Bcl-2 Antisense (G3139, Genasense) Enhances the in Vitro and in Vivo Response of Epstein-Barr Virus-associated Lymphoproliferative Disease to Rituximab

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ABSTRACT

Bcl-2 is up-regulated by EBV in immortalized lymphoblastoid B cells and is expressed in the majority of EBV-associated lymphoproliferative diseases, including posttransplant lymphoproliferative disorder (PTLD) and AIDS-related lymphoma (ARL). Given the antiapoptotic and chemoprotective effect of Bcl-2, it represents a logical target for modulation. In vitro using antisense strategies in PTLD and ARL. We previously examined the antitumor effects of a fully phosphorothioated Bcl-2 antisense oligonucleotide, G3139, in EBV + lymphoproliferative disease in vitro and in vivo using the human/severe combined immunodeficient (SCID) chimeric model of PTLD. These studies showed that G3139 treatment decreased Bcl-2 protein levels in association with antiproliferative and pro-apoptotic effects in lymphoblastoid cell lines (LCLs) in vitro. In vivo, although G3139 treatment completely abrogated EBV + lymphoid tumor engraftment in the human/SCID model of PTLD, antisense treatment alone was not curative in animals with established tumors. Because the humanized anti-CD20 antibody, rituximab, has antitumor activity in patients with PTLD and stimulates apoptosis in some lymphoid cell lines, we sought to determine whether Bcl-2 antisense treatment potentiates the antitumor effects of rituximab in EBV-associated lymphoproliferative disease in vitro and in vivo. Proliferation assays by thymidine uptake in LCLs showed that G3139 but not control oligonucleotides augmented the antiproliferative effect of rituximab. Flow cytometric terminal deoxynucleotidyltransferase-mediated nick end labeling assays confirmed that G3139 treatment enhanced the apoptotic response of LCLs to rituximab, and this interaction was oligonucleotide sequence dependent. To test the in vivo efficacy of G3139 and rituximab in the human/SCID model of PTLD, we used a delayed treatment schedule that permitted detection of enhanced antitumor activity of combination therapy. Although G3139 or rituximab treatment significantly prolonged survival compared with untreated controls, 89% of animals in the monotherapy arms died with disseminated tumors. In contrast, 79% of animals in the combined G3139 and rituximab arm remained tumor free for the duration of follow-up (>160 days) with no evidence of tumors at the time of sacrifice, indicating that G3139 in combination with rituximab was curative therapy in the majority of tumor-bearing animals. These studies demonstrated that G3139 potentiates the antitumor response of PTLD to rituximab in vivo and augments the antiproliferative and apoptotic effects of rituximab in vitro in LCLs. This is the first report of G3139 potentiating the antitumor activity of an antibody-based therapy both in vitro and in vivo. Bcl-2 antisense oligonucleotide therapy in combination with rituximab may represent a promising nontoxic and effective targeted therapy for EBV-associated lymphoproliferative diseases such as PTLD and ARL. Furthermore, this approach may have broader applications to other Bcl-2- and CD20-expressing lymphoid malignancies.

INTRODUCTION

EBV is causally linked to several human lymphoid malignancies, including Burkitt’s lymphoma, ARL, and PTLD. EBV is believed to play a central role in the pathogenesis of these malignancies because of its ability to establish latent infection and immortalize B cells (2–4). Latent EBV infection is characterized by restricted expression of viral gene products, including six nuclear antigens (EBNAs) and two transmembrane proteins (LMP-1 and LMP-2) that function cooperatively to initiate and maintain transformation (2–4). Although the pattern of latent viral gene expression (type I, II, or III) varies in the EBV-associated malignancies, with the exception of Burkitt’s lymphoma, the majority of these tumors express the EBV-encoded LMP, LMP-1 (5–7). LMP-1 is required for B-cell transformation (8) and resembles a classical oncogene in gene transfer experiments (9–12). B-cell-specific transgenic expression of LMP-1 in mice causes B-cell lymphomas, confirming that LMP-1 is oncogenic in vivo (13). LMP-1 mediates its oncogenic effects by mimicking a constitutively activated re-

