Novel Proteasome Inhibitor PS-341 Inhibits Activation of Nuclear Factor-κB, Cell Survival, Tumor Growth, and Angiogenesis in Squamous Cell Carcinoma[1]

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ABSTRACT

We have shown that activation of nuclear factor-κB (NF-κB) promotes cell survival and expression of cytokines such as growth-regulated oncogene-α, which can modulate angiogenesis, growth, and metastasis of squamous cell carcinoma (SCC). Activation of NF-κB and cytoprotective genes in cancer may result from signal-induced phosphorylation and proteasome-dependent degradation of inhibitor-κB. In this study, we examined the effects of the novel proteasome inhibitor PS-341 on activation of NF-κB and cell survival, growth, and angiogenesis in murine and human SCC cell lines. PS-341 inhibited activation of NF-κB DNA binding and functional reporter activity at concentrations between 10^{-8} and 10^{-7} M. Cytotoxicity was observed at 10^{-7} M in four murine and two human SCC lines, and followed early cleavage of poly(ADP-ribose) polymerase, a marker of caspase-mediated apoptosis. *In vivo*, PS-341 inhibited growth of murine and human SCC in mice at doses of 1–2 mg/kg given three times weekly, and dose-limiting toxicity was encountered at 2 mg/kg. Tumor growth inhibition was associated with a marked decrease in vessel density. PS-341 inhibited expression of the proangiogenic cytokines growth-regulated oncogene-α and vascular endothelial growth factor by SCC in the range at which PS-341 inhibits NF-κB. We conclude that PS-341 inhibits activation of NF-κB pathway components related to cell survival, tumor growth, and angiogenesis in SCC.

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INTRODUCTION

Constitutive activation of NF-κB[3] has been implicated in the development and progression of a number of human malignancies, including head and neck cancer (1), pancreatic cancer (2), colon cancer (3), breast cancer (4, 5), T-cell leukemia (6), and Hodgkin's lymphoma (7). We reported that the constitutive activation of NF-κB in SCCHN promotes the expression of cytokines with proinflammatory and proangiogenic activities (1). Activation of NF-κB was found to be correlated with development of a metastatic phenotype (8) and with autonomous overexpression of an NF-κB-dependent chemokine, called GRO-α (KC), which promotes tumor growth and the development of metastases *in vivo* (9). Further evidence for the significance of NF-κB activation in SCCHN comes from experiments in which transfection of a dominant-negative inhibitor of NF-κB into SCCHN cells resulted in a 70–90% reduction in cell viability (10). Thus, NF-κB activation appears to be important for the survival of SCCHN cells, as well as the conferment of a more aggressive tumor phenotype.

These observations indicate that NF-κB may be an important target for therapy. The prototypical mechanism of NF-κB activation depends on the signal-induced phosphorylation and ubiquitination of an inhibitory protein called IκB-α, which is subsequently degraded by the proteasome (11–14). A novel proteasome inhibitor, PS-341 (Millennium Pharmaceuticals, Inc.) has recently been described to be a potent inhibitor of NF-κB activation. This compound is a dipeptidyl boronic acid analogue that selectively inhibits the chymotryptic activity of the 20S proteasome. It has excellent bioavailability and stability and has been shown to have *in vivo* antitumor activity in a human prostate carcinoma xenograft model (15) and to inhibit both tumor growth and the development of metastases in an animal model of lung carcinoma (16).

In this study, we examined the effect of PS-341 on multiple murine and human SCC cell lines *in vitro* and *in vivo*. Exposure to the compound was found to inhibit activation of NF-κB and expression of the NF-κB-dependent proangiogenic chemokine GRO-α (KC) and VEGF in SCC. Significant effects of PS-341 on cell survival, tumor growth, and angiogenesis were observed.

MATERIALS AND METHODS

Cells Lines. The PAM 212 cell line is a spontaneously transformed cell line derived from neonatal BALB/c keratino-

[3] The abbreviations used are: NF-κB, nuclear factor-κB; SCCHN, squamous cell carcinoma of the head and neck; GRO-α (KC), growth-regulated oncogene-α; IκB, inhibitor of nuclear factor-κB; VEGF, vascular endothelial growth factor; EMSA, electrophoretic mobility shift analysis; PARP, poly(ADP-ribose) polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cytes *in vitro* that forms SCCs *in vivo* and was provided by Dr. Stuart Yuspa of the NCI (Bethesda, MD; Ref. 17). The PAM-LY2 cell line is a metastatic variant of the PAM 212 line isolated from lymph node metastases that formed after s.c. inoculation of PAM 212 tumor fragments in BALB/c mice. Details of the development and characterization of the PAM-LY2 variant have been described previously (18). PAM 212 and PAM-LY2 cells exhibit constitutive activation of NF-κB at low and high levels with respect to each other, respectively (8). The B4B8 and B7E3 cell lines were derived by exposure of murine oral mucosa cells to carcinogens by Dr. Fred Hendler at the University of Louisville and Dr. Giovanna Thomas in our laboratory (19). The human SCCHN cell lines UM-SCC-9 and -11B were obtained from the well-characterized UM-SCC series maintained by Dr. T. E. Carey at the University of Michigan (Ann Arbor, MI; Ref. 20). UM-SCC-9 and -11B have constitutive activation of NF-κB at low and high levels with respect to each other (1). All of the cell lines were maintained in Eagle's Minimal Essential Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.

SCC Tumor Model. BALB/c and BALB/c SCID mice were obtained from the NCI, Frederick Cancer Research and Development Center, Animal Production Area. The mice used were between 4 and 6 weeks of age, male, weight ≤20 g, and housed in a specific pathogen-free animal facility. Animal care was provided under an NIH Animal Care and Use Committee approved protocol (no. 894-99), in accordance with NIH guidelines. *In vivo* experiments with PAM-LY2 cells were conducted by injecting 5×10^6 cells s.c. over the flanks of immunocompetent syngeneic BALB/c mice. *In vivo* experiments with UM-SCC-11B cells were conducted by injecting 1×10^7 cells s.c. over the flanks of immunodeficient BALB/c SCID mice. Tumors were measured by independent animal care personnel in a blinded fashion.

Inhibitor PS-341. The synthesis and purification of PS-341 was described previously by Adams *et al.* (21). For *in vitro* studies, PS-341 was reconstituted in DMSO at a concentration of 10^{-2} M. Appropriate dilutions were performed such that the concentration of DMSO never exceeded 0.001%. For *in vivo* studies, PS-341 was carried in a vehicle containing 10% DMSO in PBS. Solutions were sterilized by filtration through a 45 μm syringe filter. PS-341 was administered using sterile technique by i.p. injections three times/week on a Monday/Wednesday/Friday schedule. For PAM-LY2 tumor-bearing mice, initiation of treatment occurred 6 days after inoculation with tumor cells and prior to the development of palpable tumors. For UM-SCC-11B tumor-bearing mice, initiation of treatment occurred after the development of palpable tumors: at least 0.02 cm^3 for the experiment in which mice received PS-341 at a dose of 1.0 mg/kg and at least 0.25 cm^3 for the experiment in which mice received a dose of 2.0 mg/kg.

