A Novel Bioluminescent Mouse Model and Effective Therapy for Adult T-Cell Leukemia/Lymphoma

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Abstract

Adult T-cell lymphomaleukemia (ATLL) is caused by human T-cell lymphotropic virus type 1 (HTLV-1). Approximately 80% of ATLL patients develop humoral hypercalcemia of malignancy (HHM), a life-threatening complication leading to a poor prognosis. Parathyroid hormone–related protein (PTHrP) and macrophage inflammatory protein-1α (MIP-1α) are important factors in the pathogenesis of HHM in ATLL, and the expression of PTHrP can be activated by nuclear factor κB (NF-κB). NF-κB is constitutively activated in ATLL cells and is essential for leukemogenesis including transformation of lymphocytes infected by HTLV-1. Our goal was to evaluate the effects of NF-κB disruption by a proteasomal inhibitor (PS-341) and osteoclastic inhibition by zoledronic acid (Zol) on the development of ATLL and HHM using a novel bioluminescent mouse model. We found that PS-341 decreased cell viability, increased apoptosis, and down-regulated PTHrP expression in ATLL cells in vitro. To investigate the in vivo efficacy, nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were xenografted with ATLL cells and treated with vehicle control, PS-341, Zol, or a combination of PS-341 and Zol. Bioluminescent imaging and tumor cell count showed a significant reduction in tumor burden in mice from all treatment groups. All treatments also significantly reduced the plasma calcium concentrations. Zol treatment increased trabecular bone volume and decreased osteoclast parameters. PS-341 reduced PTHrP and MIP-1α expression in tumor cells in vivo. Our results indicate that both PS-341 and Zol are effective treatments for ATLL and HHM, which are refractory to conventional therapy.

Introduction

Human T lymphotropic virus type 1 (HTLV-1) is the etiologic agent for adult T-cell lymphoma/leukemia (ATLL; ref. 1). Eighty percent of ATLL patients develop humoral hypercalcemia of malignancy (HHM), a severe complication resulting from increased osteoclastic bone resorption and renal calcium reabsorption (2). HHM in ATLL patients leads to a mean survival of <1 year (3); therefore, it is urgent to develop new therapies to sufficiently reduce HHM and eliminate tumor progression in ATLL.

Nuclear factor κB (NF-κB) ligand is constitutively activated in HTLV-1–infected cells, indicating the importance of NF-κB in ATLL tumorigenesis (4). In addition, NF-κB binding sites were found in the promoters of parathyroid hormone–related protein (PTHrP; refs. 5, 6) and macrophage inflammatory protein 1α (MIP-1α; ref. 7). The levels of these two proteins are increased in the plasma of ATLL patients with HHM (8, 9). PTHrP expressed in osteoblasts and cancer cells enhances the expression of the receptor activator of NF-κB ligand, an essential factor that activates osteoclastogenesis and regulates bone resorption. In the kidney, PTHrP decreases calcium and increases cyclic AMP phosphorus excretion (10). Therefore, PTHrP plays a major role in the pathogenesis of HHM (10); however, recent studies have suggested that cytokines and chemokines, such as MIP-1α, a chemoattractant and activator of monocytes during inflammation, can induce osteoclastic bone resorption and serve as a potential survival and osteoclast stimulatory factor in cancer (11). Increased concentrations of plasma PTHrP and MIP-1α are reliable clinical biomarkers of hypercalcemia in ATLL and represent potential therapeutic targets (2, 9).

Proteasome inhibitors such as PS-341 inhibit the degradation of IκB family members, resulting in a suppression of NF-κB activity (12). PS-341 has been used as a chemotherapeutic agent for relapsed multiple myeloma patients (13). In ATLL, PS-341 stabilized IκBα, IκBβ, IκBε, p21, p27, and p53 proteins and induced apoptosis and cell cycle arrest in HTLV-1–positive cells in vitro (14), but the in vivo efficacy of PS-341 is still controversial (14, 15).

