

# The synthetic triterpenoid 1-[2-cyano-3,12-dioxoleana-1,9(11)-dien-28-oyl]imidazole blocks nuclear factor- $\kappa$ B activation through direct inhibition of I $\kappa$ B kinase $\beta$

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## Abstract

The synthetic triterpenoid 1-[2-cyano-3,12-dioxoleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) is a multifunctional agent with potent anti-inflammatory, antiproliferative, cytoprotective, and apoptotic activities, whose molecular targets are unknown. Using both cell-free and cellular assays, we show that CDDO-Im is a direct inhibitor of I $\kappa$ B kinase (IKK)  $\beta$  and that it thereby inhibits binding of nuclear factor- $\kappa$ B to DNA and subsequent transcriptional activation. Pretreatment of cells with CDDO-Im prevents I $\kappa$ B $\alpha$  phosphorylation and degradation in response to tumor necrosis factor  $\alpha$ . The kinetics of this inhibition by CDDO-Im are rapid and occur within 15 min. A biotinylated analogue of CDDO-Im showed that CDDO-Im binds to the IKK signalsome. Furthermore, we show that Cys<sup>179</sup> on IKK is a target for CDDO-Im. This is the first report to show that this novel synthetic triterpenoid binds to and inhibits IKK $\beta$  directly. [Mol Cancer Ther 2006;5(12):3232–9]

## Introduction

New synthetic derivatives of the triterpenoid oleanolic acid [2-cyano-3,12-dioxoleana-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (1, 2)] are now in phase I clinical trial for treatment of leukemia and solid tumors. Preclinical studies have yielded an abundance of information relating to the cellular and subcellular mechanisms of action of these agents, particularly with respect to their antiproliferative, proapoptotic, anti-inflammatory, and cytoprotective activities (1–21). There is evidence that Keap1, which regulates the transcription factor Nrf2, is a

target for triterpenoids related to CDDO and mediates their cytoprotective effects (22). However, there is still a paucity of data on the immediate molecular targets that mediate the proapoptotic activities of CDDO and its analogues, which are observed at higher concentrations.

One of the most promising analogues of CDDO that has been synthesized is its C-28 imidazolide (CDDO-Im), which is significantly more potent than its parent, in anti-inflammatory, antiproliferative, and cytoprotective assays (3, 21, 23). CDDO-Im is highly active as an inducer of transcriptional activity of Nrf2 (22, 23) and has been used in this context *in vivo* to suppress liver carcinogenesis induced in rats by aflatoxin (24). Moreover, it is one of the most potent synthetic triterpenoids for induction of apoptosis (25–31). However, at present, there is no comprehensive understanding of the possible molecular targets of this molecule.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a proinflammatory, pro-survival, and antiapoptotic transcription factor that is constitutively activated in many tumors (32, 33). Because of the central importance of this transcription factor and its associated proteins in regulating both inflammation and cell survival (34–40), in the present study, we have investigated the interaction of CDDO-Im with the upstream kinases that regulate NF- $\kappa$ B activity [i.e., the I $\kappa$ B kinases (IKK)]. We have found that CDDO-Im directly inhibits IKK $\beta$  and thereby inhibits binding of NF- $\kappa$ B to DNA and transcriptional activation. The kinetics of this inhibition by CDDO-Im are rapid and occur within 15 min. Protein studies, which we report here, suggest that a critical cysteine residue (Cys<sup>179</sup>) on IKK $\beta$  is a required target for this action of CDDO-Im.

## Materials and Methods

### Reagents and Plasmids

The synthesis of CDDO-Im, TP301, TP303, and TP304 (see Fig. 1 for structures) has been described (21, 30, 41). Stock solutions of CDDO-Im, TP301, TP303, and TP304 (0.01 mol/L) were made in DMSO, and aliquots were frozen at  $-20^{\circ}$ . Serial dilutions were made in DMSO before addition to cell culture medium, and equivalent volumes of DMSO (<0.1%) were used as a control. Sources of reagents were as follows: I $\kappa$ B $\alpha$ , p65, and anti-HA antibodies, Santa Cruz Biotechnology (Santa Cruz, CA); phosphorylated I $\kappa$ B $\alpha$ , phosphorylated IKK, and isoform-specific IKK $\alpha$  and IKK $\beta$  antibodies, Cell Signaling (Danvers, MA); human recombinant tumor necrosis factor (TNF)  $\alpha$ , R&D Systems (Minneapolis, MN); human recombinant active IKK $\beta$  enzyme, Upstate (Charlottesville, VA); immobilized NeutrAvidin protein, Pierce Biotechnology (Rockford, IL); and biotinylated iodoacetamide (BIAM), Sigma-Aldrich

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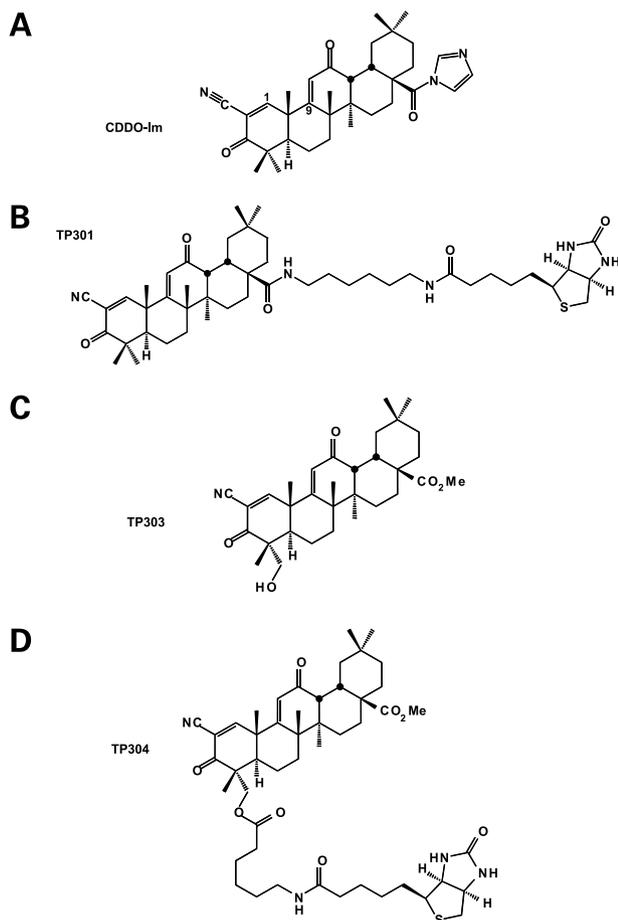
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**Figure 1.** Chemical structures of CDDO-Im, TP301, TP303, and TP304.

