Molecular and Pharmacokinetic Properties Associated with the Therapeutics of Bcl-2 Antisense Oligonucleotide G3139 Combined with Free and Liposomal Doxorubicin

Daniel E. Lopes de Menezes, Norma Hudon, Natasha McIntosh, and Lawrence D. Mayer


ABSTRACT

Bcl-2 is a key apoptosis-regulating protein that has been implicated in mechanisms of chemoresistance for a variety of malignancies by blocking programmed cell death. This study investigated the activity of the Bcl-2 antisense oligodeoxynucleotide (AS ODN) G3139 combined with free doxorubicin (F-DOX) or sterically stabilized liposomal doxorubicin (SL-DOX) to determine the role that drug pharmacodistribution properties may have on antitumor activity using a Bcl-2-expressing human breast solid tumor xenograft model. Administration of G3139 was able to delay the growth of MDA435/LCC6 cells compared with control ODN-treated animals; however, in all of the cases, tumors reestablished after AS ODN treatment. Western blot analyses of Bcl-2 levels of solid tumors showed a sequence-specific down-regulation of the Bcl-2 protein after four daily doses of G3139, which correlated with histological evidence of tumor cell death. Interestingly, the expression of Bcl-2 returned to pretreatment levels during the course of subsequent ODN administration, which suggested the development of resistance to continued Bcl-2 ODN treatment. The antitumor activity of ODN given in conjunction with either F-DOX or SL-DOX was also examined. The combination of G3139 and F-DOX was able to suppress the growth of MDA435/LCC6 cells beyond that obtained with either of the treatments given alone, indicative of synergistic action. Examination of the pharmacokinetics of F-DOX with systemic G3139 administration revealed that elevated tumor drug DOX levels were obtained with DOX treatment in the absence of G3139. This effect was sequence-specific and plasma DOX levels were unaffected by G3139 treatment, which indicated possible positive ODN-drug interactions at the tumor site. Combining G3139 with SL-DOX further increased the degree of antitumor activity. The improved efficacy of this combination was attributed to increased tumor drug levels that arise from the ability of SL-DOX to passively accumulate in solid tumors. These results suggest that additional benefits of Bcl-2 antisense ODN may be obtained when it is combined with liposomal formulations of anticancer drugs such as DOX.

INTRODUCTION

The development of chemoresistance is a major obstacle in the therapeutic intervention of most cancers. One possible mechanism of drug resistance is related to the failure of cells to engage apoptosis, a form of programmed cell death associated with many cytotoxic agents. Specifically, several members of the bcl-2 family of proto-oncogene products (in particular Bcl-2) have been implicated as antiapoptotic proteins and have been shown to be key regulators of the cell death pathway (1–5). Bcl-2 protects cells from a variety of external stresses, including chemotherapy and radiation (2, 3, 6), and its expression has been correlated with poor prognosis in a number of malignancies (7, 8). In this context, Bcl-2 has become an attractive target for molecular targeting approaches, specifically AS gene therapy. Induction of cancer cell apoptosis has been achieved in experimental systems through the use of short stretches of single-stranded DNA that can specifically hybridize complementary regions of the mRNA coding Bcl-2 (9).

The mechanism of AS3 action is believed to result from direct AS ODN-mRNA binding as well as degradation of these duplexes by RNase H both of which inhibit translation of Bcl-2 protein (10). Bcl-2 as ODN treatment of tumor cells in vitro has been shown to down-regulate Bcl-2 protein and induce increased apoptosis susceptibility (11–13). Early work with in vivo administration of phosphodiester ODNs was largely unsuccessful because of the rapid degradation of ODNs in the circulation (14, 15). The development of phosphorothioate-ODNs such as the Bcl-2 AS ODN G3139 (Genta, Inc.) has led to the clinical development of these molecules with promising early results (16–19). However, clinical trials have suggested that ODNs may not be as effective as anticipated in terms of antitumor activity (20–24).

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1 The abbreviations used are: AS, antisense; AUC, area(s) under the concentration-time curve; CHOL, cholesterol; DOX, doxorubicin; F-DOX, free DOX; [3H]CHE, cholesteryl-[1,2-3H]-hexadecyl ether; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidyl-ethanolamine; HPLC, high-pressure liquid chromatography; MM, mismatch; MTD, maximum tolerated dose; ODN, oligonucleotide; PEG, poly(ethyleneglycol); PK, pharmacokinetic; RP, reverse polarity; SCID, severely combined immunodeficient; SL, sterically stabilized liposome; SL-DOX, sterically stabilized liposomal DOX; % T/C, percent test/control; PGP, P-glycoprotein.
results (16). Combining antitumor agents with Bcl-2 AS ODN presents a supplementary therapeutic strategy. Because many anticancer drugs elicit their cytotoxic activity via apoptosis, concurrent Bcl-2 AS ODN and drug treatment could enhance drug effectiveness, thus providing an attractive strategy to overcome drug resistance in cancer (2, 17). However, only very recently have studies examined the effects of ODN in increasing the sensitivity to chemotherapeutic agents in relevant tumor models (18–20). The results of these reports provide promising indications that combinations of Bcl-2 AS ODN treatment with chemotherapy may provide significant improvements in antitumor activity, possibly in a synergistic fashion. Although these results are encouraging, many treatment characteristics such as tumor Bcl-2 expression during therapy, potential PK influences of the treatment combinations, and the effect of tumor characteristics on therapeutic activity have not been elucidated.