Bisphosphonates are potent inhibitors of bone resorption and often used for the treatments of osteoporosis, Paget’s disease, hyperparathyroidism, and tumor-induced osteolysis (16). Bisphosphonates inhibit the mevalonate pathway leading to disruption of the Ras signaling pathway. In bone, inhibition of prenylation and Ras signaling within osteoclasts inhibits intracellular vesicle transport, which is required for osteoclasts to form ruffled borders and induce osteoclastic bone resorption. In vitro, high concentrations of bisphosphonates induce S-phase cell cycle arrest and caspase-dependent apoptosis (17, 18). Zoledronic acid (Zol), a third-generation bisphosphonate, was shown to not only prevent osteolytic bone destruction but also decrease tumor burden in Tax transgenic mice (19).

Human xenograft models have been used to investigate new therapeutic agents for ATLL (20). NOD/SCID mice can be successfully engrafted with RV-ATL cells, a Tax-negative ATLL cell line derived from an ATLL patient (21). These mice develop mesenteric lymphoma and HHM 5 weeks after inoculation (22). We successfully infected RV-ATL cells with a lentivirus containing the luciferase gene and developed a bioluminescent mouse model for ATLL to noninvasively monitor tumor cell engraftment and progression and to measure the effects of treatments on tumor burden. Due to the significance of NF-κB activity in ATLL tumorigenesis and its potential role in the induction of HHM by...
increasing PTHrP and MIP-1α expression, our goal was to investigate the in vivo effect of PS-341 accompanied with the osteoclastic inhibitor Zol on tumor burden and HHM in a novel bioluminescent mouse model of ATLL. We found that the combination of PS-341 and Zol may be an effective treatment for ATLL.

Materials and Methods

Cells and drugs. RV-ATL cells derived from an ATLL patient were provided by Dr. Feuer (Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, NY; ref. 21). HTLV-1–transformed cell lines (MT2 and SLB-1), HTLV-1–negative T cells (Jurkat), and RV-ATL cells were cultured as previously described (6). PS-341 was obtained from Millennium Pharmaceuticals through the NIH. Zol was purchased from Novartis.

Transduction of RV-ATL cells with luciferase gene. RV-ATL-luc cells expressing luciferase were generated using a lentiviral vector as previously described (23). Following transduction, the cells were incubated at 37°C for 1 h and washed twice with RPMI 1640 before i.p. injections in NOD/SCID mice.

Animals and treatments. Five-week-old male NOD/SCID (NOD CB17-PRKDC-SCID/J) mice (The Jackson Laboratory) were housed and treated in accordance with the University Laboratory Animal Resources guidelines and experimental protocols were approved by the Institutional Laboratory Animal Care and Use Committee. A total of 4 × 10⁶ RV-ATL-luc cells were injected i.p. 7 days before the initiation of treatments and mice were randomly assigned into the vehicle control group or treatment groups, which received PS-341 (0.4 mg/kg twice per week, i.p.), Zol (0.1 mg/kg twice per week, s.c.), or a combination of the two drugs for 4 weeks. Tumor cells were recovered from the mice by abdominal lavage at the end of the experiment.

Cell viability and apoptosis assays. Cell viability was measured with the CellTiter 96 nonradioactive cell proliferation assay kit (Promega Corp.) and trypan blue dye exclusion assay. Cell apoptosis assay was measured with in situ cell death detection kit (Roche).

Western blotting and real-time reverse transcription-PCR. Western blotting was done using standard protocols and antibodies against IκBα (Santa Cruz Biotechnology, Inc.), phospho-IκBα (Cell Signaling Technology, Inc.), and actin (Sigma-Aldrich). Real-time reverse transcription-PCR (RT-PCR) was done as previously described (5, 24) with specific oligonucleotide primers for PTHrP (5‘-GTCCTACGCCCCCTCACA-3‘ and 5‘-GAAGAGATCTGTTGGCTAAGA-3‘; ref. 24), PTHrP P1/P2 transcript (5‘-GAAGGCAGCAGCCACCAAG-3‘ and 5‘-TGAGACCTCTCCACCAAGGC-3‘; ref. 24), MIP-1α (5‘-CTGCATCTGTGCTGTGCTCTCAAAG-3‘; ref. 25), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5‘-TGACACCAATACACTGTAGG-3‘ and 5‘-GAAGCAGGAGATGATGTCT-3‘).

