Novel therapeutic strategy targeting FLT3/ITD mutation in acute myeloid leukemia.

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Abstract:
Background: Acute Myeloid Leukemia (AML) is a heterogenous malignant disorder of hematopoietic stem cells, characterized by excessive proliferation of myeloid precursor cells, and block of cellular differentiation. Multiple genetic defects including chromosomal deletion, insertion and translocation, which result in the generation of in frame fusion proteins have been implicated in the pathogenesis of AML. Based on current detection techniques, up to 45% of AML cases in adults have normal karyotype, thus in those cases point mutations or small rearrangements may affect critical genes. One such genes mutated in AML is FLT3 gene, which express normally in early hematopoietic stem and progenitor cells and acts as a receptor FLT3 ligand. FLT3 is also highly expressed in most cases of AML, is constitutively active up to one-third of patients and is generally a poor prognostic factor. FLT3 activation in AML typically occurs due to internal tandem duplications in the juxtamembrane domain (FLT/ITD) and point mutations activation loop of FLT3receptor. Although it has been shown that FLT3 signaling prevents apoptosis, blocks myeloid differentiation, accelerates cell proliferation and produces a myeloproliferative syndrome in mice, the mechanisms of how these mutations arise and contribute to leukemic transformation are still not fully understood.
Objective: The current study is aimed at elucidating the mechanisms of cell cycle regulation of FLT3/ITD positive AML cells and also to clarify the molecular basis of cytotoxic action o PKC412, an inhibitor of FLT3 and other tyrosine kinases including protein kinase C that is currently undergoing clinical testing.
Results: PKC412 is a staurosporine derivative that inhibits several protein kinases including FLT3 and is highly anticipated as a novel therapeutic agent for acute myeloblastic leukemia (AML) carrying FLT3 mutations. In this study, we show that PKC412 exerts differential cell cycle effects on AML cells depending on the presence of FLT3 mutations. PKC412 elicits massive apoptosis without markedly affecting cell cycle patterns in AML cell lines with FLT3 mutations (MV4-11 and MOLM13), whereas it induces G2 arrest but not apoptosis in AML cell lines without FLT3 mutations (THP-1 and U937). In MV4-11 and MOLM13 cells, PKC412 inactivates Myt-1 and activates CDC25c, leading to the activation of CDC2. Activated CDC2 phosphorylates Bad at serine-128 and facilitates its translocation to the mitochondria, where Bad triggers apoptosis. In contrast, PKC412 inactivates CDC2 by inducing serine-216 phosphorylation and subsequent cytoplasmic sequestration of CDC25c in THP-1 and U937 cells. As a result, cells are arrested in the G2 phase of the cell cycle, but do not undergo apoptosis because Bad is not activated. The FLT3 mutation-dependent differential cell cycle effect of PKC412 is considered an important factor when PKC412 is combined with cell cycle-specific anticancer drugs in the treatment of cancer and leukemia.

Introduction: MS-like tyrosine kinase-3 (FLT3) is a member of class III receptor tyrosine kinases, and is expressed on hematopoietic stem/progenitor cells. The binding of FLT3 ligand induces dimerization and autophosphorylation of FLT3, and subsequently activates multiple signal transduction pathways favoring cell survival and proliferation (Lyman et al., 1993; Small et al., 1994). FLT3 is also expressed in the majority of acute leukemias and is overexpressed in many cases (Gilliland and Griffin, 2002). In addition, its mutations are found in approximately 30% of patients with acute myeloblastic leukemia (AML), and accordingly, these are the second most common genetic alterations in AML. FLT3 mutations include internal tandem duplication of the juxtamembrane domain (FLT3-ITD) and point mutations in the activating loop, both of which result in ligand-independent activation of FLT3. The constitutive activation of FLT3 appears to promote aberrant proliferation and survival of hematopoietic stem/progenitor cells, leading to leukemogenesis (Kelly et al., 2002).

Because of the high frequency of mutations and overexpression, FLT3 should be an appropriate therapeutic target in AML (Griffin, 2004). It has been shown that the staurosporine derivative PKC412 inhibits multi-target tyrosine kinases, including FLT3, protein kinase C, CDC2 and receptors for PDGF, FGF and VEGF (Levis and Small, 2005). PKC412 induces apoptosis in blasts from AML patients with FLT3 mutations, and prolongs survival in animal models of FLT3-induced myeloproliferative diseases

(Weisberg et al., 2002). Clinical trials for patients with solid tumors and AML revealed that PKC412 alone yielded limited clinical activity (Propper et al., 2001; Stone et al., 2005). To overcome this limitation, it is reasonable to combine PKC412 with other conventional antileukemic agents. Recently, we have reported that PKC412 has synergistic effects with most antileukemic agents for FLT3-mutated leukemia cell lines (Furukawa et al., 2007). In contrast, PKC412 is antagonistic to most drugs, except for those inducing mitotic arrest or active in the G2/M phase in leukemia cell lines without FLT3 mutations. This finding provides useful information for the design of effective combination therapy containing PKC412. For safe and effective clinical applications, however, it is essential to clarify the molecular basis of the FLT3 mutation-dependent divergence of leukemic cell responses to PKC412. In this study, we attempted to resolve this issue by analysing the effects of PKC412 on the expression of cell cycle regulators and Bcl-2 family proteins in AML cell lines with or without FLT3 mutations.

Materials and methods:

Reagents: PKC412 was purchased from LC Laboratories (Woburn, MA, USA), dissolved in dimethyl sulfoxide and stored at −20 °C until use.

Cells and cell culture: We used four human AML cell lines, that is, MOLM13, derived from a patient with acute monocytic leukemia (AMoL) carrying t(9;11); MV4-11, derived from a patient with AMoL carrying t(4;11); THP-1 and U937, derived from patients with AMoL without FLT3 mutations. MV4-11, THP-1 and U937 were purchased from the American Type Culture Collection (Manassas, VA, USA), and MOLM13 was provided by Dr Yoshinobu Matsuo (Grandsoul Research Institute for Immunology, Nara, Japan). These cell lines were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. Primary AML cells were isolated from the bone marrow of patients at the time of diagnostic procedure. Informed consent was obtained in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional review board. The presence of FLT3-ITD was examined as described by Kiyoi et al. (1997).

