The Combination of the Proteasome Inhibitor Bortezomib and the Bcl-2 Antisense Molecule Oblimersen Sensitizes Human B-Cell Lymphomas to Cyclophosphamide

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Abstract

Purpose: To determine whether the combination of the proteasome inhibitor bortezomib and the bcl-2 antisense molecule oblimersen can sensitize human lymphoma to cyclophosphamide.

Experimental Design: Cytotoxicity assays were conducted to determine if there was any additive or synergistic interaction between the combinations of bortezomib, oblimersen, and cyclophosphamide using a standard trypan blue exclusion assay. Based on these experiments, *in vivo* experiments in severe combined immunodeficiency beige mice were done using human lymphoma xenografts in which different schedules were explored. Bcl-2 and oblimersen levels were determined in treated tumors, some of which were resected at the end of the *in vivo* experiment and evaluated pathologically.

Results: The results suggest that the combination of bortezomib and oblimersen seem to interact in at least an additive fashion, and that the addition of cyclophosphamide to this drug combination can markedly improve tumor cell kill. In addition, it seems that these drug combinations may be schedule-dependent, with a requirement for oblimersen pretreatment. Animals treated with the triplet drug combination in a schedule-dependent manner experienced pathologic complete regression of disease, which was not observed in other treatment cohorts. The addition of bortezomib also seemed to increase the levels of intracellular oblimersen, which resulted in a marked reduction in Bcl-2. Histologic studies confirmed marked necrosis and caspase-3 activation only in the cohort receiving all three drugs.

Conclusion: The use of Bcl-2-directed therapy and a proteasome inhibitor sensitizes human lymphoma cells to cytotoxic drugs like cyclophosphamide. This combination may offer new opportunities for integrating novel targeted therapies with conventional chemotherapy.

The recent development of many novel targeted drugs has created unprecedented opportunities to treat human malignancies. Although some of these drugs may play a role in the treatment of cancer as single agents, it is more likely that these drugs will be used in combination with conventional cytotoxic agents. This integration of novel agents theoretically affords us new opportunities to modulate unique cellular targets that play a critical role in tumor growth and survival. What remains daunting is not so much the identification and validation of these targets, or even the discovery of novel small molecules that affect them, but rather, the question of how best to integrate these agents into our conventional treatment paradigms. The increasing "specificity" of many new targeted drugs suggests that these targets will be modulated in a time-dependent and cyclical manner that may be a function of several factors. These may include: the target's intracellular half-life, the target's rate of synthesis and regulation, and the kinetics of the drug-target interaction, for example. It is this discrete modulation of specific targets, often times in a temporal manner, that forms the molecular basis of drug synergy.

Hence, it is likely that many new drugs that target important growth and survival signaling pathways will be synergistic with less specific and broadly damaging conventional agents in a schedule-dependent manner. The determination of these relationships in preclinical development is essential in exploiting and optimizing therapeutic benefit. Often times, such relationships, though well defined in preclinical models, are not respected in clinical trials for "convenience's sake," giving rise to simple concurrent administration approaches in clinical

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application. Preclinical models of cancer can play a major role in clarifying these pharmacologic relationships prior to clinical study.

Proteasome inhibitors (bortezomib) and drugs targeting Bcl-2 family members (oblimersen) represent two prime examples of these principles. Studies of oblimersen on Bcl-2 over-expressing lymphoma cell lines such as DoHH2 and SU-DHL-4 *in vitro* have shown down-regulation of the message and a consequent decrease in protein expression (1, 2). Although bcl-2-targeted drugs have antitumor activity, it is the idea that they can lower the threshold for apoptosis, sensitizing cells to cytotoxic therapy, which forms a major focus for their future development. Biologically, for the cytotoxic therapy to have its best effects, levels of Bcl-2 will need to be maximally down-regulated in order for the second drug to have maximal benefit, an observation confirmed by other preclinical studies (3). Given that the half-life of Bcl-2 is ~22 hours (4), one would estimate that at least three to five half-lives would need to pass (66-110 hours) before the protein levels would be sufficiently lowered to favor induction of apoptosis. Theoretically, giving a short half-life drug at the same time as the initial dose of an anti–Bcl-2-targeted therapy may not be associated with the optimal antitumor effects. Similarly, proteasome inhibitors are known to affect a vast array of intracellular processes. Proteasome inhibitors are also known to up-regulate proapoptotic family members (5). It is this constellation of effects on both proapoptotic and antiapoptotic pathways in cancer cells that we sought to exploit by understanding the importance of these novel agents in combination with a broad DNA-damaging drug like cyclophosphamide.

Materials and Methods

Materials. Oblimersen was obtained from Genta Pharmaceuticals (Berkeley Heights, NJ) and was supplied reconstituted in normal saline at a concentration of 30 mg/mL. Bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA), supplied as a lyophilized powder which was reconstituted in PBS at a concentration of 1 mg/mL. Cyclophosphamide was supplied as lyophilized drug product from the Memorial Sloan Kettering Cancer Center pharmacy, and was reconstituted in sterile water at a final concentration of 25 mg/mL. 4-Hydroxycyclophosphamide was obtained as a lyophilized powder from Squarix Biotechnology (Marl, Germany) and was used in all *in vitro* experiments.

SKI-DLCL-1 is a diffuse large B-cell lymphoma which has been characterized as reported previously (6). RL is a transformed large B-cell lymphoma cell line that carries the t(14;18) translocation. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in suspension culture in RPMI medium plus 20% fetal bovine serum, 10 nmol/L HEPES, P + S, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 4.5 g/L L-glucose from the Memorial Sloan Kettering Cancer Center media lab.

Cytotoxicity assays. For all *in vitro* assays, cells were counted and resuspended at an approximate concentration of 3×10^5 cells per well. Bortezomib, oblimersen, 4-hydroxycyclophosphamide, or LipofectAMINE were added at varying concentrations. Cells were counted using the trypan blue exclusion assay following at least 72 hours incubation. In all experiments, between 1×10^5 and 5×10^5 cells per milliliter were counted.

Antisense transfection assay. To incorporate the oligonucleotide into the cell lines, it was necessary to transfect the cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Approximately 5×10^5 cells were plated in 12-well plates in serum-free and antibiotic-free RPMI. One microgram of oblimersen was mixed in a 1:2 ratio with the LipofectAMINE for 20 minutes at room temperature. Following the incubation period, the drug-LipofectAMINE combination was added to each well. Plates were incubated for 6 hours at 37°C at 5% CO_2, at which point 1.5 mL of RPMI containing 20% fetal bovine serum, 10 nmol/L HEPES, P + S, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 4.5 g/L L-glucose was added to each well. Cells were then incubated, and counted via trypan blue exclusion assay at 24, 48, and 72 hours to evaluate the dose-response relationship as a function of time.

