Pharmacologic inhibition of CDK4/6: mechanistic evidence for selective activity or acquired resistance in acute myeloid leukemia

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Entry into the cell cycle is mediated by cyclin-dependent kinase 4/6 (CDK4/6) activation, followed by CDK2 activation. We found that pharmacologic inhibition of the Flt3 internal tandem duplication (ITD), a mutated receptor tyrosine kinase commonly found in patients with acute myelogenous leukemia (AML), led to the down-regulation of cyclin D2 and D3 followed by retinoblastoma protein (pRb) dephosphorylation and G1 cell-cycle arrest. This implicated the D-cyclin-CDK4/6 complex as a downstream effector of Flt3 ITD signaling. Indeed, single-agent PD0332991, a selective CDK4/6 inhibitor, caused sustained cell-cycle arrest in Flt3 ITD AML cell lines and prolonged survival in an in vivo model of Flt3 ITD AML. PD0332991 caused an initial cell-cycle arrest in well-established Flt3 wild-type (wt) AML cell lines, but this was overcome by down-regulation of p27Kip1 and reactivation of CDK2. This acquired resistance was not observed in a Flt3 ITD and a Flt3 wt sample from a patient with primary AML. In summary, the mechanism of cell-cycle arrest after treatment of Flt3 ITD AML with a Flt3 inhibitor involves down-regulation of cyclin D2 and D3. As such, CDK4/6 can be a therapeutic target in Flt3 ITD AML but also in primary Flt3 wt AML. Finally, acquired resistance to CDK4/6 inhibition can arise through activation CDK2.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease at both the cytogenetic and molecular levels. Many of these alterations have prognostic impact on clinical outcome, and some also dictate therapeutic approaches. Approximately 25% of patients express a constitutively active Flt3 internal tandem duplication (ITD), a mutant form of the receptor tyrosine kinase Flt3. The Flt3 ITD is a marker for poor prognosis. The availability of cell lines expressing Flt3 ITD as well as Flt3 inhibitors has made it possible to study the signaling pathways activated by mutant Flt3. Flt3 ITD signaling regulates a multitude of proteins involved in proliferation, differentiation, and survival, reflecting a complex signaling network that promotes oncogenesis. It has also been reported that the pharmacologic inhibition of Flt3 ITD leads to cell-cycle arrest, but the underlying molecular mechanism and functional significance of this observation remains unclear.

Progression through the cell cycle from G1 to S, G2, and M phases is initiated by cyclin-dependent kinase 4 (CDK4) and the highly homologous enzyme CDK6. CDK4 and CDK6 form a complex with one of their activating subunits, which are the cyclins D1, D2, and D3. The activity of CDK4/6 is negatively regulated by the INK4 proteins. The D-cyclin/CDK4/6 complex phosphorylates the retinoblastoma protein (pRb) and the related proteins p107 and p130, as well as Smad3. In addition to being catalytically active, the cyclin-CDK4/6 complexes can also sequester the cell-cycle inhibitors p21 and p27. This promotes the activation of the Cyclin E-CDK2 complex, which further phosphorylates pRb. Hyperphosphorylated pRb loses its inhibitory effect on the E2F transcription factor family. As a result of E2F activation, the transcription of genes promoting entry into S phase is initiated. Here we show that Flt3 ITD activates the cyclin D, CDK4/6, INK4, pRb pathway by up-regulating cyclin D2 and D3 gene expression and that this pathway may constitute a drug target in AML.

Materials and methods

Compounds

THRX-165724 was obtained from Theravance (South San Francisco, CA). PD0332991 was obtained from Pfizer (La Jolla, CA). For in vitro studies, both compounds were dissolved in dimethyl sulfoxide (DMSO; 10 mM stock solutions) and stored at −20°C.

Ribonuclease protection assay

The ribonuclease protection assay was performed with the RiboQuant hCYC-1 Multi-Probe Template Set (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

Cell culture

The THP-1 and U937 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The MV4-11 and MOLM13 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell lines were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin. Frozen samples from patients with AML were obtained from the Ohio State University Comprehensive Cancer Center.

