A Synthetic Triterpenoid, CDDO-Me, Inhibits IκBα Kinase and Enhances Apoptosis Induced by TNF and Chemotherapeutic Agents through Down-Regulation of Expression of Nuclear Factor κB – Regulated Gene Products in Human Leukemic Cells

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Abstract The C-28 methyl ester of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO-Me), a synthetic triterpenoid based on naturally occurring ursolic and oleanolic acids, induces apoptosis in tumor cells, induces differentiation, and inhibits inflammatory response through a poorly understood mechanism. Because the nuclear transcription factor nuclear factor κB (NF-κB) has been shown to suppress apoptosis and promote proliferation and is linked with inflammation and differentiation, we postulated that CDDO-Me modulates NF-κB activity and NF-κB-regulated gene expression. Using human leukemia cell lines and patient samples, we show that CDDO-Me potently inhibits both constitutive and inducible NF-κB activated by tumor necrosis factor (TNF), interleukin (IL)-1β, phorbol ester, okadaic acid, hydrogen peroxide, lipopolysaccharide, and cigarette smoke. CDDO-Me was more potent than CDDO and its imidazole derivative. NF-κB suppression occurred through inhibition of IκBα kinase activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-mediated reporter gene transcription. This inhibition correlated with suppression of NF-κB-dependent genes involved in antiapoptosis (*IAP2, cFLIP, TRAF1, survivin*, and *bcl-2*), proliferation (*cyclin d1* and c-*myc*), and angiogenesis (*VEGF, cox-2*, and *mmp-9*). CDDO-Me also potentiated the cytotoxic effects of TNF and chemotherapeutic agents. Overall, our results suggest that CDDO-Me inhibits NF-κB through inhibition of IκBα kinase, leading to the suppression of expression of NF-κB-regulated gene products and enhancement of apoptosis induced by TNF and chemotherapeutic agents.

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Hematologic malignancies including leukemia, lymphoma, and myeloma account for ~10% of cancer deaths in the United States (1). The proliferation, apoptosis, and differentiation of these cells are influenced by cell signaling pathways triggered by growth factors and cytokines. The transcription factor nuclear factor κB (NF-κB) has been shown to be constitutively active in most leukemic cells and implicated in regulating the proliferation, survival, and differentiation of these cells (2–8).

Under normal conditions, NF-κB, consisting of p50, p65, and IκBα, is localized in the cytoplasm. However, when activated, this transcription factor translocates to the nucleus. In response to an activation signal, the inhibitory IκBα subunit undergoes phosphorylation, ubiquitination, and degradation, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to its nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription (9, 10). NF-κB has been shown to regulate the expression of a number of genes of which products are involved in tumorigenesis (10–14). These include antiapoptotic genes (e.g., *ciap*, *survivin*, *traf*, *cflip*, *bfl-1*, *bcl-2*, and *bcl-xl*), inflammatory genes (*cox-2*, *mmp-9*, and *VEGF*), and genes encoding adhesion molecules, chemokines, and cell cycle regulatory genes (e.g., *cyclin d1* and c-*myc*). Thus, agents that suppress NF-κB activation have therapeutic potential for leukemia and lymphoma (2–6, 8, 15).

Triterpenoids together with their close relatives, the steroids, are members of the cyclosqualenoid family (16). Natural triterpenoids are synthesized by plants as a part of their defense mechanism for regulation of physiologic processes. They have

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been used as traditional Asian medicine for centuries. The oleanolic and ursolic acids are natural triterpenoids that have been shown to possess significant anti-inflammatory and anticarcinogenic properties (17–19). Because the biological activities of some of the natural triterpenoids are relatively weak, new analogues of these molecules have been synthesized in an attempt to identify more potent agents. 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), a novel synthetic triterpenoid derived from oleanolic acid, has been shown to be more potent antitumor and anti-inflammatory agent than its natural analogues (20). The C-28 methyl ester of CDDO, CDDO-Me, has been shown to decrease the viability of leukemic cell lines, including multidrug resistance 1–overexpressing cells, and it was found to be more active than CDDO (21). 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) is another CDDO analogue more potent than its parent compound, CDDO, both *in vitro* and *in vivo* (20). Synthetic triterpenoids have been shown to induce proapoptotic Bax protein, inhibit the activation of extracellular signal–regulated kinase 1/2, block Bcl-2 phosphorylation (21), and down-regulate Flice-like inhibitory protein (FLIP; ref. 22). Synthetic triterpenoids also enhance transforming growth factor β/Smad signaling (23, 24).

The antitumorigenic, antiangiogenic, and proapoptotic effects, combined with the ability to suppress the expression of cyclooxygenase 2 (COX-2), inducible nitric oxide synthase, multidrug resistance gene 1, and FLIP, suggest that CDDO-Me mediates its effects through suppression of NF-κB. But whether CDDO-Me mediates its effects through modulation of NF-κB is not understood. In the current study, we examined the effect of CDDO-Me on NF-κB activation induced by various carcinogens in a large variety of tumor cell lines and in primary blast cells from leukemia patients. The results presented here show that CDDO-Me inhibits activation of NF-κB through suppression of IκBα kinase activation in these cells. This triterpenoid also inhibited the expression of antiapoptosis, proliferative, and angiogenesis genes, all known to be regulated by NF-κB.

Materials and Methods

Materials. CDDO, CDDO-Me, and CDDO-Im were synthesized by Dr. T. Honda at Dartmouth Medical College (25) and provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) through the Rapid Access to Intervention Development Program and by Dr. Michael Sporn (Dartmouth School of Medicine, Dartmouth, NH). They were dissolved in DMSO as a 10 mmol/L stock solution and stored at −20°C. Bacteria-derived human tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640, fetal bovine serum, and Lipofectamine 2000 were obtained from Invitrogen (Grand Island, NY). FuGENE 6 was purchased from Roche (Nutley, NJ). Cisplatin and Taxol were purchased from Sigma-Aldrich Co. (St. Louis, MO). The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65, against the epitope corresponding to amino acids mapping within the NH_2-terminal domain of human NF-κB p65; anti-p50, against a peptide 15 amino acids long, mapping at the nuclear localization sequence region of NF-κB p50; anti-IκBα, against amino acids 297-317, mapping at the COOH terminus of IκBα/MAD-3; anti-c-Rel; anti-cyclin D1, against amino acids 1 to 295, which represents full-length cyclin D1 of human origin; anti–matrix metalloproteinase (MMP)-9; anti–polyadenosine ribose polymerase; anti–inhibitor of apoptosis protein 2 (IAP2); anti-Bcl-2; and anti–TNF receptor–associated factor 1 (TRAF1). Anti-COX-2 and anti-MMP-9 antibodies were obtained from BD Biosciences (San Diego, CA) and phosphospecific anti-IκBα (Ser32) and phospho-p65 (Ser536) antibody from Cell Signaling (Beverly, MA). Anti–IκBα kinase α (IKKα) and anti-IKKβ antibodies were kindly provided by Imgenex (San Diego, CA).

