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ORIGINAL ARTICLE

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# Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapy in the A549 non-small-cell lung cancer cell line

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**Abstract** *Background*: Non-small-cell lung cancer (NSCLC) has a poor prognosis. Despite advances in therapy, survival has improved only slightly. The 26S proteasome regulates multiple cellular processes through degradation of ubiquitin-tagged proteins. The proteasome inhibitor, bortezomib (Velcade, formerly PS-341), has been shown to be an active anticancer agent both in vitro and in vivo in multiple tumor types. *Purpose*: To determine the molecular and cellular effects of the proteasome inhibitor in NSCLC as well as to evaluate the effectiveness of sequential treatment with bortezomib and gemcitabine/carboplatin (G/C) chemotherapy both in vitro and in vivo. *Methods*: All experiments were performed in the A549 NSCLC cell line. MTT assays were used to evaluate cytotoxicity. Western blotting evaluated protein levels. Measures of apoptosis included FACS analysis, DAPI staining and caspase-3 cleavage. Long-term cell viability was determined using an anchorage-dependent clonogenic assay. Sequential studies were performed in vitro and in vivo. *Results*: Bortezomib increased $p21^{waf1/cip1}$, induced $G_2$/M arrest, and triggered a small amount of apoptosis. The apoptotic effect of G/C chemotherapy was eliminated when bortezomib was administered prior to the chemotherapy; however, it was accentuated when the bortezomib was given simultaneously or after the chemotherapy. *Conclusions*: Bortezomib improves efficacy in combination with gemcitabine and carboplatin in NSCLC, but sequential effects are important and must be considered when developing therapeutic regimens.

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## Introduction

Lung cancer is the leading cause of death in both men and women today in industrialized countries, accounting for an estimated 28% of all cancer deaths in the United States [1]. Although the death rates are declining, this is more likely due to smoking cessation programs than more effective therapy [2]. Non-small-cell lung cancer (NSCLC) represents the majority of lung cancers and carries a poor prognosis with a median survival of less than 12 months [2]. Most patients present with unresectable disease and current treatment options of chemotherapy and radiotherapy are palliative at best [3]. There are multiple regimens for advanced NSCLC in use; however, these extend median survival by 2–4 months [3, 4]. New strategies are needed in the treatment of NSCLC in order to have any impact in this disease.

Inhibitors of the 26S proteasome are being investigated as a potential new class of chemotherapeutic agents [5–7]. The proteasome, a large multicatalytic protease complex, is responsible for most non-lysosomal intracellular protein degradation [8, 9]. Through protein ubiquitination and degradation, the proteasome regulates a variety of essential cell functions such as cell cycle progression, gene transcription, and apoptosis. Substrates of proteasome-mediated degradation include a variety of proteins involved in the regulation of gene transcription such as myc, fos, jun, I-$\kappa$B [10–13]; cell cycle regulators such as $p27^{kip1}$ [14] and $p21^{waf1/cip1}$ [15]; and regulators of apoptosis such as XIAP [16]. Bortezomib is a novel dipeptide boronic acid that inhibits function of the 26S proteasome and has diverse cellular effects including alteration of gene transcription, effects on cell cycle progression and induction of apoptosis [17, 18].

Experimental data have shown that bortezomib has cytotoxic effects in a variety of cancer types [19]. Its clinical utility is currently being investigated although it has been approved by the Federal Drug Administration for treatment of refractory multiple myeloma [20]. Other phase I and II studies are being conducted to determine its efficacy in a variety of solid organ malignancies [21, 22]. Recently, Ling et al. examined the effect of bortezomib in various NSCLC cell lines [23]. A $G_2$/M cell cycle arrest was consistently observed and is associated with stabilization of $p21^{waf1/cip1}$. These effects have been observed in other cancer types and some feel that this is a reliable marker of inhibition of the 26S proteasome [24, 25]. Whether this stabilization of $p21^{waf1/cip1}$ is mechanistically involved in the cytotoxic consequence of proteasome inhibition is unclear, though if so, then bortezomib may be particularly well suited for use in NSCLC [26], in which low levels of $p21^{waf1/cip1}$ are frequently observed and associated with a poor prognosis [27–29]. Stabilization of other proteins, such as p53 and $p27^{Kip1}$, are also observed and are similar markers of inhibition of the proteasome, though the relationship to induction of apoptosis remains uncertain [24, 30]. The specific mechanism(s) involved in the ability of bortezomib to sensitize various tumor cells to the apoptotic effect of chemotherapeutics, or even induce apoptosis, is unclear, though accumulation of misfolded proteins may be a central contributing mechanism [31].

Preliminary data suggest that the optimal clinical use of bortezomib is in combination with standard chemotherapeutics. Therefore, we determined the molecular and cellular response to bortezomib in a human NSCLC line and evaluated the sequence-specific interaction of bortezomib with the standard chemotherapeutics. We specifically examined gemcitabine (G) and carboplatin (C), the combination of which (G/C) is a standard regimen in NSCLC [32–34].

## Materials and methods

### Cell lines and cell culture

A549 and Calu-1 human lung cancer cell lines were obtained from the American Type Culture Collection (Rockville, Md.). All cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum. All chemical reagents were purchased from Sigma Chemical Company (St Louis, Mo.) unless otherwise specified. Gemcitabine (Gemzar, Eli Lilly Company, Indianapolis, Ind.) and carboplatin (Paraplatin, Bristol-Myers Squibb, New York, N.Y.) were obtained from the UC Davis institutional pharmacy. The 26S proteasome inhibitor PS-341 (bortezomib, Velcade) was a generous gift from Drs. Peter Elliot and Julian Adams (Millennium Pharmaceuticals, Cambridge, Mass.). This agent is a dipeptide boronic acid derivative that was developed to specifically inhibit the 26S proteasome [18].

