0026-895X/02/6101-142–149$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
*Mol Pharmacol* 61:142–149, 2002

Vol. 61, No. 1
828/951372
*Printed in U.S.A.*



# Role of Caspase 3-Dependent Bcl-2 Cleavage in Potentiation of Apoptosis by Bcl-2

YE LIANG, KAREN D. NYLANDER, CHAOHUA YAN, and NINA FELICE SCHOR

*The Pediatric Center for Neuroscience, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania*

Received January 19, 2001; accepted October 1, 2001

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

## ABSTRACT

Previous studies from our laboratory have demonstrated that Bcl-2 has a proapoptotic effect on neocarzinostatin (NCS)-treated PC12 pheochromocytoma cells. In the present study, we examine the mechanisms of this effect and demonstrate its relevance for the in vivo situation. Four hours after NCS treatment, a 23-kDa cleavage product of Bcl-2 was detected in whole cell lysates of *bcl-2*–transfected PC12 cells. In contrast, *bcl-2* transfection protected PC12 cells from cisplatin-induced apoptosis, and cisplatin treatment did not result in Bcl-2 cleavage. Similarly, Bcl-2 cleavage did not occur and Bcl-2-mediated protection from, rather than potentiation of apoptosis was observed after NCS treatment of MCF-7 breast cancer cells. The caspase 3-specific inhibitor Ac-DEVD-CHO prevented Bcl-2 cleavage and attenuated NCS-induced apoptosis in *bcl-2*–transfected PC12 cells, whereas it had no effect on NCS-induced apoptosis in mock-transfected PC12 cells. Furthermore, MCF-7 cells do not express caspase 3, a finding in concert with the lack of Bcl-2 cleavage in this line. In in vivo experiments, xenografts of *bcl-2*–transfected PC12 cells were more susceptible to NCS toxicity than were xenografts of mock-transfected PC12 cells. Caspase 3-mediated Bcl-2 cleavage therefore plays an important role in the potentiation by Bcl-2 of NCS-induced apoptosis.

In the case of most chemotherapeutic agents, overexpression of *bcl-2* leads to abrogation of apoptosis induction (Kamesaki et al., 1993; Miyashita and Reed, 1993; Dole et al., 1994; Teixeira et al., 1995). However, in the case of treatment of PC12 pheochromocytoma cells with reduction-dependent chemotherapeutic prodrugs, overexpression of *bcl-2* potentiates apoptosis induction (Cortazzo and Schor, 1996; Tyurina et al., 1997; Schor et al., 1999a,b). It was originally hypothesized that this potentiation results from a *bcl-2*-induced shift in the redox potential of the cell with increased functional thiol reserves and consequent potentiation of activation of these reduction-dependent prodrugs (Cortazzo and Schor, 1996; Schor et al., 1999a). However, if overexpression of *bcl-2* only increased the activation of the apoptosis-inciting drug, its protein product, Bcl-2, would be expected nonetheless to block apoptosis downstream of the action of this drug, presumably at the level of inhibition of cytochrome *c* release from mitochondria (Kluck et al., 1997; Yang et al., 1997). That this is not the case is demonstrated by potentiation by Bcl-2 of reduction-dependent chemotherapeutic agent-induced oxidation and externalization of membrane phosphatidylserine (Schor et al., 1999a), a late event in the apoptosis final common pathway. Furthermore, Bcl-2 prevents, rather than potentiates, apoptosis induced in MCF-7 breast cancer cells by reduction-dependent prodrugs (Schor et al., 2000). Clearly, the distal antiapoptotic effects of Bcl-2 have been thwarted in the PC12/reduction-activated prodrug system; indeed, a proapoptotic effect seems likely.

Bcl-2 is one of a family of proteins, some of which, unlike Bcl-2, are proapoptotic (for reviews, see Reed, 1994; Brady and Gil-Gomez, 1998). Previous studies have demonstrated that Bcl-2 itself can be cleaved by caspase 3 to a proapoptotic Bcl-2 fragment (Cheng et al., 1997). We have therefore examined the role of cleavage of Bcl-2 in Bcl-2-mediated potentiation of apoptosis.

## Materials and Methods

**Cells and Cell Culture.** Polyclonal mock-transfected (pBabe-puro) PC12 rat pheochromocytoma cells and PC12 cells transfected with the human *bcl-2* gene ligated into the retroviral vector pBabe, containing a puromycin resistance gene (*bcl-2*-pBabe-puro), were provided by Dale E. Bredesen (La Jolla Cancer Institute, La Jolla, CA). The ratio of Bcl-2 content of the *bcl-2* transfectants relative to mock-transfected PC12 cells is 100:1 (Kane et al., 1993). Cells were maintained as adherent monolayers in Dulbecco's modified Eagle's medium made 10% in horse serum, 5% in fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1.1% in penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were fed every 3 to 4 days; biweekly,

This work was funded by National Cancer Institute grant R01-CA74289 and the Carol Ann Craumer Endowment Fund of Children's Hospital of Pittsburgh.

**ABBREVIATIONS:** NCS, neocarzinostatin; PBS, phosphate-buffered saline; PI, propidium iodide; FACS, fluorescence-activated cell sorting; 7-AAD, 7-amino-actinomycin.

1 μg/ml puromycin was added to the medium. Cells were examined for Bcl-2 expression by Western blotting every 10 passages. Bcl-2 expression did not vary in either cell line over the course of these studies.

Mock- and *bcl-2* gene-transfected MCF-7 human breast cancer cells were generated by electroporation (300 mV; 960 mF) with pSFFV-neo and pSFFV-*bcl-2* (plasmids described in Boise et al., 1993), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis with N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Two mock- (Neo.1 and Neo. 2) and two *bcl-2*–transfected (Bcl-2.1 and Bcl-2.3) clones of MCF-7 cells were used for these studies (Schor et al., 2000). The MCF-7 cells were maintained as adherent monolayers in 75-$mm^2$ culture flasks (Invitrogen), and fed twice weekly with α-minimal essential medium (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum, 0.3% glucose, 2 mM L-glutamine (Invitrogen), and 2 μg/ml gentamicin sulfate (Biofluids, Rockville, MD).

**Neocarzinostatin and Cisplatin Treatment.** Mock- and *bcl-2*–transfected PC12 and MCF-7 cells were treated with either 0.02 μM neocarzinostatin (NCS) or 10 μM cisplatin for 1 h. Subsequently, the NCS or cisplatin was washed out and fresh medium was added. Cells were then incubated for varying lengths of time as indicated. For studies of the effects of the caspase 3 inhibitor Ac-DEVD-CHO on mock- and *bcl-2*–transfected PC12 and MCF-7 cells, 10 μM Ac-DEVD-CHO (BD PharMingen International, San Diego, CA) was added to the cells 2 h before NCS or cisplatin treatment, and maintained in the medium thereafter.

**Western Blotting Analysis of Bcl-2 and Caspase 3 proteins.** At the indicated time points, PC12 and MCF-7 cells were lysed in radioimmunoprecipitation assay buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 1 mM sodium orthovanadate). Subsequently, the protein concentrations of the lysates were estimated using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard. An aliquot of each lysate containing 500 μg of protein was loaded onto each lane and electrophoresed on a 15% SDS-polyacrylamide gel, followed by blotting on a nitrocellulose membrane (Bio-Rad). After blotting, nonspecific binding was blocked with 5% nonfat dry milk in PBS and the membrane was incubated with either anti-Bcl-2 (1:500, reactive with mouse, rat, and human Bcl-2; Santa Cruz Biotechnology) or anti-caspase 3 (1:1000; BD PharMingen) antibodies diluted in 5% nonfat dry milk in PBS at 20°C for 2 h, washed, and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Santa Cruz Biotechnology) for 1 h. The membrane was finally washed and developed with Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology) following the manufacturer's instructions. The known molecular masses of caspase 3 and Bcl-2 and its cleavage products were confirmed by coelectrophoresis of prestained molecular mass standards (Bio-Rad) on each gel.

