

Enhanced Chemosensitivity to CPT-11 with Proteasome Inhibitor PS-341: Implications for Systemic Nuclear Factor- κ B Inhibition¹

James C. Cusack, Jr.,² Rong Liu, Michael Houston, Karin Abendroth, Peter J. Elliott, Julian Adams, and Albert S. Baldwin, Jr.

Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts 02114 [J. C. C.]; Department of Biology [A. S. B.], UNC Lineberger Comprehensive Cancer Center [J. C. C., R. L., M. H., K. A., A. S. B.], and UNC Curriculum in Genetics and Molecular Biology [A. S. B.], University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599; and Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts 02139 [P. J. E., J. A.]

Abstract

Inducible activation of nuclear factor- κ B (NF- κ B) inhibits the apoptotic response to chemotherapy and irradiation. Activation of NF- κ B via phosphorylation of an inhibitor protein I κ B leads to degradation of I κ B through the ubiquitin-proteasome pathway. We hypothesized that inactivation of proteasome function will inhibit inducible NF- κ B activation, thereby increasing levels of apoptosis in response to chemotherapy and enhancing antitumor effects. To assess the effects of proteasome inhibition on chemotherapy response, human colorectal cancer cells were pretreated with the dipeptide boronic acid analogue PS-341 (1 μ M) prior to exposure to SN-38, the active metabolite of the topoisomerase I inhibitor, CPT-11. Inducible activation of NF- κ B and growth response were evaluated *in vitro* and *in vivo*. Effects on p53, p21, p27 and apoptosis were determined. Pretreatment with PS-341 inhibited activation of NF- κ B induced by SN-38 and resulted in a significantly higher level of growth inhibition (64–75%) compared with treatment with PS-341 alone (20–30%) or SN-38 alone (24–47%; $P < 0.002$). Combination therapy resulted in a 94% decrease in tumor size compared with the control group and significantly improved tumoricidal response to treatment compared with all treatment groups ($P = 0.02$). The level of apoptosis was 80–90% in the treatment group that received combination treatment compared with treatment with single agent alone (10%). Proteasome inhibition blocks chemotherapy-induced NF- κ B activation, leading to a dramatic augmentation of chemosensitivity and enhanced apoptosis. Combining proteasome inhibition with chemotherapy has significant potential to overcome the high incidence of chemotherapy resistance. Clinical studies are currently in development to evaluate the role of proteasome inhibition as an important adjuvant to systemic chemotherapy.

Introduction

The genotoxic effect of conventional anticancer therapy involving many chemotherapy agents and gamma irradiation results in the induction of apoptosis in cancer cells (1–3). The ability to inhibit apoptosis appears to be a principal mechanism by which resistant cancer cells are protected from chemotherapy and radiation (4–6). Cellular mechanisms that protect cancer cells against apoptosis include lack of a functional response mechanism to apoptotic stimuli (e.g., mutated or deleted *p53* tumor suppressor gene), presence of an

inhibitor to apoptosis (e.g., Bcl-2 and IAP³), and the expression of the multidrug resistance gene (*MDR*; Refs. 7–9). Recently, the inducible activation of the nuclear transcription factor NF- κ B has been reported to inhibit the apoptotic response to chemotherapy and irradiation (10).

NF- κ B, a key transcription factor involved with immune and inflammatory responses as well as cell growth, is regulated primarily through interactions with an inhibitor protein known as I κ B (11). Stimuli that activate NF- κ B typically induce the recently identified I κ B kinase (IKK) complex to phosphorylate I κ B on NH₂-terminal serines, which leads to ubiquitination and subsequent degradation of the inhibitor by the proteasome. After I κ B degradation, NF- κ B translocates to the nucleus, where it regulates genes involved in a variety of processes, including the suppression of apoptosis (12, 13).

Recently, a gene therapy approach using recombinant adenovirus-mediated transfer of a modified form of I κ B α resulted in significant augmentation of chemosensitivity and enhanced induction of apoptosis in a xenograft tumor model in response to chemotherapy treatment (14). These findings suggested that NF- κ B may represent an important molecular target for the purpose of enhancing the sensitivity of certain cancer cells to apoptotic stimuli. The use of gene therapy to deliver NF- κ B inhibitors is relevant to certain cancers but is limited when considering widely disseminated metastases.

An alternative strategy for the inhibition of NF- κ B activation is facilitated by inhibition of proteasome function [for a review of the proteasome function, see Elliott and Adams (15)]. The inhibition of proteolytic function effectively blocks degradation of cellular proteins that have undergone ubiquitination, such as I κ B. In fact, proteasome inhibition is a well-established mechanism to block NF- κ B in response to a variety of stimuli. A clear advantage to this therapeutic approach is the clinical availability of a systemically administered small molecule (PS-341, a potent, boronic acid dipeptide that is highly selective for proteasome inhibition; Ref. 16) that can potentially inhibit chemotherapy-induced activation of NF- κ B and enhance the apoptotic response to chemotherapy. To evaluate this therapeutic approach, we hypothesized that inactivation of proteasome function will inhibit inducible NF- κ B activation, resulting in increased levels of apoptosis in response to chemotherapy and enhanced antitumor effects *in vivo*. To test our hypothesis, we used PS-341 to evaluate the effects of proteasome inhibition on: (a) chemotherapy-induced NF- κ B activation in colorectal cancer cells; (b) the levels of apoptosis after treatment with chemotherapy; and (c) tumor growth in a xenograft model treated with chemotherapy.

