Proteasome Inhibitor PS-341, a Potential Therapeutic Agent for Adult T-Cell Leukemia

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ABSTRACT

Nuclear factor κB (NF-κB) plays a major role in the pathogenesis of human T-cell lymphotropic virus I-associated malignancy. Proteasome inhibitors provide a rational approach to control constitutively activated NF-κB in human T-cell lymphotropic virus I-infected T cells. We report that the proteasome inhibitor PS-341 decreased NF-κB DNA binding activity by preventing degradation of IκBα. In our murine model of adult T-cell leukemia, PS-341 used alone did not yield prolongation of the survival of tumor-bearing mice. However, when combined with the current clinically approved drug humanized anti-Tac, therapy with PS-341 was associated with a complete remission in a proportion of treated animals, whereas only a partial response was observed in animals treated with humanized anti-Tac alone.

INTRODUCTION

The nuclear transcription factor NF-κB plays a central role in oncogenesis induced by HTLV-I, the etiological agent of an aggressive and fatal disease termed ATL (1). The viral transcriptional activator protein Tax plays a crucial role in T-cell immortalization and factor-independent growth by activating the NF-κB pathway through its activation of IκB kinase (2, 3). The inactivation of tumor suppressor p53 in HTLV-I-positive cells is also dependent on activation of the NF-κB pathway (4). The observation that NF-κB, rather than Tax, is indispensable for the maintenance of the malignant phenotype of HTLV-I provides a molecular target for the therapy of ATL (5).

NF-κB is a family of proteins consisting of NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel. Whereas almost all combinations of homo- and heterodimers can exist, the dominant form that is activated in response to extracellular signals is the heterodimer of p65 and p50. In unstimulated cells, NF-κB is held in the cytoplasm by binding to IκBs (IκBα, IκBβ, and IκBε). On stimulation, IκB becomes rapidly phosphorylated and polyubiquitinated and consequently targets itself to the 26S proteasome for degradation, NF-κB is then released and translocates into the nucleus, where it activates transcription of responsive genes (6). In HTLV-I-infected T cells, the constitutive activation of NF-κB is associated with phosphorylation and rapid proteolytic degradation of IκBα (7) and chronic down-regulation and inactivation of IκBβ expression (8, 9) due to the interaction of HTLV-I-encoded Tax protein with IκBα kinase. Therefore, the 26S proteasome inhibitor represents a rational target for the interruption of the NF-κB pathway for ATL therapy.

PS-341, a dipeptidyl boronic acid analogue, is a selective inhibitor of the proteasome with only modest activity toward serine and thiol proteases (10). PS-341 has potent and reversible inhibitory activity against the hydrolysis of polypeptide substrates by the 20S proteasome and hydrolysis of ubiquitinated proteins by the 26S proteasome (11). By stabilizing cytoplasmic IκBα, PS-341 has shown its inhibitory effect on transactivation of NF-κB in vitro and in vivo (12). PS-341 has an inhibitory potency (Ks) of 0.6 nM against the chymotryptic activity of the 20S proteasome with an average inhibition of cell growth (GI50) across the entire NCI cell panel at 7 nM. In the Lewis lung carcinoma mouse model, oral (p.o.) administration of PS-341 (0.1–1 mg/kg) delays tumor growth and the development of metastasis in the lung (13).

In this study, we investigated the efficacy of PS-341 in ATL cells and in a murine model of ATL. We were particularly interested in the combination therapy involving PS-341 and HAT (humanized anti-IL-2Rα antibody, daclizumab, Zenapax®), which is available clinically for the treatment of ATL. The scientific basis for the use of HAT to treat ATL is based on a striking surface feature of HTLV-I-infected leukemic T cells that overexpress IL-2Rα in contrast with the lack of expression by normal resting cells (14). In a model of ATL in NOD/SCID mice, we demonstrated previously that HAT acts via an antibody-dependent cellular cytotoxicity (ADCC) mechanism involving FcγR binding of the monoclonal antibody by monocyte and polymorphonuclear leukocyte effector cells (15). The discovery that a NF-κB response element exists in the IL-2Rα promoter region (16) suggests the possible additive or synergistic efficacy of this combination through an action interfering with the interleukin 2 signaling pathway.

