

Bortezomib-Induced Apoptosis with Limited Clinical Response Is Accompanied by Inhibition of Canonical but not Alternative Nuclear Factor- κ B Subunits in Head and Neck Cancer

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Abstract Purpose: Nuclear factor- κ B (NF- κ B)/REL transcription factors promote cancer cell survival and progression. The canonical (NF- κ B1/RELA or cREL) and alternate (NF- κ B2/RELB) pathways require the proteasome for cytoplasmic-nuclear translocation, prompting the investigation of bortezomib for cancer therapy. However, limited clinical activity of bortezomib has been observed in many epithelial malignancies, suggesting this could result from incomplete inhibition of NF- κ B/RELBs or other prosurvival signal pathways.

Experimental Design: To examine these possibilities, matched biopsies from 24 h posttreatment were obtained from accessible tumors of patients who received low-dose bortezomib (0.6 mg/m²) before reirradiation in a phase I trial for recurrent head and neck squamous cell carcinoma (HNSCC). Effects of bortezomib on apoptosis and proliferation by TUNEL and Ki67 staining were compared with nuclear staining for all five NF- κ B subunits, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphorylated signal transducers and activators of transcription 3 (STAT3) in tumor biopsies, and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTP) and DNA binding assay for the five NF- κ B subunits in HNSCC cell lines.

Results: HNSCC showed increased nuclear staining for all five NF- κ B subunits, phosphorylated ERK1/2, and phosphorylated STAT3. Bortezomib treatment significantly enhanced apoptosis with inhibition of nuclear RELA in three of four tumors, but other NF- κ B subunits, ERK1/2, and STAT3 were variably or not affected, and tumor progression was observed within 3 months. In HNSCC cell lines, 10⁻⁸ mol/L bortezomib inhibited cell density while inhibiting tumor necrosis factor- α -induced and partially inhibiting basal activation of NF- κ B1/RELA, but not NF- κ B2/RELB.

Conclusions: Although low-dose bortezomib inhibits activation of subunits of the canonical pathway, it does not block nuclear activation of the noncanonical NF- κ B or other prosurvival signal pathways, which may contribute to the heterogeneous responses observed in HNSCC.

Nuclear factor- κ B (NF- κ B) is an injury, pathogen, growth factor, cytokine, and matrix signal-inducible transcription factor that normally functions to regulate cell survival and proliferation and innate and adaptive immune responses (1). NF- κ B transcription factors are heterodimers or homodimers composed from two families of REL proteins. Each member of the REL family contains an NH₂ terminal REL homology domain that mediates dimerization and domains mediating nuclear localization and binding to specific κ B DNA sequence

motifs in target genes (1). The first class, RELA (p65), RELB, and cREL, is synthesized in their mature forms, whereas the second class, consisting of NF- κ B1(p105) and NF- κ B2(p100), requires proteasome-dependent proteolysis of COOH terminal ankyrin repeats to yield p50 and p52, respectively (2). In the absence of activating stimuli, NF- κ B dimers are also bound by a family of ankyrin repeat-containing inhibitor- κ B (I κ B) proteins, sequestering them in the cytoplasm, blocking their nuclear translocation and/or DNA binding (1). The different NF- κ B heterodimers and homodimers may be preferentially activated by different ligand-receptor and I κ B kinase (IKK) signal pathways to undergo proteasome-dependent processing of subunits and I κ Bs, and cytoplasmic-nuclear translocation and activation.

The IKK signal pathways and downstream NF- κ B components have been broadly classified into canonical and non-canonical (alternative) pathways (3). The canonical pathway involves activation of a trimeric IKK complex, composed of IKK α , IKK β , and IKK γ (NEMO), in response to stimuli, such as tumor necrosis factor- α (TNF- α) and interleukin 1. Upon activation, the IKK complex phosphorylates I κ B, leading to its subsequent ubiquitination and degradation by the proteasome, allowing nuclear translocation and DNA binding of the

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NF- κ B1/RELA (p50/p65) heterodimer (4). Whereas regulation and function of NF- κ B1/cREL is believed to be similar to that of RELA (5), cREL activation is less well studied. The noncanonical (alternate) pathway involves activation of IKK α via NF- κ B–inducing kinase, leading to direct phosphorylation, ubiquitination, and degradation of the p100 precursor protein by the proteasome, allowing translocation and DNA binding by p52/RELB (6). Studies of the consequences of knockout of IKK and NF- κ B subunits in mice indicate that the canonical and noncanonical pathways may have some tissue specific developmental functions, as well as potentially redundant roles related to cell survival (3).

Consistent with the prevalence of alterations in signal pathways upstream of NF- κ B in cancer and its central role in regulating programmed responses, NF- κ B has been shown to be aberrantly activated and important in cell proliferation, survival, and malignant potential of a variety of cancers, including head and neck squamous cell carcinomas (HNSCC), in which it has been extensively studied (3, 7, 8). The canonical NF- κ B1/RELA (p50/p65) heterodimer was shown to be one of the species aberrantly activated in HNSCC and to regulate expression of cytokines, angiogenesis factors, cell cycle, and antiapoptotic genes (9–11). Increased activation of NF- κ B1/RELA was shown to occur during malignant progression in a murine SCC model (12) and to modulate an altered pattern of gene expression and more aggressive malignant phenotype (13). Consistent with this experimental murine model, increased nuclear activation of RELA has also been shown in squamous dysplasia and HNSCC tumor specimens and correlated with malignant progression and decreased survival (14). Alteration in several upstream cytokine and growth factor pathways contribute to aberrant activation of NF- κ B in HNSCC, including autocrine stimulation by interleukin-1, epidermal growth factor receptor, and TNF- α (15–18). Genetic or pharmacologic inhibition of NF- κ B was shown to restore altered gene expression and inhibit SCC proliferation, survival, angiogenesis, and resistance to TNF- α and radiotherapy (10, 13, 17–19), providing further evidence for the role of NF- κ B as a target for therapy in HNSCC.

Based upon the role of the proteasome in canonical and noncanonical NF- κ B activation, proteasome inhibitors hold potential for blockade of NF- κ B activation and for cancer therapy. Bortezomib (VELCADE) is a small molecule inhibitor that selectively blocks activity of the catalytic site of the 26S proteasome complex (20). Bortezomib has shown anticancer activity in preclinical and clinical studies and been approved for treatment refractory multiple myeloma. Bortezomib has also been tested and shown antitumor activity in preclinical studies for a number of solid tumor types, including HNSCC. Bortezomib blocked NF- κ B activation and proliferation, induced apoptosis in HNSCC cell lines, and inhibited growth, radioresistance, and angiogenesis in syngeneic murine SCC and human HNSCC xenograft models (19, 21, 22).

In a first-in-human study of bortezomib with reirradiation in patients with HNSCC, we have thus far examined the effects of a relatively low starting dose of proteasome inhibitor bortezomib (0.6 mg/m²), with different schedules of drug and reirradiation, to find tolerable regimen(s) to maximize concurrent therapy and to study effects on proteasome and NF- κ B–dependent and NF- κ B–independent prosurvival signal pathway biomarkers. Although pilot studies provided evidence

for inhibition of the proteasome together with phosphorylated RELA and increased apoptosis (23), we have subsequently observed limited clinical antitumor activity. This includes sustained partial responses in 5 of 18 patients and more temporary tumor reductions or stabilization of disease in others. The availability of pretreatment and matched posttreatment tumor biopsies and cell lines from a subset of these patients provide an opportunity to examine if these subtherapeutic responses may relate to differential effects of proteasome inhibition on canonical and alternate NF- κ B/REL or other prosurvival pathways.

