A Novel Orally Active Small Molecule Potently Induces G1 Arrest in Primary Myeloma Cells and Prevents Tumor Growth by Specific Inhibition of Cyclin-Dependent Kinase 4/6


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Abstract

Cell cycle deregulation is central to the initiation and fatality of multiple myeloma, the second most common hematopoietic cancer, although impaired apoptosis plays a critical role in the accumulation of myeloma cells in the bone marrow. The mechanism for intermittent, unrestrained proliferation of myeloma cells is unknown, but mutually exclusive activation of cyclin-dependent kinase 4 (Cdk4)-cyclin D1 or Cdk6-cyclin D2 preceeds proliferation of bone marrow myeloma cells in vivo. Here, we show that by specific inhibition of Cdk4/6, the orally active small-molecule PD 0332991 potently induces G1 arrest in primary bone marrow myeloma cells ex vivo and prevents tumor growth in disseminated human myeloma xenografts. PD 0332991 inhibits Cdk4/6 proportional to the cycling status of the cells independent of cellular transformation and acts in concert with the physiologic Cdk4/6 inhibitor p18INK4c. Inhibition of Cdk4/6 by PD 0332991 is not accompanied by induction of apoptosis. However, when used in combination with a second agent, such as dexamethasone, PD 0332991 markedly enhances the killing of myeloma cells by dexamethasone. PD 0332991, therefore, represents the first promising and specific inhibitor for therapeutic targeting of Cdk4/6 in multiple myeloma and possibly other B-cell cancers. (Cancer Res 2006; 66(15): 7661-7)

Introduction

Multiple myeloma, the second most common hematopoietic cancer, represents a clinically defined collection of plasma cell neoplasms in which malignant plasmacytoid cells are arrested at various stages of plasma cell differentiation (1). Unlike normal plasma cells, which are permanently withdrawn from the cell cycle (2, 3), multiple myeloma cells retain their self-renewing potential (4, 5). During the stable phase of the disease, myeloma cells accumulate in the bone marrow mainly due to impaired apoptosis (6). However, among the noncycling cells, there are self-renewing myeloma cells, which reenter the cell cycle and divide without restraint during relapse and drug resistance. As multiple myeloma is invariably fatal, it is imperative to define the mechanism of cell cycle deregulation in multiple myeloma.

Reentry and progression through the G1 phase of the cell cycle is driven by cyclin-dependent kinases (Cdk) in cooperation with cyclins and inhibited by Cdk inhibitors (CKI; ref. 7). Phosphorylation of the retinoblastoma protein p105 (Rb) by Cdk4 or Cdk6 together with cyclin D in early G1 and by Cdk2 in conjunction with cyclin E in late G1 leads to the release of E2F transcription factors and S-phase entry (8). In turn, the Cdk4 and Cdk6 activities are attenuated by CKIs of the INK4 family, whereas that of Cdk2 is inhibited by CKIs of the Cip/Kip family (7). Inhibition of Cdk6 by p18INK4c (p18) of the INK4 family (9, 10) is required for G1 arrest and differentiation of antibody-secreting, end-stage plasma cells from antigen-activated B cells in vivo and in vitro (2, 3). In the absence of p18, plasmacytoid cells expressing CD138 (syndecan-1), a proteoglycan present on both normal and malignant plasma cells (11), are generated, but they continue to cycle and are rapidly eliminated by apoptosis (3). Attenuation of Cdk4/6 by p18, therefore, is critical for normal plasma cell differentiation.

The mechanism that underlies cell cycle deregulation in myeloma is largely unknown. Overexpression of D cyclins has been implicated in promoting myeloma progression based on chromosomal translocation of cyclin D1 and cyclin D3 genes to immunoglobulin loci and elevation of D cyclin RNA in myeloma cells by microarray analysis (12–14). Paradoxically, cyclin D1 overexpression was associated with a more favorable clinical outcome (15–17). Analysis of Cdk4/6-specific Rb phosphorylation as an indicator of progression through early G1 in primary bone marrow myeloma cells in vivo and ex vivo has now shown that overexpression of cyclin D1 alone is insufficient to promote cell cycle progression (18). Instead, aberrant coactivation and pairing of Cdk4-cyclin D1 and Cdk6-cyclin D2 preceeds proliferation of myeloma cells and is enhanced in advanced disease, regardless of the treatment history or the clinical heterogeneity (18). The mutually exclusive pairing of Cdk4-cyclin D1 and Cdk6-cyclin D2 reveals a previously unappreciated specificity and complexity of cell cycle deregulation in myeloma and identifies Cdk4 and Cdk6 as key determinants in the loss of cell cycle control in myeloma.