MATERIALS AND METHODS

PS-341 and HAT. PS-341 was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. HAT was acquired from Hoffmann-La Roche Inc. (Nutley, NJ).

Source of ATL Cells. ATL cells (MET-1 cells) were maintained in NOD/SCID mice (Jackson Laboratories, Bar Harbor, ME), which were originally injected with purified ATL cells of a patient with acute ATL (15). The injected mice at the late stage of the disease usually had enlarged spleens, from which MET-1 cells were harvested for in vitro study or further transfer to naive NOD/SCID mice.

Whole Cell and Nuclear Extraction of ATL Cells. MET-1 cells (106 cells/ml) were cultured at 37°C in the presence of 5% CO2 in 10 ml of RPMI 1640 medium (10% fetal bovine serum) with 1 μg PS-341. Whole cell extracts and nuclear extracts were prepared from washed cells by using RIPA solution and nuclear extraction reagents (Pierce, Rockford, IL).

Western Blot Analysis. Whole cell extracts were resolved on 10% bis-Tris SDS-PAGE gels (Invitrogen, San Diego, CA) with standard procedures. Western blotting was performed using anti-IκBα antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ubiquitin antibody (1:1000; Santa Cruz Biotechnology), and anti-vinculin antibody (1:1000; Sigma Chemical Co.) in PBS with 1% Tween 20. Proteins were visualized by the enhanced chemiluminescence reaction with horseradish-peroxidase-conjugated goat antibody against mouse or rabbit IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence films (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and reagents (Pierce).

EMSA and Supershift. Analysis of NF-κB DNA binding activities by EMSA was carried out as described by McKinsey et al. (9). For the supershift assay with specific antibodies against NF-κB, nuclear extracts were preincubated with 1 μg of each antibody for 45 min at 4°C before the addition of the end-labeled double-stranded oligonucleotide probe. The antibodies used were...
anti-p50 antibody, anti-p52 antibody, anti-c-Rel antibody, and anti-p65 antibody (Santa Cruz Biotechnology). DNA-protein complexes were resolved on 6% polyacrylamide DNA retardation gels detected by autoradiography.

**Therapeutic Efficacy of PS-341 in an ATL Murine Model.** MET-1 cells (10⁴) separated from enlarged spleens of leukemic NOD/SCID mice were i.p. injected into each naïve NOD/SCID mouse (body weight, 20–22 g; age, 8 weeks old). The progression of ATL in injected mice was monitored by measuring serum levels of sIL-2Rα using an ELISA (R&D Systems, Minneapolis, MN) 1 week after injection. The mice with sIL-2Rα levels of 1,000–10,000 pg/ml were entered into the study. Groups (8 mice/group) and treatments were as follows: (a) daily i.p. administration of PBS; (b) weekly i.v. administration of 100 μg of HAT for 4 weeks; (c) daily i.p. administration of 0.06 mg/kg PS-341 on days 0–20 and 27–41; and (d) with combination treatment of group 2 and 3. The efficacy of the treatments was monitored by measuring the serum levels of β₂μ and sIL-2Rα by ELISA (R&D Systems).

We demonstrated previously that these assays represent valuable surrogate markers of the efficacy of therapeutic agents in our model (15). Kaplan-Meier plots were used to reflect the cumulative survival of mice in different treatment groups.

**Statistics.** The average levels of β₂μ and sIL-2Rα among individual treatment groups at different time points were compared using Student’s t test. The statistical differences among these treatment groups with regard to survival time were calculated with the PHREG procedure using SAS program (SAS Institute Inc., Cary, NC), which employs a discrete logistic model. P < 0.05 was considered to be statistically significant.