³ The abbreviations used are: IAP, inhibitor of apoptosis protein; NF- κ B, nuclear factor- κ B; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; ATCC, American Type Culture Collection; FBS, fetal bovine serum; MTD, maximum tolerated dose; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; HRP, horseradish peroxidase.

Received 10/19/00; accepted 3/14/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by grants from the UNC Lineberger Comprehensive Cancer Center (to J. C. C.), the National Cancer Institute [CA75528 (to J. C. C.) and CA72771, CA75080, and CA73756 (to A. S. B.)], and the American Cancer Society Clinical Oncology Career Development Award 96-21 (to J. C. C.). Additional support for this project was provided by the UNC Specialized Program of Research Excellence program in breast cancer (National Cancer Institute Grant CA58223).

² To whom requests for reprints should be addressed, at Division of Surgical Oncology, Massachusetts General Hospital, Cox Building, Room 626, 100 Blossom Street, Boston, MA 02114. Phone: (617) 724-4093; Fax: (617) 724-3895.

Materials and Methods

Chemotherapy Agents. Camptothecin is a specific inhibitor of mammalian DNA topoisomerase I. The camptothecin analogue CPT-11 and its active metabolite SN-38 were generously provided by J. Malczyn (Pharmacia and Upjohn Co., Kalamazoo, MI).

Cell Culture. The human colon cancer cell line LOVO was obtained from ATCC (Rockville, MD). The LOVO cells were grown in F-12 (Ham's) with 20% FBS. The colon cancer cell lines KM12L4 (generous gifts of J. Fidler, U. T. M. D. Anderson Cancer Center, Houston, TX), and WiDr (obtained from ATCC) were grown in MEM with nonessential amino acids (Grand Island, NY) and 10% FBS. The colon cancer cell line CCD841 (ATCC) was grown in DMEM with 10% FBS. All media were obtained from Life Technologies, Inc. (Gaithersburg, MD) and supplemented with 100 μ g/ml penicillin G and 100 μ g/ml streptomycin. Cell cultures were maintained at 37°C.

Cell Growth Response. Human colorectal cells, including LOVO, KM12L4, WiDr, and CCD841, were seeded in six-well plates at 5×10^4 cells/well. Cells were treated with PS-341 24 h after plating at the concentration of 1 μ M for 1 h. The medium was refreshed, and 3 h later, the cells were treated with SN-38, the active metabolite of CPT-11, at a dose of 10 ng/ml for LOVO and KM12L4, a dose of 5 ng/ml for WiDr, and at a dose of 0.5 n g/ml for CCD841. PBS, PS-341 alone, and SN-38 alone were used as control groups. Cells counts were performed daily.

NF- κ B Activation Assay. Activation of NF- κ B in response to treatment with chemotherapy and the proteasome inhibitor was determined by the electrophoretic mobility shift assay as described previously (17). Cells were cultured by seeding 1×10^6 cells in 100-mm dishes. Once cultures reached 50–70% confluence, they were treated with PS-341 at the dose of 1 μ M for 1 h. Three h later, cells were treated with SN-38 at the dose of 1 μ g/ml. Cells were harvested at 0, 1, 2, and 6 h after treatment with SN-38, and nuclear extracts were prepared as described previously (17). In brief, cells were harvested and suspended in hypotonic buffer. The nuclei were pelleted and resuspended in a low-salt buffer to high-salt buffer. The soluble protein released by centrifugation was collected and stored at -70°C . The DNA probe used contains an NF- κ B site (underlined) from the *H-2k^b* gene (5'-CAGGGCTGGGGATTC-CCATCTCCACAGTTTCACTTC-3'; Ref. 14). Seven μ g of nuclear extracts were preincubated with 1 μ g of poly(deoxyinosinic-deoxycytidylic acid) in binding buffer (10 mM Tris, 50 mM NaCl, 20% glycerol, 0.5 mM EDTA, and 1 mM DTT) for 10 min at room temperature. Approximately 20,000 cpm of ^{32}P -labeled DNA probe were then added and allowed to bind for 15 min. The complexes were then separated on a 5% polyacrylamide gel and autoradiographed.

Tumoricidal Response to Combination Therapy. The tumor growth response to combined treatment with the proteasome inhibitor and CPT-11 was assessed in a LOVO xenograft model. Tumors were established by injecting 5×10^6 LOVO cells s.c. into the flank of nude mice (NCR *nu/nu* athymic nude mice, 5–6 weeks of age, female, and weight of 18–20 g; Taconic, Germantown, NY). Once tumors reached 1-cm diameter in size, the animals received a tail vein injection of PS-341 at a concentration of 1 mg/kg (the MTD in mice),⁴ diluted according to the manufacturer's instructions in 0.5 ml of PBS. As a control, 0.5 ml of vehicle alone was administered by tail vein injection. Four h after administration of PS-341, 33 mg/kg of CPT-11 diluted in 0.5 ml of PBS were injected by tail vein injection. Control animals received 0.5 ml of PBS by tail vein injection. Animals were continued on twice weekly treatment, administered every Monday and Friday. Tumor diameter along two orthogonal axes was measured every other day to assess response to treatment.

To evaluate the response to increasing dosages of PS-341 when administered in combination with CPT-11 (administered at a fixed dose of 33 mg/kg), treatment groups received 1, 0.5, 0.1, or 0.01 mg/kg of PS-341 diluted in PBS. A dose of 2.5 mg/kg PS-341 was found to be lethal after a single i.v. injection in experimental animals (data not shown). Treatment groups received twice weekly i.v. administration of PS-341 and CPT-11 as described above. Tumor response was assessed by measuring tumor diameter along two orthogonal axes every other day.

