

# Antisense RNA Down-Regulation of bcl-2 Expression in DU145 Prostate Cancer Cells Does Not Diminish the Cytostatic Effects of G3139 (Oblimersen)

Anthony Raffo,<sup>1</sup> Johnathan C. Lai,<sup>3</sup> C. A. Stein,<sup>1,2</sup> Paul Miller,<sup>4</sup> Steven Scaringe,<sup>5</sup> Anastasia Khvorova,<sup>5</sup> and Luba Benimetskaya<sup>1</sup>

Departments of <sup>1</sup>Medicine, <sup>2</sup>Pharmacology, and <sup>3</sup>Biomedical Engineering, Columbia University, New York, New York; <sup>4</sup>Johns Hopkins University, School of Public Health, Baltimore, Maryland; and <sup>5</sup>Dharmacon Research, Inc., Lafayette, Colorado

## ABSTRACT

**Purpose:** Inhibition of the function of the bcl-2 protein has been postulated to sensitize cells to cytotoxic chemotherapy, and thus provides an attractive target for investigative therapies. G3139, a phosphorothioate antisense oligonucleotide targeted to the initiation codon region of the bcl-2 mRNA, is currently being evaluated in several Phase II and Phase III clinical trials. However, the mechanism of action of this molecule appears to depend on a combination of antisense plus nonantisense events. Indeed, the very idea that bcl-2 is a critical target is, at least in part, an extrapolation from experiments in which intracellular bcl-2 protein concentrations have been dramatically increased, yielding chemoresistant cells.

**Experimental Design:** In this work, we down-regulated the expression of bcl-2 protein by 80–90% by two different antisense RNA strategies (antisense RNA and small interfering RNA) in DU145 prostate cancer cells.

**Results:** Even after down-regulation of bcl-2 protein expression by either one of these strategies, the cellular phenotype induced by subsequent G3139 treatment (inhibition of cellular growth and the generation of reactive oxygen species) was essentially identical to that induced in mock-infected or wild-type DU145 cells in which bcl-2 protein expression had not been down-regulated previously.

**Conclusions:** These results strongly suggest that bcl-2 expression in DU145 cells is not strongly associated with the proliferative phenotype and that the mechanism by which G3139

produces its cytostatic effects in this cell line is bcl-2 independent.

## INTRODUCTION

Because it is an important regulator of the apoptosis process, bcl-2 is currently a frequently investigated target for antineoplastic therapeutic intervention (1–4). Elevation of expression of this antiapoptotic protein appears to contribute to resistance to a variety of antineoplastic agents, both *in vitro* and *in vivo*, which include *cis*-platinum, taxanes, mitoxantrone, Adriamycin, and dexamethasone, in addition to radiation (5–10). The bcl-2 protein, whereas expressed in very few normal tissues (*e.g.*, B lymphocytes and colon crypt cells), is in contrast, highly expressed in human tumors as diverse as prostate, colorectal, lung, gastric, non-Hodgkin's lymphoma, and in many leukemias (reviewed in Ref. 11). Use of a bcl-2 antisense RNA strategy in MCF-7 cells caused down-regulation of bcl-2 protein expression and sensitization of cells to Adriamycin (12). In HL60 cells, induction of an antisense bcl-2 mRNA caused inhibition of cell growth and autophagy, but not apoptosis (13). Similarly, Zhang *et al.* (14) demonstrated that introduction of an exogenous antisense bcl-2 mRNA increased the sensitivity of a human gastric adenocarcinoma cell line to phototoxic treatment. In human prostate cancer PC3 cells, attempted down-regulation of bcl-2 protein expression with an adenovirally delivered ribozyme (15–17) was shown to induce cellular apoptosis.

Antisense oligonucleotides such as G3139, an 18mer phosphorothioate oligodeoxynucleotide targeted to the initiation codon region of the bcl-2 mRNA (18), have also been used extensively to down-regulate the expression of bcl-2 expression (19–22), and to induce chemosensitization. The clinical utility of this strategy has been extensively demonstrated by Jansen *et al.* (23, 24) in a Phase II clinical trial in which 6 of 14 heavily pretreated patients with advanced melanoma responded to treatment with an antisense bcl-2 oligonucleotide in combination with dacarbazine. This oligonucleotide, known as G3139, Genasense, or Oblimersen, is currently being evaluated in combination with dacarbazine in Phase III clinical trials in advanced melanoma, as well as in other tumors, including chronic lymphatic leukemia, myeloma, non-small cell lung cancer, and prostate cancer.

Whereas we (25, 26) and others have demonstrated that G3139 can down-regulate, by >90%, the expression of bcl-2 protein and mRNA in several prostate cancer cell lines in tissue culture, we have also demonstrated that the effects of G3139 on the phenotype of treated PC3 cells are complex, and at least partially independent of its effects on bcl-2 expression. G3139 treatment did not produce significant cellular apoptosis in PC3 cells (as determined by lack of procaspase 3 cleavage and increase in cell surface Annexin V expression), as it does in many other cell lines. Concordant with the lack of apoptosis,

Received 9/25/03; revised 1/14/04; accepted 1/21/04.

**Grant support:** NIH Grant GM58791 (C. Stein and P. Miller).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** C. Stein and L. Benimetskaya are currently at the Albert Einstein-Montefiore Cancer Center, Department of Oncology, Montefiore Medical Center, Bronx, New York.

**Requests for reprints:** C. A. Stein, Albert Einstein-Montefiore Cancer Center, Department of Oncology, Montefiore Medical Center, 111 E. 210 Street, Bronx, NY 10467. Phone: (718) 920-8980; Fax: (718) 652-4027; E-mail: cstein@montefiore.org.

ATP production and the mitochondrial membrane potential  $\Delta\psi_m$  were preserved. Nevertheless, G3139 was highly cytostatic to PC3 cells in complete medium for up to 5 days after a 5-h treatment and also dramatically inhibited cell cloning in soft agar. In contrast, G4232, an isosequential variant of G3139 in which both CpG cytidines were C5-methylated, was only minimally antiproliferative, although it down-regulated bcl-2 expression to the same extent as G3139. In addition, a series of mismatched G3139-related oligomers could also significantly down-regulate bcl-2 protein expression, but only if the two CpG motifs were intact. Subsequently, we (27) demonstrated that the down-regulation of expression of bcl-2 protein expression and inhibition of cell growth by G3139 could be recapitulated in large part by treatment with IFN- $\beta$  and - $\gamma$ . Furthermore, it could be shown that G3139 not only activates the IFN cascade in PC3 cells after transfection (although without induction of the production of IFN proteins), but prolongs the induction to a greater extent than other related or control oligonucleotides.

In addition to these effects, G3139 also caused production of reactive oxygen species (ROS) in growth-arrested cells and oxidation of nuclear guanosine to 8-hydroxyguanosine, as determined 1F7 monoclonal antibody staining. Furthermore, we also demonstrated, via bromodeoxyuridine (BrdUrd) incorporation studies, that G3139 induced a G<sub>1</sub>-S entry block and an intra-S block in PC3 cells. These blocks could persist for as long as 3 days. Coincident with this observation, we also noted that that expression of several proteins encoded by S phase genes, including c-myb and PARP, were greatly reduced.

Due to the complexity of the phenotype induced by G3139 and remaining questions pertaining to the function of bcl-2 in prostate cancer cell models in tissue culture, we then elected to use two other, nonantisense oligodeoxynucleotide, approaches to down-regulate bcl-2 protein expression. The first involved the construction of two vectors engineered to produce truncated antisense bcl-2 RNAs. Each of these vectors was transfected into DU145 prostate cancer cells to generate clones in which bcl-2 protein expression was stably (*i.e.*, for many months) knocked down. The second approach involved the use of an anti-bcl-2 small interfering (siRNA; Refs. 28–31), which, if used at appropriate concentrations, could produce an ostensibly highly specific knockdown of bcl-2 protein expression, albeit for relatively limited periods of time (*i.e.*, ~7 days) relative to the antisense RNA approach. In brief, our data demonstrate that G3139 produces essentially the same phenotype in mock infected *versus* the bcl-2 knockdown DU145 clones, and essentially the identical phenotype in siRNA treated wild-type DU145 cells *versus* control, untreated wild type cells. In neither case was chemosensitization to cytotoxic agents induced. Thus, our data suggest that the phenotype produced after G3139 treatment of DU145 prostate cancer cells is, at the least, partially independent of its potential antisense effects.

## MATERIALS AND METHODS

**Construction and Analysis of Stably Expressed bcl-2 Antisense RNAs in Prostate Cancer Cell Lines.** bcl-2 antisense RNAs were PCR generated from a bcl-2 full-length cDNA pcDNA 3 vector obtained from S. Korsmeyer (Dana-Farber Cancer Center, Boston, MA). Primers were constructed, which



Fig. 1 Schematic showing regions of hybridization of the bcl-2 mRNA with the AS1 and AS2 antisense RNA, which were constructed to avoid a GC-rich region within the central region of the bcl-2 open reading frame.

would amplify two small regions within the bcl-2 coding sequence. The bcl-2 sequences were derived from the cDNA from human bcl-2 (accession no. M14745), and primer sets were designed to avoid a GC-rich region within the midsection of the bcl-2 open reading frame (Fig. 1).

