

# Bobel-24 and Derivatives Induce Caspase-Independent Death in Pancreatic Cancer Regardless of Apoptotic Resistance

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## Abstract

**The poor prognosis of pancreatic cancer and poor sensitivity to current therapeutics, associated with resistance to apoptosis, urge the search for new drugs. We previously described the induction of caspase-independent mitochondrial death in leukemia cells by Bobel-24 (AM-24) and derivatives. Here, we explored whether these compounds induce a similar cytotoxicity in human pancreatic carcinoma cell lines (NP18, NP9, NP31, and NP29). Bobel-24 or Bobel-16 induced cytotoxicity and DNA synthesis inhibition in all cell lines and apoptosis in all lines, except for NP9. Caspase and/or poly(ADP-ribose) polymerase-1 (PARP-1) activity inhibition experiments showed that cytotoxicity was mainly induced through apoptosis in NP18 and through a caspase-independent process in NP9. Moreover, in NP29 or NP31 cell lines, both caspase-dependent and caspase-independent cell death mechanisms coexisted. Cell death was associated with reactive oxygen species (ROS) production, mitochondrial depolarization, cytochrome *c* and apoptosis-inducing factor (AIF) release, AIF nuclear translocation, and lysosomal cathepsin release. Inhibition of ROS production, mitochondrial pore permeability, PARP-1, or phospholipase A2 partially prevented cell death. Moreover, cathepsin B inhibition or down-regulation by small interfering RNA partially blocked cell death. In conclusion, Bobel-24 and derivatives trigger caspase-independent lysosomal and mitochondrial death in all tested human pancreatic cancer lines, irrespective of their degree of apoptotic sensitivity, becoming the only active cytotoxic mechanism in the apoptosis-resistant NP9 line. This mechanism may overcome the resistance to apoptosis observed in pancreatic carcinoma when treated with current genotoxic drugs.** [Cancer Res 2008;68(15):6313–23]

## Introduction

Pancreatic cancer is one of the most lethal cancers (1). A small group of patients are cured by surgery; however, most patients are candidates to systemic chemotherapy. Nevertheless, pancreatic cancer is exceptionally resistant to the genotoxic therapy that efficiently treats other tumors (2). Gemcitabine, the standard drug

for advanced pancreatic cancer, provides clinical benefit in 20% to 30% of patients, whereas 1-year survival rate is only 18% (2).

Pancreatic carcinoma presents mutations or amplification of oncogenes and inactivation of tumor suppressors that lead to invasiveness and deregulation of cell cycle and apoptosis (2, 3). Indeed, the levels of some bcl-2 family members that regulate the mitochondrial pathway have prognostic value in pancreatic cancer patients (2, 4) and play a crucial role in mediating intrinsic or acquired cell resistance to gemcitabine or death receptors (3, 5). Moreover, the overexpression of some members of the extrinsic apoptotic pathway (FAP-1, FLIP) is also associated with resistance to death receptor-induced apoptosis in pancreatic carcinoma patients and cell lines (2, 3).

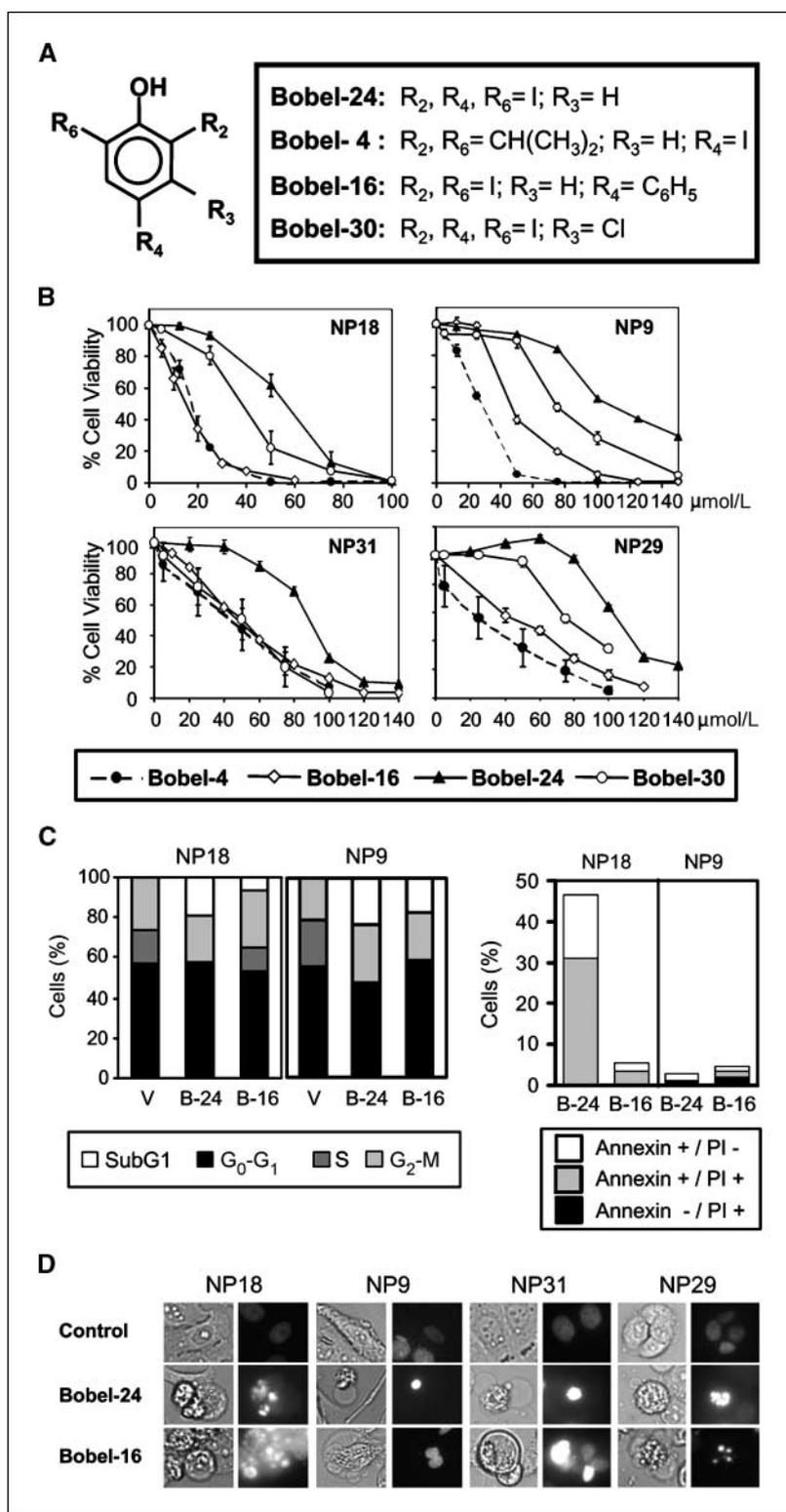
Because the intrinsic and extrinsic apoptotic pathways are both dependent on caspase activation (2, 3), the development of novel compounds able to induce caspase-independent cell death is likely to improve the therapeutic outcome in this tumor. We previously identified a series of cyclooxygenase and 5-lipoxygenase inhibitors (Bobel-24 and derivatives) that are cytotoxic on leukemia cells; however, we did not observe an association between their antitumor effect and the inhibition of these enzymes (6); thus, their mechanism of action is still currently unknown. Nevertheless, we decided to evaluate their antitumor activity in human pancreatic cancer cell lines on the basis of (a) our finding that these compounds induced bcl2-independent and caspase-independent cell death in leukemia cells (6) and (b) on the knowledge of the association between intrinsic resistance to current therapy and inhibition of apoptotic (caspase-dependent) pathways in pancreatic cancer. We reasoned that if a caspase-independent mechanism, similar to that found in leukemia cells, was in place in pancreatic cancer cells, Bobel-24 and derivatives could induce cell death regardless of the apoptotic blockade present in pancreatic cancer.

