

# Cyclophosphamide Induces Caspase 9-Dependent Apoptosis in 9L Tumor Cells

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Received April 4, 2001; accepted August 29, 2001

This paper is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

Cyclophosphamide (CPA), a widely used oxazaphosphorine anti-cancer prodrug, is inactive until it is metabolized by cytochrome P450 to yield phosphoramidate mustard and acrolein, which alkylate DNA and proteins, respectively. Tumor cells transduced with the human cytochrome P450 gene *CYP2B6* are greatly sensitized to CPA, however, the pathway of CPA-induced cell death is unknown. The present study investigates the cytotoxic events induced by CPA in 9L gliosarcoma cells retrovirally transduced with *CYP2B6*, or induced in wild-type 9L cells treated with mafosfamide (MFA) or 4-hydroperoxyifosfamide (4OOH-IFA), chemically activated forms of CPA and its isomer ifosfamide. CPA and MFA were both shown to effect tumor cell death by stimulating apoptosis, as evidenced by the induction of plasma membrane blebbing, DNA fragmentation, and cleavage of the caspase 3 and caspase 7 substrate poly(ADP-ribose) polymerase (PARP) in drug-treated cells. Caspase 9 was identified as the regulatory upstream caspase activated in 9L cells treated with CPA, MFA, or 4OOH-IFA, implicating the mitochondrial apoptotic pathway in oxazaphosphorine-induced tumor cell death. Correspondingly, expression of the

mitochondrial proapoptotic factor Bax enhanced caspase 9 activation, plasma membrane blebbing, and drug-induced cytotoxicity. Conversely, overexpression of the mitochondrial antiapoptotic factor Bcl-2 blocked caspase 9 activation, leading to an inhibition of drug-induced plasma membrane permeability and blebbing, terminal deoxynucleotidyl transferase dUTP nick-end labeling positivity, PARP cleavage, Annexin V positivity, and drug-induced cell death. Although Bcl-2 thus blocked the cytotoxic effects of activated CPA, it did not inhibit the drug's cytostatic effects. CPA induced S-phase cell cycle arrest followed by conversion to an apoptotic pre-G<sub>1</sub> state in wild-type 9L cells; by contrast, Bcl-2-expressing 9L cells accumulated in G<sub>2</sub>/M in response to CPA treatment. Intratumoral expression of Bcl-2 and related family members, including both apoptotic and antiapoptotic factors, is thus an important determinant of the responsiveness of tumor cells to CPA and ifosfamide, both in the context of conventional chemotherapy and in patients sensitized to these oxazaphosphorine drugs by the use of cytochrome P450-based gene therapy.

Cellular damage, including DNA damage induced by cancer chemotherapeutic drugs, induces programmed cell death, or apoptosis, via a complex cascade of events involving the activation of caspases, cysteine proteases which include both upstream (initiator) and downstream (effector) caspases (Nunez et al., 1998). Two major pathways of caspase-dependent apoptosis have been identified. One pathway is initiated by the formation of a death-inducing cell surface receptor signaling complex (Scaffidi et al., 1999), leading to aggregation and activation of caspase 8 via an adapter protein (Ashkenazi and Dixit, 1998; Green, 1998; Micheau et al., 1999). A second apoptotic pathway is triggered by cellular stress such as DNA damage (Green, 1998; Sun et al., 1999) and is primarily associated with the activation of caspase 9. In stress-induced cell death, signals received by mitochondria stimu-

late the release of a variety of proapoptotic molecules, including cytochrome *c* (Susin et al., 1999). Release of cytochrome *c* induces formation of the apoptosome, a multiprotein complex composed of APAF-1, caspase 9, cytochrome *c*, and ATP (Li et al., 1997; Yoshida et al., 1998; Chinnaiyan, 1999). This, in turn, leads to activation of caspase 9 via allosteric regulation by APAF-1 (Rodriguez and Lazebnik, 1999). Once activated, the initiator caspases, caspases 8 and 9, cleave and thereby activate downstream caspase family members, such as caspases 3 and 7 (Slee et al., 1999). These downstream effector caspases, in turn, cleave multiple cellular proteins, triggering a range of downstream apoptotic events.

Several cancer chemotherapeutic agents and other cytotoxic drugs, including staurosporine, etoposide, and betulinic acid (Fulda et al., 1998; Sun et al., 1999), kill tumor cells by activating the mitochondrial/caspase 9 pathway (Green,

Supported in part by National Institutes of Health Grant CA49248 (to D. J. W).

**ABBREVIATIONS:** CPA, cyclophosphamide; P450, cytochrome P450; MFA, mafosfamide; 4-OOH-IFA, 4-hydroperoxy-ifosfamide; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DTT, dithiothreitol; CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]propanesulfonate; PARP, poly(ADP-ribose) polymerase.

1998; Reed, 1999). However, other anticancer drugs, including doxorubicin (Fulda et al., 1998) and cisplatin (Seki et al., 2000), activate signaling via the cell surface and thereby activate caspase 8 as an initial apoptotic event (Micheau et al., 1999). In the case of cyclophosphamide (CPA), an anticancer alkylating agent prodrug that is metabolically activated in the liver by cytochrome P450 enzymes, the key regulators and mediators of cell death have not been identified. The activated CPA metabolites, phosphoramidate mustard and acrolein, are transported via the bloodstream to both tumor and healthy tissues, where DNA and protein damage can occur (Moore, 1991). Several studies suggest that P450-activated CPA induces apoptosis, as indicated by the ability of chemically activated derivatives of CPA to stimulate outer membrane blebbing and DNA fragmentation (Bullock et al., 1993; Hengstler et al., 1997; Story et al., 1999; Mirkes and Little, 2000). However, neither of these characteristics is sufficient to establish whether CPA induces apoptosis rather than necrosis, insofar as outer membrane blebbing alone is not sufficient evidence to establish an apoptotic pathway of cell death, and DNA fragmentation is neither necessary for nor dependent on apoptosis (Blagosklonny, 2000).

In vitro studies of the cytotoxic action of CPA have been complicated by the requirement of cytochrome P450 metabolism for formation of the active metabolites of CPA. This requirement for P450-based metabolism, coupled with the very low P450 enzyme level found in most tumor cell lines (Yu et al., 2001), has limited in vitro studies of this drug to chemically activated CPA derivatives, such as mafosfamide (MFA) (Moore, 1991). However, tumor cells treated in cell culture with MFA or other activated CPA derivatives with short half-lives may not serve as an ideal model for CPA-treated tumors. In vivo, CPA is primarily activated by cytochrome P450 in the liver, resulting in an ongoing production and slow accumulation in plasma of active metabolites (Moore, 1991; Hengstler et al., 1997). The present study addresses this issue using tumor cells transduced with CYP2B6, a liver P450 enzyme that activates CPA with high efficiency (Roy et al., 1999). Transduction of tumor cells with CYP2B6 greatly sensitizes the cells to the cytotoxic effects of CPA, both in cell culture and in vivo in solid tumor models, enabling CYP2B6 to be used in a prodrug activation, P450-based cancer gene therapy strategy (Jounaidi et al., 1998; Waxman et al., 1999). Using this approach, we compare the pathway of cell death induced by CPA when the drug is activated intracellularly by tumor cells transduced with P450 enzymes with the pathway of cell death in tumor cells exposed to a chemically activated CPA derivative, MFA, or to 4OOH-IFA, a chemically activated form of the CPA isomer, ifosfamide. An important goal of these studies is to establish whether CPA induces tumor cell death by apoptosis or by necrosis, and to determine the key regulatory steps involved. Characterization of the cytotoxic pathways activated by CPA may help identify tumors that have a greater sensitivity to CPA treatment, and may lead to development of novel treatment strategies to enhance tumor cell responsiveness to CPA-based therapies.

Our findings establish that CPA kills 9L tumor cells by inducing apoptosis; caspase 9 serves as the regulatory upstream caspase responsible for the initiation of CPA-mediated cell death. We provide evidence that Bcl-2 (Adams and

Cory, 1998; Reed, 1999), an antiapoptotic factor that prevents mitochondrial release of cytochrome *c* and thereby blocks caspase 9-dependent apoptotic events, can inhibit all of the cytotoxic effects of CPA but not its cytostatic activity. These findings highlight the importance of Bcl-2 in the responsiveness of tumor cells to CPA-based chemotherapy and suggest that Bcl-2 and related factors may play an important role in resistance of tumor cells to activated CPA.

