Eradication of Human Non-Hodgkin’s Lymphoma in SCID Mice by BCL-2 Antisense Oligonucleotides Combined with Low-Dose Cyclophosphamide

Richard J. Klasa, Marcel B. Bally, Rebecca Ng, James H. Goldie, Randy D. Gascoyne, and Frances M. P. Wong

Divisions of Medical Oncology [R. J. K.] and Pathology [R. D. G.] and Department of Advanced Therapeutics [R. J. K., M. B. B., R. N., J. H. G., F. M. P. W.], British Columbia Cancer Agency, Vancouver BC V5Z 4E6, and Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver V6T 2B5 [M. B. B., F. M. P. W.], British Columbia, Canada

ABSTRACT

Cancers overexpressing Bcl-2 protein, which prevents programmed cell death (apoptosis), are less sensitive to stresses that produce cellular damage, including chemotherapy. If the level of Bcl-2 protein can be reduced sufficiently using antisense oligonucleotides (ASOs) targeting the gene message, then cytotoxic agents may be rendered more effective in eliminating disease and increasing cure rate. Preclinical studies in SCID mice bearing Bcl-2 overexpressing systemic human B-cell lymphoma (DoHH2) were undertaken to support development of a clinical trial. These data confirm that a combination of an ASO (5 mg/kg) targeting bcl-2 and a low dose of cyclophosphamide (35 mg/kg) was an effective strategy, leading to the eradication of the DoHH2 cells in vivo and cure of the animals. When mice deficient in natural killer cell activity were treated with an ASO, similar results were observed, suggesting that ASO stimulation of the host immune system was not a significant factor in elimination of lymphoma cells. These studies indicate that therapeutic strategies involving the use of an ASO targeting bcl-2 in combination with a cytotoxic agent may improve clinical outcomes.

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INTRODUCTION

ASOs are short sequences of nucleotides complementary to coding regions of a gene of interest (1, 2). Advances in our understanding of the biological activity of ASOs have allowed development of these molecules as therapeutic agents (3, 4). Anticancer gene-targeted therapy based on reducing the level of a putative oncoprotein can be achieved by ASOs designed to bind and facilitate degradation of the mRNA that codes for that specific protein. More specifically, appropriately designed synthetic ASOs bind RNA molecules in a sequence-specific manner and either directly impair interaction with factors in the cytoplasm that are required for translation into a protein or recruit endogenous RNaseH to cleave the RNA backbone (5). Regardless of the mechanism of activity, ASOs have the potential to reduce a target protein overexpressed by tumorigenic cells.

Although elegant experiments have been carried out to define the mechanisms by which ASOs can specifically abrogate gene function, recent investigations have raised some puzzling questions regarding what factors govern their biological activity. First, ASOs can be very potent immune stimulators, by virtue of their unmethylated CpG motifs within the context of certain flanking sequences. Therapeutic activity can be attributed to systemic immune effects rather than to specific ASO/mRNA interactions (6–9). The development of control ODNs, containing similar backbone, codon length, and CpG motifs, has been useful to provide evidence for biological action linked to a specific ODN sequence. Second, for ASOs to be active delivery to the inside of a target cell must be achieved efficiently. In tissue culture, for example, this requires association with a delivery system such as cationic lipids (10, 11). In vivo, however, ASOs are active when given in free form (12–14). Although studies assessing the mechanism of ASO activity in vivo are critically important to the further advancement of this technology, our primary concern remains the identification of agents with proven therapeutic activity in the clinical setting for disseminated systemic malignant disease.

An 18 mer phosphorothioated oligonucleotide, G3139, directed against the first six codons of the open reading frame of the bcl-2 gene message has been developed by Genta Inc. (Lexington, MA) and is used in our studies. The target protein, Bcl-2, is an antiapoptotic member of a large family of genes involved in the regulation of programmed cell death (15, 16).