Different organelles and proteins mediate caspase-independent cell death (7). Thus, the mitochondrion plays a central role in caspase-dependent (apoptotic) cell death through cytochrome *c* release; nevertheless, this organelle also induces reactive oxygen species (ROS), apoptosis-inducing factor (AIF; ref. 8), and endonuclease G mediated caspase-independent death (9, 10). Poly(ADP-ribose) polymerase-1 (PARP-1) activation, after DNA damage or oxidative stress, also triggers mitochondrial dysfunction and AIF release leading to caspase-independent death (11). On the other hand, the lysosome, through the release of cathepsin B and cathepsin D, also mediates caspase-independent cell death (7, 12). Production of ROS and phospholipase-A2 (PLA<sub>2</sub>) activation prompt lysosomal permeabilization and cathepsin release (13), which, in turn, lead to enhanced mitochondrial oxidant production and dysfunction (7, 12).

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** A, chemical structures of Bobel-24 (2,4,6-triodophenol), Bobel-4 (2,6-diisopropyl-4-iodophenol), Bobel-16 (4-phenyl-2,6-diiodophenol), and Bobel-30 (3-chloro-2,4,6-triodophenol). B, cytotoxicity of Bobel-4 (B-4), Bobel-16 (B-16), Bobel-30 (B-30), or Bobel-24 (B-24) on NP18, NP9, NP31, or NP29 human pancreatic carcinoma lines after 48 h incubation. C, left, exposure to Bobel-24 or Bobel-16  $2 \times IC_{50}$  (see Table 1) for 24 h induces S-phase depletion in all lines (cell cycle fluorescence-activated cell sorting analysis). Right, Annexin assays on NP18 or NP9 cell lines treated with Bobel-24 or Bobel-16  $2 \times IC_{50}$  for 24 h. Cells were considered as early (Annexin+/PI-) or late (Annexin+/PI+) apoptotic or necrotic (Annexin-/PI+). D, membrane and chromatin modifications during Bobel-24 or Bobel-16 induced cytotoxicity on a phase-contrast microscope ( $20\times$  magnification, first column) and Hoescht nuclear fluorescent staining (second column). NP18 line was treated with vehicle (control), 100  $\mu$ mol/L Bobel-24, or 30  $\mu$ mol/L Bobel-16 for 24 h. NP9 line was treated with vehicle, 150  $\mu$ mol/L B-24 or 100  $\mu$ mol/L Bobel-16 for 24 h. NP31 and NP29 lines were exposed to vehicle, 100  $\mu$ mol/L Bobel-24, or Bobel-16 for 24 h.

On this basis, we tested whether Bobel-24 and derivatives could induce caspase-independent cell death in a panel of human pancreatic carcinoma cell lines, which display different capacities of activating an apoptotic program after genotoxic exposure. To that purpose, we evaluated the role of the mitochondria or the lysosome and their cell death mediators in the induction of caspase-independent cell death by these compounds.

Our results show that Bobel-24 and derivatives produce caspase-independent cell death in all tested human pancreatic carcinoma cell lines and a different degree or an absence of apoptosis, depending on the cell line genetic background. Moreover, this death is associated with ROS production, cathepsin B release from lysosome to cytosol, enhanced PLA<sub>2</sub> activity, PARP-1 activation, mitochondrial membrane depolarization, and AIF release from the

mitochondrion and its nuclear translocation. Our findings show that, in pancreatic carcinoma cells, these compounds activate a type of caspase-independent mitochondrial cell death that kills cells irrespective of the degree of sensitivity to apoptosis.

## Materials and Methods

**Cell culture and reagents.** NP18, NP29, NP31, and NP9 pancreatic cancer cell lines were established from Sant Pau Hospital patients. NP9 and NP29 lines were maintained in DMEM/F-12 (Ham), whereas NP18 and NP31 lines were grown in RPMI 1640 (Life Technologies-Bethesda Research Laboratories), all supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), and penicillin/streptomycin (50 units/mL).

The synthesis of Bobel-24/AM-24 (2,4,6-triiodophenol), Bobel-4 (2,6-diisopropyl-4-iodophenol), Bobel-16 (4-phenyl-2,6-diiodophenol), and Bobel-30 (3-chloro-2,4,6-triiodophenol) was previously described (6). BEL, H<sub>2</sub>O<sub>2</sub>, CsA, Tiron (ROS scavenger), and DPQ were purchased from Sigma. zVAD-fmk and zFK-mbmk were purchased from Bachem. CA-074 Me was from Calbiochem. DHE was from Molecular Probes (Invitrogen).

For cytotoxicity assays, cells were grown exponentially at a density of 100,000 cells/mL and then exposed to the indicated concentrations of Bobel-24 or its derivatives for 24 h collecting floating and adherent cells for analysis. Control cells were treated with vehicle.

**Cytotoxicity assays.** We used the Cell Proliferation Kit II for viability [2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay], following the recommended protocol (Roche). Cells were plated at 5,000 cells per well in 100  $\mu$ L of culture medium on 96-well plates. After 12 h, the tested compound was added to a final concentration in the 1 to 150  $\mu$ mol/L range and incubated for 48 h or as indicated in figure legends. Control cells were incubated with 0.5% DMSO (vehicle). In all assays, we included blanks containing the compound dissolved in media with no cells and subtracted their absorption from the experimental samples.

**Annexin assays.** For Annexin V binding versus propidium iodide (PI) staining, we treated cells for 24 h with Bobel, harvested and incubated them with Annexin V-FITC and PI for 10 min at room temperature, and immediately analyzed by flow cytometry according to BD Biosciences protocol. See figure legends for experimental detail.

**Phase contrast and fluorescent microscopy.** Cells were pretreated with 0.1  $\mu$ g Hoechst 33342/mL medium for 24 h and plated in 96-well plates, at 8,000 cells per well in 100  $\mu$ L of medium, for 12 h. Then, cells were incubated with different concentrations of tested compound for 6 to 48 h. Cell and nuclear morphology were recorded at 20 $\times$  magnification by contrast (bright field) and fluorescent microscopy over the same surface using an Axiovert-200M microscope (Zeiss).

**Cell cycle analysis.** Exponentially growing cells, exposed to Bobel-24 or Bobel-16 for 12, 24, or 48 h, were harvested and incubated in a 10  $\mu$ mol/L BrdUrd solution in medium at 37°C for 1 h. Cells were then fixed in 70% ethanol at 4°C, permeabilized and stained with FITC-anti-BrdUrd antibody

(Dako, Carpinteria, CA, USA) at room temperature for 30 min, followed by DNA staining with 5  $\mu$ g/mL PI. Cells were then analyzed on FACScan using CellQuest software (BD Biosciences).

**Mitochondrial membrane potential ( $\Delta\Psi$ ) assays.**  $\Delta\Psi$  was determined using JC-1 probes (BD MitoScreen kit, BD Bioscience). Cell lines (10<sup>6</sup> cells in 10 mL) were harvested after 16 h exposure to Bobel-24, Bobel-16, or vehicle and centrifuged, and the cell pellet was resuspended in 0.5 mL of JC-1 solution and incubated at 37°C for 10 min. After rinsing, cells were analyzed by flow cytometry (BD FACScan). A dot plot of red fluorescence (FL2, living cells with intact  $\Delta\Psi$ ) versus green (FL1) fluorescence (cells with lost  $\Delta\Psi$ ) was recorded.

**ROS detection.** A time course for ROS production was done plating 6,000 to 8,000 cells per well in 100  $\mu$ L culture medium on 96-well black plates with clear bottom (Costar). After 12 h of seeding, the evaluated compound or vehicle was added to the final indicated concentration. As a positive control for ROS production, cells were exposed to 6 mmol/L H<sub>2</sub>O<sub>2</sub>. After 30-min treatment, cells were incubated with 10  $\mu$ mol/L DHE probe in complete medium at 37°C and 5% CO<sub>2</sub> and then analyzed at 485 nm excitation and 620 nm emission wavelength over the indicated time periods using FluorStar Optima microplate reader. ROS production was expressed as relative fluorescent units, obtained by subtracting control from treated cells.

**Western blot analysis.** Western blot was done using whole protein lysates of floating plus adherent cells, as described (14). Blots were incubated with primary antibodies against PARP, caspase-3, caspase-7, caspase-8, cytochrome *c*, Bid (BD PharMingen); caspase-9, Bcl-xL (Cell Signaling Technology); actin, AIF (Santa Cruz Biotechnology); proliferating cell nuclear antigen (PCNA), Bax, Bcl-2 (BD Transduction); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International); and COXII (Molecular Probes).