(St. Louis, MO). The 3X-NF- $\kappa$ B-luciferase reporter plasmid and the IKK $\beta$  expression plasmids were gifts from Albert S. Baldwin, Jr. (University of North Carolina at Chapel Hill, Chapel Hill, NC) and Michael Karin (University of California, San Diego, CA), respectively.

#### Cell Culture

In response to various stimuli, HEK293 cells (Kathleen Martin, Dartmouth Medical School, Hanover, NH) activate NF- $\kappa$ B, and all the components of the NF- $\kappa$ B pathway are present and functional in these cells. Cells were maintained in MEM containing 10% fetal bovine serum and penicillin/streptomycin (50 units/mL penicillin and 50  $\mu$ g/mL streptomycin). For Western blotting experiments, cells were plated and treated in MEM plus 1% horse serum. All cells were incubated in a 5% CO<sub>2</sub> humidified atmosphere.

#### NF- $\kappa$ B/DNA Binding

p65/NF- $\kappa$ B binding to DNA was detected using a p65 transcription factor assay kit (Chemicon International, Temecula, CA). Briefly, HEK293 cells were treated with CDDO-Im or DMSO for 1 h followed by TNF $\alpha$  (10 ng/mL) for 30 min. Nuclear protein (10  $\mu$ g) from lysates of these cells was then added to a NF- $\kappa$ B capture probe (a

biotinylated double-stranded oligonucleotide probe containing the wild-type consensus sequence for NF- $\kappa$ B) or a NF- $\kappa$ B-specific competitor oligonucleotide (a nonbiotinylated version of the NF- $\kappa$ B capture probe) or a negative control probe (a biotinylated double-stranded oligonucleotide with a mutant NF- $\kappa$ B DNA-binding consensus sequence). The reaction mixture was then transferred to a streptavidin-coated plate for 2 h, and p65 protein bound to DNA was detected with specific anti-p65 antibodies using a colorimetric assay with 3,3',5,5'-tetramethylbenzidine.

#### Transfections and Luciferase Assays

HEK293 cells were seeded in 24-well plates at 60% confluency 1 day before transfection. For TP304-IKK binding experiments, cells were transfected with either 0.5  $\mu$ g of wild-type IKK $\beta$  or 0.5  $\mu$ g of C179A mutant IKK $\beta$  expression plasmids 24 h before TP304 treatment. For NF- $\kappa$ B transcriptional activation assays, cells were cotransfected with 0.5  $\mu$ g of a 3X-NF- $\kappa$ B-luciferase reporter plasmid and 0.5  $\mu$ g  $\beta$ -galactosidase expression plasmid using PolyFect transfection reagent (Qiagen, Valencia, CA). Twenty-four hours later, cells were treated with CDDO-Im for 1 h and then with TNF $\alpha$  for 18 h, and luciferase activity was measured on cell lysates and normalized to  $\beta$ -galactosidase activity.

#### Confocal Microscopy

HEK293 cells were plated in chamber slides at 60% confluence. Cells were treated with CDDO-Im or vehicle control for 1 h followed by stimulation with 10 ng/mL TNF $\alpha$ . After an additional 30 min, the cells were fixed with 4% formaldehyde for 10 min. Cells were then washed thrice in PBS and permeabilized with 0.1% Triton X-100 in PBS and 5% normal goat serum for 1 h. Polyclonal anti-p65 antibodies were added for 1 h followed by 1 h of incubation with Texas red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). To identify cell nuclei, coverslips were mounted in 4',6-diamidino-2-phenylindole containing Vectashield H-1000 Mounting Medium (Vector Laboratories, Burlingame, CA). Confocal images were obtained with a Leica TCS SP confocal laser scanning microscope.

#### Isolation of Proteins Labeled by TP304 and Western Blotting

HEK293 cells were treated with 2  $\mu$ mol/L TP304 for 2 h, washed with PBS to remove residual TP304 and endogenous biotin, suspended in 250 mmol/L sucrose, 50 mmol/L Tris (pH 7.4), 5 mmol/L MgCl<sub>2</sub>, 1 mmol/L EGTA, and 1 $\times$  protease inhibitor cocktail (Sigma-Aldrich), and then lysed by sonication. Centrifuged lysates containing 200  $\mu$ g of protein were incubated with 100  $\mu$ L of NeutrAvidin resin in 1 mL PBS containing 0.4% Tween 20 overnight at 4°C. NeutrAvidin-biotinylated triterpenoid complexes were washed five times with 1 mL PBS containing 0.4% Tween 20. Samples were dissolved in 50  $\mu$ L of Laemmli loading buffer, boiled, and subjected to Western blot for IKK (42).

#### In vitro IKK Kinase Assays

Constitutively active, purified recombinant IKK $\beta$  (Upstate) was diluted in enzyme buffer [20 mmol/L MOPS-NaOH (pH 7.0), 1 mmol/L EDTA, 0.01% Brij-35, 5%

glycerol, 0.1%  $\beta$ -mercaptoethanol, 1 mg/mL bovine serum albumin]. The final kinase reaction consisted of 125 ng of the diluted IKK $\beta$  enzyme, 1 mmol/L IKK substrate peptide (IKKtide, Upstate), 10 mmol/L MgAc, 0.1 mmol/L ATP, and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP and various concentrations of CDDO-Im or DMSO control. Recombinant IKK $\beta$  was preincubated with CDDO-Im or DMSO for 10 min at room temperature before addition of the MgAc/ATP/[ $\gamma$ - $^{32}$ P]ATP to initiate the reaction for 10 min at 30°C. Aliquots were spotted onto p81 phosphocellulose paper and washed thrice in 0.75% phosphoric acid followed by a single wash in acetone, and radioactivity was then measured.

#### Labeling of Protein Thiols with BIAM

HEK293 cells were treated with DMSO or 1  $\mu$ mol/L CDDO-Im for 1 h and lysed as described previously. Samples containing 200  $\mu$ g of total protein were incubated with 2 mmol/L BIAM for 1 h at 4°C. A 50% NeutrAvidin bead slurry (100  $\mu$ L) was added, and BIAM-labeled proteins were precipitated overnight at 4°C. Precipitates were washed in PBS-0.4% Tween 20 and subjected to SDS-PAGE followed by immunoblotting with an anti-IKK antibody.

