Specific Antileukemic Activity of PD0332991, a CDK4/6 Inhibitor, against Philadelphia Chromosome–Positive Lymphoid Leukemia

Atsushi Nemoto1, Satoshi Saida2, Itaru Kato2, Jiro Kikuchi3, Yusuke Furukawa3, Yasuhiro Maeda4, Koshi Akahane1, Hiroko Honna-Oshiro1, Kumiko Goi1, Keiko Kagami1, Shinya Kimura5, Yuko Satō6, Seiichi Okabe7, Akira Niwa8, Kenichiro Watanabe2, Tatsutoshi Nakahata8, Toshio Heike2, Kanji Sugita1, and Takeshi Inukai1

Abstract

S-phase progression of the cell cycle is accelerated in tumors through various genetic abnormalities, and, thus, pharmacologic inhibition of altered cell-cycle progression would be an effective strategy to control tumors. In the current study, we analyzed the antileukemic activity of three available small molecules targeting CDK4/CDK6 against lymphoid crisis of chronic myeloid leukemia (CML-LC) and Philadelphia chromosome–positive acute lymphoblastic leukemia (Ph+ ALL), and found that all three molecules showed specific activities against leukemic cell lines derived from CML-LC and Ph+ ALL. In particular, PD0332991 exhibited extremely high antileukemic activity against CML-LC and Ph+ ALL cell lines in the nanomolar range by the induction of G0–G1 arrest and partially cell death through dephosphorylation of pRb and downregulation of the genes that are involved in S-phase transition. As an underlying mechanism for favorable sensitivity to the small molecules targeting CDK4/CDK6, cell-cycle progression of Ph+ lymphoid leukemia cells was regulated by transcriptional and posttranscriptional modulation of CDK4 as well as Cyclin D2 gene expression under the control of BCR-ABL probably through the PI3K pathway. Consistently, the gene expression level of Cyclin D2 in Ph+ lymphoid leukemia cells was significantly higher than that in Ph− lymphoid leukemia cells. Of note, three Ph+ ALL cell lines having the T315I mutation also showed sensitivity to PD0332991. In a xenograft model, PD0332991, but not imatinib, suppressed dissemination of Ph+ ALL having the T315I mutation and prolonged survival, demonstrating that this reagent would be a new therapeutic modality for relapsed CML-LC and Ph+ ALL patients after treatment with tyrosine kinase inhibitors. Mol Cancer Ther; 15(1): 94–105. ©2015 AACR.

Introduction

The cyclin-dependent kinases (CDK)-retinoblastoma protein (pRb) pathway (1) is a major target of genetic alteration in a variety of tumors (2), and, the p16 (INK4A/CDKN2A) gene, which encodes a specific inhibitor of the kinase activity of CDK4/6 (1), is frequently altered in lymphoid leukemias (3). Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by Philadelphia chromosome (Ph), t(9;22) (q34;q11), and its blast crisis is a terminal phase of the disease with highly aggressive clinical features. Of note, during progression from the chronic phase to lymphoid crisis of CML (CML-LC), but not to myeloid crisis, loss of p16 is frequently observed (4–6). Ph+ ALL also frequently harbors loss of p16, and the incidence of p16 loss is significantly higher in Ph+ ALL than in Ph− ALL (7, 8). Moreover, loss of p16 is associated with more aggressive growth properties and poorer outcomes (9, 10). It has also been reported that cyclin D2 plays a role in cell-cycle progression as a downstream effector of BCR-ABL in Ph+ lymphoid leukemias (11–15). Under these circumstances, the activity of CDK4/6 could be augmented in Ph+ ALL and CML-LC, and thus, CDK4/6 kinase activity would be a therapeutic target for Ph+ ALL and CML-LC.

In the current study, we analyzed the antileukemic activity of three available small molecules targeting CDK4/CDK6, i.e., CBC219476 (16), PD0183812 (17), and PD0332991 (18–20) against CML-LC and Ph+ ALL. Of note, imatinib treatment downregulated CDK4 protein expression in some Ph+ lymphoid cell lines, and PD0332991 showed potent antileukemic activity against CML-LC and Ph+ ALL cell lines including imatinib-resistant Ph+ ALL cell lines with T315I mutation both in vitro and in vivo, suggesting its efficacy as a new therapeutic modality.