In this study, we examined the nuclear localization of the five NF- κ B/REL subunits of the canonical and alternative pathways in tumor and matched nonmalignant mucosa. Using pretreatment and posttreatment tumor biopsies, we examined the effects of bortezomib treatment on their distribution, along with that of the proteasome-independent mitogen-activated protein kinases (extracellular signal-regulated kinase 1/2, ERK1/2) and signal transduction and activating transcription factor-3 (STAT3) pathways, two important proliferative and prosurvival pathways activated in HNSCC. The potential linkage between these effects and markers of proliferation and apoptosis were examined. The effect of bortezomib on basal and inducible activation of NF- κ B/REL subunits by TNF- α , one of the factors implicated in autocrine and paracrine activation of the canonical pathway in HNSCC (17, 18), was examined. Our findings suggest that incomplete inhibition of nuclear activation of basal canonical and lack of inhibition of alternate NF- κ B/REL pathway subunits, as well as other prosurvival pathways, may contribute to the limited responsiveness observed with lower dosages of bortezomib and reirradiation.

Materials and Methods

Patient treatment and tumor specimens. Patients with histologically proved recurrent HNSCC provided informed consent before being enrolled into protocol 01-C-0104, a phase I study of bortezomib and reirradiation for recurrent HNSCC, approved by Institutional Review Board and Cancer Treatment Evaluation Program of the National Cancer Institute. Full eligibility criteria and initial study design and results are included in a previous report (23). Bortezomib produced by Millennium Pharmaceuticals was supplied through the National Cancer Institute Cancer Treatment Evaluation Program. Bortezomib was given as an i.v. bolus for 3 to 5 s on days 1 and 4 of weeks when drug was given. Radiation, delivered with a high-energy linear accelerator using 6-MV photons and electrons, was given in standard fractions of 1.8 to 2.0 Gy daily (Monday to Friday), to a total dose of 60 to 70 Gy. Among 17 patients enrolled to investigate tolerability of different schedules of bortezomib 0.6 mg/m² twice weekly with 6 to 7 wk of reirradiation, two of seven among the initial cohort progressed early and died (22), leaving 15 patients who completed treatment and were fully evaluable for toxicities. Of those who received the initial schedule of bortezomib every week with reirradiation, two of five had dose-limiting toxicities, prompting study of scheduled treatment breaks. Of those treated with a midcourse break of 2 wk from both modalities, one of six had a dose-limiting toxicity, and among those treated with a midcourse break of 2 wk from bortezomib while receiving continuous reirradiation, zero of four had dose-limiting toxicities. Overall, 8 of 17 showed reductions in tumor size of 30% or more, and 5 of 17 qualified as having durable partial responses of at least 3 mo.

Six of 17 patients consented to optional pretreatment tumor biopsies, and four of these with tumors of the oral cavity, oropharynx, or neck were safely accessible for both pretreatment

and posttreatment biopsy in the outpatient clinic. The timing of tumor sampling 24 h posttreatment from protocol patients was based upon prior pharmacodynamic data indicating proteasome inhibition by bortezomib peak to trough between 1 and 72 h, and antiproliferative and apoptotic effects observed at 24 h posttreatment in HNSCC cells and tumors in clinical and preclinical studies (19, 20, 22). Posttreatment tumor biopsies were taken 24 h after the initial bortezomib treatment, before the initiation of radiotherapy to evaluate effects of drug alone. Specimens were immediately embedded in optimum cutting temperature media and frozen at -80°C. As standard controls, tumor blocks of two sets of matched carcinoma and noncancerous epithelium from deidentified subjects from the cooperative human tissue network of National Cancer Institute were used for analysis. Frozen tissues were sectioned at a thickness of 10 μm at the largest area and placed on silanated glass slides (Histoserv) for immunostaining.

Immunoassays and scoring. Immunohistochemistry for NF-κB/REL, ERK, STAT3, apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin nick-end labeling, TUNEL) and proliferation (Ki-67) was done using standard methods (23). Scoring of nuclear phosphorylated RELA, RELB, c-REL, p105/p50, p100/p52, phosphorylated ERK1/2, and phosphorylated STAT3 was done using a modified scoring method previously reported by Nenutil et al. (24), as described in supplementary methods.

Cell culture and reagents. University of Michigan HNSCC (UMSCC) cell lines 9, 11A, and 11B were a generous gift from T.E. Carey (University of Michigan), and their characteristics and culture conditions are described in supplementary methods. Recombinant TNF-α was purchased from R&D Systems.

MTT assays. Cellular cytotoxicity was measured with an MTT calorimetric cell proliferation kit (Roche), as described by others (25) and in supplementary methods. Growth effects at different drug concentrations were analyzed for statistical significance using the Student's *t* test.

Cellular fractionation, Western blot, and DNA binding assay. Cell fractionation for Western blot and DNA binding assay were done, as recently described and as in supplementary methods (11). Antibodies used in these studies (Cell Signaling Technology) included anti-RELA, anti-RELB, anti-cREL, anti-NF-κB2/p52, anti-NF-κB1/p50, β-actin, and anti-rabbit IgG-horseradish peroxidase as the secondary antibody.

NF-κB DNA binding activity was assessed using the TransAM NF-κB family transcription factor assay kit (Active Motif) per the manufacturer's protocol, as described in supplementary methods.

Results

Origin and characteristics of tumor specimens. For comparison of immunostaining in mucosa and malignant squamous epithelia, tumor blocks from two cytokeratin-positive carcinomas and matched noncancerous epithelium from oropharynx and oral tongue from deidentified subjects were used. From the clinical trial six of 17 patients consented to pretreatment biopsies, and four had tumors of the oropharynx or oral cavity safely accessible for serial biopsies in the clinic and consented to posttreatment biopsy 24 hours after the initial bortezomib treatment, before the start of radiotherapy. The tumor location, treatments, and response to therapy of the study patients are listed in Table 1. All patients had pathologically proven recurrent HNSCC and were treated with an initial dose of bortezomib 0.6 mg/m² alone and subsequently received treatment according to schedules indicated. Proteasome assay of peripheral blood and tumor tissue showed inhibition of ~20% to 30% at 24 hours posttreatment (23). Whereas initial clinical and histologic tumor responses to bortezomib plus reirradiation varied among the biopsied patients, as described in Table 1, and below, all four patients with both pretreatment and posttreatment tumor biopsies developed progressive disease within 3 months of follow-up, making them suitable for study of potential mechanisms of incomplete response.

Different patterns of NF-κB subunit staining between HNSCC and matched noncancerous epithelium. To assess baseline patterns of NF-κB localization between HNSCC tissue and noncancerous epithelial tissue, we evaluated NF-κB subunit immunostaining patterns in matched biopsies available from two patients with HNSCC. Figure 1A illustrates staining patterns of a matched epithelial biopsy from the grossly normal tonsil (*top*) obtained opposite from a tonsillar SCC (*bottom*).