## Results

### CDDO-Im Inhibits Activation of NF- $\kappa$ B

We first examined the effect of CDDO-Im on the binding of NF- $\kappa$ B to DNA, as occurs in HEK293 cells that have been stimulated with 10 ng/mL TNF $\alpha$  (Fig. 2A). When cells were preincubated with CDDO-Im for as little as 1 h before TNF $\alpha$  stimulation, this binding was inhibited (Fig. 2A). No binding was observed in samples incubated with an oligonucleotide with a mutant NF- $\kappa$ B consensus sequence or when excess nonbiotinylated (competitor) oligonucleotide was added (Fig. 2A). The inhibitory effect of CDDO-Im on NF- $\kappa$ B binding was concentration dependent and was observed at concentrations typical for induction of apoptosis in most adherent cell lines (26, 31). The rapidity of these effects suggests that CDDO-Im is a direct inhibitor of the pathway. CDDO-Im also inhibited induction of a NF- $\kappa$ B luciferase reporter construct in HEK293 cells treated with TNF $\alpha$  (Fig. 2B). The concentrations of CDDO-Im required for inhibition of transcriptional activity correlated with those required to inhibit binding to DNA (Fig. 2A).

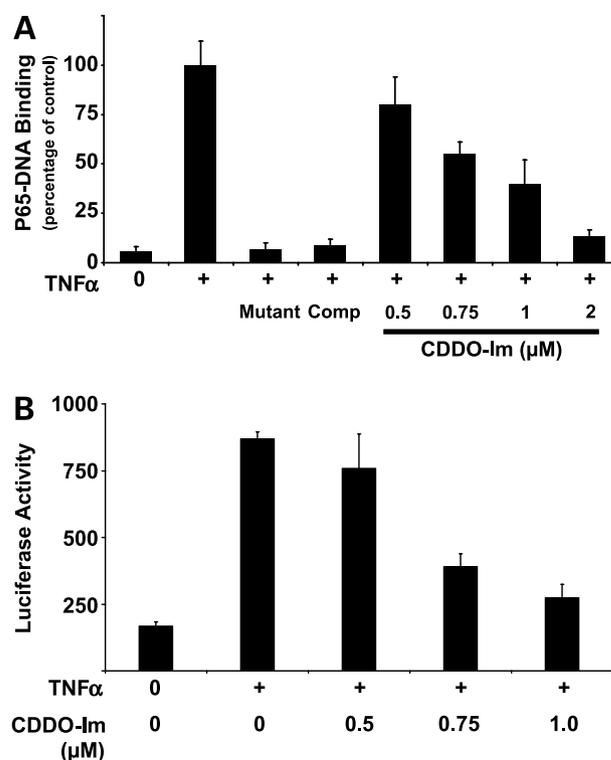
### CDDO-Im Inhibits Nuclear Translocation of NF- $\kappa$ B

Several compounds have been shown to form adducts with specific cysteine residues on NF- $\kappa$ B subunits and inhibit their binding to DNA (43). To examine whether the effects of CDDO-Im occur after NF- $\kappa$ B subunits translocate to the nucleus or whether CDDO-Im blocks their nuclear translocation, we did confocal microscopy experiments in which we monitored nuclear translocation of p65 following treatment with TNF $\alpha$ . In quiescent cells, the p65 subunit of NF- $\kappa$ B was localized mostly in the cytosol (Fig. 3A, *i*). However, p65 rapidly translocated from the cytoplasm to the nucleus following 30 min of TNF $\alpha$  stimulation (Fig. 3A, *ii*). Pretreatment with 1  $\mu$ mol/L CDDO-Im for 1 h completely blocked nuclear translocation of p65 in response to TNF $\alpha$  (Fig. 3A, *iii*). No staining was detected when slides

were incubated with either primary or secondary antibodies alone or when a secondary antibody from a different species was used (data not shown). As a control, we also showed that CDDO-Im had no effect on total p65 levels in HEK293 cells at concentrations up to 2  $\mu$ mol/L (Fig. 3B). These results clearly show that CDDO-Im affects the pathway before nuclear translocation of p65.

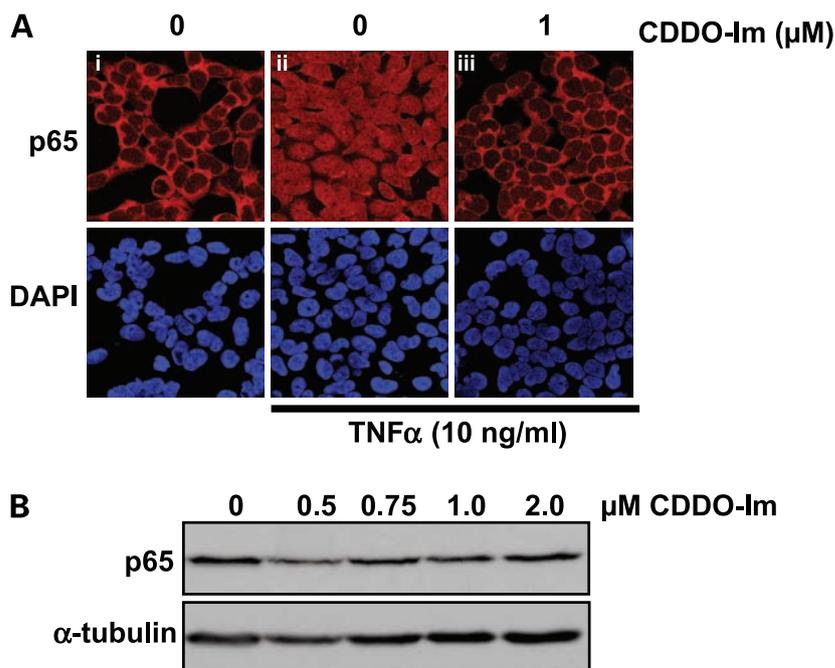
### CDDO-Im Inhibits IKK Activity in Cell Culture

To determine the target molecule for inhibition of the NF- $\kappa$ B pathway by CDDO-Im, we investigated some of the critical upstream activators of the pathway, most notably IKK. We stimulated HEK293 cells with TNF $\alpha$ , which results in IKK and I $\kappa$ B $\alpha$  phosphorylation as well as I $\kappa$ B $\alpha$  degradation (Fig. 4A; data not shown). At concentrations ranging from 500 nmol/L to 1  $\mu$ mol/L, preincubation with CDDO-Im for 1 h before TNF $\alpha$  stimulation significantly inhibited I $\kappa$ B $\alpha$  phosphorylation and resulted in concomitant stabilization of I $\kappa$ B $\alpha$  protein levels (Fig. 4A). Total IKK