Materials and Methods

Leukemia cell lines, clinical samples, and reagents

Characteristics of Ph+ and Ph− cell lines were previously reported (21, 22) and summarized in Supplementary Table S1.
SU-Ph2 and TCCY are imatinib-sensitive cell lines established from patients with Ph^+ ALL, and SU/SA and TCCY/SA are their imatinib-resistant sublines, respectively. After-long term culture with increasing concentrations of imatinib (23, 24), SK-9 was established from a Ph^+ ALL patient who relapsed after chemotherapy combined with imatinib (25). SU/SA and SK-9 were confirmed to have the T315I mutation of BCR-ABL. KOPN, KOCL, YAMN, YACL, and KOPT series of cell lines were sequentially established in our laboratory from 1980 to 2011 as previously reported (21, 22). SU-Ph2 and SU/SA were established by Y. Maeda (23) and provided in 2010, TCCY and TCCY/SA were established by Y. Sato (24) and provided in 2011, SK9 was established by S. Okabe (25) and provided in 2012, and Reh, Jurkat, and MOLT4 were purchased from ATCC in 2000. Nalm1, Nalm6, 697, IIOCB1, and HALO1 were kindly provided by Dr. Thomas Look (Dana-Farber Cancer Institute, Boston, MA) in 1997. All cell lines were cryopreserved immediately after establishment, purchase, or provision, and each frozen stock of cell line was resuscitated just before experiment. Cell lines were tested for Mycoplasma contamination by PCR kit (Takara Bio Inc) before use. Authentication of cell lines by short tandem repeat analyses was not routinely performed, but gross authentication of cell lines was performed by typing of fusion protein expression as well as microscopic observation of morphology and growth curve analyses before experiments. All cell lines were maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS). For clinical sample analysis, mononuclear cells (blasts > 90%) isolated by Ficoll-Hypaque density centrifugation were stored in liquid nitrogen and used for experiments. The use of clinical samples for analysis was approved by the ethical review board of the University of Yamanashi. PD0183812 and PD0332991 were kindly provided by Pfizer. Imatinib mesylate and GDC-0941 were purchased from Selleck, and CBC219476, AG490, U0126, LY294002, and Lactastatin were purchased from Calbiochem. Recombinant human IL7 and FL were kindly provided by Kyowa Hakko Kirin.

**Western blot analysis**

Cell lines were transferred to serum-free DMEM/F12 medium and treated with different concentrations of imatinib, AG490, U0126, or LY294002 for 2 hours. The harvested cells were treated with 1 mmol/L of AEBSF (hydrochloric acid (4-[2-Aminoethyl]benzensulfonylfluoride, HCl; Calbiochem) on ice for 10 minutes before solubilization in lysis buffer. Western blotting was performed as previously reported (22) using the antibodies listed in Supplementary Table S2.

**^3H-thymidine uptake assay and Alamar blue assay**

Cells (1 × 10^5/well) were plated on 96-well plate (Costar) in the absence or presence of imatinib, PD0183812, CBC219476, or PD0332991 at various concentrations. The plates were incubated for 36 hours, pulsed with ^3H-thymidine (1 μCi/well) for 6 hours, and harvested onto glass fiber filters. Radioactivity incorporated into DNA was measured by liquid scintillation counting. In some experiments, Alamar blue assay was performed. After 4-hour additional incubation with the Alamar blue (20 μL for each well; Life Technologies) instead of ^3H-thymidine, the plates were exposed to an excitation wavelength of 530 nm, and the emission at 590 nm was detected by microplate spectrophotometer. The cell survival was calculated by the percentages of optical densities of treated wells to those of untreated wells.

**PI staining and PI/Annexin V staining**

For cell-cycle analysis, cells were fixed with 70% ethanol on ice. After washing with PBS, the cells were stained with propidium iodide (PI, Sigma) and analyzed by flow cytometry (FACSCalibur, BD Biosciences). To detect apoptotic cell death, cells were stained with PI and fluorescein isothiocyanate-conjugated Annexin V (MBL), and analyzed by flow cytometry.

**Real-time PCR analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription and quantitative real-time PCR were performed as previously reported (22) using TaqMan probe (Applied Biosystems) listed in Supplementary Table S3. Normal human bone marrow total RNA purchased from TAKARA BIO INC (Otsu, Japan) was used as a positive control. As an internal control, quantitative real-time PCR for GAPDH (Hs 99999905_m1, Applied Biosystems) was performed.

**Lentivirus shRNA/siRNA expression vectors and infection**

PL lentiviral vector was produced to produce short hairpin RNAs (shRNA) under the control of mouse U6 promoter and coexpress GFP (22, 26). Among 5 sets of siRNA targeting p190 BCR-ABL cDNA sequence, whose specificities were confirmed by submitting to BLAST search, shRNA listed in Supplementary Table S4 was selected for further analysis due to the specificity and efficiency. Lentivirus containing pLL3.7 shRNA vector or control vector (22) was infected to KOPN7 through firep. GFP-positive population was sorted 24 hours after infection.

**Xenograft model**

NOD/SCID/γc null (NOG) mice (ref. 27; Central Institute of Experimental Animals, Kawasaki, Japan) were transplanted with SU-Ph2 or SU/SA cells (1 × 10^6 cells) through the tail vein without preconditioning. Three weeks after transplantation, when engraftment of leukemia cells was confirmed in bone marrow, imatinib (400 mg/kg/dose) or PD0332991 (150 mg/kg/dose) dissolved in deionized water or deionized water as the vehicle was orally administered through a gastric tube. Bone marrow was aspirated from the tibia at 2, 3, and 5 weeks after transplantation. Peripheral blood was collected every week from 2 to 5 weeks after transplantation.

**Phospho-pRB analysis**

Leukemia cell lines and bone marrow cells of NOG mice (5 × 10^6) were treated with Fix Buffer I (BD Biosciences) at 37°C for 10 minutes and permeabilized with Perm Buffer III (BD Biosciences) on ice for 30 minutes. After washing the cells twice with staining buffer, cells were stained with phycoerythrin (PE)-mouse anti-human CD19. PE- and FITC-conjugated mouse isotype antibodies served as the negative control in the flow cytometric analysis.