**Determination of Adherent Cell Number.** Adherent cell number was determined in control and treated cultures, as we have described previously for neuroblastoma cells (Schor, 1992; Hartsell et al., 1995, 1996; Cortazzo and Schor, 1996). Briefly, adherent cells were manually counted in each of three high-power fields from each cell culture well at each time point. Results are expressed as the mean ± S.E.M. of the three determinations. In the case of Ac-DEVD-CHO caspase 3 inhibitor treatment, 10 μM inhibitor was added 2 h before NCS treatment. The statistical significance of differences between control and treated cultures was assessed in all cases using Student's *t* test; $P \leq 0.05$ was considered significant.

**DNA Staining with Propidium Iodide.** Monolayers of mock- and *bcl-2*–transfected PC12 cells grown on coverslips were treated with either 20 nM NCS or 10 μM cisplatin for 1 h. The NCS or cisplatin was then washed away and fresh medium was added to the cells. Twenty-four hours later, the cells were washed with PBS and fixed with cold ethanol (95%) for 5 min. After washing with PBS, the cells were treated with RNase (1 mg/ml) for 1 h at 37°C. The cells were then stained with PI (20 ng/ml) for 5 min and the coverslips were mounted on slides with Gelvatol, a solution of polyvinyl alcohol (23%; Sigma Chemical, St. Louis, MO) and glycerol [50% (v/v)] in phosphate-buffered saline. A Zeiss light microscope equipped for epifluorescent illumination was used for all observations.

**Flow Cytometric Analysis of Control and NCS-Treated Cells.** Apoptotic cells were quantified by flow cytometry considering 7-amino-actinomycin D (7-AAD) staining intensity to be proportional to the DNA content (Lecoeur and Gougeon, 1996). Saponin and 7-AAD were purchased from Sigma Chemical. In short, after harvesting, the cells were washed once in PBS and once in PBS/0.05% saponin, followed by addition of 4 μg of 7-AAD in 1 ml of PBS/saponin to the samples. The cells were incubated at room temperature in the dark for 30 min, and DNA histograms were obtained using a CellQuest apparatus and CellQuest software (BD Biosciences, San Jose, CA). Data on $10^4$ cells were collected. Electronic gates were set for viable and apoptotic cells with 2N-4N DNA and subnormal DNA contents, respectively, and for exclusion of debris. Percentage of apoptosis was calculated as (number of apoptotic cells/number of total cells) × 100.

**Caspase 3 Colorimetric Protease Assay.** At different time points, both mock- and *bcl-2*–transfected PC12 cells were harvested and assayed for caspase 3 activity according to the manufacturer's instructions (Medical and Biological Laboratory Co. Ltd., Naka-ku, Japan). Briefly, 50 μg of protein in cell lysate was used for the analysis. The volume of cell lysate was adjusted to 50 μl with lysis buffer, and 50 μl of 2× reaction buffer was added to each sample. Five microliters of 4 mM DEVD-pNA substrate was added, and the samples were incubated at 37°C for 1 h. The $A_{405\ nm}$ was read in a microtiter plate reader. Fold-increase in caspase 3 activity over control samples was determined by comparing these results with the $A_{405\ nm}$ of a simultaneously incubated control (i.e., vehicle- rather than NCS-treated) sample. The background reading from cell lysates and buffers was subtracted from the readings of both NCS-treated and control samples before calculating increase in caspase 3 activity.

**In Vivo Studies of Effects of NCS, Cisplatin, or Vincristine on Tumor Growth from Mock- and *bcl-2*–Transfected PC12 Cells.** Experiments involving tumor growth were performed on 5- to 7-week-old male NIH athymic mice injected subcutaneously with $10^6$ mock- or *bcl-2*–transfected PC12 cells into the left flank on day 0 of each study. NCS (0–5 mg/kg), cisplatin (0–25 mg/kg), or vincristine (0–1.25 mg/kg) was administered intraperitoneally on day 1 ($n = 4$ mice/dose). Mice were examined daily for grossly visible tumor and, once tumors appeared, they were assessed by measurement of the largest and smallest diameter of each tumor at multiple time points during the month after implantation. Tumor volume was calculated as the product of the largest diameter and the square of the smallest diameter. Mean tumor volume for the four mice in each group was calculated for each day of measurement assuming a tumor volume of zero for those mice in which a tumor was not palpable. Statistical significance of differences in mean tumor volume was determined for each day's measurements with Student's *t* test.

## Results

**NCS and Cisplatin Induce Apoptosis in PC12 and MCF-7 Cells.** Our previous studies have demonstrated condensation, fragmentation, and margination of the nuclei (Cortazzo and Schor, 1996) and selective oxidation and externalization of membrane phosphatidylserine (Schor et al., 1999a) of mock- and *bcl-2*–transfected PC12 cells treated with NCS. We have further demonstrated a decrement in cell culture size in mock- and *bcl-2*–transfected MCF-7 cells treated with NCS (Schor et al., 2000). Sister cultures of mock- and *bcl-2*–transfected PC12 cells treated with NCS (0.020 μM) or cisplatin (10 μM) demonstrate qualitatively

similar morphological and nuclear fragmentation features (Fig. 1, A–F). In both PC12 cells and MCF-7 cells that are either mock- or *bcl-2*–transfected and treated with NCS, FACS analysis shows a DNA size distribution consistent with apoptosis [Figs. 1, H, K, and M; 2, a to d; and 6D (vehicle versus NCS alone, PC12, and MCF-7 cells, respectively)].

**Although Bcl-2 Increases Sensitivity to NCS of PC12 Cells, It Decreases Sensitivity to NCS of MCF-7 Cells. Bcl-2 also Decreases Sensitivity to Cisplatin of PC12 Cells.** Previous studies from our laboratory have demonstrated Bcl-2-mediated potentiation of apoptosis and decrease in cell culture growth in PC12 cells (Cortazzo and

