Proteasome Inhibition with Bortezomib Enhances Activity of Topoisomerase I-targeting Drugs by NF-κB-independent Mechanisms

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Abstract. *Purpose: The potentiation of topoisomerase (topo)-I-induced apoptosis by proteasome inhibitors is dependent on the treatment sequence, but not on NF-κB. In this study, alternate mechanisms modulating apoptosis induced with the topo I-targeting drug, SN-38, when followed by the proteasome inhibitor bortezomib (PS-341) were investigated. Materials and Methods: Human non-small cell lung carcinoma (NSCLC-3) cells transfected with a control vector (NSCLC-3/neo) or a vector containing dominant negative IκBα (NSCLC-3/mIκBα) were treated with SN-38 for 1 h followed by PS-341 for 4 h (SN-38→PS-341), or with either drug alone. The functional role of the anti-apoptotic protein survivin was tested using NSCLC-3 transfected with myc-tagged wild-type (NSCLC-3/myc-survivin), or dominant negative mutant T34A survivin (NSCLC-3/myc-T34A). Results: In NSCLC-3/neo or NSCLC-3/mIκBα cells, treatment with SN-38→PS-341 led to down-regulation of the survivin transcript and protein, enhanced apoptosis and reduced (>3-fold) survival compared to SN-38 or PS-341 alone. In contrast to the cells transfected with wild-type survivin, or the control NSCLC-3/neo, those cells transfected with mutant survivin and treated with SN-38→PS-341 exhibited enhanced caspase 9 activity (>2-fold), caspase 3 (>2- to 3-fold) activity and cytotoxicity compared to the NSCLC-3/neo cells. Conclusion: In contrast to inhibition of NF-κB activity, down-regulation of the anti-apoptotic survivin was correlated with modulation of the sequence-dependent synergistic effects of PS-341 in SN-38-induced apoptosis.*

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Human non-small cell lung carcinoma (NSCLC) is clinically responsive to chemotherapy with the topoisomerase I (topo I)-targeting drug irinotecan (1). The active metabolite of irinotecan, SN-38, is a potent inhibitor of the nuclear enzyme topo I (2). The antitumor activity of topo I-targeting drugs is presumed to be due to stabilization of a topoisomerase I-DNA cleavable complex leading to protein-linked DNA breaks and cell death by apoptosis (2-4). The enzyme topo I in the drug-stabilized topo I-DNA cleavable complex can be modified by SUMO-1 (5, 6). This modification by SUMO-1 has been suggested to affect the stability of the DNA-cleavable complex and its recognition for effecting downstream apoptotic pathways (5, 6).

The precise signaling pathways downstream of DNA damage induced by topo I-targeting drugs for effecting an apoptotic response have not been elucidated. NF-κB is an inducible transcription factor involved in the regulation of genes during inflammatory, acute phase and immune responses (7-10). The activation of NF-κB has been demonstrated to up-regulate anti-apoptotic genes (7-10), leading to the hypothesis that inhibiting NF-κB function can be used to promote apoptosis following chemotherapy with DNA-damaging agents. In addition to the activation of NF-κB by tumor necrosis factor, topo I-targeting drugs also induce the activation of NF-κB (11). Thus, manipulating NF-κB activation as a strategy to sensitize tumor cells to DNA-damaging agents has been actively pursued (12).

The activation of NF-κB is regulated by its association with the inhibitory protein IκBα (8). The site-specific phosphorylation at serine 32 and serine 36 of IκBα targets the protein for ubiquitination and degradation by the 26S proteasome (8-10). Therefore, pharmacological inhibition of proteasome function or the molecular strategy of transfecting a dominant negative (S32A/S36A) mutant IκBα can theoretically be used to manipulate the activation of NF-κB (11). The results of using the inhibition of NF-κB as

a strategy to improve response to cancer chemotherapy have been mixed and it is not clear whether this is related to the tumor model system being studied or the therapeutic modality being evaluated (11-13). Our previous studies have demonstrated that inhibition of NF-κB with a dominant negative mutant does not alter the apoptotic response of three human NSCLC cells treated with either topo I- and topo II-targeting drugs or ionizing radiation (14-15). These studies further revealed that the effect of proteasome inhibitors in potentiating topo-targeting drug-induced apoptosis was: (a) independent of NF-κB status and (b) dependent on the sequence of exposure to the proteasome inhibitor, either prior to or after treatment-induced damage to DNA (14). In the present study, the mechanistic basis for modulation of DNA damage induced apoptosis by the clinically active 20S proteasome inhibitor bortezomib (16) was probed in a NSCLC model expressing wild-type (NSCLC-3/neo) or mutant IκBα (NSCLC-3/mIκBα). The results suggested that the effects of bortezomib (PS-341) in promoting SN-38-induced apoptosis is possibly p53-dependent and correlated with reduced levels and activity of the anti-apoptotic protein survivin.

