Inhibition of mitochondrial metabolism by methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate induces apoptotic or autophagic cell death in chronic myeloid leukemia cells

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Abstract

The initial success of the first synthetic bcr-abl kinase inhibitor imatinib has been dampened by the emergence of imatinib-resistant disease in blast crisis chronic myeloid leukemia. Here, we report that the novel triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate (CDDO-Me) potently induced cytotoxicity in imatinib-resistant KBM5 cells expressing the T315I mutation of bcr-abl (24-h EC_{50}, 540 nmol/L). In long-term culture, CDDO-Me abrogated the growth of human parental KBM5 and KBM5-STI cells with 96-h IC_{50} of 205 and 221 nmol/L, respectively. In addition, CDDO-Me rapidly decreased the viability of murine lymphoid Ba/F3 cells expressing wild-type p210 as well as the imatinib-resistant E255K and T315I mutations of bcr-abl. The low-dose effects of CDDO-Me are associated with inhibition of mitochondrial oxygen consumption, whereas the cytotoxic effects appear to be mediated by a rapid and selective depletion of mitochondrial glutathione that accompanies the increased generation of reactive oxygen species and mitochondrial dysfunction. Interestingly, the mitochondriotoxic effects of CDDO-Me are followed by the rapid autophagocytosis of intracellular organelles or the externalization of phosphatidylserine in different cell types. We conclude that alterations in mitochondrial function by CDDO-Me can result in autophagy or apoptosis of chronic myeloid leukemia cells regardless of the mutational status of bcr-abl. CDDO-Me is in clinical trials and shows signs of clinical activity, with minimal side effects and complete lack of cardiotoxicity. Studies in leukemias are in preparation. [Mol Cancer Ther 2008;7(5):1130–9]

Introduction

Chronic myeloid leukemia (CML) is a clonal disease characterized by the accumulation of hematopoietic progenitors carrying a (9;22) chromosomal translocation commonly known as the Philadelphia chromosome that results in the expression of the oncogenic fusion kinase bcr-abl (1). bcr-abl is a constitutively activated kinase that has been shown to activate MEK/ERK (2), phosphatidylinositol 3-kinase (3), and JAK/STAT (4) signaling resulting in increased proliferation and resistance to chemotherapy (5). CML progresses from a chronic phase into a myeloid blast crisis phase accompanied by additional genetic and chromosomal abnormalities that cooperate with bcr-abl to drive disease progression. Treatment of CML with imatinib, a potent synthetic inhibitor of the bcr-abl kinase, produces high rates of hematologic and cytogenetic responses in the chronic phase of the disease, making this agent a paradigm for molecularly targeted therapies (6, 7). Unfortunately, imatinib induces only partial, short-lived responses in the blast crisis phase of the disease, and most patients develop resistance to this agent leading to disease recurrence (8). In fact, a recent long-term follow-up study of CML patients treated with imatinib reported that hematologic resistance to this agent occurred in 24% and 92% of patients in chronic and blast crisis, respectively (9). The decreased efficacy of imatinib in CML as a consequence of mutations within bcr-abl is best exemplified by the T315I mutation. Clinically, the T315I mutation is associated with a formidable therapeutic challenge because it mediates complete resistance not only to imatinib but also to many of the next generation of ABL kinase inhibitors like dasatinib (Sprycel; Bristol-Myers Squibb) and the imatinib-related compound nilotinib (Tasigna; Novartis; ref. 10). The emergence of T315I mutations in CML has given even greater urgency to develop more effective chemotherapy for this malignancy.

The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid (CDDO) is effective in inducing apoptosis in a variety of tumor cell types, including leukemia (11–14), multiple myeloma (15), breast, osteosarcoma (16), pancreatic (17), and skin (18). Furthermore, recent reports show

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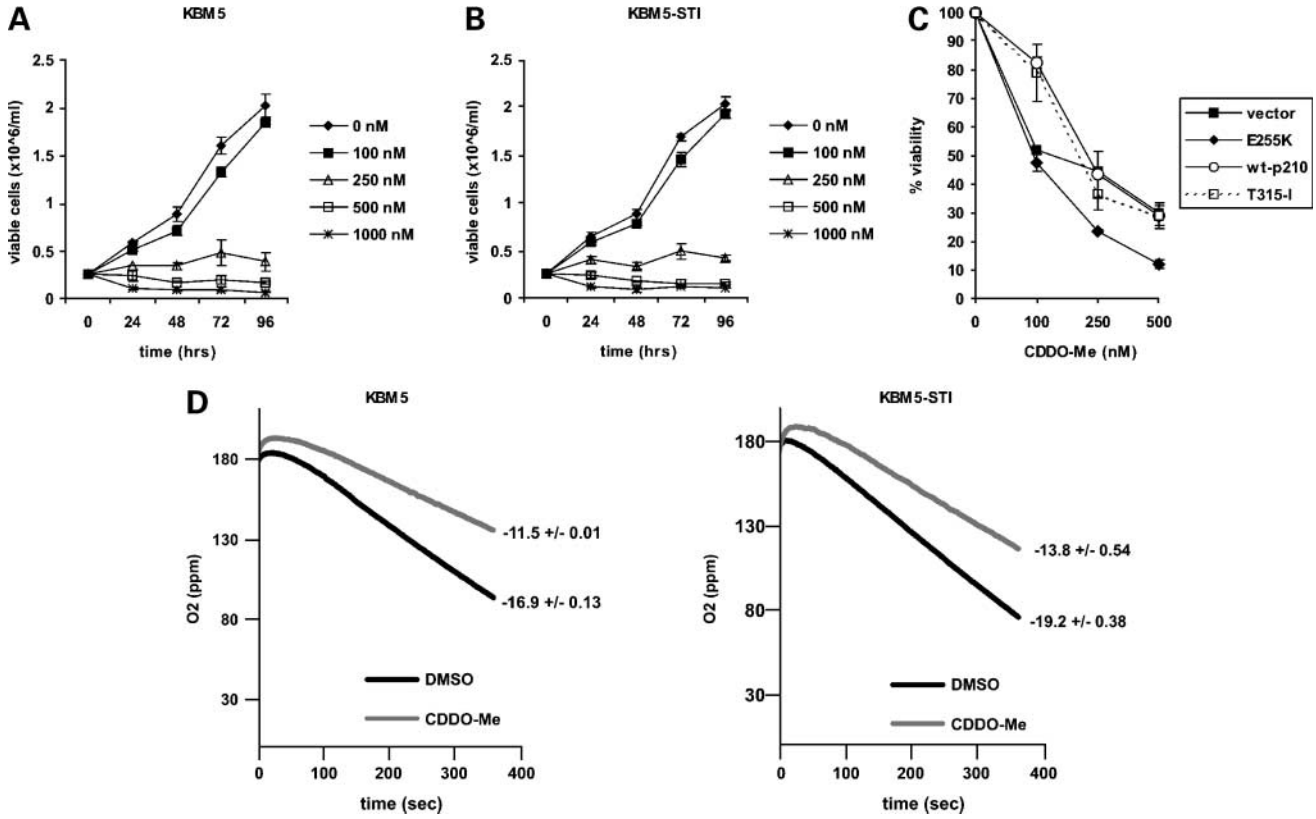