In vivo *xenograft models.* Five- to 7-week-old severe combined immunodeficiency (SCID) beige (CBSCBG-MM double) mice were obtained from Taconic Laboratories, Germantown, NY. Animals were maintained in a core animal facility under an institution-approved animal protocol. All experiments were done in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1085). Mice were injected with 1×10^7 SKI-DLCL-1 or RL cells on the flank via a s.c. route. When tumor volumes approached 50 mm^3, mice were separated into treatment groups of five mice each.

All data are expressed as the average tumor volume (mm^3) per group as a function of time. Tumors were assessed using the two largest perpendicular axes (l, length; w, width) as measured with standard calipers. Tumor volume was calculated using the formula $4/3\ \Pi r^3$, where $r = (l + w) / 4$. A formal statistical comparison between groups was done as described below. Tumor-bearing mice were assessed for weight loss and tumor volume at least twice weekly for the duration of the experiment. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm, or after loss of >10% body weight in accordance with institutional guidelines. Animals were housed in standard shoebox cages in temperature- and humidity-controlled rooms on a 12-hour light and dark cycle. Food and water were supplied ad libitum. Alzet miniature infusion pumps were used in one experiment for continuous delivery of oblimersen over a 28-day period. Alzet pumps (model 2004, Durect, Cupertino, CA) were filled with oblimersen and allowed to prime in saline for 40 hours according to the manufacturer's directions. Pumps were then implanted s.c. in the back of the mice, and removed ~32 days after implantation.

Immunohistochemistry. All immunohistochemistry samples were prepared by the Sloan Kettering Pathology Core Facility. All antibodies were purchased from Cell Signaling (Beverly, MA). Paraffin-embedded tissue sections were cut into 4 to 5 μm sections, placed on Superfrost/Plus microscope slides (Fisher, Pittsburgh, PA) and baked at 60°C for 60 minutes. Slides were then deparaffinized and hydrated with distilled water. Pretreatment protocols are used according to the primary antibody profile.

Immunoblotting. Resected xenografts were suspended in ice-cold PBS and homogenized in ice-saline. Drug-treated cells were harvested and sonicated in 1× cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2 EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium PPi, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 g/mL leupeptin; Cell Signaling Technology, Beverly, MA] supplemented with protease inhibitors (protease inhibitor cocktail set III; Calbiochem-Novabiochem Corporation, La Jolla, CA) and 2 mmol/L of phenylmethylsulfonylfluoride. After centrifugation at 16,000 × g for 5 minutes at 40°C, the total proteins in each sample were quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), denatured at 100°C for 5 minutes in 1× Laemli buffer, separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Bioscience, Piscataway, NJ). The blots were blocked in TBST [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat dried milk powder, and subsequently incubated with antibody to bcl-2 (M0887; Dako Cytomation, Carpinteria, CA) or species-specific IgG horseradish peroxidase secondary antibodies (Upstate Biotech, Waltham, MA). Proteins recognized by the antibodies were detected using the chemiluminescent detection kit (Pierce, Rockford, IL). Equivalent gel loading was confirmed by probing with antibody against β-actin.

Determination of intracellular oblimersen levels: reagents and standards. Details of these methods are as described elsewhere (7).

The standard of G3139 (5′-TCT CCC AGC GTG CGC CAT-3′) was provided by the National Cancer Institute (Bethesda, MD). The capture oligodeoxynucleotide (5′-GAATAGCGAATGGCGCACGCTGGGAGA/Biotin/-3′) and detection oligodeoxynucleotide (5′-TCG CTA TTC-3′ phosphorylated at the 5′-end and digoxigenin modified at the 3′-end) were obtained from Integrated DNA Technologies (Coralville, IA). Reacti-Bind NeutrAvidin–coated polystyrene plates were purchased from Pierce. T4 DNA ligase (Amersham Bioscience). The anti–digoxigenin-alkaline phosphate was obtained from Roche (Indianapolis, IN). Attophos and its reconstitution solution were purchased from Promega (Madison, WI). Detection was accomplished using a Gemini XS plate reader (Molecular Devices, Sunnyvale, CA). Bicinchoninic Acid kit was purchased from Pierce. The lymphoma cell lystates were sonicated and centrifuged at 10,000 × *g*, the supernatant was transferred to a new tube for the ELISA and protein assay by the Bicinchoninic Acid kit. Intracellular concentration of G3139 in cell lysates was normalized by its protein amount and expressed as pmol/mg protein. Tissue samples were weighed and pulverized while still frozen. Then, the sample was homogenized in 10:1 PBS (v/w) with a tissue homogenizer (Virtis, Gardiner, NY). Proteinase K (2 mg/mL) in buffer containing Tris-HCl (pH 8.0) and 10 mmol/L EDTA was added to the tissue homogenate to digest the tissue component. Samples were then incubated overnight at 37°C. After centrifugation at 10,000 × *g*, the supernatant was used for the ELISA assay. G3139 concentrations in tissue samples were expressed as pmol/g tissue. A fluorogenic ELISA assay was used to determine G3139 concentrations as previously described (7). Briefly, samples containing G3139 were incubated with 200 nmol/L capture probe in assay buffer [60 mmol/L phosphate buffer (pH 7.4), 1.0 mol/L NaCl, 5 mmol/L EDTA, and 0.3% Tween 20] at 42°C for 2 hours to allow hybridization. The mixture was then transferred to a NeutrAvidin-coated 96-well plate (Pierce). After washing away the matrix by washing buffer (TBS in 0.1% Tween 20), 100 nmol/L detection oligodeoxynucleotide diluted in ligation buffer [66 mmol/L Tris-HCl (pH 7.6), 10 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L ATP, 5 units/mL T4 ligase] was added to each well. The plate was incubated at 18°C overnight. After washing with buffer and treatment of 30 units of S1 nuclease (Invitrogen) in 30 mmol/L NaAc (pH 4.6), 1 mmol/L ZnAc, and 150 mmol/L of NaCl for 60 minutes at 37°C, 150 μL of anti-digoxigenin-alkaline phosphate was added into each plate, diluted in 1:2,500 bovine serum albumin block buffer in TBS (Roche) and followed by a 30-minute incubation at room temperature. The plate was washed again and 150 μL of the Attophos substrate was added in each well followed by 30 minutes of incubation at 37°C. Fluorescence intensity was measured at Ex 430 nm/Em 560 (filter = 550 nm) using a Gemini XS plate reader.

