Effects of the proteasome inhibitor PS-341 on tumor growth in HTLV-1 Tax transgenic mice and Tax tumor transplants

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Recent studies have shown that the transcription factor nuclear factor κB (NF-κB) regulates critical survival pathways in a variety of cancers, including human T-cell leukemia/lymphotrophic virus 1 (HTLV-1)–transformed CD4 T cells. The activation of NF-κB is controlled by proteasome-mediated degradation of the inhibitor of nuclear factor κBα (IκBα). We investigated the effects of PS-341, a peptide boronate inhibitor of the proteasome in HTLV-1 Tax transgenic tumors in vitro and in vivo. In Tax transgenic mice, PS-341 administered thrice weekly inhibited tumor-associated NF-κB activity. Quantitation of proliferation, apoptosis, and interleukin 6 (IL-6) and IL-10 secretion by tumor cells in culture revealed that the effects of PS-341 on cell growth largely correlated with inhibition of pathways mediated by NF-κB. However, the effect of PS-341 on the growth of tumors in Tax transgenic mice revealed heterogeneity in drug responsiveness. The tumor tissues treated with PS-341 show no consistent inhibition of NFκB activation in vivo. Annexin V staining indicated that PS-341 response in vivo correlated with sensitivity to apoptosis induced by γ irradiation. On the other hand, transplanted Tax tumors in Rag-1 mice showed consistent inhibition of tumor growth and prolonged survival in response to the same drug regimen. TUNEL staining indicated that PS-341 treatment sensitizes Tax tumors to DNA fragmentation. (Blood. 2004;104:802-809)

Introduction

Human T-cell leukemia/lymphotropic virus type 1 (HTLV-1) infection is present in 10 to 20 million people worldwide.[1,2] HTLV-1 infection results in 2 different diseases: adult T-cell leukemia/lymphoma (ATLL or ATL) and the neurologic disorder tropical spastic paraparesis/HTLV-1–associated myelopathy (TSP/HAM).[3,4] Although the epidemiology and clinical characteristics of HTLV-1 infection are defined, the molecular mechanisms used by the virus to establish persistent infection and subsequently promote lymphocyte proliferation and the host immune evasion remain poorly understood. Thus, the quest for appropriate antiviral therapy and a vaccine are difficult, but important. The vast majority of patients with ATL present with resistance to chemotherapy, limiting survival to less than a year.[5,6]

Recent investigation of the molecular events associated with HTLV progression has identified a number of molecular targets within the virus and the host that could represent excellent targets for therapeutic intervention. The 40-kDa transactivator protein, Tax, mediates the transition from latency to virion production by interacting with specific host proteins associated with cellular transcriptional pathways such as nuclear factorκB (NF-κB), cyclic adenosine monophosphate response element-binding–activating transcription factor (CREB/ATF), serum response factor (SRF), stimulatory protein 1 (SP1), and early growth response protein 1 (EGR-1). Through interaction with cellular transcription factors, Tax potently activates transcription from the viral promoter and enhancer elements of many cellular genes involved in host cell proliferation.[7,8] The oncogenic potential of Tax has been demonstrated in animal models,[9-12] and activation of NF-κB has been implicated as a critical feature of transformation.[13,14] Tax may be responsible for many of the required events necessary for HTLV-1–mediated lymphocyte immortalization and transformation.

The NF-κB family of transcription factors participates in regulation of diverse biologic processes, including immune responses, cell growth, and apoptosis.[15-19] Mammalian cells express 5 NF-κB members, RelA, RelB, c-Rel, p50, and p52, which function as various homodimers and heterodimers.[20] The NF-κB factors are normally sequestered in the cytoplasm through physical interaction with ankyrin repeat-containing inhibitors, including IκBα and related proteins.[21] A well-characterized pathway leading to NF-κB activation is through phosphorylation and subsequent degradation of IκBα.[22,23] This canonical NF-κB signaling pathway depends on a multisubunit IκB kinase (IKK), which responds to various stimuli, such as the inflammatory cytokine tumor necrosis factor α (TNF-α), the mitogen phorbol 12-myristate 13-acetate (PMA), and certain viral proteins.[15,24,25] IKK is composed of 2 catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also named NEMO, IKKAP1, or FIP-3).[26] Another level of NF-κB regulation is via processing of the NF-κB1 and NF-κB2 precursor proteins p105 and p100, a proteasome-catalyzed event required to generate p50 and p52, respectively.[20,27] Recent studies suggest that Tax physically associates with IKK [28] and stimulates the catalytic activity of this cellular kinase.[29-31] This virus-specific effect is dependent on IKKγ,[32,33] which serves as an adaptor for recruiting Tax to the IKK catalytic subunits.[34-37] Tax-induced IKK activation is responsible for the persistent phosphorylation of IκBα and nuclear expression of NF-κB in HTLV-infected T cells.[24] Tax

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protein functions as a potent inducer of p100 processing thereby inducing active production of p52. Tax-induced p100 processing does not require NF-κB–inducing kinase (NIK) but involves the noncanonical IKK component, IKKα, which phosphorylates specific serines at the C-terminal region of p100. An important mechanism of Tax action in this virus-specific pathway is to recruit IKKα to p100.[8]

IκB binds to the nuclear localization domain of NF-κB, preventing it from translocating to the nucleus and promoting expression of NF-κB target genes.[38] Ubiquitin conjugation is stimulated by phosphorylation of IκB on 2 conserved serine residues leading to proteasome-mediated degradation of IκB and consequent activation and nuclear translocation of NF-κB.[39,40] Proteasome inhibitors block IκB degradation and NF-κB activation. Importantly, proteasome inhibitors also stimulate apoptosis and inhibit angiogenesis in a variety of tumor cell types.[41-43] Given the established role of NF-κB in suppression of apoptosis, it is likely that NF-κB inhibition contributes to proteasome inhibitor–induced cell death.