These findings further suggest that Cdk4/6 may be effective targets for therapeutic intervention. To test this possibility, we inhibited Cdk4 and Cdk6 in primary bone marrow myeloma cells using PD 0332991, an orally active, water-soluble cell-permeable pyridopyrimidine, which potently inhibits recombinant Cdk4 and Cdk6 (IC50 = 0.011-0.016 μmol/L) by competing for the ATP-binding sites (19). Unlike other Cdk4/6 inhibitors that have been
used in clinical trials, such as flavopiridol, R-roscovitine (CYC202 or Seliciclib), UNC-01 (7-hydroxystauroporine), and BMS-387032 (20, 21), PD 0332991 is highly selective for Cdk4 and Cdk6, displaying little or no activity against a panel of 36 additional kinases, in particular Cdk2 (19). Inhibition of Cdk4 by PD 0332991 leads to G1 arrest in nonhematopoietic tumor cell lines in vitro (IC50 = 0.06 μmol/L), growth suppression, and marked regression of several solid tumors in xenografts (19). By contrast, treatment of human myeloma cell lines (HMCL) with flavopiridol or R-roscovitine mainly results in apoptosis without evidence of inhibition of specific Cdns (22, 23).

Here, we show that PD 0332991 potently induces G1 arrest in primary myeloma cells by inhibiting Cdk4/6 according to the cycling status of the cells and nearly completely prevents tumor growth in a disseminated xenograft human myeloma model. Furthermore, PD 0332991 enhances the killing of myeloma cells by dexamethasone. The ability of PD 0332991 to selectively inhibit Cdk4/6 and cell cycle progression of primary bone marrow myeloma cells suggests PD 0332991 as a promising candidate for mechanism-driven therapy for multiple myeloma.

Materials and Methods

Bone marrow myeloma cells and cell lines. Bone marrow specimens were obtained from multiple myeloma patients at the New York Presbyterian Hospital under informed consent as part of an Institutional Review Board–approved study. Multiple myeloma was staged according to the Salmon-Durie classification based on the criteria of monoclonal serum immunoglobulin levels, reciprocal depression of normal immunoglobulin, other peripheral blood studies, renal function, and the number of lytic bone lesions (24). Live, mononuclear cells were isolated from bone marrow aspirates by Ficoll-Hypaque density gradient centrifugation. The CD138+ bone marrow myeloma cells were then enriched from this fraction to ≥95% purity by using an automated MACS CD138 MicroBeads system (Miltenyi Biotechnology, Inc., Auburn, CA), unless otherwise indicated. CAG and MMLS human multiple myeloma cell lines were kindly provided, respectively, by Dr. J. Epstein (University of Arkansas, Little Rock, AK) and Dr. N. Krett (Northwestern University, Chicago, IL). The HS-5 human stromal cell line was obtained from the American Type Culture Collection (Manassas, VA).

Primary CD138+ multiple myeloma cells were cultured at a starting density of 2 × 10⁶/mL in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) and 2 mM L-glutamine. 100 units/ml penicillin/streptomycin, 4 mM/L HEPES, 0.1 mM/L MEM nonessential amino acid solution (all from Invitrogen), 50 μM/L β-mercaptoethanol (Sigma, St. Louis, MO), recombinant human interleukin-6 (IL-6; 40 units/mL), soluble IL-6 receptor (40 units/mL; ref. 2), insulin-like growth factor-1 (IGF-1; 100 ng/mL, R&D Systems, Minneapolis, MN), and soluble BlyS (BASF; ref. 25). Multiple myeloma cell lines were cultured in the same media at an initial density of 3 × 10⁴/mL. In some cultures, PD 0332991 (19) dissolved in dH₂O or dexamethasone (Sigma) dissolved in dimethyl sulfoxide (DMSO, Sigma) was added to the media at concentration and for the time indicated. Cell viability was determined by trypan blue exclusion in triplicate.

In some experiments, CD138+ primary bone marrow myeloma cells were cocultured at a 2:1 ratio with HS-5 stromal cells, prelabeled with the PKH67 green fluorescent cell linker kit (Sigma), and plated overnight in the presence or absence of PD 0332991 for the time indicated. For biochemical analysis, primary bone marrow myeloma cells were removed from stromal cells by gentle pipetting and rinsing with RPMI 1640. The purity of multiple myeloma cells was assessed to be ≥95% by staining with a PE-anti-human CD138 (BioSource, Camarillo, CA) antibody and flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ).

