Cancer Chemother Pharmacol (2006) 57: 357–367
DOI 10.1007/s00280-005-0054-z

ORIGINAL ARTICLE

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Bcl-2-mediated potentiation of neocarzinostatin-induced apoptosis: requirement for caspase-3, sulfhydryl groups, and cleavable Bcl-2

Received: 20 February 2005 / Accepted: 2 May 2005 / Published online: 5 July 2005

Abstract Overexpression of antiapoptotic Bcl-2 family members is thought to contribute to chemotherapeutic resistance of neural crest tumors. Paradoxical potentiation by Bcl-2 of apoptosis induced by the antineoplastic prodrug, neocarzinostatin (NCS), has been observed in PC12 pheochromocytoma cells. Prior studies have indicated that the cleavage of Bcl-2 to its proapoptotic counterpart mediated by caspase-3 is responsible for this potentiation of apoptosis. This has led to the hypothesis that induction of caspase-3 expression in *bcl-2*-transfected, caspase-3-deficient MCF-7 cells, will result in Bcl-2 cleavage and Bcl-2-dependent potentiation of NCS-induced apoptosis. These studies have further led to the hypothesis that both cleavable Bcl-2 and sulfhydryl groups are required for the activity of caspase-3 in this regard. As hypothesized, co-transfection of *bcl-2*-transfected MCF-7 cells with a caspase-3 expression construct results in cleavage of Bcl-2 and potentiation of dose-dependent, NCS-mediated cell death. Furthermore, PC12 cells transfected with an expression construct for cleavage-resistant Bcl-2 demonstrated attenuated potentiation of apoptosis relative to their counterparts transfected with wild-type *bcl-2*. Finally, irreversible oxidative titration of sulfhydryl groups resulted in concentration-dependent attenuation of apoptosis in PC12 cells, along with prevention of caspase-3 activation and Bcl-2 cleavage. These results definitively demonstrate the requirement for caspase-3, cleavable Bcl-2, and available sulfhydryl groups (separate from those required for NCS activation) in potentiation of NCS-induced apoptosis by Bcl-2.

Keywords Caspase-3 · Apoptosis · Chemotherapeutic agent · Enediyne · Reduced glutathione

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Introduction

Drug resistance is one of the major impediments to successful chemotherapy. Increasingly, therapeutic failures in this arena are hypothesized to result from altered expression of proteins that function to regulate apoptosis induction and/or enactment. It has been shown that the overexpression of *bcl-2* and the decline of Bcl-X_L/Bcl-2 ratios in certain tumors contribute to their resistance to chemotherapeutic-induced apoptosis [1–7]. Thus, understanding the unique molecular and biological consequences of altered expression of apoptosis-regulatory proteins in specific chemoresistant tumors would contribute greatly to the discovery and development of chemotherapeutic strategies that overcome drug resistance.

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in neural crest tumor cells in culture [8, 9]. Like many other naturally occurring enediynes, NCS is a prodrug that requires sulfhydryl activation for efficacy. This reduction-dependent feature makes it a promising chemotherapeutic agent for those tumors rendered resistant to other drugs by overexpression of Bcl-2, as such overexpression results in an increase in available reducing potential in these cells [10–12].

We have previously reported that transfection of some cancer cell lines with *bcl-2* and resultant overproduction of Bcl-2 results in paradoxical potentiation

of apoptosis induced by NCS. Previous reports of cleavage of Bcl-2 to its proapoptotic counterpart [13] led us to examine cleavage of Bcl-2 in NCS-treated cancer cell lines. We found that such cleavage does indeed occur in those cell lines that demonstrate Bcl-2-dependent potentiation of NCS-induced apoptosis. Inhibition of the activity of caspase-3 prevents both Bcl-2 cleavage and potentiation of apoptosis, and cells that do not express caspase-3 do not exhibit Bcl-2-mediated potentiation of NCS-induced apoptosis [13, 14]. The present studies demonstrate that induction of caspase-3 expression in cells shown in their native state to demonstrate neither caspase-3 expression nor Bcl-2-mediated potentiation of NCS-induced apoptosis results in NCS-dependent activation of caspase-3, cleavage of the overexpressed Bcl-2, and paradoxical potentiation of apoptosis. They further show that transfection of cells that express caspase-3 and overproduce Bcl-2 with an expression construct for a cleavage-resistant mutant Bcl-2 attenuates potentiation of NCS-induced apoptosis.

Previous studies of this system also demonstrated the requirement for reactive sulfhydryl groups for potentiation of apoptosis. Although the assumption has been made that these sulfhydryl groups are necessary only for activation of NCS, a reduction-dependent prodrug [15–17], attempts to titrate sulfhydryls and generate a sulfhydryl concentration-response curve suggested that this phenomenon was of an all-or-none nature, rather than governed by the linear concentration dependence expected for a simple nonenzymatic reduction like that of NCS by cellular sulfhydryls. This in turn suggested that the requirement for reactive sulfhydryl groups extends to the role of caspase-3, a cysteine-rich protease, in cleavage of Bcl-2. The present studies demonstrate that this is indeed the case.

Materials and methods

Cells and cell culture

Mock- and *bcl-2*-transfected MCF-7 human breast cancer cells were the kind gift of Dr. Charles Rudin (Department of Medicine, Northwestern University, Chicago, IL, USA; [12]). These MCF-7 cell transfectants were generated by electroporation (300 mV, 960 mFD) with pSEEV-neomycin and pSFFV-bcl-2 (plasmids described in [18]), respectively. Stably transfected MCF-7 clones were screened for Bcl-2 production by Western blot analysis using N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology). Two mock- (neo.1, neo.2) and two *bcl-2*- (Bcl-2.1, Bcl-2.3) transfected MCF-7 cell lines were obtained as monoclonal transfectants. Our previous studies have demonstrated identical behavior of the two clones relative to NCS-induced apoptosis [14]. In the present study, clone neo.1 was used for the mock- and clone Bcl-2.3 was used for the *bcl-2*-transfected MCF-7 cells. These MCF-7 transfectants were maintained as adherent monolayers in 75-mm^2 culture flasks (Life Technologies, Grand Island, NY, USA) and fed twice weekly with α-MEM (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum, 0.3% glucose, 2 mM L-glutamine (Life Technologies), and 2 mg/ml gentamicin sulfate (Biofluids, Rockville, MD, USA).

