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

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Running Title: Pharmacologic inhibition of CDK4/6 in AML

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Abstract

Entry into cell cycle is mediated by CDK4/6 activation, followed by CDK2 activation. We found that pharmacological inhibition of the Flt3 internal tandem duplication (ITD), a mutated receptor tyrosine kinase commonly found in AML patients, led to the downregulation of Cyclin D2 and D3 followed by 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 PD 0332991, 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. PD 0332991 caused an initial cell cycle arrest in well-established Flt3 wildtype (wt) AML cell lines, but this was overcome by downregulation of p27Kip and reactivation of CDK2. This acquired resistance was not observed in a Flt3 ITD and a Flt3 wt primary AML patient sample. In summary, the mechanism of cell cycle arrest following treatment of Flt3 ITD AML with a FLT3 inhibitor involves downregulation 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 of CDK2.
Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease at both the cytogenetic and molecular level. 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/G0 to S, G2 and M is initiated by 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 D-CDK4/6 complexes can also sequester the cell cycle inhibitors p21Cip1 and p27Kip1. 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 the Flt3 ITD activates the Cyclin D, CDK4/6, INK4, pRb pathway by upregulating 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, Inc (South San Francisco, CA). PD 0332991 was obtained from Pfizer, Inc (La Jolla, CA). For in vitro studies, both compounds were dissolved in 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 Biosciences Pharmingen) according to the manufacturer’s instructions.

Cell Culture. The following cell lines were obtained from the American Type Culture Collection (ATCC): THP-1 and U937. 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 RPMI1640 (GIBCO/BRL) supplemented with 10% FBS, 100 units/ mL penicillin and 100 units/ mL streptomycin. Frozen AML patient samples were obtained from the Leukemia Tissue Bank at The Ohio State University. Fresh CD34(+) cells were purchased from AllCells (Berkeley). All studies with human specimens were performed with the approval from the Ohio State University Institutional Review Board.

Immunoprecipitation and Western Blot. 1.0 x 10⁷ MV4-11 cells were incubated in 5 mL RPMI plus the indicated concentration of THRX-165724 or PD 0332991 (30 minute incubation). The cells were lysed in 0.75 mL of modified RIPA buffer [50 mM Tris-HCl, ph 7.4/ 1% Nonidet P-40/ 150 mM NaCl/ 1 mM EDTA/ 1 mM Na₃VO₄ and protease inhibitor mixture (Roche)]. The lysates were incubated with a Flt3 antibody (S-18, 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 SDS/PAGE. The proteins were transferred to a nitrocellulose membrane (Santa Cruz, CA), blocked with PBS/ 0.1% Tween20/
3% BSA, and probed with an anti-phospho-tyrosine antibody (4G10, Upstate Biotechnology, NY) overnight at 4°C. Subsequently, the blot was washed with PBS/ 0.1% Tween 20 and specific antibody binding was detected with a horseradish peroxidase-coupled secondary antibody, followed by enhanced chemiluminescence (ECL, Amersham Biosciences) and exposure to film. To reprobe the blot, the primary antibody was stripped with ImmunoPure IgG Elution Buffer (Pierce).

For Western blots not involving immunoprecipitation, cells were centrifuged, the pellet was resuspended in PBS (10 µL/ 1X10⁵ cells) and an equal volume of 2X Laemmli sample buffer was added to lyse the cells. The lysate was heated at 95°C for 10 minutes followed by centrifugation at 13,200 rpm. Typically, 20 µl of lysate were loaded on a gel for SDS/PAGE. The proteins were transferred to a nitrocellulose membrane as described above. For immunoblotting, the following antibodies were used: γ-Tubulin (Santa Cruz), β-actin (Sigma), Cyclin D2 (Cell Signaling), Cyclin D3 (Cell Signaling), pRb (4H1, Cell Signaling), CDK2 (Santa Cruz), CDK4 (Cell Signaling), CDK6 (Cell Signaling), Cyclin E (M-20, Santa Cruz), p27Kip1 (Cell Signaling), Grb2 (Cell Signaling), Foxo3a (Cell Signaling), Phospho-Foxo3a (Thr32) (Cell Signaling).