Nuclear and Cell Extracts. PAM-LY2 cells (5×10^6) were grown either in medium without PS-341 or in medium containing 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, or 1×10^{-6} M PS-341 for 12 h. The cells were harvested by scraping in PBS and resuspended in lysis buffer [10 mM Tris-HCl (pH 8.0), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, and protease inhibitor cocktail]. After 5 min on ice, the nuclei were pelleted by centrifugation and separated from the cytoplasmic extract. The nuclei were resuspended in nuclear extract buffer [20 mM Tris-HCl (pH 8.0), 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] and incubated at 4°C for 10 min. Nuclear debris was removed from the extract by high-speed centrifugation. Protein concentrations were determined using the BCA protein assay method (Protein Assay Kit; Pierce Chemical Co., Rockford, IL).

EMSA. Double-stranded DNA oligonucleotides for NF-κB and ornithine carbamyl transferase-1 were purchased from Promega (Madison, WI). The consensus sequences (underlined) were: NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and ornithine carbamyl transferase-1: 5′-TGTCGAATGCAAATCACTAGAA-3′. Oligonucleotides were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol; Amersham, Arlington Heights, IL). EMSA was performed as described previously (1). Briefly, nuclear extracts (5 μg) were added to binding buffer [100 mM HEPES-KOH (pH 7.9), 25 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, 0.5% NP-40, and 50% glycerol] containing poly(dI-dC). Competition for oligonucleotide binding was accomplished by the addition of unlabeled oligonucleotide prior to addition of ^{32}P-labeled probes. Nuclear extract proteins were incubated at 20°C for 25 min. Binding complexes were resolved on 5% polyacrylamide gels in 0.25× Tris-borate-EDTA buffer at 20°C and visualized by autoradiography.

Cell Transfection and Reporter Gene Assays. The IgκB-Luc plasmid, containing two copies of the NF-κB-binding site upstream of the minimal promoter fused with the luciferase gene, was described previously (22) and was a kind gift of Dr. U. Siebenlist (National Institute of Allergy and Infectious Disease, NIH). The pCMV-LacZ construct was made by Dr. Giovana Thomas in our laboratory and consists of a *LacZ* gene inserted between the CMV promoter and BGH poly(A) signal sequence in pcDNA3 (Invitrogen, Carlsbad, CA). PAM-LY2 cells (2×10^5/well) were transfected for 5 h at 37°C in 6-well culture plates with 2 μg of pIgk-luc and 0.1 μg of pCMVLacZ DNA plus 16 μl of Lipofectamine (Life Technologies, Gaithersburg, MD) in Optimem medium. Following transfection, the cells were grown in Eagle's Minimal Essential Medium plus 10% fetal bovine serum and exposed to PS-341 at either 10^{-8} or 10^{-7} M for 12 h or left untreated. The cells were harvested, and reporter gene activities were assayed using the Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System (Tropix, Bedford, MA), and chemiluminescence was measured by a Monolight 2010 luminometer (Analytical Luminescence Lab, San Diego, CA). The relative light units were calculated as follows:

$$\text{RLU} = \frac{\text{RLU from Luciferase}}{\text{RLU from β-Galactosidase}}$$

where RLU is relative light unit(s).

Measurement of Cell Proliferation and Viability. Cells (2×10^5/well) were grown in 6-well culture plates and exposed to PS-341 at either 10^{-8} or 10^{-7} M or left untreated. At the 4-, 12-, 24-, and 72-h time points, cells were harvested by trypsinization, stained with trypan blue dye, and visually counted. The percentage of viability was determined by the number of nonstained cells divided by the total number of cells counted.

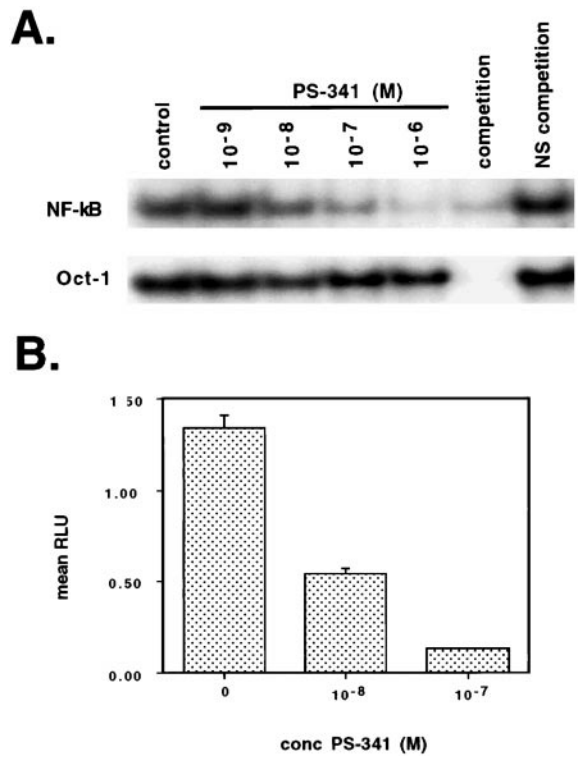

Fig. 1 Inhibition of NF-κB activation by PS-341. PAM-LY2 cells were exposed to increasing concentrations of PS-341 for 12 h, and NF-κB DNA-binding activity was measured by EMSA (*A*) and by 2xκB-luciferase reporter gene assay (*B*). *Bars*, SD.

Isolation of Whole Cell Lysates and Assay for PARP Cleavage. PAM-LY2 cells were grown to 60–90% confluency in sterile 100 × 20 mm polystyrene cell culture dishes. Cells were exposed to either medium without PS-341 or medium containing 1×10^{-7} M PS-341. At 1, 2, 3, 6, 9, and 12 h following exposure to PS-341, the cells were rinsed once with ice-cold PBS, scraped, and lysed in 250 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.2 mM Na_3VO_4, and 0.2 mM phenylmethylsulfonyl fluoride], and incubated on ice for 15 min. The lysates were passed three times through a 23-gauge needle, centrifuged at 14,000 rpm for 5 min at 4°C, and transferred to a fresh microcentrifuge tube. Total protein concentrations were determined using the BCA protein assay method (Protein Assay Kit; Pierce). Each sample (40 μg total protein) was gently vortexed with loading buffer and boiled at 100°C for 5 min. The samples were pulse centrifuged and electrophoresed through 10% Tris-Glycine precast gels (Novex, San Diego, CA) at 120 V. Proteins were transferred to nitrocellulose (Bio-Rad, Hercules, CA) for 90 min at 20 V, using the Novex gel blot module. The nitrocellulose blot was subsequently stained using Ponceau-S (Sigma Chemical Co., St. Louis, MO) to determine transfer efficiency. Blots were blocked with 5% nonfat powdered milk in Tris-buffered saline-Tween overnight at 4°C and incubated for 2 h at room temperature with a rabbit polyclonal IgG to the COOH terminus of PARP that recognizes the full-length and 89-kDa cleaved fragment (Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA). The blot was washed with three times with Tris-buffered saline-Tween and incubated with horseradish peroxidase-conjugated goat antirabbit IgG at a 1:2000 dilution (Bio-Rad) for 1 h at room temperature. Chemiluminescent detection of horseradish peroxidase was accomplished using the SuperSignal Substrate method (Pierce) followed by exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Determination of Tumor Vessel Density. Tumors from BALB/c mice receiving PS-341 (2 mg/kg) and the vehicle control were isolated and frozen in OCT compound. Frozen sections (10 μm) were fixed in formalin and stained with H&E. Adjacent sections were fixed and permeabilized in methanol-acetone (1:1, v/v) and stained with either an antibody to CD31 (PECAM-1, 2.5 μg/ml; PharMingen, San Diego, CA) or to pan-cytokeratin K6 (0.2 μg/ml; Covance Research Products, Inc., Richmond, CA) as described previously (9, 10). The density of vessels was determined by counting the number of vessels per high-powered field (×200) in four areas of each tumor section.

