c-Jun NH2-Terminal Kinase-Mediated Up-regulation of Death Receptor 5 Contributes to Induction of Apoptosis by the Novel Synthetic Triterpenoid Methyl-2-Cyano-3,12-Dioxooleana-1,9-Dien-28-Oate in Human Lung Cancer Cells

Wei Zou,1 Xiangguo Liu,1 Ping Yue,1 Zhongmei Zhou,1 Michael B. Sporn,2 Reuben Lotan,3 Fadlo R. Khuri,1 and Shi-Yong Sun1

1Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia; 2Department of Pharmacology, Dartmouth Medical School, Hanover, New Hampshire; and 3Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

ABSTRACT

Death receptor (DR) 4 or 5, on binding to its ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), triggers apoptosis via activating the caspase-8–mediated caspase cascade. Certain anticancer drugs up-regulate the expression of these receptors and thereby induce apoptosis or enhance TRAIL-induced apoptosis. In this study, we explored the ability of methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) to activate the extrinsic DR-mediated apoptotic pathway in human lung cancer cells. We found that CDDO-Me not only activated caspase-8 but also induced expression of DRs, particularly DR5, in a p53-independent mechanism. Correspondingly, CDDO-Me augmented TRAIL-induced apoptosis in these cells regardless of p53 status as evidenced by enhanced DNA fragmentation and activation of caspase cascades, suggesting that CDDO-Me–induced DRs are functionally active. Moreover, silencing of DR5 expression using small interfering RNA suppressed apoptosis induced by CDDO-Me alone or by combination of CDDO-Me and TRAIL, indicating that DR5 up-regulation is required for induction of apoptosis by CDDO-Me and for enhancement of TRAIL-induced apoptosis by CDDO-Me. CDDO-Me rapidly activated c-Jun NH2-terminal kinase (JNK) before DR up-regulation and caspase-8 activation. Moreover, application of the JNK-specific inhibitor SP600125 blocked terminal kinase (JNK) before DR up-regulation and caspase-8 activation. INTRODUCTION

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors death receptor 4 (DR4, also called TRAIL-R1) and death receptor 5 (DR5, also named Apo2, TRAIL-R2, TRICK2, or Killer/DR5) are important new proapoptotic molecules that belong to the TNF receptor gene superfamily that is defined by similar, cysteine-rich extracellular domain and additional cytoplasmic death domain (1). They are located at the cell surface, become oligomerized (trimerized) on binding to their ligand TRAIL, and then transmit death signal to the TNF receptor gene superfamily which contains either no cytoplasmic death domain or a truncated death domain and can compete with DR4 and DR5 for ligand binding and thereby act as antagonists (1). TRAIL and its receptors DR4 and DR5 are expressed widely in normal and malignant cells, whereas DcR1 and DcR2 are expressed preferentially in many normal tissues but in only a few transformed cells (1). Therefore, it was initially suggested that TRAIL’s low toxicity toward normal tissues is caused by the expression of decoy receptors in normal tissues, which can protect normal cells from induction of apoptosis by TRAIL (1).

TRAIL also can bind to three decoy receptors, DcR1 (TRAIL-R3 or TRID), DcR2 (TRAIL-R4 or TRUNDD), and osteoprotegerin (OPG), that contain either no cytoplasmic death domain or a truncated death domain and can compete with DR4 and DR5 for ligand binding and thereby act as antagonists (1). TRAIL and its receptors DR4 and DR5 are expressed widely in normal and malignant cells, whereas DcR1 and DcR2 are expressed preferentially in many normal tissues but in only a few transformed cells (1). Therefore, it was initially suggested that TRAIL’s low toxicity toward normal tissues is caused by the expression of decoy receptors in normal tissues, which can protect normal cells from induction of apoptosis by TRAIL (1).

Many studies have shown that TRAIL-induced apoptosis can be augmented by certain types of anticancer agents in a variety of cancer types in vitro and in vivo (7–10). The mechanism underlying the augmentation of TRAIL-induced apoptosis by these agents is largely related to their ability to up-regulate the expression level of TRAIL receptors (i.e., DR4 and DR5). Therefore, agents that up-regulate the expression of TRAIL receptors DR4 and DR5 may have the potential for clinical management of cancer in combination with TRAIL through augmentation of TRAIL-induced apoptosis. Because DR4 and DR5 can be regulated by either a p53-dependent or -independent mechanism (11–16), it is possible that augmentation of TRAIL-induced apoptosis by anticancer agents may be either p53 dependent or p53 independent.