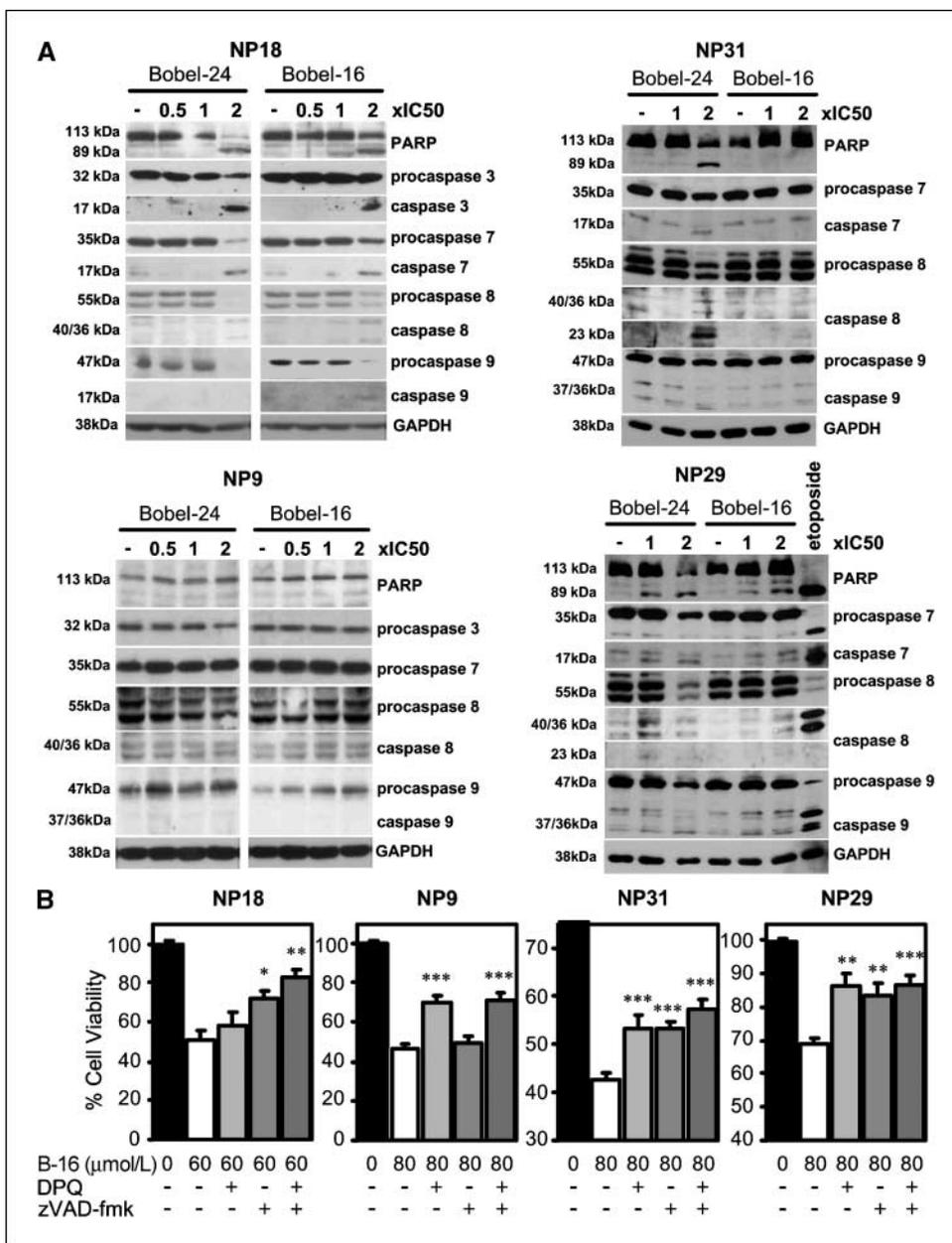
**Preparation of cytosolic and nuclear-enriched fractions.** To determine cytochrome *c* and AIF release from the mitochondria to the cytosol, floating and adherent cells (10<sup>6</sup>/10 mL) obtained after Bobel treatment were collected at 400  $\times$  *g* for 5 min, proceeding as described (6). Western blot was done using the cytoplasmic fraction.

To determine AIF translocation from the mitochondrion to the nucleus, Bobel-treated cells were washed with PBS. The adherent cells were then scrapped with buffer-A [10 mmol/L Tris-HCl (pH 8), 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 1 mmol/L 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; dichlorodiphenyltrichloroethane (DDT), 1 mmol/L phenylmethylsulfonyl-fluoride, 5  $\mu$ g/mL pepstatin-A, 1  $\mu$ g/mL leupeptin, and 2  $\mu$ g/mL aprotinin] and collected together with the floating cells. The cell pellet, obtained after centrifugation at 300  $\times$  *g* for 10 min, was gently resuspended in buffer-A + 0.1% Triton X-100, kept on ice for 10 min, centrifuged at 12,000  $\times$  *g* for 10 min, and washed again in the same buffer. The nuclear pellet was, afterwards, resuspended in buffer-B [20 mmol/L Tris-HCl (pH 8), 420 mmol/L NaCl, 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 1 mmol/L DDT, 25% glycerol, 1 mmol/L phenylmethylsulfonyl-fluoride, 5  $\mu$ g/mL pepstatin-A, 1  $\mu$ g/mL leupeptin, and 2  $\mu$ g/mL aprotinin], kept under agitation for 30 min at 4°C, and centrifuged at 20,200  $\times$  *g* for 10 min. We finally analyzed AIF nuclear

**Table 1.** Effect of Bobel-4, Bobel-16, Bobel-24, or Bobel-30 in cell survival of pancreatic carcinoma cell lines NP9, NP18, NP31, or NP29 after 48 h exposure

Cell type	IC <sub>50</sub> ( $\mu$ mol/L)			
	Bobel-4	Bobel-16	Bobel-24	Bobel-30
NP9	26.1 $\pm$ 0.2	48.2 $\pm$ 1.9	104.9 $\pm$ 0.8	75.7 $\pm$ 2.3
NP18	18.1 $\pm$ 0.7	16.2 $\pm$ 2.3	55.6 $\pm$ 3	42.6 $\pm$ 4.8
NP31	52.9 $\pm$ 8.0	44.0 $\pm$ 2.3	87.8 $\pm$ 1.0	45.6 $\pm$ 8.7
NP29	38.5 $\pm$ 10.2	55.4 $\pm$ 3.1	111.7 $\pm$ 1.5	85.7 $\pm$ 2.6

NOTE: IC<sub>50</sub> is the concentration of compound that causes the half-maximal inhibition of viability with respect to untreated cells. Values are averages of at least three independent determinations  $\pm$  SE.



**Figure 2.** A, Bobel-24 and Bobel-16 induce caspase activation and PARP cleavage in all cell lines, except for NP9. Immunoblots of whole-cell protein extracts after exposure to vehicle or to 0.5, 1, or 2  $\times$  IC<sub>50</sub> compound concentrations for 24 h. GAPDH was used to control loading. B, effect of caspase inhibition or PARP-1 inhibition on Bobel-24-induced or Bobel-16-induced death. Cells were preincubated for 1 h with 25  $\mu$ M zVAD-fmk and/or 30  $\mu$ M DPQ before 16 h exposure to Bobel-16. *P* < 0.05 (\*), *P* < 0.01 (\*\*), or *P* < 0.001 (\*\*\*) indicates significant differences of the compound versus inhibitor pretreated + compound-exposed cells.

translocation by Western blotting in the supernatant, which represents the nuclear fraction. We used anti-GAPDH (cytosolic marker) and anti-PCNA (nuclear marker) antibodies to assess the purity of the cytosolic and nuclear fractions, and the anti-COXII (mitochondrial marker) antibody to verify the lack of mitochondrial contamination of these fractions.

