The Synthetic Triterpenoid CDDO-Imidazolide Suppresses STAT Phosphorylation and Induces Apoptosis in Myeloma and Lung Cancer Cells

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Abstract

Purpose: Excessive activity of the transcription factors known as signal transducers and activators of transcription (STAT) contributes to the development and progression of malignancy in many organs. It is, therefore, important to develop new drugs to control the STATs, particularly their phosphorylation state, which is required for their transcriptional activity.

Experimental Design: Myeloma and lung cancer cells were treated with the new synthetic triterpenoid CDDO-Imidazolide, and STAT phosphorylation and apoptosis were evaluated by immunoblotting and fluorescence-activated cell sorting analysis.

Results: We now report that CDDO-Imidazolide, previously shown to be a potent agent for control of inflammation, cell proliferation, and apoptosis, rapidly (within 30-60 minutes) and potently (at nanomolar levels) suppresses either constitutive or interleukin-6-induced STAT3 and STAT5 phosphorylation in human myeloma and lung cancer cells. Furthermore, in these cells, CDDO-Imidazolide also up-regulates critical inhibitors of STATs, such as suppressor of cytokine signaling-1 and SH2-containing phosphatase-1 (a tyrosine phosphatase). Moreover, gene array studies reported here show that CDDO-Imidazolide potently regulates the transcription of important genes that are targets of the STATs.

Conclusions: Our new data thus show that CDDO-Imidazolide is a potent suppressor of STAT signaling and provide a further mechanistic basis for future clinical use of this agent to control inflammation or cell proliferation.

Studies on the role of the signal transducer and activator of transcription (STAT) family of transcription factors in carcinogenesis and on the development of novel inhibitors of the STAT pathway for both prevention and treatment of cancer are evoking increasing interest (1–3). Most notably, it is clear that the STAT transcription factors, particularly STAT3 and STAT5, are frequently overactivated in a wide variety of cancers (2, 4–7), including many hematogenous malignancies (such as leukemia, lymphoma, and multiple myeloma), and in many carcinomas at most major target sites (such as lung, breast, prostate, pancreas, ovary, and head and neck). In this context, it becomes increasingly important to discover small molecules capable of directly inhibiting the activity of both STAT3 and STAT5 (2, 3, 8, 9). STAT3 and STAT5 become active transcription factors upon their phosphorylation by a unique set of Janus-activated kinases (JAK; refs. 2, 10, 11). Their functional activity is regulated not only by these kinases but also by a set of inhibitory proteins (2, 10, 11), which either bind to activated STATs (such as protein inhibitors of activated STATs), bind to the JAKs [such as suppressor of cytokine signaling (SOCS)], or dephosphorylate activated STATs [protein tyrosine phosphatases, such as SH2-containing phosphatases (SHP)].

We now report here for the first time that a new synthetic triterpenoid, CDDO-Imidazolide, is a potent agent (active at nanomolar concentrations) for suppression of the phosphorylated state of either STAT3 or STAT5. These new results are a mechanistic extension of previous studies, in which it was first shown that CDDO-Imidazolide has marked anti-inflammatory and antiproliferative activity, both in cell culture and *in vivo* (12). The biological activity of CDDO-Imidazolide had originally been identified (13) by its ability to block the ability of IFN-γ, a known activator of STATs (14), to stimulate the *de novo* production of inducible nitric oxide synthase; subsequent studies then showed that CDDO-Imidazolide had marked antiproliferative and proapoptotic activity on a variety of human and murine tumor cells (15–19). The suppression of STAT activity, shown here for the first time, is likely an important mechanism contributing to these activities of CDDO-Imidazolide.

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Note: K. Liby and N. Voong contributed equally to this work. M.B. Sporn and J.J. Letterio are co-senior authors.

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Materials and Methods

Synthesis of CDDO-Imidazolide (1-[2-cyano-3,12-dioxooleana-1,9-dien-28-oyl] imidazole). A brief report of the synthesis of CDDO-Imidazolide has been published (13), and the structure of CDDO-Imidazolide is shown in ref. (12). Full details are as follows: Oxalyl chloride was added dropwise to a solution of CDDO (ref. 20; 3 g, 6.1 mmol) in anhydrous methylene chloride (50 mL) under nitrogen atmosphere at room temperature. The mixture was stirred at room temperature overnight. The reaction mixture was evaporated *in vacuo* to give a residue, which was dissolved in benzene (20 mL). This solution was evaporated *in vacuo* to remove excess oxalyl chloride; this procedure was repeated twice. The resultant residue was triturated with a small amount of methylene chloride to give crystals. The methylene chloride was removed *in vacuo* to give CDDO acid chloride (3.1 g; yield, 100%).

