Antitumor Efficacy of Oblimersen Bcl-2 Antisense Oligonucleotide Alone and in Combination with Vinorelbine in Xenograft Models of Human Non–Small Cell Lung Cancer

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ABSTRACT

Overexpression of Bcl-2 protein in cancer cells can inhibit programmed cell death and engender chemoresistance. Reducing Bcl-2 protein levels by using antisense oligonucleotides targeting the gene message can increase the sensitivity of cancer cells to cytotoxic agents. The objective of this work was to investigate the antitumor efficacy of the Bcl-2 antisense oligonucleotide oblimersen (Genasense; G3139), alone and in combination with vinorelbine (VNB), in an ectopic and orthotopic xenograft model of NCI-H460 human non–small-cell lung cancer. In addition to assessing therapeutic effect, Bcl-2 protein expression in tumor tissue isolated from lung and heart was measured. In the ectopic xenograft model, oblimersen at 5 and 10 mg/kg significantly inhibited tumor growth compared with saline-treated control groups, and furthermore, the antitumor effect of oblimersen was associated with down-regulation of Bcl-2 protein in isolated tumor tissue. Moreover, the combination of oblimersen with VNB was more active in inhibiting tumor growth than either drug used alone. In the orthotopic model, oblimersen treatment (5 mg/kg) increased the median survival time of mice to 33 days in comparison with a median survival time of 21 days in the control animals. With this effect of oblimersen was associated with down-regulation of Bcl-2 protein in isolated tumor tissue. Moreover, the combination of oblimersen with VNB was more active in inhibiting tumor growth than either drug used alone. In the orthotopic model, oblimersen treatment (5 mg/kg) increased the median survival time of mice to 33 days in comparison with a median survival time of 21 days in the control animals. With this model, the anticancer effect was demonstrated by assessing tumor growth in lung and heart tissues by hematoxylin and eosin staining and Bcl-2 expression by immunohistochemistry. When VNB at 5 mg/kg was combined with oblimersen administered at 5 mg/kg, 33% of mice survived more than 90 days. These data suggest that the combination of oblimersen and VNB may provide enhanced antitumor activities against non-small-cell lung cancer.

INTRODUCTION

Lung cancer causes almost a third (~30%) of all cancer-related deaths, a death toll that is greater than that attributed to colorectal, breast, and prostate cancer (the second through fourth leading causes of cancer mortality) combined (1). Treating lung cancer is a frustrating process because improvements in overall outcome remain elusive, despite the introduction of molecularly targeted therapy. A majority of patients present with advanced, incurable disease (stages IIIIB and IV) and have a very poor outcome. In this setting, the median survival with best supportive care is 4 to 6 months, with <10% of patients still alive at 1 year (2). Platinum-based doublet chemotherapy can extend the median survival to 8 to 11 months and the 1-year survival to 30% (3). Changing the natural course of advanced or metastatic non–small-cell lung cancer (NSCLC) is thus proving very difficult. However, our increased understanding of the molecular anomalies associated with NSCLC has raised hopes that targeted therapies, especially if used in combination with standard cytotoxic agents, may lead to improved outcomes (4). Recent data, for example, have demonstrated the efficacy of anti-epidermal growth factor receptor–targeted therapies (5). In this study, we have initiated preclinical drug combination studies with another molecularly targeted therapeutic agent, oblimersen, which effectively reduces expression of the antiapoptotic molecule Bcl-2 (6).

Since confirmation of its antiapoptosis role in follicular lymphoma, the role of Bcl-2 in other malignancies has been extensively explored. Initially, dysregulation and overexpression of Bcl-2 in follicular lymphoma was found to be the result of the t(14;18)(q32;q31) chromosomal translocation characteristic of the disease (7). Subsequent research has demonstrated Bcl-2 overexpression in other lymphoid malignancies as well as in solid tumors that do not exhibit the translocation. It is believed that the attenuation of apoptosis by overexpression of Bcl-2 is a contributing factor associated with malignant transformation and resistance to chemotherapy-induced apoptosis (8–10).

Although Bcl-2 overexpression is seen more commonly in small cell lung cancer, it is also seen in NSCLC tumors. Recent experience at our institution revealed that about 15% of non–small-cell tumors overexpressed Bcl-2 by immunohistochemistry (11). Several studies have examined the role of Bcl-2 expression as a predictive or prognostic marker in lung cancer, but the impact of Bcl-2 expression on outcome has not been shown to be as significant as that of p53 expression (12). Importantly, Bcl-2 overexpression in these malignancies is not associated with a specific chromosomal translocation and therefore is most likely a consequence of the general molecular dysregulation associated with lung carcinogenesis, possibly caused by mechanisms such as changes in

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promoter methylation status (13). It is believed that overexpression of Bcl-2 will impart a survival advantage to cells in the face of treatment. Furthermore, there is some evidence to suggest that down-regulation of Bcl-2 may increase sensitivity to cytotoxic chemotherapy and radiation (14). Consequently, therapeutic strategies that can directly or indirectly inhibit or suppress Bcl-2 are garnering great clinical interest.