Statistical methods. Viable cells counted using the trypan blue exclusion assay and levels of oblimersen are modeled as binomial proportions and displayed with SE bars. Treatment group comparisons were made using pairwise Fisher's exact tests. Xenograft data were analyzed by computing the "relative tumor volume," defined as tumor volume at each measurement divided by the baseline tumor volume of that mouse. This establishes an internal control to account for baseline differences. Statistical calculations are based on the relative tumor volume curve (time versus $\log_{10}$) from which the area under the time-relative volume curve for each mouse was calculated. This is considered as a measure of the total tumor burden over the experiment. We finally divided the area under the curve by the number of days the mouse was under observation, which can be interpreted as an average daily tumor burden. The average daily tumor burden was compared across groups using pairwise Wilcoxon rank-sum tests. Exact reference distributions were used to account for small samples.

Results

In vitro *assays.* Figure 1A and B present the results of three separate trypan blue exclusion assays. Concentrations of bortezomib (B), oblimersen, and 4-hydroxycyclophosphamide (CTX) were intentionally set to approximate the EC_{20} of each drug, based on previously done concentration effect curves for these agents. Overall, cells treated with the single agents produced modest cell death, although clearly some doublets (bortezomib + oblimersen, 4-hydroxycyclophosphamide + bortezomib and 4-hydroxycyclophosphamide + oblimersen) produced slightly more cell death (range, 0-50%), especially when given in an ordered sequence. Interestingly, the 72-hour exposure of all three drugs given simultaneously produced ~40% and 50% cell kill in RL and SKI, respectively, and nearly 70% to 80% cell kill when used in a schedule-dependent manner with oblimersen preceding the addition of C and B by 24 hours, although the error bars seem to overlap. Other scheduled exposures *in vitro* in which oblimersen was given concurrently with either 4-hydroxycyclophosphamide or bortezomib produced only modest cell death. There was a statistically significant difference between the cells treated with the sequenced oblimersen (oblimersen → bortezomib + CTX) compared with the simultaneous treatment cohorts (oblimersen + bortezomib + CTX; $P < 0.001$ for RL and $P = 0.06$ for SKI-DLCL-1), in favor of the sequenced treatment. In the case of RL, there was a statistically significant difference in favor of the oblimersen → bortezomib + CTX treatment cohort compared with all other treatment groups except oblimersen → CTX and CTX → bortezomib ($P < 0.01$).

In vivo *experiments.* A number of *in vivo* experiments were conducted as part of a dose-finding study in order to identify the best doses and schedule of drugs to use in the xenograft experiments. These studies showed that the standard doses of bortezomib used in murine (i.e., SCID beige mouse) experiments (i.e., 1 mg/kg on days 1, 4, 8, and 11 given by the i.p. route) was toxic in these more fastidious immunocompromised mice, and excessively toxic when given with oblimersen, with many animals experiencing irreversible weight loss of >10% of their body weight. For this reason, lower doses with less frequent dosings per cycle were explored in the single and combination cohorts. In these experiments, intentionally subtherapeutic doses of bortezomib were employed to try to circumvent some of the toxicity, and to identify potentially synergistic interactions with other drugs.

Figure 2 depicts the results of an *in vivo* experiment in which lower doses of bortezomib were given at 0.5 and 1 mg/kg only twice within the cycle (that is, at one-quarter and one-half of the maximum-tolerable dose, respectively). These curves show modest growth delay at the lower dose, and a dose-response relationship with B alone (i.e., 1 mg/kg was better than 0.5 mg/kg). The combination of bortezomib and oblimersen produced even more tumor growth delay, achieving approximately one-third of the volume seen in the control animals. Unexpectedly, however, there was minimal difference between the two doses of bortezomib when used in combination with oblimersen. A slightly greater degree of weight loss (0% versus 3-5%) was noted with the higher doses of bortezomib (i.e., 1 mg/kg × two to four doses per cycle) compared with the lower doses of bortezomib (0.5 mg/kg × two doses), prompting the use of the 0.5 mg/kg dose of bortezomib when used in combination with oblimersen. When not given in combination with oblimersen, a bortezomib dose of 1 mg/kg given twice within the cycle was used, whereas animals receiving the triplet drug combinations received one-quarter of bortezomib's maximum-tolerable dose (0.5 mg/kg twice within the cycle).