**Immunofluorescence analysis.** Cells were plated in eight-well chamber slides (BD Falcon, CultureSlides) at 10,000 to 24,000 per well in 400  $\mu$ L of medium for 12 h and were then treated with different compound concentrations for 12 to 16 h. Immunofluorescence analysis of AIF and cathepsin B were done in adherent cells and/or floating cells. Cells were rinsed with PBS and fixed with methanol for 1 min at  $-20^{\circ}$ C. They were rinsed again and blocked for 1 h in 1% bovine serum albumin on PBS at room temperature, washed with PBS, and incubated with anti-AIF antibody (1:100, Santa Cruz Biotechnology) or anti-cathepsin B (Ab-1) monoclonal antibody (1:100; Calbiochem) for 1 h. Then, cells were washed with PBS and incubated with the secondary antibody (anti-mouse-TRITC, 1:100) and Hoescht dye (50 ng/mL). Finally, cells were washed with PBS and coverslips were mounted using Fluorepre (Biomérieux).

**Small interfering RNA transient transfections.** NP9 cells were mock transfected or individually transfected with two different duplexes of 21-nucleotide small interfering RNAs (siRNA) of human cathepsin B with two 3'-overhanging TT (Ambion) or nonsilencing siRNA (Silencer Negative Control #1 siRNA, Ambion) to provide similar transfection conditions. The 5'→3' sense strand sequences of the human cathepsin B siRNA were GAGUUAUGUUUACCGAGGAt and GCUGGUCAACUAUGUCAACt for the validated (ID 105579) and the predesigned (ID 105578) siRNAs, respectively. Transfection was done in 96-well plates to a final density of  $8 \times 10^3$  cells per well and 30 nmol/L final siRNA concentration, as described in the Silencer siRNA Starter manual using the reverse transfection method with SiPORT/NeoFX reagent (Ambion). Briefly, healthy growing NP9 cells were trypsinized and resuspended in normal growth medium to  $1 \times 10^5$  cells/mL. On the other hand, 0.5  $\mu$ L of SiPORT NeoFX diluted in Opti-MeM I medium were combined with 1.5  $\mu$ L of the corresponding 2  $\mu$ M siRNA in Opti-MeM I medium to a final volume of 20  $\mu$ L per well according to the manufacturer's protocol. siRNA/SiPORT NeoFX transfection complexes (20  $\mu$ L) were dispensed into each 96-well followed by plating 80  $\mu$ L of the

cells prepared to  $1 \times 10^5$  cells/mL. Transfection mixtures were incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 24 h and then replaced with fresh growth medium before performing cathepsin B silencing analysis, Bobel exposure, and measurement of cytotoxic effect. Optimization of transfection for siRNA was done using the Silencer siRNA Starter kit (Ambion); transfection-associated toxicity was always lower than 10%.

**Quantitative reverse transcriptase-PCR.** Knockdown of cathepsin B expression was assessed by quantitative real-time reverse transcription-PCR (RT-PCR). Total RNA analyzed was extracted with Rneasy Micro kit (Qiagen GmbH) from two wells of transfected NP9 cells into 96-well plates. Briefly, transfected cells were incubated for 24 or 48 h, supernatants were removed, and cells were lysed with 75  $\mu\text{L}$  per well of a buffer containing guanidine-isothiocyanate (RLT buffer, Qiagen) per well. The lysates from two 96-well plates were collected into the same microcentrifuge tube and then processed according to the manufacturer's protocol. Reverse transcription was done with the High Capacity cDNA Archive kit (Applied Biosystems Incorp), and mRNA expression was determined using an ABIPrism 7000 Sequence Detection System (Applied Biosystems) as described (15). Predesigned Taqman Gene Expression Primer and Probe assays (Applied Biosystems) were available for human cathepsin B (HS00157194\_m1) and the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HS99999909\_m1; endogenous control). Supplementary Table S1 describes the sequence and location of their hybridization regions. All probes were FAM labeled. Gene expression results were calculated applying the comparative CT method, as previously described (15), using nontransfected NP9 cells as calibration sample.

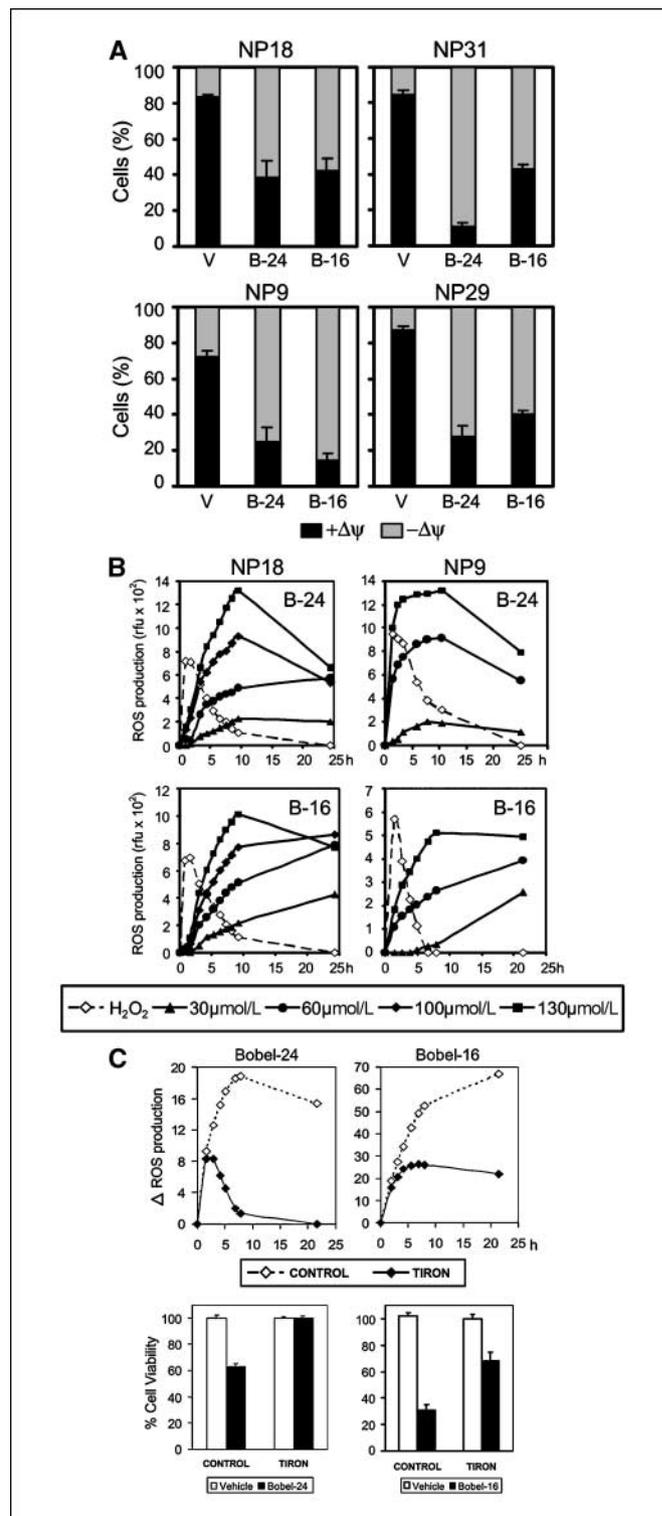
**Statistical analysis.** Data on cell viability, with or without specific inhibitors, were obtained in triplicates and expressed as mean  $\pm$  SE. Significant differences between groups were established at a  $P < 0.05$  determined using an unpaired two-tailed Student's  $t$  test.

## Results

**Bobel-24 and its derivatives show cytotoxicity in all pancreatic carcinoma cell lines.** We tested the antiproliferative activity of the leader compound Bobel-24, also named AM-24, and three derivatives (Bobel-16, Bobel-4, and Bobel-30; Fig. 1A) on four human pancreatic carcinoma cell lines (NP9, NP18, NP31, NP29) obtained from pancreatic carcinomas, previously characterized for their mutations (16) and degree of apoptotic induction by diverse genotoxic agents (gemcitabine, 5-fluorouracil, apilidin; refs. 17, 18). We treated these cell lines with Bobel-24 and its derivatives or vehicle and analyzed cell viability using the XTT assay (Fig. 1B). All compounds were cytotoxic within the micromolar range in all tested cell lines (Table 1). The antiproliferative  $\text{IC}_{50}$  for Bobel-16 and Bobel-4 were the lowest in all cell lines, whereas Bobel-24 showed the highest  $\text{IC}_{50}$ . The dose-dependent cell proliferation curves showed a cell-dependent sensitivity to Bobel-24 and derivatives; the NP18 or NP31 cell lines showed higher sensitivity than the NP9 or NP29 cell lines (Fig. 1B).