Table 1. Patient tumor and treatment characteristics and clinical outcomes

Patient	Tumor histology	Tumor location	Therapy before recurrence	Bortezomib dose	Bortezomib schedule*	Posttreatment biopsy †	Local control on treatment; by 3 mo ‡
1	Recurrent SCC	Floor of mouth and anterior triangle neck	S, C, R	0.6 mg/m ²	Twice weekly × 7 wk without break	Yes	50% tumor reduction; PD below field at 4 wk
2	Recurrent SCC	Base of tongue and anterior triangle neck	S, C, R	0.6 mg/m ²	Twice weekly × 7 wk without break	Yes	PD below field at 2 wk on treatment
3	Recurrent SCC	Left base of tongue and vallecula	S, R	0.6 mg/m ²	Twice weekly × 7 wk with 2 wk break	Yes	Stable disease on treatment; PD in field at 3 mo
4	Recurrent SCC	Anterior neck and parapharyngeal space	S, C, R	0.6 mg/m ²	Twice weekly × 7 wk without break	Yes	Stable disease on treatment PD out of field at 1 wk
5	Recurrent SCC	Anterior neck with tracheal extension	S, R	0.6 mg/m ²	Twice weekly × 7 wk without break	No	90% tumor reduction PD in field at 5 mo
6	Recurrent SCC	Base of tongue	S, R	0.6 mg/m ²	Twice weekly × 7 wk with 2 wk break	No	30% tumor reduction PD 3 mo

NOTE: S, surgery; C, chemotherapy; R, radiation; PD, progressive disease.

*Bortezomib given concurrently with fractionated radiotherapy (to 50-70 Gy) given daily (Monday to Friday).

†Timing of all posttreatment biopsies is 24 h after initial treatment before the start of radiotherapy.

‡Based upon RECIST criteria, stable disease is defined as <30% tumor reduction and partial response is defined as >30% tumor reduction.

Similarly, Fig. 1B shows a biopsy from grossly normal tongue tissue (*top*) from a site separated from an SCC of the tongue (*bottom*). Both comparisons revealed a diffuse increase in NF- κ B subunit staining in the cancer tissue when compared with that of the matched nonmalignant squamous epithelium, which showed staining restricted to the basal layers (stratum basale and spinosum). Nuclear staining for all the NF- κ B subunits seemed to be greatly reduced or lost in the suprabasal layers of the noncancerous epithelium. Diffuse NF- κ B subunit staining in the HNSCC biopsies and basal staining in the noncancerous epithelium corresponded to nuclear Ki67 staining (cell proliferation) patterns, suggesting histologic and growth pattern similarities between HNSCC and the proliferating epithelial cells of the basilar layer of the mucosa.

A panel of HNSCC specimens show variable nuclear localization of all five NF- κ B subunits. To examine the broader presence and localization of the five NF- κ B subunits in HNSCC, we compared six pretreatment tumor biopsies via immunohistochemistry. Whereas the presence or absence of cytoplasmic staining was variable between different tumor biopsies and NF- κ B subunits, six of six pretreatment biopsies show nuclear staining for all five NF- κ B subunits compared with isotype controls (Supplementary Fig. 1), ranging from weaker 1+ nuclear staining (phosphorylated RELA in patient 4) to very strong 3+ nuclear staining (cREL in patient 1). These results are consistent with nuclear localization and activation of canonical as well as noncanonical NF- κ B subunits in HNSCC.

Bortezomib inhibits nuclear localization of activated RELA and induces apoptosis in HNSCC tumors. To determine the molecular and biological effects of bortezomib *in vivo* 24 hours

after the initial treatment, we used immunohistochemistry to assess changes in NF- κ B subunit staining intensity and cellular localization, together with TUNEL and Ki67 as markers of apoptosis and proliferation. Pretreatment and posttreatment HNSCC biopsies were available from four patients for comparison. Representative staining for one of three histologic responders who showed increased apoptosis (patient 1), and one nonresponder (patient 4), are shown in Fig. 2A and B, respectively, and two additional histologic responders by TUNEL staining (patients 2 and 3) are shown in Supplementary Fig.S2. Histograms summarizing the quantitative changes in pretreatment and posttreatment apoptotic and proliferation indices and histoscores for tumors from the four patients and their significance are shown in Fig. 3 (A-D corresponds to patients 1-4).

A significant decrease in nuclear staining of phosphorylated RELA and corresponding increase in apoptosis by TUNEL staining were observed in three of four posttreatment tumor biopsies from patients 1 to 3 (Figs. 2A and 3A-C, $P < 0.05$ and Supplementary Fig. S2). Analysis of biopsies from patient 1 who showed an initial tumor reduction of $\sim 50\%$ before progression elsewhere (23), also showed a significant decrease in nuclear localization of total cREL, p50, and p52 (Figs. 2A and 3A, $P < 0.05$). Patients 2 and 3, whose disease progressed below field during and in field after treatment, respectively, also showed a significant decrease in nuclear staining of activated RELA, with or without variable inhibition of nuclear RELB, cREL, p50, or p52 (Supplementary Fig. S2A and B and Fig. 3B and C). In contrast, the tumor from patient 4 shown in Fig. 2B exhibited very weak activated RELA nuclear staining

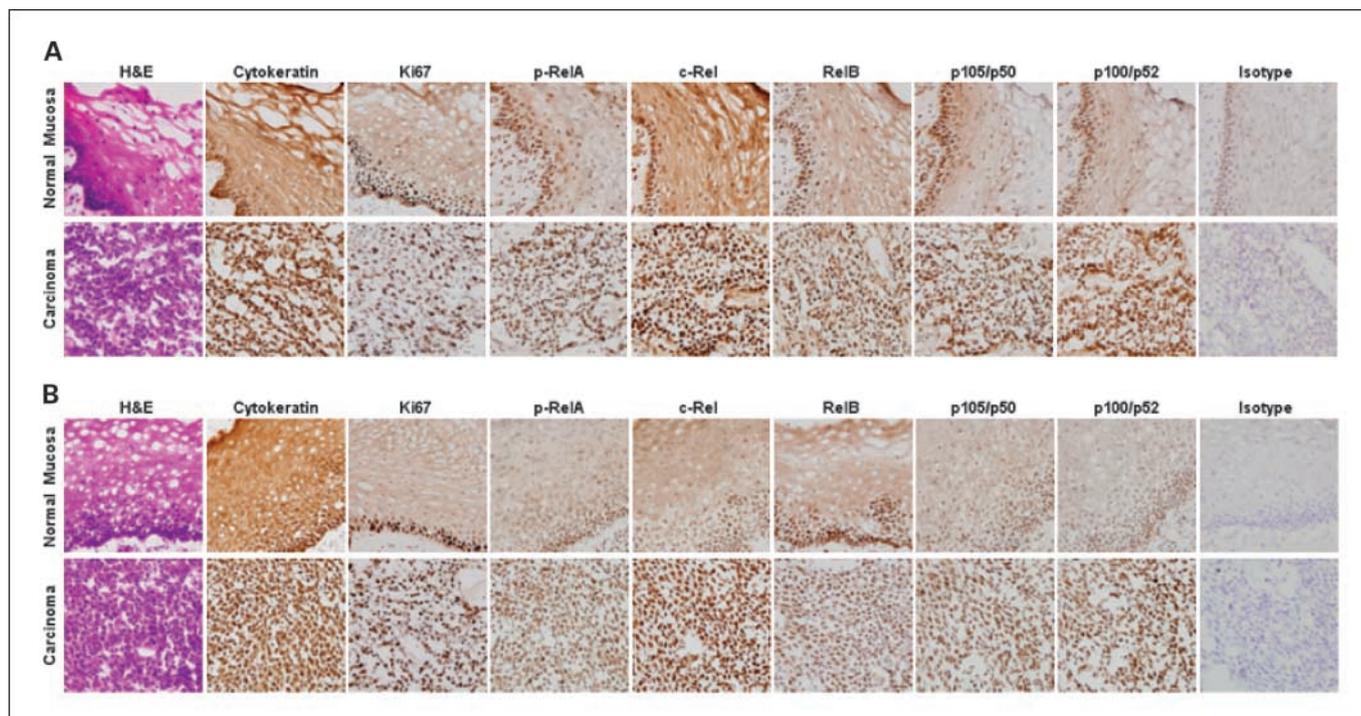


Fig. 1. Immunohistochemical analysis of NF- κ B subunits in patient matched HNSCC and noncancerous epithelium biopsies. **A.** SCC of tonsil (*bottom*; magnification, 400 \times) compared with epithelium from opposite tonsil (*top*; magnification, 400 \times). **B.** SCC of tongue (*bottom*; magnification, 400 \times) compared with biopsy from adjacent lingual epithelium (*top*; magnification, 400 \times). Both SCC biopsies show diffuse positive nuclear staining of variable intensity of all five NF- κ B subunits. NF- κ B subunit nuclear staining limited to the basal layers is shown in the matched noncancerous epithelia, with nuclear staining reduced or lost in the suprabasal layers. p-RELA, phosphorylated RELA (Ser⁵³⁶); Isotype, rabbit IgG isotype-negative control.