Cell lines. The cell lines used in our studies included chronic myelogenous leukemia (KBM-5), human monocytic leukemia (U937), human metastatic melanoma (A375), human lymphoblastic leukemia (Jurkat), human non–small-cell lung carcinoma (H1299), human multiple myeloma (U266 and MM1), human head and neck cancer (HN5 and SCC4), and human non-Hodgkin's lymphoma (HDMYZ, HDLM2, and L428). The non-Hodgkin's lymphoma cells were kindly provided by Dr. Anas Younes (M.D. Anderson Cancer Center, Houston, TX). All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified DMEM with 15% fetal bovine serum, and all other cell lines were cultured in RPMI 1640 with 10% fetal bovine serum. All media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin.

NF-κB activation. To determine NF-κB activation by TNF, we examined the NF-κB-DNA binding by electrophoretic mobility shift assay essentially as previously described (26). Briefly, nuclear extracts prepared from treated cells (1×10^6/mL) were incubated with ^{32}P-end-labeled, 45-mer, double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites), for 30 minutes at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC**CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 minutes at 37°C before the complex was analyzed by electrophoretic mobility shift assay. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantitated using Imagequant software (Amersham, Piscataway, NJ).

Western blot analysis. To determine the effect of CDDO-Me on TNF-dependent IκBα phosphorylation, IκBα degradation, p65 translocation, and p65 phosphorylation, Western blotting was done as previously described (19). Briefly, cytoplasmic or nuclear extracts were prepared and fractionated by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by enhanced chemiluminescence reagent. The bands obtained were quantitated using NIH Image (NIH, Bethesda, MD).

Immunolocalization of NF-κB p65. The effect of CDDO-Me on TNF-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) as previously described (19).

IKK assay. To determine the effect of CDDO-Me on TNF-induced IKK activation, we analyzed IKK by a method essentially as previously described (19). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKKα and IKKβ and then treated with protein A/G-Sepharose beads (Pierce, Rockford, IL). After 2 hours, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L $MgCl_2$, 2 mmol/L DTT, 20 μCi [γ-^{32}P]ATP, 10 μmol/L unlabeled ATP, and 2 μg of substrate glutathione *S*-transferase-IκBα (amino acids 1-54). After incubation at 30°C for 30 minutes, the reaction was terminated by boiling with SDS sample buffer for 5 minutes. Finally,

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the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of whole-cell proteins were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibody.

NF-κB-dependent reporter gene transcription. The effect of CDDO-Me on TNF-induced NF-κB dependent reporter gene transcription in A293 cells was measured as previously described (19). Briefly, A293 cells (5×10^5/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMVFLAG1 DNA for 24 hours. We then treated the cells for 8 hours with CDDO-Me and then stimulated them with 0.1 nmol/L TNF. The cell culture medium was harvested after 24 hours of TNF treatment. Culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA) using a Victor 3 microplate reader (Perkin-Elmer, Boston, MA).

Table 1. Clinical data for patient samples with AML

Patient no.	Diagnosis	Source	PB-Blast%	BM-blast%	WBC	FAB	Cytogenetics	Status
1	AML	BM	11	57	1.1	OD	Diploid	Primary refractory
2	AML	BM	39	61	3.1	M1	Diploid	Relapsed
3	APL	BM	0% blasts, 82% promyelocytes	7% blasts, 80% promyelocytes	58.3	M3	Pseudodiploid clone 46,XY,t(15;17) (q22;q21) [8] Diploid [11]	New
4	AML	PB	66		1.9	MI	Hypodiploid clone 45,XY,der(12)t(12;17) (p13;q11.2),-7[20]	Primary refractory
5	AML	BM	74	85	94.5	M5B	Pseudodiploid clone 46,XY,t(6;11) (q27;q23) [20]	Relapsed
6	AML	BM	73	95	87.8	OD	Insufficient yield of analyzable metaphases Ph+clone 46,XY,t(2;7) (p21;p22),t(9;22) (q34;q11.2), t(15;17) (q22;q23), del(20) (q11.2) [5]	Relapsed refractory
7	AML	BM	88	93	38.9	M4	Diploid	New
8	AML	BM	78	93	52.5	M4	Pseudodiploid clone 46,XX,del(9) (q22q33) [19]	Relapsed
9	AML	BM	71.8	62	20	M1	Hypodiploid metaphase 45,XX,-7[1],Two pseudodiploid clones 46,XX,-7,+mar[5], 46,XX,t(4;15) (p14;q24), -7,+mar[6],45-46,XX, -7,t(4;15) (p14;q24), -7,-15,+0-5mar[cp8]	Relapsed refractory
10	AML	BM	84	84	16	M1	Diploid	New
11	AML	BM	78	94	6.2	M1	Two hyperdiploid clones 47,XY,+mar[8],48,XY, +8,add(21) (p11.2), +add(21) (p11.2) [9], Diploid[3]	Relapsed refractory
12	AML	BM	94	90	82	M5a	Diploid	New
13	AML	BM	79	83	121.7	OD	Diploid	Relapsed (2nd relapse)

Abbreviations: Ph, pheresis; PB, peripheral blood; BM, bone marrow; WBC, white blood cells count; FAB, French-American-British; OD, outside diagnosis.

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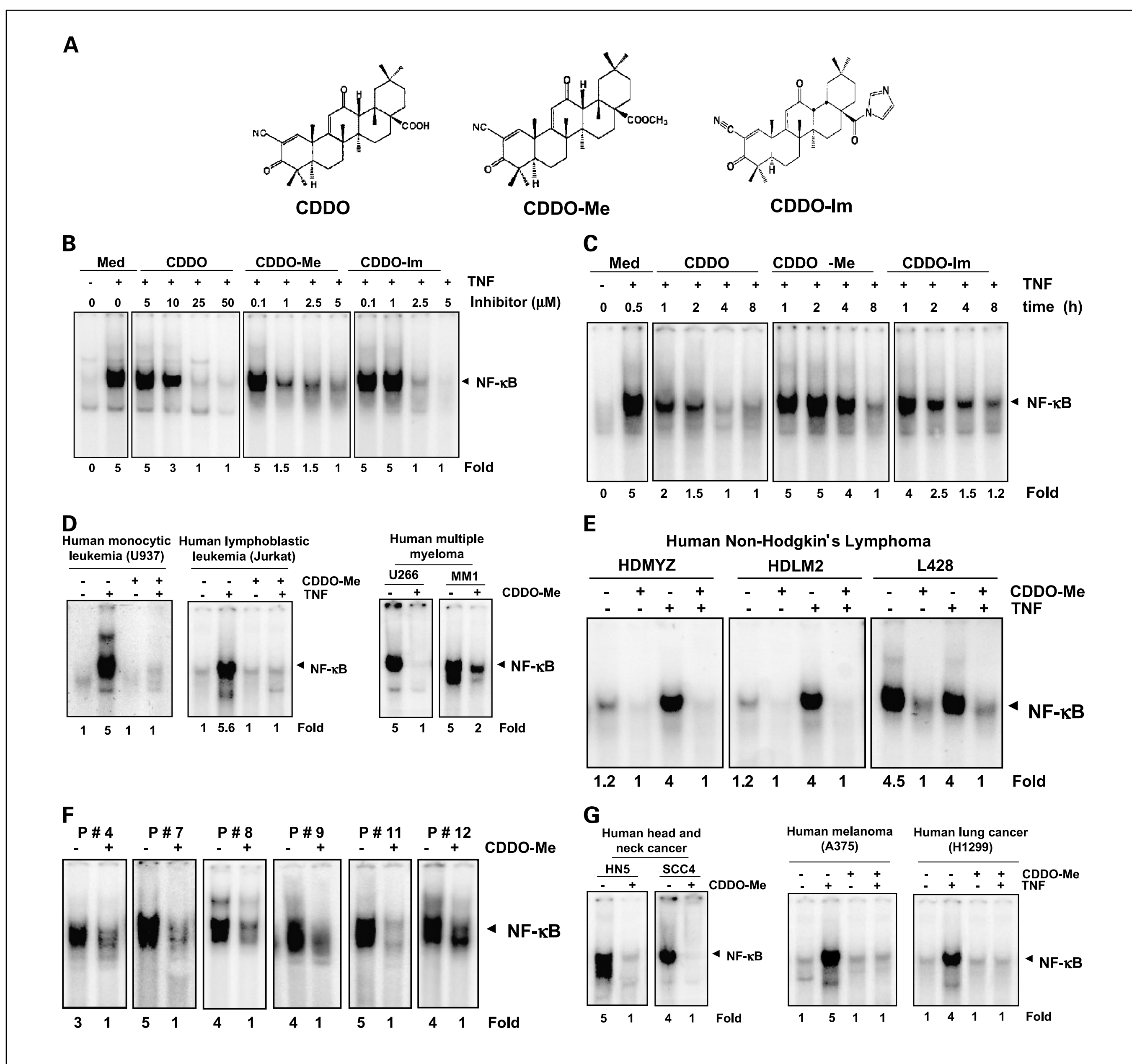