## Materials and Methods

**Generation of Stable Cell Lines by Retroviral Transduction.** Cells were grown as monolayers at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO<sub>2</sub>. Human Bcl-2 cDNA was obtained from Dr. John C. Reed (Burnham Institute, La Jolla, CA) and subcloned from the plasmid pSKII/Bcl-2 $\alpha$  into the retroviral plasmid pBabe-puro, obtained from Dr. B. Spiegelman (Dana Farber Cancer Institute, Boston MA). Human Bax cDNA was obtained from Dr. Stanley Korsmeyer (Dana Farber Cancer Institute) and subcloned from pSFV-LTR-NEO/Bax- $\alpha$  into pBabe-puro. To generate retroviral particles, the packaging cell line Bosc 23 (Pear et al., 1993) was plated at  $2.6 \times 10^6$  cells/60-mm dish. Twenty-four hours later, the cells were transfected with empty pBabe-puro or pBabe-puro containing either the cloned Bax or Bcl-2 cDNA. Fresh medium was added to the cells after 24 h; 48 h later, the medium containing retroviral particles was collected, filtered in a 0.4- $\mu$ m filter to remove any floating Bosc 23 cells, and placed on 9L cells (Hecht et al., 2000). The 9L cells were incubated with retrovirus for 48 h then selected with 2  $\mu$ g/ml puromycin for 48 h. The resultant pools of transduced 9L cells (designated 9L/Bcl-2 and 9L/Bax cells) were shown to stably express Bcl-2 or Bax by Western blot (see Fig. 3). Individual clones showing a high-level expression of Bcl-2 or Bax were selected by dilution cloning. Briefly, pools of puromycin-resistant 9L cells were plated in 96-well dishes at calculated densities of one to three cells per well. Several days later, wells containing single colonies were identified by light microscopy and then grown in 35-mm dishes. Clones expressing high levels of Bax or Bcl-2 protein on Western blots were selected for further study. The 9L/P450 cells used in this study are 9L/2B6/Reductase cells (Jounaidi et al., 1998) and were generously provided by Dr. Youssef Jounaidi of our laboratory.

**Cytostatic Assay.** 9L/pBabe, 9L/Bax, and 9L/Bcl-2 cells were treated with 12, 24, or 50  $\mu$ M MFA for 72 h. MFA was obtained from Dr. Ulf Niemeyer (Department of Medicinal Chemistry, ASTA Media AG, Frankfurt am Main, Germany). Cells remaining on the plates at 0, 24, 48, and 72 h were washed twice with cold PBS and then stained for 5 min with crystal violet [1.25 g of crystal violet (C-3886; Sigma Chemical, St. Louis, MO) dissolved in a solution containing 50 ml of 37% formaldehyde (F-8775; Sigma) and 450 ml of methanol]. The stained cells were washed three times in tap water and the plates were allowed to dry. The stain was eluted from the cells with 70% ethanol and the absorbance was then read at 595 nm (Jounaidi et al., 1998). The staining intensity of each drug-treated sample ( $A_{595}$ ) was then graphed as a percentage of the staining intensity (i.e., number of cells) at the 0-h time point.

**Long Term Survival Assay.** 9L/pBabe and 9L/Bcl-2 cells were plated at  $6 \times 10^4$  cells per well in 12-well dishes. The cells were then treated with 12 or 25  $\mu$ M MFA beginning 24 h after plating, which is designated as day 0. Cells remaining on the plates on days 0, 3, 6, and 9 were washed twice with cold PBS and then stained with crystal violet as described above. The staining intensity of each drug-treated sample ( $A_{595}$ ) was graphed as a percentage of the staining intensity (i.e., number of cells) at the 0-h time point. Additionally, on day 6, the cells treated with 12  $\mu$ M MFA were replated and allowed to grow for an additional 6 days. The cells were then stained and colonies were counted.

**TUNEL Assay.** 9L/pBabe, 9L/Bax, 9L/Bcl-2, or 9L/P450 cells were plated on 22-mm<sup>2</sup> glass coverslips (48372-049; Corning Glass, Corning, NY) at  $2.5 \times 10^4$  cells per coverslip. Coverslips were flame sterilized and placed within 35-mm dishes. Cells ( $2.5 \times 10^4$ ) were suspended in 200  $\mu$ l of media and placed onto the coverslip. Cells were allowed to attach for 2 h and then the well was filled with media. Twenty four hours after plating, the cells were treated with 0, 12, or 25  $\mu$ M MFA for 48 h or with 1 mM CPA (Sigma Chemical) for 48 h. The cells were then fixed on ice in 4% methanol-free formaldehyde solution in PBS, pH 7.4, for 25 min. The slides were then washed twice with PBS and permeabilized by a 5-min immersion in PBS containing 0.2% Triton X-100 (w/v). Slides were washed twice more with PBS. To label the DNA fragments at the 3'-OH ends with fluorescein-12-dUTP, slides were incubated for 1 h at 37°C in a humidified chamber with terminal deoxynucleotide transferase, fluorescein-12-dUTP, and equilibration buffer (200 mM potassium cacodylate, pH 6.6, 25 mM Tris-HCl, pH 6.6, 0.2 mM DTT, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride) (Apoptosis Detection System; Promega, Madison WI). The reaction was terminated by incubation in 2 $\times$  standard saline citrate solution (Promega) and the slide was immersed in 1  $\mu$ g/ml propidium iodide and washed three times with deionized water. Slides were analyzed by confocal fluorescence microscopy using an Olympus BX-50 Confocal Laser-Scanning Microscope fitted with a green fluorescence filter (520 nm) to measure fluorescein fluorescence and a red filter (620 nm) to measure propidium iodide fluorescence.

**Western Blotting.** 9L cells treated with CPA or MFA were analyzed on SDS-polyacrylamide gels (8% gel to monitor cleavage of the caspase substrate PARP, 10% gel to analyze Bcl-2, and 12% gel for Bax analysis). Antibodies to PARP (SC-7150) and Bcl-2 (reactive with both human and rat Bcl-2; SC-492) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bax antibody (reactive with both human and rat Bax) was purchased from BD PharMingen (San Diego, CA). Proteins were transferred to nitrocellulose blots, which were blocked by incubation for 1 h in 1 $\times$  PBS containing 0.3% Tween 20, and 5% nonfat powdered milk, followed by a 5-min wash with 1 $\times$  PBS containing 0.05% Tween 20. The blots were then incubated for 1 h with primary antibody at a dilution of 1:2000 in 1 $\times$  PBS containing 0.05% Tween 20 and 5% nonfat powdered milk. The blots were washed for 5 min with 1 $\times$  PBS containing 0.05% Tween 20 then incubated for 1 h with a mouse anti-rabbit horseradish peroxidase-linked secondary antibody (1:5,000 dilution) in 1 $\times$  PBS containing 0.05% Tween 20 and 5% nonfat powdered milk. The blots were washed twice with PBS containing 0.3% Tween 20 and twice with PBS containing 0.05% Tween 20 (5 min/wash), followed by a 1-min development with enhanced chemiluminescence Western blotting detection reagent from Amersham Pharmacia Biotech and exposure to Kodak X-OMAT blue film (XB-1).

**Caspase 8 and 9 Activity Measurements.** 9L cells were treated with drug for the times indicated in each experiment. Floating and attached cells were collected, pooled, resuspended in lysis buffer (10 mM HEPES buffer, pH 7.4, containing 2 mM EDTA, 0.1% CHAPS detergent, 5 mM DTT, 350 ng/ml phenylmethylsulfonyl fluoride, 10 ng/ml pepstatin A, 10 ng/ml aprotinin, and 20 ng/ml leupeptin) and lysed by three freeze-thaw cycles (alternating between a dry ice isopropanol bath and a 37°C water bath). Lysates were spun in a bench top centrifuge at full speed for 15 min and the supernatant (cell extract) fraction transferred to a new tube. Cell extracts (20  $\mu$ l) were assayed for caspase 9, caspase 8, and caspase 3 activity by incubation at 37°C for either 1 h (caspase 3) or 3 h (caspase 9 and caspase 8) in 500  $\mu$ l of reaction buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) containing 50  $\mu$ M caspase form-selective substrate: Ac-LETD-AFC (Bio-Rad) for caspase 8; Ac-LEHD-AFC (Bio-Rad) for caspase 9; and Ac-DEVD-AMC for caspase 3 (Biomol Research Labs, Plymouth Meeting, PA) (Talanian et al., 1997; Thornberry et al., 1997). Background activity was determined for each sample as follows. Cell extracts were preincubated for 15 min at room temperature, with or without caspase form-selective

inhibitor: 1  $\mu$ M z-LETD-FMK for caspase 8, 1  $\mu$ M z-LEHD-FMK for caspase 9 (BioRad Labs), and 5  $\mu$ l of Casputin for caspase 3 (Biomol). Caspase activity measured in the absence of inhibitor was divided by the background caspase activity measured in the presence of inhibitor (i.e., fold-increase in caspase activity). A value of 1 was subtracted from each measured activity, such that a caspase activity of 0 corresponds to no increase in the specific caspase activity with drug treatment. Fluorescence of the caspase product (excitation at 395 nm and emission at 525 nm for AFC substrates, and excitation at 380 nm and emission at 460 nm for the AMC substrate) was measured using a Shimadzu model RF-1501 spectrofluorophotometer and the manufacturer's PC-1501 software package (Shimadzu, Kyoto, Japan). The caspase substrates and inhibitors used in this study are believed to be caspase-selective but may not be entirely caspase form-specific. Accordingly, other caspases may partially contribute to the caspase 8, 9, and 3 activities reported here.

**Annexin V Analysis.** 9L/pBabe and 9L/Bcl-2 cells were plated at  $5 \times 10^6$  cells/100-mm dish. Cells were then treated with 25  $\mu$ M MFA or 25  $\mu$ M 4OOH-IFA for 0, 24, or 48 h. 4-OOH-IFA was a kind gift from Dr. J. Pohl (ASTA Pharma, Bielefeld, Germany). Floating and attached cells were collected, pooled, washed twice with cold 1 $\times$  PBS and then resuspended at a concentration of  $1 \times 10^6$  cells/ml of 1 $\times$  binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>). Samples (100  $\mu$ l) were incubated with 10  $\mu$ l of propidium iodide (50  $\mu$ g/ml) and 5  $\mu$ l of Annexin V-FITC (BD PharMingen) for 15 min in the dark. The cells were then analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA).

