Combination Bcl-2 Antisense and Radiation Therapy for Nasopharyngeal Cancer

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Abstract

Purpose: A wide variety of tumors depend on the dysregulation of Bcl-2 family proteins for survival. The resulting apoptotic block can often provide a mechanism for resistance to anticancer treatments, such as chemotherapy and radiation. This current study evaluates the efficacy of combining systemically delivered Bcl-2 phosphorothioate antisense (Bcl-2 ASO) and radiation for nasopharyngeal cancer therapy.

Results: Antisense uptake was unaffected by 0, 3, or 6 Gy radiation. Radiation decreased the fraction of viable C666-1 cells to 60%, with a further decrease to 40% in combination with Bcl-2 ASO. Despite a modest in vitro effect, Bcl-2 ASO alone caused the regression of established xenograft tumors in mice, extending survival by 15 days in a C666-1 and by 6 days in a C15 model. The survival time for mice treated with both Bcl-2 ASO and radiation increased by 52 days in C666-1 and by 20 days in C15 tumors. This combination resulted in a more-than-additive effect in C666-1 tumors. Less impressive gains observed in C15 tumors might be attributable to higher expression of antiapoptotic Bcl-2 family proteins and limited drug distribution in the tumor. Retreatment of C666-1 tumors with the Bcl-2 ASO-radiation combination, however, was effective, resulting in mice surviving for >80 days relative to untreated controls.

Conclusions: Our results show that the Bcl-2 ASO and radiation combination is a highly potent therapy for nasopharyngeal cancer. Further examination of combination therapy with radiation and other Bcl-2 family–targeted anticancer agents in both preclinical and clinical settings is definitely warranted.

One of the major pursuits in experimental therapeutics is the successful use of Bcl-2 family proteins for human cancer therapy. It has long been known that antiapoptotic members of the Bcl-2 family are overexpressed in a wide variety of both hematologic and solid cancers, often associated with poor outcome. Such is the case for Bcl-2 in acute myeloid leukemia (1, 2), B-cell chronic lymphocytic leukemia (3, 4), non-Hodgkin’s lymphoma (5), anaplastic large cell lymphoma (6), prostate cancer (7), small cell lung cancer (8), bladder cancer (9), and solitary fibrous tumors (10). Indeed, an increased expression of antiapoptotic Bcl-2 family members and/or a decreased expression of proapoptotic Bcl-2 family members have been found to play key roles in tumor formation, development of metastases, chemotherapy resistance, and radiation resistance (11–13).

A variety of anticancer agents using the Bcl-2 family have been developed, ranging from viral gene therapies (Bax- or BimS-overexpressing adenoviridae; refs. 14, 15), BH3 domain peptides (Antennapedia peptide-linked Bad BH3 domain and hydrocarbon-stapled BH3 peptides; refs. 16, 17), to small molecules (HA14-1 and BH3Is; refs. 18, 19). However, Bcl-2-targeted antisense oligodeoxynucleotides are the most clinically studied members of this therapeutic family. Anti-Bcl-2 oligonucleotide therapy was first introduced by Reed et al. (20) and has been further evaluated in the form of G3139 (oblimersen sodium, Genasense; Genta, Inc., Berkeley Heights, NJ). G3139 is a phosphorothioate-modified antisense sequence complementary to the first six codons of human Bcl-2 mRNA. Although clinical trials have shown that G3139 has a favorable safety profile, its efficacy is limited when given as a single agent (21, 22).
G3139 and other Bcl-2-targeted anticancer agents have been studied in combination with traditional chemotherapies (23, 24). Overexpression of Bcl-2 is known to increase clonogenic survival and inhibit radiation-induced apoptosis (25–27), but there is limited information on the in vivo efficacy of combining radiation with Bcl-2 family therapeutics for cancer therapy. Radiation is one of the most common modalities used for cancer treatment and remains the primary curative modality for malignancies such as nasopharyngeal carcinoma.

Nasopharyngeal cancer is a malignant epithelial carcinoma of the head and neck region in which the 5-year overall survival rate is only about 70% (28). Even with newer radiation techniques, such as three-dimensional intensity-modulated radiation, ~43% of patients will still develop distant metastases within 4 years (29, 30). A variety of antiapoptotic Bcl-2 proteins are overexpressed in nasopharyngeal cancer, including Bcl-2, Bcl-xL, and Bfl-1 (31–33). The EBV exists in nasopharyngeal cancer cells in a state of persistent latent infection, and EBV proteins, such as LMP1, may up-regulate antiapoptotic members of the Bcl-2 family (34).

