Combining the FLT3 Inhibitor PKC412 and the Triterpenoid CDDO-Me Synergistically Induces Apoptosis in Acute Myeloid Leukemia with the Internal Tandem Duplication Mutation

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Abstract
Mutations of the FLT3 receptor tyrosine kinase consisting of internal tandem duplications (ITD) have been detected in blasts from 20% to 30% of patients with acute myeloid leukemia (AML) and are associated with a poor prognosis. FLT3/ITD results in constitutive autophosphorylation of the receptor and factor-independent survival in leukemia cell lines. The C-28 methyl ester of the oleane triterpenoid (CDDO-Me) is a multifunctional molecule that induces apoptosis of human myeloid leukemia cells. Here, we report that CDDO-Me blocks targeting of NFκB to the nucleus by inhibiting IκB kinase β–mediated phosphorylation of IκBα. Moreover, CDDO-Me blocked constitutive activation of the signal transducer and activator of transcription 3. We report the potent and selective antiproliferative effects of CDDO-Me on FLT3/ITD-positive myeloid leukemia cell lines and primary AML cells. The present studies show that CDDO-Me treatment results in caspase-3–mediated induction of apoptosis of FLT3/ITD-expressing cells and its antiproliferative effects are synergistic with PKC412, a FLT3-tyrosine kinase inhibitor currently in clinical trials. Taken together, our studies indicate that CDDO-Me greatly enhanced the efficacy of the FLT3 inhibitor PKC412, suggesting that combining two separate pathway inhibitors might be a viable therapeutic strategy for AML associated with a FLT3/ITD mutation.

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Introduction
Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the accumulation of immature hematopoietic stem cells in the marrow with resultant bone marrow failure (1, 2). The accumulation of such immature cells is presumed to be in part due to disordered hematopoietic differentiation mechanisms, overabundant proliferation, and/or failure to undergo apoptosis. Many potentially leukemogenic pathogenetic alterations have been described, some of which provide therapeutic targets (3). One important genetic alteration that leads to accelerated proliferation in hematopoietic stem cells is an activating mutation of the FLT3 receptor tyrosine kinase, present in blasts from ~35% of patients with AML (4). Two distinct types of FLT3 mutations have been identified in patients with AML. Internal tandem duplication (ITD) mutations, characterized by a repeat of between 3 and more than 100 amino acids within the juxtamembrane domain, are found in ~25% to 30% of AML patients (5, 6). This extra sequence seems to be responsible for the disruption of the autoinhibitory activity of the juxtamembrane domain resulting in the constitutive activation of FLT3 and an adverse prognosis (particularly if present in homozygous form; ref. 7) in patients with AML (8). Blasts from another 10% of patients with AML harbor a point mutation in the tyrosine kinase domain, typically a D835Y mutation (9). The prognostic effect of tyrosine kinase domain–activating mutations is unclear (10, 11). FLT3 inhibitors (12), including PKC412 (midostaurin; ref. 13), CEP-701 (14), and sorafenib (15, 16) are being developed as potential therapeutic agents in mutant FLT3 AML.

In normal myeloid cells, ligand binding to wild-type FLT3 activates multiple signals including the phosphoinositide-3-kinase/akt, RAS/mitogen-activated protein kinase, and signal transducer and activator of transcription (STAT) pathways. In leukemic cells with FLT3-ITD, these pathways are constitutively activated (17). The mechanism of FLT3/ITD-induced proliferation is due in part to the activation via phosphorylation of the phosphoinositide-3-kinase/akt pathway, noted in both transformed BaF3 human AML cell lines and cells from AML patients (18-20).
The STAT family of transcription factors and NFκB are activated by separate upstream signals and are implicated in multiple processes required for neoplasia including transformation, tumor cell survival, and invasion/metastasis (21, 22). STAT3 activation has also been thought to be pleiotropically important in carcinogenesis (23). STAT3 is a transcription factor originally identified as a mediator of the acute phase of the inflammatory response (24). Moreover, constitutive STAT activity has been observed in ~50% of patients with AML (25) and is associated with adverse treatment outcomes (26). Upon activation, STAT3 dimerizes and translocates to the nucleus to activate target genes, including regulators of cell cycle progression and inhibitors of apoptosis (27). Inhibiting downstream pathways such as the JAK family has been suggested to be a strategy that could enhance the effects of tyrosine kinase inhibition.