Cytokine Quantitation by ELISA. PAM-LY2 cells were plated (5×10^4 cells/well) in 1.0 ml in sterile 24-well culture plates overnight. The culture medium was removed, and the cells were cultured either in medium without PS-341 or in medium containing 1×10^{-9}, 5×10^{-9}, or 1×10^{-8} M PS-341. Culture supernatants were collected at 24 h, centrifuged at 14,000 rpm for 5 min at 4°C to remove debris, and stored at −20°C until ELISA analysis. ELISAs for GRO-α (KC) and VEGF were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

RNA Preparation and Northern Blot Analysis. PAM-LY2 cells were grown to 80% confluency and exposed to either medium without PS-341 or medium containing 1×10^{-9}, 1×10^{-8}, or 1×10^{-7} M PS-341 for 12 h. Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.), and 20 μg of RNA from each sample were denatured in formaldehyde and formamide at 55°C for 10 min. The RNA was resolved on a 1.2% agarose formaldehyde denaturing gel and transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) by capillary transfer with 20× SSC. After UV cross-linking, the membrane was prehybridized for 1 h at 68°C in QuikHyb solution (Stratagene, La Jolla, CA). Labeling of KC and *GAPDH* cDNA with [^{32}P]dCTP was performed using a random-prime DNA labeling method (Prime-It RmT; Stratagene). The probes were hybridized to the filters overnight at 68°C using standard conditions, followed by autoradiography.

RESULTS

Inactivation of NF-κB by PS-341. Constitutive activation of NF-κB is observed frequently in SCC. By transient transfection of a dominant-negative IκB mutant into SCCHN cells, we previously demonstrated the significance of this constitutive NF-κB activity in cell survival (10). Because exposure to PS-341 has been shown to inhibit tumor necrosis factor α-inducible activation of NF-κB by stabilization of IκB in human umbilical vein endothelial cells (23), we examined what effect it would have on the constitutive activation of NF-κB in SCC (Fig. 1).

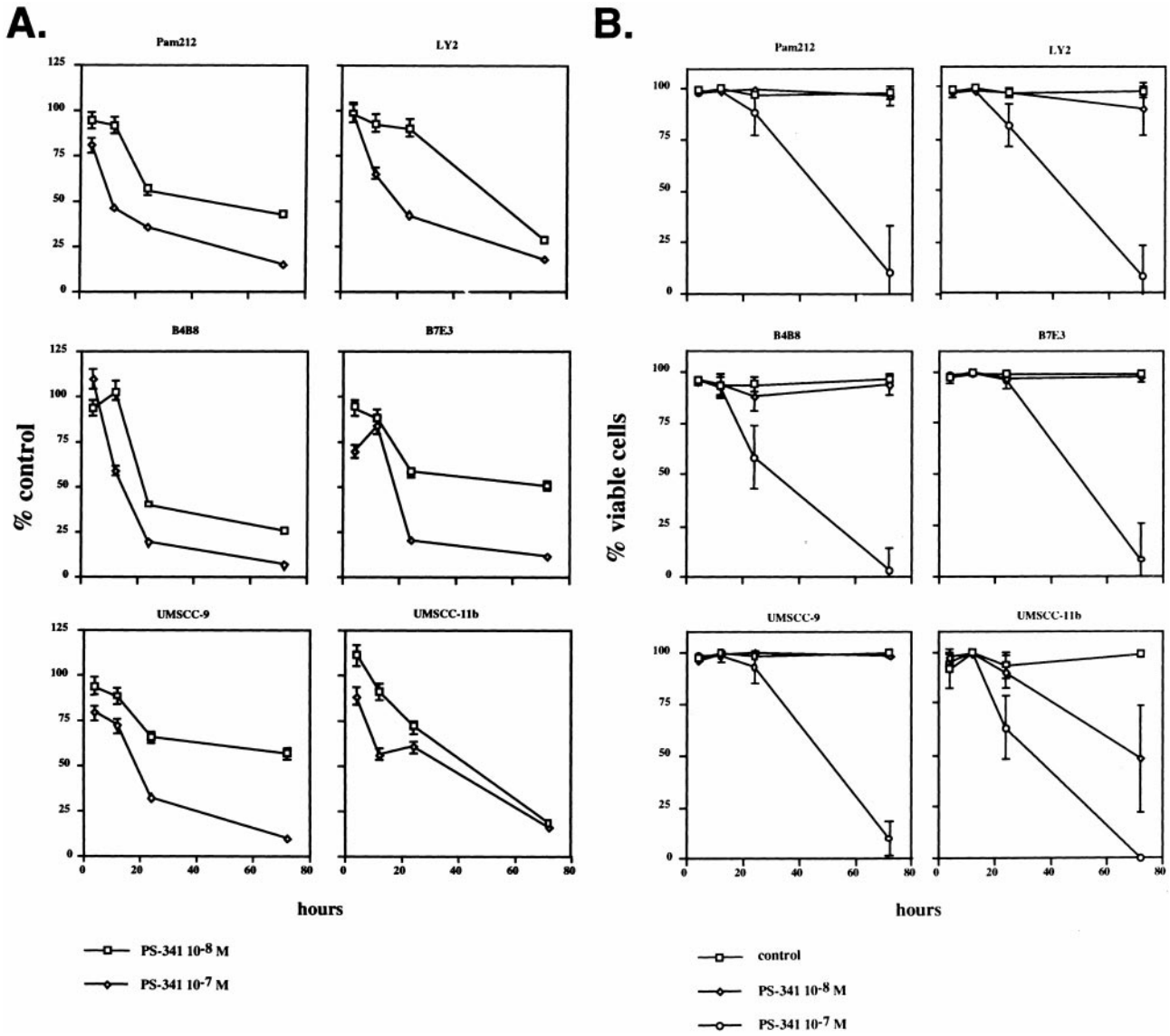

Fig. 2 Cytostatic and cytotoxic activity of PS-341. Four murine SCC cell lines (PAM 212, PAM-LY2, B4B8, and B7E3) and two human SCCHN cell lines (UM-SCC-9 and UM-SCC11B) were exposed to PS-341 at 10^{-8} and 10^{-7} M and were harvested at 4-, 12-, 24-, and 72-h time points. Both attached and detached cells were collected, stained with trypan blue dye, and counted. The percentage of PS-341-treated cells with respect to the number of untreated cells is plotted over the 72-h time course (*A*). Cell viability (*B*) was defined as the percentage of PS-341-treated cells excluding trypan blue dye with respect to the total number of cells at each time point. *Bars*, SD.