Additionally, the primers were designed to contain restriction endonuclease sites so that the fragments could be inserted into a retroviral expression system vector to express antisense RNA. The primer sequences are: 5'AS1: 5'-AGATCTCTC-GAGATGGCGCGCACGCTG-3'; 3'AS1: 5'-GGAATTGAAT-TCCCACTCGTAGCC-3'; 5'AS2: 5'-AGATCTCTCGATTCT-CCC GCCGTAC-3'; 3'AS2: 5'-GGAATTGAATTCC-CAGGG-TGATGCAAG-3'. Underlined sequences are directed against bcl-2 with 5'AS1 and 5'AS2 in the positive strand orientation and 3'AS1 and 3'AS2 in the negative strand orientation. Sequences not underlined are viral vector derived. CTCGAG denotes the *Xho*I restriction endonuclease site and GAATTC denotes the *Eco*RI site, used for the proper expression orientation in the vector. The Retroviral Gene Transfer and Expression System (BD Bioscience Clontech Labs, Inc., Palo Alto, CA) was used for these experiments in that they were specially designed to contain a 5'LTR modified from the PCC4-cell-passaged myeloproliferative sarcoma virus. These point mutations and a deletion allow high-level constitutive expression of a target sequence RNA within mammalian cells. This promoter was contained in a modified pUC plasmid, which also contained the murine stem cell virus retroviral packaging signal for high titer-defective virus production. Additionally, this vector contained the neomycin resistance gene for G418 selection of expressing colonies.

Because these retroviral expression vectors produced defective viral particles, they were initially expressed in a packaging cell line (PT67). Supernatants from these high titer viral producing clones were used to infect DU145 prostate cells, and colonies were collected and screened for inhibition of bcl-2 protein expression.

**Cells.** DU145 prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in RPMI 1640 plus 10% fetal bovine serum, supplemented by 1% nonessential amino acids, 1% pyruvate, 100 units/ml penicillin G sodium, and 100  $\mu$ g/ml streptomycin sulfate. Stock cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator.

**Reagents.** The anti-bcl-2 monoclonal antibody was purchased from Dako (Carpinteria, CA) and the anti-protein kinase C (PKC)- $\alpha$  from Transduction Laboratories (Lexington, KY). The anti-p65, p50, bax, and bcl-xL antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The XIAP, IAP-1, and c-IAP-2 antibodies were from R&D Systems (Minneapolis, MN). The anti- $\alpha$ -tubulin and catalase monoclonal antibodies,

paclitaxel and 5-bromo-2'-deoxyuridine, were from Sigma-Aldrich (St. Louis, MO). Lipofectin and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). SUPERase-In is a product of Ambion (Austin, TX). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences. 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium (HE) were purchased from Molecular Probes (Eugene, OR). Mitoxantrone is product of Immunex Corporation (Seattle, WA). Phosphorothioate oligonucleotides were synthesized and purified via standard procedures and kindly supplied by Genta (Berkeley Heights, NJ). All of the siRNAs were supplied by Dharmacon (Lafayette, CO). A list of the oligomers used is presented in Table 1.

**Oligonucleotide Transfections.** Cells were seeded the day before the experiment in six-well plates at a density of  $25 \times 10^4$  cells per well, to be 60–70% confluent on the day of the experiment. All of the transfections were performed in Opti-MEM medium (Life Technologies, Inc.) as per the manufacturer's instructions. The appropriate quantities of reagents were diluted in 100  $\mu$ l of Opti-MEM to give a final concentration of Lipofectin and 400 nM oligonucleotide. The solutions were mixed gently and preincubated at room temperature for 30 min to allow complexes to form. Then, 800  $\mu$ l of Opti-MEM was added, the solution mixed, and overlaid on the cells that had been prewashed with Opti-MEM. The incubation time for oligonucleotide/Lipofectin complexes was 5 h. The total incubation time before cell lysis and protein isolation was 72 h at 37°C.

**The siRNA Transfections.** Cells were seeded in complete medium without antibiotics the day before the experiment in six-well plates at a density of  $25 \times 10^4$  cells per well, to be 60–70% confluent on the day of the experiment. All of the transfections were performed in Opti-MEM (Invitrogen). siRNAs were diluted in 133  $\mu$ l Opti-MEM for a final concentration of 25 nM. Lipofectamine 2000 (1.33  $\mu$ l; final concentration = 1.33  $\mu$ g/ml) and 0.75  $\mu$ l of SUPERase-In (RNase Inhibitor) were resuspended in 133  $\mu$ l of Opti-MEM. The solutions were mixed gently and preincubated at room temperature for 30 min to allow complexes to form. Then, 730  $\mu$ l of complete medium without antibiotics was added, the solution mixed, and overlaid on the cells. The incubation time for siRNA/Lipofectamine 2000 complexes was 24 h. The total incubation time before cell lysis and protein isolation was 72 h at 37°C.

**Western Blot Analysis.** Cells were washed in PBS and then extracted in lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 50  $\mu$ g/ml Pefabloc SC (Roche Applied Science, Indianapolis, IN), 15  $\mu$ g/ml aprotinin, leupeptin, chymostatin, pepstatin A, 1 mM  $\text{Na}_3\text{VO}_4$ , and 1 mM NaF] at 4°C for 1 h. Cell

debris was removed by centrifugation at  $14,000 \times g$  for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts, containing 25–50  $\mu$ g of protein, were resolved by SDS-PAGE, and then transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL), and the filters incubated at room temperature for 1–2 h. For PKC- $\alpha$ , p50, p65, bcl-xL, bax, XIAP, c-IAP-1, IAP-2, catalase, and SOD, the filters were blocked in 5% nonfat milk, Tris-buffered saline containing 0.05% Tween 20 and probed with corresponding primary antibodies at 4°C overnight. To detect bcl-2, blocking was performed in 5% BSA in PBS containing 0.5% Tween 20. After washing ( $3 \times 10$  min), in Tris-buffered saline-T buffer or in PBS-T (for bcl-2), the filters were incubated for 1 h at room temperature in 5% milk in Tris-buffered saline-T or 5% milk in PBS-T (for bcl-2) with a 1:3,000 dilution of a peroxidase-conjugated secondary antibody (Amersham). After washing ( $3 \times 10$  min), enhanced chemiluminescence was performed according to the manufacturer's instructions.

**Determination of Rate of Cell Proliferation in Complete Medium.** Briefly,  $15 \times 10^4$  cells were seeded in six-well plates and allowed to attach overnight. Cells were then treated with the appropriate concentrations of oligonucleotide complexed to Lipofectin (15  $\mu$ g/ml) for 5 h 37°C. After 3–7 days of incubation at 37°C, cells were trypsinized and harvested, and their viability was determined by trypan blue exclusion. Experiments were performed in triplicate, and data are presented as the mean  $\pm$  SD. In no experiment did >10–15% of cells stain with trypan blue. In some experiments, cells were treated with siRNAs as described above, and allowed to incubate for 3 days, when they were reseeded and treated with G3139, also as described above.

**BrdUrd Incorporation.** To evaluate BrdUrd incorporation, cells treated for either 24 or 72 h with oligonucleotides were incubated for 2 h at 37°C with medium containing 10  $\mu$ M BrdUrd. After washing with PBS, the cells were fixed in cold 70% ethanol at  $-20^\circ\text{C}$  overnight. After washing in PBS, cells were resuspended in 2 N HCl/Triton X-100 at room temperature for 30 min, and resuspended in 0.1 M  $\text{Na}_2\text{B}_4\text{O}_7$  for 7 min. Then the cells were treated with an anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) in 0.5% Tween 20/1% BSA/PBS in the dark at room temperature for 45 min and costained with 0.01 mg/ml of propidium iodide (PI) for 30 min. PI and BrdUrd were excited at 488 nm. Ten thousand cells per sample were analyzed using a FACScan flow cytometer (Becton Dickinson) at a rate of 100–200 cells/s. Data were analyzed by CELLQuest software, and the percentage of cells in the phases of the cell cycle was

Table 1 Sequences of antisense oligonucleotides used in this study

Oligomer	Length	Sequence (5'-3') <sup>a</sup>	Comments
G3139	18	TCTCCAGCGTGCCCAT	Phosphorothioate, targeted to bcl-2 initiation codon
G4126	18	TCTCCAGCATGTGCCAT	Phosphorothioate, G3139 variant with single base mismatch at each CpG motif
2009	20	AATCCTCCCCAGTTCACCC	Phosphorothioate, no CpG motifs, targeted to bcl-2 coding region
G4232	18	TCTCCAGC <sup>b</sup> GTGC <sup>b</sup> GCCAT	Phosphorothioate, G3139 variant with cytosine C5-methyl at each CpG

<sup>a</sup> Bold italic represent mismatched bases.

<sup>b</sup> 5-methyl-deoxycytidine.



determined as a ratio of the appropriate fluorescent area to the total fluorescent area.

**Cell Surface Binding of Annexin V.** Cells treated for 72 h with oligonucleotides were harvested after trypsinization, and apoptotic cells were assayed with Annexin V-FITC apoptosis detection kit. Briefly,  $5 \times 10^5$  cells in 100  $\mu$ l of binding buffer were incubated with 5  $\mu$ l of Annexin V-FITC for 20 min at room temperature in the dark. Then PI (final concentration 1  $\mu$ g/ml) in 100  $\mu$ l of binding buffer were added immediately before the flow cytometry analysis (Becton Dickinson). Early apoptotic cells bound to Annexin V-FITC, but excluded PI. Cells in late apoptotic stages were labeled with both Annexin V-FITC and PI.