Triterpenoids, biosynthesized in plants by the cyclization of squa- lene, are used for medicinal purpose in many Asian countries, and some of them were reported to have anticarcinogenic activity (17–20). Because of the relatively weak biological activities of the natural triterpenoids, new analogs of these molecules were synthesized in an attempt to identify more potent agents (21–23). One of these analogs is methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), which was found to induce apoptosis in human lung cancer cells and other types of cancer cells (24–26). Because CDDO-Me has the potential as a cancer therapeutic agent, we examined the mechanism by which it induces apoptosis. We previously have shown that...
CDDO-Me induces apoptosis in human lung cancer cells by activating the cytochrome c/caspase-9–mediated signaling pathway (24). Other reports indicate that CDDO-Me and its analog CDDO induce a caspase-8–dependent apoptosis (26–30). Moreover, these agents also were reported to enhance apoptosis caused by death ligands, including TNF and TRAIL (30, 31). However, the mechanisms underlying induction of apoptosis and activation of caspase-8, as well as enhancement of TRAIL-induced apoptosis, by these novel triterpenoids are largely unclear.

In the present study, we, for the first time, showed that CDDO-Me activated the caspase-8 pathway in human lung cancer cells and that this effect is required for CDDO-Me–induced apoptosis. Furthermore, we found that CDDO-Me induced a p53-independent expression of DR5 and DR4 and enhanced TRAIL-induced apoptosis. Importantly, we showed that CDDO-Me activates the c-Jun NH2-terminal kinase (JNK) pathway and that this effect contributes to DR up-regulation, caspase-8 activation, and induction of apoptosis by CDDO-Me. Silencing of DR5 expression using siRNA attenuated CDDO-Me–induced apoptosis. Collectively, our results show a novel DR5-mediated mechanism underlying CDDO-Me–induced apoptosis in human lung cancer cells.

**MATERIALS AND METHODS**

**Reagents.** CDDO-Me was synthesized at Dartmouth College (Hanover, NH) (22). It was dissolved in DMSO at a concentration of 10 mmol/L, and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Soluble recombinant human TRAIL and the specific JNK inhibitor SP600125 were purchased from Biomek (Plymouth Meeting, PA). Recombinant soluble human DR2/Fc and OPG/Fc were purchased from Alexis Biochemicals (San Diego, CA). The caspase inhibitors CBZ-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), CBZ-Asp-Glu-Val-Ala-Asp-fluoromethyl ketone (z-DEVD-fmk), Z-Ille-Glu(OMe)-Thr-Asp(OMe)-fl-oxoromethyl ketone (z-IETD-fmk), and Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone (z-LEHD-fmk) were purchased from Enzyme System Products (Livermore, CA).

**Cell Lines and Cell Cultures.** Human non–small-cell lung carcinoma cell lines H460, A549, and H1944, which possess wild-type p53, and H522, H157, and H1792, which express mutant or no p53 (32), were purchased from the American Type Culture Collection (Manassas, VA). These cells were grown in BEGM BulletKit (Clonetics) at 37°C in 5% CO2 and 95% air.

**Human Lung Cancer Cell Lines.** H1792, which express mutant p53, and H522, H157, and H1792, which express mutant or no p53 (32), were purchased from American Type Culture Collection (Manassas, VA). These cells were grown in BEGM BulletKit (Clonetics) at 37°C in 5% CO2 and 95% air.

**Northern Blot Analysis.** Whole cellular RNA was prepared, and Northern blot analysis was performed as described previously (33). Thirty micrograms of total RNA were loaded in each lane. Human DR5 cRNA was obtained from Dr. W. S. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, PA). Human DR4 cDNA was purchased from Alexis Biochemicals, and 18S rRNA cDNA was purchased from Ambion, Inc. (Austin, TX).

**Western Blot Analysis.** Whole-cell lysates were prepared for Western blot analysis as described previously (34). Thirty micrograms of whole-cell lysates were electrophoresed through 7.5% to 12% denaturing polyacrylamide slab gels and transferred to a Hybond enhanced chemiluminescence membrane (Amersham, Piscataway, NJ) by electrophoretic analysis. The blots were probed or reprobed with the primary antibodies, and then second antibody binding was detected using the enhanced chemiluminescence system (Amersham) in accordance with the manufacturer’s instructions. Mouse monoclonal anti–caspase-3 and anti–DR4 and rabbit polyclonal anti–DR5 antibodies were purchased from IMGENEX (San Diego, CA). Rabbit polyclonal anti–caspase-9, anti–caspase-6, anti–poly(ADP-ribose) polymerase (PARP), anti–c–Jun, anti–ATF2, anti–phospho (p)-c–Jun (Ser63), and anti–phospho (p)-ATF2 (Thr21) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti–caspase-8 and rabbit polyclonal anti–DFF45 were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti–Bid antibody was purchased from Trevigen (Gaithersburg, MD). Mouse monoclonal anti–RIP and anti–caspase-7 antibodies were purchased from Pharmingen (San Diego, CA). Rabbit polyclonal anti–β-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO).

