G3139 Antisense Oligonucleotide Directed against Antiapoptotic Bcl-2 Enhances Doxorubicin Cytotoxicity in the FU-SY-1 Synovial Sarcoma Cell Line

David E. Joyner, Karen H. Albritton, Jeffrey D. Bastar, R. Lor Randall

SARC™ Laboratory, Sarcoma Services, Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, Utah 84112

Received 6 June 2005; accepted 26 September 2005

Published online 31 January 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20087

ABSTRACT: Synovial sarcoma (SS) is a highly aggressive, periartricular soft tissue sarcoma of children, adolescents, and young adults. Five- and 10-year survival rates are as low as 36 and 20%, respectively. Bcl-2, a negative regulator of apoptosis, is overexpressed in up to 90% of SS. Increased Bcl-2 expression not only leads to the development of cancer, but also to resistance of many anticancer chemotherapeutic agents. We hypothesized reducing Bcl-2 expression in SS should enhance doxorubicin cytotoxicity. Cell cultures representing two human sarcomas (FU-SY-1 SS and the pleomorphic SW982) and a primary human dermal fibroblast comparator (NHDF) were exposed in vitro to doxorubicin, or to doxorubicin preceded by Bcl-2 (G3139) antisense oligonucleotides, and assayed for cell survival, apoptosis, and modulations in Bcl-2 and Bcl-xL mRNA and protein content. SW982 sarcoma cells proved most susceptible to doxorubicin, while NHDF mesenchymal cells were least sensitive to doxorubicin. Treatment of FU-SY-1 SS with G3139 reduced Bcl-2 mRNA and protein levels, which enhanced doxorubicin-induced cell killing. There was a concurrent reduction in Bcl-xL mRNA following G3139 application in FU-SY-1 and NHDF cultures, but not in SW982. G3139 anti-Bcl-2 intervention sensitized the FU-SY-1 SS to doxorubicin, due to increased apoptosis. G3139 intervention was ineffective in the two non-SS cell lines. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:474–480, 2006

Keywords: synovial sarcoma; Bcl-2 antisense; doxorubicin; apoptosis

INTRODUCTION

Synovial sarcoma (SS) is a highly aggressive, periartricular soft tissue sarcoma of children, adolescents, and young adults. Five- and 10-year survival rates are as low as 36 and 20%, respectively. Surgical resection plus or minus adjuvant radiotherapy and/or doxorubicin (Adriamycin)-based chemotherapy are the mainstays of SS treatment. Because SS remains a very deadly form of cancer, improved therapies are necessary.

Bel-2, a negative regulator of apoptosis, is overexpressed in up to 90% of SS. Expression of Bcl-2 family members is significantly correlated with the aggressiveness of soft tissue sarcomas when controlled for the clinical tumor stage. Bcl-2 contributes to oncogenesis in a variety of human tumors and other cancer models, and may also contribute to chemotherapeutic drug resistance on the basis of its antiapoptotic activity. The role of Bcl-2 overexpression in SS oncogenesis has not been functionally assessed.

We hypothesized antisense oligonucleotides (AS ODNs) directed against antiapoptotic Bcl-2 mRNA would sensitize SS to doxorubicin chemotherapy. We therefore evaluated the in vitro efficacy of anti-Bcl-2 G3139 as a doxorubicin sensitizer for use against SS. We found that an increased incidence of apoptosis resulting from G3139 intervention was a primary factor mediating...
enhanced doxorubicin-induced cell killing in our SS cell line.

METHODS AND MATERIALS

Cell Lines and In Vitro Culture

The three human cell lines used in this study were: (1) FU-SY-1, a SS provided by Dr. Jun Nishio, Fukuoka University, Japan; (2) SW982, a cell line marketed by ATCC (HTB-93) as a SS; and (3) NHDF, a human primary dermal fibroblast cell line (Cambrex, East Rutherford, NJ), which served in previous studies as our untransformed mesenchymal comparator. The FU-SY-1 SS cell line originated from a monophasic fibrous SS. It maintains a consistent t(X;18) karyotype, has a SYT-SSX1 fusion transcript, and shows upregulation of Bcl-2. The SW982 sarcoma cell line, in contrast, lacks SYT-SSX1 or -SSX2 fusion transcripts, and has a Bcl-2 mRNA level equivalent to that of a primary fibroblast. Although SW982 is marketed as a SS, we considered it to be a pleomorphic sarcoma intermediate in morphology between normal cells and translocation-positive SS. Cell lines were screened for fusion mRNA in morphology between normal cells and translocation-positive SS. Cell lines were screened for fusion mRNA using SYT-SSX1 and SYT-SSX2 specific primers and reverse transcriptase polymerase chain reaction (RT-PCR). Cultures were maintained in mid-log phase in their appropriate culture medium within a 37°C humidified incubator containing 5% CO2. The cell lines tested negative for Mycoplasma contamination by PCR (Strategene, La Jolla, CA).