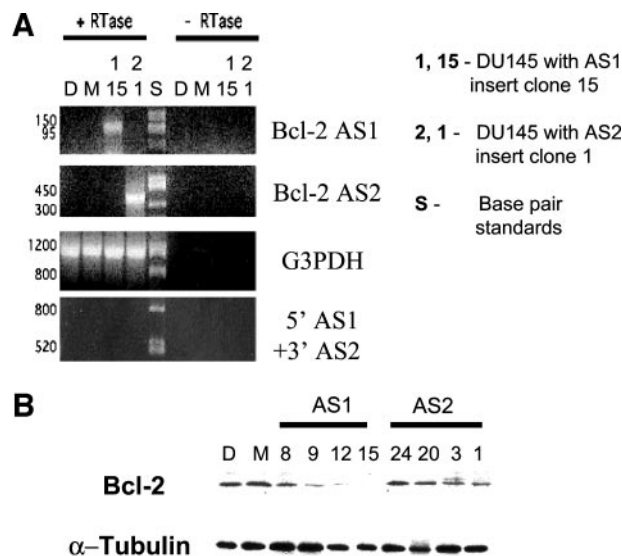
**Quantitation of Intracellular ROS Levels.** 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium (HE) were used to determine ROS and superoxide levels (32). Both dyes are nonfluorescent and can freely diffuse into cells. When HE is oxidized to ethidium (E), it intercalates into cellular DNA and fluoresces. Oxidation of 2',7'-dichlorodihydrofluorescein diacetate yields 2',7'-dichlorofluorescein (DCF), which also fluoresces, and both can be detected by flow cytometry. Cells were harvested by trypsinization, washed with PBS, and stained with 50  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate or 50  $\mu$ M HE in phenol red-free DMEM for 2 h at 37°C. The mean fluorescence channel numbers of DCF and E were analyzed by flow cytometry in the FL-1 and FL-2 channels, respectively. A minimum of 10,000 cells was acquired for each sample, and data were analyzed using CELLQuest software (Becton Dickinson). Histograms were plotted on a logarithmic scale.

## RESULTS

**Production of Stable bcl-2-Knockdown DU145 Prostate Cancer Cells.** Initially, the bcl-2 cDNA was *Eco*RI endonuclease digested from the original pcDNA3 vector and purified. Because the template for the PCR reaction was only the bcl-2 cDNA, no other products were expected. Because the primers used in the construction of these antisense RNA expressing vectors were only partially composed of bcl-2-derived sequences, the annealing temperatures used in the PCR amplification of AS1 and AS2 were low (37°C). However, this still permitted sufficient product for the subsequent unidirectional cloning of the antisense bcl-2 RNA in the pMSCV vector. The integrity of the antisense inserts in the pMSCV vector was confirmed by sequence analysis using the Columbia University sequencing core facility, and primers supplied by Clontech.

However, these same primers could also be used to detect antisense RNA produced in infected DU145 cells, because the full primer sequence, including the virally derived portion, in addition to the bcl-2 antisense fragment, was now expressed. Thus, raising the PCR annealing temperature to 58°C eliminated products derived from the sense full-length bcl-2 mRNA, whereas allowing production derived from the combined antisense bcl-2/viral-derived mRNA.

The production of the antisense bcl-2 mRNA is demonstrated by reverse transcription PCR (RT-PCR) in Fig. 2A. In all of the cases, in the absence of RTase, no PCR products were observed. As can be seen, in the bcl-2 AS1 clone, an RT-PCR product of the correct length (113 bases) was observed, but no

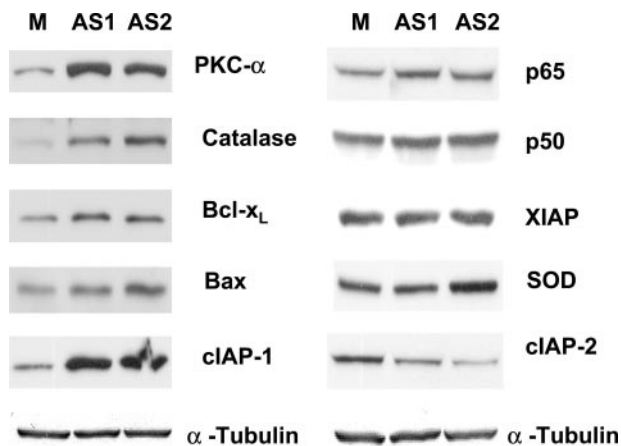


**Fig. 2** A, representative reverse transcription-PCR (RT-PCR) analysis demonstrating the production of the antisense bcl-2 mRNA in infected DU145 prostate cancer cells. In the bcl-2 AS1 clone, a 113 bp RT-PCR product was observed, which was not seen in the AS2 clone, or in the mock-infected or wild-type cells. Conversely, in the AS2 clone, a 413 bp product was observed, which was not seen in any other cell line. When a combination of the 5' AS1 and 3' AS2 primers were used, no products were observed in any cell line. A control G3PDH RT-PCR is also shown. In the absence of RTase, no PCR products were seen. B, representative Western blot analysis demonstrating down-regulation of bcl-2 expression in AS1- and AS2-infected DU145 prostate cancer cells by an antisense RNA strategy. Greatest down-regulation was observed in the AS1 clone 15 (94%) and AS2 clone 1 (83%). Protein samples (30–40  $\mu$ g of protein/lane) were analyzed as described in "Materials and Methods," with tubulin used as a control protein species. Percentage inhibition *versus* mock-transfected cells was determined by laser-scanning densitometry. D, parental DU145 cells; M, mock-infected (with empty viral vector) cells.

product of similar length was observed in the AS2 clone, or in the mock-transfected or wild-type cells. Similarly, in the AS2 clone, an RT-PCR product of the correct length (413 bases) was observed, which was not seen in any other cell line. A control G3PDH RT-PCR fragment was observed in all of the cell lines, demonstrating the integrity of full-length mRNAs. As an additional control, a combination of the 5' AS1 and 3' AS2 primers were used in a single RT-PCR reaction. No products were observed in any cell line.

Down-regulation of bcl-2 protein expression by Western blotting is demonstrated in Fig. 2B. As can be seen, this is most prominent in AS1 clone 15 (94% down-regulation by laser scanning densitometry), but is also quite marked in AS2 clone 1 (83% down-regulation). Levels of the control protein, tubulin, were essentially unchanged.

**Down-Regulation of bcl-2 Expression Is Associated with Alteration in the Expression of PKC- $\alpha$ , Catalase, cIAP-1, and cIAP-2.** In previous work (26), we demonstrated that treatment of PC3 prostate cancer cells with G3139 led not only to down-regulation of bcl-2 expression, but to bcl-2-independent down-regulation of PKC- $\alpha$  protein expression as well. This down-regulation appeared to be a consequence of the



**Fig. 3** Representative Western blot analysis demonstrating that down-regulation of bcl-2 expression in AS1 and AS2 by antisense RNA strategy leads to the up-regulation of protein kinase C (PKC)- $\alpha$ , catalase, and cIAP-1 protein expression, and down-regulation of cIAP-2. In contrast, the expression of bcl-xL and bax proteins were only slightly changed, whereas no change in the levels of expression of nuclear factor  $\kappa$ B (p50), relA (p65), XIAP, and SOD were observed. Protein samples (30–40  $\mu$ g of protein/lane) were analyzed as described in “Materials and Methods,” with tubulin used as a control protein species. The percentage of inhibition *versus* control, untreated cells was determined by laser-scanning densitometry.

induction of the IFN cascade (27, 33) by G3139. In contrast, as shown in Fig. 3, down-regulation of bcl-2 expression in AS1 and AS2 by an antisense RNA strategy leads to the up-regulation (compared with mock-infected cells) of PKC- $\alpha$  protein expression, as demonstrated by Western blotting (3–4-fold). Similarly, up-regulation of catalase (>10-fold), cIAP-1 (~4-fold), and down-regulation of cIAP-2 expression were also observed. In contrast, the expression of bcl-xL and bax proteins were only modestly altered, whereas the expression of nuclear factor  $\kappa$ B1 (p50), relA (p65), XIAP, and Mn superoxide dismutase were unchanged.

**Treatment of DU145-ASbcl-2 Cells with G3139 and Related Oligonucleotides Does Not Lead to Significant Apoptosis.** In previous experiments (26) we demonstrated that treatment of PC3 prostate cancer cells with G3139 did not lead to significant apoptosis, as demonstrated flow cytometrically by Annexin V cell surface expression. In Fig. 4, we evaluated Annexin V cell surface expression in AS1 DU145 cells *versus* mock-transfected cells. The data points, each representing a single cell, in the upper right hand box, represent cells in late apoptosis/early necrosis. When a similar experiment was performed with Taxol, approximately 50–60% of the cells were found in the upper right box.

Cells were treated with oligonucleotides (400 nM)/Lipofectin (15  $\mu$ g/ml) complexes for 5 h in Opti-MEM, and then in complete medium without complexes for an additional 67 h. In contrast to what was observed with Taxol, only 13.3% of the mock-transfected/G3139-treated, and 15.0% of AS1/G3139-treated cells could be found in late apoptosis/early necrosis. The percentage of cells in early apoptosis in G3139-treated cells (lower right box; 18.8% Mock, 19.9% AS1) was also essentially

identical. Interestingly, however, the AS1 cells appeared to be more sensitive to 2009 (Mock: 11.5% late apoptosis/early necrosis; G3139-treated 21.2%; Mock: 23.8% in early apoptosis; G3139-treated 36.7%), which down-regulates bcl-2 expression to virtually the same extent as G3139. However, 2009 is two bases longer (20mer *versus* 18mer) and probably more nonspecifically toxic. The other 18mer oligonucleotides examined (G4126 and G4232) produced similar results in both the mock and AS1 cells, *i.e.*, the production of relatively small numbers of apoptotic cells.