**Figure 3.** A, loss of mitochondrial membrane potential ( $\Delta\Psi$ ) by Bobel-24 (B-24) or Bobel-16 (B-16) treatment in NP18, NP9, NP29, or NP31 cells after 16 h exposure to  $2 \times \text{IC}_{50}$  compound concentrations or vehicle (V). Cells were then treated for 15 min with JC-1 probes and then analyzed by flow cytometry to measure the fraction of cells with polarized ( $+\Delta\Psi$ , black columns) or depolarized ( $-\Delta\Psi$ , gray columns) mitochondrial membrane. B, time courses of Bobel-16 or Bobel-24 ROS production in NP9 or NP18 cells at the indicated concentrations, measuring DHE probe oxidation by fluorometry (485 nm excitation and 620 nm emission). Treatment with 15 mmol/L  $\text{H}_2\text{O}_2$  was used as positive control. C, NP9 cell pretreatment with 5 mmol/L Tiron, a free radical scavenger, for 2 h blocked ROS generation after 3 to 5 h exposure to 130  $\mu\text{mol/L}$  Bobel (top) and rescued cell viability (bottom) compared with controls. Differences in ROS production ( $\Delta\text{ROS}$ ) were expressed as relative fluorescent units (rfu) per viable cell after background and reagent signal subtraction (top). Cell viability was analyzed after 24 h treatment in the same 96-well plates by refeeding with new media to remove probe and scavenger (bottom).

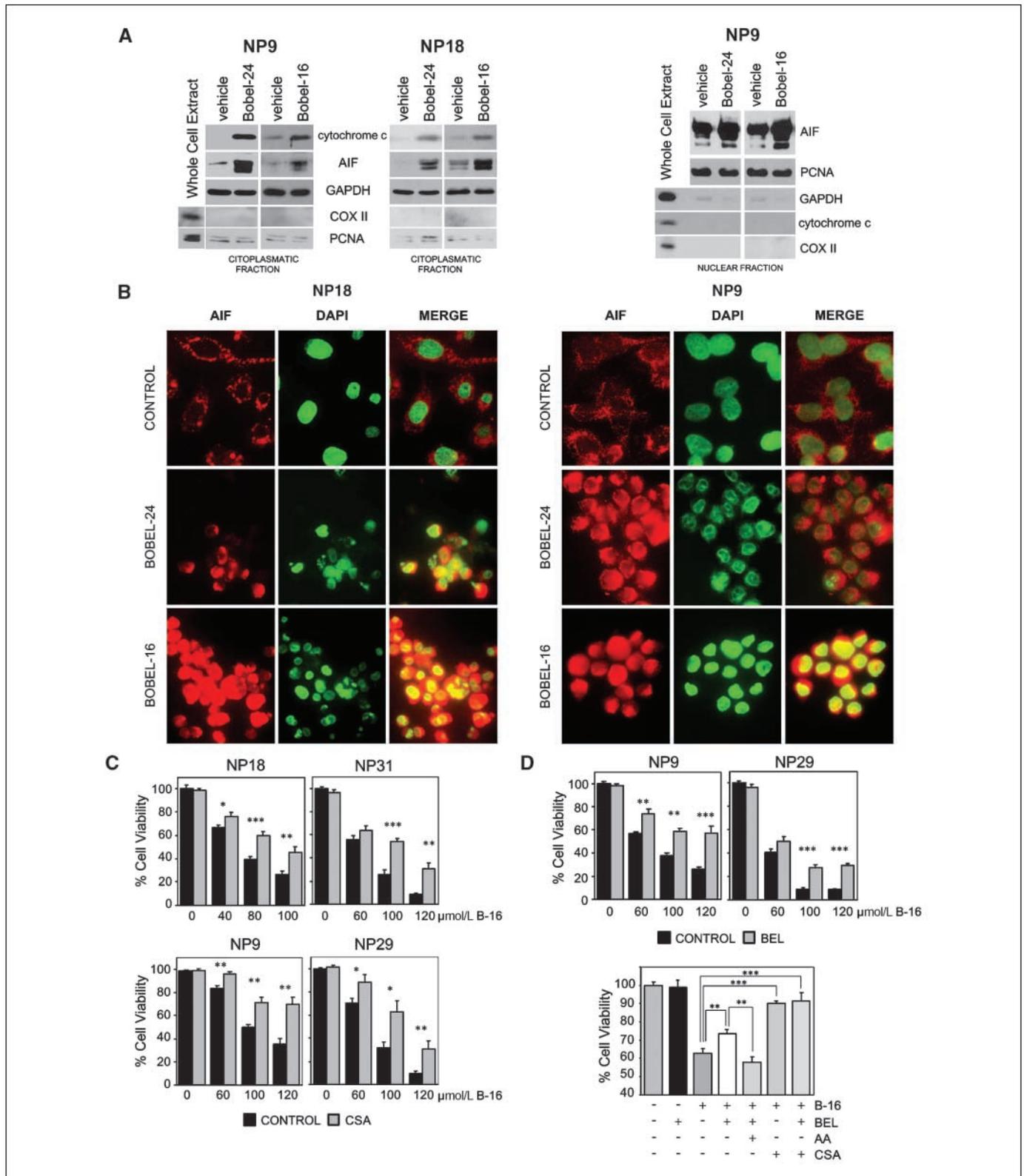
**Bobel-24 and its derivatives induce morphologic and molecular markers of apoptosis in all cell lines, except NP9.** To analyze the mechanisms of antitumor activity induced by Bobel-24 and derivatives, we assessed cell cycle distribution, Annexin V binding, and *in vivo* Hoechst staining. We focused cellular and molecular studies mostly on the effect of the leader compound, Bobel-24, and Bobel-16, because Bobel-4 was significantly more



toxic on nontransformed cells than the other derivatives (6), whereas Bobel-30 showed less antitumor activity.

The cell cycle distribution of Bobel-treated cells was analyzed by flow cytometry, after BrdUrd cell labeling followed by PI staining

(Fig. 1C, left and Supplementary Fig. S1). Consistently with their cytotoxic activity, Bobel-24 and Bobel-16 induced a significant increase in the sub-G<sub>1</sub> phase, a fraction indicative of cell death. Moreover, these compounds induced a deep repression of DNA



synthesis, as shown by S-phase reduction (detected by anti-BrdUrd-FITC) after 12, 24, or 48 h of exposure (Fig. 1C, *left* and Supplementary Fig. S1). In agreement with this, expression of cyclin-D1 and cyclin-B decreased upon Bobel treatment, whereas the cyclin-dependent kinase inhibitor p21 increased in NP9-treated cells (not shown).

The level of apoptotic induction by Bobel-24 or Bobel-16 was cell dependent. NP9 cells displayed low levels of early (~2% Annexin+/PI-) or late (~3% Annexin+/PI+) apoptotic events, as measured by Annexin V binding, after 24 h of Bobel exposure (Fig. 1C, *right*). In contrast, Bobel-24-treated NP18 cells showed classic apoptosis (16% early and 31% late apoptotic cells).

Both drugs induced nuclear condensation and chromatin fragmentation by Hoechst staining after 24-hour treatment in all cell lines, except for NP9 line, which did not show chromatin fragmentation (Fig. 1D). In contrast, NP18 line showed the highest level of fragmentation, whereas NP31 line and NP29 line showed intermediate levels of chromatin fragmentation. We also observed cell shrinkage, membrane blebbing, and cell detachment from the plate after Bobel treatment in all cell lines by brightfield (Fig. 1D). Therefore, Bobel-treated cell features were absent in necrosis (19) and have to be attributed to caspase-dependent and/or caspase-independent cell death.