The objective of this study was to characterize the molecular and pharmacological effects of G3139 Bcl-2 AS ODN treatment alone and in combination with F-DOX in a Bcl-2-expressing model of human breast cancer grown in SCID-RAG2 mice. Studies to date have not examined potential effects of chronic ODN treatment on the PK properties of coadministered anticancer drugs. Given the high DNA binding avidity of the anticancer drug DOX, possibilities exist for DOX-ODN interactions both in tissues and the circulation. To understand the pharmacological attributes of coadministering these two agents, we examined the PK properties of DOX to better understand mechanisms involved in ODN-mediated drug sensitization. Given that F-DOX has a relatively short plasma half-life, we also encapsulated DOX in small (100 nm) DSPC/CHOL liposomes containing polyethylene glycol (PEG), in which it was predicted that liposome-mediated changes in drug PKs could lead to prolonged circulation times with reduced drug-related toxicities (21–23). As a consequence of the circulation longevity, these liposomes can passively localize into solid tumors via the discontinuous capillaries, particularly during active tumor growth and angiogenesis (24), thereby exposing tumor cells to toxicities (21–23). As a consequence of the circulation longevity, these liposomes can passively localize into solid tumors via the discontinuous capillaries, particularly during active tumor growth and angiogenesis (24), thereby exposing tumor cells to toxicities (21–23).

**MATERIALS AND METHODS**

**Chemicals and Drugs.** DSPC and PEG<sub>2000</sub>-DSPE were obtained from Northern Lipids (Vancouver, British Columbia, Canada). CHOL were purchased from Avanti Polar Lipids (Alabaster, AL). [3 H]CHE was obtained from Amersham (Oakville, Canada). CHOL were purchased from Avanti Polar Lipids (Alabaster, AL) and obtained from Northern Lipids (Vancouver, British Columbia, Ontario, Canada). DOX was from Faulding Inc. (Vaudreuil, Quebec, Canada). DSPC and PEG<sub>2000</sub> -DSPE were combined with G3139.

**Preparation of Liposomes.** SLs were composed of DSPC:CHOL:PEG<sub>2000</sub>-DSPE (55:45:5 molar ratio). In some experiments, [3 H]CHE was included as a nonexchangeable, nonmetabolizable lipid tracer (26). Lipids were dissolved in chloroform, dried to a thin lipid film under high vacuum, and later hydrated by vortex-mixing in 300 mM citric acid (pH 4.0) to a final lipid concentration of 100 mg/ml. The resultant multilamellar vesicles were then freeze-thawed in liquid nitrogen for five cycles of 3 min each, to form large unilamellar vesicles. The large unilamellar vesicles were then sequentially extruded (Lipex Extruder; Lipex Biomembranes Inc., Vancouver, British Columbia, Canada) through a series of polycarbonate filters (Nucleopore; Pleasanton, CA) of pore sizes from 200 nm down to 100 nm in diameter, as described previously (27). The mean diameter of liposomes was approximately 100–110 nm measured using quasielastic light-scattering (QELS Nicomp 270 Submicron Particle sizer).

**Preparation of Liposomes.** DOX was loaded into liposomes (DOX:Lipid, 0.15:1 weight ratio) exhibiting a pH gradient (interior acidic), generated when the liposomal external citrate buffer was titrated against 0.5 M Na<sub>2</sub>CO<sub>3</sub> (28). SL-DOX was characterized for trapping efficiency using Sephadex G-50 columns and size distribution (QELS; Nicomp). The amount of DOX encapsulated was determined spectrophotometrically at λ = 480 nm.

**In Vitro AS Experiments.** Monolayers of MDA435/LCC6 cells in exponential growth phase were grown in DMEM, supplemented with 10% fetal bovine serum. A 1:1 mixture of N,N-dioleyl-N,N-dimethylammonium chloride/1,2-dioleoylsn-glycerol-3-phosphoethanolamine liposomes were used as transfecting lipids. Lipid and 800 nm ODN (1.3:1, +/- charge ratio) were mixed in serum-free media and kept on ice for 30 min to form liposome-ODN complexes. Cells were incubated with the liposome-ODN complexes for two 4-h pulses (once a day) over two consecutive days. At the end of incubation with ODNs, media was replaced after two washes with Hank’s buffer. Cell lysates were prepared 48 h after the second pulse of liposome-ODN treatment.

**Western Blot Analysis of Bcl-2 Expression.** Cells or tumor tissue were treated with ice-cold lysis buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM EDTA, and 0.1% sodium azide), containing protease inhibitors.
(Complete-Mini protease inhibitor tablets; Boehringer Mannheim GmbH, Mannheim, Germany). After incubation for 30 min on ice, samples were centrifuged at 14,000 rpm for 15 min and then stored at −70°C. Protein content in the lysed extracts was determined using a detergent-compatible Bio-Rad assay (Bio-Rad Labs, Hercules, CA). Equal amounts of protein (20 μg/lane) were subjected to 12.5% SDS-PAGE (Bio-Rad) for 45 min at 150 V in a glycerine buffer [1.92 mM glycine and 25 mM Tris (pH 8.3)] containing 1% SDS. Gels were transferred to nitrocellulose membranes in a glycerine transfer buffer with 20% methanol for 1 h at 70 V. Membranes were blocked overnight at 4°C with 5% skim milk, 0.05% sodium azide in TBS [20 mM Tris (pH 8.2) and 137 mM sodium chloride]. Mouse monoclonal antibody to human Bcl-2 (1:3,000; DAKO, Glostrup, Denmark) and mouse anti-human β-actin monoclonal antibody (1:10,000; Sigma, St. Louis, MO) were used diluted in 1% skim milk in TBS containing 0.05% Tween 20 and 0.05% sodium azide. The membranes were then incubated for 1 h with 1:3,000 horseradish peroxidase-conjugated antimouse IgG (Promega, Madison, WI). Proteins were detected by using an enhanced chemiluminescence method (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England) and visualized after exposure to Kodak film. Scanning densitometry (Molecular Dynamics, Sunnyvale, CA) was performed to quantify band intensities by volume/area integration.

