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Priority Report

A Novel Therapeutic Combination Using PD 0332991 and Bortezomib: Study in the 5T33MM Myeloma Model

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Abstract

Multiple myeloma (MM) remains incurable partly because no effective cell cycle–based therapy has been available to both control tumor cell proliferation and synergize with cytotoxic killing. PD 0332991 is an orally active small molecule that potently and specifically inhibits Cdk4 and Cdk6. It has been shown to induce rapid G_1 cell cycle arrest in primary human myeloma cells and suppress tumor growth in xenograft models. To improve therapeutic targeting of myeloma progression, we combined tumor suppression by PD 0332991 with cytotoxic killing by bortezomib, a proteasome inhibitor widely used in myeloma treatment, in the immunocompetent 5T33MM myeloma model. We show that 5T33MM tumor cells proliferate aggressively *in vivo* due to expression of cyclin D2, elevation of Cdk4, and impaired $p27^{Kip1}$ expression, despite inhibition of Cdk4/6 by $p18^{INK4c}$ and the maintenance of a normal plasma cell transcription program. PD 0332991 potently inhibits Cdk4/6-specific phosphorylation of Rb and cell cycle progression through G_1 in aggressively proliferating primary 5T33MM cells, *in vivo* and *ex vivo*. This leads to tumor suppression and a significant improvement in survival. Moreover, induction of G_1 arrest by PD 0332991 sensitizes 5T33MM tumor cells to killing by bortezomib. Inhibition of Cdk4/6 by PD 0332991, therefore, effectively controls myeloma tumor expansion and sensitizes tumor cells to bortezomib killing in the presence of an intact immune system, thereby representing a novel and promising cell cycle–based combination therapy. [Cancer Res 2008;68(14):5519–23]

Introduction

Multiple myeloma (MM), an incurable plasma cell (PC) malignancy, is characterized by the accumulation of malignant PCs in the bone marrow (BM), secretion of large amounts of monoclonal antibody, and bone lesions. Unlike normal PCs, MM cells retain their self-renewing potential due to deregulation of the cell cycle, which is particularly relevant during the aggressive phase of the disease and relapse (1). Progression through the cell cycle is driven by cyclin-dependent kinases (Cdk) in cooperation with cyclins and opposed by Cdk inhibitors (2). Aberrant coactivation of Cdk4-cyclin D1 or Cdk4/Cdk6-cyclin D2 precedes proliferation of MM cells, demonstrating that deregulation of Cdk4 and Cdk6 is central to the loss of cell cycle control in MM (3). PD 0332991 is an orally active small molecule that potently and specifically inhibits Cdk4/6 in a reversible manner (4). It has emerged as a promising agent for cell cycle–based therapy owing to its ability to rapidly and specifically inhibit Cdk4 and Cdk6 (IC_{50}, ~60 nmol/L) in primary human MM cells (5), as observed in solid tumor cell lines (4) and confirmed in mantle cell lymphoma cells and acute myloid leukemia cells (6, 7). This leads to exclusive G_1 arrest and marked tumor suppression in nonobese diabetes severe combined immunodeficient (NOD-SCID) xenografts (5). However, the antimyeloma activity of PD 0332991 in the presence of an intact immune system is unknown.

To optimize therapeutic targeting of Cdk4/6 with PD 0332991, we investigated the efficacy of PD 0332991 in inhibiting Cdk4/6 and controlling aggressive tumor growth in the immunocompetent 5T33MM model. Unlike the conventional NOD-SCID xenografts, the 5T33MM tumors are maintained by serial transfer into syngeneic immunocompetent C57BL/KaLwRij mice. Tumors develop in the BM microenvironment, thus uniquely recapitulating the properties of human myeloma (8). Furthermore, the rapid tumor development (within 4 weeks) is reminiscent of a relapsed disease, suggesting that the 5T33MM model is particularly suited for the development of a cell cycle–based therapy in the presence of an intact immune system. Here, we show that inhibition of Cdk4/6 by PD 0332991 induces G_1 cell cycle arrest in primary 5T33MM cells *in vivo* and *ex vivo*, and suppresses tumor progression. In combination with the cytotoxic drug bortezomib (Velcade), PD 0332991 both inhibits cell proliferation and enhances bortezomib killing. Combining inhibition of Cdk4/6 by PD 0332991 and cytotoxic killing by bortezomib, therefore, is a promising cell cycle–based combination therapy in myeloma.