**Bobel-24 and Bobel-16 induce caspase-dependent and/or independent cell death in a cell-dependent manner.** We also explored Bobel-induced death analyzing the level of activation of the initiator caspase-8 and caspase-9, the executor caspase-3 and caspase-7, and the cleavage of PARP, a caspase substrate (Fig. 2A). Both Bobel-24 and Bobel-16 induced different degrees of caspase-3, caspase-7, caspase-8, or caspase-9 activation and of PARP cleavage in the NP18, NP29, and NP31 cell lines. In contrast, exposure to Bobel-24 or Bobel-16 did not activate the executor caspases nor cleaved PARP in the NP9 line. Whereas the expression of proapoptotic Bid decreased in treated NP18, NP29, and NP31 cells, its apoptotic truncated form (t-Bid) was not apparent. In contrast, Bid was not regulated in NP9 cells. Moreover, we did not observe significant changes in the expression of the antiapoptotic proteins Bcl-2 or Bcl-xL or in the proapoptotic protein Bax in any of the cell lines (Supplementary Fig. S2). These results suggest a marginal or negligible participation of Bcl-2 family members in Bobel-induced cell death and are consistent with overexpression of Bcl-2 not altering cytotoxicity by Bobel-24 or derivatives in leukemic cells (6).

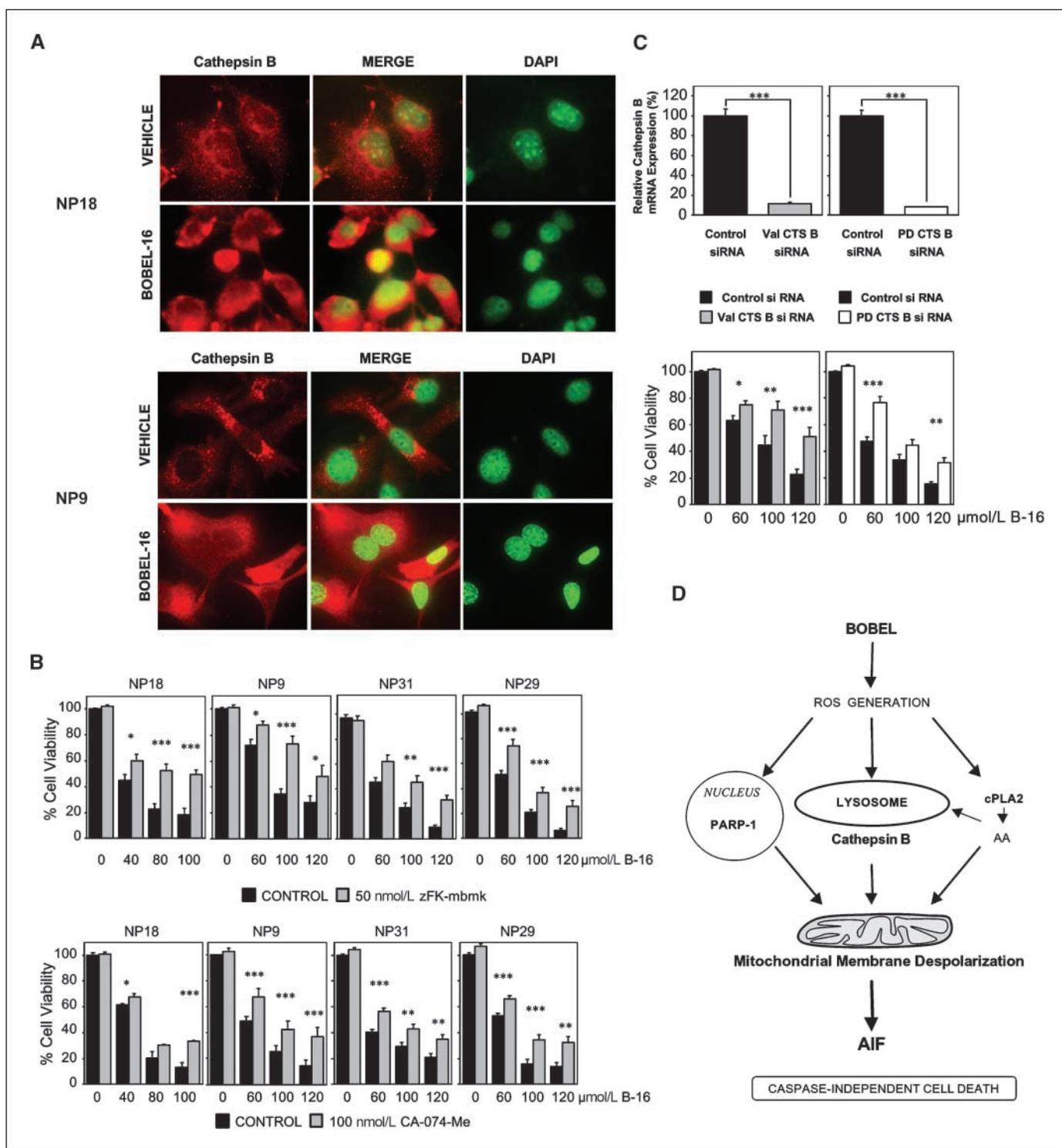
We next explored whether Bobel-16-induced death was dependent on caspase or PARP-1 activity. PARP-1 activation is involved in caspase-independent cell death (10, 11). To that purpose, we measured cytotoxicity after preincubating the cells for 1 h with zVAD-fmk (pancaspase inhibitor) and/or DPQ (PARP-1

inhibitor) and then exposed to Bobel-16 for 16 h (Fig. 2B). Treated NP18 cells showed increased cell viability, after their preincubation with zVAD-fmk alone ( $P < 0.05$ ), or with zVAD-fmk plus DPQ ( $P < 0.01$ ), but their viability remained unaltered when cells were preincubated with DPQ alone. Therefore, the main mechanism of Bobel-induced death in NP18 cells seems to be caspase-dependent, although some contribution of a caspase-independent pathway may exist. In contrast, treated NP9 cells showed increased cell viability after incubation with DPQ ( $P < 0.001$ ), but not with zVAD-fmk, suggesting that, in these cells, Bobel-induced cell death is caspase independent. Moreover, in the NP31 or NP29 cell lines, preincubation with either zVAD-fmk or DPQ significantly increased cell viability ( $P < 0.001$  to  $P < 0.01$ ), suggesting the coexistence of both death processes in these cells.

**Bobel-24 and Bobel-16 induce mitochondrial membrane depolarization and ROS-dependent cell death in all cell lines.** Since we have previously shown induction of caspase-independent cell death and mitochondrial membrane depolarization by Bobel-24 or its derivatives in leukemia cells (6), we also explored, here, whether these compounds induced these effects in pancreatic carcinoma cells. We stained cells with the JC1 dye to measure mitochondrial membrane potential because of its potential-dependent accumulation in this organelle. We found that Bobel-24 or Bobel-16 induced mitochondrial depolarization ( $-\Delta\Psi$ ) after 16 hours of exposure in all four cell lines (Fig. 3A), which prompted our search for ROS as possible upstream or downstream mediators of cell death. Thus, we measured ROS production staining Bobel-exposed cells with DHE (6), a cell-permeable fluorescence dye that reacts with a broad spectrum of ROS.  $H_2O_2$ -treated cell line was used as a positive control. Bobel-24-treated or Bobel-16-treated NP18 and NP9 cell line showed ROS production after 16 and 24 h exposure by flow cytometry (not shown). Moreover, a time course analysis of ROS production, with the same compounds in the same two cell lines (Fig. 3B), showed an increase in ROS production in a concentration and time-dependent manner. At high Bobel concentrations, maximal ROS production occurred after 5 to 10 hours of treatment. These results indicate that ROS production may mediate the mitochondrial dysfunction associated with Bobel-induced death. Consistently, pretreatment of NP9 cells for 2 hours with the free radical scavenger Tiron (a ROS scavenger) blocked ROS generation after 3 to 5 hours of exposure to Bobel (Fig. 3C, *top*) and reduced its cytotoxic effect (Fig. 3C, *bottom*), suggesting that ROS generation could mediate cell death.

