Enhanced efficacy of therapy with antisense BCL-2 oligonucleotides plus anti-CD20 monoclonal antibody in scid mouse/human lymphoma xenografts

Mitchell R. Smith, Fang Jin, and Indira Joshi
Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Abstract
Monoclonal anti-CD20 antibody (rituximab) is active, but not curative, therapy for B-cell non-Hodgkin’s lymphoma. BCL-2 is an antiapoptotic protein whose expression is dysregulated in most indolent B-cell malignancies. Antisense oligonucleotides (AS-ODNs) that down-regulate BCL-2 expression induce apoptosis and chemosensitize B-cell lymphoma cells. We hypothesized that BCL-2 down-regulation by AS-ODNs would sensitize cells to rituximab and improve therapeutic results. There is enhanced apoptosis and reduction in cell numbers when DoHH2 cells are treated in vitro with rituximab plus BCL-2 AS-ODNs, compared with either agent alone. There is little in vitro effect on WSU-FSCCL cells by rituximab, AS-ODNs that down-regulate BCL-2 by targeting the immunoglobulin portions of the BCL-2-immunoglobulin fusion molecule, or a combination of the two. The combination is more effective than either agent alone in clearing DoHH2 cells from ascites in scid mice. Combination therapy with AS-BCL-2-ODNs and rituximab significantly prolongs survival in both the DoHH2 and WSU-FSCCL models. With higher and repeated doses, this combination could be curative. We conclude that the combination of rituximab and antisense-mediated down-regulation of BCL-2 has enhanced activity against human lymphoma, prolongs survival, and could cure mice bearing human lymphoma. This merits investigation in clinical trials. [Mol Cancer Ther 2004;3(12):1693–9]

Introduction
The incidence of non-Hodgkin’s lymphoma is increasing (1). Low-grade non-Hodgkin’s lymphoma are not curable with current standard therapy (2), or even with high-dose chemotherapy with autologous stem cell support (3, 4). New treatment approaches are needed, and many interesting new biological approaches to therapy are being developed. Based on a response rate of 50% to 60%, the chimeric monoclonal anti-CD20 antibody rituximab has been approved for treatment of relapsed/refractory low-grade B-cell non-Hodgkin’s lymphoma (5). It is not, however, curative, with a median time to progression of 12 to 15 months in this patient population (5). Whereas rituximab is effective in relapsed disease, many patients (especially those with small lymphocytic subtypes) do not respond, and about 60% of responders do not respond to re-treatment (6). Even rituximab as an initial therapy with repeated dosing schedules, while prolonging disease-free survival, is not curative (7, 8). Because of these limitations, combination therapy is being investigated. Rational design of combination therapy would depend on knowledge of the precise mechanism of action of rituximab. Evidence has been reported for the role of several different mechanisms, including direct signaling of apoptosis (9–12), complement activation (13–15), and antibody-dependent cytotoxicity mediated by cytotoxic lymphocytes, monocyte/macrophages or neutrophils (16). It is likely that, depending on the system, more than one of these mechanisms plays a role in rituximab effects (17). Nonetheless, it is clear that not all CD20-positive cells are eliminated.

Regardless of the mechanism, a prerequisite for successful treatment is that lymphoma cells must ultimately undergo cell death. BCL-2 is an antiapoptotic protein (18) whose expression is dysregulated in most indolent B-cell malignancies, either by translocation near the immunoglobulin enhancer in the t(14;18) translocation (19, 20) or by other means. BCL-2 overexpression confers chemoresistance (18). Several groups, including ours, have shown that antisense oligonucleotides (AS-ODNs) that down-regulate BCL-2 expression induce apoptosis (21) and chemosensitize B-cell lymphoma cells (22, 23). We previously reported that lymphoma-specific down-regulation of BCL-2 in t(14;18)+ lymphomas by immunoglobulin-targeted AS-ODNs prolong survival of scid mice bearing human lymphoma xenografts (24). In this report, we test the hypothesis that BCL-2 down-regulation by AS-ODNs will sensitize cells to killing by rituximab and lead to improved therapeutic results in the scid/human lymphoma xenograft model.
AS-BCL-2-ODNs plus Rituximab in Lymphoma Therapy