In cells in which bcl-2 expression has been down-regulated, it might well be expected that they become increasingly sensitive, as a function of concentration, to cytotoxic chemotherapy agents. However, this was not the case in PC3 cells treated with G3139, which were not chemosensitized (26). Similarly, neither AS1 nor AS2 cells, despite their very low expression of bcl-2 protein compared with the mock-transfected cells were chemosensitized (*versus* control, mock cells, as assessed by cellular viability via trypan blue exclusion) after treatment with either Taxol or mitoxantrone (see Fig. 8).

**Bcl-2 Down-Regulation Does Not Alter the Progression through the Cell Cycle After Treatment by G3139 and Related Oligonucleotides.**

In previous experiments in wild-type PC3 cells (26), we demonstrated, via flow-cytometric examination of BrdUrd/PI uptake, that G3139 could induce a partial block of the entrance cells into S phase in addition to a partial intra-S phase block. We performed similar experiments with mock- and AS1-infected DU145 cells. However, as demonstrated in Fig. 5A and Table 2, there are only very slight differences in the percentage of cells at each phase of the cell cycle in mock-infected cells treated with G3139 *versus* cells treated with related oligonucleotides. In fact, these percentages are similar to those found in control, untreated cells, with the exception that the number of cells ostensibly in S phase, which do not take up BrdUrd (denoted S\*), increases from 4.4% to ~7%. Similar effects (Fig. 5B; Table 3) were observed in the AS1 cells after oligonucleotide treatment. No meaningful differences were found among the various oligonucleotides, but again in each case, the number of S\* cells after each oligonucleotide treatment increased, from 2.6% in control, untreated cells, to ~11% in the S\* cells.

**G3139 Causes the Production of ROS in DU145-ASbcl-2 Cells and Inhibits Cell Growth.**

In previous experiments in wild-type PC3 cells (26), we demonstrated that G3139, but not G4126, G4232, or G2009, could induce the production of ROS in treated cells. Production of ROS was maximal (3–5-fold increase *versus* control, untreated cells) ~3 days after a 5-h treatment of the cells with oligonucleotide (400 nM)/Lipofectin (15  $\mu$ g/ml) in Opti-MEM. Thus, we desired to determine whether the production of ROS after G3139 treatment would also occur in cells of which the expression of bcl-2 had been knocked down previously. Production of ROS in DU145-bcl-2 (AS1 and AS2) and in control, mock-transfected DU145 cells was evaluated flow cytometrically by two methods. In mock-transfected cells (Fig. 6A), treatment with G3139/Lipofectin (400 nM/15  $\mu$ g/ml) complexes for 5 h in Opti-MEM generated ROS, as evaluated flow cytometrically, 3 days later both by E (4-fold increase *versus* control, untreated cells) and DCF (3.3-fold increase *versus* control, untreated cells) fluorescence.

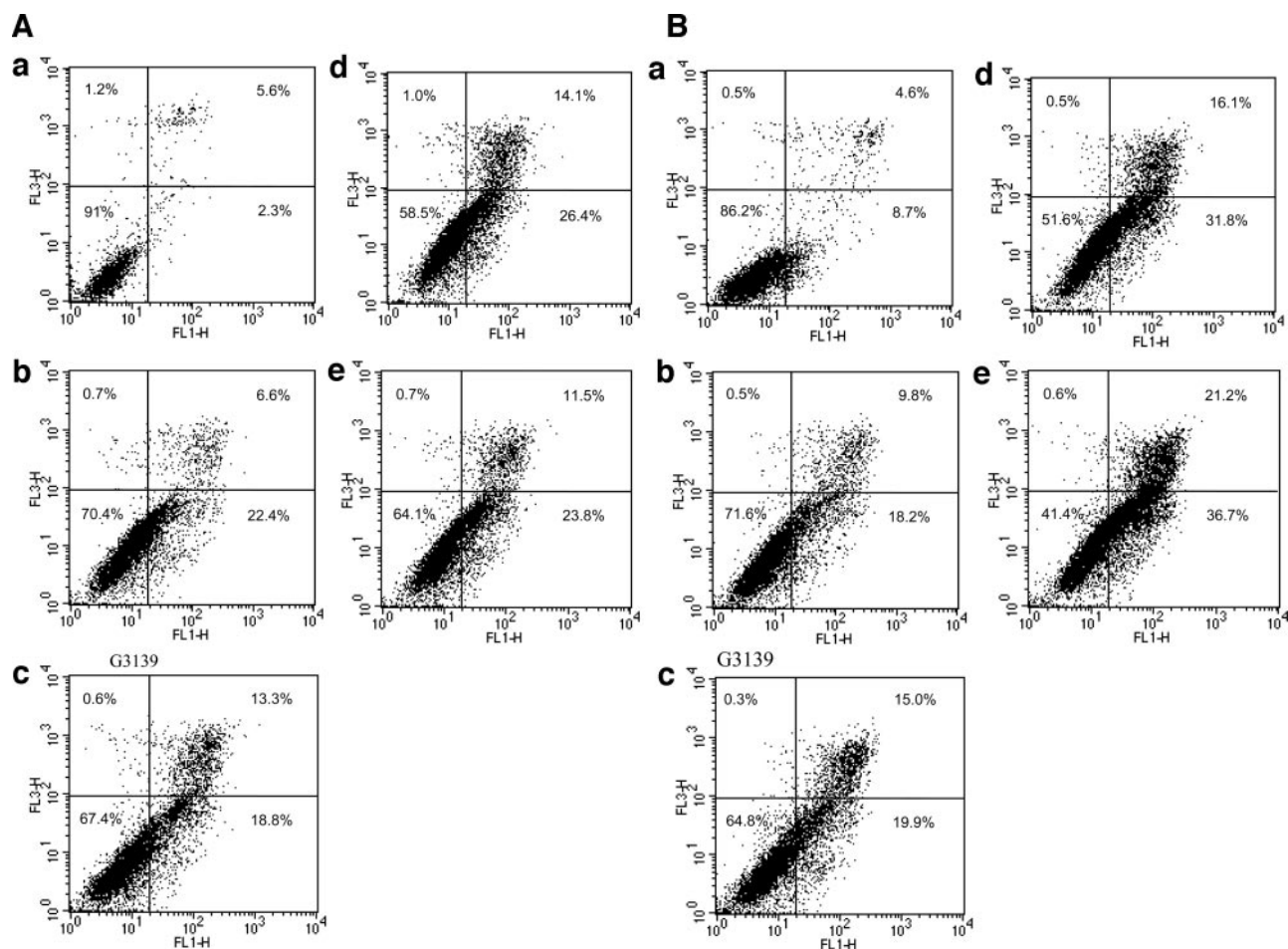


Fig. 4 Flow cytometric analysis of oligonucleotide-treated mock-transfected (A) or AS1 DU145 cells (B) analyzed after double staining with fluoresceinated anti-Annexin V monoclonal antibody staining (X axis) and propidium iodide (Y axis).  $10^4$  cells/sample were analyzed at a rate of 100–200 cells/s. Cells were treated with complexes of G3139, G4126, G4232, or 2009 (400 nM) and Lipofectin (15  $\mu\text{g}/\text{ml}$ ) for 5 h in Opti-MEM, and then for an additional 67 h in complete medium without complexes. Data points in the *top right quadrant* represent individual cells in late apoptosis/early necrosis. Early apoptosis is represented in the *bottom right quadrant*, and necrosis in the *top left quadrant*. Treatment with G3139 and related oligonucleotides did not cause significant apoptosis. a, untreated cells; b, G4126; c, G3139; d, G4232; e, 2009.

G4126 and G4232 produced only a relatively small increase (1.3-fold) *versus* control, untreated cells after 3 days, but, unlike what was seen in PC3 cells, 2009 also induced an  $\sim$ 3-fold increase in ROS production *versus* control, untreated cells.

The results obtained after oligonucleotide treatment of AS1 and AS2 cells were virtually identical to what was observed in the mock-transfected cells when monitored by E fluorescence (Fig. 6A). Although not quite as dramatic as what was observed with oxidation of HE to E, G3139 and 2009 (400 nM/15  $\mu\text{g}/\text{ml}$  Lipofectin) induced a doubling of DCF fluorescence in the mock-transfected cells 3 days after a 5-h treatment. However, G4126 and G4232 induced only very small increases. Interestingly, when monitored by DCF fluorescence, an even greater increase (up to 5-fold) in the production of ROS was observed in the AS1 and AS2 clones *versus* the mock-transfected line.