For conversion of this acid chloride to CDDO-imidazolide, 400 mg (0.78 mmol), was dissolved in anhydrous benzene (2.7 mL), and imidazole (109 mg, 1.6 mmol, 2.1 equivalents) was added under nitrogen at room temperature. The mixture was vigorously stirred at room temperature under nitrogen overnight. The reaction mixture was diluted with ethyl acetate (30 mL), washed with water (15 mL, twice) and a saturated sodium chloride solution (15 mL, once), dried over $MgSO_4$, and filtered. The filtrate gave a crystalline solid (366 mg), containing about 10% ethyl acetate. To remove the ethyl acetate, the crystalline solid was dissolved in methylene chloride, and this solution was evaporated *in vacuo* to give CDDO-Imidazolide as an amorphous solid (330 mg; yield, 78%). The purity was 99.7%, as determined by high-performance liquid chromatography (C18 column; eluant = 80% acetonitrile, 20% water).

Cell culture. CDDO-Imidazolide was dissolved in DMSO, and controls containing equal concentrations of DMSO (≤0.1%) were included in all experiments. The human myeloma cell lines RPMI 8226 and JJN3 were provided by Dr. John Shaughnessy (Myeloma Research Institute, University of Arkansas, Little Rock, AR). The mouse plasmacytoma cell line used in the present study, 1254, was developed in the laboratory of Dr. Michael Potter (National Cancer Institute, Bethesda, MD). The A549 and H358 human lung cancer cell lines were either obtained from the American Type Culture Collection (Manassas, VA) or provided by Dr. William Petty (Dartmouth Medical School), respectively. All cells were maintained in RPMI containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). For all myeloma experiments, cells were centrifuged over Lympholyte-M (Cedar Lane, Burlington, NC) to remove the dead cells and then kept in media containing 0.2% fetal bovine serum overnight before treatment with CDDO-Imidazolide, recombinant human interleukin-6 (IL-6), or calyculin A (Invitrogen).

Proliferation, apoptosis, and STAT3 ELISA assays. For proliferation assays, cells were treated with CDDO-Imidazolide, pulsed with [^{3}H]thymidine for 2 hours, and counted. Apoptosis was analyzed by fluorescence-activated cell sorting using the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems, Minneapolis, MN) and CELLQuest software (Becton Dickinson, San Jose, CA). Activation of STAT3 in nuclear lysates was measured using 10 μg of nuclear extract protein per sample and a TransAM STAT3 ELISA (Active Motif, Carlsbad, CA).

Immunoprecipitation and immunoblot analysis. Total cell lysates, nuclear extracts, or immunoprecipitated samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies against phosphorylated STAT5 (p-STAT3; Cell Signaling Technology, Beverly, MA) or p-STAT5, p-TYK2, or SHP-1/2 (Upstate, Milford, MA), as previously described (21).

Microarray analysis. A549 cells were treated with vehicle alone (control) or with 1 μmol/L CDDO-Imidazolide for 4 hours. Total RNA was isolated, reverse transcribed into biotinylated cRNA, hybridized to a human JAK/STAT Oligo GEArray containing cDNA fragments from specific genes, and detected by chemiluminescence per the manufacturer's (SuperArray, Frederick, MD) instructions.

Real-time PCR. Total RNA was isolated using TRIzol (Invitrogen), and 2-μg aliquots were reverse transcribed using Superscript II reverse transcriptase and random hexamers. PCR was done on 0.1 μg aliquots of the reverse transcription reaction, using primers and cycle conditions from SuperArray. Values were normalized to β-actin and then expressed as fold induction over vehicle control.

Results

Rapid induction of apoptosis in myeloma cells treated with CDDO-Imidazolide. CDDO-Imidazolide has been reported to induce apoptosis in a variety of human cancer cells, including myeloma, leukemia, pancreas, breast, and colon cancer cells (15–19), but in these studies, the cells were treated for ≥24 hours. In contrast, we have found that only a brief exposure to CDDO-Imidazolide is sufficient to induce apoptosis. RPMI-8226 and JJN3 human myeloma cells were treated with 500 nmol/L CDDO-Imidazolide for 4 hours, and then the drug was removed by washing the cells. Twenty-four hours after treatment, cytospins revealed membrane blebbing and nuclear fragmentation in the cells treated with CDDO-Imidazolide (Fig. 1B and D, *left*). Fluorescence-activated cell sorting analysis showed that the majority of the cells treated with CDDO-Imidazolide stained positive for Annexin V and/or propidium iodide, indicative of apoptosis.