Assessment of Apoptosis *in Vivo*. A LOVO xenograft model was used to evaluate the induction of apoptosis *in vivo* after systemic treatment with the proteasome inhibitor PS-341 and CPT-11. Nude mice bearing 1-cm diameter

s.c. tumors were treated with an i.v. injection of PS-341 at a concentration of 1 mg/kg as described above. i.v. injection of vehicle alone was used as a control. CPT-11 (33 mg/kg) was injected i.v. 4 h after administration of PS-341. Control animals received 0.5 ml of PBS by tail vein injection. Tumors were harvested from sacrificed animals 24 h after final treatment and embedded in OCT mounting medium (VWR, Sakura Finetec U.S.A. Inc., Torrance, CA; lot 1179027). Specimens were then snap-frozen in 2-methylbutane (Aldrich Chemical Co., Milwaukee, WI) over liquid nitrogen, and stored at -70°C . Four- μ m-thick sections were cut and collected on charged and pre-cleaned microscope slides (Fisher Scientific, Pittsburgh, PA). The presence of apoptosis, determined by the TUNEL assay was performed using the *in situ* cell death detection kit (Boehringer Mannheim Corp., Indianapolis, IN) according to manufacturer's instructions. The apoptotic indices of tumor sections within areas of viable tumor were estimated from the percentage of cells scored under a light microscope at $\times 100$.

Western Blot Analysis. Total cell lysates were prepared by homogenizing the cell pellets in RIPA buffer [25 mM Tris (pH 7.5), 150 μ M sodium chloride, 2 mM EDTA, 1 mM dimethyl sulfonyl fluoride, 10 mM sodium orthovanadate, 50 mM β -glycerophosphate, 1% NP40, 5% deoxycholic acid, 1% SDS, and 20% glycerol] containing a mixture of protease inhibitors [25 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 5 μ g/ml pepstatin were combined with 50 μ g of protein extract from cells treated with one of four treatment regimens including PS-341, SN-38, a combination of PS-341 and SN-38, or PBS (control)]. Four h after PS-341 treatment, the samples were electrophoresed on 10% SDS-PAGE gel overlaid with 4% acrylamide stacking gel and transferred to pure nitrocellulose membrane (0.45 μ m pore size; Schleicher & Schuell, Hercules, CA), followed by blocking with 10% dry milk in $1 \times$ Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. The first antibody P53 (Anti-P53 mouse monoclonal IgG1; Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA) was used to incubate the blot at room temperature for 1 h at a dilution of 1:2000. The second antibody was used to incubate, at room temperature, the dilution of 1:5000 for 30 min. Immunoreactive bands were visualized by ECL Western blotting analysis system (Amersham Life Sciences, Buckinghamshire, United Kingdom) and enhanced chemiluminescence. The ECL system was removed by incubating the blot in ECL strip buffer (100 mM 2-methylethanol, 2% SDS, and 62.5 mM Tris, pH 6.8) at 65°C for 30 min. P27 antibody (mouse monoclonal IgG1; Santa Cruz Biotechnology) was used to reprobe the blot at the concentration of 1:1000 for 1 h. The secondary antibody (antimouse IgG-HRP; Promega, Madison, WI) was used at a concentration of 1:2000 for 30 min at room temperature. P21 antibody (goat polyclonal IgG1; Santa Cruz Biotechnology) at a concentration of 1:1000 was used to reprobe the blot after removing the ECL system. The secondary antibody used was antigoat IgG-HRP (Santa Cruz Biotechnology) at a concentration of 1:2000. Actin, a highly conserved protein expressed in all eukaryotic cells, was used to quantitate protein loading. The ECL system was removed by incubating the blot in ECL strip buffer (100 mM 2-methylethanol, 2% SDS, and 62 mM Tris, pH 6.8) at 65°C for 30 min. The first antibody, antigoat polyclonal IgG (Santa Cruz Biotechnology), was used to reprobe the blot at the concentration of 1:500. The second antibody, antigoat IgG-HRP (Santa Cruz Biotechnology) was used at the concentration of 1:2500 to evaluate the actin expression.

Statistical Analysis. The ANOVA procedure was used to compare cell growth response and tumor growth response.

Results

Previously, it has been shown that the topoisomerase I inhibitor CPT-11, or its metabolite SN-38, induces NF- κ B activation in cancer cells *in vitro* and in tumor models (14, 18). To determine the effect of proteasome inhibition on the level of NF- κ B activation induced by exposure to chemotherapy in human colorectal cancer cells, cells were treated with proteasome inhibitor PS-341 prior to chemotherapy treatment (Fig. 1). Activation of NF- κ B, assessed by electrophoretic mobility shift assay, was observed in all cell lines treated with SN-38 alone, with peak levels occurring at either 1 or 2 h after exposure to SN-38. Pretreatment with PS-341 prior to chemotherapy completely inhibited activation of NF- κ B induced by exposure to SN-38 in all cell lines tested. Importantly, treatment with PS-341 alone did not result in activation of NF- κ B.

⁴ Personal communication, P. Elliott, Millennium Pharmaceuticals, Inc.