Materials and Methods

5TMM models. C57BL/KaLwRij mice were purchased from Harlan (Horst) and used at ages 6 to 10 wk. They were housed and maintained following the conditions approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussels (license no. LA1230281). The animal ethics meet the standards required by the United Kingdom Coordinating Committee on Cancer Research Guidelines (UKCCCR, 1998).

5TMM tumor cell isolation and analysis. The 5T33MM cells originated spontaneously in elderly C57BL/KaLwRij mice and have since been propagated *in vivo*, by i.v. transfer of the diseased marrow into young syngeneic mice (9). The development of myeloma was assessed by the level of monoclonal antibody present in the serum (paraprotein) by protein electrophoresis. When the paraprotein concentration reached 10 mg/mL, the mice were sacrificed and the BM was flushed out of femurs and tibiae and crushed out of the vertebrae. The BM cells were suspended in supplemented serum-free medium [RPMI 1640 (Life Technologies)]. The 5T33MM cells with >95% viability and >70% purity (assessed by staining with anti-idiotype antibodies) were enriched by Lympholyte M (Cedarlane)

Note: E. Menu and J. Garcia contributed equally to this work.

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gradient centrifugation at 1,000 *g* for 20 min. *Ex vivo* and *in vitro* experiments were performed in RPMI supplemented with 10% FCS (Fetal Clone I; Hyclone). The 5T33MMvt line, clonally identical to the 5T33MM tumor cells but stroma independent, is maintained in RPMI supplemented with 10% FCS (Fetal Clone I). PD 0332991 (Pfizer Global Research and Development) and bortezomib (Millennium Pharmaceuticals) were added at indicated concentrations and times.

Real-time reverse transcription-PCR. Isolation of total RNA from splenic resting B cells, PCs, and 5T33MM cells and cDNA synthesis were performed as described (10). Samples were subjected to real-time PCR analysis by using Assays-on-Demand gene expression mixes specific for mouse cyclin D1 (Mm00432359), cyclin D2 (Mm00438071), Cdk4 (Mm01624002), Cdk6 (Mm00438163), p18 (Mm00483243), p27 (Mm00438168), BLIMP-1 (Mm00476128), IRF-4 (Mm00516431), Bcl-6 (Mm00477633), Pax-5 (Mm0043501), CD138 (Mm00448918), and β-Actin (Mm00607939). Reactions were carried out in the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). The relative amount of products was determined by the comparative threshold cycle method according to the instructions of Applied Biosystems.

Immunoblotting. Preparation of whole cell lysates and immunoblotting were performed as previously described (10), using the following antibodies: mouse Mabs to Cdk4 (Cell Signaling), Rb, actin (BD PharMingen); rabbit polyclonal antibodies to $pSRb^{807/811}$ (Cell Signaling), p27, cyclin D2, and Cdk6 (Santa Cruz Biotechnology). Blots were developed with the Super-Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). The signals were determined by densitometry analysis.

BrdUrd-uptake *in vivo* and *in vitro*. For pulse labeling of 5′-bromo-2′-deoxyuridine (BrdUrd; Sigma), BrdUrd was injected intraperitoneally into mice 2 h before sacrifice, or added to the cultures *in vitro* 2 h before analysis. To assay BrdUrd uptake by immunofluorescence staining, the cells were spun onto slides, fixed, and incubated with a FITC–anti-BrdUrd antibody (Roche Diagnostics) and Rhodamine–anti-mouse total IgG (Southern Biotec), as previously described (11). To measure BrdUrd-uptake by flow cytometry, cells were stained with the same FITC–anti-BrdUrd antibody (Roche Diagnostics) and a phycoerythrin-rat anti-mouse IgG Mab (BD PharMingen). Flow cytometry was performed using a fluorescence-activated cell sorting (FACS) Calibur and the CellQuest software (BD PharMingen).