A potential inhibitor of STAT activation is the C-28 methyl ester of the oleane triterpenoid (CDDO-Me), belonging to a new class of agents that have antiproliferative and proapoptotic activity (28). CDDO-Me is being developed for use in a wide variety of human neoplasms and is currently in phase I and phase II clinical trials. CDDO-Me induces apoptosis in AML, myeloma, and diverse solid tumors (29). One mechanism of resistance to apoptosis in malignant cells may be related to inhibition of reactive oxygen species which normally accumulate during stress. However, when cells are treated with high concentrations of CDDO-Me, apoptosis is triggered by an increase in reactive oxygen species (30). CDDO-Me is also associated with a direct inhibition of IkB kinase β, thereby preventing the phosphorylation of the inhibitory IkBα moiety. Nonphosphorylated IkBα directly interacts with NFκB causing inhibition of NFκB translocation to the nucleus, interfering with the pro-proliferatory function of this moiety (31). Previous studies have also shown that treatment of U937 myelomonocytic leukemia cells with CDDO-Me is associated with significant inhibition of NFκB function (32).

The present studies have examined whether dual pathway inhibition with a FLT3/ITD inhibitor, such as PKC412, in combination with the STAT3 inhibitor CDDO-Me, might result in a synergistic level of growth arrest and apoptosis. Synergy has already been shown between protein tyrosine kinase inhibitors and downstream inhibitors such as RAD001 or rapamycin which inhibits mTOR, a critical member of the phosphoinositide-3-kinase/AKT pathway (33-35). Therefore, targeting both the mutant oncogene and the critical downstream pathway responsible for enhancing the viability of the leukemic cells may be synergistic. Our results show that BaF3/FLT3/ITD cells possess constitutive STAT3 activity and nuclear NFκB expression. Treatment of leukemic cells with CDDO-Me results in significant inhibition of STAT3 activation, loss of nuclear translocation of NFκB, and an inhibition of proliferation. Combining CDDO-Me with the FLT3/ITD inhibitor results in a synergistic loss of growth potential and induction of apoptosis in human leukemic cell lines and primary patient cells.

**Materials and Methods**

**Cell culture and reagents**

The murine interleukin 3 (IL-3)-dependent hematopoietic cell line, BaF3/FLT3-wt, the IL-3–independent BaF3/FLT3/ITD, Mv4-11 and MOLM-14 cell lines were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 ng/mL of murine IL-3 (BaF3/FLT3-wt cells only; R&D Systems, Inc.), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L of l-glutamine. The FLT3 inhibitor PKC412 (N-benzoyl-staurosporine; ref. 36), was obtained from Novartis and CDDO-Me (methyl-2-cyano-3,12 dioxooolean-1,9 diene-28-0ate; ref. 37), was provided by Reata Pharmaceuticals. BaF3/FLT3-wt, Mv4-11, MOLM-14, or BaF3/FLT3/ITD cells were cultured at a starting density of 2 × 10⁶ cells/mL in RPMI 1640 with 20 ng/mL of IL-3 (BaF3/FLT3) for 24 hours before treatments. To determine the effects of the inhibitors of FLT3/ITD or downstream pathways on proliferation or apoptosis, FLT3/ITD inhibitor PKC412 (5 and 10 nmol/L) and 1 to 5 μmol/L of CDDO-Me were added 24 hours later for different time intervals.