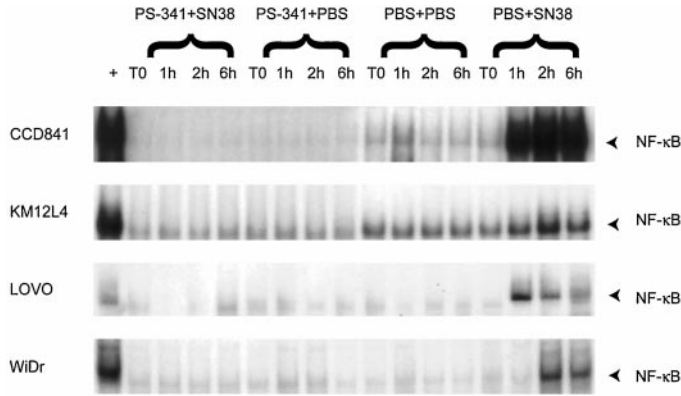


Fig. 1. The electrophoresis mobility shift assay was used to evaluate the effect of PS-341 (1 μ M) treatment on the activation of NF- κ B induced by treatment with SN-38 (1 μ g/ml) in human colorectal cancer cells. Cell cultures were treated with PS-341 or mock control for 1 h, followed 3 h later by treatment with SN-38. Cells were harvested and assayed for nuclear translocation of NF- κ B at 1, 2, and 6 h after chemotherapy treatment. Positive control (+) was KM12L4 cells treated for 2 h with 10 ng/ml tumor necrosis factor- α (a potent activator of NF- κ B). The data shown are representative of one of two independent experiments.

To determine whether pretreatment with PS-341 would enhance the anticancer effects of chemotherapy, human colorectal cancer cells were exposed to PS-341 prior to treatment with SN-38 (Fig. 2). Treatment with SN-38 or PS-341 alone resulted in 22–30% and 24–47% growth inhibition, respectively, when compared with controls receiving mock treatment. However, when PS-341 was combined with SN-38, cell counts were decreased by 64–75% compared with untreated controls, resulting in significantly improved growth inhibition when compared with treatment with PS-341 or SN-38 alone ($P < 0.002$; ANOVA). TUNEL staining for the presence of apoptosis indicates that the markedly increased growth inhibition observed in the treatment group receiving combined PS-341 and SN-38 is associated with a 4-fold increase in the level of apoptosis when compared with treatment with PS-341 or SN-38 alone (data not shown).

To determine whether the chemosensitivity of tumors could be enhanced by combining treatment with the administration of the proteasome inhibitor, a LOVO xenograft model was used (Fig. 3A). In these experiments, systemic administration of the proteasome inhibitor or CPT-11 alone resulted in a 26 and 48% decrease in tumor size, respectively, when compared with the treatment group receiving mock treatment with PBS. The treatment group that received combined systemic administration of the proteasome inhibitor and CPT-11 was the only group to undergo a true tumoricidal response, in which a persistent decrease in size of the tumor was observed throughout the course of treatment. Combined treatment with PS-341 and CPT-11 resulted in a 94% decrease in tumor size, compared with the control group, and significantly improved tumoricidal response to treatment when compared with all treatment groups ($P = 0.02$; ANOVA).

The ability of PS-341 to enhance chemosensitivity in a dose-dependent fashion was evaluated in a xenograft model by decreasing the concentration of the proteasome inhibitor while controlling the dosage of the CPT-11 (Fig. 3B). As was observed in Fig. 3A, combined treatment of CPT-11 and the MTD (1.0 mg/kg) of PS-341 resulted in a sustained tumoricidal effect in which LOVO tumors continued to decrease in size throughout the course of therapy. Increasing the dosage of PS-341 that was administered in combination with CPT-11 resulted in a dose-dependent increase in tumor growth inhibition.

The significantly decreased tumor size that resulted from combined treatment of PS-341 and CPT-11 may be explained by a growth-inhibitory effect, an increase in the level of programmed cell death, or

both. Thus, the anticancer effects of combination therapy involving the proteasome inhibitor PS-341 and SN-38/CPT-11 may be mediated by regulation of the factors that control the cell cycle and/or through modulation of the factors that regulate the induction of apoptosis. To determine whether the combination therapy effects are mediated through changes in several key cell cycle regulatory factors, we examined the effects of treatment on the cell cycle regulatory proteins p53, p21, and p27 in LOVO cells (Fig. 4). Importantly, LOVO cells are not mutated for p53 but do express oncogenic K-Ras. Treatment with either SN-38 alone and PS-341 alone resulted in stabilization of p21, p27, and p53 that was observed at 6 h and persisted until 48 h. Combination treatment, using PS-341 and SN-38, resulted in a slight increase in the level of stabilization of p21, p27, and p53 when compared with single-agent treatment alone. Staining for actin demonstrated equal protein loading in all treatment groups sampled (data not shown). These data suggest that one potential mechanism of action of the observed anticancer effects of combination therapy may be through the stabilization of p53 and the cell cycle regulatory proteins p21 and p27. This also suggested that the increased apoptosis observed in combination therapy (data not shown) may be attributable to p53-dependent apoptosis. However, as shown in Fig. 2, an equivalent reduction in cell number is achieved in either p53 wild-type or mutant cell lines.