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Leukemia Tissue Bank at The Ohio State University. Fresh CD34(+) cells were purchased from AllCells (Emeryville, CA). All studies with human specimens were performed with the approval of The Ohio State University Institutional Review Board.

**Immunoprecipitation and Western blot**

MV4-11 cells (1.0 × 10^7) were incubated in 5 mL RPMI 1640 medium plus the indicated concentration (Figure 1B) of THRX-165724 or PD0332991 (30-minute incubation). The cells were lysed in 0.75 mL of modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM NaVO_3, and protease inhibitor mixture [Roche Diagnostics, Indianapolis, IN]). The lysates were incubated with a F3H antibody (S-18; Santa Cruz Biotechnology, Santa Cruz, CA) and protein G beads (Sigma) overnight at 4°C. The immunocomplexes were recovered by centrifugation, washed with RIPA buffer, boiled in Laemmli sample buffer, and resolved by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a nitrocellulose membrane (Santa Cruz Biotechnology), blocked with phosphate-buffered saline (PBS), 0.1% Tween 20, and 3% bovine serum albumin, and probed with an anti-phosphotyrosine antibody (ECL; GE Healthcare, Chalfont St. Giles, United Kingdom) for 30 minutes at room temperature. The analysis was performed by flow cytometry.

**Cell cycle analysis**

Logarithmically growing cells were incubated with THRX-165724, PD0223991, or DMSO (vehicle control) for the indicated periods of time. The cells were washed in ice-cold PBS and fixed in 70% ethanol. Subsequently, the cells were stained with propidium iodide staining buffer (0.05 mg/ml propidium iodide [Sigma-Aldrich] and 0.1% RNase [Invitrogen]) for 30 minutes at room temperature. The analysis was performed by flow cytometry.

**Real-time PCR**

cDNA was synthesized from 500 ng of total RNA using Superscript III reverse transcriptase (Invitrogen) with oligo dT and p27^ gyr^ gene-specific primers. cDNA products were quantitated by using SYBR green fluorescence on an AB7000 thermal cycler (Applied Biosystems, Foster City, CA). Primer sets for mRNA quantitation included 2 different exon spanning primer sets. Cycle threshold results were normalized to β-Actin gene expression. p27^ gyr^ primer pair A: 913 forward, GGGAATACGAGCGACCGTCGCA; 1026 reverse, CGTTCTGCTCCACAGAAGCC. Primer pair B: 1029 forward, CAAAGAAGCTGGCCTCAAGAAG; 1176 reverse, CCAATCTCAAGTCACCGGT. Actin primer pair: forward, CCTGGAACCCAGCAAT; reverse, GCCGATCACCAGAGAGTAC.

**Phospho-pRb analysis in primary bone marrow cells**

Bone marrow cells (5 × 10^6) per mouse were treated with Fix Buffer I (BD Biosciences) for 10 minutes at 37°C and permeabilized with Perm Buffer III (BD Biosciences) for 30 minutes on ice. After washing the cells twice with staining buffer, they were stained with phycoerythrin (PE)-mouse anti-phospho pRb (pSer780) (BD Biosciences) and allophycocyanin (APC)–mouse antihuman CD45 (BD Biosciences). PE- and APC-conjugated mouse isotype antibodies served as negative controls in the subsequent analysis by flow cytometry.

**Immunohistochemistry**

Sections of formalin-fixed, decalcified, paraffin-embedded tissues were mounted on Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were dewaxed and antigen retrieval (TRIS solution [DAKO North America, Carpinteria, CA], 20 minutes in steamer, 20 minutes cooling to room temperature) was performed. Peroxidase and protein blocking was carried out using blocking solutions from DAKO as directed. Primary antibodies were applied for 30 minutes at dilutions of 1:100 for phospho-pRb (pSer870/871) (BD Biosciences) and allopbyocyanin (APC)–mouse antihuman CD45 (BD Biosciences). PE- and APC-conjugated mouse isotype antibodies served as negative controls in the subsequent analysis by flow cytometry.