Figure 1. Submicromolar concentrations of CDDO-Me abrogate the growth of imatinib-resistant CML cells in culture. **A,** KBM5 cells were cultured with 100, 250, 500, and 1,000 nmol/L CDDO-Me for the indicated times, and viable cells were quantitated as described in Materials and Methods. **B,** KBM5-STI cells were cultured with CDDO-Me (100-1,000 nmol/L) for the indicated times and analyzed as above. **C,** mouse lymphoid Ba/F3 cells expressing wild-type bcr-abl, the T315I mutant of bcr-abl, the E255K mutant of bcr-abl, and a vector control were treated with increasing concentrations of CDDO-Me as indicated and viability was measured by trypan blue exclusion. **D,** cells were cultured with 100 nmol/L CDDO-Me for 24 h and oxygen consumption was monitored in 1×10^7 cells as described in Materials and Methods.

that the C28 methyl ester derivative of CDDO, methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate (CDDO-Me), is 5-fold more potent than CDDO as an antitumor agent *in vitro* (11, 19). CDDO and CDDO-Me reportedly disrupted intracellular redox balance in U937 cells and multiple myeloma cells, thereby activating the intrinsic apoptotic pathway (11, 15), and CDDO-Me exhibited some selectivity in apoptosis induction between tumor and normal cells (19). Interestingly, recent evidence from our group indicates that CDDO induced the release of cytochrome *c* from isolated mitochondria via a cyclosporine A–independent permeability transition, suggesting that this organelle may be a direct target of this agent (14, 20).

Here, we report that the CDDO derivative CDDO-Me is effective in abrogating the growth of imatinib-resistant CML cells of human and mouse origin and that the antiproliferative effects of this oleanic acid derivative appear to be initiated by rapid perturbations in mitochondrial function associated with increased oxidative stress. Interestingly, cytotoxic doses of CDDO-Me induced apoptotic or autophagic cell death in different cell types, and this is to our knowledge the first report showing that the mitochondriotoxic effects of CDDO-Me can also activate autophagy. Autophagy, or programmed cell death II, is a pathway that recruits the endolysosomal system to digest intracellular components, presumably as a mode of survival during nutrient deprivation, but was more recently reported to be a form of cellular demise in cancer cells after a variety of chemotherapeutic insults (21). We hypothesize that CDDO-Me may be effective in treating CML, regardless of bcr-abl mutational status, by inducing programmed cell death (either apoptosis or autophagy) via the disruption of mitochondrial function.

Materials and Methods

Chemicals and Biochemicals

CDDO-Me was kindly provided by Dr. Edward Sausville (National Cancer Institute) under the RAID program and by Dr. Michael Sporn (Dartmouth Medical College). NAC was purchased from Sigma. CMH_2DCF-DA, CMXRos, and TMRM were all obtained from Molecular Probes. Z-VAD-fmk was purchased from Alexis Biochemicals. Phospho-p38 and p38 antibodies were purchased from Cell Signaling Technologies. Heme oxygenase-1 antibody was purchased from BD Biosciences and