PK and Tissue Distribution. Female RAG2 mice bearing MDA435/LCC6 tumors (0.1–0.15 g) were injected i.p. with G3139 (5 mg/kg) on days 1–4. On day 4, 3 h after ODN treatment, the mice were injected with a single i.v. bolus dose of 5 mg/kg of either F-DOX or SL-DOX (labeled with [3H]CHE).

RESULTS

In Vivo Antitumor Activity. Efficacy experiments were conducted in RAG2 mice bearing MDA435/LCC6 tumors, randomly assigned into 4–6 mice/group. Treatments were commenced on either day 3 (early treatment) or day 17 (~0.1–0.15-g tumors) after cell inoculation. Saline (controls), Bcl-2 AS G3139 or RP ODNs (5 or 10 mg/kg) were administered i.p. daily for five doses a week over a 1-, 3-, or 6-week regimen. F-DOX or SL-DOX (5 or 10 mg/kg; 1, 3, or 6 injections once a week) was administered i.v. via the tail vein, either alone or in conjunction with ODNs. When G3139 was administered in combination with either F-DOX or SL-DOX, the drug treatment was staggered 3 h after ODN treatment.

Mice were observed daily and mouse body weights as well as signs of stress (e.g., lethargy, ruffled coat, ataxia, and so forth) were used to detect possible toxicities. Animals with ulcerated tumors or >25% loss of body weight were killed. Caliper measurements of tumors were converted into mean tumor weight (g) using the formula: \( \frac{1}{2} \times \text{length (cm)} \times \text{width (cm)} \times 0.5236 \). An average tumor weight per mouse was calculated from the mean of the two bilateral tumors and was used to calculate the group mean tumor weight ± SE (n = 6–12 mice) from at least two independent experiments per group.

Tumor and Tissue Processing. Mouse tissues/tumors were collected at selected times and fixed in paraformaldehyde. Paraffin-embedded tissues were sectioned and were subjected to gross histopathology using H&E staining. For Western analysis, tumors were homogenized in lysis buffer using a Polytron homogenizer (Kinematica).

Statistical Analyses. All of the linear regression was done using Microsoft Excel (Seattle, WA). Student’s t test was used to measure statistical significance between two treatment groups. Multiple comparisons were done using one-way ANOVA, and posttests that compared different treatment means were done using Bonferroni’s test (Statistica release 4.5; StatSoft Inc., Tulsa, OK). Data were considered significant for \( P < 0.05 \).

RESULTS

Growth Properties of MDA435/LCC6 Tumor Xenograft Mice Given Bcl-2 AS G3139. We tested the antitumor effects of Bcl-2 AS G3139 in SCID RAG2 mice bearing MDA435/LCC6 human breast solid tumors implanted bilaterally in the mammary pads. Saline-treated control tumors grew reproducibly to a size of 0.5 g within approximately 35 days, with >95% mouse tumor-takes (Fig. 1). Treatments were initiated 3 days after cell inoculation or when the tumors were established to a size of 0.1–0.15 g (approximately day 17). G3139 was administered using a treatment schedule of i.p. injections (1 a day) given on days 3–7, 10–14, and 17–21. Early treatments with 5 mg/kg Bcl-2 AS G3139 significantly delayed tumor growth: Day-35 tumor weights were 0.44 g and 0.17 g in control and AS-treated groups, respectively (Fig. 1A). Bcl-2 AS G3139 was administered at a maximum of 10 mg/kg, because higher doses exceeded the MTD in SCID RAG2 mice, which is approximately 10–15 mg/kg G3139. Mice that were given 10 mg/kg G3139 exhibited marginal therapeutic improvements compared with the 5-mg/kg G3139 dose; hence, for most in vivo treatment regimens, a dose of 5 mg/kg was selected. Administration of 5 mg/kg G3139 exhibited marginal therapeutic improvements compared with the 5-mg/kg G3139 dose; hence, for most in vivo treatment regimens, a dose of 5 mg/kg was selected. Administration of 5 mg/kg G3139 exhibited marginal therapeutic improvements compared with the 5-mg/kg G3139 dose; hence, for most in vivo treatment regimens, a dose of 5 mg/kg was selected.
Bcl-2 AS and LS-DOX in vitro on MDA435/LCC6 cells, using tumor cell specimens without contamination from Bcl-2 derived lowed for determination of human Bcl-2 levels obtained from lysates from tumor homogenates were electrophoresed, and membranes were probed with Bcl-2 and actin antibodies. Because these reactions during the course of G3139 treatment (Fig. 2). Mice implanted with established (0.1–0.15 g) bilateral MDA435/LCC6 tumors were treated with saline, RP-ODN, or G3139. Lysates from tumor homogenates were electrophoresed, and membranes were probed with Bcl-2 and actin antibodies. Because these antibodies were directed against the human proteins, this allowed for determination of human Bcl-2 levels obtained from tumor cell specimens without contamination from Bcl-2 derived from mouse cells.