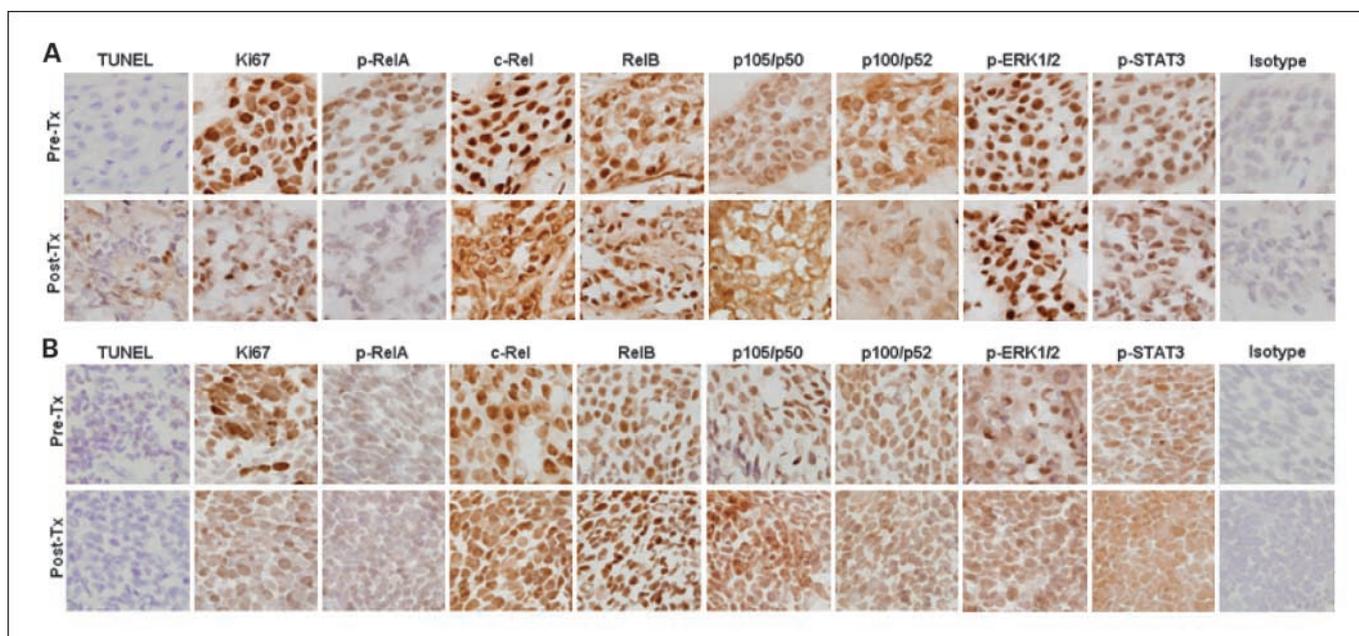


Fig. 2. Immunohistochemistry analysis of pretreatment and 24-h posttreatment biopsies from patients 1 and 4 after 0.6 mg/m² bortezomib. Patient 1 (*A*) shows decreased staining from pretreatment (*top*; magnification, 1,000 \times) to posttreatment (*bottom*; magnification, 1,000 \times) in phosphorylated RELA, c-REL, p50, p52, and Ki67, as well as increased TUNEL staining, whereas RELB seems to be unaffected. Conversely, patient 4 (*B*) shows very weak pretreatment nuclear phosphorylated RELA and no change after bortezomib therapy in NF- κ B subunit nuclear localization or TUNEL staining, but did show decreased Ki67. In both patients, activated ERK1/2 and STAT3 are unaffected by bortezomib therapy. p-ERK1/2, phosphorylated ERK (Thr²⁰²/Tyr²⁰⁴); p-STAT3, phosphorylated STAT3 (Tyr⁷⁰⁵); pre-tx, pretreatment; post-tx, posttreatment.

pretreatment and showed no significant change in nuclear staining of any NF- κ B subunit or TUNEL staining posttreatment. No reduction in size or sustained local control was observed in this patient. Based upon these patterns, the proapoptotic effects of bortezomib detected by TUNEL staining seemed to correspond most consistently with inhibited nuclear localization of RELA and other subunits of the canonical, rather than the alternative, NF- κ B activation pathway. Proliferation of tumor cells as measured by Ki67 immunostaining decreased after bortezomib therapy in all four tumors, including patient 4, potentially related to NF- κ B-independent effects of bortezomib and proteasome inhibition on turnover of other proteins regulating cell proliferation (20).

In addition to functional and NF- κ B subunit immunostaining, we assessed activation and effects of bortezomib on other signal pathways implicated in proliferation and survival of HNSCC, including ERK and STAT3 (26, 27). Immunohistochemical analysis of pretreatment and posttreatment tumor biopsies 24 hours post-bortezomib therapy revealed no change in activated ERK1/2 or activated STAT3 nuclear staining (Fig. 2 and Supplementary Fig. S2). We also saw no significant increase in nuclear cJUN implicated in proteasome-dependent apoptosis in multiple myeloma in three specimens for which tissue was available for additional stains (Supplementary Fig. S3). These results suggest that the proapoptotic and antiproliferative effects of bortezomib observed in these HNSCC tumor specimens were not related to effects on these other progrowth and prosurvival pathways and that their coactivation could potentially contribute to the lack of complete dependence on NF- κ B and response to proteasome inhibition.

NF- κ B subunits are detected in the nucleus and exhibit variable DNA binding activity in HNSCC cell lines. To further charac-

terize the nuclear activation and function of NF- κ B components in HNSCC, we investigated the cellular localization and basal DNA binding activity of NF- κ B subunits in a panel of HNSCC cell lines. Figure 4A illustrates results of Western blot analysis of NF- κ B subunits in cytoplasmic and nuclear extracts from three HNSCC cell lines. Both RELA and RELB were detected in the nucleus of all three cell lines, with the greatest relative amount present in UMSSC-11B. Similarly, all three cell lines had variable amounts of p50 and p52 in the nucleus with 11B showing the darkest bands. Variable amounts of nuclear cREL were present with the greatest relative amount present in UMSSC-9. Compared with the nuclear fraction, relatively greater amounts of precursor proteins NF- κ B1(p105) and NF- κ B2(p100) appeared in the cytoplasm, consistent with known mechanisms of processing required before nuclear localization of the p50 and p52 NF- κ B subunits (3, 6).