**Cell Cycle Analysis.** 9L/pBabe and 9L/Bcl-2 cells were plated at  $5 \times 10^6$  cells/100-mm dish and 24 h later treated with 25  $\mu$ M MFA for either 0, 24, or 48 h. The floating and attached cells were collected, pooled, and washed with 1 $\times$  PBS and then fixed in 70% ethanol for at least 2 h. The ethanol was removed, the cells were washed with 1 $\times$  PBS and then resuspended in PBS containing 20  $\mu$ g/ml propidium iodide, 1% (v/v) Triton X-100 and 0.2 mg/ml RNase (Sigma; RNase was boiled for 5 min to inactivate any DNase contaminant). The content of propidium iodide per cell was determined using a FACSCalibur flow cytometer and Cell Quest software.

## Results

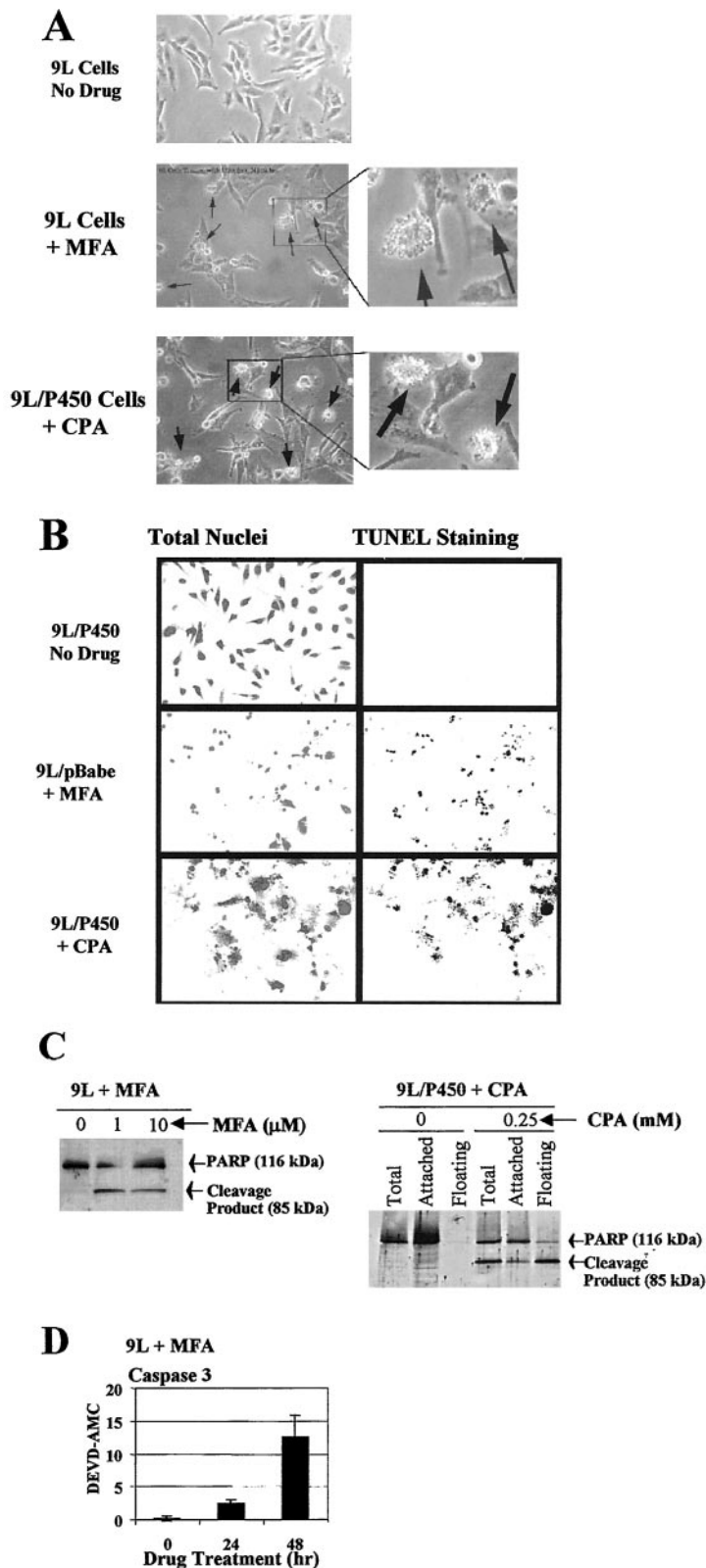
### CPA and MFA Induce Apoptosis of 9L Tumor Cells.

Wild-type 9L gliosarcoma cells, like most tumor cells, are deficient in P450 enzymes capable of converting the prodrug CPA into its active chemotherapeutic metabolite, phosphoramidate mustard, and thus are insensitive to this chemotherapeutic drug in culture. However, 9L cells transduced with the P450 gene *CYP2B6* activate CPA efficiently and are sensitive to CPA toxicity (Jounaidi et al., 1998). To model the pathway of tumor cell death stimulated by activated CPA produced extracellularly (i.e., in the liver), 9L cells were treated with MFA, a chemically activated form of CPA. To model the effects of intracellular CPA activation, such as occurs in the context of P450-based cancer gene-therapy (Waxman et al., 1999), 9L/P450 cells were treated with CPA. In both cases (i.e., treatment of 9L cells with MFA or treatment of 9L/P450 cells with CPA), drug exposure induced outer membrane blebbing, which was readily visualized by light microscopy (Fig. 1A). Drug treatment also led to DNA fragmentation, as revealed by TUNEL staining of free 3'-OH DNA ends (Fig. 1B). Drug treatment also induced cleavage of the caspase 3 and caspase 7 substrate PARP, visualized by Western blot analysis (Fig. 1C). Additionally, we have shown that MFA activates caspase 3, assayed using the fluorogenic caspase 3 substrate DEVD-AMC (Fig. 1D). These findings

indicate that CPA and MFA both activate caspase-dependent apoptotic pathways.

To ascertain which of the upstream (i.e., initiator) caspases is responsible for the activation of these downstream apoptotic events, the effects of drug treatment on caspase 8 and caspase 9 activities were analyzed. Extracts of the drug-

treated cells were assayed using the fluorometric caspase 8 substrate Ac-LETD-AFC and the caspase 9 substrate Ac-LEHD-AFC (Gurtu et al., 1997). Caspase 9 was activated to a greater extent than caspase 8, both in 9L cells treated with MFA (Fig. 2A) and in 9L/P450 cells treated with CPA (Fig. 2B). Moreover, activation of caspase 9 was typically detect-



**Fig. 1.** CPA and MFA induce plasma membrane blebbing (A), DNA fragmentation (B), PARP cleavage (C), and caspase 3 activation (D). 9L and 9L/P450 cells were treated with 50  $\mu$ M MFA or 0.5 mM CPA, respectively, for 48 h. The cells were photographed to visualize outer membrane blebbing, marked by arrows (A) or were assayed for DNA fragmentation using the TUNEL assay as described under *Materials and Methods* (B). Slides were analyzed using a confocal microscope using a fluorescein green fluorescence filter (520 nm) and propidium iodide red filter (620 nm). C, Western blots of extracts prepared from cells treated for 48 h with the indicated concentrations of MFA and CPA and probed with anti-PARP antibody. D, caspase 3 activity assayed in extracts of 9L cells treated with 25  $\mu$ M MFA for 0, 24, or 48 h. Caspase activity was determined by dividing the activity of the extract when assayed with substrate alone by the activity of the extract in the presence of the caspase 3-selective inhibitor Casputin then subtracting a value of 1 (see *Materials and Methods*).

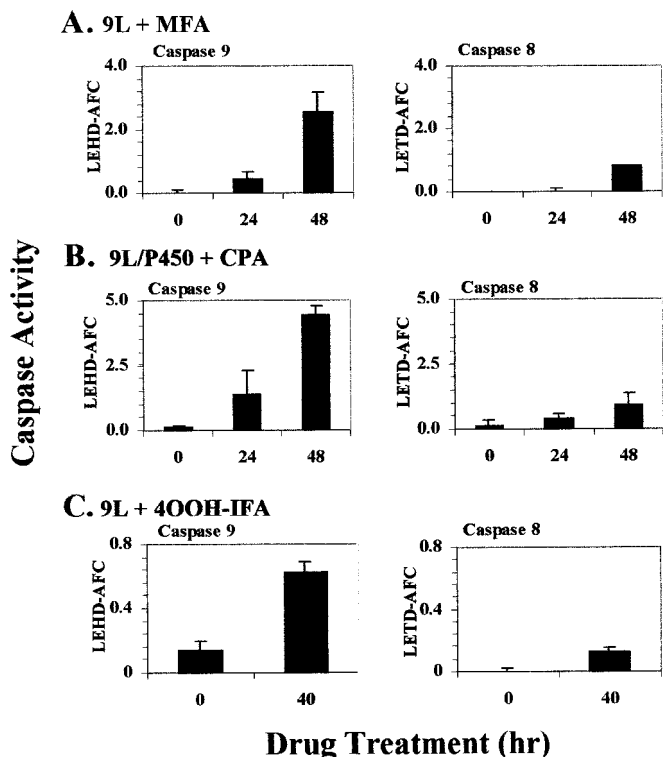
able before the onset of the activation of caspase 8 (see 24-h time points). A similar result was obtained in cells treated with 4OOH-IFA, a chemically activated form of ifosfamide (Fig. 2C). Ifosfamide is an isomer of CPA that is also activated by P450-catalyzed 4-hydroxylation but yields a chemically distinct DNA cross-link. Although 4OOH-IFA induced the same pattern of caspase activation, it activated caspase activity to a lesser extent than MFA. This reflects the lower intrinsic cytotoxicity of activated ifosfamide compared with activated CPA in 9L cells (Jounaidi et al., 1998).