Apoptosis assays. DNA content per cell was determined by FACS immediately after the addition of propidium iodide (PI; 1 μg/mL). To detect mitochondrial depolarization, cells were incubated with Mitotracker (330 nmol/L; Invitrogen) at 37°C for 30 min. The retention of Mitotracker was assayed by FACS according to the manufacturer's specification.

Kaplan-Meier curve. After transplantation of the 5T33MM tumors, mice (n = 9) were treated with vehicle or PD 0332991 (4, 5) between days 7 and 19 in the single-agent study, or treated with vehicle or PD 0332991 between days 4 and 11 and with bortezomib between days 12 and 24 in combination therapy (n = 10). Additional naive mice were included as negative controls. Animals were sacrificed when they showed signs of morbidity, namely hind limb paralysis.

Statistical analysis. For statistical analysis of the *in vitro* data, the Student's *t* test was used. Kaplan-Meier analysis was used to determine the effect of treatment on the survival of the mice. *P* values of ≤0.05 were considered significant.

Results and Discussion

Elevation of Cdk4 and impaired $p27^{Kip1}$ expression in 5T33MM cells. Loss of G_1 cell cycle control precedes unrestrained proliferation in primary human BM myeloma cells (3). To determine the basis for the rapid growth of 5T33MM tumors, we characterized the expression of core G_1 cell cycle regulators in primary BM 5T33MM tumor cells by quantitative real-time reverse transcription-PCR (RT-PCR). Compared with resting mouse B cells, which are positioned at the G_0-G_1 boundary, normal mouse PCs isolated from an antibody response expressed cyclin D2 but not cyclin D1, and Cdk4 but not Cdk6 (Fig. 1*A*). However, normal PCs are arrested in early G_1 because of increases in $p18^{INK4c}$, a specific inhibitor of Cdk4 and Cdk6 (12, 13), and $p27^{Kip1}$, which inhibits cyclin E-Cdk2 in late G_1 and early S phase (Fig. 1*A*; refs. 14, 15). Primary 5T33MM cells expressed comparable levels of cyclin D2 and $p18^{INK4c}$ mRNA but 4-fold higher Cdk4 mRNA and less than half of $p27^{Kip1}$ mRNA relative to PCs (Fig. 1*A*). This is reminiscent of findings in aggressively growing primary human BM MM cells (3). Collectively, these results suggest that together with cyclin D2 expression, overexpression of Cdk4 and insufficient $p27^{Kip1}$ expression in 5T33MM tumor cells overrides inhibition by $p18^{INK4c}$ to promote cell cycle progression through G_1 and S phase entry.

The PC transcription program is maintained in 5T33MM cells. Aberrant expression of cell cycle genes in 5T33MM cells may stem from an impaired PC transcriptional program. Apart from expressing a high level of CD138, PCs were elevated in the expression of Blimp-1 (16) and IRF-4 (17), two transcription factors required for PC differentiation, and reduced in Bcl-6 (18) and Pax-5 (19), targets of Blimp-1 repression, when comparing with resting B cells (Fig. 1*B*). CD138, Blimp-1, and IRF-4 were similarly elevated, and Bcl-6 and Pax-5 were accordingly reduced in primary 5T33MM tumor cells. The appropriate expression of key PC transcription factors in 5T33MM cells suggests that the PC transcriptional program is maintained and that deregulation of the cell cycle is due to inappropriate Cdk4 and p27 expression.