PC12 cells that stably overexpress the wild-type and the D34A Bcl-2 constructs (see below) were maintained as adherent monolayers in Dulbecco's modified Eagle's medium supplemented with 10% in horse serum, 5% in fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1.1% in penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were fed every 3 to 4 days. PC12 cells transfected with the D34A cleavage resistant mutant *bcl-2* gene [13] are referred to henceforth as D34A-PC12 cells; PC12 cells transfected with the wild-type *bcl-2* gene are referred to henceforth as wtBcl-2-PC12 cells; and PC12 cells transfected with the control plasmid are referred to as Mock-PC12 cells. Cells were examined for Bcl-2 expression by Western blotting for each experiment.

Plasmids and transfection

Caspase-3 plasmid

The full-length human caspase-3 was amplified by RT-PCR using forward (5′-gatatcatggagaacactgaaaactcagtgg-3′) and reverse (5′-ttagtgataaaaatagagttcttttgtg-3′) primers and human brain cDNA as a template. The purified PCR product was inserted into pGEM-easy T vector. The caspase-3 fragment was cut out with EcoR V and Not I and subcloned into pcDNA3.1/Zeo(+) (Invitrogen Life Technologies). The successful cloning was confirmed by sequencing the plasmid.

bcl-2 plasmids

Both the wild-type and the cleavage-resistant D34A mutant *bcl-2* plasmids were previously described in detail [13] and obtained from Dr. Emily Cheng (Washington University, St. Louis, MO, USA).

Transfection with caspase-3 or Bcl-2 expression construct

MCF-7 cells that were stably transfected with *bcl-2* (*bcl-2*-MCF-7) or a control plasmid (mock-MCF-7) were transfected transiently with a plasmid containing the gene for caspase-3 or an empty plasmid using a Nucleofector instrument and Nucleofector kit V (Amaxa Biosystem, Koeln, Germany) optimized for use with MCF-7 cells following the manufacturer's instructions. The resultant four double transfectants are referred as bcl-2/casp3, bcl-2/zeo, neo/casp3, and neo/zeo, respectively. The expression of caspase-3 and *bcl-2* was confirmed for each experiment using Western blot analysis.

PC12 cells were transfected with the plasmids containing the genes for wild-type or cleavage-resistant Bcl-2, respectively, or a control plasmid. Transfected cells were selected as stable cell lines using selection antibiotic.

NCS treatment

Mock- and *bcl-2*-transfected MCF-7 cells co-transfected with a control or caspase-3 expression construct and PC12 cells overexpressing the D34A or wild-type Bcl-2 expression construct or an empty plasmid were treated with 10–50 nM NCS (Kayaku Pharmaceuticals Ltd., Japan) or an equivalent volume of vehicle for 1 h. Subsequently, the cells were rinsed twice with and maintained in fresh medium. Percent apoptosis and cell viability were determined by flow cytometric analysis 24 h and Alamar blue assay (Biosource, Sacramento, CA, USA) 48 h after completion of NCS treatment.

ThioGlo-1 treatment

NCS activation

Potentiation of NCS-induced apoptosis is sulfhydryl-dependent [11]. In order to define the concentration-response relationship of this sulfhydryl dependency, we first manipulated the cellular sulfhydryl content in *bcl-2*-overexpressing PC12 cells growing in 96-well plate using the sulfhydryl binding agent, ThioGlo-1. For each experiment, the intracellular reduced glutathione (GSH) content was determined as described [19] to calculate the amount of the ThioGlo-1 needed to titrate the desired amount of GSH. ThioGlo-1 (0.5–20 nmol/well), or an equivalent volume of vehicle was added to sister cultures of *bcl-2*-transfected PC12 cells prior to the addition of NCS. After 10–20 min incubation with ThioGlo-1, cells were then washed with fresh medium and treated with NCS (10, 20 and 50 nM; 1 h). Cell viability was determined as described 24 h after the addition of NCS.

Bcl-2 cleavage

Our previous studies [14] demonstrated that, while thiol-dependent activation of NCS takes place within 1 h of incubation with NCS, cleavage of Bcl-2 does not take place until 4 h after initiation of NCS treatment (i.e., 3 h after NCS is washed out). To test the requirement for free sulfhydryl groups for activation of caspase-3 and cleavage of Bcl-2 (separate and distinct from the sulfhydryl requirement for activation of NCS), ThioGlo-1 was added to NCS-treated, *bcl-2*-transfected PC12 cells immediately after washout of NCS. Three hours later, cells were studied by Western blot analysis for cleavage of Bcl-2.

Western blot analysis of Bcl-2 and caspase-3

Twenty-four hours after the completion of NCS treatment, cells were lysed in RIPA buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 mg/ml aprotinin, 1 mM sodium orthovanadate). Subsequently, the protein concentration was estimated in triplicate samples using the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA) with bovine serum albumin as a standard. Two hundred microgram of protein was loaded in each lane of a 12% SDS-polyacrylamide gel and electrophoresed followed by blotting onto a nitrocellulose membrane (Bio-Rad Laboratories). After blotting, nonspecific binding was blocked by 5% non-fat dry milk in PBS for 1 h and the membrane was incubated for 2 h at 20°C with either anti-Bcl-2 or anti-caspase-3 antibodies (1:500 for Bcl-2 and 1:1000 for caspase-3; Santa Cruz Biotechnology) diluted into 5% nonfat dry milk in PBS, washed, and incubated with secondary horseradish peroxidase conjugated antimouse IgG antibody or antirabbit IgG (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.

Cell viability assay

Sensitivity to NCS was examined as follows: cells were plated on flat-bottomed 96-well plates at a density of 2×10^4 cells/100 µl, and preincubated for 24 h prior to NCS (10, 20 and 50 nM; 1 h) treatment. Forty-eight hours after NCS treatment, Alamar blue was added to the medium, and 1 h later fluorescence was determined using excitation at 530 nm and emission at 590 nm in a cytofluorometer (Cytofluor II; PerSeptive Biosystems, Framingham, MA). NCS concentration-response curves were constructed for each cell line. The statistical significance of the effect of transfection upon the concentration-response curve of *bcl-2*- and mock-transfected cells in turn was determined using Student's *t* test at each concentration point.

