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PRINCIPAL INVESTIGATOR: Rita Nahta, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M. D. Anderson Cancer Center
Houston, TX 77030

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14. ABSTRACT The anti-apoptotic protein Bcl-2 is overexpressed in a majority of breast cancers, and is associated with a diminished apoptotic response and resistance to various anti-tumor agents. Bcl-2 inhibition is currently being explored as a possible strategy for sensitizing breast cancer cells to standard chemotherapeutic agents. Antisense (AS) Bcl-2 oligonucleotides represent one method for blocking the anti-apoptotic effects of Bcl-2. In this study we demonstrate that AS Bcl-2 efficiently blocks Bcl-2 expression, resulting in apoptosis of breast cancer cells. AS Bcl-2-mediated cytotoxicity was associated with induction of the B-cell translocation gene 1 (BTG1). Importantly, knockdown of BTG1 reduced AS Bcl-2-mediated cytotoxicity in breast cancer cells. Further, BTG1 expression appears to be negatively regulated by Bcl-2, and exogenous expression of BTG1 induced apoptosis. These results suggest that BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells, and that its induction contributes to AS Bcl-2-mediated cytotoxic effects.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Appendices.....	12

INTRODUCTION

Chemoresistance is a major clinical problem in the treatment of breast cancer, and is associated with decreased apoptosis in response to chemotherapeutic agents [1]. Apoptosis occurs through two major pathways- the extrinsic or cytoplasmic pathway, which is regulated by the Fas death receptor, and the intrinsic pathway, which is controlled in part by the Bcl-2 family of proteins [2,3]. Overexpression of Bcl-2 occurs in 40-80% of primary invasive breast carcinomas, depending on the methods of measurement and quantification [4-8]. Although Bcl-2 expression is associated with estrogen receptor (ER)-positive disease, which has a highly favorable prognosis, its overexpression is associated with a diminished apoptotic response and resistance to various anti-tumor agents [9-11]. Thus, therapeutic suppression of Bcl-2 levels is a feasible approach toward improving the outcome of current standard chemotherapies in breast cancer. The use of antisense (AS) Bcl-2 oligonucleotides is one approach for downregulating Bcl-2 levels and activity. AS Bcl-2 has been shown to effectively suppress Bcl-2 expression in vitro and in vivo [12-15], and to promote chemosensitization and tumor regression of human breast cancer xenografts [12,13]. We found that AS Bcl-2-mediated cytotoxicity of breast cancer cells was associated with induction of B-cell translocation gene 1 (BTG1). Our purpose was to examine the role of BTG1 in Bcl-2-regulated apoptosis and in chemosensitivity. Through this study, we observed that knockdown of BTG1 reduced AS Bcl-2-mediated cytotoxicity, suggesting that induction of BTG1 is an important molecular mechanism contributing to the cytotoxic effects of AS Bcl-2 therapy. In addition, BTG1 promoter activity was suppressed by Bcl-2, and BTG1 induced apoptosis, suggesting that BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells [16].

BODY

The research accomplishments are described below according to each task listed in the approved Statement of Work.

Task 1: Investigate the role of BTG1 in apoptosis.

The results of this aim are published [16]; the manuscript can be found in appendix 2 of this document. The major results from this publication are described below.

BTG1 is induced by antisense Bcl-2.

MCF-7 breast cancer cells transfected with the antisense (AS) oligonucleotide G3139 (GenasenseTM, Genta, Inc.), which is targeted against the first six codons of the Bcl-2 transcript, demonstrate increased cell death (Figure 1 in reference 16). Gene changes in MCF-7 cells transfected with 400 nM AS Bcl-2 G3139 versus 400 nM of a control oligonucleotide G4126, which has a two base pair mismatch in comparison to AS Bcl-2 G3139, were examined by Affymetrix gene chip array (Figure 2 in appendix 2, reference 16). Hierarchical gene cluster analysis with subsequent validation by reverse transcriptase (RT) PCR demonstrated that the B cell translocation gene 1 (BTG1) transcript was induced 3- to 4-fold by AS Bcl-2 in MCF-7 cells (Figure 2 and Table 1 in appendix 2, reference 16). Induction of BTG1 was also observed by RT-PCR in MDA231 breast cancer cells (approximately 3-fold) and in an MDA231-derived stable Bcl-2-overexpressing clone (approximately 2-fold).

BTG1 contributes to AS Bcl-2-induced cell death.

To determine if induction of BTG1 contributes to AS Bcl-2-mediated cytotoxic effects, we suppressed BTG1 expression using BTG1 siRNA and then examined effects of AS Bcl-2 on cell viability. MCF-7 cells were transfected with 400 nM control siRNA or 200 nM or 400 nM BTG1 siRNA for 48 hours (h). Western blotting demonstrated that BTG1 levels were reduced with 400 nM BTG1 siRNA, but not with 200 nM BTG1 siRNA or control siRNA (Figure 3A in appendix 2, reference 16). Hence, treatment of cells with 400 nM BTG1 siRNA for 48 h was used for subsequent experiments.

MCF-7 cells were transfected with 400 nM control siRNA or 400 nM BTG1 siRNA. After 24 h, control oligonucleotide G4126 or AS Bcl-2 G3139 was

transfected at 400 nM each. Cell viability was measured after an additional 48 h using trypan blue exclusion (Figure 3B in appendix 2, reference 16). Knockdown of BTG1 partially inhibited AS Bcl-2-mediated cell death.

To specifically assess effects on apoptosis, ELISA-based analysis of DNA fragmentation was performed. Similar to trypan blue assays, MCF-7 cells were transfected with 400 nM control siRNA or BTG1 siRNA for 24 h, then with G4126 or AS Bcl-2 for an additional 48 h. Protein lysates were then evaluated for levels of DNA fragmentation (Figure 3C in appendix 2, reference 16). Knockdown of BTG1 blocked AS Bcl-2-mediated DNA fragmentation, indicating that BTG1 contributes to AS Bcl-2-mediated apoptosis.

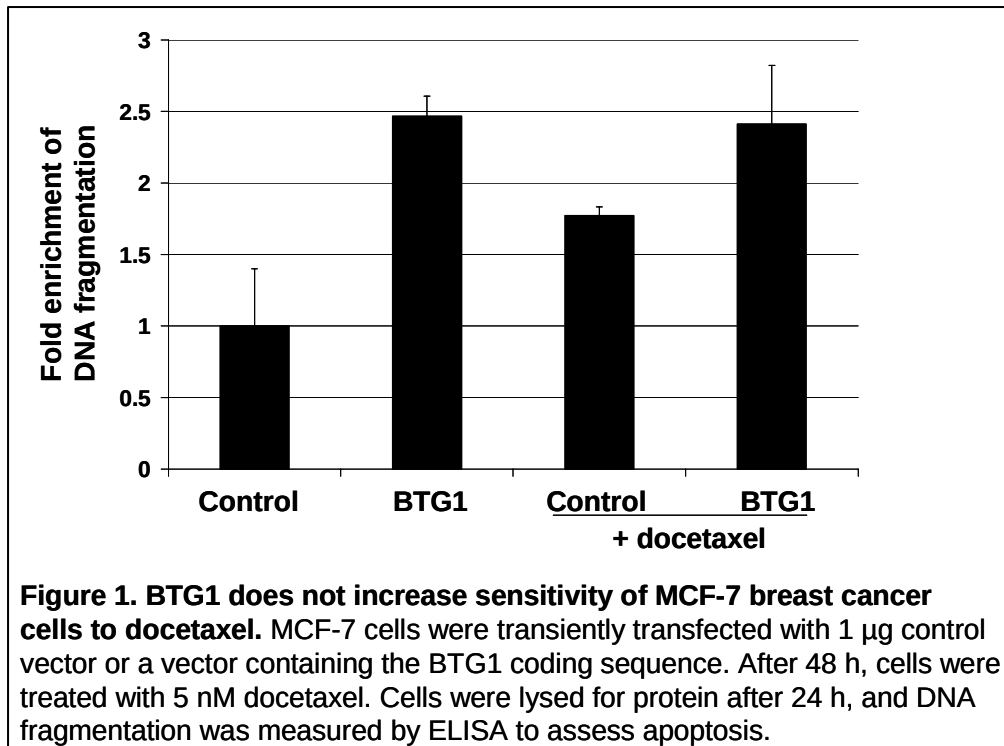
BTG1 is a Bcl-2-regulated mediator of apoptosis.