### Tetrazolium dye methylthiotetrazole (MTT) cytotoxicity assay

For cytotoxicity assays, cells were seeded onto 96-well microculture plates at $1\times10^4$ cells/well and allowed to adhere for 24 h. The medium was removed and replaced with fresh medium with or without increasing concentrations of cytotoxic agents. The cells were then incubated for an additional 48 h. The medium was again removed and replaced with fresh medium without additional cytotoxic agents and the cells incubated for 24 h. Cell survival was then quantified using the tetrazolium dye MTT as described previously [35]. Each experimental data point represents the average value obtained from six replicates, and each experiment was repeated at least three times.

### Western blotting

Following treatment, cells were harvested by trypsinization (trypsin 0.25%; 1 m*M* EDTA), washed with PBS, and lysed at −20°C in lysis buffer (150 m*M* NaCl, 1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride, and 25 m*M* Tris (pH 7.5). Debris was sedimented by centrifugation and the protein concentration of the supernatant was determined using a Bio-rad protein detection assay kit (Bio-Rad Laboratories, Hercules, Calif.). Protein (75–100 μg) was solubilized at 100°C in Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 15% 2-mercaptoethanol. The protein was separated on 10% or 15% SDS-PAGE gel by electrophoresis at 100 V for 1.5 h and electrophoretically transferred to 0.2 mm nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) for 1 h at 100 V. Membranes were blocked for 1 h in a TBS-T buffer (25 m*M* Tris, pH 8.0, 150 m*M* NaCl, and 0.05% Tween-20) containing 5% (w/v) non-fat dried milk. Blots were then probed overnight with antibodies to $p21^{waf1/cip1}$ (Upstate USA, Charlottesville, N.C.), caspase-3 (Biosource International, Camarillo, Calif.), and actin (Santa Cruz Biotechnology, Santa Cruz, Calif.). The blots were developed using species-specific secondary antibodies. Immunoreactive material was visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, N.J.).

### 4,5-Diamidino-2-phenylindole hydrochloride (DAPI) staining

To identify and quantitate the induction of apoptosis, cells underwent DAPI staining following experimental treatments. DAPI is a fluorescent stain that allows examination of nuclei in a fluorescence microscope for morphologic assessment of changes during apoptosis [36]. In brief, following 24 h of treatment, cells were harvested by trypsinization and washed with PBS. The pellet was then fixed in 4% formaldehyde. The cells were

resuspended in 500 μl DAPI solution (0.2 μg/ml DAPI (Roche, Indianapolis, Ind.), 0.1% Triton X-100, 2% paraformaldehyde, 1× PBS) for 30 min. Cells were then spotted onto a microscope slide and allowed to air-dry. Images were obtained using an Olympus BX61 fluorescent microscope with digital camera output using Slidebook TM 3.0 software. Cell counts were performed by an independent observer over four individual low-power fields.

### Long-term cell viability

A clonogenic assay was used to quantitate long-term anchorage-dependent cell proliferation that may not be accurately reflected by immediate measurements following treatment [37]. Following the treatment outlined above, cells were collected by gentle trypsinization and washed in PBS. Cells were diluted (1:1000) in complete medium and were subsequently plated in six-well tissue culture dishes with each treatment group plated in triplicate. The medium was then changed biweekly over a 6-week course. Cells were stained with crystal violet and total colonies per well were counted.

### Determination of apoptotic cells by FACS analysis

Quantification of apoptosis by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis was carried out as described previously [38]. Bortezomib (50 n*M*), gemcitabine (1 μ*M*), and carboplatin (250 μ*M*) treatments in 12-h increments over a 24-h period were as described above. Each experiment was repeated on three separate occasions and representative data are shown.

### Sequential in vitro assays

To determine the effect of sequence difference on cellular response, cells were treated with bortezomib (50 n*M*), carboplatin (250 μ*M*) and gemcitabine (1 μ*M*) in various schedules. After 12 h of initial treatment, the medium was changed to fresh medium containing the other treatment. The control cell medium was changed at similar time points. For the simultaneous treatment, after an initial 12 h in medium, cells were treated with bortezomib, carboplatin and gemcitabine. After the second 12-h treatment, the medium was change and the cells then were harvested for FACS analysis or Western blotting after an additional 24 h. Therefore, all groups received the same duration of exposure to each agent, and assays were performed at the same point following the last treatment.

### Effect of varying sequential regimens in vivo

Athymic mice at 6–8 weeks of age were maintained in a dedicated barrier facility according to institutional guidelines, and experiments were conducted under an approved animal protocol. Subcutaneous xenograft tumors were established in the flank bilaterally by the injection of 200 μl cell suspension of $10\times10^6$ A549 cells mixed 60:40 with sterile culture medium and Matrigel (Becton-Dickinson Biosciences, San Diego, Calif.). Four mice per treatment group were used yielding eight tumors per treatment. Tumors had started exponential growth 30 days following A549 cell suspension injection, and mice were assigned randomly to one of four treatment regimens: saline (control), bortezomib followed by combination G/C 8 h later, G/C first followed by bortezomib 8 h later, and bortezomib/G/C administered simultaneously. Bortezomib was injected intraperitoneally (i.p.) at a dose of 0.50 mg/kg in a volume of 0.5 ml [39], gemcitabine was injected i.p. at a dose of 100 mg/kg, and carboplatin was injected i.p. at a dose of 30 mg/kg in a combined volume of 0.5 ml. Treatments were administered biweekly. Injections of 0.5 ml 1% DMSO (to serve as the control for the bortezomib) and 0.5 ml 0.9% normal saline (to serve as the control for the G/C combination) were used for the control group. Tumors were measured biweekly and tumor areas were calculated using the cross-sectional diameter. At the completion of the experiment the animals were killed and the tumors weighed.