Fig. 1. *A,* structure of CDDO, CDDO-Me, and CDDO-Im. *B,* CDDO, CDDO-Me, and CDDO-Im inhibit TNF-dependent NF-κB activation in a dose-dependent manner. KBM-5 cells (2×10^6/mL) were preincubated with the indicated concentrations of CDDO, CDDO-Me, or CDDO-Im for 8 hours at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. *C,* CDDO, CDDO-Me, and CDDO-Im inhibit TNF-dependent NF-κB activation in a time-dependent manner. KBM-5 cells (2×10^6/mL) were preincubated with CDDO (25 μmol/L), CDDO-Me (1 μmol/L), and CDDO-Im (2.5 μmol/L) for the indicated times at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. *D,* CDDO-Me suppresses NF-κB activation in a non cell-type specific manner. Two million U937, Jurkat, U266, or MM1 cells were pretreated with 1 μmol/L CDDO-Me for 8 hours and then U937 and Jurkat cells were treated with 0.1 nmol/L TNF for 30 minutes as indicated. The nuclear extracts were then prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *E,* two million human non-Hodgkin's lymphoma cells were exposed to 1 μmol/L CDDO-Me for 8 hours, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *F,* two million blast cells from AML patients were exposed to 1 μmol/L CDDO-Me for 8 hours, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *G,* two million HN5, SCC4, A375, or H1299 cells were pretreated with 1 μmol/L CDDO-Me for 8 hours and then A375 and H1299 cells were treated with 0.1 nmol/L TNF for 30 minutes as indicated, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods.

COX-2 promoter–dependent reporter luciferase gene expression. To determine the effect of CDDO-Me on *cox-2* promoter activity, a *cox-2* reporter assay was done as previously described (27). Briefly, A293 cells were seeded at a concentration of 1.5×10^5 per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg of DNA consisting of *COX-2* promoter-luciferase reporter plasmid, along with FuGENE6 reagent, according to the protocol of the manufacturer. After a 24-hour exposure to the transfection mixture, the cells were incubated in medium containing CDDO-Me for 12 hours. The cells were exposed to TNF (0.1 nmol/L) for 24 hours and then harvested. Luciferase activity was measured by using the Luclite (Perkin-Elmer) luciferase assay system according to the protocol of the

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manufacturer and detected with a luminometer (Victor 3, Perkin-Elmer). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

Cytotoxicity assay. Cytotoxicity was assayed by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (28). Briefly, 5,000 cells were treated in triplicate in 96-well plates at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was then added to each well. After a 2-hour incubation at 37°C, extraction buffer (20% SDS, 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and the absorbance was then measured at 570 nm using a 96-well multiscanner (Dynex Technologies, MRX Revelation, Chantilly, VA).

Polyadenosine ribose polymerase cleavage assay. For detection of cleavage products of polyadenosine ribose polymerase, whole-cell extracts were prepared by subjecting CDDO-Me-treated cells to lysis in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 μg/mL aprotinin, 0.005 μg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L $NaVO_4$]. Lysates were spun at 14,000 rpm for 10 minutes to remove insoluble material, resolved by 10% SDS-PAGE, and probed with polyadenosine ribose polymerase antibodies.

Live and dead assay. To measure apoptosis, we used the Live and Dead assay (Molecular Probes, Carlsbad, CA) as previously described (28). Briefly, 1×10^5 cells were incubated with 1 μmol/L CDDO-Me, alone or in combination with 1 nmol/L TNF, for 16 hours at 37°C. Cells were stained with the Live and Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcein-AM) and then incubated at 37°C for 30 minutes. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon).

Results

We investigated the effect of a synthetic triterpenoid, CDDO, and its methyl ester and imidazole analogues on the NF-κB-regulated gene products and the NF-κB signaling pathway in a variety of leukemia cell lines and primary blast cells from patients with acute myelogenous leukemia (AML; see Table 1), as well as in other tumor cell lines. Although all the derivatives of CDDO tested (Fig. 1A) were effective in suppressing NF-κB activation, CDDO-Me was the most effective among the three; thus, we examined the effect of this particular compound on NF-κB signaling pathway. The concentration of CDDO-Me used and the duration of exposure for DNA-binding and Western blot analysis had minimal effect on the viability of these cells as determined by trypan blue dye exclusion test (data not shown). To determine the mechanism of action of CDDO-Me, human chronic myelogenous leukemia KBM-5 cells were used. We used TNF to activate NF-κB because the NF-κB signaling pathway activated by TNF is well understood.

CDDO, CDDO-Me, and CDDO-Im block NF-κB activation in a dose- and time-dependent manner. We first examined all the three compounds for their effectiveness in blocking TNF-induced NF-κB activation. As shown by electrophoretic gel shift mobility assay, all of them inhibited NF-κB activation in a dose- and time-dependent manner; however, CDDO-Me was the most potent among the three (Fig. 1B and C). At a dose of 1 μmol/L, CDDO-Me inhibited >90% of NF-κB activation, whereas at this concentration, CDDO and its imidazole analogues were minimally active (Fig. 1B). None of these synthetic triterpenoids activated NF-κB by themselves. Although 25 μmol/L CDDO was sufficient to inhibit NF-κB activation within 1 hour, CDDO-Me required only 1 μmol/L, the lowest among the three. Therefore, we used CDDO-Me for the remainder of the study.