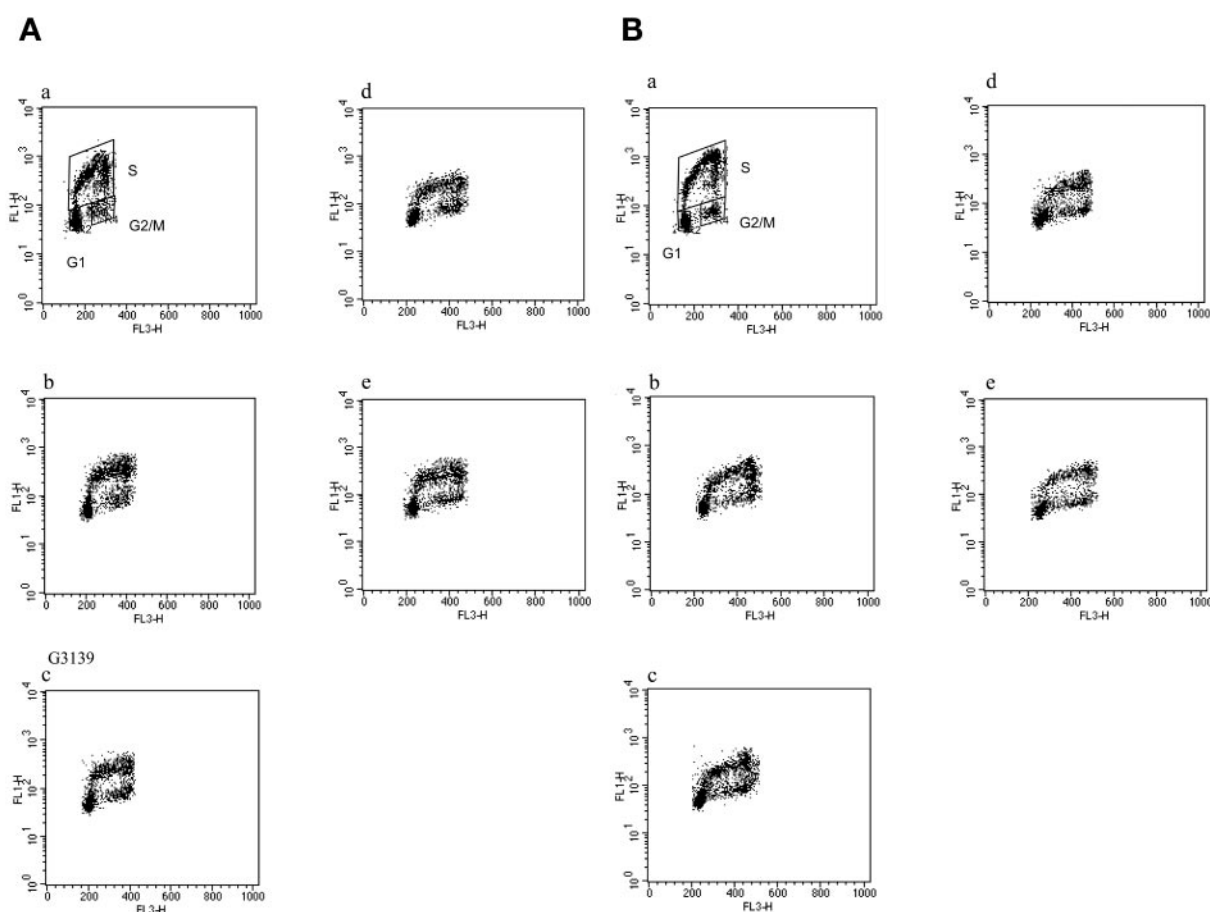
We then, by trypan blue exclusion (Fig. 6B), evaluated the ability of G3139 and related oligonucleotides to inhibit cellular growth over time. Similar to what we observed previously in oligonucleotide-treated wild-type PC3 cells, G3139 (400 nM/

Lipofectin 15  $\mu\text{g}/\text{ml}$ ) is the best suppressor of mock-transfected, AS1 and AS2 cell growth. In each case, the difference between G3139 and G4126 is highly statistically significant.

#### A siRNA Targeted to the bcl-2 mRNA Causes Down-Regulation of the Expression of bcl-2 Protein But No Apparent Phenotypic Change in Wild-Type DU145 Cells or Chemosensitization.

The production of AS1 and AS2 involved a clonal selection process with all of its attendant uncertainties. To additionally define the role of bcl-2 in the chemoresistant phenotype, we then determined what changes would be produced if highly specific down-regulation of bcl-2 expression in wild-type DU145 cells via a siRNA strategy was used. We examined a panel of 10 siRNAs and selected D6 as the most active (90–100% down-regulation of bcl-2 protein at 25 nM duplex oligonucleotide/1.33  $\mu\text{g}/\text{ml}$  Lipofectamine 2000 (Fig. 7A). The expression of other proteins, such as PKC- $\alpha$ , bcl-xL, and bax, was not meaningfully affected. Down-regulation of bcl-2 expression persisted for at least 7 days after a 24-h incubation with the complexes in Opti-MEM (Fig. 7B). No





**Fig. 5** Flow cytometric analysis of bromodeoxyuridine incorporation (Y axis; FL1 channel) and DNA content as determined by propidium iodide staining (X axis; FL3 channel) in oligonucleotide-treated mock (A) and AS1 transfected (B) DU145 cells. Cells were either untreated (a); or treated for 5 h with complexes of G4126 (b), G3139 (c), G4232 (d), or 2009 (e; 400 nm) and Lipofectin (15  $\mu$ g/ml) for 5 h in Opti-MEM, and then for an additional 67 h in complete medium without complexes. Nineteen h later, as described in “Materials and Methods,” cells were pulsed with 10  $\mu$ M bromodeoxyuridine for 2 h, harvested, and analyzed (A and B; and region gates for the quantitative analysis are indicated). Integrated data are presented in Tables 2 and 3.

**Table 2** BrdUrd<sup>a</sup> incorporation in mock-transfected DU145 cells

Oligo	G <sub>1</sub>	S	G <sub>2</sub> /M	S <sup>b</sup>
Control	42.8%	43.8%	9.0%	4.4%
G4126	38.5%	44.2%	10.1%	7.2%
G3139	43.7%	37.7%	11.6%	7.0%
G4232	35.1%	45.0%	12.1%	7.8%
2009	37.6%	43.3%	11.5%	7.6%

<sup>a</sup> BrdUrd, bromodeoxyuridine.

<sup>b</sup> S, cells in S phase that do not take up BrdUrd.

effects on cellular growth *versus* untreated, control wild-type cells, again as assessed by trypan blue exclusion, were observed. In addition, treatment with siRNA D6 did not induce any changes in the expression of either PKC- $\alpha$  or bcl-xL proteins, unlike what was observed in AS1 and AS2. No changes in progression through the cell cycle, as determined flow cytometrically via BrdUrd/PI uptake, were observed. Furthermore, wild-type DU145 cells were not sensitized to treatment with

paclitaxel, mitoxantrone, or thapsigargin (Fig. 8) after siRNA D6 treatment, nor did cells treated with this duplex produce ROS as determined flow cytometrically by measurement of E or DCF fluorescence (data not shown).

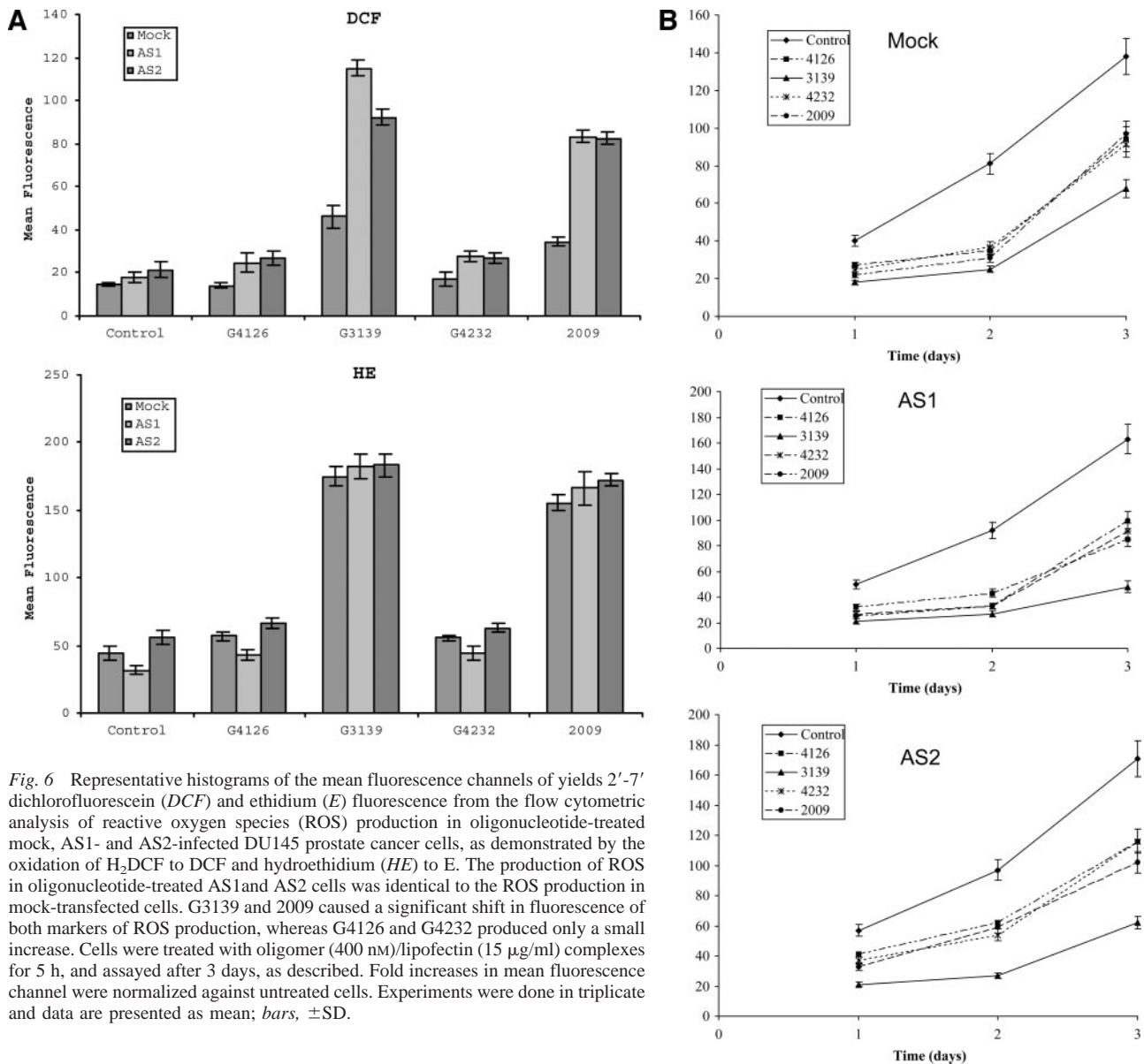
We then determined whether the same phenotype induced by G3139 would also be induced in wild-type DU145 cells in which bcl-2 protein expression had already been knocked down by treatment with siRNA D6. Thus, 3 days after treatment with

**Table 3** BrdUrd<sup>a</sup> incorporation in AS1-infected DU145 cells

Oligo	G <sub>1</sub>	S	G <sub>2</sub> /M	S <sup>b</sup>
Control	39.0%	49.7%	8.7%	2.65%
G4126	38.0%	41.9%	9.0%	11.1%
G3139	36.3%	42.5%	11.3%	9.9%
G4232	39.3%	36.9%	11.2%	12.6%
2009	38.4%	39.5%	12.0%	10.1%

<sup>a</sup> BrdUrd, bromodeoxyuridine.