Materials and Methods

Materials. The topo I-targeting agent SN-38 (active metabolite of irinotecan, CPT-11) was obtained from Pharmacia & Upjohn Co. Stock solutions of SN-38 were prepared in dimethylsulfoxide (Sigma Chemical Co.) and stored frozen at –20˚C. The dominant negative IκB (S32A/S36A) cDNA cloned into pUSEamp(+) expression vector and the empty control pUSEamp(+) expression vector were obtained from Upstate Biotechnology (Lake Placid, New York, USA). The antibody to p53 and survivin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The fluorogenic substrates LEHD-AFC and DEVD-AFC for determining caspase 9 and caspase 3 activities, respectively, were obtained from BioVision Inc. (Palo Alto, CA, USA). The cell culture medium and fetal bovine serum (FBS) were obtained from BioWhittaker Inc. (Gaithersburg, MD, USA). All other chemicals of analytical grade were obtained from commercial sources.

Cell lines and transfection. The human NSCLC model systems, NSCLC-3 and the selection of NSCLC-3/neo or NSCLC-3/mIκBα have been previously described (14). The parental wild-type NSCLC-3/wt and NSCLC-3/neo or NSCLC-3/mIκBα were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine and maintained at 37˚C in a humidified 5% CO_2 plus 95% air atmosphere. The NSCLC-3/neo or NSCLC-3/mIκBα cells were cultured in the presence of 1 mg/ml of G418. The doubling time *in vitro* for the NSCLC-3/wt and NSCLC-3/neo or NSCLC-3/mIκBα cells was ~35 h.

Mutant (threonine34alanine) survivin from a pcDNA3 vector containing full length wild-type survivin, was carried out by site-directed mutagenesis with the Quikchange Site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA). The primers used for the mutation were as follows: 5'-GCT GCG CCT GCG CCC CGG AGC GGA TGG-3' and 5'-CCA TCC GCT CCG GGG CGC AGG CGC AGC-3'. The site-directed mutagenesis of T34A was confirmed by PCR and DNA sequence analysis. Transfection of the NSCLC-3 cells with mutant (threonine34alanine) survivin (T34Asurvivin) in pcDNA3 expression vector under the control of the CMV promoter or the empty pcDNA3 expression vector was carried out using 4 μg DNA/5x10^5 cells/1.0 ml culture medium containing 10 ml of Lipofectamine 2000 (GIBCO/BRL, Gaithersburg, MD, USA). Stable transfectants were selected in 1 mg/ml G418.

Measurement of apoptosis and caspase activity. The apoptosis in drug-treated cells was determined using the technique of Muscarella *et al.* (17). Briefly, 2x10^5 control or treated cells were re-suspended in 100 μl staining solution (70 μg/ml Hoechst 33342 and 100 μg/ml propidium iodide in phosphate buffered saline) and were incubated at 37˚C for 15 min. The stained cells were viewed using a fluorescence microscope with the appropriate filters, so as to simultaneously visualize the blue fluorescence from Hoechst 33342 and the red fluorescence from propidium iodide. Normal viable cells fluoresce blue within the nucleus and the apoptotic cells show condensation of chromatin and formation of small masses of varying sizes. However, necrotic cells stain pink, are swollen and the chromatin is not condensed and fragmented as in apoptotic cells. Lysates prepared from aliquots of the control and treated cells were tested for caspase 9 and caspase 3 activity using the fluorogenic substrate leucine-glutamic acid-histidine-aspartic acid (LEHD) and aspartic acid-glutamic acid-valine-aspartic acid (DEVD), respectively, peptide substrate coupled to 7-amino-4-trifluoromethylcoumarin (AFC). Flow cytometry for cell cycle traverse perturbations was carried out following staining with propidium iodide, as described earlier (18). The cytotoxicity induced by SN-38 ± PS-341 was determined by a soft agar colony-forming assay. The cells were treated with a range of drug concentrations for 60-240 min at 37˚C in a humidified 5% CO_2 plus 95% air atmosphere. Following treatment, the cells were washed and 3x10^4 cells were plated in triplicate in 35x10-mm Petri dishes using RPMI 1640 supplemented with 2 mM L-glutamine and 20% FBS. Colonies (>50 cells) were counted following incubation of the Petri dishes for 10-12 days in a humidified 5% CO_2 plus 95% air atmosphere.