**Bobel-24 and Bobel-16 induce cytochrome *c* and AIF release from the mitochondrial membrane and AIF nuclear translocation.** Mitochondrial membrane depolarization was a common

**Figure 4.** Cytochrome *c* and AIF mitochondrial release to the cytoplasm and AIF nuclear translocation in NP18 or NP9 cells treated with Bobel-24 or Bobel-16 (A and B). *A, left*, cytosolic fraction analysis of cytochrome *c* and AIF in cells incubated with  $2 \times IC_{50}$  of compound or vehicle for 16 or 20 h. GAPDH was used as a cytoplasmic fraction marker, as well as a control for loading. The negligible level of PCNA indicated no nuclear contamination, and the absence of COXII expression indicated no mitochondrial contamination of the cytoplasmic fraction. *Right*, analysis of AIF nuclear translocation, as measured in the nuclear fraction after 20 h Bobel-24 or Bobel-16  $2 \times IC_{50}$  treatment of NP9 cells. PCNA was used as a nuclear fraction marker and as a control for loading. The lack of GAPDH expression indicated no cytoplasmic contamination, and the absence of cytochrome *c* or COXII expression indicated no mitochondrial contamination of the nuclear fraction. *B*, immunolocalization of AIF after Bobel exposure. NP18 cells (*left*) were treated with vehicle, Bobel-24  $2 \times IC_{50}$  or Bobel-16  $1 \times IC_{50}$  for 20 h. NP9 cells (*right*) were treated with vehicle, Bobel-24  $1 \times IC_{50}$  or Bobel-16  $2 \times IC_{50}$  for 20 h. AIF was stained with anti-AIF-TRITC (red, first column), the nucleus was stained with Hoescht (green, second column), and colocalization was detected by merge (orange or yellow, third column). AIF has granulous mitochondrial pattern in control and diffuse distribution in cytosol and nucleus in Bobel-treated cells. *C* and *D*, inhibition of MPP or PLA<sub>2</sub> activity blocks Bobel-induced cytotoxicity. *C*, partial suppression of cytotoxicity after cell pretreatment with 2  $\mu$ mol/L CsA (MPP inhibitor) for 30 min and exposure to Bobel-16 for 6 h in NP18 or for 16 h in NP9, NP29, or NP31 cell lines. *D, top*, partial suppression of cell death after cell preincubation with 20  $\mu$ mol/L BEL (PLA<sub>2</sub> inhibitor) for 1 h followed by 6 h exposure to Bobel-16 treatment in NP9 or NP29 cells. *Bottom*, AA reverses the protective effect of PLA<sub>2</sub> inhibition in Bobel-treated cells. NP9 cells were preincubated with 20  $\mu$ mol/L BEL, 100  $\mu$ mol/L AA, or 2  $\mu$ mol/L CsA or their combination for 1 h, followed by 6 h Bobel-16 treatment.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), or  $P < 0.001$  (\*\*\*) indicates significant differences between Bobel versus inhibitor pretreated + Bobel-treated cells.



**Figure 5.** A, cathepsin B release from the lysosome to the cytoplasm in NP18 or NP9 cells associates with Bobel-16–induced cell death. NP18 (A, top) or NP9 (A, bottom) cells were incubated with vehicle or Bobel-16 at 30 or 60  $\mu\text{mol/L}$ , respectively, for 12 h. Cathepsin B staining with anti–cathepsin B–TRITC (first column, red), nuclear staining with Hoescht (third column, green), and their colocalization (overlay in second column, orange or yellow). Cathepsin B (red) shows a granulo-lyosomal pattern in control cells (first line) and has a mainly diffuse cytosolic (red) and to a less degree nuclear (orange or yellow) distribution after Bobel treatment (second line). B, inhibition of cathepsin B, by cell preincubation with 50 nmol/L zFK-mbmk (top) or 100 nmol/L CA-074 Me (bottom) for 1 h, partially blocked in all cell line cytotoxicity induced by 6 h (in NP18) or 16 h (in the rest of cell lines) exposure to Bobel-16.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*) mean significant differences between Bobel versus zFK-mbmk or CA-074 Me pretreated + Bobel-treated cells. C, silencing of cathepsin B expression by siRNA *CTS B* partially abolished cytotoxicity induced by Bobel-16 in NP9 cells. NP9 cells were transfected with two different siRNA of cathepsin B [validated (*Val*) or pre-designed (*PD*) *CTS B* siRNA] or with a negative control siRNA, using a 30 nmol/L final siRNA concentration. Twenty-four (Supplementary Fig. S3) and 48 h (top) after transfection, knockdown of cathepsin B expression was determined by quantitative PCR; then, NP9 cell viability was determined after additional exposure for 16 h to Bobel-16 (bottom). Results shown are representative of three or more independent experiments. The mRNA levels of cathepsin B were normalized with the housekeeping gene *HRPT1*. More details are described in Material and Methods ( $P < 0.05$  (\*),  $P < 0.01$  (\*\*), and  $P < 0.001$  (\*\*\*) identify significant differences between negative control siRNA#1 transfected cells versus *CTS B* siRNA transfected cells). D, diagram of Bobel-induced caspase-independent cell death in pancreatic carcinoma cells.

finding in caspase-dependent and caspase-independent pancreatic carcinoma cell death induced by Bobel-24 or its derivatives, as we have previously described in caspase-independent cell death in leukemic cells (6). Consequently, we explored whether pancreatic carcinoma cell death by Bobel-24 or Bobel-16 was mediated by cytochrome *c* release (common in caspase-dependent death) and/or mitochondrial release and nuclear translocation of AIF (common in caspase-independent death). Bobel-24 or Bobel-16 exposure induced cytochrome *c* and AIF release to the cytosol because both proteins accumulated in the cytosolic fractions (Fig. 4A, *left*). Moreover, analysis of AIF localization by immunofluorescence showed a granulose (mitochondrial) distribution in control vehicle-treated NP18 cells, whereas treatment with Bobel-24 or Bobel-16 led to a loss of the granulose pattern in the cytosol (red) and to AIF detection in the nucleus (orange or yellow; Fig. 4B, *left*). Similarly, in untreated NP9 cells (Fig. 4B, *right*), AIF showed a filamentous pattern in the mitochondria, which, after Bobel exposure became diffused and localized in both the cytosol and the nucleus (yellow or orange). Nuclear translocation was less evident at lower Bobel-24 concentrations (Fig. 4B, *right, second line*). We confirmed the nuclear translocation of AIF, by Western blot in nuclear-enriched fractions of NP9-treated cells (Fig. 4A, *right*).

**Inhibition of the mitochondrial pore permeability or PLA<sub>2</sub> activity block Bobel cytotoxicity in all cell lines.** Loss of  $\Delta\Psi$  associated with Bobel-induced cell death prompted the analysis of its requirement in this process. Thus, we preincubated NP9 or NP18 cells with an inhibitor of the mitochondrial pore permeability (MPP), CsA, before exposure for 6 or 16 h to Bobel-16 (Fig. 4C). Its inhibition delayed and partially rescued cell death induced by Bobel-16 (Fig. 4C), suggesting the involvement of the mitochondrial depolarization through the alteration of MPP in Bobel-induced cell death.

PLA<sub>2</sub> activity may mediate nuclear shrinkage in caspase-independent death by various stimuli (20), producing arachidonic acid (AA) and ROS, which may also mediate this type of death (21). Therefore, we tested if the PLA<sub>2</sub> inhibitor BEL could block the nuclear shrinkage, in the absence of fragmentation, that occurred during the Bobel-16-induced death of NP9 cells. Cell pretreatment with BEL did not inhibit nuclear shrinkage in NP9 line, but partially inhibited Bobel-induced death in NP9 and NP29 cell lines (Fig. 4D, *top*). We also observed that the preincubation of NP9 cells with BEL plus AA, a product of PLA<sub>2</sub> activity, before their exposure to Bobel-16, reversed the protective effect of BEL on Bobel cytotoxicity (Fig. 4D, *bottom*). This suggests that both PLA<sub>2</sub> activity and AA production mediate Bobel-16-induced cell death. In summary, the alteration of the mitochondrial membrane permeability, PLA<sub>2</sub> activity, and AA production seem to mediate Bobel-induced cell death.