Antisense oligonucleotides (ASOs) are one category of agents capable of affecting Bcl-2 expression. These short sequences of nucleotide bases complementary to coding sequences of a gene of interest can be designed to bind RNA molecules in a sequence-specific manner (6, 15–17). Binding can directly impair interaction with factors in the cytoplasm that are required for translation of the message into protein and recruit endogenous RNase H to cleave the RNA backbone (18). The latter results in a reduction in the targeted RNA pool, which subsequently leads to reductions in the target protein encoded by that RNA. For ASOs to be active, delivery to the inside of a target cell must be achieved efficiently and, in tissue culture, for example, this requires association with a delivery system such as cationic lipids (19, 20). In vivo, however, ASOs are active when given in free form, possibly due to interactions with plasma lipoproteins (21).

Anti-Bcl-2–based treatment using oblimersen, an 18-mer oligonucleotide sequence targeting the first six codons of the open reading frame of the Bcl-2 message, is being tested in phase III clinical trials as a chemo-sensitizing agent in melanoma and other tumors (22, 23). It has been shown previously that Bcl-2 ASOs have an inhibitory effect on the growth of Bcl-2–overexpressing lung cancer cell lines (24, 25), and clinical studies evaluating the use of Bcl-2 ASOs in patients with lung cancer have been reported (26). Here, we present data demonstrating inhibition of tumor growth, anticancer effect, and prolongation of survival of tumor-bearing animals by oblimersen in two murine xenograft models of NSCLC: a subcutaneous model and an orthotopic model. Furthermore, tumor inhibition is shown to be associated with down-regulation of Bcl-2 protein in tumor tissue. In addition, we demonstrate greater antitumor effects when oblimersen was given in combination with vinorelbine, a chemotherapeutic agent used in the treatment of NSCLC.

MATERIALS AND METHODS

Tumor Cell Lines and Drugs. NCI-H460 human NSCLC cells were obtained from the National Cancer Institute tumor repository and maintained in RPMI 1640 supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO2. Cells were used in exponential growth phase, up to a maximum of 10 in vitro passages. Phosphoro-thioate 18-mer ASO with a sequence complementary to the first six codons of the open reading frame of Bcl-2 mRNA (5'-tctccc-agg-gtg-ccg-ctt-3; 18-mer) was generously provided by Gentra Inc. (Berkeley Heights, NJ). This was used as the ASO (oblimersen; G3139) to Bcl-2. A reverse polarity phosphoro-thioate sequence, 5'-tac-cgc-gtg-cca-ccc-tct-3', was used as control (RPO; Gentra Inc.). Vinorelbine (VNB; GSK Inc. Mississauga, Ontario, Canada) was purchased from the British Columbia Cancer Agency Pharmacy.

Tumor Models. Male severe combined immunodeficient (SCID)-RAG2 mice (7–9 weeks old; 23–26 g) were obtained from the British Columbia Cancer Agency Joint Animal Facility breeding colony and maintained in a pathogen-free environment. Two NCI-H460 models were used in these studies: A solid tumor model was developed for antitumor efficacy studies by subcutaneous implantation of 105 NCI-H460 cells in the flank of SCID-RAG2 mice; and an “orthotopic” NCI-H460 lung tumor model was established to assess the effects of treatment on systemic disease. 106 NCI-H460 cells were implanted through the chest wall into the left pleural space of SCID-RAG2 mice in a volume of 100 μL using a 26-gauge needle. The depth of needle penetration through the intercostal muscles was controlled to avoid lung injury and hemorrhage into the pleural space. All animal protocols were approved by the University of British Columbia Animal Care Committee, and all studies were completed in accordance with the current guidelines of the Canadian Council on Animal Care.

In vivo Antitumor Activity. Efficacy experiments were conducted in male SCID-RAG2 mice bearing established (0.05 cm3) NCI-H460 tumors (6 mice per group). Day 1 was defined as the day treatments started, typically 12 to 14 days after tumor cell inoculation. Oblimersen or reverse polarity control (RPO) was administered intraperitoneally daily at 5 or 10 mg/kg for 5 doses per week (Monday to Friday) over a 3-week period. Saline was administered using the same schedule for control mice. VNB (5 or 10 mg/kg) was administered intravenously every 4th day x 3 (days 1, 5, and 9) via a lateral tail vein, either alone or in combination with ASO (administered as described above). The dose schedules used were based on previous reports demonstrating that phosphoro-thioate ASOs have elimination half-lives of 12 to 72 hours (27). When ASOs were administered in combination with VNB, the intravenous drug treatment was staggered 4 hours after intraperitoneal ASO treatment.