Isolation of resting and activated primary mouse B cells. Splenocytes were isolated from p18+/+ and p18−− mice (7-8 weeks, age-matched, either sex; ref. 26). High-density (resting B) and low-density (activated B and plasma) cells were isolated from splenocytes from the 60% to 70% and 50% to 60% interfaces of a discontinuous Percoll gradient, respectively, as previously described (27). Resting B cells were cultured at 4 × 10⁶/mL in RPMI 1640 containing 10% FBS, as previously described (25) in the presence or absence of F(ab)2 anti-IgM (7A/4 μg/mL; Zymed, South San Francisco, CA) together with recombinant soluble human BlyS (BACE; 50 ng/mL).

Analysis of bromodeoxyuridine uptake, DNA content, and apoptosis. Bromodeoxyuridine (BrdUrd; 5 μg/mL; Sigma) was added to cultures of CD138+ bone marrow myeloma cells and HMCL cells at 3 × 10⁶/mL for the time indicated. BrdUrd uptake was measured by flow cytometry as described (27), using a FITC-anti-BrdUrd (Roche Diagnostics, Pleasanton, CA) or an APC-anti-BrdUrd (Becton Dickinson) monoclonal antibody (mAb). For DNA content analysis, cells were fixed in 70% ethanol at 4°C for 30 minutes and incubated in PBS containing 50 μg/mL propidium iodide (Sigma) and 100 units/mL RNase A (Sigma) for 30 minutes at 37°C in the dark. The DNA content per cell was analyzed by flow cytometry.

Immunoblotting. Preparation of whole-cell lysates and immunoblotting was as previously described (25). The following antibodies were used for immunoblotting: mouse mAbs to human Rb, Cdk6, Cdk4, cyclin D1 (all from Cell Signaling, Beverly, MA), mouse Rb (Becton Dickinson), or actin (Becton Dickinson); rabbit polyclonal antibodies to p53R273 or p53R287 (Cell Signaling), cyclin D2 (Santa Cruz Biotechnology, Santa Cruz, CA), and poly(ADP-ribose) polymerase-1 (PARP; Cell Signaling). Membranes were rinsed in TBS-T [10 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.02% Tween 20], incubated with horseradish peroxidase–linked goat anti-mouse or anti-rabbit (1:5,000) secondary antibodies for 60 minutes, and developed with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL). The signals were determined by densitometry analysis.

Establishment of disseminated myeloma xenografts and therapy. Establishment of the disseminated xenograft nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse multiple myeloma model has been described (28). In brief, 10 × 10⁶ CAG cells stably expressing the HSV-TK-eGFP-luciferase fusion protein (28) were injected i.v. via tail vein to NOD/SCID (NOD/LtSzPrkO/j) mice (The Jackson Laboratory, Bar Harbor, ME) at 8 to 9 weeks of age. The tumor distribution was followed by serial whole-body noninvasive imaging of visible light emitted by luciferase-expressing myeloma cells upon injection of mice with luciferin. Seven days after tumor injection, a group of NOD/SCID mice with established disseminated multiple myeloma were divided into two cohorts, with statistically equivalent tumor burden between cohorts (as evaluated by bioluminescence imaging). PD 0332991 was dissolved in sodium lactate buffer (50 mmol/L, pH 4) and was given daily at 150 mg/kg by gavage. The control mice received the same amount of sodium lactate buffer solution through the same route. The overall survival of mice was defined as the time between injection of tumor cells and death, or sacrifice upon development of hind leg paralysis, and was compared with the treatment group with Kaplan-Meier survival analysis.

Statistical analysis. All analyses were done using the Stata 7.0 statistical software (Stata Corp., College Station, TX), with P < 0.05 considered to be significant. For comparing tumor-associated variables, the nonpaired Student's t test was used unless otherwise specified. Log-rank tests were used to calculate the statistical significance of difference of Kaplan-Meier survival curves.

Luciferase assay. Luciferase activity was measured in HSV-TK-eGFP-luciferase+ CAG cells using the Dual-Glo luciferase assay system according to manufacturer’s instructions (Promega, Madison, WI). Luciferase activity was measured using the LMax (Molecular Devices, Sunnyvale, CA) microplate reader and the Softmax Pro Software (Molecular Devices).