3 The abbreviations used are: ASO, antisense oligonucleotide; ODN, oligodeoxynucleotide; RPO, reverse-polarity ODN; MMO, mismatch ODN; MW, molecular weight; LUV, large unilamellar vesicle; DOPE, dioleoyl-sn-glycero-3-phosphoethanolamine; CPA, cyclophosphamide; QD, every day treatment; QOD, every other day treatment; SCID, severe combined immunodeficient; NHL, non-Hodgkin’s lymphoma.
Studies of G3139 on the Bcl-2-overexpressing lymphoma cell lines DoHH2 and SU-DHL-4 in vitro have shown down-regulation of message and resultant decrease in protein expression (14). Tumor xenograft models in SCID mice using G3139 alone have demonstrated therapeutic activity that is specific when compared with control antisense sequences (3, 14). Pharmacokinetic as well as toxicity studies have been performed identifying a dose range with a good therapeutic index (17, 18). Used as a single agent in a Phase I study in patients with relapsed NHL G3139 has been reported to modify Bcl-2 levels in clinical samples of lymphoid cells using doses that were also associated with objective responses (19).

More recently, in vitro experiments have suggested that Bcl-2 plays a major role in the response of malignant cells to a variety of stresses that produce cellular damage, including chemotherapy (20–22). Malignant cell lines transfected with the bcl-2 gene, with resultant overexpression of the protein product, demonstrate increased resistance to various chemotherapeutic agents (23–26). Additionally, cell lines overexpressing Bcl-2 are rendered more sensitive to killing by chemotherapeutic agents, either with introduction of ASOs directed at the antisense sequence (27, 28). This resultant chemosensitization has been correlated with down-regulation of Bcl-2 expression.

The studies reported here assess the in vivo therapeutic potential of combining ASOs targeting bcl-2 with a low dose of a cytotoxic agent commonly used in the treatment of lymphoma. It is believed that this chemosensitizing effect correlates with specific ASO-mediated down-regulation of bcl-2 mRNA message and, subsequently, Bcl-2 protein. This is supported by PCR data and immunohistochemical evaluation of bone marrow obtained from DoHH2-bearing mice. Furthermore, results in perforin-deficient mice demonstrate that elimination of lymphoma cells is not a result of natural killer cell function.

MATERIALS AND METHODS

G3139 (ASO), G3622 (RPO), and G4126 (MMO) are fully phosphorothioated, linear, single-stranded 18 mer oligodeoxyri-bonucleotides (Genta Inc.). G3139 (sequence, 5'-tctccagctg-gcccat; MW, 5764 g/mol) is complementary to the first six codons of the human bcl-2 open reading frame. G3622 (sequence, 5'-tacgccgtgccacctt; MW, 5764 g/mol) is the reverse-polarity sense control of G3139, whereas G4126 (sequence, 5'-tctccagctgccat; MW, 5683 g/mol) has a two-base mismatch to G3139.

DoHH2 is an EBV-negative human B-cell NHL cell line that carries the t(14;18) and has been extensively studied (29). Following the initial description, it was found that a complex translocation involving chromosomes 8, 14, and 18 resulted in a derivative 8, which contained both the c-myc and bcl-2 oncogenes juxtaposed to the immunoglobulin heavy chain region with resultant overexpression of both protein products (30). DoHH2 cells were grown in RPMI media containing 5% horse serum and 5% fetal bovine serum with 10 units/mL and 0.1 mg/mL penicillin/streptomycin and 2 mM L-glutamine. Cells were used for experiments between passages 8 and 17.

Preparation of LUV/ODN Complexes. Bcl-2 expression could not be down-regulated with ASOs in the absence of a synthetic oligonucleotide delivery agent, as demonstrated by our results and others (11, 31). For this reason, cationic liposomes consisting of DODAC and DOPE were used to enhance ASO intracellular delivery. ODNs in sterile water were diluted to a 5 μM concentration. DODAC:DOPE LUVs (Inex Pharmaceuticals Corp., Vancouver, British Columbia) were diluted in sterile water to a concentration of 4.6 mM total lipid to an equal volume to the ODNs solution. The LUVs had a mean diameter of 80 ± 20 nm before the formation of LUV/ODN complexes, as determined by quasi-elastic light scattering (Nicomp submicron particle sizer operating with an argon laser at 675.8 nm). ODNs were combined with the diluted LUV solution in one addition at 4°C, and LUV/ODN complexes immediately formed as indicated by a change in sample turbidity. Final charge ratio of ODN:cationic lipids was 1.3:1 (+/-), and final ODN concentration was 2.5 μM. After incubation for at least 30 min, LUV/ODN complexes (200–400 nm using quasi-elastic light scattering) were added to DoHH2 cells.