Bioluminescent imaging. Bioluminescent imaging was done with the in vivo imaging system (IVIS, Xenogen Corp.) as previously described (23). Photon signals were quantified with LivingImage software version 2.2 (Xenogen).

Measurement of plasma calcium and MIP-1α concentrations. Total calcium concentration in plasma from each mouse was measured with the Quantichrom Calcium Assay kit (Bio/Assay Systems). Human MIP-1α levels in plasma were measured with Human CCL3/MIP-1α Quantikine ELISA kit (R&D systems) on pooled plasma from each group. Six non–tumor-bearing age- and sex-matched mice were used as controls.

Histopathology, immunohistochemistry, enzymatic histochemistry analyses, and histomorphometry. On sacrifice, a complete necropsy was done on each animal. Tibias were removed, defleshed, fixed in 10% neutral-buffered formalin, decalcified, and stained with HE for histopathologic evaluation. Enzymatic histochemistry for tartrate-resistant acid phosphatase (Sigma-Aldrich) was done as previously described (22). Bone histomorphometry was done with the Image-Pro Plus, version 5.0 software (Media Cybernetics). Total bone volume, trabecular bone volume, trabecular bone perimeter, and osteoclast perimeter and numbers were measured.

Figure 1. PS-341 and Zol reduced RV-ATL cell viability and induced apoptosis in vitro. A and B, RV-ATL cells were treated with indicated amounts of PS-341 for 48 h. MTT assay (A) and trypan blue exclusion assay (B) were done in triplicate. *, P < 0.05, compared with vehicle (ANOVA and Holm’s method to adjust for multiplicity). C, RV-ATL cells were treated with indicated amounts of Zol for 48 h. MTT assay was done in four replicates. *, P < 0.0001, compared with vehicle (ANOVA and Dunnet’s method to adjust for multiplicity). D, RV-ATL cells were treated with indicated amounts of Zol and PS-341 for 48 h. Numbers indicate the TUNEL-positive cells measured by flow cytometry. Gating was based on the positive and negative controls. Ten thousand cells were acquired for analysis. All experiments were done in triplicate. *, P < 0.05, compared with vehicle (ANOVA and Dunnet’s method to adjust for multiplicity).
**Statistical analysis.** The main outcome variables in this study were cell counts, apoptosis, percentage of viable cells, total calcium concentration, PTHrP and MIP-1α expression, total bone volume, trabecular bone volume, osteoclast perimeter, and trabecular bone perimeter. These are all continuous variables. These variables were log transformed to carry out the statistical analyses because the distributions in the original scale were skewed. For all the variables that data were collected at a single time point, ANOVA and nonparametric tests (Kruskal-Wallis) were used for the analyses. Pairwise comparisons were tested by Tukey’s, Holm’s, or Dunnett’s methods to adjust for multiplicity. P values are reported in the caption of the corresponding figures. Error bars indicate SDs unless otherwise mentioned.

**Results**

PS-341 and Zol significantly reduced cell viability and induced apoptosis in ATLL cells. To examine the efficacy of PS-341 and Zol in vitro, RV-ATL cells were treated with PS-341 and Zol for 48 h and cell viability was measured with 3-(4,5-Dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue exclusion assays. PS-341 induced a dose-dependent inhibition of cell viability by MTT assay (Fig. 1A) and trypan blue exclusion assay (Fig. 1B). Similar results were found in other HTLV-1-positive (MT-2 and SLB-1) or HTLV-1-negative (Jurkat) cell lines treated with PS-341 (data not shown). There was also a decrease of viability in cells treated with Zol at 50 to 200 μmol/L for 48 h (Fig. 1C) by MTT assay. To examine the effects on apoptosis, terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays were done on RV-ATL cells treated with PS-341 and/or Zol for 48 h. PS-341 (20 nmol/L) significantly induced apoptosis in 20.4% of the cells when compared with vehicle control (10.4%), whereas Zol induced apoptosis in 22.2% of the treated cells. When RV-ATL cells were treated with both PS-341 and Zol, no further increase in apoptotic cells was observed (25%; P < 0.0001). These data showed the apoptotic potential of PS-341 and Zol and showed that both drugs induced apoptosis similarly in RV-ATL cells.