Materials and Methods

Cell Culture

DoHH2, a t(14;18)\(^+\) transformed lymphoma cell line, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DMSZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). WSU-FSCCL, as previously reported (25), was isolated from the pleural fluid of a patient with follicular grade 1 lymphoma, contains t(14;18), is EBV-negative, and behaves in scid mice as a more indolent disease (24), although it does contain a c-myc translocation. Cells are incubated under standard conditions and cell numbers are determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and apoptosis by Apo2.7 staining by flow cytometry as before (24). Briefly, exponentially growing cells plated on day –1 in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum at a cell density of 1.0 × 10\(^6\) cells per milliliter in 0.16 mL of 96-well plates are treated with 20 \(\mu\)g/mL (7 \(\mu\)M) phosphorothioate-modified oligonucleotides and/or 30 \(\mu\)g/mL anti-CD20 monoclonal antibody by direct delivery on day 0. No lipofection or transfection agents are utilized and no further oligonucleotide or antibody additions are made. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done and absorbance values converted to viable cell numbers and apoptosis is assayed by Apo2.7 staining.

Oligonucleotides

Oligonucleotides with a phosphorothioate backbone were synthesized at the Fox Chase Cancer Center DNA Synthesis Core Facility by standard chemistry, deprotected, and resuspended in sterile modified Tris-HCl buffer. Oligonucleotides are injected i.p. Sequences are antisense C\(_p\) heavy chain for WSU-FSCCL, and to codons 2 to 7 of BCL-2 for DoHH2 (21), using control oligonucleotides with 8 bp substitutions that retain the overall ATGC content and the single CpG dinucleotide for each AS-ODN. Sequences, as previously reported (21), are obtained with antisense gaagacgctcactttgg (28), antisense gtcggcccctagggctc (27), C\(_p\) mutant gtacaggcactgttagc. The control oligonucleotides, denoted in prior publications as mutated or (mut), have sequences containing 8 bp changes that retain the same overall number of adenine, thymine, guanine, and cytosine bases.

Anti-CD20 Monoclonal Antibody

Murine anti-CD20 (clone C2B8) and chimeric anti-CD20 (clone C2B8) were obtained from IDEC (San Diego, CA). These were injected i.p. We did not observe any difference in efficacy between the murine and chimeric forms.

Scid/Human Xenograft

Female CB17 scid mice were bred, housed, and treated in the Fox Chase Cancer Center Laboratory Animal Facility under an approved protocol. Mice 4 to 8 weeks old were injected i.p. with either 1 \(\times\) 10\(^6\) WSU-FSCCL cells or 5 \(\times\) 10\(^6\) DoHH2 cells. Mice were observed daily and euthanized when they seemed ill. Lymphoma involving diffuse adenopathy, splenomegaly, ascites, and infiltration of the liver and marrow developed in untreated mice with each model, at 8 to 11 weeks with WSU-FSCCL (24), and at 4 to 6 weeks with DoHH2. Cells were serially collected from asites by peritoneal washings to monitor lymphoma development. As discrete measurable tumors did not consistently develop with any route of injection, survival was used as the end point for efficacy of treatment.

Apoptosis and Cell Cycle

Cell cycle was analyzed by DNA content per cell, by propidium iodide staining of nuclei from hypotonically lysed cells (29). Apoptosis was estimated as the percentage of hypodiploid cells, indicating DNA fragmentation. Apoptosis was also determined by staining of intact cells with Apo2.7 (30).

Results

In vitro Effects of Anti-CD20 ± AS-ODNs

We previously reported in vitro effects of down-regulation of BCL-2 by AS-ODNs targeted to either BCL-2 or the C\(_p\) portion of the BCL-2-immunoglobulin fusion transcript in WSU-FSCCL cells (21). In that report, we confirmed that the predicted sequence-specific down-regulation of BCL-2 did induce apoptosis. Similar effects are obtained with AS-BCL-2-ODN treatment of DoHH2 cells (not shown).