This current study thus investigates the role of phosphorothioate-modified Bcl-2 oligodeoxynucleotide antisense (Bcl-2 ASO) therapy, in combination with radiation for nasopharyngeal cancer. Furthermore, we have evaluated the properties of antisense distribution as it relates to this solid tumor model.

**Materials and Methods**

**Antisense oligonucleotides.** All oligodeoxynucleotides were 2× high-performance liquid chromatography–purified phosphorothioates obtained from TriLink Biotechnologies (San Diego, CA). Similar to G3139, Bcl-2 ASO corresponds to the first six codons of the human Bcl-2 mRNA translation initiation site (5'-TCTCCCAGCAGTGTGCCCCAT-3'; ref. 35). RC is the reverse control oligonucleotide (5'-TACCGCGTGAGACCCTCT-3'), and MMC is a two-base antisense mismatch control (5'-TCTCCAGCAGTGTGCCCCAT-3', mismatches underlined). FITC-MMC oligonucleotides consisted of a FITC molecule labeled on the 5' end of a MMC oligonucleotide.

For in vitro experiments, lyophilized antisense oligonucleotides were diluted in PBS (PBS without Ca²⁺ and Mg²⁺ was used throughout this study) to ~3 μg/μL and added to regular growth medium (with serum). The growth medium/antisense solutions were changed daily. For in vivo experiments, antisense molecules were administered in 100 μL PBS.

**Administration of ionizing radiation.** For in vitro experiments, cells were irradiated at room temperature using a 137Cs unit (Gamma-cell 40 Extractor; Nordion International, Inc., Ontario, Canada) at a dose rate of 1.1 Gy/min. For in vivo experiments, the mice were immobilized in a Lucite box, and the tumor-bearing mouse legs were exposed to 100 kV (10 mA) at a dose rate of 10 Gy/min.

**Fig. 1.** C666-1 cells uptake antisense oligonucleotides. A, C666-1 cells were incubated with 5 μmol/L of FITC-MMC for 24 hours and compared with an untreated control.
Cells and culture conditions. The nasopharyngeal cancer cell line C666-1 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc., Quebec, Canada) and antibiotics (100 mg/L penicillin and 100 mg/L streptomycin) as previously described (36). The latent presence of EBV has been consistently shown in this cell line (37).

Antisense uptake assays. C666-1 cells were seeded in T-25 flasks (1.5 x 10^6 per flask). At 70% confluency, experimental and control cells were given either 0, 3, or 6 Gy radiation. Fresh medium containing 5 μmol/L FITC-MMC was then added 0, 3, or 6 hours after radiation. Twenty-four hours after antisense administration, the cells were examined under the Axiovert S 100 microscope (Zeiss, Gottingen, Germany) at 488 nm. Subsequently, the cells were trypsinized, pelleted, resuspended in PBS, and analyzed using CellQuest software (Becton Dickinson, Mountain View, CA) with a FACSCalibur flow cytometer (Becton Dickinson).

Real-time PCR. Forward (5'-CATGTGTGGAGACGGTCGTAA-3') and reverse (5'-GCCGGTTCAGGTACTCAGTC-3') primer pairs were designed for human Bcl-2 mRNA using the Primer Express software (Applied Biosystems, Foster City, CA). β-Actin control primers were purchased from Applied Biosystems. C666-1 cells were seeded (3 x 10^6 per T-75 flask), and 1 day later, were incubated with 5 μmol/L of either RC or Bcl-2 ASO. After 24 hours, cells were lysed, and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Chatworth, CA). Reverse transcription was done using SuperScript II Reverse Transcriptase (Invitrogen, San Diego, CA) as specified by the manufacturer. Real-time PCR was done using SYBR Green (Applied Biosystems) and a Perkin-Elmer/ABI Prism 7900 sequence detection system (PE Biosystems, Foster City, CA). The following cycling profile was used: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The mean fold change in mRNA expression was calculated using the 2^-ΔΔCt method (38). Each sample was analyzed in triplicate, with the experiment done a total of three times.

Western blot analysis. Cells were seeded (3 x 10^6 per T-75 flask) 1 day before a 4-day incubation with RC or Bcl-2 ASO, with the antisense/growth medium solution changed daily. Cell extracts were prepared using a lysis buffer [0.1 mol/L Tris-HCl (pH 8), 1% SDS, 10 mmol/L EDTA, 20 mmol/L DTT], and protein concentration was determined using the Bio-Rad Detergent-Compatible Protein Assay (Hercules, CA). Immunoblotting was done as previously described (36). Briefly, 30 μg of protein were loaded into Novex Tris-glycine (4-20%) protein gels (Invitrogen) for electrophoresis, semidry transfer was done using nitrocellulose membranes and a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad), and blocking was done using PBST (0.1% Tween 20 in PBS) containing 5% low-fat milk. The monoclonal mouse anti-human Bcl-2 oncoprotein clone 124 (1:1,000 dilution; DakoCytomation, Carpinteria, CA) and monoclonal mouse anti-β-actin clone AC-15 (1:10,000 dilution; Sigma-Aldrich, St. Louis, MO) antibodies were used.