**Statistical analysis**

Gene expression level and the IC_{50} values of CDK4/6 inhibitors between different categories of cell lines and primary samples were compared using the Mann–Whitney test, whereas the others were compared using the Student t test if not otherwise specified. The probabilities of survival in xenograft...
models were analyzed by Kaplan–Meier analysis and log-rank test.

Results
Specific antileukemic activity of PD0332991 against Ph⁺ lymphoid leukemia cell lines

We first tested the antileukemic activity of two available small molecules targeting CDK4/6, i.e., CBC219476 (16) and PD0183812 (17), in a variety of lymphoid leukemia cell lines using the ³H-thymidine uptake assay. The IC₅₀ of CBC219476 was relatively lower in Ph⁺ lymphoid leukemia cell lines than Ph⁻ B-cell precursor ALL (BCP-ALL) and T-cell ALL (T-ALL) cell lines (Fig. 1A), and the IC₅₀ of PD0183812 was significantly lower in Ph⁺ lymphoid leukemia cell lines than Ph⁻ B-ALL and T-ALL cell lines (Fig. 1B). We next analyzed the activity of PD0332991, an orally active inhibitor of CDK4/6 with selective ability to block pRb phosphorylation at serine 780 and 795 in the low nanomolar range (18–20). PD0332991 is currently undergoing phase I/II clinical trials in solid tumor patients (28–31), and was recently designated for Breakthrough Therapy (32) for potential treatment of patients with breast cancer by FDA as palbociclib. In the previous reports regarding the PD0332991 sensitivity of breast cancer cell lines (33) and ovarian cancer cell lines (34) in vitro, 23% and 10% of cell lines showed good response (IC₅₀ < 50 nmol/L), whereas 36% and 50% of cell lines showed resistance (IC₅₀ > 500 nmol/L), respectively. Of note, PD0332991 revealed extremely high potential against Ph⁺ lymphoid leukemia cell lines (Fig. 1C). The IC₅₀ determined with ³H-thymidine uptake assay was less than 25 nmol/L in 6 of 9 Ph⁺ lymphoid leukemia cell lines, and none of Ph⁺ lymphoid leukemia cell lines were resistant to PD0332991 (Supplementary Table S5). Although less sensitive in comparison with Ph⁺ lymphoid leukemia cell lines, 8 (40%) of 20 Ph⁻ B-ALL cell lines were highly sensitive (IC₅₀ < 50 nmol/L) to PD0332991, whereas only 1 Ph⁻ B-PC-ALL cell line (5%) was resistant (IC₅₀ > 500 nmol/L). The IC₅₀ of Ph⁺ lymphoid leukemia cell lines (Fig. 1D) were significantly lower than those of MLL+ALL cell lines (mean IC₅₀: 100 nmol/L,
Treatment with PD0332991 dramatically dephosphorylated Rb in Ph⁺ lymphoid leukemia cell lines except for KOPN30bi (Fig. 2A), which lost the Rb gene and exceptionally showed relative resistance to PD0332991 as well as PD0183812 and CBC219476. Consistent with dephosphorylation of pRb, gene expression levels of TK1 and PCNA, which had been reported to be the direct target genes of E2F transcriptional factor and are involved in the S-phase transition (35–37), were markedly downregulated in Ph⁺ lymphoid leukemia cell lines (Fig. 2B). As a result, G₀–G₁ arrest was markedly induced after PD0332991 exposure (Fig. 2C and Supplementary Fig. S1A). Moreover, longer (120-hour) exposure induced significant accumulation of cells in the sub-G₀–G₁ phase in 4 of 9 cell lines (Fig. 2D), and induction of apoptosis was confirmed in two cell lines (Supplementary Fig. S1B).

We next analyzed the possibility of combination therapy of PD0332991 and antileukemic agents on Ph⁺ ALL using an Alamar blue assay. First, we analyzed the effect of PD0332991 on the sensitivity to vincristine (VCR) as a representative chemotherapeutic agent in three sequences (Supplementary Fig. S1C), and found an antagonistic effect of PD0332991 particularly when cells were pretreated with PD0332991 (Supplementary Fig. S1D). Both KOPN57bi and SU-Ph2 were sensitive to VCR (cell viabilities; around 40%–60%) or simultaneous treatment (cell viabilities; 20%–30%) with PD0332991 induced marked resistance to VCR. We next analyzed the combination of imatinib and PD0332991 in three sequences of exposure (Fig. 2E). In contrast to VCR, PD0332991 showed an additive effect on imatinib-sensitivities of KOPN57bi and SU-Ph2 regardless of sequences (Fig. 2F).