Doxorubicin Treatment

Log-phase cells were inoculated into 25 cm² tissue culture flasks 1 day prior to treatment. Immediately preceding doxorubicin application, cultures were washed with Hanks Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) and doxorubicin (Sigma, St. Louis, MO), diluted to 50 to 1000 nM in serum-free medium, was then added to each treatment flask. Control flasks received serum-free medium only. Cultures were incubated at 37°C for 24 h, after which the doxorubicin was removed, the cells washed with HBSS, and culture medium containing 10% fetal bovine serum was added to each flask. Cultures were incubated at 37°C for an additional 24 h prior to assaying by trypan blue dye exclusion. A minimum of three independent experiments involving three or more flasks per treatment dose were conducted with each cell line. Results (mean ± SE) are reported as viable cells as a percentage of untreated controls.

AS/Doxorubicin Combined Treatment

Phosphorothionate oligonucleotides G3139 (anti-Bcl-2) and G3622 (reversed polarity control) were supplied by Genta, Inc. (Berkeley Heights, NJ). A second Bcl-2 nonsense control (5’-ACACCCCAAATTCTTCCGCC-3’) was synthesized by the DNA/Pepptide Core Facility at the University of Utah. Intracellular/intrnuclear delivery of AS and control ODNs was achieved using the cationic carrier Oligofectamine (Invitrogen). Transfection conditions were defined using fluoroscence microscopy, and cytotoxicity assays according to the manufacturer’s recommendations.

AS ODN intervention consisted of seeding 25 cm² culture flasks 24 h prior to exposure. Following a wash with HBSS, AS or control ODNs diluted in OptiMEM (Invitrogen) were added to treatment flasks and the cultures incubated at 37°C for 4 h. Control cultures received Oligofectamine in OptiMEM. Upon completion, cultures were washed with HBSS, covered with culture medium, and returned to the incubator pending cell harvest. For combined AS/doxorubicin therapy, treatment and control cultures exposed 24 h earlier to ODNs (30 or 300 ng/mL for 4 h) were washed, then incubated in 50 to 1000 nM doxorubicin diluted in serum-free culture medium, or in serum-free medium without doxorubicin (controls). Cultures were washed subsequently with HBSS to remove the doxorubicin, and returned to the incubator for an additional 24 h prior to assaying by trypan blue dye exclusion. Exponentially decaying dose–response curves (Survival = 100 [a + (1-a)exp(-cdose)]) were fitted to treatment groups and a Wald Test was used to define significant differences in treatment survival.

RNA and Protein Preparation

Total RNA and proteins were extracted and purified from cell cultures using TRIzol Reagent (Invitrogen) and stored at −80°C pending use. RNA concentrations and integrity were assessed photometrically (A260/A280) and by visualization on 1% agarose gels. Proteins concentrations were determined using the colorimetric BCA protein assay (Pierce Chemical Co., Rockford, IL).

Real-Time RT-PCR

Bcl-2 and Bcl-xL mRNA were quantified by real-time RT-PCR using transcript-specific primers (Bcl-2 forward: 5’-CGCCCCCTGGATGACTGAGT-3’, reverse: 5’-GGGCCGTACAGTTCCACAA-3’; Bcl-xl forward: 5’TCCCTTTGTCTACGCTTTCCACG-3’, reverse: 5’GGTGCGATTGCGGCTTT-3’), a FastStart DNA Master SYBR Green I kit (Roche, Indianapolis, IN) and a Roche LightCycler with related software, as described by Perreard et al. Cell cultures exposed to G3139 were harvested for RT-PCR analysis at three time intervals: (1) immediately following the 4-h G3319 incubation (designated 4 h postapplication), (2) 24 h post-G3139 application (corresponding to the initiation of doxorubicin treatment), and (3) 48 h postapplication (termination
of doxorubicin treatment). First-strand cDNA used for real-time RT-PCR was generated from TRizol-purified total RNA using the ProStar First-Strand RT-PCR kit from Stratagene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference mRNA for standardizing transcript concentrations.