Flow cytometric analysis of apoptotic cells

Flow cytometric analysis was performed on each of the four NCS- and vehicle-treated MCF-7 cell transfectants and on NCS- and vehicle-treated wild-type *bcl-2*, D34A mutant *bcl-2*, and mock PC12 cell transfectants. The incidence of apoptosis was quantified by flow cytometry considering 7-AAD staining intensity to be proportional to the DNA content [20]. Saponin and 7-amino-actinomycin D (7-AAD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, after harvesting, the cells were washed once in PBS and once in PBS/0.05% saponin, followed by addition of 4 mg 7-AAD in 1 ml

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 (Becton Dickinson). Data on 10^4 cells were collected. Electronic gates were set for viable and apoptotic cells with $2N \pm 4N$ DNA and subnormal DNA contents, respectively, and for exclusion of debris. Percent apoptosis was calculated as (number of apoptotic cells/number of total cells)×100. For each cell line, the percent apoptosis under vehicle-treated conditions was subtracted from the percent apoptosis under NCS-treated conditions. For MCF-7 cell transfectants, Student's *t* test was used to compare the ratio of the average percent apoptosis in NCS-treated cells corrected for control (i.e., vehicle-treated) cells in bcl-2/casp3 vs. bcl-2/zeo cells with the analogous ratio in neo/casp3 vs. neo/zeo cells, with $P < 0.05$ considered to indicate statistical significance. For PC12 cells, one-way ANOVA followed by Fisher's PLSD test was used to compare the difference in the average incidence of cell death among wtBcl-PC12 cells, D34A-PC12 cells and mock-PC12 cells.

Results

Requirement for caspase-3

Expression of Bcl-2 and caspase-3 in mock- and bcl-2-transfected MCF-7 cells co-transfected with a caspase-3 expression construct or its corresponding empty vector

Lysates of four double transfectants for *bcl-2* and caspase-3 and their corresponding mock (*neo* and *zeo*, respectively) transfectants (i.e., bcl-2/casp3; bcl-2/zeo; neo/casp3; neo/zeo cells) were examined by Western blotting for their contents of Bcl-2 (Fig. 1a) and caspase-3 (Fig. 1b), respectively. Figure 1a depicts the content of Bcl-2 in *bcl-2*- (lanes 1 and 2) and mock- (lanes 3 and 4) transfected MCF-7 cells. The Bcl-2 content of *bcl-2*-transfected MCF-7 cells is approximately twice that of mock-transfected cells. Figure 1b shows the expression of caspase-3 in control vector-transfected and caspase-3-transfected MCF-7 cells 24 h after transfection. While vector-transfected MCF-7 cells (lanes 2 and 4) did not contain any immuno-detectable caspase-3, caspase-3-transfected MCF-7 cells contained a significant amount of the caspase-3 protein (lanes 1 and 3).

Expression of caspase-3 results in cleavage of Bcl-2 in NCS-treated MCF-7 cells

To examine if expression of caspase-3 results in NCS treatment-triggered cleavage of Bcl-2 in MCF-7 cells, we transfected stable *bcl-2* transfectants (Bcl-2-MCF-7 cells) with either a caspase-3 expression construct or a control vector, and then treated each resulting double transfectant with 20 nM NCS for 1 h. While NCS treatment of Bcl-2-MCF-7 cells transfected with a control vector resulted in no detectable cleavage of Bcl-2 (Fig. 2a), NCS treatment of bcl-2/casp3 cells resulted in production of the 23 kD Bcl-2 cleavage product previously reported [14] for PC12 pheochromocytoma cells treated with NCS (Fig. 2b). In accordance with this, NCS treatment of bcl-2/casp3 cells results in activation of caspase-3 (Fig. 2c, arrow). Although the fraction of the total Bcl-2 that is cleaved is small under these conditions, similar fractional cleavage of Bcl-2 in MCF-7 cells has been associated with enhanced release of cytochrome C from mitochondria and enactment of apoptosis [21].

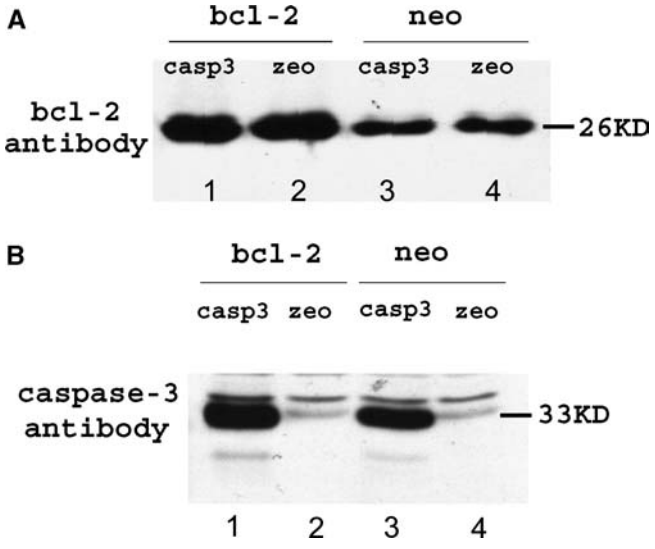


Fig. 1 Content of Bcl-2 and caspase-3 in MCF-7 cells transfected with either or both *bcl-2* (**a**) and caspase-3 (**b**). 200 μg of protein from whole cell lysates of bcl-2/casp3 (lane 1), bcl-2/zeo (lane 2), neo/casp3 (lane 3) and neo/zeo (lane 4) MCF-7 cells were separated by 12% SDS-PAGE, and different portions of the same blot were analyzed with antibodies to Bcl-2 (**a**) and caspase-3 (**b**), respectively. **a** Bcl-2 content in the four MCF-7 cell double transfectants. The content of Bcl-2 in *bcl-2*-transfected MCF-7 cells is about twice that in mock-transfected MCF-7 cells. **b** Caspase-3 content of caspase-3- and control vector-transfected MCF-7 cells. *bcl-2*- or mock-transfected MCF-7 cells were transiently transfected with either a caspase-3 expression construct or a control vector (1–2 μg/μl). Cells were harvested and lysed for Western blot analysis 24 h after transfection. Control vector-transfected MCF-7 cells do not show any detectable caspase-3 (lanes 2 and 4), whereas caspase-3-transfected MCF-7 cells are positive for caspase-3 staining (lanes 1 and 3)

Expression of caspase-3 results in Bcl-2-mediated potentiation of NCS-induced apoptosis in MCF-7 cells

To investigate whether or not the expression of caspase-3 converts the protective effect of Bcl-2 to one of potentiation of apoptosis, we constructed an NCS concentration-cell death response curve using the Alamar blue assay. As is seen in Fig. 3a, *bcl-2*-transfected cells demonstrate potentiation of response to NCS ($P < 0.001$ when 10 nM and 20 nM NCS were used and $P < 0.05$ when 50 nM NCS was used; Fig. 3b) only after transfection with a caspase-3 expression construct.

