Inhibition of Glutathione Synthesis Overcomes Bcl-2-Mediated Topoisomerase Inhibitor Resistance and Induces Nonapoptotic Cell Death via Mitochondrial-Independent Pathway

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Abstract

Bcl-2 protein plays a critical role in inhibiting anticancer drug–induced apoptosis. We found that Bcl-2 overexpression is associated with a nearly 3-fold increase in cellular glutathione levels and with increased resistance to cell death after treatment with etoposide or SN-38, a derivative of camptothecin, in leukemia 697 cells with wild-type p53. Treatment of Bcl-2-overexpressing 697 cells (697-Bcl-2) with buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis, reduced cellular glutathione levels and completely abolished Bcl-2-mediated drug resistance. Morphologic studies revealed that nonapoptotic cell death was induced in 697-Bcl-2 cells after treatment with BSO plus etoposide or SN-38. Activation of caspase-3/7 and cytochrome c release could not be detected in 697-Bcl-2 cells after these drug treatments. Notably, we showed that proteasome-mediated down-regulation of Puma and Noxa proteins occurs in 697-Bcl-2 cells after treatment with BSO plus topoisomerase inhibitor, although there is an increase in the protein levels of p53 in these 697-Bcl-2 cells. In contrast, parental 697 cells underwent typical apoptosis with up-regulation of Puma and Noxa proteins, followed by cytochrome c release and caspase-3/7 activation after treatment with topoisomerase inhibitor in the presence or absence of BSO. Our data suggest that BSO may possess a unique activity to overcome Bcl-2-mediated drug resistance by stimulating the signals that can bypass mitochondrial process in Bcl-2-overexpressing cells. (Cancer Res 2006; 66(11): 5772-80)

Introduction

The most challenging hurdle to the treatment of cancer is the resistance of tumor cells to currently used chemotherapeutic drugs. The fact that defects in apoptosis can promote drug resistance downstream of the drug-target interaction strongly suggests a causal relationship between apoptosis and drug-induced cytotoxicity (1). The expression of genes that regulate apoptotic cell death plays an important role in determining the sensitivity of tumor cells to chemotherapy. In this context, up-regulation of the antiapoptotic Bcl-2 family members in tumors has been associated with drug resistance (2–6). High expression of the antiapoptotic protein Bcl-2 is found in a number of human hematologic malignancies and solid tumors (7, 8). The functional blockade of Bcl-2 or other antiapoptotic proteins, such as Bcl-xL, could either induce apoptosis in cancer cells or sensitize these cells for chemotherapy (9, 10). Thus, antiapoptotic members of the Bcl-2 family have attracted intensive interest in drug discovery to develop a new class of anticancer agents.

It has been reported that the action of Bcl-2 is related to antioxidant protection against cellular damage (11–14). Previous studies have shown that Bcl-2 overexpression raises cellular glutathione levels (15–17). Glutathione is the most abundant intracellular thiol, present at concentration of 0.1 to 10 mmol/L, and serves as a critical cellular antioxidant (18, 19). Glutathione is required for the maintenance of protein sulfhydril groups in a reduced state and for resistance to oxidative stress through detoxification of reactive oxygen species (ROS). Glutathione can detoxify alkylating agents, including cisplatin, through the formation of glutathione adducts (20, 21). It is known that depletion of glutathione by incubation with buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, increases the cytotoxicity of alkylating drugs (22, 23). It is possible to consider that the increased glutathione levels in Bcl-2-overexpressing cells may contribute to the resistance to chemotherapeutic agents. However, Meurette et al. (24) recently reported that Bcl-2-mediated antiapoptotic effect occurs independently of intracellular glutathione level. Thus, it remains unclear whether glutathione contents in Bcl-2-overexpressing cells are actually involved in its antiapoptotic function.

It is known that topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide induce covalent protein DNA complex formation (25, 26). The signaling pathway from topoisomerase inhibitor–induced DNA damage to irreversible apoptotic commitment has not been fully defined, although ROS produced by topoisomerase inhibitor may be involved in this process (27, 28). Overexpression of Bcl-2 or related antiapoptotic Bcl-2 family members can lead to drug resistance, suggesting that the mitochondrial events regulated by Bcl-2, including cytochrome c release, are critical events of the DNA damage–initiated apoptotic pathway (8). Apoptosis provoked by DNA damage requires the p53 tumor suppressor, but which of the many p53-regulated genes are required has remained unknown. Recently, Noxa (29) and Puma (Bbc3; refs. 30–32) merit particular attention because BH3-only proteins [the proteins related to the Bcl-2 family only by the BH3 (Bcl-2 homology region 3) interaction domain] are essential triggers for the evolutionarily conserved path to apoptosis.