In vitro Delivery Assays. DoHH2 cells were plated at a concentration of 1 × 10⁶ cells/ml in 6-well tissue culture-treated plates. All cells were plated in RPMI media in the absence of serum. Cells were incubated for 4 h at 37°C and, subsequently, RPMI media was replaced with 5% fetal bovine and 5% horse serum-containing media. After 72 h, cell lysates were obtained using 0.15% SDS in 50 mM Tris (pH 8.0) and the expression of Bcl-2 protein was determined using Western blot analysis.

Western Blot. Western blot analysis was completed as described previously (32). Protein concentrations in the obtained cell lysates were determined by absorbance at 280 nm. Approximately 75–100 μg of protein were separated using polyacrylamide gel electrophoresis. Western transfer was completed on nitrocellulose membrane and blocked with 5% skim milk. Bcl-2 primary antibody was diluted to 12 ng/ml, and β-actin primary antibody (DAKO, Carpenteria, CA) was diluted to 10 ng/ml in Tris-buffered saline containing 1% skim milk and 0.1% Tween 20 for 1 h. Antibody binding was detected via chemiluminescence using enhanced chemiluminescence reagent from Amersham (Baie D’Urfé, Quebec, Canada). Membrane was exposed to X-Omat Kodak film (Mandel Scientific, Guelph, Ontario, Canada) for 30 s-1 min.

In vivo Model. Male SCID/Rag-2 mice used for these studies were obtained from a breeding colony at our institution when they were 6–9 weeks of age, weighed 22 g and were maintained in a pathogen-free environment. Viable DoHH2 cells (5 × 10⁶ cells in 200 μl) were injected i.v. via the tail vein of each animal, and disease was allowed to establish for 4 days. Cohorts of at least three animals were then treated in the following groups: (a) untreated control (injected with saline); (b) CPA (Carter-Horner Inc., Mississauga, Ontario, Canada) at 15, 35, 75, or 150 mg/kg i.p. on days 4, 8, and 12; (c) ASO, RPO, or MMO at 5.0 or 12.5 mg/kg QD or QOD i.p. for 14 treatments; and (d) ASO, RPO, or MMO QD or QOD i.p. for 14 treatments in combination with CPA i.p. on days 4, 8, and 12. Animals were assessed for illness by nonbiased technicians and terminated at signs of illness including, but not limited to,
paralysis in the hind region, scruffy coat, lethargy, weight loss of >20%, or if they survived past 90 days.

Pfp/Rag-2 male mice were obtained from Taconic (Germantown, NY). These are SCID/Rag-2 mice deficient in perforin synthesis. Although natural killer cells are present in these animals, they are not capable of lysing cells. Animals were used within 2 weeks of arrival at our facility. Cohorts of five or six animals received injections of 5 × 10^6 DoHH2 cells in 200 µl i.v. via the tail vein. On day 4 after tumor cell inoculation animals began treatment with ASO alone (5 mg/kg for 14 treatments QOD) or in combination with CPA (35 mg/kg; days 4, 8, and 12) injected i.p. Animals were assessed and terminated at signs of illness, as described above.

**Immunohistochemistry.** Microscopic sections of a femur for controls or treated animals were processed routinely for H&E staining and reviewed by an experienced hematopathologist (R.D.G.). Paraffin section immunohistochemistry was performed to analyze Bcl-2 expression, as described previously (33).

**Molecular Genetics.** High MW DNA was extracted from all tissue specimens and the DoHH2 cell line with an automated DNA extractor (Applied Biosystems Model 341; Perkin-Elmer Corp., Foster City, CA). PCR for immunoglobulin heavy chain and the presence of a bcl-2 (mbr) rearrangement was performed, as described previously (34). The presence of amplifiable DNA in the reactions was confirmed in all cases by a parallel amplification of a 155-bp and a 510-bp segment of the p53 and β-globin genes, respectively.