Transgenic mice expressing Tax from the human granzyme B promoter in lymphoid cells develop tumors at peripheral sites of the body at a median of 7 months of age.[44] These tumors consist primarily of large granular lymphocytes (LGLs) that subsequently infiltrate secondary lymphoid organs, liver, and lungs. The tumors exhibit constitutive activation of NF-κB and elevated expression levels of NF-κB–inducible cytokines, including interleukin 6 (IL-6), IL-10, IL-15, and interferon γ (IFN-γ).[13] Inhibitors of NF-κB activation, sodium salicylate and cyclopentenone prostaglandins, blocked spontaneous proliferation of Tax transgenic mouse spleen cells. In addition, Tax-induced tumor cells, which are resistant to γ irradiation–induced apoptosis, undergo apoptosis in the presence of sodium salicylate and prostaglandins. These results strongly suggest that Tax-mediated induction of NF-κB activity contributes to tumorigenesis in vivo. Thus, this tumor system is an excellent animal model to assess the effects of NF-κB inhibitors.

PS-341 is a dipeptide boronate antagonist of the proteasome that was recently developed by Millennium Pharmaceuticals (Cambridge, MA) for use in cancer therapy. PS-341 forms a covalent, reversible complex with the proteasome and it is much more potent than its peptide aldehyde predecessors (ie, MG-132 and calpain inhibitor-I).[45,46] PS-341 has been demonstrated to be a potent and selective proteasome inhibitor in clinical trials for a variety of tumor types, including myeloma, chronic lymphocytic leukemia, prostate cancer, pancreatic cancer, breast cancer, and colon cancer.[47-49] Proteasome inhibitors provide a rational approach to control constitutively activated NF-κB in HTLV-1–infected T cells.[50] This study describes the activity of this drug on Tax transgenic mouse tumors and Tax-induced tumors transplanted into Rag-1 mice.

Materials and methods

Animals and cell lines

Transgenic mice expressing HTLV-1 Tax (C57/Bl6.SJL) have been described previously.[44] Rag-1 mice in the C57/Bl6 background and wild-type C57/Bl6 were purchased from Taconic Farms (Germantown, NY) and used at 8 weeks of age. Mice were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine throughout the study. Progress of tumor development was monitored every week and animals were killed at the end of the experiment or if tumors grew to more than 35 mm in diameter. The F8 and SC cell lines are derived from a Tax tumor in the F8 founder line and have been described previously.[11] Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), antibiotics, vitamins, and pyruvate, under an atmosphere of 5% CO_2 in air. Single-cell suspensions of tumor tissues were harvested and plated in tissue-culture dishes. Murine splenocytes were subjected to red blood cell (RBC) lysis and grown in the presence of 10 μg/mL phytohemagglutinin (PHA) and 200 U recombinant human IL-2 (rhIL-2).

Reagents and drug regimen

Sodium salicylate was obtained from Sigma (St Louis, MO) and 1 M stock solutions were prepared. PS-341 was a gift from Millennium Pharmaceuticals and a 10^{-6} M stock was prepared in saline for in vitro experiments. Three groups of mice, drug-treated Tax-positive, vehicle-treated Tax-positive, and drug-treated Tax-negative, consisting of at least 10 animals each, were studied. Tax transgenic mice at age 7 months with no tumors or tumors less than 4 mm in diameter were used. The longest tumor diameter and the perpendicular diameter were measured with vernier calipers for each animal at 2, 4, 6, and 8 weeks following the start of the drug administration. Tumor volume was calculated by the formula: volume in $mm^3 = ab^2/2$ where a is the longest tumor diameter and b the perpendicular diameter. For tumor transplantation experiments, 3 groups of Rag-1 mice, with 5 to 6 animals each, were given injections with tissue culture–derived SC cells via the subcutaneous route and treated with drug or vehicle. PS-341 was administered at 0.1 mg/kg 3 times a week via the subcutaneous route. Sterile saline was used for vehicle injections.

Thymidine incorporation assays

Mouse spleen cells or tumor cells (3×10^6 cells/well) were added to 6-well plates in 3 mL complete RPMI and cultured for 4 hours following 10 mM sodium salicylate treatment or 10^{-7} M PS-341 treatment. Nontransgenic mouse spleen cells were cultured in complete media containing 10 μg/mL PHA and 200 U/mL rhIL-2. Then, 1 μCi/well (1.1 MBq/well) of [^{3}H] thymidine was added to the cultures, and incubated for 14 hours at 37°C. Cells were harvested onto glass filters, and thymidine incorporation was quantitated by liquid scintillation counting.

Cell viability

Cells were seeded into 96-well microculture plates at 5000 cells/well and treated with either sodium salicylate or PS-341 for 20 hours. Viability measurements were conducted by trypan blue exclusion. Each experimental data point represents the average value obtained from 5 tumors or 5 spleens and each experiment was performed in triplicate.