**Figure 2.** CDDO-Im inhibits binding of NF- $\kappa$ B to DNA and subsequent transcriptional activity. **A**, HEK293 cells were treated with CDDO-Im at the concentrations indicated for 1 h followed by the addition of TNF $\alpha$  (10 ng/mL) for 30 min. Nuclear extracts were prepared for analysis in a p65 transcription factor binding assay with biotinylated NF- $\kappa$ B, biotinylated mutant NF- $\kappa$ B (*Mutant*), or nonbiotinylated competitor NF- $\kappa$ B (*Comp*) probes. **B**, cells were transfected with 0.5  $\mu$ g of a 3X-NF- $\kappa$ B-luciferase reporter construct and 0.5  $\mu$ g of a  $\beta$ -galactosidase expression plasmid. CDDO-Im or DMSO was added to the culture medium 24 h after transfection, and TNF $\alpha$  (10 ng/mL) was added 1 h after CDDO-Im treatment. Cells were lysed 18 h after TNF stimulation. Luciferase activities were measured and normalized to  $\beta$ -galactosidase activity. *Columns*, average of triplicate transfections with similar results obtained in at least three independent experiments.

**Figure 3.** CDDO-Im inhibits TNF-induced nuclear translocation of NF- $\kappa$ B. **A**, HEK293 cells were treated with 1  $\mu$ mol/L CDDO-Im or DMSO control for 1 h and then stimulated with 10 ng/mL TNF $\alpha$  for 20 min. Cells were fixed and p65 cellular location was determined by confocal microscopy. **B**, HEK293 cells were treated with DMSO or CDDO-Im at the concentrations indicated for 1 h. Whole-cell lysates were prepared and total p65 levels were determined by Western blot.



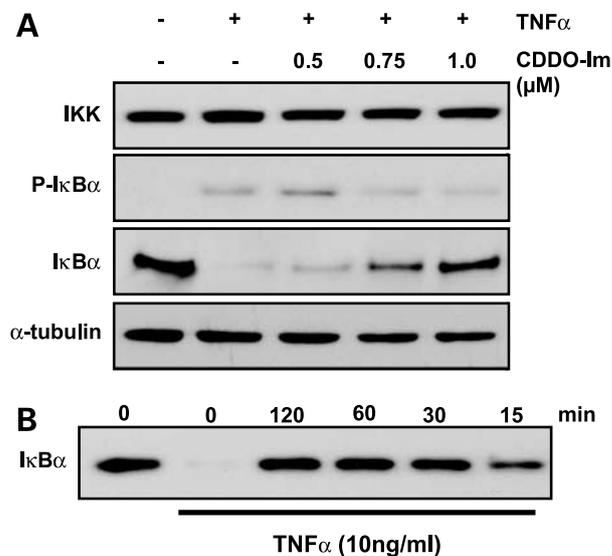
protein levels were not affected by these concentrations of CDDO-Im, indicating that CDDO-Im directly suppresses IKK activity (Fig. 4A). The effect of CDDO-Im on IKK activity induced by TNF $\alpha$  and on I $\kappa$ B $\alpha$  degradation was dependent on the concentration of the CDDO-Im (Fig. 4A); at least 1  $\mu$ mol/L was required to inhibit IKK completely, which correlated with the optimal concentration required to inhibit NF- $\kappa$ B DNA binding, transcriptional activity, and p65 nuclear translocation (Figs. 2A and B and 3A). Next, we determined the kinetics for this inhibition of IKK activity by pretreating with CDDO-Im for varying times before TNF $\alpha$  stimulation and examining I $\kappa$ B $\alpha$  stability as an indication of IKK activity. As little as 15-min pretreatment with CDDO-Im resulted in a significant increase in the stability of I $\kappa$ B $\alpha$  protein (Fig. 4B). Similar effects on IKK, I $\kappa$ B $\alpha$ , NF- $\kappa$ B DNA binding, and transcriptional activity were observed in H460 non-small cell lung carcinoma cells (data not shown). These results strongly suggest that a protein within the IKK complex may be the molecular target of CDDO-Im.

#### CDDO-Im Interacts Directly with IKK

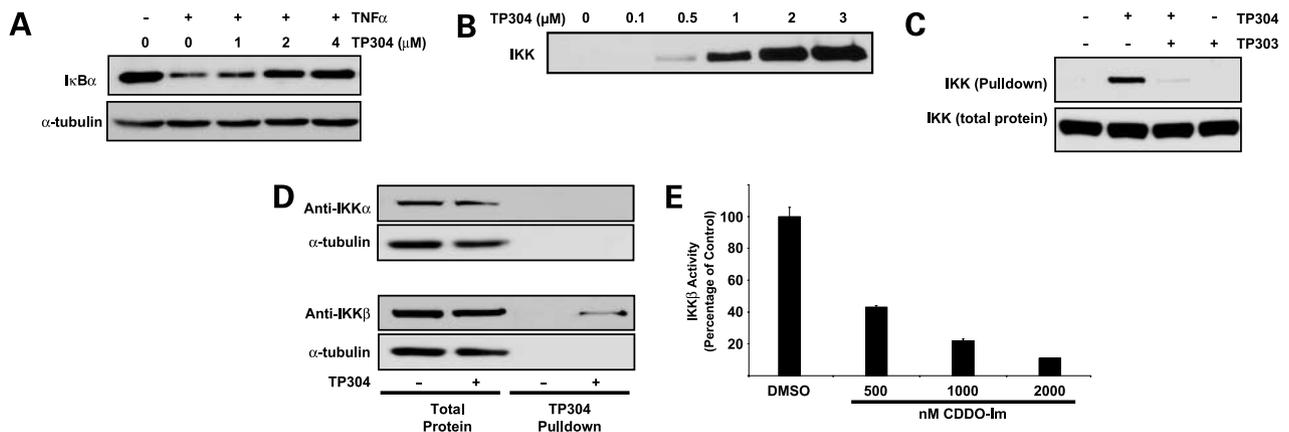
Agents that interact with sulfhydryl groups, such as parthenolide, the cyclopentenone prostaglandins 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> and PGA<sub>1</sub>, and trivalent arsenic, have been shown to bind covalently to a critical cysteine residue (Cys<sup>179</sup>) within the activation loop of IKK and inhibit its kinase activity (44–46). Because CDDO-Im could potentially form covalent bonds with sulfhydryl groups through a Michael addition at carbon 1 or 9 (47), we examined if a biotinylated analogue of CDDO (TP304; Fig. 1D) could interact with the IKK complex.