Activity of CDK4/6 Inhibitor against Ph⁺ Leukemia

Because of the high sensitivities of Ph⁺ lymphoid leukemia cell lines to CDK4/6 inhibitors, we analyzed changes in CDK4 expression in 5 Ph⁺ lymphoid leukemia cell lines after treatment with imatinib (Fig. 3A). As previously reported (11–15), imatinib downregulated cyclin D2 in all cell lines except for KOPN30bi and upregulated p27 in all cell lines except for KOPN57bi (Fig. 3A), suggesting that the involvement of cyclin D2 in the cell-cycle progression of Ph⁺ lymphoid leukemia cells could be one of the mechanisms for good sensitivities to CDK4/6 inhibitors. Expression levels of CDK6, Cyclin D1, and Cyclin D3 were almost unchanged in all cell lines. Of note, imatinib also downregulated CDK4 in 3 cell lines (KOPN55bi, KOPN57bi, and KOPN72bi; Fig. 3A). In these three cell lines, downregulation of CDK4 was accompanied by dephosphorylation of pRb at serine 780 and 795 (Supplementary Fig. S2A). Gene expression level of CDK4 was also downregulated (Fig. 3B), but, particularly in KOPN57bi, downregulation of CDK4 gene expression was less significant compared with changes in protein expression level of CDK4 and gene expression level of c-MYC, one of the downstream molecules of BCR-ABL (38). Lactacystin, a proteasome inhibitor, partly blocked imatinib-induced downregulation of CDK4, but not of c-MYC, in KOPN57bi and KOPN72bi (Fig. 3C). These observations suggested that both transcriptional and posttranscriptional regulations are involved in the CDK4 expression under the control of BCR-ABL. In KOPN57bi, imatinib-induced growth arrest was partially rescued by IL7 and FLT3 ligand (FL; ref. 39), and PD0332991 counteracted this rescue effect of IL7/FL (Supplementary Fig. S2B). Thus, we analyzed an effect of IL7 and/or FL on the expression of CDK4 in an imatinib-treated KOPN57bi (Supplementary Fig. S2C). Imatinib-induced CDK4 downregulation was partially recovered by FL alone, but not by IL7 alone, and was almost fully recovered by costimulation with IL7 and FL (Supplementary Fig. S2C). Next, to verify the pathway(s) that regulates CDK4 expression of the imatinib-treated KOPN57bi cells in response to growth factor stimulation, we analyzed an effect of a series of inhibitors on CDK4 expression (Fig. 3D). LY294002, an inhibitor of the PI3K pathway, most effectively downregulated CDK4 expression of KOPN57bi, whereas LY294002 and AG490, an inhibitor of the Jak/STAT pathway, but not U0126, an inhibitor of the MAPK pathway, blocked IL7/FL–induced CDK4 upregulation in imatinib-treated cells (Fig. 3D). Both 10 μmol/L of LY294002 and 0.1 μmol/L of GDC-0941, another inhibitor of the PI3K pathway with higher selectivity, also downregulated CDK4 expression in KOPN55bi and KOPN72bi almost as efficiently as imatinib did (Supplementary Fig. S2D and S2E).

Although further confirmation by genetic experiments is required to conclude, these observations suggested that the PI3K pathway might be involved in the regulation of CDK4 expression in Ph⁺ lymphoid leukemia cells as a downstream pathway of BCR-ABL and the Jak/STAT pathway might also play a role in the rescue expression of CDK4 by IL7/FL.

We next tested the effect of shRNA against BCR-ABL in KOPN57bi cells. By the lentiviral introduction of shRNA against junctional sequences of BCR-ABL, gene expression level of BCR-ABL in the shRNA introduced cells was significantly downregulated to around 60% of the level expressed in the control shRNA-transfected cells (Fig. 3E). As a result, gene expression levels of Cyclin D2 and c-MYC were significantly downregulated (Fig. 3E). Gene expression level of CDK4 was weakly but significantly downregulated by shRNA against BCR-ABL, and protein expression of CDK4 was also downregulated by shRNA against BCR-ABL (Fig. 3F). These observations provided another evidence that BCR-ABL mediates upregulation of CDK4 in Ph⁺ lymphoid cell lines.

Antileukemic activity of PD0332991 against a Ph⁺ lymphoid leukemia cell line with a T315I mutation in BCR-ABL