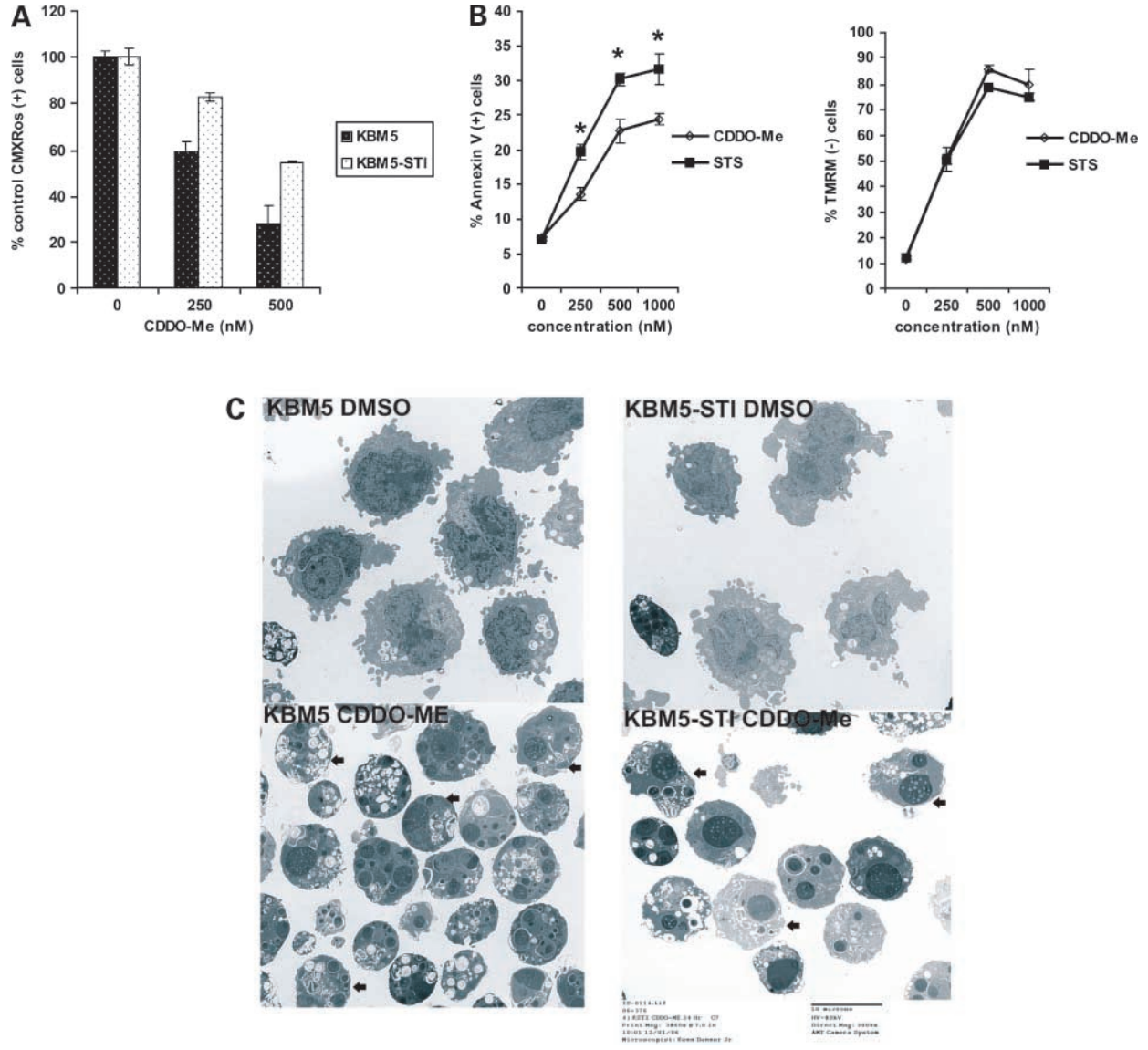

Figure 2. CDDO-Me induces programmed cell death that can be manifested by apoptosis or autophagy. **A,** KBM5 and KBM5-STI cells were treated with CDDO-Me as above for 24 h and $\Delta\Psi_m$ was measured using CMXRos as described in Materials and Methods. **B,** KBM5 cells were treated with CDDO-Me or staurosporine (*STS*) for 24 h and phosphatidylserine externalization and $\Delta\Psi_m$ were analyzed by flow cytometry. *, $P < 0.007$. **C,** KBM5 and KBM5-STI cells were treated with 500 nmol/L CDDO-Me for 24 h and processed for TEM as described in Materials and Methods. *Arrows,* autophagosomes.

glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Chemicon. Poly(ADP-ribose) polymerase-1 antibody was purchased from Santa Cruz Biotechnology, and goat anti-mouse and anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Bio-Rad. All other chemicals used were of the highest purity available.

Cell Lines

KBM5 cells were derived from a patient with myeloid blastic phase of CML; the cells contain multiple copies of the Philadelphia chromosome while lacking the normal *ABL* gene. KBM5 cells resistant to imatinib (KBM5-STI) were derived by Ricci et al. by chronic exposure of KBM5 cells to imatinib (22). KBM5-STI cells were able to grow in the presence of 2.0 μmol/L STI571 and were maintained at this concentration. Cells were grown in RPMI 1640 supplemented with 10% FCS, 1% glutamine, and 100 units/mL penicillin in a 37°C incubator containing 5% CO_2. Interleukin-3-dependent murine pro-B cell line BaF3 transfected with vector, wild-type p210 (expressing p210 bcr-abl), E255K, or T315I were kindly provided by Dr. C. Sawyers and were cultivated in RPMI 1640 complemented with 10% FCS, 1% glutamine, 2 ng/mL interleukin-3 (vector only), and 2 μmol/L puromycin (23). Viable cell numbers were quantitated in a Vi-Cell Cell Viability Analyzer (Beckman-Coulter).

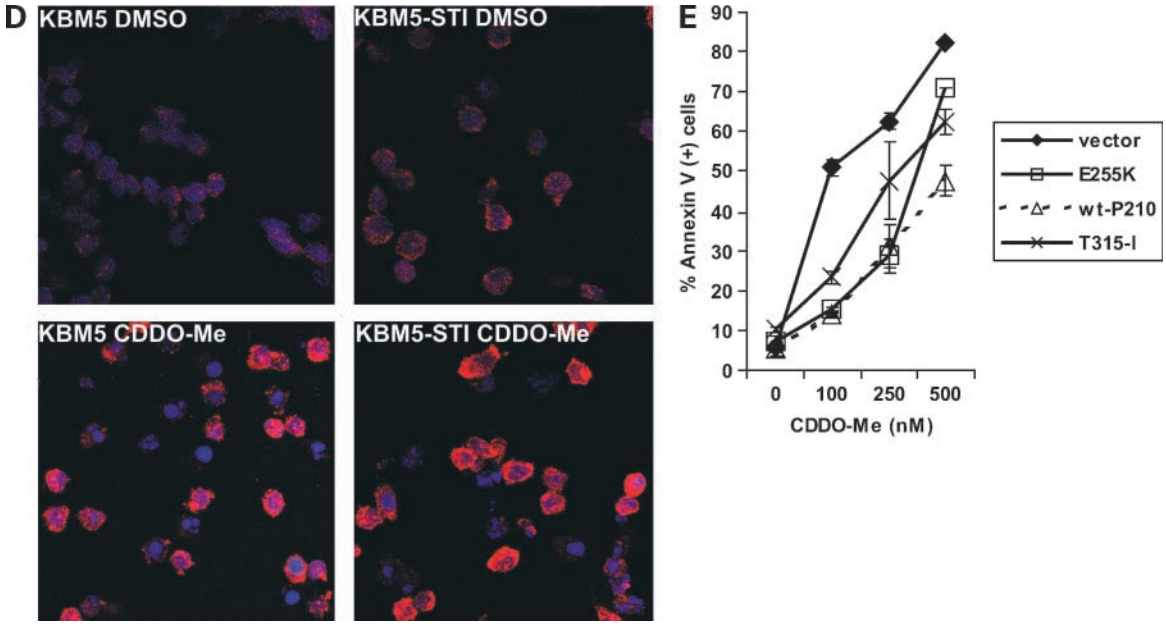

Figure 2 *Continued.* **D,** immunohistochemical expression of the autophagosomal protein LC3B was investigated in cells treated as above. **E,** mouse lymphoid Ba/F3 cells expressing wild-type bcr-abl, the T315I mutant of bcr-abl, the E255K mutant of bcr-abl, and a vector control were treated with increasing concentrations of CDDO-Me as indicated and phosphatidylserine externalization and $\Delta\Psi_m$ were analyzed by flow cytometry.