We examined the effect of PS-341 on NF-κB activation in cell line PAM-LY2, which exhibits a high constitutive NF-κB activity (8). Cells were exposed to increasing concentrations of PS-341 for 12 h, and NF-κB activity was determined by EMSA and by the κB-luciferase reporter assay. Fig. 1*A* shows that a dose-dependent inhibition of DNA binding activity was observed with an IC_{50} of $\sim 10^{-8}$ M. PAM-LY2 cells transfected with a 2xκB-luciferase reporter construct (IgκB-Luc) also showed inhibition of NF-κB transactivation activity with a similar IC_{50} of $\sim 10^{-8}$ M (Fig. 1*B*). Thus, PS-341 inhibits both DNA binding and functional activation of NF-κB in PAM-LY2 SCC cells.

Cytostatic and Cytotoxic Effects of PS-341. The effect of PS-341 on cell growth was examined in four murine SCC cell lines (PAM 212, PAM-LY2, B4B8, and B7E3) and two human SCCHN cell lines (UM-SCC-9 and UM-SCC-11B). These cells were exposed to PS-341 concentrations of 10^{-8} and 10^{-7} M and were harvested at 4-, 12-, 24-, and 72-h time points. Both attached and detached cells were collected and counted. A reduction in the number of cells was observed in the majority of the cell lines exposed to the higher concentration by 12 h (Fig. 2*A*). By 24 h, a dramatic reduction in the total number of cells was seen in all of the cell lines. A range of sensitivity to the compound was observed. Upon exposure to PS-341 at the higher

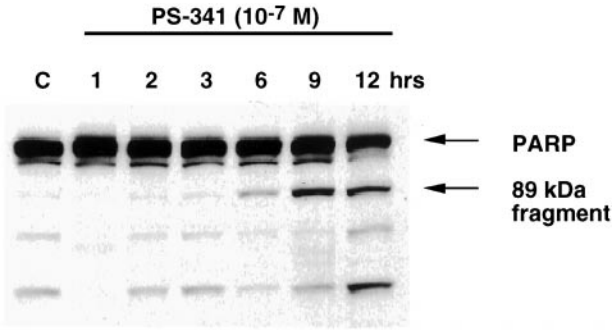

Fig. 3 Activation of PARP cleavage by PS-341. Whole-cell lysates of PAM-LY2 cells were prepared at 0, 1, 2, 3, 6, 9, and 12 h after exposure to 10^{-7} M PS-341. Proteins (40 μg) were separated by PAGE, and immunoblot analysis was performed with polyclonal antibodies to PARP. The 112-kDa intact PARP protein and the 89-kDa cleaved fragment are indicated.

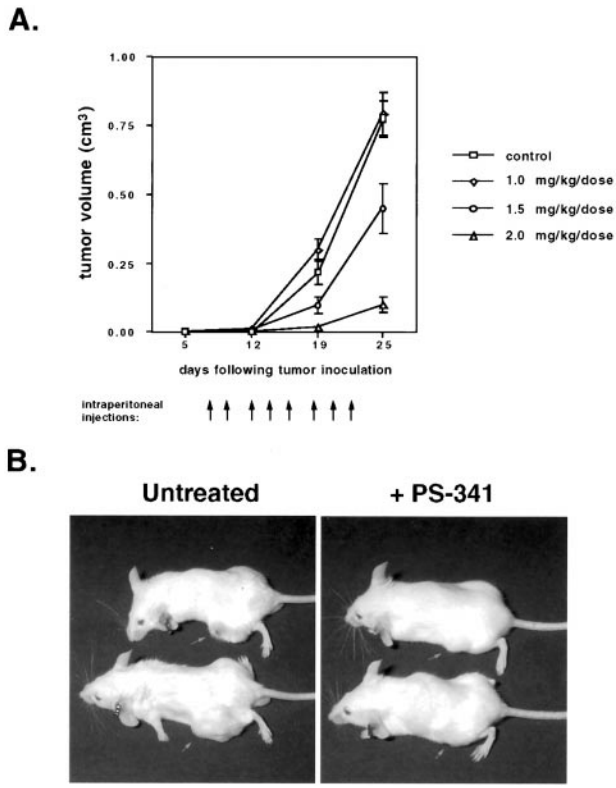

Fig. 4 Antitumor activity of PS-341 in PAM-LY2 tumor-bearing mice. PAM-LY2 cells (5×10^6 cells/mouse) were injected s.c. over the flanks of immunocompetent syngeneic BALB/c mice and allowed to develop tumors. *A*, i.p. injections of PS-341 were administered on a Monday, Wednesday, Friday schedule, with the time of each dose indicated by an *arrow*. Tumors were measured in a blinded fashion by an independent technician. Tumor volume was calculated using the following equation: volume = $(0.5 \times L^2 \times W)$, where L is length and W is width. Mean tumor volumes ± SD (*bars*) for $n = 5$ mice/group are shown. Vehicle for compound delivery was 10% DMSO in PBS. *B*, photographs of mice were taken at 25 weeks after tumor inoculation, just before sacrifice. *Arrows* indicate tumors. The PS-341-treated mice in the photograph received the compound at doses of 2.0 mg/kg. The untreated mice received 10% DMSO in PBS.

concentration (10^{-7} M), a significant reduction in viability was observed. This higher concentration correlated with the concentration at which near-complete inhibition of constitutive NF-κB activation was observed (Fig. 1).

The reduction in total cell number induced by exposure to 10^{-8} M PS-341 did not correlate with a reduction in cell viability in most cell lines as determined by trypan blue exclusion assay (Fig. 2*B*). Specifically, among the murine cell lines, PAM 212 and B4B8 exhibited an earlier and more significant reduction in cell number with the lower concentration of PS-341. Among the human cell lines, UM-SCC-11B exhibited a greater sensitivity to the lower concentration of PS-341 and was the only line that showed a reduction in the percentage of viable cells at this concentration. This indicated that the reduction in cell number at this concentration is more a reflection of decreased cell proliferation than of cell death. Proteasome inhibition with PS-341 has been reported to cause accumulation of cell cyclin-dependent kinase inhibitor p21 (CIP1/WAF1), and inhibition of cell cycle progression and proliferation in PC-3 cells (15). Consistent with this, we observed a 5-fold increase in p21 at both 10^{-7} and 10^{-8} M by Western blot, which was accompanied by increased accumulation of cells in the S and G_2-M phase by 24 h, prior to the appearance of sub-G_0-G_1 fragments of dead cells at 72 h by DNA flow cytofluorometry (data not shown).

Activation of the caspase cascade was examined in PAM-LY2 cells following exposure to 10^{-7} M PS-341, which resulted in cell death, by immunoblotting for PARP and cleaved PARP fragments (Fig. 3). PARP is a 112-kDa nuclear protein that is specifically cleaved by caspase-3, and the detection of an 89-kDa cleaved fragment is indicative of apoptosis (24, 25). Whole-cell lysates of PAM-LY2 cells were prepared at 0, 1, 2, 3, 6, 9, and 12 h following exposure to 10^{-7} M PS-341. PARP cleavage was evident by 6 h, a time point well before morphological evidence of cell death (Fig. 2).