Quantitation of apoptosis induced by γ irradiation

Fresh tumor and spleen cell suspensions were incubated in the presence or absence of 10 mM sodium salicylate or 10^{-7} M PS-341 for 20 hours prior to treatment with 30 Gy (3000 rad) γ irradiation. Five hours after irradiation, 1×10^5 cells were dual-stained with fluorescein isothiocyanate–conjugated antibody against annexin V and propidium iodide as described by the manufacturer (Pharmingen, San Diego, CA). Apoptotic cells were measured by fluorescence-activated cell sorting (FACS) analysis on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

p65 ELISA

Cells from mouse spleens or primary tumor tissues (3×10^6 cells/well) were added to 6-well plates in 3 mL RPMI and cultured for 16 hours at 37°C in the presence or absence of 10 mM sodium salicylate or 10^{-7} M PS-341, and cell extracts were collected. Using an enzyme-linked immunosorbent assay (ELISA), p65-p50 DNA-binding activity was measured with 10 μg total cell extract with the Trans-AM kit according to the manufacturer's recommendations (Active Motif, Carlsbad, CA).

Immunoblotting

F8 or SC cells were cultured with 10^{-7} M PS-341 or 10 mM sodium salicylate, harvested, washed, and lysed using lysis buffer: 50 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA [ethylenediaminetetraacetic acid], 5 mM NaF, 2

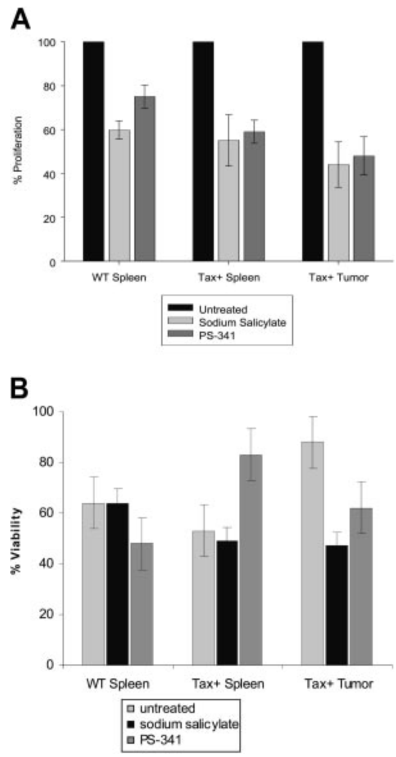

Figure 1. PS-341 inhibits spontaneous proliferation of Tax transgenic mouse tumors in culture. (A) A total of 3×10^6 splenocytes or tumor cells from 5 Tax transgenic and 5 nontransgenic mouse splenocytes were cultured for 4 hours following drug or mock treatment. Nontransgenic splenocytes were also stimulated with 200 U/mL IL-2 plus 10 μg/mL PHA. Then, 1 μCi/well (1.1 MBq/well) [^{3}H] thymidine was added to cultures, and thymidine incorporation was measured after a 16-hour incubation. Percent proliferation is reported ± SEM considering the [^{3}H] thymidine incorporation of untreated samples as 100%. The statistical significance of the inhibition was evaluated by a paired *t* test: $P \leq .01$ for untreated compared to sodium salicylate treatments and $P \leq .05$ for untreated compared to PS-341 treatments. (B) Cell viability was measured by trypan blue exclusion after 20 hours of culture with sodium salicylate or PS-341 and values from spleens or tumors from 5 Tax transgenic and 5 nontransgenic mouse spleens are reported ± SEM.

mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/mL leupeptin, and 5 μg/mL aprotinin. For detection of phospho-IκB, IκB, and actin, cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL), and immunoblotted with antiphospho-IκB, IκB (Cell Signaling Technology, Beverly, MA), and actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies as described earlier.[51]

Quantitation of IL-6 and IL-10 secretion by ELISA

To evaluate IL-6 and IL-10 expression after treatment with PS-341, cells were plated in 12-well plates. Four hours later, cells were exposed to 10^{-7} M PS-341 for 24 hours. Supernatants were collected after 16 hours and IL-6 and IL-10 protein levels were determined using OptEIA IL-6 and IL-10 ELISA kits (PharMingen). Cell numbers were equivalent in control and PS-341–treated triplicate samples.

Quantitation of apoptosis in situ

Tumors were established in Rag-1 mice by subcutaneous injections of 5×10^6 SC cells and allowed to grow for 3 days before the PS-341 or vehicle injections were started. Tumors (n = 5) were harvested after 8 weeks and fixed for paraffin embedding and tissue sectioning. Analysis of DNA fragmentation by fluorescent TUNEL was performed using a commercial kit (Promega, Madison, WI) as described in the manufacturer's instructions. For each group at least 4 independent fields were selected at random from different tumors so that the comparison among groups would involve roughly equivalent numbers of cells. The apoptotic index in 6 random fields from 3 drug-treated and 3 vehicle-treated tumors were counted at × 600 magnification.

Results

Analysis of the mechanisms of Tax-mediated oncogenesis using an inhibitor of NF-κB activation, PS-341

Cell-culture assays were performed with splenocytes and tumors from 5 Tax transgenic animals and control splenocytes from 5 nontransgenic animals.[13] The effect of 10^{-7} M PS-341 was compared to no treatment or treatment with 10 mM sodium salicylate as a positive control. Cell proliferation was examined by [^{3}H] thymidine incorporation, after 16 hours of incubation. Treatment with PS-341 partially inhibited the proliferation of tumor cells, as well as PHA- and IL-2–activated nontransgenic splenocytes (Figure 1A). Cell viability assays show that there are small but insignificant differences in viability, indicating minimal drug toxicity with treatment (Figure 1B).