**Western Blots**

Cell lysates (SW982 and NHDF = 30 μg total protein/lane, FU-SY-1 = 10 μg/lane) and positive controls (Bcl-2: Sigma B1182; Bcl-xL: Sigma B0934) were separated on 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) and blotted onto polyvinylidene fluoride membranes (Millipore, Bradford, MA) using a Bio-Rad Trans-Blot SD Semi-Dry transfer cell according to the manufacturer’s recommendations. Proteins were probed overnight at 4°C with mouse antihuman Bcl-2 (Sigma; Clone Bcl-2-100), mouse antihuman Bcl-xL (BD PharMingen, San Diego, CA; clone 2H12), or mouse anti-β-tubulin (loading control; BD PharMingen; clone 5H1) antibodies. Horseradish peroxidase-conjugated antimouse IgG (Sigma) was used to detect primary antibodies. Bcl-2, Bcl-xL, and β-tubulin bands were detected using enhanced chemiluminescence reagents supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein bands were quantified by densitometric analysis using Scion Image β 4.0.2 software (Scion Corp., Frederick, MD). The resulting Bcl-2 and Bcl-xL values were adjusted relative to their respective cell line-specific Oligofectamine (OF)-β-tubulin controls, which had been normalized to a value of 1.00.

**Apoptosis**

We tested for apoptosis-related phosphatidylserine (PS) externalization by means of flow cytometry using the Vybrant Apoptosis Assay Kit (Alexa Fluor® 488 annexin V/Propidium Iodide kit #2) supplied by Molecular Probes, Inc. (Eugene, OR). A 4-h incubation of cells in 5 μg/mL etoposide (Sigma) served as a positive control.

**RESULTS**

**Constituent Levels of Bcl-2/Bcl-xL mRNA**

Because SS reportedly overexpress Bcl-2, Bcl-2 mRNA levels were assessed by real-time RT-PCR. Untreated FU-SY-1 SS cells contained approximately 15 times more Bcl-2 mRNA than did SW982 sarcoma or NHDF mesenchymal cells, which had equivalent concentrations of Bcl-2 mRNA (data not shown). Constituent levels of Bcl-xL mRNA, in contrast, were approximately three times greater in NHDF cells than in either sarcoma cell line.

**Doxorubicin Cytotoxicity**

Doxorubicin cytotoxicity was defined by subjecting cell cultures to a 24-h treatment with doxorubicin in serum-free culture medium. Cell survival was assayed by trypan blue dye exclusion 24 h after doxorubicin removal. Dose–response curves for cultures treated with doxorubicin at concentrations of 50 to 1000 nM are illustrated in Figure 1. Both sarcoma cell lines were sensitive to doxorubicin, with cell survival less than 40% of their respective controls at the maximum doxorubicin dose. NHDF cells, in comparison, demonstrated greater than 75% cell survival at the maximum doxorubicin dose of 1000 nM. Thus, doxorubicin sensitivity did not correlate exclusively with constituent Bcl-2 mRNA concentrations.

**AS/Doxorubicin Combined Treatment**

We hypothesized combined G3139/doxorubicin therapy should be more effective at killing SS cells than would an application of doxorubicin alone. To test this hypothesis, cell cultures initially exposed to 30 or 300 ng/mL G3139 for 4 h were incubated in 50 to 1000 nM doxorubicin, assayed for cell survival, and the results compared against cultures exposed only to doxorubicin. For combined G3139/doxorubicin experiments, control cultures (without doxorubicin) were also treated with 30 or 300 ng/mL G3139, and control
cell survival adjusted to 100% for comparative purposes. We found only the FU-SY-1 SS showed a G3139 dose-dependent enhancement in doxorubicin cytotoxicity (Fig. 2), with cell survival approximating 20% of controls at a combined dose of 30 ng/mL G3139 and 1000 nM doxorubicin. FU-SY-1 cell survival at 300 ng/mL G3139 and 1000 nM doxorubicin was 10% of controls, and represented a significant $(F = 4.59, p = 0.035, \text{Wald Test})$ reduction in cell survival relative to doxorubicin treatment alone. The NHDF and SW982 cell lines, in contrast, were unaffected by G3139 intervention. No enhancement in doxorubicin cytotoxicity was evident following application of either Bcl-2 control ODN.

