CDK4/6 Inhibitor PD 0332991 Sensitizes Acute Myeloid Leukemia to Cytarabine-Mediated Cytotoxicity

Chenyi Yang, Cynthia A. Boyson, Maurizio Di Liberto, Xiangao Huang, Jeffrey Hannah, David C. Dorn, Malcolm A.S. Moore, Selina Chen-Kiang, and Pengbo Zhou

Abstract

Cyclin-dependent kinase (CDK)4 and CDK6 are frequently overexpressed or hyperactivated in human cancers. Targeting CDK4/CDK6 in combination with cytotoxic killing therefore represents a rational approach to cancer therapy. By selective inhibition of CDK4/CDK6 with PD 0332991, which leads to early G1 arrest and synchronous S-phase entry upon release of the G1 block, we have developed a novel strategy to prime acute myeloid leukemia (AML) cells for cytotoxic killing by cytarabine (Ara-C). This sensitization is achieved in part through enrichment of S-phase cells, which maximizes the AML populations for Ara-C incorporation into replicating DNA to elicit DNA damage. Moreover, PD 0332991 triggered apoptosis of AML cells through inhibition of the homeobox (HOX)9 oncogene expression, reducing the transcription of its target PIM1. Reduced PIM1 synthesis attenuates PIM1-mediated phosphorylation of the pro-apoptotic BAD and activates BAD-dependent apoptosis. In vivo, timely inhibition of CDK4/CDK6 by PD 0332991 and release profoundly suppresses tumor growth in response to reduced doses of Ara-C in a xenograft AML model. Collectively, these data suggest selective and reversible inhibition of CDK4/CDK6 as an effective means to enhance Ara-C killing of AML cells at reduced doses, which has implications for the treatment of elderly AML patients who are unable to tolerate high-dose Ara-C therapy.

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with the median age of 69 years (1). Among genes that are implicated in the leukemogenesis of AML, high HOX gene expression has been associated with unfavorable prognosis, and lower HOX9 expression correlates with a more favorable progression-free survival and response to therapy (2–4). However, despite intensive investigation of the etiology of AML and recent advances in targeted therapy, the nucleoside analog cytarabine (Ara-C) remains the first-line chemotherapy drug for AML for the last 40 years. Unfortunately, older patients are generally intolerant to high-dose Ara-C due to high toxicity and frequent resistance (5).

Cyclin-dependent kinase (CDK)4 and CDK6 are rarely mutated but frequently overexpressed or hyperactivated in human cancers (6, 7). PD 0332991 (palbociclib), a cell permeable pyridopyrimidine with oral bioavailability, is an exceptionally selective and potent inhibitor of CDK4 and CDK6 (IC50~10 nmol/L for recombinant proteins; ref. 8). Unlike other CDK inhibitors, at concentrations specific for inhibition of CDK4/CDK6, PD 0332991 has little or no activity against other CDK kinases, including CDK2, due to steric hindrance (8). Providing the first evidence for PD 0332991’s bioactivity in primary cancer cells, we showed that PD 0332991 inhibited CDK4/CDK6 in primary human multiple myeloma cells (IC50, 60 nmol/L) in the presence of bone marrow stromal cells, leading to early G1 arrest (9). PD 0332991 was similarly effective in inhibiting CDK4/CDK6 in AML, mantle cell lymphoma (MCL), glioblastoma, and many other cancer types ex vivo (10–12), and suppressed tumor growth in myeloma, MCL and AML tumor xenografts (8, 9, 11), and in an immunocompetent mouse myeloma model (13).

Induction of early G1 arrest by PD 0332991 requires retino-blastoma (Rb), the substrate of CDK4 and CDK6, but not p53, and this is reversible in vitro and in vivo (14). In the first-in-human single-agent phase I clinical trial, PD 0332991 effectively inhibited CDK4/6 and induced early G1 arrest within tolerable doses, resulting in a favorable clinical response in relapse/refractory MCL patients (15). When used in combination with letrozole, PD 0332991 more than tripled the progression-free survival of metastatic breast cancer patients treated with letrozole alone in a phase II clinical trial (16). The selectivity and reversibility of cell-cycle inhibition by PD 0332991 and its clinical efficacy suggest a unique opportunity to target specific phases of the cell cycle in cancer.