To determine if TP304 had similar physiologic properties as CDDO-Im, we pretreated cells for 1 h with TP304 before IKK/NF- $\kappa$ B induction by TNF $\alpha$  and found that this biotinylated analogue inhibited the activity of TNF $\alpha$  in a

dose-dependent manner (Fig. 5A). The concentrations required to inhibit I $\kappa$ B $\alpha$  degradation were slightly higher than those required by CDDO-Im (Fig. 4A). These observations correlate with the known potency differences between CDDO-Im and TP304 in inhibition of cellular proliferation (41), although both compounds can inhibit



**Figure 4.** CDDO-Im inhibits TNF-mediated IKK activity *in vivo*. **A**, HEK293 cells were treated with DMSO or CDDO-Im for 1 h followed by treatment with TNF $\alpha$  (10 ng/mL) for 5 min [phosphorylated I $\kappa$ B $\alpha$  (P-I $\kappa$ B $\alpha$ )] or 15 min (IKK and I $\kappa$ B $\alpha$ ). Whole-cell lysates were prepared and subjected to Western blot analysis. **B**, HEK293 cells were treated with DMSO or 2  $\mu$ mol/L CDDO-Im followed by stimulation with TNF $\alpha$  (10 ng/mL) for 15 min. Whole-cell lysates were prepared and subjected to Western blot analysis.



**Figure 5.** CDDO-Im interacts with the IKK complex and inhibits its kinase activity. **A**, HEK293 cells were treated with various concentrations of biotinylated triterpenoid (TP304) for 1 h followed by stimulation with TNF $\alpha$  for 15 min. IkB $\alpha$  levels were assessed by Western blot. **B**, cells were treated with TP304 for 2 h. TP304-protein complexes were precipitated from cell lysates with NeutrAvidin resin. The presence of IKK was detected by Western blotting with anti-IKK antibodies. **C**, HEK293 cells were treated with DMSO or varying concentrations of TP304, TP303, or both for 2 h. NeutrAvidin precipitations were done as described, and TP304-IKK complexes were detected by Western blot. *Bottom*, a 30  $\mu$ g sample of the cell lysate used for the TP304 precipitation was also run on the same gel to show the presence of equal IKK concentrations among the different lysate fractions. **D**, analysis of TP304 binding to cellular proteins as in **(B)**. Western blotting was done with isoform-specific antibodies for IKK $\alpha$  and IKK $\beta$ . **E**, *in vitro* kinase assay done with recombinant active IKK $\beta$  and various concentrations of CDDO-Im as described in Materials and Methods. Reactions were conducted in duplicate, and similar results were obtained in at least three independent experiments.

degradation of IkB $\alpha$ . To determine if TP304 bound directly to the IKK complex, we used immobilized NeutrAvidin to isolate TP304-protein complexes. Cells were treated with TP304, and lysates were precipitated with NeutrAvidin before Western blotting. TP304 binds to the IKK complex in a dose-dependent manner (Fig. 5B). Importantly, binding of TP304 to the IKK complex was significantly reduced when cells were pretreated with TP303 (the nonbiotinylated parent compound used to prepare TP304) or CDDO-Im, indicating that both TP303 and CDDO-Im compete with TP304 for binding to the same region of IKK (Fig. 5C; data not shown). Furthermore, no IKK precipitated in cells treated with control vehicle or cells treated with another control moiety [i.e., the portion of the TP304 molecule that contains the biotin as well as the esterified linker but from which the triterpenoid nucleus is absent (data not shown)]. Finally, pretreatment of HEK293 cells with sodium arsenite blocked TP304 binding to the IKK complex, suggesting that both reagents bind to the same reactive cysteine residue(s) (data not shown). In addition, a second less potent (41) biotinylated triterpenoid (TP301; Fig. 1B) bound to the IKK complex, but the binding affinity of TP301 was less than that of TP304 (data not shown). The biotin group of TP301 is attached via a linker to carbon 28 of the triterpenoid parent structure, in contrast to TP304 in which biotin is attached via a linker to carbon 23 (Fig. 1B and D; ref. 41).

To probe this interaction between IKK and CDDO-Im further, we used isoform-specific antibodies to discern whether CDDO-Im has a specific affinity for either IKK $\alpha$  or IKK $\beta$ . Following the NeutrAvidin precipitation described above, we blotted back with isoform-specific antibodies for either IKK $\alpha$  or IKK $\beta$ . Only IKK $\beta$  was pulled down by TP304 (Fig. 5D); measurement of  $\alpha$ -tubulin confirmed equal protein loading between samples. Importantly, no  $\alpha$ -tubulin

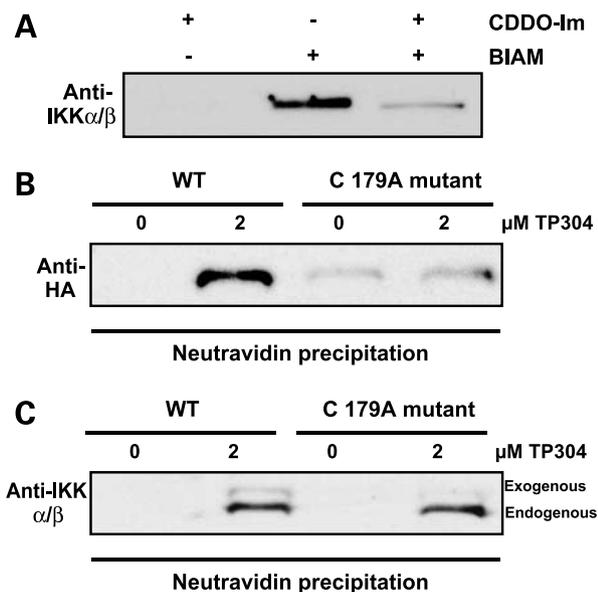
protein was detected in either NeutrAvidin precipitation, confirming that the pull-down is specific for IKK (Fig. 5D). In addition, the IKK $\beta$  antibody used in these experiments is isoform specific and directed toward a different epitope than that of the antibody used in Fig. 5B and C, confirming that detection of IKK within this system is not due to antibody cross-reactivity.

