Relative Bcl-2 Independence of Drug-Induced Cytotoxicity and Resistance in 518A2 Melanoma Cells

Luba Benimetskaya,1 Johnathan C. Lai,2 Anastasia Khvorova,3 Sijian Wu,1 Emily Hua,1 Paul Miller,4 Li-Ming Zhang,1 and Cy A. Stein1

1Albert Einstein-Montefiore Cancer Center, Department of Oncology, Montefiore Medical Center, Bronx, New York; 2Department of Biomedical Engineering, Columbia University, New York, New York; 3Dharmacon Research, Lafayette, Colorado; and 4Johns Hopkins University, School of Public Health, Baltimore, Maryland

ABSTRACT

Purpose: Inhibition of the function of Bcl-2 protein has been postulated to sensitize cells to cytotoxic chemotherapy. G3139 (Genasense) is a phosphorothioate anti–Bcl-2 antisense oligonucleotide, but its mechanism of action is uncertain. The aim of the present work is to investigate inhibition of Bcl-2 expression in 518A2 melanoma cells, the cell line on which recent phase II and phase III clinical trials employing this agent were based.

Experimental Design: We down-regulated the expression of Bcl-2 protein by two different strategies in these cells: one employing G3139 and controls, and the other using a small interfering RNA approach. Cell viability after treatment with oligonucleotides or small interfering RNA and cytotoxic agents including gemcitabine, DDP, docetaxel, and thapsigargin was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. A 518A2 melanoma cell line stably overexpressing Bcl-2 protein was constructed and treated with either these cytotoxic agents or G3139.

Results: The cytotoxic effects of either G3139 or small interfering RNA treatment of 518A2 melanoma cells are Bcl-2 independent. In addition, in the Bcl-2–overexpressing cells, only a modest increment in chemoresistance was observed, and treatment with G3139 not only did not down-regulate Bcl-2 expression but produced essentially identical toxicity as was observed in the wild-type or mock-transfected cells.

Conclusions: Our results suggest that the mechanism whereby G3139 produces drug-induced cytotoxicity in the 518A2 melanoma line is not dependent on levels of Bcl-2. These findings emphasize the nonsequence specific effects of this phosphorothioate oligonucleotide and call into question the validity of Bcl-2 as a target in this cell line.

INTRODUCTION

G3139 (Oblimersen; ref. 1) is an 18-mer phosphorothioate oligodeoxynucleotide targeted to the initiation codon region of the Bcl-2 mRNA, which has been examined as a chemosensitizing agent for advanced melanoma (2). This work, in turn, was dependent on earlier observations (3) that mice deficient in Bcl-2 tended to become hypopigmented (3) and on a retrospective analysis of the negative effect on survival of increased Bcl-2 expression in the lymph node metastases of patients with malignant melanoma (4). However, these data (4) were obtained from only 15 samples immunostaining positive for bcl-2 by flow cytometry of 42 originally obtained from patients.

G3139 in combination with dacarbazine did not produce a statistically significant increase in the primary end point of overall survival versus dacarbazine alone in a randomized phase III clinical trial. However, tantalizing increases were observed in progression-free survival (74 days versus 49 days; \( P = 0.0003 \)), in the proportion of patients who achieved a major antitumor response (13% versus 7%; \( P = 0.006 \)), and in the number of durable complete responses, although the numbers are small (10 [2.8%] versus 1 [0.5%]; ref. 5). These data must be noted in the context of the lack of Food and Drug Administration drug approvals for advanced melanoma since 1975, when dacarbazine was approved.

There is substantial agreement that increased Bcl-2 expression is directly related to chemoresistance in at least some cell lines (6, 7). Down-regulation of Bcl-2 expression has also been accomplished by an antisense RNA strategy in MCF-7 breast cancer cells (8) and in DU145 prostate cancer cells (9) and by an adenovirally delivered anti–bcl-2 ribozyme in PC3 prostate cancer cells (10). In the MCF-7 cells, increased sensitivity to doxorubicin and estrogen withdrawal was noted, and apoptosis was produced in the ribozyme-transfected PC3 cells. However, in the DU145 cells, chemosensitization was produced neither by the antisense RNA approach nor by small interfering RNA down-regulation of Bcl-2 protein expression. Other work (11, 12) in PC3 prostate cancer cells has demonstrated that treatment with G3139 did produce down-regulation of Bcl-2 protein and mRNA expression. However, cellular cytostasis, rather than chemosensitization or apoptosis, was observed. Furthermore, a small interfering RNA targeted to the Bcl-2 mRNA of PC3 cells also did not produce chemosensitization (9).