Inhibition of inducible NF-κB activation by CDDO-Me is not cell type specific. It has been reported that the mechanism of NF-κB induction varies among different cell types (29); thus, we examined whether CDDO-Me was effective in blocking NF-κB activation in five human cell lines from a variety of tumors: monocytic leukemia (U937), lymphoblastic leukemia (Jurkat), non-Hodgkin's lymphoma, melanoma (A375), and non–small-cell lung carcinoma (H1299). CDDO-Me completely inhibited TNF-induced NF-κB activation in all five cell lines [Fig. 1D (*left*), 1E (*left* and *middle*), and 1G (*right*)]. Thus, CDDO-Me was effective in inhibiting TNF-inducible NF-κB in a wide variety of tumor types.

CDDO-Me inhibits constitutive NF-κB activation. It has been reported that NF-κB is constitutively active in a wide variety of tumor cells (7). We therefore examined the effect of CDDO-Me in a number of tumor cells where NF-κB is known to be constitutively active. CDDO-Me inhibited the constitutive NF-κB activation in human multiple myeloma (U266 and MM1, Fig. 1D, *right*), non-Hodgkin's lymphoma (L428, Fig. 1E), and human head and neck squamous cell carcinoma cell lines (Fig. 1G, *left*). CDDO-Me also inhibited constitutively active NF-κB in blast cells from AML patients (Fig. 1F). Thus, the NF-κB inhibitory activity of CDDO-Me was not cell type specific.

CDDO-Me blocks NF-κB activation induced by various agents. A wide variety of cytokines, tumor promoters, oxidative stress inducers, and carcinogens have been shown to induce the activation of NF-κB through mechanisms that differ. We therefore examined the effect of CDDO-Me on the activation of NF-κB induced by cigarette smoke condensate, phorbol myristate acetate, okadaic acid, H_2O_2, lipopolysaccharide, IL-1β, and TNF in KBM-5 cells. DNA-binding assay (electrophoretic mobility shift assay) showed that CDDO-Me suppressed the NF-κB activation induced by all these agents (Fig. 2A). These results suggest that CDDO-Me acted at a step in the NF-κB activation pathway that is common to all these agents.

Suppressed NF-κB consists of both p50 and p65. The NF-κB family of proteins comprises five members that combine in different sets to exert a wide variety of effects. The most predominant combination involved in tumorigenesis is a heterodimer of p50(NF-κB1) and p65(RelA). When nuclear extracts from TNF-activated cells were incubated with antibodies to p50 and p65 subunits of NF-κB, the resulting bands were shifted to higher molecular masses (Fig. 2B), suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum did not have any effect. Addition of excess unlabeled NF-κB (cold oligo; 100-fold) caused complete disappearance of the band whereas mutated oligo had no effect on the DNA-binding. These results suggest that the NF-κB combination suppressed by CDDO-Me was composed of the p50 and p65 subunits.

CDDO-Me does not directly affect binding of NF-κB to the DNA. Because some NF-κB inhibitors, including *N*-tosyl-L-phenylalanine chloromethyl ketone (the serine protease inhibitor), herbimycin A (protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester, directly modify NF-κB to suppress its DNA binding (30–32), we investigated whether CDDO-Me

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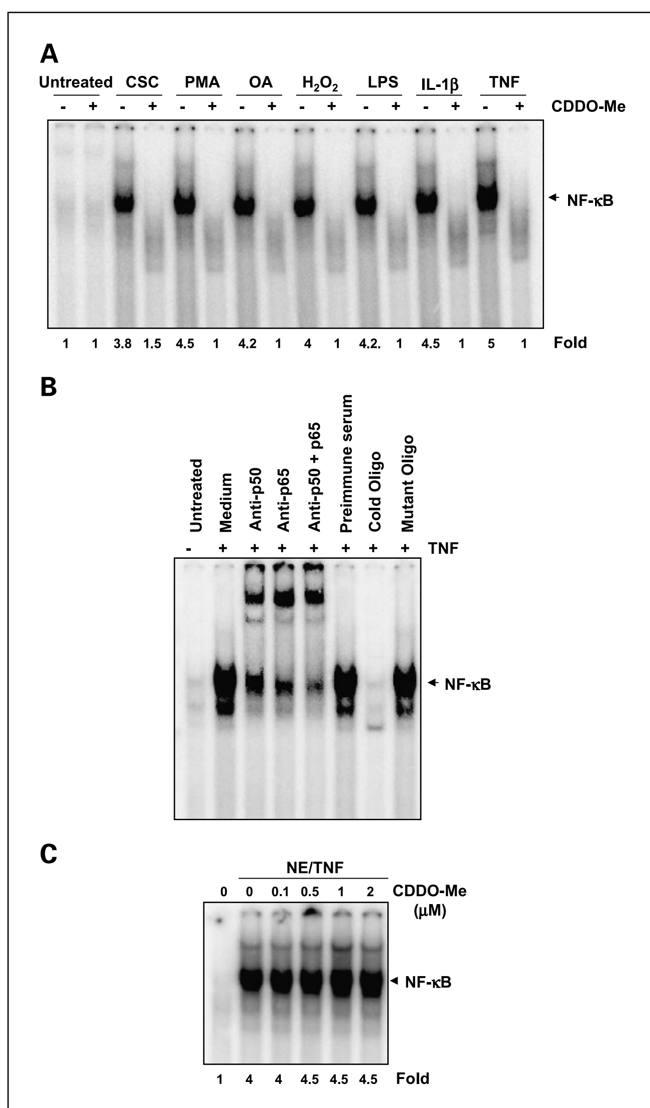

Fig. 2. *A,* CDDO-Me blocks NF-κB activation induced by cigarette smoke condensate (*CSC*), phorbol myristate acetate (*PMA*), okadaic acid (*OA*), H_2O_2, lipopolysaccharide (*LPS*), IL-1β, and TNF. KBM-5 cells (2×10^6/mL) were preincubated for 8 hours at 37°C with 1 μmol/L CDDO-Me and then treated with cigarette smoke condensate (10 μg/mL, 30 minutes), phorbol myristate acetate (100 ng/mL, 1 hour), okadaic acid (500 nmol/L, 4 hours), H_2O_2 (250 μmol/L, 1 hour), lipopolysaccharide (10 μg/mL), IL-1β (100 ng/mL), or TNF (0.1 nmol/L). Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. *B,* TNF-induced NF-κB consists of p50 and p65 subunits. Nuclear extracts from KBM-5 cells (2×10^6/mL), treated or not treated with 0.1 nmol/L TNF for 30 minutes, were incubated with the antibodies indicated for 30 minutes at room temperature, and the complex was analyzed by supershift assay. *C,* CDDO-Me does not modulate the ability of NF-κB to bind to the DNA. Nuclear extracts from KBM-5 cells (2×10^6/mL), treated or not treated with 0.1 nmol/L TNF for 30 minutes, were treated with the indicated concentrations of CDDO-Me for 2 hours at room temperature and then assayed for DNA binding by electrophoretic mobility shift assay.

also followed a similar mechanism. We did an *in vitro* NF-κB-DNA-binding assay in the presence of various concentrations of CDDO-Me. The dose of CDDO-Me used in our experiments did not directly modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells; thus, CDDO-Me seemed to inhibit NF-κB activation by a mechanism other than direct binding (Fig. 2C).

CDDO-Me inhibits TNF-induced IKK activation. Activation of IKK has been shown to be critical for TNF-induced NF-κB activation. As shown in Fig. 3A (*top*), CDDO-Me completely suppressed TNF-induced activation of IKK. Neither TNF nor CDDO-Me had any direct effect on the expression of IKK proteins (Fig. 3A, *middle* and *bottom*).