Fig. 1 Continued. C, cells were treated as in (B), but antisense was administered 6 hours after radiation (0, 3, or 6 Gy). % FITC-positive cells. Representative results. Each experiment has been done thrice independently.

Fig. 2. Bcl-2 ASO decreases Bcl-2 expression. C666-1 cells were incubated with RC or Bcl-2 ASO. A, cells were harvested for real-time PCR analysis 24 hours later. Bcl-2 mRNA levels were normalized to β-actin levels, and the fold change between Bcl-2 ASO and RC-treated cells are shown. Columns, mean; bars, SE. *, P < 0.05, a significant difference from RC treated cells. B, cells were harvested for Western blot analysis after 4 days of treatment. Representative blot. Each experiment has been done thrice independently.
Determination of cell viability. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt viability assay (Promega, Madison, WI) was used to determine cell viability, as C666-1 cells were not amenable to clonogenic assays. C666-1 cells, 1 day after being seeded in 96-well plates (2 × 10^4 per well), were incubated with Bcl-2 ASO or RC for 4 days and then given 6 Gy radiation (where indicated). The cells were kept in Bcl-2 ASO or RC for an additional 5 or 7 days. The growth medium was not changed during the last 2 days of treatment. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt viability assay was done in accordance with the manufacturer's specifications. Each experiment was done in triplicate, with three independent experiments conducted.

Morphologic assessment of apoptosis. C666-1 cells were seeded (1.5 × 10^6 per T-25 flask) 24 hours before treatment. The cells were then treated with antisense oligonucleotides for 4 days, given 6 Gy radiation, and incubated with antisense for another 6 days. The growth medium was not changed during the last 2 days of treatment. Apoptosis was evaluated morphologically using Hoechst 33342 (Calbiochem, La Jolla, CA) fluorescence staining. Briefly, adherent as well as any detached cells were collected, washed in PBS, gently resuspended in a 10 μmol/L Hoechst 33342/4% formalin-PBS solution, and then placed in the dark at 4°C overnight. Afterwards, cells were resuspended, placed onto blood smear slides, coverslipped, and immediately visualized under UV light using a Zeiss Axioskop HBO 100 microscope (Zeiss). The proportion of cells showing the
morphic features of apoptosis, such as condensed areas of chromatin or nuclear fragmentation, were then scored. For each sample, at least 1,000 cells were scored from >10 microscopic fields. Three independent experiments were done.

Sub-G<sub>1</sub> flow cytometric analysis. C666-1 cells, seeded in six-well plates (0.3 × 10<sup>6</sup> per well), were treated with antisense and radiation as discussed above. Six days after radiation, adherent and any detached cells were collected, pelleted at 200 × g, resuspended in 1.5 mL hypotonic fluorochrome solution (50 μg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100; Sigma-Aldrich), and left in the dark at 4°C overnight. Cells treated with 700 nmol/L staurosporine (Sigma-Aldrich) for 5 hours were used as a positive control. Flow cytometric analysis was then done (FACSCalibur, Becton Dickinson) and analyzed with CellQuest (Becton Dickinson). Sub-G<sub>1</sub> peaks were identified by ModFit LT (Verity Software House, Inc., Topsham, ME).

Animal experiments. All animal experiments were conducted in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network (Ontario, Canada). In all cases, 6- to 8-week-old severe combined immunodeficient (SCID) BALB/c female mice obtained from the Animal Research Colony, Ontario Cancer Institute were used.

Nasopharyngeal cancer xenografts were established in SCID mice, and the tumor-plus-leg diameter

Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining. C666-1-harboring mice were treated as described above, except that treatment began when the tumor-plus-leg diameter was ~9.5 mm. The mice were sacrificed on either day 4 or 5 of treatment, which corresponded to 24 or 48 hours, respectively, after the third injection of antisense. Tumors were immediately fixed in 10% formalin (in PBS) for 48 hours, placed in 70% alcohol for 48 hours, paraffin embedded, and sectioned (5 μm). Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining was done by Clinical Research Program Services, Toronto General Hospital, University Health Network.