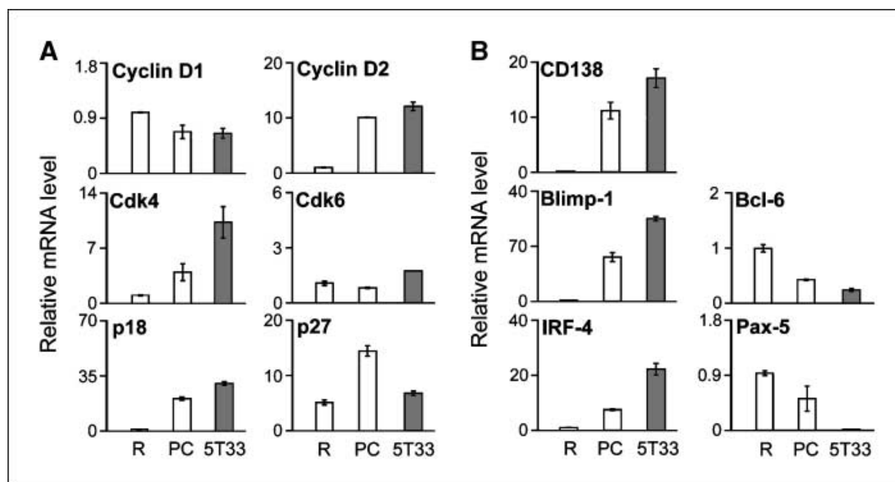

Figure 1. RNA analysis of the 5T33MM primary tumor cells. Real-time RT-PCR analysis of the relative expression in resting B cells, normal PCs, and 5T33MM cells of (*A*) RNA encoding cell cycle core proteins and (*B*) RNA encoding CD138, Blimp-1, IRF4, Bcl-6, and Pax-5. Resting splenic B cells (*R*) and PC were isolated from normal C57BL6 mice after immunization with 4-hydroxy-3-nitrophenyl linked to chicken γ-globulin as previously described (10). The data are representative of three independent experiments.

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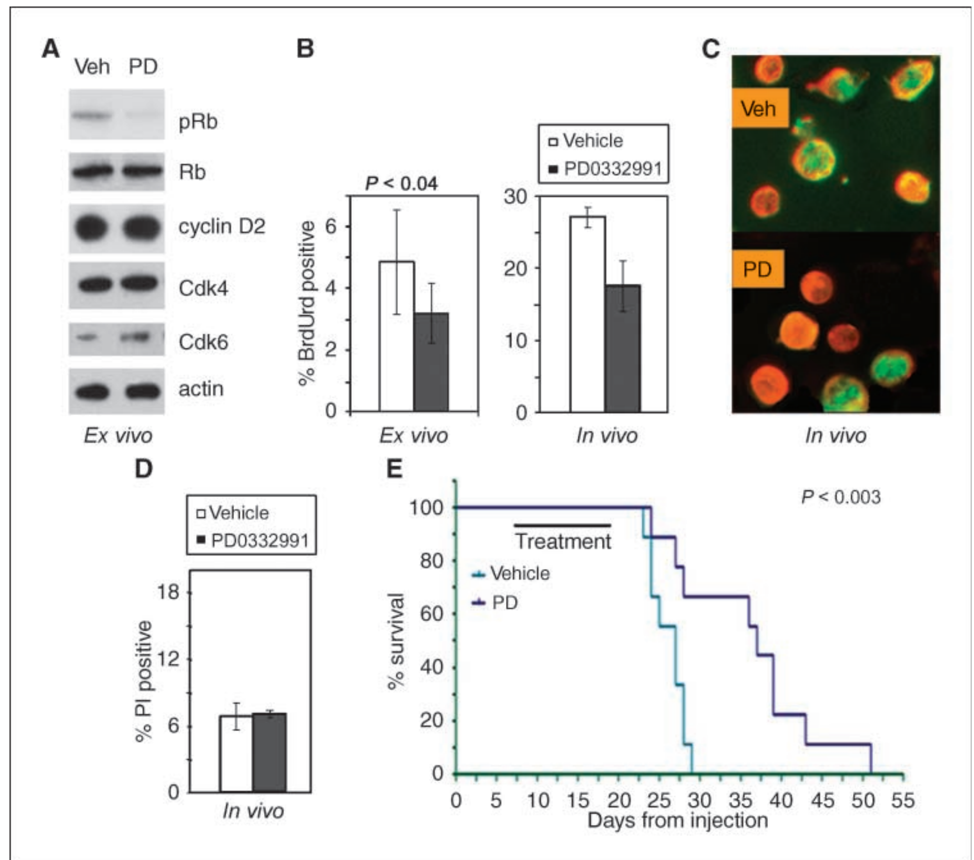