The functional effects of Bcl-2 G3139 were first confirmed in MDA435/LCC6 cells, using in vitro assays with Bcl-2 AS G3139 delivered to cells via ODN-lipid complexes. Bcl-2 protein in cells was determined after 48 h by using Western analysis (Fig. 2A). Relative Bcl-2 levels were compared using scanning densitometry after normalization to actin. No significant reduction in Bcl-2 levels was observed in MDA435/LCC6 cells given either a 2-base MM ODN or RP ODN treatments (Fig. 2A, Lanes 2 and 3), and these levels were consistently maintained at approximately 75–95% of controls (Fig. 2A, Lane 1). In vitro exposure of MDA435/LCC6 cells to G3139 led to a reduction in Bcl-2 protein levels by approximately 80–95% (Fig. 2A, Lane 4) compared with saline-treated controls (Fig. 2A, Lane 1), which confirmed the sequence specificity of Bcl-2 AS G3139 in down-regulating the target protein in an in vitro culture system.

In tumor samples from mice, systemic administration of G3139 (5 mg/kg) caused a pronounced down-regulation of Bcl-2 expression within 4 days of initiating G3139 treatment in which Bcl-2 protein levels were reduced to approximately 3% (Fig. 2, B–D) of control tumors (P < 0.001; Fig. 2C, Lane 2 versus Lane 1). No down-regulation of Bcl-2 protein was observed with either RP ODN or F-DOX treatments (Fig. 2B). With the AS treatment, Bcl-2 protein levels returned to pretreatment levels within 2 days after the final ODN treatment of the first weekly schedule (Fig. 2, C and D). Of particular interest was the observation that the solid tumors were insensitive to subsequent courses of G3139 treatment. Specifically, no detectable decrease in Bcl-2 expression was observed in the second or third weekly course of treatment (Fig. 2, C and D).

Histopathology of Tumor Specimens. To evaluate whether Bcl-2 AS (G3139) administration results in direct tumor cell kill, we examined the histology of tumors undergoing G3139 treatment (Fig. 3). Experiments were performed in parallel with the Western blot studies for tumor Bcl-2 protein levels to correlate the two features (Fig. 2, B and C, versus Fig. 3). Mice with established 0.1–0.15-g MDA435/LCC6 tumors were given saline, 5 mg/kg G3139, or RP ODN i.p. on days 17–21, 23–27, and 29–33, or F-DOX (5 mg/kg) on days 20, 27, and 34. Excised tumors were sectioned and stained with H&E. Tumors of control mice or RP ODN-treated controls were composed of densely packed tumor cells with varying amounts of vasculature, evenly surrounded by epithelial and fibrous tissue (Fig. 3A). F-DOX treatment resulted in marginal tumor-cell kill (Fig. 3C). Despite the small change in tumor volume observed with approximately 75–95% of controls (Fig. 2A, Lane 1). In vitro exposure of MDA435/LCC6 cells to G3139 led to a reduction in Bcl-2 protein levels by approximately 80–95% (Fig. 2A, Lane 4) compared with saline-treated controls (Fig. 2A, Lane 1), which confirmed the sequence specificity of Bcl-2 AS G3139 in down-regulating the target protein in an in vitro culture system.

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Bcl-2 AS G3139 treatment (Fig. 1B), the histology of tumors given Bcl-2 treatment revealed an increased fraction of dead cells (identified by their amorphous shape and condensed nuclei) reflecting a large percentage cell kill (Fig. 3, D–F). Dead tumor cells and areas of degenerative tissue were observed by day 20, appearing as loosely arranged cells with the occurrence of vacuolated structures (Fig. 3D). By day 23, tumor cells were surrounded by areas of dead tissue and surrounding stromal cells, creating regions (Fig. 3E) of tumor growth that, by day 28 (Fig. 3F) to day 34 (not shown), developed into isolated tumor pockets among large areas of dead tissue within the solid tumor. Areas of tumor cells were heterogeneous throughout the tumor section (both peripheral and central parts of tumor; Fig. 3, D–F). In addition, these tumor pockets were generally devoid of vasculature, although some blood vessels were present in contiguous areas of necrotic tissue (Fig. 3F).

**Combined Bcl-2 AS G3139 with F-DOX or SL-DOX.** To evaluate whether combined treatments of F-DOX or SL-DOX and G3139 in MDA435/LCC6 tumors results in any cooperative effects, we compared the therapeutic efficacy of F-DOX (Fig. 4) or SL-DOX (Fig. 5) in the presence and absence of G3139. In these experiments, dosages were adjusted so as not to exceed the MTD, particularly when combined with chemotherapy. A maximum of 10 mg/kg G3139 combined with 10
mg/kg DOX could be tolerated by SCID RAG2 mice without showing significant signs of toxicity (i.e., 25% weight loss). Treatment regimens were initiated either early, on day 3 after cell inoculation (Fig. 4 and 5), or when tumors weighed approximately 0.1–0.15 g (day 17, Fig. 6). Fig. 4A presents the in vivo efficacy results for 5 mg/kg and 10 mg/kg doses of F-DOX to MDA435/LCC6 tumor-bearing mice compared with saline controls in an early-treatment schedule. F-DOX administered at a dose of 5 mg/kg i.v. caused only minor delays in tumor growth between days 25 and 40, whereas 10 mg/kg F-DOX induced significant tumor growth suppression beyond day 40 (Fig. 4A).