Immunohistochemistry. Immunohistochemistry was done on 4-μm sections of paraffin-embedded vertebrae from the CAG xenograft using a TechMate500 Bi tec automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer’s specification. CD138+ CAG cells were detected using an anti-CD138 mAb (Serotec, Oxford, England) and a red chromogen. The nuclei were visualized by counterstaining with

Cancer Res 2006; 66: (15). August 1, 2006 7662 www.aacrjournals.org
hematoxylin (blue). Simultaneous expression of other proteins was detected with mAbs to Rb (Cell Signaling), polyclonal rabbit antibodies to pSRb\textsuperscript{807/811} of Rb and cleaved caspase-3 (Cell Signaling). Controls include pretreating tissue sections with calycin A (Cell Signaling) to prevent dephosphorylation during or after antigen retrieval or with calf intestine phosphatase (Cell Signaling) to verify phosphorylation. Simultaneous detection of apoptosis of CD138\textsuperscript{+} cells by the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was done using the ApopTag kit (Intergen, Purchase, NY) and a brown chromogen.

Results

PD 0332991 potently inhibits Cdk4/6 and induces G\textsubscript{1} arrest in primary bone marrow myeloma cells. The ability of PD 0332991 to inhibit Cdk4/6 was examined in freshly isolated CD138\textsuperscript{+} (a cell surface marker for normal and malignant plasma cells) bone marrow myeloma cells by detecting Cdk4/6-specific phosphorylation of Rb on Ser\textsuperscript{780} (pSRb\textsuperscript{780}) and Ser\textsuperscript{807/811} (pSRb\textsuperscript{807/811}; refs. 29, 30). Primary bone marrow CD138\textsuperscript{+} myeloma cells retain their cycling capacity ex vivo only within the initial hour of isolation. However, even in the absence of continuous cycling, PD 0332991 inhibited Cdk4/6 phosphorylation regardless of the disease stage or treatment history in all multiple myeloma cases tested (n = 14; Fig. 1A; Supplementary Table S1), because deregulation of Cdk4/6 is a frequent event in multiple myeloma (18). Consistent with previous observations in nonhematopoietic cell lines (19), PD 0332991 inhibits Cdk4/6 within 4 to 6 hours in primary bone marrow myeloma cells (Supplementary Table S1; data not shown). These findings show directly, for the first time, that PD 0332991 potently inhibits Cdk4/6 in primary myeloma cells.

To study the inhibition of Cdk4/6 by PD 0332991 in cycling primary myeloma cells, we optimized a coculture system with human HS-5 bone marrow stromal cells (BMSC; ref. 31), in which primary myeloma cells, we optimized a coculture system with human HS-5 bone marrow stromal cells (BMSC; ref. 31), in which primary myeloma cells or treatment history in all multiple myeloma cases tested (Pt 1, stage II, untreated) and cultured ex vivo with PD 0332991 (PD) for 12 hours. Similar results were obtained with PD 0332991 treatment for 4 to 6 hours. B, bone marrow myeloma cells from multiple myeloma patient (Pt 3, stage III, untreated) were cocultured ex vivo with HS-5 stromal cells and PD 0332991 for 24 hours. The ratios of pSRb to total Rb were determined by densitometry analysis. C, fluorescence-activated cell sorting analysis of BrdUrd (BrdU) uptake in freshly isolated CD138\textsuperscript{+} bone marrow myeloma cells (Pt 4, stage III, untreated) cocultured with PKH67 green-labeled HS-5 human stromal cells and PD 0332991 for 13 hours, with BrdU present in the last 10 hours. BrdUrd uptake was measured using an APC-anti-BrdUrd antibody. Representative of 14 experiments (A and B). Representative of three experiments (C).

PD 0332991 potently inhibits Cdk4/6 and induces G\textsubscript{1} arrest in HMCls. We next investigated the mechanism of PD 0332991 action in MMLS (IgG secreting) and CAG (IgA secreting) cell lines ex vivo. Both HMCls were established from end-stage multiple myeloma patients, when myeloma cells expanded without restraint independent of stromal support. They coexpressed high levels of cyclin D2 and Cdk6, along with Cdk4 but not cyclin D1 (Fig. 2A), as is the case with most other 25 HMCLs tested (data not shown). PD 0332991 potently and rapidly inhibited Cdk4/6-specific phosphorilation of Rb (IC\textsubscript{50} = 0.06-0.07 \textmu M/L; Fig. 2A; data not shown) in both MMLS and CAG cells.

