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Effects of Bcl-2 modulation with G3139 antisense oligonucleotide on human breast cancer cells are independent of inherent Bcl-2 protein expression

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Summary

We have investigated the effects of transient Bcl-2 down-regulation induced by the Bcl-2 antisense oligodeoxynucleotide (ODN) G3139 (Genta Incorporated) in high Bcl-2 protein expressing, estrogen receptor (ER) positive MCF-7 and low Bcl-2 expressing, ER negative MDA435/LCC6 human breast cancer cells. Treatment with Bcl-2 antisense ODN *in vitro* caused $>$ 80% reduction of Bcl-2 protein levels in a sequence specific manner for both cell lines. Maximum mRNA reduction was achieved within 24 h of the first antisense ODN exposure whereas full protein down-regulation required antisense exposure over 48 h. This Bcl-2 reduction was associated with 80–95% loss of viable cells compared to untreated cells. Similar cytotoxic effects were observed in both cell lines despite a nine-fold intrinsic difference in Bcl-2 protein expression suggesting that the relative degree of down-regulation of Bcl-2 is more important than the absolute reduction. Cell death associated with G3139 exposure exhibited properties indicative of apoptosis such as mitochondrial membrane depolarization and caspase activation. Combined treatment with G3139 and cytotoxic agents resulted in additive cytotoxicity in both cell lines. However, under most conditions studied, the direct cytotoxic activity of G3139 antisense was not synergistic with the cytotoxic agents. These results suggest that while Bcl-2 clearly constitutes an attractive therapeutic target due to its role in regulating apoptosis in breast cancer cells, additional mechanisms are important in the control of apoptosis arising from exposure to anticancer agents *in vitro*.

Introduction

The role of Bcl-2 as an important pro-survival factor has been associated with aggressive tumor phenotypes as well as resistance to various treatment modalities including chemotherapy and radiation [1]. Given that the majority of anticancer drugs exert their cytotoxic effects through the induction of apoptosis, Bcl-2 has been implicated as a potential mediator of the multidrug resistance phenotype. This has been supported by reports that increasing Bcl-2 expression through gene transfection can protect cells from some cytotoxic agents whereas decreasing Bcl-2 by antisense gene transfection can lead to increased chemosensitivity [1–3]. Increased levels of Bcl-2 have been correlated with inhibition of mitochondrial membrane depolarization and other indicators of apoptosis activation [4, 5].

While the studies described above suggest therapeutic implications of Bcl-2 manipulation in cancer treatment, it is unlikely that a gene therapy approach would be workable for widespread chemosensitization of disseminated tumor cells. However, temporary reduction of Bcl-2 protein levels in tumor cells using antisense oligonucleotides (ODN) may represent a more feasible option to manipulate Bcl-2 expression due to

the fact that ODNs are better suited for systemic administration compared to many types of gene therapy. In this context, studies have demonstrated that Bcl-2 antisense ODN treatment alone can induce apoptotic tumor cell death in cell culture and preclinical tumor models [6–11]. Furthermore, there is evidence suggesting that phosphorothioate antisense ODN-induced Bcl-2 down-regulation can chemosensitize tumors *in vivo* [6, 9, 10]. However, the fact that Bcl-2 antisense ODNs exhibit inherent cytotoxic activity as well as possible chemosensitizing properties on these high Bcl-2 expressing tumor lines complicates resolving the degree to which Bcl-2 down-regulation and chemotherapy act synergistically.

In contrast to ODN treatment, Bcl-2 antisense gene transfected tumor cell systems commonly used for *in vitro* analysis of Bcl-2 manipulation effects are selected on the basis of stable surviving clones which may not exhibit the cytotoxic properties of Bcl-2 down-regulation. Compensatory mechanisms responding to changes in Bcl-2 levels could potentially conceal important processes relevant to Bcl-2 functions in apoptosis control that would be normally present. Therefore, these lines may not allow the direct consequences of transient alterations in Bcl-2 levels to be examined, thereby confounding *in vitro*/*in vivo* correlations.

In the studies presented here, we have investigated the effects of transient Bcl-2 down-regulation using the phosphorothioate antisense ODN G3139 in estrogen receptor (ER) positive MCF-7 and the lower Bcl-2 expressing ER negative MDA435/LCC6 human breast cancer cells. These cell lines were selected to reflect the clinical observation that ER positive tumors tend to have high expression of Bcl-2 while ER negative tumors have lower expression [19, 20].

G3139 is directed against the first six codons following the translation initiation site of Bcl-2 mRNA and has been shown to effectively reduce both Bcl-2 mRNA and protein expression in a sequence specific manner [12]. Various molecular and cell survival analyses were performed on tumor cells exposed to G3139 alone and in combination with a variety of apoptosis inducing agents in order to resolve the direct cytotoxicity and chemosensitizing potential of Bcl-2 antisense ODN treatment in high and low Bcl-2 expressing breast cancer cell lines. The results of these studies provide useful information regarding the laboratory investigation of Bcl-2 antisense ODNs for therapeutic purposes in breast and other types of cancer.

Materials and methods

Cell lines

MCF-7 (ER positive) and MDA-435/LCC6 (ER negative) human breast cancer cell lines were obtained from the NCI (Bethesda, MD) and Dr. R. Clarke (Georgetown Univ.), respectively. Cells were maintained in RPMI or DMEM, respectively, with 10% fetal bovine serum at 37°C and 5% CO_2.

Antisense oligodeoxynucleotides (ODNs)

Antisense ODNs directed against the open reading frame of bcl-2 mRNA (5′-TCTCCCAGCGTGCGCCAT-3′, G3139, Genta Incorporated, San Diego, CA) were introduced into the breast cancer cells using liposomes composed of 50%/50% (by wt.) dioleoyldimethylammonium chloride (DODAC)/dioleoylphosphatidyl-ethanolamine (DOPE) purchased from Northern Lipids (Vancouver, BC). To ensure sequence specificity, both a sham (buffer) treatment (control) and a 2-base mismatch ODN (5′-TCTCCCAGC*A*TG*T*GCCAT-3′, G4126, Genta Incorporated, San Diego, CA) were used as controls. Monolayers of cells in exponential growth were incubated with the liposome-ODN complex in serum-free media for 4 h every 24 h on two consecutive days.

Western analysis

Bcl-2, Bcl-X_L, Bax, and Mcl-1 protein expression was evaluated by western analysis using commercially available antibodies. Cell lysates were prepared by washing cells once with ice cold phosphate buffered saline followed by the addition of lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 0.02% sodium azide) containing Complete^{TM}, mini protease inhibitor cocktail (Boehringer Mannheim/Roche Diagnostics, Indianapolis, IN). Samples were centrifuged and the supernatant stored at −70°C. Lysates equalized for protein content were separated in 0.1% SDS, 12.5% polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride or nitrocellulose membranes. Membranes were blocked (1% BSA, 0.02% Tween-20, 0.05% sodium azide, and 1X PBS for Immobilon membranes; 5% milk, 0.05% sodium azide, and 1X TBS for nitrocellulose membranes) then probed with antibodies for Bcl-2 (DAKO, Carpinteria, CA), Bcl-X (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Bax (Santa Cruz Biotechnology Inc., Santa

Cruz, CA) and Mcl-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies (DAKO), then visualized using an enhanced chemiluminescence western blotting analysis system (Amersham Pharmacia Biotech).