<sup>b</sup> S, cells in S phase that do not take up BrdUrd.



**Fig. 6** Representative histograms of the mean fluorescence channels of yields 2'-7' dichlorofluorescein (DCF) and ethidium (E) fluorescence from the flow cytometric analysis of reactive oxygen species (ROS) production in oligonucleotide-treated mock, AS1- and AS2-infected DU145 prostate cancer cells, as demonstrated by the oxidation of H<sub>2</sub>DCF to DCF and hydroethidium (HE) to E. The production of ROS in oligonucleotide-treated AS1 and AS2 cells was identical to the ROS production in mock-transfected cells. G3139 and 2009 caused a significant shift in fluorescence of both markers of ROS production, whereas G4126 and G4232 produced only a small increase. Cells were treated with oligomer (400 nM)/lipofectin (15 μg/ml) complexes for 5 h, and assayed after 3 days, as described. Fold increases in mean fluorescence channel were normalized against untreated cells. Experiments were done in triplicate and data are presented as mean; bars, ±SD.

siRNA D6 (or with control siRNA D3), the cells were transfected with G3139 (400 nM)/Lipofectin (15 μg/ml). Control cells were transfected with G4126, and other controls consisted of cells not transfected with siRNAs. Two characteristic aspects of the G3139-induced phenotype were examined, cell number (determined daily for 4 days after either G3139 or G4126 transfection) and production of ROS (determined flow cytometrically 3 days after either G3139 or G4126 transfection). The results are depicted in Fig. 9, A and B, where it is shown that there is no change in G3139 phenotype (*i.e.*, either in cell number or in production of ROS) whether or not the expression of bcl-2 protein has been down-regulated previously by siRNA D6. We believe that this experiment strongly suggests that the phenotype induced in prostate cancer cells by G3139 is independent of its ability to down-regulate bcl-2 protein expression,

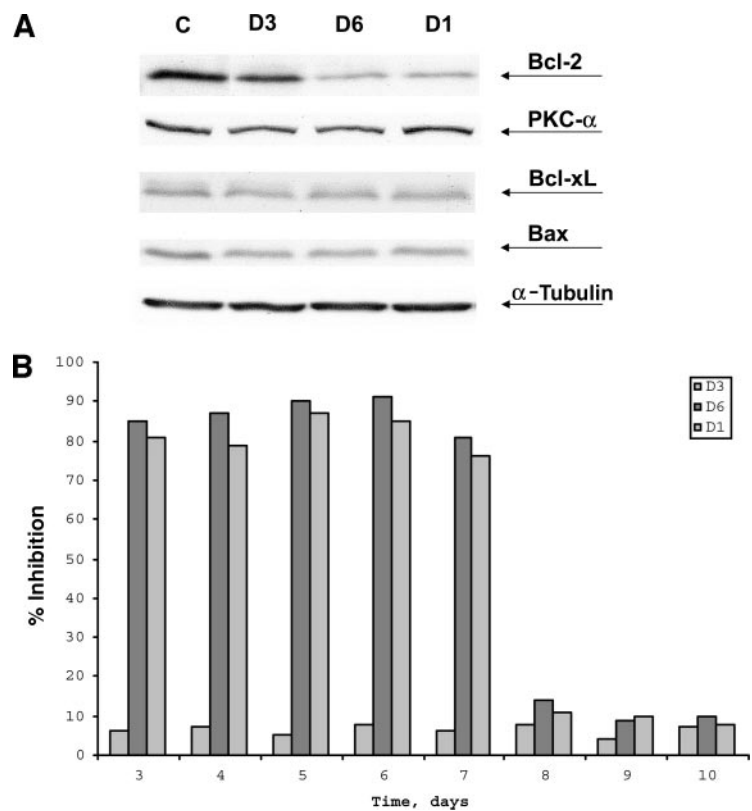
at least under conditions that provide for at least 90% bcl-2 down-regulation.

## DISCUSSION

G3139 was designed and is used as an antisense effector molecule, and there cannot any longer be any doubt that this molecule can down-regulate the expression of bcl-2, ostensibly by an RNase H-dependent antisense mechanism of action, at least in tissue culture experiments using prostate cancer cell models. Nevertheless, it is also becoming clear, as delineated by Benimetskaya *et al.* (27), and Lai *et al.* (26), that G3139 can interact with prostate cancer cells in additional, nonantisense ways. In fact, G3139 can down-regulate the expression of bcl-2 protein by both antisense and nonantisense mechanisms simul-



**Fig. 7** **A**, representative Western blot analysis demonstrating that small interfering RNA bcl-2 (*D6* and *D1*) down-regulates bcl-2 protein expression. In contrast, essentially no change in the levels of expression of protein kinase C (*PKC*)- $\alpha$  or bax proteins were observed. DU145 cells were treated either with complexes of *D6*, *D1*, or *D3* (25 nM) and Lipofectamine 2000 (1.33  $\mu$ g/ml) for 24 h in Opti-MEM and then for an additional 48 h in complete medium without complexes, and harvested. Protein samples (30–40  $\mu$ g of protein/lane) were analyzed by Western blotting as described in “Materials and Methods,” with tubulin used as a control protein species. The percentage of inhibition *versus* control, untreated cells was determined by laser-scanning densitometry. **B**, time dependence of the effects of siRNA bcl-2 (*D6* and *D1*) on bcl-2 protein expression. DU145 cells were treated either with complexes of *D6*, *D1*, or *D3* (25 nM) and Lipofectamine 2000 (1.33  $\mu$ g/ml) for 24 h in Opti-MEM and then for an additional 2–9 days in complete medium without complexes. Cells were harvested, and protein samples (30–40  $\mu$ g of protein/lane) were analyzed by Western blotting 3–10 days after the treatment as described in “Materials and Methods,” with tubulin used as a control protein species. The percentage of inhibition *versus* control, untreated cells was determined by laser-scanning densitometry.



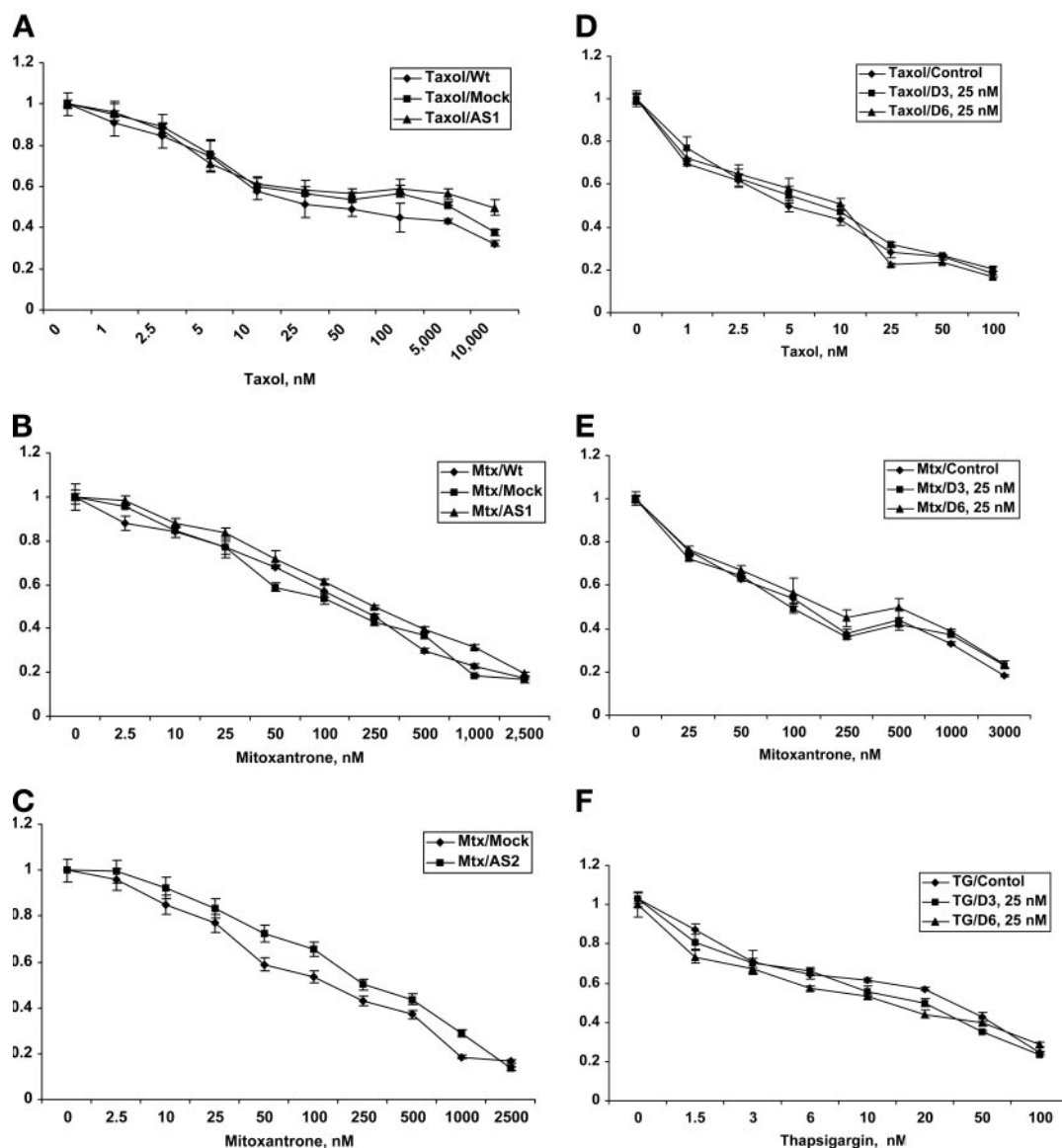
taneously, and it has been very difficult, if not impossible, to separate the effects of each mechanism on cellular phenotype. On the other hand, these mechanisms can potentially be distinguished by down-regulation of bcl-2 expression before G3139 treatment is administered.