This led to G\textsubscript{1} cell cycle arrest, as evidenced by the profound reduction in the proportion of MMLS cells that took up BrdUrd in a 30-minute pulse following treatment with 0.1 \textmu M/L PD 0332991, from 36% to 18% by 8 hours and to 7% by 12 hours (Fig. 2B). This reduction was not enhanced by higher PD 0332991 concentrations, suggesting that induction of G\textsubscript{1} arrest by PD 0332991 was saturated at 0.1 \textmu M/L (IC\textsubscript{50} ~ 0.05 \textmu M/L). Although the uptake of BrdUrd was also not reduced further by 24 hours of treatment with 0.1 \textmu M/L PD 0332991, presumably due to depletion of PD 0332991, it was abolished by treatment with 0.25 \textmu M/L PD 0332991 at this time (Fig. 2B). The reduction of BrdUrd in response to PD 0332991 was corroborated by the increases in cells in G\textsubscript{1}, as determined by fluorescence-activated cell sorting analysis of DNA content (Supplementary Fig. S1). Similar results were obtained with the CAG cells (data not shown). PD 0332991, therefore, effectively induces G\textsubscript{1} arrest in a time- and dose-dependent manner in cycling HMCLs.

PD 0332991 did not induce apoptosis at concentrations that specifically inhibit Cdk4/6 because at concentrations below 5 \textmu M/L, PD 0332991 did not increase the cleavage of PARP, indicative of caspase activation, or Annexin V–binding activity (Fig. 2C; top; data not shown). However, the expansion of total live myeloma cells was reduced with increasing concentrations of PD 0332991, as a consequence of induction of apoptosis in addition to G\textsubscript{1} arrest (Fig. 2C, bottom). Taken together, these results show that at concentrations that specifically inhibit Cdk4/6, PD 0332991 potently induces G\textsubscript{1} arrest without inducing apoptosis in cycling myeloma cells.

\textsuperscript{8} M. Di Liberto, R. Gottschalk, and S. Chen-Kiang, unpublished.
PD 0332991 inhibits Cdk4/6 proportional to the activation status of the cells in concert with p18\(^{INK4c}\). The ability of PD 0332991 to inhibit Cdk4/6 in proportion to the cycling status in primary myeloma cells suggests that PD 0332991 may preferentially target myeloma cells, particularly during relapse. To address this possibility further, we asked whether PD 0332991 also inhibits Cdk4/6 proportional to the cell activation status in nontransformed primary B cells. Resting (G0-G1) mouse splenic B cells were isolated and treated with increasing concentrations of PD 0332991 before or after activation ex vivo by anti-IgM, which functions as a surrogate antigen to induce cell cycle reentry and progression. In resting B cells, the levels of Cdk4/6-specific phosphorylation of Rb, Cdk4, and cyclin D2 were all below detection by immunoblotting, as reported previously (25). However, albeit extremely low, pSRb and a gradual diminution with increasing amounts of PD 0332991 were seen following prolonged exposure of the same blot (1 hour; Fig. 3, *). By contrast, in anti-IgM–activated B cells, Rb was highly phosphorylated as a consequence of elevated Cdk4 and cyclin D2 synthesis. PD 0332991 potently inhibited Cdk4 phosphorylation of Rb (IC\(_{50}\) ~ 0.05 \(\mu\)m/L), with an efficiency comparable with that in cycling HMCLs (Fig. 2) and cycling primary bone marrow myeloma cells (Fig. 1). PD 0332991, therefore, inhibits Cdk4 proportional to the activation status of the cells independent of cellular transformation.

The physiologic Cdk4/6 inhibitor p18\(^{INK4c}\) may have a role in myeloma pathogenesis as it is deleted in some HMCLs (32) and is not detectable by immunohistochemistry in primary CD138\(^+\) bone marrow myeloma cells in 37% of multiple myeloma cases (n = 245).\(^7\) To further investigate the mechanism of PD 0332991 action, we asked whether PD 0332991 inhibits Cdk4/6 independent of p18\(^{INK4c}\). In resting B cells, the absence of p18 seemed to lower the threshold of Cdk4 activation because Cdk4 phosphorylation of Rb in p18\(^+/−\) cells (detected in a 5-minute exposure of the same blot, *) was substantially higher than in their normal counterparts (Fig. 3), although it had no effect on the expression of Cdk4 or cyclin D2. Importantly, following activation of the cell cycle by anti-IgM, inhibition of Cdk4 phosphorylation of Rb by PD 0332991 was compromised in p18\(^+/−\) B cells (IC\(_{50}\) ~ 0.12 \(\mu\)mol/L) compared with their normal counterparts (Fig. 3). PD 0332991, therefore, inhibits Cdk4 in concert with the physiologic Cdk4/6 inhibitor p18 in nontransformed B cells.