Immunohistochemistry. C666-1- or C15-harboring mice were established. When the tumor-plus-leg diameter reached ~9.5 mm, the mice were sacrificed. Their tumors were immediately fixed in 10% formalin (in PBS) for 48 hours, placed in 70% alcohol for 48 hours, paraffin embedded, and sectioned (5 μm). Immunohistochemistry was done using either a prediluted rabbit polyclonal anti-Bcl-x<sub>L</sub> antibody (1:4 dilution; Abcam, Inc., Cambridge, MA) or a rabbit polyclonal anti-Bcl-1/A1 antibody (1:300 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For Bcl-2 staining, a FITC-conjugated mouse monoclonal anti-human Bcl-2 antibody (1:50 dilution; BD Biosciences, San Jose, CA) was used in combination with a biotinylated goat anti-fluorescein antibody (Vector Laboratories, Inc., Burlingame, CA).

In vivo imaging. EBV-positive nasopharyngeal cancer xenograft tumors were established in SCID mice, and the tumor-plus-leg diameter

Fig. 3 Continued. C. the proportion of cells displaying morphologic features of apoptosis was quantified. Columns, mean from three independent experiments, with at least 1,000 cells counted for each experiment; bars, SE. **, P < 0.01, a significant difference from radiation; ***, P < 0.05, a significant difference from RC + radiation.

Fig. 3 Continued. D. C666-1-cells were incubated with RC or Bcl-2 ASO for 4 days, before the administration of 6 Gy radiation (where indicated). The cells were harvested for sub-G<sub>1</sub>, flow cytometry 6 days later. Representative experiment from three independent experiments. % Apoptotic cells: sub-G<sub>1</sub> fraction (black), the G<sub>0</sub>-G<sub>1</sub> fraction (horizontal stripes), the S fraction (gray), and the G<sub>2</sub>-M fraction (vertical stripes).
was allowed to grow until ~8.5 mm (for the imaging of small tumors), ~12.5 mm (for the imaging of large tumors), or ~14 mm (for the imaging of drug penetration and vasculature colocalization). FITC-MMC (10 mg/kg in 100 μL PBS) was injected i.v. via the tail vein. One day after injection, Hoechst 33342 (6 μg/μL in 100 μL PBS) was injected i.v. via the tail vein for visualization of functional blood vessels (39). The mice were sacrificed 1 minute later. The major organs and the tumors were immediately removed and cryofixed in the dark using the Tissue-Tek ornithine carbamyl transferase compound (Bayer Corp., Pittsburgh, PA). Sections (5 μm) were cut and stored in the dark at ~70°C. Tumors were imaged using a Zeiss Axiovert 200M (Zeiss) for wide-field microscopy (FITC: excitation, 480 nm; beam splitter, 505 nm; emission, 535 nm; Hoechst 33342: excitation, 360 nm; beam splitter, 395 nm; emission, 460 nm). Major organs were imaged using the Zeiss Axioskop HBO 100.

**Statistical analyses.** Unless otherwise stated, all experiments were conducted independently thrice, and data are reported as mean ± SE. The Wilcoxon signed rank test was used for the real-time PCR analysis. The Wilcoxon rank sum test was used for the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt cell viability; Hoechst 33342 evaluation of apoptosis; C666-1 xenograft treatment; and C15 xenograft treatment analyses. For the xenograft treatment experiments, comparisons were made when the tumor-plus-leg diameter was 15 mm.

**Results**

**C666-1 cell uptake of antisense oligonucleotides.** To determine the efficiency of antisense oligonucleotide uptake in vitro, C666-1 cells were incubated with 5 μmol/L FITC-MMC.
MMC for 1 day. Using flow cytometry, C666-1 cells were found to have high levels of uptake, with 84.0% of cells having a fluorescent signal of >95.0% of untreated cells (Fig. 1A and B). When 0, 3, or 6 Gy of irradiation was given 0, 3, or 6 hours before FITC-MMC administration, antisense uptake remained unaffected (Fig. 1C; additional data not shown). Thus, antisense molecules may be effectively administered after radiation in C666-1 cells.

**Bcl-2 ASO effectively decreases Bcl-2 expression in nasopharyngeal cancer.** The effect of Bcl-2 ASO on C666-1 cell Bcl-2 expression was determined in vitro using real-time PCR and Western blotting. Relative to RC-exposed cells, treatment with 5 or 10 μmol/L Bcl-2 ASO significantly decreased Bcl-2 mRNA levels by 0.37- or 0.03-fold ($P < 0.05$), respectively (Fig. 2A). Bcl-2 protein levels also decreased relative to RC and untreated controls (Fig. 2B).