### Statistical analysis

Analysis of the effect of the various treatments on cell numbers (from the MTT cytotoxicity assays and the DAPI apoptosis assays) or xenograft tumor weight was performed using two-tailed Student's *t*-test assuming unequal variance. Statistical significance was assumed for a $P$ values less than 0.05.

## Results

### Biologic effects of bortezomib

We first investigated the effect of bortezomib on A549 NSCLC cells. A dose-response curve of the cytotoxic effect was produced using the MTT assay. These data demonstrate that bortezomib was cytotoxic to these cells with a $LD_{50}$ of approximately 30 n*M* (Fig. 1a). Bortezomib has been consistently demonstrated to induce stabilization of $p21^{waf1/cip1}$ in a variety of cell types and we therefore determined whether a similar effect could be observed in the A549 NSCLC cell line. Stabilization of $p21^{waf1/cip1}$ in a dose-dependent fashion was observed, with effects noted at doses as low as 1 n*M* (Fig. 1b). The effect of bortezomib on cell cycle progression has also been examined in a variety of cell types and $G_2$/M arrest has frequently been observed. When cells were treated with bortezomib there was a marked increase in the number of cells in the $G_2$/M phase (72.3% vs 15.3% in the control). There was also a small percentage of cells, approximately 5.2%, in the sub-$G_0$ phase compared to

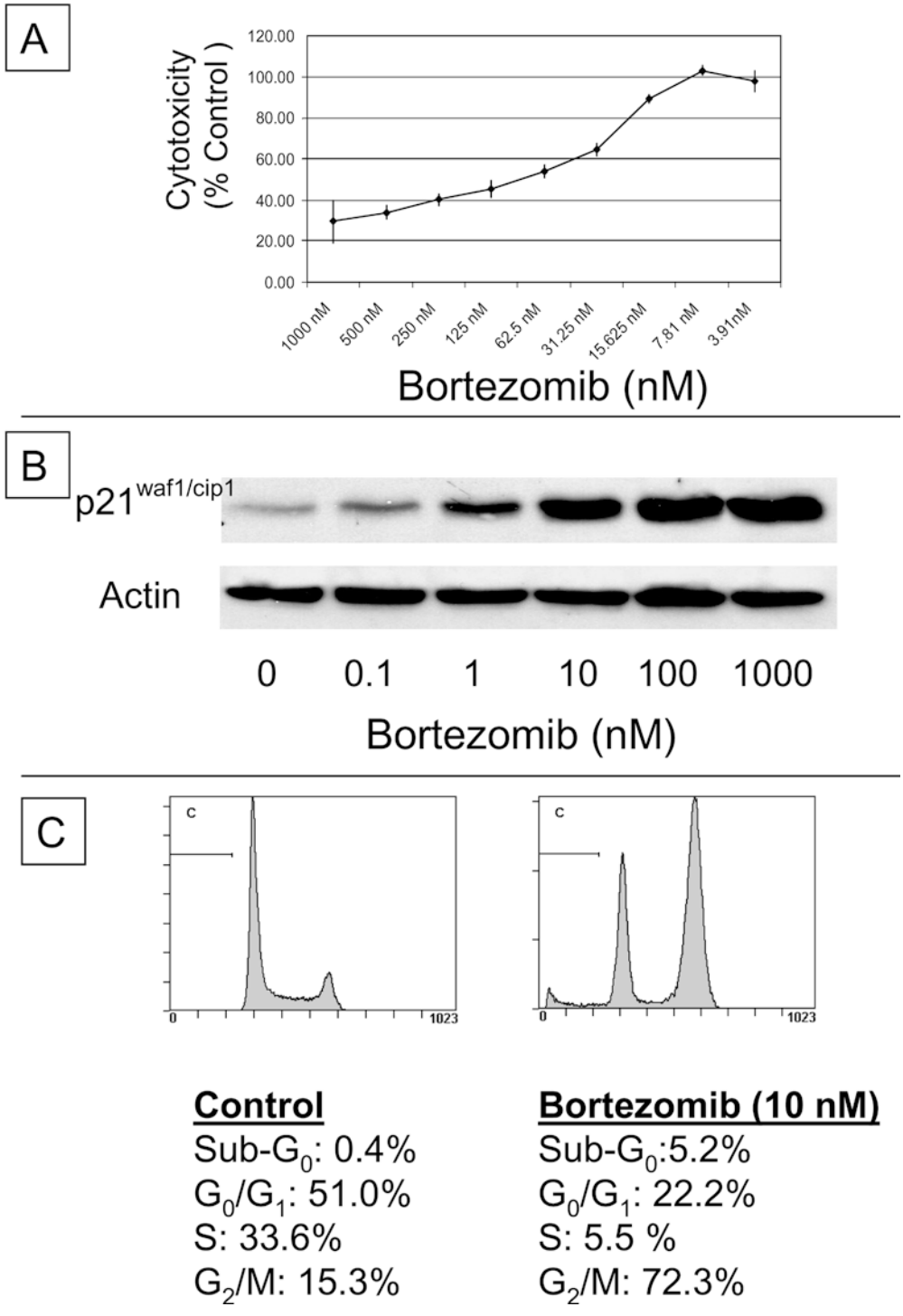



**Fig. 1a–c** Cellular and molecular effects of bortezomib on A549 cells. **a** Cytotoxicity following treatment over a wide dose range of bortezomib using the MTT assay. **b** Effect on $p21^{waf1/cip1}$ levels following 24 h of bortezomib treatment. **c** Effect of bortezomib (10 n*M*) on cell cycle distribution

0.4% in the untreated group, indicative of a mild induction of apoptosis (Fig. 1c).