**RESULTS**

**PS-341 Blocked the Degradation of IκBα in the Cytoplasm and Weakened NF-κB DNA Binding Activity in the Nucleus.** Using a thymidine incorporation assay, we found that PS-341 inhibited the growth of the Jurkat and C81-66-45 cell lines (data not shown). A luciferase reporter plasmid with the κB enhancer region was transfected into Jurkat cells. Results showed that after an 18-h incubation with 10 nm PS-341, the luciferase activity was abolished (data not shown). Similar findings were reported recently in other cell culture systems (11). Transactivation of NF-κB was one but not the only target of PS-341 to arrest cell growth.

The targeting of IκBα, the cytoplasmic inhibitory subunit complexed to NF-κB, for degradation by the proteasome is known to occur in response to a variety of stress activators (6). In this study, we attempted to investigate whether PS-341 stabilized IκBα by inhibiting the 26S proteasome, thereby decreasing the translocation of NF-κB into the nucleus. Western blot analysis revealed that when MET-1 cells were treated with 1 μM PS-341, levels of IκBα remained unchanged. Meanwhile, the slower-migrating form of phosphorylated IκBα accumulated gradually. Incubation with PS-341 resulted in increased levels of the ubiquitinated proteins in whole cells, suggesting the impaired processing activity of the proteasome (Fig. 1).

To examine the effect of PS-341 on NF-κB DNA binding, EMSA was performed. Supershift results showed that the predominant NF-κB protein species consisted of p50/p50 homodimer and p65/p50 heterodimer. Incubation of the nuclear extracts with an anti-p50 antibody led to an almost complete shift of the upper and lower migrating bands. Incubation with anti-p65 antibody reduced the upper migrating band (Fig. 2A). An anti-c-Rel antibody and an anti-p52 antibody failed to shift the mobility of any band (data not shown). The incubation of 1 μM PS-341 with MET-1 cells resulted in a weakening of NF-κB DNA binding activity (Fig. 2B).

**PS-341 Enhanced Anti-ATL Efficacy of HAT.** Based on our preliminary pharmacokinetic study, i.p. administered PS-341 was equally as bioavailable as i.v. administered PS-341 and manifested a prolonged half-life (data not shown); PS-341 was therefore i.p. administered to ATL-bearing NOD/SCID mice in our study. Due to apparent toxicity, e.g., weight loss, PS-341 (0.06 mg/kg, i.p.) was given daily for 3 weeks. There was a 4–7% loss of body weight in all mice of both the PS-341 alone group and the combination group, whereas <2% change of body weight was seen in mice of both the PBS control group and the HAT alone group. After a 1-week recovery period, mice in the PS-341-treated groups regained body weight, and the dosing regimen was then resumed for another 2 weeks. The efficacy of the treatment was reflected in a decrease of the surrogate marker sIL-2Rα and/or β₂μ levels in the serum (Fig. 3, A and B).

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**Fig. 1.** Effect of PS-341 on levels of IκBα, phosphorylated IκBα, and ubiquitinated proteins in MET-1 cells. MET-1 cells were incubated in the absence or presence of 1 μM PS-341 for the time periods indicated above each lane. Proteins from whole cell extracts were resolved by SDS-PAGE and immunoblotted with antibody against either IκBα, ubiquitin, or vinculin, as described in “Materials and Methods.” With PS-341 treatment, the phosphorylated form of IκBα, the slower-migrating form of IκBα, accumulated markedly over the 4-h incubation period. The inhibition of 26S proteasome activity with PS-341 treatment was reflected in increased levels of ubiquitinated proteins. All samples had comparable levels of vinculin.

