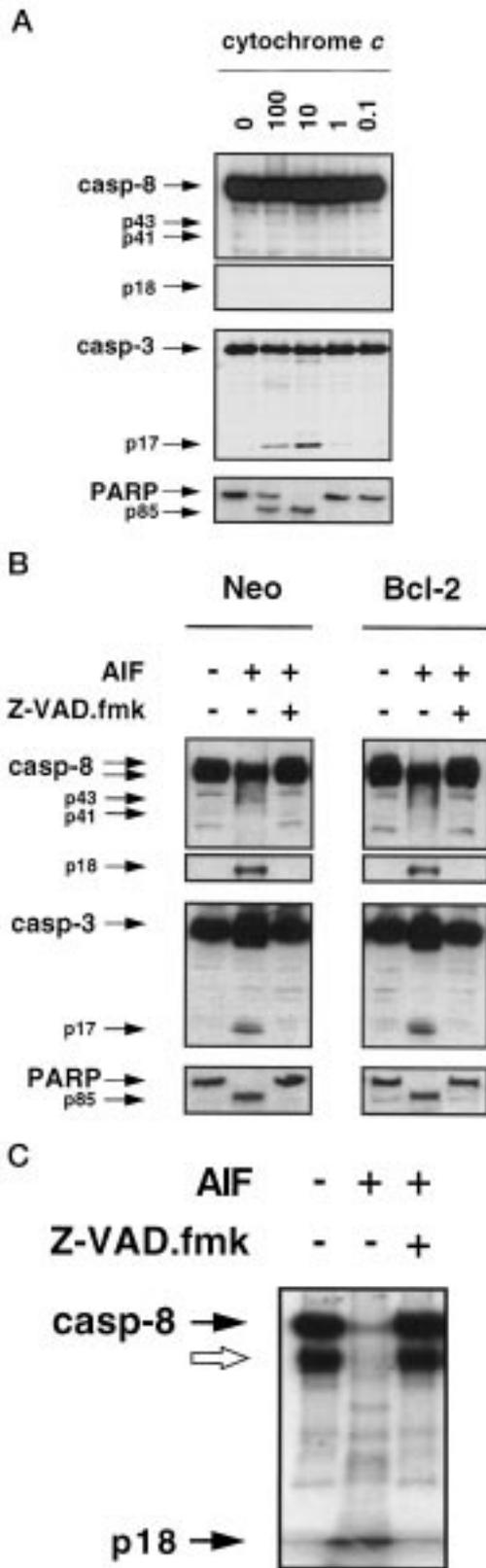


**FIG. 4. BetA causes release of apoptogenic factor(s) from isolated mitochondria.** *A*, soluble factor(s) released from mitochondria undergoing PT induce cleavage of caspases. Mitochondria isolated from SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only were treated with 10  $\mu\text{g/ml}$  BetA or 5 mM atractyloside (*Atra*) for 0.5 h in the presence or absence of 50  $\mu\text{M}$  BA or 60  $\mu\text{M}$  Z-VAD-fmk. Mitochondrial supernatants obtained by high speed centrifugation were incubated with cytosolic extracts for 6 h at 37  $^{\circ}\text{C}$ . Cytosolic extracts incubated with supernatants of untreated mitochondria were used as a control. Western blot analysis was performed as described for Fig. 3*A*. *B*, soluble factor(s) released from mitochondria undergoing PT induce nuclear fragmentation. Mitochondria isolated from SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only were treated with 10  $\mu\text{g/ml}$  BetA or 5 mM atractyloside for 0.5 h in the presence or absence of 50  $\mu\text{M}$  BA or 60  $\mu\text{M}$  Z-VAD-fmk. Mitochondrial supernatants obtained by high speed centrifugation were incubated with nuclei for 2 h at 37  $^{\circ}\text{C}$ . Nuclei incubated with supernatants of untreated mitochondria were used as a control. Nuclear apoptosis was determined by FACS analysis of propidium iodide-stained DNA content. *C*, BetA-induced cytochrome *c* release. Mitochondria isolated from SHEP cells transfected with *bcl-2*, *bcl-X<sub>L</sub>*, or a neomycin resistance vector only (*Neo*) were treated with 10  $\mu\text{g/ml}$  BetA. Mitochondria (*mito*) or cytosolic extracts (*cytosol*; S100 fraction) were prepared as described under "Experimental Procedures." Proteins (5  $\mu\text{g/lane}$ ) were separated by 15% SDS-PAGE. Immunodetection of cytochrome *c* (*cyt c*) was performed by mouse anti-cytochrome *c* mAb and ECL. *cas*p, caspase.