**Bcl-2 Inhibits the Apoptotic Events Activated by MFA.** The antiapoptotic factor Bcl-2 and the Bcl-2-related proapoptotic factor Bax both localize at the mitochondrial membrane in apoptotic cells (Riparbelli et al., 1995; Wolter et al., 1997). Bcl-2 inhibits the mitochondrial transition and release of cytochrome *c* in cells treated with apoptotic stimuli, although Bax augments these events (Adams and Cory, 1998; Green, 1998; Green and Reed, 1998; Chinnaiyan, 1999). To further investigate the role of caspase 9 activation and associated mitochondrial events in MFA-induced cell death, 9L tumor cells that stably express either Bax or Bcl-2 were prepared by retroviral transduction (see *Materials and Methods*). Western blotting confirmed the expression of Bax and Bcl-2 in these cell lines compared with a control 9L cell line transduced with empty retroviral vector (9L/pBabe cells) (Fig. 3). 9L cells express a low level of endogenous rat Bax, which migrates faster than the exogenous human Bax transduced into the cells, whereas endogenous rat Bcl-2 expres-

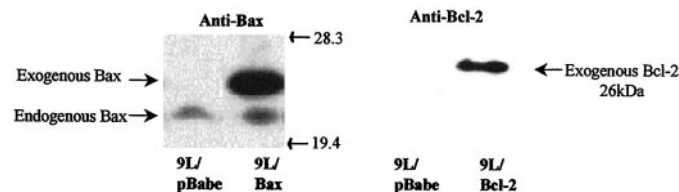
sion could not be detected (Fig. 3). Using these cell lines, we investigated whether the expression of Bax or Bcl-2 modulates plasma membrane blebbing induced by MFA. Figure 4 shows that MFA treatment for 48 h induces outer membrane blebbing of 9L/pBabe cells and that this effect is extensive at 25  $\mu$ M MFA. By contrast, 9L/Bax cells exhibited enhanced blebbing at 12  $\mu$ M MFA, whereas 9L/Bcl-2 cells were strikingly resistant to this morphological change, even at 25  $\mu$ M MFA.

These findings were confirmed and extended by monitoring the effects of Bax and Bcl-2 expression on MFA-induced DNA fragmentation, visualized by TUNEL assay. Figure 5A shows that 25  $\mu$ M MFA (48 h exposure) was required to induce significant TUNEL positivity in 9L/pBabe cells. By contrast, DNA fragmentation was strongly induced in 9L/Bax cells at 12  $\mu$ M MFA (Fig. 5B) and was totally suppressed in 9L/Bcl-2 cells, even at 25  $\mu$ M MFA (Fig. 5C). To visualize total nucleic acid content, cells were stained with propidium iodide (Fig. 5, lower sections). Bcl-2 does not typically alter the rates of drug uptake or efflux, drug metabolism, or the extent of drug-induced damage to macromolecules such as DNA (Fisher et al., 1993; Bullock et al., 1996). Consequently, the TUNEL staining activity inhibited by Bcl-2 is a reflection of apoptosis-induced DNA fragmentation rather than direct DNA alkylation induced by MFA.

An early event in many apoptotic pathways is the flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This redistribution of phosphatidylserine can be detected in live cells using the membrane-impermeable, phosphatidylserine-specific binding protein Annexin V (Fadok et al., 1992). Annexin V binding serves as a specific indicator of early-stage apoptosis in cells whose plasma membrane is still intact, as demonstrated by the exclusion of the nuclear DNA stain propidium iodide. In the late stages of cell death, however, the integrity of the plasma membrane becomes compromised during both apoptosis and necrosis, and cells can no longer exclude Annexin V or propidium iodide. Treatment of 9L/pBabe cells with 25  $\mu$ M MFA or 25  $\mu$ M 4OOH-IFA for 24 h induced 4.5- and 7-fold increases, respectively, in the number of cells actively undergoing early-stage apoptosis, identified as cells stained with Annexin V but not propidium iodide (Fig. 6, A and B, apoptotic cell fraction; dark bars). After 48 h, the total number of apoptotic and dead cells (both Annexin V-positive and propidium iodide-positive), increased to ~30% of the total cell population in both MFA- and 4OOH-IFA-treated 9L/pBabe cells. By contrast, in 9L/Bcl-2 cells, neither MFA nor 4OOH-IFA increased the



**Fig. 2.** Treatment of 9L or 9L/P450 cells with MFA (A), CPA (B), or 4OOH-IFA (C) activates caspase 9 earlier and to a greater extent than caspase 8. Caspase 8 and 9 activities were assayed in extracts of drug-treated cells using fluorometric caspase 8 or caspase 9 substrates in the presence or absence of their respective peptide inhibitors as described under *Materials and Methods*. Cells were incubated with 25  $\mu$ M MFA (9L), 1 mM CPA (9L/P450), or 25  $\mu$ M 4OOH-IFA (9L) for the times indicated.

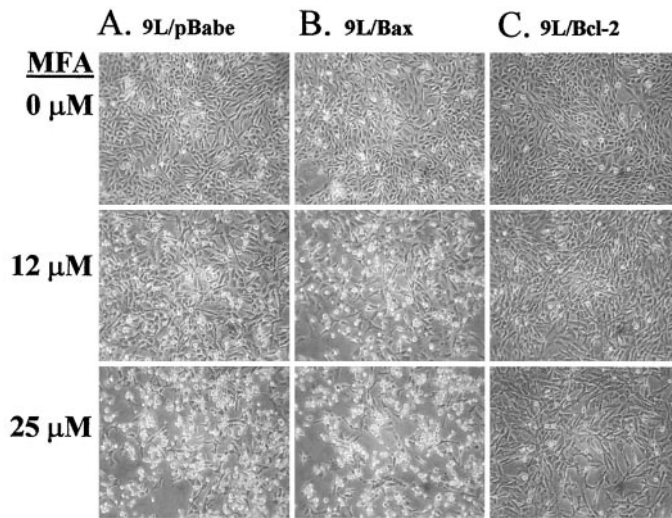


**Fig. 3.** Protein expression in 9L/Bax and 9L/Bcl-2 cell lines. 9L/Bax, 9L/Bcl-2, and 9L/pBabe (control) cell lines were prepared by retroviral transduction and characterized with regard to Bax and Bcl-2 expression by Western blotting. 9L cells express an endogenous rat Bax protein, which migrates faster than the retrovirally transduced human Bax protein. Human Bax (i.e., Bax- $\alpha$ ) migrates at 26 kDa, consistent with the migration of the exogenous human Bax protein shown here. Endogenous rat Bcl-2 expression was not detected in the 9L cells, despite the reported cross-reactivity of the anti-Bcl-2 antibody with rat Bcl-2.

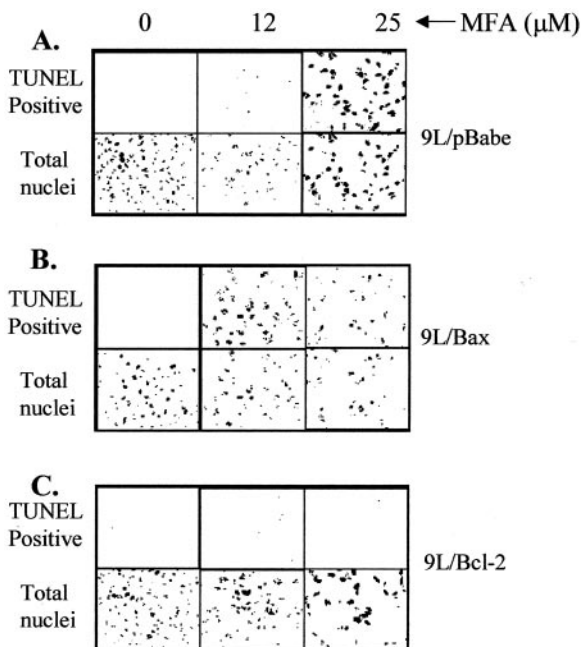
fraction of apoptotic cells; moreover, the total number of apoptotic + dead cells did not increase above the basal level of ~5%. This inhibition of propidium iodide staining by Bcl-2 indicates that Bcl-2 is also able to inhibit the outer membrane permeability induced by MFA and 4OOH-IFA. These results were confirmed by confocal microscopy visualization of the Annexin V- and propidium iodide-staining of MFA- and 4OOH-IFA-treated 9L/pBabe, 9L/Bax and 9L/Bcl-2 cells (data not shown). These findings lend strong support to our conclusion that MFA and 4OOH-IFA both induce apoptosis rather than necrosis in 9L tumor cells.