PD 0332991 prevents tumor growth in a disseminated xenograft human myeloma model. Our finding that PD 0332991 inhibits Cdk4/6 according to the cell activation status further supports investigating the antitumor effect of PD 0332991 in vivo. We addressed this possibility in a newly developed NOD/SCID xenograft human multiple myeloma model in which disseminated tumors develop aggressively following injection of the HMCL CAG cells stably expressing the HSV-TK-eGFP-luciferase fusion protein. The tumor distribution can be quantified within days of tumor induction by serial whole-body noninvasive bioluminescence imaging of visible light emitted by luciferase-expressing multiple myeloma cells upon luciferin injection (28).

PD 0332991 rapidly inhibited myeloma tumor growth to 2.1% (dorsal) and 3.9% (ventral) by the end of the 12-day treatment (Fig. 4A). As a control, the luciferase activity in HSV-TK-eGFP-luciferase\(^+\) CAG cells was sustained following overnight incubation with PD 0332991 in vitro (Supplementary Fig. S2). Kaplan-Meier survival curves (P = 0.0007) further showed that the untreated mice...
died of tumor burden around 35 days after tumor induction, whereas the PD 0332991-treated mice survived (Fig. 4B). They also recovered completely from the loss of body weight (15%) during treatment (Fig. 4C). However, tumor growth resumed after discontinuation of PD 0332991 treatment, and the treated mice succumbed to death ~12 days later than the untreated mice (Fig. 4B).

Confirming that PD 0332991 rapidly inhibits the Cdk4/6 activities in vivo, treatment of tumor-induced mice with PD 0332991 overnight was sufficient to drastically reduce Cdk4/6-specific Rb phosphorylation in CD138+ CAG cells present in the vertebrae (Fig. 4D). PD 0332991 did not induce apoptosis of CAG cells in vivo based on the absence of TUNEL and caspase-3 activity (Fig. 4D; data not shown). There were also no overt changes in the spleen architecture (Fig. 4D; data not shown). Thus, through specific inhibition of Cdk4/6, PD 0332991 nearly completely prevents myeloma tumor growth in the aggressive, disseminated NOD/SCID xenograft human myeloma model.

PD 0332991 enhances killing of myeloma cells by dexamethasone. As a therapeutic agent, PD 0332991 can be used either at low concentrations to specifically and potently inhibit Cdk4/6 without induction of apoptosis, or at higher doses (>5 μmol/L) to induce apoptosis (Fig. 2C). These features suggest that PD 0332991 is an ideal molecule to specifically target Cdk4/6 and induce G1 arrest in combination with a second cytotoxic agent. This may lead to synergistic killing of myeloma cells, with a lower concentration of the cytotoxic agent required to bring about the same level of killing, or, with the same dose of each agent, a greater level of killing. We chose as the second agent dexamethasone, a glucocorticoid that is currently used as an antitumor agent in the treatment of myeloma as well as other cancers (33).

Induction of G1 arrest in MM1.S myeloma cells by treatment with 2 μmol/L PD 0332991 for 24 hours did not induce apoptosis as anticipated (Fig. 5A and B). However, when this was followed by treatment with dexamethasone for 48 hours, both the proportion and the total number of live cells were markedly reduced. The dose of dexamethasone required to achieve the same level of killing was 10 times lower when used in combination with PD 0332991 than alone (Fig. 5B, top, compare 0.01 μmol/L dexamethasone/PD 0332991 with 0.1 μmol/L dexamethasone alone).

**Figure 4.** PD 0332991 prevents tumor growth in a disseminated NOD/SCID xenograft human myeloma model. A, HSV-TK-eGFP-luciferase–positive CAG cells were injected in NOD/SCID mice; 150 mg/kg of PD 0332991 was given on day 7 after tumor induction, and the tumor mass (photons/s/cm²/steradian) was evaluated. % Ventral and dorsal tumor burden relative to that of vehicle-treated (untreated) mice. Columns, tumor burden from six individual mice. B, Kaplan-Meier survival curves of six PD 0332991–treated mice and six vehicle-treated (untreated) mice. C, % body weight of PD 0332991–treated and untreated mice relative to the start of PD 0332991 treatment. D, immunohistochemical analysis of simultaneous expression of CD138 (red) and Rb (brown), pSRb807/811 (brown), or TUNEL (brown) in vertebrae of tumor-induced mice 12 hours after administration of PD 0332991 or vehicle. % CD138+ cells expressing total Rb or pSRb807/811. As a control, spleen sections from the same mice were stained with hematoxylin. Representative of two independent experiments.