In the present study, we showed that introduction of Bcl-2 into human B-cell leukemia 697 cells is associated with up-regulated intracellular content of glutathione. This Bcl-2-overexpressing 697 cells show increased resistance to cell death after treatment with etoposide or SN-38, a derivative of camptothecin. It was shown here that BSO, an inhibitor of glutathione synthesis, normalized...
glutathione levels in 697-Bcl-2 cells and completely abolished Bcl-2-mediated drug resistance. Interestingly, we found that BSO plus topoisomerase inhibitor induced nonapoptotic cell death only in Bcl-2-overexpressing 697 cells, but not in parental 697 cells. We also investigated the mechanism of nonapoptotic cell death in Bcl-2-overexpressing cells from the aspect of the regulation of p53 target genes, such as Puma (p53 up-regulated modulator of apoptosis) and Noxa. Notably, we showed that proteasome-mediated down-regulation of Puma and Noxa proteins occurs in Bcl-2-overexpressing cells after treatment with BSO plus topoisomerase inhibitor. These Bcl-2-overexpressing cells underwent nonapoptotic cell death without cytochrome c release and caspase-3/7 activation. Taken together, our data suggest that inhibition of glutathione synthesis by BSO may overcome Bcl-2-mediated drug resistance by stimulating the signals that can bypass mitochondrial process in Bcl-2-overexpressing cells.

Materials and Methods

Cell culture. Cells (697) are a cloned human pre-B leukemic cell line derived from acute lymphoblastic leukemia, which carries the wild-type p53 (wt-p53; ref. 33). The 697 cells are also termed EU-3 in the literature. The 697-Neo and 697-Bcl-2 cells are infected with control retrovirus and recombinant Bcl-2 retrovirus, respectively (34). Cells were cultured in RPMI 1640 supplemented with 10% FCS (Sigma, St. Louis, MO) at 37°C under 5% CO2 in a humidified atmosphere. Exponentially growing cells were exposed to drugs for the indicated time periods.

Chemicals and antibodies. Etoposide was a kind gift from Nippo Kayaku, Co., Ltd. (Tokyo, Japan). SN-38 was obtained from Daiichi Sankyo, Co., Ltd. (Tokyo, Japan). BSO, propidium iodide, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. Ac-DEVD-MCA, Ac-VEID-MCA, and MG-132 were obtained from Peptide Institute (Osaka, Japan). Carboxy-2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). Antibodies used for immunoblotting were purchased as follows: rabbit anti-human Puma and monoclonal anti-p53 antibodies were from Sigma; mouse anti-human Noxa antibody was from Imgenex (San Diego, CA); mouse anti-human Bcl-2 antibody (clone 124) was from DakoCytomation (Glostrup, Denmark); rabbit anti-human topoisomerase I antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-human topoisomerase II a/β was from Cell Signaling Technology (Danvers, MA); and rabbit anti-human Bax antibody was from Upstate Biotechnology (Lake Placid, NY). Purified mouse anti-human cytochrome c monoclonal antibody was from PharMinGen (San Diego, CA).

Cytotoxicity assays. Cytotoxicity of topoisomerase inhibitor (etoposide or SN-38) was evaluated using a tetrazolium 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) colorimetric assay. All assays were done in triplicate and all experiments were done multiple times. Cells were incubated at 37°C in the continuous presence of drug for 24 hours. After drug treatment, cells were incubated at 37°C with XTT reagent for 4 hours and processed according to the instructions of the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

Assessment of cytotoxicity to CD34+ progenitor cells. Bone marrow CD34+ cells were obtained from Cambrex Corporation (New Jersey, PA). CD34+ cells were cultured in StemPro-34 medium with supplement (Invitrogen, Carlsbad, CA) plus the interleukin-3 (50 ng/mL), stroma cell factor (100 ng/mL), and granulocyte macrophage colony-stimulating factor (25 ng/mL; Sigma-Aldrich Japan, Tokyo, Japan). CD34+ cells were pre-cultured at 37°C for 2 days to stimulate growth. Then, BSO (50 μmol/L) was added to StemPro-34 medium for 16 hours. CD34+ cells were further incubated at 37°C with etoposide or SN-38 in the presence of BSO for 24 hours. The cell number of control group increased by 1.7-fold for 24 hours. The growth rates of CD34+, 697-Neo, and 697-Bcl-2 cells were almost same. After drug treatment, cytotoxicity was measured using XTT reagent as described above.