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3 The abbreviations used are: ARL, AIDS-related lymphoma; SCID, severe combined immunodeficient; LCL, lymphoblastoid cell line; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling; LMP, latent membrane protein; ODN, oligodeoxynucleotide; PTLD, posttransplant lymphoproliferative disorder; BsdUrd, bromodeoxyuridine.
ceptor of the Tumor Necrosis Factor Receptor family, thereby activating an array of cellular genes that affect cell growth and survival (14, 15). One important transforming function of LMP-1 is to protect cells from programmed cell death by up-regulating antiapoptotic genes, including Bcl-2 (16–18). Gene transfer studies in EBV− Burkitt cells have demonstrated that LMP-1 induces Bcl-2 expression and that this effect confers resistance to apoptosis.

Because the majority of EBV+ PTLDs and ARLs express LMP-1 (5–7), it is not surprising that Bcl-2 is overexpressed in these tumors (19, 20). Although Bcl-2 expression in follicular lymphomas bearing the 14;18 translocation is recognized as a key pathogenetic feature, the pathogenetic significance of Bcl-2 overexpression in EBV-associated lymphomas such as ARL and PTLD is less clear. Nonetheless, given the antiapoptotic function of Bcl-2 and its role in conferring resistance to chemotherapeutic agents, it represents a rational target for modulation using antisense strategies in Bcl-2-expressing EBV+ tumors.

G3139 (oblimersen sodium; Genasense) is a fully phosphorothioated antisense ODN targeted to the first six codons of the Bcl-2 open reading frame. Preclinical studies have demonstrated that G3139 mediates sequence-dependent antitumor effects and enhances chemosensitivity in murine xenograft tumor models (21–24). In Phase I testing, G3139 has had an excellent toxicity profile in humans (25–28). In previously published studies we have shown that G3139 mediates sequence-dependent antitumor effects in EBV+ B-cell lymphoproliferations both in vitro and in vivo (29). G3139 treatment of EBV-immortalized LCLs was associated with sequence-dependent depletion of Bcl-2 protein, inhibition of proliferation, and stimulation of apoptosis. To determine whether Bcl-2 antisense had antitumor effects in EBV-associated lymphoproliferative disease in vivo, we tested G3139 in the human/SCID chimeric model of PTLD. In this animal model, i.p. injection of established LCLs in SCID mice gives rise to fatal intra-abdominal EBV+ lymphoid tumors after a latent period of 30–50 days. These tumors exhibit the histopathological and molecular characteristics of EBV+ PTLD in allograft recipients (30, 31). G3139 treatment of LCL-bearing SCID mice completely abrogated tumor engraftment when treatment was initiated on the first day after tumor inoculation, whereas the control ODNs had no effect on tumor engraftment. However, G3139 treatment of animals with established tumors (i.e., treatment was delayed for 10 days after tumor inoculation) did not prevent the development of tumors, although survival of G3139-treated animals with established tumors was significantly prolonged compared with that of untreated or control ODN-treated animals. Thus, these studies showed that Bcl-2 antisense ODN treatment has significant sequence-dependent antitumor effects in vivo in the human SCID model of PTLD, but it is not curative in animals with established tumors.

Recently, the humanized monoclonal anti-CD20 antibody, rituximab, has been shown to have single-agent activity in PTLD (32, 33). Because rituximab stimulates apoptosis in some lymphoid cell lines in vitro (34, 35) and may mediate its effects through down-regulation of Bcl-2 (36, 37), we hypothesized that Bcl-2 antisense ODN treatment may augment the antitumor activity of rituximab in EBV+ lymphoproliferative disease. To test this hypothesis, in this study we have examined the antitumor effects of G3139 and rituximab in vitro in LCLs and in vivo in the human SCID model of PTLD, using a delayed treatment schedule that permitted detection of enhanced antitumor activity. The in vitro studies demonstrated that G3139 augmented the antiproliferative and proapoptotic effects of rituximab in LCLs, and this in vitro interaction was oligonucleotide sequence dependent. Moreover, G3139 in combination with rituximab was a curative treatment in SCID mice bearing established LCL-derived tumors, whereas monotherapy (G3139 or rituximab) prolonged survival but was not curative. Our studies have shown that G3139 treatment potentiates the antitumor response of EBV+ lymphoproliferative disease to rituximab both in vitro and in vivo. These findings form the basis for further evaluation of this strategy in patients with EBV+ Bcl-2-expressing lymphoproliferative diseases and may have broader applicability to other CD20+ Bcl-2-expressing lymphomas.