To achieve this, we used two antisense RNA technologies. Initially, we transfected DU145 cells with a vector capable of producing a truncated antisense RNA strand. It has been postulated that such a molecule can inhibit gene expression by a variety of mechanisms, including the direct physical blockade of ribosomal read-through, and the promotion of degradation of double-stranded RNA (34), perhaps by Dicer. In fact, bcl-2 protein expression has been down-regulated previously by an antisense RNA or ribozyme (16, 17) approach by a number of experimenters (13, 14, 33). An advantage of the antisense RNA approach is the long-term stability of the clones (*e.g.*, AS1 and AS2) with bcl-2 substantially knocked down. A disadvantage is the requirement for clonal selection. In fact, as we have shown recently (27), transfection of prostate cancer cells, at least with a variety of 18mer phosphorothioate oligonucleotides, causes the sequence-independent induction of the IFN cascade, which at least transiently (1–3 days) decreases bcl-2 mRNA and protein expression, as well as up-regulates the expression of many other growth-related genes. Thus, it is not clear *a priori* how bcl-2 protein expression in transfected prostate cancer cells would fare in an antibiotic-mediated clonal selection process.

To knock down bcl-2 protein expression without the problem of clonal selection, we used two 21mer double-stranded oligoribonucleotides (siRNA *D1* and *D6*; reviewed in Refs.

35–39). These siRNAs were selected from a panel of 10 as the most effective, and could achieve highly reproducible bcl-2 protein expression knockdown of >90% at a concentration of 25 nM.

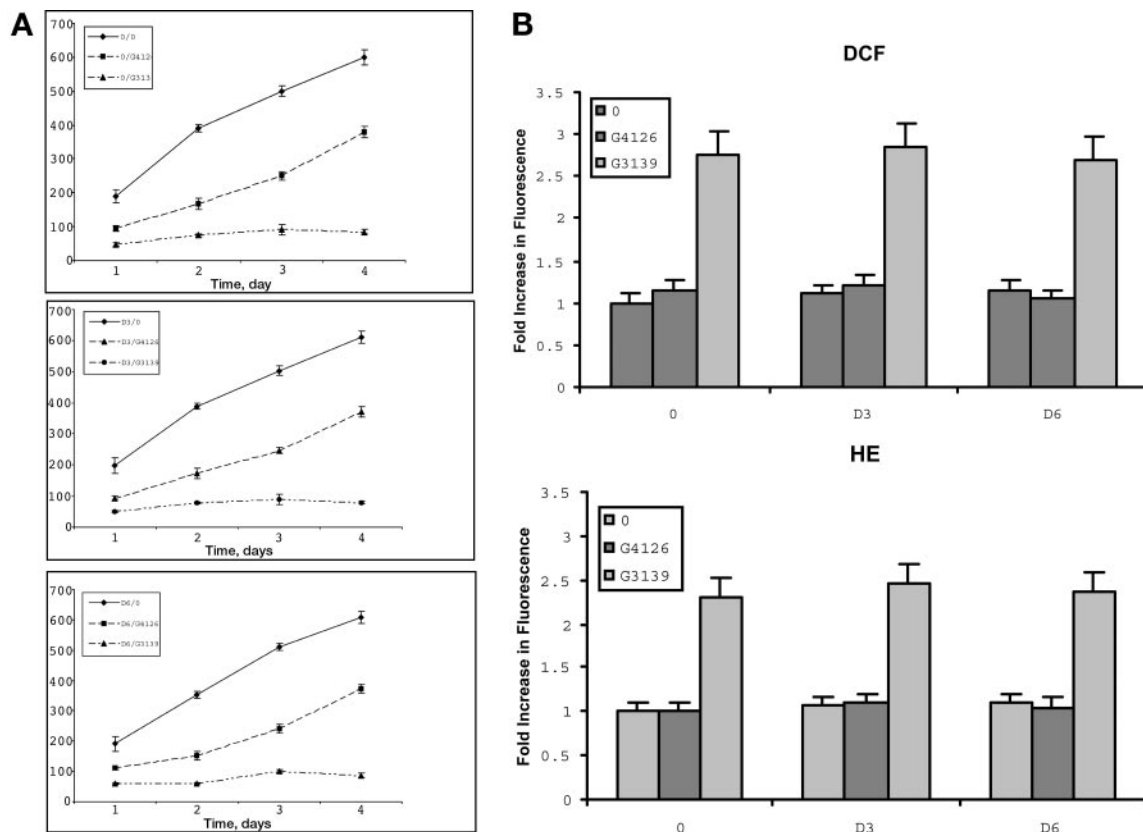
G3139 did not cause any dramatic difference in the number of AS1 *versus* mock-transfected DU145 cells undergoing apoptosis. The AS1 cells, however, seemed to be more sensitive to treatment by 2009 than the mock-transfected cells, which may have to do with the fact that 2009 is somewhat longer than G3139 (20mer *versus* 18mer) and, thus, potentially somewhat more nonsequence specifically toxic. More importantly, however, is that we could demonstrate no increase in chemosensitization (to docetaxel or mitoxantrone) in the AS1 or AS2 *versus* mock-infected cells. Concordantly, treatment of wild-type DU145 cells with siRNA *D6*, which can consistently down-regulate bcl-2 protein expression by as much as 90%, also did not sensitize these cells to these cytotoxic agents. In addition, major aspects of the G3139-induced phenotype (*e.g.*, growth suppression and ROS generation) were identical in the presence (as in the wild-type cells) or absence (as in the siRNA *D6*-treated wild-type cells) of bcl-2 expression. Taken together, these data strongly suggest that despite experiments that might imply the contrary (15, 16), the expression of basal levels of DU145 prostate cancer cells do not appear to contribute in any major way that we can determine to a chemoresistant phenotype (at least with respect to the cytotoxic agents used in this study) in DU145 cells in tissue culture. Moreover, we have obtained data recently (27) that leads to very much the same conclusions in PC3 prostate cancer cells.



**Fig. 8** DU145 cells in which bcl-2 protein expression has been down-regulated previously are not sensitized to treatment with cytotoxic agents. Mock-transfected and the AS1 or AS2 clones were treated for 72 h with Taxol (A) or mitoxantrone (B and C) at the indicated concentrations. Cellular viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in "Materials and Methods," and absorbance was measured at 570 nm. Cellular viability was determined as a ratio of the absorbance of the treated cells to that of the corresponding untreated cells. Data are presented as the average; bars,  $\pm$ SD;  $n = 4$ . Wild-type DU145 cells were also not sensitized to cytotoxic agents after small interfering RNA D6 treatment (D-F).  $25 \times 10^4$  cells were seeded in six-well plates, treated either with complexes of D6 or control D3 small interfering RNAs (25 nM) and Lipofectamine 2000 (1.33  $\mu$ g/ml) for 24 h in Opti-MEM, and then for an additional 2 days in complete medium without complexes. The cells were then reseeded in 96-well plates ( $0.4 \times 10^4$  cells/well) at the same density, and next day treated for 72 h with Taxol (D), mitoxantrone (E), or thapsigargin (F) at the indicated concentrations. Cellular viability was determined as a ratio of the absorbance of the treated cells to that of the corresponding untreated cells. Data are presented as the average; bars,  $\pm$ SD;  $n = 4$ .

Our data do not call into question experiments in which prostate cancer cells have been genetically engineered to "over-express" bcl-2 or related antiapoptotic proteins (5–10), resulting in chemoresistance. However, the process of extrapolation of chemoresistance at "high" bcl-2 expression to chemosensitization at "low" bcl-2 expression seems to be a flawed one, at least in prostate cancer cell lines in tissue culture. Furthermore, it is difficult, if not impossible, to claim that an observed cellular

phenotype is a direct cause of the down-regulation of a bcl-2 if G3139 is used as the down-regulator. The molecule simply induces too many molecular and cellular events (*e.g.*, ROS production and inhibition of cellular proliferation) that are not bcl-2 dependent because their patterns of expression cannot be recapitulated by treatment with the ostensibly bcl-2-specific siRNA D6. However, one caveat that must be considered is that at least some of the differences observed between the AS1 and



**Fig. 9** A, the rate of proliferation of oligonucleotide-treated DU145 prostate cancer cells, as determined by trypan blue exclusion, did not change whether or not the expression of bcl-2 protein has been down-regulated previously by small interfering RNA (siRNA).  $25 \times 10^4$  cells were seeded in six-well plates, treated either with complexes of D6 or control D3 (25 nM) and Lipofectamine 2000 (1.33  $\mu\text{g}/\text{ml}$ ) for 24 h in Opti-MEM and then for an additional 2 days in complete medium without complexes. The cells were then reseeded in six-well plates ( $25 \times 10^4$  cells/well) and next day treated either with complexes of G3139 or G4126 (400 nM) and Lipofectin (15  $\mu\text{g}/\text{ml}$ ) for 5 h in Opti-MEM, and then for an additional 67 h in complete medium without complexes. Cells were counted daily. Data are presented as the average; bars,  $\pm$ SD;  $n = 3$ . B, representative histograms of the mean fluorescence channels of 2',7'-dichlorofluorescein (DCF) and ethidium (E) fluorescence from flow cytometric analysis of reactive oxygen species production in oligonucleotide-treated DU145 prostate cancer cells as demonstrated by the oxidation of  $\text{H}_2\text{DCF}$  to DCF and hydroethidium (HE) to E. The production of reactive oxygen species did not change whether or not the expression of bcl-2 protein has been knocked down previously by siRNA. Three days after treatment with siRNA (D6 or D3) cells were reseeded in six-well plates ( $25 \times 10^4$  cells/well) and the next day transfected with G3139 or G4126 as described in A. The fold increase in mean fluorescence channel was normalized against untreated cells. Experiments were done in triplicate and data are presented as mean; bars,  $\pm$ SD.