PS-341 inhibited PTHrP expression and induced the accumulation of phosphorylated IκBα. The PTHrP P2 promoter was shown to be activated by NF-κB (5, 6), PS-341, a proteasome inhibitor, inhibits the degradation of IκBα, a NF-κB inhibitor, in multiple T-cell lines (14). To determine whether PS-341 had a similar mechanism of action in RV-ATL cells, we treated RV-ATL cells with PS-341 and measured the expression level of PTHrP transcripts specifically transcribed from the P2 promoter by real-time RT-PCR. As shown in Fig. 2, 20 nmol/L PS-341 downregulated PTHrP gene expression by 49% compared with vehicle controls. PS-341 did not reduce MIP-1α significantly in vitro (data not shown). To determine whether PS-341 was able to inhibit NF-κB activity by inducing IκBα accumulation in RV-ATL cells, whole-cell lysates were extracted from RV-ATL cells treated with

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**Figure 2.** PS-341 inhibited the expression of PTHrP and induced the accumulation of phosphorylated IκBα in RV-ATL cells in vitro. A, RV-ATL cells were treated with PS-341 at 20 nmol/L for 48 h. Total RNA was extracted and real-time RT-PCR was done in triplicate with the TaqMan gene expression assay kit. The expression levels were normalized to an internal control and compared with the vehicle using the ΔΔCt method. *, P < 0.05, compared with vehicle (rank-sum test). B, RV-ATL cells were treated with 20 or 100 nmol/L PS-341 for the indicated times, followed by protein extraction. Whole-cell extracts of treated cells were immunoblotted with specific antibodies against phosphorylated IκBα (p-IκBα), total IκBα (t-IκBα), and actin. Lane 1, untreated controls; lanes 2 to 5, RV-ATL cells treated with vehicle; lanes 6 to 9, RV-ATL cells treated with 20 nmol/L PS-341; lanes 10 to 13, RV-ATL cells treated with 100 nmol/L PS-341. C, quantitative analysis of Western blots by densitometry. The levels of phosphorylated IκBα were normalized to total IκBα (top) or actin (bottom). RV-ATL cells were treated with vehicle and 20 or 100 nmol/L PS-341 for indicated times. Results were generated from three independent experiments. Bars, SE. *, P < 0.05, compared with vehicle (adjusted ANOVA models); **, P < 0.05, compared with vehicle (t test). P = 0.16 and P = 0.11 for the 90 and 120 min, respectively, after adjusting for multiple comparisons by ANOVA and Dunnett’s method.

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20 or 100 nmol/L of PS-341 for 30 to 120 min and Western blotting was done with antibodies specific for phosphorylated, total IκBα, and actin. As shown in Fig. 2B and C, PS-341 treatment resulted in an accumulation of phosphorylated IκBα in a time-dependent manner. The accumulation of phosphorylated IκBα has been correlated with the inhibition of NF-κB activity in ED(−) cells, another Tax-negative ATLL cell line (26), suggesting that PS-341 may have a similar mechanism of action to inhibit NF-κB activity in RV-ATL cells.

**PS-341 and Zol decreased tumor burden.** To monitor in vivo tumor growth noninvasively, we injected NOD/SCID mice with RV-ATL-luc cells generated by transducing RV-ATL cells with lentiviral vectors encoding luciferase/yellow fluorescent protein. Signals from bioluminescent imaging successfully represented the progression and location of tumor growth and tumor burden over time (Fig. 3). Mice bearing RV-ATL-luc cells developed HHM and lymphoma predominantly in the mesenteric lymph nodes 5 weeks after inoculation. To determine the in vivo efficacy of PS-341 and Zol, mice were treated with PS-341 alone, Zol alone, or a combination of the two drugs beginning 1 week after inoculation. Bioluminescent imaging was done 21 and 28 days after treatments began. Signals from bioluminescent imaging at day 28 were significantly lower in the mice treated with PS-341, Zol, or the combination compared with the vehicle group (Fig. 3B and C; \( P < 0.0001, P = 0.002, \) and \( P < 0.0001, \) respectively). Signals from day 21 showed similar results (data not shown). However, a wide range of tumor cell counts extracted from the abdominal cavity using abdominal lavage after sacrifice. \( *, P < 0.0001; **, P = 0.009, \) compared with vehicle (one-way ANOVA and Tukey’s method to adjust for multiplicity).