RNase protection assays

Total RNA was isolated using Trizol (Life Technologies, Rockville, MD). Assays were carried out using the RiboQuant multiprobe (Pharmingen, San Diego, CA) protection assay system with the template set hApo-2c as described in the manufacturer's protocol. Quantitation was determined using phosphoimaging analysis (Phosphoimager and ImageQuant v5.0 software, Molecular Dynamics, Sunnyvale, CA).

Viability assays

Equal numbers of cells ($2–5 \times 10^3$) were seeded in 96-well microtiter plates and allowed to adhere overnight. After ODN treatments, 48–72 h exposures to varying concentrations of cytotoxics (doxorubicin, paclitaxel, cisplatin) and direct apoptosis inducing agents (C6 ceramide) were performed. Direct counts were performed utilizing hemocytometer and trypan blue dye exclusion. Cell viability after ODN and/or cytotoxic treatment was also assessed for total number of viable cells using a conventional 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay and optical density determined at 570 nm. Clonogenicity was assessed using plating efficiencies for serial dilutions of single cell suspensions as detected by methylene blue staining of single adherent colonies. All assays were performed in triplicate.

Caspase-3 activity assay

Cells were harvested at various times after ODN treatment and caspase-3 levels in cells determined using a caspase-3 apoptosis detection kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Caspase-3 activity in cell lysates was determined fluorometrically utilizing a synthetic substrate, DEVD-AFC (tetrapeptide DEVD-7-amino-4-trifluoromethyl coumarin AFC) which is cleaved to a fluorescent AFC analog. The measured fluorescence of free AFC (Excitation 400 nm / Emission 505 nm) was used to determine the amount of caspase-3 activated in ODN-treated cells.

Mitochondrial membrane potential measurement and cell cycle analyses

Alterations in mitochondrial membrane potential following ODN treatment was monitored using a cationic lipophilic dye 3,3′-dihexiloxa dicarbocyanine (DiOC6) [13, 14]. At selected times after ODN treatment, 5×10^5 cells were harvested and incubated with DiOC6 (40 nM) for 30 min at 37°C. In some experiments, simultaneous cell cycle analysis was evaluated using Hoechst staining (20 μg/ml) for 30 min at 37°C. After incubation with appropriate dyes, cells were washed twice with cold PBS containing 0.1% BSA (PBS-B), re-suspended in PBS containing 0.5 μg/ml PI, and were immediately analyzed by flow cytometry (Coulter Epics, Miami, FL). Changes in the membrane potential were normalized for the cellular mitochondrial mass by staining with 100 nM nonylacridine orange (NAO).

For cell cycle analyses, 1×10^6 cells were washed with ice-cold PBS and then slowly permeabilized with 70% ethanol, dropwise at 4°C for 30 min. Cells were then washed with PBS-B and RNAase-treated (1 mg/ml, DNAase-free) for 30 min. at 37°C. Finally, cells were stained with 50 μg/ml PI in PBS and the PI was allowed to equilibrate in cells on ice for 30 min prior to analysis on flow cytometer (Coulter). Files of a minimum of 10,000 ungated events were collected and later analyzed using Expo analysis software (Coulter).

Statistical analysis:

Data were analyzed by the Student's *t*-test or one way analysis of variance (ANOVA). The levels of statistical significance were set at $p < 0.05$ (two-sided). All statistical calculations were performed using SPSS for Windows, version 7.5.1 software.

Results

Effect of transient Bcl-2 manipulation on growth properties of ER negative and positive breast cancer cells

The effects of the Bcl-2 antisense ODN G3139 and its two base mismatch control (G4126) on Bcl-2 protein levels in MCF-7 and MDA435/LCC6 cells were examined over the time course utilized for ODN treatment and chemosensitivity assays. Preliminary experiments revealed that ODN-DODAC/DOPE liposomal complexes were necessary for significant Bcl-2