**Cell viability and apoptosis assays**

The cells were grown and treated with different inhibitors for varying intervals of time as described above. Cell counts for proliferation studies were determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega), according to the instructions of the manufacturer. The cells were stained with Annexin V-FITC and propidium iodide before flow cytometry analysis was done. The analysis of protein using flow cytometry included cell fixing, permeabilization, and immunostaining. The fix buffer, permeabilizing buffer, and washing/staining buffer were purchased from Santa Cruz Biotechnology and the process was done according to the recommendations of the manufacturer.

**AML patient cells**

Patient one was an 89-year-old man with AML (FLT3 wt, complex karyotype) whose marrow showed background dysplasia; patient two was a 74-year-old male with M1 AML (FLT3 wt, normal karyotype), and patient three was a 54-year-old male with M4 AML (FLT3-ITD 165 bp, normal karyotype). The patient’s blood or bone marrow samples were diluted 2:1 with PBS or with basic medium without serum, gently mixed by inverting the tubes 8 to 10 times, carefully layered on Ficoll-Paque, and spun down at room temperature for 20 minutes at 2,000 rpm. The layer of cells was removed using a sterile transfer pipette and transferred to a 15 mL tube. The cells were mixed with 50 mL of ice-cold PBS and spun down at 1,000 rpm for 5 minutes. After washing twice with PBS or medium, cells were counted using a hemocytometer. Cells were then cultured in RPMI 1640 with 15% fetal bovine serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol/L of l-glutamine. Cell survival for patient sample studies was determined using the trypan blue exclusion assay.
Colony assays
Plates of 5 × 10^4 BaF3/FLT3/ITD, Mv4-11, or MOLM-14 cells in methylcellulose medium in RPMI 1640 containing fetal bovine serum were prepared. These plates also contained CDDO-Me with or without PKC412 at different concentrations. The plates were incubated for more than 1 week at 37°C in 5% CO₂ and colonies were then counted on an inverted microscope.

Isolation of nuclear and cytoplasmic fractions
Subcellular fractionation was done as described (38). In brief, BaF3/FLT3-wt or BaF3/FLT3/ITD cells were washed twice with ice-cold PBS and resuspended in 1 mL of fractionation lysis buffer (1 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L β-glycerophosphate, 0.5 mmol/L sodium orthovanadate, 2 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L DTT, 40 mg/mL phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL aprotenin; pH 7.2). Following incubation on ice for 1 hour, the cells were disrupted by Dounce homogenizer using 20 strokes. The homogenate was layered onto 1 mL of 1 mol/L sucrose in lysis buffer and centrifuged at 1,600 × g for 15 minutes to pellet the nuclei. The supernatant above the sucrose cushion was collected and centrifuged at 150,000 × g for 30 minutes at 4°C to collect the soluble or cytoplasmic fraction. Purity of the fractions was monitored by immunoblot analysis with anti-1kBα or anti-lamin B antibodies.

Immunoblot analysis
Cells were harvested and rinsed with ice-cold PBS. Ice-cold lysis buffer [0.5 mL; 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 mg/mL leupeptin, and 1 mmol phenylmethylsulfonyl fluoride] was added to 1 × 10^7 cells and sonicated on ice four times for 5 seconds each, followed by microcentrifugation for 10 minutes at 4°C. The soluble proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blotted with primary antibodies and then with secondary antibodies as described (38). The protein bands were then detected by enhanced chemiluminescence (Amersham Biosciences). The same membranes were blotted with primary antibodies (NFκB, IκBα, STAT3, PARP, and caspase-3) were purchased from Santa Cruz Biotechnology. Phospho-IKKβ and phospho-STAT3 were purchased from Cell Signaling Technology. Lamin B and β-actin antibodies were purchased from BD Biosciences and Sigma, respectively.

Statistical analysis
Isobologram analysis was done using the CalcuSyn Software Program (Biosoft). A combination index of <1.0 indicates synergism and a combination index of 1 indicates additive activity (19).