Mice were observed daily, and body weight measurements and signs of stress (e.g., lethargy, ruffled coat, and ataxia) were used to detect toxicities. Animals with ulcerated tumors or whose tumors exceeded 1 cm3 were euthanized. Electronic caliper (Mitutoyo Corp., Kawasaki, Japan) measurements of tumors were converted into mean tumor size (cm3) using the following formula: \( \frac{1}{2} \times \text{length (cm)} \times \text{width (cm)} \). An average tumor size per mouse was used to calculate the group mean tumor size ± SE (n = 6–12 mice) from two independent experiments.

The effects of treatment on progression of systemic disease and survival time of mice were studied using the intrapleural xenograft model. Treatments were initiated 7 days after tumor cell injection. Histologic analysis had demonstrated consistent involvement of lung and heart by tumor by this stage. Saline, oblimersen (Bcl-2 ASO), or Bcl-2 RPO sequence was administered intraperitoneally daily at 5 or 10 mg/kg for 5 doses per week over a 3-week regimen. VNB (2.5 and 5 mg/kg) was administered intravenously on days 7, 11, and 15, after tumor implant, via a lateral tail vein, either alone or in combination with ASOs, which were administered intraperitoneally. When ASOs were administered in combination with VNB, the drug treatment was staggered 4 hours after intraperitoneal ASO treatment. Each treated group consisted of 6 to 12 mice, and control groups consisted of 26 mice, pooled from independent experiments. All experiments designed to assess therapeutic activity included control animals (which were treated with saline). Because the NCI-H460 model was highly robust and reproducible, these controls exhibited very comparable growth curves and survival times. For this reason, all controls were aver-
aged, and experimental groups were compared with these controls. Where experimental groups were repeated, the results of these studies were also averaged. In general, studies done in this laboratory consist of smaller \( n \) (typically 6 mice per treatment arm), but the studies are repeated to demonstrate consistency of the results. When the studies are repeated, the sample size is considered to be 12. Animal health was checked daily, and antitumor activity was evaluated as follows: treated \textit{versus} control \% = median survival time (MST) of treated group/MST of control group \( \times 100 \).

**Histologic Study.** Cohesive NCI-H460 tumors were harvested on the last day of treatment for the subcutaneous xenograft model. For the intrapleural xenograft model, lung and heart tissue was removed at day 7 after tumor implantation and the day of last treatment. All tumors and tissues were fixed in formalin and embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin (H&E) and assessed for immunohistochemical expression of Bcl-2 (mouse monoclonal antihuman Bcl-2; DAKO, Glostrup, Denmark) using the ABC peroxidase labeling procedure (DAKO). Microscopic evaluation and scoring were carried out by a pathologist blinded to the treatment group.

**Statistical Analyses.** An unpaired Student’s \( t \) test (parametric) was used to measure statistical significance between two treatment groups. Multiple comparisons were done using a one-way analysis of variance and \textit{post hoc} test that compared different treatment groups by the Scheffe test criteria (Statistica release 4.5; Soft Inc., Tulsa, OK). A comparison of the survival curves among all of the treated and control groups was performed with Kaplan-Meier survival analysis, which takes censored values into account (SPSS release 12.0; SPSS Inc., Chicago, IL). Data were considered significant for \( P < 0.05 \).

**RESULTS**

**Antitumor Efficacy of Oblimersen in the Subcutaneous Tumor Model.** The antitumor effects of oblimersen in SCID-RAG2M mice bearing established subcutaneous NCI-H460 human NSCLC tumors are summarized in Fig. 1. Saline-treated control tumors grew reproducibly from a measurable size of 0.05 cm\(^3\) to 0.60 cm\(^3\) within approximately 13 days (\( \sim \) 25–27 days after tumor cell inoculation). Comparing treatment effects measured on day 13 of this study indicated that oblimersen significantly \( (P < 0.05) \) inhibited tumor growth. The therapeutic effects were dose dependent; the mean tumor sizes on day 13 were 0.60, 0.27, and 0.16 cm\(^3\) in saline and 5 and 10 mg/kg oblimersen treatment groups, respectively. In contrast, administration of Bcl-2 RPO at 10 mg/kg provided no therapeutic activity. None of the mice treated with oblimersen displayed any significant signs of toxicity.