The G3139-induced cytostasis was demonstrated (11), via Affymetrix oligonucleotide microarray, to be related to the induction of members of the interferon cascade, which occurred in the absence of induction of interferon proteins. It was also demonstrated that direct treatment of PC3 cells with recombinant interferon-\( \beta \) and/or interferon-\( \gamma \) protein recapitulated many elements of the G3139-induced phenotype, including Bcl-2 and...
protein kinase C-α (PKC-α) protein down-regulation, an increase in cell surface major histocompatibility complex class I expression, down-regulation of Cip1 expression, and profound cellular cytostasis without apoptosis.

The capacity for the production of profound off-target (or nonspecific) effects (13, 14) by G3139 (as a member of the phosphorothioate class of oligonucleotide) had not previously been examined in 518A2 melanoma cells, the line on which the clinical phase II trial of DTIC ± dacarbazine was originally based (2). However, significant nonsense specificity has been observed in vitro and in SCID mice containing SK-2 melanoma xenografts (15).

In this study, we demonstrate that neither treatment of this melanoma cell line with a small interfering RNA targeted to Bcl-2 nor Bcl-xL or both simultaneously produces chemosensitization. We also show that down-regulation of Bcl-2 expression by use of a small interfering RNA does not alter the G3139-induced cellular phenotype and that up-regulation of Bcl-2 expression does not substantially alter the G3139-induced loss of cellular viability as a function of time. We thus believe that these data (a) strongly suggest that the Bcl-2 protein present in this melanoma cell line in tissue culture is not a critical target, and (b) point out the value of cross-validation of antisense oligonucleotide experiments by the use of the small interfering RNA technology.

MATERIALS AND METHODS

Cells. The mycoplasma-free human melanoma cell line 518A2 was a kind gift of Dr. Volker Wacheck (University of Vienna, Austria). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. The stock culture was maintained at 37°C in a humidified 5% CO2 incubator.

518A2 cells were transfected with the neomycin-selectable pSFFV/Bcl-2 plasmid (courtesy of S. Korsmeyer, Dana-Farber Cancer Center, Boston, MA) or with the control, neomycin-resistant expression vector pSFFV. Aliquots containing 10 μg of plasmid and 5 μL/mL LipofectAMINE 2000 in serum-free minimal essential medium (Opti-MEM, Invitrogen) were added to the cultured cells. The transfection medium was replaced 5 hours later with complete medium. Individual colonies were selected from the plates after approximately 3 to 4 weeks of routine maintenance in complete medium containing 0.6 μg/mL G418 sulfate (Invitrogen).

Reagents. The anti–Bcl-2 monoclonal antibody was from Dako (Carpinteria, CA), and the PARP-1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-α-tubulin monoclonal antibody and thapsigargin were from Sigma-Aldrich (St. Louis, MO). Docetaxel is a product of Avenex (Bridgewater, NJ) and gencitabine is a product of Eli Lilly (Indianapolis, IN). LipofectAMINE 2000 was purchased from Invitrogen. SUPERase In is a product of Ambion (Austin, TX). The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from BD Biosciences (San Jose, CA). Phosphorothioate oligonucleotides were synthesized and purified via standard procedures and kindly supplied by Genta (Berkeley Heights, NJ). G4126 is a two-base mismatch control for G3139. All small interfering RNAs were supplied by Dharmacon (Lafayette, CO).

Oligonucleotide Transfections. Cells were seeded the day before the experiment in six-well plates at a density of 15 × 10⁴ cells per well, to be 60 to 70% confluent on the day of the experiment. All transfections were performed in Opti-MEM medium (Invitrogen) plus complete medium without antibiotics, as described previously (12). The incubation time for oligonucleotide/LipofectAMINE 2000 complexes was 5 hours. The total incubation time before cell lysis and protein isolation was 48 hours at 37°C.