As cyclin D2 and CDK4 play a role in cell-cycle progression of Ph⁺ lymphoid leukemias as the downstream pathway of BCR-ABL, a CDK4/6 inhibitor could offer antileukemic activity against imatinib-resistant Ph⁺ lymphoid leukemias having mutation of the ABL kinase domain of BCR-ABL. SU-Ph2 is an imatinib-sensitive Ph⁺ ALL cell line with double Ph-chromosome, and representatively showed a favorable sensitivity to antileukemic activity of PD0332991 (Fig. 1C). SU/SR is an imatinib-resistant subline of SU-Ph2 established after long-term culture of SU-Ph2 in the presence of increasing concentrations of imatinib (23) and has the T315I mutation of BCR-ABL in one of two BCR-ABL genes (Supplementary Fig. S3A; ref. 40). In SU-Ph2, imatinib dephosphorylated BCR-ABL (ref. 40Supplementary Fig. S3A) and downregulated gene and protein expression level of CDK4 and cyclin D2 (Fig. 4A and B), as confirmed before in 3 Ph⁺ lymphoid
Figure 2.
Antileukemic activity of PD0332991 against Ph⁺ lymphoid leukemia cell lines. A, induction of dephosphorylation of pRb. Representative results of flow cytometric analysis of phosphorylated pRb in two cell lines are shown at left. In flow cytometric histograms, vertical axis indicates numbers of detected event. Changes in the percentage of phosphorylated pRb in triplicate analysis of each cell line cultured in the absence or presence of PD0332991 (100 nmol/L) for 24 hours are indicated at right. B, downregulation of TK1 and PCNA. Gene expression levels of TK1 and PCNA in each cell line cultured in the absence or presence of PD0332991 (100 nmol/L) for 24 hours were semiquantified by real-time RT-PCR analysis using gene expression of GAPDH as an internal control. C, induction of G0–G1 arrest. Representative results of flow cytometric analysis of PI staining in two cell lines are shown at left. In flow cytometric histograms, vertical axis indicates numbers of detected event. Changes in mean percentage of cells in the G0–G1 phase in triplicate analysis of each cell line cultured in the absence or presence of PD0332991 (100 nmol/L) for 24 hours are indicated at right. (Continued on the following page.)
leukemia cell lines (Fig. 3A and B), resulting in blockage of cell proliferation (Fig. 4C). In contrast, imatinib failed to down-regulate both CDK4 and cyclin D2 expression (Fig. 4A and B) and block proliferation of SU/SR (Fig. 4C). Of note, in the \(^{3}H\)-thymidine uptake assay, SU/SR was sensitive to PD0332991 (IC\(_{50}\) 30 nmol/L; Fig. 4D) like other Ph\(^+\) lymphoid cell lines, although slightly less sensitive when compared with SU-Ph2 (IC\(_{50}\) <25 nmol/L). Dephosphorylation of pRB and accumulation into G0–G1 phase by PD0332991 were confirmed in both SU-Ph2 (Fig. 4E) and SU/SR (Fig. 4E). Induction of apoptosis after longer exposure to PD0332991 was observed in both SU-Ph2 and SU/SR (Supplementary Fig. S3B). We also analyzed the activities of PD0332991 against another set of Ph\(^+\) ALL cell line, TCCY, and its imatinib-resistant subline having the T315I mutation, TCCY/SR, which was established after long-term culture in the presence of increasing concentrations of imatinib (ref. 24; Supplementary Fig. S3C). As shown in Fig. 4F, TCCY/SR was moderately sensitive to PD0332991 (IC\(_{50}\) 80 nmol/L), although less sensitive when compared with TCCY (IC\(_{50}\) 50 nmol/L). Finally, we analyzed SK-9, an imatinib-resistant Ph\(^+\) ALL cell line (Supplementary Fig. S3C) that was established from the Ph\(^+\) ALL patient relapsed after chemotherapy combined with imatinib, whose leukemia cells had the naturally occurring T315I mutation (25). SK-9 was highly sensitive to PD0332991 (IC\(_{50}\) <25 nmol/L) as Ph\(^+\) lymphoid leukemia cell lines not having the T315I mutation (Fig. 4F). Dephosphorylation of pRB and accumulation into G0–G1 phase by PD0332991 were confirmed in TCCY, TCCY/SR, and SK-9 (Supplementary Fig. S3D). These observations indicated that PD0332991 is active against imatinib-resistant Ph\(^+\) lymphoid leukemias having the T315I mutation.

**Antileukemic activity of PD0332991 in a xenograft model**

We tested the antileukemic activity of PD0332991 in a xenograft model using NOD/SCID/\(\gamma\)c null (NOG) mice (ref. 27; Fig. 5 and Supplementary Fig. S4). First, we confirmed the in vivo activity of imatinib as a control (Fig. 5A and B). Two-week oral administration of imatinib (400 mg/kg/dose) blocked rapid dissemination of leukemia in mice transplanted with SU-Ph2 (SU-Ph2 mice), but only minimally in mice transplanted with SU/SR (SU/SR mice), into both bone marrow (Fig. 5A) and peripheral blood (Supplementary Fig. S4A). As a result, prolonged survival of 11 days (median) was observed in SU-Ph2 mice, but not in SU/SR mice (Fig. 5B). We next tested the in vivo activity of PD0332991 (Fig. 5C–F). Two doses of orally administered PD0332991 (150 mg/kg/dose) led to significant reduction in pRB phosphor-ylation in SU-Ph2 and SU/SR (Fig. 5C), and two weeks of orally administered PD0332991 blocked rapid dissemination of SU/SR as well as SU-Ph2 into bone marrow (Fig. 5D and E) and peripheral blood (Supplementary Fig. S4B and S4C). As a result, prolonged survival of 16 days and 13 days was observed in SU-Ph2 mice and SU/SR mice, respectively (Fig. 5F). These observations indicated that PD0332991 is active in vivo against Ph\(^+\) lymphoid leukemias even having the T315I mutation.