To advance mechanism-based targeting of CDK4/CDK6 in AML, we now show that induction of early G1 arrest by PD 0332991 inhibition of CDK4/CDK6 resulted in efficient synchronization of AML cells. After release of PD 0332991-induced early G1 arrest, AML cells progress synchronously into S-phase, thereby...
creating a defined time window, during which AML cells are poised for incorporation of Ara-C. Moreover, sequential PD 0332991 + Ara-C treatment resulted in a dramatic increase in cytotoxic killing of AML cells in vitro. At the molecular level, induction of early G1 arrest by PD 0332991 led to reduced expression of HOXA9 and the HOXA9-target gene Pim1. This resulted in a loss of inhibitory phosphorylation of BAD, and subsequent activation of BAD-mediated apoptosis in response to Ara-C. Our results suggest that the antitumor activity of PD 0332991 stems from both reversible G1 arrest that sensitizes AML cells to Ara-C–based therapy, and effective downregulation of HOXA9 expression that leads to the derepression of a proapoptotic response.

Materials and Methods

Cell culture and reagents

The human AML cell line HL-60 and the U937 histiocytic lymphoma with monocytic features were obtained from the ATCC. The cell lines were maintained in RPMI1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. The human primary CD34+ AML cells were purified using MACS CD34 MicroBeads (Miltenyi Biotec), and cocultured with mitomycin-treated HS-5 human stromal cells (17). The peripheral blood mononuclear cells were isolated using Ficoll Density Centrifugation (GE Healthcare Life Sciences). Cytarabine (Ara-C) was purchased from Sigma. PD 0332991 was obtained from Pfizer.

Cell-cycle analysis

HL-60 cells were incubated with PD 0332991, or PBS (vehicle control) for the indicated periods of time. 5-bromo-2-deoxyuridine (BrdUrd, 5 µg/mL; Sigma) was added to AML cells for 30 minutes and centrifuged. The cells were washed in ice-cold PBS and fixed in 70% ethanol. BrdUrd uptake was measured by flow cytometry as described (17, 18), using a FITC-anti-BrdUrd (Roche Diagnostics) monoclonal antibody. Subsequently, cells were stained with 0.05 mg/mL propidium iodide (Sigma Aldrich) and 0.1% RNase (Invitrogen) for 30 minutes at room temperature, and subjected to analysis by flow cytometry.

Western blot analysis

HL-60 cells (1.0 × 10⁶) were lysed in 0.5 mL of modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, and protease inhibitor mixture (Roche Diagnostics)]. The lysates were boiled in Laemmli sample buffer, and resolved by SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane, blocked with PBS, 0.1% Tween 20, and 5% BSA, and probed with a primary antibody overnight at 4°C. Subsequently, the blot was washed with PBS and 0.1% Tween 20, and specific antibody binding was detected with a horseradish peroxidase–coupled secondary antibody, followed by enhanced chemiluminescence (GE Healthcare) and exposure to film.

For immunoblotting, the following antibodies were used: β-actin (Santa Cruz Biotechnology), RB, phospho-RB (Ser 807/811), BAD, HOXA9 (Cell Signaling Technology).

Cell apoptosis and viability

Apoptotic and dead cells were analyzed by staining the cells with MitoTracker Red CMXRos (33 nmol/L; Invitrogen) to detect mitochondrial outer membrane permeabilization. Live cells were determined by Trypan blue exclusion in triplicate.

Quantitative real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen). The first-strand cDNA was synthesized from 500 ng of total RNA using Superscript III reverse transcriptase (Invitrogen). cDNA products were quantitated using the SYBR green fluorescence reagent on the ABI PRISM 7900 HT Sequence Detection System. Cycle threshold results were normalized to β-actin gene expression.

Establishment of myeloid leukemia xenografts and therapy

A total of 500 HL-60 cells stably expressing the HSV-TK-eGFP-luciferase fusion protein were injected via the lateral tail vein into NOD/SCID/IL-2Rγc−/− mice. The tumor distribution was followed by serial whole-body noninvasive imaging of visible light emitted by luciferase-expressing HL-60 cells upon injection of mice with luciferin. Seven days after tumor injection, the NOD/SCID/IL-2Rγc−/− mice with established myeloid leukemia were divided into four groups. PD 0332991 was dissolved in vehicle (50 mmol/L sodium lactate, pH 4.0) and was given daily by gavage at 150 mg/kg for 7 days indicated. Ara-C (1.6 or 10 mg/kg) was administered intraperitoneally as indicated. The control mice received the vehicle through the same route and on the same schedule. Survival times were compared with Kaplan–Meier survival analysis.