Our results demonstrating that AS Bcl-2 induces BTG1 suggest that BTG1 is negatively regulated by Bcl-2. To test this hypothesis, MDA-231-derived Neo stable control, Bcl-2-overexpressing clone 4, and Bcl-2-overexpressing clone 5 stable transfectants were transiently transfected with a BTG1 promoter-luciferase reporter construct. Increased expression of Bcl-2 in the stable transfectants resulted in an approximately two-fold reduction in BTG1 promoter activity versus Neo control cells (Figure 4A in appendix 2, reference 16). These results demonstrate that Bcl-2 suppresses BTG1 promoter activity, supporting a role for Bcl-2 as a negative transcriptional regulator of BTG1.

Our results suggest that BTG1 contributes to AS Bcl-2-mediated apoptosis. To determine if BTG1 itself induces apoptosis, MCF-7 cells were transiently transfected with a control vector or a vector containing the BTG1 coding sequence. Immunoblotting confirmed increased expression of BTG1 after 48 h transfection (Figure 4B in appendix 2, reference 16). To assess BTG1-mediated apoptosis, cells were lysed and analyzed for DNA fragmentation after 48 h transfection with BTG1 or control vector. BTG1 induced a 2.5-fold increase in fragmented DNA (Figure 4C in appendix 2, reference 16), indicating induction of apoptosis. These results support BTG1 as a novel Bcl-2-regulated mediator of apoptosis in breast cancer.

Task 2: Demonstrate that BTG1 increases the chemosensitivity of breast cancer cells.

Having established that BTG1 induces apoptosis of breast cancer cells, we proposed that BTG1 may further enhance chemotherapy-induced apoptosis. MCF-7 cells were transiently transfected with a control vector or BTG1 expression vector, and then treated with docetaxel (Figure 1). DNA fragmentation was measured as an indicator of apoptosis. While BTG1 induced a 2.5-fold increase in apoptosis, addition of docetaxel did not further enhance this level of cell death. This preliminary data may suggest that BTG1 does not increase chemosensitivity of breast cancer cells. However, several important points must be taken into consideration. 1) We tested only one concentration of docetaxel. 2) Only one cell line was examined. 3) Only one chemotherapeutic agent was assessed. Therefore, ongoing studies are examining additional concentrations of docetaxel and other agents in MCF-7 cells and in other breast cancer lines. In addition, BTG1 siRNA prior to chemotherapy treatment is being used as a strategy to determine if downregulation of BTG1 results in chemoresistance.



KEY RESEARCH ACCOMPLISHMENTS

- Identification of BTG1 as an inducer of apoptosis in breast cancer cells
- Evidence that BTG1 is negatively regulated by Bcl-2 at the transcriptional level
- Evidence that BTG1 contributes to cell death produced by antisense Bcl-2 oligonucleotides in breast cancer

REPORTABLE OUTCOMES

1. Nahta R, Yuan LXH, Fitterman DJ, Zhang L, Ueno NT, Symmans WF, and Esteva FJ. (2006) B-Cell Translocation Gene 1 (BTG1) Contributes to Antisense Bcl-2-Mediated Apoptosis in Breast Cancer Cells. *Molecular Cancer Therapeutics* 5: 1593-1601.

CONCLUSIONS

Summary

The following novel findings were demonstrated in this study. 1) Induction of BTG1 contributed at least in part to the cytotoxic activity of AS Bcl-2, as knockdown of BTG1 partially inhibited AS Bcl-2-mediated cell death. 2) BTG1 expression was negatively regulated by Bcl-2, since AS Bcl-2 induced BTG1 and overexpression of Bcl-2 suppressed BTG1 promoter activity. 3) BTG1 induced apoptosis in breast cancer cells. Thus, a model is proposed where Bcl-2 decreases *btg1* promoter activity, such that antisense oligonucleotide-mediated reduction in Bcl-2 levels results in increased BTG1 levels, which, in turn, mediates increased apoptosis of breast cancer cells. These results support BTG1 as a novel Bcl-2-regulated mediator of apoptosis in breast cancer cells, whose induction contributes to AS Bcl-2-mediated cell death.

Implications and significance

This work has identified a novel mediator of apoptosis in breast cancer cells. The implications are important, as apoptosis is one of the central processes abnormally regulated in cancers, and one of the most important pathways

targeted by anti-neoplastic agents. Correlative studies have provided preliminary evidence that BTG1 expression may also be associated with response to certain anti-cancer treatments and with less aggressive forms of cancer. For example, BTG1 was expressed in acute myeloid leukemia (AML) cells from patients in complete remission, but not in patients that did not achieve remission. Thus, BTG1 was proposed to be a marker of remission in AML [17]. BTG1 expression was also shown to correlate with an androgen-dependent state in prostate cancer, with reduced BTG1 expression observed when cells progressed to an androgen-independent state [18]. In addition, BTG1 was induced upon treatment of AML cells with retinoids [17]. Based on these findings and on the new findings presented here, further studies examining the roles of BTG1 in predicting treatment response and in cancer progression are warranted.

Future directions

Future experiments will examine apoptosis in BTG1 transfectants and BTG1 siRNA transfectants treated with chemotherapeutic agents. Stable transfectants will also be developed, as transfection efficiency varies considerably between transient transfectants. In addition to exploring the chemosensitization activity of BTG1, we will examine levels of BTG1 in breast tumors to determine if other correlates exist, for example with respect to grade, stage, or hormone receptor status. The results emanating from this Concept Award, as published in reference 16, provide the principal investigator with a good foundation on which to further build a project focused on BTG1, apoptosis, and breast cancer.

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APPENDICES

Please see the following list attached as Appendix 1:

List of personnel receiving pay from the research effort

Please see the following publication attached as Appendix 2:

Nahta R, Yuan LXH, Fiterman DJ, Zhang L, Ueno NT, Symmans WF, and Esteva FJ. (2006) B-Cell Translocation Gene 1 (BTG1) Contributes to Antisense Bcl-2-Mediated Apoptosis in Breast Cancer Cells. Mol Cancer Ther 5: 1593-1601.

SUPPORTING DATA

N/A

APPENDIX 1

List of personnel receiving pay from the research effort:

Rita Nahta, Ph.D.

APPENDIX 2

Attached Publication:

Nahta R, Yuan LXH, Fiterman DJ, Zhang L, Ueno NT, Symmans WF, and Esteva FJ. (2006) B-Cell Translocation Gene 1 (BTG1) Contributes to Antisense Bcl-2-Mediated Apoptosis in Breast Cancer Cells. Mol Cancer Ther 5: 1593-1601.