The in vitro effects of anti-CD20 are cell line–dependent. For DoHH2 cells, we found minimal direct induction of apoptosis by anti-CD20 alone (Fig. 1, left). Cross-linking the antibody with goat anti-human IgG slightly enhanced apoptosis (not shown). Complement-mediated lysis of DoHH2 cells by anti-CD20 is also minimal. WSU-FSCCL cells are also not sensitive to direct induction of apoptosis by anti-CD20 (Fig. 1, right). In contrast, the large cell lymphoma cell lines, SU-DHL-4 and SU-DHL-6, are very sensitive to both direct anti-CD20–induced apoptosis and complement-mediated lysis (not shown). Because we have used DoHH2 and FSCCL cells and the corresponding scid mouse models to test AS-ODNs (24), we used these cells to investigate the effects of combining AS-ODNs and anti-CD20. The data in Fig. 1 show that there is a significant increase in apoptosis in DoHH2 cells in vitro when AS-ODNs and chimeric anti-CD20 are combined, compared with either agent alone (P < 0.03 versus anti-CD20 alone, P < 0.10 versus AS-ODNs alone). These data were derived using concentrations that gave maximal single agent effects. AS-ODNs, anti-CD20, or the combination had no significant effects on direct induction of apoptosis in WSU-FSCCL cells in vitro.

The induction of apoptosis in DoHH2 cells translates into a decrease in cell number after 72 hours of incubation in vitro. Expressed as the percentage of untreated control cells, AS-BCL-2-ODNs and anti-CD20 had minimal effects (84 ± 2\% and 84 ± 1\%, respectively), anti-CD20 plus control oligonucleotides were 72 ± 3\%, whereas anti-CD20 plus AS-BCL-2-ODNs reduced cell numbers to 42 ± 4\% (P = 0.0006 compared with anti-CD20 plus control oligonucleotides).
AS-ODNs plus Anti-CD20 Induces Apoptosis and Reduces Non-Hodgkin's Lymphoma Cells in Ascites

DoHH2 cells injected i.p. into scid mice were allowed to grow for 3 weeks. At this stage, mice had detectable ascites and had begun to appear ill. We have previously shown the diminished efficacy of AS-ODNs when lymphoma was allowed to grow prior to treatment (24). Groups of three mice were then given 400 µg of AS-ODNs i.p. or control oligonucleotides, followed 1 hour later with or without 100 µg (approximately 5 mg/kg) of anti-CD20. The oligonucleotide dose, which we used in our prior report (24), was based on findings that 400 µg of oligonucleotides, regardless of these mice. After a single 500 µg oligonucleotide injection, mice seemed ill for several days and some died. As we have shown previously, the 400 µg AS-ODN dose led to maximal decrease in BCL-2 protein levels and induction of apoptosis in vivo. Ascites was collected 16 hours later and analyzed for human CD45-positive cells that were also stained with anti-CD20, the antibody is active (24), was based on findings that 400 µg of oligonucleotides, regardless of these mice. After a single 500 µg oligonucleotide injection, mice seemed ill for several days and some died. As we have shown previously, the 400 µg AS-ODN dose led to maximal decrease in BCL-2 protein levels and induction of apoptosis in vivo. Ascites was collected 16 hours later and analyzed for human CD45-positive cells that were also stained with the marker of early apoptosis Apo2.7 (30). Apoptosis was assayed by Apo2.7 staining of triplicate wells of a 96-well plate.

AS-ODNs plus Anti-CD20 Induces Apoptosis and Reduces Non-Hodgkin's Lymphoma Cells in Ascites

For survival experiments, DoHH2 cells were allowed to grow for 3 weeks prior to the start of therapy. In the first experiment, single doses of AS-BCL-2-ODNs and anti-CD20 were used to permit detection of a positive interaction of the treatments. A single dose of AS-BCL-2-ODNs or a single 5 or 20 µg dose of anti-CD20 is essentially ineffective in this model, whereas the combination markedly prolongs survival (Fig. 4). Using a treatment program with AS-ODNs on day 3 and anti-CD20 on day 5, a single injection of AS-ODNs prolonged survival from 29 to 32 days to 50 days, but all mice were dead by day 55, whereas a single 5 or 20 µg dose of antibody had no effect. The combination prolonged survival in a dose-dependent manner for the anti-CD20, to 60 days with the lower dose of antibody, and to 87 days with the higher dose. In this case, two of five mice remained disease-free. The P value for 20 µg anti-CD20 plus AS-ODNs versus 20 µg antibody alone was 0.028.