## Cellular effects of bortezomib in combination with chemotherapy

We next examined the cellular effect of adding bortezomib to gemcitabine or carboplatin in A549 cells. Using DAPI staining to quantitate apoptosis, we noted that bortezomib, carboplatin and gemcitabine are independently capable of inducing apoptosis in A549 cells (Fig. 2a). While the induction of apoptosis by bortezomib alone was modest, the addition of either chemotherapy increased the apoptotic effect (Fig. 2a). While we did not perform extensive dose studies to obtain isobolograms for drug interactions, there would appear to be at least additive effects of bortezomib in the induction of apoptosis by either chemotherapy. We then examined the effect of bortezomib on the apoptotic and cell cycle

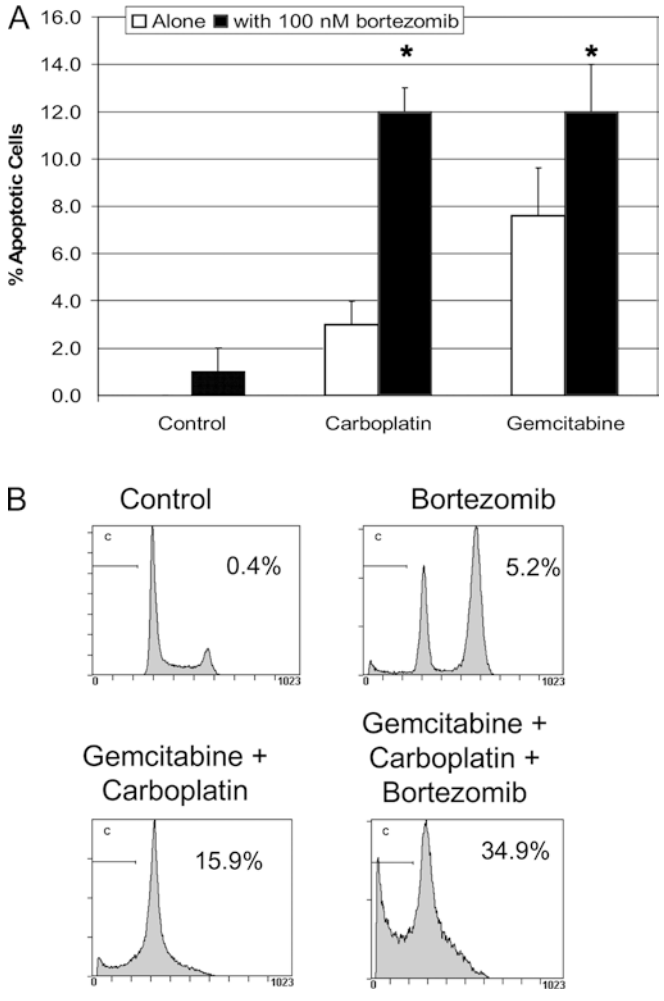



**Fig. 2 a** Apoptotic effect of adding bortezomib (100 n*M*) to carboplatin (250 μ*M*) or gemcitabine (1 μ*M*) determined by cell counts using DAPI staining following 24-h treatments (*$P < 0.05$ vs without bortezomib). **b** Cell cycle effect of bortezomib on the G/C combination with quantitation of the percentage of cells in the sub-$G_0$ fraction representing apoptotic cells

changes of the G/C combination using FACS analysis. Gemcitabine has been reported to induce $G_0$ arrest, while carboplatin induces S-phase arrest. When these two agents were administered simultaneously to A549 cells, we observed a primarily $G_0$ phase arrest with a minor increase in S phase and a moderate induction of apoptosis (15.9%). When bortezomib was added to the G/C combination, there was a further increase in apoptosis (34.9%) but without any $G_2$/M arrest as observed with bortezomib alone (Fig. 2b). In fact, the cell cycle distribution with this three-drug regimen was similar to that with the G/C combination and did not resemble the effect of isolated bortezomib administration.

## Sequential effects of bortezomib in combination with chemotherapy

As sequence of administration may be important when combining bortezomib with other chemotherapeutics agents, we next evaluated the molecular and cellular effects of sequential treatment (Fig. 3a). FACS analysis of sequential treatments showed that when bortezomib was administered before the G/C combination, there continued to be an increase in $G_2$/M arrest as seen with bortezomib treatment alone with only a small increase in the sub-$G_0$ fraction (8.7%). When bortezomib was administered simultaneously or after the G/C combination, there was no $G_2$/M arrest but a dramatic increase in the sub-$G_0$ phase (30.2% and 34.9%, respectively). We confirmed the induction of apoptosis in these variations of sequential therapy by examining caspase-3 cleavage. Caspase-3 cleavage was increased primarily following bortezomib administered simultaneously or after the G/C combination, and was notably absent when bortezomib was administered prior to the chemotherapies (Fig. 3b).