B cell translocation gene 1 contributes to antisense Bcl-2-mediated apoptosis in breast cancer cells

Rita Nahta,^{1,6} Linda X.H. Yuan,^{1,6}
Derek J. Fitterman,² Li Zhang,³
W. Fraser Symmans,² Naoto T. Ueno,^{4,6,7}
and Francisco J. Esteva^{1,5,6,7}

Departments of ¹Breast Medical Oncology, ²Molecular Pathology, ³Biostatistics, ⁴Blood and Marrow Transplantation, and ⁵Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center; ⁶Breast Cancer Translational Research Laboratory; and ⁷The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas

Abstract

The antiapoptotic protein Bcl-2 is overexpressed in a majority of breast cancers, and is associated with a diminished apoptotic response and resistance to various antitumor agents. Bcl-2 inhibition is currently being explored as a possible strategy for sensitizing breast cancer cells to standard chemotherapeutic agents. Antisense Bcl-2 oligonucleotides represent one method for blocking the antiapoptotic effects of Bcl-2. In this study, we show that antisense Bcl-2 efficiently blocks Bcl-2 expression, resulting in the apoptosis of breast cancer cells. Antisense Bcl-2-mediated cytotoxicity was associated with the induction of the B cell translocation gene 1 (BTG1). Importantly, knockdown of BTG1 reduced antisense Bcl-2-mediated cytotoxicity in breast cancer cells. Furthermore, BTG1 expression seems to be negatively regulated by Bcl-2, and exogenous expression of BTG1 induced apoptosis. These results suggest that BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells, and that its induction contributes to antisense Bcl-2-mediated cytotoxic effects. [Mol Cancer Ther 2006;5(6):1593–601]

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Requests for reprints: Rita Nahta or Francisco J. Esteva, Department of Breast Medical Oncology, Unit 1354, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: 713-792-2817; Fax: 713-563-0739. E-mail: rnahta@mdanderson.org or festeva@mdanderson.org
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Introduction

Chemoresistance is a major clinical problem in the treatment of breast cancer, and is associated with decreased apoptosis in response to chemotherapeutic agents (1). Apoptosis occurs through two major pathways—the extrinsic or cytoplasmic pathway, which is regulated by the Fas death receptor, and the intrinsic pathway, which is controlled in part by the Bcl-2 family of proteins (2, 3). This family is composed of various proapoptotic and antiapoptotic proteins that heterodimerize and modulate each other's function. Thus, the relative concentration of each Bcl-2 family member is thought to determine whether cell suicide will occur. The ratio of antiapoptotic Bcl-2 to proapoptotic Bax is a critical determinant of apoptosis, as Bcl-2 heterodimerizes with Bax, blocking apoptosis (4).

Overexpression of Bcl-2 occurs in 40% to 80% of primary invasive breast carcinomas, depending on the methods of measurement and quantification (5–9). Although Bcl-2 expression is associated with estrogen receptor–positive disease, which has a highly favorable prognosis, its overexpression is associated with a diminished apoptotic response and resistance to various antitumor agents (10–12). Thus, the therapeutic suppression of Bcl-2 levels is a feasible approach toward improving the outcome of current standard chemotherapies in breast cancer.

Therapeutic approaches targeting Bcl-2 include antisense strategies to inhibit translation of the Bcl-2 protein and small-molecule inhibitors that recognize the surface pocket of Bcl-2, blocking the interaction of Bcl-2 with the BH3 domain of related death agonists such as Bax. Antisense Bcl-2 has been shown to effectively suppress Bcl-2 expression *in vitro* and *in vivo* (13–16), and to promote chemosensitization and tumor regression of human breast cancer xenografts (13, 14). Interestingly, the quantity of endogenous Bcl-2 was not a critical determinant of antisense efficacy in breast cancer models, as cell survival and Bcl-2 levels were reduced by >80% in breast cancer lines expressing high (MCF-7) or low (MDA435/LCC6) Bcl-2 levels (15, 16).

In the present study, we sought to determine the biological effects of antisense Bcl-2 in breast cancer cells, and to investigate the molecular mechanisms associated with these effects. We found that antisense Bcl-2-mediated cytotoxicity of breast cancer cells was associated with induction of the B cell translocation gene 1 (BTG1). Knockdown of BTG1 reduced antisense Bcl-2-mediated cytotoxicity, suggesting that the induction of BTG1 is an important molecular mechanism contributing to the cytotoxic effects of antisense Bcl-2 therapy. In addition, BTG1 expression was suppressed by Bcl-2, and BTG1 induced apoptosis, suggesting that BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells.

Materials and Methods

Materials

The antisense Bcl-2 molecule used here is an 18-mer phosphorothioate DNA oligonucleotide that is complementary to the first six codons of the human Bcl-2 open reading frame (G3139, Genasense, oblimersen sodium), and the control oligonucleotide G4126 contains a 2 bp mismatch versus G3139. Antisense Bcl-2 and control oligonucleotide were provided by Genta, Inc. (Berkeley Heights, NJ) at 100 $\mu\text{mol/L}$ stock concentrations. The sequences of the oligonucleotides are as follows: Bcl-2 antisense G3139 (5'-TCTCCCAGCGTGCGCCAT-3'); mismatch control G4126 (5'-TCTCCCAGCATGTGCCAT-3'). Small interfering RNA (siRNA) against BTG1 was purchased from Dharmacon (Lafayette, CO) with the following sequence: sense strand, 5'-UUGUUGGGUCUCACACUCAA-3'; antisense strand, 5'-CAACCCAGAGUGUGAGUUCUU-3'. Negative control siRNA was purchased from Ambion (Austin, TX). OligofectAMINE transfection reagent (Invitrogen, Carlsbad, CA) was used according to the manufacturer's guidelines.

Cell Culture

Estrogen receptor-positive MCF-7 and MDA-231 (MDA-MB-231) breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). Bcl-2-overexpressing MDA-231 clones (referred to as clone 4 and clone 5 cells) and the Neo control stable transfectant were created as previously described (17). Briefly, the pCl-neo/Bcl-2 (CMV-Bcl-2) plasmid encoding the full-length Bcl-2 coding sequence or the backbone vector pCl-neo was transfected into MDA-231 cells with subsequent neomycin (G418) antibiotic selection and screening of clones by immunoblot. All cells were maintained in DMEM supplemented with 1% penicillin-streptomycin and 10% FCS.

Immunofluorescence

MCF-7, MDA-231, and MDA-231 Bcl-2-overexpressing clone 5 cells were seeded at 10,000 cells per well in chamber slides. Cells were transfected the next day using OligofectAMINE with antisense Bcl-2 oligonucleotide labeled with 6-fluorescein on the 5'-T residue (FAM-G3139) at doses ranging from 150 to 600 nmol/L. After 48 hours, cells were fixed in 4% formaldehyde, washed with PBS, and viewed by fluorescence microscopy.

Immunoblotting

Total protein lysates were obtained using 1% NP40 lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 8), 1% NP40], and immunoblotted (50 μg) with an anti-Bcl-2 monoclonal antibody (Oncogene Research Products, San Diego, CA) diluted 1:1,000, anti-BTG1 polyclonal (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:100, or an anti- β -actin monoclonal antibody (Santa Cruz Biotechnology) at 1:5,000. Secondary antibodies were chosen according to species of origin and detected using the Odyssey Imaging system (Li-Cor Biosciences, Lincoln, NE).

Analysis of Cell Death

Cell Survival Assays. MCF-7, MDA-231 parental, and MDA-231 clone 5 cells were seeded at 5×10^4 cells/well in 12-well dishes. After 24 hours, cells were transfected with

2-fold serial dilutions of mismatch control oligonucleotide or antisense Bcl-2 using OligofectAMINE reagent. Transfection control cultures were treated with OligofectAMINE alone. Cells were trypsinized after 72 hours and counted by trypan blue exclusion. For each dose, the ratio of viable antisense Bcl-2-transfected cells to viable mismatch control oligonucleotide-transfected cells was determined, and then graphed as a percentage of viable cells in the OligofectAMINE control group. All experiments were done in triplicate (at least twice). Error bars represent SD between replicates.