Ectopic expression of human HOXA9

To express human HOXA9, the HOXA9 retroviruses were produced by cotransfecting 293T cells with the PINCO-HOXA9 plasmid and the pAmpho and pQE plasmids (19). The PINCO retroviral vector was used as the control retrovirus. HL-60 cells were spin-infected with viral supernatants at 2,000 rpm for 2 hours at room temperature.

shRNA knockdown

To knockdown BAD, the pKLO.1 vector carrying BAD or the GFP control shRNA (Sigma) was cotransfected with pVSVG and Delta-8.9 plasmids into 293T cells to generate the desired shRNA lentivirus (17). Knockdown of each target was validated by quantitative real-time PCR (qRT-PCR) and immunoblotting at 72 hours after transduction.

Statistical analysis

For statistical analysis of the in vitro data, the Student t test was used. Kaplan–Meier analysis was used to determine the effect of treatment on the survival of the mice.

Results

Cell-cycle synchronization of AML cells by PD 0332991

Ara-C is a deoxynucleoside analog that is incorporated into replicating DNA only during the S-phase of the cell cycle. We posit that strategies to enrich asynchronously growing AML cells in S-phase will maximize the AML population poised for Ara-C incorporation, thereby increasing the efficiency of cytotoxic killing at a reduced Ara-C dose. To this end, PD 0332991 has been shown to be a selective and potent inhibitor for CDK4 and CDK6 in vitro and in vivo in animal models, and in a clinical trial of recurrent MCL (9–13, 15). We first assessed the impact of PD 0332991-dependent cell-cycle inhibition on enhancement of Ara-C cytotoxicity in
Cell-cycle synchronization by PD 0332991 enhances Ara-C killing of AML cells

Ara-C is converted to cytosine arabinoside triphosphate by deoxycytidine kinase (dCK), and to a lesser extent, by deoxyguanosine kinase, which elicits cytotoxicity when incorporated into replicating DNA in S-phase through termination of DNA strand elongation (20, 21). To determine whether S-phase synchronization sensitizes AML cells to Ara-C, HL-60 cells were arrested in G_1 by PD 0332991, and then released to fresh medium to allow synchronized entry into S-phase. Ara-C was administered at 8 hours after removal of PD 0332991 when maximal S-phase enrichment was achieved, and induction of apoptotic cell death was determined by the MitoTracker assay that measures the induction of early G_1 arrest by PD 0332991 followed by synchronous S-phase entry upon release of the G_1 block would allow more AML cells to incorporate Ara-C than those of asynchronous-growing cells, thereby enhancing the efficiency of cytotoxic killing (Fig. 1B). The human HL-60 AML cells were cultured in the presence of 0.5 μmol/L PD 0332991 or the DMSO vehicle. PD 0332991 completely inhibited CDK4/CDK6 within 24 hours, as indicated by the loss of CDK4/CDK6-specific phosphorylation of Rb on serine^807-811 (pRb), arrested the cell cycle in G_1, and marked reduction of DNA replication as measured by BrdUrd pulse labeling (Fig. 1C and E). After washout of PD 0332991 from the medium, HL-60 cells began to enter S-phase by 4 hours, with concomitant increase in pRb. By 8 hours, the majority of HL-60 cells were progressing into S-phase (Fig. 1C and E). Similar results were obtained in the U937 histiocytic lymphoma with acute monocytic leukemia morphology in response to PD 0332991 treatment and release (Supplementary Fig. S2). Thus, PD 0332991 effectively arrested AML cells in early G_1, and synchronized them in S-phase after PD 0332991 withdrawal.