Whereas FSCCL cells are not sensitive in vitro to anti-CD20, the antibody is active in vivo, perhaps due to immune clearance mechanisms such as antibody-dependent cellular cytotoxicity (16). Therefore, we proceeded to test the combination in vivo in FSCCL/scid mice. The survival of mice in this model was significantly prolonged by treatment with the combination of AS-ODNs targeted

![](Image)

**Figure 1.** Induction of apoptosis by chimeric anti-CD20 and/or AS-BCL-2-ODNs. Logarithmically growing DoHH2 cells (left) or WSU-FSCCL cells (right) were treated with 30 µg/mL chimeric anti-CD20 or 20 µg/mL AS-BCL-2-ODNs or mut-oligonucleotide. Oligonucleotides were added 2 hours after the antibodies, and incubation continued for a total of 24 hours. Apoptosis was assayed by Apo2.7 staining of triplicate wells of a 96-well plate.

**Figure 2.** Induction of apoptosis in ascites cells. DoHH2 cells injected i.p. into scid mice were allowed to grow for 3 weeks. Mice then received 400 µg of control oligonucleotides or AS-BCL-2-ODNs i.p., followed 1 hour later by 100 µg of chimeric anti-CD20. Ascites cells were collected 16 hours after oligonucleotides were given and analyzed for Apo2.7-positive cells. P value < 0.02 for anti-CD20 + AS-BCL-2-ODNs versus anti-CD20 + control (mut)-oligonucleotides.
to immunoglobulin sequences fused to BCL-2 plus anti-CD20 (21), compared with single doses of either treatment alone (Fig. 5). In comparison, P values are less than 0.005 for AS-ODNs plus anti-CD20 versus control oligonucleotides plus anti-CD20.

In an attempt to improve the cure rate, we gave additional and/or increased doses of each agent, using up to 100 μg anti-CD20 per mouse. Data in Fig. 6 shows the dose-dependent prolongation of survival with increasing amounts of AS-ODNs, up to the maximum tolerated dose of 400 μg per mouse, in combination with 100 μg anti-CD20. In this experiment, the combination of the maximal tolerated dose of 400 μg AS-ODNs + 100 μg anti-CD20 per mouse led to long-term survival for four of the five mice. These mice were sacrificed on day 193 and were negative for the presence of lymphoma cells by histology and by PCR for the t(14;18) translocation.

In two additional experiments (Fig. 7), we utilized repeated doses of AS-ODNs, which we have previously found to be therapeutically effective in this model (24). In these experiments, four of the five mice (Fig. 7, left) and all six mice (Fig. 7, right) treated with 4 × 400 μg doses of AS-ODNs and 2 × 100 μg doses of anti-CD20 remained free of lymphoma. At these higher doses, there seemed to be some protective effect from the control oligonucleotides, reflecting non-antisense effects, perhaps on an immune stimulation basis (45, 46). Nonetheless, AS-ODN markedly enhanced the effect of the antibody in terms of therapeutic efficacy.

**Discussion**

Standard chemotherapy is not curative in low-grade non-Hodgkin’s lymphoma. Even in first remission, dose-intense therapy requiring autologous stem cell support does not cure this disease (3, 4). Cure will likely require biological and/or immunologic approaches. Rituximab is clearly an active, but noncurative therapy. Promising early results for rituximab combinations with chemotherapy have been presented (e.g., refs. 31, 32, including phase III randomized trials, and refs. 33, 34).