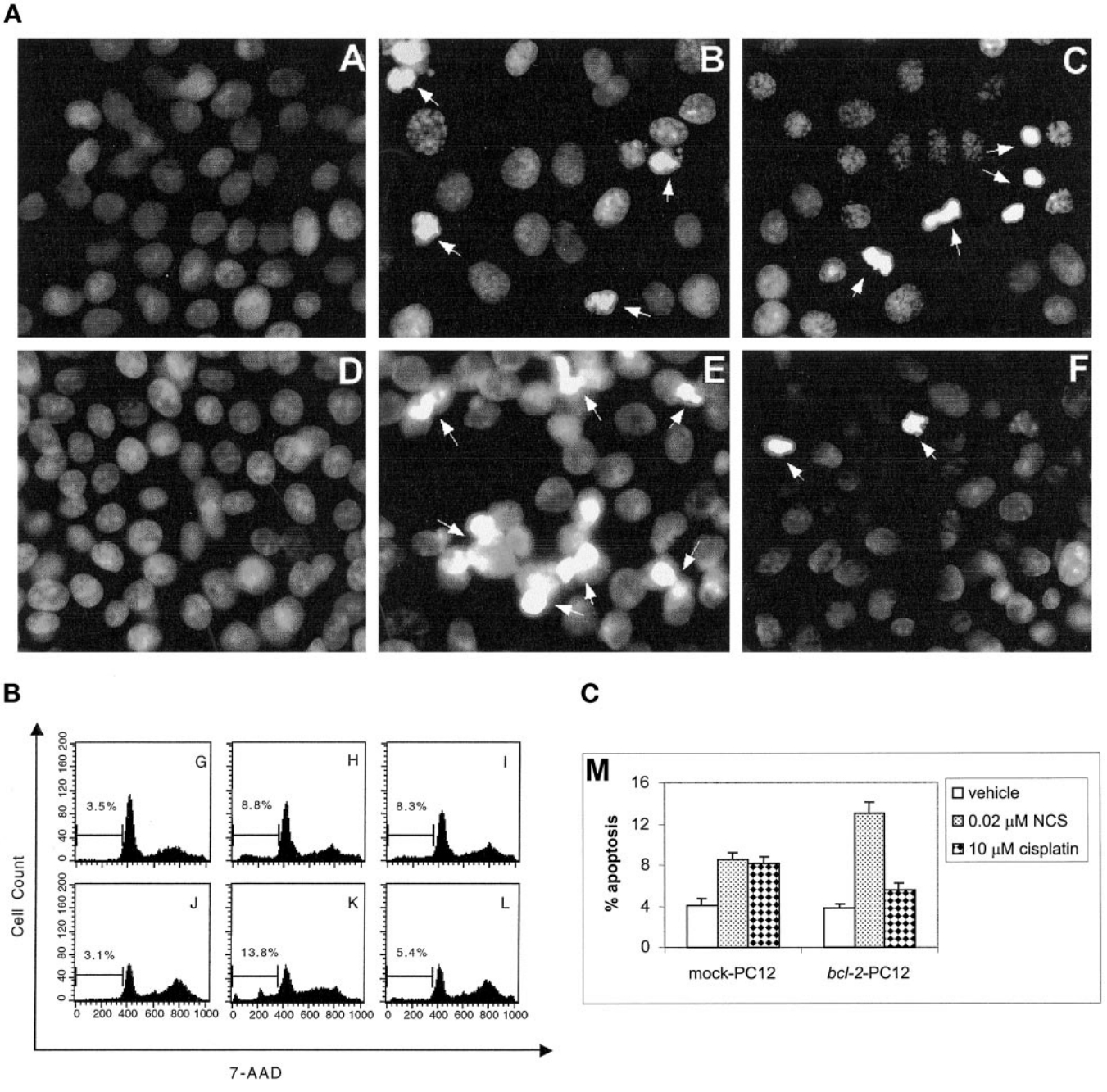


**Fig. 1.** Top, NCS and cisplatin induce apoptosis in both mock- and *bcl-2*–transfected PC12 cells. Both mock- and *bcl-2*–transfected PC12 cells were treated with either 0.02 $\mu$M NCS or 10 $\mu$M cisplatin for 1 h then washed and fed with fresh medium. After 24 h of incubation, the cells were fixed and stained with PI. A, control mock-transfected PC12 cells. B, NCS-treated mock-transfected PC12 cells. C, cisplatin-treated mock-transfected PC12 cells. D, control *bcl-2*–transfected PC12 cells. E, NCS-treated *bcl-2*–transfected PC12 cells. F, cisplatin-treated *bcl-2*–transfected PC12 cells. Arrows indicate apoptotic cells with condensed and fragmented nuclei. Magnification, 400×. Bottom left, flow cytometric analysis of NCS- and cisplatin-treated PC12 cells. Both mock- and *bcl-2*–transfected PC12 cells were treated with either 0.02 $\mu$M NCS or 10 $\mu$M cisplatin for 1 h then washed and fed with fresh medium. After 24 h of incubation, the cells were harvested and stained with 7-AAD (see *Materials and Methods*) and then analyzed by FACS. G, Control mock-transfected PC12 cells. H, NCS-treated mock-transfected PC12 cells. I, cisplatin-treated mock-transfected PC12 cells. J, control *bcl-2*–transfected PC12 cells. K, NCS-treated *bcl-2*–transfected PC12 cells. L, cisplatin-treated *bcl-2*–transfected PC12 cells. Bcl-2 potentiates NCS-induced apoptosis and attenuates cisplatin-induced apoptosis. Bottom right (M), Flow cytometric analysis of NCS- and cisplatin-treated PC12 cells. The mean ± S.D. for three independent experiments performed as described in G to L, above, is shown.

Schor, 1996; Schor et al., 1999a) and Bcl-2-mediated protection from decrease in cell culture growth in MCF-7 cells (Schor et al., 2000). These findings resulted in a downward shift of the $EC_{50}$ of NCS in PC12 cells and an upward shift of the $EC_{50}$ of NCS in MCF-7 cells transfected with *bcl-2* relative to mock-transfected cells. Figures 1 and 2 demonstrate the effect of *bcl-2* transfection of PC12 and MCF-7 cells, respectively, on apoptosis induction by NCS as determined by FACS analysis 24 h after NCS treatment. An increase in the prevalence of apoptosis is produced by *bcl-2* transfection of PC12 cells. In contrast, Bcl-2 renders MCF-7 cells less sensitive to apoptosis induction by NCS. Furthermore, consistent with our previously reported studies (Cortazzo and Schor, 1996), unlike the case for NCS, Bcl-2 decreased the incidence of apoptosis in PC12 cells treated with cisplatin [Fig. 1, I, L, and M].

**NCS Induces Bcl-2 Cleavage *in bcl-2*–Transfected PC12 Cells.** Previous reports have demonstrated cleavage of Bcl-2 to its proapoptotic counterpart in genetically engineered cell systems (Cheng et al., 1997). To begin to examine the possibility that cleavage of the Bcl-2 protein plays a role in the potentiation of NCS treatment-induced apoptosis, we looked for cleavage of Bcl-2 in PC12 cells treated with NCS. For this study, *bcl-2*- and mock-transfected PC12 cells were subjected to NCS treatment and the cell lysate was harvested and analyzed by Western blotting. As shown in Fig. 3A, 4 h after NCS treatment, Bcl-2 was cleaved to a 23-kDa protein in the *bcl-2*–transfected cells. It is of particular note that cleavage of Bcl-2 is observed 12 to 20 h before the cells demonstrate light microscopic evidence of apoptosis (data not shown). The cleavage product was detected at 24 but not at 48 h after a 1-h treatment with NCS.

Mock-transfected PC12 cells have 100-fold lower levels of Bcl-2 protein than *bcl-2*–transfected cells (Kane et al., 1993). Neither whole Bcl-2 nor Bcl-2 cleavage product is detected at any time point under these blotting conditions in the mock transfectant (Fig. 3A).

**NCS Did Not Induce Bcl-2 Cleavage in bcl-2–Transfected MCF-7 Cells.** To determine whether Bcl-2 cleavage after NCS treatment cosegregated only with potentiation of

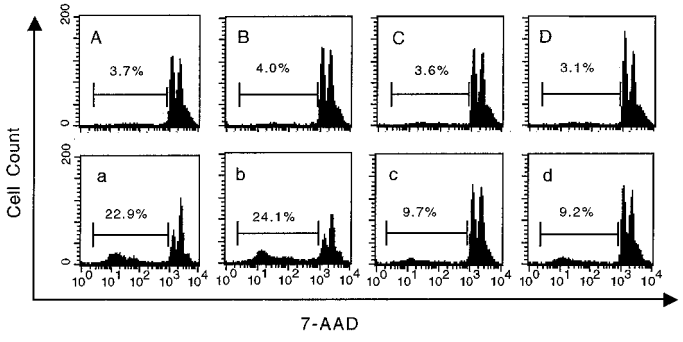


**Fig. 2.** Transfection with *bcl-2* leads to decreased sensitivity of MCF-7 cells to NCS. In all cases, cells were treated for 1 h with NCS (0.02 $\mu$M; 37°C) and FACS analysis was performed 24 h later as described under *Materials and Methods*. A, control neo.1 MCF-7 cells. B, control neo.2 MCF-7 cells. C, control bcl-2.1 MCF-7 cells. D, control bcl-2.3 MCF-7 cells. a, NCS-treated neo.1 MCF-7 cells. b, NCS-treated neo.2 MCF-7 cells. c, NCS-treated bcl-2.1 MCF-7 cells. d, NCS-treated bcl-2.3 MCF-7 cells. Transfection and selection of these clonal cell lines are described under *Materials and Methods*. A representative set of FACS results is shown here. Figure 6D (vehicle versus NCS alone) shows the mean ± S.D. for three independent such experiments.