**Animal survival studies**

All mouse experiments were performed with the approval of the Institutional Animal Care and Use Committee at The Ohio State University. Twenty nonobese diabetic (NOD)/severely compromised immunodeficient (SCID) mice (age, 12 weeks) were conditioned with 300 rads of total body irradiation and immediately injected with 6 × 10^6 log-phase MOLM-13 cells via the lateral tail vein. The mice were divided randomly into 2 groups and dosed once daily with PD0332991 (150 mg/kg, 10 μL/g) or an equivalent volume of vehicle (lactic acid buffer, pH 4.0). Dosing was performed by oral gavage starting on day 6 after engraftment. Survival times were compared with the logrank test using Prism (GraphPad Software, San Diego, CA).

**Kinase assay**

Cells were treated with DMSO or PD0332991 (500 nM) for 24 or 96 hours. The cells were centrifuged, washed with ice-cold PBS, and then lysed for 10 minutes in ice-cold lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Roche Diagnostics) and 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM β-glycerophosphate. Lysates were cleared by centrifugation at 13,000g for 5 minutes at 4°C. The protein concentration of the cleared lysates was determined by the bicinchoninic acid protein assay. The immunoprecipitation was performed using the in-house spreadsheet according to the median-effect method introduced by Chou and Talalay and as described previously.

**Cell proliferation and viability**

Single agent effects on cell proliferation and viability were assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics). The drug combination studies were also performed using the MTT assay. The analysis was executed with an in-house spreadsheet according to the median-effect method introduced by Chou and Talalay and as described previously.
Results
THRX-165724 and SU14813 Were Potent Inhibitors of Flt3

To study Flt3 ITD signaling, we chose 4 AML cell lines as a model system: THP-1 and U937, which express Flt3 wild type (wt), and MV4-11 and MOLM13, which express Flt3 ITD. THRX-165724 and SU14813,24 inhibitors of the platelet-derived growth factor receptor family, which includes Flt3, were used as pharmacologic tools (Figure 1A).

To characterize the activity and potency of THRX-165724, we first investigated the compound’s effect on Flt3 autophosphorylation. AML cell lines expressing Flt3 wt (THP-1) and Flt3 ITD (MV4-11) were incubated with increasing concentrations of THRX-165724. In THP-1 cells, Flt3 autophosphorylation was stimulated by the addition of Flt3 ligand (FL). In MV4-11, Flt3 ITD is constitutively phosphorylated and therefore did not require activation with FL. Flt3 was immunoprecipitated, and an immunoblot with an anti-phosphotyrosine antibody was performed. The experiment revealed that THRX-165724 is an equipotent inhibitor of Flt3 wt and Flt3 ITD autophosphorylation with an half-maximal inhibitory concentration (IC50) of approximately 30 nM (Figure 1B). Using the MV4-11 cell line, we found that the IC50 of SU14813-mediated inhibition of Flt3 autophosphorylation in this assay was approximately 10 nM (Figure 1B). An MTT assay was performed to investigate the effect of THRX-165724 and SU14813 on proliferation and cell viability. Both compounds affected MV4-11 and MOLM13, the 2 cell lines with Flt3 ITD. The IC50 in this assay was 50-100 nM for THRX-165724 and 10-20 nM for SU14813. In contrast, THP-1 and U937 cells, which express Flt3 wt, were not affected by either THRX-165724 or SU14813 up to 5 μM (Figure 1C). The effect caused by THRX-165724 and SU14813 is a result of apoptosis because MOLM13 and MV4-11 cells (Flt3 ITD) became positive for the apoptosis marker Annexin V, whereas U937 and THP-1 cells (Flt3 wt) did not (Figure 1D). To learn whether the induction of apoptosis by THRX-165724 and SU14813 is reversible, we performed a drug wash-out experiment. This experiment revealed that the removal of THRX-165724 or SU14813 after a 24-hour incubation period rescues the cells from cell death (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). In summary, these experiments demonstrate that THRX-165724 and SU14813 are potent inhibitors of Flt3 autophosphorylation that can induce programmed cell death in AML cells with Flt3 ITD.