Using the pRb signal on the film of a Western Blot, densitometry was used to quantify the percentage of dephosphorylated pRb versus total pRb.

**Kinase Assay.** Cells were treated with DMSO or PD 0332991 (500 nM) for 24 hours 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, 0.5% NP40) which contained a protease inhibitor cocktail (Roche Diagnostics) and 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycerophosphate. Lysates were cleared by centrifugation at 13,000 x g for five minutes at 4°C. The protein concentration of the cleared lysates was determined by the BCA protein assay (Pierce). For immunoprecipitation 200 µg of total protein from each lysate
was brought to 600 µl with lysis buffer. Antibodies were added to each lysate at a concentration of 1µg. The immunoprecipitation was performed at 4°C for 1 hour. Immunocomplexes were captured by Protein G Agarose beads (Invitrogen). Agarose beads were collected and washed three times with kinase buffer (50 mM Tris pH7.5, 10 mM MgCl₂, 1 mM DTT). CDK2 immunoprecipitates were incubated in 30 µl of kinase buffer with 5 µg histone H1 and 10 µCi [γ-32P]-ATP (3000 Ci/mmol, Amersham) for 30 min at 30˚C. After addition of Laemmli sample buffer, samples were analyzed by SDS-PAGE followed by Western Blot transfer. Phosphorylated histone H1 was visualized by autoradiography.

**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-diphenyltetrazolium bromide (MTT) assay (Roche). 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 Talalay²² and as described previously²³.

**Flow Cytometry.** Cell Cycle Analysis: Logarithmically growing cells were incubated with THRX-165724, PD 0223991 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 PI staining buffer (propidium iodide 0.05 mg/ml, Sigma-Aldrich, 0.1% RNase, Invitrogen) for 30 min at room temperature. The analysis was performed by flow cytometry.

**Real-Time PCR.**
cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase (Invitrogen) with oligo dT and p27Kip1 gene specific primers. cDNA products were quantitated by using SYBR green fluorescence on an AB7000 thermal cycler (Applied
Biosystems). Primer sets for mRNA quantitation included two different exon spanning primer sets. Cycle threshold results were normalized to β-Actin gene expression.

p27Kip1 primer pair A: 913F: GGAATAAGGAAGCGACCTGCA;
1026R: CGTCTGCTCCACAGAACC. Primer pair B:
1029F: CAAGAAGGCCTGGCCTACAAG; 1176R: CCATTCCATGAAGTCAGCGAT.
Actin primer pair: F: CCTGGACCCAGCACAAT; R: GCCGATCCACACGGAGTACT

**Phospho-pRb analysis in primary bone marrow cells:** Five million bone marrow cells per mouse were treated with Fix Buffer I (BD Cat. No.557870) for 10 minutes at 37°C and permeabilized with Perm Buffer III (BD Cat. No.558050) for 30 minutes on ice. After washing the cells twice with staining buffer, they were stained with PE-mouse anti-phospho pRb (pSer780) (Cat. No.558548) and APC-mouse anti human CD45 (Cat. No.555483). 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). Slides were deparaffinized and antigen retrieval (DAKO TRS solution, 20 min in steamer, 20 min 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 min at dilutions of 1:100 for phospho-pRb (pSer807/11) and 1:250 for human CD45. Appropriate secondary antibodies were from Vector Elite kits. They were incubated with the sections for 30 min. The chromagen was DAB and sections were lightly counterstained with hematoxylin. After dehydration, coverslips were applied using a xylene-based mounting medium.
Animal efficacy studies. All mouse experiments were performed with the approval of the Institutional Animal Care and Use Committees at The Ohio State University. Survival study: Twenty NOD/SCID mice (age: twelve weeks) were conditioned with 300 RAD total body irradiation and immediately injected with $6 \times 10^6$ log-phase MOLM-13 cells via the lateral tail vein. The mice were divided randomly into two groups and dosed once daily with PD 0332991 (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 six after engraftment. Survival times were compared with the logrank test using Graphpad Prism software.
RESULTS