Small Interfering RNA Transfections. Cells were seeded in complete medium without antibiotics the day before the experiment in six-well plates at a density of 15 × 10⁴ cells per well, to be 60 to 70% confluent on the day of the experiment. All transfections were performed in Opti-MEM medium plus complete medium without antibiotics, as described previously (9).

Western Blot Analysis. Cells treated with oligonucleotide–lipid complexes were extracted in lysis buffer (10) at 4°C for 1 hour. Aliquots of cell extracts, containing 25 to 40 μg of protein, were resolved by SDS-PAGE and then transferred to Hybond ECL filter paper (Amersham Biosciences, Arlington Heights, IL). After treatment with appropriate primary and secondary antibody, ECL was performed, as described previously (12).

Determination of Rate of Cell Proliferation in Complete Medium. In brief, 0.4 × 10⁴ cells were seeded in 96-well plates and allowed to attach overnight. Cells were then treated with the appropriate concentrations of drugs. After 2 to 6 days of incubation at 37°C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was performed as per the manufacturer’s instructions. Absorbance was determined at 570 nm with a Benchmark plus Microplate spectrophotometer (Bio-Rad, Hercules, CA). Experiments were performed in quadruplicate, and data are presented as the average ± SD. In some experiments, cells were treated with oligonucleotide–lipid complexes or small interfering RNAs as described above and allowed to incubate for 2 or 3 days, when they were reseeded and treated with drugs.

Annexin V Cell Surface Binding. Cells treated for 72 hours with oligonucleotides were harvested after trypanosinization, and apoptotic cells were assayed by flow cytometry as described previously (12) using the Annexin V-FITC apoptosis detection kit (Becton Dickinson, San Jose, CA). Early apoptotic cells bound to anti-Annexin V-FITC, but excluded propidium iodide. Cells in late apoptotic stages were labeled with anti-Annexin V-FITC and propidium iodide.

Analysis of DNA Content. Cells were treated with oligonucleotide–lipid complexes as described in the text. After 3 days, they were fixed with ethanol and stained with 0.01 mg/mL propidium iodide (PI), and flow cytometry performed. Propidium iodide was excited at 488 nm. Data were analyzed by the CELLQuest software, and the percentage of cells in each cell cycle phase was determined as a ratio of the fluorescent area of the appropriate peaks to the total fluorescent area.
**Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction.** Total RNA was isolated from 518A2 melanoma cells using the RNeasy kit (Qiagen, Valencia, CA). RNA was reverse-transcribed with Platinum Taq (Invitrogen). All reverse transcription-PCR reactions (50 μL final volume) were performed as described previously. The forward and reverse primers for amplification of Bcl-2 and a 246-bp β-actin fragment used to normalize for RNA concentration have been noted (9). The MxA, OAS-1, and Cip1 were 5'-GCTACACACCGTGACGGATATGG-3' (forward) and 5'-TCAGAAGAGAAGCCACGTGA-3' (reverse); 5'-CGGAGACAGCGAGGGTAAAT-3' (forward) and 5'-CTGCCCAAGCTCTACCTTCC-3' (reverse); and 5'-CAGTCTCCATGTTCCCT-3' (reverse), respectively.

**Flow Cytometric Analysis of Mitochondrial Membrane Potential and Mitochondrial Mass.** Mitochondrial membrane potential was determined by JC-1 fluorescence and analyzed in FL-1 and FL-2 channels of a FACSCalibur (Becton Dickinson) flow cytometer equipped with a single 488 nm argon laser, as described previously (12).

**Subcellular Fractionation.** Cells were harvested by trypsinization and were washed with cold PBS. Cell pellets were resuspended in 300 μL of buffer A [250 mmol/L sucrose; 10 mmol/L Tris-HCl (pH 7.4); 1 mmol/L EGTA; 50 μg/mL Pefabloc; and 15 μg/mL leupeptin, aprotinin, and pepstatin]. Cells were then homogenized on ice in a Dounce homogenizer until ~90% of cells were disrupted, as judged by trypan blue staining. Crude lysates were centrifuged at 1,000 g for 10 minutes at 4°C twice to remove nuclei and unbroken cells. The supernatant was collected in a new tube and was subjected to a 10,000 x g centrifugation for 30 minutes at 4°C. The supernatant was collected as the cytosolic fraction, and the mitochondrial pellets were resuspended in 30 μL of buffer A.