These findings were confirmed in the Calu-1 NSCLC cell line. Bortezomib induced a similar $G_2$/M arrest (data not shown). When bortezomib was combined with G/C, the sequence-dependent induction of apoptosis was as dramatic as that observed in A549 cells (Fig. 3c). The sequence of bortezomib $\rightarrow$ G/C induced very little increase in the sub-$G_0$ phase (6.7%; left panel, Fig. 3c) while bortezomib administered simultaneously with the G/C combination induced a moderate increase in apoptotic cell numbers (21.5%; right panel, Fig. 3c). However, the sequence of G/C $\rightarrow$ bortezomib was the most efficient sequence for induction of apoptosis (33.6%; middle panel, Fig. 3c). Therefore, the sequence-dependent induction of immediate apoptosis may possibly be generalized beyond the A549 cell line and potentially to NSCLC in general.

Figure 4a shows that similar results were obtained from the MTT assay as in previous experiments, in which the bortezomib increases the cytotoxic effect to G/C therapy but only if administered simultaneously or following the chemotherapies. In fact, bortezomib treatment before the combination chemotherapies eliminated the efficacy of this treatment with results similar to those in untreated cells. A long-term cell viability assay was performed with each of the sequential treatment groups. Again, the bortezomib $\rightarrow$ G/C treatment regimen was inferior to the G/C combination alone, or the other two sequences of bortezomib and G/C (Fig. 4b).

Several groups have reported that stabilization of $p21^{waf1/cip1}$ may be a potential mediator of the chemosensitizing effect of bortezomib. We therefore determined levels of $p21^{waf1/cip1}$ and p53 following treatment with the various sequences (Fig. 5). As noted before, bortezomib treatment led to higher levels of $p21^{waf1/cip1}$ while the G/C combination had minimal effect. Interestingly, the degree to which the various sequences of treatment increased $p21^{waf1/cip1}$ did not correlate with the apoptotic effects observed previously. We also examined p53 induction, and noted that all treatments increased p53 levels, though the regimen with the greatest increase (G/C $\rightarrow$ bortezomib) was also the sequence that induced the greatest apoptosis. As the increase in levels of $p21^{waf1/cip1}$