**THRX-165724 and SU14813 are potent inhibitors of Flt3.** To study Flt3 ITD signaling we chose four AML cell lines as a model system: THP-1 and U937 which express Flt3 wildtype (wt) and MV4-11 and MOLM13 which express Flt3 ITD. THRX-165724\(^{24}\) and SU14813\(^{25}\), inhibitors of the PDGFR receptor family which includes Flt3, were used as pharmacological 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 IC50 of about 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 is about 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 two 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. The effect caused by THRX-165724 and SU14813 is a result of apoptosis since 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 if 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 (Supplemental Figure 1). 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 leads to the downregulation of Cyclin D2 and D3.** It has been reported that the inhibition of Flt3 ITD signaling affects the cell cycle prior to 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 reenter the cell cycle after an incubation with THRX-165724 and SU14813 for 24 hours followed by a wash-out of the inhibitors (Supplemental Figure 2). 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 three 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 downregulation of Cyclin D2 and D3 transcript levels after treating the cells with THRX-165724 (Figure 2B).

As shown by Western Blot 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 \(^{26}\). pRb is known to be hyperphosphorylated when CDK4/6 is active, so the loss of CDK4/6 activity leads to the dephosphorylation of pRb \(^{26}\) 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 downregulation of Cyclin D2 and D3 as well as the dephosphorylation of pRb was also observed in Flt3 ITD MOLM13 cells (Supplemental Figure 3). 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 downregulation 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 upregulation of Cyclin D2 and D3, and that inhibition of the Flt3 ITD inhibits activation of CDK4/6.

**PD 0332991 is a potent CDK4/6 inhibitor.** In order to evaluate the role of CDK4/6 in the AML cell lines, we used PD 0332991, a small molecule inhibitor of CDK4/6 that is currently in phase II clinical trials (Figure 3A). PD 0332991 has little or no activity against a panel of 30 kinases, including CDK2, suggesting that this inhibitor is highly selective for CDK4/6. Importantly, we found that at 500 nM, PD 0332991 has 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 PD 0332991. A Western Blot of cell lysates showed decreasing pRb phosphorylation, confirming CDK4/6 inhibition by PD 0332991 (Figure 3C). The IC50 of PD 0332991 in this assay in both cell lines is 30 nM.

We next tested the effect of PD 0332991 on the cell cycle of our model cell lines after an incubation period of 24 hours 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 reentered 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 reentered the cell cycle at 120 hours (Figure 4B). However, despite reentering the cell cycle, PD 0332991 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 PD 0332991 on the cell cycle also led to an effect on proliferation (Figure 4C). MOLM13 and MV4-11 cells incubated with PD 0332991 as a single agent for eight 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 PD 0332991 following prolonged exposure as a single agent. We next tested if treatment of the four AML model cell lines with PD 0332991 can enhance the effect of the Flt3 inhibitor SU14813. In an MTT assay, the four AML cell lines as well as primary blasts from patient 17 (Flt3 ITD) and patient 75 (Flt3 wt) were incubated for three days with DMSO (vehicle control), PD 0332991, SU14813 or a combination of the compounds. PD 0332991 strongly enhanced the effect of SU14813 in MOLM13 and MV4-11 (both Flt3 ITD) and to a lesser degree also 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 Talalay\textsuperscript{22} was performed to determine if 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 IC50 values of these compounds in the two cell lines, the resulting combination index (CI) is 0.78 for MV4-11
and 0.67 for MOLM13. A CI value of under 0.9 is defined as synergistic activity, hence, PD0332991 and SU14813 act synergistically in this assay.

PD 0332991 also strongly enhanced the pro-apoptotic 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 PD 0332991 and SU14813 did not increase in Annexin V staining compared to SU14813 alone. In fact, Annexin V staining was somewhat reduced in the combination treatment. THP-1, 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 4E and the primary flow cytometric data is presented in Supplemental Figure 4.