Figure 1.
PD 0332991 synchronization enhanced cytotoxic killing of HL-60 promyelocytic leukemia cells by Ara-C. A, human purified CD34^+ cells from AML patients were cultured with mitomycin-treated HS-5 human stromal cells. Left, primary AML cells were cotreated with PD 0332991 (0, 0.5, and 1 μmol/L) and Ara-C (0, 1, and 5 μmol/L) for 24 hours and cell viability was determined by Trypan blue staining. Right, the same AML cells were treated with PD 0332991 (0, 0.5, and 1 μmol/L) for 24 hours to induce G_1 arrest and then cultured in fresh medium for 12 hours to allow re-entry into S-phase before the addition of Ara-C (0, 1, and 5 μmol/L). Cell viability was determined by Trypan blue staining after Ara-C 24-hour treatment. B, schematic diagram of the protocol for cell-cycle synchronization by reversible PD 0332991 arrest in G_1 and subsequent cytotoxic killing by Ara-C upon release into S-phase. C, HL-60 cells were cultured in 0.5 μmol/L PD 0332991 for 24 hours, then released into fresh media and harvested at the indicated time points. BrdUrd was added at each time point indicated for 30 minutes, followed by propidium iodide staining and FACS analysis of BrdUrd uptake (left) and DNA content (right). Numbers indicate the percentages of BrdUrd-positive S-phase cells. D, HL-60 cells were arrested by 0.5 μmol/L PD 0332991 and released into fresh medium. Ara-C (50 μmol/L) was added at 8 hours after PD 0332991 removal, and apoptosis induction was measured by loss of mitochondrial membrane potential using the Mito Tracker Red CMXRos assay (Invitrogen). The time course of PD 0332991 arrest, release, and Ara-C administration is indicated on the right. Numbers indicate the percentages of MT-negative cells. E, inhibition of CDK4/6-dependent Rb phosphorylation on serine^807/811 (pRb) as an indication of induction of early G_1 arrest 24 hours post-PD 0332991. Release of PD 0332991 arrest and S-phase entry was marked by rephosphorylation of Rb. pRb and overall Rb levels were detected by immunoblotting at the indicated time points by the anti-phospho-Rb and anti-Rb antibodies (Cell Signaling Technology). F, Actin was also measured as the loading control. G, HL-60 cells were arrested in early G_1 by PD 0332991 for 24 hours and released synchronously into S-phase for 8 hours. Ara-C was added at the indicated doses for 48 hours, and cell viability was determined by Trypan blue staining. These data are representative of three independent experiments; error bars, SD. * P < 0.05.
shown that cell-cycle synchronization of AML cells.

Reversible CDK4/CDK6 inhibition by PD 0332991 enhanced tumor suppression by Ara-C.

We next evaluated the antitumor effect of sequential PD 0332991 and Ara-C administration in disseminated AML xenografts in NOD/SCID/IL2Rγ−/− (NSG) mice. Previously, we have shown that cell-cycle synchronization in vivo can be achieved by timely administration of PD 0332991 that induced G1 arrest, followed by discontinuation of PD 0332991 to allow S-phase entry (14).

In this AML xenograft model, a total of 500 HL-60 cells stably expressing the HSV-TK-eGFP-luciferase transgenes (HL-60-LUC+) were injected via tail vein into NSG mice (5 groups, 5 mice per group) for induction of disseminated tumors, which were monitored by serial noninvasive bioluminescence imaging (BLI). PD 0332991 (150 mg/kg) was administered daily by gavage on days −6 to −4 after tumor induction to arrest cell cycle in G1. After 4 days of PD 0332991 withdrawal, which we previously determined to achieve maximal S-phase entry (14), Ara-C was administered intraperitoneally at low dose (1.6 mg/kg) or high dose (10 mg/kg) daily for 3 consecutive days. Tumor load was measured by BLI weekly between day 2 and day 23 (Fig. 2A). As a control, NSG mice with HL-60-LUC+ xenografts were treated either with PD 0332991 or Ara-C alone at low or high dose in parallel. Disseminated tumors developed by 14 days following the injection of HL-60-LUC+ cells (Fig. 2B and C).