**Detection of Cell Surface DR4 and DR5 Using Flow Cytometry.** The procedure for direct antibody staining and subsequent flow cytometric analysis for cell surface protein was described previously (35). The mean fluorescence intensity that represents antigenic density on a per cell basis was used to represent the DR4 or DR5 expression. Phycocerythrin (PE)-conjugated mouse anti-human DR4 monoclonal antibody (D1R1), PE mouse antihuman DR5 monoclonal antibody (D1R2–4), and PE mouse IgGl isotype control (MOPC-21/P3) were purchased from eBioscience (San Diego, CA).

**Detection of Apoptosis.** Apoptosis was primarily evaluated by measuring cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) formed during apoptosis using a Cell Death Detection ELISA plus kit (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer’s instructions. We also measured the sub-G1 population as another indicator of apoptosis as described previously (36).

**Silencing of DR5 Expression Using Small Interfering RNA.** The target sequence of DR5 small interfering RNA (siRNA) was 5′-AAGACCCCTTTGT-GCTGTTTGTGTC-3′, which was described previously (37). The target sequence of control (nonsilencing) siRNA was 5′-AATTCTCCGAACGTGTCACGT-3′. These siRNAs were ordered from Qiagen (Valencia, CA). The transfection of siRNA was conducted in a 24-well plate (1 μg/well) using RNAiFect transfect reagent purchased from Qiagen following the manufacturer’s instructions. The cells were either harvested for preparation of whole-cell lysates and Western blot analysis after 72 hours to detect DR5 expression or reseeded in a 96-well plate for additional treatment with CDDO-Me on the second day to detect apoptosis. After a given time, cells in the 96-well plate exposed to different treatments then were subjected to detection of apoptosis using a Cell Death Detection ELISA plus kit as described previously.

**RESULTS**

**CDDO-Me Activates Caspase-8–Mediated Apoptotic Signaling Pathway.** To determine whether CDDO-Me activates the caspase-8–mediated apoptotic signaling pathway, we treated H460 and A549 cells with increasing concentrations of CDDO-Me (0.25 to 1.0 μmol/L) for 15 hours and harvested floating and attached cells for preparation of whole-cell lysates. Using Western blot analysis, we found that CDDO-Me decreased the procaspase-8 levels and increased the active (cleaved) caspase-8 levels in a concentration-dependent manner (Fig. 1A). Bid and RIP, two known substrates of caspase-8 (38–40), were activated or cleaved as indicated by the decreases in their expression levels (Fig. 1A) during CDDO-Me treatment. Thus, it appears that CDDO-Me activates caspase-8 and its mediated downstream apoptotic events. Additional experiments showed that the activation of caspase-8 could be detected as early as 9 hours in H460 cells and at 3 hours in H157 cells, respectively, after exposure to CDDO-Me (Fig. 1B), indicating that caspase-8 activation is an early event in CDDO-Me–induced apoptosis. We also noted that the activation of caspase-8 by CDDO-Me was more apparent in H460 and H157 cells than in A549 cells (Fig. 1A), which corresponds to our previous finding that H460 and H157 cells were more sensitive than A549 cells to CDDO-Me–induced apoptosis (24).

Furthermore, we determined whether caspase-8 activation is required for CDDO-Me–induced apoptosis by examining the ability of CDDO-Me to induce apoptosis in the presence of the caspase-8 inhibitor z-IETD-fmk. As the dose of z-IETD-fmk increased, the level of CDDO-Me–induced DNA fragments dramatically decreased (Fig. 1C), indicating that z-IETD-fmk suppresses CDDO-Me–induced apoptosis. This result shows that caspase-8 activation is required for induction of apoptosis by CDDO-Me.