Figure 1. Treatment of 697-Bcl-2 cells with BSO abolishes Bcl-2-mediated drug resistance. A, Western blot analysis of Bcl-2, topoisomerase I, and topoisomerase II a/β proteins. Total cellular protein extracts were prepared from untreated 697-Neo or 697-Bcl-2 cells for Bcl-2 protein analysis. In the case of topoisomerase I and topoisomerase II a/β protein analysis, 697-Bcl-2 cells were untreated (control) or treated with BSO (50 μmol/L) at 37°C for 16 hours. Cell extracts were subjected to Western blotting. Actin was used as loading control. B, Bcl-2 overexpression increases glutathione concentration in 697 cells. Total cellular glutathione from control transfectants (697-Neo) and Bcl-2 transfectants (697-Bcl-2) were measured as described in Materials and Methods. +BSO, mean glutathione content in 697-Neo or 697-Bcl-2 cells incubated with 50 μmol/L BSO for 16 hours. Columns, mean from three separate experiments; bars, SD. C, effect of BSO treatment on the cytotoxicity of etoposide (VP-16) or SN-38 in 697-Neo or 697-BSO cells. 697-Neo or 697-BSO cells were preincubated with BSO (50 μmol/L) for 16 hours. Then, cells were incubated with various concentrations of etoposide or SN-38 at 37°C for 24 hours. Cell survival rate was determined by XTT assay. Cell survival after etoposide exposure in the presence (A, 697-Neo; B, 697-Bcl-2) or absence (Δ, 697-Neo; C, 697-Bcl-2) of BSO (left). Cell survival after SN-38 exposure in the presence (A, 697-Neo; B, 697-Bcl-2) or absence (Δ, 697-Neo; C, 697-Bcl-2) of BSO (right). Points, mean from three separate experiments; bars, SD. D, effect of BSO treatment on the cytotoxicity of etoposide or SN-38 in CD34+ progenitor cells. CD34+ cells were cultured in StemPro-34 medium with various cytokines as described in Materials and Methods. Cells were preincubated with BSO (50 μmol/L) for 16 hours. Then, cells were incubated with various concentrations of etoposide or SN-38 at 37°C for 24 hours. Cell survival rate was determined by XTT assay. Cell survival after etoposide exposure in the presence (●) or absence (□) of BSO (left). Cell survival after SN-38 exposure in the presence (●) or absence (□) of BSO (right). Points, mean from three separate experiments.

Morphologic examination by confocal laser scanning microscope. Propidium iodide (50 μmol/L) or DAPI (2 μg/mL) was directly added to culture flask after drug treatment under nonpermeabilized conditions. Cell morphology was analyzed by confocal laser microscopy (TCS-SP2-AOBS, Leica Microsystems, Wetzlar, Germany). Under these conditions, membrane-damaged cells exhibited orange fluorescence-derived from
propidium iodide. In the case of DAPI staining, nonmembrane-damaged cells (normal or apoptosis) were also stained.

**Measurement of cellular glutathione content.** Total cellular glutathione content was measured by the method of Tietze (35), with a minor modification. Briefly, 5 × 10^6 cells were prepared and washed twice with cold PBS and suspended in 125 mM/L sodium phosphate buffer containing 6.3 mM/L EDTA (pH 7.5). After sonication, 12% 5-sulfosalicylic acid was added to the cell lysates, and the mixture was allowed to precipitate for 2 hours at 4°C. After centrifugation at 10,000 × g for 15 minutes, protein-free lysates were obtained. The reaction mixture for determination of reduced glutathione (GSH) content consisted of lysates, 0.3 mM/L NADPH, 6 mM/L 5,5′-dithiobis-(2-nitrosoeic acid), and 0.5 unit of GSH reductase. The absorbance at 412 nm was monitored for 6 minutes using a plate reader (SPECTRA Max250; Molecular Devices, Sunnyvale, CA).