![Figure 3](https://cancerres.aacrjournals.org/content/67/24/11862/F3.large.jpg)

**Figure 3.** PS-341 and Zol decreased tumor burden in vivo. A, serial bioluminescent imaging of mice injected i.p. with \( 4 \times 10^7 \) RV-ATL-luc cells. The highest level of photon emission corresponded to the orange/red end of the color spectrum. B, representative bioluminescent imaging from mice treated with vehicle, PS-341 (0.4 mg/kg i.p.), Zol (0.1 mg/kg, s.c.), or a combination of both. Bioluminescent imaging was done 28 d after treatments were begun. C, quantitative analysis of bioluminescent imaging at day 28. \( *, P < 0.0001; **, P = 0.002, \) compared with vehicle (one-way ANOVA and Tukey’s method to adjust for multiplicity). D, cell counts of tumor cells extracted from the abdominal cavity using abdominal lavage after sacrifice. \( *, P < 0.0001; **, P = 0.009, \) compared with vehicle (one-way ANOVA and Tukey’s method to adjust for multiplicity).
of signal intensity was observed in mice treated with Zol alone (Fig. 3C). To confirm the measurement of tumor burden by bioluminescent imaging, cells were extracted by abdominal lavage and counted immediately after sacrifice (Fig. 3D). Similar to the bioluminescent imaging results, there were significant differences in cell numbers in mice treated with PS-341 alone, Zol alone, or the combination compared with vehicle controls (*P* < 0.0001, **P** = 0.009, and ***P*** < 0.0001, respectively). A wide range of cell numbers was again observed in Zol alone group. Pearson correlation coefficient analyses identified a strong correlation between bioluminescent imaging signal intensities and cell numbers (Supplementary Table S1; *r* = 0.921, *P* < 0.0001), indicating that tumor burden can be well represented by bioluminescent imaging. These data showed that both PS-341 and Zol were able to reduce tumor burden in mice bearing RV-ATL-luc cells.

**PS-341 and Zol decreased HHM.** To examine the efficacy of PS-341 and Zol on HHM, we measured total plasma calcium concentrations in the mice. Six non–tumor-bearing age- and sex-matched mice were used as controls. Tumor-bearing mice treated with vehicle had significantly higher plasma calcium concentrations (mean, 13.0 mg/dL; *P* = 0.0002) compared with the control mice (mean, 10.6 mg/dL). Significant differences were found in mice treated with PS-341 alone, Zol alone, or the combination compared with vehicle controls (*P* < 0.0001, *P* = 0.009, and *P* < 0.0001, respectively). A wide range of cell numbers was again observed in Zol alone group. Pearson correlation coefficient analyses identified a strong correlation between bioluminescent imaging signal intensities and cell numbers (Supplementary Table S1; *r* = 0.921, *P* < 0.0001), indicating that tumor burden can be well represented by bioluminescent imaging. These data showed that both PS-341 and Zol were able to reduce tumor burden in mice bearing RV-ATL-luc cells.

**Zol decreased osteoclastic bone resorption in mice with RV-ATL-luc.** To examine the effect of the treatments on osteoclastic bone resorption in vivo, bone histopathology and histomorphometry were done. Increased trabecular bone volume was present in tibias from mice treated with Zol alone compared with vehicle (Fig. 5A). Mice treated with a combination of the two drugs had a similar phenotype (results not shown). Bone histomorphometry revealed an ~2.5-fold increase in trabecular bone volume/total bone volume in Zol-treated and combination-treated mice (Fig. 5B). A decrease in osteoclast perimeter/trabecular bone perimeter was also present in combination-treated mice (Fig. 5C), indicating that Zol inhibited osteoclastic bone resorption in mice bearing RV-ATL-luc cells.