caspase activation and nuclear fragmentation were observed when atractyloside was used instead of BetA (Fig. 4, *A* and *B*). This indicates that BetA triggers the mitochondrial release of soluble apoptogenic factor(s). Accordingly, BetA directly induced cytochrome *c* release in isolated mitochondria (Fig. 4*C*). This BetA-driven release of cytochrome *c* was blocked by BA or in mitochondria from Bcl-2- or Bcl-X<sub>L</sub>-overexpressing cells (Fig. 4*C*).

*Caspase-8 Cleavage Is Mediated by AIF, but Not by Cyto-*

*chrome c*—To assess whether activation of caspases was mediated by cytochrome *c* released from BetA-treated mitochondria, purified cytochrome *c* was added to cytosolic extracts, and cleavage of caspases was monitored by Western blot analysis. As shown in Fig. 5*A*, cytochrome *c* triggered the proteolytic processing of caspase-3 to its active subunits and caused caspase-mediated cleavage of PARP. However, addition of cytochrome *c* to cytosolic extracts did not induce caspase-8 cleavage (Fig. 5*A*). In contrast, when mitochondrial supernatants or



**FIG. 5. Apoptogenic proteins released from mitochondria induce cleavage of caspases.** *A*, cytochrome *c* induces cleavage of caspase-3 (*casp-3*). Cytosolic extracts from SHEP cells were incubated with 0.1–100  $\mu\text{M}$  cytochrome *c*. Immunodetection of caspase-3, caspase-8, and PARP was performed as described for Fig. 3*A*. *B*, AIF induces cleavage of both caspase-8 and caspase-3. Cytosolic extracts from SHEP cells transfected with a neomycin resistance vector only (*Neo*) or *bcl-2* were incubated with partially purified AIF. Immunodetection of caspase-3, caspase-8, and PARP was performed as described for Fig. 3*A*. *C*, AIF cleaves recombinant caspase-8. *In vitro* translated,

partially purified (cytochrome *c*-free) AIF was used instead of cytochrome *c*, both caspase-3 and caspase-8 were cleaved in cytosolic extracts (Fig. 5*B*). In addition, partially purified AIF induced cleavage of *in vitro* translated, radiolabeled caspase-8 to the active p18 subunits (Fig. 5*C*). These findings demonstrate that distinct mitochondrial proteins released by BetA differ in their capacity to activate different caspases. Cleavage of caspase-8 downstream of mitochondria seems to require AIF activity.

#### DISCUSSION

Cytotoxic drugs have been reported to act primarily by inducing apoptosis in sensitive target cells (1). Triggering of apoptosis by anticancer drugs involves simultaneous or subsequent activation of death receptor systems, perturbation of mitochondrial function, and proteolytic processing of caspases, the death effector molecules of apoptosis (25). Thus, the cell death pathway may be entered at multiple sites, and most drugs may hit various targets, although the precise molecular mechanisms have not been characterized in detail. Here, we report that one class of anticancer agents exemplified by BetA may act by directly targeting mitochondria, resulting in caspase activation downstream of mitochondria.

Using a cell-free system, we found that BetA directly triggered PT in isolated mitochondria, and induction of PT appears to be the initial event in BetA-triggered apoptosis. Inhibition of PT by overexpression of Bcl-2 or Bcl-X<sub>L</sub> or by the mitochondrion-specific inhibitor BA prevented all manifestations of apoptosis in intact cells and in a cell-free system such as disruption of  $\Delta\Psi_m$ , activation of caspases, cleavage of substrates (PARP), and nuclear fragmentation. In contrast to BetA, classical cytotoxic drugs such as doxorubicin, *cis*-platinum, or etoposide did not induce mitochondrial perturbations in isolated mitochondria, suggesting that mitochondrial PT, which occurred in intact cells during apoptosis triggered by these substances (20), was the consequence of a primary activation of other pathways or systems. BetA specifically kills neuroectodermal tumor cells (37). When added to intact cells, BetA specifically induced mitochondrial alterations in SHEP neuroblastoma cells, but not in lymphoid cell lines (data not shown). However, BetA-treated mitochondria isolated from the B lymphoblastoid cell line SKW6.4 triggered mitochondrial PT and mediated nuclear fragmentation, similar to mitochondria from SHEP cells. Thus, the specificity of BetA for neuroectodermal tumors may be explained by cell-specific uptake and/or translocation of the compound to the mitochondrial compartment rather than by differences in mitochondria themselves.