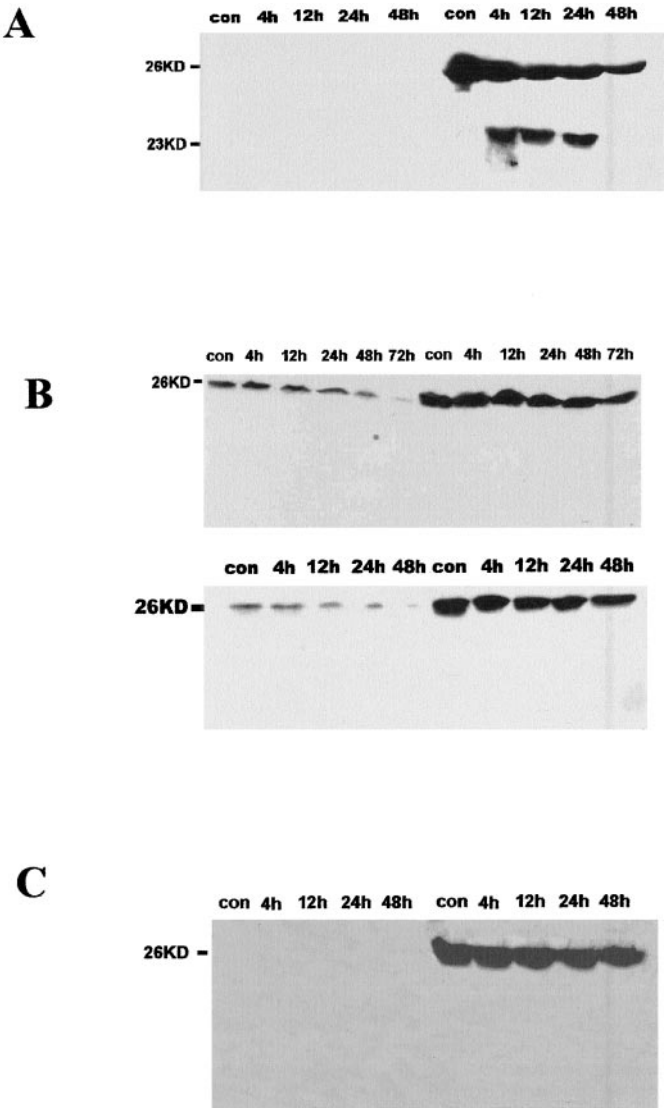


**Fig. 3.** A, NCS induces Bcl-2 cleavage in *bcl-2*–transfected PC12 cells. Mock- and *bcl-2*–transfected PC12 cells (polyclonal) were treated with NCS (0.02 $\mu$M; 1 h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western Blot analysis. Five hundred micrograms of protein was loaded per lane. Results of staining with an antibody for Bcl-2 (Santa Cruz Biotechnology) are shown. Mock-transfected PC12 cells, leftmost five lanes; *bcl-2*–transfected PC12 cells, rightmost five lanes. Note that mock-transfected PC12 cells contain 100-fold less Bcl-2 than *bcl-2*–transfected PC12 cells (Kane et al., 1993). The known molecular masses of the Bcl-2 band and that of its cleavage product ($\Delta$N34) were confirmed using simultaneously electrophoresed prestained standards (Bio-Rad). B, NCS did not induce Bcl-2 cleavage in *bcl-2*–transfected MCF-7 cells. Mock- and *bcl-2* transfected MCF-7 cells (two independent clones each) were treated with NCS (0.02 $\mu$M; 1 h). At various time points after completion of NCS treatment, the cells were harvested and lysed for Western blot analysis. Five hundred micrograms of protein was loaded per lane. Results of staining with an antibody for Bcl-2 (Santa Cruz Biotechnology) are shown. Top, neo.1 mock-transfected cells, leftmost five lanes; bcl-2.1 *bcl-2*–transfected cells, rightmost five lanes. Bottom, neo.2 mock-transfected cells, leftmost five lanes; bcl-2.3 *bcl-2*–transfected cells, rightmost five lanes. The molecular mass of the band detected (26 kDa) was confirmed as described in the legend for A. C, Cisplatin did not induce Bcl-2 cleavage in *bcl-2*–transfected PC12 cells. Mock and *bcl-2*–transfected PC12 cells were treated with 10 $\mu$M cisplatin for 1 h. At various time points, cells were harvested for Western blot analysis for Bcl-2. Five hundred micrograms of protein was loaded per lane. Mock-transfected PC12 cells, leftmost five lanes; *bcl-2*–transfected PC12 cells, rightmost five lanes. The molecular mass of the band detected (26 kDa) was confirmed as described in the legend for A.

(as opposed to protection from) apoptosis, we examined the effects of NCS treatment on Bcl-2 in MCF-7 cells. Our previous studies demonstrated a Bcl-2-mediated shift in the NCS concentration-cell culture growth curve of MCF-7 cells (Schor et al., 2000), and Bcl-2 seemed to protect MCF-7 cells from apoptosis [Figs. 2 and 6D (vehicle versus NCS alone)]. After NCS treatment, Bcl-2 was not cleaved in either *bcl-2-* or mock-transfected MCF-7 cells (Fig. 3B).

**Cisplatin Did Not Induce Bcl-2 Cleavage in Mock- or *bcl-2*–Transfected PC12 Cells.** Unlike the case for NCS, *bcl-2* transfection protects PC12 cells from cisplatin-induced apoptosis (Fig. 1M; Cortazzo and Schor, 1996). We therefore determined whether cisplatin induced Bcl-2 cleavage in *bcl-2*–transfected PC12 cells. As is shown in Fig. 3C, there was no Bcl-2 cleavage in mock- or *bcl-2*–transfected PC12 cells after cisplatin treatment. Again, at time points when apoptotic morphology and FACS DNA distribution were evident (Fig. 1), no cleavage of Bcl-2 could be detected.

**MCF-7 Cells Do Not Express Caspase 3.** Caspase 3 has been shown to cleave Bcl-2 to proapoptotic peptides in other systems (Cheng et al., 1997). In an effort to link the cleavage of Bcl-2 in PC12 cells and the lack thereof in MCF-7 cells, we measured caspase 3 expression in each of our transfectants of PC12 and MCF-7 cells. As shown in Fig. 4, caspase 3 is expressed in *bcl-2-* and mock-transfected PC12 cells, but not in either *bcl-2-* or mock-transfected MCF-7 cells. Interestingly, caspase 3 is clearly not required for apoptosis induction [Figs. 2 and 6D (vehicle versus NCS alone)] in the latter four transfected lines.