MATERIALS AND METHODS

Cell Lines and Reagents. 11-23 and Sweig are EBV-immortalized LCLs derived by infecting umbilical cord lymphocytes with the FF41 strain and adult B cells with the B958 strain of EBV, respectively. LCLs were maintained in RPMI 1640 + 10% heat-inactivated FCS. Eighteen-mer fully phosphorothioated ODNs corresponding to the first six codons of the human Bcl-2 open reading frame were generously provided by Genta, Inc. (Berkeley Hills, NJ). The sequences of the ODNs are as follows: Bcl-2 antisense G3139, 5′-TCTCCACGCTGCGCCAT-3′; reverse sequence control (RV), 5′-TACCGGTCGCCACCTCT-3′; and two-base mismatch control (MM), 5′-TCTCCACGATGTGCGCCAT-3′. The lyophilized ODNs were resuspended just before use. Rituximab (InVid Pharmaceuticals, La Jolla, CA) and the isotype-matched control antibody, Herceptin (Genentech, Inc., San Francisco, CA), were reconstituted according to the manufacturer’s protocol and stored at −20°C.

Incubation of Cells with ODNs and Antibody. For in vitro studies, cells in log phase of growth (2 × 10⁵ cells/ml) were exposed to rituximab (or mock treated) and then cultured with or without ODN for 3 days. For the rituximab treatment, cells were incubated with rituximab (0.5 mg/ml) in culture medium for 1 h at 4°C, washed, and then cultured in medium with goat antihuman IgG (Pierce, Rockford, IL) for 8 h at 37°C. Mock-treated cells were exposed to goat antihuman IgG without rituximab. Subsequently, cells were washed and then cultured with ODN (G3139 or control ODN) for 3 days as described previously (29). ODN (10 μM) was added directly to the culture medium after the cells were washed every 24 h. In some experiments, cells were exposed to ODN in the presence of cationic lipid (Eu-fectin 8; JBL, San Luis Obispo, CA) in serum-free artificial medium (Opi-MeM I; Life Technologies, Inc.). Antibody, Herceptin (Genentech, Inc., San Francisco, CA), was added directly to the culture medium after the cells were washed every 24 h. In some experiments, cells were exposed to ODN in the presence of cationic lipid (Eu-fectin 8; JBL, San Luis Obispo, CA) in serum-free artificial medium (Opi-MeM I; Life Technologies, Inc., Gaithersburg, MD) to enhance uptake, essentially as described previously (29).

Cellular Proliferation Assays. Cells (2 × 10⁵/ml) were plated in triplicate in 0.2 ml of medium in microtiter wells and cultured for 72 h with or without rituximab and ODN as described above. During the last 16 h of culture, each well was pulsed with 1 μCi of [3H]thymidine. Cells were harvested with a multiple automated sample harvester, and [3H]thymidine uptake was measured by scintillation counting and expressed as
the mean ± SD of triplicate assays. The two-tailed unpaired t test was used to determine significance of differences in thymidine uptake.

**Apoptosis Assays.** To detect and quantitate apoptosis, a flow cytometric TUNEL assay was performed with the APO-BrdUrd kit (PharMingen, San Diego, CA) according to the manufacturer’s protocol, as described previously (29), using 1 × 10^6 cells/sample after culture with or without rituximab and ODN for 72 h as described above. This method uses terminal deoxynucleotidyltransferase and bromo-dUTP to label exposed 3'-OH DNA ends in fixed cells; BrdUrd-tagged DNA is then quantitated by flow cytometry using fluorescein-conjugated anti-BrdUrd antibody. The percentage of cells stimulated to undergo apoptosis by treatment was calculated as follows: (percentage of apoptotic treated cells) − (percentage of apoptotic control cells)/(100 − percentage of apoptotic control cells) × 100, where the control cells were either untreated LCLs or reverse control ODN-treated cells.