Figure 2. PD 0332991 inhibits Cdk4/6 and G_1 progression in 5T33MM tumor cells and improves survival. *A,* immunoblotting of Cdk4/6-specific pSRb in 5T33MM tumor cells treated *ex vivo* with 1 μmol/L of PD 0332991 for 16 h. The 5T33MM cells isolated were >70% pure based on IgG staining. The data are representative of three independent experiments. *B,* flow cytometric analysis of BrdUrd uptake in 5T33MM cells, *ex vivo,* in cells cultured for 16 h with 1 μmol/L of PD 0332991, and *in vivo* in animals treated with 150 mg/kg PD 0332991 for 36 h. BrdUrd was given 2 h before analysis at a concentration of 5 μg/mL (*ex vivo*) or 40 mg/kg (*in vivo*). Mean of three independent experiments is shown; $P < 0.04$. *C,* immunofluorescent staining of BrdUrd-uptake in 5T33MM cells in mice treated *in vivo* with vehicle (*veh*) or PD 0332991 (150 mg/kg; *PD*). Cells were double stained with anti–IgG-TRITC (*red*) and anti-BrdUrd–FITC (*green*). *D,* PI staining of 5T33MM cells treated *in vivo* with vehicle or PD 0332991 (150 mg/kg). *E,* Kaplan Meier survival curve. Mice were treated daily by gavage with either 150 mg/kg PD 0332991 or the vehicle (lactate buffer; ref. 5) between the 7th and 19th day after injection with 5T33MM cells. The onset of morbidity in the vehicle group was on the 22nd day. On average, the PD 0332991 group lived 10 d longer (25 versus 35 d; $P < 0.003$). The data are representative of two independent experiments.

PD 0332991 inhibits Cdk4/6 and G_1 progression in 5T33MM cells *in vivo* and *ex vivo*. The ability of PD 0332991 to inhibit Cdk4 in 5T33MM cells was determined by assaying Cdk4/6-specific phosphorylation of Rb on serine$^{807/811}$ (pSRb; ref. 3). PD 0332991 (1 μmol/L) inhibited pSRb in primary 5T33MM cells *ex vivo* within 16 hours while maintaining the levels of cyclin D2, Cdk4, and Cdk6 (Fig. 2*A*).

Phosphorylation of Rb by Cdk4 and Cdk6 in early G_1 and cyclin E/Cdk2 in late G_1 facilitates the release of E2F transcription factors necessary for S phase entry (2). To determine whether inhibition of Cdk4 by PD 0332991 leads to G_1 arrest in 5T33MM cells, BrdUrd-uptake during the last 2 hours of PD 0332991 treatment (14–16 hours) *ex vivo* was measured by flow cytometry. Although 5T33MM cells survive and cycle only for a limited period of time *ex vivo*, the uptake of BrdUrd was reduced by 40% (5 versus 3%) after PD 0332991 treatment (Fig. 2*B*). Validating this result, treating terminally diseased mice with PD 0332991 (150 mg/kg) for 36 hours led to a 35% reduction of BrdUrd uptake (27 versus 18%) in the 5T33MM tumor cells *in vivo* (Fig. 2*B*). Corroborating the FACS analysis, dual immunofluorescence staining shows a similar reduction in the proportion of BrdUrd-positive cells among the IgG-positive tumor cells (Fig. 2*C*). Because BrdUrd was present *in vivo* only for the last 2 hours before analysis, the high rate of BrdUrd-uptake further showed that 5T33MM tumor cells cycled at an extraordinarily high rate *in vivo*, thereby resembling relapsed disease. PD 0332991 did not induce cell death at the time and