**Bcl-2 ASO reduces cell viability in combination with radiation.** The cytotoxic effects of Bcl-2 ASO and radiation were assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay and compared with the effects of RC or no treatment (Fig. 3A). A time-dependent and dose-dependent reduction in cell viability was observed when Bcl-2 ASO was given in combination with 6 Gy radiation. The fraction of viable cells decreased to 0.62 (2.5 μmol/L Bcl-2 ASO) and 0.59 (5 μmol/L Bcl-2 ASO) 5 days after radiation, which further decreased to 0.50 (2.5 μmol/L Bcl-2 ASO) and 0.40 (5 μmol/L Bcl-2 ASO) on day 7. In contrast, C666-1 cells treated with RC, Bcl-2 ASO alone, radiation, or RC plus radiation showed much smaller reductions in cell viability.

**Bcl-2 ASO increases radiation-induced apoptosis.** The combination of Bcl-2 ASO and radiation was anticipated to induce apoptosis. Thus, 6 days after radiation, treated cells were examined for morphologic changes in nuclear structure using Hoechst 33342 staining (Fig. 3B). C666-1 cells that were mock, RC, or Bcl-2 ASO treated had an apoptotic fraction of 1.1%, 0.9%, or 1.4%, respectively (Fig. 3C).

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**Fig. 5.** Bcl-2 ASO and local radiation produce an *in vivo* therapeutic benefit. A, C666-1 xenografts were established in SCID mice and randomized into the following groups: no treatment, RC, MMC, Bcl-2 ASO, radiation (RT), RC + radiation, MMC + radiation, or Bcl-2 ASO + radiation. Six injections of antisense (10 mg/kg) with or without two doses of radiation (2 × 4 Gy) were given. Three independent experiments were conducted, with three mice per group in each experiment, for a total of nine mice per group. Points, means; bars, SE. * $P < 0.05$, a significant difference from untreated, RC or MMC mice; ** $P < 0.05$, a significant difference from radiation, RC + radiation, or MMC + radiation mice. B, photographs of untreated or Bcl-2 ASO + radiation – treated mouse tumors on day 15. Temporary hair loss was observed with all radiation-treated mice.
apoptotic fraction increased in cells treated with radiation or RC plus radiation to 3.5% or 5.0%, respectively, but the largest increase was observed in cells treated with Bcl-2 ASO plus radiation at 10.7% (P < 0.05).

The presence of a sub-G_1 DNA peak/population was detected by flow cytometry, confirming the morphologic assessment of apoptosis (Fig. 3D). Similar results were obtained, with Bcl-2 ASO plus radiation increasing the apoptotic fraction to 7.40%, RC, RC plus radiation, and Bcl-2 ASO alone showed 0.90%, 3.70%, and 0.97% apoptosis, respectively.

**FITC-labeled antisense distributes largely to the tumor, liver, and kidney.** To track antisense distribution in tumor-bearing SCID mice, FITC-MMC was administered (10 mg/kg), and major organs were harvested 24 hours later. Antisense was found to distribute largely to tumor cells, hepatocytes, sinusoid endothelial cells in the liver, and kidney cortex tubular cells (Fig. 4A). When either RC or Bcl-2 ASO were injected (10 mg/kg) into SCID mice, rare (<1:1,000 cells) hepatocyte mitosis and apoptosis were observed (Fig. 4B).

**Bcl-2 ASO and radiation inhibit in vivo tumor growth.** To determine the therapeutic efficacy of the Bcl-2 ASO plus radiation combination in nasopharyngeal cancer, C666-1 xenograft tumors were established in SCID mice. When the tumor-plus-leg diameter reached 8.25 to 8.5 mm, six i.v. tail vein injections of 10 mg/kg antisense were given (one injection daily), along with two radiation treatments (2 \times 4 Gy for C666-1). Untreated, RC-treated, and MMC-treated groups had to be sacrificed after \sim 15 days due to tumor growth.

To determine the therapeutic efficacy of the Bcl-2 ASO plus radiation combination in nasopharyngeal cancer, C666-1 xenograft tumors were established in SCID mice. When the tumor-plus-leg diameter reached 8.25 to 8.5 mm, six i.v. tail vein injections of 10 mg/kg antisense were given (one injection daily), along with two radiation treatments (2 \times 4 Gy for C666-1). Untreated, RC-treated, and MMC-treated groups had to be sacrificed after \sim 15 days due to tumor growth.