**CDK2 is activated by p27Kip1 downregulation.** To investigate if the differential effect of PD 0332991 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 PD 0332991 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 five days of incubation with PD 0332991 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 PD 0332991, neither p27Kip1 nor Cyclin E protein levels changed significantly (Figure 5B). However, CDK2 protein was slightly
downregulated in all four cell lines. Interestingly, after four days of incubation with PD 0332991, the CDK2 inhibitor p27Kip1 was strongly downregulated in Flt3 wt THP-1 and U937. Furthermore, in U937 Cyclin E was upregulated. 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 PD 0332991 for multiple days, the selective downregulation 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 hours and 96 hours with PD 0332991 (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\textsuperscript{29-32}. We found, however, no changes in Foxo3a phosphorylation status in any of the four AML cell lines treated for 96 hours with PD 0332991 (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 two different sets of p27Kip1 specific primers for this experiment. This experiment showed p27Kip1 mRNA is modestly reduced between 2-3 fold in THP-1, U937 and MOLM13 cells treated with PD 0332991. There was no significant change of p27Kip1 transcript levels in MV4-11 cells.

**Primary AML cells are sensitive to PD 0332991.** To study the effect of CDK4/6 inhibition on primary cells we used two AML patient samples and CD34(+) hematopoietic progenitor cells from a normal donor. Patient 28 expressed Flt3 wt whereas patient 82 expressed Flt3 ITD. Figure 6A and 6B demonstrate that PD 0332991 treatment for four days significantly reduced patient cells that are in S or G2 of the cell cycle. The effect of PD 0332991 on the cell cycle of CD34(+) cells is less pronounced. PD 0332991 treatment for four 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 thirty four percent of pRb in its dephosphorylated form after treatment with PD 0332991 (Figure 6B). A CDK2 in vitro kinase assay revealed that the treatment with PD 0332991 for four days greatly reduces CDK2 activity in the two patient samples but not in the CD34(+) cells (Figure 6C). The reduction in \(^{32}\)P 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 6D). Hence, in contrast to the two well-established Flt3 wt cell lines THP-1 and U937 that display autonomous growth ex vivo, the two primary AML patient samples, regardless of the Flt3 status, displayed strong sensitivity to PD 0332991.

**PD 0332991 is active in a chimeric mouse-human model of AML.** In the first mouse experiment we tested if PD 0332991 could induce the dephosphorylation of pRb in vivo. NOD/SCID mice engrafted with MOLM13 were treated 24 and 4 hours prior to sacrificing with PD 0332991 or buffer (vehicle control) by oral gavage. The bone marrow cells were isolated and co-stained with anti-human CD45 to identify the leukemic MOLM13 cells and with human specific anti-phospho-pRb. The engraftment with human CD45 cells was at least 50% in all mice tested. Figure 7A demonstrates that CD45 cells from mice treated with PD 0332991 showed a significant reduction in pRb phosphorylation as compared to CD45 cells from mice treated with buffer as a control. Hence, the compound is active in the desired cell population in vivo. In the subsequent aggressive survival experiment we found that single agent PD 0332991 could prolong the survival of NOD/SCID mice engrafted with MOLM13 by 4-5 days \((p=0.0003)\) (Figure 7B). In the final experiment we engrafted mice with MOLM13, treated the mice for five days starting on day five after engraftment and performed immunohistochemistry on the bone marrow. Staining with H&E and anti-human CD45 demonstrated the presence of MOLM13 cells in the
bone marrow of control mice but to a lesser extent in the bone marrow of mice treated with PD 0332991. Furthermore, the MOLM13 cells in the control mice showed strong phospho-pRb staining (Figure 7C).

**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 downregulation 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.

In order to study the role of CDK4/6 in AML with Flt3 ITD as well as in AML with Flt3 wt we obtained PD 0332991, a selective CDK4/6 inhibitor with activity in model systems for multiple myeloma, mantle cell lymphoma and rhabdomyosarcoma. In our AML model system PD 0332991 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 likely the result of the observed reactivation of CDK2 activity. This reactivation may be caused by p27Kip1 downregulation which we found to be significant in U937 and THP-1 cells after PD 0332991 treatment for four days. The downregulation 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 did not observe an increase in Foxo3a phosphorylation in THP-1 or U937 in response to PD 0332991 which suggests that a decrease in Foxo3a transcriptional activity is not involved in the downregulation of p27Kip1 protein. Interestingly, however, 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 PD 0332991. The changes in p27Kip1 transcript levels were modest, about 2-3 fold. The reduction in p27Kip1 gene transcription in THP-1 and U937 cells could account for the reduction in p27Kip1 protein levels. However, since 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 PD 0332991 mediated reduction in p27Kip1 levels.