CDDO-Imidazolide suppresses either constitutive or inducible STAT phosphorylation in myeloma cells. Because of the rapid induction of apoptosis in myeloma cells treated with CDDO-Imidazolide, and because of the importance of STAT phosphorylation for suppression of apoptosis (22), we measured levels of STAT phosphorylation after treatment of JJN3 cells with CDDO-Imidazolide (250 nmol/L) for 1 to 3 hours. IL-6 is an autocrine growth factor in these cells. As shown in Fig. 2A, the level of p-STAT5 in whole-cell extracts decreased in a time-dependent manner, with virtually no p-STAT5 protein detected after 3 hours of treatment with CDDO-Imidazolide. CDDO-Imidazolide treatment also reduced the level of total STAT5 protein in JJN3 cells (data not shown), but this reduction was not observed until 4 hours after treatment. STATs regulate their own expression (10), and the delayed reduction in total STAT5 is consistent with the suppression of STAT-mediated transcription. Moreover, in whole-cell extracts of a murine plasmacytoma, CDDO-Imidazolide also blocked the ability of IL-6 to increase the level of both p-STAT3 (Fig. 2B) and the level of p-TYK2, a member of the JAK kinase family that phosphorylates STATs (Fig. 2C). Within 30 minutes, p-TYK2 levels were drastically diminished, and almost no p-STAT3 was observed after 1 hour of treatment with 250 nmol/L CDDO-Imidazolide. In addition to suppressing constitutive p-STAT5 levels in total cell extracts of JJN3 cells, CDDO-Imidazolide also reduced the levels of nuclear p-STAT5 in these cells in a time- and dose-dependent manner (Fig. 2D). As shown in Fig. 2E, CDDO-Imidazolide rapidly decreased STAT3 binding to consensus STAT3 DNA sequences in an ELISA assay of JJN3 nuclear lysates.

CDDO-Imidazolide decreases constitutive STAT phosphorylation, inhibits proliferation, and induces apoptosis in lung cancer cells. STAT phosphorylation resulting from the autocrine action of IL-6 is a known important survival pathway in myeloma (22). Constitutive STAT3 phosphorylation has also been implicated as a growth-promoting, survival pathway in