**Figure 4.** PS-341 and Zol decreased plasma total calcium concentrations. Total calcium concentrations in plasma from mice treated with vehicle, PS-341, Zol, or a combination of the two drugs. Six non–tumor-bearing age- and sex-matched mice served as controls. *, *P* = 0.014; **, *P* = 0.004; ***, *P* < 0.0001; ****, *P* = 0.0002, compared with vehicle (one-way ANOVA and Tukey’s method to adjust for multiplicity).

**Figure 5.** Zol inhibited osteoclastic activity in vivo. A, representative H&E-stained sections of tibias taken from mice in the Zol alone or vehicle group. B and C, trabecular bone volume/total bone volume and osteoclast perimeter/trabecular bone perimeter in tumor-bearing mice treated with vehicle, PS-341, Zol, or the combination and in non–tumor-bearing control mice were measured using Image-Pro plus software. *P* = 0.0009 and *P* < 0.0001, respectively (ANOVA). *, *P* < 0.0001; **, *P* = 0.015, compared with vehicle (one-way ANOVA and Dunnett’s adjustment).
PS-341 decreased PTHrP and MIP-1α in vivo. Expression of PTHrP and MIP-1α in cells from the abdomen of tumor-bearing and non–tumor-bearing mice (controls) was measured by real-time RT-PCR. High levels of PTHrP and MIP-1α expression were found in the cells from vehicle-treated mice (Fig. 6A and B). PTHrP and MIP-1α expression was decreased in mice treated with PS-341 alone and the drug combination but not in mice treated with Zol alone. These data correlated with the circulating plasma MIP-1α concentrations in the mice. The MIP-1α concentration in the vehicle-treated mice was very high (2,000 pg/mL) compared with the PS-341–treated mice (<10 pg/mL), the combination group (<10 pg/mL), or the control group (non–tumor-bearing mice; <10 pg/mL). Zol did not significantly decrease the plasma MIP-1α concentration (1,870 pg/mL).

Discussion

Successful treatments for ATLL remain elusive despite medical advances in the development of chemotherapeutic regimens and targeted chemotherapy. Cyclophosphamide, hydroxydaunorubicin, Oncovin, and prednisolone; nucleoside analogues; topoisomerase inhibitors; IFN; zidovudine; arsenic trioxide; and monoclonal antibodies have been used to treat ATLL patients, but the prognosis is still poor. The first potential “cure” of ATLL was associated with bone marrow transplantation, but patients suffered from opportunistic infections (3). ATLL patients also develop severe HHM, causing life-threatening hypercalcemia. Therefore, it is important to investigate new treatments for ATLL, especially in patients that have developed or have the potential to develop HHM. We provide new data supporting the in vivo effects of a proteasome inhibitor (PS-341) and an osteoclastic inhibitor (Zol) on tumor burden and HHM using a bioluminescent mouse model of human ATLL.

One of the major challenges of preclinical studies is the development of relevant animal models. Xenograft mouse models have proved to be useful in examining new treatment strategies for ATLL. HHM occurs in 80% of the ATLL patients but is not recapitated in most of the ATLL animal models. In the RV-ATL transplantation model, an immunodeficient mouse strain (NOD/SCID) that lacks functional T cells and B cells was used for inoculation. Mice bearing RV-ATL cells not only developed ascites and lymphoma in mesenteric lymph nodes but, importantly, they developed HHM 5 weeks after inoculation of tumor cells. Tumor cells obtained by peritoneal lavage were used for passaging RV-ATL cells in vivo due to the fact that the cells have a low level of viability and proliferation in vitro. Because lymphoma/leukemia is not a readily accessible cancer and it is not easy to measure tumor burden noninvasively in vivo, the RV-ATL cells were successfully transduced with a lentivirus containing the luciferase gene. Tumor progression and burden were measured by bioluminescent imaging; therefore, we are able to detect and monitor lymphoma cell growth in abdominal organs before the presentation of clinical manifestations. This represents the first bioluminescent animal model of human ATLL that induces HHM and can be used for preclinical studies.