We assessed the consequences of oblimersen treatment by examining tumor tissue by histologic (H&E staining) and immunohistochemical analysis of Bcl-2 expression in sections derived from tumors obtained on the last day of treatment (day 19). Tumors from mice treated with 10 mg/kg oblimersen, Bcl-2 RPO, or saline were evaluated, and representative sections are shown in Fig. 2. Tumors from control (saline-treated) mice or RPO-treated mice were composed of densely packed, viable tumor cells with a very small number of dead cells (Fig. 2A, left and middle panels). The tumors also exhibited extensive Bcl-2 protein expression (Fig. 2B, left and middle panels). In contrast, tumors from animals treated with oblimersen showed an increase in the number of dead cells and exhibited regions of necrosis compared with controls. Necrosis was identified morphologically by areas of cells characterized by amorphous shape and condensed nuclear material (Fig. 2A, right panel). Bcl-2 protein expression in these tumor sections was reduced (Fig. 2B, right panel), compared with control groups.

Mice bearing established NCI-H460 tumors were treated with a combination of oblimersen and VNB. Treatment was initiated when tumor size was approximately 50 mm\(^3\). Fig. 3A presents the \textit{in vivo} efficacy results obtained after treatment with VNB given Q4D \( \times 3 \) at 5 and 10 mg/kg doses. The two regimens induced significant tumor growth suppression \( (P < 0.05) \) in a dose-dependent manner (Fig. 3A) without showing significant signs of undesirable toxicity (i.e., body weight loss). On day 13, mean tumor sizes were 0.60, 0.28, and 0.10 cm\(^3\) in saline and 5 and 10 mg/kg VNB treatment groups, respectively (Fig. 3A). The antitumor activities of VNB were partially associated with down-regulation of Bcl-2 protein expression in these tumors, as judged by immunohistochemistry (data not shown).

When oblimersen treatment was combined with VNB (5 mg/kg), an even more pronounced delay of NCI-H460 tumor growth was observed compared with either treatment administered alone (Fig. 3B). On day 13, mean tumor sizes were 0.60, 0.16, and 0.11 cm\(^3\) in groups treated with saline alone or with 5 or 10 mg/kg oblimersen in combination with VNB (5 mg/kg), respectively. The mean tumor sizes in mice treated with 5 mg/kg VNB in the presence or absence of oblimersen (5 and 10 mg/kg) on day 17 were also compared (Fig. 3B). The tumor sizes in the group of combined VNB and 5 or 10 mg/kg oblimersen were 0.28 and 0.15 cm\(^3\), respectively.
which were significantly ($P < 0.05$) smaller than the tumor size of 0.42 cm$^3$ in the groups treated with 5 mg/kg VNB alone (Fig. 3B). Bcl-2 RPO did not enhance the activity of VNB. These data suggest one of two possibilities: (a) Oblimersen increased the chemosensitivity of the NSCLC subcutaneous tumor model to VNB; or (b) VNB increased the sensitivity of the NSCLC tumor model to the antitumor effects of oblimersen. These mice did not show any significant signs of overt toxicity, and weight loss was not increased by combination with oblimersen. However, in all treatment groups, tumor growth persisted after an initial response, despite concurrent oblimersen and/or VNB treatment.

**Fig. 2** Histopathology of NCI-H460 tumors implanted in SCID-RAG2M mice after 10 mg/kg systemic dosing of oblimersen (Bcl-2 AS; right panels), RPO control sequence (Bcl-2 RP; middle panels), or saline control (left panels) for 19 days. A. Tumor sections were stained with H&E. Representative tumor pictographs are shown (taken with a $\times 10$ objective). B. Bcl-2 protein expression in tumor sections stained using immunohistochemistry; representative tumor pictographs are shown (taken with a $\times 40$ objective).

![Histopathology of NCI-H460 tumors](image)

**Fig. 3** Treatment of NCI-H460 tumors xenografts in SCID-RAG2M mice. Methods of tumor inoculation, treatment schedules, and tumor measurement were as described in Material and Methods. A, antitumor efficacy of VNB (5 or 10 mg/kg). VNB treatment resulted in significantly lower tumor weights on day 13 compared with controls (5 mg/kg VNB, $P < 0.03$; 10 mg/kg VNB, $P < 0.0018$). B, antitumor efficacy of VNB (5 mg/kg) or oblimersen (5 or 10 mg/kg) alone and in combination. The combination of VNB (5 mg/kg) + oblimersen (5 or 10 mg/kg) was significantly ($P < 0.05$) better than VNB alone when comparisons were made based on tumor sizes determined on day 13 and 17. All data are expressed as means $\pm$ SE ($n = 6$ mice per group). Arrows, days of VNB or oblimersen + VNB administration.