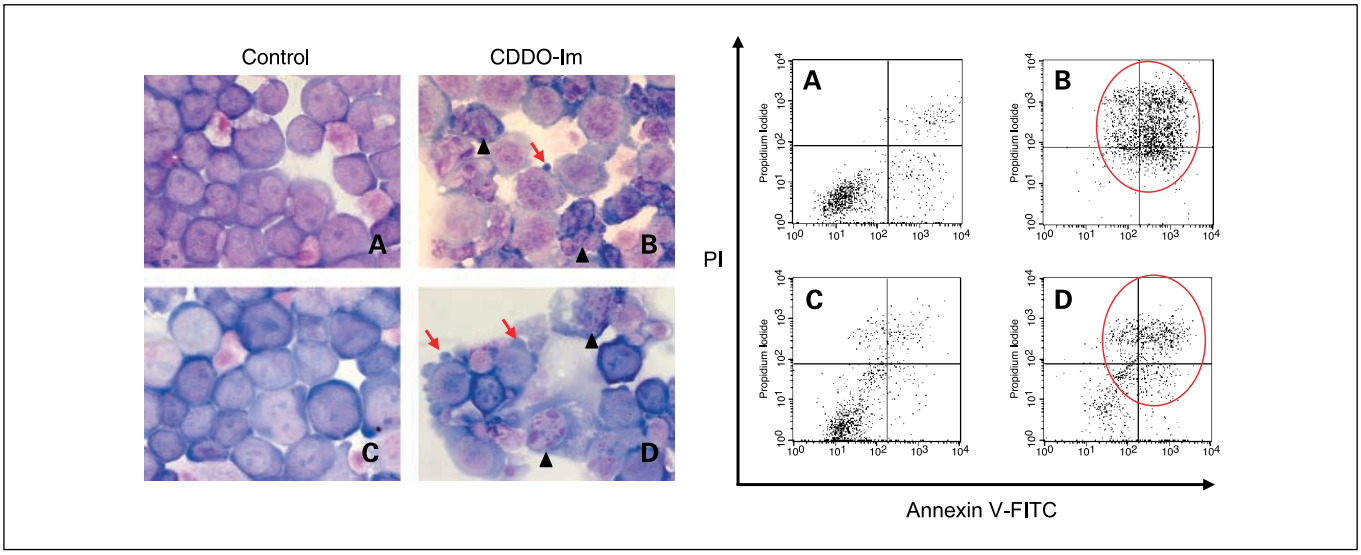

Fig. 1. CDDO-Imidazolide (*CDDO-Im*) rapidly induces apoptosis in myeloma cells. Human myeloma cells (*A* and *B*, RPMI-8226; *C* and *D*, JJN3) were treated with 500 nmol/L CDDO-Imidazolide for 4 hours followed by washout of the drug. At 24 hours, cells were analyzed by cytospin (*left*) or fluorescence-activated cell sorting (*right*) for Annexin V and propidium iodine staining. Red arrows, membrane blebbing; black arrowheads, nuclear fragmentation.

lung cancer (4). As shown in Fig. 3, STAT3 phosphorylation is constitutive in both A549 (Fig. 3A) and H358 (Fig. 3B) human lung cancer cells. Notably, 1 μmol/L CDDO-Imidazolide rapidly decreased p-STAT3 levels in these cells in a time- and dose-dependent fashion, although levels of STAT3 itself did not change (data not shown). Consistent with the role of p-STAT3 as a growth/survival factor, the same concentrations of CDDO-Imidazolide that reduced constitutive STAT3 phosphorylation also inhibited proliferation (Fig. 3C) and induced apoptosis (Fig. 3D) in A549 cells. The percentage of Annexin V–positive cells, indicative of early apoptosis, increased from 3% in the control cells to 16% and 8%, respectively, in cells treated with either 300 or 1,000 nmol/L CDDO-Imidazolide. Moreover, CDDO-Imidazolide increased the percentage of cells positive for both Annexin V and propidium iodide (hallmarks of late apoptosis) from 3% in the controls to either 9% and 26%. The induction of apoptosis in the A549 lung cancer cells was confirmed by PARP cleavage (data not shown). Analogous results were obtained with H358 cells (data not shown). Although the concentrations required for blocking STAT phosphorylation and inducing apoptosis are higher in the adherent lung cancer cells than in the suspension myeloma cells, the kinetics are similar.

CDDO-Imidazolide regulates STAT target genes and induces SOCS-1 and SHP-1. Further studies were done using gene array technology. Thus, A549 lung cancer cells were treated with 1 μmol/L CDDO-Imidazolide for 4 hours, and RNA from these samples were compared with RNA from untreated control cells. Some of the genes in the STAT pathway either up-regulated or down-regulated by the treatment with