Next, we examined whether CDDO-Me directly interacted with IKK to block its activity *in vitro*. An *in vitro* kinase assay of TNF-treated extracts in the presence of various concentrations of CDDO-Me showed that CDDO-Me did not directly interfere with the IKK activity (Fig. 3B). Because cellular treatment inhibits IKK activity, CDDO-Me could possibly block the upstream kinases that have been implicated in the activation of IKK.

CDDO-Me inhibits TNF-dependent IκBα phosphorylation. Because IκBα phosphorylation is required for NF-κB activation, we next determined whether CDDO-Me affected TNF-induced IκBα phosphorylation, another condition for NF-κB translocation. Western blot analysis using antibody that detects only the serine-phosphorylated form of IκBα indicated that CDDO-Me completely suppressed TNF-induced IκBα phosphorylation (Fig. 3C). Thus, CDDO-Me inhibited TNF-induced NF-κB activation by inhibiting phosphorylation of IκBα.

CDDO-Me inhibits TNF-dependent IκBα degradation. Because IκBα degradation is typically required for translocation of NF-κB to the nucleus (33), we determined whether inhibition of TNF-induced NF-κB activation by CDDO-Me was due to inhibition of IκBα degradation. We found that TNF induced IκBα degradation in control cells and CDDO-Me completely blocked TNF-induced IκBα degradation (Fig. 3D).

CDDO-Me inhibits TNF-induced phosphorylation of p65. Phosphorylation of p65 is also required for the transcriptional activity of NF-κB (34). Therefore, we also tested the effect of CDDO-Me on TNF-induced phosphorylation of p65. CDDO-Me almost completely suppressed the phosphorylation of p65 (Fig. 3E, *top*).

CDDO-Me inhibits TNF-induced nuclear translocation of p65. Western blot analysis of nuclear extracts (Fig. 3E, *middle*) and an immunocytochemical assay (Fig. 3F) indicated that CDDO-Me inhibited TNF-induced nuclear translocation of p65. We conclude that CDDO-Me inhibited the phosphorylation as well as the nuclear translocation of the p65 subunit of NF-κB.

CDDO-Me represses TNF-induced NF-κB-dependent reporter gene expression. Our results up to this point show that CDDO-Me inhibited the translocation of p65 into the nucleus. Because DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (35), we assayed NF-κB-dependent gene transcription using the SEAP reporter construct. We transiently transfected A293 cells with the NF-κB-regulated construct and then stimulated them with TNF. TNF produced an almost 18-fold increase in SEAP activity over vector control (Fig. 4A), which was abolished by dominant-negative IκBα, indicating specificity. When the cells were pretreated with CDDO-Me, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results show that CDDO-Me inhibits NF-κB-dependent reporter gene expression induced by TNF.

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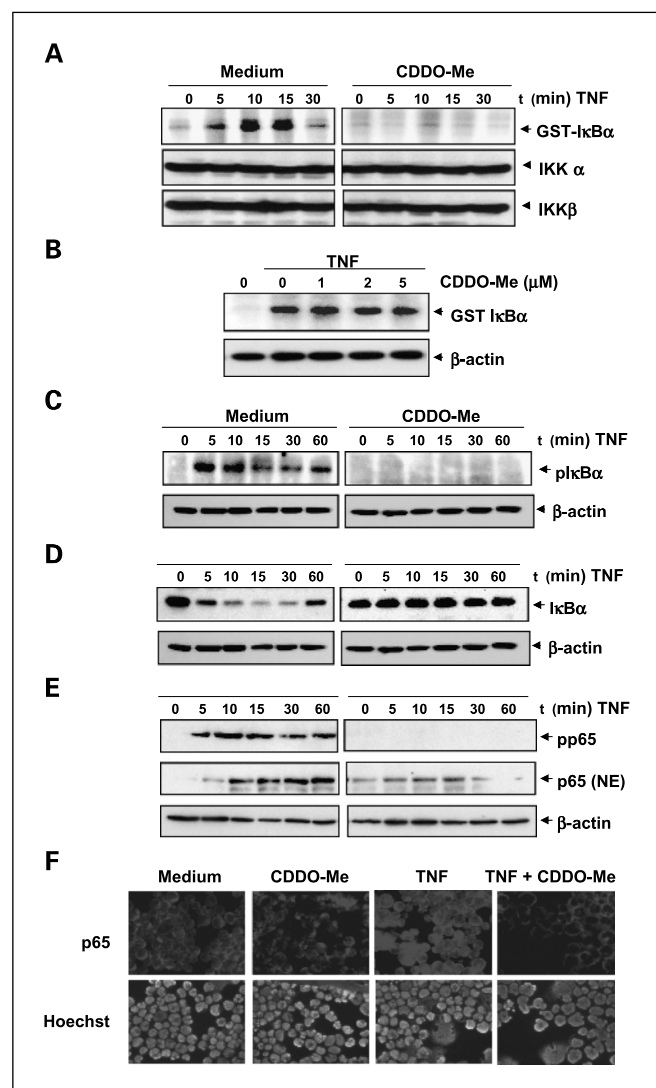

Fig. 3. *A,* CDDO-Me inhibits TNF-induced IκBα kinase activity. KBM-5 cells ($2 \times 10^6/10^6/10^6$/mL) were treated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared and 200 μg of extract were immunoprecipitated with antibodies against IKKα and IKKβ. Thereafter, immune complex kinase assay was done as described in Materials and Methods. To examine the effect of CDDO-Me on the level of expression of IKK proteins, 30 μg of whole-cell extract were run on 10% SDS-PAGE, electrotransferred, and immunoblotted with indicated antibodies as described in Materials and Methods. *B,* CDDO-Me has no direct effect on IKK activity. Whole-cell extracts were prepared from untreated and TNF (0.1 nmol/L) – treated KBM-5 cells (2×10^6/mL); 200 μg of protein/sample whole-cell extract were immunoprecipitated with antibodies against IKKα and IKKβ. The immune complex was treated with the indicated concentrations of CDDO-Me for 30 minutes at 30°C, and then a kinase assay was done as described in Materials and Methods. Equal protein loading was evaluated by IKKβ. *C,* CDDO-Me inhibits TNF-induced phosphorylation of IκBα. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for phosphorylated IκBα in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (*bottom*). *D,* CDDO-Me inhibits TNF-induced degradation of IκBα. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for IκBα degradation in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (*bottom*). *E,* CDDO-Me inhibits TNF-induced phosphorylation and translocation of p65. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for the indicated times. The cytoplasmic extracts were analyzed by Western blotting using antibodies against the phosphorylated form of p65 (*top*) and nuclear extracts were analyzed using antibodies against p65 (*middle*). β-Actin was examined to show equal protein loading. *F,* CDDO-Me inhibits TNF-induced nuclear translocation of p65. KBM-5 cells (1×10^6/mL) were first treated with 1 μmol/L CDDO-Me for 8 hours at 37°C and then exposed to 0.1 nmol/L TNF for 30 minutes. After cytospin, immunocytochemical analysis was done as described in Materials and Methods.