The inhibition of Flt3 ITD led to the down-regulation of cyclin D2 and D3

It has been reported that the inhibition of Flt3 ITD signaling affects the cell cycle before inducing apoptosis.11,12 We investigated the effect of THRX-165724 and SU14813 on the cell cycle by incubating MOLM13, MV4-11, THP-1, and U937 cells with the inhibitors for 24 hours. THRX-165724 as well as SU14813 induced a specific G1 cell-cycle arrest in MOLM13 and MV4-11 (Flt3 ITD) but not in THP-1 and U937 (Flt3 wt) (Figure 2A). The cell-cycle arrest was reversible. MOLM13 and MV4-11 cells were able to re-enter the cell cycle after an incubation with THRX-165724 and SU14813 for 24 hours followed by a wash-out of the inhibitors (Figure S2). To gain insight into the cell-cycle genes controlled by Flt3 ITD signaling, we isolated total RNA from MV4-11 cells.
that had been treated with THRX-165724 or DMSO (vehicle control) for 3 hours. This relatively short incubation time is expected to identify changes in the expression of genes that are directly downstream of Flt3 ITD signaling. A ribonuclease protection assay was performed using various sets of probes for genes involved in proliferation and the cell cycle. Using this approach we observed significant down-regulation of cyclin D2 and D3 transcript levels after treating the cells with THRX-165724 (Figure 2B).

As shown by Western blotting, the change in gene expression level correlates with a change in cyclin D2 and D3 protein level (Figure 2C). After 4 hours of treatment with THRX-165724, cyclin D2 and D3 are greatly reduced. Cyclin D1 protein could not be detected by Western blot. It follows that the reduction of cyclin D2 and D3 protein leads to the reduction of CDK4/6 activity.25 pRb is known to be hyperphosphorylated when CDK4/6 is active, so the loss of CDK4/6 activity leads to the dephosphorylation of pRb,25 which is reflected in a shift of the apparent molecular weight of the protein. We found that at 4 hours after the start of THRX-165724 treatment, as cyclin D2 and D3 levels decrease, pRb phosphorylation also decreases. At 12 hours, when cyclin D2 and D3 levels are very low, pRb is almost completely dephosphorylated. The THRX-165724 mediated down-regulation of cyclin D2 and D3 as well as the dephosphorylation of pRb was also observed in Flt3 ITD MOLM13 cells (Figure S3). THRX-165724 had no effect on the D-cyclin protein level and pRb phosphorylation status in the Flt3 wt cell lines THP-1 and U937 (Figure 2D). Like THRX-165724, the second Flt3 inhibitor, SU14813, induced the down-regulation of cyclin D2 and D3 as well as pRb dephosphorylation in MV4-11 and MOLM13 (Flt3 ITD) but not in THP-1 and U937 (Flt3 wt) (Figure 2D). These data suggest that CDK4/6 is activated by Flt3 ITD signaling through the up-regulation of cyclin D2 and D3 and that inhibition of the Flt3 ITD inhibits activation of CDK4/6.