Real-time RT-PCR analysis of survivin transcript. Total RNA was isolated from approximately 2x10^6 control or treated cells using the Qiagen Rneasy isolation kit (Qiagen, Valencia, CA, USA). Single-strand cDNA was synthesized from 1 mg of RNA using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. PCR reactions were carried out using 25 ng cDNA template, and SybrGreen PCR Reagents (ABI, Foster City, CA, USA). The forward survivin 5'-GG ACCACCGCATCTCTACATTCAA-3' and reverse 5'-CGTTCTCA GTGGGGCAGTGGAT-3' primers amplified a 110-bp product. The forward β_2-microglobulin 5'-CTTGTCTTTCAGCAAGG ACTGG-3'and reverse 5'-CATGATGCTGC-TTACATGTCTC-3' primers amplified a 120-bp product. The PCR reaction was performed and quantified on the ABI Prism 7700 sequence detection system using Sequence Detection System Software 1 .7a (ABI). Relative survivin gene expression was determined by comparison of the critical amplification threshold values of the treated cells against those of the untreated control cells following normalization with β_2-microglobulin expression, according to the manufacturer's protocol.

Cell lysis and Western blotting. The samples were electrophoresed on 10% Bis-Tris or 12% Tris-HCl gels according to the manufacturer's protocol. The separated proteins were transferred to PVDF membranes in transfer buffer (Invitrogen) containing 10-15% methanol at 30 volts for 1-1.5 h. For immunoprecipitation and immunoblot analysis, cell lysates were prepared in the radio-immunoprecipitation assay buffer (RIPA) containing 20 mM Tris-HCl, pH 8.0, 0.425M NaCl, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 2 mM EDTA, 0.5 mM EGTA and β-mercaptoethanol plus protease and phosphatase inhibitors (14, 19). An aliquot of the cell lysate was electrophoresed and transferred to PVDF membrane. The membrane was blocked in 5% non-fat dry milk in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20 (TBST) at room temperature for 1 h. The membrane was then incubated with an appropriate dilution of the primary antibody at 4˚C (usually overnight). Following washing, the membrane was incubated with peroxidase-labelled secondary antibody, diluted 40,000-fold, for 1 h at room temperature. The relative intensity of the signal was determined for the protein band of interest and normalized with the relative intensity of actin.

Data analysis. Analysis of variance was used to compare the various treatment conditions in the wild-type transfected NSCLC-3 cells. No adjustments were made for multiple comparisons, thus $p<0.05$ was used to indicate statistical significance.

Results

Apoptotic effects of PS-341 are similar in NSCLC-3/neo and NSCLC-3/mIκBα cells. The results in Figure 1A demonstrate that PS-341 was a potent inducer of apoptosis in NSCLC-3/neo or NSCLC-3/mIκBα cells. The apoptosis induced by PS-341 was both concentration- and time-dependent. Notably, the effects of PS-341 in inducing apoptosis were NF-κB-independent, since comparable levels of apoptosis were obtained in both the NSCLC-3/neo and NSCLC-3/mIκBα cells, which differ in their DNA binding activity of NF-κB, when treated with topoisomerase I- or II-targeting drugs (14).

Treatment with PS-341 following SN-38-induced DNA damage leads to synergistic enhancement in apoptosis and reduced accumulation of cells in S + G_2/M fraction. In order to test whether PS-341 was capable of modulating DNA damage-induced apoptosis, the effects of treatment with 25 nM PS-341 for 4 h following exposure to SN-38 (40 nM and 100 nM) for 60 min was determined. The results in Table I demonstrate that post-treatment with PS-341 enhanced the apoptosis induced by SN-38 in NSCLC-3/neo or NSCLC-3/mIκBα cells. Using the criteria for quantifying *in vitro* drug interactions (19), the data demonstrated that post-treatment with PS-341 following SN-38 induced DNA damage, leading to a synergistic enhancement of apoptosis. The analysis of cell cycle phase distribution by flow cytometry revealed that, although PS-341 alone did not significantly induce cell cycle traverse perturbations, accumulation of cells in the S + G2/M fraction observed following treatment with SN-38 alone was reduced >2-fold when PS-341 followed SN-38 treatment (SN-38→PS-341). A graphical presentation of the cell cycle traverse perturbations induced by PS-341, SN-38 or SN-38→PS-341 is outlined in Figure 1B. The role of PS-341 in enhancing SN-38-induced apoptosis at comparable levels in NSCLC-3/neo or NSCLC-3/mIκBα cells, suggested that the inhibitory effect of PS-341 on NF-κB activation was not involved.