Combining Bcl-2 G3139 treatment (5 mg/kg) with F-DOX (5 mg/kg) inhibited tumor growth to a greater degree than either treatment administered alone (Fig. 4B versus Fig. 4A and 3A). When G3139 and F-DOX doses were escalated to those approaching their MTDs in RAG2 mice (i.e., 10 mg/kg G3139 and 10 mg/kg F-DOX), an even more pronounced delay in tumor progression was observed (Fig. 4B). However, at these doses, mice displayed signs of toxicity (~12% transient body-weight loss).

To test for possible synergism between the G3139 and F-DOX, we compared the mean tumor weights of G3139 or RP ODN with or without F-DOX on days 25 and 35 (Fig. 1A versus Fig. 4A and B). On day 25, no difference in tumor weights were observed between control mice (0.21 ± 0.03 g), RP ODN treatment (0.19 ± 0.03 g; 90% T/C), and F-DOX treatment (0.17 ± 0.03 g; 81% T/C; P > 0.05; Fig. 1A versus Fig. 4A). G3139 (0.08 ± 0.02 g; 38% T/C) was able to suppress mouse tumor growth compared with controls, F-DOX, or RP ODN (P < 0.05; Fig. 1A versus Fig. 4A). When administered in combination, G3139 and F-DOX resulted in day-35 tumor weights of 0.03 g ± 0.005 (14% T/C), which were significantly smaller than for equivalent doses of the individual treatments (P < 0.01). Similarly, day-35 tumor weights for the combined G3139 + F-DOX treatment group were significantly (P < 0.01) less than those observed for either individual treatment (Fig. 4B versus 4A and 1A). We then examined the antitumor effects of SL-DOX on MDA435/LCC6 tumors when administered in the early therapeutic schedule in RAG2 mice. Tumors were treated with SL-DOX (5 mg/kg i.v. on days 6, 13, and 20) with or without

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**Fig. 3 Histopathology of MDA-435/LCC6 tumors implanted in SCID-RAG2 mice after Bcl-2 AS G3139 treatment.** SCID-RAG2 mice were given injections of 2 × 10⁶ cells into the mammary fat pad and were treated when tumors were approximately 0.1–0.15 g (day 17) with PBS (controls), 5 mg/kg RP ODN, or 5 mg/kg Bcl-2 AS ODN given i.p. on days 17–21, 23–27, and 29–33 or with F-DOX (5 mg/kg) on days 20, 27, and 34. Tumor sections at indicated times were stained with H&E. Representative tumor pictographs: A, saline-treated controls (day 20); B, RP ODN (day 23); C, F-DOX (day 23); D, G3139 treatment (day 23); E, G3139 treatment (day 28). Letter symbols in the pictographs: T, tumor cells; BV, blood vessel showing RBCs; V, vacuolated areas of degenerative tissue; D, areas of dead tumor cells; ×200.
SL-DOX alone exhibited antitumor effects and was able to substantially decrease tumor growth compared with saline controls \( (P < 0.01; \text{day } 25) \) and was superior to equivalent doses of F-DOX \( (P < 0.01; \text{day } 25) \) using the same treatment schedule (Fig. 4A versus Fig. 5). Treatments of combined G3139 (5 g/kg) and SL-DOX (5 mg/kg) were slightly more effective in suppressing tumor growth compared with SL-DOX up to day 45; however, later-stage tumor growth rates were similar (Fig. 5).

The results above, taken together, indicate that early-treatment schedules of combined G3139 and SL-DOX did not represent a significant improvement over G3139 plus F-DOX (Fig. 5 versus 4B). Because the pharmacology of SL-DOX is dependent on the presence of permeable tumor blood vasculature, we then examined the efficacy of combined G3139 and SL-DOX on established MDA435/LCC6 tumors that exhibit an intratumoral capillary system (Fig. 3A) that may allow selective extravasation of SL-DOX in tumor sites. RAG2 mice with 0.1–0.15-g MDA435/LCC6 solid tumors (day 17) were treated with 5 mg/kg Bcl-2 AS G3139 (15 doses over 3 weeks) with or without F-DOX or SL-DOX (given on days 20, 27, and 34). In this established solid tumor model with weekly DOX administration, there was no difference in the tumor growth curves of mice treated with either F-DOX (day-35 tumor weight, 0.35 ± 0.04 g) or saline controls (day-35 tumor weight, 0.39 ± 0.05 g; Fig. 6A), and the antitumor activity of Bcl-2 AS G3139 (5 mg/kg) alone was marginal (day-35 tumor weight, 0.27 ± 0.02 g; Fig. 6A). Combined Bcl-2 AS G3139 (5 mg/kg) and F-DOX (5 mg/kg) delayed tumor growth and was superior to equivalent doses of either treatment given alone (day-35 tumor weight, 0.27 ± 0.03 g; Fig. 6A). In comparison, SL-DOX (5 mg/kg) alone (day-35 tumor weight, 0.31 ± 0.04 g; Fig. 6B versus 6A) was able to delay tumor growth, and this effect was equivalent to that of G3139 + F-DOX (0.27 ± 0.03 g; Fig. 6A versus 6B). G3139 + SL-DOX (day-35 tumor weight, 0.25 ± 0.03 g) only slightly increased antitumor activity compared with SL-DOX (day-35 tumor weight, 0.25 ± 0.03 g) using the same treatment schedule (Fig. 5 versus 4B).
tumor weight, 0.31 ± 0.04 g; Fig. 6B) and was equivalent to the combined G3139 + F-DOX treatment (day-35 tumor weight, 0.27 ± 0.03; Fig. 6B versus 6A). The antitumor effect of combined Bcl-2 and SL-DOX treatment was enhanced when the dose of SL-DOX was increased to 10 mg/kg (SL-DOX day-35 tumor weight, 0.21 ± 0.01 g, versus SL-DOX + G3139 day-35 tumor weight, 0.12 ± 0.01 g; Fig. 6C), which indicated synergistic activity. In addition to tumor growth suppression, G3139 (5 mg/kg) + SL-DOX (10 mg/kg) therapy exhibited transient tumor shrinkage (Fig. 6) after the first SL-DOX dose (day 20). However, with all of the treatments, tumor growth persisted despite concurrent G3139 and/or DOX treatment after an initial response.