#### CDDO-Im Inhibits Recombinant IKK Activity

Further and more definitive confirmation of IKK $\beta$  as a target of CDDO-Im comes from *in vitro* assays with constitutively active, recombinant IKK $\beta$  kinase. Because free thiols can interact with CDDO-Im by Michael addition (22, 47), which sequesters this triterpenoid and limits its bioavailability, we titrated the concentration of reducing agent used in the enzyme assay to the minimum amount required for IKK to retain its activity (data not shown). We also used the less reactive monothiol, 2-mercaptoethanol, instead of the more commonly used dithiol, DTT, to lessen the sequestration of CDDO-Im. CDDO-Im dose dependently inhibited the *in vitro* kinase activity of IKK $\beta$  (Fig. 5E). The concentrations required to inhibit IKK activity in this cell-free assay correlated closely with those concentrations required for the same effect in a cell-based assay (compare Fig. 5E with Fig. 4A). The ability of CDDO-Im to inhibit the activity of this constitutively active kinase confirms that inhibition of the NF- $\kappa$ B pathway mediated by CDDO-Im is at the level of IKK and independent of upstream activators of the kinase.

#### Sulfhydryl Groups in IKK Are the Targets for Modification by CDDO-Im

To determine if CDDO-Im interacts with reactive cysteine residues on IKK, we treated cells with iodoacetamide, a classic reagent for alkylating thiol groups. Iodoacetamide forms covalent adducts with reactive cysteine residues with



**Figure 6.** Cysteine residues on IKK are targets for CDDO-Im. **A**, HEK293 cells were treated in the presence or absence of 1  $\mu\text{mol/L}$  CDDO-Im for 1 h followed by cell lysis and labeling of thiol residues with BIAM and precipitation of BIAM-labeled proteins by NeutrAvidin. The presence of IKK was detected by Western blot. **B**, HEK293 cells transfected with HA-tagged wild-type or C179A IKK $\beta$  expression plasmids were treated with 2  $\mu\text{mol/L}$  TP304 for 1 h followed by NeutrAvidin pull-down of TP304-protein complexes. Following the NeutrAvidin pull-down, the presence of the exogenous IKK protein was detected by Western blot with anti-HA mouse monoclonal antibodies. **C**, the blot in **(B)** was reprobated with rabbit anti-IKK antibodies to confirm that equivalent levels of endogenous IKK are precipitable. Top, exogenous HA-tagged IKK $\beta$  protein.

low  $pK_a$  (48). If, however, a triterpenoid is bound to reactive cysteine residues on target proteins, these residues will not be alkylated by iodoacetamide. We used a biotin-tagged version of iodoacetamide (i.e., BIAM) for the following experiments. Free thiol residues in whole-cell lysates from DMSO- or CDDO-Im-treated cells were labeled with BIAM and precipitated with NeutrAvidin resin. BIAM precipitated IKK as detected by Western blot (Fig. 6A). However, significantly less IKK was precipitated by BIAM when cells were pretreated with 1  $\mu\text{mol/L}$  CDDO-Im (Fig. 6A). These results indicate that CDDO-Im binds to reactive cysteine residues on IKK and blocks the subsequent interaction of BIAM with these residues. Although IKK $\beta$  contains 18 cysteine residues, many of these form stable disulfide bridges and are inaccessible to agents that react with free cysteine. However, there is a highly reactive free cysteine residue at position 179 in the activation loop of the kinase. Cys<sup>179</sup> is exquisitely reactive, and numerous agents that interact with sulfhydryl groups inhibit the activity of IKK $\beta$  through interaction with this residue (44–46). To investigate whether Cys<sup>179</sup> plays a role in the CDDO-Im/IKK interaction, we transfected HEK293 cells with expression plasmids containing either wild-type HA-IKK $\beta$  or a mutant in which Cys<sup>179</sup> is replaced by alanine (C179A). These cells were then treated with TP304, and NeutrAvidin was used to precipitate TP304-protein

complexes. Significantly less binding of exogenous IKK $\beta$  was detected in the C179A mutant compared with wild-type (Fig. 6B). When the same blot was reprobated with an IKK antibody, both endogenous and exogenous IKK could be detected. Although equivalent amounts of endogenous IKK bound to TP304, significantly less binding was detected to the C179A mutant compared with the wild-type protein (Fig. 6C). Furthermore, sodium arsenite, which binds covalently to Cys<sup>179</sup> (44), effectively competed for binding with TP304 to the IKK complex in the NeutrAvidin pull-down experiments (data not shown). These results show that sulfhydryl groups in IKK are the targets for modification by CDDO-Im and that interaction of CDDO-Im with Cys<sup>179</sup> may be responsible for inhibiting the kinase function of IKK $\beta$ .

## Discussion

We have examined the effects of a synthetic triterpenoid, CDDO-Im, on the NF- $\kappa$ B pathway and show for the first time that CDDO-Im is a direct and potent inhibitor of IKK $\beta$ , an upstream kinase responsible for activation of this pathway. Thus, CDDO-Im (a) inhibits IKK activity in cell culture, (b) prevents p65 nuclear translocation, (c) interacts with the IKK signalsome, and (d) inhibits the kinase activity of a recombinant active IKK $\beta$  enzyme *in vitro*. Furthermore, we have shown that Cys<sup>179</sup> on IKK $\beta$  is a target for modification by CDDO-Im.

What are the overall implications of the above findings? NF- $\kappa$ B is a transcriptional hub in cells, a central node that serves to coordinate both inflammatory and survival responses to a wide variety of stimuli and plays a critical role in tumor chemoresistance. There is major interest in developing new drugs that will regulate its activity (39, 40, 49), and we show here that CDDO-Im is a highly potent agent for this purpose. Because NF- $\kappa$ B is a survival factor for tumor cells, its inhibition by CDDO-Im may contribute to the potent proapoptotic effects of this compound. The mid to high nanomolar concentrations at which CDDO-Im inhibits IKK $\beta$  correlate with those typically required for this agent to induce apoptosis in most adherent tumor cell lines (26, 31). Not surprisingly, CDDO-Im and its analogues have previously been shown to down-regulate many NF- $\kappa$ B target genes (50, 51). CDDO-Im has also been shown to act synergistically with TNF-related apoptosis-inducing ligand in inducing apoptosis in human cancer cells by sensitizing TNF-related apoptosis-inducing ligand-resistant cells to TNF-related apoptosis-inducing ligand treatment. This sensitization occurs by promoting caspase-8 processing through down-regulation of its endogenous antiapoptotic antagonist protein, cellular FADD-like interleukin-1 $\beta$ -converting enzyme inhibitory protein (long), a well-defined NF- $\kappa$ B target (27). Consistent with these observations, CDDO-Im failed to induce apoptosis in RC-K8 lymphoma cells, which harbor a mutated *I $\kappa$ B $\alpha$*  gene, resulting in constitutively increased expression of p65/NF- $\kappa$ B (25). The concentrations required to inhibit IKK $\beta$  are higher than those required to inhibit

*de novo* transcription of inducible nitric oxide synthase and cyclooxygenase-2 by CDDO-Im and its synthetic triterpenoid analogues. Whereas high concentrations of triterpenoids can inhibit NF- $\kappa$ B and contribute to their anti-inflammatory activities, at lower concentrations it is more likely that these anti-inflammatory activities occur through mechanisms similar to those responsible for phase 2 enzyme induction. This hypothesis is substantiated by recent results showing a close correlation between phase 2 gene induction and suppression of inducible nitric oxide synthase synthesis (22, 23).