**Caspase 3 Activity in Mock- and *bcl-2*–Transfected PC12 Cells after NCS Treatment.** To examine caspase 3 activation over time we used the chromogenic caspase 3 substrate DEVD-pNA. As shown in Fig. 5, caspase 3 activity was elevated 30 min after NCS treatment in both mock- (Fig. 5A) and *bcl-2*–transfected PC12 (Fig. 5B) cells. The activity decreased to near baseline by 24 h after completion of NCS treatment.

**Caspase 3 Inhibitor Ac-DEVD-CHO Attenuates Sensitivity to NCS of *bcl-2*–Transfected PC12 cells, but Has No Effect on That of *bcl-2*–Transfected MCF-7 Cells.** To further demonstrate the role of caspase 3 in the cleavage of Bcl-2 after NCS treatment, we pretreated *bcl-2-* and mock-transfected PC12 cells with the caspase 3 inhibitor Ac-DEVD-CHO (10 $\mu$M) for 2 h before NCS treatment (0.02 $\mu$M; 1 h). As shown in Fig. 6B, pretreatment with Ac-DEVD-CHO results in attenuation of the sensitivity to NCS treatment of *bcl-2*–transfected PC12 cells; in contrast, Ac-DEVD-CHO did not alter the sensitivity to NCS of mock-transfected PC12 cells (Fig. 6A). Note that day 2 cell counts obtained at an NCS concentration of zero did not differ significantly between control and Ac-DEVD-CHO-treated cells ($P > 0.05$; Student's $t$ test). Furthermore, 2 h of pretreatment of *bcl-2*–transfected PC12 cells with Ac-DEVD-CHO abolished NCS-induced cleavage of Bcl-2 (Fig. 6C). The sister culture control gel (i.e., cells treated with NCS in the absence of Ac-DEVD-CHO) is shown in Fig. 3A.

In contrast, Ac-DEVD-CHO had no effect on the susceptibility to apoptosis induction by NCS of any of the four (two mock and two bcl-2) MCF-7 transfectants, as measured by FACS analysis 24 h after completion of NCS treatment (Fig. 6D).

**Unlike the Case for Cisplatin Treatment, Xenografts of *bcl-2*–Transfected PC12 Cells Are More Susceptible to NCS Toxicity Than Are Xenografts of Mock-Transfected PC12 Cells.** Nude mice ($n$ = 4 for each transfectant

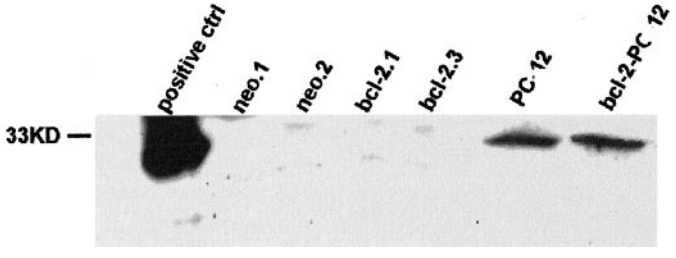


**Fig. 4.** Caspase 3 expression in PC12 and MCF-7 cells. Five hundred micrograms of protein was loaded per lane. The lysate of the A431 cell line represents a known positive control. No caspase 3 was detected in any of the four different MCF-7 cell transfectants (neo.1, neo.2, bcl-2.1, bcl-2.3). In contrast, the two PC12 transfectants express caspase 3 in comparable amounts. The electrophoretic position of a simultaneously run 33-kDa molecular mass standard is indicated to the right of the blot photograph.

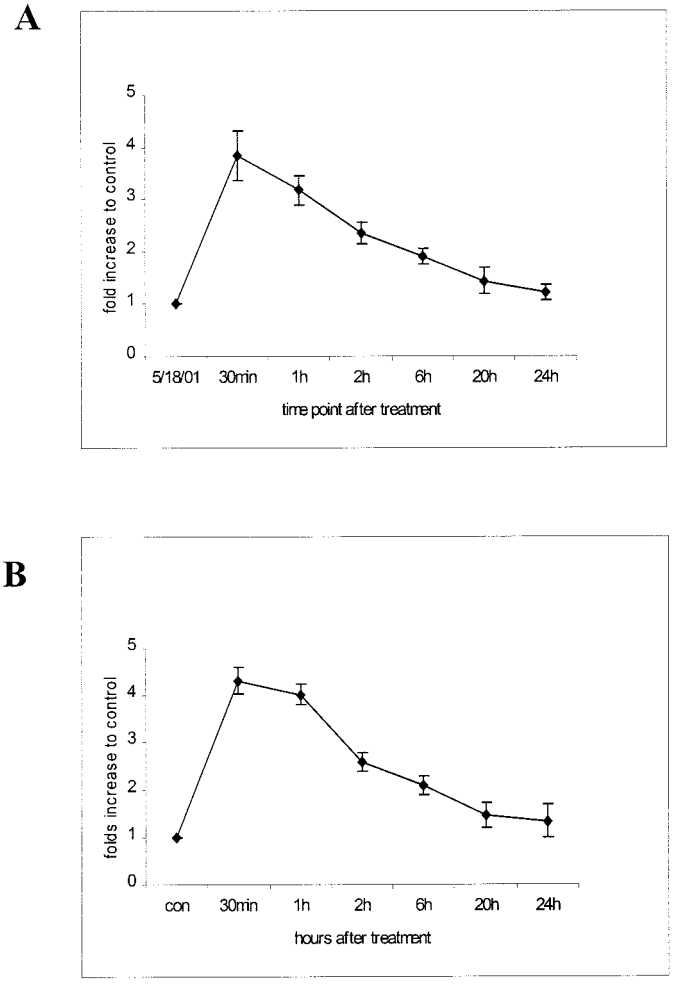


**Fig. 5.** Caspase 3 is activated in both mock- (A) and *bcl-2*– (B) transfected PC12 cells after NCS treatment (0.02 $\mu$M; 1 h). At the indicated time points, both mock- and *bcl-2*–transfected PC12 cells were harvested and assayed for caspase 3 activity by using the chromogenic substrate DEVD-pNA, according to the manufacturer's instructions (Medical and Biological Laboratory Co. Ltd.). The background reading from cell lysates and buffers was subtracted from the readings of both NCS-treated and control samples before calculating fold increase in caspase 3 activity. In both transfectants, caspase 3 activity was elevated 30 min after completion of NCS treatment, and slowly decreased to near baseline by 24 h later.