Mice bearing RV-ATL-luc cells developed hypercalcemia, and bone histomorphometric analyses revealed increased osteoclast numbers in vehicle-treated tumor-bearing mice compared with control mice. No significant differences in the trabecular bone volume occurred between control and tumor-bearing mice. Hypercalcemia observed in this model was likely multifactorial and contributed by all the target organs of calcium regulation, including bone, kidney, and the intestines. Osteolytic lesions that occur in human patients are typically the result of disease progression for months or years. To evaluate the efficacy of therapeutic agents, 40 million cells were injected i.p. in the mice. At the end of week 5, most tumor-bearing mice without treatment met the guidelines for euthanasia. Therefore, we believe that significant bone loss would have occurred if the mice were kept for longer periods of time. In addition, Dr. Uchiyama’s lab reported a xenograft model of ATLL cells with hypercalcemia and found decreased bone formation rates in the absence of significant differences in bone resorption. This suggested that hypercalcemia in these mice was likely due to other target organs of calcium regulation in addition to bone (27).

The complete mechanism of HHM development in ATLL is not clear; however, in many types of cancer, PTHrP plays a central role by stimulating osteoclasts and increasing bone resorption. It has also been shown that PTHrP enhances cell growth (28) and protects against apoptosis (29). We observed a dramatic increase in

Figure 6. PS-341 reduced PTHrP and MIP-1α expression in vivo. mRNA was extracted from the cells obtained from abdominal lavage of tumor-bearing mice treated with vehicle, PS-341, Zol, or the combination and from non–tumor-bearing control mice. Real-time RT-PCR was done with primers specific for human PTHrP (A) or MIP-1α (B). For MIP-1α expression levels, mRNA from MT-2 cells was used as a calibrator. GADPH was used as an internal control. *, P = 0.0002; **, P = 0.0009; ***, P < 0.0001, compared with vehicle (one-way ANOVA and Dunnett’s adjustment).
and the direct effect of PS-341 on osteoclasts and the indirect osteoclast numbers and inhibited bone resorption in mouse models of multiple myeloma (34–36), the effects were transient and the direct effect of PS-341 on osteoclasts and the indirect effects via inhibition of myeloma cells were not examined. In our model, we speculate that PS-341 mediated disruption of tumor cells and a reduction of tumor-related humoral factors, which impaired osteoclast function (shown by a decrease in hypercalcemia). PS-341, however, was not able to decrease the osteoclast numbers that were previously induced by humoral factors secreted from the tumor cells. In contrast to Zol, little is known about the distribution or action of PS-341 in bone, which may have direct or indirect effects on osteoclasts. In addition, PS-341 is a nonspecific 26S proteasome inhibitor that affects multiple signaling pathways. Its potential effects on calcium homeostasis due to regulation by the kidney or intestines are unknown. Therefore, PS-341 may have decreased hypercalcemia in this model through effects on the kidney or intestines. In bone, it may have inhibited osteoclast function or reduced osteoclast apoptosis, although it did not change osteoclast numbers. Additional studies investigating the effects of PS-341 on the kidney, intestines, and bone are required.

In addition to the well-known effect of Zol on inhibition of osteoclastic bone resorption, its effect on cell viability and apoptosis in cancer cells has been intensively studied. Direct cytotoxicity of Zol has been shown in breast cancer, prostate cancer, and myeloma cells (18, 37). Clinical trials for investigating the effects of Zol on prevention and treatment of bone metastases are ongoing. Importantly, we found that Zol induced ATLL cell apoptosis in vitro and partially decreased tumor burden in vivo, in addition to its inhibition of osteoclastic activity and HHM. There was a wide variability in the tumor burden in Zol-treated mice in our investigation and the reasons remain unknown; however, the bone response to Zol was consistent as expected. The combination of Zol with a chemotherapeutic agent has been used in several clinical trials. It would be important to investigate whether PS-341 and Zol function synergistically in the future.

In conclusion, we developed a bioluminescent mouse model to investigate new therapies for HHM and ATLL. We showed that the combination of PS-341 and Zol was an effective treatment for a mouse model of human ATLL and may prove to be an effective therapy for human patients.

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