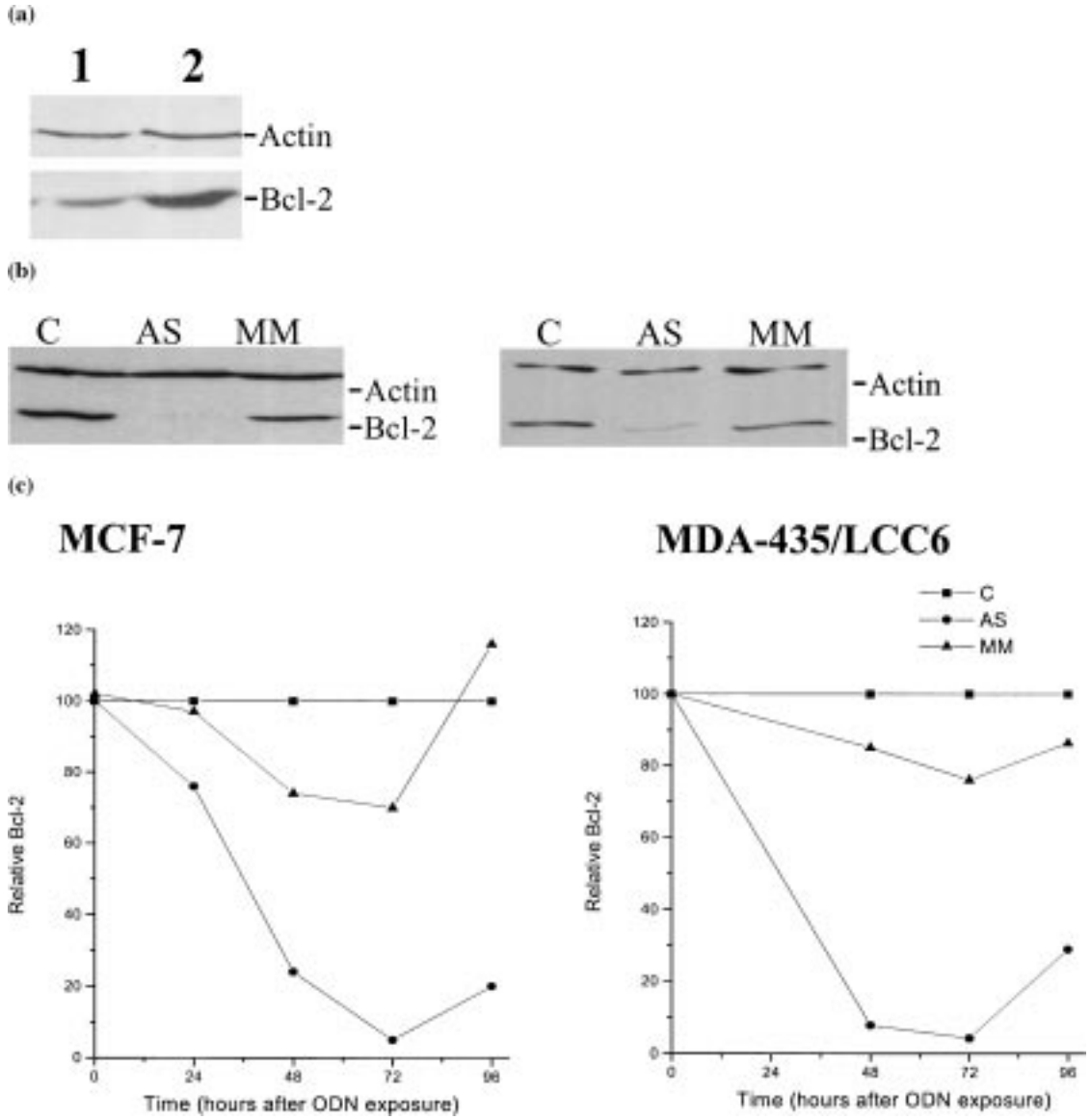

Figure 1. Bcl-2 antisense ODN (G3139) decreases Bcl-2 protein expression in MCF-7 and MDA-435/LCC6 cell lines. (A) Western analysis of intrinsic Bcl-2 protein expression in MDA-435/LCC6 (lane 1) and MCF-7 (lane 2) cells. (B) Western analysis of Bcl-2 protein in (A) MCF-7 and (B) MDA-435/LCC6 cells 48 h after exposure to sham control (C), G3139 antisense (AS) or G4126 mismatch (MM) ODN treatments. (C) Timeline of relative Bcl-2 protein expression in (A) MCF-7 and (B) MDA-435/LCC6 cells after C, AS, or MM treatment.

protein reduction and that optimal down-regulation was achieved using a lipid to DNA charge ratio of 1.3:1 (lipid/ODN wt. ratio of 5). Maximum Bcl-2 reduction was obtained at a G3139 concentration of 800 nM in both cell lines; reduced ODN concentrations led to decreased ($< 50\%$) down-regulation and concentrations beyond 1 μM resulted in non-sequence specific increased cell death, consistent with toxicity arising from cationic lipid/DNA complexes. Experimental results presented herein were performed using oligonucleotides at a concentration 800 nM in both cell lines. As shown in Figure 1A, direct comparison between ER positive MCF-7 and ER negative MDA435/LCC6 cells revealed that the former breast cancer cells exhibit 9-fold higher intrinsic (untreated control) Bcl-2 protein levels compared to the MDA435/LCC6 cell line. Extensive reductions in Bcl-2 protein levels are obtained for both MCF-7 and

MDA435/LCC6 breast cancer cells after two 4 h exposure to G3139 compared to control, untreated cells whereas minimal effects are observed with the 2-base mismatch control ODN (Figure 1B). Densitometry analysis revealed that Bcl-2 levels were suppressed 80–95% by G3139 compared to $\leq$20% down-regulation in the presence of the mismatch control. The sequence specific Bcl-2 effect was maintained between 48 and 72 h after ODN treatment and protein levels increased to within 50% of control after 96 h (Figure 1C). Western analysis for Bcl-X_L, Bax, and Mcl-1 protein revealed no significant changes in protein expression levels 24–72 h after G3139 treatment relative to mismatch ODN or untreated controls (data not shown).

The levels of Bcl-2 mRNA were assessed by ribonuclease protection assay (RPA) analysis after ODN treatment. Phosphoimaging analysis indicated that Bcl-2 mRNA expression was decreased by the antisense treatment. The reduction in Bcl-2 mRNA observed in MCF-7 cells at 24 h (Figure 2) was observed for an additional 48 h, after which time Bcl-2 mRNA increased back to control levels. In the MDA435/LCC6 cells the level of bcl-2 mRNA was decreased by approximately 60–80% in antisense treated cells compared with a less than 25% decrease in mismatch treated cells. The bcl-2 mRNA reduction was evident even after 96 h.

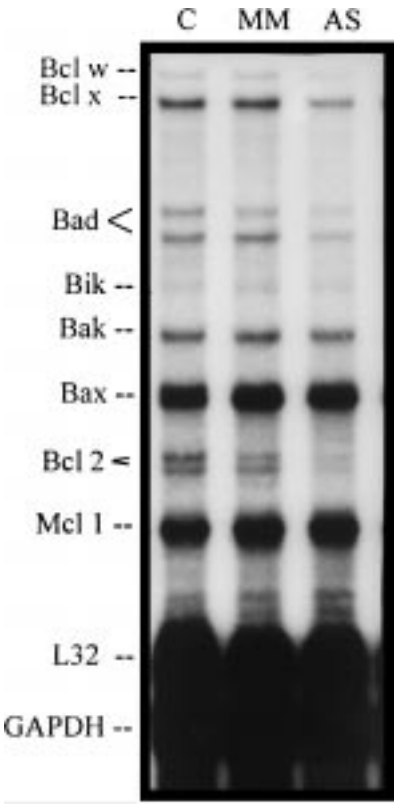

Figure 2. G3139 reduces Bcl-2 mRNA expression in the MCF-7 cell line. Ribonuclease protection assay analysis of MCF-7 mRNA 24 h after treatment with sham control (C), G3139 antisense ODN (AS) or mismatch ODN (MM).

In MCF-7 cells, the decrease in Bcl-2 mRNA expression was accompanied by marginal decreases in Bcl-x and Bad expression, however, no significant changes in Bcl-w, Bak or Bax expression were observed. There was also a trend towards increased expression of Mcl-1 RNA in antisense treated MCF-7 cells. In MDA435/LCC6 cells, treatment with G3139 resulted in a trend towards decreased expression levels of Bad, but the level of Mcl-1, Bcl-w, Bcl-x Bak and Bax were unchanged. However, in all cases these trends were not statistically significant.

Ribonuclease protection assay analysis was carried out using the hAPO-2c probe kit (Pharmingen) which allowed cell line-specific differences to be noted. Expression of the survival antagonist Bik was observed in MCF-7 cells while no expression of the apoptosis inhibitor Bfl-1 mRNA was detected. In contrast, for MDA435/LCC6 cells the situation was reversed with Bfl-1 expression being clearly indicated with no detectable expression of Bik observed. This cell line-specific gene expression was unaffected by the treatment with either antisense ODN or doxorubicin (data not shown).

The direct cytostatic/cytotoxic effects of G3139 on MCF-7 and MDA435/LCC6 breast cancer cells were determined by monitoring the number of live cells and percent viability before and at various times after treatment with G3139, mismatch ODN or control buffers. The growing adherent cells were exposed to ODNs for 4 h upon initiation of the experiment and a repeated exposure 24 h later. As shown in Figure 3, in MDA435/LCC6 cells, down-regulation of Bcl-2 using 800 nM G3139 resulted in significant ($p < 0.05$) reduction in the number of viable cells over 72 h after initiation of treatment. Viable cell numbers for cells treated with mismatch ODN were comparable to those for control cells at earlier time points ($p > 0.05$) and reducing the G3139 concentration below 600 nM resulted in growth curves similar to mismatch ODN. At 24 h after the first exposure to G3139, cell numbers were similar to those initially plated. However, at later times, cell numbers were reduced by approximately 50% relative to the initial cell count and 90% relative to cell counts for the control group (Figure 3). Sim-