Because CDDO-Me caused Bid cleavage (Fig. 1A), we speculated that caspase-8 activation resulted in caspase-9 activation via a Bid-mediated mechanism. Therefore, we further examined whether the
caspace-8 inhibitor z-IETD-fmk blocked CDDO-Me–induced caspace-9 activation. As shown in Fig. 1D, z-IETD-fmk, as expected, decreased level of the cleaved form of caspace-8 induced by CDDO-Me, indicating that it suppresses CDDO-Me–induced caspace-8 activation. Correspondingly, we detected the cleaved form of caspace-9 in cells treated with CDDO-Me alone but not in cells treated with CDDO-Me in the presence of z-IETD-fmk, indicating that z-IETD-fmk blocks CDDO-Me–induced caspace-9 activation. Collectively, these results show that CDDO-Me induces caspace-8 activation upstream of caspace-9 activation.

CDDO-Me Induces DR5 and DR4 Expression. It is known that caspace-8 plays a central role in apoptosis mediated by death receptors, such as Fas, DR4, and DR5 (40–43). Therefore, we wished to determine whether CDDO-Me could affect the expression of these death receptors. We first examined the effects of CDDO-Me on the expression of Fas, DR4, and DR5 in H460 and A549 cells, both of which have wild-type p53 (32). CDDO-Me increased Fas mRNA levels in the A549 cells but not in the H460 cells. This increase was weak at 6 hours and reached a peak at 18 hours (data not shown). When we expanded this examination to more lung cancer cell lines, including H157, H522, H292, and H1944 cells, we failed to see such an effect on Fas expression (data not shown). However, CDDO-Me induced DR5 and DR4 expression more rapidly in H460 and A549 cells, with peak levels detected at 12 hours (Fig. 2A). Considering a late and limited effect of CDDO-Me on Fas expression, we then focused our analyses on DR4 and DR5 expression in the subsequent experiments.

Because DR4 and DR5 can be regulated by p53 (11, 12, 14), we wanted to identify any relationship between DR4 and DR5 expression and p53 status. To do so, we evaluated the effects of CDDO-Me on DR4 and DR5 expression in more lung cancer cell lines with different p53 status. CDDO-Me increased the expression levels of DR4 and DR5 mRNAs in the four additionally tested cell lines (H157, H1792, H522, and H1944) regardless of p53 status (Fig. 2). Collectively, these results suggest that CDDO-Me induced a p53-independent expression of DR4 and DR5 in human lung cancer cells.

It should be noted that DR5 induction generally was more apparent than DR4 up-regulation by CDDO-Me in all of the tested lung cancer cell lines (Fig. 2A). This suggests that DR5 may play a more important role than DR4 in mediating CDDO-Me–induced apoptosis if they are involved in CDDO-Me–induced apoptosis. By examining the expression of DR4 and DR5 at the protein level, we found that DR5 protein expression increased after treatment with 0.5 μmol/L and 1 μmol/L of CDDO-Me in all of the tested cell lines, albeit to various degrees. In contrast, DR4 protein was weakly elevated in one (H157) of six cell lines only when treated with 1 μmol/L CDDO-Me (Fig. 2B). Moreover, CDDO-Me increased cell surface DR5 by 68% (mean fluorescent intensity increased from 26.9 to 45.3) and 78% (mean fluorescent intensity increased from 27.1 to 48.3) in A549 and H1792 cell lines, respectively, compared with DMSO control, whereas it either did not increase (A549) or even decrease (H1792) cell surface DR4 (H1792; Fig. 2C). These results further support a more important role for DR5 than DR4 in CDDO-Me–induced apoptosis.

CDDO-Me Augments TRAIL-Induced Apoptosis. If CDDO-Me induces the expression of DR5 gene leading to increased amount of DR5 on the cell surface, the treatment of cells with CDDO-Me combined with exogenous TRAIL should result in augmented induction of apoptosis. TRAIL at concentrations ranging from 25 to 100 ng/mL (H1792 and H522) or 25 to 1000 ng/mL (A549 cells) weakly increased DNA fragmentation in A549, H1792, and H522 cells, whereas the addition of CDDO-Me augmented DNA fragmentation, which reached levels much greater than those induced by each agent alone (Fig. 3A). Similarly, the sub-G1 population induced by CDDO-Me and TRAIL combination (39%) was much greater than those induced by either CDDO-Me (17.5%) or TRAIL (13.6%) alone (Fig. 3B). These results indicate that the combination of CDDO-Me...
and TRAIL augments induction of apoptosis. Moreover, the augmentation of apoptosis by the combination of CDDO-Me and TRAIL was suppressed in the presence of recombinant human decoy receptors DcR2 or OPG (Fig. 3C). This shows that induced DR5 is functional (in terms of ligation with TRAIL), and CDDO-Me enhances TRAIL-induced apoptosis by up-regulating its receptors. Importantly, neither TRAIL alone nor the combination of CDDO-Me and TRAIL increased DNA fragmentation in NHBE cells (Fig. 3A), suggesting that the combination of CDDO-Me and TRAIL might not be toxic to normal cells.