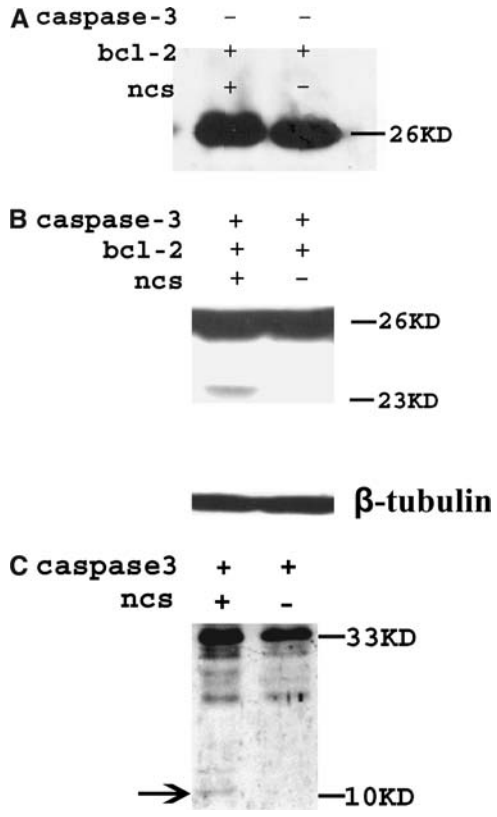


Fig. 2 NCS-induced cleavage of Bcl-2 in MCF-7 cells is caspase-3 dependent. Bcl-2- and/or caspase-3-transfected MCF-7 cells were treated with NCS (20 nM; 1 h). 14 h after completion of NCS treatment, the cells were harvested and lysed for Western blot analysis. Five hundred microgram (**a**, **b**) or 200 μg (**c**) of protein was loaded in each lane. Results of staining with antibodies for Bcl-2 (**a** and **b**) and caspase-3 (**c**) are shown. **a** NCS did not induce Bcl-2 cleavage in bcl-2/zeo cells. **b** Transfection with caspase-3 results in Bcl-2 cleavage in *bcl-2*-transfected MCF-7 cells treated with NCS. β-tubulin staining was performed as a loading control. **c** NCS induces activation of caspase-3 in *bcl-2*-transfected MCF-7 cells (*arrow*)

Such Bcl-2-induced potentiation is not seen in mock-transfected cells transfected with the same caspase-3 construct ($P > 0.05$ at all NCS concentrations; Fig. 3b). Note that, although the fraction of cells killed by NCS increases with increasing NCS concentration, even with caspase-3 transfection, it plateaus at approximately 50%. This is most likely because only those cells that undergo DNA replication in the presence of NCS are susceptible to NCS-induced cell death [15] and the transfection effected with casp3 in this study is transient, implying that some fraction of the cells may revert to wild-type during the study.

In addition, we used flow cytometric analysis to quantify apoptotic cell death in NCS-treated double transfectants of MCF-7 cells. Figure 4a shows that, while the expression of caspase-3 resulted in an increase in cell death in both neo/casp3 and bcl-2/casp3 cells, the increase in bcl-2/casp3 cells is much higher than that in neo/casp3 cells relative to their respective *zeo*-transfected counterparts. In this particular study, caspase-3 transfection results in a sevenfold increase in NCS-induced cell death for bcl-2/casp3 cells and only a 1.2-fold increase for neo/casp3 cells when each value for NCS-treated cells is corrected for the corresponding value for vehicle-treated cells. Figure 4b shows the average incidence of cell death determined by FACS analysis in bcl-2/casp3, bcl-2/zeo, neo/casp3, and neo/zeo cells from three independent experiments. When the ratio of percent cell death in bcl-2/casp3 versus bcl-2/zeo cells was compared with the ratio of cell death in neo/casp3 versus neo/zeo cells using Student's *t* test, the calculated *P* value was 0.03, indicating that the expression of caspase-3 results in statistically significant potentiation of NCS-induced cell death in bcl-2/casp3 cells.

Requirement for cleavable Bcl-2

Attenuation of incidence of NCS-induced apoptosis in D34A-PC12 cells relative to wtBcl-2-PC12 cells

Cell lysates from D34A-PC12 cells and wtBcl-2-PC12 cells treated with NCS were analyzed by Western blotting analysis. While NCS treatment of wtBcl-2-PC12 cells resulted in an enhanced production of the 23 kD Bcl-2 cleavage product relative to the untreated cells, NCS treatment of D34A-PC12 cells resulted in no detectable cleavage of Bcl-2 (Fig. 5). As is shown in Fig. 6, transfection of PC12 cells with an expression construct for the D34A mutant of Bcl-2 decreases susceptibility of these cells to NCS treatment relative to PC12 cells that overexpress wild-type Bcl-2. This increased cell viability was seen at 10 nM ($P < 0.001$) and 20 nM ($P < 0.01$) NCS. Treatment with 50 nM NCS was sufficiently toxic that the two transfectants demonstrate equivalent susceptibility to NCS-induced death. The apoptotic fraction of 20–25% represents the replicating fraction of the PC12 cells for which NCS, a mitosis-dependent chemotherapeutic agent, is toxic [22, 23]. The data depicted in Fig. 7 further demonstrate that the incidence of cell death in the D34A mutant cells is intermediate between that seen in the wild-type Bcl-2-producing and mock-transfected cells. This is in keeping with the relative cleavage resistance of the mutant Bcl-2 [13].

Requirement for reactive sulfhydryl groups for the activation of NCS and cleavage of Bcl-2

Titration of GSH content with ThioGlo-1

Addition of ThioGlo-1 to *bcl-2*-transfected PC12 cells results in dose-dependent oxidative inactivation of intracellular free sulfhydryl groups. In this particular experiment, as shown in the legend to Fig. 8, treatment with 1 and 2 nmol/well ThioGlo-1 resulted in depression of free sulfhydryl content by 25% and 38%, respectively.