In BetA-treated neuroblastoma cells, activation of different caspases and nuclear fragmentation were found only in cells with perturbed mitochondrial function, and BetA-induced loss of  $\Delta\Psi_m$  could not be inhibited by the caspase inhibitor Z-VAD-fmk. This suggests that caspase activation occurs downstream of mitochondria and that activation of mitochondria is sufficient to trigger all downstream events leading to apoptosis. In contrast, in doxorubicin-treated cells, loss of  $\Delta\Psi_m$  was inhibited by Z-VAD-fmk, indicating that activation of mitochondria is preceded by upstream caspase activation.<sup>2</sup> Thus, BetA seems to define a class of cytotoxic agents whose apoptosis-inducing

<sup>2</sup> Fulda, S., Susin, S. A., Kroemer, G., and Debatin, K.-M. (1998) *Cancer Res.* **58**, 4453–4460.

<sup>35</sup>S-labeled caspase-8 was incubated with partially purified AIF for 16 h at 4 °C in the presence or absence of 60  $\mu\text{M}$  Z-VAD-fmk. The reaction products were separated by 15% SDS-PAGE and visualized by autoradiography. The migration position of an N-terminal truncated caspase-8 is labeled by the open arrow.

effect is predominately initiated by activation of mitochondria.

Our studies on BetA-induced apoptosis provide a molecular sequence of the interplay between activation of different caspases and mitochondrial PT. Following death receptor triggering, the receptor proximal caspase-8 becomes activated, which in turn mediates full activation of the caspase cascade, cleavage of substrates, and concomitant triggering of mitochondrial PT (40–43). Therefore, upon CD95 triggering, activation of caspase-8 occurs even in cells in which mitochondrial PT and processing of downstream caspases are blocked by overexpression of Bcl-2 or Bcl-X<sub>L</sub> (2). In contrast, in BetA-treated cells, activation of both caspase-8 and caspase-3 was secondary to mitochondrial PT. Apoptogenic proteins such as cytochrome *c* and AIF released from mitochondria upon permeability transition have been shown to directly induce cleavage of caspases (2, 3, 6). BetA-triggered mitochondrial PT and apoptosis involved cleavage of caspase-8 and caspase-3 independent of CD95 ligand/receptor interaction. Thus, in BetA-treated cells, caspase-8 became activated by mitochondria undergoing PT in the absence of CD95 death-inducing signaling complex formation. AIF released from mitochondria may mediate caspase-8 cleavage following BetA treatment since partially purified AIF could cleave recombinant caspase-8. In contrast, cytochrome *c* did not induce activation of caspase-8, although it induced caspase-3 cleavage, indicating that the mechanism of caspase-8 activation downstream of mitochondria differed from that of caspase-3. Caspases might appear to be dispensable for mediating DNA fragmentation since partially purified AIF has been described to induce DNA fragmentation and since we found fragmentation of nuclei upon incubation with isolated mitochondria. However, these preparations may still contain small amounts of cytosolic fractions that may mediate activation of downstream targets such as PARP, DNA fragmentation factor, or caspase-activated deoxyribonuclease (44, 45).

Different classes of anticancer drugs may enter the apoptotic pathway at distinct entry sites before they eventually induce a Bcl-2/Bcl-X<sub>L</sub>-controlled  $\Delta\Psi_m$  disruption that marks the initiation of a common effector phase of apoptosis. Our findings may have implications for tumor therapy directed toward activation of apoptosis effector systems. Direct activation of mitochondrial PT, as exemplified by cytotoxic drugs such as BetA, may be sufficient for induction of apoptosis in cancer cells and may bypass the requirement for upstream signaling. Those drugs could still be effective against tumor cells that have a defect in upstream apoptosis pathways. In this context, it appears intriguing that tumors cells with a defect in the CD95 system (24), which fail to respond to classical chemotherapeutic agents, are fully susceptible to BetA-induced cell death (37). Thus, our findings may be important to define and develop a new class of cytotoxic agents with direct mitochondrial effects that could overcome some forms of tumor-associated mechanisms of chemoresistance.

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