**Upregulation of Cyclin D2 and loss of p16 in Ph\(^+\) lymphoid leukemia cells**

As it was extremely difficult to get conclusive observations regarding the PD0332991-sensitivity of primary samples of Ph\(^+\) lymphoid leukemias due to poor proliferative potential without growth factors in vitro, we simply compared the gene expression profile of cell-cycle regulators between cell lines (Fig. 6A) and primary samples (Fig. 6B) of Ph\(^+\) lymphoid leukemia. We first analyzed gene expression levels of Cyclin D2, CDK4, and p16 by real time RT-PCR in Ph\(^+\) lymphoid leukemia cell lines and compared with those in Ph\(^-\) BCP-ALL cell lines (Fig. 6A). Of note, consistent with the above notion that the gene expression of Cyclin D2 was regulated by BCR-ABL in Ph\(^+\) lymphoid leukemia cell lines, it was significantly higher in Ph\(^-\) lymphoid leukemia cell lines than Ph\(^+\) BCP-ALL cell lines (Fig. 6A). In contrast, the gene expression level of CDK4 was similar between Ph\(^+\) lymphoid leukemia cell lines and Ph\(^-\), BCP-ALL cell lines (Fig. 6A). Finally, the p16 gene expression level was significantly lower in Ph\(^+\) lymphoid leukemia cell lines than in Ph\(^-\) BCP-ALL cell lines (Fig. 6A). Due to biallelic loss of the p16 gene (Supplementary Table S5) as we previously reported (41). Among Ph\(^-\) BCP-ALL cell lines, there was no significant difference in the IC\(_{50}\) of PD0332991 between the cell lines with p16 gene expression and those without it (Supplementary Fig. S5A). Gene expression levels of p27, Cyclin D1, and CDK6 were identical between Ph\(^+\) lymphoid leukemia cell lines and Ph\(^-\), BCP-ALL cell lines (Supplementary Fig. S5B). As KOPN30hi, which lost the Rb gene, was exceptionally less sensitive to PD0332991 among Ph\(^+\) lymphoid leukemia cell lines (Fig. 1C), gene expression level of Rb was compared between Ph\(^+\) lymphoid leukemia cell lines and Ph\(^-\), BCP-ALL cell lines. Although Ph\(^-\), BCP-ALL cell lines were significantly less sensitive to PD0332991 than Ph\(^+\) lymphoid leukemia cell lines (Fig. 1D), gene expression level of Rb was similar between two groups (Supplementary Fig. S5C).