**Real-Time RT-PCR**

Real-time RT-PCR was used to verify temporal (4, 24, and 48 h post-G3139 application) alterations in mRNA concentrations in response to AS intervention. A 4-h application of G3139 at 300 ng/mL reduced Bcl-2 mRNA concentrations 14-fold within 24 h in the FU-SY-1 SS (Table 1), suggesting this SS cell line is susceptible to anti-Bcl-2 intervention. G3139 intervention was less effective in SW982 and NHDF cells. Peak depression of Bcl-2 mRNA occurred in both sarcoma cell lines within 24 h after G3139 application, compared to 48 h in NHDF cells. Bcl-2 transcript levels then rebounded in the sarcoma cells during the last 24 h of the 48-h test period. Thus, initiation of the 24-h doxorubicin treatment in combined therapy corresponded temporally with maximum (or near maximum) G3139-induced depression of Bcl-2 mRNA.

G3139 application also depressed Bcl-xL transcript levels in FU-SY-1 cells, reflecting a ninefold reduction in Bcl-xL mRNA by 48 h, compared to controls (Table 1). NHDF Bcl-xL levels were also affected by G3139 application, although at 4 and 24 h post-G3139 application, concentrations of Bcl-xL mRNA in treated NHDF cells actually exceeded that of controls. The SW982 sarcoma, in contrast, showed little fluctuation in Bcl-xL mRNA in response to G3139, and at all three time points (4, 24, and 48 h), Bcl-xL levels in G3139-treated cultures exceeded transcript concentrations in SW982 controls.

**Western Blots**

Western blots were used to assess changes in Bcl-2 and Bcl-xL protein concentrations induced by G3139 intervention. Protein bands were quantified using Scion software, then normalized against their respective Oligofectamine (OF) $\beta$-tubulin controls. The resulting protein concentration estimates were then compared within each cell line over the three collection time points (4, 24, and 48 h post-G3139 application). Reductions in Bcl-2 protein were apparent in all three cell lines within 24 h after G3139 application (Fig. 3), and protein levels continued to decrease in treated cultures for an additional 24 h. Thus, Western blots confirmed the deleterious effect of G3139 on Bcl-2 content in these cell lines, and

| Table 1. Fold Changes in Bcl-2 and Bcl-xL mRNA Content in FU-SY-1, SW982, and NHDF Cells following a 4-h Application of 300 ng/mL anti-Bcl-2 G3139<sup>a</sup> |
|---------------------|-------|-------|-------|
| Cell Line/mRNA     | 4     | 24    | 48    |
| FU-SY-1             |       |       |       |
| Bcl-2               | -5    | -14   | -6    |
| Bcl-xL              | -2    | -6    | -9    |
| SW982               |       |       |       |
| Bcl-2               | -5    | -7    | -3    |
| Bcl-xL              | +2    | +3    | +1    |
| NHDF                |       |       |       |
| Bcl-2               | +2    | -9    | -12   |
| Bcl-xL              | +8    | +1    | -1    |

<sup>a</sup> Fold changes in mRNA content compared to Oligofectamine (OF) controls as defined by real-time RT-PCR. Treated cultures assayed at 4, 24, and 48 h post-G3139 initiation. Positive (+) infers overexpression, while negative (−) infers under expression relative to controls.
demonstrate that Bcl-2 protein levels were reduced when compared to OF controls at the time of doxorubicin application.

Bcl-xL protein, in contrast, increased in concentration in the two sarcomas over the 48 h test period (Fig. 2), but decreased in NHDF cells. Furthermore, Bcl-xL protein levels in G3139-treated SW982 and NHDF cultures always exceeded their respective untreated OF controls.

**Induction of Apoptosis**

An Annexin V (phosphatidylserine externalization)/flow cytometry assay was used to document enhanced apoptosis resulting from G3139 intervention followed by doxorubicin. In confirmation of our hypothesis, we found that an increased incidence of apoptosis resulting from G3139 intervention was a primary factor mediating enhanced doxorubicin-induced cell killing in the FU-SY-1 SS cell line (Fig. 4). The enhanced sensitivity to doxorubicin evident in FU-SY-1 SS cells represented a threefold increase in the percentage of apoptotic cells present relative to the percentage resulting from doxorubicin treatment alone. The difference between the two treatments was statistically significant (Students two-tailed t-test with equal variances, n = 5 flasks/treatment; t = 6.1, p < 0.001). The two non-SS cell lines, in contrast, showed a reduced level of apoptosis rather than an enhancement following combined therapy, when compared to doxorubicin treatment alone. However, this reversal in effect was significant for NHDF cultures only (Students two-tailed t-test with unequal variances, n = 8 flasks/treatment; t = 2.74, p = 0.028).