To establish the importance of BTG1 to antisense Bcl-2-mediated cytotoxicity, 400 nmol/L of control siRNA or 400 nmol/L of BTG1 siRNA were transfected into MCF-7 cells. After 24 hours, 400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 was transfected for an additional 48 hours, at which point cells were trypsinized and counted by trypan blue exclusion. Cell viability was determined as a percentage of the control siRNA/mismatch control oligonucleotide transfection group. All experiments were done in triplicate (at least twice). Error bars represent SD between replicates.

DNA Fragmentation Cell Death Detection ELISA. To establish the importance of BTG1 to antisense Bcl-2-mediated cytotoxicity, 400 nmol/L of control siRNA or 400 nmol/L of BTG1 siRNA were transfected into MCF-7 cells. After 24 hours, 400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 was transfected for an additional 48 hours, at which point protein lysates were obtained and analyzed for cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) representative of apoptosis using the Cell Death Detection ELISA (Roche, Indianapolis, IN) according to manufacturer guidelines. Absorbance was measured at 490 nm. The DNA fragmentation enrichment factor was determined as a ratio of absorbance per group to absorbance of the control siRNA/mismatch control oligonucleotide group. All experiments were done in triplicate, with error bars representing the SD between replicates.

To establish the effect of BTG1 on apoptosis, MCF-7 cells were transfected with an empty control vector or pXT-BTG1 expression plasmid (a gift from Dr. Cabello, Montpellier, France; ref. 18). After 72 hours, protein lysates were obtained and analyzed for cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) representative of apoptosis using the Cell Death Detection ELISA (Roche) according to manufacturer guidelines. Absorbance was measured at 490 nm. The DNA fragmentation enrichment factor was determined as a ratio of absorbance of the BTG1-transfected group to the absorbance of the control transfectant group. All experiments were done in triplicate, with error bars representing the SD between replicates.

Microarray Analysis

MCF-7 cells were transfected with 200 nmol/L of mismatch control oligonucleotide for 24 hours or 200, 400, and 600 nmol/L of antisense Bcl-2 oligonucleotide for 24 and 48 hours. Total RNA was extracted using the RNeasy

Mini Kit (Qiagen, Valencia, CA), converted to cRNA and hybridized (10 µg per sample) onto human genome array U133A (Affymetrix, Santa Clara, CA) containing ~16,900 well-characterized gene sequences. Hierarchical gene cluster analysis was done, in which genes were filtered according to a mean log expression level >5.5.

Reverse Transcriptase-PCR

MCF-7, MDA-231 parental, and clone 5 cells were transfected for 48 hours with 400 of nmol/L mismatch control oligonucleotide or 400 of nmol/L antisense Bcl-2 oligonucleotide. Total RNA was extracted using RNeasy Mini Kit (Qiagen). Expression levels of β -actin, RTP801, and BTG1 were measured by reverse transcription-PCR (RT-PCR). For the reverse transcription procedure, 200 ng of thawed RNA from the extracted RNA was combined with 0.5 µL of RNase inhibitor (Sigma, St. Louis, MO), 1.0 µL of anchored oligo (dT)₂₃ (Sigma), and 6.5 µL of RNase-free dH₂O. This mixture was incubated for 10 minutes at 70°C. This solution was then added to 2.0 µL of reverse transcriptase buffer, 1.0 µL of 5 mmol/L deoxynucleotide mix (combination of dATP, dCTP, dGTP, dTTP—5 mmol/L of each), 1.0 µL of Sensiscript reverse transcriptase (all from Qiagen Sciences, Baltimore, MD), and 6.0 µL of RNase-free dH₂O. This was followed by incubation at 25°C for 15 minutes, then 50 minutes of incubation at 42°C.

The following primers (Sigma Genosys, The Woodlands, TX) were used for PCR: RTP801 forward, 5'-GGGGTAC-CATGCCTAGCCTTGG-3'; RTP801 reverse, 3'-TAAAGCGG-CCGCTCACAACATGTCAATGAGCAGCTG-5'; BTG1 forward, 5'-ACTAGTAAGCATGACCTGGGA-3'; BTG1 reverse, 5'-ACAAAATAGATGGTGGTTTGTGG-3'; β -actin forward, 5'-GCGGGAAATCGTGCGTTGACATT-3'; β -actin reverse, 3'-GTGCTTTGATGGAAGTTGAGGTAG-5'. The PCR mixture consisted of 3.0 µL of cDNA from the above reverse transcriptase procedure, 5.0 µL of PCR buffer (100 mmol/L Tris-HCl, 500 mmol/L KCl, 15 mmol/L MgCl₂, and 0.01% gelatin; Sigma), 1.0 µL of 10 mmol/L deoxynucleotide mix (combination of dATP, dCTP, dGTP, dTTP—10 mmol/L of each; Sigma), 1.0 µL of REDTaq DNA Polymerase (Sigma), 38.0 µL of RNase-free dH₂O, 1.0 µL each of 15 mmol/L forward and reverse primers. For PCR conditions, the mixture was incubated in a PCR MasterCycler Personal (Eppendorf Scientific, Inc., Westbury, NY) with the first denaturation at 94°C for 2 minutes, the following denaturation at 94°C for 15 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and final extension at 68°C for 5 minutes for a total of 35 amplification cycles. The final PCR product was visualized in a 2% agarose gel containing ethidium bromide. Quantitation of bands was calculated as the band net intensity, and was done using the Kodak DC-290 digital camera with Kodak 1D Image Analysis software (Kodak, Rochester, NY). The band net intensity for the PCR product band of RTP801 or BTG1 was divided by the band net intensity of the β -actin PCR product band for that RNA sample run in a parallel PCR reaction at the same time to yield a relative band net intensity ratio as previously described (19).

Luciferase Reporter Assay

MDA-231 Neo, Bcl-2 clone 4, and Bcl-2 clone 5 cells were transfected with 1 µg of pGL3-BTG1 (a gift from Dr. von Lindern, Erasmus Medical Center Rotterdam, Rotterdam, the Netherlands), which encodes the -1033/+82 BTG1 promoter (and part of the first exon) upstream of the luciferase reporter gene, as previously described (20), or 1 µg of pGL3 control luciferase reporter plasmid (Promega, Madison, WI). All cells were also transfected with 1 ng of hRL4 (Promega), a *Renilla* luciferase plasmid. After 48 hours, cells were lysed and luciferase values were measured using the Dual Luciferase Reporter Kit (Promega) and luminometer. All pGL3 or pGL3-BTG1 promoter luciferase values were normalized to the *Renilla* luciferase value per group. The ratio of normalized BTG1 promoter-luciferase to normalized pGL3 control luciferase was then determined, and graphed as the BTG1 promoter-luciferase activity.

Results

Antisense Bcl-2 Down-Regulates Bcl-2 and Decreases Breast Cancer Cell Viability

The MDA-231 breast cancer cell line, stable control, and Bcl-2 transfectants derived from MDA-231 (17), and MCF-7 breast cancer cells were used for our study. Parental MDA-231 and control Neo MDA-231-derived stable transfectant cells express relatively low levels of endogenous Bcl-2, whereas MCF-7 cells overexpress Bcl-2 (Fig. 1A). The stable MDA-231-derived clone 5 cells overexpress Bcl-2 at a level similar to MCF-7 cells, whereas clone 4 cells show significantly higher levels. Using these cell lines as models of Bcl-2 overexpression in breast cancer, we studied the cellular and molecular effects of antisense Bcl-2 treatment.