**Statistical Analysis.** Cohorts were determined by combining several experiments together. Survival analysis data were evaluated with censored regression, with median survival times determined using the Cox’s f-test. This was done because several of the treated groups consisted of long-term survivors (>90 days). For these groups median survival times were estimated based on the assumption that the surviving animals died on day 91. Groups with >50% 90-day median survival were labeled not obtained (NO). Cohorts with less than three uncensored data points were labeled not determined (ND) because estimated parameters were not reliable. Survival curves were computed using the Kaplan-Meier method. Treatment groups were subsequently analyzed using Statistica software and compared using a two-sample log-rank test. p-values were derived from the log-rank test comparing two samples and were reported if p < 0.05 or were determined to be not significant (NS).

**RESULTS**

Confirmation that the DoHH2 cell line expresses the Bcl-2 protein and that this expression can be down-regulated in vitro with bcl-2 antisense oligonucleotides is demonstrated by Western blot analysis (Fig. 1). Cells were treated with either ASO or two control ODNs consisting of a full-length reverse-polarity sense sequence (RPO) as well as a two-base mismatch sequence to G3139 (MMO). Of note, these control oligonucleotides contain similar backbone, codon length, and CpG motifs as G3139. ODNs were delivered in free form or with cationic liposomes (DODAC:DOPE LUVs) as a carrier. The concentration of ODNs used (2.5 µM) was the minimum concentration, following complexation with cationic lipids, that clearly demonstrated down-regulation of Bcl-2 protein with minimal toxicity (data not shown). The cell toxicity that was observed following ASO addition was not due to associated lipids. DoHH2 cells expressed the Bcl-2 protein as indicated by the 26 kDa band, whereas delivery of LUV/ASO complexes specifically down-regulated Bcl-2 protein expression. This effect was sequence specific because it was not seen with the control oligonucleotides, where results were indistinguishable from untreated cells. This antisense effect correlated with a small decrease in cell number and could only be obtained when using a formulation method involving ASO complexation with cationic liposomes to engender ASO delivery.

**In vivo** studies evaluated therapeutic activity in a SCID-human xenograft model where DoHH2 cells, a human B-cell lymphoma, were injected i.v. Mice that received inoculations of 5 × 10^6 cells i.v. were terminated as a consequence of tumor progression within 35 days if left untreated. Cell titration data (not shown) indicate that survival time is cell concentration dependent, as reported previously (35). For example, our data show that a two-log reduction in cell number (i.e. injection of 5 × 10^6 cells) results in 100% death by day 79. Untreated, control animals exhibited paralysis, disorientation, and lethargy, among other symptoms, whereas gross observation during ne-
cropsy showed s.c. masses as well as enlarged retroperitoneal and perigastric lymph nodes in some, but not all, animals. ODN-treated animals, regardless of whether the ODN was antisense or controls, presented enlarged spleens (at least two times larger than untreated animals) consistent with the mitogenic activity observed for ODNs in toxicology studies with mice. Fig. 2A shows the representative section through a femur in untreated control animals, and Fig. 2B shows the corresponding Bcl-2 immunoperoxidase staining. Extensive infiltration with Bcl-2-expressing human lymphoma is observed with complete replacement of murine marrow, and Bcl-2 immunoperoxidase staining is strikingly positive in control femur and lymph nodes. Similarly, bcl-2 and immunoglobulin heavy chain PCR demonstrated a single dominant band in the untreated control animals of approximately 100 bp and 365 bp, respectively (Fig. 2E, Lane 2).