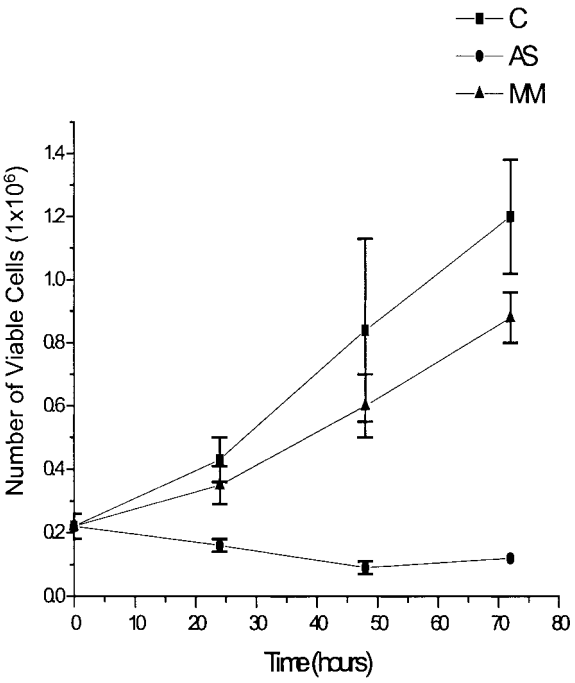

Figure 3. G3139 treatment reduces viable cell number. Direct counts of viable MDA435/LCC6 cells utilizing trypan blue dye exclusion after treatment with sham control (C), G3139 antisense ODN (AS), or mismatch ODN (MM).

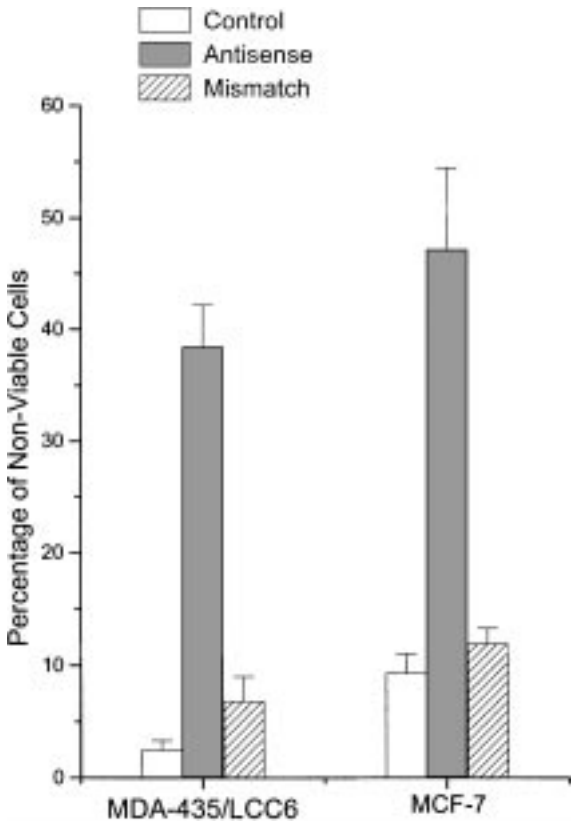

Figure 4. G3139 induces cytotoxic effects on MCF-7 and MDA435/LCC6 cells. Percentage of nonviable cells utilizing direct cell counts and trypan blue dye exclusion 48 h after sham control, G3139 antisense or mismatch ODN treatment.

ilar results were obtained with MCF-7 cells where the number of viable cells 48 h after initiation of ODN treatment were 14% and 76% relative to untreated controls for G3139 and mismatch groups, respectively.

The results from cell growth determination suggest that Bcl-2 down-regulation with G3139 results in cytotoxicity rather than simply cytostasis with the concentrations used in these experiments. This interpretation was corroborated by examining the viability of adherent cells using trypan blue dye exclusion. Control and mismatch ODN treated cells exhibited high cell viability where less than 12% of the adherent cells were unable to exclude trypan blue. In contrast, the number of non-viable MCF-7 and MDA435/LCC6 cells 48 h after G3139 treatment were 47% and 38% of the total adherent cell population, respectively (Figure 4). In addition, replating equal numbers of viable control, mismatch and G3139 treated cells after ODN treatment resulted in differential growth rates for the various groups. At 72 h after replating, the number of tumor cells in the G3139 treatment group were 42% of that for control cells whereas the number of mismatch treated cells were 72% of control (Figure 5).

Analysis of mechanisms associated with G3139 induced cytotoxicity

Experiments were performed to reveal the nature of the direct cytotoxicity caused by exposing MCF-7 and MDA435/LCC6 breast cancer cells to G3139. Particular attention was given to monitoring morphological and molecular endpoints typically associated with apoptosis. A hallmark feature of apoptotic cell death is the development of a cell morphology where the nuclei condense and often fragment or bleb into numerous vacuoles. We examined this by performing fluorescence microscopy of DAPI stained tumor cells exposed to G3139, mismatch ODN and untreated controls in both cell lines. As demonstrated in Figure 6, DAPI fluorescence associated with control or mismatch treated cells was strong and evenly distributed within the nuclear compartment for the vast majority of cells. In contrast, cells treated with Bcl-2 antisense ODN exhibited more punctated fluorescence

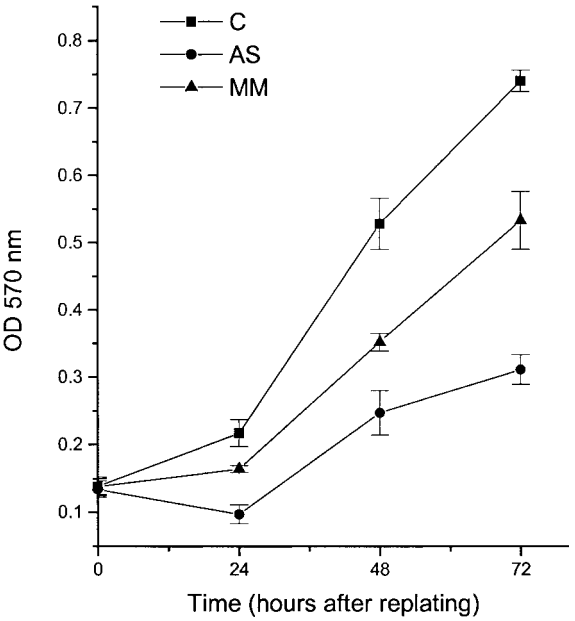

Figure 5. G3139 reduces growth rate in MCF-7 cells. Total number of viable cells determined by MTT dye reduction assay after treatment with sham control (C), G3139 antisense (AS) or mismatch (MM) ODN.

with condensed nuclei, many of which displayed features consistent with nuclear blebbing. Cells with very faint DAPI staining in what appeared to be nuclear remnants could also be detected in this group. Another feature characteristic of apoptotic cell death is the activation of caspase-3. MDA435/LCC6 cells exposed to G3139 had 2.3 fold higher caspase-3 activity than an equal number of mismatch ODN treated cells and 3.9 fold higher activity than untreated controls ($p < 0.0001$). Mismatch ODN treated cells showed only marginal activation of caspase-3 relative to controls (Figure 7).