An ELISA of p65-p50 DNA-binding activity with nuclear extracts from treated cells showed that PS-341 effectively blocked activation of

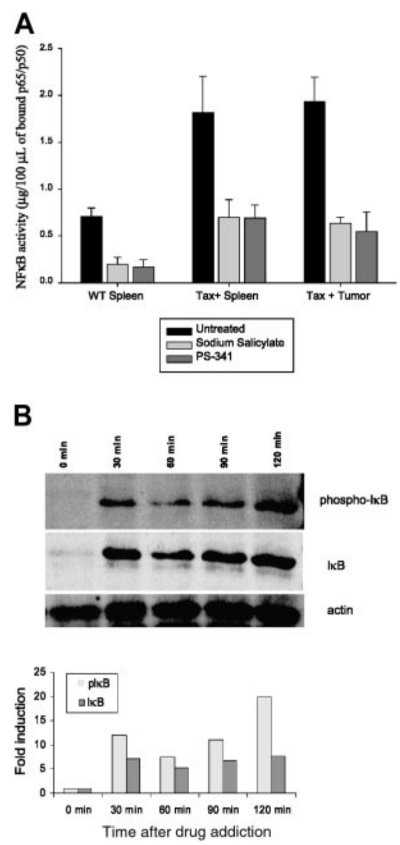

Figure 2. NF-κB activation is reduced in Tax transgenic mouse tumors treated with PS-341 in vitro. Nuclear extracts were prepared from Tax transgenic tissues, and representative results from 5 transgenic spleens and tumors and 5 nontransgenic splenocytes are shown. Tissues used included tumors arising on the nose, leg, or foot, as well as spleens with tumor involvement or nontransgenic splenocytes. (A) ELISA was performed as described in "Materials and methods." Values are represented as means of 1 of 3 separate experiments. Error bars correspond to SEM between 5 animals in each group. The statistical significance of the inhibition was evaluated by a paired *t* test: $P \leq .05$ for untreated compared to sodium salicylate treatments and $P \leq .05$ for untreated compared to PS-341 treatments. (B) F8 cells were cultured with 10^{-7} M PS-341, harvested at 0, 30, 60, 90, and 120 minutes, washed, and lysed to prepare total cell extracts. For detection of phospho-IκB, IκB, and actin, cell lysates were subjected to electrophoresis on a 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with antiphospho-IκB, IκB, and actin (loading reference) antibodies. Blots were developed using a chemiluminescent substrate.

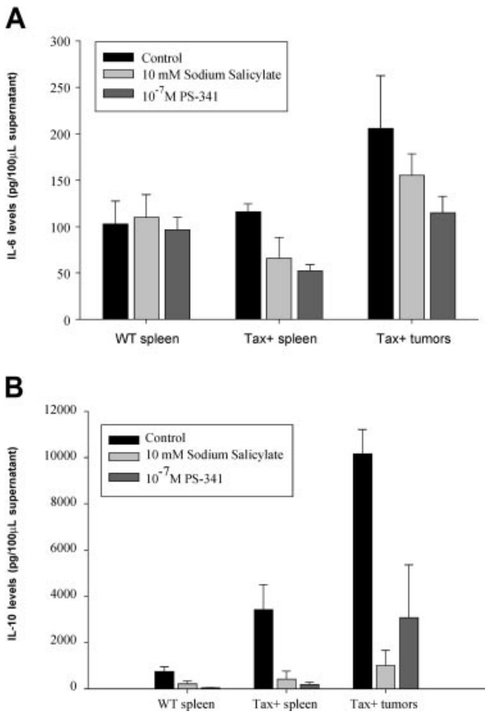

Figure 3. Effects on IL-6 and IL-10 production in Tax transgenic mouse tumors treated with PS-341. A total of 3×10^6 cells from 5 nontransgenic and 5 Tax transgenic mouse spleen or primary tumor tissues were cultured for 16 hours. Supernatants were used in ELISA to measure (A) IL-6 and (B) IL-10 production. Error bars represent SEM of 1 of 3 separate experiments using tissues from 5 Tax transgenic and 5 nontransgenic mice each. The statistical significance of the inhibition was evaluated by a paired *t* test: $P \leq .2$ for untreated compared to sodium salicylate treatments and $P \leq .1$ for untreated compared to PS-341 treatments. The statistical significance for IL-10 production is $P \leq .03$ for untreated compared to sodium salicylate treatments and $P \leq .03$ for untreated compared to PS-341 treatments.

NF-κB in Tax tumors in culture (Figure 2A). Western blots performed with cell extracts from Tax-expressing tumor cell line, F8, indicate that the levels of IκB and phospho-IκB are increased in the presence of sodium salicylate or PS-341 in vitro (Figure 2B). Induction of NF-κB–responsive IL-6 and IL-10 expression was quantitated using ELISA on culture supernatants and results show that IL-6 production by transgenic splenocytes is not significantly reduced (Figure 3A), whereas IL-10 levels were significantly reduced after PS-341 treatment (Figure 3B). IL-10 secretion by Tax tumors was induced to a greater extent than that of IL-6. We have previously shown a decrease in NF-κB–inducible cytokines in Tax tumors in response to sodium salicylate and prostaglandin treatments. Although statistically not significant, a trend in the decrease in the levels of IL-6 is clear. Although not as prominent as repression of IL-10 secretion in these tumor and spleen samples, the inhibition of IL-6 may be due to inhibition of NFκB activation.