**The CDDO-Me and TRAIL Combination Exhibits Enhanced Activation of Caspase Cascades.** Caspase activation is known to be a critical event in signaling the death receptor-mediated apoptotic pathway (1). We sought to determine which caspases are involved in apoptosis induced by the combination of CDDO-Me and TRAIL by analyzing the effects of these agents on caspase activation in A549 cells. CDDO-Me and TRAIL alone at the tested concentrations did not activate or only slightly activated the initiator caspases, caspase-8 and caspase-9, and the caspase-8 substrate Bid. However, the combination of CDDO-Me and TRAIL activated both caspases and Bid as evidenced by decreased level of the proform of each caspase, appearance of the cleaved (active) forms, or both (Fig. 4A and B). A similar analysis of effector caspases revealed that combined CDDO-Me and TRAIL enhanced the cleavage of caspase-3, caspase-6, and caspase-7 and the subsequent cleavage of their substrates PARP and DFF45 (Fig. 4A and B). Furthermore, the enhanced induction of apoptosis by CDDO-Me and TRAIL combination was suppressed by the pan-caspase inhibitor z-VAD-fmk, the caspase-3 inhibitor z-DEVD-fmk, the caspase-8 inhibitor z-IETD-fmk, and the caspase-9 inhibitor z-LEHD-fmk, respectively (Fig. 4C). Collectively, these results show that the activation of caspase cascades, including initiator caspases, caspase-8 and caspase-9, and effector caspases, caspase-3, caspase-6, and caspase-7, mediates the augmented apoptosis induced by the CDDO-Me and TRAIL combination.

**DR5 Up-Regulation Is Required for CDDO-Me–Mediated Apoptosis and Enhancement of TRAIL-Induced Apoptosis.** The aforementioned experiments clearly indicate that CDDO-Me induces DR5 expression and a caspase-8–dependent apoptosis. We next wanted to know whether DR5 up-regulation was required for CDDO-Me–induced apoptosis and enhancement of TRAIL-induced apoptosis. To do so, we silenced DR5 expression using a DR5 siRNA and then compared the effects of CDDO-Me on induction of apoptosis between cells expressing DR5 and cells with silenced DR5 expression. In H1792 cells, DR5 expression was successfully knocked down with the DR5 siRNA but not with the control siRNA (Fig. 5A). As a related gene, DR4 expression was not altered by the DR5 siRNA (Fig. 5A). The cells with silenced DR5 expression (transfected with the DR5 siRNA) had a much lower level of DNA fragments than those expressing normal level of DR5 when exposed to CDDO-Me (Fig. 5B), indicating that DR5 silencing causes cell resistance to CDDO-Me–induced apoptosis. Therefore, we conclude that DR5 up-regulation is required for CDDO-Me–induced apoptosis. Moreover, CDDO-Me cooperated with TRAIL to augment apoptosis in control siRNA-transfected cells but not in DR5 siRNA-transfected cells (Fig. 5C), indicating that.
DR5 up-regulation also is required for enhancement of TRAIL-induced apoptosis by CDDO-Me.

**CDDO-Me Induces DR5 Up-regulation and Apoptosis via a JNK-Mediated Mechanism.** Although CDDO-Me induces the expression of DR5 and DR4 in few cell lines, we did not know how it up-regulates their expression. It is well known that many therapeutic agents trigger apoptosis via activation of stress-related signaling pathways including p53 and JNK-mediated ones (44). We and others have shown that DR5 and/or DR4 can be regulated via stress-related mechanisms such as activation of p53 (11, 12, 14), activator protein (AP-1; ref. 16), or nuclear factor κB (NFκB; ref. 15). Considering that CDDO-Me induces a p53-independent induction of DR5 in this study and that its analog CDDO inhibits NFκB signaling (30) but induces JNK activation (26) in leukemia cells, we examined the possibility that CDDO-Me activates JNK pathway and, if so, whether JNK activation contributes to CDDO-induced DR5 induction and apoptosis in human lung cancer cells. As shown in Fig. 6A, CDDO-Me increased the levels of p-c-Jun and p-ATF2, two well-known JNK substrates, in a time-dependent manner in H157 cells. The increases in p-c-Jun and p-ATF2 occurred rapidly at 30 minutes or 50 minutes after treatment with CDDO-Me. It transiently and weakly increased the total ATF2 level but enhanced the total c-Jun level in a similar fashion as it increased the p-c-Jun level because phosphorylation of c-Jun regulates its transcriptional activity (45). These results indicate that CDDO-Me activates JNK pathway.