Results
CDDO-Me inhibits NFκB activation by blocking IKKβ phosphorylation in FLT3/ITD myeloid leukemic cells
NFκB activates the transcription of diverse genes that regulate cell proliferation and survival. In the absence of growth stimulation, NFκB localizes in the cytoplasm in complex with members of the IkB family of inhibitor proteins (39, 40). Phosphorylation of IkBα by IkB kinase β (IKKβ) in the presence of growth factor stimuli induces the degradation of IkBα, which results in the release of free NFκB to the nucleus which then promotes proliferation (39, 40). To assess the localization of NFκB in BaF3-wt cells grown in the presence of IL-3, we stimulated BaF3-wt cells with this growth factor and analyzed nuclear lysates by immunoblotting with anti-NFκB antibody. Nuclear lysates from BaF3/FLT3/ITD cells were also analyzed by immunoblotting with anti-NFκB. Our results showed that NFκB is present in the nucleus of BaF3/FLT3/ITD cells or in IL-3–induced BaF3-wt cells (Fig. 1A). Equal loading was confirmed by immunoblots with nuclear lamin B (Fig. 1A). To determine the localization of NFκB in response to CDDO-Me, we treated BaF3/FLT3/ITD cells with 1 μmol/L of CDDO-Me for different time intervals. Nuclear and cytosolic lysates were analyzed by immunoblotting with anti-NFκB antibody. The results show significant inhibition of the nuclear levels of NFκB in cells treated with CDDO-Me (Fig. 1B). For equal loading and to assess the purity of nuclear lysates, the immunoblots were also analyzed with anti-lamin B and anti-IκBα antibodies (Fig. 1B). To ensure that the nuclear fractions were pure, we showed that IkBα (limited to cytosol) was not present (Fig. 1B, bottom).

NFκB is released from cytosolic IkBα and is targeted to the nucleus in response to phosphorylation by IKKβ kinase and subsequent ubiquitination of phosphorylated IkBα. To determine whether CDDO-Me affects IKKβ phosphorylation, whole cell lysates from control and CDDO-Me–treated BaF3/FLT3/ITD cells were analyzed by immunoblotting with anti–phosphorylated IKKβ. The results show that CDDO-Me inhibits constitutive phosphorylation of IKKβ (Fig. 1C) and thereby inhibits the phosphorylation of IkBα. There was no change in the levels of total IKKβ protein (Fig. 1C). In concert with these results, CDDO-Me also inhibited the degradation of IkBα (data not shown), and thereby, translocation of NFκB to the nucleus (Fig. 1B).

CDDO-Me inhibits constitutive activation of STAT3 in cells expressing FLT3/ITD
Studies have shown that the JAK1-STAT3 pathway is constitutively activated in multiple leukemia cell lines (41). Constitutive activation of STAT3 contributes to tumor cell proliferation and inhibits apoptosis (41). To determine whether CDDO-Me–induced apoptosis is mediated by inhibiting constitutive activation of STAT3, we analyzed lysates of BaF3/FLT3/ITD cells growing in the absence...
of IL-3 with anti–phosphorylated STAT3 antibodies and found that STAT3 is constitutively phosphorylated (Fig. 1D, control lane). CDDO-Me significantly inhibits the constitutive phosphorylation of STAT3 within 4 hours (Fig. 1D). There was no change in the levels of non-phosphorylated STAT3 protein (Fig. 1D). Taken together, these findings suggest that CDDO-Me inhibits both STAT3 phosphorylation and blocks nuclear translocation of NFκB.