Antitumor Activity of PS-341 *in Vivo*. The ability of PS-341 to inhibit tumor growth was examined *in vivo*. PAM-LY2 cells were injected s.c. over the flanks of immunocompetent syngeneic BALB/c mice and allowed to form tumors. These tumor-bearing mice were treated with i.p. injections of PS-341 at dosages of 1, 1.5, and 2.0 mg/kg or vehicle alone as a control ($n = 5$ mice/group) on a Monday, Wednesday, Friday schedule as indicated in Fig. 4. A dose-dependent inhibition of tumor growth was observed. Significant inhibition was noted at doses ≥1.5 mg/kg at days 19 and 25 ($P < 0.05$). Toxicities, noted at doses of 2.0 mg/kg, included dehydration, lethargy, and weight loss; at this dose, two of the five mice died following the last treatment on days 23 and 25, and the final tumor measurements of these mice are included at day 25.

In two additional experiments, the human cell line UM-SCC-11B, was injected s.c. over the flanks of immunodeficient BALB/c SCID mice, and once tumors developed, i.p. injections of PS-341 or vehicle alone as a control were administered as shown in Fig. 5. Significant inhibition of tumor growth was observed in mice receiving 1 mg/kg (Fig. 5*A*; $P < 0.05$, days 19–22; $n = 5$ mice/group), and mice receiving the lower dose appeared healthy throughout the treatment. Tumor regression was observed in mice receiving doses of 2.0 mg/kg *versus*

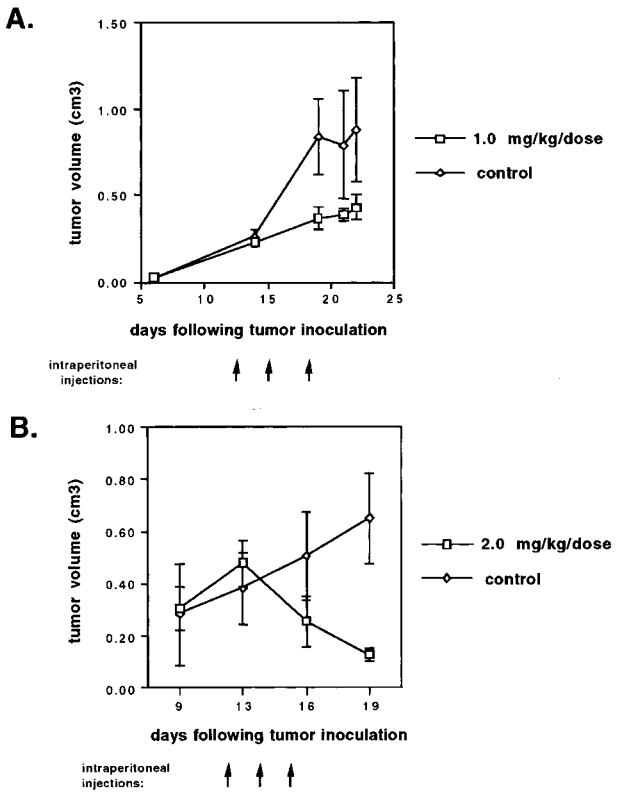

Fig. 5 Antitumor activity of PS-341 in human tumor xenograft-bearing mice. UM-SCC-11B cells (1×10^7 cells/mouse) were injected s.c. over the flanks of immunodeficient BALB/c SCID mice and allowed to develop tumors. i.p. injections of PS-341 were administered on a Monday, Wednesday, Friday schedule, with the time of each dose indicated by an *arrow*. Two experiments were conducted: one using PS-341 at doses of 1.0 mg/kg (*A*) and the other at doses of 2.0 mg/kg (*B*). Tumors were measured in a blinded fashion by an independent technician. Tumor volume was calculated using the following equation: volume = $(0.5 \times L^2 \times W)$, where L is length and W is width.. Mean tumor volumes ± SD (*bars*) for doses of 1.0 mg/kg ($n = 5$ mice/group; *A*) and 2.0 mg/kg ($n = 6$ mice/group; *B*) are shown. Vehicle for compound delivery and control mice was 10% DMSO in PBS.

controls (Fig. 5*B*; $P < 0.05$, day 19; $n = 6$ mice/group). Of note, these human xenograft tumors appeared much more sensitive to the compound than the PAM-LY2 tumors and required only three doses at 2.0 mg/kg to achieve regression. This relative sensitivity is consistent with the *in vitro* data shown in Fig. 2. Again, mice receiving the higher dose exhibited the toxicities observed previously, and three of the six mice receiving this dose died just prior to termination of the experiment. The final tumor measurements of four of six evaluable mice were included at day 19. Similar toxicity of PS-341 has been observed in toxicity studies in mice receiving doses ≥2 mg/kg.[4]

Decreased Blood Vessel Density in Tumors Treated with PS-341. Tumors harvested from PAM-LY2-bearing mice were sectioned and examined for histological changes by H&E staining and for blood vessel density by immunohistochemical staining for the endothelial cell specific antigen CD31. The photomicrographs in Fig. 6, *A* and *B*, shows tumor from control group mice, which demonstrates the typical histology of Pam-LY2, including vessels (Fig. 6, *A* and *B*, *arrows*). Fig. 6 shows that regression following PS-341 treatment is accompanied by decreased cellular density and necrosis (Fig. 6*D*, *arrow*) in association with decreased vessel density as measured by CD31 staining (Fig. 6*E*). The decrease in intratumoral blood vessel density in mice receiving PS-341 at a dose of 2.0 mg/kg was significant when compared with untreated tumor-bearing mice ($P < 0.0001$, paired t test). These results suggest that in addition to direct cytotoxic activity, PS-341 may have antiangiogenic activity in tumors.

Inhibition of Proangiogenic Cytokine Expression by PS-341. Previous observations by our laboratory have indicated significantly higher production of the NF-κB-dependent proangiogenic cytokines GRO-α (KC; Ref. 26) and VEGF[5] by the PAM-LY2 cell line compared with the parental PAM 212 cell line. We have recently demonstrated the importance of KC in promoting angiogenesis, tumor growth, and metastasis (9). In that study, introduction of the KC transgene into PAM 212 cells specifically promoted the growth and metastasis of tumors in immunocompetent syngeneic mice. In addition, enhanced leukocyte infiltration and intratumoral vessel formation was observed. Because PAM-LY2-bearing mice treated with PS-341 in this study were found to have tumors with a significant reduction in blood vessel density and because inhibition of NF-κB by PS-341 was observed in this cell line, we examined the effect of PS-341 on the expression of KC and VEGF.

PAM-LY2 cells were treated ± PS-341 for 24 h at concentrations of 1×10^{-9}, 5×10^{-9}, and 1×10^{-8} M. A significant dose-dependent reduction in the constitutive production of both KC and VEGF was measured by immunoassay of cell supernatants (Fig. 7). To verify that the decrease in cytokines was not simply a result of PS-341-induced cell death, PAM-LY2 cells were treated with or without PS-341 for 8 h, and KC mRNA expression was examined by Northern analysis (Fig. 8). A dose-dependent inhibition of KC expression relative to the expression of the *GAPDH* housekeeping gene was noted with an IC_{50} of $\sim 10^{-8}$ M. This is consistent with the IC_{50} seen with PS-341-induced NF-κB inhibition (Fig. 1). Thus, PS-341 inhibits the expression of NF-κB-dependent proangiogenic cytokines, possibly contributing to the decreased blood vessel density observed in PAM-LY2 tumors treated with the compound.