**Bcl-2 Expression Blocks Activation of Initiator Caspases 8 and 9 and Effector Caspase 3.** We next ex-

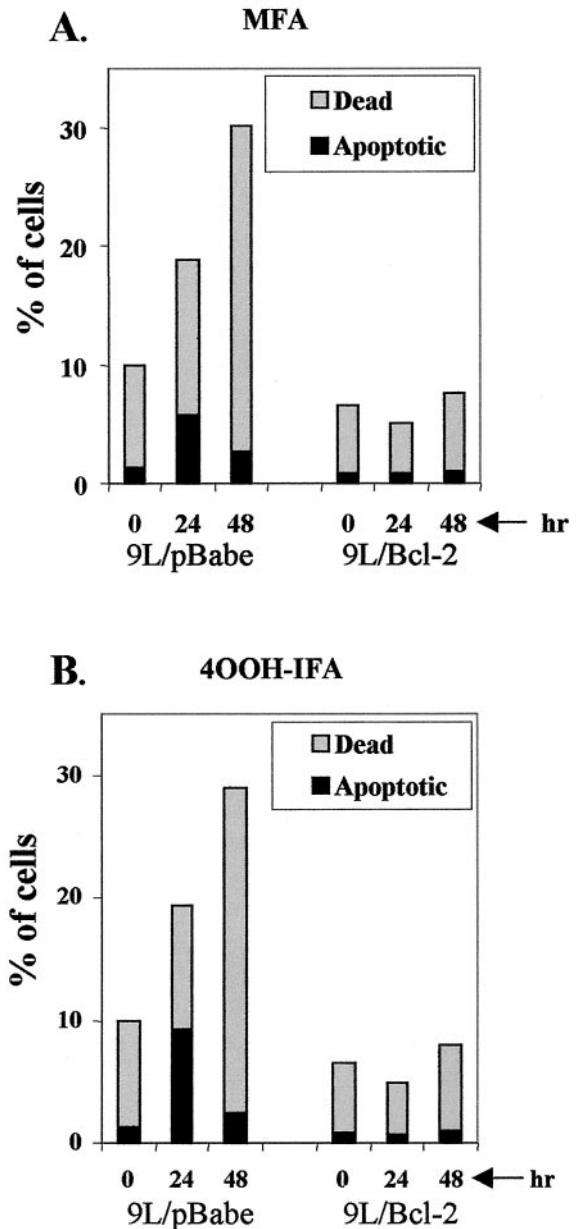
amined whether the expression of Bax or Bcl-2 alters the activation of the initiator caspases, caspases 8 or 9, after MFA treatment. In 9L/pBabe control cells, MFA activation of caspase 9, but not caspase 8, was detected 24 h after drug treatment (25  $\mu$ M). Both caspases were activated at 48 h, with caspase 9 activated to a greater extent than caspase 8 (Fig. 7A), in agreement with the data obtained earlier for 9L wild-type cells (Fig. 2). Bax expression enhanced the activation of caspase 9 at both time points and of caspase 8 at 48 h. Most strikingly, Bcl-2 expression fully blocked the activation of both initiator caspases as well as the effector caspase 3 in cells treated with activated CPA (Fig. 7A). Bcl-2 also blocked



**Fig. 4.** Bcl-2 inhibits while Bax enhances MFA-induced outer membrane blebbing. 9L/pBabe (A), 9L/Bax (B), and 9L/Bcl-2 (C) cells were treated with 0, 12, or 25  $\mu$ M MFA. After 48 h of drug treatment the cells were photographed to visualize outer membrane blebbing.



**Fig. 5.** Bcl-2 inhibits while Bax enhances MFA-induced DNA fragmentation. 9L/pBabe (A), 9L/Bax (B), and 9L/Bcl-2 (C) cells were treated with 0, 12 or 25  $\mu$ M MFA for 48 h. DNA fragmentation was visualized by TUNEL staining. Total nuclei shown for each sample were visualized by staining with propidium iodide.

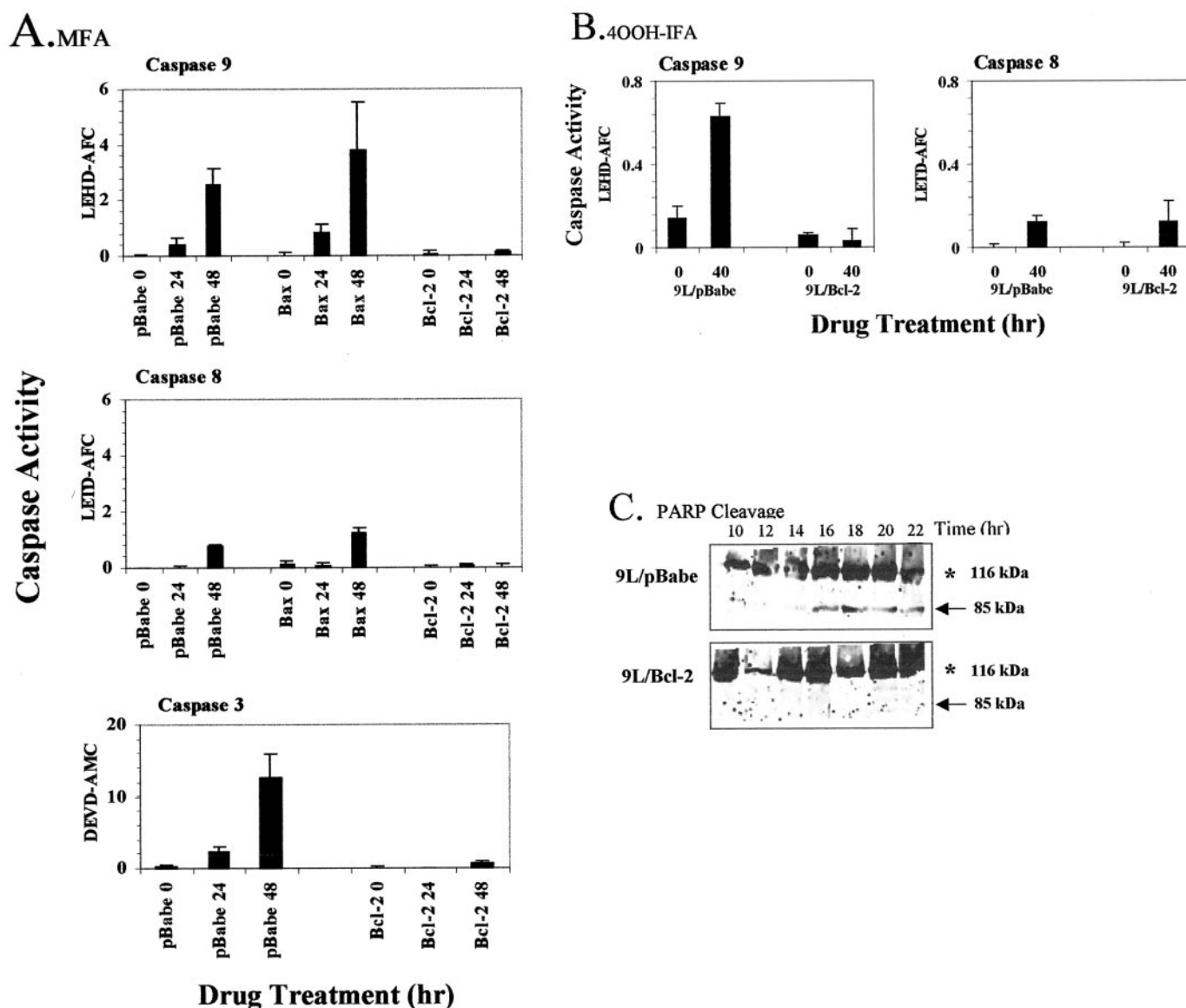


**Fig. 6.** Bcl-2 inhibits MFA-induced Annexin positivity and outer membrane permeability. 9L/pBabe and 9L/Bcl-2 cells were treated with 25  $\mu$ M MFA or 25  $\mu$ M 4OOH-IFA for 0, 24, or 48 h. The cells were then incubated with 5  $\mu$ l of Annexin-FITC and 5  $\mu$ g/ml propidium iodide. Live cells ( $1 \times 10^4$ ) were analyzed within 1 h of exposure to Annexin V by flow cytometry. Cells which were Annexin-positive only, were scored as apoptotic (dark bars), while those that were both Annexin- and propidium iodide-positive were counted as dead (light, upper portion of each bar).

caspase activation in cells treated with activated ifosfamide (Fig. 7B) and MFA-induced cleavage of the downstream caspase substrate PARP (Fig. 7C). Caspase inhibition was also observed when a pool of retrovirally transduced 9L/Bcl-2 cells was treated with MFA, demonstrating that our findings with 9L/Bcl-2 cells are not an artifact associated with selection of an apoptosis-resistant 9L clone (data not shown). Given the specificity of Bcl-2 for inhibition of the mitochondrial-dependent apoptotic pathway that leads directly to activation of caspase 9 (Green, 1998; Reed, 1998; Chinnaiyan, 1999), the block in caspase 8 activation seen in 9L/Bcl-2 cells suggests that the activation of caspase 8 is downstream of the activation of caspase 9.