**PD0332991 was a potent CDK4/6 inhibitor**

To evaluate the role of CDK4/6 in the AML cell lines, we used PD0332991, a small molecule inhibitor of CDK4/6 that is currently in phase II clinical trials (Figure 3A). PD0332991 has little or no activity against a panel of 30 kinases, including CDK2, suggesting that this inhibitor is highly selective for CDK4/6.26,27 It is noteworthy that we found 500 nM PD0332991 to have no effect on Flt3 ITD autophosphorylation, which makes this compound a suitable tool to study CDK4/6 in our AML model system (Figure 3B). We incubated Flt3 ITD+, MV4-11, and MOLM13 cells with increasing concentrations of PD0332991. A Western blot of cell lysates showed decreasing pRb phosphorylation, confirming CDK4/6 inhibition by PD0332991 (Figure 3C). The IC50 of PD0332991 in this assay in both cell lines was 150 nM. We next tested the effect of PD0332991 on the cell cycle of our model cell lines after incubation periods of 24 and 120 hours. Figure 4A demonstrates that MOLM13 cells (Flt3 ITD positive) are strongly arrested in G1 at 24 and 120 hours. Furthermore, a sub-G1
peak at 120 hours suggests that some cells are undergoing apoptosis. In contrast, U937 cells (Flt3 wt) were arrested in G1 at the 24-hour time point, but these cells had re-entered the cell cycle after 120 hours. Like MOLM13, the Flt3 ITD positive MV4-11 cell line was arrested in G1 at 24 and 120 hours whereas Flt3 wt THP-1 cells were arrested in G1 at the 24-hour time point but had re-entered the cell cycle at 120 hours (Figure 4B). However, despite re-entering the cell cycle, PD0332991 treated THP-1 and U937 were still showing significantly fewer cells in S and G2 of the cell cycle than the respective control cells. As expected, the effect of PD0332991 on the cell cycle also led to an effect on proliferation (Figure 4C). MOLM13 and MV4-11 cells incubated with PD0332991 as a single agent for 8 days decreased in cell number, and although THP-1 and U937 cell numbers increased, the increase was considerably smaller than for the same cells incubated with the vehicle control. Hence, the inhibition of entry into the cell cycle and proliferation in these well-established Flt3 wt AML cell lines is only partial, consistent with an acquired mechanism of resistance to PD0332991 after prolonged exposure as a single agent.

We next tested whether treatment of the 4 AML model cell lines with PD0332991 can enhance the effect of the Flt3 inhibitor SU14813. In an MTT assay, the 4 AML cell lines as well as primary blasts from patient 17 (Flt3 ITD) and patient 75 (Flt3 wt) were incubated for 3 days with DMSO (vehicle control), PD0332991 (500 nM), or a combination of the compounds. PD0332991 strongly enhanced the effect of SU14813 in MOLM13 and MV4-11 (both Flt3 ITD) and to a lesser degree in cells from patient 17 (Flt3 ITD). Neither SU14813 nor PD0332991 alone or in combination had a significant effect on THP-1 and U937 (Flt3 wt) cells or cells from patient 75 (Flt3 wt) (Figure 4D). A median effect analysis according to the method of Chou and Talalay21 was performed to determine whether the enhanced activity of the drug combination in MV4-11 and MOLM13 was synergistic. We found that at 50 nM PD0332991 plus 25 nM SU14813, concentrations close to the IC_{50} values of these compounds in the 2 cell lines, the resulting combination index (CI) is 0.78 for MV4-11 and 0.67 for MOLM13. A CI value of less than 0.9 is defined as synergistic activity; hence, PD0332991 and SU14813 act synergistically in this assay.

PD0332991 also strongly enhanced the proapoptotic activity of SU14813 in MOLM13 and MV4-11 as assayed by Annexin V and 7-AAD staining. In contrast to the cell lines, in primary blasts from patient 17 (Flt3 ITD), the combination of PD0332991 and SU14813 did not increase in Annexin V staining compared with SU14813 alone. In an MTT assay, the 4 AML cell lines as well as primary blasts from patients 17 (Flt3 ITD) and 75 (Flt3 wt) were incubated in triplicate with DMSO (vehicle control), THRX-165724 (500 nM), and PD0332991 (500 nM) for 30 minutes.

**Figure 3.** PD0332991 is a potent inhibitor of CDK4/6 but does not inhibit Flt3 ITD. (A) Structure of PD0332991. (B) MV4-11 cells were incubated with DMSO (vehicle control), THRX-165724 (500 nM), and PD0332991 (500 nM) for 30 minutes. The cells were lysed, Flt3 ITD was immunoprecipitated, and an immunoblot was performed with an anti-phosphotyrosine antibody. The blot was subsequently stripped and reprobed with anti-Flt3. (C) Western blot. MV4-11 and MOLM13 cells were incubated with increasing concentrations of PD0332991 for 16 hours. Lysates were resolved by SDS-PAGE followed by immunoblotting with anti-pRb.
CDK2 is activated by p27Kip1 down-regulation