Although the antisense Bcl-2 used in this study has been shown to down-regulate Bcl-2 in various breast cancer cell lines (15, 16), we wanted to ensure that the cell lines used in this study were able to internalize the antisense Bcl-2 oligonucleotide with subsequent down-regulation of Bcl-2. The breast cancer cell lines MCF-7, MDA-231 parental, and MDA-231-derived Bcl-2-overexpressing stable clone 5 were transfected with antisense Bcl-2 oligonucleotide labeled with 6-fluorescein on the 5'-T residue (FAM-G3139). After 48 hours, cells were fixed and viewed by fluorescence microscopy (Fig. 1B). All cell lines efficiently internalized antisense Bcl-2 at low concentrations (200–250 nmol/L), confirming that this antisense Bcl-2 could be delivered to breast cancer cells *in vitro* using liposome-mediated transfection.

Next, Bcl-2 down-regulation by antisense Bcl-2 was assessed by immunoblotting (Fig. 1C). MCF-7 cells showed decreased Bcl-2 levels upon transfection with 600 nmol/L of antisense Bcl-2. Similar to MCF-7 cells, concentrations of 400 to 800 nmol/L of antisense Bcl-2 reduced Bcl-2 protein levels in MDA-231 parental cells. In contrast, clone 5 cells required much higher doses (1,500 nmol/L) to down-regulate Bcl-2. Complete suppression of Bcl-2 expression was not achieved in clone 5 cells even with such high doses

of antisense Bcl-2. However, MCF-7 cells, which express similar levels of Bcl-2 as clone 5 cells, showed more significant down-regulation of Bcl-2 upon treatment with a lower dose (600 nmol/L) of antisense Bcl-2. Thus, factors other than baseline Bcl-2 levels seem to affect antisense-mediated down-regulation of Bcl-2.

The dose-response profiles of MCF-7, MDA-231 parental, and clone 5 cells to antisense Bcl-2 were obtained by trypan blue exclusion assays. Cells were transfected with 2-fold serial dilutions of mismatch control oligonucleotide or antisense for 72 hours. For each dose, the ratio of viable antisense Bcl-2-transfected cells to viable mismatch control oligonucleotide-transfected cells was determined, and this ratio was then graphed as a percentage of the viable cells in the OligofectAMINE control group. MCF-7 cells showed an IC_{50} (50% inhibition of viability) at ~400 to 600 nmol/L (Fig. 1D), which is consistent with immunoblots that showed reduced Bcl-2 expression in MCF-7 cells treated

with 600 nmol/L antisense Bcl-2 (Fig. 1C). Also consistent with immunoblots of Bcl-2 down-regulation, MDA-231 parental cells showed significantly higher sensitivity to antisense Bcl-2, with an IC_{50} of ~200 nmol/L and essentially all cells nonviable by 1,600 nmol/L, compared with clone 5 cells, which maintained ~50% cell viability at 1,600 nmol/L (Fig. 1E).

Antisense Bcl-2 Induces the Expression of *BTG1*

To better understand the molecular mechanisms by which antisense Bcl-2 induces a cytotoxic response in breast cancer cells, we did microarray analysis of MCF-7 cells transfected with antisense Bcl-2 in comparison to MCF-7 cells transfected with mismatch control oligonucleotides. Hierarchical gene cluster analysis was done to examine relative changes in gene expression per treatment group (Fig. 2A). Only 10 genes were predicted by microarray to be induced by 48 hours of antisense Bcl-2 transfection (Table 1). Gene induction did not seem to be

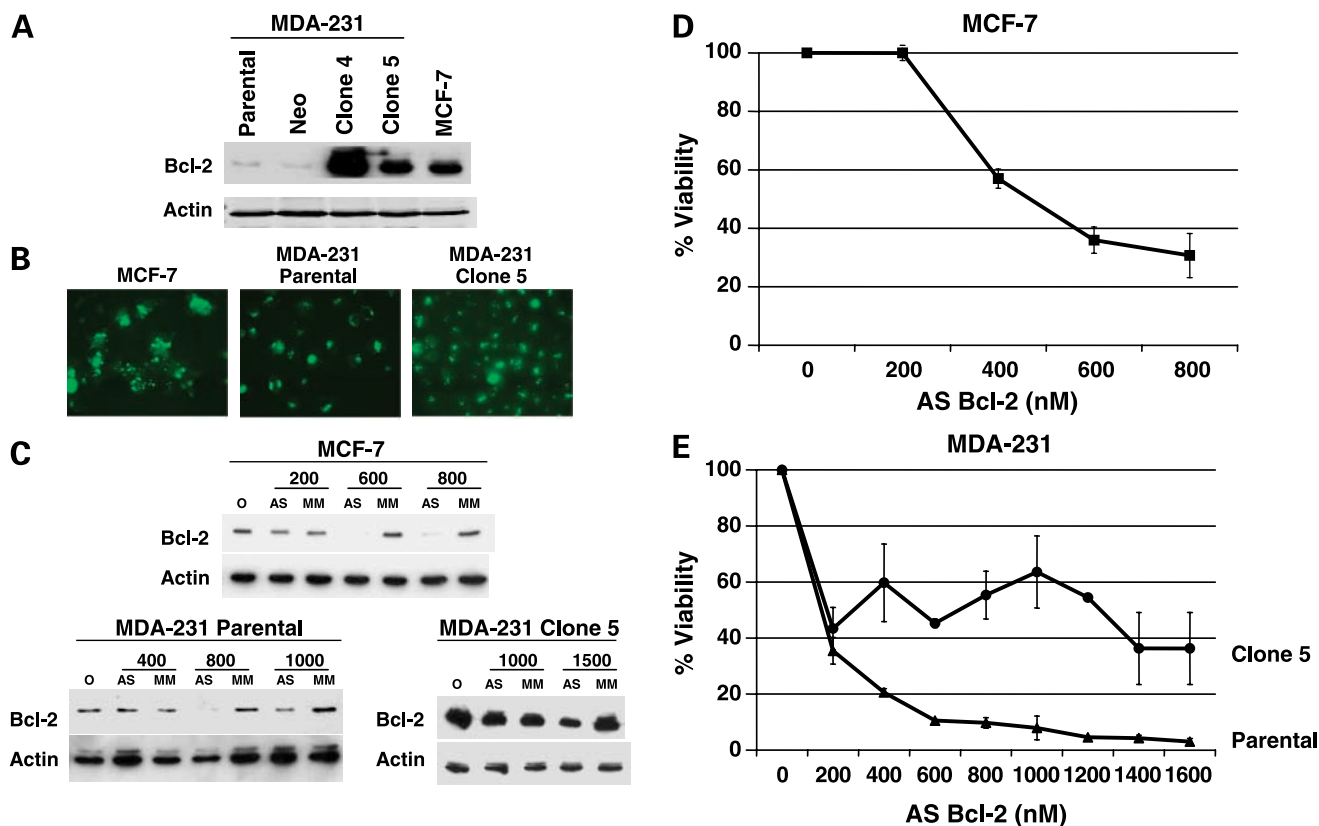


Figure 1. Antisense Bcl-2 down-regulates Bcl-2 protein levels and inhibits cell survival. **A**, to assess baseline expression of Bcl-2 protein, MDA-231 parental, MDA-231/Neo control stable transfectant, MDA-231/Bcl-2 clone 4 stable transfectant, MDA-231/Bcl-2 clone 5 stable transfectant, and MCF-7 cells were lysed for protein and immunoblotted (50 μ g) for Bcl-2 and actin. **B**, to show internalization of antisense Bcl-2, cells were transfected with antisense Bcl-2 labeled with 6-fluorescein (FAM-G3139) at doses ranging from 150 to 600 nmol/L. After 48 h, cells were fixed in 4% formaldehyde, washed with PBS, and viewed by fluorescence microscopy. Representative fields are shown for MCF-7 cells transfected with 250 nmol/L of FAM-G3139, MDA-231 parental cells with 200 nmol/L of FAM-G3139, and clone 5 cells with 200 nmol/L of FAM-G3139. **C**, MCF-7 and MDA-231 parental and clone 5 cells were transfected with mismatch control oligonucleotide or antisense Bcl-2 at doses ranging from 200 to 1,500 nmol/L for 72 h or treated with OligofectAMINE alone (O) for 72 h. Total protein lysates were immunoblotted for Bcl-2 and actin. MCF-7 (**D**) and MDA-231 (**E**) parental and clone 5 cells were transfected with mismatch control oligonucleotide or antisense Bcl-2 at doses ranging from 200 to 1,500 nmol/L for 72 h. Cell viability was assessed by trypan blue exclusion. For any given concentration of antisense Bcl-2 oligonucleotide, cell viability of antisense Bcl-2-transfected cells is shown as a percentage of cells transfected with the same dose of mismatch control oligonucleotide. All assays were done in triplicate and repeated at least thrice. Bars, SD between replicates.