Treatment of DoHH2-bearing SCID mice was initiated 4 days after tumor cell injection, with both ODNs and CPA always given via i.p. injection. The animals were treated with ODNs alone, CPA alone, or ODNs together with CPA. The experiments, summarized in Tables 1 and 2 and Fig. 3, assessed a range of doses of both ODNs and CPA. Two dosing schedules of ODNs were evaluated, each schedule consisting of 14 total i.p. injections, one given over a period of 18 days and the other over a period of 28 days. Dose scheduling was based on previous reports that phosphorothioate ODNs have elimination half-lives of ~12–72 h (17). CPA was given consistently i.p. 4, 8, and 12 days after tumor cell injection. Untreated control animals, as well as the RPO- and MMO-treated control groups, were terminated or died with progressive tumor at a median of 33–37 days after tumor cell inoculation (see Table 1). No long-term survivors were observed in these groups. CPA-treated
animals (Table 2) showed a dose-response correlation with no effect (median survival time, 36 days) seen at a dose of 15 mg/kg and modest effect (median survival time, 47 days) at 35 mg/kg. The DoHH2-bearing animals could be treated at higher dose (75 and 150 mg/kg), and at these doses 100% survival of animals (Table 2) showed a dose-response correlation with no effect (median survival time, 36 days) seen at a dose of 15 mg/kg and modest effect (median survival time, 47 days) at 35 mg/kg. The DoHH2-bearing animals could be treated at higher dose of CPA (75 and 150 mg/kg), and at these doses 100% long-term (>90 days) survival was observed. Therefore, the two lower doses (15 and 35 mg/kg) were selected for studies evaluating combinations with ASO.

Free ASOs were given at doses of 2.5, 5, and 12.5 mg/kg/day for 14 doses QOD starting 4 days after cell inoculation (Table 1). In comparison with untreated animals, those treated with even the lowest dose of ASO showed a significant (p < 0.000001) increase, when compared with control, in median survival from 33 days to at least 62 days. Dose-dependent increases in therapeutic efficacy were most easily observed by monitoring the percentage of long-term (>90 days) survivors after treatment. As the dose increased from 2.5 to 5 to 12.5 mg/kg/day, the number of long-term survivors increased from 17% to 48% to 58%, respectively. The differences in response between the 5 and 12.5 mg/kg dose levels were not significant. If the dosing schedule was changed to QD injections there was a slight, but not significant, decrease in therapeutic activity noted. The QD schedule at 12.5 mg/kg gave a long-term survival rate of 44% and a median survival time of 85 days, whereas the QOD schedule resulted in a long-term survival rate of 58%. There was no statistically significant difference between results when animals were treated QD or QOD.

It is worth noting that pathological evaluation at necropsy was completed in all long-term survivors and those animals showed no evidence of tumor either on gross inspection or on histological examination of tissues. Molecular studies, designed to detect the human bcl-2 gene by PCR, failed to detect residual disease in these mice (Fig. 2E, Lane 4). In contrast, those animals that were terminated during the course of these studies because of disease progression, whether in an ASO or a CPA alone treatment group, were found to have enlarged lymph nodes and tumor nodules consistent with those of untreated mice, as well as molecular evidence of disease as demonstrated by PCR (Fig. 2E, Lane 2). Immunohistochemistry was used to confirm the presence of bcl-2-expressing human lymphoma in the lymph nodes, spleen, and femoral bone marrow. The findings established that the progression of the human lymphoma was the cause of death in these animals. It was concluded on the basis of these results that death during the 90-day time course was always associated with progression of the DoHH2 tumor and that animals surviving beyond 90 days were free of any residual disease. Fig. 2C and D, shows a representative femoral bone marrow of a combination treated animal (5.0 mg/kg ASO and 35.0 mg/kg CPA) that survived past 90 days. It was observed that there was no human lymphoma cell infiltrate (Fig. 2C) and Bcl-2 staining was negative (Fig. 2D). Regardless, immunohistochemistry was routinely performed on samples from all animals that survived or were terminated as a consequence of tumor development. Some animals were maintained in the vivarium for time periods in excess of 120 days. In addition, selected mice that survived as a consequence of treatment received reinoculations of 5 × 10^6 DoHH2 cells to assess whether the SCID animals had developed immune resistance to tumor cell challenge (results not shown). These animals died within 35 days, typical of control animals, and exhibited Bcl-2-expressing human lymphoma in spleen, lymph nodes, bone marrow, and elsewhere.