Because Tax tumors are resistant to apoptosis after 3000 rads γ irradiation, the effects of PS-341 treatment in rendering Tax tumor cells sensitive to apoptosis was also examined by an annexin V staining assay (Figure 4).[13] PS-341 reversed the resistance to γ irradiation–mediated apoptosis (Figure 4).

Evaluation of PS-341 in Tax tumor–positive transgenic mice

PS-341 was injected subcutaneously 3 times a week over an 8-week period at a dose of 0.1 mg/kg in 11 transgenic animals with no tumors or tumors less than 4 mm in diameter and 7 nontransgenic animals. As a vehicle control, 10 transgenic animals were given injections of phosphate-buffered saline (PBS) subcutaneously, 3 times a week. Preliminary studies indicated that for PS-341 toxicity in control and tumor-bearing mice, doses of the drug higher than 0.1 mg/kg were not tolerated and led to rapid lethality (data not shown). The dose 0.1 mg/kg was selected as the maximum tolerated dose for these studies. Tumor growth depicted as fold changes in tumor volume in 11 transgenic mice treated with drug and 7 vehicle-treated transgenic mice are shown in Figure 5 for each time point. All 10 Tax-nontransgenic mice were healthy with no significant weight changes throughout the period of drug administration (data not shown). Tumors in animals treated with PS-341 do not show significantly different growth rates than tumors in vehicle-treated animals (Figure 5). Overall, this study shows that PS-341 has limited effects in transgenic animals. At the end of the 8-week period, the animals were killed and blood counts and differentials were performed. No differences in the drug-treated or vehicle control animals were evident, indicating that the animals displayed no adverse responses to PS-341 administration. The Tax tumors manifested divergent responses to PS-341. Two of the 11 drug-treated animals manifested partial remissions, 3 animals had stable disease, and 6 animals showed tumor progression. Among 7 vehicle-treated animals, 1 animal showed partial remission, and 6 animals exhibited tumor progression over the time course of the study. Statistical analysis of the PS-341 treatment cohort in comparison with the vehicle-treated group indicates that PS-341 has a variable effect on Tax tumors. We observed more animals with stable disease or partial remissions in drug-treated groups as compared to the vehicle groups. The increase of tumor volume, however, exhibited no statistically significant differences. Drug injections in animals with more advanced tumors showed no effect on tumor growth, indicating that PS-341 was active only in early stages of tumor development (data not shown). There appeared to be no significant histologic changes in tumors from any of the drug- or vehicle-treated groups including those animals exhibiting partial remissions or stable disease (data not shown). Similarly, no significant inhibition of NFκB activity could be demonstrated in vivo in any of the drug-treated tumor tissues as compared to vehicle control tissues. However, there is a clear

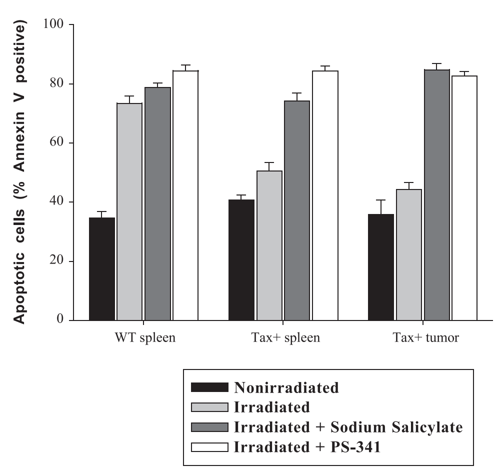

Figure 4. NF-κB inhibitors induce apoptosis in Tax transgenic tumor cells. Fresh tumor and spleen cell suspensions from nontransgenic and Tax transgenic mice were incubated in the presence or absence of 10 mM sodium salicylate or PS-341 for 20 hours prior to treatment with 30 Gy (3000 rads) irradiation. Five hours after irradiation, 10^5 cells were stained with fluorescein isothiocyanate–conjugated annexin V (FL1) to evaluate cells in early apoptosis. Total numbers of dead cells were measured by propidium iodide staining (FL-2). Apoptotic cells are indicated by annexin V–positive population, and the percentage apoptotic cells is shown. Error bars represent SEM between 5 mice in 1 representative experiment of 3 experiments consisting of 5 nontransgenic and 5 transgenic mice each. The statistical significance of the inhibition was evaluated by a paired *t* test: $P \leq .01$ for untreated compared to sodium salicylate treatments and $P \leq .03$ for untreated compared to PS-341 treatments.