We next analyzed primary samples of Ph\(^+\) and Ph\(^-\) lymphoid leukemias (Fig. 6B). As observed in the cell lines, the Cyclin D2, but not CDK4, gene expression level was significantly higher in Ph\(^+\) lymphoid leukemia samples than in Ph\(^-\) BCP-ALL samples (Fig. 6B), suggesting that Cyclin D2 might play a role in the proliferation of primary Ph\(^+\) lymphoid leukemia cells. Although the difference in p16 gene expression level was not significant between Ph\(^+\) and Ph\(^-\) leukemia samples, lower gene expression level (<0.5 of relative expression) was observed more frequently in Ph\(^+\) ALL and CML-CLC samples than in Ph\(^-\) BCP-ALL samples (7/12 samples vs. 3/10 samples; \(P = 0.039\) in \(\chi^2\) test; Fig. 6B). In addition, the Rb gene expression level was similar between Ph\(^+\) lymphoid
Figure 3.
Downregulation of CDK4 expression in Ph⁺ lymphoid leukemia cell lines by imatinib treatment. A, downregulation of CDK4 and cyclin D2 expression and upregulation of p27 expression. Western blot analysis of BCR-ABL, phosphorylated BCR-ABL, CDK4, CDK6, Cyclin D1, Cyclin D2, Cyclin D3, p27, and Tubulin in five Ph⁺ lymphoid leukemia cell lines treated with imatinib (0.5 μmol/L) for 24 hours. B, gene expression levels of CDK4 and c-Myc in three lymphoid leukemia cell lines treated with imatinib (0.5 μmol/L) for the indicated periods. Real-time RT-PCR was performed using gene expression of GAPDH as an internal control. C, effect of a proteasome inhibitor on CDK4 expression in Ph⁺ lymphoid leukemia cell lines treated with imatinib. Western blot analysis of BCR-ABL, phosphorylated BCR-ABL, CDK4, c-MYC, and Tubulin was performed in three cell lines treated with imatinib (0.5 μmol/L) in the absence or presence of lactastatin (20 nmol/L) for 24 hours. D, effect of inhibitors against signaling pathways of BCR-ABL on CDK4 expression in a Ph⁺ lymphoid leukemia cell line. Western blot analysis of CDK4, BCR-ABL, and Tubulin was performed in KOPN57bi cells treated with imatinib (0.5 μmol/L) in the absence or presence of lactastatin (20 nmol/L) for 24 hours. E, effect of inhibitors against signaling pathways of BCR-ABL on CDK4 expression in a Ph⁺ lymphoid leukemia cell line. Western blot analysis of CDK4, BCR-ABL, and Tubulin was performed in KOPN57bi cells treated with imatinib (0.5 μmol/L) in the absence or presence of lactastatin (20 nmol/L) for 24 hours. F, gene expression levels of BCR-ABL, Cyclin D2, CDK4, and c-Myc in the GFP-positive populations of KOPN57bi cells 24 hours after infection of the lentivirus vectors containing control shRNA or shRNA specific for BCR-ABL. Real-time RT-PCR was performed using gene expression of GAPDH as an internal control. Asterisks indicate significant difference (***: p < 0.01; **: p < 0.05; t-test).
Figure 4.
Antileukemic activity of PD0332991 against a Ph⁺ ALL cell lines with a T315I mutation of BCR-ABL in vitro. A and B, gene (A) and protein (B) expression of CDK4 and cyclin D2 in SU-Ph2 and SU/SR cells cultured in the presence or the absence of 0.5 μmol/L of imatinib for 24 hours. C, in vitro imatinib sensitivity of parental SU-Ph2 and its imatinib-resistant subline, SU/SR, that has the T315I mutation of BCR-ABL. Vertical axis indicates percentage of uptake of 3H-thymidine and horizontal axis indicates the concentration of imatinib. D, in vitro PD0332991 sensitivity of SU-Ph2 and SU/SR assayed by 3H-thymidine uptake. E, induction of dephosphorylation of pRb and G0–G1 arrest by PD0332991. Flow cytometric analysis of phosphorylated pRb (vertical axis) and PI staining (horizontal axis) are shown. SU-Ph2 and SU/SR were cultured in the absence or presence of PD0332991 (100 nmol/L) for 24 hours. F, in vitro sensitivity to PD0332991 of SK-9, TCCY, and TCCY/SR. SK-9 is an imatinib-resistant Ph⁺ ALL cell line having the T315I mutation. TCCY/SR has the T315I mutation as an imatinib-resistant subline of TCCY, a Ph⁺ ALL cell line. Vertical axis indicates percentage of uptake of 3H-thymidine and horizontal axis indicates the concentration of PD0332991.
Figure 5.
Antileukemic activity of PD0332991 against a Ph⁺ ALL cell line with a T315I mutation of BCR-ABL in a xenograft model using NOG mice. A, effect of imatinib in a xenograft model. NOG mice transplanted with SU-Ph2 or SU/SR were treated with imatinib (400 mg/kg/dose; n = 5) or vehicle (n = 5) for 2 weeks (5 days a week) after 3 weeks of inoculation, and the percentages of human CD19-positive/mouse CD45-negative population in bone marrow were monitored by flow cytometry before and after imatinib treatment. Asterisks indicate significant difference in \( t \) test (\(^*\), \( P < 0.05 \)). B, survival of NOG mice treated with imatinib. Survival of NOG mice transplanted with SU-Ph2 or SU/SR and treated with imatinib (400 mg/kg/dose) or vehicle for 2 weeks after 3 weeks of inoculation was analyzed by the Kaplan-Meier method. \( P \) in log-rank test is indicated when it is significant. C, dephosphorylation of pRb by PD0332991 in leukemia cells transplanted into NOG mice. Phosphorylation of pRb was monitored in human CD19-positive population of bone marrow mononuclear cells in NOG mice transplanted with SU-Ph2 or SU/SR, in which two doses of PD0332991 (150 mg/kg/dose; n = 3) or vehicle (n = 3) were orally administered after 3 weeks of inoculation. Asterisks indicate significant difference in \( t \) test (\(^*\), \( P < 0.05 \)). D and E, effect of PD0332991 in a xenograft model. NOG mice transplanted with SU-Ph2 (D) or SU/SR (E) were treated with PD0332991 (150 mg/kg/dose; n = 5) or vehicle (n = 5) for 2 weeks (5 days a week) after 3 weeks of inoculation, and the percentages of human CD19-positive/mouse CD45-negative population in bone marrow were monitored by flow cytometry before and after PD0332991 treatment. Asterisks indicate significant difference in \( t \) test (\(^*\), \( P < 0.05 \)). F, survival of NOG mice treated with PD0332991. Survival of NOG mice transplanted with SU-Ph2 or SU/SR and treated with PD0332991 (150 mg/kg/dose) or vehicle for 2 weeks after 3 weeks of inoculation was analyzed by the Kaplan-Meier method. \( P \) value in log-rank test is indicated.
leukemia samples and Ph+ BCP-ALL samples, and there were no samples that lost the Rb gene (Supplementary Fig. SSD). These observations indicated the gene expression profile of cell-cycle regulators was basically identical between cell lines and primary samples of Ph+ lymphoid leukemia, suggesting that PD0332991 might be effective on primary Ph+ lymphoid leukemia cells.

**Discussion**

In hematologic malignancies, loss of p16 gene is frequently observed especially in lymphoid malignancies (3), suggesting that CDK4/6 inhibitors would be effective to control lymphoid leukemias lacking p16 gene function. Surprisingly, however, Ph− BCP-ALL cell lines with loss of p16 gene expression were not as sensitive as Ph+ lymphoid leukemia cell lines; the IC50 of PD0332991 in Ph+ BCP-ALL cell lines with loss of p16 gene expression ranged from 30 nmol/L to 200 nmol/L (median 100 nmol/L), whereas the IC50 of PD0332991 was largely <25 nmol/L in Ph+ lymphoid cell lines. Conversely, a Ph+ ALL cell line (YAMN73) with exceptionally intact p16 gene and protein expression was also sensitive to PD0332991 (IC50 < 25 nmol/L). These observations suggested that there might be underlying mechanisms for the higher sensitivity of Ph+ lymphoid leukemia cell lines to CDK4/6 inhibitors other than loss of p16. Indeed, we found that CDK4 expression level was regulated by both transcriptional and posttranscriptional modulations probably through the PI3K pathway in most of Ph+ lymphoid leukemia cell lines. Considering two previous reports that c-MYC is one of downstream targets of BCR-ABL (38) and that CDK4 is one of transcriptional targets of c-MYC (42), it would be feasible to predict that CDK4 expression is transcriptionally regulated by BCR-ABL through c-MYC at least in part. These findings suggest that upregulation of CDK4 expression by BCR-ABL in Ph+ lymphoid leukemias is critically involved in their higher sensitivity to CDK4/6 inhibitors. Moreover, we also confirmed that cyclin D2 expression was regulated by BCR-ABL in Ph+ lymphoid leukemia cell lines as previously reported (11–15). Consistently, we newly discovered that gene expression level of cyclin D2 was significantly higher in Ph+ lymphoid leukemia cells than in Ph+ lymphoid leukemia cells both in the cell lines and in the primary samples. These findings strongly suggest that upregulation of cyclin D2 gene expression by BCR-ABL in Ph+ lymphoid leukemias is also involved in their higher sensitivity to CDK4/6 inhibitors.