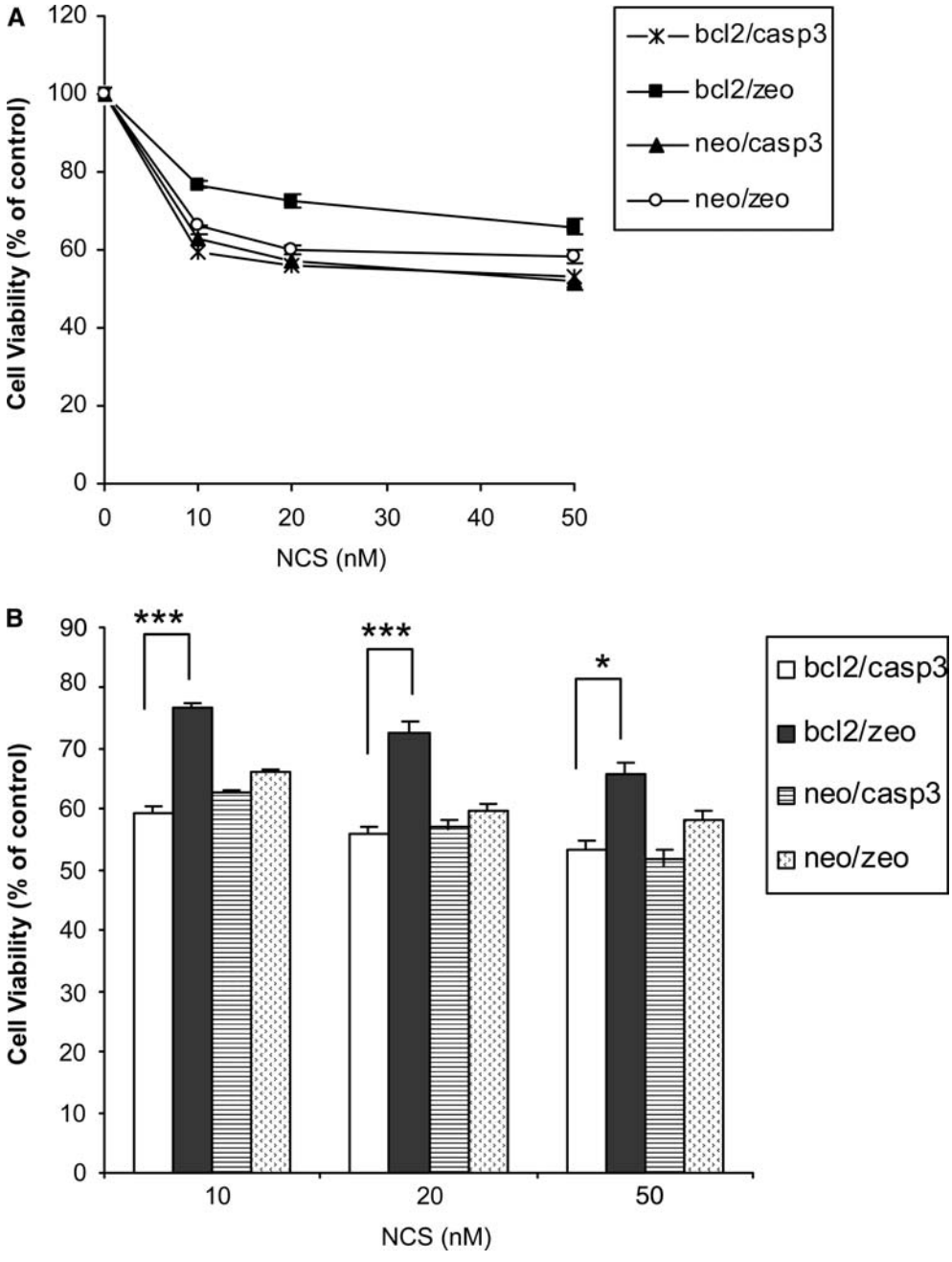


Fig. 3 Transfection of caspase-3 potentiates NCS induced cell death in Bcl-2 overexpressing MCF-7 cells. Cell viability was determined in all four double transfectants 48 h after NCS treatment by Alamar blue assay as described in Materials and methods. Viabilities are presented as the mean ± SE for all doses in three independent experiments (**a**). Transfection of caspase-3 resulted in a significant decrease of cell viability in *bcl-2*-transfected MCF-7 cells (bcl-2/casp3) as compared with Bcl-2-MCF-7 cells transfected with a control construct (bcl-2/zeo) at all doses (**b**). $*P < 0.05$; $***P < 0.001$

Effect of GSH content on cell susceptibility to NCS-induced death

In view of the requirement for reductive activation for antineoplastic efficacy of NCS [15–17] and potentiation of NCS-induced apoptosis [11], we examined the effects of titration of GSH with ThioGlo-1 on PC12 cells susceptibility to NCS-induced death. This differs considerably from previous studies [11] that have examined NCS efficacy in cells the GSH content of which had been driven to zero with 24 h of incubation with buthionine sulfoximine. Figure 8 demonstrates the threshold effect of small changes in GSH content on NCS efficacy. At control GSH levels, the surviving fraction of PC12 cells is reduced by 20% 24 h after treatment with 50 nM NCS. Depression of GSH content to 75% of control levels does not alter the efficacy of NCS in this regard. However, further depression of GSH content to 62% of control levels renders NCS completely ineffective in inducing death in PC12 cells.

Irreversible oxidation of cellular sulfhydryl groups after NCS activation but before caspase-3 activation prevents caspase-3 activation and Bcl-2 cleavage

Our previous studies demonstrated that depletion of cellular glutathione before NCS activation prevents

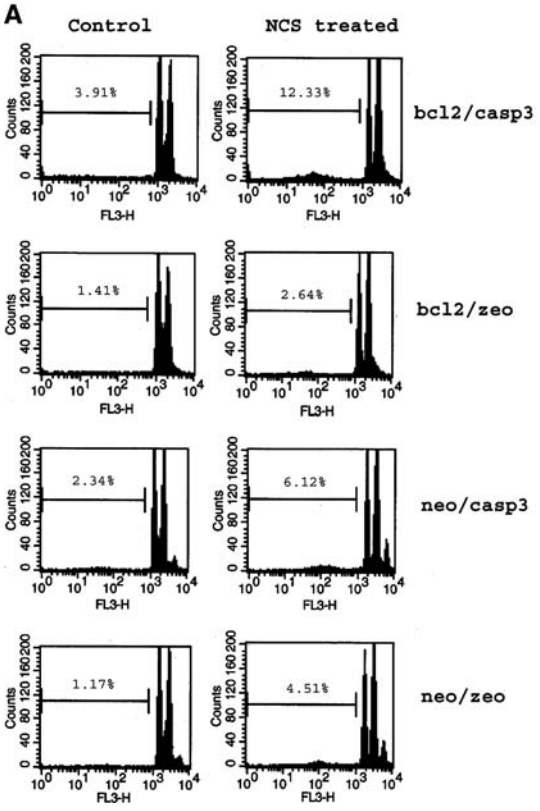


Fig. 4 Flow cytometric analysis of NCS-treated MCF-7 cells. All four double transfectants were treated with NCS (20 nM) for 1 h then rinsed with and maintained in fresh medium. After 24 h of incubation, the cells were harvested and stained with 7-AAD and then analyzed by flow cytometry. **a** A representative set of flow cytometric results from vehicle- (*left panels*, control) and NCS- (*right panels*, NCS-treated) treated MCF-7 cells that were transfected with either or both *bcl-2* and caspase-3 as indicated. Numbers in each panel represent percent cell death calculated from 10,000 cells. **b** Incidence of cell death induced by NCS in MCF-7 double transfectants. The average incidence of cell death was calculated after correction for the percent cell death in each simultaneously-run vehicle-treated sample. Although transfection with caspase-3 increased the incidence of cell death in both *bcl-2*- and mock-transfected MCF-7 cells, the increase is significantly higher in *bcl-2*-transfected cells than in mock-transfected cells. The mean ± SD for three independent experiments performed as described in **a** is shown. When the ratio of the average cell death in bcl-2/casp3 vs. bcl-2/zeo MCF-7 cells was compared with the analogous ratio in neo/casp3 versus neo/zeo MCF-7 cells using Student's *t* test, the calculated P value is 0.03, indicating that the expression of caspase-3 potentiated NCS-induced cell death in *bcl-2*-transfected MCF-7 cells