It appears that activation of JNK pathway occurs (30 minutes to 1 hour) before death receptor induction (3 to 6 hours; Fig. 2A), caspase-8 activation (3 to 9 hours; Fig. 1B), and apoptosis (after 12 hours; ref. 24) can be induced after cells are exposed to CDDO-Me, thus representing a early event. The question was raised as to whether JNK activation plays any role in mediating CDDO-Me–induced death receptor up-regulation and apoptosis. To address this issue, we examined the effects of CDDO-Me on death receptor expression, caspase activation, and apoptosis in the presence of the JNK-specific inhibitor SP600125 (46). SP600125 at concentrations of 10 to 30 μmol/L, as expected, completely abolished not only CDDO-Me–induced increase of p-c-Jun but also the basal level of p-c-Jun in the H157 cell line. Correspondingly, the elevated c-Jun level induced by CDDO-Me, as well as the basal level of c-Jun, was suppressed by SP600125 (Fig. 6B). These results confirm that SP600125 is a JNK inhibitor in our cell system.

We next examined the effect of SP600125 on DR5 and DR4 induction by CDDO-Me in the H157 cell line. As shown in Fig. 6C, CDDO-Me increased DR5 and DR4 expression. However, SP600125 at concentrations of 10 to 30 μmol/L, which are effective in inhibiting JNK activation, blocked CDDO-Me–induced up-regulation of DR5 and DR4 in a concentration-dependent fashion. This result indicates that CDDO-Me induces DR expression via a JNK-mediated mechanism.

Furthermore, we studied whether JNK activation is required for CDDO-Me–induced caspase-8 activation and apoptosis. In the same cell line, we analyzed the effects of CDDO-Me on activation of caspase-8 and caspase-3, DNA fragmentation, and sub-G1 population formation in the absence and presence of SP600125. It is well known that activation of caspase-3 (a downstream effector caspase of caspase-8) and cleavage of its substrate PARP are hallmarks of apoptotic cell death. In the absence of SP600125, CDDO-Me appeared to activate caspase-8 and caspase-3 and to increase cleavage of PARP as indicated by the appearance of their cleaved forms detected by Western blot analysis. In the presence of SP600125, CDDO-Me

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**Fig. 3.** Enhanced apoptosis induced by combined CDDO-Me and TRAIL in human lung cancer cells but not in normal lung epithelial cells (A and B) and suppression of CDDO-Me/TRAIL-induced apoptosis by decoy receptor proteins (C). A. The indicated cell lines were seeded in 96-well cell culture plates and treated on the second day with 1 μmol/L (A549 and NHBE cells) or 0.5 μmol/L (H1792 and H522 cells) CDDO-Me alone, the indicated concentrations of TRAIL alone, or their combinations for 16 hours. DNA fragmentation was estimated using ELISA. Columns, means of triplicate determinations; bars, SD. A549 and NHBE cells were treated with 0.5 μmol/L CDDO-Me, 50 ng/mL TRAIL, alone, and the combination of CDDO-Me and TRAIL. After 19 hours, cells were harvested and subjected to sub-G1 analysis using flow cytometry. C. A549 cells were seeded in 96-well cell culture plates. On the second day, the cells were pretreated with 2 μg/ml rhOPG:Fc or rhDcR2:Fc for 30 minutes and then cotreated with rhOPG:Fc or rhDcR2:Fc, 1 μmol/L CDDO-Me, and 25 ng/mL TRAIL for 16 hours. Apoptosis then was measured using ELISA. Columns, means of triplicate determinations; bars, SD; rh, recombinant human.
failed to do so (Fig. 7A). These results show that CDDO-Me–mediated caspase-8 and caspase-3 activation and PARP cleavage are JNK dependent. In a similar manner, CDDO-Me increased the amount of DNA fragments (Fig. 7B) and sub-G₁ population (Fig. 7C), respectively, in the absence of SP600125. However, these effects were suppressed in the presence of SP600125. These results indicate that JNK activation also is required for CDDO-Me–induced DNA fragmentation and sub-G₁ formation. Collectively, these results show that JNK activation is required for CDDO-Me–induced apoptosis in human lung cancer cells.