Human Subjects

Bone marrow or peripheral blood samples were obtained for *in vitro* studies from patients with CML; samples were collected during routine diagnostic procedures after informed consent was obtained in accordance with regulations and protocols approved by the Human Subjects Committee of The University of Texas M. D. Anderson Cancer Center. Mononuclear cells were separated by Ficoll-Hypaque (Sigma) density gradient centrifugation.

Measurement of Mitochondrial Membrane Potential

After appropriate treatments, cells were washed twice in PBS and then resuspended in 100 μL PBS containing 0.5 μg/mL MitoTracker CMXRos and 15 ng/mL MitoTracker Green and incubated at 37°C for 45 min. Cells were then washed twice in PBS and analyzed by flow cytometry in a FACSCalibur flow cytometer using a 488 nm argon excitation laser. Alternatively, for confocal microscopy or short time-point measurements of mitochondrial membrane potential ($\Delta\Psi_m$), cells were loaded with 50 nmol/L of the potentiometric probe TMRM, treated as indicated, and analyzed by confocal microscopy or flow cytometry. Results presented are mean ± SE of three independent experiments.

Western Blot Analysis

Cells were harvested by centrifugation, washed twice in PBS, and resuspended in ice cold lysis buffer [1% Triton X-100, 45 mmol/L KCl, 10 mmol/L Tris (pH 7.5)] supplemented with protease and phosphatase inhibitors and then subjected to SDS-PAGE in 10% or 12% polyacrylamide gels followed by protein transfer to a Hybond-P membrane (Amersham Pharmacia Biotech) and immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase blots were run in parallel as loading controls. Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics).

Transmission Electron Microscopy

After appropriate treatments, samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.3) for 1 h. After fixation, the samples were washed and treated with 0.1% Millipore-filtered cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 30 min, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in Spurr's low viscosity medium. The samples were polymerized in a 70°C oven for 2 days. Ultrathin sections were cut in a Leica Ultracut microtome (Leica), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL USA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques).

Measurement of Intracellular Reduced GSX by Flow Cytometry

Cells (3×10^5 cells/mL; 0.5 mL) were treated with CDDO-Me as indicated or with 2 mmol/L diethylmaleate for 30 min. Cells were then collected by centrifugation, washed in PBS once, and resuspended in 0.2 mL PBS containing 400 nmol/L Cell Tracker Green (Molecular Probes) and incubated on ice protected from light for 10 min. Cells were then washed in PBS several times and Cell Tracker Green fluorescence was quantitated by flow

cytometry. The mean Cell Tracker Green fluorescence from diethylmaleate-treated samples was considered to be background and subtracted accordingly. All experiments were done in duplicate and repeated at least thrice.

Measurement of Oxygen Consumption

Cells were resuspended in 1 mL fresh warm medium preequilibrated with 21% oxygen and placed in the sealed respiration chamber equipped with a thermostat control, a microstirring device, and a Clark-type oxygen electrode disc (Oxytherm; Hansatech Instrument). Both oxygen content in the cell suspension medium and oxygen consumption rate were constantly monitored, and the signals were integrated using the software supplied by the manufacturer.

LC3B Immunohistochemistry

Anti-LC3B antibody against a synthetic peptide corresponding to the NH_2-terminal 14 amino acids of isoform B of human LC3 and an additional cysteine (PSEKTFKQRRTFEQC) was prepared by immunization of a rabbit and then affinity purified on an immobilized peptide-Sepharose column (Covance). After appropriate treatments, cells were harvested by centrifugation and fixed in 4% paraformaldehyde at room temperature for 10 min. Cells were then permeabilized by 0.1% Triton-X and washed extensively in PBS followed by incubation in 5% normal goat serum in PBS with 0.1% Tween 20 at 37°C for 1 h. After incubation, LC3B antibody was added (1:100 dilution) and immune complexes were allowed to form at 4°C overnight. Cells were washed in PBS twice followed by incubation with an APC-conjugated anti-rabbit IgG for 30 min at room temperature. Cells were then mounted on 4′,6-diamidino-2-phenylindole containing medium (ProLong Gold; Invitrogen) and analyzed by confocal microscopy on an Olympus IX71 inverted microscope.

Results

Submicromolar Concentrations of CDDO-Me Inhibit the Growth of Imatinib-Resistant CML Cells in Culture

Ricci et al. reported the development of an imatinib-resistant CML cell line by chronic exposure of the previously described KBM5 CML cell line (24) to increasing concentrations of imatinib (22). This imatinib-resistant cell line, KBM5-STI, was shown to carry the T315I mutation in the ATP-binding pocket of bcr-abl that has also been reported in a proportion of imatinib-resistant patients (22, 25). To investigate if CDDO-Me would be effective in preventing the proliferation of this clinically relevant imatinib resistance model, we cultured KBM5 and KBM5-STI cells in the presence of increasing concentrations of CDDO-Me (100-1,000 nmol/L) for 24, 48, 72, and 96 h. Under our experimental conditions, KBM5-STI cells were resistant to imatinib doses as high as 2 μmol/L, whereas parental KBM5 cells underwent rapid apoptosis at this concentration of imatinib (data not shown). Our results presented in Fig. 1A and B show that CDDO-Me decreased the numbers of viable cells of both KBM5 and KBM5-STI cells, with 96-h IC_{50} of 205 and 221 nmol/L, respectively. To investigate if decreased cell cycle progression contributes to the antiproliferative action of CDDO-Me, we analyzed cell cycle distribution in cells treated with this agent and found that at 72 h post-treatment CDDO-Me induced a similar increase (1.7- to 1.8-fold at 300 nmol/L; data not shown) in the G_1 phase of the cell cycle in both KBM5 and KBM5-STI cells. Finally, we investigated if imatinib resistance in different cell types would result in decreased sensitivity to CDDO-Me. For these experiments, we treated mouse lymphoid Ba/F3 cells expressing wild-type bcr-abl and the imatinib-resistant E255K and T315I bcr-abl mutants with increasing concentrations of CDDO-Me. As illustrated in Fig. 1C, CDDO-Me rapidly decreased the viability of mouse Ba/F3 cells expressing wild-type bcr-abl (24-h IC_{50}, 250 nmol/L), the E255K bcr-abl mutant (24-h IC_{50}, 94 nmol/L), or the T315I bcr-abl mutant (24-h IC_{50}, 210 nmol/L). Taken together, these data show that CDDO-Me effectively prevents the proliferation of imatinib-resistant CML cells.