To investigate whether the differential effect of PD0332991 on the cell cycle of MV4-11 and MOLM13 (both Flt3 ITD) versus THP-1 and U937 (both Flt3 wt) is reflected in pRb phosphorylation status, we performed a Western blot. After 24 hours of exposure to PD0332991, pRb is completely dephosphorylated in all cell lines (Figure 5A). The complete dephosphorylation of pRb at 24 hours correlates well with the strong inhibitory effect on the cell cycle at this time point in all AML cell lines (Figure 4B). After 5 days of incubation with PD0332991, pRb is still completely dephosphorylated in the MOLM13 and MV4-11 Flt3 ITD AML cell lines. In contrast, pRb is partly phosphorylated again in the Flt3 wt U937 and THP-1 cell lines (Figure 5A), reflecting the reentry into the cell cycle as was detected by flow cytometry (Figure 4B).

To explore the possibility that CDK2 activity can compensate for the loss of CDK4/6 activity in THP-1 and U937 cells, we investigated the expression level of proteins involved in CDK2 regulation. After a 24-hour incubation period with PD0332991, neither p27Kip1 nor Cyclin E protein levels changed significantly (Figure 5B). However, CDK2 protein was slightly down-regulated in all 4 cell lines. It is noteworthy that after 4 days of incubation with PD0332991, the CDK2 inhibitor p27Kip1 was strongly down-regulated in Flt3 wt THP-1 and U937. Furthermore, in U937 Cyclin E was up-regulated. In contrast, no significant changes in the protein level of p27Kip1 and Cyclin E were observed in Flt3 ITD MOLM13 and MV4-11 (Figure 5B). These data led us to hypothesize that after treatment with PD0332991 for multiple days, the selective down-regulation of p27Kip1 in Flt3 wild-type THP-1 and U937 cells is responsible for higher CDK2 activity than is seen in MOLM13 and MV4-11 cells. To test this hypothesis, we performed a CDK2 in vitro kinase assay with cells treated for 24 and 96 hours with PD0332991 (Figure 5C). After treatment for 24 hours, CDK2 kinase activity was reduced in all cell lines, suggesting that CDK2 activation is dependent on CDK4/6 activity at this early time point. At the 96-hour time point, however, CDK2 activity in THP-1 and U937 cells was restored.

p27Kip1 transcription can be promoted by the transcription factor Foxo3a and the phosphorylation of Foxo3a leads to its inactivation.28-31 We found, however, no changes in Foxo3a phosphorylation status in any of the 4 AML cell lines treated for 96 hours with PD0332991 (Figure 5D). We used mRNA prepared from cells of the same experiment to perform a real-time PCR analysis to detect changes in p27Kip1 transcript levels. We applied 2 different sets of p27Kip1-specific primers for this experiment. This experiment showed that p27Kip1 mRNA is modestly reduced between 2- and 3-fold in THP-1, U937, and MOLM13 cells treated with PD0332991. There was no significant change of p27Kip1 transcript levels in MV4-11 cells.

Primary AML cells are sensitive to PD0332991

To study the effect of CDK4/6 inhibition on primary cells, we used 2 AML patient samples and CD34+ hematopoietic progenitor cells from a healthy donor. Patient 28 expressed Flt3 wt, whereas patient 82 expressed Flt3 ITD. Figure 6A,B demonstrates that PD0332991 alone. In fact, Annexin V staining was somewhat reduced in the combination treatment. THP-1 and U937 cells, as well as cells from patient 75, showed no enhanced Annexin V staining in response to any of the compound treatments. The level of induction of apoptosis is depicted in a bar graph representation in Figure S4.
treatment for 4 days significantly reduced patient cells that are in S or G2 of the cell cycle. The effect of PD0332991 on the cell cycle of CD34+/H11001 cells is less pronounced. PD0332991 treatment for 4 days leads to the almost complete dephosphorylation of pRb in cells of patient 82 (Flt3 ITD) (98% of pRb is dephosphorylated) and strong dephosphorylation of pRb in cells from patient 28 (Flt3 wt) (68% of pRb dephosphorylated). In contrast, CD34+/H11001 cells show only 34% of pRb in its dephosphorylated form after treatment with PD0332991 (Figure 6C). A CDK2 in vitro kinase assay revealed that the treatment with PD0332991 for 4 days greatly reduces CDK2 activity in the 2 patient samples but not in the CD34+ cells (Figure 6D). The reduction in 32P incorporation into the CDK2 substrate histone H1 in this assay was 50% for patient 28 and 39% for patient 82, but only 3% in CD34+ cells (Figure 6E). Hence, in contrast to the 2 well-established Flt3 wt cell lines THP-1 and U937, which display autonomous growth ex vivo, the 2 primary AML patient samples, regardless of the Flt3 status, displayed strong sensitivity to PD0332991.