**Fig. 5 Continued.** C. C666-1-bearing mice were treated with three daily injections (10 mg/kg i.v.) of antisense, and radiation was given before the second injection. Tumors were harvested for terminal deoxynucleotidyl transferase-mediated nick-end labeling staining 24 or 48 hours after the last injection. Representative results from the 24-hour group. White arrows point to examples of apoptotic cells (an enlarged example is in the inset). Bar, 20 \mu m. D. quantification of apoptotic staining. At least 1,000 cells were counted for each sample, with three mice in each group. Columns, means; bars, SD.
burden (Fig. 5A). In contrast, mice treated with Bcl-2 ASO remained alive until day 26 ($P < 0.05$). After each set of three i.v. injections, distinct tumor regression could be observed, specifically from days 5 to 6 and from days 10 to 11. Radiation extended survival until day 48. Bcl-2 ASO plus radiation extended survival until day 67, significantly

Fig. 6. Bcl-2 ASO and local radiation produce an in vivo therapeutic benefit in C15 tumors. A, C15 xenografts were established in SCID mice and randomized into the following groups: untreated, RC, MMC, Bcl-2 ASO, radiation, RC + radiation (RT), MMC + radiation, or Bcl-2 ASO + radiation. Six injections of antisense (10 mg/kg) and two doses of radiation ($2 \times 2$ Gy) were given. Three independent experiments were conducted, with three mice per group in each experiment, for a total of nine mice per group. Points, means; bars, SE. *, $P < 0.05$, a significant difference from untreated, RC or MMC mice; **, $P < 0.05$, a significant difference from radiation, RC + radiation, or MMC + radiation mice. B, C666-1 and C15 tumors have different levels of Bcl-2 family proteins. Nasopharyngeal cancer xenografts were evaluated for Bcl-2, Bcl-xL, and Bfl-1 levels using immunohistochemistry. C15 tumors have higher expression of all three evaluated Bcl-2 family proteins. White arrow points to cells with more intense staining. Bar, 20 μm.
prolonging survival time by 19 days over mice treated with radiation alone and by 52 days over untreated mice (P < 0.05). This combination treatment seemed to produce a more-than-additive therapeutic benefit in C666-1 tumors. Temporary local hair loss was consistently observed at the irradiated region for all mice treated with radiation (Fig. 5B). With Bcl-2 ASO plus radiation treatment, hair loss was more marked and occurred for a longer period of time but was reversible as some regrowth was observed.

Treated xenograft tumors display an increase in apoptosis. To determine the mode of tumor cell death in vivo, C666-1-bearing mice were first established and treated with three injections of antisense (10 mg/kg) and one dose of radiation (4 Gy, given before the second injection of antisense). Then, tumors were harvested for terminal deoxynucleotidyl transferase–mediated nick-end labeling staining 24 or 48 hours after the last injection of antisense. Bcl-2 ASO or Bcl-2 ASO plus radiation treatments increased apoptosis to 4.6% or 2.3% after 24 hours, respectively (Fig. 5C and D). After 48 hours, the rate of apoptosis was 1.6% or 2.0%, respectively. Radiation and RC plus radiation treated groups showed a minimal increase in apoptosis at the 24-hour time point.

Bcl-2 ASO and radiation inhibit C15 tumor growth in vivo. The therapeutic efficacy of the Bcl-2 ASO plus radiation...
combination was assessed in a second EBV-positive nasopharyngeal cancer model, the C15 xenograft. Mice were treated similarly to the C666-1 xenografts, but $2 \times 2$ Gy radiation was administered. In untreated C15 tumors, mice had to be sacrificed on day 11 (Fig. 6A). With Bcl-2 ASO or radiation alone, the mice survived until day 17 (a significant difference, $P < 0.05$) or day 23.5, respectively. Bcl-2 ASO plus radiation significantly extended survival time until day 31 ($P < 0.05$). Compared with the C666-1 xenograft treatment experiment, Bcl-2 ASO seemed less effective, and the Bcl-2 ASO plus radiation combination seemed to only have an additive effect.

**C666-1 and C15 xenograft tumors display different Bcl-2 family protein expression.** One possible explanation for the different responses of C666-1 and C15 xenografts to Bcl-2 ASO is that the tumors may harbor different levels of Bcl-2 and/or other antiapoptotic Bcl-2 family members. Using immunohistochemistry, it was determined that C15 tumors had higher levels of Bcl-2, Bcl-$x_L$, and Bfl-1 expression (Fig. 6B). Bcl-2 staining was observed in almost all tumor cells in both nasopharyngeal cancer models but was significantly more intense in C15 tumors. Bcl-$x_L$ was visible in C15 tumor cells but only stained minimally in C666-1 tumors. Bfl-1 staining was present in almost all tumor cells, but very intense staining was observed in 15% to 20% of C15 and in 5% to 10% of C666-1 tumor cells.