To determine whether apoptosis was increased in the xenograft

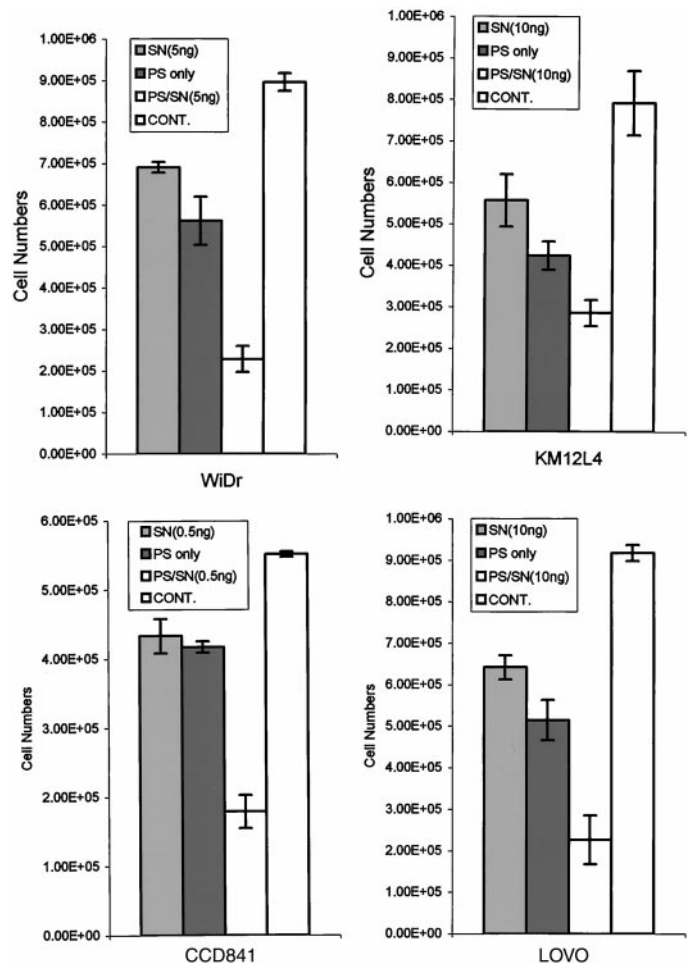


Fig. 2. The effect of proteasome inhibition on the sensitivity of colorectal cancer cell cultures to treatment with SN-38 was determined. The IC_{20} (corresponding to the concentration of SN-38 at which 20% growth inhibition was observed) was determined for each cell line. The corresponding IC_{20} was used in each set of experiments for the individual cell lines, as indicated in parentheses. Cell counts obtained at 96 h after drug treatment are reported as the means obtained from triplicate experiments; bars, 1 SD.

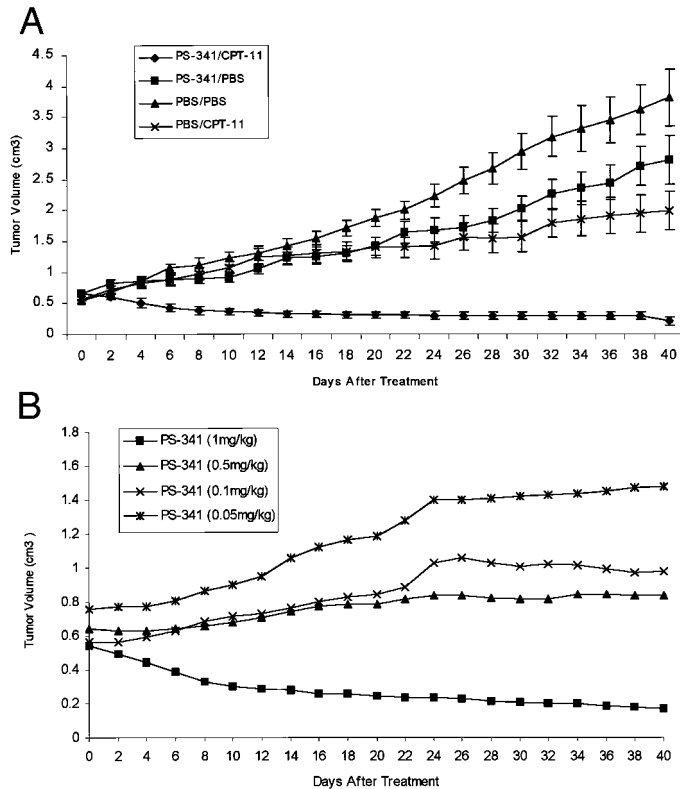


Fig. 3. The effects of combination therapy using the proteasome inhibitor PS-341 and CPT-11 were assessed in a xenograft model. One-cm-diameter LOVO tumors were grown in the flanks of nude mice. The tumoricidal effects of systemic treatment was assessed (A) after pretreatment with PS-341 (1 mg/kg i.v. bolus injection) or vehicle alone, followed by i.v. bolus administration of CPT-11 (33 mg/kg) or vehicle. Treatment was administered biweekly (Monday and Thursday), and tumor diameter along two orthogonal axes was recorded every other day. Tumor volume was calculated by assuming a spherical shape of the tumor, using the formula: volume = $4/3 \pi r^3$, where r is $1/2$ (mean diameter of the tumor), and recorded as the means for each treatment group ($n = 8-10$); bars, SE. The dose response to increasing concentrations of PS-341 when used in combination with a fixed dosage of CPT-11 (33 mg/kg) was assessed in a LOVO xenograft model (B). The tumoricidal response to different concentrations of PS-341, including the MTD (1 mg/kg) in nude mice, was determined by pretreatment with an i.v. bolus administration of PS-341, followed by an i.v. bolus administration of CPT-11. Treatment was administered biweekly, and tumor diameter along two orthogonal axes was recorded every other day ($n = 8-10$). Tumor volume was calculated as described above.

tumor model with the combination therapy, tumor sections were stained for the presence of TUNEL-positive cells after treatment (Fig. 5). The TUNEL-positive tumor cells at 24 h after treatment with CPT-11 or PS-341 accounted for $\sim 10\%$ of the total number of cells stained. This relatively low level of apoptosis is not significantly different from that observed in tumor sections obtained from untreated controls (data not shown). In comparison, the number of tumor cells that stained positively for apoptosis was 80–90% in the treatment group that received combined treatment with PS-341 and CPT-11. Thus, these findings suggest that the tumoricidal response to combined treatment with PS-341 and CPT-11 appears to result from markedly increased levels of apoptosis when compared with treatment with either agent alone.