DISCUSSION

Proteasome inhibitors are emerging as a promising class of chemotherapeutic agents in the treatment of cancer. *In vitro* screening data from a panel of 60 cell lines derived from human tumors indicated that PS-341 has significant growth-inhibitory activity against a wide variety of malignancies (15). Two studies examining prostate and lung carcinoma animal models have also

[4] P. Elliott, unpublished observations.

[5] Z. Chen, unpublished observations.

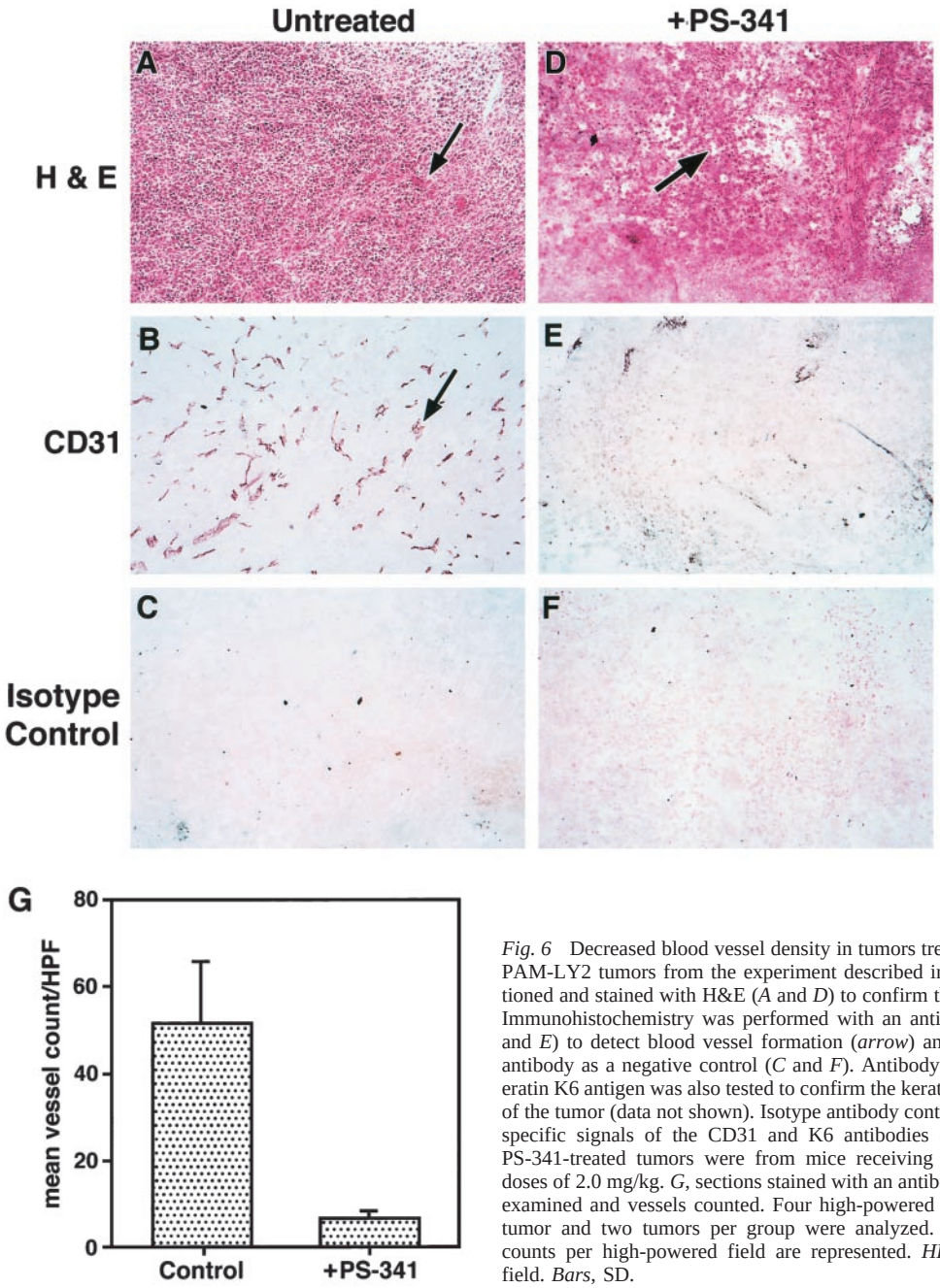

Fig. 6 Decreased blood vessel density in tumors treated with PS-341. PAM-LY2 tumors from the experiment described in Fig. 4 were sectioned and stained with H&E (*A* and *D*) to confirm the SCC histology. Immunohistochemistry was performed with an antibody to CD31 (*B* and *E*) to detect blood vessel formation (*arrow*) and with an isotype antibody as a negative control (*C* and *F*). Antibody to the pan cytokeratin K6 antigen was also tested to confirm the keratinocyte derivation of the tumor (data not shown). Isotype antibody controls confirmed the specific signals of the CD31 and K6 antibodies (not shown). The PS-341-treated tumors were from mice receiving the compound at doses of 2.0 mg/kg. *G*, sections stained with an antibody to CD31 were examined and vessels counted. Four high-powered fields (×200) per tumor and two tumors per group were analyzed. The mean vessel counts per high-powered field are represented. *HPF*, high-powered field. *Bars*, SD.

demonstrated that PS-341 has significant antitumor activity *in vivo* (15, 16). In the study presented here, we have shown that PS-341 has potent cytotoxic activity in a number of SCC cell lines and inhibits tumor growth *in vivo*. Although morphological cell death, measured by trypan blue exclusion assay, was not evident until after 24 h of exposure to PS-341, processes shown to be involved in apoptotic cell death appeared to be activated much earlier. Specifically, we found that commitment to apoptosis caused by PS-341 treatment appears to occur as early as 6 h, because this is the time at which we observed cleavage of PARP in cells treated with PS-341.