Figure 8 presents flow cytometry plots of MDA435/LCC6 cells for DiOC6 detection of the mitochondrial membrane potential (normalized for mitochondrial content using nonylacridine orange). Untreated control cells are characterized by strong DiOC6 staining indicative of a large mitochondrial membrane potential (Figure 8A). Minimal effects were observed during or after the exposure of the cells to mismatch ODN (Figure 8B). In contrast, G3139 induced significant depolarization (low DiOC6 staining) in cells 8 h (data not shown) and 24 h after Bcl-2 antisense ODN treatment (Figure 8C).

Simultaneous Hoechst 33342/PI determination for cell cycle *versus* viability was also performed (Figure 9). In control cells, approximately 65% of cells exhibited a characteristic live-cell DNA profile (Figure 9A, quadrant A1) with very few apoptotic cells (Figure 9A, quadrants A2 and A3). A minor effect was observed during or after the exposure of the cells to mismatch ODN (Figure 9B). For G3139 treated cells, the relative number of live cells was reduced to 17% (Figure 9C, quadrant A1) with a significant increase in the hypodiploid cell population (Figure 9C, quadrants A2 and A3) compared to untreated control or mismatch-treated cells.

Flow cytometric cell cycle analyses using PI staining showed similar cell cycle distributions for control and mismatch-treated MDA435/LCC6 cells (Figure 10A-*a* vs. *b*). G3139 treatment resulted in significantly increased numbers of apoptotic cells (61%, sub-Go/G1) (Figure 10B-*c*) compared to controls.

Similar results were observed for MCF-7 cells; however, the increased fragility of these cells to processing steps resulted in elevated non-viable cells in control and ODN treatment groups, which precluded quantitative comparisons. It is also important to note that G3139 did not have any effects on mitochondrial membrane potential, PI profile or Hoechst/PI staining in K562 cells which display negligible amounts of Bcl-2 protein.

Combined antisense ODN and chemotherapy treatment

Down-regulation of Bcl-2 with the antisense ODN leads to direct cytotoxic activity in both ER positive and ER negative human breast cancer cells without the need to provide additional apoptotic stimuli. However, a great deal of interest exists in the ability of decreasing Bcl-2 levels to sensitize tumor cells to conventional chemotherapy regimens. We therefore evaluated the effects of combining Bcl-2 down-regulation through G3139 with exposure to various anticancer drugs and the apoptosis initiating agent C6-ceramide. In Figure 11, the number of viable cells relative to untreated controls are presented for MCF-7 and MDA435/LCC6 tumor cells exposed 48 h to various concentrations of doxorubicin. Pretreatment of cells with mismatch ODN prior to doxorubicin exposure had a modest effect on the cytotoxicity of the anticancer agent in MCF-7 cells (Panel A) and minimal effects in MDA435/LCC6 cells (Panel B). However, cells pre-treated with G3139 exhibited significantly increased cytotoxicity when incubated with doxorubicin. This effect was most notable at low drug

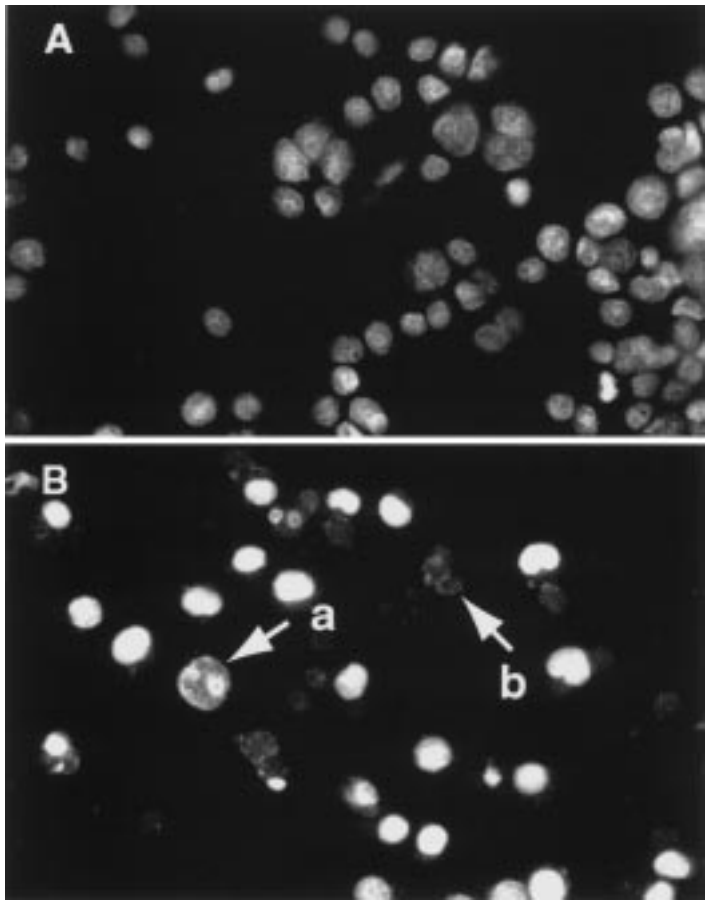

Figure 6. MDA435/LCC6 cells treated with G3139 display typical apoptotic morphology. Fluorescent micrographs of MDA435/LCC6 cells stained with DNA dye 4′-6′ diamino-2-phenyl indole (DAPI) and cytocentrifuged onto glass slides (Leica, 400×). Cells treated with Bcl-2 antisense G3139 (panel B) demonstrate striking apoptotic morphology (punctated fluorescence with condensed nuclei (arrow a) and faint DAPI staining with nuclear remnants (arrow b)) compared to relatively normal MM-treated cells (panel A).

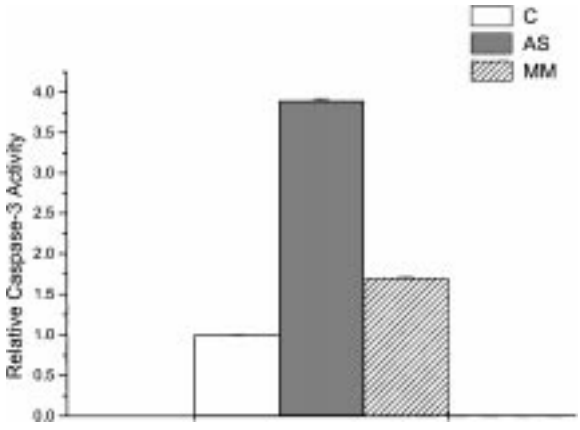

Figure 7. Increased casapase-3 activity in MDA435/LCC6 cells after G3139 exposure. MDA435/LCC6 cells were treated with either sham control (C), G3139 antisense (AS) or mismatch (MM) ODN and the cellular activity of caspase-3 was analyzed 48 h after using a fluorometric apoptosis detection kit.

concentrations (below 0.1 μM doxorubicin) where the number of viable cells in the G3139 treated group were 40–50% that observed in control cells.