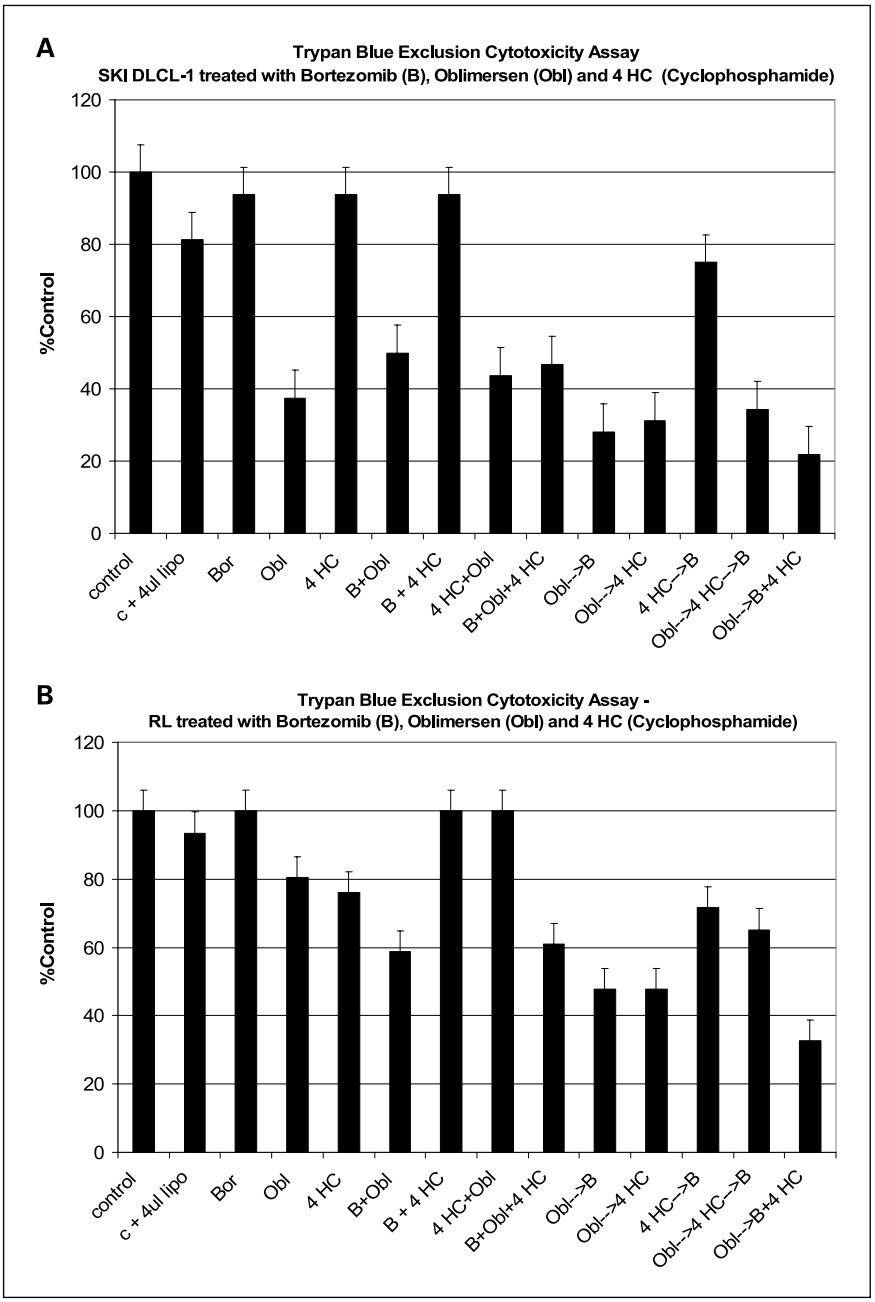

Fig. 1. *A,* results of a trypan blue exclusion assay with the cell line SKI-DLCL-1. *B,* the same experiment with the cell line RL. *Columns,* mean of three different assays; *bars,* ±SE. Bortezomib and cyclophosphamide were added at a concentration of 1 nmol/L, which was estimated to approximate the EC_{20} from the concentration effect curves of these agents in these cell lines. Both assays were evaluated following 72 hours of incubation. In all experiments, between 1×10^5 and 5×10^5 cells per milliliter were counted. Cells were seeded at a density of 3×10^5.

Figure 3 shows the results of a same-day treatment (i.e., simultaneous administration) of all three drugs. These particular experiments are slightly different from all others in that these animals received all drugs on the same day, at dose intensities that are the same as for all other experiments. These studies show that the simultaneous administration of all drugs is associated with a compromise in the treatment outcome. In addition, there was no statistically significant difference between the triplet containing cohort and any other treatment cohort ($P > 0.1$-0.84). Cyclophosphamide, whereas clearly producing the best single agent activity, was still inferior to any doublet except the bortezomib plus oblimersen cohort. Integration of cyclophosphamide clearly improved the activity of bortezomib and oblimersen, although the triplet seemed only modestly more active than any doublet. Animals receiving the triplet drug combination experienced slightly more weight loss (~10-14%) during treatment, although all these animals regained their lost weight during the "rest" week in between cycles.

Figure 4 presents the results of a scheduled treatment experiment in which a 48-hour preexposure of oblimersen was used prior to treatment with CTX and/or bortezomib. This particular experiment produced the best results in favor of the triplet combination, showing that the doublet of oblimersen and bortezomib was similar to what had been noted earlier, and that single agent cyclophosphamide again produced the best results of any singlet, although it was inferior to any doublet. Again, the favorable benefit of the bortezomib plus oblimersen doublet was statistically significant compared with the control ($P < 0.002$). Interestingly, three of five animals in

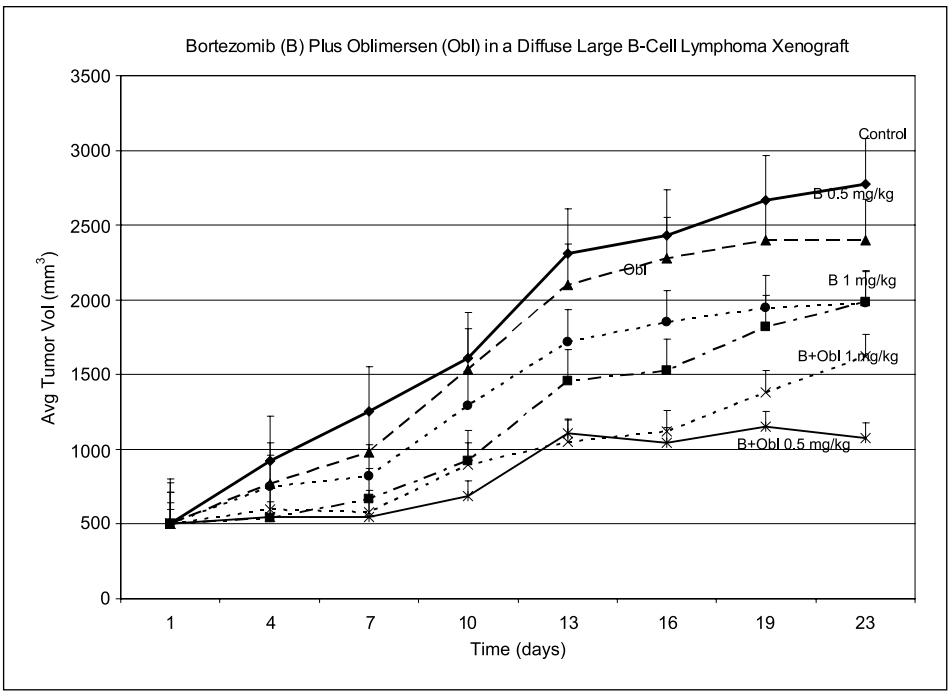

Fig. 2. Bortezomib dose optimization. Changes in tumor volume as a function of time in SCID beige mice implanted with 1×10^7 SKI-DLBCL cells in the s.c. flank. Treatment began ~30 days postimplantation of the lymphoma. Control animals received saline only, animals treated with bortezomib only received 0.5 or 1 mg/kg on days 3 and 7, animals treated with oblimersen only received 3 mg/kg on days 1, 3, 5, and 7, or a combination of bortezomib and oblimersen on the days noted in the single agent cohorts. All drug injections were by the i.p. route.

the triplet cohort experienced pathologically documented complete regression of their disease, whereas one complete regression was also appreciated in the doublet containing CTX and bortezomib. Again, animals in the triplet experienced slightly more weight loss (although <10% of the total body mass) compared with animals receiving only one or two drugs. These results seemed to be sensitive to the dose of cyclophosphamide, in that the same exact experiment conducted with 25 mg/kg of cyclophosphamide produced no cures in any treatment arm, and did not produce significant differences between any doublet or triplet cohort (figure not shown). There was a statistically significant difference between the triplet cohort and every other treatment cohort ($P < 0.01$) in favor of the triplet combination. For example, the P values for the triplet compared with CTX alone, CTX + bortezomib and CTX + oblimersen were 0.009, 0.041, and 0.002, respectively.