A single regimen of Ara-C at a low or high dose had little or only marginal effect on the tumor load, whereas PD 0332991 alone initially suppressed the tumor growth (day 9), but was unable to sustain the inhibitory effect (days 16–23). Strikingly, the sequential PD 0332991–Ara-C treatment markedly reduced tumor load at all time points compared with the control groups (Fig. 2B). Notably, inhibition of tumor development by Ara-C at low dose (1.6 mg/kg) in PD 0332991-synchronized HL-60-LUC+ xenografts was 9 times greater on day 9 and 16 times greater on day 16 than in asynchronous HL-60-LUC+ xenografts (Fig. 2B and C). Although the PD 0332991–high Ara-C dose regimen decreased the tumor load further (Fig. 2B, compare the 3rd and 6th groups and Fig. 2C), mortality occurred, as did the single high-dose Ara-C treatment (Fig. 2D). In comparison, none of the mice treated with the combined PD 0332991–low-dose Ara-C regimen succumbed to death while on therapy, demonstrating improved survival over other treatment groups (Fig. 2B–D). Together, these results indicate that PD 0332991 synchronization markedly enhances the killing of AML cells at a significantly reduced Ara-C dose that was otherwise ineffective.

Cell-cycle–coupled regulation of HOXA9 expression mediates Ara-C killing.

Disregulation of the leukemogenic HOXA9 oncoprotein is frequently observed in AML and is associated with poor prognosis and therapy response (2–4). To elucidate the mechanisms that underlie cell-cycle enhancement of Ara-C killing, we investigated the PD 0332991 response of HOXA9. In prolonged early G1 arrest induced by 24 hours of PD 0332991 treatment, there was a sharp decline of both the mRNA and protein levels of HOXA9 (Fig. 3A and B), which coincided with dephosphorylation of RB (Fig. 1E).

The HOXA9 mRNA levels began to recover at 8 hours after the release of the G1 block when the majority of HL-60 cells were
entering S-phase, peaked at 12 hours (S–G2), and declined as cells entered the next G1 phase of the cell cycle (Fig. 3A, compare with Fig. 1A; Fig. 1E). The HOXA9 protein levels also began to recover at 8–12 hours after PD 0332991 removal in middle-late S-phase, which paralleled that of HOXA9 mRNA (Fig. 3B). However, even with the HOXA9 mRNA levels declining sharply between 12 and 24 hours, the HOXA9 protein continued to increase in G2–M, reaching the pretreatment level as cells entered the next G1 phase of cell cycle (Fig. 3B and 1A). These data indicated that the expression of HOXA9 mRNA, but not protein, is coupled to the cell cycle: low in G1 and G1–S transition, elevated in S-phase, and declined as the cells entered G2–M. The basis for the uncoupling of HOXA9 mRNA and protein expression is currently unknown, but induction of prolonged early G1 arrest likely led to perturbation of translational, posttranslational modification, or degradation pathways that governs the steady state levels of HOXA9 protein.

This led us to investigate whether the PD 0332991-mediated uncoupling of HOXA9 protein expression in S-phase contributes to enhanced Ara-C killing. HL-60 cells were infected with a recombinant PINCO-HOXA9 retrovirus to establish stable cell lines in which ectopic expression of HOXA9 did not fluctuate during the cell cycle (Fig. 3C). Despite the striking cell-cycle dependence of endogenous HOXA9 mRNA expression, and the ability of HOXA9 to stimulate proliferation of primitive hematopoietic cells (22–24), enforced expression of HOXA9 did not affect the kinetics of PD 0332991-dependent G1 cell-cycle arrest or the subsequent S-phase entry upon release of PD 0332991 (Fig. 3D).