**Evaluation of G3139 and Rituximab Effects in Vivo.** For in vivo studies we used the human/SCID chimeric model of EBV⁺ lymphoproliferative disease (29–31). We have shown previously that i.p. injection of 20 million LCLs (Sweig) results in the reproducible development of fatal lymphoproliferative disease involving the abdominal cavity and viscera 30–50 days postinoculation (29). These tumors share remarkable similarities with EBV⁺ PTLD in that they are diffuse large cell lymphomas of human B-cell origin, EBV is present with a broad pattern of viral gene expression, and Bcl-2 is abundantly expressed. Female SCID/NCR mice (5–7 weeks old) were obtained from the National Cancer Institute breeding colony (Bethesda, MD) and housed in a pathogen-free environment. Animals were randomly assigned to experimental groups of six to seven mice. Each mouse was given an i.p. injection with 20 million LCLs (Sweig) in 0.5 ml of sterile PBS on day 0. Animals were monitored daily and sacrificed when they developed clinical signs of disease. All animals were subjected to necropsy to determine the gross pattern of tumor development. Animals that did not develop clinical signs of disease were sacrificed at 168, 159, 191, and 160 days (experiments 1, 2, 3, and 4, respectively) and examined for evidence of gross tumor. The ODN was administered i.p. in 0.5 ml of sterile saline at a dose of 10 mg/kg/day over 12 days (total dose, 125 mg/kg) in five divided doses at 72-h intervals. Rituximab (or Herceptin) was administered i.p. at a dose of 20 mg/kg weekly for four doses. Treatment was started on day 1 immediately before or day 15 (delayed treatment) after injection of LCLs. The end point for the therapeutic trial was survival, based on the day of sacrifice for each animal. Survival of each group was described by a Kaplan-Meier plot. Experimental groups were statistically compared with the log-rank (Mantel-Cox) test for analysis of thymidine uptake.

**RESULTS**

**In Vitro Effect of G3139 and Rituximab on Proliferation of LCLs.** In previously published studies we demonstrated that G3139 treatment depleted Bcl-2 protein levels, inhibited proliferation, and stimulated apoptosis in EBV⁺ LCLs compared with control ODNs (29). Because rituximab has been shown to inhibit cell growth in some lymphoid cell lines in vitro (34, 35), we sought to determine whether G3139 in combination with rituximab represents a more potent antitumor treatment in EBV⁺ LCLs than either agent alone. Although rituximab has growth-inhibitory and proapoptotic effects in some B-lymphoid cell lines, including Ramos, EBV⁺ LCLs are relatively resistant to the antiproliferative and proapoptotic effects of rituximab in vitro. We have found previously that the antiproliferative effect of rituximab in LCLs is minimal (<20%) over a broad range of concentrations and is not significantly impacted by the addition of cross-linking antibodies (goat antihuman) or complement.

To determine whether G3139 augments the antitumor effects of rituximab in vitro, we performed proliferation assays on LCLs treated with ODN (G3139 or control ODN), rituximab, or ODN in combination with rituximab. As we have shown previously, G3139 significantly inhibited proliferation of LCLs relative to mock-treated (48–57%; P < 0.01) or control ODN-treated LCLs (43–51%; P < 0.01), as shown in three representative experiments (Fig. 1). Rituximab treatment had minimal antiproliferative effects compared with mock-treated cells (10–17%; P > 0.14). When rituximab was combined with G3139, there was significant enhancement of antiproliferative effect compared with G3139 alone (45–65%; P < 0.017) or rituximab + control ODN (67–80%; P < 0.015). In contrast, there was no significant difference in proliferation between LCLs treated with control ODN + rituximab and LCLs treated with control ODN alone (2–15%; P > 0.13) or rituximab alone (0–14%; P > 0.29). These results demonstrated that G3139, but not the control ODNs, augmented the antiproliferative effect of rituximab in LCLs in vitro. When the effects of G3139 and rituximab treatment were compared with mock-treated LCLs, the growth-inhibitory effect of the combination was more than additive; the antiproliferative effect of G3139 + rituximab (71–85%) was greater than the sum of the inhibitory effect of either agent alone in individual experiments (62–67%). These data demonstrated that G3139 enhances the antiproliferative effect of rituximab in LCLs and that this effect is oligonucleotide sequence dependent. Furthermore, at the doses used in these experiments, the inhibitory effect of combined treatment was more than additive, suggesting a synergistic interaction.