Figure 5 presents the results of a continuous infusion experiment of oblimersen using the same dose intensity of oblimersen. Oblimersen was given at a concentration of 10 mg/kg per day for 28 days using a scheduled administration, such as that employed in Fig. 4. These results essentially mirror the earlier observations noted in Fig. 4. Once again, single agent bortezomib produced modest growth delay relative to the control, although in these experiments, the single agent

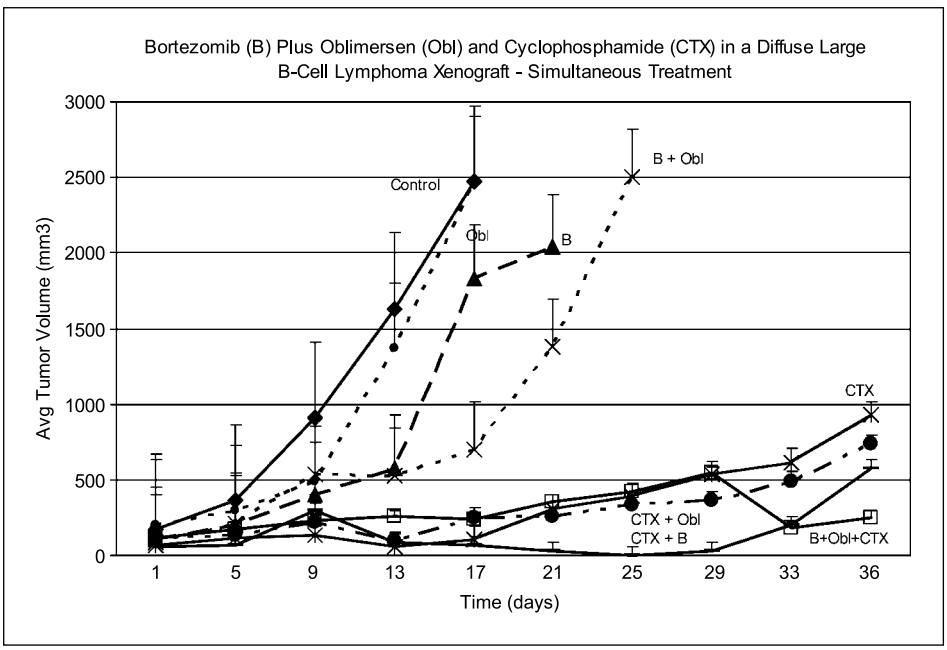

Fig. 3. Simultaneous treatment. Measurement of average tumor volume in SCID beige mice implanted with 1×10^7 SKI-DLBCL cells in the s.c. flank. Treatment began once tumors reached ~250 mm^3. Control animals were treated with saline only, animals treated with bortezomib only received 1 mg/kg on days 1, 4, 7, and 10; animals treated with oblimersen only received 3 mg/kg on days 1, 4, 7, and 10; animals treated with cyclophosphamide only received 50 mg/kg on days 1, 4, 7, and 10. All combination-treated cohorts received the noted drugs on the same days noted above, except that when bortezomib was given in combination with oblimersen, the dose of bortezomib was 0.5 mg/kg. All injections of drug were by the i.p. route.

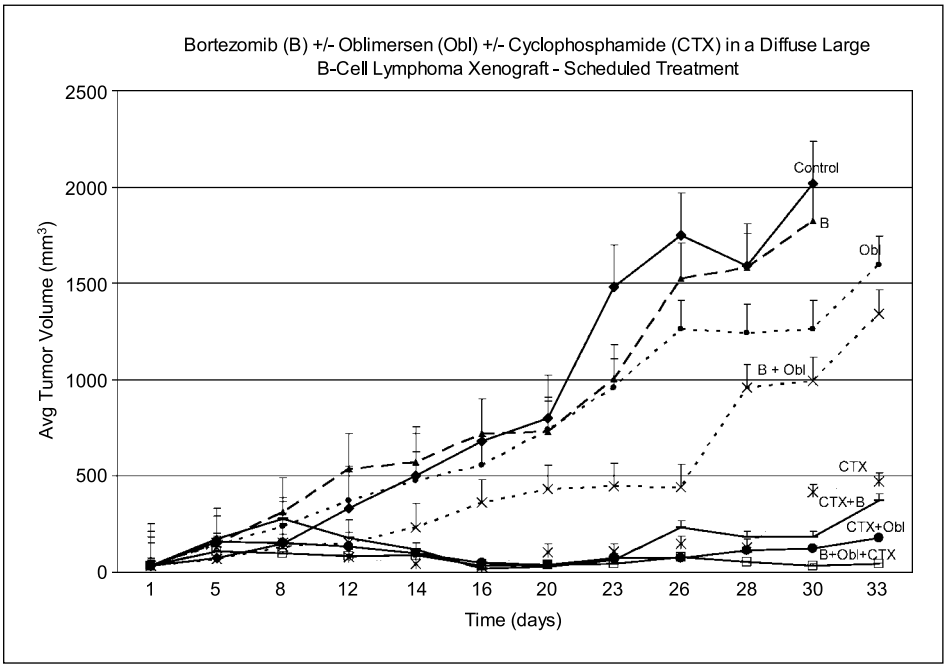

Fig. 4. Scheduled sequential treatment. Measurement of average tumor volume in SCID beige mice implanted with 1×10^7 SKI-DLBCL cells in the s.c. flank. Treatment began once tumors reached ~ 250 mm^3. Control animals received saline only; animals treated with bortezomib only received a dose of 1 mg/kg on days 3 and 7; animals treated with oblimersen only received a dose of 3 mg/kg on days 1, 3, 5, and 7; animals treated with cyclophosphamide only received a dose of 50 mg/kg on days 2, 4, 6, and 8. All combination-treated cohorts received the drugs on the same days noted above, except when bortezomib was given in combination with oblimersen, the dose of bortezomib was always 0.5 mg/kg. All injections of drug were by the i.p. route.

oblimersen seems slightly better. There was a statistically significant difference between the triplet cohort in this experiment and every other treatment cohort ($P < 0.05$), except when compared with the oblimersen + CTX treatment cohort ($P = 0.13$). The continuous infusion approach produced only one mouse with a pathologic complete regression of disease in the triplet combination. However, the burden of surgery and the presence of the large pumps in these immunocompromised mice produced excessive infectious comorbidities in many animals. Under these circumstances, in these particular mouse models, although feasible, this method of drug delivery may be suboptimal compared with more frequent i.p. administrations of oblimersen. The differences between the results seen with the continuous infusion pump and the alternate day administration of oblimersen may be related to differences in the pharmacokinetic profiles achieved with these methods of drug delivery.