Although the results above demonstrate that combining Bcl-2 antisense ODNs with anticancer drugs such as doxorubicin can provide significant increases in cytotoxicity over the individual agents, this in itself does not necessarily establish the presence of synergy between the two treatments. A series of studies were performed where the cytotoxicity of various agents known to induce tumor cell death through apoptosis were combined with mismatch ODN or G3139 treatment and the degree of cell kill was normalized to that obtained with ODN exposure alone (relative viable cells = # $\text{cells}_{+\text{drug}+\text{ODN}}$ ÷ # $\text{cells}_{-\text{drug}+\text{ODN}}$). In this case, exposure of MCF-7 and MDA435/LCC6 cells to the anticancer drugs doxorubicin, paclitaxel and cisplatin as well as the apoptosis inducing C6-ceramide

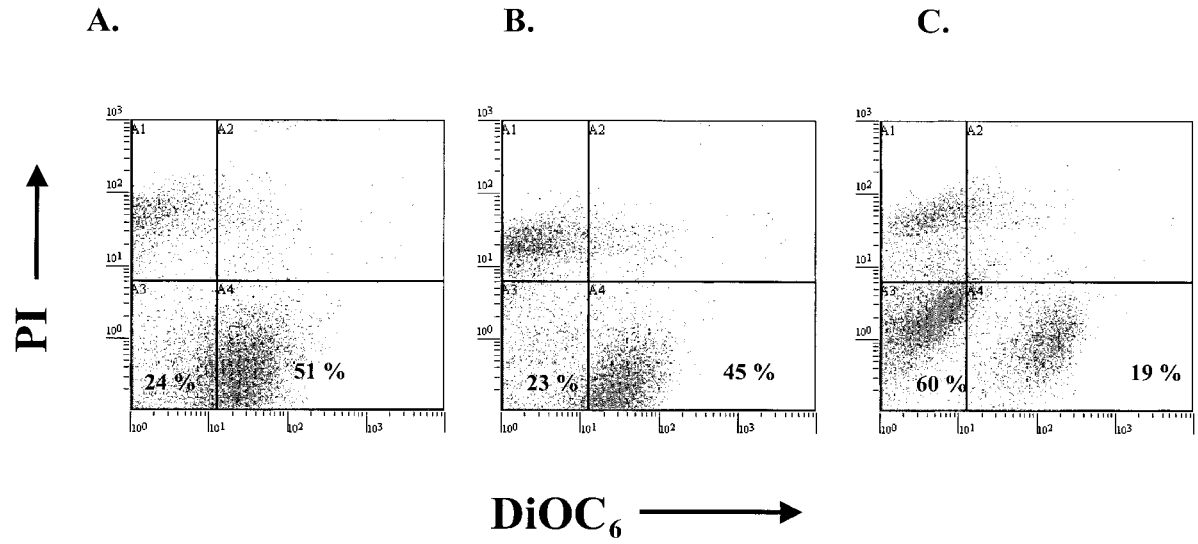

Figure 8. G3139 induces depolarization of the mitochondrial membrane potential. Flow cytometric histograms of MDA435/LCC6 cells for DiOC6 detection of mitochondrial membrane potential after sham control (panel A), mismatch (panel B) or G3139 antisense (panel C) ODN treatment.

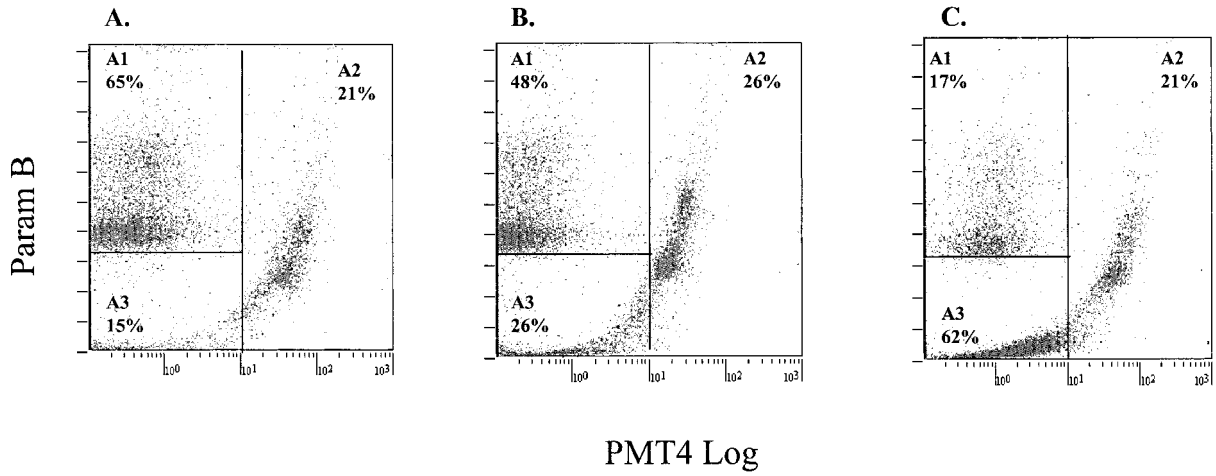

Figure 9. G3139 treatment results in direct cell apoptosis measured by flow cytometry analyses. (column A) MDA435/LCC6 cells stained with Hoechst (Param B) and PI (PMT4). Based on the Hoechst/PI staining, quadrants A1: shows normal cells with typical DNA profile, A2: apoptotic and necrotic cells, and A3: late apoptotic or debris. G3139 treated cells (panel C) have reduced numbers of normal/live cells and higher percentages of apoptotic cells as compared to sham control (panel A) or Bcl-2 mismatch ODN treatment (panel B).

did not reveal any differences in the chemosensitivity of cells that would not have died from G3139 alone (Figure 12). IC_{50} values for control (no ODN) MDA435/LCC6 cells were virtually identical to those obtained for tumor cells pretreated with G3139 or the mismatch ODN control (IC_{50} values $\cong 0.3\,\mu M$, 1.0 nM, 10 μM, and 8 μM for doxorubicin, paclitaxel, cisplatin and C6-ceramide, respectively). Very similar results were obtained with MCF-7 cells.

In order to ensure that the observations above were not a result of the culturing, drug exposure or cytotoxicity assay conditions utilized, experiments examining synergy between anticancer agents and G3139 induced down-regulation of Bcl-2 were conducted using a variety of experimental designs. Cytotoxicity curves were generated for doxorubicin and paclitaxel with ODN pretreatment using a wide variety of tumor cell plating densities (10–80% confluent), drug exposure