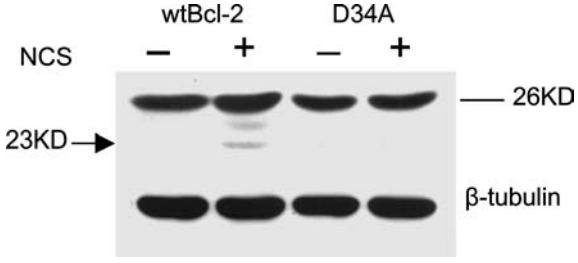


Fig. 5 Transfection of PC12 cells with an expression construct for mutant Bcl-2 [changing Asp^{34} to Ala; D34A] abolishes the NCS-induced cleavage of Bcl-2 in PC12 cells. PC12 cells that are stably overproducing wild-type Bcl-2 (wtBcl-2) and mutant Bcl-2 (D34A) were treated with NCS (20 nM; 1 h). Four hours after completion of NCS treatment, the cells were harvested and lysed for Western blot analysis. Two hundred microgram of protein was loaded in each lane. Results of staining with antibodies for Bcl-2 are shown. β-tubulin staining is used as a loading control, demonstrating that both loading and *bcl-2* overexpression are similar in the two transfectants

induction of apoptosis by NCS in PC12 cells [11]. However, the cysteine-rich nature of caspase-3 raises the question of whether reactive sulfhydryl groups are necessary for caspase-3 activation and, therefore, Bcl-2 cleavage distal to the activation of NCS. NCS activation takes place within 1 h of incubation with PC12 cells, while cleavage of Bcl-2 does not occur until 3 h after completion of incubation [14]. We therefore reasoned that oxidative inactivation of cellular sulfhydryl groups by ThioGlo-1 added in the temporal window between NCS activation and Bcl-2 cleavage would impact only the need for sulfhydryl reduction in caspase-3-mediated Bcl-2 cleavage. We hypothesized that addition of ThioGlo-1 1 h after addition and immediately following washout of NCS would prevent Bcl-2 cleavage 3 h later by preventing activation of caspase-3.

As we have shown in our previous publication [14], 4 h after initiation of NCS treatment of PC12 cells, a Bcl-2 cleavage product was detected by Western blotting (Fig. 9a). In contrast, when ThioGlo-1 was added 1 h after initiation of NCS treatment and immediately following NCS washout, no Bcl-2 cleavage product was

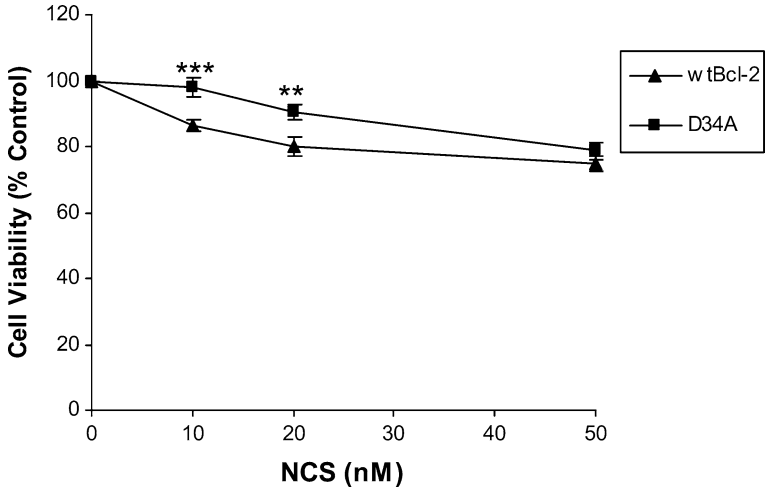


Fig. 6 PC12 cells transfected with mutant Bcl-2 (D34A) have increased cell viability after NCS treatment relative to cells transfected with wild-type Bcl-2. wtBcl-2-PC12 and D34A-PC12 cells were treated with 10, 20, and 50 nM NCS for 1 h, then rinsed with and maintained in fresh medium. Cell viability was determined 48 h after NCS treatment by Alamar blue assay as described in Materials and methods. Viability is presented as the mean ± SE for each dose of NCS in three independent experiments. Mutation of *bcl-2* resulted in a significant increase in cell viability in D34A-PC12 cells as compared with wtBcl-2-PC12 cells at 10 and 20 nM NCS. $^{**}P < 0.05$; $^{***}P < 0.001$ (Student's *t* test)

detected. Furthermore, treatment with NCS alone resulted in activation of caspase-3 as evidenced by the detection of a caspase-3 cleavage product on Western blots (Fig. 9b). On the other hand, no caspase-3 cleavage product was detected in PC12 cells that were treated with NCS followed by ThioGlo-1.

Discussion

Previous studies demonstrated that an overexpression of Bcl-2 in PC12 pheochromocytoma cells resulted in altered handling of reactive oxygen species and relative resistance to the consumption of glutathione (GSH; [12]). Our recent studies have shown that Bcl-2-overproducing PC12 cells demonstrate caspase-3-dependent cleavage of Bcl-2 to its pro-apoptotic counterpart. Blocking activation of caspase-3 abolishes both cleavage of Bcl-2 and Bcl-2-mediated potentiation of apoptosis in PC12 cells [14]. In contrast to the case for PC12 cells, MCF-7 breast cancer cells do not express caspase-3 or demonstrate Bcl-2-mediated potentiation of apoptosis [24].

In the present study, we directly tested whether the expression of caspase-3 in MCF-7 cells results in Bcl-2-mediated potentiation of NCS-induced apoptosis. Our results demonstrate that transfection of *bcl-2*-transfected MCF-7 cells with a caspase-3 expression construct results in cleavage of Bcl-2 and a sevenfold increase in the induction of apoptosis after NCS treatment relative to casp3/zeo cells ($P < 0.03$). This is consistent with the hypothesis that caspase-3 expression and caspase-3-dependent cleavage of Bcl-2 are necessary and sufficient for potentiation of apoptosis by Bcl-2.