DISCUSSION

Activation of caspases plays an important role in apoptosis triggered by various proapoptotic signals (47, 48). It generally is recognized that there are two major apoptotic pathways: one involves death signals transduced through death receptors, and the other relies on a signal from the mitochondria (48, 49). Both pathways are involved in an ordered activation of a set of caspases, which in turn cleave cellular substrates leading to the morphologic and biochemical changes of apoptosis. The activation of caspase-8 and caspase-9 has been documented to play central roles in mediating apoptosis signaled by death receptors and by mitochondria, respectively (48, 49). However, caspase-8 can activate caspase-9–mediated apoptotic pathway via activating or cleaving Bid protein (48, 49). In the present study, we found that CDDO-Me activated caspase-8 and that the presence of the caspase-8 inhibitor z-IETD-fmk abrogated apoptosis induced by CDDO-Me, indicating that caspase-8 activation is required for CDDO-Me–induced apoptosis in human lung cancer cells. Moreover, CDDO-Me up-regulated DR5 expression, and silencing of DR5 expression using a DR5 siRNA abrogated CDDO-Me–induced apoptosis, showing that DR5 up-regulation also is required for CDDO-Me–induced apoptosis. Therefore, we conclude that CDDO-Me induces DR5-mediated caspase-8 activation and induction of apoptosis in human lung cancer cells. Our previous study showed that CDDO-Me also activated caspase-9–mediated apoptotic pathway (24). In the present study, we found that the caspase-8 inhibitor z-IETD-fmk blocked CDDO-Me–induced caspase-9 activation, indicating that caspase-8 activation is an early event upstream of caspase-9 activation. Because Bid, a substrate of caspase-8, was
cleaved (activated) during CDDO-Me–induced apoptosis, it is likely that the downstream caspase-3 also can be indirectly activated by the caspase-8–dependent activation of caspase-9 via the Bid-mediated mechanism during CDDO-Me–induced apoptosis. Thus, our results, for the first time, highlight a novel death receptor-mediated mechanism by which CDDO and possibly other triterpenoids induce apoptosis in human cancer cells (Fig. 8).

Although CDDO-Me induced the expression of DR5 and DR4 at the mRNA level, DR5 but not DR4 was universally increased at the protein level in human lung cancer cells. Moreover, CDDO-Me increased the amount of DR5 but not DR4 on the cell surface. Thus, DR5 induction should play a more important role than DR4 up-regulation in mediating CDDO-Me–induced apoptosis. The expression of DR4 and DR5 can be regulated by a p53-dependent (11–14) or by a p53-independent mechanism (13, 15–17). In this study, CDDO-Me induced the expression of DR4 and DR5 (i.e., at the mRNA level) in all of the tested lung cancer cell lines, regardless of p53 status, indicating that CDDO-Me up-regulates their expression through a p53-independent mechanism.

It recently was reported that novel triterpenoids, including CDDO and CDDO-Me, activate the JNK pathway, which is required for induction of apoptosis by these triterpenoids in human leukemia cells (26). However, it has not been elucidated how JNK activation results in induction of apoptosis. In agreement with this report, we found that CDDO-Me rapidly increased the levels of p-c-Jun and p-ATF2, two substrates of JNK, revealing that CDDO-Me rapidly activates JNK pathway in human lung cancer cells. Moreover, the JNK inhibitor SP600125, which blocked CDDO-Me–induced JNK activation, also attenuated CDDO-Me–induced apoptosis including caspase-3 activation, PARP cleavage, and DNA fragmentation, showing that JNK activation is required for CDDO-Me–induced apoptosis in human lung cancer cells. For the first time, we have shown that JNK activation mediates DR5 and DR4 up-regulation and caspase-8 activation induced by CDDO-Me because SP600125 effectively suppressed CDDO-Me–induced death receptor up-regulation and caspase-8 activation. Together with the result that silencing of DR5 expression using siRNA decreased cell sensitivity to CDDO-Me–induced apoptosis, we conclude that CDDO-Me induces apoptosis through a
JNK-mediated up-regulation of DR5 and DR4 (in some cell lines) in human lung cancer cells (Fig. 8).