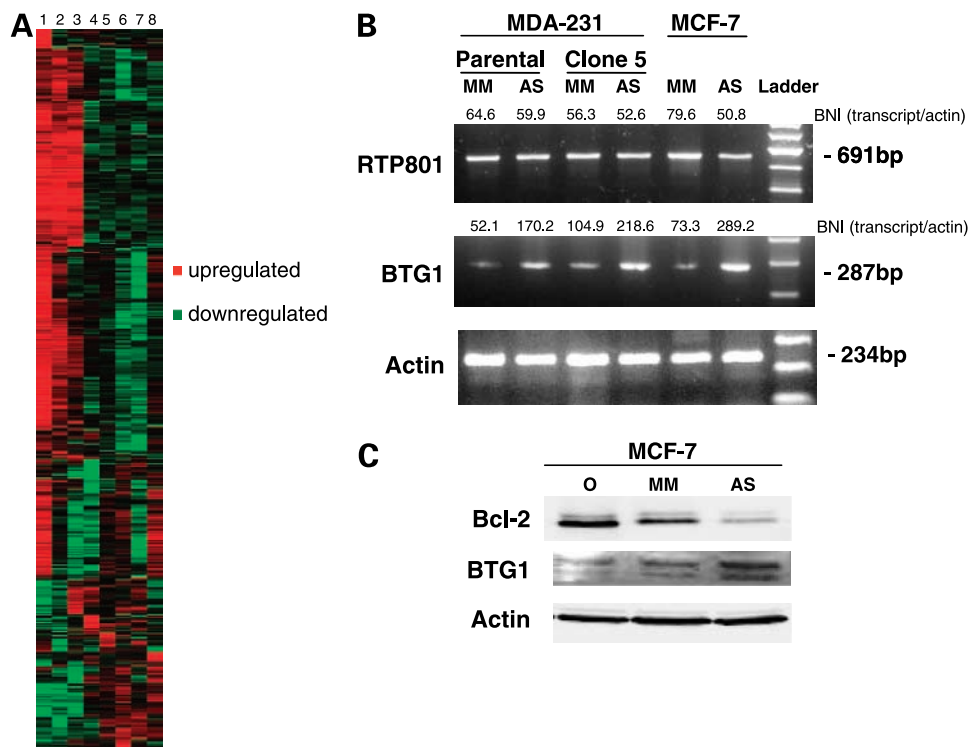


Figure 2. Antisense Bcl-2 induces expression of BTG1. **A**, MCF-7 cells were treated with OligofectAMINE alone (lane 6), transfected with 200, 400, or 600 nmol/L of antisense Bcl-2 for 24 h (lanes 1–3) or 48 h (lanes 4, 5, and 8), or transfected with 200 nmol/L of mismatch control oligonucleotide for 24 h (lane 7). Microarray analysis was done using human genome array U133A (Affymetrix), which contains ~16,900 well-characterized gene sequences. Hierarchical gene cluster analysis was done, in which genes were filtered according to a mean log expression level > 5.5. Red, gene up-regulation; green, gene down-regulation. **B**, MDA-231 parental, clone 5, and MCF-7 cells were transfected with 400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 for 48 h, at which time total RNA was extracted. RT-PCR analysis of transcripts for RTP801 and BTG1 were done to validate the microarray findings. The band net intensity (BNI) was determined as the ratio of RTP801 or BTG1 to actin per sample. RTP801 induction was not observed by RT-PCR. BTG1 induction was validated in all cell lines on transfection with 400 nmol/L of antisense Bcl-2 on the order of 2- to 3-fold versus cells transfected with 400 nmol/L of mismatch control oligonucleotide. **C**, immunoblot analysis of MCF-7 cells (100 µg) treated with OligofectAMINE alone (O), or transfected with 400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 for 48 h confirmed that antisense Bcl-2 induces BTG1 protein expression.

dose-dependent. Two of the genes have a putative role in growth arrest and apoptosis. RTP801 is a hypoxia-inducible factor-1-responsive gene whose overexpression is associated with apoptosis, particularly in response to cellular

stress (21). According to microarray studies, the RTP801 transcript was induced ~5-fold in antisense Bcl-2-treated MCF-7 cells (Table 1). RT-PCR analysis of MCF-7, MDA-231 parental, and clone 5 cells transfected for 48 hours with

Table 1. Genes predicted by microarray to be induced by antisense Bcl-2

Gene	Fold increase (dose)		
	200 nmol/L	400 nmol/L	600 nmol/L
Hypoxia-inducible factor-1-responsive RTP801 gene	4.93	4.52	4.95
Solute carrier family 7, member 11	3.02	3.00	3.77
Phospholipid scramblase 1	2.69	4.06	3.74
Asparagine synthetase	3.79	2.17	3.61
BTG1	2.74	2.98	3.10
Stanniocalcin 2	2.58	2.91	2.74
Amino acid transporter 2	2.50	2.29	2.78
Ornithine decarboxylase inhibitor	2.20	2.17	2.66
CD24 antigen	2.77	1.89	2.58
v-myc	2.74	1.94	2.53

NOTE: Genes that were predicted by microarray analysis to be induced with a fold increase >2.5 relative to mismatch control oligonucleotide-transfected cells are shown for MCF-7 cells transfected with 200, 400, or 600 nmol/L antisense Bcl-2 for 48 hours.

400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 was done to validate microarray findings. RT-PCR failed to show induction of RTP801 by antisense Bcl-2 oligonucleotide (Fig. 2B). The second gene with a putative role in cell death predicted to be induced 3-fold by antisense Bcl-2 on microarray was *BTG1*, which is a potential mediator of chemotherapy-induced apoptosis (22). RT-PCR analysis of MCF-7, MDA-231 parental, and clone 5 cells transfected for 48 hours with 400 nmol/L of mismatch control oligonucleotide or 400 nmol/L of antisense Bcl-2 validated induction of *BTG1* on the order of 2- to 4-fold (Fig. 2B). Immunoblotting further confirmed up-regulation of *BTG1* in response to antisense Bcl-2 treatment (Fig. 2C). Thus, down-regulation of Bcl-2 resulted in increased expression of *BTG1*.

BTG1 siRNA Reduces Antisense Bcl-2-Mediated Apoptosis of Breast Cancer Cells

To determine if the induction of *BTG1* contributes to antisense Bcl-2-mediated cytotoxic effects, we suppressed *BTG1* expression using *BTG1* siRNA and then examined the effects of antisense Bcl-2 on cell viability. MCF-7 cells were transfected with 200 or 400 nmol/L of *BTG1* siRNA or 400 nmol/L of control siRNA for 48 hours each. *BTG1* siRNA at 400 nmol/L reduced *BTG1* protein levels, whereas the equivalent concentration of control siRNA did not alter *BTG1* levels (Fig. 3A).

