Triterpenoid CDDO-Methyl Ester Inhibits the Janus-Activated Kinase-1 (JAK1) → Signal Transducer and Activator of Transcription-3 (STAT3) Pathway by Direct Inhibition of JAK1 and STAT3

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Abstract

The C-28 methyl ester of the oleane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO-Me) induces apoptosis of human cancer cells by disrupting redox balance and is in clinical trials. CDDO-Me contains unsaturated carbonyl groups that form reversible adducts with thiol nucleophiles. The present studies show that CDDO-Me blocks interleukin-6 (IL-6)–induced and constitutive activation of the Janus-activated kinase 1 (JAK1) in cells. In support of a direct mechanism, CDDO-Me forms adducts with JAK1 at Cys1077 in the kinase domain and inhibits JAK1 activity. In concert with these results, CDDO-Me blocked IL-6–induced and constitutive activation of signal transducer and activator of transcription 3 (STAT3). Moreover, we show that CDDO-Me (a) binds directly to STAT3 by a mechanism dependent on the alkylation of Cys259 and (b) inhibits the formation of STAT3 dimers. These findings indicate that CDDO-Me inhibits activation of the JAK1 → STAT3 pathway by forming adducts with both JAK1 and STAT3. [Cancer Res 2008;68(8):2920–6]

Introduction

The synthetic oleane triterpenoids are a new class of agents that have antiproliferative and proapoptotic activity (1). One of the synthetic oleane triterpenoids, C-28 methyl ester of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO-Me), is under study in phase I and II clinical trials for the treatment of patients with hematologic malignancies and solid tumors. In this regard, CDDO-Me and other derivatives with modifications at the C-28 position induce apoptosis of human myeloid leukemia (2–6), multiple myeloma (7–9), osteosarcoma (10), lung cancer (11, 12), breast cancer (13, 14), and pancreatic cancer (15). The synthetic oleane triterpenoids activate the phase II response of cells at low nanomolar concentrations and thereby protect against oxidative stress by decreasing levels of reactive oxygen species (1). By contrast, at low micromolar concentrations, CDDO-Me and related derivatives induce apoptosis in vitro by increasing reactive oxygen species and decreasing intracellular glutathione (5, 7, 15, 16). How the synthetic oleane triterpenoids disrupt redox balance and induce apoptosis at low micromolar concentrations is not known. However, structure-activity analysis has shown that α,β-unsaturated carbonyl groups on rings A and C confer Michael addition with a nucleophilic target (17, 18). In this context, nuclear factor κB activates the transcription of diverse genes that regulate cell survival (19), and CDDO directly inhibits the IκB kinase (20, 21). These findings have indicated that CDDO-Me induces apoptosis, in part, by alkylating critical cysteines in proteins that regulate survival.

Materials and Methods

Cell culture. Human Hela cervical cancer and MDA-MB-468 breast cancer cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM/L L-glutamine. Cells were treated with CDDO-Me (provided by Reata Pharmaceuticals) and human IL-6 (20 ng/mL; R&D Systems).

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doi:10.1158/0008-5472.CAN-07-3036
Figure 1. CDDO-Me inhibits IL-6–induced and constitutive JAK1 activity. A and B, HeLa cells were pretreated with 1 μmol/L CDDO-Me for 6 h and then left unstimulated (control) or stimulated with IL-6 for 15 min. Whole-cell lysates were subjected to immunoblotting with the indicated antibodies. C, MDA-MB-468 cells were treated with the indicated concentrations of CDDO-Me for 6 h. Lysates were immunoblotted with the indicated antibodies. D, MDA-MB-468 cells were treated with 1 μmol/L CDDO-Me for the indicated times. Lysates were immunoblotted with the indicated antibodies.