**RESULTS**

**Down-Regulation of Bcl-2 mRNA and Protein Expression in 518A2 Melanoma Cells Does Not Produce Chemosensitization.** Both G3139 (100 nmol/L; 2 days) and the small interfering RNA D6 (10 nmol/L, 3 days) complexed with cationic lipids efficiently down-regulate the expression of Bcl-2 protein (by Western blotting) and mRNA (by reverse transcription-PCR; Fig. 1), as opposed to the two-base mismatched oligomer G4126 and the control small interfering RNA D3. However, in contrast to what was observed after treatment of PC3 and DU145 prostate cancer cells with G3139 (11), essentially no increase was observed in mRNA expression (by reverse transcription-PCR) for the MxA, OAS-1, and Cip1 mRNAs. Each of these genes can be induced by direct treatment with interferons or by induction of the interferon cascade.

518A2 cells were then treated with G3139 or G4126 (100 nmol/L)/LipofectAMINE 2000 (1.9 μg/mL; 5-h transfection) and then with increasing concentrations of docetaxel, DDP, gemcitabine, or the endoplasmic reticulum Ca2+-ATPase inhibitor thapsigargin (chosen because of its lack of cell cycle specificity). Little or no chemosensitization, as assayed by MTT, was produced after 3 (Fig. 2) or even 4 days (data not shown). Even if the cytotoxic chemotherapy was administered 2 days

![Fig. 1](image-url)
after G3139 treatment, when Bcl-2 protein down-regulation was maximal, chemosensitization still was not observed at any subsequent time. Similarly, and in accord with what we have observed in DU145 prostate cancer cells, down-regulation of Bcl-2 protein expression by the D6 small interfering RNA does not greatly alter 518A2 cellular viability. However, neither the D6 nor control D3 small interfering RNA leads to chemosensitization to increasing concentrations of either docetaxel or thapsigargin as assayed after 3 days by MTT (Fig. 3).

We then determined whether down-regulation of Bcl-2 expression by the D6 small interfering RNA before treatment with either G3139 or G4126 could alter the cytotoxicity of these agents. Thus, 518A2 cells were treated with 10 nmol/L D6 or control D3 small interfering RNA for 3 days (transfection time 24 hours) and then with increasing concentrations of either G3139 or G4126 in complex with LipofectAMINE 2000. As assessed by MTT (Fig. 4), there were no differences in the phosphorothioate oligonucleotide-induced cytotoxicity regardless of whether approximately 90% or more of the baseline quantity of Bcl-2 protein was present.

**Simultaneous Down-Regulation of Bcl-2 and Bcl-xL Protein Expression by Small Interfering RNA Does Not Lead to Chemosensitization in 518A2 Melanoma Cells.** Because down-regulation of Bcl-2 protein expression is in itself insufficient for chemosensitization of 518A2 melanoma cells, we then asked whether simultaneous down-regulation of Bcl-xL and Bcl-2 protein expression would suffice. We thus evaluated the small interfering RNA DX4 (control DX1; both 10 nmol/L/ LipofectAMINE 2000 [1.33 μg/mL], 24-hour transfection). As shown in Fig. 5, neither DX1 nor DX4 significant affects cellular viability after 3 days. Neither DX1 nor DX4 affects expression of Bcl-2 protein (assayed by Western blotting 3 days after the transfection), but only DX4 down-regulates the expression of Bcl-xL protein. On the other hand, whereas D6 targets Bcl-2 protein expression successfully, it does not affect Bcl-xL protein expression. The simultaneous combination of D6 + DX1, however, successfully targets Bcl-2 and Bcl-xL protein expression. Nevertheless, the simultaneous down-regulation of both of these strongly anti-apoptotic proteins also does not lead to chemosensitization to increasing concentrations of either DDP or docetaxel. However, (Fig. 4) the combination of the two small interfering RNAs is significantly growth suppressive, which may significantly blunt the effects of cytotoxic chemotherapy.