**Measurement of caspase activity.** Activity of caspase-3/7 and caspase-6 was analyzed by the cleavage of the fluorometric substrates Ac-DEVD-MCA and Ac-VEID-MCA, respectively. Cell lysates were prepared by washing cells thrice in ice-cold PBS (without Ca^2+ and Mg^2+) and then incubating the cells for 15 minutes on ice at a density of 1 × 10⁷/mL in TKM buffer [50 mM/L Tris-HCl (pH 7.5), 5 mM/L MgCl₂, and 50 mM/L KCl] containing 0.25% Triton X-100. The cell lysates were centrifuged (10,000 × g for 2 hours), and the mixture was allowed to precipitate for 2 hours at 4°C and the pellets were discarded. The supernatants were aliquoted and frozen at −80°C. Enzyme reactions were done in 96-well plates with 100 μL cell lysates, and DEVD-MCA or VEID-MCA (final concentration of 20 μM/L) as described previously (36). Each sample was seeded in triplicate. After incubation at 37°C for 90 minutes, 7-amino-4-methyl coumarin released from the substrates was measured using a fluorescence microplate reader.

**DNA fragmentation assay.** The cells were lysed in a solution containing 500 mM/L Tris-HCl (pH 8.0), 20 mM/L EDTA, 10 mM/L NaCl, 1% (w/v) SDS, and 100 μg/mL proteinase K at 37°C for 48 hours. DNA was extracted by standard phenol-chloroform-isooamyl alcohol extraction procedures as described previously (37) and treated with 100 μg/mL RNase A at 37°C for 30 minutes. DNA samples were separated on 1.2% agarose gel electrophoresis. Gels were stained with ethidium bromide.

**Detection of ROS.** ROS generation was measured by using a fluorescent probe, DCFH-DA, as described previously (38). After drug treatment, cells were incubated with 20 μM/L DCFH-DA for 12 minutes at 37°C. The cells were then washed twice with PBS and analyzed by EPICS X1 flow cytometer (Coulter, Hialeah, FL).

**Immunoblotting.** Cells were washed in cold PBS and lysed in Laemmli buffer containing protease inhibitors (3 μg leupeptin, 3 μg aprotinin, and 2 μmol/L phenylmethylsulfonyl fluoride). Cell lysates were electrophoresed at 125 V in 12% SDS-polyacrylamide gels. After transfer to Immobilon-P membranes (Millipore, Bedford, MA), membranes were blocked overnight in PBS-Tween containing 5% nonfat dried milk, probed for 1 hour with primary antibody, and probed for 1 hour with secondary antibody (1:1,000 dilution). Visualization was achieved using enhanced chemiluminescence (SuperSignal West Pico Substrate, Pierce, Rockford, IL) according to the instructions of the manufacturer. Band intensities were semiquantitatively analyzed using the AlphAEase FC software (Alpha Innotech Corporation, San Leandro, CA).

**Quantitative real-time reverse transcription-PCR analysis.** Total cellular RNA was prepared using RNAeasy minikit (Qiagen, Hilden, Germany). RNA was reverse transcribed to cDNA using cDNA superscript preamplification system (Invitrogen) with oligo(dT) as a primer. To assess mRNA expression of Puma and Noxa, a quantitative real-time PCR was done using SYBER premix Ex Taq (Takara, Kyoto, Japan) in a Light Cycler (Roche Diagnostic). The primer pair used for amplifying Puma was a forward primer, 5′-CGACCCTAACGCACGATCAGA-3′ and a reverse primer, 5′-GGCAACTTAAATGGGCTCTCCAC-3′. The primer pair used for amplifying Noxa composed of a forward primer, 5′-ATTACCCTGTCGCTACTCTGGAAG-3′, and a reverse primer, 5′-CAATGTCGTGACGAGCTACTCG-3′. A standard curve was generated for each primer pair as well as for glyceraldehyde-3-phosphate dehydrogenase to which gene expression levels were normalized. Finally, a ratio was calculated comparing normalized gene expression values in treated versus untreated control for each cell sample.

**Results**

Bcl-2 overexpression significantly reduces the cell death after etoposide or SN-38 exposure in 697 cells. Human pre-B leukemia cell line 697 was transduced using retroviral vector designed to express human Bcl-2 (34). Immunoblot analysis showed ~15-fold elevated levels of Bcl-2 protein in 697-Bcl-2 cells (Fig. 1A), comparable with the levels found in cell line SU-DHL-4 with t(14; 18) that deregulates Bcl-2 (34). Human leukemia 697 cells possess wt-p53 gene (33). Previous data indicated that this cell line was highly sensitive to killing by a variety of chemotherapeutic agents.
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Figure 3. Generation of ROS in 697-Neo and 697-Bcl-2 cells incubated with topoisomerase inhibitor with or without BSO. 697-Neo cells (A and B) or 697-Bcl-2 cells (C and D) were incubated with etoposide (1 μmol/L; A and C) or SN-38 (0.1 μmol/L; B and D) at 37°C for 4 hours in the presence or absence of 50 μmol/L BSO. BSO was added to culture medium 16 hours before etoposide or SN-38 treatment. ROS generation was measured by using a fluorescent probe, DCFH-DA, as described in Materials and Methods.