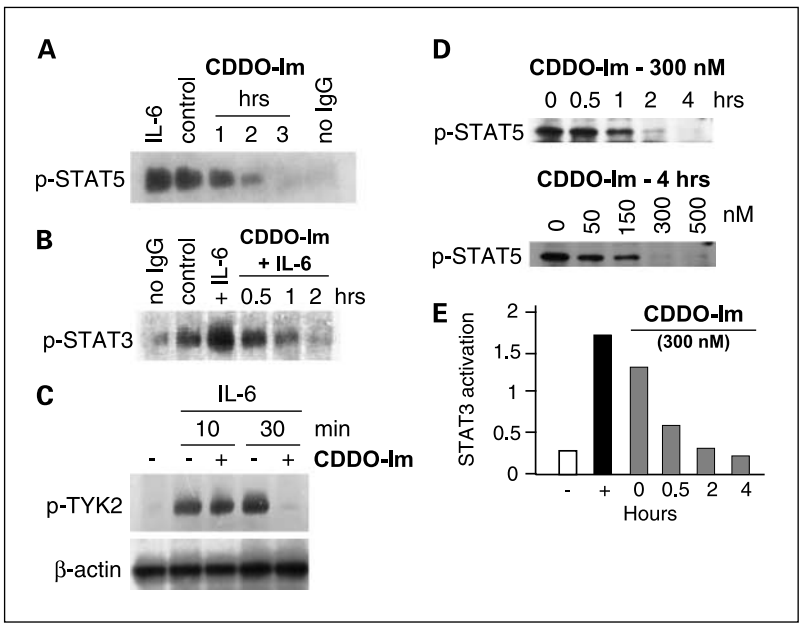

Fig. 2. CDDO-Imidazolide suppresses STAT phosphorylation (*A-C*) and its nuclear action (*D-E*) in myeloma cells. JJN3 human myeloma cells (*A*, *D*, and *E*), in which IL-6 is an autocrine growth factor, or murine 1254 plasmacytoma cells (*B* and *C*), which were stimulated with IL-6 (10 ng/mL), were treated with CDDO-Imidazolide (250 nmol/L unless indicated otherwise) for various times. Cell lysates were immunoprecipitated with phosphotyrosine antibodies (*A* and *B*), whereas whole-cell extracts (*C*) or nuclear lysates (*D*) were immunoblotted with specific antibodies to p-STAT5. Nuclear lysates of JJN3 cells were used to measure STAT3 binding to consensus DNA sequences (*E*); cells were treated with 300 nmol/L CDDO-Imidazolide, and an ELISA that used immobilized oligonucleotides was done on these lysates. Negative (−) and positive (+) controls included in the kit (Active Motif).

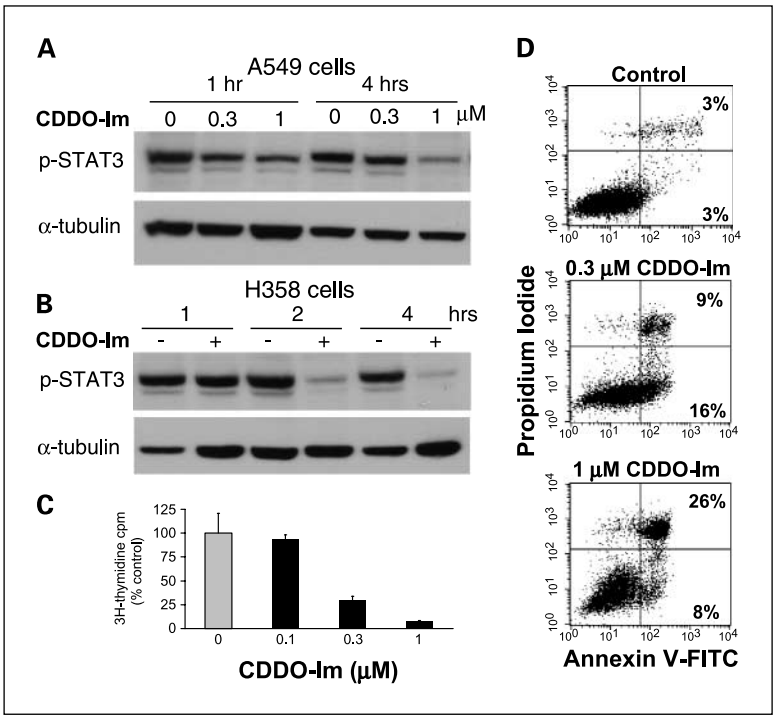

Fig. 3. CDDO-Imidazolide decreases constitutive STAT phosphorylation, inhibits proliferation, and induces apoptosis in lung cancer cells. A549 (*A*) and H358 (*B*) human lung cancer cells were treated with 0.3 to 1 μmol/L (*A*) or 1 μmol/L CDDO-Imidazolide (*B*) for 1 to 4 hours, and whole-cell extracts were immunoblotted with p-STAT3 and tubulin antibodies. To measure cell proliferation, A549 cells were treated with CDDO-Imidazolide for 48 hours, and evaluated by a [^{3}H]thymidine incorporation assay. A549 cells also were treated with CDDO-Imidazolide for 24 hours and then analyzed for apoptosis by flow cytometry for Annexin V and propidium iodide staining (*D*).