**In Vitro Effect of G3139 and Rituximab on Apoptosis in LCLs.** We showed previously that G3139 treatment of LCLs stimulated apoptosis relative to untreated or control ODN-treated cells under conditions of serum deprivation (29). Although rituximab has been reported to stimulate apoptosis in some B-lymphoid lines (34, 35), we have been unable to demonstrate a proapoptotic effect in LCLs, with or without cross-linking or complement, using a variety of assays including the TUNEL assay, propidium iodide staining, and poly(ADP-ribose) polymerase cleavage. To determine whether the enhanced antiproliferative effect of rituximab in the presence of G3139 in LCLs was attributable, in part, to increased apoptotic cell death, we performed TUNEL assays in LCLs treated with ODN and/or rituximab, both in the presence of serum and under

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4 J. Lacy and R. Loomis, unpublished observations.
conditions of serum deprivation (with cationic lipid). Under conditions of serum deprivation, G3139 stimulated apoptosis relative to untreated cells [or reverse control ODN-treated cells (57%)], whereas rituximab had minimal effect on apoptosis (5%; Fig. 2, A and B; Table 1). Rituximab in combination with G3139 dramatically increased apoptosis relative to untreated controls (83%; Fig. 2A), and this effect was more than additive (5 + 57% versus 83%). The proapoptotic effect of rituximab in the presence of ODN was oligonucleotide sequence dependent because reverse control ODN did not increase apoptosis of rituximab-treated cells (Fig. 2B). The interaction of G3139 and rituximab was also apparent in the presence of serum (Fig. 2, C and D; Table 1). Under these conditions, G3139 or rituximab treatment of LCLs had minimal or no effect on apoptosis. However, G3139 in combination with rituximab markedly stimulated apoptosis (83%; Fig. 2A), and this effect was more than additive (5 + 57% versus 83%). The proapoptotic effect of rituximab in the presence of ODN was oligonucleotide sequence dependent because reverse control ODN did not increase apoptosis of rituximab-treated cells (Fig. 2B). The interaction of G3139 and rituximab under these conditions was oligonucleotide sequence dependent. These in vitro studies suggested that G3139, but not control ODNs, interacts with rituximab in vitro in LCLs to promote apoptotic cell death.

Antitumor Effect of G3139, Rituximab, and G3139 + Rituximab in the Human/SCID Model of PTLD. The in vitro studies of G3139 in combination with rituximab in LCLs demonstrated that G3139 enhanced the antitumor effects of rituximab and that this interaction was oligonucleotide sequence dependent. To determine whether G3139 augments the antitumor activity of rituximab in vivo in EBV+ lymphoproliferative disease, we tested the effects of G3139 in combination with rituximab in the human/SCID model of PTLD. In studies published previously, we demonstrated that G3139 monotherapy had significant sequence-dependent antitumor activity in vivo in the human/SCID model of PTLD (29). G3139 treatment completely abrogated tumor engraftment in the majority of animals and significantly prolonged survival compared with untreated or control ODN-treated animals (Ref. 29; Fig. 3A). However, in animals with established disease (treatment delayed for 10 days after inoculation of tumor cells), treatment was not curative; five of six animals died with tumors, despite significant prolongation of survival compared with the controls (Ref. 29; Fig. 3A). Thus, as a single agent, G3139 had antitumor effects in vivo in the human/SCID model of PTLD but was not a curative treatment in animals with established disease.

Rituximab has been shown to have antitumor activity in patients with PTLD (32, 33). Because rituximab is a humanized antibody that may mediate its effects through immune-based mechanisms, it was not known whether rituximab would have antilymphoma activity in SCID mice. In a pilot experiment to determine the antitumor activity of rituximab as monotherapy in the human/SCID model of PTLD, we found that rituximab treatment (but not control antibody treatment) completely abrogated tumor engraftment if initiated on day 1 (Fig. 3B). In animals with established tumors (treatment delayed until day 15), rituximab significantly prolonged survival, but all animals (six of six) died with tumors. Thus, rituximab, like G3139, demonstrated antitumor activity in the human/SCID model of PTLD, but rituximab monotherapy was not curative in animals.