It is clear, as assessed by immunohistochemistry and PCR, that ASO treatment of lymphoma-bearing mice eliminated Bcl-2 expression associated with the DoHH2 cell. This is most likely a consequence of eradication of the tumor cells and may not be directly linked to down-regulation of bcl-2 expression. It is possible that the therapeutic activity is linked to ASO-induced immune stimulation and tumor cell loss by activated killer cells. Reports from many investigators have suggested that the mechanism of action for ASOs may be, in fact, a result of their potency as immune stimulators rather than specific down-regulation of protein expression (6–9). Although the SCID/Rag-2 mice used in these studies were deficient in B- and T-cell maturation, we further examined the effect of ASOs on immunostimulation by treating lymphoma-bearing mice deficient also in perforin production (Fig. 4 and Table 3). Plp/Rag-2 mice received injections of DoHH2 i.v. and were treated with ASO at 5 mg/kg alone for 14 treatments (QOD) or in combination with CPA (35 mg/kg; three treatments, days 4, 8, and 12). Median survival of animals treated with ASOs increased from 27 days (control) to 37 days (p < 0.01). When mice were treated with CPA in addition to ASO, median survival times increased to 61 days when compared with animals treated with ASO only (p < 0.01). Furthermore, 17% of the animals in the combination-treated cohort survived past 90 days.

The aim of these studies was to determine whether there was a clear therapeutic advantage provided when ASOs targeting the bcl-2 gene product were used in combination with CPA. Because the experimental model was sensitive to the activity of each agent alone, doses where minimal activity was defined

### Table 1 Effect of oligonucleotide treatment on SCID/Rag-2 male mice that received inoculations of 5 × 10^6 DoHH2 cells i.v. Animals were treated with None (saline only), RPO, MMO, or ASO for 14 treatments i.p.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>n</th>
<th>Median survival</th>
<th>% 90-day survival</th>
<th>p*</th>
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* p-values were derived from using the log-rank test comparing each group versus control.

NA, not applicable; QOD, every other day treatment, animals were treated alternate days, including weekends; QD, every day treatment, animals were treated every day either 14 days consecutively or excluding weekends; NO, not obtained, if greater than 50% of the animals survived past 90 days then median survival could not be obtained; NS, not significant, p > 0.05 when compared with control animals.

*% 

It is worth noting that pathological evaluation at necropsy was completed in all long-term survivors and those animals showed no evidence of tumor either on gross inspection or on histological examination of tissues. Molecular studies, designed to detect the human bcl-2 gene by PCR, failed to detect residual
(CPA at 15 and 35 mg/kg and ASOs at 2.5 and 5 mg/kg injection) were used in the combination studies. These studies have been summarized in Table 2 and Fig. 3B. The benefits achieved by using the bcl-2 ASOs in combination with CPA are best illustrated at the lowest doses. In the absence of ASOs, a dose of CPA at 15 mg/kg resulted in no measurable increase in the life span of mice bearing the DoHH2 tumors. Alternatively, ASO given alone at a dose of 2.5 mg/kg/injection increased median survival to 62 days, and one of six animals survived beyond 90 days. In combination, the median survival times were 72 and 84 days when treated with CPA at 15 mg/kg and ASO at 2.5 mg/kg and 5 mg/kg, respectively. Both of these treatment groups exhibited long-term survival rates of 50% or more. A similar pattern was also seen in animals treated at the 35 mg/kg dose of CPA. When animals were treated with 35 mg/kg CPA or ASO at 5 mg/kg/injection, they exhibited median survival times of 47 and 79 days, respectively, with no long-term survivors for CPA and 48% for ASO. When treated with both drugs the median survival times could not be determined because long-term survival exceeded 60% (range, 61–100%, depending on cohort), regardless of whether the ASO dose was 2.5 or 5 mg/kg/injection. CPA administered in combination with the

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**Table 2**  Treatment of SCID/Rag-2 male mice that received inoculations of $5 \times 10^6$ DoHH2 cells i.v. Animals were treated with None (saline only), CPA alone, or in combination with RPO, MMO, or ASO. CPA was injected i.p. on days 4, 8, and 12 after tumor inoculation, and ODNs were given 14 treatments total i.p.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>n</th>
<th>Median survival</th>
<th>% 90-day survival</th>
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</table>

* P's were derived from using the log-rank test comparing each group versus the corresponding CPA concentration. CPA only treated animals were compared with the control group.

b NA, not applicable; QOD, every other day treatment, animals were treated alternate days, including weekends; QD, every day treatment, animals were treated every day either 14 days consecutively or excluding weekends; NO, not obtained, if greater than 50% of the animals survived past 90 days then median survival could not be obtained.