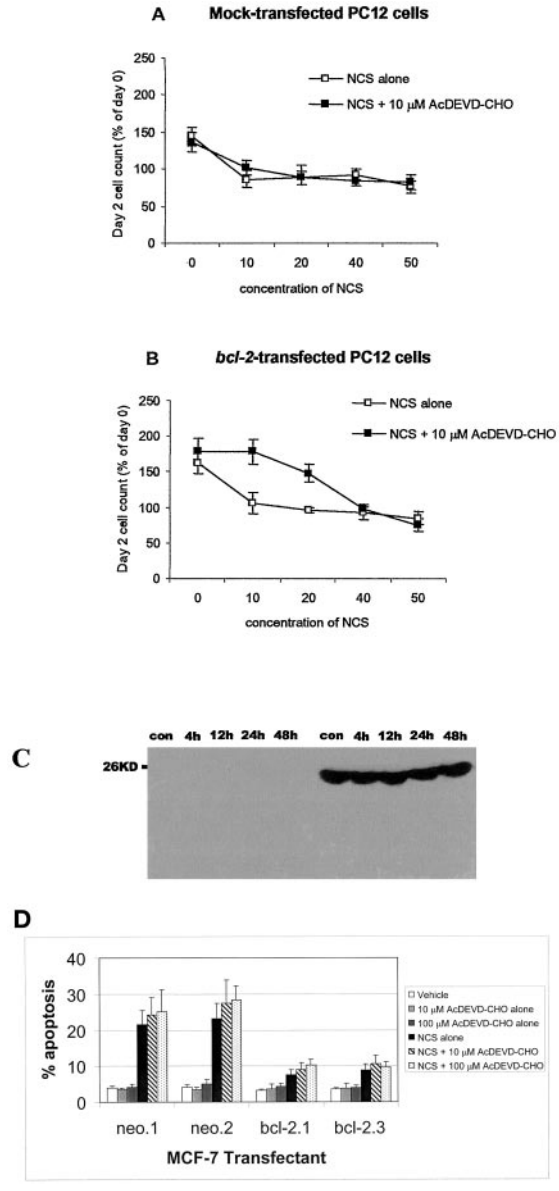


**Fig. 6.** Caspase 3 inhibitor Ac-DEVD-CHO increased the day 2 cell culture size after NCS treatment of *bcl-2*–transfected, but not mock-transfected PC12 cells. Both mock- and *bcl-2*–transfected PC12 cells were incubated with 10 $\mu$M Ac-DEVD-CHO for 2 h before NCS treatment (0.02 $\mu$M; 1 h). The cells were then washed and incubated with fresh medium on day 0. Cells were counted daily as we have described previously (Falcione et al., 1993; Cortazzo and Schor, 1996), and the day 2 cell counts are plotted as a reflection of cells remaining after completion of apoptosis evident by fluorescence microscopy and FACS analysis 24 h after completion of NCS treatment (day 1; Fig. 1). Plotted cell counts represent the mean counts for three high-power fields, and are expressed as a percentage of the count on day 0 ± S.E.M. Results are shown from a single representative experiment of three performed. A, mock-transfected PC12 cells. B, *bcl-2*–transfected PC12 cells. C, Western blot analysis of Bcl-2 cleavage in Ac-DEVD-CHO pretreated mock- and *bcl-2*–transfected PC12 cells. Five hundred micrograms of protein was loaded per lane. The sister culture control gel (i.e., cells treated with NCS in the absence of Ac-DEVD-CHO) is shown in Fig. 3A. D, FACS analysis performed 24 h after completion of NCS treatment of mock (two neo clones) and *bcl-2* (two *bcl-2* clones) transfectants of MCF-7 cells treated with NCS (0.02 $\mu$M) ± Ac-DEVD-CHO (10 or 100 $\mu$M) as described under *Materials and Methods*. In all cases, although NCS induces apoptosis and *bcl-2* transfection significantly diminishes MCF-7 cell sensitivity to NCS-induced apoptosis ($P < 0.05$ for all transfectants, vehicle compared with NCS alone; $p < 0.05$, NCS-treated condition for each mock transfectant compared with each *bcl-2* transfectant; Student's $t$ test), Ac-DEVD-CHO has no significant effect on apoptosis induction by NCS in these cell lines.

at each NCS dose) were administered subcutaneous injections of mock- or *bcl-2*–transfected PC12 cells as described under *Materials and Methods*. Subcutaneous implants of *bcl-2*–transfected PC12 cells more readily generate gross tumors than their mock-transfected counterparts. That is, the tumors that form from them are palpable earlier and ultimately larger than those formed from implanted mock-transfected PC12 cells. Nonetheless, as is the case for in vitro treatment of *bcl-2*- and mock-transfected PC12 cells, implants from *bcl-2*–transfected cells are more susceptible to NCS (0–5 mg/kg)-induced inhibition of tumor growth rate than are implants from mock-transfected cells (Fig. 7; Table 1; tumor volume at day 32 differs as follows by Student's $t$ test: mock-transfected cells, 3 mg/kg compared with 5 mg/kg, $p < 0.025$; *bcl-2*–transfected cells, saline compared with 1 mg/kg, $P < 0.05$).

An analogous study performed with intraperitoneal administration of cisplatin (0–25 mg/kg) demonstrated comparable depression of tumor growth rate in murine xenografts of mock- and *bcl-2*–transfected PC12 cells (Fig. 7; Table 1; tumor volume at day 27 differs between 5 and 10 mg/kg with $P = 0.05$ for both transfectants). At doses of cisplatin below 10 mg/kg, no depression of tumor growth rate was seen in either xenografted transfectant. In no case was Bcl-2-mediated potentiation of the antitumor effect seen. At doses of cisplatin above 10 mg/kg, lethal toxicity of the drug, evident before tumors were grossly detectable in control animals, precluded evaluation of the effect of higher-dose cisplatin on tumor growth.

Whereas NCS is a cell cycle phase-specific, proliferation-dependent drug, cisplatin is cell cycle-nonspecific and proliferation-independent (Carter et al., 1982). To exclude the possibility that the difference in in vivo sensitivity to NCS between mock- and *bcl-2*–transfected PC12 cells was merely the result of the faster growth rate of tumors in the latter transfectants, we also examined tumor growth in xenograft-bearing nude mice treated with vincristine (0–1.25 mg/kg), another cell cycle-specific, proliferation-dependent chemotherapeutic agent. As was the case for cisplatin, and in contrast to the case for NCS, mock- and *bcl-2*–transfected PC12 cell xenografts exhibited comparable sensitivity to vincristine (Table 1). This makes it unlikely that differential sensitivity to NCS is merely the result of the more rapid cycling of *bcl-2*–transfected cells.

## Discussion

Our previous studies have demonstrated that *bcl-2* overexpression potentiates the enediyne-induced decrease in cell number in some cell lines and prevents it in others. We now show that potentiation of enediyne sensitivity of *bcl-2*–transfected PC12 cells is associated with enhanced incidence of apoptosis and cleavage of Bcl-2 protein. Conversely, decreased sensitivity to enediynes of *bcl-2*–transfected MCF-7 cells and decreased sensitivity to cisplatin of *bcl-2*–transfected PC12 cells are associated with protection from apoptosis and the absence of Bcl-2 cleavage. Although Bcl-2 itself is an antiapoptotic protein, it has recently been reported that the cleavage product of this protein is proapoptotic (Cheng et al., 1997; Kirsch et al., 1999). Bcl-2 cleavage has been reported to occur after Asp-34, and to result in production of a proapoptotic cleavage product lacking the N-terminal 34

amino acids of Bcl-2 (Cheng et al., 1997). This is consistent with the 23-kDa cleavage product that was detected in response to NCS treatment. Cleavage of Bcl-2 was not detected in cisplatin-treated, *bcl-2*–transfected PC12 cells or NCS-treated, *bcl-2*–transfected human MCF-7 cells.

That this Bcl-2 cleavage requires the activity of caspase 3 is suggested by two lines of evidence. NCS treatment of MCF-7 cells, which have been described previously and demonstrated herein not to express caspase 3, does not result in cleavage of Bcl-2. [The absence of caspase 3 expression in MCF-7 cells is the result of a 47-base pair deletion within exon 3 of the caspase 3 gene (Janicke et al., 1998).] Furthermore, the caspase 3-specific inhibitor Ac-DEVD-CHO blocks both the cleavage of Bcl-2, resulting from NCS treatment and the potentiation of NCS-induced apoptosis seen with *bcl-2* overexpression in PC12 cells, but has no effect on MCF-7 cells. This is consistent with previous reports of the abrogation of cleavage of Bcl-2 when caspase 3 was immunodepleted from extracts of 293 cells. Conversely, immunodepletion of caspase 7 did not affect Bcl-2 cleavage in the 293 cell system, and transfection of caspase 3-deficient MCF-7 cells with the gene for caspase 3 resulted in cleavage of Bcl-2 (Kirsch et al., 1999). That caspase 7 seems not to cleave Bcl-2 is particularly interesting in light of the related structures and substrate specificities of caspases 3 and 7 and the differential substrate selectivities of the two, hypothesized to be the result of significant differences in sequence around their S4-binding sites (Sgorbissa et al., 1999; Wei et al., 2000). It is clear that caspase 3 plays a critical role in the cleavage of Bcl-2 in *bcl-2*–transfected PC12 cells. Although this role is likely to be a direct one, it is also possible that caspase 3 activates a downstream protease that, in turn, directly cleaves Bcl-2. The disappearance of the Bcl-2 cleavage product by 48 h after NCS treatment may represent degradation of this fragment with or without new Bcl-2 synthesis or may reflect a selection phenomenon (i.e., that those cells still alive at 48 h are those that sustained little enough Bcl-2 cleavage that they did not undergo apoptosis). Either scenario is consistent with completion of the effects of a single 1-h exposure to NCS by 48 h after treatment.