Flow cytometric analysis: The cell cycle profile was obtained by staining DNA with Vindelov's solution (0.04 mg ml−1 propidium iodide in 5 mM Tris–HCl, 5 mM NaCl and 0.005% nonidet P-40) in preparation for flow cytometry. The size of the sub-G1, G0/G1 and S+G2/M fractions was calculated as a percentage by analysing DNA histograms with the ModFitLT 2.0 program (Verity Software, Topsham, ME, USA). Surface marker analysis was carried out according to the standard method.

Immunocytochemistry: After collecting on glass slides using a Cytospin centrifugator (Shandon Scientific, Cheshire, England), cells were fixed in 4% paraformaldehyde in phosphate-buffered saline, and stained with a rabbit anti-tubulin polyclonal antibody (Sigma, St Louis, MO, USA) and Alexa 488-conjugated goat antibody to rabbit immunoglobulin (Molecular Probes, Eugene, OR, USA) as primary and secondary antibodies, respectively. Finally, nuclear DNA was stained with 100 ng ml−1 propidium iodide in phosphate-buffered saline for 5 min.

Detection of apoptosis: Apoptosis was detected in situ by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method using MEBSTAIN apoptosis kit (MBL International, Woburn, MA, USA). In brief, cells were fixed on glass slides as described above, and treated with terminal deoxynucleotidyl transferase (TdT) in the presence of biotin-dUTP to label 3′-ends of fragmented DNA. The labeled ends were visualized with avidin-conjugated fluorescein isothiocyanate (FITC). Finally, intact DNA was counterstained with propidium iodide.

Confocal laser microscopy: Confocal microscopic analysis was performed using the following antibodies: anti-CDC25c (C2-2; BD Pharmingen, San Jose, CA, USA), antiphosphorylated CDC25c at serine-216 (no. 4901; Cell Signalling Technology, Beverly, MA, USA), anti-human 14-3-3 (C23-1; BD Pharmingen), anti-Bad (no. 9292; Cell Signalling Technology) and anti-human mitochondria (AE-1; Leinco Technologies Inc., St Louis, MO, USA). We used Alexa 488-conjugated goat antibody to mouse immunoglobulin (Molecular Probes) and Cy3-conjugated goat antibody to rabbit immunoglobulin (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as secondary antibodies.

Immunoprecipitation and immunoblotting: Immunoprecipitation and immunoblotting were carried out according to the standard methods using the following antibodies: anti-FLT3 (8F2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiphosphorylated FLT3 at tyrosine-591 (no. 3461; Cell Signalling Technology), antiphosphorylated protein kinase C (pan) at serine-660 (no. 9371; Cell Signalling Technology), antiphosphorylated c-Kit at tyrosine-719 (no. 3391; Cell Signalling Technology), antiphosphorylated PDGF receptor β at tyrosine-751 (no. 3161; Cell Signalling Technology), antiphosphorylated CDC2 at tyrosine-15 (no. 9111; Cell Signalling Technology), anti-Bad monoclonal (clone 48; BD Transduction Laboratories, San Jose, CA, USA), anti-Bad polyclonal (no. 9292; Cell Signalling Technology), antiphosphorylated Bad at serine-136 (no. 9295; Cell Signalling Technology), antiphosphorylated Bad at serine-128 (AB3567; Chemicon International, Temecula, CA, USA), anti-Bcl-2 (clone 4D7; BD Transduction Laboratories), anti-Bcl-x (clone 44; BD Transduction Laboratories) and anti-β-actin (C4; ICN Biomedicals, Aurora, OH, USA).

Results:

Effects of PKC412 on growth kinetics of AML cell lines with or without FLT3 mutations

We first compared the sensitivity to PKC412 of four AML cell lines: MOLM13, MV4-11, THP-1 and U937. It has been shown that MOLM13 and MV4-11 cell lines carry FLT3-ITD), whereas FLT3 is in wild-type configuration in THP-1 and U937 cell lines (Quentmeier et al., 2003). THP-1 cells express a large amount of FLT3 but U937 cells do not express FLT3 (Yao et al., 2003). In pilot experiments, we determined the dose–response curves at day 3 (data not shown), and calculated the IC50 values for MOLM13, MV4-11, THP-1 and U937 to be 38±4, 34±6, 116±26 and 207±41 nM, respectively. On the basis of this result, we treated these cell lines with 100 nM PKC412 and observed the changes in growth kinetics. As shown in Figure 1a, PKC412 almost completely inhibited an increase in cell

numbers of MOLM13 and MV4-11 cell lines. Growth inhibition was accompanied by the abrogation of constitutive phosphorylation of FLT3 (Figure 1b). The effects of PKC412 on other possible targets were variable and not correlated with cytotoxicity (Figure 1c and Supplementary Figure 1), suggesting that FLT3 is a principal target of PKC412-mediated growth inhibition of MOLM13 and MV4-11 cells. The proliferation of THP-1 cells was also markedly suppressed by PKC412, whereas U937 cells were relatively resistant to the drug (Figure 1a, lower panel). It is of note that the steady-state growth of AML cell lines with FLT3 mutations (MOLM13 and MV4-11) was significantly slower than that of AML cell lines without FLT3 mutations (THP-1 and U937) (Figure 1a, upper panel)