NS, not significant. p > 0.05 when compared with control.

C Not significant. p > 0.05 when compared to the corresponding CPA alone concentration.

D Not determined. There were less than three uncensored samples, and estimated parameters were not reliable.

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**Fig. 3** Survival curves of SCID/Rag-2 male mice that received inoculations of $5 \times 10^6$ DoHH2 cells. A, treatment groups of 5 mg/kg ODN alone (injected i.p. QOD for 14 treatments). B, treatment groups with 35 mg/kg CPA (injected i.p. on days 4, 8, and 12 after tumor cell inoculation) and 5 mg/kg ODN (injected i.p. QOD for 14 treatments). Groups of animals were control ( ), ASO ( ), RPO (△), MMO (▲), and CPA only ( ).
Table 3  Treatment of pfp/Rag-2 male mice that received inoculations of $5 \times 10^6$ DoHH2 cells i.v. Animals were treated with None (saline only), ASO only (5 mg/kg injected i.p. QOD for 14 treatments beginning day 4), or in combination with CPA (injected i.p. on days 4, 8, and 12)  

<table>
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<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>n</th>
<th>Median survival</th>
<th>% 90-day survival</th>
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<td>NA</td>
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<td>QOD</td>
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<td>ASO + CPA</td>
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<td>QOD + 4,8,12</td>
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<td>17</td>
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</table>

* p-values were derived from using the log-rank test comparing animals treated with ASO alone versus control and ASO + CPA versus ASO alone.

NA, not applicable; QOD, every other day treatment, animals were treated alternate days, including weekends.

Fig. 4  Survival curves of pfp/Rag-2 mice that received inoculations of $5 \times 10^6$ DoHH2 cells i.v. Cohorts of mice were control (■), treated with 5 mg/kg ASO i.p. (●), or treated with 5 mg/kg ASO and 35 mg/kg CPA i.p. (▲). ASO treatment began on day 4 and continued QOD for 14 treatments, and CPA treatments were on days 4, 8, and 12.

day and 53 days, respectively, and this was not significantly different than the activity observed for CPA given alone (median survival of 47 days). It is worth noting that long-term survival increased significantly when CPA was combined with ASOs given by the less effective QD dosing schedule. As summarized in Table 2, mice treated with ASO at 5 and 12.5 mg/kg/injection (QD) in combination with a 35-mg/kg dose of CPA exhibited long-term survival rates of 100% and 83%.

**DISCUSSION**

These preclinical in vitro and in vivo studies have attempted to model the behavior of human lymphoma using the DoHH2 cells implanted i.v. in SCID mice. This cell line carries the t(14;18) translocation associated with Bcl-2-overexpressing lymphomas (29). Chemotherapy alone in a moderate dose range did have an impact on the disease in prolonging median survival modestly, but with no long-term survivors. Treatment with G3139 alone resulted in some long-term disease-free survivors. However, when ASO was combined with low-dose CPA the majority of animals were long-term disease-free survivors with no histological or molecular evidence of persistence of lymphoma. We noted that animals treated with ODNs, whether alone or in combination, exhibited splenomegaly, which correlated with dose. This effect did not seem to cause distress in the animals, and no evidence of ASO-related toxicity was observed. This splenic hyperplasia may be specific to murine species, because it was not observed in primates or after clinical therapy with ASO (19).