Our previous studies (Cortazzo and Schor, 1996; Schor et al., 2000) demonstrated the role of altered glutathione handling and antioxidant potential in mediating the potentiation of sensitivity to NCS by Bcl-2. These previous studies arose from the need for reductive activation of NCS and most other enediynes for their biological effects (DeGraff and Mitchell, 1985). Interestingly, caspase 3 activation depends critically on redox state. Its active site cysteine residue must be reduced for caspase activity, and the caspase 3-activating activity of the protein thioredoxin is proportional to the number

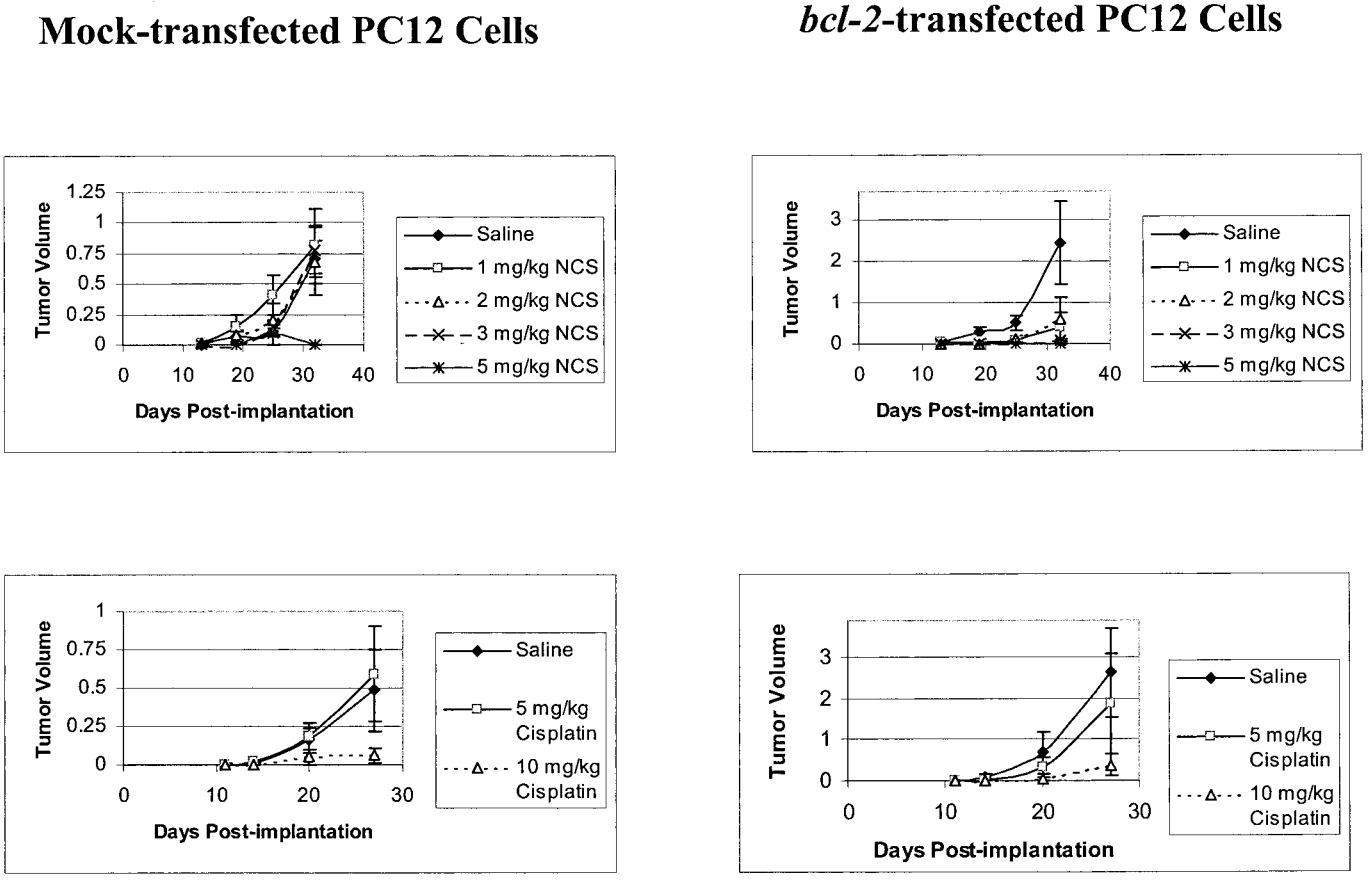


**Fig. 7.** NCS treatment is more effective against tumors derived from *bcl-2*–transfected cells than against those derived from mock-transfected cells. PC12 cells were xenografted into nude mice ($n = 4$/group) by subcutaneous injection of $10^6$ cells on day −1. Saline (vehicle control), NCS, or cisplatin was administered as a single intraperitoneal dose on day 0. Tumor volume was computed at multiple time points after xenograft implantation as the product of the length (longest dimension) and square of the width (smallest dimension) of each tumor that developed. Mice in which tumors did not develop were assigned a tumor volume of zero for that day. For values for mock transfectants treated with NCS, 3 mg/kg differs from 5 mg/kg at the $P < 0.025$ level (Student's $t$ test). For values for *bcl-2* transfectants treated with NCS, saline differs from 1 mg/kg at the $P < 0.05$ level. For both mock and *bcl-2* transfectants treated with cisplatin, 5 mg/kg values differ from 10 mg/kg values at the $P = 0.05$ level.

TABLE 1

$ED_{50}$ for tumor size reduction (day 27–32) relative to saline-injected control mice

For each treatment group, the $ED_{50}$ for tumor size reduction (day 27–32) was defined as the drug dose at which the day 27 to 32 mean tumor volume was one half that of the simultaneously saline-injected group. In most cases, the $ED_{50}$ falls between two actually administered doses; it is therefore expressed as the range of doses in which it falls. See *Materials and Methods* for further detail.

| Drug | Mock Transfectants | bcl-2 Transfectants |
|---|---|---|
| | *mg/kg* | |
| NCS | 3–4 | <1 |
| Cisplatin | 5–10 | 5–10 |
| Vincristine | 0.75–1 | 0.75–1 |

of reduced cysteine residues in the thioredoxin (Baker et al., 2000). Increased reducing potential via *bcl-2* overexpression may therefore provide the impetus for increased activation of both NCS and caspase 3.