**Bcl-2 Inhibits the Cytotoxic Effects but Not the Cytostatic Effects of Activated CPA.** Anticancer alkylating agents such as CPA can, in principle, exert cytostatic and/or cytotoxic effects on tumor cells. To evaluate the influences of

Bax and Bcl-2 on these cellular responses to activated CPA, the growth inhibitory effects of MFA were assayed. Initial experiments showed that MFA inhibited the growth of 9L/Bcl-2 cells somewhat less extensively than that of 9L/Bax cells or 9L/pBabe controls (e.g., 56% relative growth rate of 20  $\mu$ M MFA-treated 9L/Bcl-2 cells versus 18 and 30% relative growth rates of 9L/Bax and 9L/pBabe cells, respectively, with similar results obtained using 9L/Bcl-2 and 9L/Bax cell pools; data not shown). To distinguish the cytostatic effects and the cytotoxic effects of MFA, the effect of drug treatment on the increase in cell number was analyzed over time. In the absence of drug treatment, all three cell lines displayed similar growth rates (Fig. 8, first panel). Upon treatment with MFA, 9L/pBabe and 9L/Bax cells continued to grow for 24 h, at which time the cell number began to decrease, evidencing significant cell death by 48 and 72 h. Greater cytotoxicity was seen in the case of the Bax-expressing cells than in the



**Fig. 7.** Caspase activation in 9L cells expressing Bax and Bcl-2. Activation of caspases in 9L cells treated with 25  $\mu$ M MFA (A) or 25  $\mu$ M 4OOH-IFA (B) was indicated by Bax and inhibited by Bcl-2. Cells were treated with 25  $\mu$ M MFA for 0, 24, or 48 h, as indicated. Cell extracts were then assayed for the indicated caspase activities as described under *Materials and Methods*. PARP cleavage was detected on Western blots of 9L/pBabe cells treated with 50  $\mu$ M MFA for >16 h, whereas this cleavage was inhibited in the Bcl-2 expressing cell lines for at least 22 h (C). The full-length PARP protein band is indicated by an asterisk; the PARP cleavage produce is marked by an arrow.

9L/pBabe control cells; the difference was most prominent at 12  $\mu\text{M}$  MFA. In contrast, the Bcl-2-expressing 9L cells continued to grow over the 72-h time period, albeit at a substantially diminished rate compared with drug-free control cells (Fig. 8). These findings demonstrate that MFA exerts cytotoxic effects that are enhanced by Bax and are blocked by Bcl-2. Moreover, MFA exerts cytostatic effects that are manifested even in Bcl-2-expressing cells (Fig. 8).

To better understand the significance of the cytostatic effects of MFA seen in 9L/Bcl-2 cells, the effects of MFA were evaluated in long-term survival assays, extending over a 9-day period. Additionally, MFA-treated cells were trypsinized and replated 6 days after beginning drug treatment and allowed to grow for an additional three days to determine their ability to reattach to the plate, an indication of cell viability. The drug-treated cells were also assayed for their ability to form colonies. Over a 9-day growth period, 9L/pBabe cells markedly decreased in number at both 12 and 25  $\mu\text{M}$  MFA (Fig. 9A). In contrast, 9L/Bcl-2 cells treated with 12  $\mu\text{M}$  MFA continued to grow, albeit at a slower rate than drug-free control cells, whereas the cells were static at 25  $\mu\text{M}$  MFA, with no significant increase or decrease in cell number observed (Fig. 9A, bottom). Essentially all of the 9L/Bcl-2 cells (12  $\mu\text{M}$  MFA treatment) were still viable at 6 days, as judged by their cell reattachment capability (Fig. 9B; compare cell number 3 days after trypsin treatment and replating to cell number without replating). Efficient reattachment was also observed for the few remaining 9L/pBabe cells, suggesting that these latter cells might have stochastically escaped MFA-induced cell death.

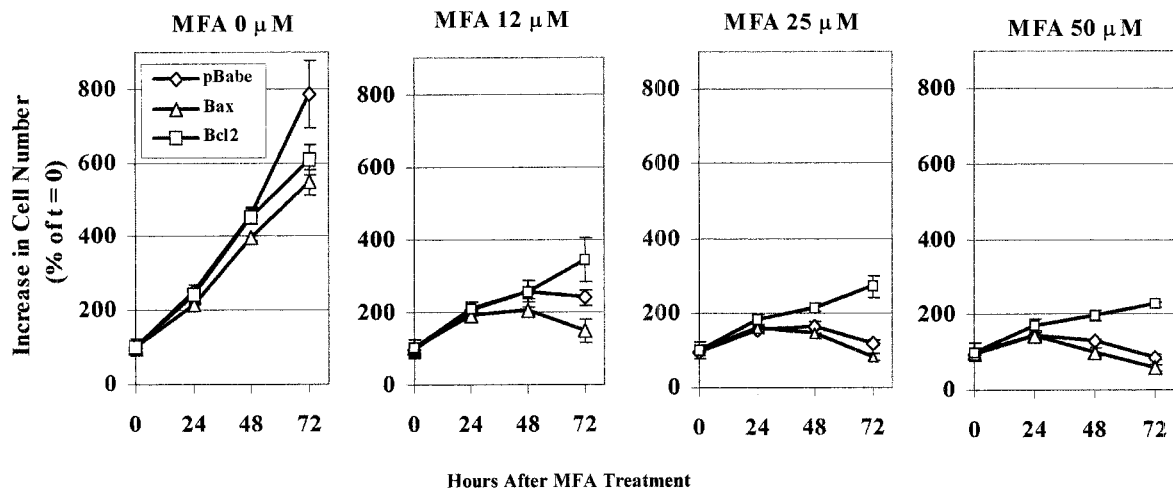
In studies comparing the ability of the 6-day MFA-treated cells to form colonies, Bcl-2 expression resulted in  $\sim 3$  times the number of colonies compared with the control cells (Fig. 9C), whereas the untreated cells showed no difference in colony formation between Bcl-2-expressing and nonexpressing cells (data not shown). Although the expression of Bcl-2 conferred sustained cell survival to a large number of cells (Fig. 9, A and B), the fraction of these cells able to form colonies was low. Thus, there was a 7-fold increase in the number of viable 9L/Bcl-2 cells compared with 9L/pBabe control cells (Fig. 9B) but only a 3-fold increase in the number

of 9L/Bcl-2 cells that were able to form colonies (Fig. 9C). The ultimate fate of the Bcl-2-expressing cells that are arrested by MFA treatment (i.e., cells able to reattach but unable to proliferate and form colonies) is not known. These cells may eventually die or they may somehow circumvent their arrest and begin to proliferate.

To further understand the cytotoxic and cytostatic effects of MFA, cell cycle analysis was carried out. Figure 10, left, shows that 9L/pBabe cells treated with MFA arrest in S-phase within the first 24 h of drug treatment. By 48 h, many of these cells have died and are visualized in the pre- $G_1$ , apoptotic peak. Bcl-2-expressing 9L cells also undergo S-phase arrest within the first 24 h but then migrate to the  $G_2/M$  phase of the cell cycle by 48 h, with few or no cells present in the pre- $G_1$  apoptotic peak.