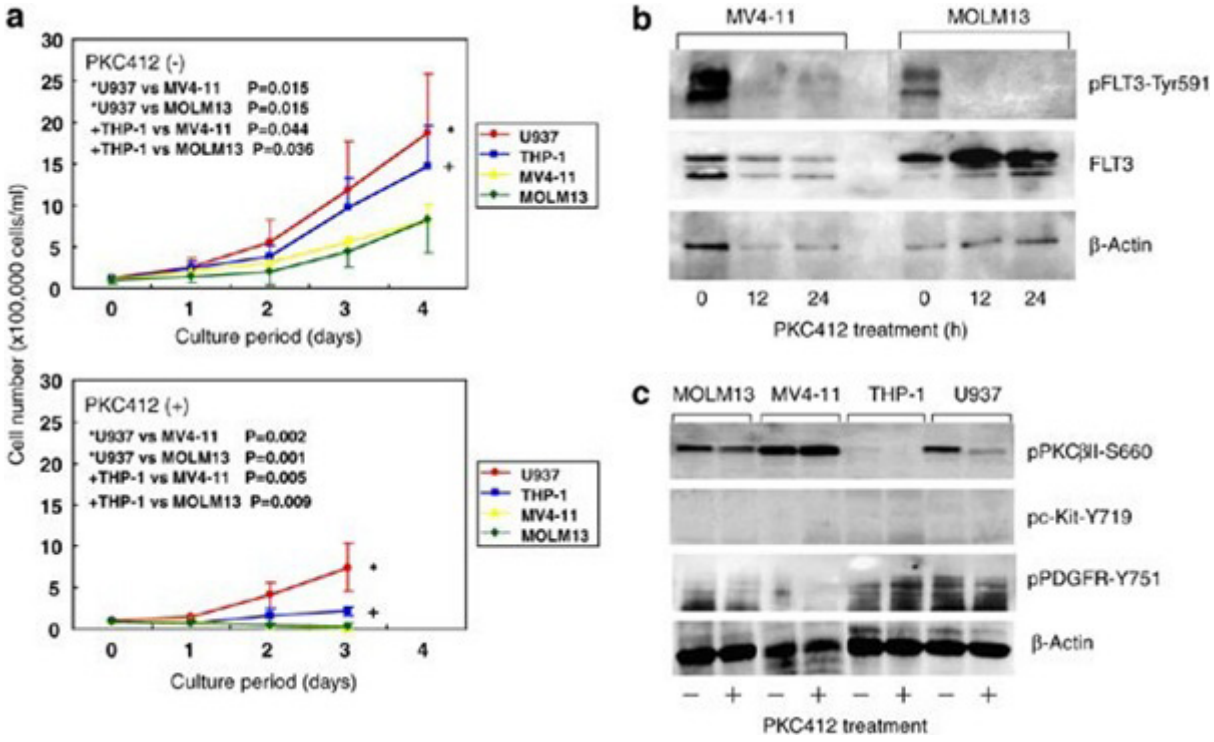


Figure 1. Effects of PKC412 on cell growth and FLT3 phosphorylation of acute myeloblastic leukemia (AML) cell lines.

(a) We seeded AML cell lines at an initial concentration of 1 × 105 cells ml−1and cultured in the absence (upper panel) or presence (lower panel) of 100 nM PKC412 for up to 4 days. (b) Whole cell lysates were prepared from MV4-11 and MOLM13 cells cultured with 100 nM PKC412 at the indicted time points and subjected to immunoblotting with an antibody that specifically recognizes FLT3 phosphorylated at tyrosine-591 (pFLT3-Tyr591).

Cell cycle analysis of PKC412-treated AML cell lines

Next, we attempted to clarify the underlying mechanisms of PKC412-mediated growth inhibition of AML cell lines using cell cycle analysis. PKC412-induced apoptosis, as judged by an increase in the size of the sub-G1 fraction (see Figure 2a for representative results, and Table 1 for quantification and statistical analysis of three independent experiments), DNA fragmentation (Figure 2b) and the appearance of apoptotic bodies (Figure 2c), in MOLM13 and MV4-11 cell lines after 24 h of culture without markedly affecting cell cycle distribution except for a minor decrease in the fraction of cells in the growth phases (S and G2/M phases). In contrast, THP-1 and U937 cells were arrested in the G2 phase by PKC412 after 12 h of treatment, and underwent mitotic slippage to become hyperploid after 48 h, especially in the latter (Figure 2 and Table 1). Apoptosis was barely observed in these cell lines up to 48 h of culture.

The difference of the changes in cell cycle distribution between PKC412-treated AML cell lines with FLT3-ITD and those with wild-type FLT3 may provoke the differential responses of cells to the combination of PKC412 and cell cycle-modifying drugs. To verify this hypothesis, we examined the combined effects of PKC412 and either aphidicolin, which induces G1/S arrest by inhibiting DNA polymerase α, or colchicine, which arrests cells at mitosis by disrupting mitotic spindle. As shown in Figure 2d, PKC412 was synergistic with both aphidicolin and colchicine in MV4-11 cells, but only with colchicine in U937 cells. This is fully consistent with the pattern of cell cycle changes induced by PKC412 in each cell line.