AS2 cells after G3139 treatment and the wild-type DU145 cells after siRNA D6 treatment relate to long-term bcl-2 suppression in the former *versus* short-term (*i.e.*, 7 day) bcl-2 suppression in the latter. Nevertheless, it is still clear from the siRNA D6 experiments that bcl-2 protein plays no apparent role in the generation of ROS, in the rate of cell growth, or in the chemoresistant phenotype in DU145 cells.

Finally, although our data suggest that bcl-2 down-regulation alone, at least in DU145 prostate cancer cells, contributes relatively little to a proapoptotic phenotype, and it is perhaps more likely that the combination of bcl-2 down-regulation plus the nonantisense effects of G3139 produce the desired, antineoplastic phenotype. This, in turn, suggests that attempts to dramatically decrease the nonspecificity of phosphorothioate antisense oligonucleotides may be accomplished at the cost of clinical efficacy. This in no way, however, devalues the antisense approach for therapeutic purposes. Instead, we believe

that it points this area in a somewhat new, and more complex direction, but one that nevertheless will hopefully ultimately lead to improved clinical efficacy.

## REFERENCES

- Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–911.
- Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. *FEBS Lett* 2000;466:6–10.
- Reed JC, Jurgensmeier JM, Matsuyama S. Bcl-2 family proteins and mitochondria. *Biochim Biophys Acta* 1998;1366:127–37.
- Dias N, Stein CA. Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides. *Eur J Pharm Biopharm* 2002;54:263–9.
- Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 1993;81:151–7.
- Kamesaki S, Kamesaki H, Jorgensen TJ, et al. Bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to

- topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res* 1993;53:4251–6.
7. Baffy G, Miyashita T, Williamson JR, Reed JC. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J Biol Chem* 1993;268:6511–9.
  8. Walton MI, Whysong D, O'Connor PM, et al. Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res* 1993;53:1853–61.
  9. Fisher TC, Milner AE, Gregory CD, et al. Bcl-2 modulation of apoptosis induced by anticancer drug: resistance to thymidylate stress is independent of classical resistance pathways. *Cancer Res* 1993;53:3321–6.
  10. Tang C, Willingham MC, Reed JC, et al. High levels of p26BCL-2 oncoprotein retard taxol-induced apoptosis in human pre-B leukemia cells. *Leukemia (Baltimore)* 1994;8:1960–9.
  11. Reed JC. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol Oncol Clin N Am* 1995;9:451–73.
  12. Teixeira C, Reed JC, Pratt MA. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res* 1995;55:3902–7.
  13. Saeki K, Yuo A, Okuma E, et al. Bcl-2 down-regulation causes autophagy in a caspase-independent manner in human leukemic HL60 cells. *Cell Death Differ* 2000;7:1263–9.
  14. Zhang WG, Ma LP, Wang SW, Zhang ZY, Cao GD. Antisense Bcl-2 retrovirus vector increases the sensitivity of a human gastric adenocarcinoma cell line to photodynamic therapy. *Photochem Photobiol* 1999;69:582–7.
  15. Dorai T, Perlman H, Walsh K, et al. A recombinant defective adenoviral agent expressing anti-bcl-2 ribozyme promotes apoptosis of bcl-2-expressing human prostate cancer cells. *Int J Cancer* 1999;82:846–52.
  16. Dorai T, Goluboff ET, Olsson CA, Buttyan R. Development of a hammerhead ribozyme against BCL-2. II. Ribozyme treatment sensitizes hormone-resistant prostate cancer cells to apoptotic agents. *Anti-cancer Res* 1997;17:3307–12.
  17. Dorai T, Olsson CA, Katz AE, Buttyan R. Development of a hammerhead ribozyme against bcl-2. I. Preliminary evaluation of a potential gene therapeutic agent for hormone-refractory human prostate cancer. *Prostate* 1997;32:246–58.
  18. Klasa RJ, Bally MB, Ng R, et al. Eradication of human non-Hodgkin's lymphoma in SCID mice by BCL-2 antisense oligonucleotides combined with low-dose cyclophosphamide. *Clin Cancer Res* 2000;6:2492–500.
  19. Kitada S, Takayama S, De Riel K, Tanaka S, Reed JC. Reversal of chemoresistance of lymphoma cells by antisense-mediated reduction of bcl-2 gene expression. *Antisense Res Dev* 1994;4:71–9.
  20. Gleave ME, Miyake H, Goldie J, Nelson C, Tolcher A. Targeting bcl-2 gene to delay androgen-independent progression and enhance chemosensitivity in prostate cancer using antisense bcl-2 oligodeoxynucleotides. *Urology* 1999;54:36–46.
  21. Campbell MG, Dawson M, Koeffler HP. Growth inhibition of DU-145 prostate cancer cells by a Bcl-2 antisense oligonucleotide is enhanced by N-(2-hydroxyphenyl)all-trans retinamide. *Br J Cancer* 1998;77:739–44.
  22. Bilim V, Kasahara T, Noboru H, Takahashi K, Tomita Y. Caspase involved synergistic cytotoxicity of bcl-2 antisense oligonucleotides and Adriamycin on transitional cell cancer cells. *Cancer Lett* 2000;155:191–8.
  23. Jansen B, Schlagbauer-Wadl H, Brown BD, et al. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med* 1998;4:232–4.
  24. Jansen B, Wacheck V, Heere-Ress E, et al. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet* 2000;356:1728–33.
  25. Benimetskaya L, Miller P, Benimetsky S, et al. Inhibition of potentially anti-apoptotic proteins by antisense protein kinase C- $\alpha$  (Isis 3521) and antisense bcl-2 (G3139) phosphorothioate oligodeoxynucleotides: relationship to the decreased viability of T24 bladder and PC3 prostate cancer cells. *Mol Pharmacol* 2001;60:1296–307.
  26. Lai JC, Benimetskaya L, Santella R, et al. G3139 (Oblimersen) may inhibit prostate cancer cell growth in a partially bis-CpG-dependent, non-antisense manner. *Mol Cancer Ther* 2003;2:1031–43.
  27. Benimetskaya L, Wittenberger T, Stein CA, et al. Changes in gene expression induced by G3139 in prostate carcinoma cells are recapitulated at least in part by treatment with interferon- $\beta$  and - $\gamma$ . *Clin Cancer Res* 2004, in press.
  28. Elbashir SM, Harborth J, Lendeckel W, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (Lond)* 2001;411:494–8.
  29. Zamore PD, Tuschl T, Sharp PA, Bartel D. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000;101:25–33.
  30. Tuschl T. Expanding small RNA interference. *Nat Biotechnol* 2002;20:446–8.
  31. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 2001;15:188–200.
  32. Tarpey MM, Fridovich I. Methods of detection of vascular reactive species: nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res* 2001;89:224–36.
  33. Feng L, Balakir R, Precht P, Horton WE. Bcl-2 regulates chondrocyte morphology and aggrecan gene expression independent of caspase activation and full apoptosis. *J Cell Biochem* 1999;74:576–86.
  34. Vilenchik M, Raffo AJ, Benimetskaya L, Shames D, Stein CA. Antisense RNA down-regulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents. *Cancer Res* 2002;62:2175–83.
  35. Rickford AS, Cogoni C. RNA-mediated gene silencing. *Cell Mol Life Sci* 2003;60:871–2.
  36. Scherr M, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr Med Chem* 2003;10:245–56.
  37. Shuey DJ, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today* 2002;15:1040–6.
  38. Timmons L. The long and short of siRNAs. *Mol Cell* 2002;10:435–7.
  39. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* 2002;3:737–47.



# Clinical Cancer Research

## Antisense RNA Down-Regulation of bcl-2 Expression in DU145 Prostate Cancer Cells Does Not Diminish the Cytostatic Effects of G3139 (Oblimersen)

Anthony Raffo, Johnathan C. Lai, C. A. Stein, et al.

*Clin Cancer Res* 2004;10:3195-3206.

**Updated version** Access the most recent version of this article at:  
<http://clincancerres.aacrjournals.org/content/10/9/3195>

**Cited articles** This article cites 37 articles, 13 of which you can access for free at:  
<http://clincancerres.aacrjournals.org/content/10/9/3195.full#ref-list-1>

**Citing articles** This article has been cited by 9 HighWire-hosted articles. Access the articles at:  
<http://clincancerres.aacrjournals.org/content/10/9/3195.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://clincancerres.aacrjournals.org/content/10/9/3195>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.