Figure 2. CDDO-Me directly inhibits JAK1 activity. A, HeLa cells were left untreated as the control or stimulated with IL-6 for 15 min (left). MDA-MB-468 cells were analyzed without stimulation (right). Anti-JAK1 precipitates were incubated with GST-STAT3 and [γ-32P]ATP in the absence and presence of 1 μmol/L CDDO-Me. The reaction products were analyzed by SDS-PAGE and autoradiography (top). The precipitates were also analyzed by immunoblotting with anti-JAK1 (bottom). B, MDA-MB-468 cells were transfected with GFP-JAK1. At 48 h after transfection, lysates were precipitated with anti-GFP. The precipitates were incubated with GST-STAT3 and [γ-32P]ATP in the absence and presence of 1 μmol/L CDDO-Me. The reaction products were analyzed by SDS-PAGE and autoradiography (top). The precipitates were also analyzed by immunoblotting with anti-GFP (bottom). C, lysates from HeLa cells were incubated with biotin or CDDO-Me-biotin and then precipitated with streptavidin-Sepharose beads. The adsorbates were immunoblotted with anti-JAK1 and anti-JAK2 (left). Intact MDA-MB-468 cells were pretreated with the indicated concentrations of CDDO-Me for 2 h and then incubated with 1 μmol/L CDDO-Me-biotin. Lysates were precipitated with streptavidin-Sepharose beads and the adsorbates were immunoblotted with anti-JAK1 (right). D, recombinant JAK1-KD or JAK1-KD(C1077A) was incubated with 1 μmol/L CDDO-Me-biotin for 30 min. The complexes were isolated with streptavidin-Sepharose beads and the precipitates were subjected to immunoblotting with anti-JAK1 (top). Input of the proteins was determined by immunoblotting with anti-JAK1 (bottom).
Figure 3. CDDO-Me inhibits IL-6-induced STAT3 activation. A and B, HeLa cells were pretreated with the indicated concentrations of CDDO-Me for 6 h and then stimulated with IL-6 for 15 min. Whole-cell lysates were immunoblotted with the indicated antibodies (A). Nuclear lysates were immunoblotted with anti-STAT3 and, as controls for equal loading and purity, with antibodies against nuclear lamin B and cytosolic IkBα (B). WCL, whole-cell lysate. C, HeLa cells were transfected with pSTAT3-Luc and SV40-Renilla-Luc. At 24 h after transfection, cells were pretreated with 1 μmol/L CDDO-Me for 6 h, stimulated with IL-6 for 15 min, and then assayed for luciferase activity. Columns, mean fold activation relative to that obtained for the control (assigned a value of 1) from three separate experiments; bars, SD. D, HeLa cells were treated with 1 μmol/L CDDO-Me for 2 h and then IL-6 was added for the indicated times. Lysates were immunoblotted with the indicated antibodies.

Figure 4. CDDO-Me inhibits constitutive activation of the STAT3 pathway. A and B, MDA-MB-468 cells were treated with the indicated concentrations of CDDO-Me for 6 h or 1 μmol/L CDDO-Me for the indicated times. Whole-cell lysates were immunoblotted with the indicated antibodies (A). Nuclear lysates were immunoblotted with anti-STAT3 and, as controls for equal loading and purity, with antibodies against nuclear lamin B and cytosolic IkBα (B). C, MDA-MB-468 cells were transfected with pSTAT3-Luc and SV40-Renilla-Luc. At 24 h after transfection, cells were treated with the indicated concentrations of CDDO-Me for 6 h and then assayed for luciferase activity. Columns, mean fold activation relative to that obtained for cells treated with 2 μmol/L CDDO-Me (assigned a value of 1) from three separate experiments; bars, SD. D, MDA-MB-468 cells were left untreated or treated with 1 μmol/L CDDO-Me for 6 h. Lysates were immunoblotted with the indicated antibodies.
CDDO-Me Directly Inhibits Both JAK1 and STAT3

Results

CDDO-Me inhibits JAK1 activity. To determine whether CDDO-Me affects JAK signaling, we first studied HeLa cells that were stimulated with IL-6. Phospho-JAK1 levels were low to undetectable in control HeLa cells and were increased with IL-6 stimulation (Fig. 1A). Notably, treatment of the HeLa cells with CDDO-Me suppressed IL-6–induced JAK1 activation (Fig. 1A). IL-6 stimulation was also associated with increases in phospho-JAK2 levels (Fig. 1B). However, in contrast to JAK1, CDDO-Me treatment had no apparent effect on JAK2 activation (Fig. 1B). There was no detectable activation of JAK3 in the IL-6–stimulated HeLa cells (data not shown). In contrast to HeLa cells, JAK1 is constitutively activated in human MDA-MB-468 cells (Fig. 1C). Treatment of MDA-MB-468 cells with CDDO-Me was associated with downregulation of phospho-JAK1 levels (Fig. 1C). Moreover, the effect of CDDO-Me on phospho-JAK1 was detectable within 2 hours of treatment (Fig. 1D). These findings indicate that CDDO-Me inhibits JAK1, but not JAK2, activation.