CDDO-Me and PKC412 inhibit proliferation of FLT3/ITD-positive AML cells

To confirm that CDDO-Me would inhibit proliferation in our model cell line, we treated the murine BaF3/FLT3/ITD cells with different concentrations of CDDO-Me for varying time intervals. BaF3/FLT3/ITD cells were sensitive, in a dose-dependent manner to CDDO-Me, with an IC50 of 1 μmol/L at 3 days of treatment (Fig. 2A). Furthermore, the human myeloid leukemia cell lines which have FLT3 ITD mutations, Mv4-11 and MOLM-14, were treated with different concentrations of CDDO-Me and were analyzed for proliferation. CDDO-Me inhibited the proliferation of these cell lines at 1 to 5 μmol/L (Fig. 2B and C). We had previously shown that the proliferation of cells made growth factor independent by transduction of a FLT3-ITD tyrosine kinase (19), is inhibited by the FLT3 inhibitor, PKC412. We next assessed the effect of PKC412 and CDDO-Me alone and in combination on colony formation of BaF3/FLT3-ITD cells. Results show that CDDO-Me and PKC412 synergistically inhibited colony formation of murine BAF3/FLT3-ITD cells (Fig. 2D). Isobologram analysis was done and the combination index was 0.56 at doses of 10 and 100 nmol/L of PKC412 and CDDO-Me, respectively. Similar results were obtained when human Mv4-11 and MOLM-14 (FLT3-ITD expressing cell lines) were treated with both agents.

CDDO-Me in combination with PKC412 induces apoptosis in myeloid leukemia cells expressing FLT3-ITD

To determine the combinatorial activity of CDDO-Me and PKC412, we treated BaF3/FLT3-ITD cells with low doses of both agents alone and in combination for 2 or 3 days and assessed apoptosis by flow cytometry. The data showed that treatment of cells with 5 nmol/L of PKC412 + 1 μmol/L of CDDO-Me was associated with over 80% induction of apoptosis (Fig. 3A). The results from combined treatment were also compared with cells treated with PKC412 or CDDO-Me separately. Isobologram analysis showed moderate synergism in apoptosis in cells treated with PKC412 + CDDO-Me compared with either agent alone (Fig. 3A). These results were also duplicated in the human leukemic cell lines, Mv4-11 and MOLM-14 (Fig. 3B and C). To extend these findings, we treated cells from three different AML patients with diverse concentrations of CDDO-Me and PKC412 and analyzed proliferation. Patient one was an 89-year-old man with AML with background dysplasia (FLT3 wt, complex karyotype); patient two was a 74-year-old male...
with M1 AML (FLT3 wt, normal karyotype), and patient three was a 54-year-old male with M4 AML (FLT3-ITD 165 bp, normal karyotype). In concert with the leukemia cell lines, treatment of patient cells with CDDO-Me and PKC412 was also associated with significant inhibition of cell survival (Fig. 3D).

**CDDO-Me in combination with PKC412 synergistically promotes the cleavage of PARP and caspase-3**

To determine the mechanism of apoptosis mediated by both of these drugs, we asked whether the combination of low concentrations of CDDO-Me and PKC412 would induce the intrinsic apoptosis pathway by measuring the cleavage of PARP and caspase-3. There was little cleavage of PARP in BaF3/FLT3/ITD cells treated with low concentrations of CDDO-Me (100 nmol/L) or PKC412 (5 nmol/L) alone (Fig. 4A). However, there was a significant induction in PARP cleavage in cells treated with CDDO-Me plus PKC412 (Fig. 4A). Based on densitometric scanning of the immunoblots, we noted a 4- to 5-fold induction of cleaved PARP fragments in cells treated with the combination of PKC412 and CDDO-Me to that compared with either agent alone. Similar results were obtained when BaF3/FLT3-ITD cells were treated with PKC412 and CDDO-Me and analyzed by immunoblotting with anti–caspase-3 (8- to 9-fold induction; Fig. 4B). We also showed that treatment of Mv4-11 cells with CDDO-Me alone or in combination with PKC412 was associated with significant cleavage of PARP (Fig. 4C). Taken together, our results therefore showed that combining CDDO-Me with PKC412 is associated with highly significant activation of caspase-3 and PARP.