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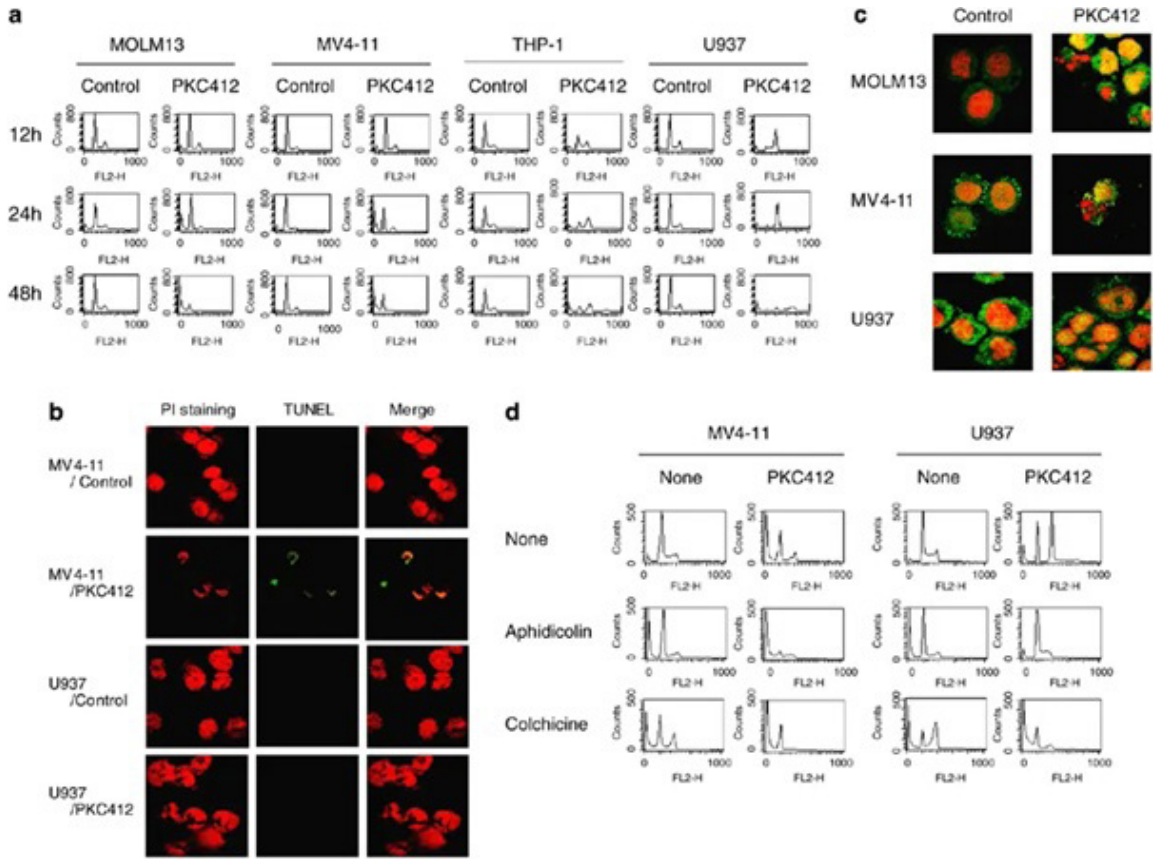


Figure 2. Differential effects of PKC412 on the cell cycle of acute myeloblastic leukemia (AML) cell lines with or without FLT3 mutations.

MOLM13, MV4-11, THP-1 and U937 cells were seeded at 1 × 105 cells ml−1 and cultured in the absence (control) or presence (PKC412) of 100 nM PKC412. (a) Cells were harvested at the indicated time points,and stained with propidium iodide in preparation for cell cycle analysis. b) After 24 h of culture, cells were harvested for the detection of DNA fragmentation by the TUNEL method.

PKC412 differentially modulates the phosphorylation of CDC2 in AML cell lines depending on the presence of FLT3 mutations

The above results indicate that PKC412 inhibits the growth of AML cells via distinct mechanisms depending on the presence of FLT3 mutations. We investigated the underlying mechanisms of differential responses to PKC412. First, we checked the phosphorylation status of CDC2, which reflects its activity in vivo, in PKC412-treated AML cell lines. As shown in Figure 3a, CDC2 was heavily phosphorylated at tyrosine-15, an ATP-binding site critical for the kinase function of CDC2 (Watanabe et al., 1995), in untreated MOLM13 and MV4-11 cells. In contrast, CDC2-tyrosine 15 phosphorylation was not prominent in untreated THP-1 and U937 cells. These results suggest that CDC2 is relatively inactive in AML cell lines with FLT3 mutations but is constitutively active in AML cell lines with wild-type FLT3. This pattern is fully compatible with the steady-state growth kinetics of these cells (Figure 1a). PKC412 modified the phosphorylation state of CDC2 in a reciprocal manner dependent on the presence of FLT3 mutations: CDC2 phosphorylation decreased in MOLM13 and MV4-11 and increased in THP-1 and U937 after treatment with the drug. The inhibition of CDC2 kinase activity may underlie PKC412-induced G2 arrest of THP-1 and U937 cells.