While this manuscript was in preparation, it was reported that the methyl ester of CDDO (CDDO-Me) also inhibited IKK (51). However, in these experiments, the authors concluded that CDDO-Me targets a protein upstream of IKK rather than IKK itself, resulting in loss of IKK activation and subsequent kinase activity. This conclusion was based on the inability of CDDO-Me to inhibit an active IKK protein in their *in vitro* kinase assay; however, the buffer in their assay contained 2 mmol/L DTT, which is a thousand fold excess over the concentration of CDDO-Me used. We have repeatedly found that high concentrations of DTT and 2-mercaptoethanol in assay buffers sequester triterpenoids and, thus, effectively antagonize their activity. Furthermore, recent reports show that CDDO and its analogues form Michael adducts with both protein and nonprotein thiols (22, 31, 47). In our experiments, similar concentrations of CDDO-Im were required to inhibit IKK $\beta$  *in vitro* and *in vivo*. However, if we added excess DTT into our *in vitro* kinase buffer, much greater concentrations of CDDO-Im were required to inhibit the kinase activity of IKK $\beta$ .

Due to the electrophilicity of CDDO-Im and its relatives and their diverse array of physiologic effects, they may have multiple targets, which may involve reactive cellular nucleophiles, particularly cysteine. Previous work has shown that interaction with specific cysteine residues on Keap1 is responsible for induction of the cytoprotective Keap1/Nrf2 pathway by electrophilic compounds (52). CDDO-Im exhibits biphasic dose responses; it is cytoprotective at low concentrations and apoptotic at higher concentrations. Induction of the Keap1/Nrf2 pathway is required for the antioxidant/cytoprotective effects of this compound (23). At higher concentrations, CDDO-Im may bind to reactive cysteine residues on other target proteins, such as IKK, to induce apoptosis. Interestingly, all the direct targets of CDDO-Im and its analogues identified to date, including PPAR $\gamma$ , Keap1, and  $\beta$ -tubulin, contain reactive cysteine residues, which play a critical role in the cellular function of the respective proteins (4, 18, 22). In our current studies, we have identified Cys<sup>179</sup> on IKK as a critical target of CDDO-Im. This cysteine residue has a role as a target for many of the electrophilic compounds, such as arsenic and the cyclopentenone prostaglandins, which inactivate IKK. Overall, the inhibition of IKK $\beta$  by CDDO-Im may have important pharmacologic implications because it is the  $\beta$  isoform of IKK that is predominantly involved in inflammation and antiapoptosis (35, 36, 53–55).

For CDDO-Im, IKK is the first direct target identified to date. Due to the ability of this compound to undergo Michael addition with reactive sulfhydryl groups, CDDO-Im most likely interacts with a network of protein sulfhydryl targets to elicit its pharmacologic effects. To identify the full spectrum of targets with which this clinically promising compound interacts with, broad-scale proteomic assays have been initiated.

## Note Added In Proof

After this article was submitted, another report appeared that also showed inhibition of IKK by a different synthetic TRITERPENOID (56).

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## References

- Honda T, Rounds BV, Gribble GW, et al. Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* 1998;8:2711–4.
- Honda T, Rounds BV, Bore L, et al. Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *J Med Chem* 2000;43:4233–46.
- Place AE, Suh N, Williams CR, et al. The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth *in vivo*. *Clin Cancer Res* 2003;9:2798–806.
- Couch RD, Ganem NJ, Zhou M, et al. 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid disrupts microtubule polymerization: a possible mechanism contributing to apoptosis. *Mol Pharmacol* 2006;69:1158–65.
- Ikeda T, Nakata Y, Kimura F, et al. Induction of redox imbalance and apoptosis in multiple myeloma cells by the novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid. *Mol Cancer Ther* 2004;3:39–45.
- Ito Y, Pandey P, Sporn MB, et al. The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol Pharmacol* 2001;59:1094–9.
- Kim KB, Lotan R, Yue P, et al. Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate, that potentially induces caspase-mediated apoptosis in human lung cancer cells. *Mol Cancer Ther* 2002;1:177–84.
- Konopleva M, Elstner E, McQueen TJ, et al. Peroxisome proliferator-activated receptor  $\gamma$  and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias. *Mol Cancer Ther* 2004;3:1249–62.
- Konopleva M, Tsao T, Estrov Z, et al. The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-dependent and -independent apoptosis in acute myelogenous leukemia. *Cancer Res* 2004;64:7927–35.
- Konopleva M, Contractor R, Kurinna SM, et al. The novel triterpenoid CDDO-Me suppresses MAPK pathways and promotes p38 activation in acute myeloid leukemia cells. *Leukemia* 2005;19:1350–4.
- Konopleva M, Zhang W, Shi YX, et al. Synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest in HER2-overexpressing breast cancer cells. *Mol Cancer Ther* 2006;5:317–28.
- Pedersen IM, Kitada S, Schimmer A, et al. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood* 2002;100:2965–72.
- Samudio I, Konopleva M, Pelicano H, et al. A novel mechanism of action of methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate: direct permeabilization of the inner mitochondrial membrane to inhibit electron transport and induce apoptosis. *Mol Pharmacol* 2006;69:1182–93.