Inhibiting drug (34). Our results showed that 697 cells were also sensitive to etoposide and SN-38 (Fig. 1C). To examine the capacity of Bcl-2 to inhibit cell death after etoposide or SN-38 exposure, 697-Neo and 697-Bcl-2 cells were incubated in the presence of variable concentrations of etoposide or SN-38, and cell viability was monitored by XTT assay. Bcl-2 was found to markedly reduce the cell death after etoposide or SN-38 exposure (Fig. 1C).

Measurement of cellular glutathione levels in 697-Neo and 697-Bcl-2 cells. It has been reported that cellular glutathione level is an important determinant for the sensitivity of anticancer agents. In addition, Bcl-2 overexpression may increase cellular glutathione contents (15–17). Thus, we measured the intracellular levels of total glutathione in 697-Neo and 697-Bcl-2 cells. As shown in Fig. 1B, Bcl-2 overexpression in 697-Bcl-2 cells was associated with a nearly 3-fold increase in cellular glutathione levels.

BSO pretreatment abrogates Bcl-2-mediated drug resistance in 697-Bcl-2 cells. We investigated whether elevated cellular glutathione levels may cause drug resistance in 697-Bcl-2 cells. BSO can reduce intracellular glutathione level through inhibition of γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione synthesis. Indeed, BSO treatment reduced glutathione levels in 697-Neo cells. However, reduction of cellular glutathione by BSO was particularly evident in 697-Bcl-2 cells (Fig. 1B). XTT assays were done after BSO plus topoisomerase inhibitor treatment. As shown in Fig. 1C, pretreatment of 697-Bcl-2 cells with BSO almost completely abrogated Bcl-2-mediated etoposide or SN-38 resistance. In contrast, BSO slightly potentiated the cytotoxicity of etoposide or SN-38 in 697-Neo cells (Fig. 1C). Treatment with BSO alone did not affect the cell growth of 697-Neo or 697-Bcl-2 (data not shown). It has been known that the cytotoxic effects of SN-38 and etoposide are closely associated with the cellular levels of topoisomerase I and topoisomerase II, respectively. We examined the possibility whether BSO pretreatment may increase the cellular levels of topoisomerase I and topoisomerase II α/β in 697-Bcl-2 cells. Western blotting revealed that there were no significant changes in the protein amounts of topoisomerase I and topoisomerase II α/β in 697-Bcl-2 cells after BSO exposure (Fig. 1A). Next, we investigated the effect of BSO on the cytotoxicity of etoposide and SN-38 in normal CD34+ progenitor cells. We observed a minor enhancement of the cytotoxicity of etoposide and SN-38 by BSO pretreatment in normal CD34+ cells (Fig. 1D). These data indicate that BSO pretreatment is particularly effective in Bcl-2-overexpressing leukemia cells with high levels of glutathione.

BSO plus etoposide or SN-38 treatment induces cell death without caspase-3/7 activation in 697-Bcl-2 cells. Next, we examined the mode of cell death induced by BSO plus etoposide or SN-38 treatment. The cell membrane integrity was determined using trypan blue dye exclusion method. When 697-Bcl-2 cells were incubated with etoposide for 9 hours in the presence of BSO, ~23% of cells died. After 12-hour treatment, cell viability rapidly decreased to 22% (Fig. 2A). Similar results were obtained in the case of SN-38 (Fig. 2A). It is known that activation of caspase-3/7 is an important event during apoptosis. We measured the activities of caspase-3/7 in the cell lysates from etoposide- or SN-38-treated cells. As shown in Fig. 2B, caspase-3/7 activity increased in 697-Neo cells after treatment with etoposide or SN-38 in a time-dependent manner. However, we could not detect significant increase in caspase-3/7 activity in 697-Bcl-2 cells treated with etoposide or SN-38 in the presence or absence of BSO (Fig. 2B), although a rapid decrease in cell viability could be found in these cells after treatment with BSO plus etoposide or SN-38 (Fig. 2A). Caspase-6 is known as another executioner caspase. We also measured the caspase-6 activity. Activation of caspase-6 was observed only in 697-Neo cells, but not in 697-Bcl-2 cells after these drug treatments.
We hypothesized that glutathione depletion by BSO may facilitate ROS accumulation in cells treated with etoposide or SN-38. ROS generation was determined by measuring the H$_2$DCF-DA-derived fluorescence. In parental 697-Neo cells, an increase in ROS generation was observed after treatment with etoposide (Fig. 3A) or SN-38 (Fig. 3B) in the absence of BSO. Minor enhancement of ROS generation by BSO pretreatment was detected in 697-Neo cells incubated with etoposide (Fig. 3A) or SN-38 (Fig. 3B). In the case of 697-Bcl-2 cells, we observed only a slight increase in ROS levels after exposure to etoposide (Fig. 3C) or SN-38 (Fig. 3D). However, a significant increase in ROS generation in 697-Bcl-2 cells treated with etoposide (Fig. 3C) or SN-38 (Fig. 3D) was caused by preincubation with BSO. These data indicate that augmentation of ROS generation by BSO is particularly evident in 697-Bcl-2 cells incubated with etoposide or SN-38.