Immunohistochemical staining. H&E staining was done on tissue from animals used in the scheduled treatment experiment (Fig. 4). Tumors were removed 6 hours posttreatment midway through cycle 2. These tissues revealed marked necrosis of the lymphoma mass obtained from those animals that did not achieve complete regression of disease in the triplet

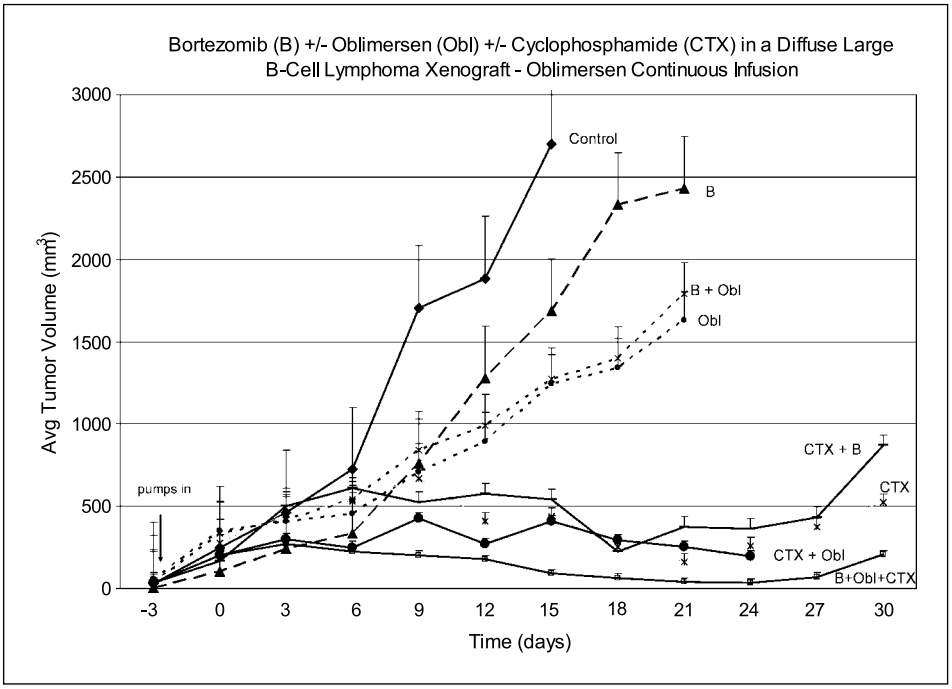

Fig. 5. Continuous infusion experiment. Measurement of average tumor volume in SCID beige mice implanted with 1×10^7 SKI-DLBCL cells in the s.c. flank. Treatment began once tumors reached ~ 250 mm^3. Control animals were treated with saline only; animals treated with bortezomib only received a dose of 1 mg/kg on days 3 and 7; animals treated with oblimersen only received a dose of 10 mg/kg/d by continuous infusion for 28 days; animals treated with cyclophosphamide only received a dose of 50 mg/kg on days 2, 4, 6, and 8. All combination-treated cohorts received the drugs on the same days noted above, except when bortezomib was given in combination with oblimersen, the dose of bortezomib was always 0.5 mg/kg. All injections of drug were by the i.p. route, except for oblimersen, which was given by the s.c. flank pump.