CDDO-Me interacts directly with JAK1. To further assess the effects of CDDO-Me, we immunoprecipitated JAK1 from control and IL-6–stimulated HeLa cells and performed in vitro kinase assays with GST-STAT3 as substrate. IL-6 stimulation was associated with increases in JAK1-mediated phosphorylation of STAT3 (Fig. 2A, left). However, addition of CDDO-Me to the reactions blocked STAT3 phosphorylation, indicating that CDDO-Me directly blocks JAK1 activity (Fig. 2A, left). In vitro kinase assays with constitutively activated JAK1 immunoprecipitated from MDA-MB-468 breast cancer cells also showed that addition of CDDO-Me to the reactions blocks JAK1-mediated phosphorylation of STAT3 (Fig. 2A, right). To confirm these findings, we expressed GFP-tagged JAK1 in MDA-MB-468 cells. Analysis of anti-GFP precipitates showed that phosphorylation of GST-STAT3 is inhibited by CDDO-Me (Fig. 2B). To determine whether CDDO-Me interacts with JAK1, lysates from HeLa cells were incubated with CDDO-Me conjugated to biotin. Analysis of the adsorbates by immunoblotting with anti-JAK1 showed binding to CDDO-Me and not biotin (Fig. 2C, left). By contrast, there was no detectable binding of CDDO-Me to JAK2 (Fig. 2C, left). Incubation of intact MDA-MB-468 cells showed binding of JAK1 to CDDO-Me-biotin and not biotin (Fig. 2C, right). In addition, pretreatment of intact cells with CDDO-Me competitively blocked binding of JAK1 to CDDO-Me-biotin (Fig. 2C, right).

JAK1 contains a cysteine residue at position 1077 in the kinase domain that is not present in JAK2. Binding of CDDO-Me-biotin to JAK1 and STAT3.

Luciferase assays. Cells were transfected with pSTAT3-Luc (Panomics) and SV40-Renilla-Luc (Promega) in the presence of Lipofectamine 2000 (Invitrogen). At 24 h after transfection, lysates were analyzed with the Dual Luciferase Assay Kit (Promega).

Analysis of STAT3 dimerization. Cell lysates were incubated with anti-STAT3 (Santa Cruz Biotechnology) and precipitated with protein A/G beads. The precipitates were immunoblotted with anti-STAT3 (BD Transduction Laboratories). In other studies, MDA-MB-468 cells were cotransfected with pCMV-STAT3-Flag and pCMV-STAT3-HA (30) in the presence of Lipofectamine 2000 (Invitrogen). At 48 h after transfection, lysates were incubated with anti-hemagglutinin (HA; Sigma) and the precipitates analyzed by immunoblotting with anti-Flag (Sigma).

[20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, and 100 μmol/L ATP] with 1 μg of recombinant glutathione S-transferase (GST)-STAT3 and [γ-32P]ATP (Perkin-Elmer Life Sciences), as described (35), in the absence and presence of 1 μmol/L CDDO-Me for 30 min at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Binding of CDDO-Me-biotin to JAK1 and STAT3. CDDO-Me and CDDO were biotinylated as described (36). Lysates were incubated with 1 μmol/L biotin (Calbiochem) or CDDO-Me-biotin for 2 h, followed by addition of streptavidin-Sepharose beads. The beads were washed and then subjected to immunoblotting with anti-STAT3 (BD Transduction Laboratories). In other studies, MDA-MB-468 cells were cotransfected with pCMV-STAT3-Flag and pCMV-STAT3-HA (30) in the presence of Lipofectamine 2000 (Invitrogen). At 48 h after transfection, lysates were incubated with anti-hemagglutinin (HA; Sigma) and the precipitates analyzed by immunoblotting with anti-Flag (Sigma).