**Figure 5.** PD 0332991 enhances killing of myeloma cells by dexamethasone. A, MM1.S cells were incubated in the presence or absence of 2 μmol/L PD 0332991 for 24 hours before addition of dexamethasone. Analysis of total live cells by trypan blue staining (B), and DNA content per cell by propidium iodide analysis (C) was done at 48 hours after addition of dexamethasone. Representative of three independent experiments.
DNA content analysis indicated that dexamethasone treatment alone resulted in an increase in dead cells (<2N) and a corresponding reduction of cells in S and G2-M phases but not in G1 (Fig. 5C, top). These results were corroborated with the reduction in viability and loss of total live cells (Fig. 5B), confirming that dexamethasone acts mainly as a cytotoxic agent. Consistent with specific inhibition of Cdk4/6, PD 0332991 treatment alone led to a time-dependent increase in the proportion of MM1S cells in G1, from 55% to ~80% by 72 hours, and a corresponding reduction of cells in S and G2-M phases (Fig. 5C, bottom). The subsequent combination with dexamethasone treatment resulted in a striking dose-dependent increase in the proportion of dead cells (<2N; Fig. 5C). The killing of MM1S cells by 0.01 μmol/L dexamethasone in combination with PD 0332991 was comparable to that by 0.1 μmol/L dexamethasone alone. With increasing dexamethasone concentrations, the killing was further augmented by combining with PD 0332991.

Thus, as a proof of concept, sequential treatment with PD 0332991 and dexamethasone leads to synergistic killing of myeloma cells. This finding strongly suggests that by combining the ability of PD 0332991 to specifically inhibit Cdk4/6 with a second cytotoxic agent, a PD 0332991–based combination therapy represents a novel and effective therapeutic strategy for myeloma.

Discussion

**Inhibition of Cdk4/6 by PD 0332991 prevents myeloma tumor progression.** PD 0332991, a newly developed small-molecule inhibitor of Cdk4 and Cdk6, is superior to other Cdk4 or Cdk6 inhibitors that have been used in clinical trials in its oral activity, high potency, and specificity (19, 34). Treatment of HMCLs or myeloma cells in vitro with the Cdk inhibitor flavopiridol, which also inhibits Cdk2 (35), or R-roscovitine, which poorly inhibits Cdk4 and Cdk6 (IC50 > 100 μmol/L; ref. 36), mainly leads to apoptosis (22, 23). It is unclear whether either compound inhibits Cdk4 and Cdk6 in multiple myeloma cells selectively or has antitumor activity in vivo. We showed in this study that PD 0332991 potently inhibits Cdk4/6 in primary bone marrow myeloma cells (Fig. 1) and nearly completely prevents the growth of human myeloma tumors in aggressive, disseminated xenografts without gross side effects (Fig. 4).

Moreover, the high specificity of PD 0332991 action lies in part in its ability to inhibit Cdk4 and Cdk6 according to the activation status of the cells independent of cellular transformation. PD 0332991 inhibits Cdk4/6 much more effectively in cycling primary bone marrow myeloma cells and in HMCL cells than in noncycling primary bone marrow myeloma cells (Figs. 1-2) and in activated primary mouse B cells more than in resting B cells (Fig. 3).

Of interest, PD 0332991 inhibits Cdk4/6 in concert with the physiologic CKI p18 (Fig. 3). p18 inhibits Cdk4/6 mainly by forming a stable inactive binary complex, thereby precluding the activation of Cdk4/6 by D cyclins (9). It can also form a ternary complex with Cdk4/6-cyclin D and inhibit Cdk4/6 (37). PD 0332991 inhibits Cdk4/6 by competing for the ATP-binding sites on Cdk4 and Cdk6, even when they are in complex with the D cyclin (19). Whether the partial dependence on p18 for PD 0332991 inhibition of Cdk4/6 stems from cooperative binding of p18 and PD 0332991 to distal sites on the same kinase, or from differential inhibition of free Cdk4/6 and cyclin D–bound Cdk4/6 remains to be determined. In either case, p18 protein expression is below detection by immunohistochemistry in primary bone marrow myeloma cells in ~37% of multiple myeloma cases. The genes encoding p18 (32) and other INK4 family CKIs have been reported to be deleted or inactivated by hypermethylation in myeloma cell lines (38, 39). Understanding the mechanism by which PD 0332991 and INK4 Cdk inhibitors, in particular p18, cooperate in inhibiting Cdk4/6 should provide novel insights into targeting Cdk4/6 in myeloma.