CDDO-Me represses NF-κB-dependent reporter gene expression induced by TNF receptor 1, TNF receptor–asociated death domain, TRAF2, NF-κB-inducing kinase, and IKK. We next determined where CDDO-Me acts in the sequence of TNF receptor 1, TNF receptor–asociated death domain (TRADD), TRAF2, NF-κB-inducing kinase (NIK), and IKK recruitment that characterizes TNF-induced NF-κB activation (36, 37). In cells transfected with TNF receptor 1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, NF-κB-dependent reporter gene expression was induced; CDDO-Me suppressed SEAP expression in all cells except those transfected with p65 (Fig. 4B). Because IKK activation can cause the phosphorylation of IκBα and p65, we suggest that CDDO-Me inhibits NF-κB activation through inhibition of IKK.

CDDO-Me represses TNF-induced COX-2 promoter activity. COX-2 promoter has been shown to be regulated by NF-κB (38); thus, we next determined the effect of CDDO-Me on NF-κB-regulated COX-2 promoter activity. As shown in Fig. 4C, CDDO-Me abolished the TNF-induced COX-2 promoter activity in a dose-dependent manner.

CDDO-Me inhibits TNF-induced COX-2, MMP-9, and vascular endothelial growth factor expression. COX-2, MMP-9, and vascular endothelial growth factor (VEGF) are known to be regulated by NF-κB (38–40); thus, the effect of CDDO-Me on the expression of these NF-κB-regulated genes was also examined. TNF treatment induced the expression of COX-2, MMP-9, and VEGF gene products and CDDO-Me abolished the TNF-induced expression of these gene products (Fig. 5A).

CDDO-Me inhibits TNF-induced cyclin D1 and c-myc expression. Both c-myc and cyclin D1 regulate cellular proliferation and are regulated by NF-κB (41). Whether CDDO-Me controls the expression of these gene products was also examined. Our results show that CDDO-Me abolished in a dose-dependent fashion the TNF-induced expression of cyclin D1 and c-myc (Fig. 5B).

CDDO-Me inhibits TNF-induced activation of antiapoptotic gene products. NF-κB up-regulates the expression of a number of genes implicated in facilitating tumor cell survival, including *cIAP2*, *cFLIP*, *TRAF1*, *survivin*, and *bcl-2* (42–50). We found that CDDO-Me inhibited the TNF-induced expression of all of these antiapoptotic proteins (Fig. 5C).

We next examined whether CDDO-Me inhibited the expression of these antiapoptotic proteins in blast cells derived from AML patients. We found that the expression of cIAP1, cFLIP, and Bcl-2 was down-regulated by CDDO-Me (Fig. 5D).

CDDO-Me potentiates the cytotoxic effects of TNF. Because NF-κB-regulated gene products suppress TNF-induced apoptosis (51), we examined the effects of CDDO-Me on the apoptotic effects of TNF. TNF by itself did not induce a significant amount of apoptosis; however, in combination with CDDO-Me, the cytotoxic effects of TNF were enhanced (Fig. 6A, *top left*). CDDO-Me also potentiated the caspase-induced cleavage of polyadenosine ribose polymerase activated by TNF (Fig. 6B).

We next determined the effect of CDDO-Me on TNF-induced cytotoxicity using the Live and Dead assay that determines intracellular esterase activity and plasma membrane integrity. Our result showed that CDDO-Me enhanced the cytotoxic effects of TNF (Fig. 6C).

CDDO-Me potentiates the cytotoxic effects of chemotherapeutic drugs. Chemotherapy-induced apoptosis has also been shown to be suppressed by NF-κB-regulated gene products

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(52); therefore, we examined the effects of CDDO-Me on the apoptotic effects of the chemotherapeutic drugs 5-fluorouracil, Taxol, and doxorubicin. CDDO-Me enhanced the cytotoxic effects of all three (Fig. 6A).

CDDO-Me suppresses NF-κB activation induced by chemotherapeutic drugs. To further determine whether CDDO-Me potentiates the cytotoxic effects of chemotherapeutic drugs through the suppression of NF-κB, we examined the effect of CDDO-Me on cisplatin, doxorubicin, and Taxol-induced NF-κB activation in KBM-5 cells. We found that all the three chemotherapeutic agents activated NF-κB and CDDO-Me pretreatment suppressed the activation (Fig. 6D). Our observations suggest that CDDO-Me potentiated the apoptotic effects of chemotherapeutic agents most likely through the suppression of NF-κB activation.

Discussion

The aim of this study was to determine the effect of CDDO-Me on NF-κB and NF-κB-regulated gene products involved in cellular survival, proliferation, differentiation, and inflammation. We found that CDDO-Me is a potent inhibitor of both constitutive and inducible NF-κB activation. The suppression of NF-κB occurred through inhibition of activation of IκBα kinase. The triterpenoid also suppressed NF-κB activated by TNF receptor 1, TRADD, TRAF2, NIK, and IKK and NF-κB-dependent gene products that mediate cell survival (IAP2, cFLIP, TRAF1, survivin, and Bcl-2), cell proliferation (cyclin D1, COX-2, and c-myc), and angiogenesis (VEGF and MMP-9). We believe that these effects on the NF-κB pathway account for the ability of the molecule to potentiate the cytotoxic effects of TNF and chemotherapeutic agents.

Our results show that CDDO-Me is more active than CDDO or CDDO-Im in suppression of NF-κB activation. These results are in agreement with other reports (23, 53) that both CDDO-Me and CDDO-Im exhibit higher activity than CDDO in suppression of lipopolysaccharide and IFN-γ-induced nitric oxide production. Our results are also consistent with those of Kim et al. (54) who found that CDDO-Me was more potent than CDDO in decreasing the survival of human non–small-cell lung carcinoma cells. Interestingly, Wang et al. (55) showed that CDDO is a peroxisome proliferator–activated receptor γ agonist in adipocytes but CDDO-Me is a peroxisome proliferator–activated receptor γ antagonist. However, we recently showed agonistic effects of all three compounds, with CDDO-Me and CDDO-Im being the most active in transactivation assays and in recruiting coactivators to peroxisome proliferator–activated receptor γ (56).

Several leukemia, lymphoma, myeloma, and epithelial tumors have been shown to have constitutively active NF-κB (2, 4, 7, 57, 58). Our results indicate that CDDO-Me suppresses NF-κB activation in human leukemia (U937 and Jurkat) cells, multiple myeloma (U266 and MM1) cells, non-Hodgkin's lymphoma (HDMYZ, HDLM2, and L428) and epithelial cell carcinomas of the head and neck, lung cancer, and melanoma cells. Primary blast cells from AML patients had constitutively active NF-κB that was also suppressed by CDDO-Me.