We then examined the effects of DDP and other cytotoxic agents on the 518A2 melanoma cells. By Western blotting, increasing concentrations of DDP (2 and 3 days) cause progressive increases in the cleavage of PARP-1 and, at the higher drug concentrations, down-regulation of Bcl-2 protein expression itself (not shown). As an example, the apoptosis induced by DDP treatment was demonstrated by the concentration-dependent cell surface expression of Annexin V expression, which is consistent with the induced PARP-1 cleavage. At 10 μmol/L DDP, approximately 62% of the cells are either in early or late apoptosis (not shown). These DDP data suggest that the down-regulation of Bcl-2 protein expression may be indeed a consequence, and not a cause of, cellular apoptosis.

---

**Fig. 2** Down-regulation of Bcl-2 by G3139 does not produce chemosensitization to increasing concentrations of cytotoxic agents. Cells were treated with G3139 and G4126 at a 100 nmol/L concentration, as described in Materials and Methods. Cells numbers were normalized and reseeded in 96-well plates in medium containing various concentrations of docetaxel (A), thapsigargin (B), gemcitibine (C), or DDP (D) 2 days after transfection. MTT assay was performed after 3 additional days of incubation. Cellular viability was determined as a ratio of the absorbance at 570 nm of the treated cells to the absorbance of the untreated cells. Data are presented as the average ± SD, n = 3.

---

8374 G3139 Induces bcl-2–Independent Cytotoxicity in Melanoma Cells
Forced Overexpression of Bcl-2 Protein in 518A2 Melanoma Cells Produces Only a Relatively Small Amount of Chemoresistance.

Transfection of 518A2 melanoma cells with the plasmid pSFFV-Bcl-2 generated a series of clones (B1–B8) that stably overexpress Bcl-2 protein. One clone (B3) as well as a mock-transfected clone (M4) were further analyzed (Fig. 6A). By serial dilutions and Western blotting, it could be determined that the increase in Bcl-2 protein expression in the B3 clone was >1000-fold. However, this enormous overexpression did not, surprisingly, affect the growth rate of the clones relative to the M4 clone and the wild-type cells. As shown in Fig. 6A, cytoplasmic and mitochondrial levels of Bcl-2 protein were greatly elevated (clone B3). We then treated the B3 clone with increasing concentrations of various cytotoxic chemotherapeutic agents [docetaxel; Fig. 6D; DDP, gemcitabine docetaxel, and thapsigargin (not shown)] and determined cell viability at 4 and 6 days by MTT. Although some resistance could be observed for thapsigargin and DDP only, it must be considered to be relatively minimal, with values of IC50 never increasing by more than a factor of 2 versus the M4 clone or wild-type cells. The MTT results with DDP are almost exactly paralleled by examination of DDP-induced mitochondrial membrane depolarization (ΔΨm) as assessed flow cytometrically by employing the dye JC-1 (Fig. 6B and C). The difference in the number of cells with depolarized ΔΨm is no more than a factor of 2, and at some DDP concentrations, not even that.

The B3 clone was also treated with increasing concentrations of G3139 and G4126, but the cytotoxicity induced by G3139 was only slightly reduced relative to the wild-type cells and the M4 clone. Again, examination of JC-1 fluorescence by flow cytometry demonstrates that there was no more than a 25% difference in the number of B3 cells versus M4 (mock transfected) cells with depolarized ΔΨm. In fact, the data suggest that the B3 clone is even more protected against G4126 than G3139.
G3139. Regardless, it seems clear that even in the presence of extremely high levels of mitochondrial and cytoplasmic Bcl-2, the cytotoxic effects of G3139 (and G4126) in 518A2 melanoma cells are substantially independent of Bcl-2 protein concentration.

**DISCUSSION**

Even though Bcl-2 expression can be related to resistance to cytotoxic chemotherapy, it has also been suggested that the levels of Bcl-2 protein expression are not necessarily predictive of cellular resistance to pro-death stimuli (16). For example, it has been demonstrated in ovarian and lung cancer cell lines (17–20) that basal levels of Bcl-2 expression do not necessarily correlate with resistance to apoptosis after treatment with cytotoxic chemotherapy. Similar to our observations in 518A2 melanoma cells, forced overexpression of Bcl-2 in the A2780 ovarian cancer cell line resulted in only a low level of resistance to DDP, and in some delay in apoptosis (21). Moreover, there is no obvious correlation in breast cancer cell lines between Bcl-2 overexpression and sensitivity to DNA-damaging agents (17). In fact, in glioma cells, Bcl-2 at very high levels of expression may be pro-apoptotic (22), perhaps because of caspase-3 cleavage of Bcl-2 protein at Asp34 to a pro-apoptotic fragment (23).