Discussion

A growing body of evidence suggests that protection of cancer cells from cell death facilitates tumorigenesis by conveying a selection advantage to cancer cells, thereby disrupting the homeostatic balance of cell death and cell growth in favor of cells that use antiapoptotic survival mechanisms (19). In addition to its role in tumorigenesis, protection against apoptosis may also play a role in resistance to conventional treatments, such as chemotherapy and irradiation (19,

20). Survival mechanisms that function by the inhibition of the apoptotic response may be distinguished by their activity as either acquired or inducible. Acquired mechanisms of antiapoptosis are associated with genetic mutations involved in malignant transformation, such as mutation of the *p53* tumor suppressor gene (21), overexpression of *Bcl-2* (8), and overexpression of P-glycoprotein in multidrug resistance (7). In contrast, inducible mechanisms of antiapoptosis, are not directly involved in tumorigenesis and are only transiently expressed after exposure to genotoxic stimuli such as anticancer therapies (10, 14).

Recently, we and others have described the inducible antiapoptotic function of the transcription factor NF- κ B as a principle mechanism by which cancer cells are protected from undergoing programmed cell death after exposure to tumor necrosis factor- α (10, 22, 23), gamma irradiation, and some chemotherapy agents (10). We have also reported that protection from apoptosis resulting from inducible NF- κ B activation may be overcome by selective NF- κ B inhibition using the super-repressor $\text{I}\kappa\text{B}\alpha$ (10, 14, 18). In those studies, infection of tumor cells with a recombinant adenovirus that expresses a mutated form of the highly specific NF- κ B inhibitor $\text{I}\kappa\text{B}\alpha$ (super-repressor $\text{I}\kappa\text{B}\alpha$) significantly decreased inducible NF- κ B activation in response to treatment with the topoisomerase I inhibitor CPT-11 in a wide variety of human colorectal cancer cell lines and the breast cancer cell line MCF-7 (18). The inhibition of inducible NF- κ B resulted in enhanced chemosensitivity *in vitro* and in xenograft models of both colorectal cancer and sarcoma (14, 18). It was also demonstrated that the enhanced response to chemotherapy was associated with markedly increased levels of apoptosis after combination treatment (14, 18). The mechanisms responsible for the antiapoptotic effects of NF- κ B activation after exposure to chemotherapy and gamma irradiation

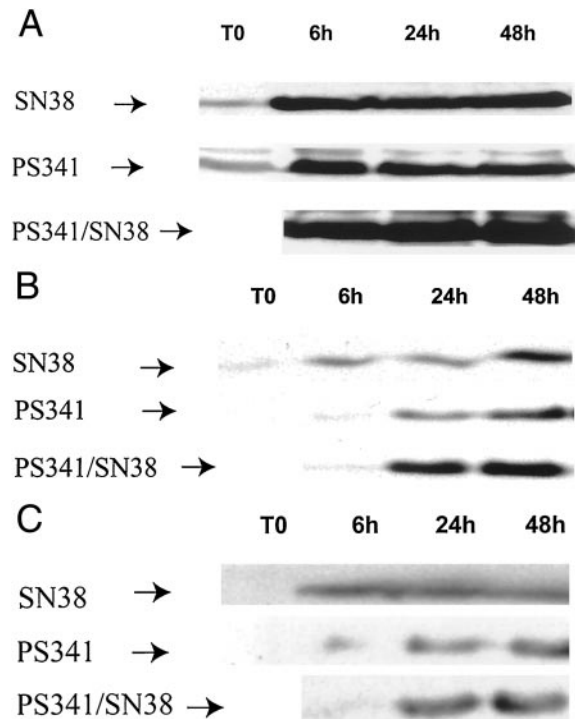
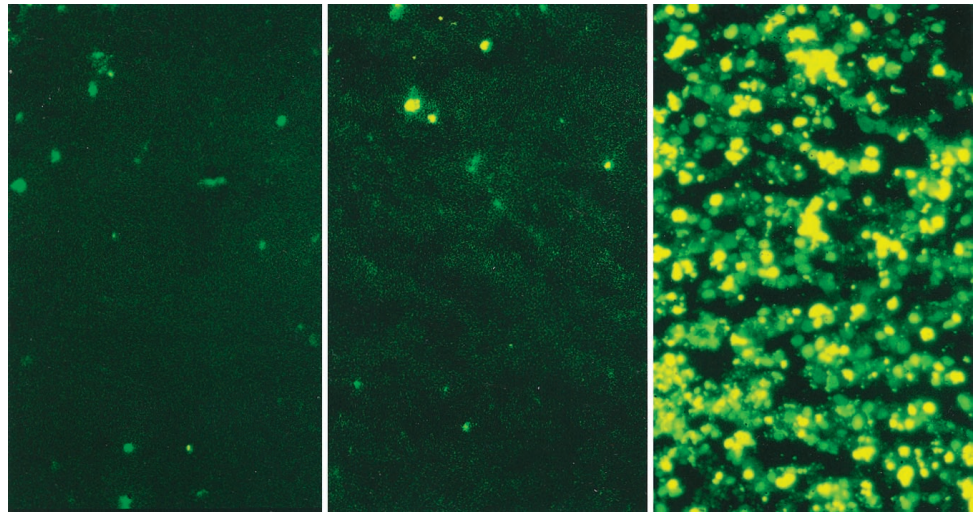


Fig. 4. Stabilization of cellular proteins mediated by combination therapy using proteasome inhibitor PS-341 and SN-38. A, Western blots of p53 in LOVO cells at 0, 6, 24, and 48 h after treatment with SN-38. The treatment group labeled SN-38 was pretreated with PBS-341 prior to chemotherapy treatment; treatment group PS-341 was pretreated with PS-341 4 h prior to mock treatment; treatment group PS341/SN-38 was pretreated with PS-341 4 h prior to treatment with SN-38. Immunoblot analysis was used to detect p21 (B) and p27 (C) from protein obtained from whole cell lysates using LOVO cells treated in a similar fashion to that described above.