Up-regulation of p53, Puma, and Noxa in 697-Neo cells after treatment with etoposide or SN-38 in the presence or absence of BSO. Bcl-2 family proteins are essential regulators of apoptosis in various experimental models. In particular, the BH3-only proteins Noxa and Puma are known to contribute directly to mitochondrial membrane depolarization (29–31), followed by release of cytochrome c and apoptosis. Using anti-Puma and Noxa antibody, Western blot analysis was done. Interestingly, isoform Puma-α protein levels increased significantly in 697-Neo cells 5 hours after etoposide treatment (Fig. 4A). In addition, an increase of Noxa protein could be detected in 697-Neo cells after etoposide treatment (Fig. 4A). We also observed the up-regulation of Bax protein in 697-Neo cells after drug treatment (Fig. 4A). In the case of SN-38 treatment, induction of Puma-α, Noxa, and Bax were also detected after 5-hour treatment (Fig. 4B). Notably, both etoposide and SN-38 induced up-regulation of Puma and Noxa at very low concentrations. As shown in Fig. 4C, similar up-regulations of Puma and Noxa were detected in 696-neo cells incubated with BSO plus etoposide. Real-time reverse transcription-PCR (RT-PCR) analysis also revealed the up-regulation of Puma and Noxa mRNA (Fig. 5A and B) in 697-Neo cells incubated with etoposide. We also tested whether p53 protein may increase after treatment with etoposide or SN-38, because 697 cells possess wt-p53 gene (33). As shown in Fig. 4D, up-regulation of p53 protein could be detected in etoposide or SN-38-treated 697-Neo cells.

Down-regulation of Puma and Noxa protein levels and its inhibition by proteasome inhibitor MG-132 in 697-Bcl-2 cells treated with BSO plus etoposide or SN-38. It was observed that treatment of 697-Bcl-2 cells with BSO plus etoposide or SN-38 induces cell death without caspase-3/7 activation. We hypothesized that induction of cell death without caspase-3/7 activation may be because of the absence of up-regulation of Puma and Noxa, and deficient in cytochrome c release into cytosol in 697-Bcl-2 cells incubated with BSO plus etoposide or SN-38. Using Western blotting, we examined the changes of the protein amounts of

![Figure 4](https://www.aacrjournals.org) Immuno blotting analysis. A and B, effect of etoposide or SN-38 treatment on p53, Puma, Noxa, and Bax protein levels in 697-Neo cells. Cells were untreated or treated with etoposide (1 μmol/L; A) or SN-38 (0.1 μmol/L; B) at 37°C for 5 or 10 hours. Cell extracts were subjected to Western blotting. C, effect of BSO plus etoposide treatment on p53, Puma, and Noxa protein levels in 697-Neo cells. Cells were untreated or treated with etoposide (1 μmol/L) at 37°C for 7 hours in the presence or absence of BSO. BSO was added 16 hours before etoposide treatment. Cell extracts were subjected to Western blotting. D, effect of etoposide treatment on p53, Puma, Noxa, and Bcl-2 protein levels in 697-Bcl-2 cells. Cells were untreated or treated with 1 μmol/L etoposide in the absence or presence of 50 μmol/L BSO. BSO was added 16 hours before etoposide treatment. In addition, the effect of MG-132 on p53, Puma, Noxa, and Bcl-2 protein levels in 697-Bcl-2 cells was examined. Cells were treated with etoposide (1 μmol/L) plus MG-132 (20 μmol/L) in the presence of BSO. Western blotting was done as described in Materials and Methods. A representative example of three independent experiments giving very similar results is shown. After Western blotting, band intensities of Puma and Noxa were semiquantitatively analyzed using the AlphaEase FC software (Alpha Innotech). The relative densitometric intensities of the signals are shown above the corresponding bands. Results are mean values from three separate experiments.
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Puma, Noxa, Bcl-2, and p53 in 697-Bcl-2 after these drug treatments. There was only a slight increase in the levels of Puma and Noxa proteins in 697-Bcl-2 cells incubated with etoposide for 5 hours in the presence or absence of BSO. BSO was added to culture medium 16 hours before etoposide treatment. After drug treatment, total RNA was prepared. The relative expression of Puma (A and C) and Noxa (B and D) mRNA was quantitated by real-time RT-PCR analysis as described in Materials and Methods. Columns, mean from three separate experiments.