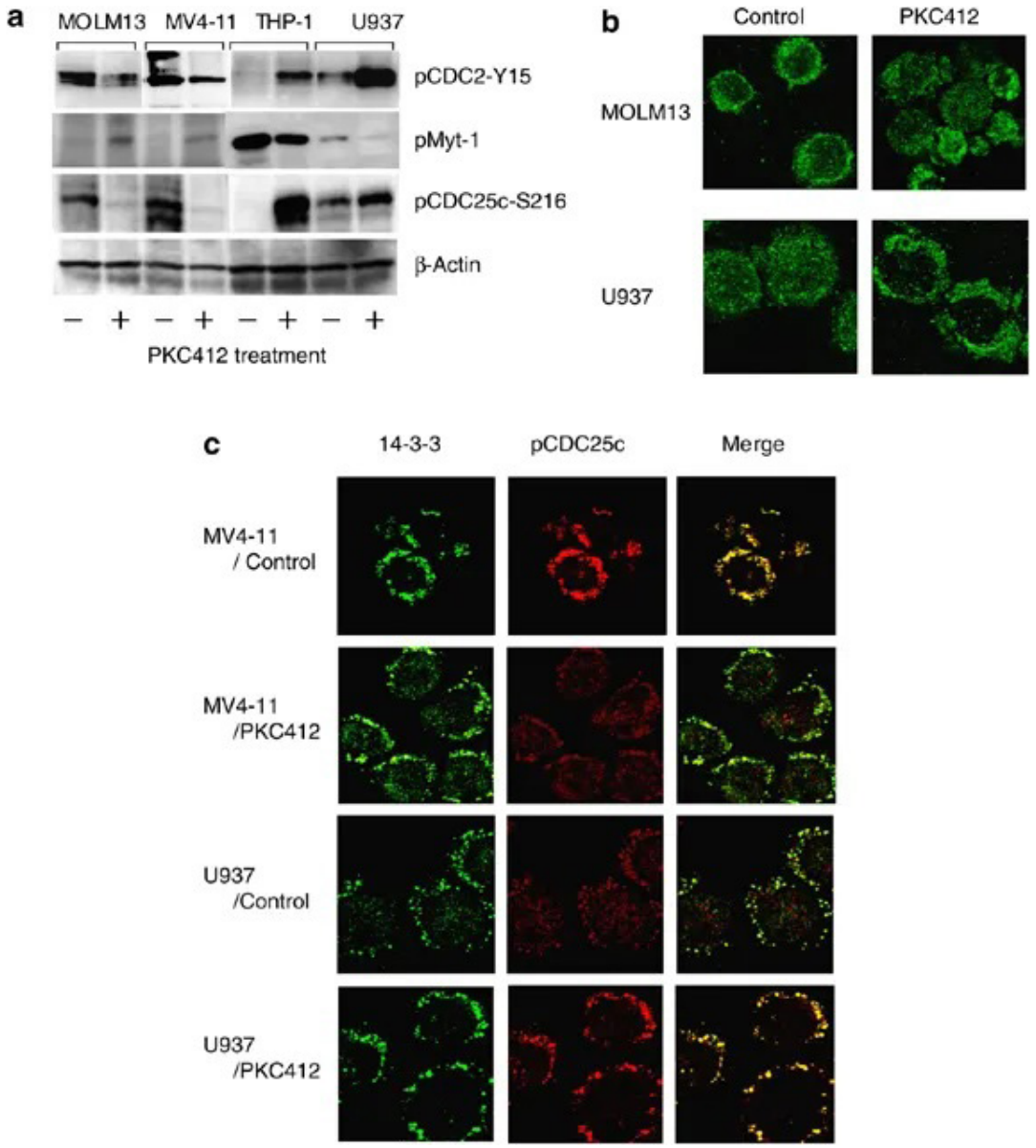


Figure 3. PKC412 differentially modulates CDC2 phosphorylation in acute myeloblastic leukemia (AML) cell lines depending on the presence of FLT3 mutations.

(a) Whole cell lysates were prepared from AML cell lines before (−) and after (+) culture with 100 nM PKC412 for 24 h and subjected to immunoblot analysis for the indicated proteins. (b) Intracellular localization of CDC25c was examined by immunocytochemistry in MOLM13 and U937 cells cultured in the absence (control) or presence (PKC412) of 100 nM PKC412 for 24 h. (c) Colocalization of CDC25c and 14-3-3 proteins was examined by confocal microscopy in MV4-11 and U937 cells cultured in the absence (control) or presence (PKC412) of 100 nM PKC412 for 24 h.

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s Myt-1 kinase is responsible for CDC2 phosphorylation at tyrosine-1, we examined the activation status of Myt-1 in these cells by immunoblotting with an antibody specifically recognizing the inactive/phosphorylated species of Myt-1. Consistent with the phosphorylation status of CDC2, Myt-1 was active (underphosphorylated) in AML cell lines with FLT3-ITD and inactive (phosphorylated) in AML cell lines without FLT3-ITD before treatment with PKC412 (Figure 3a). Myt-1 became phosphorylated and thereby inactivated by PKC412 in the former, whereas it became dephosphorylated and thereby activated in the latter, leading to the increased phosphorylation of CDC2. It is of note that Wee1, another kinase involved in tyrosine-15 phosphorylation, was below the detection limit in FLT3 mutation-positive leukemic cells as previously described (Neben et al., 2005).

Next, we investigated the effects of PKC412 on the activity and intracellular localization of CDC25c, which activates CDC2 by removing phosphates from tyrosine-15. CDC25c was phosphorylated at serine-216 in untreated MOLM13 and MV4-11 cells, and was dephosphorylated by PKC412 (Figure 3a). In contrast, PKC412 increased the serine-216 phosphorylation of CDC25c in THP-1 and U937 cells. Upon phosphorylation at serine-216, CDC25c binds to 14-3-3 proteins and is translocated from the nucleus to the cytoplasm (Lopez-Girona et al., 1999). We therefore confirmed the cytoplasmic sequestration of CDC25c phosphorylated at serine-216 using immunocytochemistry. As shown in Figure 3b, CDC25c was mostly retained in the cytoplasm in untreated MOLM13 cells, and was translocated to the nucleus after treatment with PKC412. In contrast, CDC25c was distributed throughout the entire cells in untreated U937 cells, and became sequestered in the cytoplasm and thus inactivated by the addition of PKC412.

These observations are fully consistent with the pattern of serine-216 phosphorylation shown in Figure 3a. Furthermore, we demonstrated the binding of phosphorylated CDC25c to 14-3-3 proteins in cytoplasm using confocal microscopy. As shown in Figure 3c, phosphorylated CDC25c colocalized with 14-3-3 in untreated MV4-11 cells, and the interaction was dissociated after culture with PKC412. In U937 cells, PKC412 increased the amounts of phosphorylated CDC25c, which translocated from the nucleus and bound to 14-3-3 proteins in the cytoplasm. These results at least partly explain why PKC412 arrests cells in the G2 phase of the cell cycle exclusively in AML cell lines without FLT3 mutations.

PKC412-induced apoptosis of AML cell lines with FTL3 mutations is mediated via selective phosphorylation of Bad at serine-128