To assess functionality of the nuclear NF- κ B subunits, we measured subunit binding activity to a consensus κ B-binding site using a quantitative NF- κ B DNA-binding assay (11). Consistent with Western blot data, RELA, RELB, p50, and p52 from UMSSC-9, UMSSC-11A, and UMSSC-11B (Fig. 4B, C, and D, respectively) showed significant binding to consensus κ B oligonucleotide, with UMSSC-11B generally showing the highest binding activity, relative to negative controls and wild-type free oligonucleotide competition. Binding of cREL to κ B oligonucleotide was significantly greater than negative control and after wild-type oligonucleotide competition only in UMSSC-9, in which higher protein levels were detected by Western blot. Although statistically significant, cREL DNA binding in UMSSC-9 was weaker than binding shown by the positive control Raji cell nuclear extracts, indicating cREL DNA binding across the three HNSCC cell lines was relatively weak or undetectable.

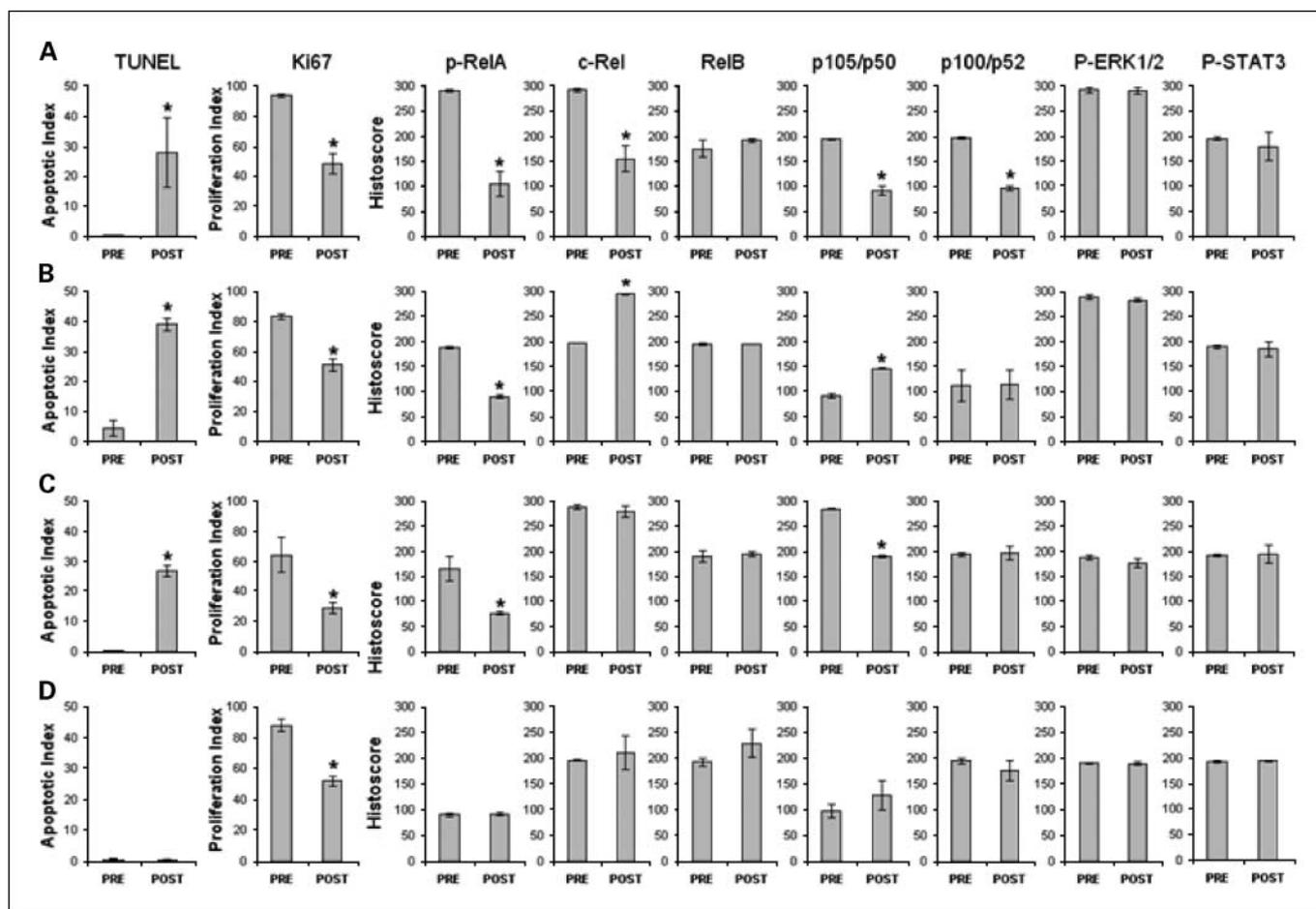


Fig. 3. Panel of histograms summarizing the pretreatment and posttreatment apoptotic indices, proliferation indices, and histoscores for four patients with evaluable biopsies. In patients 1 to 3 (A-C), decreased activated nuclear RELA correlated with increased apoptosis measured via TUNEL assay. Patient 4 (D) showed no decrease in activated RELA and no increase in TUNEL. Change in nuclear localization of total c-REL, RELB, p50, and p52 was variable among the four patients. Decreased proliferation, measured via Ki67 staining, was consistently observed after bortezomib therapy. Nuclear staining of activated ERK1/2 and STAT3 was unaffected by bortezomib in four of four patients. Staining scores shown as a mean of three high power fields. *, statistically significant differences between pretreatment and posttreatment staining scores ($P < 0.05$, Student's t test).

Bortezomib effects on cytotoxicity, basal, and TNF- α inducible RELA and p50 DNA binding activity. To evaluate the *in vitro* cytotoxicity of bortezomib, we exposed HNSCC cell lines to concentrations of 10^{-9} , 10^{-8} , and 10^{-7} mol/L bortezomib for 5 days and evaluated cell death via MTT assays. The lower concentrations chosen for exposure (10^{-9} to 10^{-8} mol/L) were based on approximate peak serum concentrations of bortezomib levels estimated to result after treatment with 0.6 mg/m^2 i.v. bortezomib, using results of previous pharmacokinetic studies (28). At 10^{-9} mol/L, no effect on cell growth or survival was observed in UMSSC-9 or UMSSC-11A and significant inhibition was not observed with 10^{-9} mol/L in UMSSC-11B until day 5 (Fig. 5A). However, with increasing concentrations of bortezomib, dose-dependent cytotoxicity was observed in all three cell lines. Early cell death in UMSSC-9 and UMSSC-11B was observed beginning day 2 with 10^{-8} mol/L, but no significant cytotoxicity was seen in UMSSC-11A until day 3. Significant cell killing was observed in all cells with 10^{-7} mol/L by day 1. Such data indicate variable sensitivity of these HNSCC cell lines to 10^{-9} and 10^{-8} mol/L bortezomib and that only one of three HNSCC lines studied (UMSSC-11B) shows

sensitivity to inhibition by bortezomib in the range estimated to be achieved at concentrations in serum in the present study based on previous pharmacokinetic data (28).