**Bobel-induced cell death is dependent on cathepsin B activity and release from the lysosome.** Lysosomal cathepsin B has been proposed as a possible initiator of cell death (7, 22). Moreover, ROS production and PLA<sub>2</sub> activation may also induce permeabilization of the lysosome (12, 13, 23). To explore the possible role of this organelle in Bobel-16-induced cell death, we analyzed by immunofluorescence if the compound induces the release of cathepsin B from the lysosomes and whether the use of specific inhibitors of cathepsin B activity reverts its cytotoxicity. Cathepsin B release from the lysosome occurred after 12 hours of exposure to Bobel in adherent cells before their detachment. Cathepsin B (red) showed a granulose (lysosomal) distribution in control NP18 (Fig. 5A, *top*) or NP9 (Fig. 5A, *bottom*) cells; whereas

after exposure to Bobel-16, cathepsin B diffused to the cytosol (red), losing this granulose pattern. Moreover, preincubation with low concentrations of the highly specific cathepsin B inhibitors, zFK-mbmk (Fig. 5B, *top*) or CA-074Me (Fig. 5B, *bottom*) partially blocked Bobel-induced cell death in all four cell lines, individually. To further confirm the importance of cathepsin B release and activity in Bobel-induced cell death, we silenced cathepsin B expression in NP9 cells. We chose the NP9 cell line because it shows a type of cell death that is exclusively caspase independent. The expression of cathepsin B in NP9 cells achieved a 90% inhibition, as determined by real-time RT-PCR at 24 (Supplementary Fig. S3) or 48 hours (Fig. 5C, *top*) after transfection of the validated or of the predesigned cathepsin B siRNA in these cells. Moreover, down-regulation of cathepsin B expression by each of these siRNAs partially abolished cytotoxicity induced by Bobel-16 in NP9 cells, compared with cells transfected with a nontargeting siRNA (negative control; Fig. 5C, *bottom*). These results indicate the requirement of cathepsin B release from the lysosomes and cathepsin B activity for Bobel-induced cell death in pancreatic carcinoma cell lines.

## Discussion

We describe the induction of caspase-independent cell death by Bobel-24 (AM-24) and its derivative Bobel-16 in all tested pancreatic carcinoma cell lines, bearing different genetic backgrounds, regardless of a concomitant and cell line-dependent degree of apoptotic induction.

**Cell line-dependent induction of apoptosis.** We observed wide differences in apoptosis induction by Bobel-24 or derivatives depending on the tested cell line despite their potent cytotoxic effect in all lines. Thus, NP18 showed maximal induction of chromatin condensation and fragmentation, phosphatidyl-serine exposure, activation of caspases and PARP proteolysis, and partial reversion of cytotoxicity by a pancaspase inhibitor, suggesting that cytotoxicity occurred mainly by classic apoptosis. In the NP29 or NP31 cell lines, the less intense caspase activation and PARP proteolysis indicated an intermediate level of apoptosis. In contrast, Bobel-induced cytotoxicity was not apoptotic in the NP9 cell line because the pancaspase inhibitor was unable to reverse cell death, which occurred without phosphatidyl-serine exposure, chromatin fragmentation, caspase activation, or PARP cleavage despite the alteration of mitochondrial potential and cytochrome *c* release. The disconnection between mitochondrial alteration and caspase activation in the NP9 line is consistent with its inability to enter apoptosis by genotoxic treatment (17, 18) and with growth rate changes, by implantation site in an animal model, depending on cell cycle rather than on apoptotic regulation (14).

**Activation of caspase-independent cell death in all pancreatic cell lines.** Despite wide differences in apoptosis among the tested cell lines, Bobel-24 or Bobel-16 induced mediators of caspase-independent death in all tested cell lines. ROS generation was an early (peak at 5–10 h) event in Bobel-induced cell death, in all lines, occurring earlier than other mediators of cell death and it may be a critical inductor of cell death because the free radical scavenger Tiron blocked both ROS generation and cytotoxicity.

Lysosomal permeabilization and cathepsin B activity were also important in Bobel-mediated cytotoxicity, as inhibition of its activity with specific inhibitors or down-regulation of cathepsin B expression by siRNA partially prevented cell death. This is in

agreement with ROS-induced lysosomal permeabilization (13, 24) triggering cathepsin B release, mitochondrial dysfunction, and AIF release (12, 13) leading to caspase-independent death. PARP-1 activation contributed also to cytotoxicity, since a specific inhibitor partially reversed Bobel-induced cell death. Consistently, ROS is able to activate PARP-1, a nuclear sensor of DNA damage (which agrees with our observation of S-phase reduction), generating the PAR polymer that triggers mitochondrial dysfunction, AIF release, and caspase-independent death (11, 25).

In addition, Bobel-induced ROS may activate PLA<sub>2</sub> and generate its product AA, which could mediate cytotoxicity, because PLA<sub>2</sub> inhibition partially prevented cell death, whereas AA restored cytotoxicity. This is in agreement with oxidative stress activating PLA<sub>2</sub> and producing AA (22, 26), PLA<sub>2</sub> activation destabilizing lysosomes and mitochondria (13, 23), and AA inducing mitochondrial damage and permeabilization (21, 26, 27). Moreover, Bobel inhibition of LOX and COX (6) could increase the available AA, enhancing lysosomal permeability.

Therefore, Bobel-24 and derivatives seem to induce redox damage in several organelles, in all pancreatic cancer cell lines, activating the following caspase-independent mitochondrial cell death pathway (Fig. 5D): ROS generation triggers lysosomal permeabilization and cathepsin B release to the cytosol, PARP-1 activation in the nucleus, and PLA<sub>2</sub> activation in the cytosol, all leading to dissipation of mitochondrial potential and permeabilization and release and nuclear translocation of AIF.

Based on previous reports that high levels of oxidative stress trigger lysosomal membrane permeabilization, leading to caspase-independent cell death (28) or to autophagy (29), we believe autophagy is a possible mechanism for the caspase-independent lysosomal and mitochondrial cell death we have observed. However, the confirmation of this proposal will require a functional evaluation of the involvement of the pathways known to regulate autophagy in cell death induced by Bobel-24 and derivatives.

**Hierarchical activation of cell death mechanisms in pancreatic cancer?** Bobel-24 and derivatives seem to induce concomitantly caspase-dependent and caspase-independent death in pancreatic carcinoma cells. However, there may be a hierarchy of cell death mechanisms: when apoptosis is not blocked, it is the preferred and almost exclusive mechanism, despite of also showing features of caspase-independent death (e.g., NP18); when it is partially blocked (e.g., NP29 or NP31), it coexists with caspase-independent death; and when it is completely blocked (e.g., NP9 apoptotic resistance), cell death is only induced by a caspase-independent mechanism. Caspase and PARP-1 inhibition experiments support this argument. This is consistent with the faster

apoptotic death being dominant over caspase-independent death (30) when several death programs coexist (7, 12) and with our observation that CsA inhibition of MPP reverts NP18 apoptosis sooner than NP9 caspase-independent death.

In addition, our results agree with the selection for antiapoptosis that occurs during tumor progression (31), relating to K-ras activation and p53 and p16 inactivation in pancreatic carcinoma (2), since NP18 (Kras wt, p16 wt, p53 mut; ref. 32) dies by apoptosis, NP9 (Kras mut, p16 mut, p53 mut; ref. 32) is resistant to apoptosis, and the NP29 or NP31 cell lines (both Kras mut, p16 mut, p53 wt; ref. 32) die by apoptotic and nonapoptotic mechanisms.

The introduction of this type of compounds in the clinic for the purpose of treating pancreatic cancer could be feasible, especially for Bobel-24, because dosage regimens of this compound, achieving therapeutic concentrations, are well tolerated in humans. Thus, a phase I study conducted to develop the drug as antiinflammatory found no moderate or serious adverse events (only mild, transient, and subclinical hypothyroidism) in healthy volunteers when given at dose schedules yielding 250 to 300 μmol/L plasma concentrations (see ref. 33). These concentrations are twice to thrice higher than those needed to induce cell death in human pancreatic carcinoma cell lines (IC<sub>50</sub> range, 55–111 μmol/L; see Table 1). In summary, Bobel-24 and its derivatives induce mitochondrial and caspase-independent death, mediated by ROS, cathepsin B, PARP-1, PLA<sub>2</sub>, and AIF, in all tested human pancreatic cancer cell lines, irrespective of their degree of sensitivity to apoptosis. This represents a distinct cell death mechanism that has been proposed to overcome apoptosis resistance (12, 27), either intrinsic (2, 3) or acquired by genotoxic treatment (2, 5), which seems to associate with its high rate of K-ras mutations (34).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Bobel-24 and Derivatives Induce Caspase-Independent Death in Pancreatic Cancer Regardless of Apoptotic Resistance

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