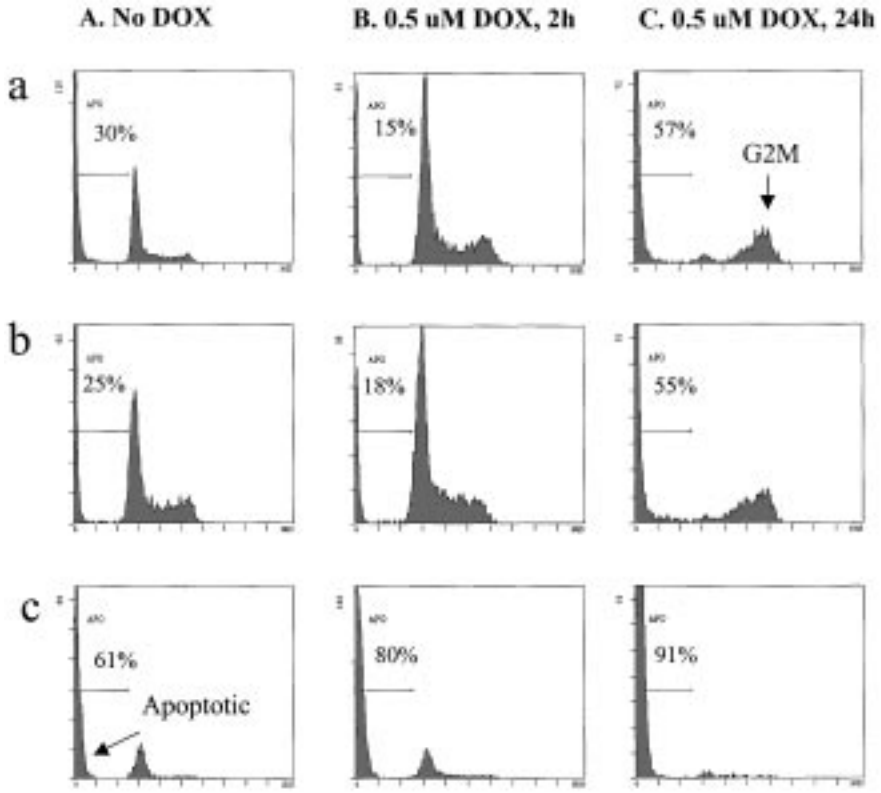

Figure 10. Quantitative cell-cycle analysis of G3139 and doxorubicin treated cells using propidium iodide staining and flow cytometry. MDA435/LCC6 cells were treated with either sham control (row *a*), mismatch (row *b*) or G3139 antisense ODN (row *c*) followed by exposure to doxorubicin. (column A) 24 h after ODN/sham treatment and no doxorubicin exposure; (column B) after a 2-h exposure to 0.5 μM doxorubicin following ODN/sham treatment; (column C) after a 24-h exposure to 0.5 μM docorubicin following ODN/sham treatment.

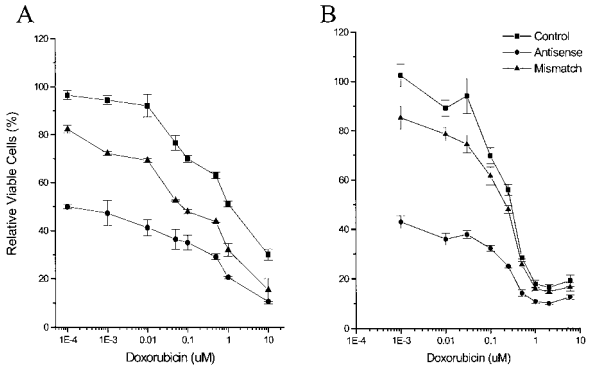

Figure 11. Increased cytotoxic effect of doxorubicin after pre-treatment with G3139. Equal numbers of MCF-7 (panel A) and MDA435/LCC6 (panel B) were pre-treated with sham control, Bcl-2 antisense or mismatch ODN. Relative viable cells were determined by MTT dye reduction assays after a 48-h exposure to doxorubicin.

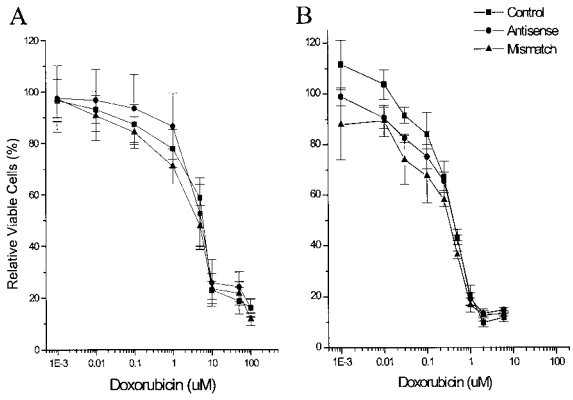

Figure 12. Pre-treatment with Bcl-2 antisense ODN does not result in a synergistic increase in the chemosensitivity of MCF-7 and MDA435/LCC6 cells. Cells were pre-treated with sham control, Bcl-2 antisense or mismatch ODN. After replating at equivalent cell number and density, MCF-7 (panel A) and MDA435/LCC6 (panel B) cells were exposed for 48-h to doxorubicin. Relative viable cells were determined by MTT dye reduction assay.

times (4–120 h, continuous and pulsed), concomitant drug and ODN exposure as well as clonogenicity, MTT and direct cell counting (using trypan blue) assays for determining the number of viable cells. Cytotoxicity curves with negligible differences in drug IC_{50} values between G3139, mismatch ODN and ODN-free tumor cell groups were obtained under all culturing conditions and drug exposure times as well as for clonogenic and total viable cell detection assays. Also, no differences were observed for either breast cancer cell line when lower doses of ODN were employed which led to attenuated Bcl-2 down-regulation (data not shown).

In the case of concomitant exposure of the breast cancer cells to drugs during ODN treatment (drugs removed from media during the 4-h ODN pulses and subsequently returned), a trend toward decreased drug IC_{50} values (increased chemosensitivity) was observed for G3139 treated cells (normalized to G3139 alone) compared to mismatch ODN or control cells. However, the approximately 2-fold sensitization consistently obtained under these conditions could not be shown to be statistically significant (data not shown). In addition, MDA435/LCC6 cells pretreated with G3139 appeared to die faster when exposed to doxorubicin compared to mismatch ODN treated cells. Specifically, the percent reduction of viable cells 8 and 24 h after addition of 5 μM doxorubicin was 23% and 84%, respectively, for the G3139 group compared to 0% and 75% for mismatch ODN treated cells. At $\geq$ 48 h post drug treatment as well as in clonogenic assays, no differences in the drug cytotoxicity curves were observed between the two ODN treatment groups.

Assays for endpoints indicative of apoptosis were also completed on MDA435/LCC6 cells to allow comparisons of the process of drug induced cell death with tumor cells exposed to G3139 and mismatch ODN. In the presence of 0.5 μM doxorubicin, flow cytometric cell cycle analysis using quantitative PI staining showed that the relative numbers of apoptotic cells following either sham control or mismatch ODN treatments increased in a similar manner from 2 to 24 h (Figure 10B vs. C). At 24 h, an arrest in G2/M phase of the cell cycle was observed (Figure 10C-*a,b*). Increased numbers of apoptotic cells were observed after G3139 treatment and this effect was even more pronounced in the presence of doxorubicin (Figure 10B,C-*c*). Flow cytometric analysis demonstrated increasing mitochondrial membrane potential depolarization in tumor cells after addition of doxorubicin in a manner qualitatively similar to that achieved with G3139 treatment (data not shown).