CDDO-Me Decreases Oxygen Consumption in CML Cell Lines

We have reported previously that submicromolar concentrations of CDDO-Me inhibit oxygen consumption in AML cell lines and that this effect precedes activation of the mitochondrial permeability transition (20). To investigate if CDDO-Me similarly affects mitochondrial metabolism in CML cell lines, we monitored oxygen consumption in KBM5 and KBM5-STI cells after treatment with subcytotoxic doses of CDDO-Me. As shown in Fig. 1D, after 24-h treatment with 100 nmol/L CDDO-Me, oxygen consumption was decreased in KBM5 and KBM5-STI by 32% (16.9-11.5 nmol/mL min) and 28% (19.2-13.8 nmol/mL min), respectively ($P < 0.008$). Interestingly, KBM5-STI cells had a 14% higher basal rate of oxygen consumption than parental KBM5 cells ($P < 0.002$), suggesting the possibility that the imatinib resistance may be associated with differences in mitochondrial function.

CDDO-Me Can Induce Apoptosis or Autophagy in CML

To investigate if apoptosis contributes to the antiproliferative effects of CDDO-Me in CML cells, we examined $\Delta\Psi_m$ and phosphatidylserine externalization in cells treated with increasing concentrations of CDDO-Me for 24 h. As illustrated in Fig. 2A, CDDO-Me induced a dose-dependent loss of $\Delta\Psi_m$ in both KBM5 (24-h IC_{50}, 303 nmol/L) and KBM5-STI (24-h IC_{50}, 540 nmol/L) cells. Surprisingly, in contrast to staurosporine, CDDO-Me induced significantly ($P < 0.007$) less externalization of phosphatidylserine in KBM5 cells at doses sufficient to induce marked loss of $\Delta\Psi_m$ (Fig. 2B), suggesting the possibility that this agent is activating alternative modes of cell death. Similar findings were obtained in KBM5-STI cells after 24 and 96 h of exposure to CDDO-Me (data not shown). Indeed, transmission electron microscopy revealed that both KBM5 and KBM5-STI cells formed extensive double membrane vesicles after exposure to cytotoxic concentrations of CDDO-Me, and this was accompanied by a decrease in cell size ruling out the possibility of oncosis (Fig. 2C). Furthermore,

immunohistochemistry revealed an increased cytoplasmic staining of LC3B, a protein commonly associated with autophagosomes (Fig. 2D). In contrast, mouse Ba/F3 cells expressing wild-type bcr-abl, the E255K bcr-abl mutant, or the T315I bcr-abl mutant rapidly externalized phosphatidylserine in response to CDDO-Me (Fig. 2E), and similar observations were made in K562 cells (data not shown), suggesting that autophagic cell death induced by this agent is not modulated by bcr-abl per se.

CDDO-Me Induces Rapid Generation of Reactive Oxygen Species That Is Associated with Decreased $\Delta\Psi_m$ and Precedes the Loss of Intracellular Reduced GSX

Because reactive oxygen species (ROS) is a component of oxidative stress-induced autophagy (26), we investigated if CDDO-Me induced the accumulation of ROS in KBM5 and KBM5-STI cells. Our results illustrate that 1 μmol/L CDDO-Me induced a significant ($P < 0.02$) 2.8-fold increase in the ROS-dependent CM-H_2DCF fluorescence in KBM5 cells and a significant ($P < 0.008$) 4.2-fold increase in KBM5-STI cells after a 3-h treatment (Fig. 3A), suggesting that ROS is indeed a component of the cytotoxicity of this agent in CML cells. Consistent with a mitochondriotoxic effect of ROS, the increase in ROS accompanied a decrease in $\Delta\Psi_m$ of 50% ($P < 0.002$) and 36% ($P < 0.002$) in KBM5 and KBM5-STI cells, respectively (Fig. 3B). To investigate if oxidative stress in CML cells treated with CDDO-Me is associated with a decrease in the levels of reduced GSX (GSH), we measured the levels of intracellular GSH in KBM5 and KBM5-STI cells treated with 1 μmol/L CDDO-Me. The results illustrated in Fig. 3C show that 1 μmol/L CDDO-Me decreased the levels of GSH in a time-dependent manner in KBM5 cells (30% loss at 6 h; $P < 0.01$) and KBM5-STI cells (80% loss at 6 h; $P < 0.001$), albeit the latter cell type displayed a more rapid and pronounced decrease in the levels of GSH. These results are congruent with our observation that KBM5-STI cells generated more ROS in response to CDDO-Me treatment than KBM5 cells (Fig. 3A) and suggest that the increased generation of ROS induced by this agent contributes to oxidation of the GSX pool in CML cells. Our observations thus show that increased generation of ROS and oxidation of intracellular GSH are associated with CDDO-Me-induced mitochondrial dysfunction and precede the onset of autophagy.

CDDO-Me Induces a Rapid and Selective Depletion of Mitochondrial GSX That Leads to Oxidation of Cardiolipin

Cholesterol has been shown to accumulate in the mitochondrial membrane and prevent the import of GSX from the cytosol leading to mitochondrial dysfunction (27, 28). Because the triterpenoid structure of CDDO-Me shares some similarity to cholesterol, we hypothesized that the rapid generation of ROS and decreased membrane potential induced by CDDO-Me may be mediated by perturbations in mitochondrial GSX (GSXm). We therefore quantitated biochemically the levels of GSXm and whole-cell GSX in CML cells treated with CDDO-Me. The results presented in Fig. 4A show that 300 nmol/L CDDO-Me