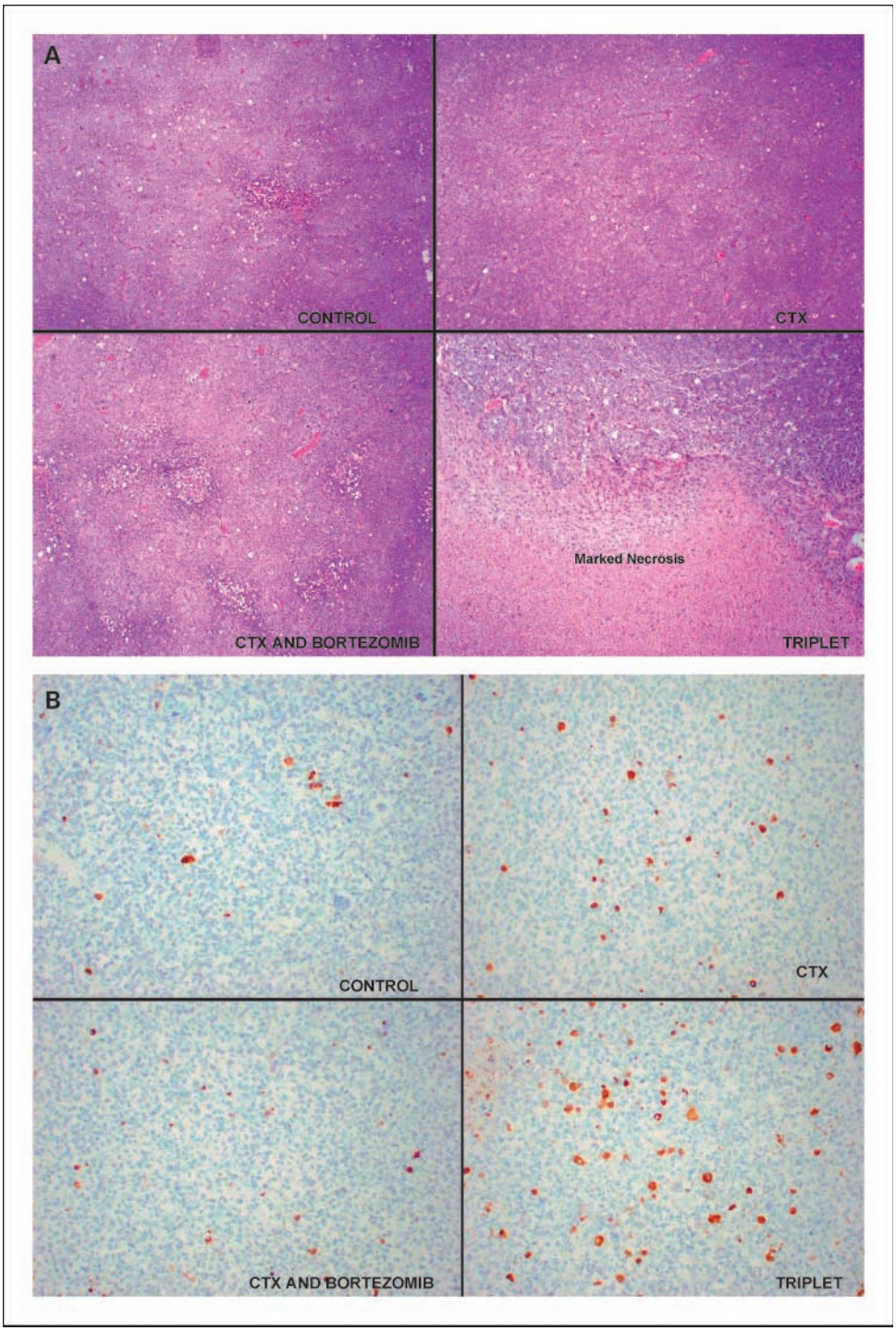

Fig. 6. *A,* H&E staining done on tumor samples from a mouse experiment (*areas in pink,* necrosis). *B,* caspase-3 staining done on tumor samples from a mouse experiment (*areas in red,* presence of apoptotic bodies and increased caspase-3).

combination (i.e., $n = 2$). The pictures from each slide are representative of the entire slide. Tissue from the control animals revealed diffuse infiltration by a large B-cell lymphoma, with no necrosis. Areas of necrosis are seen in pink, whereas normal tumor tissue staining appears dark purple. Animals treated with any single drug exhibited little to no necrosis, whereas those animals receiving any doublet exhibited only mild necrosis (Fig. 6A). Interestingly, markedly more necrosis, in fact, the most seen in any tumor specimen, is clearly appreciated in the resected tumors from the mice receiving the three-drug regimen. In addition, markedly greater caspase-3 activation was appreciated in the triplet cohorts compared with any single or doublet cohort (Fig. 6B). Caspase-3 activation slides show an increased presence of apoptotic bodies in the triplet cohort compared with any doublet, singlet, or control.

Effects of bortezomib on intracellular oblimersen and Bcl-2 levels. Table 1 shows the average concentration of oblimersen (pmol/g tissue) in the RL tumors extracted from mice treated with oblimersen ± bortezomib. These data first show the rapid loss of oblimersen in tumor as a function of time, with the

average levels changing from 5.3 ± 1.5 to 1.3 ± 0.86 pmol/g tissue after only 12 hours (a 75% decrease). Interestingly, combined treatment with bortezomib seems to increase the levels of oblimersen at 12 and 24 hours by 32% and 55%, respectively, an increase that was statistically significant in the tumor samples analyzed 24 hours after treatment (P = 0.02). Samples of these same tumors were also analyzed by Western blot analysis for Bcl-2. These studies show that there seems to be more reduction in the levels of Bcl-2 in the tumors treated with oblimersen at 24 hours compared with the 12-hour samples and the control tumor. Interestingly, the levels of Bcl-2 are at the lowest levels in the tumors treated with both bortezomib and oblimersen at 24 hours (Fig. 7A). Bortezomib alone did not affect Bcl-2 levels in the lymphoma (Fig. 7B). Collectively, these finding support the contention that bortezomib may increase intracellular oblimersen levels, which seem to be associated with a marked reduction in Bcl-2. Furthermore, it suggests that a lead in time of oblimersen exposure might be advantageous, in that the effects of other cytotoxic agents are likely to have their greatest effect when Bcl-2 levels are at their nadir.

Discussion

The concept of targeting both the proteasome and Bcl-2 may represent a unique platform to sensitize tumor cells to the effects of conventional chemotherapy, effects which seem to be schedule-dependent. The relationship between the induction of apoptosis and proteasome inhibition is likely to involve both direct and indirect influences (8). Nuclear factor κB (NF-κB) itself is known to elicit an antiapoptotic effect by suppressing tumor necrosis factor-α. Interestingly, the inhibition of NF-κB nuclear translocation has been found to enhance apoptotic killing by radiation or chemotherapy (9), which may explain some of the observations made in the experiments presented here.

These studies show that the combination of even smaller doses of bortezomib in conjunction with oblimersen seem to affect the growth delay of a diffuse large B-cell lymphoma in SCID beige mice. Integration of cyclophosphamide into this combination adds to the treatment benefit of bortezomib + oblimersen, and when delivered in a schedule-dependent manner, seems to produce pathologic complete remissions. The observation that bortezomib might augment the effects of oblimersen by increasing its intracellular retention was an unexpected finding. Even more encouraging and supportive of the findings in general, is the observation that oblimersen + bortezomib can lead to marked reductions of Bcl-2 in these tumor specimens at 24 hours, which might help explain the need for oblimersen preexposures. Although an explanation for this observation remains elusive at this point, it is conceivable that the proteasome inhibitor might facilitate cellular uptake of the oligodeoxynucleotides, or might even lead to the accumulation of some intracellular protein that might inhibit the degradation of the antisense molecule. Once internalized, oligodeoxynucleotides are sequestered in the endosomal-lysosomal compartment, where only a small proportion of oligodeoxynucleotides actually escapes degradation in the vesicles (7). Proteasome inhibitors could alter this aspect of the oligodeoxynucleotide pharmacology leading to increased intracellular half-life, resulting in more oligodeoxynucleotides actually reaching its intended target. Regardless, the collective *in vitro*, *in vivo*, and correlative studies support the broader conclusion that oblimersen + bortezomib may sensitize lymphoma cells to the lethal effects of cyclophosphamide by markedly lowering Bcl-2 levels. Interestingly, Pei et al. (10) noted that the sequential (not simultaneous) treatment of multiple myeloma cells with bortezomib followed by the small-molecule Bcl-2 inhibitor HA14-1 resulted in a marked increase in mitochondrial injury. This injury was manifested as a loss of the mitochondrial transmembrane potential, and release of cytochrome *c*, resulting in the induction of apoptosis. These affects were blocked by the free radical scavenging agent *N*-acetyl-L-cysteine, implicating a role for reactive oxygen species in the process.

What seems consistent in these studies is that the most favorable treatment outcome, regardless of schedule, always appeared in the "triplet" combinations, although all "doublets" were superior to any singlet, except for the simple B + G doublet. With regard to schedule, it is also interesting that the most favorable results occurred in the experiments in which an ordered sequence was studied. In fact, pathologic complete remissions were only seen when the triplet combinations were given in an ordered fashion. Whereas an emphasis was not placed on deciphering the panoply of molecular changes that occurred following exposure to all the different combinations of agents, it is likely that there are a multitude of effects that could well explain these observations that include effects on bcl-2, proapoptotic family members, cell cycle arrest, and NF-κB.