**Fig. 2.** Effect of PS-341 on NF-κB DNA binding activity in MET-1 cells. A, in a supershift assay, nuclear extracts from MET-1 cells were preincubated with NF-κB subunit-specific antibody as indicated above each lane before the addition of a ³²P-radiolabeled probe. Results showed that the components of NF-κB protein in MET-1 cells consisted of p50/p50 homodimer and p65/p50 heterodimer. B, after treatment for the indicated time periods with 1 μM PS-341, MET-1 cells were lysed, and nuclear extracts were analyzed by EMSA as described in “Materials and Methods.” Compared with the control samples, the samples with PS-341 treatment showed weakened NF-κB DNA binding activity, although they all had similar Oct-1 DNA binding activity.
were disease free (negative sIL-2Rα) for up to 140 days after initial infection with ATL, and two of these animals lived as long as 186 and 236 days after the initiation of the experiment. In contrast, in the HAT alone treatment group, only three of eight mice were still alive at day 126, and they manifested sIL-2Rα levels ranging from 8,000–500,000 pg/ml. These three mice died by day 138. These results indicate that although HAT was effective in treating ATL, it could only result in partial remissions of the disease. Although PS-341 by itself was not able to control the progression of ATL, the combination of PS-341 and HAT offered an effective therapeutic regimen for ATL, as defined by yielding a complete response in a proportion of treated animals.

**DISCUSSION**

The aim of this study was to explore the potential use of PS-341, a synthetic dipeptidyl boronic acid, in the treatment of ATL. Due to its potent and reversible inhibition of the 26S proteasome, we reasoned that PS-341 would add therapeutic value to the treatment of ATL because NF-κB is constitutively active in the ATL cells (17). Recent studies have provided some insights into the multiple aspects of NF-κB involvement in oncogenesis, including the control of apoptosis, the cell cycle, cellular differentiation, and cell migration (6). By targeting the nuclear translocation and activation of NF-κB, proteasome inhibitors have the potential to affect multiple cascades of events, thereby yielding anticancer activities (18). On the other hand, because ubiquitin-proteasome proteolysis is the principle pathway for intracellular turnover of numerous key regulatory proteins in all eukaryotic cells (19), the inhibition of this ubiquitous pathway may be inevitably accompanied by severe side effects. Moreover, it may not be feasible to block the NF-κB pathway for extended periods because NF-κB has an indispensable role in the maintenance of proper host defense responses. Indeed, in our NOD/SCID mouse model, i.p. administration of PS-341 at 0.1 mg/kg twice a day was very toxic to the mice and caused a 100% death rate within a 2-week period following 6 days of treatment. The treatment dose of PS-341 at 0.06 mg/kg (i.p., daily for 5 weeks) used in the present study was tolerated, although there was still noticeable toxicity. Nevertheless, PS-341 is tolerable at a dose up to 1 mg/kg when the drug is p.o. or i.p. administered to Balb/C mice twice a day for 2–3 weeks (13). It can be p.o. administered to Lewis rats at 0.3 mg/kg daily for 3–4 weeks with acceptable toxicity (12).

During days 14–35, the group receiving PS-341 had lower sIL-2Rα and β2μ levels when compared with the PBS control group (P < 0.05). However, by day 42, the β2μ levels were no longer lower in the PS-341 alone treatment group than in the PBS control group. The insignificant efficacy of PS-341 when used alone, which was possibly due to the limited dosing schedule, was also reflected in Kaplan-Meier survival plots (Fig. 4) when compared with the control group (P = 0.5). During the entire course of the study, both the HAT alone treatment group and the combination treatment group had significantly lower sIL-2Rα and β2μ levels than the PBS control group and the PS-341 alone treatment group (P < 0.05). The superior efficacy of these treatment groups was reflected in prolonged animal life spans (P = 0.05).

In addition, we addressed the issue of whether PS-341 enhanced the efficacy of HAT. After day 35, the levels of sIL-2Rα and β2μ in the combination treatment group were significantly lower than those of the HAT alone treatment group (P < 0.05). Although there was no statistical difference with regard to survival between these two groups (P = 0.6), three of eight mice in the combination treatment group
The failure of PS-341 alone to prolong survival of the tumor-bearing mice at the dosing regimen adopted in the present study may reflect the short duration of therapy that could be used. Nevertheless, PS-341 enhanced the anti-ATL activity of simultaneously administered HAT monoclonal antibody. PS-341 is currently undergoing several Phase I/II human clinical trials to evaluate its anticancer activity in patients with advanced cancer. The results of this study support a trial of this drug in ATL patients, preferably as an agent combined with HAT therapy.

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