Bcl-2 plays an important role in t(14;18)+ non-Hodgkin’s lymphoma, as well as in other indolent B cell malignancies such as chronic lymphocytic leukemia. Bcl-2 over-expression is a mechanism of resistance to drug-induced apoptosis. We (21, 24, 27), and others (22, 35–37), have been investigating pharmacologic down-regulation of BCL-2 by AS-ODNs as a potential therapeutic modality in low-grade non-Hodgkin’s lymphoma. Such BCL-2-targeted...
AS-ODNs may not only have inherent efficacy, but may also act as chemosensitizing agents (22, 23). Here, we have explored the ability of BCL-2 modulation by AS-ODNs to sensitize with a biological, rather than a chemotherapeutic agent. We have showed that the combination of these biological approaches, in two different human lymphoma xenograft/scid mouse models, is more effective than either agent alone in terms of induction of apoptosis, clearance of lymphoma cells from ascites, and, most importantly, in prolongation of survival and ability to eradicate the lymphoma. Single doses of these agents show the enhanced efficacy of the combination. More intense schedules show the ability to cure these mice. There is a prior report of enhanced efficacy of the combination of AS-BCL-2-ODNs and rituximab (38). Those data differ from ours in using an EBV-positive model. Furthermore, our prior report in in vivo models (24) showed the sequence-specific down-regulation of BCL-2 and induction of apoptosis by AS-ODNs.

The mechanism or mechanisms of the interaction remain to be fully elucidated. The precise means by which rituximab exerts its antilymphoma effects remains a matter of debate (17, 39); therefore, it is difficult to assign an exact role for the AS-ODNs in this combination. Considering the various, and not mutually exclusive, mechanisms of rituximab action, antisense-mediated down-regulation of BCL-2 may, for example, sensitize the cells to direct induction of apoptosis by the antibody (40). Recent data that rituximab down-regulates BCL-2 (41) indicates another potential mechanism of interaction. Complement lysis

Figure 5. Survival of WSU-FSCCL/scid mice treated with single doses of murine anti-CD20 and/or AS-ODNs. WSU-FSCCL cells injected i.p. were allowed to grow for 7 days. AS-ODNs or control (mut)-oligonucleotides (200 μg) were injected i.p. on day 7, whereas 20 μg anti-CD20 or control medium were injected i.p. on day 9. For WSU-FSCCL cells, AS-ODNs were targeted to the immunoglobulin heavy chain constant region C\textsubscript{\textalpha} (21). Mice (five per group) were followed for survival. The sole remaining mouse was sacrificed on day 190 and was without gross or microscopic evidence of disease. For control (mut)-oligonucleotides + anti-CD20 versus AS-ODNs + anti-CD20, \(P < 0.005\).

Figure 6. Effects of higher doses of murine anti-CD20 in addition to AS-BCL-2-ODNs on survival of DoHH2/scid mice. DoHH2 cells injected i.p. were allowed to grow for 3 days. The indicated dose of AS-BCL-2-ODNs (0, 200, or 400 μg) was injected i.p. on day 3, and 100 μg anti-CD20 was injected i.p. on day 5. Mice (five per group, four for the antibody alone group) were followed for survival. Surviving mice were sacrificed on day 193 and were histopathologically negative for lymphoma.

Figure 7. Effects of multiple doses of AS-BCL-2-ODNs and murine anti-CD20 on survival of DoHH2/scid mice. Results of replicate survival experiments in which DoHH2 cells were injected i.p. into scid mice, allowed to grow for 3 days, and then mice were treated with 400 μg of the indicated oligonucleotides on days 3, 5, 7, and 17, and ± 100 μg anti-CD20 on days 10 and 19. Top, six mice per group; bottom, five mice per group.
 More importantly, however, when assayed both by number AS-BCL-2-ODNs enhanced efficacy of the combination of anti-CD20 and furthermore, these AS-ODNs induce apoptosis and pro-

ODNs, and AS-C \textit{A}

sequence-specific manner in \textit{t}(14;18)+ lymphoma cell lines. Our previous reports (21, 24) showed that AS-BCL-2-

DNA's for \textit{bcl-2} and a hybrid \textit{bcl-2/immnoglobulin} transcript resulting from

unique EBV-negative low-grade lymphoma line (WSU-FSCCL) exhibiting

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