CDDO-Imidazolide are listed in Table 1; the up-regulation of SOCS-1, SMAD7, JUNB, PIM-1, and ISFG3 mRNA has been confirmed by reverse transcription-PCR (data not shown). Notably, the array showed that the mRNA for SOCS-1, a negative regulator of the STATs (2, 10), was increased almost 6-fold. Furthermore, the ability of CDDO-Imidazolide to regulate SOCS-1 expression in a time-dependent (Fig. 4A) and dose-dependent (data not shown) manner was confirmed in separate experiments. Moreover, in JJN3 myeloma cells, CDDO-Imidazolide rapidly increased expression of SHP-1 phosphatase protein (Fig. 4B). Finally, in JJN3 cells treated with CDDO-Imidazolide for 1 to 2 hours, immunoprecipitated with SHP antibodies, and then immunoblotted for p-STAT5, the formation of a SHP-STAT5 complex was observed (Fig. 4C). SHP-1 binds to immunoreceptors (such as the receptors for interleukins and IFNs) through interaction of SH2 domains with an "immunoreceptor tyrosine-based inhibition motif" within the receptor proteins (23). However, these SH2-containing phosphatases have also been shown to dephosphorylate STATS directly (24), Inhibition of IFN-γ-induced p-STAT1 by transforming growth factor-β (TGF-β) in T cells is a direct consequence of the formation of a STAT-SHP-1 complex, induced rapidly after exposure to TGF-β. CDDO-Imidazolide enhances TGF-β signaling, but it is not known whether the suppression of STAT phosphorylation by CDDO-Imidazolide is directly mediated by TGF-β. As expected, treatment with the serine/threonine–specific phosphatase inhibitor calyculin A (25) had no effect on the formation of the SHP-STAT5 complex.

Discussion

We have shown for the first time that a synthetic triterpenoid, such as CDDO-Imidazolide (which is a known potent antiproliferative and proapoptotic agent), is a potent suppressor of the activation of both STAT3 and STAT5 in human and mouse tumor cells. These results have been obtained with nanomolar levels of CDDO-Imidazolide. They occur in cells in which the STATs are either constitutively phosphorylated or in which their phosphorylation has been induced by a cytokine, such as IL-6. IL-6 is known to induce both inflammatory and proliferative states in a wide variety of cells, both malignant and nonmalignant, and has long been known to be an autocrine growth factor for human myeloma cells (26). The suppression of STAT phosphorylation in our studies was accompanied by a rapid induction of apoptosis, which is in accord with the known ability of activated STATs to suppress apoptosis (2–4, 6, 7). Moreover, we show for the first time that

Table 1. Genes in the STAT pathway either up-regulated or down-regulated by the treatment with CDDO-Imidazolide

Gene	Fold induction
CEBPB	2.1
IL22RA1	0.5
IL2RG	0.6
ISGF3G	5.7
JUNB	2.2
NMI	0.2
PIM1	3.2
SMAD1	2.4
SMAD7	3.0
SOCS1	5.9

NOTE: A549 cells were treated with 1 μmol/L CDDO-Imidazolide for 4 hours, and total RNA was isolated and analyzed using a human JAK/STAT Oligo GEArray blot. The expression of the listed genes changed following treatment with CDDO-Imidazolide compared with untreated control cells.

CDDO-Imidazolide induces the expression of two negative regulators of the STATs (i.e., SOCS-1 and SHP-1), thereby diminishing STAT activity.

The immediate proximate molecular target for CDDO-Imidazolide in these studies is not yet known, although it is clear that its effects on the STAT pathway occur very rapidly, as we show suppressive effects on levels of p-TYK2 (a JAK), p-STAT3, or p-STAT5 within 30 to 60 minutes of treatment of either human or murine myeloma or lung cancer cells. Likewise, increased levels of the phosphatase SHP-1 were found within 30 minutes of treatment of myeloma cells. From what is known of the chemistry of the interaction of triterpenoids, such as CDDO-Imidazolide, with putative targets, it may be difficult to implicate a single target. The A-ring of a molecule such as CDDO-Imidazolide is known to form adducts (which may be reversible) with reactive thiol groups, either with model targets, such as DTT (27), or with a specific cysteine-rich protein target, such as Keap1, the molecular inhibitor of the transcription factor Nrf2 (28). The triterpenoid, thus, may have a transient interaction with a critical cysteine on one of its target proteins, but this interaction may be so short-lived that it will be difficult to show covalent Michael addition (27); this would be a "hit-and-run" mechanism (29). Moreover, it has been difficult to evaluate the effects of CDDO-Imidazolide and its relatives in standard cell-free kinase assays because almost all of these measurements require a reducing agent, such as mercaptoethanol or DTT, to maintain the enzymatic activity of the respective kinase being assayed. These reducing agents, in turn, form complexes with CDDO-Imidazolide and its congeners, effectively "chelating" the triterpenoid and blocking its activity.