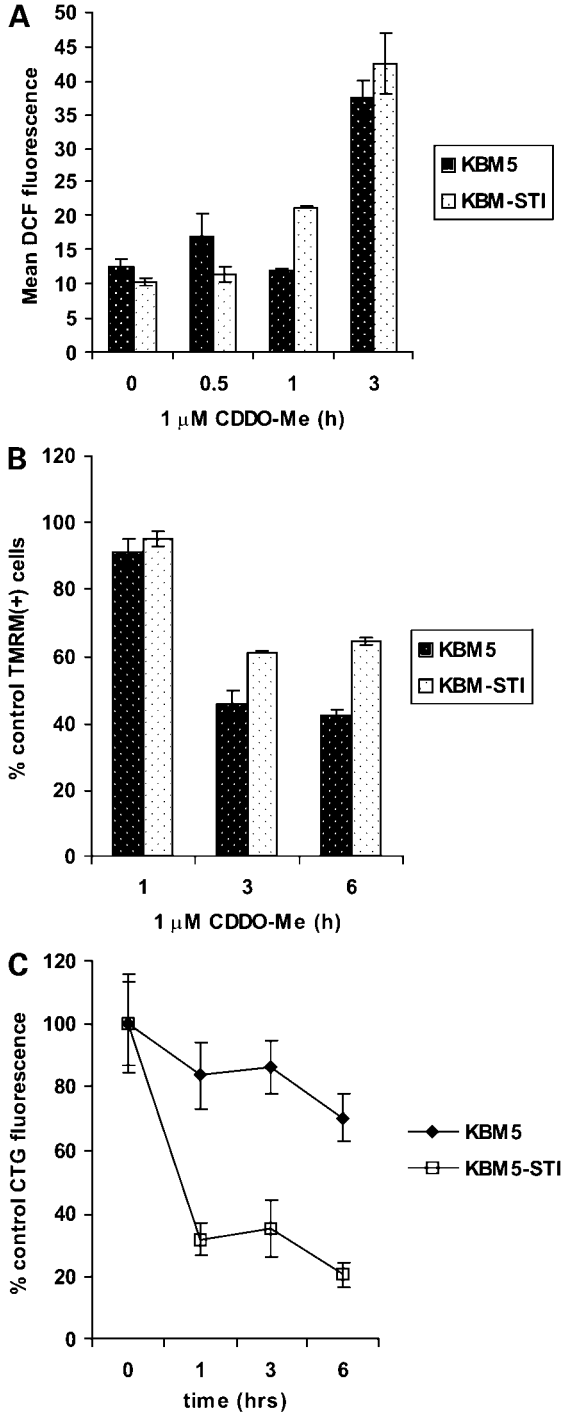

Figure 3. CDDO-Me induces rapid generation of ROS, mitochondrial dysfunction, and oxidation of intracellular GSH. **A,** cells were treated with CDDO-Me as indicated and the generation of intracellular ROS was quantitated by flow cytometry as described in Materials and Methods. **B,** cells were loaded with the potentiometric probe TMRM and treated with CDDO-Me as indicated, and $\Delta\Psi_m$ was quantitated by flow cytometry as described in Materials and Methods. **C,** cells were treated with CDDO-Me as indicated and loaded with the GSH-specific probe Cell Tracker Green followed by incubation in ice for 10 min. Cells were then extensively washed in PBS, and the GSH-specific fluorescence of Cell Tracker Green was measured as described in Materials and Methods.

selectively depleted GSXm by 69% ($P < 0.003$) in KBM5 cells, and a similar effect was seen in KBM5-STI cells (65%; $P < 0.02$) after 3-h treatment without any marked effect in whole-cell GSX, supporting the notion that the triterpenoid structure of CDDO-Me may mediate perturbations in GSXm flux. Cardiolipin is the most abundant phospholipid in the mitochondrial membrane and has been shown to modulate the initiation of the intrinsic apoptotic pathway (29, 30). Because GSXm is critical for the maintenance of cardiolipin (31–33), we examined the levels of cardiolipin in cells treated with CDDO-Me by staining with the cardiolipin-selective fluorochrome nonyl acridine orange (NAO). NAO binds with high affinity and selectivity to reduced but not oxidized cardiolipin, making it a useful probe to study the redox status and quantity of this lipid in mitochondria (34). Notably, CDDO-Me potently increased the proportion of cells displaying decreased NAO staining [NAO(-)] in KBM5 cells (7.4-fold; $P < 0.00001$) and to a lesser but significant extent in KBM5-STI cells (2.7-fold; $P < 0.005$; Fig. 4B). Because it has been shown previously that depletion of GSXm results in increased expression of the oxidative stress-responsive protein heme oxygenase-1 (35), we investigated if a similar response was elicited by CDDO-Me in CML cell lines. Western blot analysis of KBM5 and KBM5-STI cells treated with CDDO-Me indeed shows that this agent markedly induces the expression of heme oxygenase-1 in both cell lines (Fig. 4C). Taken together, the above data suggest that CDDO-Me-induced mitochondrial dysfunction in CML cells is mediated by the selective loss of GSXm that results in accumulation of ROS and the loss of cardiolipin in KBM5 and KBM5-STI cells.