The ability to compensate at least partially for the pharmacological inhibition of CDK4/6 by using CDK2 may be one way for established AML cell lines to escape the anti-proliferative effect of an inhibitor like PD 0332991. We found it, however, encouraging that both the Flt3 ITD(+) and the Flt3 wt primary AML samples that we tested were sensitive to PD 0332991 and did not upregulate CDK2 activity following prolonged exposure. In the Flt3 ITD primary AML sample the observed cell cycle arrest, the pronounced dephosphorylation of pRb, and CDK2 downregulation 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 downregulation 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 in 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 PD 0332991. However, since 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 PD 0332991 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, PD 0332991 as a single agent did not induce Annexin V staining in the three day incubation period of this experiment. The enhancement of SU14813 activity by PD 0332991 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 as compared to 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 PD 0332991/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 downregulation of p27Kip1 and CDK2 activation.
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Acknowledgments

The authors would like to thank Cristina Lewis and Gerrit Los at Pfizer for providing PD0332991 and SU14813 as well as Mathai Mammen and Patrick Humphrey at Theravance for providing THRX-165724. Thanks to Audrey Papp and Zunyan Dai for technical help with the Real-Time PCR experiments. This work was supported by a grant from the Leukemia & Lymphoma society to R.B.

Conflict of interest statement: R.B. has declared a financial interest in Theravance whose potential product was studied in the present work. L.W., J.W., B.W.B, A.M.D., D.K., T.L. and M.A.C. declare no conflict of interest.

Authorship

Contribution: L.W. conducted research and analyzed data; J.W. conducted research and analyzed data; B.W.B. designed and conducted research, 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 paper; R.B. designed research, analyzed data and wrote the paper.
FIGURE LEGENDS

Fig. 1. **THRX-165724 and SU14813 are potent inhibitors of Flt3 autophosphorylation and induce apoptosis in AML cells with Flt3 ITD.**

A, Chemical structure of THRX-165724 and SU14813. B, Inhibition of Flt3 autophosphorylation with THRX-165724 and SU14813. MV4-11 cells were incubated with increasing concentrations of THRX-165724 and SU14813 for 30 minutes. THP-1 cells were incubated with increasing concentrations of THRX-165724 and Flt3 wild-type autophosphorylation was stimulated with FL (50 ng/mL for 5 minutes). Flt3 phosphorylation status was determined by Flt3 immunoprecipitation followed by SDS-PAGE and Western Blot with an anti-phosphotyrosine antibody. C, MTT assay. MV4-11, MOLM13, THP-1 and U937 cells were incubated with increasing concentrations of THRX-165724 and SU14813 for 72 hours. Cell viability and proliferation was assessed using the MTT assay. D, Induction of apoptosis. MV4-11, MOLM13, THP-1 and U937 cells were incubated for 72 hours with THRX-165724 (300 nM) or SU14813 (100 nM). Apoptosis was assessed by staining with Annexin V-PE and 7-AAD.

Fig. 2. **The inhibition of Flt3 ITD induces a G1 cell cycle arrest and downregulation of D-Cyclins.** A, MV4-11, MOLM13, THP-1 and U937 cells were treated with THRX-165724 (300 nM) or SU14813 (100 nM) for 24 hours. Following propidium iodide staining, cell cycle analysis was performed by flow cytometry. B, Ribonuclease Protection Assay. MV4-11 cells were treated with THRX-165724 (300 nM) or with vehicle (DMSO) for 3 hours. Changes in mRNA levels of a set of Cyclin genes was determined by ribonuclease protection assay (RPA) (P: Probe). C, Western Blot. MV4-11 cells were treated with THRX-165724 for 0, 1, 2, 4, 8 and 12 hours. Lysates were immunoblotted with the indicated antibodies. Probing with anti-tubulin was used to assure equal loading. D, Western Blot. MOLM13, MV4-11, U937 and THP-1 cells were treated with THRX-165724 (300 nM) or DMSO (vehicle control) as well as SU14813 (100 nM) or
DMSO (vehicle control) for 16 hours. A Western Blot was performed with cell lysates probing for Cyclin D2, D3 and pRb. Actin served as marker for equal loading.