BSO plus etoposide or SN-38 treatment induces nonapoptotic cell death in 697-Bcl-2 cells. We investigated whether release of cytochrome c into cytosol may occur in 697-Neo or 697-Bcl-2 cells after drug treatment. The cytochrome c release into cytosol could be detected in 697-Neo cells treated with etoposide in the presence or absence of BSO (Fig. 6A). In 697-Neo cells incubated with etoposide, we observed typical morphologic features of apoptosis, such as nuclear fragmentation and bleb formation (Fig. 6C). Similar morphologic changes were detected in 697-Neo cells incubated with BSO plus etoposide (Fig. 6D). In these cells, enlarged cell size and small nuclei without chromatin fragmentation were observed (Fig. 6C). These morphologic features are not consistent with apoptosis or necrosis. Agarose gel electrophoresis showed a smear pattern of DNA fragmentation in 697-Bcl-2 cells incubated with etoposide plus BSO (Fig. 6B), although internucleosomal DNA fragmentation could be detected in 697-Neo cells treated with etoposide in the presence or absence of BSO (Fig. 6B). In addition, as shown in Fig. 5, mRNA levels of Puma and Noxa increased in 697-Bcl-2 cells incubated with etoposide plus BSO. Furthermore, up-regulation of p53 was observed in 697-Bcl-2 cells, suggesting that the mode of cell death is not passive necrosis. Thus, we...
Discussion

In the present study, we found that overexpression of Bcl-2 in 697 cells is associated with up-regulated intracellular content of glutathione and with increased resistance to cell death after topoisomerase inhibitor treatment. We also observed that BSO treatment normalized glutathione level and completely abolished Bcl-2-mediated drug resistance against SN-38 or etoposide. These data indicate that cellular glutathione may be a determinant for the sensitivity to topoisomerase inhibitor. Glutathione depletion by BSO may facilitate ROS accumulation in cells treated with topoisomerase inhibitor, which, in turn, increases their cytotoxicity. Indeed, it was shown here that enhancement of ROS generation by BSO was particularly evident in 697-Bcl-2 cells incubated with topoisomerase inhibitor. This observation may explain the selective sensitization of the 697-Bcl-2 cells by BSO to topoisomerase inhibitor.

Also of special interest is our observation that topoisomerase inhibitor plus BSO treatment induced nonapoptotic cell death only in Bcl-2-overexpressing 697 cells, but not parental 697 cells. This observation suggests that induction of nonapoptotic cell death of 697-Bcl-2 is not fully explained by the direct pharmacologic action of BSO alone. It is possible to consider that cell death pathway may be modulated by Bcl-2 overexpression in 697-Bcl-2 cells after exposure to BSO plus topoisomerase inhibitor. In the present study, the molecular mechanism of cell death in 697-Bcl-2-overexpressing cells was examined from the aspect of the regulation of p53 target genes, such as Puma and Noxa.