To characterize functional effects of bortezomib treatment on NF- κ B subunit inhibition, we assessed DNA binding activity of all five NF- κ B subunits in HNSCC cells at 4-hour, 12-hour, and 24-hour time points after treatments of 10^{-9} and 10^{-8} mol/L bortezomib with and without TNF- α , a stimulus implicated in autocrine activation of canonical NF- κ B pathway in HNSCC (17, 18). Treatment with TNF- α resulted in significant induction of DNA binding activity of the RELA and p50 subunits only, consistent with its predominant specificity for activation of the canonical pathway. Also, as expected, treatment with bortezomib resulted in time-dependent and concentration-dependent inhibition of TNF- α inducible RELA and p50 DNA binding. Inhibition of inducible DNA binding of RELA and p50 at the 4-hour time point by 10^{-9} and 10^{-8} mol/L bortezomib was equivalent or nearly equivalent (Fig. 5B and C). However, significant differences in binding inhibition between the two bortezomib concentrations were apparent by the 12-hour time point in RELA and by the 12-hour or 24-hour time

points in p50. Generally, inhibition of inducible RELA and p50 DNA binding increased from the 4-hour to 24-hour time points with 10⁻⁸ mol/L bortezomib, whereas inhibition was constant or decreased with 10⁻⁹ mol/L bortezomib. These data indicate that, while there may be no early (4 hours) difference between interruption of RELA and p50 function by 10⁻⁹ and 10⁻⁸ mol/L bortezomib, a significantly greater degree and sustained inhibition of DNA binding function is observed with the higher concentration by 24 hours posttreatment. Our results also indicate that neither 10⁻⁹

nor 10⁻⁸ mol/L bortezomib had any effect on baseline constitutive DNA binding activity of any of the NF-κB subunits by the 24-hour time point in our three HNSCC cell lines (24-hour data shown in Supplementary Fig. S4A and B). However, partial inhibition of the baseline constitutive binding of RELA could be seen at the higher concentration of bortezomib after 36 hours in UMSSC-11B (Supplementary Fig. S5), consistent with the kinetics of inhibitory effects seen in MTT assay by day 2 (Fig. 5A, right). Whereas a similar lack of effect of bortezomib on baseline constitutive cREL, RELB,

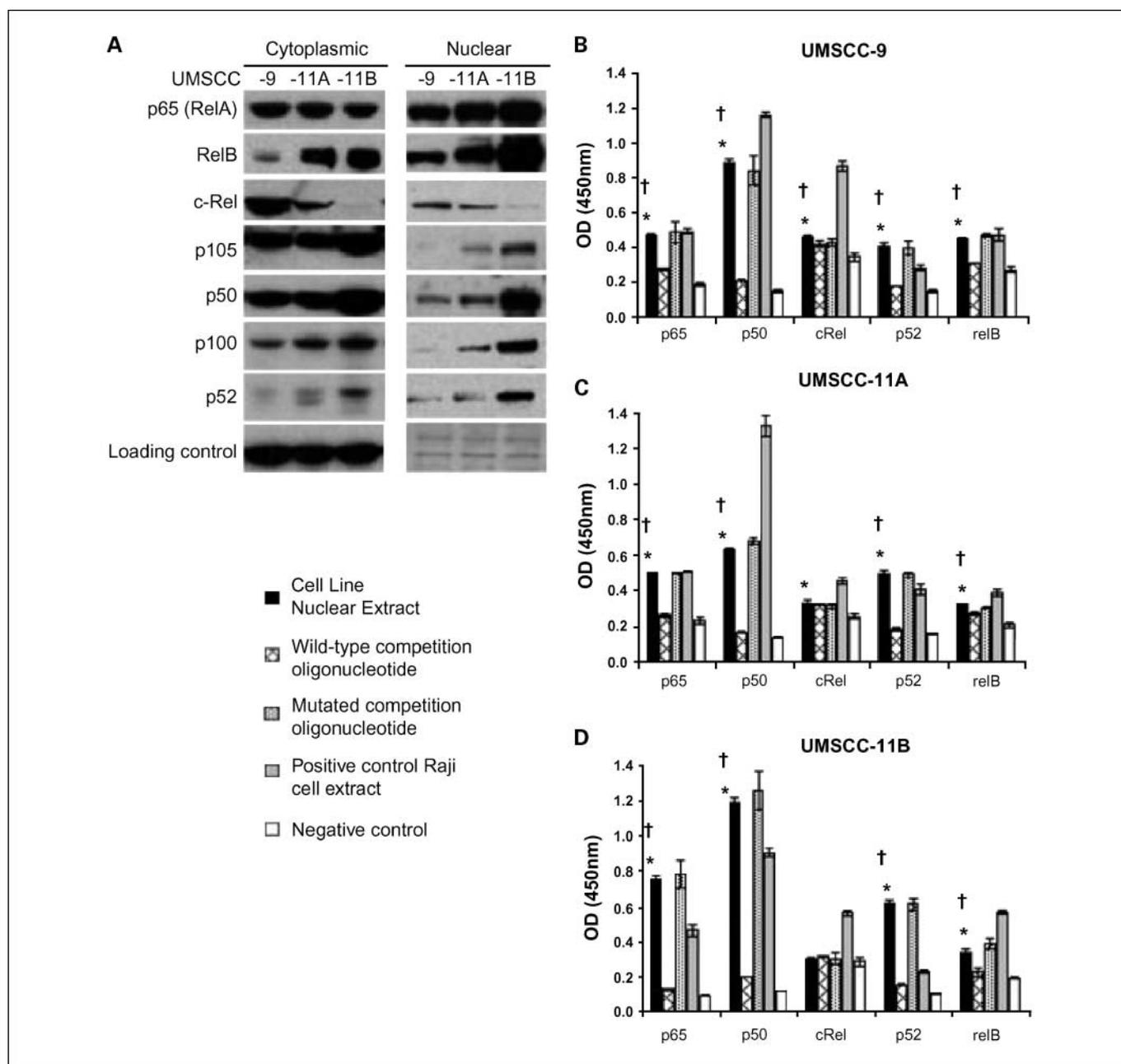


Fig. 4. Baseline NF-κB subunit localization and DNA binding activity of HNSCC cell lines. *A*, Western blot analysis of cytoplasmic and nuclear fractions reveals variable by positive nuclear localization of total RELA, RELB, c-REL, p50, and p52. Whereas UMSSC-11B shows the greatest relative quantity of nuclear RELA, RELB, p50, and p52, UMSSC-9 shows the greatest relative amount of c-REL. Greater relative amounts of the precursor proteins p105 and p100 were found in the cytoplasm compared with the nucleus. *B*, compared with negative and wild-type oligonucleotide controls, all three cell lines show significant constitutive DNA binding activity of RELA, p50, RELB, and p52, whereas only UMSSC-9 shows weak but significant constitutive c-REL DNA binding. *, significant ($P < 0.05$) increase in DNA binding above the negative control; †, significant ($P > 0.05$) increase in DNA binding above the wild-type competition oligonucleotide.