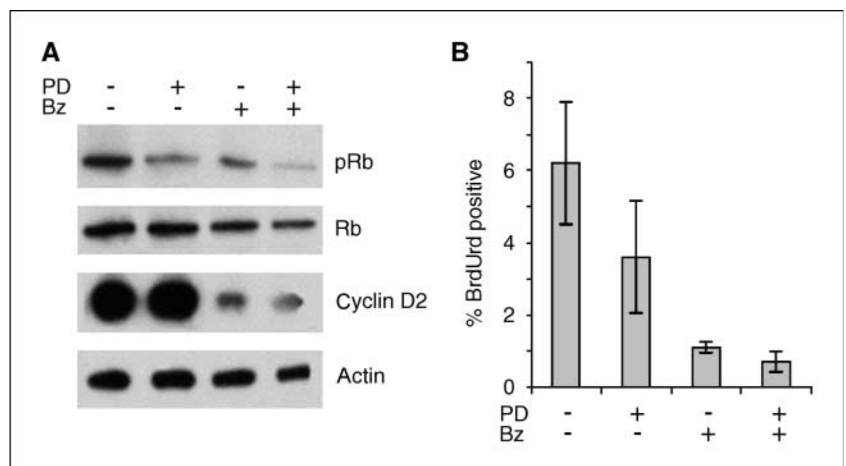

Figure 3. Synergistic induction of G_1 cell cycle arrest by PD 0332991 and bortezomib (*Bz*) in 5T33MM cells. *A,* immunoblotting of Cdk4/6-specific pSRb and cyclin D2 and (*B*) Flow cytometric analysis of BrdUrd uptake in 5T33MM cells treated *ex vivo* with 1 μmol/L of PD 0332991 and/or 3 nmol/L bortezomib for 16 h. BrdUrd was given 2 h before analysis at a concentration of 5 μg/mL. Mean of three independent experiments is shown.

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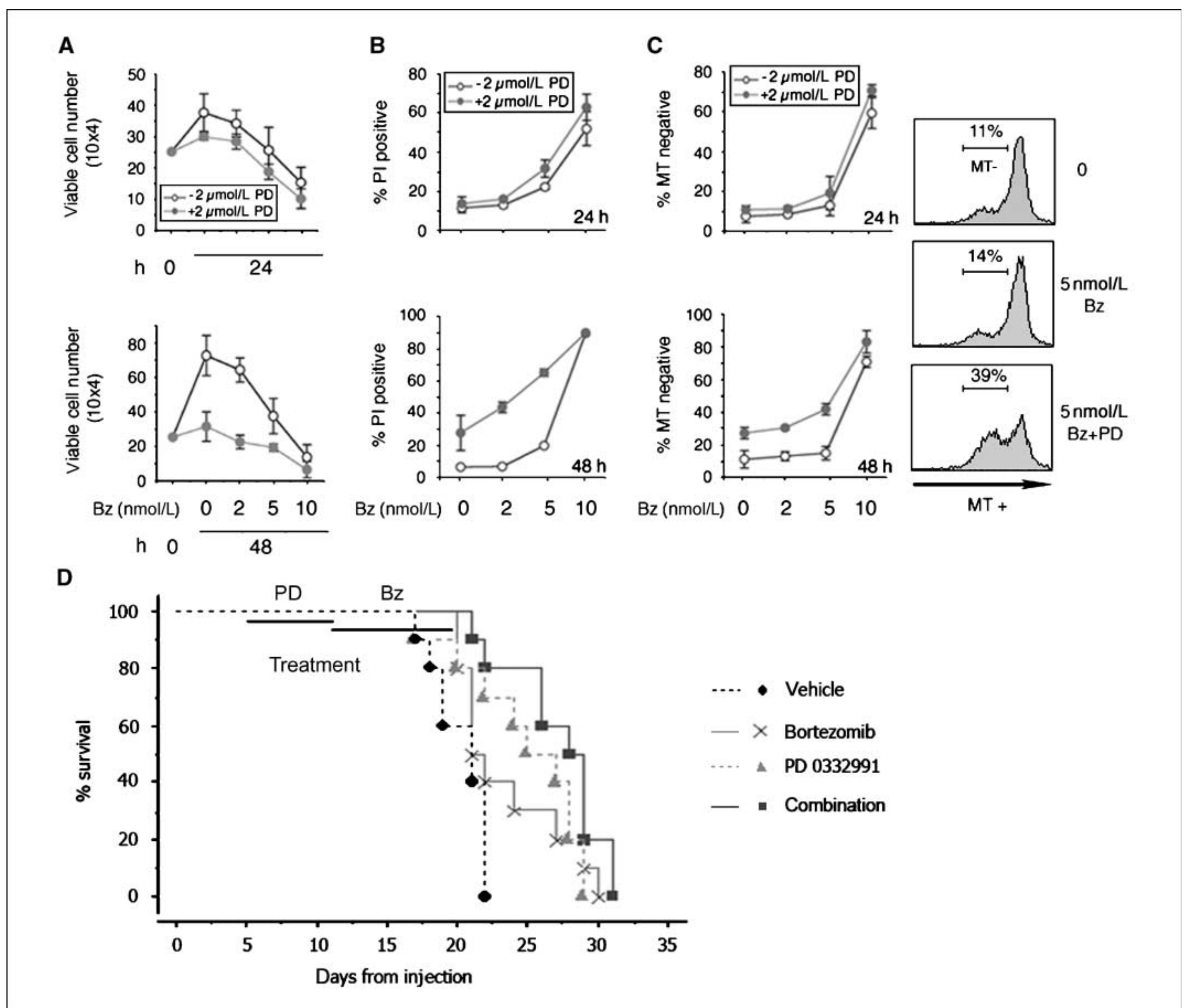