Table I. *PS-341 synergistically enhanced the apoptosis induced by SN-38 in NSCLC-3/neo or NSCLC-3/mIκBα cells.*

Treatment	NSCLC-3/neo		NSCLC-3/mIκBα	
	% Cells in S - G_2+M Fraction	% Apoptosis	% Cells in S - G_2+M Fraction	% Apoptosis
Control	36%	4±1	36%	4±1
25 nM PS-341	32%	17±1	33%	15±2
40 nM SN-38	73%	9±5	71%	9±4
40 nM SN-38 → PS-341	28%	42±4	21%	55±13
100 nM SN-38	75%	8±1	73%	8±2
40 nM SN-38 → PS-341	30%	49±4	20%	56±9

The synergistic effect (19) of post-treatment with PS-341 on SN-38-induced apoptosis was further confirmed based on enhanced caspase 9 and caspase 3 activities (data not shown), as well as when the survival of cells was determined by a soft-agar colony assay (Figure 1C). The survival of NSCLC-3/neo or NSCLC-3/mIκBα cells treated with SN-38→PS-341 was significantly reduced ($p<0.05$) compared to treatment with PS-341 or SN-38 alone. However, the survival of NSCLC-3/neo or NSCLC-3/mIκBα cells treated with SN-38→PS-341 were not different, suggesting that the PS-341-induced modulation of SN-38-induced cytotoxicity was NF-κB independent.

Potentiation of DNA damage-induced apoptosis by post-treatment with PS-341 involves down-regulation of survivin and can be enhanced by mutant threonine34alanine (T34A) survivin in NSCLC-3 cells. Since the majority of inhibitors of apoptosis proteins (IAP), with the exception of survivin (20-22), are NF-κB-regulated, our efforts were focussed on this anti-apoptotic protein, since it was reported to be negatively regulated by wild-type p53 (23, 24). Furthermore, the expression of survivin is cell cycle phase-dependent (22, 25). The anti-apoptotic function of survivin was reported to involve binding to caspase 9 (26). The phosphorylation of survivin on threonine 34 by the $p34^{cdc2}$-cyclin B complex is also required for the association of survivin with caspase 9 and for the inhibition of caspase 9 activity (26).

To determine the level of mRNA transcript turnover, real-time RT-PCR experiments were performed to