It is interesting that, despite their lack of caspase 3 expression, MCF-7 cells still undergo apoptosis induced by a host of exogenous stimuli, including vitamin D (Mathiasen et al., Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. 1999), staurosporine (Janicke et al., 1998), the diazo radical initiator 2,2′-azobis(2,4′-dimethylvaleronitrile) (Schor et al., 1999b), and NCS (Schor et al., 2000). Similarly, cisplatin treatment of PC12 cells results in apoptosis, but not in cleavage of Bcl-2. Both caspase 3 and Bcl-2 cleavage are probably not involved in apoptosis in these systems; rather, activation of an alternative pathway involving caspase 7, as has been described previously for prostate carcinoma cells (Marcelli et al., 1998, 1999; Liang et al., 2001), is probable. Thus, although cleavage of Bcl-2 is clearly not required for enactment of apoptosis, it seems to potentiate this process in enediyne-treated PC12 cells.

The mechanism of apoptosis induction by NCS in MCF-7 cells may involve proapoptotic changes other than those mediated by caspase 3. Studies from our laboratory indicate that treatment of MCF-7 cells with NCS results in down-regulated expression of Bcl-2 and up-regulated expression of its proapoptotic analog, Bax (Liang et al., 2001). The resulting decrease in the Bcl-2/Bax ratio has been shown in other systems to trigger the release of cytochrome *c* from the mitochondria (Nakatsuka et al., 2000), and to thereby induce apoptosis (Putcha et al., 1999; Liang et al., 2000). Modulation of the Bcl-2/Bax ratio perhaps represents an alternative mechanism of potentiation of apoptosis in cells lacking caspase 3.

## References


Baker A, Santos BD, and Powis G (2000) Redox control of caspase-3 activity by thioredoxin and other reduced proteins. *Biochem Biophys Res Commun* **268:**78–81.

Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, and Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74:**597–608.

Brady HJ and Gil-Gomez G (1998) Bax. The pro-apoptotic Bcl-2 family member, Bax. *Int J Biochem Cell Biol* **30:**647–650.

Carter SK, Glatstein E, and Livingston RB (1982) *Principles of Cancer Treatment.* McGraw-Hill, New York.

Cheng EH-Y, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, and Hardwick JM (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. Science *(Wash DC)* **278:**1966–1968.

Cortazzo M and Schor NF (1996) Potentiation of enediyne-induced apoptosis and differentiation by Bcl-2. *Cancer Res* **56:**1199–1203.

DeGraff WG and Mitchell JB (1985) Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. *Cancer Res* **45:**4760–4762.

Dole M, Nunez G, Merchant AK, Maybaum J, Rode CK, Bloch CA, and Castle VP (1994) Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Res* **54:**3253–3259.

Falcione M, Milligan KD, Schwartz MC, and Schor NF (1993) Prevention of neocarzinostatin-induced cell death and morphologic change in SK-N-SH human neuroblastoma cells by continuous exposure to nerve growth factor. *Biochem Pharmacol* **46:**731–738.

Hartsell TL, Hinman LM, Hamann P, and Schor NF (1996) Determinants of the response of neuroblastoma cells to DNA damage: the roles of pretreatment cell morphology and chemical nature of the damage. *J Pharmacol Exp Ther* **277:**1158–1166.

Hartsell TL, Yalowich JC, Ritke MK, Martinez AJ, and Schor NF (1995) Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. *J Pharmacol Exp Ther* **275:**479–485.

Janicke RU, Sprengart ML, Wati MR, and Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* **273:**9357–9360.

Kamesaki S, Kamesaki H, Jorgensen TJ, Tanizawa A, Pommier Y, and Cossman J (1993) bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res* **53:**4251–4256.

Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, and Bredesen DE (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Wash DC)* **262:**1274–1277.

Kirsch DG, Doseff A, Chau BN, Lim DS, de Souza-Pinto NC, Hansford R, Kastan MB, Lazebnik YA, and Hardwick JM (1999) Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. *J Biol Chem* **274:**21155–21161.

Kluck RM, Bossy-Wetzel E, Green DR, and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science (Wash DC)* **275:**1132–1136.

Lecoeur H and Gougeon ML (1996) Comparative analysis of flow cytometric methods for apoptosis quantitation in murine thymocytes and human peripheral lymphocytes from controls and HIV-infected persons. Evidence for interference by granulocytes and erythrocytes. *J Immunol Methods* **198:**87–99.

Liang Y, Jorgensen A, Kaestel C, Wiencke AK, Lui GM, la Cour M, Ropke C, and Nissen MH (2000) Bcl-2, Bax, and c-Fos expression correlates to RPE cell apoptosis induced by UV-light and daunorubicin. *Curr Eye Res* **20:**25–34.

Liang Y, Yan C, and Schor NF (2001) Apoptosis in the absence of caspase 3. *Oncogene* **20:**6570–6578.

Marcelli M, Cunningham GR, Haidacher SJ, Padayatty SJ, Sturgis L, Kagan C, and Denner L (1998) Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. *Cancer Res* **58:**76–83.

Marcelli M, Cunningham GR, Walkup M, He Z, Sturgis L, Kagan C, Mannucci R, Nicoletti I, Teng B, and Denner L (1999) Signaling pathway activated during apoptosis of the prostate cancer cell line LNCaP: overexpression of caspase-7 as a new gene therapy strategy for prostate cancer. *Cancer Res* **59:**382–390.

Mathiasen IS, Lademann U, and Jaattela M (1999) Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. *Cancer Res* **59:**4848–4856.

Miyashita T and Reed JC (1993) Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* **81:**151–157.

Nakatsuka H, Ohta S, Tanaka J, Toku K, Kumon Y, Maeda N, Sakanaka M, and Sakaki S (2000) Cytochrome c release from mitochondria to the cytosol was suppressed in the ischemia-tolerance-induced hippocampal CA1 region after 5-min forebrain ischemia in gerbils. *Neurosci Lett* **278:**53–56.

Putcha GV, Deshmukh M and Johnson EMJr (1999) BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J Neurosci* **19:**7476–7485.

Reed JC (1994) Bcl-2 and the regulation of programmed cell death. *J Cell Biol* **124:**1–6.

Schor NF (1992) Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin. Studies in murine neuroblastoma cells. *J Clin Invest* **89:**774–781.

Schor NF, Rudin CM, Hartman A-R, Thompson CB, Tyurina YY, and Kagan VE (2000) Cell line dependence of Bcl-2-induced alteration of glutathione handling. *Oncogene* **19:**472–476.

Schor NF, Tyurina YY, Fabisiak JP, Tyurin VA, Lazo JS, and Kagan VE (1999a) Selective oxidation and externalization of membrane phosphatidylserine: Bcl-2-induced potentiation of the final common pathway for apoptosis. *Brain Res* **831:**125–130.

Schor NF, Tyurina YY, Tyurin VA, and Kagan VE (1999b) Differential membrane antioxidant effects of immediate and long-term estradiol treatment of MCF-7 breast cancer cells. *Biochem Biophys Res Commun* **260:**410–415.

Sgorbissa A, Benetti R, Marzinotto S, Schneider C, and Brancolini C (1999) Caspase-3 and caspase-7 but not caspase-6 cleave Gas2 in vitro: implications for microfilament reorganization during apoptosis. *J Cell Sci* **112:**4475–4482.

Teixeira C, Reed JC, and Pratt MA (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res* **55:**3902–3907.

Tyurina YY, Tyurin VA, Carta G, Quinn PJ, Schor NF, and Kagan VE (1997) Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells. *Arch Biochem Biophys* **344:**413–423.

Wei Y, Fox T, Chambers SP, Sintchak J, Coll JT, Golec JM, Swenson L, Wilson KP, and Charifson PS (2000) The structures of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. *Chem Biol* **7:**423–432.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng T-I, Jones DP, and Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science (Wash DC)* **275:**1129–1132.


**Address correspondence to:** Dr. Nina Felice Schor, Division of Child Neurology, Children's Hospital of Pittsburgh, 3705 Fifth Ave., Pittsburgh, PA 15213. E-mail: nfschor@pitt.edu