Figure 4. Synergistic induction of cell death by PD 0332991 and bortezomib in 5T33MMvt cells. *A,* 5T33MMvt cells were cultured in the presence or absence of 2 μmol/L PD 0332991 and bortezomib at indicated concentrations for 24 or 48 h. *A,* the total number of live cells were determined by trypan blue staining. *B,* the proportion of dead cells was determined by PI uptake. *C,* the proportion of cells losing retention of Mitotracker (*% MT-negative*) was determined by flow cytometry. Mean of five independent experiments (*left*) and one representative (*right*) is shown. *D,* Kaplan Meier survival curve. Mice were treated daily by gavage with either 150 mg/kg PD 0332991 or the vehicle (lactate buffer) between the 4th and 11th day after injection with 5T33MM cells. Thereafter, they were treated 4 times with 0.4 mg/kg bortezomib (s.c. in physiologic water) every 3 d. The onset of morbidity in the vehicle group was on the 17th day. On average, the PD 0332991 group lived 4 d longer than the vehicle (20 versus 24 d; $P < 0.005$) and the combination group 7 d longer (27 d).

concentration used based on the analysis of DNA fragmentation (Fig. 2*D*). Together, these data show that PD 0332991 induces G_1 arrest in the rapidly cycling 5T33MM tumor cells, *ex vivo* and *in vivo*.

Inhibition of Cdk4/6 by PD 0332991 prolongs the survival of 5T33MM diseased mice. To examine whether inhibition of Cdk4/6 by PD 0332991 in 5T33MM cells could suppress tumor progression and prolong survival, mice were inoculated with 5×10^5 5T33MM cells and treated for 2 weeks with 150 mg/kg PD 0332991 daily by gavage after the onset of disease (~day 7). PD 0332991 treatment led to a significant increase in survival, to a mean of 35 days in the PD 0332991–treated mice compared with 25 days in vehicle-treated mice ($n = 9$; $P < 0{,}003$; Fig. 2*E*). No severe side effects from the PD 0332991 treatment were observed, although tumor growth resumed after discontinuation of the treatment because PD 0332991 acts reversibly (4). Thus, by targeting Cdk4 and Cdk6, PD 0332991 suppressed tumor growth in the aggressive, immunocompetent 5T33MM model and significantly improved the survival of the diseased mice.

Inhibition of Cdk4/6 by PD 0332991 sensitizes 5T33MM cells to killing by bortezomib. We then asked whether targeting Cdk4/6 by PD 0332991 would also enhance cytotoxic killing of 5T33MM cells. As a proof of concept, we chose the proteasome inhibitor bortezomib, which is widely used in myeloma treatment but as a single agent effective in only ~30% of cases. pSRb was reduced in primary 5T33MM tumor cells by 16 hours of *ex vivo* treatment with PD 0332991 (1 μmol/L) or bortezomib (3 nmol/L) alone, and further reduced by PD 0332991 and bortezomib

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combined. Although the cyclin D2 level remained unchanged after PD 0332991 treatment *ex vivo*, as observed (Fig. 2*A*), it was drastically reduced by bortezomib treatment (Fig. 3*A*). The uptake of BrdUrd in 5T33MM cells *ex vivo* was reduced proportional to inhibition of pSRb in the PD 0332991 response but reduced further in the bortezomib response (Fig. 3*B*). Taken together, these results confirm that Cdk4 phosphorylation of Rb is a major determinant for G-S transition in primary 5T33MM tumor cells and suggest that reduction of pSRb by bortezomib was due to preferential killing of S phase cells, whereas that by PD 0332991 was a consequence of inhibition of Rb phosphorylation.