**PK and Tissue Distribution Studies.** To understand the mechanisms involved in AS-mediated drug sensitization, we examined the PK properties of F-DOX and SL-DOX after

![Figure 6](image-url)
G3139 administration. Established MDA435/LCC6 tumors (0.1–0.15 g) in RAG2 mice were treated with 5 mg/kg G3139 on days 17–20. F-DOX or SL-DOX (5 mg/kg) was administered on day 20, 3 h after the injection of G3139, corresponding to a similar dosing schedule used in the efficacy studies. For comparisons, we also evaluated the PK characteristics of F-DOX and SL-DOX in the absence of G3139 treatment. PK profiles of F-DOX and SL-DOX in plasma and tumor with or without G3139 are illustrated in Fig. 7, and the descriptive PK parameters are summarized in Table 1. F-DOX has a very short residence time in plasma, as manifested by its short distribution half-life ($t_{1/2a}$ = 0.06 h), large volume of distribution ($V_d = 2536$ ml), and small AUC ($2.99 \mu$g·h/ml; see Table 1). Encapsulation of DOX in liposomes significantly alters the PK of the encapsulated DOX, characterized by its long circulation times ($t_{1/2b}$ = 13 h). The retention of drug in liposomes was confirmed by monitoring the plasma drug:lipid ratios (data not shown).

The PK of F-DOX when coadministered with G3139 or RP ODN showed minimal differences in plasma drug distribution and elimination characteristics (Fig. 7A). F-DOX in the presence of G3139 demonstrated a marginal change in the AUC ($3.2 \mu$g·h/ml) and no change in the distribution $t_{1/2a}$ (0.06 h) and displayed a slightly lower $V_d$ (36.5 ml), which reflected minor ODN-DOX interactions in plasma (Table 1). In comparison, the plasma drug profiles of SL-DOX in the absence and presence of G3139 showed similar drug plasma-time curves with small changes in their descriptive PK attributes (Fig. 7A and Table 1). The AUC of SL-DOX with G3139 was decreased 1.3-fold and the distribution $t_{1/2b}$ decreased 2-fold, which indicated relatively small effects on these two parameters. The observed $V_d$ was close to the plasma volume of mice, which indicated limited distribution of liposomal drug into tissues, regardless of the presence of ODN in plasma (Table 1).

Examination of the tumor levels of F-DOX and SL-DOX revealed very interesting tumor-uptake profiles (Fig. 7B). F-DOX in the presence of G3139 treatment was found to rapidly distribute into tumors at levels approximately 2-fold higher than in the absence of G3139 ($P < 0.001$). The difference in the tumor DOX uptake properties were AS-specific and not observed with the RP ODN + F-DOX combination (Fig. 7B). In contrast, the G3139 effect on mediating tumor localization of SL-DOX was smaller in comparison; however, significantly greater levels were ob-

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**Table 1** Summary of selected pharmacokinetic parameters of plasma DOX administered as either F-DOX or SL-DOX in the presence or absence of Bcl-2 antisense oligonucleotides

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC$_{0-t}$ (µg · h/ml)</th>
<th>$V_d$ (ml)</th>
<th>$t_{1/2a}$ (h)</th>
<th>$t_{1/2b}$ (h)</th>
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<tbody>
<tr>
<td>F-DOX</td>
<td>2.99</td>
<td>2536</td>
<td>0.057</td>
<td>52.5</td>
</tr>
<tr>
<td>F-DOX + Bcl-2 RP ODN</td>
<td>3.14</td>
<td>2278</td>
<td>0.055</td>
<td>49.5</td>
</tr>
<tr>
<td>F-DOX + Bcl-2 AS ODN</td>
<td>3.19</td>
<td>1648</td>
<td>0.057</td>
<td>36.5</td>
</tr>
<tr>
<td>SL-DOX</td>
<td>1040.8</td>
<td>2.3</td>
<td>4.41</td>
<td>13.4</td>
</tr>
<tr>
<td>SL-DOX + Bcl-2 AS ODN</td>
<td>767.4</td>
<td>1.8</td>
<td>2.25</td>
<td>12.6</td>
</tr>
</tbody>
</table>

$V_d$, volume of distribution; $t_{1/2a}$, distribution half-life; $t_{1/2b}$, elimination half-life.
served in the presence of G3139 beyond 4 h (Fig. 7C). Furthermore, monitoring tumor liposomal lipid confirmed that SL-DOX drug accumulation in tumors was attributable to tumor uptake of liposomal drug rather than drug release from liposomes in plasma (not shown).