**PD0332991 is active in a chimeric mouse-human model of AML**

In the first mouse experiment we tested whether PD0332991 could induce the dephosphorylation of pRb in vivo. NOD/SCID mice engrafted with MOLM13 were treated 24 and 4 hours before sacrificing with PD0332991 or buffer (vehicle control) by oral gavage. The bone marrow cells were isolated and stained with propidium iodide, and cell-cycle analysis was performed by flow cytometry. The effect of PD0332991 on the cell cycle of CD34+ cells is less pronounced. PD0332991 treatment for 4 days leads to the almost complete dephosphorylation of pRb in cells of patient 82 (Flt3 ITD) (98% of pRb is dephosphorylated) and strong dephosphorylation of pRb in cells from patient 28 (Flt3 wt) (68% of pRb dephosphorylated). In contrast, CD34+ cells show only 34% of pRb in its dephosphorylated form after treatment with PD0332991 (Figure 6C). A CDK2 in vitro kinase assay revealed that the treatment with PD0332991 for 4 days greatly reduces CDK2 activity in the 2 patient samples but not in the CD34+ cells (Figure 6D). The reduction in 32P incorporation into the CDK2 substrate histone H1 in this assay was 50% for patient 28 and 39% for patient 82, but only 3% in CD34+ cells (Figure 6E). Hence, in contrast to the 2 well-established Flt3 wt cell lines THP-1 and U937, which display autonomous growth ex vivo, the 2 primary AML patient samples, regardless of the Flt3 status, displayed strong sensitivity to PD0332991.

**Discussion**

This study began with a focus on the molecular basis by which pharmacologic inhibition of Flt3 ITD autophosphorylation antagonizes the entry into the cell cycle. We found that the inhibition of Flt3 ITD signaling leads to a significant down-regulation of cyclin D2 and D3 gene and protein expression levels, thus affecting CDK4/6 activity as reflected in the dephosphorylation of pRb. Hence, CDK4/6 is a downstream effector of Flt3 ITD-mediated oncogenic pathways.

To study the role of CDK4/6 in AML with Flt3 ITD as well as in AML with Flt3 wt, we obtained PD0332991, a selective CDK4/6 inhibitor with activity in model systems for multiple myeloma, mantle-cell lymphoma, and rhabdomyosarcoma.32-34 In our AML...
model system, PD0332991 established a tight and sustained cell-cycle block in the Flt3 ITD MV4-11 and MOLM13 cell lines but only a transient cell-cycle block in Flt3 wt THP-1 and U937. In contrast to MV4-11 and MOLM13 cells, THP-1 and U937 cells were able to rephosphorylate pRb. The molecular basis for the rephosphorylation of pRb in THP-1 and U937 is probably the result of the observed reactivation of CDK2 activity. This reactivation may be caused by p27Kip1 down-regulation, which we found to be significant in U937 and THP-1 cells after PD0332991 treatment for 4 days. The down-regulation of p27Kip1 could occur post-translationally or at the transcriptional level. p27Kip1 gene transcription can be controlled by the Forkhead transcription factor Foxo3a (FKHR-L1), which is inactivated by phosphorylation. We observed no increase in Foxo3a phosphorylation in THP-1 or U937 in response to PD0332991, which suggests that a decrease in Foxo3a transcriptional activity is not involved in the down-regulation of p27Kip1 protein. It is noteworthy, however, that in measuring p27Kip1 mRNA levels by real-time RT-PCR, we observed a reduction of p27Kip1 gene expression in MOLM13, THP-1, and U937 in response to PD0332991. The changes in p27Kip1 transcript levels were modest, approximately 2- to 3-fold. The reduction in p27Kip1 gene transcription in THP-1 and U937 cells could account for the reduction in p27Kip1 protein levels. However, because a similar reduction in p27Kip1 gene expression without the concomitant drop in protein is observed in MOLM13, additional post translational effects in THP-1 and U937 may contribute to the PD0332991-mediated reduction in p27Kip1 levels.