**Antisense oligonucleotides colocalize to the vasculature and display systemic tumor penetration effects.** Some systemically administered molecules have limited penetration into tumors. Thus, mice with very large (tumor-plus-leg diameter, $\sim 14$ mm) tumors were injected with FITC-MMC and Hoechst 33342 (for functional blood vessel visualization), and then their tumors were imaged using wide-field microscopy. The antisense was observed to distribute...
throughout the tumor, with at least some fluorescence evident throughout all areas of the tumor (Fig. 7A). A much more intense fluorescence was observed at the periphery of the tumor, with a lower apparent distribution observed in the center of the tumor. The antisense distribution was consistent with the high number of small blood vessels (probably capillaries) around the periphery of the tumor (Fig. 7B). At least five sections were imaged from different levels throughout the tumor, and consistent drug/blood vessel distributions were observed (data not shown).

Another potential reason for the dissimilar therapeutic responses between the C666-1 and C15 tumors might have been related to the antisense distribution. Thus, FITC-MMC and Hoechst 33342 were used to assess the antisense and blood vessel distribution in both small (tumor-plus-leg diameter, \(f\) 8.5 mm) and large (tumor-plus-leg diameter, \(f\) 12.5 mm) nasopharyngeal cancer xenografts. Both small and large C666-1 tumors had a diffuse antisense and blood vessel distribution (Fig. 7C). On the other hand, C15 tumors had large blood vessels (not capillaries) and many areas in which very little antisense could be seen. Thus, antisense seems to have a more limited distribution in C15 than in C666-1 tumors.

Retreatment of C666-1 tumors with Bcl-2 ASO plus radiation. In C666-1-harboring mice treated with Bcl-2 ASO plus radiation, the tumors began to regrow on day 18. To determine if large repopulated tumors were still susceptible to Bcl-2 ASO plus radiation, mice were retreated on day 37, when the tumor-plus-leg diameter was over 12 mm (Fig. 8). Retreatment with Bcl-2 ASO plus radiation was very effective, decreasing the diameter to \(f\) 9 mm. Moreover, mice survived for over 95 days, which was 80 days beyond that of untreated mice, 69 days more than mice treated with one cycle of Bcl-2 ASO, and 28 days more than mice treated with one cycle of Bcl-2 ASO plus radiation. Finally, these retreated tumors seemed to have plateaued in their size. Control-treated tumors could not be retreated due to their large size on day 37.

Discussion

This is the first \textit{in vivo} demonstration of the efficacy of a Bcl-2 family–targeted anticancer agent, specifically Bcl-2 ASO, in combination with radiation for cancer therapy. As shown in our two nasopharyngeal cancer models, the \textit{in vivo} tumor response seems more than additive, but this will depend on the balance of proapoptotic and antiapoptotic Bcl-2 family proteins, as well as the distribution of the ASO molecule.

Bcl-2 family proteins have been known to play essential roles in a variety of malignancies, including nasopharyngeal cancer. Several antiapoptotic Bcl-2 proteins, such as Bcl-2, Bcl-x\(_L\), and Bfl-1, have been found to be essential in the development and progression of nasopharyngeal cancer (31, 33, 34). Moreover, radiation is the primary curative treatment modality for nasopharyngeal cancer, and Bcl-2 proteins can contribute to radioresistance (25–27). Despite this knowledge, \textit{in vivo} therapeutic studies have not focused on the anticancer combination of mitochondrial-mediated apoptosis targeting and radiation. In this current study, we show that Bcl-2 ASO effectively decreased Bcl-2 mRNA and protein levels and achieved a modest therapeutic effect as a single agent \textit{in vitro}. In combination with radiation, however, Bcl-2 ASO decreased the fraction of viable cells to 0.40 after 7 days and increased the apoptotic fraction to \(f\) 10.7% (Fig. 3). Bcl-2 ASO may be functioning \textit{in vitro} as a radiosensitizer, and this is corroborated by a recent report using a dual Bcl-2 and Bcl-x\(_L\) antisense oligonucleotide (40).
Targeting the mitochondrial-mediated apoptosis pathway can facilitate the cytotoxic effects of radiation or chemotherapy (13, 40). In addition, pursuing an apoptotic therapeutic strategy targets the tumor cells for elimination, thereby preventing such cells from mutating into a more malignant variant. In contrast, genotoxic agents, such as radiation or chemotherapy, may induce further genetic instability without cell death, in the presence of an apoptotic block (12). Finally, targeting apoptosis, unlike necrosis, can also facilitate cancer cell death without inducing inflammation or damage to the surrounding normal tissues (12).