Fig. 1 Effect of G3139 and rituximab treatment on proliferation of EBV+ LCLs. Proliferation was assayed by [3H]thymidine uptake in LCLs. Cells were cultured in the absence (Mock) or presence [control (Con) ODN and G3139] of oligonucleotide with and without rituximab (Rit), as described in “Materials and Methods.” In A and B, cells were treated with the reverse control ODN; in C, cells were treated with the two-base mismatch control ODN. ODN was added directly to the medium in A and C; in B, cells were exposed to ODN in the presence of cationic lipid. Values represent the mean ± SD of triplicate assays.
with established disease. In fact, treatment with either G3139 or rituximab gave very similar survival curves as single agents with immediate or delayed treatment.

Because our in vitro studies suggested that G3139 treatment may enhance the antitumor effects of rituximab in EBV+ LCLs, we sought to determine whether G3139 in combination with rituximab represents a more potent antitumor treatment in vivo in the human/SCID model of PTLD. To detect enhanced antitumor efficacy of combined G3139 and rituximab therapy, we used a delayed treatment schedule. SCID mice were given inoculations of LCLs on day 0, and on day 15 treatment was initiated with G3139 alone (7), rituximab alone (7), or G3139 + rituximab (7).

Untreated control animals (seven of seven) died with tumors 43–47 days after LCL injection (Fig. 3C). The survival of both the G3139 and the rituximab monotherapy arms was significantly prolonged compared with the untreated control arm [median survival = 59 days for rituximab (P = 0.0003) and 71 days for G3139 (P = 0.0006)]. However, all animals (seven of seven) in the rituximab arm died with tumors, six of seven animals in the G3139 arm died with tumors, and the difference in tumor incidence between untreated and G3139- or rituximab-treated animals was not significant. In contrast, when G3139 was combined with rituximab, five of seven animals remained tumor free at the time of sacrifice at 191 days (P = 0.04 versus G3139; P = 0.026 versus rituximab). The median survival for the combined treatment arm was >191 days, and the difference in survival between the combined arm and untreated or rituximab arm was significant (P = 0.0003 and P = 0.0033, respectively). These results have been replicated in an additional experiment using an identical study design (Fig. 3D). In this experiment, after 160 days of follow-up, seven of seven untreated animals had died with tumors (median survival = 53 days), five of six G3139-treated animals had died with tumors (median survival = 83 days), and six of seven rituximab-treated animals had died with tumors (median survival = 88 days). In the combined G3139 +

Table 1  Percentage of LCLs stimulated to undergo apoptosis by G3139 and/or rituximab treatment relative to untreated or control ODN-treated cells

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<tr>
<th>Treatment</th>
<th>Experiment (control)</th>
<th>G3139</th>
<th>Rituximab</th>
<th>G3139 + Rituximab</th>
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<tr>
<td>Experiment 1 (untreated)</td>
<td></td>
<td>57</td>
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<td>Experiment 1 (control ODN-treated)</td>
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rituximab arm, only one of seven animals developed tumors (at day 122), and the remaining six animals remained free of disease, with a median survival exceeding 160 days. The difference in survival between the combined treatment arm and G3139 or rituximab arm was significant ($P = 0.0052$ and $P = 0.0035$, respectively). The data from these two in vivo experiments demonstrate not only that G3139 treatment in combination with rituximab significantly prolongs survival of LCL-bearing SCID mice compared with G3139 or rituximab treatment alone but also that it is also curative in the majority of LCL-bearing SCID mice. Furthermore, these data indicate that Bcl-2 antisense ODN treatment significantly enhances the anti-tumor activity of rituximab in EBV+ lymphoproliferative disease.

**DISCUSSION**

Preclinical and clinical studies using the phosphorothioated Bcl-2 antisense ODN G3139 (Genasense) have demonstrated sequence-dependent antitumor effects in a wide array of murine xenograft tumor models (21–24) as well as an excellent toxicity profile in humans in Phase I testing (25–28). These studies have shown that G3139 effectively depletes Bcl-2 protein both in vitro and in vivo, decreases resistance to apoptotic stimuli, and enhances the cytotoxic effects of cytotoxic chemotherapeutic agents and radiation therapy. We now report that G3139 enhances the antitumor effects of rituximab in EBV+ Bcl-2-expressing lymphoblastoid cells. This is the first report of G3139 potentiating the antitumor activity of an antibody-based therapy both in vitro and in vivo.