**PD 0332991 as a novel mechanism-driven therapeutic strategy for myeloma.** PD 0332991 is orally active, water soluble, and seems to selectively inhibit Cdk4/6 without inducing drug resistance in solid tumor xenografts (19). These are favorable features for therapeutic application. Cdk4 and Cdk6 are dispensable for development (40). We showed that PD 0332991 inhibits Cdk4/6 according to the activation status of the cells, in particular in cycling primary bone marrow myeloma cells freshly isolated from both new and relapsed patients (Fig. 1). These findings provide a strong rationale for targeting myeloma progression with PD 0332991.

In support of this possibility, we showed by noninvasive bioimaging in our aggressive, disseminated CAG-NOD/SCID xenograft human myeloma model (28) that PD 0332991 rapidly and effectively prevents myeloma tumor growth, with reversible weight loss and without discernible side effects (Fig. 4). When PD 0332991 was similarly given in rats, the maximum concentration of PD 0332991 in plasma was estimated to be ~5 μmol/L (34). Assuming that the pharmacokinetics of PD 0332991 in mice is comparable with that in rats, this plasma concentration would allow PD 0332991 to act as a specific Cdk4/6 inhibitor without inducing cell death in vivo, as observed in vitro (Figs. 1-2). Consistent with this possibility, we found that PD 0332991 prevented tumor progression in xenografts without causing overt side effects (Fig. 4). Together, these observations support the potential to inhibit Cdk4/6 with specificity by PD 0332991 in myeloma therapy.

**Inhibition of Cdk4/6 by PD 0332991 in combination therapy.** There has been renewed interest in the development of a successful Cdk4/6 inhibitor in cancer therapy. Two recently published studies showed a role for Cdk4-cyclin D1 kinase activity in ErbB-2-induced mammary tumors (41, 42). Because cyclin overexpression and Cdk activation is a common feature in breast tumors and other cancers, including myeloma, the development of a successful Cdk inhibitor could have significant benefit in cancer treatment.

Here, we show that killing of myeloma cells by dexamethasone can be augmented by inhibition of Cdk4/6 and induction of G1 cell cycle arrest with PD 0332991 (Fig. 5). How a steroid, such as dexamethasone, functions as a cytotoxic agent is unknown. However, recent evidence suggests that it may involve the phosphatidylinositol 3-kinase (PI3K)/Akt pathway because inhibition of this pathway seems to enhance the killing of a human follicular lymphoma cell line by dexamethasone (43). Consistent with this notion, prolonged treatment of MM1S cells with 2 μmol/L PD 0332991 (48 hours) led to a modest reduction in the phosphorylation of Akt. It is tempting to speculate that as a secondary effect of prolonged G1 arrest in response to PD 0332991, reduction of the PI3K/Akt pathway may contribute to enhanced killing of myeloma cells by dexamethasone. This possibility awaits future exploration.

Although as a proof of concept we have presented evidence here that PD 0332991 can enhance the killing of myeloma cells by dexamethasone, it may also be possible to achieve greater synergy by combining PD 0332991 with other cytotoxic agents. The pro tease inhibitor Bortezomib (44) is one attractive possibility.

8 L.B. Baughn and S. Chen-Kiang, unpublished.
Although Bortezomib is widely used in the treatment of myeloma (45), only 30% of multiple myeloma patients respond (44), and the basis for Bortezomib resistance remains unknown. Multiple myeloma remains incurable in part due to a lack of a mechanism-defined combination therapy that will not only suppress tumor progression but also prevent residual disease. The functional evidence presented in this study strongly suggests that in combination with a second agent, PD 0332991 is a novel candidate for mechanism-defined combination therapy in myeloma and also possibly other B-cell malignancies.

Acknowledgments

Received 3/24/2006; revised 5/30/2006; accepted 6/1/2006.

Grant support: NIH T32 postdoctoral fellowship (L.B. Baughm), NIH grant ROI AR 49436 (S. Chen-Kiang), Translational Research Program grants (S. Chen-Kiang and M.A.S. Moore), and a Specialized Center of Research grant from the Leukemia and Lymphoma Society.

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We thank Dr. Josephina Garcia for advice on the studies of primary mouse B cells; Dr. Stephen Eck for helpful comments and suggestions; David Jayabalan, Khan Zhang, Yi-Fang Liu, and Plinio Silva for technical assistance; and members of the Chen-Kiang laboratory for stimulating discussions.

References

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