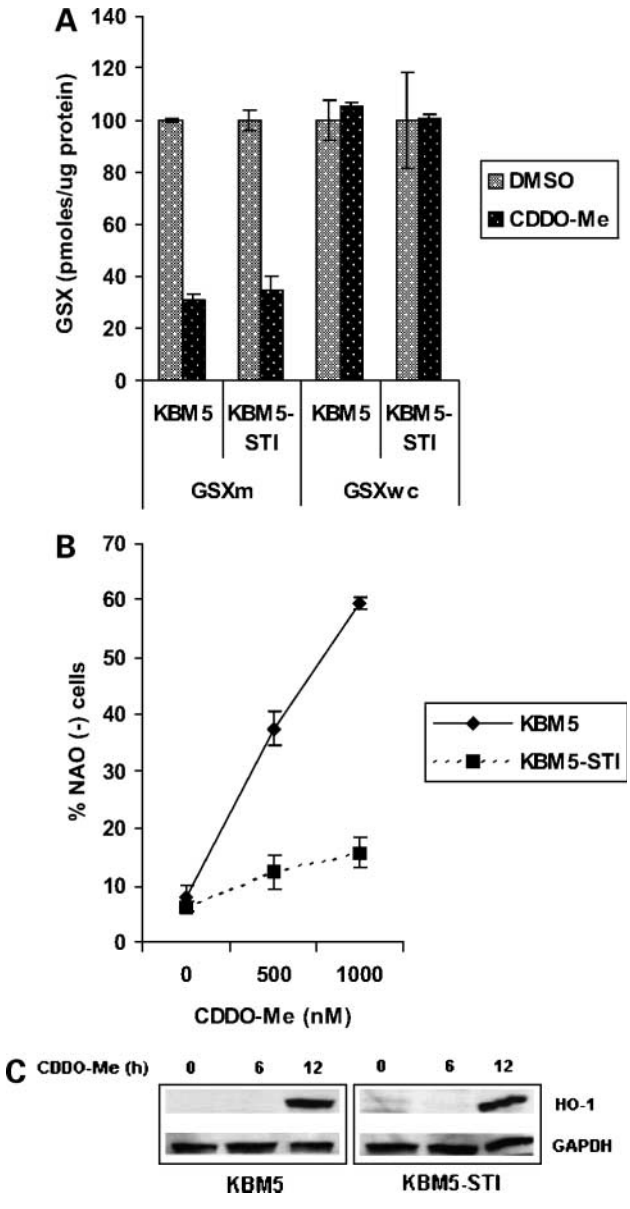

Figure 4. CDDO-Me induces early depletion of GSXm and oxidation of the mitochondrial phospholipid cardiolipin. **A,** KBM5 and KBM5-STI cells were treated with 300 nmol/L CDDO-Me for 3 h and the levels of GSX in whole-cell and mitochondrial extracts were quantitated biochemically as described in Materials and Methods. **B,** cells were treated as indicated and incubated with 10 nmol/L of the cardiolipin-specific dye NAO. Cells were then washed in PBS and NAO FL1 fluorescence was quantitated by flow cytometry. **C,** cells were treated with 500 nmol/L CDDO-Me for 6 and 12 h and heme oxygenase-1 expression was investigated by Western blotting as described in Materials and Methods.

CDDO-Me Decreases Viability, Induces Apoptosis, and Provokes the Loss of Intracellular GSH in Primary CML Cells

To investigate if CDDO-Me would effectively decrease the viability of primary CML blast crisis cells in culture, we exposed *ex vivo* leukemic cells derived from three patients in the blast crisis phase of CML. The results presented in Fig. 5A show that CDDO-Me decreases the viability of primary CML cells with a mean 24-h IC_{50} concentration of 599.7 ± 78.6 nmol/L. Moreover, when the levels of intracellular GSH were measured after a short time exposure to CDDO-Me (3 h), we found that, consistent with our observations in CML cell lines, CDDO-Me decreased the levels of GSH in all three patient samples with a mean EC_{50} concentration of 637 ± 27.3 nmol/L (Fig. 5B). Flow cytometric analysis of these samples, as well as a T315I CML sample (no. 4), indicated that CDDO-Me induced loss of $\Delta\Psi_m$ and externalization of phosphatidylserine (Fig. 5C), suggesting that apoptosis and not autophagy is the preferential mode of cell death induced by CDDO-Me in primary CML samples regardless of the mutational status of bcr-abl. The doses required for cytotoxicity were not significantly different from the doses required to decrease intracellular GSH ($P > 0.1$). Our results suggest that oxidative stress, represented by decreased GSH levels, is indeed associated with the loss of viability induced by CDDO-Me in primary CML cells.

Discussion

The emergence of imatinib resistance in CML patients has been associated with the development of point mutations in the kinase domain of bcr-abl. Clinically, imatinib resistance may be overcome by (*a*) novel bcr-abl inhibitors, such as