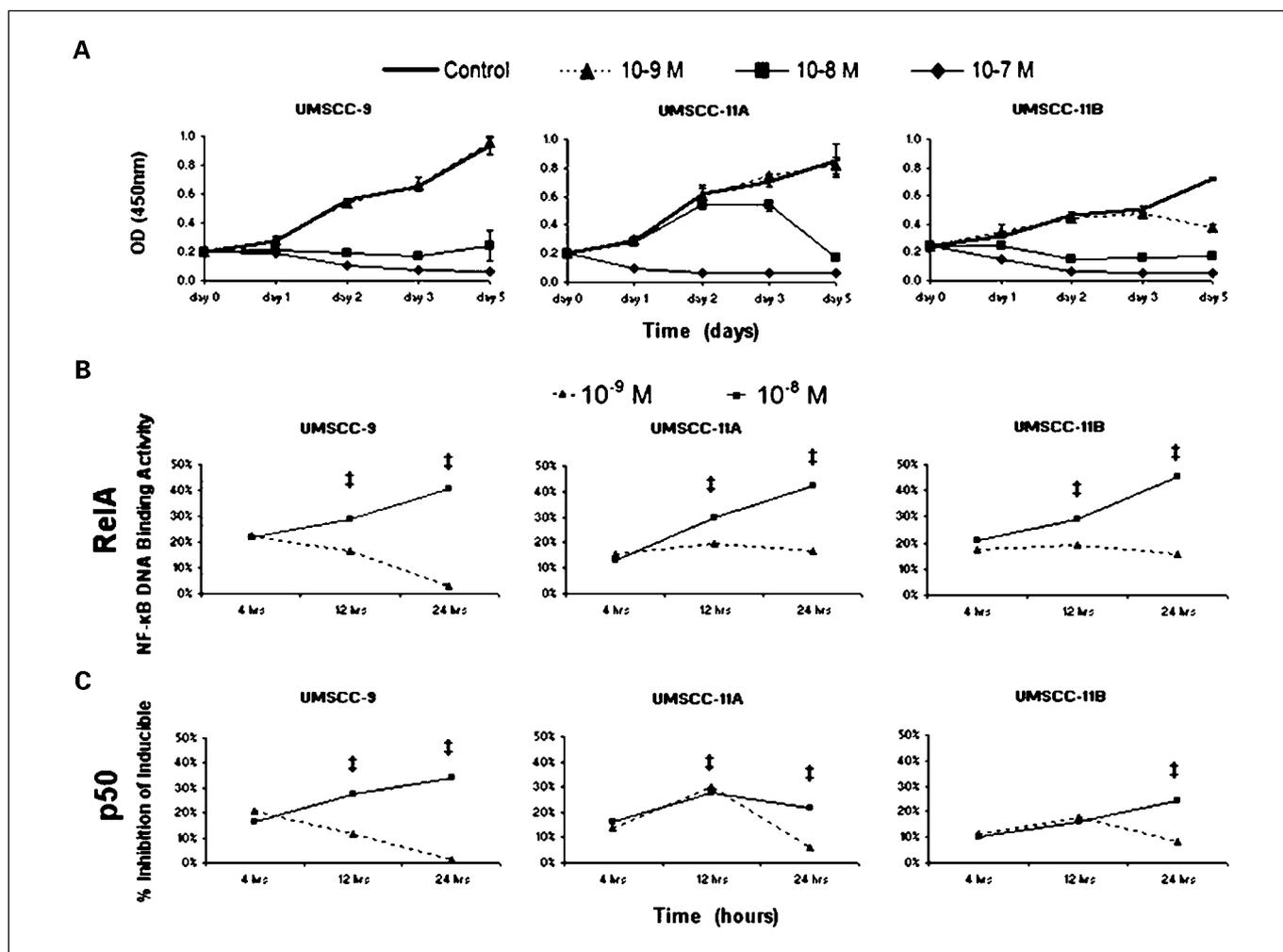


Fig. 5. Cytotoxicity and altered DNA binding activity after bortezomib treatment in HNSCC cell lines. *A*, MTT assays reveal variable cytotoxicity to low dose bortezomib. High dose (10^{-7} mol/L) bortezomib caused significant cell death universally by day 1, low dose (10^{-9} mol/L) caused significant cell death in UMSSC-11B by day 5 only, and intermediate dose (10^{-8} mol/L) caused significant cytotoxicity by day 1 in UMSSC-9 and UMSSC-11A and by day 3 in UMSSC-11A. *, first time point at which 10^{-9} mol/L bortezomib shows significant ($P < 0.05$) cytotoxicity compared with control (untreated) cells; **, first time point at which 10^{-8} mol/L bortezomib shows significant cytotoxicity; †, first time point at which 10^{-7} mol/L bortezomib shows significant ($P < 0.05$) cytotoxicity. *B* and *C*, inhibition of TNF- α inducible RELA and p50 DNA binding, respectively. There seems to be no early (4 h) difference in inhibition of inducible RELA or p50 DNA binding by 10^{-9} or 10^{-8} mol/L bortezomib. However, as inhibition by 10^{-8} mol/L increased with time, inhibition by 10^{-9} mol/L remains constant or decreases with time creating significant differences in inhibition by the 12-h or 24-h time points. ‡, significant ($P < 0.05$) difference in inhibition of inducible activity.

or p52 DNA binding was observed, we were unable to identify ligands able to induce further cREL, RELB, or p52 DNA binding activity to assess the effects of bortezomib on inducible activation of these subunits in HNSCC.

Discussion

Aberrant activation of the NF- κ B/REL family members has been reported in a variety of types of cancer, including HNSCC (7, 29–31). NF- κ B1/RELA, the major component of the canonical pathway, has been most extensively studied and implicated in pathogenesis and resistance of cancers to standard chemotherapy and radiation (8, 31). As a consequence of the well-studied role of the proteasome in κ B degradation and activation of NF- κ B1/RELA, the clinical potential and effects of proteasome inhibitors on cancers demonstrating aberrant activation of NF- κ B1/RELA has also been most extensively examined (5–8, 19, 20, 22, 23, 31). We and other investigators

have detected nuclear activation and DNA binding of NF- κ B and REL subunits of the canonical and alternate pathways (refs. 9, 18 and this study), as well as activation of other signal pathways (9, 27, 32) implicated in cell proliferation and survival, but the activation and effects of proteasome inhibition on these pathways in patient tumors has not been reported.

Here, we studied all five NF- κ B/REL subunits and show that they exhibit aberrant nuclear localization in a panel of HNSCC tumor specimens when compared with the basilar pattern observed in matched noncancerous mucosal epithelium. Nuclear localization of these NF- κ B/REL family members, and binding to consensus κ B oligonucleotide of NF- κ B1, RELA and NF- κ B2, RELB, but not cREL, was also detected in HNSCC cell lines. Together, these observations are consistent with activation and DNA binding of both canonical and noncanonical NF- κ B/REL pathway subunits in HNSCC.

Proteasome inhibitor bortezomib at the lower dosage examined results in histologic evidence of apoptosis but

limited and transient clinical activity outside or within the reirradiation field, suggesting the possibility that the limited peak of proteasome inhibition of $\sim 30\%$ attained at 0.6 mg/m^2 (23) may have differential effects on components of the canonical and alternate pathways or other prosurvival pathways in HNSCC. Bortezomib was found to have inhibitory effects on phosphorylated S536-RELA or p50 together with proapoptotic TUNEL activity in three of four and two of four biopsies, respectively, consistent with contribution of ongoing activation of the canonical pathway to cell survival. However, except in patient 1 who had a rapid initial 50% tumor reduction, no inhibitory effect was observed on nuclear localization of NF- κ B2, RELB, or cREL subunits, nor upon ERK1/2 and STAT3 in any patient. Also, no increase in cJUN implicated in apoptosis in myelomas was observed. These observations suggest that inhibition of RELA/p50 enhanced apoptosis, but incomplete or lack of inhibition of these other NF- κ B and REL subunits, and components of other prosurvival signal pathways could contribute to survival and escape of cancer cells, resulting in the incomplete tumor responses observed.