Finally, we attempted to clarify the molecular mechanisms by which only AML cells with FLT3 mutations underwent apoptosis in the presence of PKC412. To this end, we screened for the expression of proapoptotic members of the Bcl-2 family by immunoblotting. As the expression of Bax and Bak was constitutive and unaffected by PKC412 in four AML cell lines (data not shown), we focused on BH3-only members of the Bcl-2 family, direct inducers of apoptosis (Galonek and Hardwick, 2006; Green, 2006). Among BH3-only proteins examined, only Bad was moderately expressed, and Bim, Bid, Puma and Noxa were undetectable in AML cell lines used in this study (data not shown). As PKC412 did not change the abundance of Bad protein, we examined the phosphorylation status of Bad using phosphorylation site-specific antibodies. It has been shown that Bad phosphorylation at various residues represents a checkpoint for the cell fate decision. For instance, CDC2 enhances the proapoptotic ability of Bad by promoting serine-128 phosphorylation and mitochondrial translocation of Bad (Konishi et al., 2002). In contrast, Akt promotes cell survival by phosphorylating Bad at serine-136, which provides a canonical binding site for 14-3-3 proteins to disrupt interaction with Bcl-2/Bcl-x on mitochondrial membranes (Zha et al., 1996). Bad was phosphorylated at serine-136 and was inactive in untreated AML cells (Figure 4a, upper panel). Marginal levels of phosphorylation at serine-128 were observed in THP-1 and U937 cells, which may be due to the hyperactivation of CDC2. PKC412 induced Bad phosphorylation at serine-128 selectively in AML cell lines with FLT3-ITD along with dephosphorylation at serine-136 (Figure 4a, lower panel). AML cell lines with wild-type FLT3 did not show serine-128 phosphorylation. Next, we examined the intracellular localization of Bad using confocal microscopy. In untreated MV4-11 and U937 cells, Bad was distributed throughout the cytoplasm and did not colocalize with mitochondria (Figure 4b, Control). PKC412 induced the translocation of Bad to the mitochondria in MV4-11 cells but not in U937 cells (Figure 4b, PKC412). Intracellular localization of Bad is fully compatible with the alteration of site-specific phosphorylation of Bad, and at least partly explains the selective induction of apoptosis in AML cells with FLT3 mutations by PKC412.
Next, we determined the effects of PKC412 on the expression of antiapoptotic members of the Bcl-2 family. Immunoblot analyses revealed that AML cell lines used in this study expressed Bcl-2 and Bcl-x but not Mcl-1 and Bcl-w (data not shown). As shown in Figure 4c, Bcl-2 was readily downregulated by PKC412 in all four cell lines. The effects of PKC412 on Bcl-x expression were variable, suggesting that downregulation of Bcl-2 and Bcl-x is not a primary determinant of the sensitivity of AML cells to PKC412. However, it is possible that the reduced levels of Bcl-2 expression cooperate with the activation of Bad to enhance apoptosis in PKC412-treated MOLM13 and MV4-11 cells.

PKC412 induces apoptosis in primary AML cells with FTL3 mutations via the CDC2-Bad pathway

To testify the clinical relevance of our findings, we carried out the same experiments using primary AML cells from three patients with AML carrying FLT3-ITD. As shown in Figure 5a, PKC412 induced apoptosis with a decrease in the fraction of cells in the S phase in two cases and G1 arrest with a moderate degree of apoptosis in one case. The effects of the drug on the phosphorylation status of FLT3, CDC2, Myt-1, CDC25c and Bad were almost identical to those observed in MOLM13 and MV4-11 cell lines (Figure 5b). There results indicate that our findings are not only valid in the cell line models, but have more general meanings.

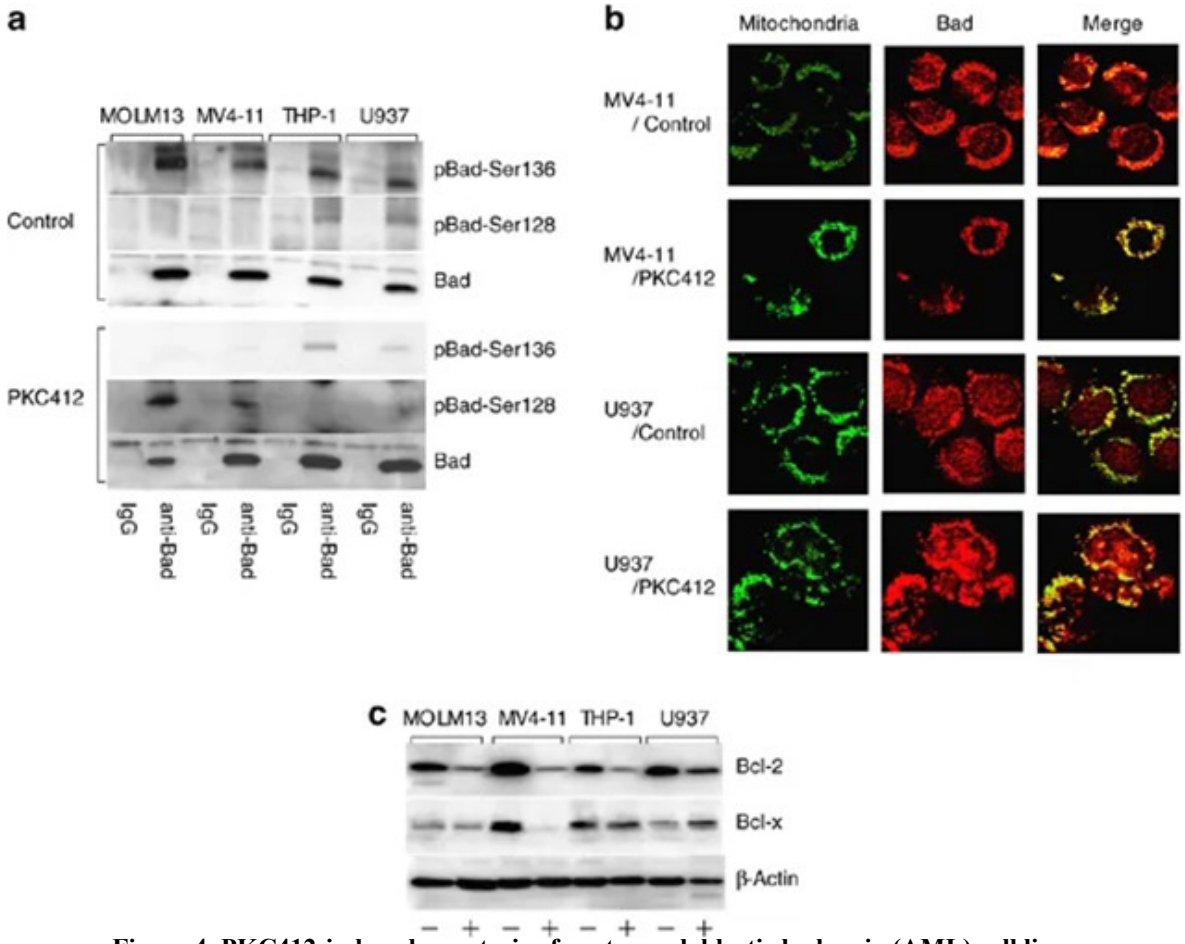


Figure 4. PKC412-induced apoptosis of acute myeloblastic leukemia (AML) cell lines with FTL3 mutations is mediated via phosphorylation of Bad at serine-128.