Discussion

The studies described here were undertaken in order to characterize the *in vitro* direct cytotoxic and chemosensitizing effects of Bcl-2 antisense ODN treatment in a pharmacological context and to correlate such observations with molecular endpoints for specific cellular processes associated with apoptosis. Although transfecting tumor cells with the Bcl-2 gene has provided mechanistic information concerning its role in controlling apoptosis, demonstrating protection from apoptosis with gain of Bcl-2 does not a *priori* establish that down-regulation in a treatment setting will provide therapeutic benefits. Similarly, Bcl-2 antisense gene expressing transfectants may have limited predictive value pharmacologically due to the selection of surviving clones in the face of a pro-apoptotic manipulation. In view of these considerations, we investigated the effects of Bcl-2 antisense ODN exposure using a wide variety of culturing conditions, drug concentrations and exposure times as well as several methods for assessing cytotoxicity. Pharmacological correlations focused on quantitating the absolute number of viable cells remaining after treatment since this is ultimately the parameter that will reflect therapeutic response.

The results obtained here demonstrate that the Bcl-2 antisense ODN G3139 is very effective in reducing Bcl-2 mRNA and protein levels for both ER negative and positive human breast cancer cell lines. Protein levels remained depressed for over 72 h after ODN exposure in the MCF-7 and MDA/435/LCC6 cells and this down-regulation was sequence specific, similar to observations in studies employing this antisense molecule with other cell lines [6–10]. It appears that decreasing Bcl-2 protein levels by $\geq$ 50% results in significant cell death that is mediated through apoptosis and also leads to decreased growth of the surviving cell population. Very similar results were obtained for MCF-7 and MDA435/LCC6 cell lines, which is of interest since they display large differences in inherent Bcl-2 expression. This suggests that the relative degree of Bcl-2 down-regulation, possibly coupled with the baseline ratio of Bcl-2 to other pro- or anti-apoptotic proteins, may be more important than absolute decreases in dictating Bcl-2 antisense-

induced cytotoxicity. Therefore, the therapeutic benefits of Bcl-2 antisense ODNs may not be restricted to tumor populations that are selected due to overexpression of this protein, although it is anticipated that some finite level of protein expression would be required for activity.

Our results demonstrated that both G3139 and anticancer drug treatments induced cell death through classical apoptosis pathways associated with mitochondrial membrane depolarization, caspase activation, and nuclear condensation/blebbing. When these two treatment modalities were combined, increased cytotoxicity was observed as reflected by reduced numbers of viable cells. The decreased percent viability of tumor cells observed in the combined treatment suggested enhanced sensitivity to chemotherapy induced by Bcl-2 reduction. However, from a pharmacological standpoint, this implied synergy should be reflected by decreased viable cell numbers compared to the survival probabilities arising from combining two active cytotoxic agents. This anticipated result was not observed in our investigations under a wide spectrum of conditions using metabolic and clonogenicity assays for cell survival. In general, we were unable to demonstrate that MCF-7 and MDA/LCC6 breast cancer cells surviving G3139 treatment were more sensitive to anticancer drugs or C6-ceramide, all of which induced apoptosis individually. It could be argued that the viable cells remaining after G3139 treatment do not display decreased Bcl-2 levels and therefore would not be expected to be more chemosensitive. However, the cells recovered for Bcl-2 protein determination are the same cells that are used for cytotoxicity studies. The fact that Bcl-2 protein levels are down-regulated by $>80\%$ at this point argues against explanations based on cell heterogeneity with respect to Bcl-2 expression.

We are unable at this time to clearly elucidate how G3139 induced Bcl-2 down-regulation may be uncoupled from apoptotic cell death induced by the anticancer agents studied here. Apoptosis induced independent of Bcl-2 has been documented in other systems [15]. However, this does not account for the apparent discrepancies between the investigations here and others suggesting the presence of synergistic Bcl-2 antisense-chemotherapy effects [7, 16, 17]. It is important to note that most investigations in this area have focused more on mechanistic studies utilizing Bcl-2 gene (and antisense gene) transfected cells rather than identifying specific therapeutic applications. Furthermore, these systems often utilized short duration exposure of tumor cells to high concentrations of cytotoxic agents in order to follow endpoints of apoptosis and assays used to detect cytotoxicity were often more qualitative in nature [2]. In other cases, attempts to reveal chemosensitization effects of Bcl-2 down-regulation utilized simultaneous titrations of antisense ODN and chemotherapeutic agents [16]. Although increased cell kill was shown with such combination treatments, normalization of that data for the survival probability of the individual agents (compare Figures 10 and 11) generates cytotoxicity curves very comparable to those obtained here.

Previous studies have provided encouraging indications that combining Bcl-2 antisense ODN treatment with chemotherapy may result in enhanced antitumor activity *in vivo* due to increased chemosensitivity associated with Bcl-2 down-regulation [6, 9, 10]. Unfortunately, obtaining definitive evidence of synergy in animal models is complicated by difficulties in correlating tumor growth changes with actual tumor cell kill. These complications can arise from *in vivo* tumor model features such as combination treatment-related changes in drug pharmacodistribution properties, the presence of nonmalignant cells in the tumor mass and increased fractions of dead tumor cells associated with hypoxic regions within solid tumors. This is particularly important when antitumor activity is observed for both agents being combined *in vivo*. For example, in a previous study, dacarbazine treatment of SCID mice bearing melanoma solid tumors provided a 72% reduction in tumor mass 21 days after cell inoculation and Bcl-2 antisense caused 60% tumor growth inhibition [6]. The fact that the mean tumor weight of animals treated with antisense and dacarbazine in combination was decreased by 98% strongly supports synergistic action since additive effects would predict an 89% reduction in tumor growth. However, given the additional environmental factors that may influence therapeutic activity *in vivo*, such indications must be somewhat tempered due to the significant therapy provided by each treatment component individually. It is important to note that a recent study combining a Bcl-2 ODN with mitoxantrone in a murine androgen sensitive breast tumor model provided clear evidence for synergistic antisense-related chemosensitization [10]. Such synergy could be demonstrated due to the lack of appreciable direct cytotoxicity of ODN against this cell line with the conditions employed. Taken together with the results reported here, future investigations focused on chemosensitization by modulation of Bcl-2 must define experimental conditions

and models where reduction of Bcl-2 in itself does not independently lead to significant apoptosis.

Ultimately, the therapeutic utility of antisense ODNs against Bcl-2 will be dictated by the ability to achieve significant tumor Bcl-2 down-regulation and therapeutic activity in patients. Early indications from clinical trials suggest that G3139 may directly induce tumor cell death leading to therapeutic responses [18]. The strong cytotoxic effects of Bcl-2 down-regulation demonstrated here with G3139 on breast cancer cells exhibiting both high and low levels of inherent Bcl-2 expression suggest that such genetic manipulations may have therapeutic potential in this patient population. This lack of antisense ODN dependence on inherent Bcl-2 levels may also mean that the utility of Bcl-2 as a therapeutic target is not directly linked to its prognostic utility in breast cancer where its expression has been shown in some studies to be a positive prognostic marker [19, 20]. Whether chemosensitization can be achieved clinically is yet to be determined; however, early results from a clinical trial of G3139 plus dacarbazine in melanoma patients with resistant disease are encouraging [21].

The results of the studies completed here indicate that care should be given to avoid oversimplification of the complex apoptosis pathways that may be targeted for therapeutic intervention. Clearly, our ability to use *in vitro* and *in vivo* preclinical studies predictively in the design of clinical trials with agents directed at apoptosis factors will require further elucidation of the pathways governing cell death and how these controls will compare and contrast moving from the laboratory to human studies.

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