Consistent with findings in the tumor biopsies, variable levels of all five NF- κ B subunits were also detected in nuclear extracts from patient-derived HNSCC cell lines *in vitro*, enabling analysis of their functional DNA binding activity and effects of bortezomib. All, except for cREL, showed significant competition-specific DNA binding activity to consensus κ B oligonucleotide. Also consistent with the molecular responses observed *in vivo*, we found that bortezomib could inhibit TNF- α -induced DNA binding of canonical pathway subunits RELA and NF- κ B1 (p65, p50). However, bortezomib had minimal effect on baseline binding activity of these subunits or alternative pathway components NF- κ B2 or RELB, over a similar time course *in vitro*. However, partial inhibition of baseline constitutive binding of RELA was observed at higher concentrations of bortezomib after 36 hours, consistent with the kinetics of inhibitory effects in MTT assay for UMSSC 11B (Supplementary Fig. S5 and Fig. 5A). Differential and incomplete inhibition of cell proliferation and survival was also detected among these cell lines. These results further support the hypothesis that bortezomib can interrupt TNF- α -induced and partially inhibit constitutive activation of the RELA-containing components of the canonical pathway and enhance apoptosis, without completely inhibiting the baseline nuclear localization or DNA binding of NF- κ B1/RELA, other NF- κ B/REL family members activated by noncanonical pathways, or other prosurvival pathways, such as ERK1/2 and STAT3. Together, the findings from patient-derived HNSCC tumor specimens and cell lines indicate that further studies are warranted to examine the contribution of noncanonical NF- κ B and coactivated pathways to cell survival and as targets for combined therapy with inhibitors of the proteasome and noncanonical IKK α pathway.

In cancer, an increasingly complex role and function of the canonical and noncanonical IKK-NF- κ B pathways and their components are emerging (8). Consistent with the role of canonical pathway in activation of NF- κ B1/RELA and the effects observed with bortezomib, we showed that expression of I κ B α mutants lacking NH₂ terminal S32 and S36 phosphorylated acceptor sites for TNF- α -induced activation by the canonical IKK complex (I κ B α M or super repressor) inhibits NF- κ B

activation, cell survival and tumorigenesis by these cell lines and tumor xenografts (10). We and others have also shown that interruption of interleukin-1, TNF, epidermal growth factor, and intermediate kinase signaling involved in canonical pathway activation attenuates activation of NF- κ B in HNSCC (15–18). Of note, tobacco-associated and human papillomavirus-positive HNSCC tumors both seem to have high binding activity of the NF- κ B1/RELA dimer, suggesting that activation may be dependent on pathogenic context and stimuli (8, 14, 30). Furthermore, inhibition of RELA by p65 small interfering RNA inhibited p65 and partially inhibited p50 and NF- κ B target genes regulating cell cycle and antiapoptosis while inducing cell cycle arrest and apoptosis in HNSCC (11). This finding is consistent with our previous and present observations that bortezomib can inhibit p65 nuclear localization, cell cycle, and antiapoptotic genes, reduce proliferation, and induce apoptosis in HNSCC *in vitro* and *in vivo* (19, 23). Together, these results confirm that canonical NF- κ B components are important contributors to cell proliferation and survival.

Evidence of activation of NF- κ B2/RELB components of the alternative pathway in the present study suggests that activation of the alternative IKK-NF- κ B pathway may also contribute to pathogenesis and resistance to apoptosis. Consistent with this, we recently showed that kinase-deficient K44A mutants of IKK α , as well as IKK β , implicated in activation of alternative and canonical pathways, respectively, can each partially block NF- κ B reporter and target gene expression in HNSCC (33). IKK α has also recently been shown to have other noncanonical functions as a nuclear factor that can enhance or inhibit NF- κ B activation and NF- κ B-independent differentiation in different contexts (reviewed in refs. 8, 34). These findings further indicate that analysis of the function of canonical and alternative roles of IKKs and NF- κ B pathway components in cell survival and malignant phenotype are warranted.

Based upon increased understanding of overlapping and differential specificity and roles of NF- κ B subunits for binding promoters of target genes, the composition of different NF- κ B subunits should also be taken into account when evaluating inhibition of NF- κ B. The RELA and p50 subunits have been shown to mediate overexpression of certain genes and proteins, including the cell cycle regulatory gene *cyclin D1* and antiapoptotic genes *BCL-X_L* and *cIAP* in HNSCC (11). Furthermore, RELA activation through overexpression in HNSCC induces resistance to radiotherapy (35). Homodimers of p50 mediate expression of the antiapoptotic protein *bcl-2* (36), also shown to be overexpressed in HNSCC (37). Similar to RELA, cREL modulates expression of *BCL-X_L* and *A1* in B lymphocytes (38) and p52 has been shown to mediate expression of cyclin D1 through complex formation with the coactivator BCL3 in hepatocellular carcinoma (39). Our results indicating nuclear localization but low DNA binding activity of cREL to a typical κ B promoter sequence may also be potentially important. Although its binding and functional role in HNSCC has not yet been established, specific overexpression of cREL has been documented in several hematologic and solid tumor types, suggesting it plays a role oncogenesis (40). These and other data suggest that, while inhibition of the conventional p50/RELA NF- κ B heterodimer in HNSCC is desirable, it is likely that clinically significant effects on HNSCC tumor growth and survival will be affected by alternative NF- κ B family members as well.

Our results also indicate bortezomib has no or limited effects upon two other prosurvival pathways commonly activated in HNSCC and other cancers. HNSCC carcinogenesis is known to involve aberrant activation of several signaling pathways, including the progrowth and prosurvival NF- κ B, Ras-mitogen-activated protein kinase-ERK1/2 and interleukin-6-STAT3 pathways (16, 27, 32, 41, 42). NF- κ B has recently been reported to promote coactivation of STAT3 in HNSCC (42). At the low-dose and early posttreatment time points evaluated here, we found no significant inhibition of ERK1/2 and STAT3 pathway by bortezomib. These data suggest that a treatment approach that targets common mechanisms or combines therapies to target the multiple prosurvival pathways commonly coactivated in these tumors is needed. Combination molecular therapy that targets multiple pathways may be needed to obtain significant clinically apparent effects on tumor growth and survival, and future studies using such an approach will be of great interest.

In conclusion, this is a pilot study, and several limitations should be addressed in future studies. First, having tumor biopsies from patients treated with a relatively low dose of bortezomib and only four patients with pretreatment and posttreatment tumor biopsies limits our ability to draw stronger conclusions regarding the role of the proteasome inhibition in the less well-characterized cREL, RELB, and p52 activation pathways *in vivo*. Second, although we show consistent inhibition of TNF- α induced DNA binding of RELA and p50 subunits, we have been unable, thus far, to identify the ligand(s) or intermediate alterations that stimulate cREL, RELB, and p52 binding activity, preventing the ability to draw conclusions regarding mechanisms of activation or blockade

of inducible binding of these subunits in HNSCC cell lines. However, based on our binding assay data, attainment of higher concentrations needed to show strong inhibition of these pathways may not be possible, given previous pharmacokinetic data and dose-limiting toxicities we have encountered with combination bortezomib and radiotherapy (23). In future studies with bortezomib and in other combinations in a larger series of patients, identifying stimuli that activate the alternative pathway in HNSCC lines will likely provide further insight into the role of the proteasome in disrupting activation of non-canonical NF- κ B components. An additional possible limitation involves the use of tumors from patients with recurrent HNSCC as a model for bortezomib activity *in vivo*. Although limited to recurrent disease by our phase I trial, these tumors are highly aggressive and may by nature show up-regulated NF- κ B activity and/or therapeutic resistance due to activation of multiple pathways. Further studies evaluating the biological activity of bortezomib, in combination with agents targeting other pathways in previously untreated HNSCC tumors, will be valuable, and such clinical trials will soon be under way.

Disclosure of Potential Conflicts of Interest

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Bortezomib-Induced Apoptosis with Limited Clinical Response Is Accompanied by Inhibition of Canonical but not Alternative Nuclear Factor- κ B Subunits in Head and Neck Cancer

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