HOXA9 is also known to have antiapoptotic activity (25). We therefore posit that the reduction of HOXA9 protein levels by prior PD 0332991-induced G1 arrest may influence the survival of synchronized HL-60 cells in response to Ara-C. Indeed, enforced expression of HOXA9 dramatically inhibited Ara-C–induced apoptosis, as determined by the reduction of mitochondrial permeabilization in Ara-C–treated HL-60 cells, more pronounced in PD 0332991–Ara-C combination treatment, as measured by the percentage of Mito Tracker-negative cells at the indicated time points. These data are representative of two independent experiments, and error bars indicate SD.
turn, Pim1 phosphorylates the proapoptotic protein BAD on Serine112, which inactivates BAD and attenuation BAD-mediated apoptosis (25). The PIM1 mRNA was reduced approximately 60%, in parallel with the reduction of HOXA9 mRNA and protein expression following prolonged early G1 arrest induced by PD 0332991 treatment for 24 hours (Fig. 4A). Ectopic expression of HOXA9 in HL-60 cells effectively prevented the reduction of PIM1 mRNA in PD 0332991-induced G1-arrested cells, consistant with PIM1 being a direct transcriptional target of HOXA9 (Fig. 4A; ref. 25). Knocking down BAD expression by shRNA, markedly increased the resistance of HL-60 cells to apoptosis induced by the PD 0332991–Ara-C combination therapy (Fig. 4B–E). Notably, the shGFP-expressing HL60 cells exhibited high basal level rates of apoptosis (15%) compared with the native HL60 cells (5%) under normal growth conditions (compare Figs. 4D and 1D), and the fold-increase of Ara-C–induced apoptosis was less pronounced upon exposure to Ara-C. Nevertheless, the survival of these PD 0332991-synchronized shGFP-HL60 cells was markedly reduced after Ara-C treatment. Together, these results provide compelling evidence that PD 0332991-induced sensitization of AML cells to Ara-C cytotoxicity is mediated, at least in part, by the reduction of HOXA9 and its transcriptional target PIM1, thereby enhancing the proapoptotic effect of BAD.

Discussion

The CDK4/CDK6-specific inhibitor PD 0332991 (palbociclib) is under intense investigation for its clinical efficacy in cell-cycle control and enhancing tumoricidal activity in combination therapy (9, 12, 14, 15, 26, 27). This study took advantage of the reversibility of PD 0332991 inhibition of CDK4 and CDK6 to synchronize AML cells in S-phase to maximize the percentage of AML cells poised for incorporating Ara-C into replicating DNA at a defined time point, thereby potentiating the tumoricidal activity of Ara-C. It is noteworthy that high-dose Ara-C in combination with anthracyclines remains the major induction chemotherapy for AML patients below 60 years of age (5). However, the median ages of the majority of AML patients at the time of diagnosis are typically between 65 and 70 years when they are less tolerant of the toxic effect of the intensive Ara-C regimen. As such, there is an urgent unmet need to develop novel therapies that effectively manage the leukemic disease for elderly AML patients.

Our studies demonstrated that PD 0332991-mediated enrichment of AML cells at the S-phase markedly enhances cytotoxic killing of AML cells at much reduced doses of Ara-C (Fig. 5). PD 0332991-synchronized HL-60 xenograft mice responded favorably to low dose Ara-C (1.6 mg/kg) in reducing tumor load and
induction of prolonged early G1 arrest and sensitization of cancer cytotoxic killing by induction of prolonged early G1 arrest. However, the delay in HOXA9 expression apparently contributes to cell-cycle sensitization to Ara-C killing.

Suppression of HOXA9 expression led to concomitant reduction of expression of the HOXA9-target Pim1 gene, and the subsequent derepression of phosphorylation of BAD, resulting in promotion of apoptosis. Indeed, enforced expression of HOXA9 restored Pim1 expression and attenuated the Ara-C–induced death of AML cells. Brumatti and colleagues recently reported that HOXA9 is also required to maintain the expression of BCL2, which is critical for the leukemogenic activity of HOXA9 in myeloid progenitors (28), although it is yet unclear whether HOXA9 acts directly on the transcription of BCL2, as seen with Pim1. HOXA9 appears essential for survival of HL60 cells, as silencing of HOXA9 by lentiviral shRNA led to massive cell death (Chen and Zhou, unpublished data). This precludes a direct assessment of how reduced HOXA9 expression may affect Ara-C–induced cell death. It is noteworthy that HL-60 cells with enforced expression of HOXA9 were not fully refractory to Ara-C, and prolonged treatment of Ara-C resulted in loss of viability, suggesting that the combined PD 0332991–Ara-C therapy disabled other survival mechanisms required for drug resistance. Future genome-wide interrogations of cellular pathways such as RNAi knockdown or enforced expression of cDNA libraries in AML cells will likely discover these new cellular pathways and targets that aid the design of more effective therapeutic strategies for AML.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C.A. Boyson, M.D. Liberto, X. Huang, D.C. Dorn, M.A.S. Moore, S. Chen-Kiang, P. Zhou Development of methodology: C. Yang, C.A. Boyson, M.D. Liberto, D.C. Dorn, M.A.S. Moore, P. Zhou Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Yang, C.A. Boyson, J. Hannah, D.C. Dorn Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Yang, C.A. Boyson, J. Hannah, D.C. Dorn, S. Chen-Kiang, P. Zhou Writing, review, and/or revision of the manuscript: C. Yang, C.A. Boyson, X. Huang, J. Hannah, M.A.S. Moore, S. Chen-Kiang, P. Zhou Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.A. Boyson, D.C. Dorn, S. Chen-Kiang Study supervision: D.C. Dorn, S. Chen-Kiang, P. Zhou Other (provided funding support): S. Chen-Kiang