**DISCUSSION**

The optimal treatment for SS is open to debate, and may differ according to the age of the patient. Surgical resection in combination with radiotherapy and/or chemotherapy remains the primary treatment for localized SS, with doxorubicin the principal chemical agent. Despite the slightly higher response rates achieved in some studies, no multidrug regime has demonstrated a survival advantage when compared against single-agent doxorubicin. Multidrug chemotherapy, to minimize myelotoxicity, necessitates application of lower doxorubicin doses than have proven optimal for SS (~75 mg/m²). Herein we suggest an alternative solution to this problem: enhance efficacy in SS by employing agents that predispose cellular target(s) to doxorubicin’s toxic effects. Increased efficacy promotes single-chemical application, with potentially lower doses. With
this in mind, we evaluated the efficacy of Bcl-2 antisense intervention for use with doxorubicin against SS.

The two sarcoma cell lines were impacted more severely by doxorubicin than was the NHDF comparator. Malignant cells, which often lack the mitotic controls necessary to facilitate drug-induced damage repair, should be more sensitive to antineoplastic drugs than are normal cells. FU-SY-1 SS cells, supporting a higher concentration of Bcl-2 mRNA and protein and an equivalent level of Bcl-xL, proved less sensitive to doxorubicin cytotoxicity than did SW982 sarcoma cells. The NHDF cell line was least sensitive to doxorubicin-induced cytotoxicity, perhaps partially resulting from elevated levels of Bcl-xL, compared to the sarcoma cell lines.

G3139 functioned as a doxorubicin sensitizer only with the FU-SY-1 SS. This interaction was evidenced through a dose-dependent increased rate of doxorubicin-induced apoptosis resulting from G3139 degradation of Bcl-2. Reductions in Bcl-2 mRNA and protein following G3139 application were also evident in SW982 and NHDF cultures, but neither cell line responded favorably to G3139 intervention.

G3139 application impacted both Bcl-2 and Bcl-xL mRNA, making it difficult to attribute the enhanced G3139/doxorubicin response shown by FU-SY-1 cultures to reductions in Bcl-2 alone. G3139 targets the first six codons of the human Bcl-2 mRNA open reading frame (Genta Inc.), and we were unable to find a nucleotide sequence within the Bcl-xL transcript that is receptive to the G3139 anti-Bcl-2 oligonucleotide. When the G3139 nucleotide sequence (TCT CCC AGC GTG CGC CAT) was processed through NIH BLAST, only Bcl-2 mRNA was reported; there was no inference to Bcl-xL (Bcl2L1).

Under our experimental conditions, Bcl-2 protein levels in FU-SY-1 cells decreased following G3139 intervention, while Bcl-xL concentrations increased. The loss of Bcl-2 protein, with no reduction in Bcl-xL protein, suggests Bcl-2 was the principal target of G3139 intervention in the FU-SY-1 SS. G3139 intervention also reduced Bcl-2 mRNA and protein content in the SW982 cell line, but had minimal impact on Bcl-xL, and did not enhance doxorubicin efficacy. G3139 intervention, therefore, was ineffective in SW982 sarcoma cells. We suspect Bcl-xL probably serves as the dominant antiapoptotic protein in the SW982 cell line. NHDF mesenchymal cells also proved unresponsive to G3139 intervention, and were only moderately impacted by doxorubicin.

This study demonstrated G3139 intervention effectively sensitized SS cells to doxorubicin-based chemotherapy, primarily through an enhanced rate of apoptosis. G3139, applied alone or coupled with doxorubicin, exerted little or no influence on apoptosis in the SW982 sarcoma cell line, possibly due to the confounding influence of antiapoptotic Bcl-xL. NHDF mesenchymal cells, in contrast, may have benefited from G3139 intervention, because survival of NHDF cells subjected to combined therapy exceeded that of equivalent cultures treated only with doxorubicin.

ACKNOWLEDGMENTS

Funding was supplied through the Terri Anna Perine Sarcoma Fund and a Primary Children’s Medical Center Research Foundation Grant. We thank S. Lessnick and J. Yost for critically reviewing the manuscript.

REFERENCES