The ability to compensate at least partially for the pharmacologic inhibition of CDK4/6 by using CDK2 may be one way for established AML cell lines to escape the antiproliferative effect of an inhibitor such as PD0332991. We found it encouraging, however, that both the Flt3 ITD+ and the Flt3 wt primary AML samples that we tested were sensitive to PD0332991 and did not up-regulate CDK2 activity after prolonged exposure. In the Flt3 ITD primary AML sample, the observed cell-cycle arrest, the pronounced dephosphorylation of pRb, and CDK2 down-regulation in response to CDK4/6 inhibition are consistent with the observations noted in Flt3 ITD AML cell lines. An identical pattern of cell-cycle arrest and CDK2 down-regulation observed in the Flt3 wt primary AML patient sample was in contrast to that observed in the well-established Flt3 wt AML cell lines. However, the Flt3 wt patient sample and Flt3 wt AML cell lines were similar with respect to the observation that pRb dephosphorylation was less complete than in Flt3 ITD cells. The dephosphorylation of pRb in the Flt3 wt patient cells was 68% versus 98% in the Flt3 ITD patient cells.

The partial dephosphorylation of pRb in CD34+ cells (only 34% of pRb is dephosphorylated) could be due to incomplete inhibition of CDK4/6 by PD0332991. However, because we treated the CD34+ cells in parallel and under the same conditions as the strongly responding primary AML cells, we believe that the basis for the remaining pRb phosphorylation in CD34+ cells is a result of compensatory CDK2 activity that we were able to measure by an in vitro kinase assay.

Finally, an MTT assay and Annexin V staining demonstrated that the inhibition of CDK4/6 by PD0332991 strongly enhanced the activity of the Flt3 inhibitor SU14813 in the AML cell lines MV4-11 and MOLM13 (both are Flt3 ITD positive). In contrast to SU14813, PD0332991 as a single agent did not induce Annexin V staining in the 3-day incubation period of this experiment. The enhancement of SU14813 activity by PD0332991 was also observed in blasts from a patient with Flt3 ITD in the MTT assay but...
not by Annexin V staining. In fact, Annexin V staining was slightly reduced in cells treated with the drug combination compared with cells treated with SU14813 alone. The improved efficacy of the drug combination in the MTT proliferation/viability assay but not in the Annexin V apoptosis assay may indicate that in primary cells with Flt3 ITD, the advantage of a P0D332991/SU14813 combination treatment may result from a tighter cell-cycle block and not necessarily from enhanced apoptosis as observed in cell lines.

In summary, we provide evidence that CDK4/6 is a downstream effector of Flt3 ITD signaling. The inhibition of CDK4/6 alone or in combination with inhibition of Flt3 ITD may have a therapeutic effect in AML. However, resistance to CDK4/6 inhibition may arise through down-regulation of p27Kip1 and CDK2 activation.

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Authorship

Contribution: L.W. conducted research and analyzed data; J.W. conducted research and analyzed data; B.W.B. designed and conducted research and analyzed data; A.-M.D. conducted research and analyzed data; D.K. conducted research and analyzed data; T.L. conducted research and analyzed data; M.A.C. analyzed data and wrote the manuscript; R.B. designed research, analyzed data, and wrote the manuscript. L.W. and J.W. contributed equally to this work.

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