![Treatment of NCI-H460 tumors](image)
Antitumor Activity of Oblimersen and Vinorelbine in the Orthotopic NCI-H460 Model. After inoculation of 10^6 NCI-H460 tumor cells into the pleural cavity, numerous tumor nodules were observed on the inner surface of ribs, diaphragm, lung, and heart within 1 week. Local invasion of lung and heart tissues by tumor cells was observed in 100% of mice and confirmed by histologic analysis on day 7 (data not shown). When these animals were treated with oblimersen given daily at 5 and 10 mg/kg for 15 doses, a significant therapeutic response was observed (Fig. 4). In contrast with untreated animals, oblimersen treatment at 5 and 10 mg/kg significantly increased the MST of these NCI-H460 tumor-bearing mice from 21 days to 33 and 36 days, respectively (Table 1). Nonetheless, no long-term survivors were observed. In contrast to oblimersen-treated animals, however, administration of Bcl-2 RPO at 10 mg/kg provided no therapeutic activity (Fig. 4). Saline- and Bcl-2 RPO-treated control animals were terminated or died of progressive tumor growth by day 29 after tumor cell inoculation with a MST of 21 days (Table 1).

An evaluation of H&E-stained sections of lung and heart of these control animals demonstrated that tumors intensively invaded these tissues (Fig. 5A, left and middle panels). In contrast, these tissues exhibited little tumor growth and reduced invasion in animals given oblimersen treatment at 10 mg/kg (Fig. 5A, right panel). As noted in the methodology, all H&E-stained sections from groups of six control and treated mice were scored qualitatively by a pathologist blinded to the treatment groups. Results showed limited disease in the lung and no detectable disease in the heart. In addition, Bcl-2 protein expression was assessed by immunohistochemical methods. The results demonstrated that lung and heart tissues from control mice or RPO-treated controls exhibited obvious Bcl-2 protein expression (see Fig. 5B, left and middle panels). In contrast, the lung and heart tissue from oblimersen-treated mice showed a complete lack of Bcl-2 protein expression (Fig. 5B, right panel), suggesting that the oblimersen antitumor effect was mediated through down-regulation of Bcl-2 protein expression. Positive-staining leukocytes within tissue provided positive internal controls of Bcl-2 expression. These cells, normal benign bronchial columnar cells and light staining striated muscle, were not included in the Bcl-2 score.

To determine whether improved therapeutic effects could be provided when oblimersen was used in combination with VNB in the NCI-H460 orthotopic model, the therapeutic effect of oblimersen at 5 mg/kg with VNB (2.5 and 5 mg/kg) was compared with the effect of the agents individually. In the absence of oblimersen, VNB treatment at 2.5 and 5 mg/kg significantly increased the MST of NCI-H460 tumor-bearing mice from 21 days to 24 and 35 days, respectively (Table 2). The combination of oblimersen at 5 mg/kg with VNB (2.5 and 5 mg/kg) produced an even more pronounced increase in MST (Table 2). In addition, more long-term survivors were observed among animals treated with the combination (Fig. 6). The combined treatment resulted in 17% and 33% long-term survivors (≥90 days) and dose-dependent therapeutic activity when VNB was administered at 2.5 and 5 mg/kg in combination with oblimersen at a dose of 5 mg/kg (every day × 5 for 3 weeks). In contrast, administration of Bcl-2 RPO at 5 mg/kg with VNB at 5 mg/kg did not provide any increase in MST compared with VNB treatment alone (Table 2). 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 histologic examination of tissues. Selected mice that survived as a consequence of treatment received reinoculations of 1 × 10^6 NCI-H460 cells to assess whether the SCID animals had developed an immune response to the inoculated tumor cells. These animals died within 28 days.

DISCUSSION

Most anticancer drugs induce activation of apoptotic pathways as part of their cytotoxic activity (28). Relevant progress has been made in understanding the events underlying drug-induced apoptosis, and the Bcl-2 family of proteins plays a crucial role in regulating this process (29, 30). Overexpression of the antiapoptotic molecule Bcl-2 can engender resistance to anticancer drugs (29, 31), and, conversely, use of oblimersen to reduce Bcl-2 levels has been proven to be directly cytotoxic and to enhance chemosensitivity. These effects have been documented in vitro for morphologically distinct NSCLC cell lines (14, 25), small-cell lung cancer cell lines (32), and cell lines derived from other tumor types (6). Several studies on the in vivo biological effects of oblimersen have also been reported, and only a few of these are referenced here (22, 32–36). In this study, the therapeutic effects of oblimersen, alone and in combination with VNB, are described using a subcutaneous and an orthotopic NSCLC tumor model. These results are correlated with ASO-mediated suppression of Bcl-2 protein in tumor tissue. The studies presented suggest that the combination appears to be more effective than either individual agent used alone.
The immunohistochemical results data presented here demonstrate that daily intraperitoneal oblimersen (QD × 5 days for 3 weeks) is capable of achieving qualitatively substantial down-regulation of Bcl-2 expression in the subcutaneous solid tumor model as well as in the orthotopic model, where cells grew in the lung and heart (Figs. 2B and 5B). Furthermore, treatment was also associated with significant antitumor activity. The effects appeared to be specific for oblimersen because no therapeutic response was observed in animals treated with the reverse sequence control Bcl-2 RPO. Importantly, the RPO sequence used did contain the immunostimulatory CpG motifs, suggesting that the activity seen in this NCI-H460 model was not due to nonspecific immune stimulation. Histologic assessment of tumors derived from mice treated with oblimersen demonstrates significant levels of tumor cell death and suggests that the decrease in tumor size and increase in survival of animals treated with oblimersen were caused by tumor cell kill associated with down-regulation of Bcl-2 protein (Figs. 1, 2, 4, and 5).