Although Bcl-2 is present in normal melanocytes, benign nevi, primary melanoma, and melanoma metastases (24), its role in the pathogenesis and prognosis of clinical melanoma is not clear (25). In several older studies (26–29), bcl-2 expression was found to decrease in melanoma cells versus normal melanocytes. However, other studies (for example refs. 24, 30, and 31) have found little or no difference in the expression of Bcl-2 in human melanocytes and melanoma cells. Because of this lack of difference, it has been suggested (31) that Bcl-2 cannot be a prognostic factor in melanoma. However, this analysis does not take into account the differing molecular contexts of melanocytes and melanoma cells in which Bcl-2 might function.

On balance, it seems overall that about one-third of the data in advancing melanoma suggest an increase in Bcl-2 expression, whereas one-third actually suggest a decrease (25). However, the quantity of expressed Bcl-2 may not be nearly as important as is functional importance, which at this point is not well understood over large numbers of human melanomas as opposed to what is thought to be true in model systems. In 518A2 melanoma cells, however, our data demonstrate that the functional importance of Bcl-2 appears to be low despite relatively high levels of expression.

In solid tumor cells that lack the t(14;18) translocation, additional mechanisms may lead to up-regulation of bcl-2 protein expression (17, 32). It has been suggested, for example, that the ability of p53 to negatively regulate Bcl-2 expression (33, 34) is abolished in p53 mutant cells (17). However, it is doubtful

---

**Fig. 5** Simultaneous down-regulation of Bcl-2 and Bcl-xL protein expression in 518A2 melanoma cells does not lead to chemosensitization to increasing concentrations of DDP or docetaxel. A. Western blot analysis of the effects of small interfering RNAs targeted to the Bcl-2 and Bcl-xL mRNAs. B. Western blot analysis of combinations of anti–Bcl-2 and anti–Bcl-xL small interfering RNAs. 518A2 cells were treated with the small interfering RNAs (10 nmol/L D6 or D3 and 50 nmol/L DX4 or DX1) as described in Materials and Methods. Cell numbers were normalized, and cells were reseeded in 96-well plates in medium containing either 3 μmol/L DDP (C) or 5 μmol/L docetaxel (D) 2 days after transfection. MTT assays were performed after 4 or 6 additional days of incubation, respectively. Cellular viability was determined as a ratio of the absorbance at 570 nm of the treated cells to the absorbance of the untreated cells. Data are presented as the average ± SD, n = 3.
that 518A2 cells contain wild-type p53. In additional experiments, we found that basal p53 expression can easily observed after Western blotting, unlike the difficult to detect levels usually observed in p53 wild-type cells (35). In addition, treatment of the 518A2 cells with cytotoxic agents causes a sharp decrease in Cip1 expression (not shown), not the increase that might be observed in p53 wild-type cells because of the positive regulation of Cip1 expression by p53 (36, 37). Finally, a small interfering RNA that successfully down-regulates p53 expression does not alter the extent of diminished viability in cells treated with either cytotoxic agents or G3139 (data not shown).

Our data demonstrate that Bcl-2 phosphorylation does not occur either basally or after treatment with a variety of cytotoxic agents. It has been shown, for example, in a murine, interleukin-3–dependent hematopoietic cell line (38), that Bcl-2 phosphorylation may be required for its anti-apoptotic function. On the other hand, data have also suggested that the anti-apoptotic activity of Bcl-2 is actually diminished after serine phosphorylation in response to treatment with paclitaxel (39). However, phosphorylation of Bcl-2 may, under these circumstances, be only a marker of M-phase events and not necessarily a regulator of apoptosis (40).

In theory, the use of small interfering RNA oligonucleotides (41, 42) provides a facile, relatively specific way of down-regulation of gene expression, at least when compared with the use of phosphorothioate oligonucleotides. We previously used this strategy (9) in combination with an antisense RNA approach to demonstrate the lack of chemosensitization produced when Bcl-2 protein expression was knocked-down in DU145 prostate cancer cells. Down-regulation of Bcl-2 mRNA and protein expression by the D6 small interfering RNA in 518A2 melanoma cells also does not produce chemosensitization to a variety of cytotoxic agents after 5 days, which is close to the maximum time that D6 will down-regulate Bcl-2 protein expression. In 607B melanoma cells, however (43), chemosensitization to DDP was demonstrated after small interfering RNA down-regulation of Bcl-2 protein expression. Nevertheless, it should be noted that in
this case, only a single drug (DDP) at a single concentration (1 μmol/L) was used.