The possibility that inhibition of Cdk4/6 and induction of G_1 arrest may enhance the killing of tumor cells by bortezomib was then addressed in the 5T33MMvt cells, which are clonally identical to the 5T33MM tumor cells. Treatment of 5T33MMvt cells with bortezomib (2–10 nmol/L) alone led to a dose- and time-dependent reduction of live 5T33MMvt cells, as shown by the profound cell death (DNA fragmentation) in cells treated with 5 nmol/L of bortezomib (Fig. 4*A–B*). The combination of PD 0332991 with bortezomib induced synergistic apoptosis, which prevented the accumulation of live cells by 48 hours at low bortezomib concentration (2–5 nmol/L) and led to eradication of live cells at 10 nmol/L bortezomib. Accordingly, mitochondrial depolarization indicated by the loss of retention of the Mitotracker dye was increased by treatment with PD 0332991 and bortezomib compared to bortezomib alone (Fig. 4*C*). Thus, inhibition of Cdk4/6 by PD 0332991 leads to G_1 arrest and enhances the killing of 5TMM cells to bortezomib through increased mitochondrial depolarization. These results are similar to those reported by Huang and colleagues (20) in primary human BM MM cells.

To determine if the enhanced killing *in vitro* could lead to prolonged survival *in vivo,* mice were treated earlier in tumor development with 150 mg/kg PD 0332991 for 7 days instead of 12 days, and then with a suboptimal concentration of bortezomib (0.4 mg/kg), 4 times in 12 days (Fig. 4*D*). With the reduced schedule, the PD 0332991 treatment still led to a modest prolongation in survival (a mean of 24 days compared with 20 days in vehicle treated mice; $P < 0{,}05$). At the suboptimal concentration, bortezomib also led to a mean survival of 23 days. The mice tolerated the PD 0332991/bortezomib combination therapy and survived even longer (mean of 27 days) than those treated with either agent alone, although this difference failed to reach statistical significance. In addition, it seems that there is an initial survival advantage after the combination treatment, which however is not sustained. This is not surprising given that PD 0332991 acts reversibly (4), and suggests that repetitive cycles of alternating PD 0332991 and bortezomib treatments might be necessary to achieve optimal therapeutic effects. This observation notwithstanding, our data provide evidence in an immunocompetent myeloma model that PD 0332991 can enhance the antimyeloma effect of bortezomib in combination therapy *in vivo*.

This study defines the cell cycle defects in 5T33MM cells, and provides the first direct evidence that PD 0332991 potently inhibits Cdk4/6 and cell cycle progression through G_1 in rapidly cycling myeloma tumor cells in the presence of an intact immune system. PD 0332991 is orally bioavialable and acts reversibly. The substantial improvement in the survival of the PD 0332991–treated mice and the absence of overt side effects show that PD 0332991 is a powerful cytostatic agent when used alone. When combined with a cytotoxic agent such as bortezomib, targeting Cdk4 and Cdk6 with PD 0332991 enhanced the killing of tumor cells by bortezomib both *in vitro* and *in vivo*, in part through induction of mitochondrial depolarization. Taken together, our findings suggest that combining tumor suppression by targeting Cdk4 and Cdk6 with cytotoxic killing is a promising strategy for developing an effective cell cycle–based combination therapy in myeloma. This would be greatly accelerated by further preclinical studies in the immunocompetent myeloma models such as the 5T2MM and 5T33MM models.

Disclosure of Potential Conflicts of Interest

I. Chen, Employment, Pfizer, Inc.; Ownership Interest, Pfizer, Inc.

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