It is known that DR5 and DR4 can be regulated in p53-independent mechanisms (13, 15–17). However, it is largely unknown how p53-independent mechanisms regulate DR5 and DR4 expression. In the current study, we, for the first time, have shown that JNK activation up-regulates DR5 and DR4 expression. It is known that c-Jun/AP-1 is a major target of the JNK signaling pathway by phosphorylating the transactivation domain of c-Jun, leading to an increase in its transactivation potential (45). Thus, by increasing c-Jun phosphorylation, JNK promotes c-Jun expression transcriptionally, leading to an increase in AP-1 activity. We have shown that DR5 can be regulated by AP-1 activation via a functional AP-1 binding site in its promoter region (16). We also recently identified a functional AP-1 binding site in the DR5 promoter region and thus showed that DR5 can be up-regulated through an AP-1–mediated mechanism.4 In the current study, CDDO-Me increased not only p-c-Jun level but also total c-Jun level. Therefore, we suggest that CDDO-Me activates JNK, which in turn enhances AP-1 activity via increasing c-Jun expression, thus leading to up-regulation of DR5 and DR4 expression (Fig. 8).

TRAIL binds to DR4 and/or DR5, leading to a tumor-selective induction of apoptosis, and thus is considered to be a promising candidate for cancer treatment (2, 3). However, human cancer cells exhibit a heterogeneous response to TRAIL-induced apoptosis (2, 7, 8, 50). Some cancer cells, including lung cancer cells, are resistant to TRAIL. Fortunately, many therapeutic agents can enhance cancer cell response or sensitize cancer cells to TRAIL treatment to overcome this resistance (2, 7, 8). Our finding that CDDO-Me up-regulates the expression of DR5, and possibly DR4 in some cell lines, independently of p53 has important clinical implications because CDDO-Me combined with TRAIL or other TRAIL-inducing agents will augment induction of apoptosis by TRAIL–death receptor interaction. We found that combined CDDO-Me and TRAIL exhibited a more than additive induction of apoptosis in several lung cancer cell lines, regardless of p53 status. Because silencing of DR5 expression using siRNA abrogated the apoptosis induced by the combination of CDDO-Me and TRAIL, we conclude that CDDO-Me enhances or augments TRAIL-induced apoptosis in a p53-independent fashion by up-regulating DR5 expression. In A549 cells, TRAIL alone even at 1 μg/mL weakly increased DNA fragmentation, indicating that they are not sensitive to TRAIL-induced apoptosis. However, in the presence of CDDO-Me, TRAIL at 25 ng/mL was sufficient to induce high level of DNA fragmentation. Therefore, it appears that TRAIL combined with CDDO-Me can overcome cell resistance to TRAIL-induced apoptosis.

The ability of CDDO and CDDO-Me to enhance apoptosis induced by death ligands recently has been shown in several other types of cancer cell lines. Stadheim et al. (30) reported that CDDO potently enhanced TNF-induced apoptosis in human leukemia cells by inhibiting TNF-mediated expression of antiapoptotic genes. In another study that is more relevant to our report, Kim et al. (31) have shown that CDDO-Me and CDDO augmented TRAIL-induced apoptosis in a panel of human cancer cell lines, including prostate, ovarian, colon, and cervical cancer cells. Importantly, this combination was not cytotoxic to normal cells, including primary cultures of hepatocytes, endothelial cells, peripheral blood leukocytes, and bone marrow. In agreement with their findings, we also found that the combination of CDDO-Me and TRAIL did not show enhanced apoptosis in NBHE cells. However, they did not find that CDDO up-regulated DR4 or DR5 expression using Western blot analysis in these cancer cell lines. They showed that the enhanced apoptosis induction by this combination is mediated by the ability of CDDO or CDDO-Me to decrease FLIP protein level through ubiquitination and proteasome-dependent degradation. A more recent study from the same group (51) showed that CDDO or CDDO-Me induced a caspase-8–dependent apoptosis and enhanced TRAIL-induced apoptosis in leukemia cell lines involving down-regulation of FLIP. They also found that CDDO induced a slight increase in DR4 and DR5 protein levels in U937 cells (51); however, they did not examine the effects of CDDO-Me on the expression of DR4 and DR5 (31, 51). Our results clearly showed that CDDO-Me activated caspase-9 (24) and caspase-8 (current study) in human lung cancer cells, whereas the report by Suh et al. (51) showed that CDDO-Me activated caspase-8 but not caspase-9 in leukemia cells. Therefore, it is possible that CDDO-Me may have some distinct effect on apoptosis from CDDO or that these triterpenoids may have cell-type specific effects.

In summary, the current study has shown that CDDO-Me induces a JNK-dependent up-regulation of DR5 expression, leading to activation of caspase-8 and induction of apoptosis in human lung cancer cells. Thus, our findings in this study reveal a novel JNK-dependent, DR5-mediated mechanism by which CDDO-Me, as well as possibly other triterpenoids, induces apoptosis in human cancer cells.

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Wei Zou, Xiangguo Liu, Ping Yue, et al.


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