**Discussion**

Previous studies with FLT3 inhibitors as single agents in patients with mutant FLT3 AML showed biological activity but few clinical responses (14, 15, 42). For example, in a 20-patient, proof of concept trial in which patients with abnormal FLT3 mutant AML were given the multi-targeted kinase inhibitor PKC412, 70% experienced a reduction in the peripheral blood blast count but no complete remissions were noted (14). Many reasons have been postulated to explain the lack of more pronounced clinical activity, including insufficiently prolonged inhibitory drug levels, elaboration of survival factors in a
protected leukemic stem cell niche, or activation of alternative/downstream pathways.

It has been suggested that it may be possible to counteract resistance to FLT3 inhibitors by inhibiting more than one pathway. Inhibiting tyrosine kinase activation in conjunction with AKT/mTOR inhibition might represent one such approach (43). Our results show that inhibiting both FLT3 activation and the JAK-STAT pathway, which in neoplastic cells, results in activation of NFκB via simultaneous treatment with FLT3 inhibitor PKC412 plus CDDO-Me, results in synergistic cessation of cell growth. Such results were seen in both murine leukemia lines transfected with FLT3/ITD, human leukemic mutant FLT3 cell lines as well as primary patient cells. Additionally, other studies have shown that CDDO-Me induces the generation of reactive oxygen species from both non-mitochondrial or mitochondrial sources, which is associated with the induction of apoptosis (44). Therefore, there is also the possibility that simultaneous FLT3 inhibition increases oxidative injury. Because both CDDO-Me and PKC412 are being developed independently as potential antineoplastic agents, these studies suggest that combination clinical trials with these two agents are indicated.

After demonstrating that the FLT3 inhibitor PKC412 and the STAT3 inhibitor CDDO-Me synergistically kill FLT3 mutant leukemia cells, we attempted to determine the mechanism of this synergy. NFκB is commonly involved in maintaining the survival of cancer cells in general (44) and AML specifically (45-47). Our earlier studies have shown that CDDO-Me blocks tumor necrosis factor α–induced targeting of NFκB p65 to the nucleus (48, 49).

**FIGURE 3.** A, BaF3/FLT3/ITD cells were treated with 1 μmol/L of CDDO-Me and 5 nmol/L of PKC412, alone or in combination (P + C), for 2 d (closed bars) and 3 d (open bars) and analyzed for apoptosis by flow cytometry. Assessment of cells by Annexin V/propidium iodide staining was used as a measure for apoptosis. B, MOLM-14 cells were treated with 1 μmol/L of CDDO-Me and 5 nmol/L of PKC412, alone or in combination (P + C), for 2 d (closed bars) and 3 d (open bars) and analyzed for apoptosis by flow cytometry. C, Mv4-11 cells were treated with 1 μmol/L of CDDO-Me and 5 nmol/L of PKC412, alone or in combination (P + C) for 3 d, and analyzed for apoptosis by flow cytometry. D, cells from three different AML patients were treated with 1 μmol/L of CDDO-Me and 5 nmol/L of PKC412, either alone or in combination (P + C) for 3 d. The cells were assessed for viability using trypan blue exclusion and are shown as a percentage of survival of cells.
The ability of NFκB to activate proneoplastic genes depends on it being free of the inhibitor IκBα, which in turn, is negatively regulated by phosphorylation by IKKβ kinase. Therefore, agents which inhibit the phosphorylation of IκBα and thereby prevent NFκB activation, might potentially be antineoplastic. STAT3 activation activates IKKβ kinase, which indirectly allows NFκB translocation to the nucleus, thereby promoting survival and proliferation. We showed that interrupting the STAT3 pathway with CDDO-Me in conjunction with inhibiting FLT3 by PKC412 might be useful as a synergistic antileukemic approach. Our preclinical experiments detail a potentially useful combination which could have relevance in patients whose myeloblasts have activating mutations in FLT3. The mechanisms of this synergy between CDDO-Me and PKC412 may be due to an enhancement of NFκB inactivation.

Disclosure of Potential Conflicts of Interest

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Synergistic Induction of Apoptosis in AML Cells


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