A recent study [25] has demonstrated Bax translocation, caspase 9 activation, and cytochrome C release prior to the cleavage of Bcl-2 in *bcl-2*-transfected PC12 cells after NCS treatment, indicating that the cleavage of Bcl-2 by caspase-3 is not the initial stimulus for apoptosis, but rather an amplification mechanism for facilitating the apoptotic pathways in those cells. With regard to the molecular pathway from NCS treatment to cytochrome C release, recent studies have provided insight into the question of how DNA damage induced by chemotherapeutic drugs leads to the induction of apoptosis. Drugs such as methotrexate activate the mitochondrial/caspase 9 pathway [26], whereas other drugs, including doxorubicin primarily activate the death receptor/caspase 8 pathway [27]. In some cases, the pathway of apoptotic death is tumor cell type-dependent [28–31]. In the case of NCS treatment of native MCF-7 cells, data suggest that NCS treatment activates the mitochondrial/caspase 9 pathway, as inhibition of caspase 9, but not caspase 8, protected MCF-7 cells from NCS-induced apoptosis [24]. Although the molecular events directly downstream of NCS treatment are less well understood, Banin et al. [32] have reported enhanced phosphorylation of p53 by ATM in response to treatment with NCS in a lymphoblastoid cell line, indicating that p53 is involved in NCS-induced apoptosis in some cells. However, our previous study failed to detect p53 in both mock- and *bcl-2*-transfected MCF-7 cells before or after NCS treatment [24], suggesting involvement of a p53-independent pathway in this system. The observations that NCS treatment increased the level of p53 in HPV-positive (HeLa, INBL, and CaSki) as well as ViBo cell lines but not C33A cells [33] supports the argument that p53 involvement is cell line-dependent.

While the present study makes it clear that cleavage of Bcl-2 is critical for potentiation of NCS-induced apoptosis, the mechanism by which the Bcl-2 fragment

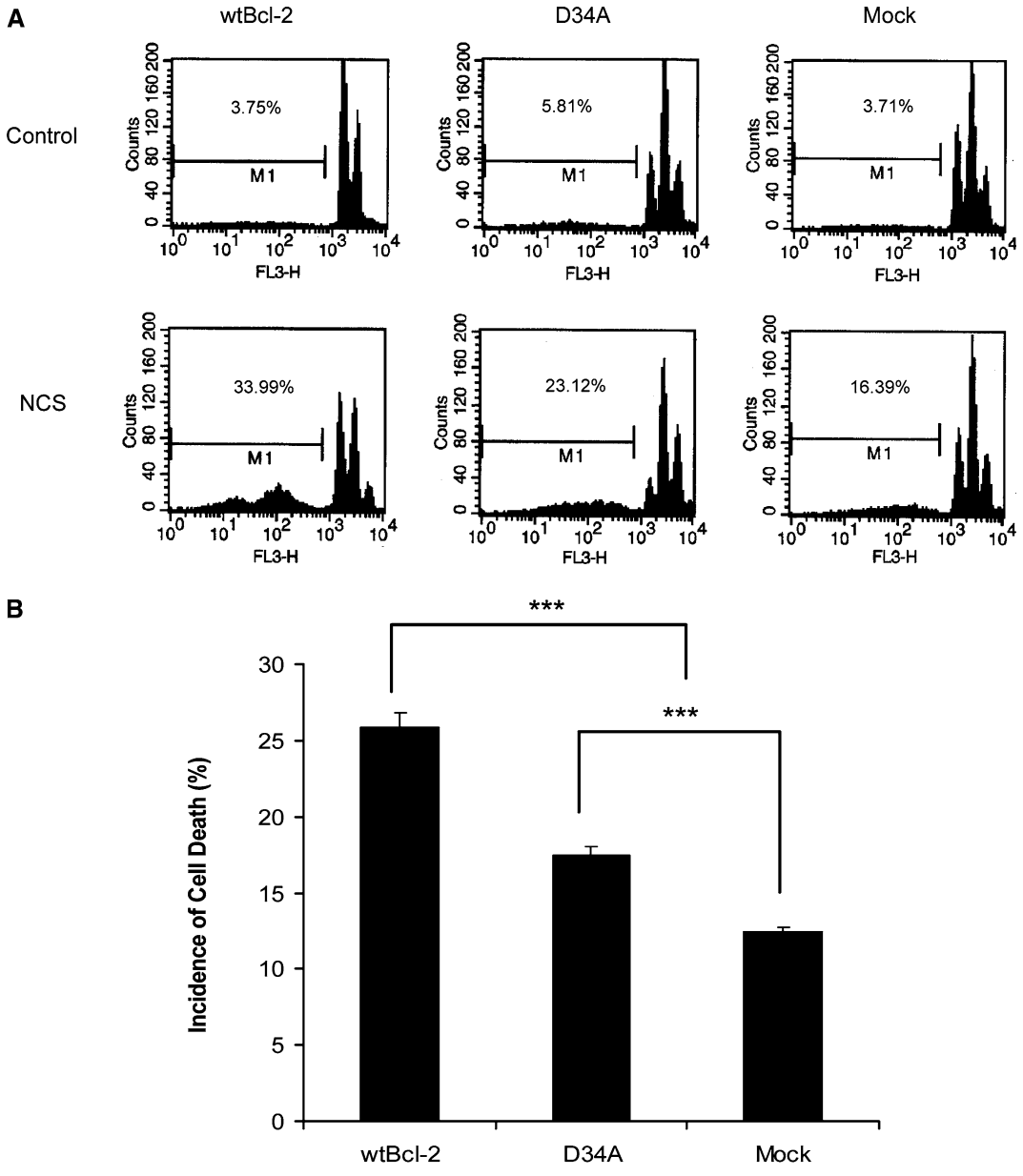


Fig. 7 Flow cytometric analysis of NCS-treated PC12 cell transfectants. PC12 cells transfected with wtBcl-2, D34A, and a mock (empty) construct were treated with NCS (20 nM) for 1 h then rinsed with and maintained in fresh medium. After 24 h of incubation, the cells were harvested and stained with 7-AAD and then analyzed by flow cytometry. **a** A representative set of flow cytometric results from vehicle- (*top panels*, control) and NCS- (*bottom panels*, NCS) treated PC12 cells that were transfected with wtBcl-2, D34A, and mock plasmids, respectively. Numbers in each panel represent percent cell death calculated from 10,000 cells. **b** Incidence of cell death induced by NCS in each set of PC12 cell transfectants. The average incidence of cell death was calculated after correction for the percent cell death in each simultaneously-run vehicle-treated sample. The mean ± SE for three independent experiments performed as described in **a** is shown. The average incidence of cell death in D34A-PC12 cells is significantly lower when compared with that seen in wtBcl-2-PC12 cells; it is still significantly higher than that seen in mock-PC12 cells treated with NCS. ***$P < 0.001$ [one-way ANOVA followed by Fisher's PLSD test]

functions as a proapoptotic protein is still unknown. One possibility is that the BH4 domain of Bcl-2 stabilizes a voltage-dependent anion channel in the mitochondria, preventing the release of cytochrome C, a critical molecule that binds to Apaf1 and caspase 9 to form an apoptosome that, in turn, leads to the activation of caspase-3 and subsequent apoptosis. Another possibility is that when the BH4 domain is cleaved, the 23 kD cleavage product of Bcl-2 may heterodimerize with Bax or allow Bax to homodimerize to release cytochrome c, as both Bcl-2 and Bax are capable of creating pores in an artificial membrane [34].