Low-dose chemotherapy with an ASO that is directed at bcl-2 has the potential to lower the amount of antineoplastic agent required to eliminate disease and, therefore, reduce the associated toxicity. It is important to note that down-regulation of the specific antisense target protein need only be transient to minimize the growth advantage of the tumor cell such that the effect of chemotherapy is maximal. We have demonstrated that G3139 shows specific down-regulation of Bcl-2 and enhances the activity of low doses of CPA in this model and in perforin-deficient mice. When cured SCID/Rag-2 mice were challenged with DoHH2 cells, all animals exhibited symptoms of Bcl-2-expressing lymphoma and died within 35 days, similar to control. This would indicate that ASO treatment did not stimulate a lasting immune response. Furthermore, the studies in the pfp/Rag-2 mice strongly suggest that immunostimulation is not a factor in eradication of lymphoma cells in vivo. Rather, specific ASO/mRNA interactions leading to down-regulation of Bcl-2 protein may, in fact, prime the tumor cells for death pathways (i.e., apoptosis), leading to chemosensitization. Follicular lymphomas overexpress Bcl-2 as a survival advantage, and, in many instances, this overexpression is the sole determinant keeping at least a percentage of the malignant cells from going down the apoptotic pathway. If the apoptotic barrier is relieved, these cells appear sufficiently damaged for death.

Our studies conclusively demonstrate that combination treatment with CPA and ASO renders SCID/Rag-2 mice curable of human lymphoma in a large fraction of the animals. The interaction between the two agents shows dose-response correlations (Tables 1 and 2). For both doses of CPA, increasing the dose of ASO from 2.5 to 5 mg/kg resulted in longer median survivals and an overall increase in long-term survivors. A rather striking result was achieved when a completely ineffective dose of CPA (15 mg/kg; median survival 36 days and no long-term survivors) was combined with a modestly effective dose of ASO (2.5 mg/kg: 61 day median survival and 16% long-term survivors) to produce a 72-day median survival and 50% long-term survivors. These results suggest that chemotherapy at very modest doses could be made much more effective with use of ASOs without increasing the toxicity to normal tissues. Such an increase in the efficacy of currently available chemotherapeutic agents could significantly alter the prognosis of a large number of modestly to moderately sensitive human
tumors, resulting in improved clinical outcomes or increasing the potential for cure.

The model has direct relevance to the clinical situation faced in NHL where patients typically present with what appears to be a chemotherapy-sensitive tumor at diagnosis that regresses only to recur within months to years after treatment. Clinically, the prevalence of Bcl-2 overexpression in NHL is high, consisting of 90% in follicular and mantle cell histologies and 50% of diffuse large cell disease. The DoHH2 cell line was derived from a follicular lymphoma carrying a t(14;18) that results in constitutive bcl-2 gene overexpression. The aggressive nature of the disease in this model is, however, more suggestive of a transformation to a higher-grade histology, a common event in follicular lymphoma. Indeed, a recent reexplanation of the molecular and cytogenetic features of the cell line, using more sensitive detection techniques, has revealed a second translocation involving the c-myc oncogene with a resultant derivative chromosome 8 carrying t(8;14;18) (30). We have just recently described this clinical phenomenon of double translocation and constitutive overexpression of both bcl-2 and c-myc in a subset of patients with small noncleaved cell (Burkitt-like) lymphoma, which represents a very aggressive form of the disease (36).

The data presented here are the first to address the potential role of ASOs directed at the bcl-2 gene message in enhancing the therapeutic efficacy of a cytotoxic agent in NHL. In a model of human melanoma implanted s.c. in SCID mice, Jansen et al. (10) have shown chemosensitization to dacarbazine (DTIC) with ASOs directed at bcl-2, resulting in reduced tumor volumes. The present study, however, is the first example of successfully using this strategy to increase the actual cure rate of any systemically distributed metastatic malignancy in a xenograft model. Moreover, the data suggest that improved clinical outcomes could be achieved with standard, or even lower, doses of anticancer drugs when combined with ASOs, potentially impacting overall clinical tolerance and costs of care. As a single agent, G3139 has entered clinical testing and promising initial results have been published, with a number of responses seen, in addition to down-regulation of Bcl-2 protein in clinical samples from patients treated for NHL (19). Based directly on our results, we have initiated Phase I/II clinical trials of chemotherapy in combination with G3139 in patients with NHL.

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REFERENCES