Bcl-2 ASO as a single agent seemed more potent in vivo than predicted based on the in vitro data, extending the survival of C666-1 or C15-harboring mice by 11 or 6 days, respectively. It is well known that “naked” antisense oligonucleotides are delivered into cells with much higher efficiencies in vivo than in vitro (41, 42). Another possible reason for the greater effect observed in vivo may be related to the three-dimensional tumor environment, which is “stressful,” with hypoxia, nutrient deprivation, absence of growth-stimulating signals, presence of growth-inhibitory signals, surrounding tissue barriers, and host immune responses (43, 44). These microenvironmental stresses may result in the tumor cells being more dependent upon the antiapoptotic Bcl-2 family members for survival; hence, targeting this pathway using Bcl-2 ASO would render these cells more vulnerable to apoptosis. Logically, Bcl-2 ASO efficacy seems to be a function of the intrinsic antiapoptotic signals within the tumor, given that the C15-bearing mice seemed more resistant compared with the C666-1 tumors. Immunohistochemistry corroborates that there was a greater antiapoptotic signal in the C15 tumors, shown by higher expression of Bcl-2, Bcl-xL, and Bfl-1 (Fig. 6B). This suggests that the screening of critical antiapoptotic signals can be used to determine optimal patient selection for Bcl-2 ASO therapy. When Bcl-2 ASO is combined with radiation in vivo, this interaction seemed more than additive, extending the survival of C666-1-bearing mice by 52 days compared with untreated mice (Fig. 5A and B). One mechanism for this interaction is apoptosis, which was observed to occur with both the Bcl-2 ASO alone and when combined with radiation, as indicated by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining (Fig. 5C and D). The in vivo apoptotic fraction is often low and is considered a measurement of the rate of apoptosis and not total apoptosis. An even more impressive result is the repeat treatment of the C666-1 tumors with this combined approach, which extended survival for >80 days (Fig. 8). The increased susceptibility of C666-1 tumors to a second round of treatment may suggest that the partially damaged cells from the first round are now being eliminated, providing the rationale for cycles of repeated treatments in the clinic.

Similar to clinical trial data (45), Bcl-2 ASO therapy seemed well tolerated. Rare hepatocyte mitoses and apoptosis were observed with either RC or Bcl-2 ASO treated mice, suggesting that this may be a nonspecific response to the phosphorothioate backbone (Bcl-2 ASO does not inhibit Mus musculus Bcl-2). This would be consistent with the observation of transient liver function abnormalities in some G3139 clinical trial patients (45). We did observe a more pronounced alopecia at the irradiated site with the Bcl-2 ASO-radiation combination than with any other treatment group. This hair loss was reversible; nevertheless, this underscores the potential for Bcl-2 ASO to radiosensitize both normal and tumor tissue. Close monitoring will be required when such clinical trials are being conducted.

This current study is also the first to report the evaluation of antisense distribution as a function of tumor vasculature. Drug distribution in tumors is often a challenge for systemic therapies, whether using small molecules, chemotherapies, or antibodies (46). The antisense distribution is clearly different between the C666-1 and C15 tumors (Fig. 7), wherein in the former, antisense seemed to colocalize with the vasculature, being significantly more visible at the tumor periphery. However, in the latter C15 tumors, particularly in the larger-sized tumors, there seems to be a physical separation between the antisense and the tumor vasculature. The reason behind these discrepancies is unclear, but we are currently evaluating this further using other human xenograft tumor models.

Antisense oligonucleotides have gained popularity as rationally designed therapeutics. Recently, the antisense field has experienced dampened enthusiasm due to G3139’s inability to achieve Food and Drug Administration approval after disappointing results from phase III clinical trials in advanced melanoma and multiple myeloma (47, 48). However, in a recently conducted chronic lymphocytic leukemia phase III trial involving G3139 and fludarabine/cyclophosphamide, the proportion of patients achieving a major response was significantly increased (49). These trials underscore the importance for developing methods that can identify the susceptibility of individual tumors for different therapies. For example, the Bcl-2 ASO/radiation combination may be less effective in prostate cancers, which may preferentially undergo terminal growth arrest rather than apoptosis after radiation (50). Differences in tumor response may be observed even within the same tumor type, as shown by our C666-1 and C15 xenograft experiments. The currently used phosphorothioate antisense molecules, such as G3139, represent the first generation of chemical oligonucleotide modifications. Newer 2′O-methyl and locked nucleic acid modifications, as well as the use of chimeric antisense molecules, provide for increased target binding affinity and improved pharmacokinetics (24, 41). These newer modifications give reason for optimism in the antisense field.

Bcl-2 ASO is one of many available agents that can target the Bcl-2 family. When combined with radiation, such agents show radiosensitizing tumoricidal effects in vivo. This study provides insight into the importance of drug biodistribution and Bcl-2 family member expression, which could affect the efficacy of this very promising combinatorial therapeutic strategy for human cancers.

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