DISCUSSION

Most anticancer drugs have been shown to induce activation of apoptotic pathways as part of their cytotoxic activity (2, 5, 17). This has generated a great deal of interest for therapeutic intervention in which manipulations aimed at shifting the balance of apoptosis control in favor of cell death may lead directly to tumor cell death and/or sensitize tumor cells to chemotherapeutic agents (19, 31). In this context, proteins encoded by the bcl-2 gene family have been extensively studied and demonstrated to play an important role in apoptosis regulation (2, 5). These gene products either antagonize apoptosis (e.g., Bcl-2, Bcl-X L, Mcl-1) or function as death agonists (e.g., Bax, Bcl-X S, Bad; Refs. 32, 33). The ratio of these proteins within a tumor cell dictates the nature of homo- and heterodimerization between antagonists and agonists, which ultimately regulates the response to an apoptotic trigger such as chemotherapy (5, 6, 34).

Attempts to achieve therapeutic pro-apoptotic manipulations in tumor cells have focused primarily on the down-regulation of apoptosis antagonists such as Bcl-2 and Bcl-X L using AS ODNs. This is because of the difficulties associated with systemic applications of gene therapy designed to provide gain of functional changes in tumor cells (35). In contrast, phosphorothioate or mixed chemistry-backbone ODNs are amenable to development as pharmaceutical agents and have been shown to be well tolerated when administered as systemic infusions to humans (14). In particular, AS ODNs directed against Bcl-2 have been pursued as therapeutic agents, and one such ODN, G3139 (Genta, Inc.) has progressed to the stage of Phase II clinical testing (8).

Numerous studies have documented the effect of Bcl-2 manipulation using gene or AS ODN therapy on the susceptibility of tumor cells to apoptotic stimuli (36). Indications of both direct cytotoxicity and chemosensitization with Bcl-2 AS ODNs have been documented in vitro for a range of tumor types (19, 31, 37). Far fewer studies on the biological effects of Bcl-2 AS ODNs in vivo have been reported. Bcl-2 AS ODNs have been shown to induce tumor growth suppression independent of additional chemotherapy in murine and human xenograft solid-tumor models (19, 31, 35). However, in few reports have therapeutic effects been correlated with altered Bcl-2 expression in tumor specimens (19). Furthermore, pharmacological analyses have not been performed when Bcl-2 AS ODN have been coadministered with anticancer agents. It is important to identify potential drug PK alterations in combined therapies that could complicate resolving the AS-specific chemosensitizing influence of Bcl-2 manipulations.

In the studies described here, we evaluated the in vivo activity of the Bcl-2 AS ODN G3139 in the MDA435/LCC6 human xenograft solid-tumor model. This model was chosen to assess how Bcl-2 manipulations may affect tumor growth/chemosensitivity in a tumor line exhibiting low inherent Bcl-2 expression, because previous preclinical studies have used high Bcl-2 protein-expressing tumors (31), and we have demonstrated activity in the MDA435/LCC6 line in vitro.4 Also, the investigations here were designed to elucidate both the direct antitumor and chemosensitizing activity of G3139 when combined with the anticancer drug DOX by correlating Bcl-2 expression in tumor specimens and drug PK properties with therapeutic activity.

Recently, AS ODNs containing unmethylated CpG motifs within the ODN sequence have been shown to be potent immune stimulators (38, 39). To address the possibility that therapeutic activity was attributable to AS-mRNA interaction and not to immunostimulatory effects, we compared the efficacy of G3139 against a control Bcl-2 RP sequence of the same codon length, containing the same nucleotides with CpG motifs. In our studies, we demonstrated that daily i.p. G3139 treatment administered over 3 weeks was capable of achieving significant Bcl-2 protein down-regulation in human breast xenograft solid tumors, and this was associated with antitumor activity. A maximum activity was obtained at a dose of 5–10 mg/kg per injection. This effect was specific for the Bcl-2 AS G3139 sequence, and there was no observable activity with the control Bcl-2 RP ODN. These results confirm observations by Klasa et al. (40) that showed specific efficacy of this G3139 molecule and not Bcl-2 RP control in perforin-knockout (natural killer-deficient) mice and argued against possible immunostimulatory action, which is dependent on functional natural-killer activity (40). The degree of tumor growth suppression was attenuated when treatment was initiated after the tumors reached a size of 0.1–0.15 g compared with initiation before palpable tumor formation. This is an interesting result because Western analysis of tumor samples (0.1–0.15 g) during the course of G3139 treatment indicated >90% Bcl-2 protein reductions during the first course of treatment. Furthermore, the histology of these tumors revealed significant induction of cell death, which indicated that small changes in tumor volume could actually reflect a large percentage cell kill (Fig. 1B and Fig. 3, D–F).

The fact that tumor growth suppression, Bcl-2 down-regulation, and histological evidence of cytotoxicity were transient in the face of continued G3139 treatment is a very interesting and potentially important observation. Several potential mechanisms could account for the development of an apparent Bcl-2 AS ODN-resistant tumor state. These include the selection of tumor cells that either overexpress Bcl-2 protein after AS ODN treatment or result from possible mutation in the mRNA message that decreases sensitivity to the targeted ODNs. Reduced tumor ODN accumulation could arise from poor access of ODN to tumor cells, particularly those situated in areas devoid of vasculature (Fig. 3, D–F). Alternatively, the PKs and solid tumor uptake of G3139 may be altered in later treatment courses. At this time, we are unable to resolve what is responsible for this change in G3139 responsiveness; however, it is clearly a feature that

has significant implications for therapeutic applications and warrants further investigation.