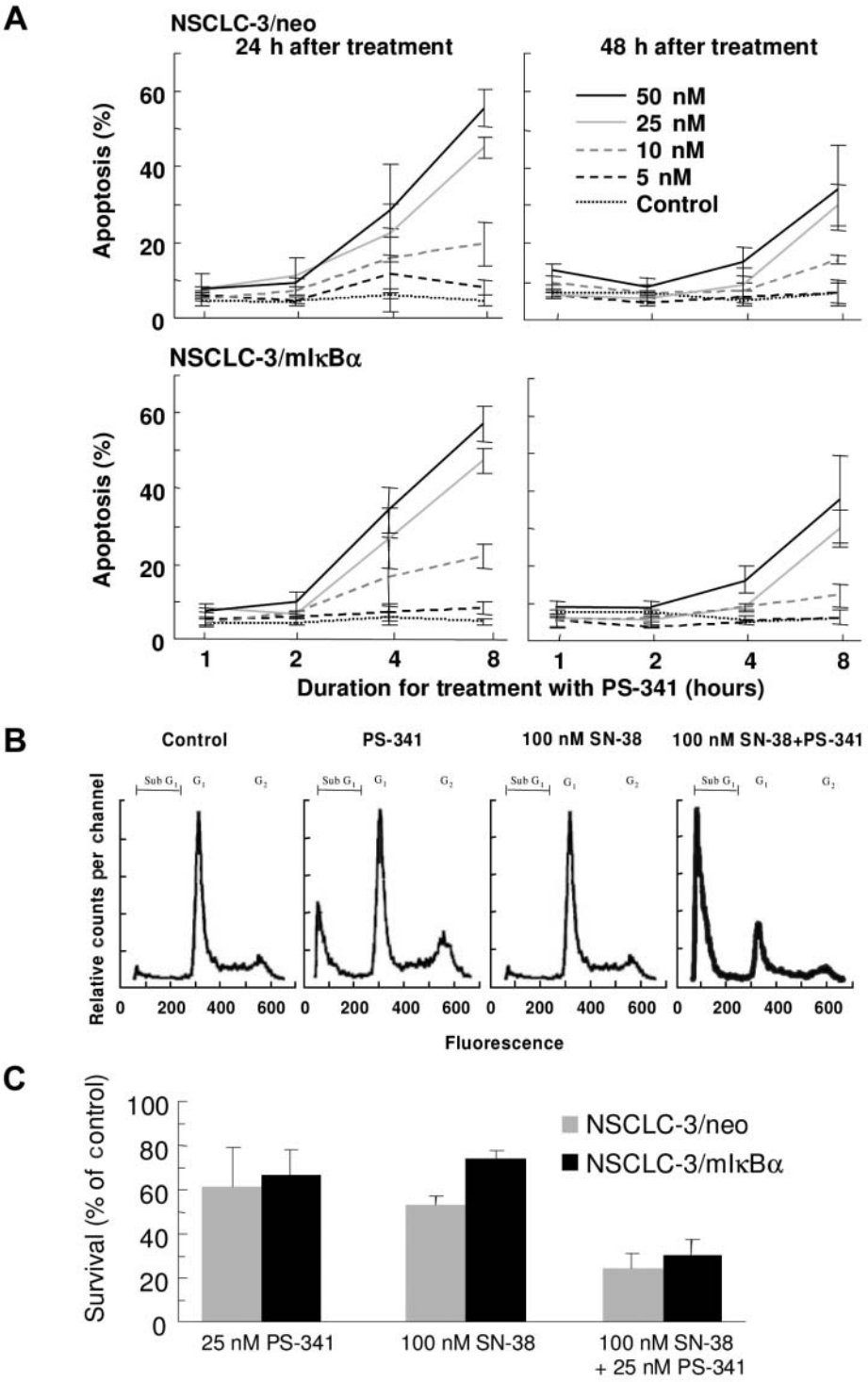

Figure 1. *A: Effect of PS-341 on apoptosis in NSCLC-3/neo and NSCLC-3/mIκBα cells. The cells were treated with the indicated concentrations of PS-341 for 1-8 h, were washed, re-suspended in drug-free medium and apoptosis was determined by fluorescent microscopy (18) at 24 h and 48 h. B: Cell cycle traverse perturbations in NSCLC-3/mIκBα cells treated with PS-341, SN-38 or SN-38→PS-341. The NSCLC-3/mIκBα cells were treated with 25 nM PS-341 (4 h), 100 nM SN-38 (1 h) or 100 nM SN-38 (1 h)→25 nM PS-341 (4 h), washed, re-suspended in drug-free medium and the cell cycle traverse perturbations were determined by flow cytometry at 24 h. C: Effect of PS-341 on survival of NSCLC-3/neo or NSCLC-3/mIκBα cells treated with PS-341, SN-38 or SN-38→PS-341. The survival of cells treated with SN-38→PS-341 was significantly lower ($p<0.05$) compared to those treated with PS-341 or SN-38 alone. The cells were treated as indicated earlier (Figure IB) and the survival was determined by colony-formation in soft agar. The colony-forming efficiencies of NSCLC-3/neo and NSCLC-3/mIκBα were 11.1% and 8.9%, respectively.*

examine changes in survivin mRNA. For these experiments, survivin mRNA levels were determined at different times after treatment with 25 nM PS-341 alone for 120 min, 100 nM SN-38 alone for 60 min, or 100 nM SN-38 for 60 min followed by 25 nM PS-341 for 120 min (Figures 2 A and B). While both SN-38 and SN-38→PS-341 treatments led to decreases in survivin mRNA levels up to 8 h after removal of the drug, the decrease in the mRNA was maintained only up to 24 h for the SN-38→PS-341 treatment. In the SN-38-treated cells, survivin mRNA had recovered back to control levels between 8 and 24 h post-treatment. At later time-points, the survivin mRNA levels were significantly lower ($p<0.03$) in cells treated with SN-38→PS-341 compared to with PS-341 or SN-38 alone. PS-341 alone did not alter the levels of survivin mRNA and no differences were observed between the NSCLC-3/neo and NSCLC-3/mIκBα cells. The immunoblot analysis of survivin protein at 18 h demonstrated down-regulation (>3-fold) of survivin protein in both the NSCLC-3/neo and NSCLC-3/mIκBα cells treated with SN-38→PS-341 (Figure 2C). These data suggest that DNA damage caused by SN-38 initiates down-regulation of the survivin transcript. Furthermore, while this down-regulation is a reversible process, it can be sustained with proteasome inhibition.