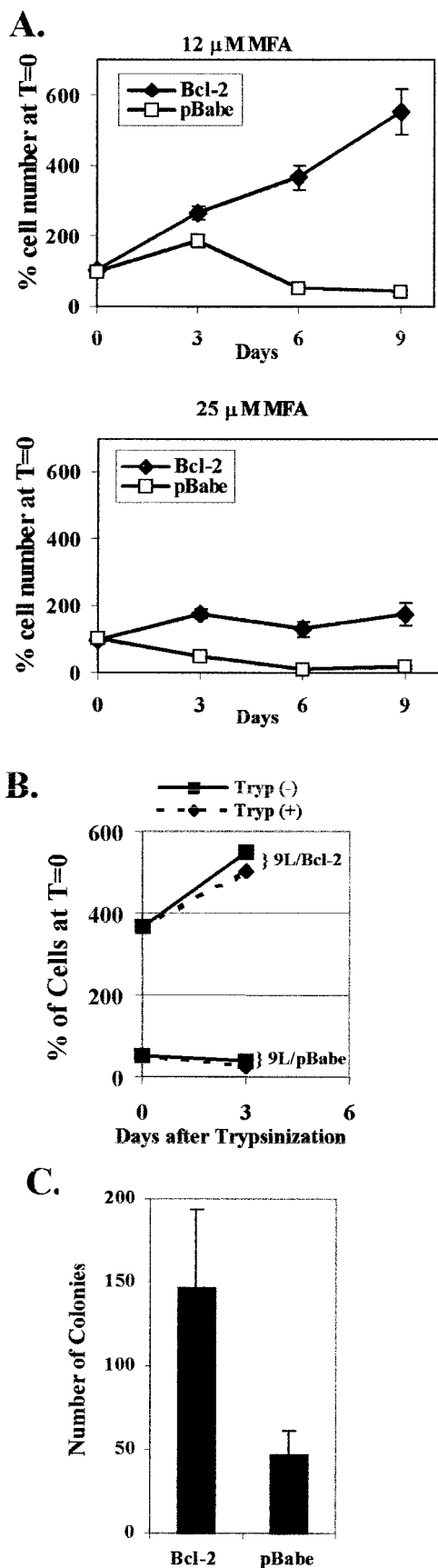
## Discussion

This study establishes that the anticancer prodrugs CPA and ifosfamide, acting via their activated 4-hydroxy metabolites, induce a mitochondrial, caspase 9-dependent apoptotic pathway in 9L gliosarcoma cells. The same apoptotic pathway of cell death was induced in 9L/P450 cells treated with the prodrug CPA as in 9L wild-type cells exposed extracellularly to chemically activated metabolites of CPA or ifosfamide. Thus, CPA-treated tumor cells that are subject to intratumoral, P450-dependent prodrug activation undergo the same caspase 9-dependent mechanism of cell death as do cells exposed to activated CPA metabolites generated extracellularly (e.g., in the liver). In addition, we have shown that Bcl-2 expression is an important determinant of the susceptibility of tumor cells to CPA-induced cytotoxicity. 9L cells that overexpress Bcl-2 do not undergo caspase 9 activation, do not exhibit downstream caspase 3 activity, and are resistant to cytotoxicity induced by treatment with MFA, a chemically activated form of CPA. Bcl-2 inhibition of caspase activation was also seen when the tumor cells were treated with chemically activated ifosfamide, 4OOH-IFA, demonstrating that CPA and ifosfamide, which form distinct DNA cross-links and strand lesions, both induce a mitochondrially regulated pathway of cell death. Bcl-2 was also able to inhibit



**Fig. 8.** Cytotoxic and cytostatic effects of MFA on 9L tumor cells. Shown are the effects of MFA on the growth of 9L/pBabe, 9L/Bax, and 9L/Bcl2 cells. Cells were treated with MFA at the concentrations indicated for 0, 24, 48, and 72 h. The increase in cell number is graphed as a percent of cells that were attached to the plate at the zero time point.





**Fig. 9.** Bcl-2 expression confers cell viability. A, 9L/pBabe and 9L/Bcl-2 cells were treated with either 12 or 25  $\mu$ M MFA and allowed to grow for 9 days. Data are graphed as a percentage of the cells originally plated on

both Annexin V and propidium iodide staining of intact cells in response to drug treatment, indicating that Bcl-2 blocks early apoptotic events, represented by Annexin V positive cells, as well as late-stage apoptotic events, represented by cells that additionally stain with propidium iodide. Together, these findings strongly support the proposal that the mitochondrial-dependent activation of caspase 9 is a key regulatory event in CPA- and ifosfamide-induced cell death in 9L tumor cells.

The maintenance of mitochondrial function and the inhibition of drug-induced caspase 9 activity in 9L tumor cells transduced with Bcl-2 severely inhibited the appearance of all downstream apoptotic responses, including PARP cleavage, DNA fragmentation, outer membrane blebbing, and cell detachment. Bcl-2 inhibition of caspase 9 activation is most likely caused by its ability to block mitochondrial release of cytochrome *c* (Adams and Cory, 1998; Green and Reed, 1998; Chinnaiyan, 1999). The finding that Bcl-2 expression can additionally inhibit the activation of caspase 8 (Fig. 7) raises the possibility that caspase 8 activation is downstream of and dependent on the initial activation of caspase 9. Caspase 9-dependent activation of caspase 8 has been reported to occur via the activation of caspase 3 in response to either cytochrome *c* release or chemical-induced apoptosis (Slee et al., 1999; Sun et al., 1999). In contrast, the activation of caspase 9 downstream of caspase 8 is typically seen in death receptor-mediated apoptosis (Scaffidi et al., 1999).

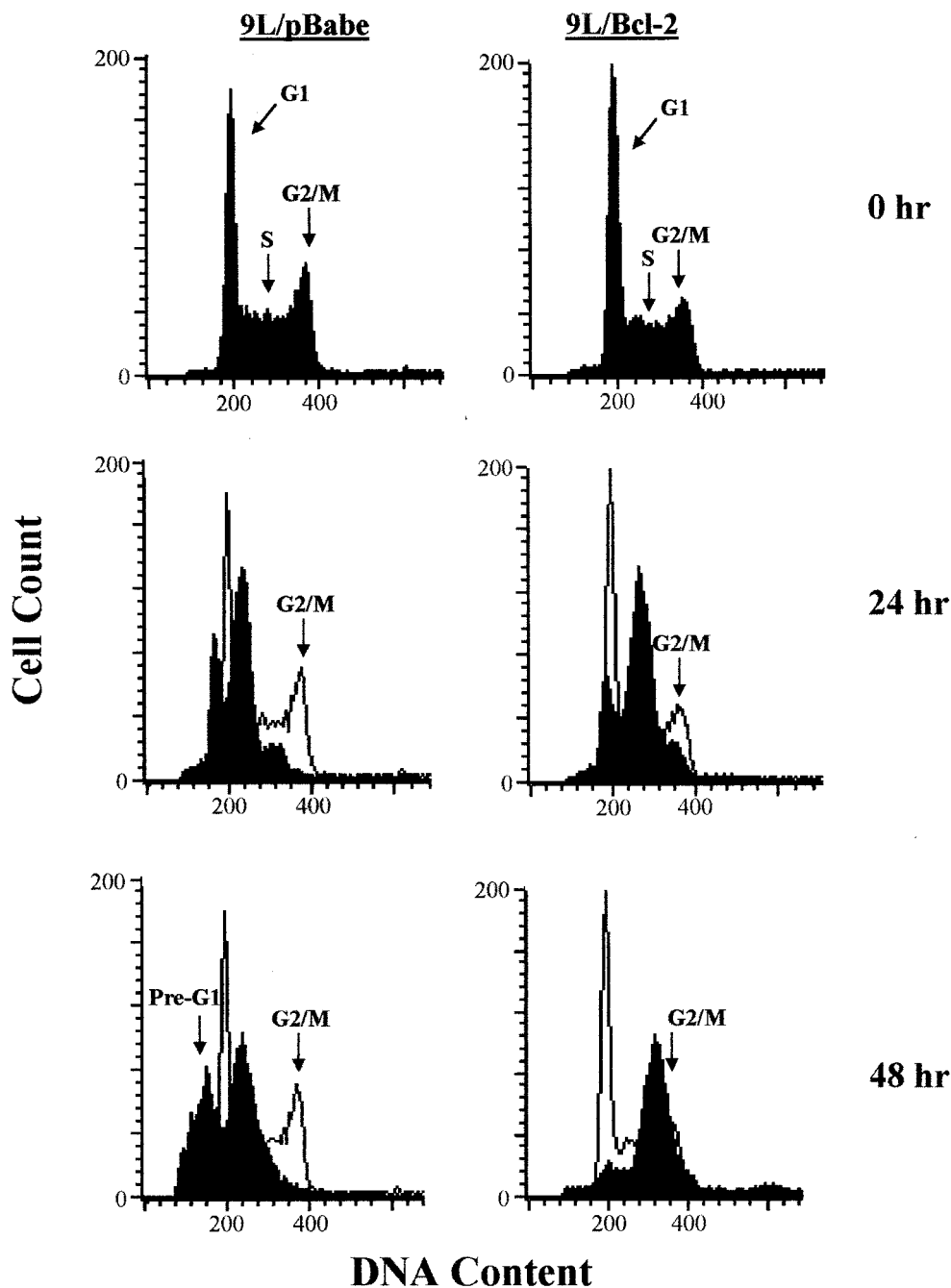
The present data demonstrate an important role for caspase 9 in the apoptotic cascade leading to CPA-induced cell death, suggesting that caspase 9 may be the initiating caspase. This finding is in agreement with the report that a caspase 8-specific peptide inhibitor blocks cisplatin-induced but not CPA-induced apoptosis in human osteosarcoma cells (Seki et al., 2000). Moreover, in teratogenesis studies of developing mouse embryos, activated CPA induced the release of cytochrome *c* from mitochondria followed by the activation of caspases 2 and 3 (Mirkes and Little, 2000), supporting the conclusion that the activation of these downstream caspases is dependent on the mitochondrial/caspase 9 pathway, and not on caspase 8-mediated cell death. It is nevertheless possible, however, that the initial caspase activation event does involve caspase 8, at least in certain tumor cells. Two classes of cells can be distinguished based upon their response to cell surface, death receptor-stimulated cell death (Scaffidi et al., 1998). In type I cells, binding of ligand to a death receptor induces sufficient caspase 8 activity to directly initiate the caspase cascade. In type II cells, however, the stimulation of caspase 8 activation by death receptor ligands is barely detectable and is insufficient to initiate the caspase cascade without the downstream activation of caspase 9. This downstream activation of caspase 9 and the ensuing mitochondrial apoptotic responses in death receptor ligand-treated type II cells can be blocked by Bcl-2 (Scaffidi et al., 1998, 1999). In the case of 9L tumor cells, apoptosis induced by TNF $\alpha$  under conditions of protein synthesis inhibition (2 ng/ml TNF $\alpha$  for 12 h in the presence of 10 ng/ml cycloheximide) is partially

day 0. Cells remaining after 6 days treatment with 12  $\mu$ M MFA were assayed for viability using a trypsinization and reattachment assay (B; see *Materials and Methods*) or for colony formation activity (C). Cells were trypsinized, replated, and allowed to grow for 6 days before they were stained and colonies larger than  $\sim$ 30 cells were counted.

blocked by Bcl-2 (our unpublished observations). Accordingly, 9L cells appear to be type II in nature, insofar as the expression of Bcl-2 moderated TNF $\alpha$ -mediated cell death (Scaffidi et al., 1998; Scaffidi et al., 1999). It is thus conceivable that the activation of caspase 9 by activated CPA seen in the present study occurs downstream of an earlier, low-level cell surface receptor/caspase 8 activation event. Regardless of whether caspase 8 or caspase 9 is the true initiating caspase, however, it is clear from the present study that the key regulatory caspase with respect to CPA induction of cell death is caspase 9.