We next transfected MCF-7 cells with 400 nmol/L of control siRNA or 400 nmol/L of *BTG1* siRNA. After 24 hours, mismatch control oligonucleotide or antisense Bcl-2 was transfected at 400 nmol/L each. Cell viability was measured after an additional 48 hours by trypan blue exclusion (Fig. 3B). Knockdown of *BTG1* partially inhibited antisense Bcl-2-mediated cell death. To specifically assess effects on apoptosis, ELISA-based analysis of DNA fragmentation was done. Similar to trypan blue assays, MCF-7 cells were transfected with 400 nmol/L of control siRNA or *BTG1* siRNA for 24 hours, then with mismatch control oligonucleotide or antisense Bcl-2 for an additional 48 hours. Protein lysates were then evaluated for levels of DNA fragmentation (Fig. 3C). Knockdown of *BTG1* blocked antisense Bcl-2-mediated DNA fragmentation, indicating that *BTG1* contributes to antisense Bcl-2-mediated apoptosis.

BTG1 Is a Bcl-2-Regulated Mediator of Apoptosis

Our results demonstrating that antisense Bcl-2 induces *BTG1* suggest that *BTG1* is negatively regulated by Bcl-2. To test this hypothesis, MDA-231-derived Neo control, Bcl-2-overexpressing clone 4, and Bcl-2-overexpressing clone 5 stable transfectants were transiently transfected with a *BTG1* promoter-luciferase reporter construct. Increased expression of Bcl-2 in the stable transfectants resulted in an ~2-fold reduction in *BTG1* promoter activity versus Neo control cells (Fig. 4A). These results show that Bcl-2 suppresses *BTG1* promoter activity, supporting a role for Bcl-2 as a negative transcriptional regulator of *BTG1*.

Our results suggest that *BTG1* contributes to antisense Bcl-2-mediated apoptosis. To determine if *BTG1* itself induces apoptosis, MCF-7 cells were transiently transfected

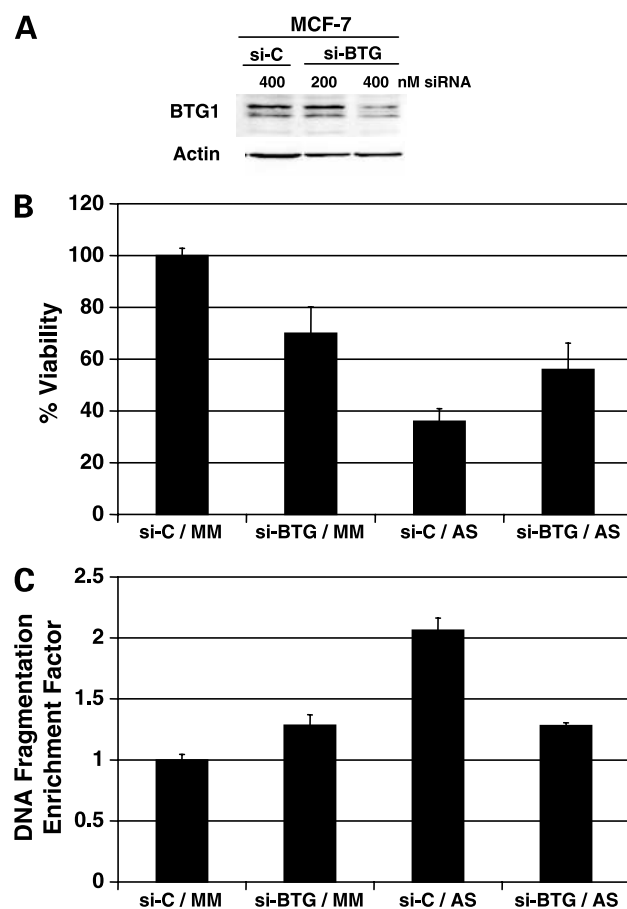


Figure 3. *BTG1* knockdown blocks antisense Bcl-2-mediated cell death. **A**, MCF-7 cells were transfected with control siRNA (*si-C*) at 400 nmol/L, or *BTG1* siRNA (*si-BTG1*) at 200 or 400 nmol/L, each for 48 h. Protein lysates were immunoblotted (100 μ g) for *BTG1* and actin, and showed knockdown of *BTG1* using 400 nmol/L *BTG1* siRNA. **B**, MCF-7 cells were treated in the following groups: transfection of control siRNA or *BTG1* siRNA for 24 h, followed by transfection of mismatch control oligonucleotide or antisense Bcl-2 for an additional 48 h. Cells were counted by trypan blue exclusion. All experiments were done in triplicate (at least twice). Cell viability is expressed as a percentage of the control siRNA/mismatch control oligonucleotide transfection control group. Bars, SD between triplicates. Knockdown of *BTG1* reduced antisense Bcl-2-mediated cell death. **C**, the same transfection groups as in **B** were lysed for protein and analyzed by ELISA for DNA fragmentation. DNA fragmentation is shown relative to the control siRNA/mismatch control oligonucleotide transfection control group. Error bars represent SD between triplicates. The control siRNA/antisense Bcl-2 group showed 2-fold induction of DNA fragmentation. Knockdown of *BTG1* inhibited antisense Bcl-2-mediated DNA fragmentation.

with a control vector or a vector containing the *BTG1* coding sequence. Immunoblotting confirmed increased expression of *BTG1* after 48 hours of transfection (Fig. 4B). To assess *BTG1*-mediated apoptosis, cells were lysed and analyzed for DNA fragmentation after 48 hours of transfection with *BTG1* or control vector. *BTG1* induced a 2.5-fold increase in fragmented DNA (Fig. 4C), indicating the induction of apoptosis. These results support *BTG1* as a novel Bcl-2-regulated mediator of apoptosis in breast cancer.

Discussion

Overexpression of Bcl-2 is associated with decreased apoptosis and resistance to multiple therapeutic agents (11, 12). Over the past several years, inhibition of Bcl-2 has emerged as a potential strategy for increasing sensitivity to currently used chemotherapeutic agents. One method of inhibiting Bcl-2 is through the delivery of antisense oligonucleotides that inhibit translation of the Bcl-2 mRNA, such that Bcl-2 protein expression is suppressed. The prototypical antisense Bcl-2 used in the clinic was G3139 (Genasense), which has been tested clinically in multiple solid tumor types including breast tumors. *In vitro* and *in vivo* studies showed that G3139 antisense Bcl-2 inhibited mammary tumor growth and increased the chemosensitivity of mammary carcinomas (13–16). In this article, we have studied the molecular mechanisms contributing to antisense Bcl-2-mediated cell death in breast cancer cells.

The following novel findings were shown in this study. (a) Induction of BTG1 contributed, at least in part, to the cytotoxic activity of antisense Bcl-2, as the knockdown of BTG1 partially inhibited antisense Bcl-2-mediated cell death. (b) BTG1 expression was negatively regulated by Bcl-2, as antisense Bcl-2 induced BTG1 and overexpression of Bcl-2 suppressed BTG1 promoter activity. (c) BTG1 induced apoptosis in breast cancer cells. Thus, we propose a model (Fig. 4D) in which Bcl-2 decreases *btg1* promoter activity, such that antisense-mediated reduction in Bcl-2 levels results in increased BTG1, which, in turn, mediates the increased apoptosis of breast cancer cells. These results support BTG1 as a novel Bcl-2-regulated mediator of apoptosis in breast cancer cells, whose induction contributes to antisense Bcl-2-mediated cell death.