Fig. 5. Representative photomicrographs demonstrating TUNEL staining of LOVO tumor sections after treatment with CPT-11 (*left*), PS-341 (*center*), and combination PS-341 and CPT-11 (*right*). Tumors were harvested 24 h after final treatment.



have not been fully elucidated. However, it is likely that downstream gene targets that are NF- κ B regulated, such as the IAP proteins c-IAP1 and c-IAP2, are involved in this inducible survival mechanism (9).

Several methods of inhibiting NF- κ B activation are currently under investigation in preclinical studies in our laboratory. One of the first and most effective methods of NF- κ B inhibition uses recombinant adenovirus-mediated overexpression of the *I κ B α* gene, described previously. However, the clinical feasibility of gene therapy approaches for inhibiting NF- κ B are currently limited by the requisite intratumoral delivery of the recombinant virus that expresses the NF- κ B inhibitor. Alternatively, a systemic means of inhibiting NF- κ B may be applicable in the treatment of widely disseminated metastatic disease. In this setting, i.v. administered agents that block the antiapoptotic response mediated by chemotherapy-induced NF- κ B activation in tumor cells may enhance the response of metastatic disease to conventional drug treatment. To examine the potential role of systemic NF- κ B inhibition to enhance chemosensitivity of human cancer cells, we have selected the proteasome inhibitor PS-341, which has been shown to block NF- κ B activation through stabilization of *I κ B α* , to combine with the chemotherapy agent SN-38/CPT-11.

The ubiquitin-proteasome pathway (specifically, the 26S proteasome) is the principle mechanism by which cellular proteins, including ubiquitinated *I κ B α* , are degraded (24–26). Inhibition of the ubiquitin-proteasome pathway results in dysregulation of cellular proteins involved in cell cycle control, promotion of tumor growth, and induction of apoptosis (16). Recently, a novel class of anticancer agents called proteasome inhibitors that yield effective anticancer responses both *in vitro* and *in vivo* have been introduced into the treatment of malignancy (16, 27). The dipeptide boronic acid analogue PS-341, used in these experiments, inhibits the chymotryptic activity of the proteasome in a potent, reversible, and selective manner (16). Proteasome inhibition using this compound has demonstrated significant cytotoxic activity against a range of human tumor cell lines that comprise the National Cancer Institute *in vitro* screen and is well tolerated in preclinical animal studies and in Phase I clinical trials (16). The ability of proteasome inhibitors to regulate NF- κ B activation has led many to speculate that these new compounds may have important clinical implications in the management of both inflammatory disease and malignancy (28).

This report addresses the issue of whether pretreatment of cancer cells with PS-341 can effectively block the activation of NF- κ B that is induced by exposure to SN-38/CPT-11. As a second objective, we

have sought to determine whether NF- κ B inhibition using PS-341 may augment the sensitivity of tumor cells to chemotherapy. Similar to the findings we reported using the super-repressor *I κ B α* (14, 18), proteasome inhibition using PS-341 potentially blocked the early activation of NF- κ B at 2 h after chemotherapy treatment. In addition, combining proteasome inhibition with chemotherapy treatment resulted in a significant anticancer effect compared with treatment with single agent alone. TUNEL assays, performed to determine whether this increased anticancer effect from combination treatment was attributable in part to regulation of apoptosis, demonstrated markedly increased levels of apoptosis compared with single agent treatment alone. This observed response is comparable with the response observed when NF- κ B was selectively inhibited using the super-repressor *I κ B α* in our previous reports (14, 18).

Our findings suggest that the enhanced anticancer effects, observed when PS-341 is combined with chemotherapy, are attributable to inhibition of inducible NF- κ B activation. However, potentially other cell cycle regulatory processes and apoptotic response mechanisms impacted by proteasome inhibition play a contributing role:

(a) Proteasome inhibition may result in prolonged binding of the camptothecin to the topoisomerase-1 enzyme in a complex that under physiological conditions would be degraded by the proteasome (29). Enhanced sensitivity to SN-38/CPT-11 when a proteasome inhibitor is coadministered may therefore result in part from the impaired metabolism and longer half-life of the topoisomerase 1-camptothecin complex, resulting from impaired proteasome function. The addition of the proteasome inhibitor to chemotherapy would thereby effectively increase the duration of activity of the SN-38/CPT-11.

(b) The findings that p21, p27, and p53 are stabilized by chemotherapy or PS-341 treatment suggest that stabilization of these key cell cycle regulatory proteins may be involved in the antitumor effects of these molecules when used alone or in combination. The ability to enhance chemosensitivity by proteasome inhibition, however, was observed in cells expressing both wild-type p53 (CCD841 and LOVO), mutant p53 (KM12L4 and WiDR), and Bcl-2 (KM12L4), suggesting that inhibition of chemotherapy-induced NF- κ B activation using proteasome inhibition may occur independently of the functional status of p53 in colorectal cancer cell lines.