Figure 5. CDDO-Me blocks dimerization of STAT3. A and B. HeLa cells were stimulated with IL-6 for 15 min (A). MDA-MB-468 cells were untreated (B). Lysates were immunoprecipitated with anti-STAT3 (polyclonal, anti–NH2-terminal). The precipitates were incubated with 0 or 1 μmol/L CDDO-Me for 2 h, washed, suspended in nonreducing sample buffer, boiled for 5 min, and immunoblotted with anti-STAT3 (monoclonal, anti–NH2-terminal). C. MDA-MB-468 cells were transfected with the empty pCMV vector or cotransfected with Flag-STAT3 and HA-STAT3. At 48 h after transfection, lysates were precipitated with anti-HA. The precipitates were incubated with 0 or 1 μmol/L CDDO-Me for 2 h and then immunoblotted with the indicated antibodies.
stimulates STAT3-mediated transcription and that CDDO-Me inhibits this response (Fig. 3C). STAT3 activates transcription of the cyclin D1 and survivin genes. Consistent with inhibition of STAT3-mediated transcription, IL-6–induced increases in cyclin D and survivin expression were inhibited by CDDO-Me treatment (Fig. 3D). These results and our findings with JAK1 indicate that CDDO-Me blocks IL-6–induced activation of the JAK1→STAT3 pathway. CDDO-Me blocks constitutive activation of the JAK1→STAT3 pathway. The JAK1→STAT3 pathway is constitutively activated in MDA-MB-468 breast cancer cells (37). Consistent with the results obtained in IL-6–stimulated HeLa cells, treatment of MDA-MB-468 cells with CDDO-Me down-regulated constitutive phosphorylation of STAT3 (Fig. 4A, left). Analysis of the kinetics of this response showed decreases in phospho-STAT3 levels within 2 hours of CDDO-Me treatment (Fig. 4A, right). Furthermore, CDDO-Me inhibited the constitutive nuclear localization of STAT3 in MDA-MB-468 cells (Fig. 4B). Constitutive activation of the pSTAT3-Luc reporter was inhibited by CDDO-Me (Fig. 4C). In addition, CDDO-Me treatment was associated with down-regulation of cyclin D1 and survivin expression (Fig. 4D). These results indicate that CDDO-Me blocks constitutive activation of the JAK1→STAT3 pathway in MDA-MB-468 cells.

CDDO-Me blocks STAT3 dimerization. JAK1-mediated phosphorylation of STAT3 is associated with the targeting of phospho-STAT3 dimers to the nucleus for activation of STAT3-dependent genes. Thus, IL-6 stimulation of HeLa cells results in the formation of STAT3 dimers (Fig. 5A). To determine if CDDO-Me affects STAT3 dimerization, lysates from IL-6–stimulated HeLa cells were incubated with CDDO-Me. The results show that CDDO-Me disrupts the STAT3 dimers (Fig. 5A). Constitutive STAT3 dimerization in MDA-MB-468 cells was also disrupted by CDDO-Me (Fig. 5B). To confirm these results, we transfected MDA-MB-468 cells with HA-tagged STAT3 (HA-STAT3) and Flag-tagged STAT3 (Flag-STAT3). Immunoblot analysis of anti-HA precipitates with anti-FLAG showed the formation of Flag-STAT3-HA-STAT3 dimers (Fig. 5C). Moreover, incubation of the anti-HA precipitates with CDDO-Me resulted in disruption of the Flag-STAT-HA-STAT3 dimers (Fig. 5C). These results indicate that CDDO-Me blocks STAT3 dimerization.

CDDO-Me forms adducts with STAT3 that are dependent on Cys259. Incubation of HeLa cell lysates with CDDO-Me-biotin showed that CDDO-Me forms complexes with STAT3 (Fig. 6A, left). Incubation of intact MDA-MB-468 cells showed binding of STAT3 to CDDO-Me-biotin and not biotin (Fig. 6A, right). In addition, pretreatment of intact MDA-MB-468 cells with different concentrations of CDDO-Me showed inhibition of the binding of CDDO-Me-biotin to STAT3 (Fig. 6A, right). We also incubated recombinant STAT3 with CDDO-Me-biotin. Analysis of the adsorbates with anti-STAT3 showed that CDDO-Me interacts directly with STAT3 (Fig. 6B). The parent compound CDDO similarly formed direct complexes with recombinant STAT3 (Fig. 6B). By contrast, the interaction was blocked when recombinant STAT3(C259A) was incubated with CDDO-Me-biotin (Fig. 6C). These findings indicate that CDDO-Me inhibits STAT3 by direct alkylation of Cys259.