Based on these data demonstrating that survivin mRNA and protein levels were down-regulated in NSCLC-3/neo or NSCLC-3/mIκBα cells treated with SN-38→PS-341, whether there was a correlation with increased caspase 9 activity, which is inhibited by phosphorylated survivin (26), was determined. For these experiments, NSCLC-3 cells were stably transfected with myc-tagged wild-type or dominant negative mutant T34A survivin. The data in Figure 3A and 3B clearly demonstrate the overexpression of myc-tagged wild-type and myc-tagged mutant T34A survivin, respectively. In addition, the data also show equivalent levels of endogenous survivin in the NSCLC-3/neo, NSCLC-3/myc-survivin and NSCLC-3/myc-T34A survivin transfected cells. Comparison of caspase 9 activity in NSCLC-3/myc-T34A survivin mutant cells and in NSCLC-3/neo or NSCLC-3/myc-survivin cells, treated with PS-341 alone, SN-38 alone or SN-38→PS341 (Table II) revealed that caspase 9 activity was significantly increased in the NSCLC/myc-T34A survivin cells treated with either PS-341 alone ($p<0.01$) or SN-38→PS-341 ($p<0.001$). The comparison of caspase 3 activity in the NSCLC-3/myc-T34A survivin mutant cells and in the NSCLC-3/neo or NSCLC-3/myc-survivin cells, treated with PS-341 alone, SN-38 alone or SN-38→PS341 (Table II), revealed that caspase 3 activity was significantly ($p=0.001$) increased only in NSCLC-3/myc-T34A survivin cells treated with SN-38→PS-341. The enhanced caspase 9 and caspase 3 activity in the NSCLC-3/myc-T34A survivin mutant cells compared to the NSCLC-3/neo or NSCLC-3/myc-survivin cells treated with SN-38 followed by PS-341 also correlated with significantly reduced survival in a soft-agar colony assay of NSCLC-3/myc-T34A survivin mutant cells treated with PS-341 ($p=0.002$) and SN-38→PS-341 ($p=0.012$).

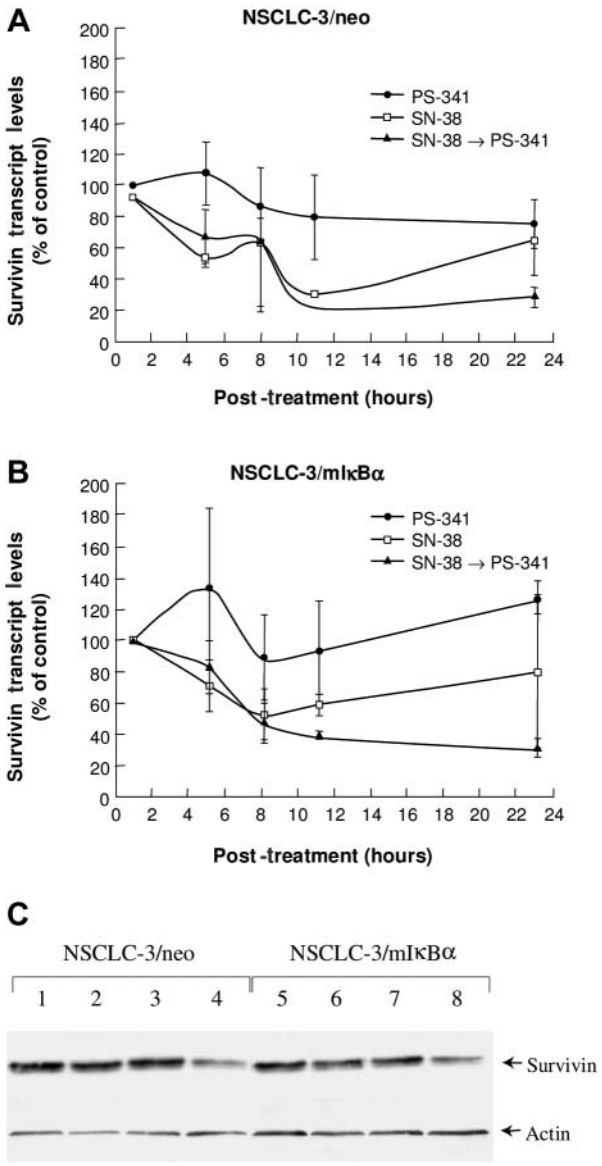

Figure 2. *Real-time PCR of survivin expression in NSCLC-3/neo (A) and NSCLC-3/mIκBα (B) cells. The cells were treated as described earlier in the legend to Figure 1B and the survivin transcript levels were determined at the indicated time-intervals. Immunoblot of survivin protein expression in NSCLC-3/neo and NSCLC-3/mIκBα cells (C) treated with SN-38 ± PS-341. Control (lanes 1 & 5); 25 nM PS-341 for 4 h (lanes 2 & 6); 100 nM SN-38 for 1 h (lanes 3 & 7); and 100 nM SN-38 (1 h) →25 nM PS-341 for 4 h (lanes 4 & 8).*