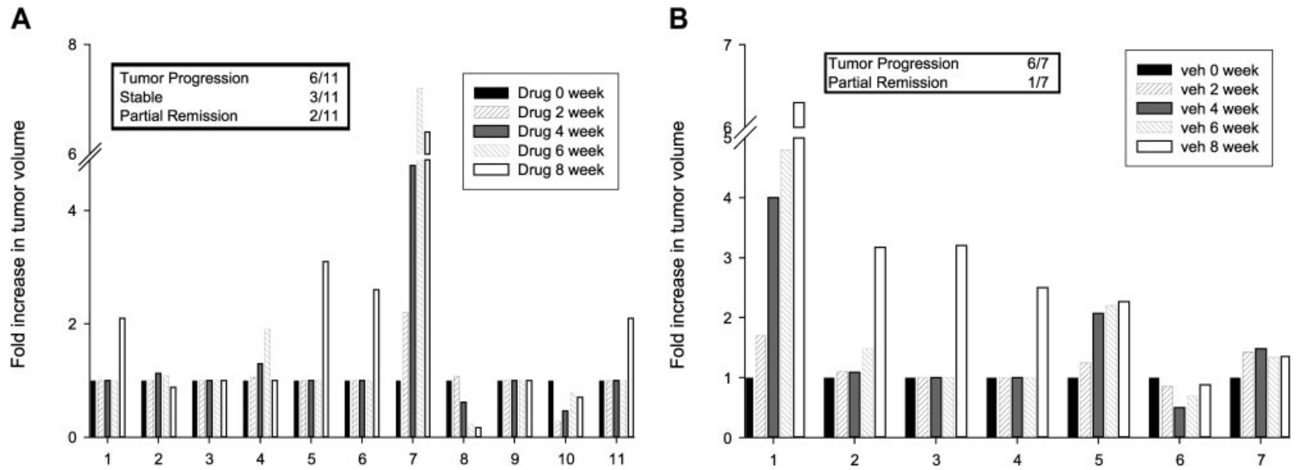

Figure 5. Fold increase in tumor volume of PS-341–treated and untreated tumors in Tax transgenic animals. Tax-positive and Tax-negative mice were injected with drug or vehicle thrice weekly via the subcutaneous route as described in "Materials and methods." Tumor volumes were measured on Tax tumor–positive mice every other week for 8 weeks during the treatment using vernier calipers. The fold increase in tumor volume at 0 week at the start of treatment compared to that at different time intervals is depicted here for 0, 2, 4, 6, and 8 weeks of treatment. Tax-negative mice received drug as a control for toxicity. The numbers correspond to different mice with tumors treated with either PS-341 or vehicle. Mice exhibiting tumor volume increases of 25% or more of initial volume were considered to be tumor progressors, those with 50% or more overall decrease were considered to be partial remissions, and all forms of tumors in between indicate a stable disease. The statistical significance of the response to PS-341 treatment on tumor growth was evaluated by a paired *t* test: $P \leq .9$ for vehicle treated compared to PS-341 treated groups.

correlation between the response of tumors to the PS-341 regimen and their ability to respond to decreased NFκB activity in response to PS-341 in cell culture (Table 1).

Effects of PS-341 on transplanted Tax tumors in Rag-1 mice

The activity of PS-341 in a transplant model of Tax tumors was also analyzed. The drug was delivered thrice weekly in a regimen similar to that for the transgenic mice. Animals treated at this dose level displayed no significant weight changes or other signs of toxicity (data not shown). The effects of PS-341 treatment on tumorigenicity were assessed for a total of 8 weeks. PS-341 did not affect tumor incidence but slowed the growth of the transplanted tumors significantly (Figure 6A). Drug-treated tumor-positive animals also demonstrated prolonged survival as compared to vehicle-treated mice (Figure 6B). The animals were humanely killed after the completion of 8 weeks of drug regimen, and tumor sections were evaluated for apoptosis by TUNEL staining. Drug treatment induced DNA fragmentation in tumors, indicating that the inhibition of tumor growth was due to enhanced cell death on PS-341 treatment (Figure 7A). Representative fields containing apoptotic cells from 3 tumors of each group are shown, which clearly depict the higher rate of apoptosis in mice treated with PS-341. The number of apoptotic cells in 6 random fields each from 3 different tumors from the drug-treated and the vehicle-treated groups were counted and the apoptotic index is shown in Figure 7B.

Table 1. NFκB activity in Tax transgenic mouse tissues

Treatment	Mean NFκB activity in vivo, OD ± SEM*	Inhibition of NFκB activity in vitro†
Vehicle-treated Tax$^+$ tumors	0.270 ± 0.084	Tumor progressors, 1%
PS-341–treated Tax$^+$ tumors	0.312 ± 0.120	Tumor progressors, 0%-28%
		Stable/remissions/ nonprogressors, 92%-99%

OD indicates optical density.

*Nuclear extracts were prepared from 8 PS-341–treated tumors and 5 vehicle-treated tumors, and p50-p65 NFκB DNA-binding activity was measured in ELISA format as described in "Materials and methods."

†Cells from tissues were cultured in the presence of PS-341 for 20 hours, and NFκB activity was determined using p65 ELISA. Percent inhibition in both groups corresponding to status of disease is listed.

Discussion

Increasing evidence indicates that a series of discrete molecular alterations underlies the establishment and progression of tumorigenesis. We have shown that the transcription factor, NF-κB, is constitutively activated in Tax tumors and cell lines. We found elevated expression levels of NF-κB–inducible cytokines, including IL-6, IL-10, IL-15, and IFN-γ, in freshly isolated primary

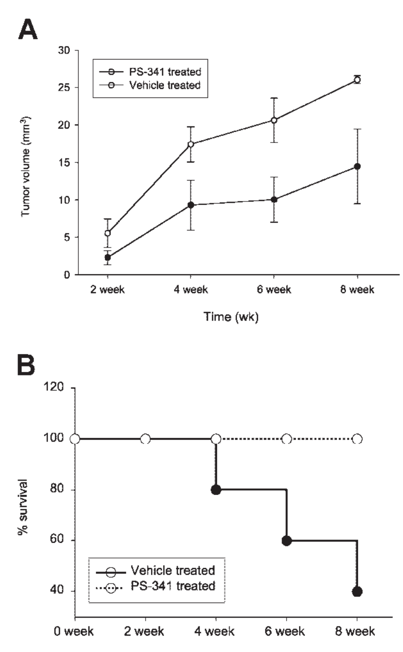

Figure 6. Inhibition of tumor growth in mice given transplants of Tax tumor. (A) Five Rag-1 immunodeficient mice at 8 weeks of age in each group were injected with SC cells subcutaneously and 2 days later were treated with PS-341 similarly for 8 weeks as described in Figure 5. The tumor volumes for all animals in each group at weeks 2, 4, 6, and 8 are plotted with SE measurements. The statistical significance of the response to PS-341 treatment on tumor growth was evaluated by a paired *t* test: $P \leq .01$ for vehicle treated compared to PS-341 treated groups. (B) Survival curves of animals under PS-341 or saline treatments were monitored and statistical significance determined using a paired *t* test: $P \leq .05$ for vehicle-treated compared to PS-341–treated groups.