14. Stadheim TA, Suh N, Ganju N, Sporn MB, Eastman A. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* 2002;277:16448–55.
15. Suh N, Wang Y, Honda T, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res* 1999;59:336–41.
16. Suh N, Roberts AB, Birkey RS, et al. Synthetic triterpenoids enhance transforming growth factor  $\beta$ /Smad signaling. *Cancer Res* 2003;63:1371–6.
17. Suh WS, Kim YS, Schimmer AD, et al. Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. *Leukemia* 2003;17:2122–9.
18. Wang Y, Porter WW, Suh N, et al. A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor  $\gamma$ . *Mol Endocrinol* 2000;14:1550–6.
19. Yue P, Zhou Z, Khuri FR, Sun SY. Depletion of intracellular glutathione contributes to JNK-mediated death receptor 5 upregulation and apoptosis induction by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me). *Cancer Biol Ther* 2006;5:492–7.
20. Konopleva M, Tsao T, Ruvolo P, et al. Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. *Blood* 2002;99:326–35.
21. Honda T, Honda Y, Favaloro FG Jr, et al. A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bioorg Med Chem Lett* 2002;12:1027–30.
22. Dinkova-Kostova AT, Liby KT, Stephenson KK, et al. Extremely potent triterpenoid inducers of the phase 2 response: correlations of protection against oxidant and inflammatory stress. *Proc Natl Acad Sci U S A* 2005;102:4584–9.
23. Liby K, Hock T, Yore MM, et al. The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer Res* 2005;65:4789–98.
24. Yates MS, Kwak MK, Egner PA, et al. Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole. *Cancer Res* 2006;66:2488–94.
25. Chauhan D, Li G, Podar K, et al. The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance. *Blood* 2004;103:3158–66.
26. Chintharlapalli S, Papineni S, Konopleva M, et al. 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells through peroxisome proliferator-activated receptor  $\gamma$ -dependent and -independent pathways. *Mol Pharmacol* 2005;68:119–28.
27. Hyer ML, Croxton R, Krajewska M, et al. Synthetic triterpenoids cooperate with tumor necrosis factor-related apoptosis-inducing ligand to induce apoptosis of breast cancer cells. *Cancer Res* 2005;65:4799–808.
28. Ikeda T, Sporn M, Honda T, Gribble GW, Kufe D. The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. *Cancer Res* 2003;63:5551–8.
29. Ikeda T, Kimura F, Nakata Y, et al. Triterpenoid CDDO-Im down-regulates PML/RAR $\alpha$  expression in acute promyelocytic leukemia cells. *Cell Death Differ* 2005;12:523–31.
30. Liby K, Voong N, Williams CR, et al. The synthetic triterpenoid CDDO-imidazolide suppresses STAT phosphorylation and induces apoptosis in myeloma and lung cancer cells. *Clin Cancer Res* 2006;12:4288–93.
31. Samudio I, Konopleva M, Hail N, Jr., et al. 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) directly targets mitochondrial glutathione to induce apoptosis in pancreatic cancer. *J Biol Chem* 2005;280:36273–82.
32. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 1986;46:705–16.
33. Richmond A. NF- $\kappa$ B, chemokine gene transcription and tumour growth. *Nat Rev Immunol* 2002;2:664–74.
34. Arlt A, Schafer H. NF- $\kappa$ B-dependent chemoresistance in solid tumors. *Int J Clin Pharmacol Ther* 2002;40:336–47.
35. Greten FR, Eckmann L, Greten TF, et al. IKK $\beta$  links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004;118:285–96.
36. Karin M, Delhase M. The I $\kappa$ B kinase (IKK) and NF- $\kappa$ B: key elements of proinflammatory signalling. *Semin Immunol* 2000;12:85–98.
37. Karin M. Nuclear factor- $\kappa$ B in cancer development and progression. *Nature* 2006;441:431–6.
38. Karin M. NF- $\kappa$ B and cancer: mechanisms and targets. *Mol Carcinog* 2006;45:355–61.
39. Luo JL, Kamata H, Karin M. IKK/NF- $\kappa$ B signaling: balancing life and death—a new approach to cancer therapy. *J Clin Invest* 2005;115:2625–32.
40. Nakanishi C, Toi M. Nuclear factor- $\kappa$ B inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005;5:297–309.
41. Honda T, Janosik T, Honda Y, et al. Design, synthesis, and biological evaluation of biotin conjugates of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid for the isolation of the protein targets. *J Med Chem* 2004;47:4923–32.
42. Suh N, Honda T, Finlay HJ, et al. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res* 1998;58:717–23.
43. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor  $\kappa$ B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. *J Biol Chem* 2001;276:32008–15.
44. Kapahi P, Takahashi T, Natoli G, et al. Inhibition of NF- $\kappa$ B activation by arsenite through reaction with a critical cysteine in the activation loop of I $\kappa$ B kinase. *J Biol Chem* 2000;275:36062–6.
45. Rossi A, Kapahi P, Natoli G, et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of I $\kappa$ B kinase. *Nature* 2000;403:103–8.
46. Kwok BH, Koh B, Ndubuisi MI, Elofsson M, Crews CM. The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits I $\kappa$ B kinase. *Chem Biol* 2001;8:759–66.
47. Couch RD, Browning RG, Honda T, et al. Studies on the reactivity of CDDO, a promising new chemopreventive and chemotherapeutic agent: implications for a molecular mechanism of action. *Bioorg Med Chem Lett* 2005;15:2215–9.
48. Kim JR, Yoon HW, Kwon KS, Lee SR, Rhee SG. Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 2000;283:214–21.
49. Karin M, Yamamoto Y, Wang QM. The IKK NF- $\kappa$ B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17–26.
50. Han SS, Peng L, Chung ST, et al. CDDO-imidazolide inhibits growth and survival of c-Myc-induced mouse B cell and plasma cell neoplasms. *Mol Cancer* 2006;5:22.
51. Shishodia S, Sethi G, Konopleva M, Andreeff M, Aggarwal BB. A synthetic triterpenoid, CDDO-Me, inhibits I $\kappa$ B $\alpha$  kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor  $\kappa$ B-regulated gene products in human leukemic cells. *Clin Cancer Res* 2006;12:1828–38.
52. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A* 2002;99:11908–13.
53. Li ZW, Chu W, Hu Y, et al. The IKK $\beta$  subunit of I $\kappa$ B kinase (IKK) is essential for nuclear factor  $\kappa$ B activation and prevention of apoptosis. *J Exp Med* 1999;189:1839–45.
54. Li ZW, Omori SA, Labuda T, Karin M, Rickert RC. IKK $\beta$  is required for peripheral B cell survival and proliferation. *J Immunol* 2003;170:4630–7.
55. Lawrence T, Bebiën M, Liu GY, Nizet V, Karin M. IKK $\alpha$  limits macrophage NF- $\kappa$ B activation and contributes to the resolution of inflammation. *Nature* 2005;434:1138–43.
56. Ahmad R, Raina D, Meyer C, Kharbanda S, Kufe D. Triterpenoid CDDO-Me blocks the NF- $\kappa$ B pathway by direct inhibition of IKK $\beta$  on Cys-179. *J Biol Chem*. In press, 2006.

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## The synthetic triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole blocks nuclear factor- $\kappa$ B activation through direct inhibition of I $\kappa$ B kinase $\beta$

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