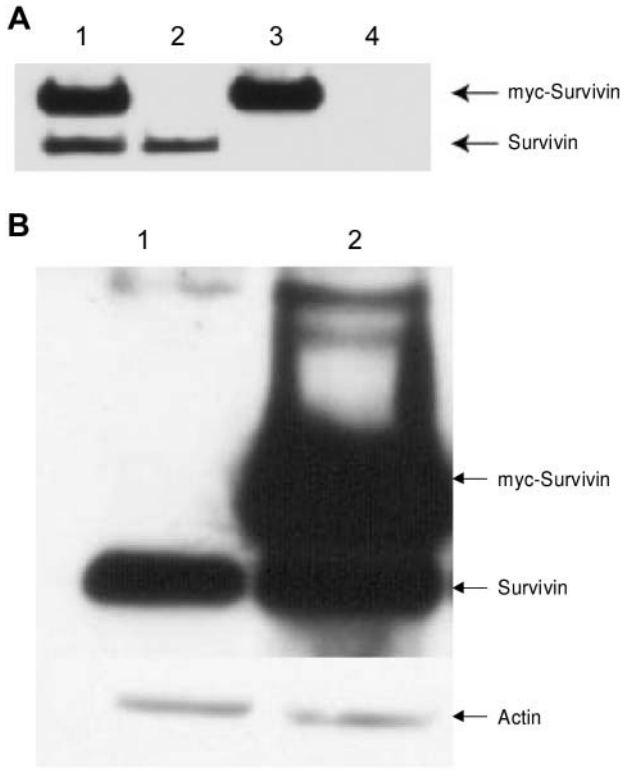

Figure 3. *Expression of myc-tagged wild-type and mutantT34A survivin in transfected NSCLC-3 cells. (A) Protein levels of survivin following transfection of NSCLC-3 cells with pcDNA3 myc-survivin. Lane 1, total cell lysate of NSCLC-3/myc-survivin; Lane 2, total cell lysate of NSCLC-3/neo; Lane 3, immunoprecipitate of cell lysate from NSCLC-3/myc-survivin cells using myc epitope-specific antibody; and Lane 4, immunoprecipitate of cell lysate from NSCLC-3/neo cells using myc epitope-specific antibody. (B) Expression of survivin and myc-tagged T34A mutant survivin in NSCLC-3/neo (Lane 1) and NSCLC-3/T34A survivin (Lane 2) cells.*

Table II. *Effect of transfected wild-type or mutant T34A survivin in NSCLC-3 cells on caspase activity and survival following treatment with SN-38 ± PS-341.*

Treatment	NSCLC-3/ neo	NSCLC-3/ myc-survivin	NSCLC-3/myc-T34A survivin
Caspase 9			
25 nM PS-341	0.79±0.07[a]	0.93±0.11	1.29±0.12 (p=0.01)[d]
100 nM SN-38	0.8±0.06	1.22±0.17	1.16±0.15
100 nM SN-38 → 25 nM PS-341	1.13±0.20	1.16±0.15	2.16±0.52 (p=0.001)[d]
Caspase 3			
25 nM PS-341	1.79±0.44[b]	1.78±0.27	2.20±0.45
100 nM SN-38	0.94±0.08	1.35±0.17	1.38±0.03
100 nM SN-38 → 25 nM PS-341	2.79±0.49	2.38±0.30	3.94±0.63 (p=0.001)[d]
Survival			
25 nM PS-341	62±15.4[c]	54±12.1	30±4.6 (p=0.002)[d]
100 nM SN-38	65±15.9	61±11.5	53±4 (p=0.19)[d]
100 nM SN-38 → 25 nM PS-341	36±14.6	24±4.9	12±3.1 (p=0.012)[d]

The cells were treated as described in Materials and Methods. [a]fold-increase in caspase 9 activity determined using substrate LEHD-AFC; [b]fold-increase in caspase 3 activity determined using substrate DEVD-AFC; [c]cell survival (% of control) in soft-agar colony assay; [d]statistical significance for differences compared to NSCLC-3/neo cells. No significant differences in caspase 9 activity, caspase 3 activity or cell survival between NSCLC-3/neo and NSCLC-3/myc-survivin were found with the various treatments.