Acknowledgments
The authors thank Gail J. Roboz and Monica L. Guzman for primary AML cells, members of the Chen-Kiang and Zhou labs for helpful discussions, and Kang Zhang for technical assistance.

Grant Support
This work was supported in part by an Investigator Initiated Research Project from Pfizer, the STARR Cancer Consortium grant #13-A162 and NIH grant RO1 CA 120531 (S. Chen-Kiang), the Gar Reichman Fund of the Cancer Research Institute (M.A.S. Moore), and NIH grants SR01 CA038210 and RO1 CA159925 (P. Zhou).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 9, 2014; revised December 23, 2014; accepted January 13, 2015; published OnlineFirst March 5, 2015.

Figure 5.
PD 0332991 sensitizes AML cells to Ara-C killing by at least two mechanisms: (i) S-phase synchronization for maximal Ara-C incorporation in replicating DNA and (ii) dismantling the HOXA9-dependent antiapoptotic pathway.

displayed overall improved survival (Fig. 2). The increased mortality in mice treated with high dose Ara-C (10 mg/kg), with or without PD 0332991 synchronization, further highlights the benefit of cell-cycle synchronization by PD 0332991 that markedly enhanced the tumoricidal effect of Ara-C at significantly reduced doses. These findings implicate that cell-cycle sensitization with PD 0332991 in combination with reduced dose of Ara-C may improve the treatment outcomes of AML patients who are otherwise ineligible to the current high-dose Ara-C regimen.

Sensitization of AML cells to Ara-C by PD 0332991 treatment likely results from alterations of multiple cellular signaling pathways, such as the DNA damage response and survival pathways that are secondary to induction of prolonged early G1 arrest. First, induction of prolonged early G1 arrest and sensitization of cancer cells to cytotoxic killing by PD 0332991 is due to inhibition of CDK4/CDK6 as it absolutely requires Rb, the substrate of CDK4 and CDK6 (14, 27). Second, induction of prolonged early G1 by PD 0332991 sensitizes cancer cells to cytotoxic killing by a partner agent by forcing an imbalance in genes expression. In prolonged early G1 arrest that exceeds the early G1 transit time by PD 0332991 inhibition of CDK4/CDK6, only genes programmed for early G1, and not other cell-cycle phase are expressed, and the cell-cycle–coupled gene expression is incompletely restored upon release from the G1 block (14). Consistent with reprogramming of cancer cells for cytotoxic killing by induction of prolonged early G1 arrest, PD 0332991 treatment alone (for 21 days) resulted in tumor regression and durable complete and partial responses in some recurrent MCL patients in the first single-agent phase I clinical trial (15).

At the mechanistic level, we observed a striking downregulation of the leukemogenic HOXA9 oncogene as one such response of AML cells to PD 0332991 treatment. Our studies established HOXA9 as a cell-cycle–coupled gene, with increased mRNA expression in S-phase over other cell-cycle phases. Surprisingly, while the HOXA9 protein level paralleled that of the mRNA level in G1, S-phase albeit with a delay, it did not follow the reduction of mRNA as cells exit S-phase. It is possible that the prolonged early G1 arrest induced by PD 0332991 altered the translational efficiency of HOXA9 or the posttranslational modification pathways that rendered HOXA9 refractory to degrada-
References


CDK4/6 Inhibitor PD 0332991 Sensitizes Acute Myeloid Leukemia to Cytarabine-Mediated Cytotoxicity

Chenyi Yang, Cynthia A. Boyson, Maurizio Di Liberto, et al.

Cancer Res 2015;75:1838-1845. Published OnlineFirst March 5, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2486

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/03/07/0008-5472.CAN-14-2486.DC1

Cited articles
This article cites 26 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/75/9/1838.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/75/9/1838.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.