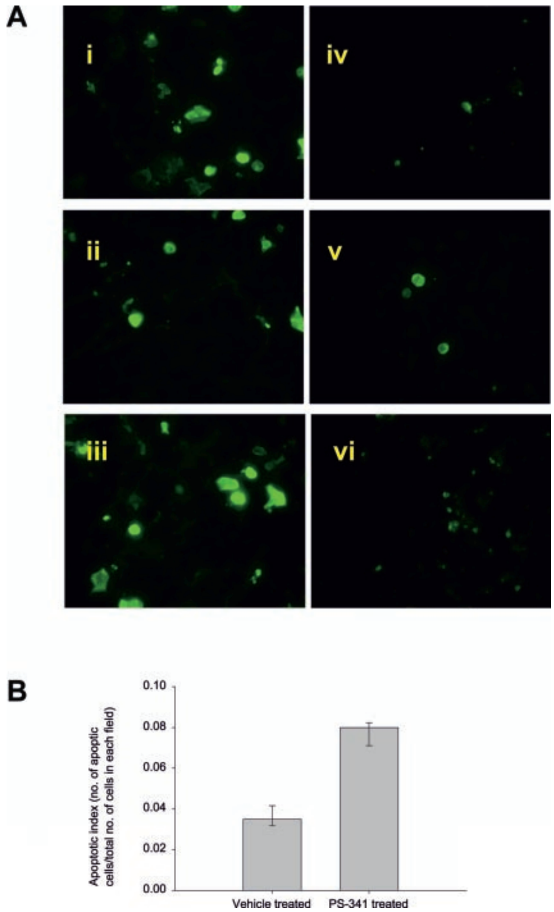

Figure 7. Effects of PS-341 on apoptosis in transplanted Tax tumors. (A) Control animals or animals treated with 0.1 mg/kg PS-341 (subcutaneously) or drug-treated mice with no tumor transplants were killed at 8 weeks of treatment. Apoptosis was measured on paraffin-embedded sections by TUNEL staining as described in "Materials and methods." Levels of apoptosis were significantly higher in PS-341–treated tumors compared with controls. Analysis of DNA fragmentation is shown for 3 representative fields obtained from PS-341–treated tumors (i-iii) or vehicle-treated tumors (iv-vi). (B) The apoptotic index was calculated by dividing the number of $TUNEL^+$ cells by the total number of cells in 6 random fields in 3 tumors. The statistical significance of apoptosis in response to PS-341 treatment was evaluated by a paired *t* test: $P \leq .002$ for untreated compared to PS-341–treated tumors. Error bars correspond to SEM.

tumors from Tax transgenic mice. Inhibitors of NF-κB activity, sodium salicylate and cyclopentenone prostaglandins, blocked spontaneous proliferation of Tax transgenic mouse spleen cells. In addition, Tax-induced tumor cells, which are resistant to irradiation-induced apoptosis, became sensitive to apoptosis in the presence of sodium salicylate and prostaglandins. These results suggest that Tax-mediated induction of NF-κB activity contributes to tumorigenesis in vivo. Therefore, this transcription factor is an attractive therapeutic target for several reasons. NF-κB regulates the expression of a wide variety of genes implicated in proliferation, angiogenesis, invasion, and metastasis,[52,53] and interruption of this signaling is expected to inhibit these processes. Furthermore, many studies have shown that NF-κB inhibits apoptosis,[54] and several of the genes involved in this death inhibitory activity have been identified.[55] Finally, many conventional cancer therapies activate NF-κB as a byproduct of their effects on cancer cells, and this effect can limit their efficacy.[45] The ubiquitin-proteasome pathway plays an important role in regulating the cell cycle, neoplastic growth, and metastasis. A number of key regulatory proteins are temporally degraded during the cell cycle by the ubiquitin-proteasome pathway, and the ordered degradation of these proteins is required for the cell to progress through the cell cycle and to undergo mitosis.[46,56]

One of the targets of ubiquitin-proteasome–mediated degradation is the tumor suppressor p53, which acts as a negative regulator of cell growth.[57] This suppressor is required for the transcription of a number of genes involved in cell-cycle control and DNA synthesis (ie, E2F, $p21^{CIP1/WAF1}$) and also plays an important function in apoptosis induced by cellular damage, including ionizing radiation. Cyclins and the cyclin-dependent kinase inhibitors p21 and $p27^{KIP1}$ are another set of growth regulatory proteins that are regulated by proteasome-dependent proteolysis. Both p21 and p27 can induce G_1 cell-cycle arrest by inhibiting the cyclin D-, E-, and A-dependent kinases. Furthermore, the ubiquitin-proteasome pathway is required for transcriptional regulation. NF-κB is a key transcription factor whose activation is regulated by proteasome-mediated degradation of the inhibitor protein IκB. Cell adhesion molecules (CAMs) such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) are regulated by NF-κB and are involved in tumor metastasis and angiogenesis in vivo. During metastasis, these molecules direct the adhesion and extravasation of tumor cells from the vasculature to distant tissue sites within the body. Thus, tumor cell metastasis is also limited by the down-regulation of NF-κB–dependent expression of cell adhesion molecules.[58] Moreover, NF-κB is also required in a number of cell types to maintain cell viability as an antiapoptotic controlling factor. Inhibiting NF-κB activation by stabilizing the IκB protein makes cells more sensitive to environmental stress and cytotoxic agents, ultimately leading to apoptosis.