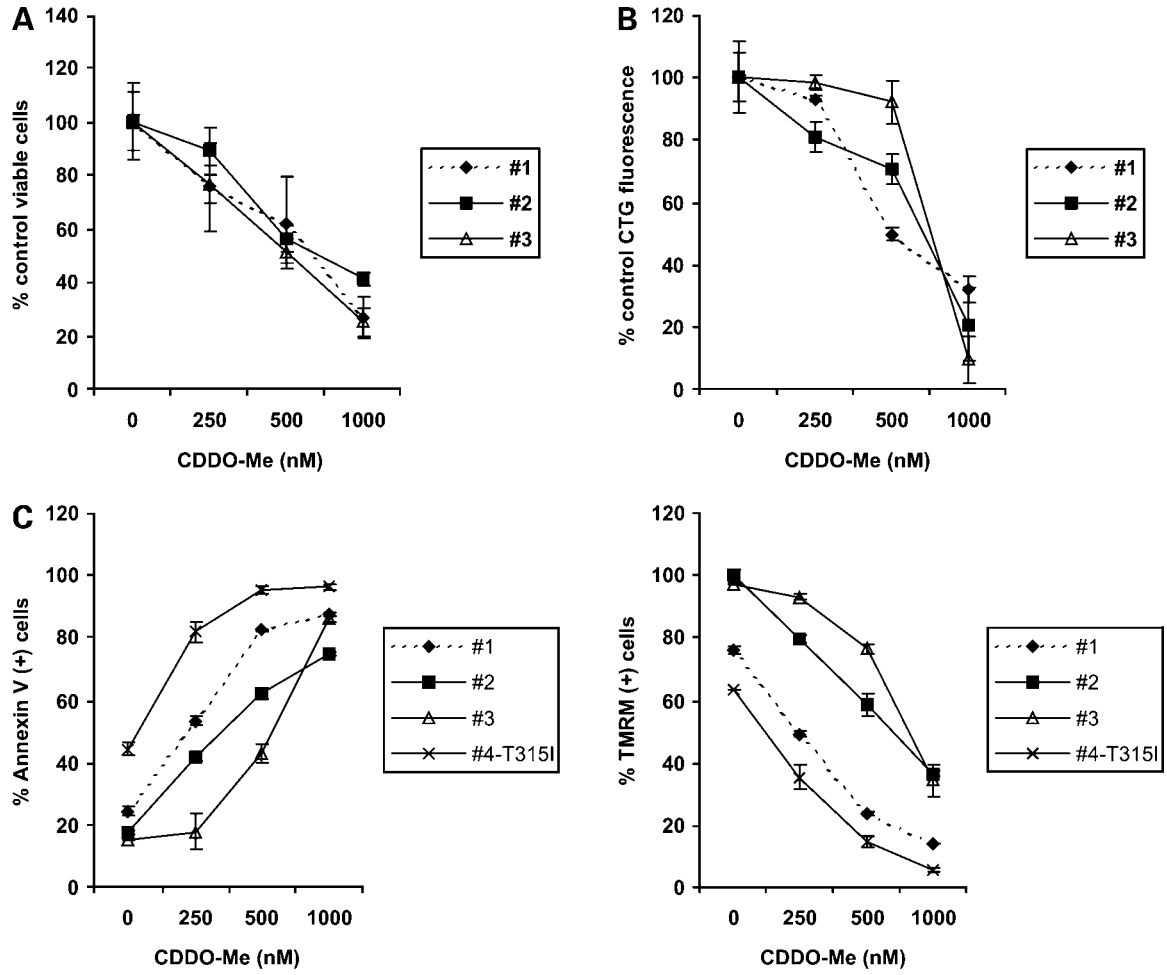

Figure 5. CDDO-Me induces GSH depletion and apoptosis in primary CML blast crisis cells. **A,** cells obtained from patients in blast crisis CML were cultured *ex vivo* with increasing concentrations of CDDO-Me (0-1,000 nmol/L) for 24 h and viable cells were counted after trypan blue staining using a hemocytometer. **B,** patient samples were treated with increasing concentrations of CDDO-Me (0-1,000 nmol/L) for 3 h and intracellular GSH was quantitated by flow cytometry as described in Materials and Methods. **C,** patient samples were treated as in **A** and phosphatidylserine externalization and $\Delta\Psi_m$ were quantitated by flow cytometry as described in Materials and Methods.

nilotinib and dasatinib, which inhibit most clinically relevant bcr-abl mutants, except the T315I mutation, or (*b*) modulation of targets independent of bcr-abl, like farnesyl transferases or Aurora kinase (36–39). Albeit these approaches have shown promising preliminary results, identifying additional targets for the treatment of CML is of utmost importance.

CDDO-Me has been reported to prevent the proliferation of AML cells, but the effects of this novel triterpenoid on CML cells in the context of imatinib resistance have not been investigated. Here, we report that CDDO-Me effectively abrogates the growth of both parental and imatinib-resistant human KBM5 (KBM5-STI) CML cells that express the clinically relevant T315I mutation. This single base-pair substitution (C1308T) was originally identified in six of nine patients who displayed imatinib resistance (25), and subsequent work showed that this mutation, as well as the E255K mutation identified previously in an imatinib-resistant patient (40), resulted in a constitutively active kinase resistant to inhibition by imatinib *in vitro* (41). Notably, the T315I mutation also confers resistance to the novel bcr-abl inhibitor nilotinib and the src inhibitor dasatinib that have been reported to be 25 to 30 times more potent that imatinib against a variety of bcr-abl mutants (42). Albeit CDDO-Me at doses above 300 nmol/L equally decreased the viability of both KBM5 and KBM5-STI cells, parental KBM5 cells appeared to be more sensitive to the growth inhibitory effects of low doses (100-300 nmol/L) of CDDO-Me than KBM5-STI cells (120-h IC_{50}, 57 versus 126 nmol/L), and this correlated with the increased sensitivity to cardiolipin oxidation induced by CDDO-Me (7.4-fold versus 2.7-fold). In contrast, murine lymphoid Ba/F3 cells expressing wild-type bcr-abl or the T315I mutant were equally sensitive to low doses of CDDO-Me, and in fact, Ba/F3 cells bearing the E255K bcr-abl mutant were more sensitive to the growth-inhibitory effects

of 500 nmol/L of this agent ($P < 0.02$). Taken together, our results indicate that molecular alterations associated with imatinib resistance, rather than imatinib resistance per se, modulate the cytotoxicity of CDDO-Me but do not confer resistance to this agent.

Mechanistically, our data indicate that the antileukemia effects of CDDO-Me are mediated in large part by the induction of programmed cell death, which may manifest itself as apoptosis or autophagy. In addition, a moderate G_1-S cell cycle block may also contribute to the growth-inhibitory effects of CDDO-Me in CML cells. In agreement with previous findings in AML, subcytotoxic doses of CDDO-Me induced a decrease in oxygen consumption in KBM5 and KBM5-STI cells, suggesting that a critical mitochondrial event is associated with the effects of this agent. At higher cytotoxic doses, CDDO-Me induced the rapid dissipation of $\Delta\Psi_m$, and this was accompanied by the increased generation of ROS that preceded a marked decrease in the levels of intracellular GSH. Our results suggest the possibility that a common mitochondrial target may mediate both apoptosis and autophagy, and this is the first report to show that CDDO-Me can induce both types of programmed cell death.

The rapid dissipation of GSXm preceding the generation of ROS is in complete agreement with our previous observations in pancreatic cancer cell lines (17), and similar to our observation in AML cell lines, albeit in AML cell lines CDDO-Me did not generate ROS, suggesting inherent mitochondrial differences in AML and CML cells (20). In support of this notion, an earlier report showed that bcr-abl conferred increased antioxidant capacity to CML cells (43), and Trachootham et al. recently showed that overexpression of bcr-abl in hematopoietic cells caused elevation of ROS levels, rendering these cells susceptible to apoptosis induction by agents that inactivated antioxidant defenses (44). Taken together, the above suggest that CDDO-Me may target bcr-abl-expressing CML cells via its ability to decrease GSXm and GSH. Further studies are ongoing to investigate the mechanism of CDDO-Me-induced GSXm depletion in CML cells and how this effect contributes to apoptosis induction by this class of agents. Additionally, the role of mitochondrial respiration in modulating apoptosis or autophagy in the context of CDDO-Me is being investigated.

In summary, our findings indicate the potential clinical utility of CDDO-Me in CML regardless of bcr-abl mutational status. Notably, phase I clinical trials of this oral agent are currently ongoing at The University of Texas M. D. Anderson Cancer Center in patients with solid tumors, with first indications of clinical activity and notable lack of toxicity, particularly any cardiotoxicity (45). The elucidation of the mechanism of CDDO-Me-induced cell killing will provide useful information to optimize antileukemic strategies targeting CML (especially among refractory cases) in upcoming clinical trials using this novel triterpenoid.

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