### Table 1 Effect of saline, RPO control sequence, or oblimersen (Bcl-2 ASO) treatment on SCID-RAG2M male mice bearing NCI-H460 cells inoculated intrapleurally

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>n</th>
<th>MST (days)</th>
<th>% 90-day survival</th>
<th>T/C % *</th>
<th>P †</th>
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<tr>
<td>None</td>
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<td>NA</td>
<td>26</td>
<td>21</td>
<td>0</td>
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<tr>
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<td>QD</td>
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<tr>
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<td>QD</td>
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<td>33</td>
<td>0</td>
<td>157</td>
<td>&lt;0.0001</td>
</tr>
<tr>
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<td>10</td>
<td>QD</td>
<td>9</td>
<td>36</td>
<td>0</td>
<td>171</td>
<td>&lt;0.0001</td>
</tr>
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</table>

Abbreviations: MST, median survival time; NA, not applicable; QD, daily treatment for 15 days, excluding weekends.

* T/C (%) = MST of treated mice/MST of untreated mice × 100%.

† P values were derived by using the log-rank test comparing each group versus control.

‡ Not significant when compared with control animals.

Fig 5 Histopathology of lung and heart tissues from SCID-RAG2M mice that received intrapleural inoculation with 1 × 10⁶ NCI-H460 cells and were treated with oblimersen (Bcl-2 ASO), RPO control sequence, or saline control. A. Lung (top panels) and heart (bottom panels) sections were stained with H&E. Representative pictographs are shown (taken with a ×10 objective). B. Bcl-2 protein expression in lung (top panels) and heart (bottom panels) sections stained using immunohistochemistry; representative tissue pictographs are shown (taken with a ×40 objective). Saline-treated control (left panels), Bcl-2 RPO sequence treatment (middle panels), and oblimersen Bcl-2 ASO treatment (right panels).
As stated above, some ASOs containing unmethylated CpG motifs within their sequence have been shown to be potent immune stimulators (37, 38). Our results suggest that the therapeutic effects obtained were specific for the oblimersen sequence, confirming the observations by Klasa et al. (35), which likewise showed specific efficacy due to the G3139 molecule and not Bcl-2 RPO in perforin-knockout (natural killer-deficient) SCID mice bearing systemic follicular lymphoma. In combination, these results argue against possible immunostimulatory action, which is dependent on functional natural killer activity for oblimersen used here. Furthermore, others have shown that a substitution of unmethylated CpG motifs within the oblimersen sequence with 5-methylation of cytosine (Genta G4232) to abrogate immune stimulation resulted in an antitumor activity similar to that of the oblimersen (39). It should be noted that mice treated with oblimersen had significantly increased spleen weight relative to those treated with G4232 or saline, a phenomenon that has been associated with immune stimulation (40). We have observed that when oblimersen was given to non–tumor-bearing and tumor-bearing SCID mice, spleen weight increased significantly in both groups. Spleen weight in both groups returned to normal within 1 month after the end of treatment (data not shown). It is important to note that this oblimersen-associated splenic hyperplasia may be specific to murine species because it was not observed in primates and has not been witnessed in the clinical setting in humans. It is also important to note that the RPO ASO control sequence used in these studies caused increases in spleen weight that were not significantly different than those seen in the oblimersen-treated group. Although spleen weight increases are a very crude indication of immune stimulation, by this measurement both sequences used were immunostimulatory, yet only oblimersen exhibited significant therapeutic effects.

The results presented do not exclude the possibility that some of the therapeutic effects of oblimersen are due to immune stimulation. Perhaps more importantly, it is acknowledged here that the therapeutic potential of oblimersen is enhanced due to the fact that it likely mediates antitumor activity through several mechanisms. As shown here and by others (34, 41–43), oblimersen effectively inhibits expression of Bcl-2. It is accepted that the oligonucleotide sequence used can stimulate the immune system in a manner that can indirectly result in antitumor effects. It is also possible that the immune effects engendered by administration of oblimersen may directly influence induction of apoptosis (44).