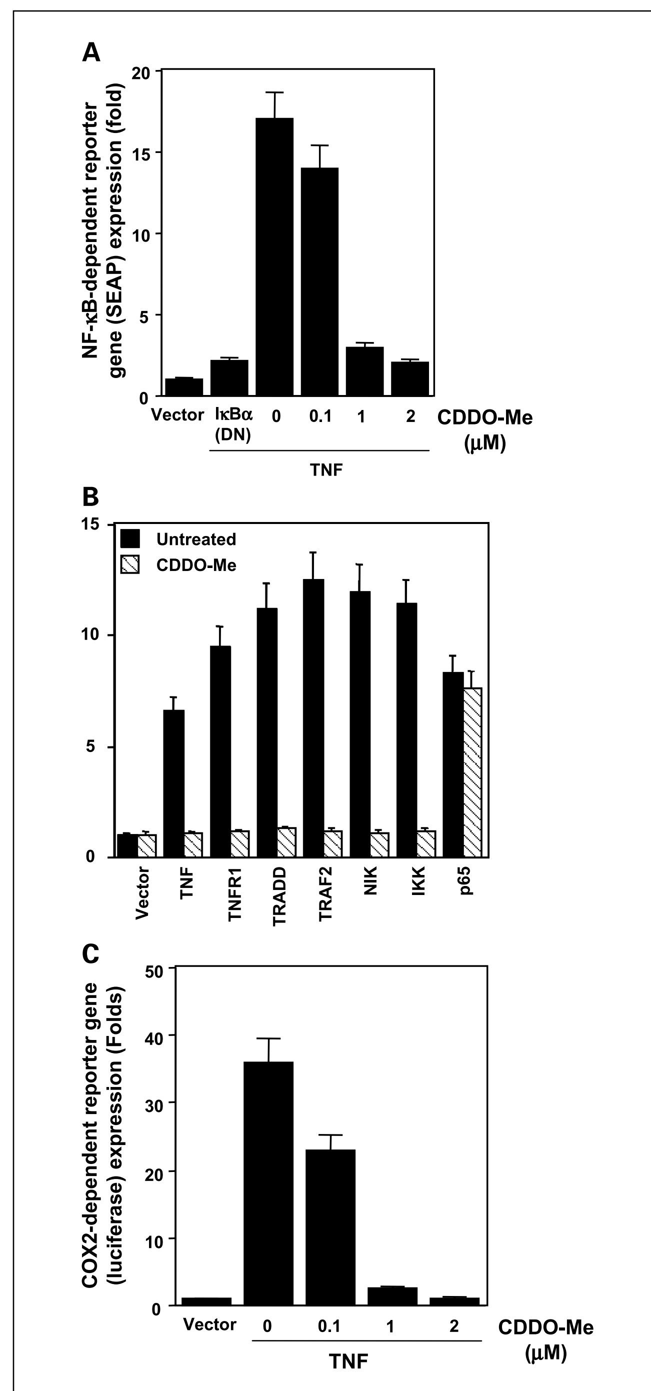

Fig. 4. *A,* CDDO-Me inhibits TNF-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with an NF-κB-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of CDDO-Me for 8 hours. After 12 hours in culture with 0.1 nmol/L TNF, cell supernatants were collected and assayed for SEAP activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. *B,* CDDO-Me inhibits NF-κB-dependent reporter gene expression induced by TNF receptor 1 (*TNFR1*), TRADD, TRAF2, NIK, and IKKβ. A293 cells were transiently transfected with the indicated plasmids along with an NF-κB-containing plasmid linked to the SEAP gene and then left either untreated or treated with 1 μmol/L CDDO-Me for 24 hours. Cell supernatants were assayed for secreted alkaline phosphatase activity as described in Materials and Methods. Columns, fold activity over the activity of the vector control; bars, SD. *C,* CDDO-Me inhibits TNF-induced COX-2 promoter activity. A293 cells were transiently transfected with a COX-2 promoter plasmid linked to the luciferase gene and then treated with the indicated concentrations of CDDO-Me. After 24 hours in culture with 0.1 nmol/L TNF, cell supernatants were collected and assayed for luciferase activity as described in Materials and Methods. Columns, fold activity over the activity of the vector control.

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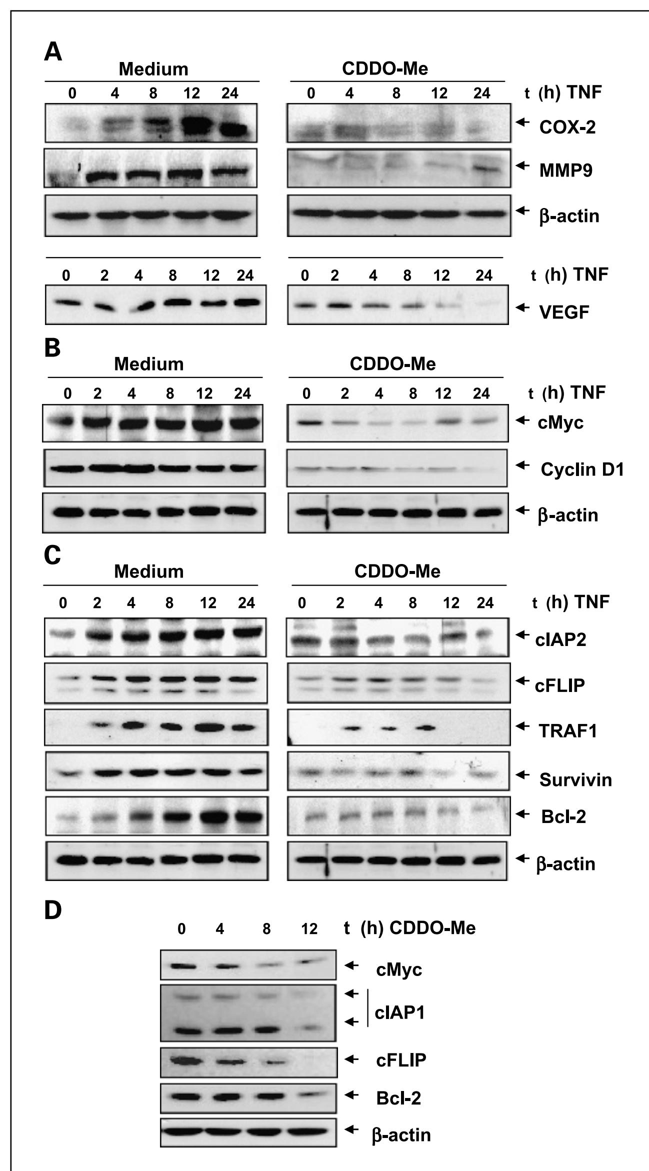

Fig. 5. *A,* CDDO-Me inhibits MMP-9, COX-2, and VEGF expression induced by TNF. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against VEGF, MMP-9, and COX-2. *B,* CDDO-Me inhibits cyclin D1 and c-myc expression induced by TNF. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against cyclin D1 and c-myc. *C,* CDDO-Me inhibits the expression of antiapoptotic gene products cIAP2, cFLIP, TRAF1, survivin, and Bcl-2. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against proteins as indicated. *D,* CDDO-Me inhibits the expression of antiapoptotic gene products cIAP1, cFLIP, and Bcl-2 in blast cells from AML patients. AML blast cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for different time points as indicated. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against proteins as indicated.