These observations prompted us to study chemical inhibitors that could interfere with constitutive NF-κB activation in Tax tumors and T cells in patients with ATL. The proteasome inhibitor, PS-341, was selected because proteasome-mediated degradation of the physiologic inhibitor of NF-κB, IκB, is a well-known component of NF-κB activation. PS-341 is also attractive because it has already been evaluated in clinical trials in other cancers where it displayed promising activity and minimal toxicity.

The effects of PS-341 in cultures of Tax tumors from transgenic mice were examined first. Our results are consistent with the effects of PS-341 observed in several other tumor models in that PS-341 inhibited proliferation, NF-κB activity, and induction of NF-κB–inducible cytokines, IL-6 and IL-10, resulting in the induction of apoptosis via γ irradiation in Tax-induced tumors. In this in vitro model PS-341 appears to have a significantly greater effect on the reversal of apoptosis (Figure 4) than on the control of proliferation (Figure 1), as observed in the transplantation studies in mice (Figure 7). Although the levels of IL-6 secreted by tumor and spleen cells in vitro were not significantly altered by PS-341 treatment, as compared to inhibition of IL-10 release, we have previously demonstrated a prominent role of NF-κB inhibition on both these cytokines.[13] The wild-type splenocytes stimulated with PHA showed a significant level of IL-6 secretion that was not affected by sodium salicylate or PS-341. The inhibitory effects of PS-341 on Tax tumors are apparent in that PS-341 treatment resulted in the levels of IL-6 similar to those of wild-type splenocytes.

The results of PS-341 in the transgenic animal study were heterogeneous, with some tumors displaying sensitivity to PS-341 and other tumors more refractory to the drug. Nonetheless, given that complete 20S proteasome inhibition cannot be achieved in vivo without mortality,[45] our data suggest that some tumor cells will display inherent resistance to the drug with the dose levels that can be achieved in vivo. Using a 10-fold higher concentration of drug led to lethal toxicity, indicating that this drug did have toxicity above a tolerated dose of 0.1 mg/kg. Direct measurement of NF-κB activity confirmed that PS-341 produced equivalent inhibition of NF-κB activation in drug-sensitive and drug-resistant tumor cells, indicating that drug resistance was not due to differences in drug uptake or activity. There appears to be no consistent inhibition of NF-κB activity in vivo in tumors treated with PS-341. The sensitivity to PS-341 in vivo, however, appears to correlate with the sensitivity of these tumors cells to inhibition of NFκB activation in vitro (Table 1).

The effects of PS-341 on the growth of transplanted Tax tumors were also examined. The effects of the NF-κB inhibition were more significant in this model, in comparison to the transgenic mice study. This may be attributed to the fact that Tax expression is restricted in transgenic tumor cells lines, whereas Tax is expressed at high levels in the transgenic mouse tumors. This expression may affect one or more modifications on the genetic and physiologic makeup of the transgenic mice. Our results confirmed earlier reports showing that PS-341, in combination with antibodies to IL-2 Rβ, blocks NF-κB activation in ATL cancer cells and inhibits proliferation.[50] However, the observation that PS-341 inhibited growth of tumors to varying degrees demonstrates that inhibition of NF-κB per se is not always sufficient to induce apoptosis. On the other hand, it is possible that NF-κB inhibition lowers the threshold for apoptosis induced by other stimuli, including conventional cancer chemotherapeutic agents. Although we did not see dramatic effects of PS-341 on transgenic Tax tumors, the data on transplanted Tax tumors indicates that PS-341 is an active drug alone or in combination with other therapeutic interventions in this murine model of large granular cell lymphoma. The disparity in responses of the Tax transgenic tumors may be due to the fact that multiple cellular pathways are targeted by Tax leading to immortalization, and blockade of NF-κB does not prevent tumor incidence or progression completely. Also, the dosage tolerated by these mice without obvious toxicity may result in insufficient inhibitor activity to block NF-κB activation. This is clear from the lack of inhibition of NF-κB activity in vivo during treatment, although the tumors display a decrease in NF-κB in response to PS-341 or sodium salicylate in vitro. Also, there are several documented pathways for IκB-independent NF-κB activation, which may also be prevalent the Tax tumors.[58] Thus, it is possible that in the transplanted tumors PS-341 may be exerting its tumor inhibitory effects by one or more mechanisms, including effects on angiogenesis, cell-cycle arrest, and apoptosis in an NF-κB–independent manner. The drug was able to inhibit the growth of tumors derived from the Tax cell line by increased apoptosis as has been observed in other tumor systems.[59,60] This study has paved the way for the trials of more specific NF-κB inhibitors in isolation or combinations with other inhibitors of Tax function in our transgenic mice and subsequently in humans affected by HTLV. These results provide a valuable lesson about the basic biology of Tax and HTLV as a slow but potent pathogen, which will be useful for designing better treatment options as models for ATL and other malignancies.

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