VNB, a Vinca alkaloid antineoplastic agent derived from Madagascan periwinkle leaves, functions by interfering with microtubule assembly (45). It appears to have preferential selectivity for mitotic microtubules as opposed to axonal microtubules (46). VNB is approved for use as a single agent and in combination with cisplatin for patients with stage IV NSCLC, where it is associated with a 15% to 25% and a 30% to 35% overall response rate, respectively (47). Moreover, its acceptable side effect profile as a single agent makes it suitable for patients of poorer performance status (Eastern Cooperative Oncology Group performance status = 2; ref. 48). The combination of the anticancer agent VNB with oblimersen presents an additional therapeutic strategy for the treatment of this challenging disease.

Table 2  Treatment of SCID-RAG2M male mice that received inoculations of 10⁶ NCI-H460 cells in pleural cavity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Schedule</th>
<th>n</th>
<th>MST (days)</th>
<th>% 90-day survival</th>
<th>T/C% *</th>
<th>P †</th>
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<tbody>
<tr>
<td>Saline</td>
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<td>VNB</td>
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<td>Days 7, 11, 15</td>
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<td>0</td>
<td>114</td>
<td>0.0051</td>
</tr>
<tr>
<td>VNB</td>
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<td>Days 7, 11, 15</td>
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<td>35</td>
<td>0</td>
<td>167</td>
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</tr>
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<td>QD + days 7, 11, 15</td>
<td>6</td>
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<td>0</td>
<td>167</td>
<td>&gt;0.05 ‡</td>
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<td>ASO + VNB</td>
<td>5 + 2.5</td>
<td>QD + days 7, 11, 15</td>
<td>6</td>
<td>37</td>
<td>17</td>
<td>154</td>
<td>0.019</td>
</tr>
<tr>
<td>ASO + VNB</td>
<td>5 + 5</td>
<td>QD + days 7, 11, 15</td>
<td>6</td>
<td>62</td>
<td>33</td>
<td>177</td>
<td>0.00005</td>
</tr>
</tbody>
</table>

Note. Animals were treated with saline control, VNB alone, or in combination with RPO control sequence or oblimersen (Bcl-2 ASO). VNB was injected intravenously for three doses after tumor inoculation, and ASOs were given intraperitoneally.

As stated above, some ASOs containing unmethylated CpG motifs within their sequence have been shown to be potent immune stimulators (37, 38). Our results suggest that the therapeutic effects obtained were specific for the oblimersen sequence, confirming the observations by Klasa et al. (35), which likewise showed specific efficacy due to the G3139 molecule and not Bcl-2 RPO in perforin-knockout (natural killer-deficient) SCID mice bearing systemic follicular lymphoma. In combination, these results argue against possible immunostimulatory action, which is dependent on functional natural killer activity for oblimersen used here. Furthermore, others have shown that a substitution of unmethylated CpG motifs within the oblimersen sequence with 5-methylation of cytosine (Genta G4232) to abrogate immune stimulation resulted in an antitumor activity similar to that of the oblimersen (39). It should be noted that mice treated with oblimersen had significantly increased spleen weight relative to those treated with G4232 or saline, a phenomenon that has been associated with immune stimulation (40). We have observed that when oblimersen was given to non–tumor-bearing and tumor-bearing SCID mice, spleen weight increased significantly in both groups. Spleen weight in both groups returned to normal within 1 month after the end of treatment (data not shown). It is important to note that this oblimersen-associated splenic hyperplasia may be specific to murine species because it was not observed in primates and has not been witnessed in the clinical setting in humans. It is also important to note that the RPO ASO control sequence used in these studies caused increases in spleen weight that were not significantly different than those seen in the oblimersen-treated group. Although spleen weight increases are a very crude indication of immune stimulation, by this measurement both sequences used were immunostimulatory, yet only oblimersen exhibited significant therapeutic effects.

The results presented do not exclude the possibility that some of the therapeutic effects of oblimersen are due to immune stimulation. Perhaps more importantly, it is acknowledged here that the therapeutic potential of oblimersen is enhanced due to the fact that it likely mediates antitumor activity through several mechanisms. As shown here and by others (34, 41–43), oblimersen effectively inhibits expression of Bcl-2. It is accepted that the oligonucleotide sequence used can stimulate the immune system in a manner that can indirectly result in antitumor effects. It is also possible that the immune effects engendered by administration of oblimersen may directly influence induction of apoptosis (44).