As also observed in DU145 cells (9), down-regulation of Bcl-2 protein expression by the D6 small interfering RNA before treatment with G3139 or G4126 did not block the cytotoxic effects of these phosphorothioate oligonucleotides. We believe that this result clearly demonstrates that G3139 can produce cellular cytotoxicity in 518A2 cells that is independent of Bcl-2 activity. This conclusion is strengthened by the data in the B3 (Bcl-2-overexpressing) clone, in which Bcl-2 protein is highly overexpressed in cytoplasm and mitochondria. There, the decrease in cellular viability induced by either G3139 or cytotoxic chemotherapy (dosed at the identical concentrations in the B3 clone as in the mock-transfected M4 clone or the wild-type cells, and assessed by depolarization of ΔΨm) occurs in the absence of any significant diminution of Bcl-2 protein expression. In addition, given (a) the lack of cytotoxicity of the D6 small interfering RNA in context of its 90% knock-down of bcl-2 protein expression, and (b) the decrease in Bcl-2 protein expression during the induction of apoptosis by G3139 and 10 μmol/L DDP, it is possible, in some cases, that the down-regulation of Bcl-2 protein expression in 518A2 cells is a consequence of apoptosis. However, this is clearly not always the case, because the induction of apoptosis by either gemcitabine or docetaxel is not associated with Bcl-2 down-regulation in these cells (not shown).

Bcl-xL has been postulated as a chemoresistance factor in Mel Juso cells, at least to DDP (44) dosed at a single concentration. In contrast, our data in 518A2 melanoma cells suggest that down-regulation of expression of this strongly anti-apoptotic protein by itself (45, 46) does not, similar to Bcl-2 down-regulation, lead to chemosensitization. Moreover, simultaneous down-regulation of Bcl-2 + Bcl-xL protein expression by the small interfering RNA D6 does also not lead to chemosensitization. Previously, however, Olie et al. (47) synthesized a single 20-mer phosphorothioate 2′-O-methoxyethoxy gap-mer (48) antisense oligonucleotide targeted to the Bcl-2 and Bcl-xL mRNAs. They and others (49) were able to demonstrate, in several primary melanoma cell cultures, normal melanocytes and in the established A375 cell line, that this “bi-specific” oligomer dramatically reduced cellular viability and induced cellular apoptosis in the absence of an added cytotoxic agent. In addition, treatment of Bcl-2-overexpressing clones derived from the M14 melanoma line by this oligomer blocked hypoxia-induced vascular endothelial growth factor secretion and diminished angiogenesis (50). Nevertheless, the apparent discrepancy between these data and the results presented herein may be explained by the differences in cell lines used and the use of 20-mer phosphorothioate oligonucleotides in the previously published studies. In fact, in subsequent studies, we demonstrate that the induction of apoptosis in 518A2 melanoma cells by G3139 as a single agent is at least partially a phosphorothioate class effect.5

Finally, it must be noted that this analysis applies at this point only to the 518A2 melanoma cell line in tissue culture and cannot reasonably be extrapolated to any in vivo situation. Additional extensive preclinical and clinical studies will be required to determine the true value of Bcl-2 as a target in clinical melanoma.

REFERENCES


Relative Bcl-2 Independence of Drug-Induced Cytotoxicity and Resistance in 518A2 Melanoma Cells


Updated version  Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/10/24/8371](http://clincancerres.aacrjournals.org/content/10/24/8371)

Cited articles  This article cites 49 articles, 18 of which you can access for free at: [http://clincancerres.aacrjournals.org/content/10/24/8371.full#ref-list-1](http://clincancerres.aacrjournals.org/content/10/24/8371.full#ref-list-1)

Citing articles  This article has been cited by 8 HighWire-hosted articles. Access the articles at: [http://clincancerres.aacrjournals.org/content/10/24/8371.full#related-urls](http://clincancerres.aacrjournals.org/content/10/24/8371.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.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.