In addition to the evidence for direct antitumor activity of G3139 against human breast cancer solid-tumor xenografts, we obtained indications that Bcl-2 down-regulation may provide some level of chemosensitization. Synergistic effects of Bcl-2 AS G3139 combined with chemotherapy were observed only under certain conditions, i.e., Bcl-2 AS G3139 and F-DOX in the early-treatment schedule and Bcl-2 AS G3139 and SL-DOX in the larger solid tumors. For example, on day 35 in the early-treatment model, G3139-related tumor-growth inhibition was 48% relative to control tumors, whereas the tumor size was reduced by 22% in mice treated with F-DOX at 5 mg/kg in the absence of G3139. If tumors responded to these two therapies independently when they are coadministered, then one would predict a mean tumor weight of 0.19 g on day 35. This is 2.7-fold larger than the observed tumor weight of 0.07 g, which agrees with the degree of chemosensitization observed in a previous human melanoma xenograft study (19) and supports the presence of chemosensitizing activity. Similar results were obtained for SL-DOX at 10 mg/kg combined with G3139 in the treatment of 0.1–0.15-g size tumors. However, in other experiments performed here, clear evidence of chemosensitization could not be established.

The above results highlight the complexities of resolving issues of chemosensitization when both of the agents used displayed significant antitumor activity individually. It should be noted that a recent report documented the ability of Bcl-2 down-regulation in a Shionogi murine solid-tumor model to sensitize these tumors to mitoxantrone (35, 37). The observers’ identification of chemosensitization was simplified by the lack of direct cytotoxicity of Bcl-2 AS ODNs on this tumor cell line and established sound evidence for Bcl-2 AS ODN-related chemosensitization in solid tumors. Our results at this time cannot unambiguously establish whether Bcl-2 down-regulation with G3139 chemosensitizes MDA435/LCC6 tumors to DOX; however, this would seem to be possible.

We investigated the use of long-circulating formulations of SL-DOX to determine whether increasing the level and duration of tumor drug delivery may improve the effects of combining DOX with G3139-induced Bcl-2 down-regulation. Increased antitumor activity was observed with SL-DOX compared with F-DOX for established (0.1–0.15 g) tumors both in the absence and presence of G3139 coadministration. This enhanced therapy seemed related primarily to the increased activity of the SL-DOX formulation itself, although indications of G3139-induced chemosensitization were obtained for a SL-DOX dose of 10 mg/kg. Given the transient effects of Bcl-2 down-regulation observed by Western analysis, it is possible that chemosensitization effects are masked by the fact that this may occur for only the first week of therapy when Bcl-2 suppression is achieved.

No therapeutic benefit over F-DOX was observed when SL-DOX was administered using the early-treatment schedule (initiated on day-3 after tumor inoculation) either in the absence or presence of G3139. Because the pharmacology of SL-DOX is dependent on the presence of an active tumor neovascularature (22–24), the lack of activity of SL-DOX in this early-treatment regimen is most likely related to the fact that the neovascularature through which liposomes mediate the enhanced delivery to solid tumors would not be well established shortly after tumor inoculation compared with the physiological situation of solid tumors in the size range of 0.1–0.15 (24). This implies that simply increasing the circulation lifetime of the anticancer agent is insufficient to increase antitumor activity when combined with G3139.

One issue regarding combining AS ODN therapy with anticancer drugs for chemosensitization purposes that has not been previously addressed is the potential for PK, metabolic, or tissue distribution interactions between the ODN and coadministered chemotherapy agent. This phenomenon has created complications in interpreting the results of chemosensitization trials that use PGP inhibitors when therapeutic improvements were associated with PK alterations as well as PGP inhibition. Our results here demonstrate that DOX tumor exposure is affected by the presence of G3139 coadministration in the absence of plasma PK alterations. However, in contrast to the results from PGP modulation studies, the PK interactions between G3139 and F-DOX seem to favor selective delivery to tumor tissue. The plasma exposure of F-DOX in mice treated with G3139 was minimally reduced compared with no G3139 treatment, whereas tumor exposure to DOX increased 2- to 3-fold. The reasons for these changes are not fully understood but could be related to enhanced drug permeability of tumor tissue treated with G3139 (based on histology slides), increased tumor uptake of DOX-G3139 complexes that could form in the circulation, and increased binding of DOX to tumor cells arising from G3139-induced DNA lesions that are induced by apoptosis activation. The evidence of chemosensitizing activity by G3139 combined with SL-DOX in established tumors argues in favor of molecular-based chemosensitization because improved therapy was achieved in the absence of significant PK changes. More extensive investigations focusing on this issue will be required to establish the molecular basis and therapeutic implications of such ODN-mediated effects.

In summary, although the Bcl-2 AS ODN G3139 can cause effective reductions in tumor cell Bcl-2 protein levels that lead to increased antitumor activity, its therapeutic utility may depend on various aspects of tumor physiology in addition to the pharmacological properties of the anticancer drug(s) with which it may be combined. Such information may enhance our ability to apply therapies targeted at apoptosis regulation with conventional cytotoxic agents in a manner that will significantly improve the management of cancer with chemotherapy.

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REFERENCES


Molecular and Pharmacokinetic Properties Associated with the Therapeutics of Bcl-2 Antisense Oligonucleotide G3139 Combined with Free and Liposomal Doxorubicin

Daniel E. Lopes de Menezes, Norma Hudon, Natashia McIntosh, et al.


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