Finally, the present results suggest that further studies are needed on the interface between STATs and TGF-β. Both STATs and TGF-β have profound effects on the initiation of inflammation (the original context for the discovery of the STATs) and on the proliferation and apoptosis of cells (2, 10, 14, 30–33). Furthermore, recent gene array studies indicate that STAT3 regulates a common set of genes involved in wound healing and cancer (34). TGF-β is the paradigmatic wound-healing cytokine (32). Recent studies also indicate that CDDO-Imidazolide is a potent enhancer of TGF-β signaling (35); thus, triterpenoids, such as CDDO-Imidazolide, may serve to elucidate mechanistic relationships between the STAT and the TGF-β signaling pathways. TGF-β has previously been shown to inhibit T-cell receptor–mediated signaling by up-regulating tyrosine phosphatases in T cells (36). The finding that both CDDO-Imidazolide and TGF-β share the ability to induce formation of a SHP-1/STAT complex highlights the potential significance of TGF-β signaling in mediating the potent anti-inflammatory effects of the synthetic triterpenoids. Furthermore, the effects of CDDO-Imidazolide on STAT function described here suggest that important effects of this agent on angiogenesis will be found because STATs are critical regulators of angiogenesis (37–39).

The recent focus on the development of Janus kinase inhibitors emphasizes the relevance of the STAT pathway as a therapeutic target. The ability of triterpenoids to affect JAK/STAT signaling predicts the potential for their application in a number of disease settings other than cancer in which aberrant activation of JAK/STAT pathways is observed. These include organ transplants and autoimmune diseases in which Th1-mediated inflammatory responses underlie the progressive immune-mediated tissue destruction (11, 40). For example, TYK2 has been shown to play an essential role in IL-12 signaling, and the resistance of *Tyk2*$^{-/-}$ mice to arthritis predicts that inhibitors of TYK2 activation may be useful clinically. CDDO-Imidazolide is the first small molecule to show activity as an inhibitor of TYK2 activation. The importance of JAK/STAT signaling in malignancies of both hematopoietic and epithelial origin clearly adds further evidence that triterpenoids should be significant anticancer agents. Future preclinical studies should focus on both the chemopreventive and therapeutic activity of triterpenoids in disease models in which the suppression of JAK/STAT signaling may be clinically relevant.

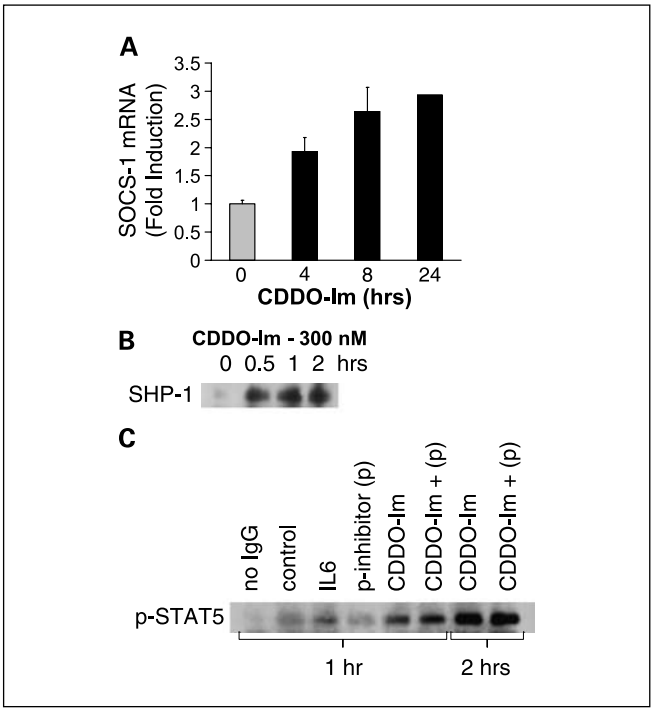

Fig 4. CDDO-Imidazolide regulates STAT target genes and induces SOCS-1 and SHP-1. *A,* SOCS-1 mRNA expression from A549 cells treated with 1 μmol/L CDDO-Imidazolide for 0 to 24 hours was analyzed by real-time PCR. Lysates of JJN3 cells treated with 300 nmol/L CDDO-Imidazolide for 0 to 2 hours were immunoprecipitated with SHP antibodies and then immunoblotted for SHP-1 (*B*) or p-STAT5 (*C*). p-inhibitor, calyculin A (1 nmol/L).

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