Discussion

Drugs that target the enzymes topo I or topo II stabilize topo - DNA cleavable complex formation, which leads to protein-linked DNA strand breaks and cell death (3, 4). Although, it is generally accepted that topo I- or topo II-targeting drugs produce DNA damage and induce cell death by apoptosis, it remains to be addressed whether the signaling pathways that regulate the initiation of apoptosis induced by topo poisons are dependent on this DNA damage. Since it is well established that the activation of NF-κB is an inducible stress response and topo poisons are effective in stimulating this pathway (11), we previously examined the functional role of NF-κB activation in apoptosis induced by the topo I inhibitor SN-38 or the topo II inhibitor VP-16 (14). Our results (14) indicated that NF-κB (based on an increase in DNA binding activity) was indeed activated following treatment with SN-38 or VP-16. However, in contrast to the published literature (11), our results obtained with the neo- and mIκBα-transfected NSCLC-3 or NSCLC-5 cells, indicated that the differential activation of NF-κB did not alter apoptosis or clonogenic cell survival following treatment with SN-38 or VP-16. Since proteasome inhibitors can markedly inhibit the activation of NF-κB, a systematic analysis of the effects of proteasome inhibition on apoptosis following DNA damage induced by topo-targeting drugs demonstrated that post-treatment with the proteasome inhibitor MG-132 significantly enhanced apoptosis in either neo or dominant negative mIκBα transfected NSCLC-3 or NSCLC-5 cells (14).

In order to systematically characterize the signaling pathways regulating DNA damage-induced apoptosis by proteasome inhibition, we selected PS-341, a novel and potent proteasome inhibitor. Our choice of PS-341 as a substitute for MG-132 in the present studies was based on the criterion that unlike MG-132, which inhibits other proteases, *e.g.,* lysosomal cysteine proteases and calpains, PS-341 is 500-fold more selective for inhibition of 20S proteasome (16). PS-341 is also a significantly more potent

inducer of apoptosis (Figure 1A) and this effect did not appear to be dependent on the NF-κB status of the cells since comparable results were obtained in NSCLC-3/neo or NSCLC3/mIκBα cells. Although PS-341 induced apoptosis in a dose-dependent manner, it had minimal effects on cell cycle traverse perturbations. It is well recognized that in response to DNA damage, cells accumulate in the G2-phase of the cell cycle to possibly repair DNA prior to replication and that apoptosis is a consequence in cells attempting to divide with damaged DNA. Based on the present results on the modulation of DNA damage-induced apoptosis by PS-341, unlike treatment with SN-38 alone, the treatment with SN-38 followed by PS-341 led to increased apoptosis without accumulation of cells in the G_2 + M-phase of the cell cycle. This is supported by the results on the early increase in caspase activity (6 h) and the absence of significant cell cycle progression beyond the S-phase when cells were analyzed by flow cytometry at varying time-intervals following treatment.

Altering the activation of NF-κB did not significantly affect the modulation of DNA damage-induced apoptosis by PS-341. This suggests that anti-apoptotic proteins not regulated by NF-κB may be involved. The enhanced apoptosis and decreased accumulation of cells in the G_2-phase with SN-38→PS-341 treatment also suggested a possible role of survivin in this process. The anti-apoptotic function of survivin is related to its inhibitory effects on the initiator caspase 9 (26). This inhibitory effect is also dependent on the phosphorylation of survivin at threonine 34 by $p34^{cdc2}$ kinase (26). Thus, inhibiting the phosphorylation of survivin by transfection of a myc-T34A mutant survivin is reported to not only attenuate the anti-apoptotic effects of survivin, but also inhibit phosphorylation of the constitutive survivin (27). Using real-time RT-PCR analysis, it was demonstrated that recovery of survivin transcript expression was strongly inhibited only with the SN-38→PS-341 treatment. This was also correlated with a decrease in survivin protein levels. The requirement for down-regulation of survivin to elicit the enhanced apoptosis with SN-38→PS-341 treatment was supported by enhanced caspase activation and reduced survival in NSCLC-3 cells transfected with the myc-T34A mutant survivin but not with wild-type survivin.

In summary, we have shown that inhibition of proteasome function after DNA damage can significantly enhance apoptosis induced by topo I-targeting drugs. The effect of proteasome inhibition on modulating DNA damage-induced apoptosis is not dependent on NF-κB activation, but due to the down-regulation of survivin. Current studies are focussed on characterizing the role of proteasome inhibition in activating apoptosis with topo I-targeting drugs to improve the therapeutic benefit in cancer chemotherapy.

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