(a) Whole cell lysates subjected to immunoprecipitation with either anti-Bad monoclonal antibody (anti-Bad) or mouse IgG (IgG). (b) For in situ visualization of Bad, we used anti-Bad polyclonal antibody and Cy3-conjugated goat antibody to rabbit immunoglobulin as primary and secondary antibodies, respectively. (c) Protein samples in (a) were subjected to immunoblot analyses for Bcl-2, Bcl-x and β-actin (loading control). Data shown are representative of multiple independent experiments.

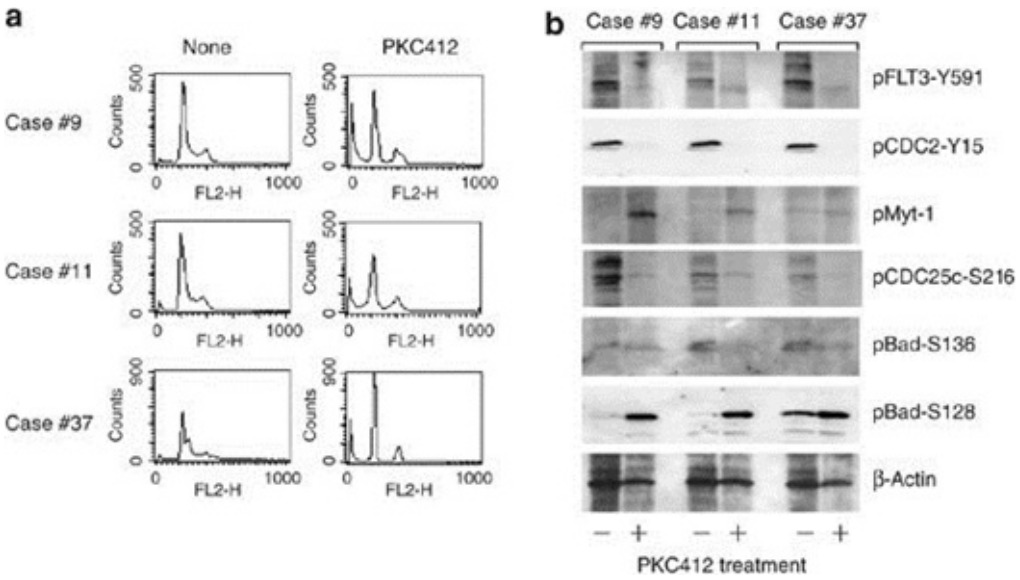


Figure 5. PKC412 induces apoptosis and cell cycle alterations in primary acute myeloblastic leukemia (AML) cells via the CDC2-Bad pathway.

Bone marrow mononuclear cells from patients with AML carrying FLT3-ITD were cultured in the absence (none) or presence (PKC412) of 100 nM PKC412 for 24 h and subjected to cell cycle analysis (a) and immunoblotting (b).

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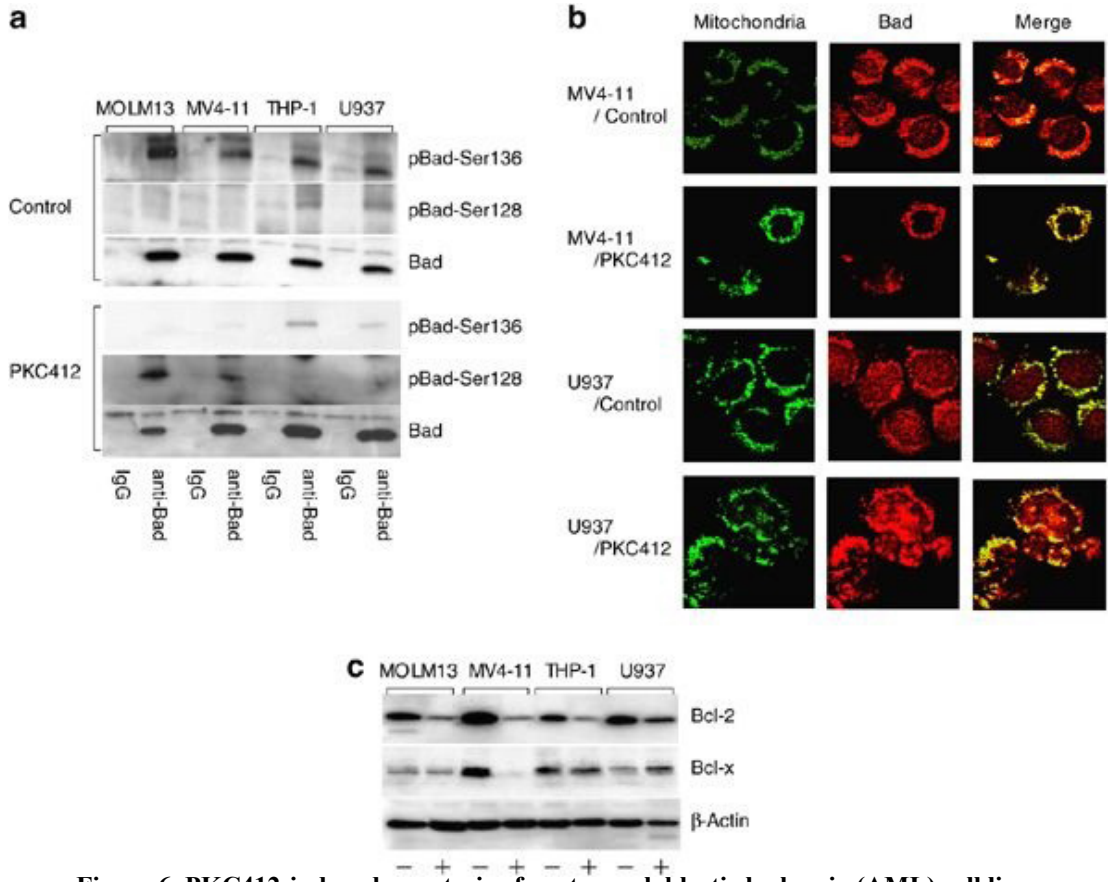


Figure 6. PKC412-induced apoptosis of acute myeloblastic leukemia (AML) cell lines with FTL3 mutations is mediated via phosphorylation of Bad at serine-128.