EBV+ lymphoproliferative diseases represent a significant cause of morbidity and mortality in immunosuppressed allograft recipients and in immunocompromised patients with HIV infection (38–40). These tumors are highly associated with EBV and have been shown to express EBV latent viral proteins, including the oncogenic EBV-encoded LMP, LMP-1 (5–7). A critical transforming function of LMP-1 is up-regulation of the anti-apoptotic cellular protein, Bcl-2 (17). Not surprisingly, strong expression of Bcl-2 has been shown to be a consistent feature of EBV-associated PTLD (19, 20). Given the known function of Bcl-2 in promoting cell survival and protecting cells from apoptotic stimuli, including DNA-damaging chemotherapeutic agents, overexpression of Bcl-2 likely plays a role in the pathogenesis and chemoresistance of PTLD.

Given the potential role of Bcl-2 in the pathogenesis of PTLD, we have tested the hypothesis that antisense-mediated...
reductions in Bcl-2 expression in PTLD will promote cell death and mediate antitumor effects (29). We demonstrated previously that exposure of LCLs to Bcl-2 antisense ODN G3139 in vitro caused a sequence-dependent decline in Bcl-2 protein levels, inhibition of proliferation, and stimulation of apoptosis. Moreover, we demonstrated a profound antitumor effect of G3139 in vivo, using the human/SCID chimeric model of EBV+ PTLD. G3139 treatment of LCL-bearing SCID animals completely prevented the development of fatal tumors in the majority of animals. However, if the initiation of antisense treatment was delayed, the majority of these animals ultimately succumbed to tumor despite significant prolongation of survival.

Recently, rituximab has been shown to have activity in PTLD (32, 33). In vitro studies suggest that the rituximab may have direct proapoptotic and antiproliferative effects mediated by CD20 signal transduction (34, 35) as well as immune-based cytotoxic effects (41, 42). We now present findings demonstrating that G3139 in combination with rituximab has potent antitumor effects in vitro in LCLs and in vivo in EBV+ tumor-bearing SCID mice. The in vitro studies of G3139 in combination with rituximab demonstrated that G3139 enhanced both the antiproliferative and apoptotic effects of rituximab in LCLs and that these effects were oligonucleotide sequence dependent. Moreover, in the human SCID model of PTLD, G3139 treatment in combination with rituximab was curative in the majority of animals. Although G3139 or rituximab as single agents prolonged the survival of tumor-bearing SCID mice, monotherapy was not curative, and treated animals died with extensive intra-abdominal lymphoma. In contrast, the majority (11 of 14) of tumor-bearing animals that received combined G3139 and rituximab treatment were cured and had no evidence of tumors at the time of sacrifice. The animals treated with G3139 and rituximab that died with tumors (n = 3) had extensive intra-abdominal disease at autopsy, suggesting that the treatment did not alter the pattern of spread or tumor distribution.