**Fig. 3.** **PD 0332991 is a potent inhibitor of CDK4/6 but does not inhibit Flt3 ITD.** A, Structure of PD 0332991. 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-phospho-tyrosine 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 PD 0332991 for 16 hours. Lysates were resolved by SDS-PAGE followed by immunoblotting with anti-pRb.

**Fig. 4.** **PD 0332991 affects entry into the cell cycle and proliferation of AML model cell lines.** A, Cell Cycle Analysis. Flow cytometric analysis of MOLM13 and U937 cells treated with DMSO (vehicle control) for 120 hours and PD 0332991 (500 nM) for 24 and 120 hours and stained with propidium iodide. B, Bar graph representation of cell cycle analysis of MOLM13, MV4-11, THP-1 and U937 cells treated for 24 (no fill) and 120 hours (grey) with PD 0332991 (500 nM). The cells were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells in S/G2 as compared to the control cells which were normalized to 100% (treated with DMSO for 24 and 120 hours, respectively) was plotted (mean ± standard error of three independent experiments). C, Proliferation assay. MV4-11, MOLM13, THP-1 and U937 cells were plated in the presence of DMSO (vehicle control) or PD 0332991 (500 nM) at a density of 0.5X10^6 / mL. The cells were counted every two days and the cell number was adjusted to 0.5X10^6 / mL. (mean ± standard error of three independent experiments). D. MTT assay. MV4-11, MOLM13, U937, THP-1 cells as well as primary patient blasts from two patients (Pt 17 (Flt3 ITD) and Pt 75 (Flt3 wt)) were incubated in triplicate for three days with DMSO (control), PD0332991 (500 nM) (light grey), SU14813 (30 nM for cell lines and 100 nM for
patient samples) (medium grey) or PD0332991 (500 nM) plus SU14813 (30 nM for cell lines and 100 nM for patient samples) (dark grey). The DMSO control is normalized to 100% (mean ± standard error of three independent experiments for the cell lines and one experiment with triplicate data points for the patient samples). E. Apoptosis assay. MV4-11, MOLM13, THP-1 and U937 cells were incubated for three days with DMSO (control), PD0332911 (250 nM) (light grey), SU14813 (100 nM) (medium grey) or PD0332991 (250 nM) plus SU14813 (100 nM) (dark grey). The DMSO control is normalized to 100%. The cells were stained with Annexin V-PE and 7-AAD followed by flow cytometric analysis (See Supplemental Figure 4 for primary data).

**Fig. 5.** THP-1 and U937 cells can rephosphorylate pRb after multiple days of PD 0332991 treatment by reactivating CDK2. A, Western Blot. The indicated AML cell lines were treated with DMSO (vehicle control) or PD 0332991 (500 nM) for 24 or 120 hours. Lysates were resolved by SDS-PAGE followed by immunoblotting with anti-pRb. B, Western Blot. Cells were treated for 24 or 96 hours with PD0332991 (500 nM) or DMSO (vehicle control). Lysates were subjected to SDS-PAGE followed by Western Blot analysis with the indicated antibodies. An antibody against actin was used as a loading control. C, CDK2 in vitro Kinase Assay: Cells were incubated for 96 hours with DMSO (vehicle control) or PD 0332991 (500 nM). Cell lysates were prepared and normalized by protein concentration. CDK2 was immunoprecipitated to perform the in vitro kinase assay. Incorporation of $^{32}$P in the CDK2 substrate histone H1 was assessed by SDS-PAGE followed by autoradiography. As a control for the kinase assay, a lysate from THP-1 cells was incubated with an isotype matched antibody to form an immunocomplex. To confirm equal protein amounts in each normalized extract, aliquots were separated by SDS-PAGE and analyzed by Western blot with an anti-Grb-2 antibody. D, Western Blot and Real-time PCR. The indicated AML cell lines were treated with DMSO (vehicle control) or PD 0332991 (500 nM) for 96 hours. Lysates were prepared and resolved by SDS-PAGE followed by immunoblotting with phospho-Foxo3a (Thr32), Foxo3a, p27 and actin as a loading control. The cells were also used to
prepare mRNA to determine by Real-Time PCR the pairwise changes in p27 mRNA levels between DMSO and PD0332991 treated cells. Two Primer Pairs were used for the analysis.