Our previous studies [11, 12] demonstrated that the potentiation of NCS-induced apoptosis by Bcl-2 is dependent on the altered metabolism of GSH. In the present study, we show that this potentiation is also

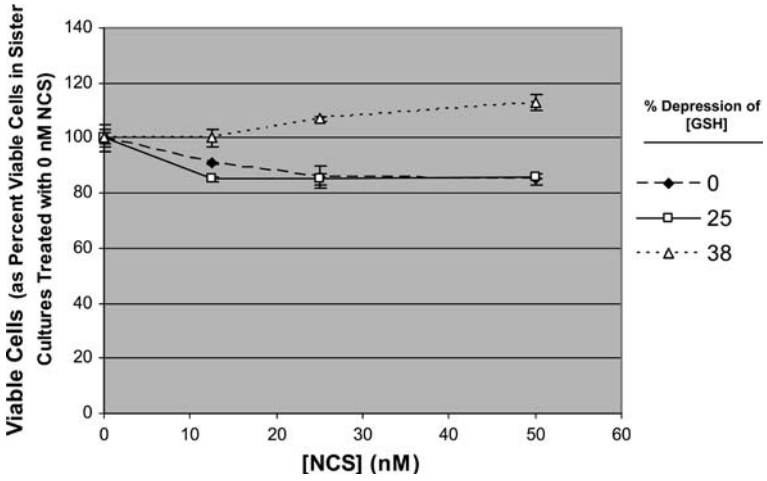
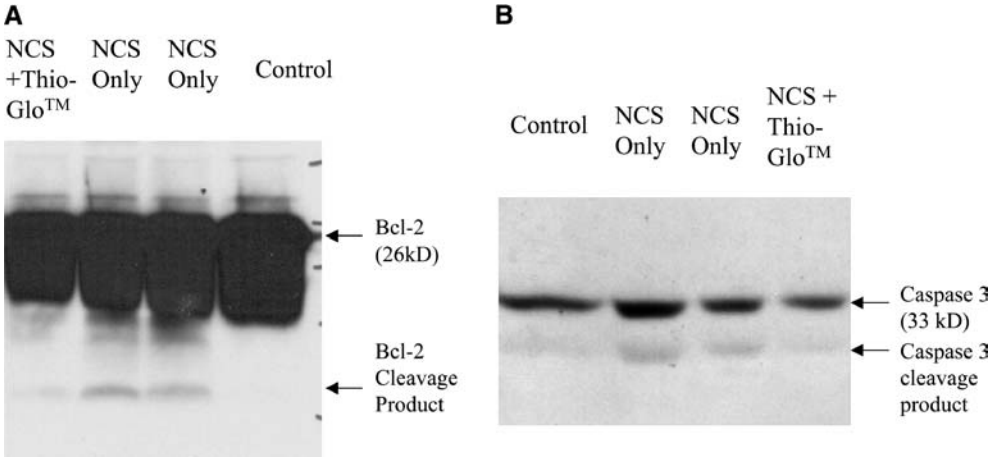


Fig. 8 Effect of GSH content on cell susceptibility to NCS-induced death. Bcl-2-PC12 cells cultured in 96-well plates were treated with 0, 1 and 2 nmols/well of ThioGlo-1, respectively, prior to the addition of NCS (10, 20, and 50 nM, 1 h). Forty-eight hours after NCS treatment, cell viability was determined using Alamar blue assay. Treatment with 1 and 2 nmol/well ThioGlo-1 resulted in depression of GSH content by 25 and 38% (i.e., to 75% and 62% of control values), respectively. Cell viability is presented as the mean ± SE for each dose of NCS at each intracellular concentration GSH in three independent experiments

dependent on caspase-3-mediated cleavage of Bcl-2. While the relationship between Bcl-2 cleavage and altered metabolic handling of GSH is not known, it is intriguing that caspase-3 activation, like NCS activation, is in some sense sulfhydryl reduction-dependent. Its active site cysteine residue must be reduced for caspase activity, and the caspase-3-activating activity of the protein thioredoxcin is proportional to the number of reduced residues in the thioredoxcin [35]. The current study demonstrates the requirement for reactive sulfhydryl groups beyond the timeframe of NCS activation. Increased reducing potential via Bcl-2 overexpression may therefore provide the impetus for increased activation of both NCS and caspase 3. Understanding the mechanistic connections among Bcl-2 overproduction, enhanced GSH turnover, Bcl-2 cleavage, and potentiation of NCS-induced apoptosis will not only facilitate the development of more efficacious pharmacological agents for *bcl-2*-overexpressing, chemoresistant cancer, but will also shed light on the regulation of apoptosis in general.

Fig. 9 **a** *bcl-2*-transfected PC12 cells were treated with 20 nM NCS for 1 h, while the control cells were untreated. Some of the NCS-treated cells were then treated with 80 nmol of ThioGlo-1. Samples were run on an 8–16% acrylamide gradient gel and immunostaining with anti-Bcl-2 antibodies revealed Bcl-2 cleavage products in the NCS only-treated cells. **b** *bcl-2*-transfected PC12 cells were treated with 20 nM NCS for 1 h, while the control cells were untreated. NCS-treated cells were then treated with 80 nmol of ThioGlo-1 or an equivalent volume of vehicle. Samples were run on a 12.5% SDS gel and immunostaining with anti-caspase-3 antibodies revealed caspase-3 cleavage products in NCS only-treated cells. In both **a**and **b**, a representative blot of three performed is shown

Acknowledgements The authors thank Carmel Portugal and Karen D. Nylander for expert technical assistance in the performance of the studies described. They also thank Dr. Guodong Cao for his help in making the caspase-3 expression plasmid. These studies were supported by a grant from the National Institutes of Health (R01-CA74289) and the Carol Ann Craumer Endowment Fund of the Children's Hospital of Pittsburgh Foundation.



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