The precise mechanism by which G3139 enhances the antitumor effects of rituximab in PTLD is not known. The sequence dependency of the effects of G3139 both in vitro and in vivo in diverse tumor models, including our model of EBV-associated lymphoproliferative disease, is consistent with an antisense mechanism of action, resulting in Bcl-2 depletion and decreased resistance to apoptotic stimuli (21–24, 29). Although we were unable to evaluate the in vivo effect of G3139 on Bcl-2 levels in the human SCID model of PTLD because it is not a solid tumor model with accessible tumor during G3139 treatment, other investigators have shown consistently that G3139 treatment results in Bcl-2 depletion in diverse tumor types in vivo (21–24). The precise mechanism of action of rituximab remains uncertain and may include direct stimulation of apoptosis by CD20 cross-linking (34, 35) as well as complement-dependent cell lysis and antibody-dependent cellular cytotoxicity (41, 42). However, there is marked variability in susceptibility of different B-cell lines to rituximab-mediated, complement-dependent cell lysis and apoptosis (37, 41), and our studies indicate that LCLs are relatively resistant to rituximab in vitro. Recent studies by Alas et al. (36, 37) demonstrated that rituximab causes down-regulation of Bcl-2 expression and sensitization of some lymphoid cell lines to proapoptotic stimuli. These studies suggest that rituximab inhibits the interleukin 10 paracrine/autocrine loop and Janus-activated kinase/signal transducers and activators of transcription signaling pathway, resulting in inactivation of signal transducers and activators of transcription 3 and decreased transcription of Bcl-2. Interestingly, Baiocchi et al. (43) have shown that lymphomagenesis in the human/SCID model of PTLD involves abundant production of interleukin 10. We have also found that rituximab treatment of LCLs in vitro is associated with modest decreases in Bcl-2 protein expression and, interestingly, that the effect of G3139 in combination with rituximab on Bcl-2 protein depletion is at least additive in vitro (data not shown). Thus, it is possible that Bcl-2 antisense in combination with rituximab leads to potent suppression of Bcl-2 via distinct mechanisms in EBV+ lymphoproliferative diseases, resulting in enhanced susceptibility to apoptotic stimuli. Our finding that enhancement of the antitumor activity of rituximab by G3139 in vitro is oligonucleotide sequence dependent suggests that the antisense effect of G3139 may be important in this interaction. Although our previous in vivo studies using G3139 in the human/SCID model of PTLD have demonstrated oligonucleotide sequence-dependent antitumor effects (29), we cannot yet exclude the possibility that G3139 in combination with rituximab in vivo mediates additive or supra-additive effects via non-sequence-dependent mechanisms.

Our studies support the concept that the Bcl-2 antisense ODN, G3139, in combination with rituximab may represent an effective new treatment option for EBV+ Bcl-2-expressing lymphoproliferative diseases, including PTLD and ARL. EBV-associated lymphomas remain a significant cause of morbidity and mortality in immunosuppressed allograft recipients and in patients with HIV infection (38–40). Although an array of treatment options have been used to treat PTLD or ARL, including chemotherapy, rituximab, IFN, radiation therapy, and restoration of immune function through modifications in immunosuppressive drugs (PTLD), the overall mortality of the EBV+ lymphoproliferative diseases remains in excess of 50% (38–40). Despite the initial enthusiasm regarding the efficacy rituximab in treatment of PTLD, approximately half of treated patients are either unresponsive or relapse after an initial complete or partial response (33). Because the morbidity and mortality of PTLD and ARL result from both unresponsive disease and treatment-related complications in these immunocompromised patient populations, the concept of using nontoxic targeted therapies in combination is appealing. G3139 and rituximab are both remarkably well tolerated in humans with minimal and nonoverlapping toxicities. G3139 has been extensively evaluated in Phase I/II studies, and although thrombocytopenia has been reported as a dose-limiting toxicity with some schedules, G3139 does not cause leukopenia or end-organ damage (25–28). Furthermore, given the excellent toxicity profiles of G3139 and rituximab, it may be feasible to combine this treatment with cytotoxic drugs. In vitro and in vivo studies using G3139 have demonstrated that depletion of Bcl-2 protein levels is associated with chemosensitization (21–24). Similarly, in vitro studies suggest that rituximab sensitizes some B-cell lines to chemotherapeutic agents (36, 44). Thus, the use of G3139 in combination with rituximab may not only enhance the antitumor efficacy of
cytotoxic drugs but may also permit the use of less toxic doses without compromising efficacy.

In conclusion, we have demonstrated that the Bcl-2 antisense ODN G3139 enhances the in vitro and in vivo antitumor effects of rituximab in EBV+ lymphoproliferative disease. G3139 in combination with rituximab may represent a potentially nontoxic yet effective targeted treatment strategy for EBV+ B-cell lymphomas, including PTLD and ARL. Our preclinical studies of G3139 and rituximab in the human SCID model of PTLD support the investigation of this combination in patients with Bcl-2-expressing lymphoproliferative diseases.

REFERENCES


Bcl-2 Antisense (G3139, Genasense) Enhances the \textit{in Vitro} and \textit{in Vivo} Response of Epstein-Barr Virus-associated Lymphoproliferative Disease to Rituximab

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