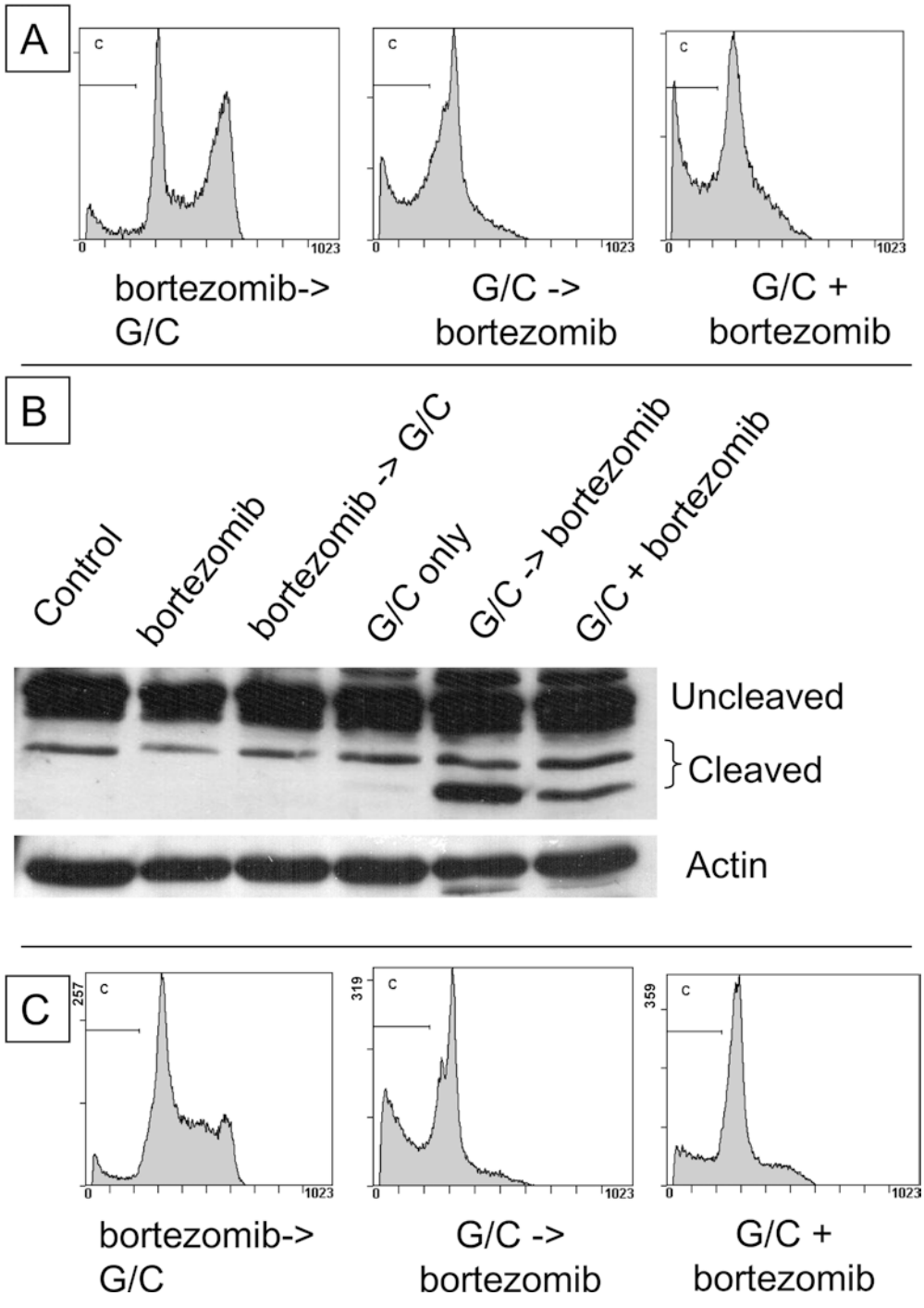



**Fig. 3** **a** Apoptotic effect of different schedules of bortezomib and the gemcitabine/carboplatin (G/C) combination including bortezomib for 12 h then G/C (*left panel*), G/C for 12 h then bortezomib (*middle panel*), or all three agents simultaneously (*right panel*) in the A549 cell line. **b** Western blotting for caspase-3 cleavage in response to treatments with bortezomib, G/C, or different schedules of combination treatment in the A549 cell line. **c** Apoptotic effect of the same schedules of bortezomib and G/C combination as in **a**, but in the Calu-1 cell line

by bortezomib is mediated by protein stabilization rather than p53-mediated transcription, it would be anticipated that the $p21^{waf1/cip1}$ and p53 changes would be independent, which in fact they were.

## Xenograft experiment with sequential treatment

These preliminary data led us to conduct an in vivo experiment utilizing subcutaneous xenografts of A549 in athymic mice. Following the successful establishment of tumors, mice were treated systemically with one of three regimens of bortezomib/G/C that reflected the same three sequential regimens used in the in vitro studies. The growth of tumors treated with the sequence of bortezomib $\rightarrow$ G/C was essentially the same as untreated tumors, while the other two treatments led to a significant reduction of tumor growth (Fig. 6). These results validate the preliminary in vitro results that demonstrate that bortezomib treatment prior to the combination of G/C eliminates the efficacy of this chemotherapy regimen.

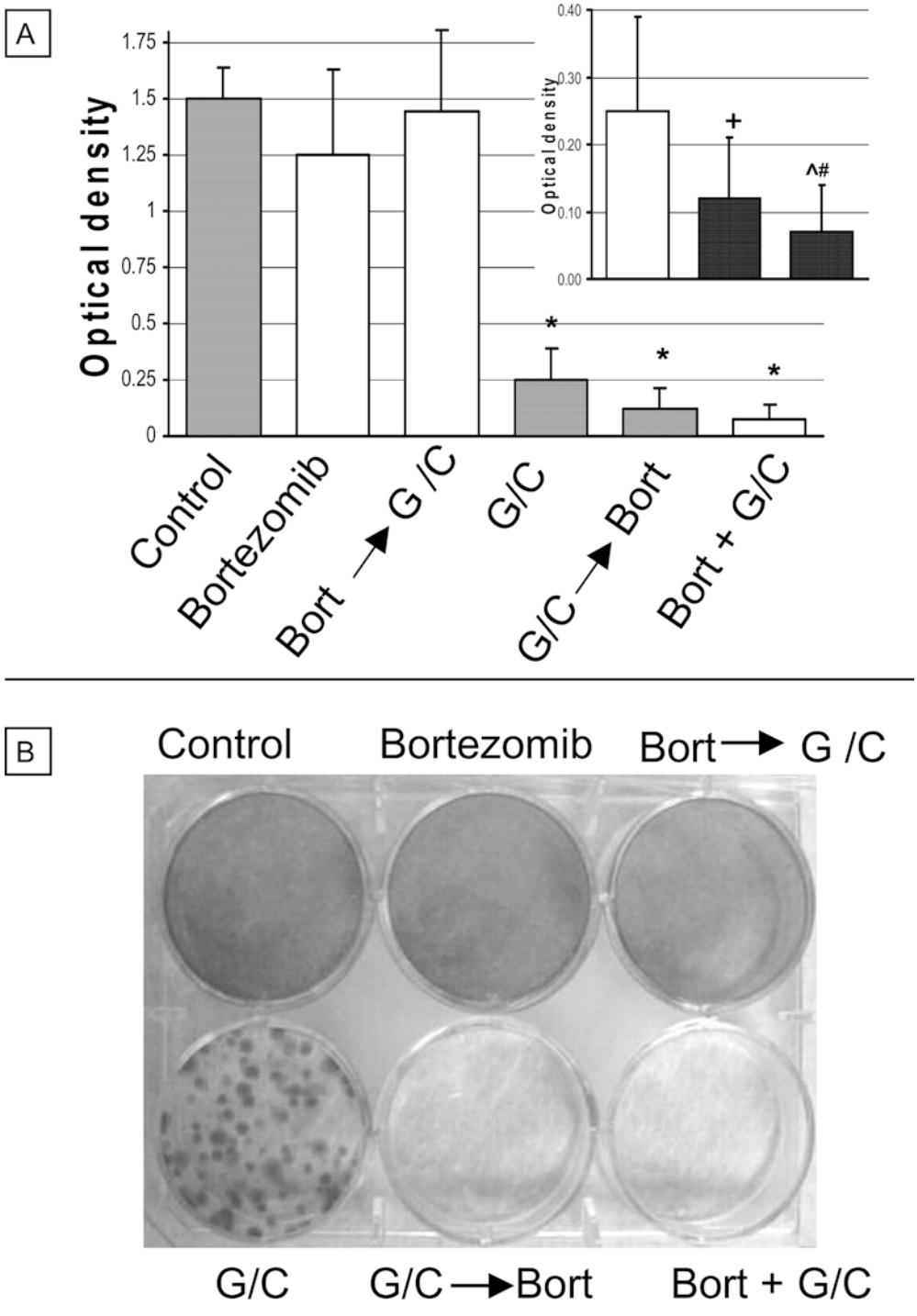



**Fig. 4** **a** Cytotoxicity of bortezomib (*Bort*), the gemcitabine/carboplatin (*G*/*C*) combination, or different schedules of combination treatment as measured by the MTT assay with the *inset* demonstrating expanded views of the last three treatment groups (*$P < 0.01$ vs bortezomib or bortezomib → G/C; $^{+}P = 0.01$ vs G/C alone; ^$P = 0.004$ vs G/C alone; $^{\#}P = 0.16$ vs G/C → bortezomib). **b** Representative plate following crystal violet staining of the clonogenic, long-term cell viability assay following the same treatments as in **a**