Fig. 6. Effect of PD 0332991 on primary AML cells. CD34(+) and primary AML cells from patients 28 (Flt3 wt) and 82 (Flt3 ITD) were plated in methylcellulose and treated with PD 0332991 (500 nM) or DMSO (vehicle control) for four days. The cells were fixed and stained with propidium iodide and cell cycle analysis was performed by flow cytometry. A. Representative flow cytometric cell cycle analysis of one experiment with CD34(+) cells as well as cells from patient 28 and 82. B. Bar graph analysis of the percentage of PD 0332991 treated cells in S and G2 as compared to control cells using data from three independent experiments (mean ± standard error). B, Western Blot. CD34(+) and primary AML cells from patients 28 and 82 were treated as in A. Cell lysates were prepared, resolved by SDS/PAGE and an immunoblot was performed with the indicated antibodies. C. Cells were treated as in A. Cell lysates were prepared and normalized by protein concentration. CDK2 was immunoprecipitated to perform the in vitro kinase assay. Incorporation of $^{32}$P in the CDK2 substrate histone H1 was assessed by SDS-PAGE followed by autoradiography. As a control for the specificity of the kinase assay, a lysate from CD34(+) cells was incubated with an isotype matched antibody to form an immunocomplex. To confirm equal protein amounts in each normalized extract, aliquots were separated by SDS-PAGE and analyzed by Western blot with an anti-Grb-2 antibody. D. Quantitative representation of $^{32}$P-histone H1 (cpm) of the experiment depicted in C.

Fig. 7. AML mouse model to study the efficacy of PD 0332991 in vivo. A, Phospho-pRb staining in human CD45 cells. Six NOD/SCID mice were inoculated with MOLM13 cells. On day nine after inoculation, three mice were dosed with PD 0332991 (150 mg/kg) and three mice were dosed with vehicle (lactic acid buffer). On day ten, the mice were dosed a second time with PD 0332991 (150 mg/kg) or vehicle four hours prior to sacrificing them. The bone marrow was
isolated, stained with anti-human CD45 plus anti-phospho-pRb and analyzed by flow cytometry.

B, Kaplan Meyer survival plot. NOD/SCID mice were inoculated with MOLM13 cells. Starting on day six after inoculation, one group (ten mice) was dosed with PD 0332991 (150 mg/kg), the other group (ten mice) was dosed with vehicle (lactic acid buffer). The survival of both groups was analyzed by Kaplan-Meyer plot. The survival benefit of mice treated with PD 0332991 was statistically significant (p=0.0003, logrank test). C, Immunohistochemistry. Ten NOD/SCID mice were engrafted as above. Five mice each were dosed with PD 0332991 (150 mg/kg) or lactic acid buffer (control) starting on day 5 after inoculation. On day 10, all mice were sacrificed. Sections of the sterna were prepared and stained as indicated with H&E, anti-human CD45 and anti-phospho pRb. The arrows point at the human leukemia cells.
Figure 1
Figure 3
Figure 4.
Figure 5.
Figure 6.
A. 

Mouse 1

Mouse 2

Mouse 3

Mouse 4

Mouse 5

Mouse 6

PD0332991

10^6

10^5

10^4

10^3

10^2

10^1

10^0

phospho-pRb

human CD45

23%

31%

12%

3%

44%

Phospho-pRb

B. 

Dosing Interval

Percent survival

Buffer

PD, 150 mg/kg

Days after tumor inoculation

0 2 4 6 8 10 12 14 16 18 20

C. 

PD 0332991

Control

H&E

CD45

Phospho-pRb

Figure 7.
Pharmacologic inhibition of CDK4/6: mechanistic evidence for selective activity or acquired resistance in acute myeloid leukemia

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