For example, other more direct influences of proteasome inhibition on the induction of apoptosis have been shown in leukemic cells following exposure to the proteasome inhibitor lactacystin (11). For example, in both Jurkat (T-cell line) and Namala (B-cell line) cell lines, inhibition of the proteasome has been shown to differentially up-regulate proapoptotic Bcl-2

Table 1. Analysis of oblimersen levels (pmol/g tissue) in large cell lymphoma (RL) from SCID beige mice treated with oblimersen ± bortezomib

Cohort	Average ± SD	Change in oblimersen with bortezomib (%)	*P*
Control	0	0	0
Oblimersen (12 h post)	5.3 ± 1.5	not applicable	—
Oblimersen + bortezomib (12 h post)	7.72 ± 2.1	32	0.1
Oblimersen (24 h post)	1.3 ± 0.86	0.86	not applicable
Oblimersen + bortezomib (24 h post)	2.9 ± 0.76	55	0.02

NOTE: The percentage of change in oblimersen levels is based on a comparison to those cells treated with oblimersen alone at the same time point (i.e., 12 or 24 hours).

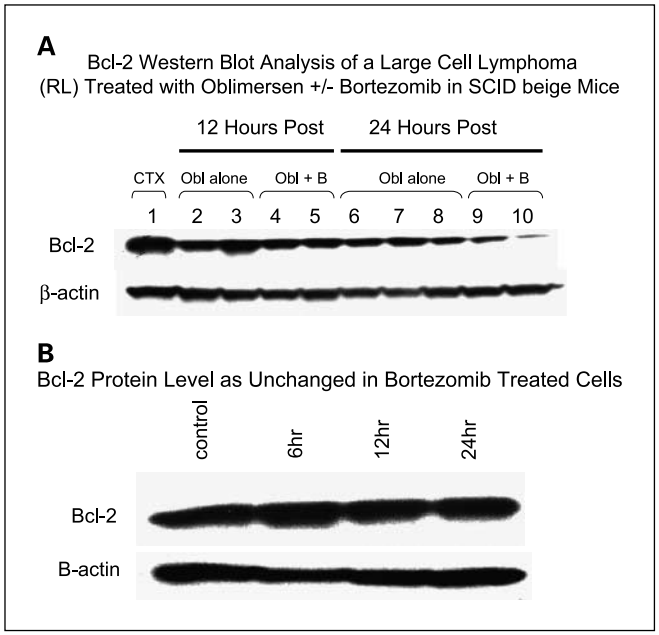

Fig. 7. *A,* Bcl-2 immunoblotting. SCID beige mice carrying SKI-DLCL-1 were treated with one dose of oblimersen ± bortezomib. *Lanes 9* and *10*, the level of Bcl-2 was markedly lower in the oblimersen + bortezomib – treated cohorts at 24 hours. *B,* RL cells treated with 5 nmol/L bortezomib show no changes in Bcl-2 levels at relatively high doses of bortezomib.

family members including Bik, by decreasing its proteolytic degradation, whereas other Bcl-2 family members in this model, including Bax, Bak, and Bad were not similarly affected. The accumulation of Bik alone was shown to be sufficient in inducing apoptosis in these leukemic cells (11). Additionally, it is apparent that proper functioning of the electron transport chain is highly dependent on proteasome activity. These same authors showed that the abrupt interruption of protein turnover changes the trans-mitochondrial membrane potential in a way that favors the induction of apoptosis. Marshanksy et al. (11) showed that Bik and the antiapoptotic member Bcl-$_{XL}$ coprecipitated, leading to the hypothesis that excess Bik could trap and theoretically nullify the antiapoptotic influences of Bcl-$_{XL}$ because the level of Bcl-$_{XL}$ is not changed following proteasome inhibition. This "trapping" of the Bcl-$_{XL}$ then offers a theoretical mechanism for overriding the antiapoptotic effects of Bcl-$_{XL}$, leading to eventual cell death.

Ling et al. (5) have nicely shown a number of time-dependent downstream effects on apoptosis mediated by proteasome inhibition. These authors showed that treatment of the H460 cell line with bortezomib resulted in both a time-dependent and concentration-dependent set of effects on Bcl-2 phosphorylation and cleavage. Treatment of H460 cells with bortezomib resulted in the cleavage of Bcl-2, with the identification of a unique cleavage product (M_r 25,000). The generation of this cleavage product was not prevented by caspase inhibitors, which was the case with the typically seen M_r 23,000 cleavage product seen following exposure to conventional cytotoxic therapies, raising the possibility of a caspase-independent pathway. The Bcl-2 cleavage products accumulated in the mitochondrial membrane early, usually 12 hours after exposure, whereas poly(ADP-ribose) polymerase cleavage and DNA fragmentation was seen ~36 hours postexposure. The authors concluded that inhibition of the proteasome resulted in a prompt phosphorylation of Bcl-2, leading to the formation of a unique cleavage product which was associated with G_2-M arrest and the induction of apoptosis (5).

Of course, NF-κB can also play an important role in the induction of apoptosis. Heckman et al. (12) established a link between NF-κB and apoptosis, which may provide some mechanistic basis for the activity of bortezomib in follicular lymphoma (13). The disease is well characterized by the translocation of the bcl-2 proto-oncogene from chromosome 18q21 to the immunoglobulin heavy chain locus at chromosome 14q32 (14–16), leading to constitutive overexpression of bcl-2 protein, protecting cells from programmed cell death. In addition to the overexpression of bcl-2, many lymphoma cells that carry the t(14:18) also overexpress NF-κB. These authors showed that cell lines expressing an IκBα super-repressor exhibited marked reductions in bcl-2 protein, implicating a role for NF-κB in the regulation of bcl-2. These observations could provide a rationale for employing proteasome inhibitors and obviously bcl-2-targeted drugs in follicular lymphoma (12, 13). A similar example has also been shown in mantle cell lymphoma, in which inhibition of the constitutive activation of NF-κB leads to the induction of both cell cycle arrest and apoptosis through the down-regulation of bcl-2 family members and activation of multiple caspases (17).

These data provide a compelling argument for exploring the importance of schedule in the administration of agents that might have discrete time-dependent effects on tumor cell biology. Furthermore, although only informally addressing some of the biological effects of these agents, it provides a molecular rationale for why these two classes of drugs might be complementary. Clearly, there is much to learn about the sequence of molecular events that occur inside the cell in order to better understand how such rational drug combinations can be developed. Future efforts will be directed toward understanding these downstream events and the importance of these temporal relationships, and beginning to translate them into the conduct of clinical trials that acknowledge these preclinical findings.

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