Considering the critical role of cyclin D2 and CDK4 in the cell growth of Ph+ lymphoid leukemias as one of the downstream targets of BCR-ABL, CDK4/6 inhibitors could be effective against Ph+ lymphoid leukemias even if they have the imatinib-resistant BCR-ABL mutations. In fact, we confirmed the antileukemic activity of PD0332991 against an imatinib-resistant Ph+ ALL cell lines (SU/SR, SK-9, and TCCV/SR) having the T315I mutation. Two-week administration of PD0332991 effectively blocked rapid dissemination of SU/SR in NOG mice, whereas administration of imatinib only showed marginal effects. The sensitivity to PD0332991 of SU/SR cells in vivo was almost identical to that of the parental SU-Ph2 cells. As a result, two-week administration of PD0332991 significantly prolonged the survival of both mice transplanted with SU/SR (median, 13 days) and mice transplanted with SU-Ph2 (16 days). Moreover, the in vivo activity of two-week administration of PD0332991 against mice transplanted with SU-Ph2 was as potent as that of imatinib (median, 11 days) as estimated by prolonged survival. Considering that PD0332991 exerts its antileukemic activity against Ph+ lymphoid leukemia cells by targeting the downstream pathway of BCR-ABL, although we have not tested it directly, PD0332991 could be effective against Ph+ lymphoid leukemias having BCR-ABL mutants such as F317L, E255K/V, and Y253H, which are resistant to second-generation TKIs as well as imatinib (43). Overall, the data presented here suggest that CDK4/6 inhibitors are attractive second-line agents for CML-BC and Ph+ ALL patients, in particular those who relapse after TKI therapy.

In clinical testing of solid tumor patients, PD0332991 was generally well tolerated at least as monotherapy (28–30), and a combination trial with bortezomib (44) is currently under way in patients with relapsed mantle cell lymphoma. Thus, PD0332991, probably in combination with chemotherapy (34), will be a new candidate for clinical studies that will hopefully result in advances in the therapy of TKI-resistant CML-BC and Ph+ ALL. However, we found that PD0332991 antagonized the antileukemic activity of VCR particularly when pretreated. As PD0332991 strongly

---

**Figure 6.** Upregulation of Cyclin D2 and loss of p16 in Ph+ lymphoid leukemia cells. A and B, gene expression levels of cell-cycle regulators in Ph+ and Ph− lymphoid leukemia cells. Gene expression in Ph+ lymphoid leukemia cell lines (closed circles) and Ph− lymphoid cell lines (MLL+ ALL cell lines, closed boxes; other BCP-ALL cell lines, open boxes; A) and that in primary samples (Ph+ ALL, closed circles; CML-BC, open circles; MLL− ALL, closed boxes; other BCP-ALL, open boxes; B) were semiquantified by real-time RT-PCR using normal human bone marrow mononuclear cells as a control. As an internal control for relative gene expression, real-time PCR for GAPDH was performed.
induced Go-G1 arrest, it is likely to be even detrimental to schedule PD0323991 in combination with S-phase–dependent chemotherapeutic agents in the clinical setting. In contrast, it should be noted that PD0323991 showed additive effect on antileukemic activity of imatinib. Interestingly, it has also been reported that the combination of PD0323991 with imatinib effectively induced cell-cycle arrest not only in a Ph+ lymphoid cell line but also in an Abl– transduced murine leukemia virus–transformed Pro-B cell line that acquired imatinib resistance due to forced expression of CDK6 (45). These observations suggest that the combination of PD0323991 with TKIs would be effective against Ph+ ALL and CML-IC, although further analysis both in vivo and in vitro is required.

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

Authors’ Contributions
Conception and design: K. Akahane, T. Nakahata, T. Heike, T. Inukai
Development of methodology: S. Saida, I. Kato, T. Nakahata

References

Acknowledgments
The authors thank Masako Abe for technical support. 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 December 15, 2014; revised September 28, 2015; accepted September 29, 2015; published OnlineFirst December 4, 2015.


Molecular Cancer Therapeutics

Specific Antileukemic Activity of PD0332991, a CDK4/6 Inhibitor, against Philadelphia Chromosome–Positive Lymphoid Leukemia


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-1065

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2015/12/04/1535-7163.MCT-14-1065.DC1

Cited articles
This article cites 45 articles, 29 of which you can access for free at:
http://mct.aacrjournals.org/content/15/1/94.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/1/94.full#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.