Although activated CPA does not induce apoptosis in 9L tumor cells expressing Bcl-2, it does induce a cytostatic response. This cytostatic effect is evidenced by the diminished cell growth of 9L/Bcl-2 cells with retention of cell viability

after drug treatment. The ability of Bcl-2 and related anti-apoptotic factors to maintain CPA-treated tumor cells in a cytostatic state may be a feature that is characteristic of CPA treatment, as suggested by the prolonged cell survival and reduction in cell proliferation in preB leukemic cells expressing Bcl-2 (Miyashita and Reed, 1993) and by the maintenance of cell viability in neuroblastoma cells expressing Bcl-X1 (a Bcl-2-related antiapoptotic factor) when treated with activated CPA (Dole et al., 1995). However, when these latter cells were treated with the anticancer drug etoposide, Bcl-X1 conferred only a short-term delay in the onset of apoptosis with no long-term survival advantage (Dole et al., 1995). The combination of CPA-induced DNA damage with a Bcl-2-dependent cytostatic response may allow tumor cells to circumvent drug-induced cell death. If the drug-treated



**Fig. 10.** 9L cells undergo MFA induced S-phase arrest and cell death, whereas 9L/Bcl-2 cells undergo MFA-induced G<sub>2</sub>/M-phase cell cycle arrest. 9L/pBabe and 9L/Bcl-2 cells were treated with 25  $\mu$ M MFA for 0, 24, and 48 h. Cells were then fixed, stained with propidium iodide, and DNA content for each cell in the population was determined by flow cytometry. The phases of the cell cycle are labeled on the 9L/pBabe histogram at time 0. Histograms depicting the 24 and 48 h treatments (filled area) are overlaid on the respective histogram of the zero time point control for the corresponding cell line (9L/pBabe or 9L/Bcl-2) (open area). The pre-G<sub>1</sub> phase peak is labeled on the 9L/pBabe cells treated with drug for 48 h. Pre-G<sub>1</sub> cells contain a lower DNA content than cells in G<sub>1</sub>, and are considered to be dead cells.

tumor cells subsequently begin to divide (i.e., after repair of the CPA-induced lesion), this could lead to propagation of cells that would now be chemoresistant (Miyashita and Reed, 1993). Indeed, Bcl-2 expression resulted in a 7-fold increase in the number of viable 9L tumor cells present 6 days after MFA treatment and a 3-fold increase in colony-forming activity (Fig. 9). These findings demonstrate that Bcl-2 overexpression increases the viability of 9L tumor cells damaged by treatment with activated CPA, and that some of these cells are able to circumvent the DNA damage that they sustain and are able to proliferate.

Investigations of a possible correlation between Bcl-2 expression in tumors and patient prognosis have shown a coincidence between elevated Bcl-2 levels and a chemoresistant phenotype in neuroblastoma (Castle et al., 1993), prostate cancer (McDonnell et al., 1992), non-Hodgkin's lymphoma and various leukemias (Campos et al., 1993; Strasser et al., 1997). Murine lymphoma cells engineered to express Bcl-2 become resistant to chemotherapy-induced apoptosis and display increased clonogenic survival after exposure to various anticancer drugs *in vitro*. Of all the drugs tested, however, CPA was the only one for which Bcl-2 expression inhibited drug-induced tumor growth delay *in vivo*. Interestingly, for most of the other drugs examined, Bcl-2 expression resulted in an increase in tumor sensitivity to drug treatment (Story et al., 1999). The seeming contradiction, that Bcl-2 can inhibit chemotherapy-induced apoptosis but cannot inhibit tumor growth delay for the majority of the drugs studied, may be due to the cytostatic effects of Bcl-2 (Fisher et al., 1993; Kamesaki et al., 1993) coupled with the possibility that each drug induces a distinct pathway of cell death. Accordingly, Bcl-2 may be unable to confer tumor growth delay for those drugs in which the mode of action eventually circumvents Bcl-2 protection, enabling the cells to go through a prolonged cell death process (Reed, 1999). The present finding that CPA-induced cytotoxicity can be fully blocked by Bcl-2 supports our proposal that Bcl-2 expression and its ability to block caspase 9 activation may be a significant indicator of CPA chemoresistance and suggests that this resistance may be more characteristic of CPA than other anticancer drugs.

Cell cycle analysis revealed that activated CPA induced S-phase arrest in 9L tumor cells. This arrest is most likely caused by DNA damage induced by the active metabolite phosphoramidate mustard. Activated CPA induces DNA crosslinks and strand lesions in Chinese hamster lung fibroblasts; however, the DNA crosslinks are transient and are converted to alkali labile apurinic sites (Hengstler et al., 1997). Interestingly, when Chinese hamster lung fibroblasts are treated with ionizing radiation or CPA, both of which induce DNA strand lesions, the cells repair only the radiation-induced lesions, indicating that these two types of DNA damage are different from each other and probably undergo repair by different repair mechanisms. Consistent with our findings in 9L cells, MFA-induced S-phase arrest in leukemic cells occurs progressively earlier in S-phase with higher doses of MFA (Davidoff and Mendelow, 1993). This correlation between S-phase progression and drug concentration suggests that the extent of DNA damage directly impacts the cell's ability to advance through S-phase. Our study further shows that MFA-treated tumor cells do not fully progress through S-phase; rather, they arrest halfway through S-phase before undergoing apoptosis. This suggests that there

is a threshold level of DNA damage that needs to be reached before the cells commit to apoptosis. It is not entirely surprising that Bcl-2 cells, which arrest in S-phase, progress to G<sub>2</sub>/M with cross-links present in their DNA. Eukaryotic chromosomes have multiple origins of replication, each of which can initiate DNA replication independently, thereby providing the possibility of DNA replication, even in the presence of a drug-induced cross-link. It seems likely that when DNA damage is induced by activated CPA in Bcl-2-deficient 9L cells, the damage signals the cell death pathway before the completion of DNA replication, whereas in 9L/Bcl-2 cells the presence of Bcl-2 allows for sustained cell viability and continued DNA replication.

Cytochrome P450-based anticancer gene therapy confers major chemosensitivity to CPA and substantially enhances tumor growth delay in several *in vivo* tumor models, including 9L gliosarcoma cells (Waxman et al., 1999). This chemotherapeutic strategy can lead to nearly complete regression of large solid tumors grown subcutaneously in severe combined immune deficiency mice when combined with an antiangiogenic schedule of CPA administration (Jounaidi and Waxman, 2001). The present finding that 9L tumor cells have little or no endogenous Bcl-2 protein suggests that this factor may contribute to their responsiveness to CPA and to CPA/P450-based gene therapy. MCF-7 breast carcinoma cells, which do express Bcl-2 (Piche et al., 1998) are, however, also strongly sensitized to CPA by expression of a CPA-activated P450 gene (Chen and Waxman, 1995), indicating that the absence of Bcl-2 is not a prerequisite for effective prodrug activation-based P450 gene therapy. A further increase in chemosensitivity may, however, be achieved in the case of Bcl-2-expressing tumors using antisense oligonucleotide targeting Bcl-2, which effectively reduces the viability of leukemia cells and small-cell lung cancer cells even in the absence of drug treatment (Reed et al., 1990; Ziegler et al., 1997). In addition, intracellular expression of anti-Bcl-2 antibodies, although having no effect on the growth rate of MCF-7 breast cancer cells, enhances drug-induced cytotoxicity (Piche et al., 1998). Accordingly, investigation of the utility of these or other Bcl-2 targeting strategies in combination with CPA/P450-based gene therapy may be warranted.

Finally, it may be advantageous to investigate drug treatment combinations that enhance the chemosensitivity of tumor cells to CPA via effects on the expression of Bcl-2 or Bcl-2 family members. For example, breast cancer cells pretreated with low doses of etoposide respond by increasing their expression of Bax. Subsequent treatment of these cells with activated CPA results in a marked decrease in cell viability (Gibson et al., 1999). Human myeloid leukemia HL60 cells treated with activated CPA exhibit a decrease in Bcl-2 expression (Bullock et al., 1993), and this down-regulation of Bcl-2 levels could be an important mechanism for CPA chemosensitization. Given the effectiveness of CPA-dependent cytochrome P450 gene therapy seen in preclinical studies and the apparent specific inhibitory action of Bcl-2 on CPA- and ifosfamide-induced apoptosis, modulators or other treatments that decrease Bcl-2 protection may serve as useful adjuncts for cancer gene therapeutic strategies using oxazaphosphorine anti-cancer drugs.

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