We have demonstrated that inhibition of cell proliferation occurs at 10^{-8} M in SCC and precedes morphological cell death induced by PS-341 at 10^{-7} M. A cell cycle blockade induced by PS-341 has been observed at the S-phase and G_2-M transition in PC-3 prostate carcinoma cells (15). The molecular mechanism of the PS-341-induced G_2-M block probably involves the dysregulated processing and degradation of cell cycle regulatory proteins by the proteasome. The cyclin-dependent kinase inhibitor p21, which normally is ubiquitinated and degraded by the proteasome, has been shown to accumulate along with an increase in cells in S and G_2-M upon PS-341 exposure (15). We

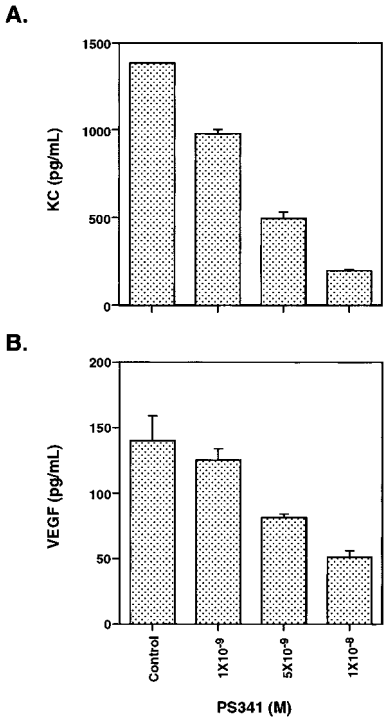

Fig. 7 PS-341 inhibits production of proangiogenic factors. PAM-LY2 cells, which constitutively express high levels of GRO-α (KC) and VEGF, were incubated in medium without PS-341 or in medium containing 1×10^{-9}, 5×10^{-9}, or 1×10^{-8} M PS-341. Culture supernatants were collected at 24 h, centrifuged at 14,000 rpm for 5 min at 4°C to remove debris, and stored at −20°C until ELISA analysis. *Bars*, SD.

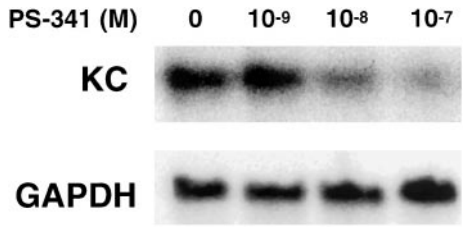

Fig. 8 PS-341 inhibits the mRNA expression of GRO-α (*KC*). PAM-LY2 cells were treated in the absence or presence of PS-341 for 8 h at concentrations of 10^{-9}, 10^{-8}, and 10^{-7} M. Total RNA was isolated, and 20 μg of RNA/lane were separated by gel electrophoresis. Northern analysis was performed with ^{32}P-labeled probes for KC and *GAPDH*.

observed similar accumulation of p21 and cells in the S and G_2-M phase in PAM 212 and PAM-LY2 SCC cells cultured with 10^{-8} M PS-341.[6] We examined the effect of PS-341 on p27 and cyclin D1 but did not observe any changes in the levels of these cell cycle regulatory proteins by immunoblot analysis (data not shown). Thus, it seems likely that the effects of PS-341 observed reflect the effects of more than one proteasome-dependent mechanism, particularly at concentrations below those that have significant effects on activation of NF-κB and apoptotic mechanisms.

We have shown that PS-341 is a potent inhibitor of NF-κB activation in SCC cells. The mechanism of this inhibition has been shown to involve stabilization of the NF-κB "inhibitor" protein IκB-α, which in unstimulated cells sequesters NF-κB to the cytoplasm (23, 27). Previous work by our laboratory has demonstrated the importance of NF-κB activation in the survival and progression of SCCHN (1, 10). The inhibition of NF-κB by PS-341 probably contributes to the compound's antitumor effects seen in SCC-bearing mice treated in this study. It is interesting that the UM-SCC-11B human cell line, which has a much higher constitutive activation of NF-κB than the UM-SCC-9 cell line (1), has a greater sensitivity to PS-341. We have previously shown that the survival of UM-SCC-11B is highly dependent on activation of NF-κB, supporting the hypothesis that NF-κB is an important biological target of PS-341 (10). The antiapoptotic effects associated with NF-κB activation may involve a variety of NF-κB-regulated genes such as *IAP-1/2* and *Bcl* family members (28, 29). *IAP-1* was recently found to be activated in PAM-LY2 cells, which exhibit increased activation of NF-κB.[7] Although NF-κB appears to be a significant target of PS-341, inhibition of the proteasome affects a wide variety of regulatory proteins (30), and inhibition of NF-κB activity is probably only one of a number of factors contributing to cell death. From a therapeutic point of view, this multifaceted effect of PS-341 may explain the broad spectrum of antitumor activity seen in the NCI *in vitro* screening of multiple cancer cell lines (15).

In addition to direct cytotoxicity toward tumor cells, PS-341 may be exerting its effects on tumor growth through anti-angiogenic mechanisms, as observed in the CD31 staining of tumors from mice treated with the compound. One possible mechanism may be the down-regulation of KC and VEGF production by PS-341. We have shown that the NF-κB-inducible chemokine KC specifically promotes intratumoral blood vessel formation, tumor growth, and metastasis (9). The murine chemokine KC shares sequence homology and chemotactic activity with the human C-X-C family chemokines GRO-α and interleukin-8. Consistent with this possibility, Huang *et al.* (31) reported that inhibition of NF-κB by a dominant-negative IκB mutant can inhibit interleukin-8 expression and growth, angiogenesis, and metastasis of human melanoma xenografts in SCID mice. Harbison *et al.* (32) have recently found that PS-341 treatment also reduces survival and proliferation of endothelial cells in human pancreatic carcinoma xenografts and is modulated by NF-κB-dependent expression of VEGF. By examining early effects of PS-341, they have obtained evidence that endothelial cell apoptosis may precede tumor cell apoptosis. However, PS-341 may also be directly cytotoxic to endothelial cells,[6]

[6] J. Sunwoo *et al.*, unpublished observations.

[7] G. Dong *et al.*, Molecular profiling of transformed and metastatic murine squamous carcinoma cells by differential display and cDNA microarray reveals altered expression of multiple genes related to growth, apoptosis, angiogenesis and the NF-κB signal pathway, submitted for publication.

and therefore, multiple mechanisms may contribute to the decrease in vascularity observed.

Doses up to 1.5 mg/kg were tolerated by the mice in this study, and the toxicities seen with the higher doses are consistent with previous animal studies (15). One problem with s.c. xenograft models such as these is the fact that the distribution of PS-341 to the skin is limited, and therefore higher doses are probably needed to achieve delivery to the tumor, creating a narrow therapeutic window (15). It is possible that efficacy may occur at doses well below the toxic range in the clinical setting.

Inhibition of NF-κB has been shown to sensitize transformed cells to tumor necrosis factor-α and various chemotherapeutic agents such as CPT-11 (33, 34). The use of PS-341 in combination with 5-fluorouracil, cisplatin, paclitaxel, and Adriamycin has been explored and appears to have an additive antiproliferative effect and to inhibit the formation of metastases (16). Although we have demonstrated the significant antitumor activity of PS-341 in this study, it may be possible to achieve greater antitumor effects at lower less toxic doses of the compound when it is used in combination with other therapies. Further studies examining these interactions are warranted.

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REFERENCES

1. Ondrey, F. G., Dong, G., Sunwoo, J., Chen, Z., Wolf, J. S., Crowl-Bancroft, C. V., Mukaida, N., and Van Waes, C. Constitutive activation of transcription factors NF-(κ)B, AP-1, and NF-IL6 in human head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines. Mol. Carcinog., *26:* 119–129, 1999.

2. Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. The nuclear factor-κ B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res., *5:* 119–127, 1999.

3. Bours, V., Dejardin, E., Goujon-Letawe, F., Merville, M. P., and Castronovo, V. The NF-κ B transcription factor and cancer: high expression of NF-κ B- and I κ B-related proteins in tumor cell lines. Biochem. Pharmacol., *47:* 145–149, 1994.

4. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol., *17:* 3629–3639, 1997.

5. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig., *100:* 2952–2960, 1997.

6. Mori, N., Fujii, M., Ikeda, S., Yamada, Y., Tomonaga, M., Ballard, D. W., and Yamamoto, N. Constitutive activation of NF-κB in primary adult T-cell leukemia cells. Blood, *93:* 2360–2368, 1999.

7. Bargou, R. C., Emmerich, F., Krappmann, D., Bommert, K., Mapara, M. Y., Arnold, W., Royer, H. D., Grinstein, E., Greiner, A., Scheidereit, C., and Dorken, B. Constitutive nuclear factor-κB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J. Clin. Investig., *100:* 2961–2969, 1997.

8. Dong, G., Chen, Z., Kato, T., and Van Waes, C. The host environment promotes the constitutive activation of nuclear factor-κB and proinflammatory cytokine expression during metastatic tumor progression of murine squamous cell carcinoma. Cancer Res., *59:* 3495–3504, 1999.

9. Loukinova, E., Dong, G., Enamorado-Ayalya, I., Thomas, G., Chen, Z., and Van Waes, C. Growth-regulated oncoprotein-α expression by murine squamous-cell carcinoma promotes tumor growth, metastasis, leukocyte infiltration, and angiogenesis by a host CXC receptor-2-dependent mechanism. Oncogene, *19:* 3477–3486, 2000.

10. Duffey, D. C., Chen, Z., Dong, G., Ondrey, F. G., Wolf, J. S., Brown, K., Siebenlist, U., and Van Waes, C. Expression of a dominant-negative mutant inhibitor κBα of nuclear factor-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo*. Cancer Res., *59:* 3468–3474, 1999.

11. Ghosh, S., and Baltimore, D. Activation *in vitro* of NF-κ B by phosphorylation of its inhibitor I κ B. Nature (Lond.), *344:* 678–682, 1990.

12. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. The ubiquitin-proteasome pathway is required for processing the NF-κ B1 precursor protein and the activation of NF-κ B. Cell, *78:* 773–785, 1994.

13. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. A proteasome inhibitor prevents activation of NF-κ B and stabilizes a newly phosphorylated form of I κB-α that is still bound to NF-κ B. EMBO J., *13:* 5433–5441, 1994.

14. Baldwin, A. S., Jr. The NF-κ B and Iκ B proteins: new discoveries and insights. Annu. Rev. Immunol., *14:* 649–683, 1996.

15. Adams, J., Palombella, V. J., Sausville, E. A., Johnson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., and Elliott, P. J. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res., *59:* 2615–2622, 1999.

16. Teicher, B. A., Ara, G., Herbst, R., Palombella, V. J., and Adams, J. The proteasome inhibitor PS-341 in cancer therapy. Clin. Cancer Res., *5:* 2638–2645, 1999.

17. Yuspa, S. H., Hawley-Nelson, P., Koehler, B., and Stanley, J. R. A survey of transformation markers in differentiating epidermal cell lines in culture. Cancer Res., *40:* 4694–4703, 1980.

18. Chen, Z., Smith, C. W., Kiel, D., and Van Waes, C. Metastatic variants derived following *in vivo* tumor progression of an *in vitro* transformed squamous-cell carcinoma line acquire a differential growth advantage requiring tumor-host interaction. Clin. Exp. Metastasis, *15:* 527–537, 1997.

19. Thomas, G. R., Chen, Z., Oechsli, M. N., Hendler, F. J., and Van Waes, C. Decreased expression of CD80 is a marker for increased tumorigenicity in a new murine model of oral squamous-cell carcinoma. Int. J. Cancer, *82:* 377–384, 1999.

20. Krause, C. J., Carey, T. E., Ott, R. W., Hurbis, C., McClatchey, K. D., and Regezi, J. A. Human squamous-cell carcinoma. Establishment and characterization of new permanent cell lines. Arch. Otolaryngol., *107:* 703–710, 1981.

21. Adams, J., Behnke, M., Chen, S., Cruickshank, A. A., Dick, L. R., Grenier, L., Klunder, J. M., Ma, Y. T., Plamondon, L., and Stein, R. L. Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. Bioorg. Med. Chem. Lett., *8:* 333–338, 1998.

22. Kanno, T., Brown, K., and Siebenlist, U. Evidence in support of a role for human T-cell leukemia virus type I Tax in activating NF-κ B via stimulation of signaling pathways. J. Biol. Chem., *270:* 11745–11748, 1995.

23. Palombella, V. J., Conner, E. M., Fuseler, J. W., Destree, A., Davis, J. M., Laroux, F. S., Wolf, R. E., Huang, J., Brand, S., Elliott, P. J., Lazarus, D., McCormack, T., Parent, L., Stein, R., Adams, J., and Grisham, M. B. Role of the proteasome and NF-κB in streptococcal cell wall-induced polyarthritis. Proc. Natl. Acad. Sci. USA, *95:* 15671–15676, 1998.

24. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature (Lond.), *371:* 346–347, 1994.

25. Duriez, P. J., and Shah, G. M. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem. Cell Biol., *75:* 337–349, 1997.

26. Smith, C. W., Chen, Z., Dong, G., Loukinova, E., Pegram, M. Y., Nicholas-Figueroa, L., and Van Waes, C. The host environment pro-

motes the development of primary and metastatic squamous cell carcinomas that constitutively express proinflammatory cytokines IL-1α, IL-6, GM-CSF, and KC. Clin. Exp. Metastasis, *16:* 655–664, 1998.

27. Karin, M. How NF-κB is activated: the role of the IκB kinase (IKK) complex. Oncogene, *18:* 6867–6874, 1999.

28. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science (Wash. DC), *281:* 1680–1683, 1998.

29. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol. Cell. Biol., *19:* 5923–5929, 1999.

30. Ciechanover, A., Orian, A., and Schwartz, A. L. The ubiquitin-mediated proteolytic pathway: mode of action and clinical implications. J. Cell. Biochem., *77:* 40–51, 2000.

31. Huang, S., DeGuzman, A., Bucana, C. D., and Fidler, I. J. Constitutive NF-κB activity regulates growth, angiogenesis, and metastasis of cutaneous human melanoma. Proc. Am. Assoc. Cancer Res., *41:* 636, 2000.

32. Harbison, M. T., Bruns, C. J., Bold, R. J., Elliot, P. J., Adams, J., Abbruzzese, J. L., and McConkey, D. J. Proteasome inhibitor PS-341 effective as an anti-angiogenic agent in the treatment of human pancreatic carcinoma via the inhibition of NF-κB and subsequent inhibition of vascular endothelial growth factor production. Proc. Am. Assoc. Cancer Res., *41:* 71, 2000.

33. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science (Wash. DC), *274:* 784–787, 1996.

34. Wang, C. Y., Cusack, J. C., Jr., Liu, R., and Baldwin, A. S., Jr. Control of inducible chemoresistance: enhanced antitumor therapy through increased apoptosis by inhibition of NF-κB. Nat. Med., *5:* 412–417, 1999.

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