Human pre-B leukemia 697 cells possess wt-p53 gene (33). Although the mechanism(s) of p53-dependent apoptosis is still not completely understood, it has been established that p53 activates transcription of proapoptotic genes, including Puma, Noxa, Bax, and Bam (39). Recently, it has been reported that the BH3-only proteins Puma and Noxa are critical mediators of the apoptotic responses induced by p53 and other stimuli (40, 41). The protein encoded by Puma was found to be exclusively mitochondrial and to bind Bcl-2 and Bcl-X₁ through a BH3 domain (30, 31). Overexpression of Puma induces cytochrome c release and activates the mitochondrial process in Bcl-2-overexpressing cells. These Bcl-2-overexpressing cells underwent nonapoptotic cell death without cytochrome c release and caspase-3/7 activation. In contrast, treatment with BSO plus etoposide or SN-38 induces a significant down-regulation of Puma and Noxa proteins in Bcl-2-overexpressing cells. These Bcl-2-overexpressing cells underwent nonapoptotic cell death without cytochrome c release and caspase-3/7 activation. Inhibition of glutathione synthesis by BSO may overcome Bcl-2-mediated drug resistance by generating the signals that can bypass mitochondrial process in Bcl-2-overexpressing cells.
rapid induction of programmed cell death (30, 31). Mouse embryonic fibroblasts deficient in Noxa showed notable resistance to oncogene-dependent apoptosis in response to DNA damage. Very recently, Erlacher et al. (42) showed that Puma plays a critical role in induction of apoptosis in response to γ-radiation and glucocorticoid treatment in lymphoid cells, whereas Noxa is dispensable.

In this study, we observed an apparent up-regulation of Puma and Noxa proteins in 697-Neo cells after treatment with etoposide or SN-38. Especially, prominent up-regulation of Puma protein could be detected. These 697-Neo cells underwent typical apoptosis with cytochrome c release and caspase-3/7 activation, followed by internucleosomal DNA fragmentation. In contrast, we showed that a significant down-regulation of Puma and Noxa proteins occurs in Bcl-2-overexpressing cells after treatment with BSO plus etoposide or SN-38. These Bcl-2-overexpressing cells underwent nonapoptotic cell death without cytochrome c release and caspase-3/7 activation. Our data suggest that cotreatment with BSO plus topoisomerase inhibitor shows a unique activity to induce cell death via mitochondrial-independent pathway in Bcl-2-overexpressing cells (Fig. 6D). Induction of nonapoptotic cell death may be adequately explained by the lack of cytochrome c release and caspase-3/7 activation in 697-Bcl-2 cells. The defect of cytochrome c release may be the consequence of lack of up-regulation of Puma and Noxa. It is likely that typical apoptotic morphologic changes, such as cell shrinkage and nuclear fragmentation, may not be produced in the absence of caspase-3/7 activation in 697-Bcl-2 cells.

The molecular mechanism of cell death in Bcl-2-overexpressing cells remains unclear. To our knowledge, there has been no report to see whether expression of proapoptotic proteins Puma and Noxa may increase after DNA-damaging agent treatment in Bcl-2-overexpressing cells with wt-p53. It was shown here that up-regulation of Puma and Noxa proteins was significantly suppressed in 697-Bcl-2 cells after etoposide exposure in the absence or presence of BSO. In the case of cotreatment with BSO plus topoisomerase inhibitor, an apparent degradation of these proteins was detected. We also observed that the protein levels of p53 increased after treatment with BSO plus etoposide or SN-38 in 697-Bcl-2 cells. It is known that p53 binds the promoter region of Puma and Noxa, and transactivates these genes (29, 32). In spite of up-regulation of mRNA expression of Puma and Noxa, protein levels of Puma and Noxa were markedly down-regulated in 697-Bcl-2 cells after exposure with BSO plus etoposide or SN-38. We assumed the possibility that Noxa and Puma may be degraded by proteasomal activity. Our data showed that proteasome inhibitor MG-132 inhibits degradation of Puma and Noxa during cell death in 697-Bcl-2 cells, suggesting that levels of Noxa and Puma may be regulated by the proteasome system. Down-regulation of Puma and Noxa proteins by proteasome might occur in Bcl-2-overexpressing cells to protect mitochondria when the cells were exposed to genotoxic stress.

In conclusion, we have shown that glutathione depletion by BSO in Bcl-2-overexpressing cells alters cellular responses to etoposide and SN-38 and may provide the basis for improving the therapeutic efficacy of these agents in human leukemia cells.

Acknowledgments

Received 11/1/2005; revised 2/23/2006; accepted 3/22/2006.

Grant support: Grant-in-aid for Scientific Research (C 17590986) from Japan Society for Promotion of Science.

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We thank Sayaka Hayashi for her excellent technical assistance.

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Inhibition of Glutathione Synthesis Overcomes Bcl-2-Mediated Topoisomerase Inhibitor Resistance and Induces Nonapoptotic Cell Death via Mitochondrial-Independent Pathway

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