VNB, a Vinca alkaloid antineoplastic agent derived from Madagascan periwinkle leaves, functions by interfering with microtubule assembly (45). It appears to have preferential selectivity for mitotic microtubules as opposed to axonal microtubules (46). VNB is approved for use as a single agent and in combination with cisplatin for patients with stage IV NSCLC, where it is associated with a 15% to 25% and a 30% to 35% overall response rate, respectively (47). Moreover, its acceptable side effect profile as a single agent makes it suitable for patients of poorer performance status (Eastern Cooperative Oncology Group performance status = 2; ref. 48). The combination of the anticancer agent VNB with oblimersen presents an additional therapeutic strategy for the treatment of this challenging disease.

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It is interesting to note that treatment with VNB did cause an apparent down-regulation of Bcl-2, as judged by immunohistochemistry. This was observed in both subcutaneous and intrapleur al tumor models. There is no evidence that Bcl-2 expression is a predictor of response to VNB in the clinical setting (49). Despite this, there is indirect evidence associating VNB with altered apoptosis signal transduction pathways (50), and it has been suggested that VNB induces apoptosis via Bcl-2 phosphorylation in MCF7 cells (51). The precise means by which VNB induces a decrease in Bcl-2 protein expression in this model warrants further study, but the observation suggests that oblimersen and VNB act at least in an additive manner on a Bcl-2-dependent apoptotic pathway in bringing about cell death. To better define the type of interactions these two agents exhibit, further dose titration information is required. As noted by others, dose effects in vitro and in vivo are often nonlinear and a dose that is synergistic (or superadditive) at one affect level may not be synergistic at another dose level. The only way to gain this understanding is through dose-effect curves, and it is critical to identify combinations that appear to be additive or synergistic at maximum effect levels.

The data presented here add weight to the assertion that anticancer drugs are more effective in combination than as single agents. Traditional anticancer treatment protocols contain mixtures of several non–cross-resistant cytotoxic agents (52), but their application is limited by broad toxicities, and their use is precluded in patients with poor performance status. The possibility of combining cytotoxic chemotherapy with more rational molecularly targeted treatment has excited medical oncologists and cancer biologists alike, but attempts at doing so in clinical trials have shown inconsistent results. Most recently, promising results have been obtained using a combination of oblimersen with dacarbazine in the setting of metastatic melanoma (23), where the response rate and progression-free survival were significantly longer in the G3139 treatment arm with an early trend to improved survival (53). The data presented here suggest that such a combination can likewise be effective in the setting of metastatic NSCLC, a malignancy also associated with a poor outcome. Currently, single-agent VNB in the NSCLC setting is reserved for the more elderly and frail patient deemed not to be a candidate for “doublet” chemotherapy. The possibility of improving response rate and prolonging survival in such patients without incurring significant additional side effects is obviously attractive.

To date, clinical trials involving oblimersen have used a schedule that predoses patients for several days with the ASO, occasionally using continuous infusion pumps, before administering the cytotoxic drug. The intention is to cause sufficient down-regulation of Bcl-2 protein and lower the apoptotic threshold in the malignant cells and improve cell kill (6). In this study, no such pretreatment was given, and the drugs were administered concurrently with impressive results in inhibiting tumor growth and down-regulating Bcl-2 protein expression. Whether this effect is specific only for this combination or suggests that oblimersen can lower the apoptotic threshold more rapidly than previously assumed is unclear. However, in designing a schedule for clinical trials of this combination of drugs, it may be possible to dispense with the pretreatment protocol.

Predicting which tumors will respond to treatment has always been an important goal in cancer therapeutic agents, not only to minimize unwarranted side effects but also to reduce the costs incurred by health care systems. This study did not address the issue of correlating response to oblimersen to the level of Bcl-2 protein or mRNA. The NCI-H460 NSCLC cell line is known to be a express high levels of Bcl-2 and may not be typical of all NSCLC tumors (4). However, it has been shown that anti Bcl-2 strategies are effective in cells that express relatively low amounts of Bcl-2 protein (33). With this in mind, further work is required to assess potential predictive molecular markers of response to oblimersen.

The data presented here are the first to address the potential role of ASO directed at the Bcl-2 gene message alone in enhancing the therapeutic efficacy of VNB in both models of NSCLC. This study successfully used this strategy to increase the number of long-term survivors (33%) when mice were treated at a point when they had systemically distributed disease. Moreover, the data suggest that improved clinical outcomes could be achieved with standard or even lower doses of anticancer drugs when combined with oblimersen, potentially impacting overall clinical tolerability and costs of care. Further investigations, possibly leading to clinical trials of these drugs in combination in metastatic NSCLC, are warranted.

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REFERENCES

Bcl-2 Antisense against Xenograft Lung Cancer Models


Antitumor Efficacy of Oblimersen Bcl-2 Antisense Oligonucleotide Alone and in Combination with Vinorelbine in Xenograft Models of Human Non–Small Cell Lung Cancer

YanPing Hu, Gwyn Bebb, Sophia Tan, et al.


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