## Discussion

Bortezomib was developed as an antineoplastic agent to specifically inhibit the 26S proteasome, which is involved in homeostasis of a variety of proteins involved in gene transcription and cell cycle regulation. Preliminary experimental data have consistently demonstrated the ability of bortezomib to sensitize a variety of cancer cells to the apoptotic effects of diverse chemotherapeutic agents, though the specific mediator(s) of chemosensitization remain unclear. Similar to the effect observed in other cancer cell lines [40–43], we noted in the A549 NSCLC line that bortezomib sensitized cells to the apoptotic effect of either gemcitabine or carboplatin. The optimal sequence of chemotherapies with disparate mechanisms of action is an intense area of investigation [44]. We consistently observed that when bortezomib treatment preceded the combination chemotherapy, the cytotoxic effect of G/C therapy was abrogated or eliminated.

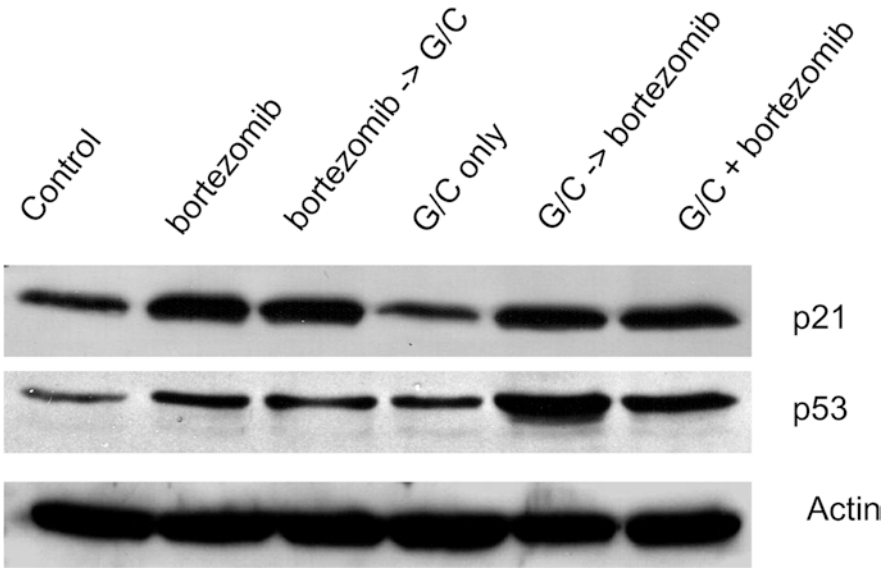
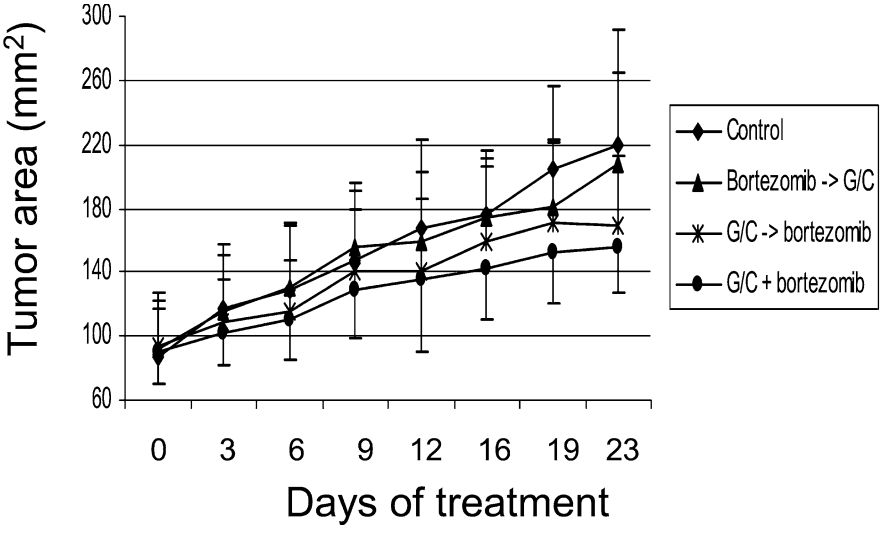



**Fig. 5** The effect of bortezomib and gemcitabine/carboplatin (*G*/*C*) administered singly, simultaneously or in sequence (as described in the "Materials and methods") on protein levels of $p21^{waf1/cip1}$ and p53. Western blotting for actin was also performed to demonstrate equivalency of sample loading