Discussion

The synthetic oleanane triterpenoids disrupt redox balance and thereby induce apoptosis at low micromolar concentrations (5, 7, 15, 16). The mechanism(s) responsible for such activity remain unclear. However, certain insights are being obtained from the
findings that the synthetic oleane triterpenoids can confer Michael addition with nucleophilic targets (17, 18). In this regard, a direct interaction of a synthetic oleane triterpenoid was found spectroscopically with thiol groups of the Keap1 sensor (18). Subsequent studies have shown that CDDO and its C-28 derivatives interact directly with I-B kinase β at Cys179 in the activation loop (20, 21). Otherwise, to our knowledge, there are no reports of CDDO or its derivatives forming adducts with other protein targets. The cyclopentenone prostaglandins also contain an α,β-unsaturated carbonyl moiety and react with the I-B kinase β Cys179 residue (38). However, few targets of the cyclopentenone prostaglandins have been identified to date, even when using an unbiased proteomic approach (39). Nonetheless, the findings that the cyclopentenone prostaglandins react with H-Ras (40), heat shock protein 90 (39), and estrogen receptor α (41) provide potential leads for additional targets of the synthetic oleane triterpenoids.

Constitutive activation of STAT3 contributes to tumorigenesis by promoting proliferation and inhibiting apoptosis (42). Tyrosine phosphorylation of STAT3 confers dimerization through binding of the SH2 domain of one monomer to a P-pY-LKTK motif on the other STAT3 monomer (31, 43–45). The present results show that CDDO-Me inhibits IL-6–induced and constitutive Jak1 activity. The results support a model in which CDDO-Me binds directly to Jak1 at Cys107 in the kinase domain and thereby blocks Jak1 as an upstream effector of STAT3 phosphorylation (Fig. 6D). The triterpenoids have been shown to decrease pSTAT3 levels (9, 32, 33) and the present findings show that this response is associated with the direct inhibition of Jak1.

Dimerization of STAT3 is necessary for its activation and oncogenic function (25). STAT3 target genes confer diverse processes of transformation, including proliferation, apoptosis, angiogenesis, and metastasis (46). In this regard, agents that inhibit STAT3 dimerization and activation exhibit anti tumor activity (30, 31). Previous studies have shown that STAT3 dimerization is also mediated by interchain disulfide bridging involving Cys259 (37). Notably, the present results show that CDDO-Me disrupts STAT3 dimers. Moreover, we show that CDDO-Me binds directly to STAT3 by forming adducts with Cys259 (Fig. 6D). Thus, CDDO-Me–mediated disruption of STAT3 dimerization is consistent with alklylation of Cys259 and thereby loss of interchain bridging. CDDO-Me treatment was also associated with down-regulation of cyclin D1 and survivin, both encoded by STAT3 target genes. Notably, the inhibition of Jak1 would be sufficient to decrease STAT3 phosphorylation and dimerization. However, the results also show that CDDO-Me directly inhibits STAT3. These findings thus collectively indicate that CDDO-Me blocks the Jak1–STAT3 pathway by directly inhibiting both Jak1 and STAT3 (Fig. 6D).

Acknowledgments
Received 8/7/2007; revised 1/25/2008; accepted 2/7/2008.

Grant support: National Cancer Institute grants CA42802, CA100707, and CA80628.

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We thank Dr. Jianyu Lin (Ohio State University) for providing the Flag-STAT3 and HA-STAT3 vectors, Dr. Robert Schreiber (Washington University) for the GPP-JAK1 vector, Dr. Nancy Reich (Stony Brook University) for the GST-STAT3 vector, and Dr. Ratu Ray (St. Louis University) for the MYC-JAK1 vector. D. Raina is a consultant to Beata.

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Cancer Research 2008; 68: (8). April 15, 2008 2926 www.aacrjournals.org

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