Although a variety of mechanisms in addition to inhibition of NF- κ B may potentially contribute to the efficacy of combination therapy using PS-341 and SN-38/CPT-11, additional studies will be necessary to further elucidate this potentially complex response. Furthermore, the ability of proteasome inhibition to enhance the sensi-

tivity of other genotoxic anticancer therapies without increasing toxicity in preclinical animal studies warrants further evaluation and is the focus of ongoing studies in our laboratory. Nevertheless, the dramatic augmentation of sensitivity to SN-38/CPT-11 observed both *in vitro* and *in vivo* reported here has significant potential to overcome the relatively high incidence of chemotherapy resistance seen in patients with metastatic disease. Clinical studies to evaluate the role of proteasome inhibition as an important adjuvant to systemic chemotherapy are currently in development.

Acknowledgments

We thank Yuhua Lin for assistance with statistical analysis and Gail Somodi for assistance in the preparation of the manuscript.

References

- Kastan, M. B. Molecular determinants of sensitivity to antitumor agents. *Biochim. Biophys. Acta*, *1424*: R37–R42, 1999.
- Schmitt, C. A., and Lowe, S. W. Apoptosis and therapy. *J. Pathol.*, *187*: 127–137, 1999.
- Wu, G. S., and El-Deiry, W. S. Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or bcl-2. *Clin. Cancer Res.*, *2*: 623–633, 1996.
- Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat. Med.*, *2*: 574–577, 1996.
- Fulda, S., Susin, S. A., Kroemer, G., and Debatin, K. M. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. *Cancer Res.*, *58*: 4453–4460, 1998.
- Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. *Cell*, *78*: 539–542, 1994.
- Huang, Y., Ibrado, A. M., Reed, J. C., Bullock, G., Ray, S., Tang, C., and Bhalla, K. Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. *Leukemia*, *11*: 253–257, 1997.
- Lotem, J., and Sachs, L. Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells. *Cell Growth Differ.*, *4*: 41–47, 1993.
- Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S. NF- κ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science (Wash. DC)*, *281*: 1680–1683, 1998.
- Wang, C. Y., Mayo, M., and Baldwin, A. S. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF- κ B. *Science (Wash. DC)*, *274*: 784–787, 1996.
- Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baeuerle, P. A. Rapid proteolysis of I κ B α is necessary for activation of transcription factor NF- κ B. *Nature (Lond.)*, *365*: 182–185, 1993.
- 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.
- Baldwin, A. S. The NF- κ B and I κ B proteins: new discoveries and insights. *Annu. Rev. Immunol.*, *14*: 649–683, 1996.
- Wang, C. Y., Cusack, J. C., Liu, R., and Baldwin, A. S. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF- κ B. *Nat. Med.*, *5*: 412–417, 1999.
- Elliott, P. J., and Adams, J. Recent advances in understanding proteasome function. *Curr. Opin. Drug Discovery Dev.*, *2*: 484–490, 1999.
- 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.
- Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S. Characterization of an immediate-early gene induced in adherent monocytes that encodes I κ B-like activity. *Cell*, *65*: 1281–1289, 1991.
- Cusack, J. C., Liu, R., and Baldwin, A. S. Inducible chemoresistance to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin (CPT-11) in colorectal cancer cells and a xenograft model is overcome by inhibition of nuclear factor- κ B activation. *Cancer Res.*, *60*: 2323–2330, 2000.
- Naik, P., Karrim, J., and Hanahan, D. The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. *Genes Dev.*, *10*: 2105–2116, 1996.
- Dixon, S. C., Soriano, B. J., Lush, R. M., Borner, M. M., and Figg, W. D. Apoptosis: its role in the development of malignancies and its potential as a novel therapeutic target. *Ann. Pharmacother.*, *31*: 76–82, 1997.
- Bellamy, C. O., Clarke, A. R., Wyllie, A. H., and Harrison, D. J. p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage. *FASEB J.*, *11*: 591–599, 1997.
- Beg, A. A., and Baltimore, D. An essential role for NF- κ B in preventing TNF- α -induced cell death [see comments]. *Science (Wash. DC)*, *274*: 782–784, 1996.
- Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF- α -induced apoptosis by NF- κ B [see comments]. *Science (Wash. DC)*, *274*: 787–789, 1996.
- Goldberg, A. L., Stein, R., and Adams, J. New insights into proteasome function: from archaeobacteria to drug development. *Chem. Biol.*, *2*: 503–508, 1995.
- King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. How proteolysis drives the cell cycle. *Science (Wash. DC)*, *274*: 1652–1659, 1996.
- Coux, O., Tanaka, K., and Goldberg, A. L. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.*, *65*: 801–847, 1996.
- Orlowski, R. Z., Eswara, J. R., Lafond-Walker, A., Grever, M. R., Orlowski, M., and Dang, C. V. Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. *Cancer Res.*, *58*: 4342–4348, 1998.
- Grisham, M. B., Palombella, V. J., Elliott, P. J., Conner, E. M., Brand, S., Wong, H. L., Pien, C., Mazzola, L. M., Destree, A., Parent, L., and Adams, J. Inhibition of NF- κ B activation *in vitro* and *in vivo*: role of 26S proteasome. *Methods Enzymol.*, *300*: 345–363, 1999.
- Desai, S. D., Liu, L. F., Vazquez-Abad, D., and D'Arpa, P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J. Biol. Chem.*, *272*: 24159–24164, 1997.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Enhanced Chemosensitivity to CPT-11 with Proteasome Inhibitor PS-341:: Implications for Systemic Nuclear Factor- κ B Inhibition

James C. Cusack, Jr., Rong Liu, Michael Houston, et al.

Cancer Res 2001;61:3535-3540.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/61/9/3535>

Cited articles This article cites 29 articles, 13 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/61/9/3535.full#ref-list-1>

Citing articles This article has been cited by 100 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/61/9/3535.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/61/9/3535>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.