Besides suppressing activation of constitutively expressed NF-κB, CDDO-Me also suppressed NF-κB activation induced by carcinogens, tumor promoters, and inflammatory stimuli in leukemia cells. Because the pathway through which these various agents activate NF-κB may vary, these results suggest that CDDO-Me must act at a common step. We found that CDDO-Me suppressed the TNF-induced phosphorylation and degradation of IκBα, leading to inhibition of p65 translocation into the nucleus. Our results differ from that of Stadheim et al. (59) who showed that CDDO did not affect the TNF-induced phosphorylation and degradation of IκBα or nuclear p65 translocation. Their results, however, suggest that CDDO inhibits NF-κB-dependent IκBα resynthesis at a level downstream of p65(RelA) accumulation in the nucleus by exerting a destabilizing effect on specific mRNA transcripts. In their study, authors did not investigate the effect of CDDO on TNF-induced NF-κB DNA binding or on the NF-κB reporter assays. Whether

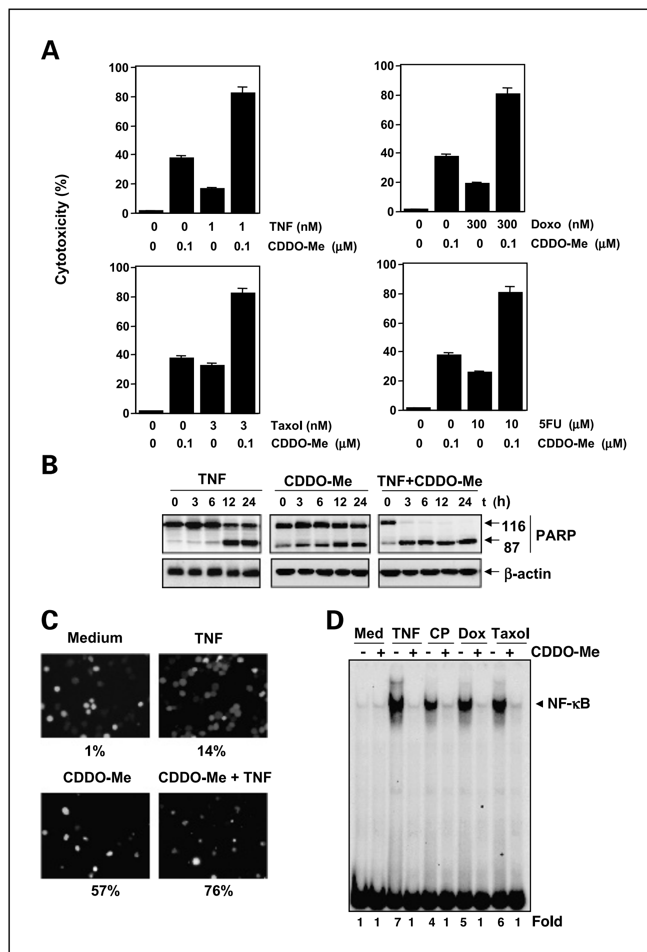

Fig. 6. CDDO-Me enhances apoptosis induced by TNF and chemotherapeutic agents. *A,* KBM-5 cells (5,000/0.1 mL) were incubated at 37°C with TNF, Taxol, 5-fluorouracil (*5-FU*), or doxorubicin in the presence and absence of 0.1 μmol/L CDDO-Me, as indicated, for 72-hour duration, and the viable cells were assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Columns, mean cytotoxicity from triplicate cultures; bars, SD. *B,* KBM-5 cells (2×10^6/mL) were serum starved for 24 hours and then incubated with TNF (1 nmol/L), alone or in combination with CDDO-Me (1 μmol/L), for the indicated times and polyadenosine ribose polymerase (*PARP*) cleavage was determined by Western blot analysis as described in Materials and Methods. *C,* KBM-5 cells (2×10^6/mL) were serum starved for 24 hours and then incubated with TNF (1 nmol/L), alone or in combination with CDDO-Me (0.1 μmol/L), as indicated for 24 hours. Cell death was determined by calcein-AM – based Live and Dead assay as described in Materials and Methods. *D,* KBM-5 cells (2×10^6/mL) were preincubated with CDDO-Me (1 μmol/L) for 8 hours and then treated with chemotherapeutic agents, cisplatin (*CP*; 20 μg/mL, 4 hours), doxorubicin (*Dox*; 50 μmol/L, 4 hours), and Taxol (50 μmol/L, 16 hours) at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation.

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the differences in results are due to cell type, dose, or time of exposure to CDDO is unclear.

We found that CDDO-Me also inhibited TNF-induced activation of IKK. Our *in vitro* kinase assay results show that CDDO-Me is not a direct inhibitor of IKK. Thus, it seems that CDDO-Me blocks the activation of IKK indirectly by interfering with some upstream regulatory kinases; this possibility requires further investigation. Akt, NIK, mitogen-activated protein kinase kinase kinase 1, and atypical protein kinase C are the potential candidates because they are some of the upstream kinases that regulate IKK (60–62).

Besides NF-κB activation, the NF-κB reporter activity induced by TNF, TNF receptor 1, TRADD, TRAF2, NIK, and IKK was also abrogated by CDDO-Me. The NF-κB reporter activity induced by p65, however, was unaffected by CDDO-Me, suggesting that this agent acts upstream to p65. NF-κB activation leads to the expression of genes that are involved in the proliferation and metastasis of cancer (13, 14, 40). In this report, we show that CDDO-Me inhibits the expression of cyclin D1 and c-myc, which are both regulated by NF-κB. Our results are also consistent with a recent report by Lapillonne et al. (63) who showed that CDDO down-regulates cyclin D1 and Bcl-2.

Our results also show that the expression of COX-2, MMP-9, and VEGF, also known to be regulated by NF-κB, is down-regulated by CDDO-Me. Our observations are consistent with previous reports that CDDO suppresses the abilities of various inflammatory cytokines, such as IFNγ, IL-1, and TNF, to induce *de novo* formation of the enzymes inducible nitric oxide synthase and inducible COX-2 in mouse peritoneal macrophages, rat brain microglia, and human colon fibroblasts (20). CDDO inhibits IL-1-induced MMP-1 and MMP-13 expression in human chondrocytes. CDDO also inhibits the expression of Bcl-3, an IL-1-responsive gene that preferentially contributes to MMP-1 gene expression (64). These results further imply that CDDO-Me exercises its anticancer properties through the inhibition of NF-κB.

NF-κB is known to regulate the expression of IAP1, X-linked IAP, Bfl-1/A1, TRAF1, Bcl-2, cFLIP, and survivin, and their overexpression in numerous tumors has been linked to tumor cell survival, chemoresistance, and radioresistance. Our results indicate that CDDO-Me treatment down-regulates all of these gene products. CDDO-Me has been shown to inhibit the growth of wide variety of tumor cells including leukemic cells and non–small-cell lung carcinoma cells (21, 54, 65). This inhibition of growth may be mediated through down-regulation of various genes as shown here. Down-regulation of cFLIP by CDDO-Me as described here is consistent with a previous report in AML described using CDDO (22).

We found that CDDO-Me also suppressed the NF-κB activation induced by the chemotherapeutic drugs, cisplatin, doxorubicin, and Taxol, and potentiated apoptosis. The down-regulation of various antiapoptotic gene products by CDDO-Me also sensitized the cells to the apoptotic effects of TNF. The sensitization of leukemic and lung cancer cell lines to TRAIL by CDDO (22, 66) may also be due to down-regulation of various gene products as reported here. Overall, our results indicate that apoptotic, antiproliferative, anti-invasive, anti-angiogenic, antimetastatic, and anti-inflammatory activities assigned to CDDO-Me may be mediated through suppression of NF-κB and NF-κB-regulated gene products.

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