The *btg1* coding sequence was originally cloned from a chromosomal translocation t(8;12)(q24;q22) of a B cell chronic lymphocytic leukemia (23). It is a member of the Tob/Btg family, which consists of seven antiproliferative

proteins. BTG1 is expressed predominantly in quiescent cells at the G₀/G₁ phase transition, with levels declining as cells enter S phase (24). Exogenous expression of BTG1 resulted in reduced proliferation with G₁ arrest and/or apoptosis in several cell types, including NIH3T3 murine fibroblasts (18, 24), microglia (25), and myoblasts (26). A role for BTG1 in cellular differentiation has been proposed based on experiments showing that BTG1 expression stimulates myoblast differentiation (26), and that BTG1 is up-regulated in leukemic cells upon treatment with chemicals that induce differentiation (27). Thus, BTG1 seems to play roles in inhibiting proliferation, promoting

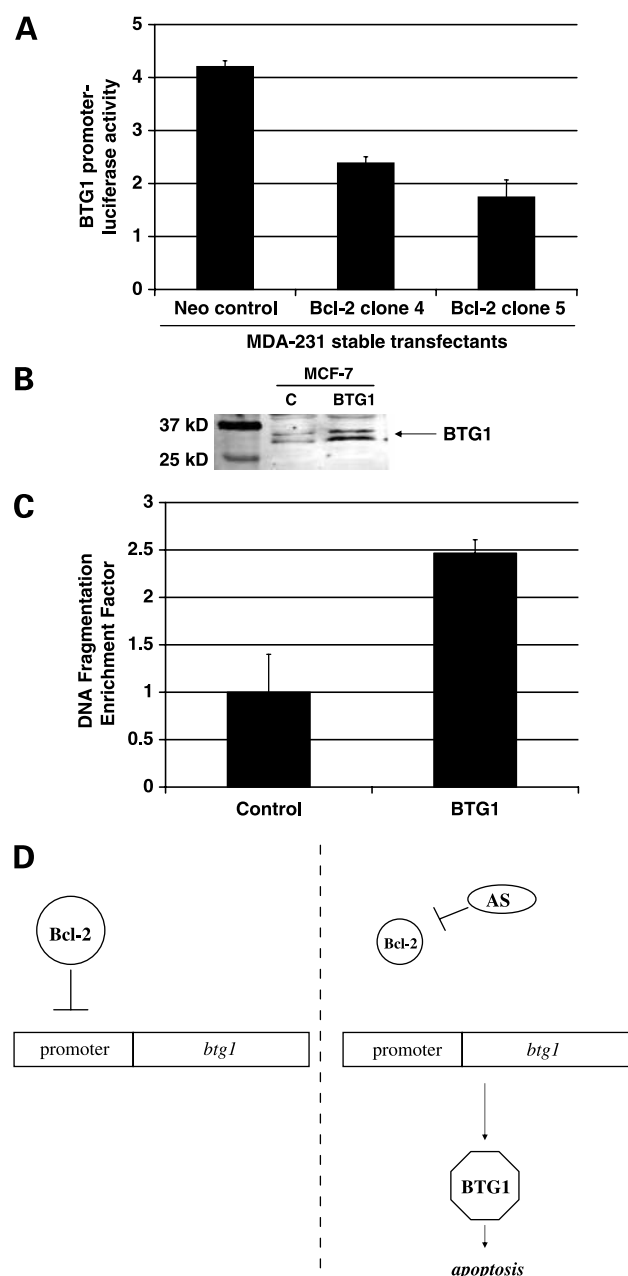


Figure 4. BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells. **A**, MDA-231 stable transfectants Neo control, Bcl-2 clone 4, and Bcl-2 clone 5 were transfected with either pGL3 luciferase reporter plasmid (1 μ g) or pGL3-BTG1 promoter-luciferase reporter construct (1 μ g). All cells were cotransfected with *Renilla* luciferase internal control plasmid (1 ng). After 48 h, lysates were obtained and luciferase values were read on a luminometer. All pGL3 or pGL3-BTG1 promoter luciferase values were normalized to the *Renilla* luciferase value per group. The ratio of normalized BTG1 promoter-luciferase to normalized pGL3 control luciferase was then determined, and is shown as BTG1 promoter-luciferase activity in the graph. Bars, SD between triplicates. Stable overexpression of Bcl-2 suppressed BTG1 promoter activity. **B**, MCF-7 cells were transfected with an empty backbone control vector (C) or the vector containing the BTG1 coding sequence (BTG1) for 48 h. Protein lysates were immunoblotted (100 μ g) for BTG1 to confirm transfection. **C**, as in **B**, MCF-7 cells were transfected with an empty backbone control vector or the vector containing the BTG1 coding sequence for 48 h. Protein lysates were analyzed by ELISA for DNA fragmentation, which is shown relative to control transfectants. Bars, SD between triplicates. BTG1 transfectants showed ~2.5-fold increase in DNA fragmentation. **D**, proposed model showing that Bcl-2 protein blocks *btg1* promoter activity, and that down-regulation of Bcl-2 by antisense Bcl-2 results in increased BTG1, which promotes apoptosis.

apoptosis, and stimulating cellular differentiation in multiple cell types. Our study is the first to show that BTG1 induces apoptosis, contributing to antisense Bcl-2-mediated cytotoxic effects in breast cancer cells.

Correlative studies have provided preliminary evidence that BTG1 expression may also be associated with response to certain anticancer treatments and with less aggressive forms of cancer. For example, BTG1 was expressed in acute myeloid leukemia cells from patients in complete remission, but not in patients that did not achieve remission. Thus, BTG1 was proposed to be a marker of remission in acute myeloid leukemia (27). BTG1 expression was also shown to correlate with an androgen-dependent state in prostate cancer, with reduced BTG1 expression observed when cells progressed to an androgen-independent state (28). In addition, BTG1 was induced upon treatment of leukemic cells with retinoids (27). Based on these findings and on the new findings that we present, further studies examining the roles of BTG1 in predicting treatment response and in cancer progression are warranted.

Previous studies focusing on the regulation of BTG1 suggested that phosphorylation and cellular localization affect BTG1 function. The cyclin-dependent kinase p34cdc2, in complex with either cyclin E or cyclin A, was shown to phosphorylate BTG1 on serine 159, facilitating the interaction of BTG1 with the human carbon catabolite repressor protein-associative factor 1 (29). Phosphorylation promoted the nuclear localization of BTG1, blocking cellular proliferation and resulting in growth inhibition (29, 30). In addition, the Forkhead transcription factor FoxO3a was shown to directly induce the *btg1* promoter, up-regulating the expression of BTG1 and stimulating differentiation of erythroid cells (20).

In our study, the identification of BTG1 as a Bcl-2 target gene is a novel finding. Interestingly, BTG1 expression was previously shown to be induced in microglia via activation of the Janus-activated kinase/signal transducers and activators of transcription pathway (25). In that case, BTG1 expression was associated with the inhibition of proliferation. Importantly, an independent study showed that signal transducers and activators of transcription-1 inhibited the activation of the *bcl-2* promoter in neuroblastoma cells (31). Hence, Janus-activated kinase/signal transducers and activators of transcription-1 signaling was independently associated with reduced Bcl-2 levels, increased BTG1 levels, and decreased proliferation. These data are consistent with our findings of negative regulation of BTG1 by Bcl-2, and support the inverse relationship between these two proteins. Further studies examining the regulation of Bcl-2 and BTG1 by the signal transducers and activators of transcription-1 pathway are also warranted, as it has been shown to mediate chemotherapy-induced apoptosis in breast cancer cells (32).

Bcl-2 is an important therapeutic target for breast cancer and other solid tumors. In addition to improving the delivery of small molecules targeting Bcl-2, future studies will focus on the identification of predictive markers of

response to bcl-2-directed therapy. The role of BTG-1 induction by Bcl-2 blockade should be explored in clinical trials using antisense oligonucleotides or small molecules directed against Bcl-2.

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