(a) Whole cell lysates wered subjected to immunoprecipitation with either anti-Bad monoclonal antibody (anti-Bad) or mouse IgG (IgG). Immunoprecipitants were resolved on 12% acrylamide gels, followed by immunoblotting with polyclonal antibodies against Bad phosphorylated at serine-136, Bad phosphorylated at serine-128 and Bad. (b) For in situ visualization of Bad, we used anti-Bad polyclonal antibody and Cy3-conjugated goat antibody to rabbit immunoglobulin as primary and secondary antibodies, respectively. For co-labeling of mitochondria, we used a combination of anti-human mitochondria monoclonal antibody and Alexa 488-conjugated goat antibody to mouse immunoglobulin. (c) Protein samples in (a) were subjected to immunoblot analyses for Bcl-2, Bcl-x and β-actin (loading control). Data shown are representative of multiple independent experiments.

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Discussion

In this study, we obtained a clue pertinent to the molecular basis of the synergism and antagonism of PKC412 with other antileukemic agents. PKC412 alone induced G2 arrest but not apoptosis in AML cell lines without aberrant FLT3 activation (THP-1 and U937) at a clinically achievable concentration. Because of the ability to cause G2 arrest, PKC412 is synergistic with drugs that are active in the G2/M phase, such as colchicine, in these cell lines. This may be an important advantage for the usage of PKC412 over other FLT3 inhibitors, because such effects have not been demonstrated with other FLT3 inhibitors (Levis et al., 2004; Yee et al., 2004). For the same reason, PKC412 may be antagonistic with drugs that synchronize target cells in late G1 to early S phase, such as aphidicolin, in FLT3 mutation-negative leukemias. In contrast, PKC412-induced massive apoptosis without markedly affecting cell cycle patterns in AML cell lines harboring FLT3 mutations, which may underlie the synergism of PKC412 with most antileukemic agents.

Next, we attempted to elucidate the underlying mechanisms whereby PKC412 differentially affects the cell cycle distribution depending on the presence of FLT3 mutations. We obtained evidence suggesting that the cell cycle effect is mediated through the modulation of CDC2 activity by PKC412. The kinase activity of CDC2 is regulated by the opposing effects of specific kinases (Myt-1 and Wee1) and a specific phosphatase (CDC25c) on tyrosine-15, an ATP-binding site of CDC2 (Galaktionov et al., 1995; Watanabe et al., 1995). In untreated AML cell lines harboring FLT3-ITD, CDC2 was found to be at least partially inactivated by phosphorylation at tyrosine-15. This may be attributable to FLT3-ITD-mediated activation of Myt-1 kinase and inactivation of CDC25c by serine-216 phosphorylation and subsequent binding to 14-3-3 proteins. We are now trying to elucidate the signaling path

ways of FLT3-ITD causing these changes. The partial inactivation of CDC2 may underlie the observation that AML cells with FLT3-ITD grow slower than those with wild-type FLT3. PKC412 reversed this process and activated CDC2 by promoting Myt-1 inactivation and facilitating nuclear translocation of CDC25c. In AML cells without FLT3-ITD, CDC2 is mostly in an active conformation and allows rapid cell cycling. PKC412 seems to inhibit CDC2 activity through CDC25c inactivation, which results in G2 arrest. Although the precise mechanisms underlying this phenomenon are at present unknown, similar observations have been reported in non-small cell lung cancer and glioblastoma (Ikegami et al., 1996; Begemann et al., 1998).

Finally, we went on to identify target molecules responsible for selective induction of apoptosis in PKC412-treated AML cells with FLT3 mutations. To this end, we investigated the expression and function of Bcl-2 family proteins. Apoptotic cell death usually occurs through mitochondrial outer membrane permeabilization (MOMP), which is controlled by the balance between pro- and antiapoptotic members of the Bcl-2 family. Bad is a member of the BH3-only subfamily, and is known to promote MOMP by activating membrane-anchored proapoptotic Bcl-2 family proteins Bax and Bak (Galonek and Hardwick, 2006; Green, 2006). The function of Bad is tightly regulated by phosphorylation. For instance, the PI3 kinase-Akt pathway mediates phosphorylation of Bad at serine-136 and abrogates its proapoptotic activity (Zha et al., 1996). It is well known that FLT3-ITD phosphorylates Bad through the Akt pathway and suppresses apoptosis of hematopoietic stem cells, which contribute to the development of leukemia (Kim et al., 2006). In contrast, serine-128 phosphorylation promotes apoptosis in primary neurons by antagonizing survival functions of growth factors (Konishi et al., 2002). Serine-128 phosphorylation is shown to be catalysed by CDC2 and c-Jun N-terminal kinase (Donovan et al., 2002). Our results suggest that PKC412 causes phosphorylation of Bad at serine-128 via CDC2 activation along with dephosphorylation at serine-136 via blocking FLT3-ITD signaling, which results in apoptosis of AML cells with FLT3 mutations. In AML cells without FLT3-ITD, Bad phosphorylation at serine-128 did not occur because of PKC412-mediated inactivation of CDC2. Although serine-136 was dephosphorylated in some degree, these cells did not undergo apoptosis in response to PKC412. This is in line with a previous report that dephosphorylation of Bad is not sufficient and downregulation of Bcl-x is necessary for the induction of apoptosis (Yang et al., 2005). In addition, Bcl-x inhibits CDC2 kinase activity at the G2/M checkpoint by direct binding and causes G2 arrest (Schmitt et al., 2007). As Bcl-x was not downregulated in PKC412-treated THP-1 and U937 cells, it is possible that Bcl-x serves as an additional factor to determine the responses to PKC412.

This thesis containing original research materials that have been published next peer reviewed journals with impact factor:

Leukemia	*9.9*
Oncogene	*7.5*
Oncology Research	*4.6*
Atherosclerosis,	*4.5*

Original papers

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