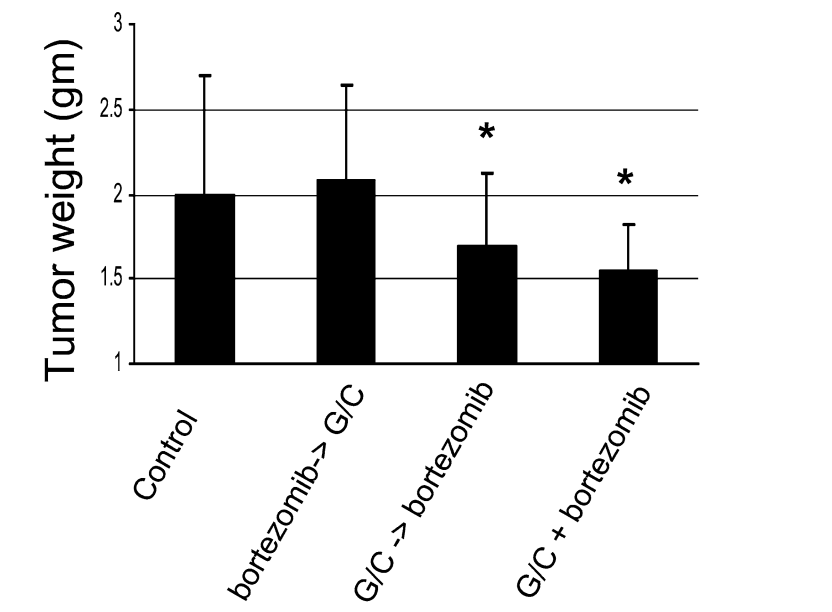




**Fig. 6** Growth of A549 xenografts in athymic mice during biweekly treatment with the three different schedules of bortezomib and the gemcitabine/carboplatin (G/C) combination compared to control treatment, and tumor weight at the conclusion of the treatment ($*P < 0.05$ vs control)

Although bortezomib treatment increased $p21^{waf1/cip1}$ levels, this effect did not seem to correlate with the $G_2$/M arrest or the efficacy of bortezomib to alter apoptosis in response to the G/C combination. Although $p21^{waf1/cip1}$ is involved in the checkpoint mechanisms of various points in the cell cycle through binding/inhibition of various cyclin-dependent kinases, Schreiber et al. noted only a $G_0/G_1$ arrest when $p21^{waf1/cip1}$ was exogenously expressed in A549 cells [45]. Although the mechanism(s) of the sequence-specific interactions with chemotherapy may be different from the apoptotic mechanism of bortezomib, one potential explanation involves the distinct cell cycle arrests: bortezomib, $G_2$/M arrest [10]; gemcitabine, $G_0/G_1$ arrest [46]; and carboplatin, $S/G_2$ arrest [47]. As both of these chemotherapies have effects on DNA synthesis (gemcitabine is a nucleoside analog and carboplatin is an alkylating agent), the effect of each agent is observed only in specific phases of the cell cycle. Therefore when bortezomib is administered first, the $G_2$/M arrest prevents cells from entering the part of the cell cycle during which gemcitabine and carboplatin are functioning. Conversely, when G/C are administered first and induce $G_0$ arrest, bortezomib may alter secondary targets that are independent of the $G_2$/M cell cycle arrest and therefore accentuate the postmitotic cell death induced by the G/C combination. The timing between the therapies was arbitrarily chosen and may have an additional impact on the interaction of bortezomib and the G/C combination. The time interval between the administration of other chemotherapies has been examined and shown to be very critical [48–51], and this is an area of ongoing investigation in our laboratory.

The majority of research that determines the apoptotic efficacy of novel cytotoxic agents focuses on the early induction of cell death using a variety of techniques such as FACS analysis and Western blotting for biochemical events such as PARP cleavage and caspase activation. There is increasing evidence that most cells die by a mechanism of delayed cell death, and evaluation of this consequence may be more reflective of the efficacy of agents when administered to patients [52]. The data from the clonogenic assay and MTT studies are important as they reflect the sequence-dependence of bortezomib with the G/C combination on the delayed induction of cell death beyond the early apoptosis noted in the FACS analysis.

One potential secondary target of bortezomib that may be involved in these sequence-specific effects is the stabilization of I-$\kappa$B. This effect allows persistent NF-$\kappa$B sequestration in the nucleus and prevention of initiation of transcription of various target genes [53]. Bortezomib has been demonstrated to inhibit the chemotherapy-induced activation of NF-$\kappa$B, which is a common cellular response to diverse chemotherapeutics, including both gemcitabine and carboplatin [40, 41, 54, 55]. When bortezomib is administered prior to the G/C combination, it had no efficacy on the chemotherapy-induced cell survival signals, such as NF-$\kappa$B, which have been shown to mediate resistance to these agents. Reports of the sequence-dependent effects of bortezomib are limited; Fahy et al. demonstrated that the optimal apoptotic effect in pancreatic cancer cells occurs with the sequence gemcitabine $\rightarrow$ bortezomib [56], and Mack et al. reported preliminary observations that the greatest apoptosis occurs with the sequence docetaxel $\rightarrow$ bortezomib in the Calu-1 NSCLC line [26]. Therefore, the effect of bortezomib may not be sensitization of cancer cells to the apoptotic effect of chemotherapy, but may be modulating the cellular response to the chemotherapeutics and thereby accentuating cell death.

Bortezomib is already in phase I and II trials and has been found to be well tolerated both alone and in combination with various chemotherapeutics. Many of these regimens were developed empirically with regard to sequence of treatment. These data provide evidence that the schedule of combination treatment must be considered carefully in the design of trials and should take into account preclinical data. While it is important to confirm sequential effects in each cancer type studied, it appears that bortezomib given prior to chemotherapy may yield inferior results.

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