
Kevin B. Kim, Reuben Lotan, Ping Yue, Michael B. Sporn, Nanjoo Suh, Gordon W. Gribble, Tadashi Honda, Gen Sheng Wu, Waun Ki Hong, and Shi-Yong Sun

Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [K. B. K., R. L., P. Y., W. K. H., S.-Y. S.]; Department of Pharmacology [M. B. S., N. S.], Dartmouth Medical School, and Department of Chemistry [G. W. G., T. H.], Dartmouth College, Hanover, New Hampshire 03755; and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201 [G. S. W.]

Abstract

Lung cancer continues to be the leading cause of cancer-related death in the United States. Therefore, new agents targeting prevention and treatment of lung cancer are urgently needed. In the present study, we demonstrate that a novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) is a potent inducer of apoptosis in human non-small cell lung carcinoma (NSCLC) cells. The concentrations required for a 50% decrease in cell survival (IC50) ranged from 0.1 to 0.3 μM. CDDO-Me induced rapid apoptosis and triggered a series of effects associated with apoptosis including a rapid release of cytochrome c from mitochondria, activation of procaspase-9, -7, -6, and -3, and cleavage of poly(ADP-ribose) polymerase and lamin A/C. Moreover, the caspase-3 inhibitor DEVD-FMK and the pan caspase inhibitor Z-VAD-FMK suppressed CDDO-Me-induced apoptosis. These results indicate that CDDO-Me induced apoptosis in human NSCLC cells via a cytochrome c-triggered caspase activation pathway. CDDO-Me did not alter the level of Bcl-2 and Bcl-xL proteins, and no correlation was found between cell sensitivity to CDDO-Me and basal Bcl-2 expression level. Furthermore, overexpression of Bcl-2 did not protect cells from CDDO-Me-induced apoptosis. These results suggest that CDDO-Me induces apoptosis in NSCLC cells irrespective of Bcl-2 expression level. In addition, no correlation was found between cell sensitivity to CDDO-Me and p53 status, suggesting that CDDO-Me induce a p53-independent apoptosis. Our results demonstrate that CDDO-Me may be a good candidate for additional evaluation as a potential therapeutic agent for human lung cancers and possibly other types of cancer.

Introduction

In the United States, lung cancer is the leading cause of cancer mortality among both men and women. It has been estimated that there will be 169,500 new cases and 157,400 deaths from lung cancer in 2001 (1). Unfortunately, the severe morbidity of lung cancer and the poor 5-year relative survival rate (only 14%) have not been improved by current treatments. Therefore, intense efforts are being mounted to find effective new agents and treatments against lung cancer.

Triterpenoids, biosynthesized in plants by the cyclization of squalene, are used for medicinal purpose in many Asian countries, and some of them were reported to have anticarcinogenic activity (2–5). Because the biological activities of some of the natural triterpenoids are relatively weak, new analogues of these molecules have been synthesized recently in an attempt to identify more potent agents (6–8). One of these analogues, CDDO,3 was found to inhibit proliferation of many human cancer cells and to suppress the ability of various inflammatory cytokines, such as IFN-γ, interleukin-1, and tumor necrosis factor-α. CDDO also induce de novo formation of the enzymes, inducible nitric oxide synthase, and inducible cyclooxygenase, showing a potential for either chemopreventive or chemotherapeutic activity against malignancy (9). Moreover, CDDO was reported recently to induce apoptosis via a caspase-8-dependent mechanism in human osteosarcoma and myeloid leukemia cells (10, 11).

CDDO-Me is a derivative of CDDO (Fig. 1). This compound was as active as CDDO in suppressing the increased production of nitric oxide by IFN-γ in mouse macrophages (7). Interestingly, CDDO-Me was identified recently as a PPARγ antagonist, whereas CDDO was recognized as a PPARγ agonist (12). In this report, we compared the effects of CDDO and CDDO-Me on the growth of human NSCLC cell lines. We

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2 To whom requests for reprints should be addressed, at Department of Thoracic/Head and Neck Medical Oncology, Box 432, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-5062; Fax: (713) 792-7976; E-mail: ssun@mdanderson.org.

3 The abbreviations used are: CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oate; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate; NSCLC, non-small cell lung carcinoma; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling; SRB, sulforhodamine B; PI, propidium iodide; PPARγ, peroxisome-proliferator-activated receptor γ.
found that CDDO-Me was much more potent than CDDO in decreasing cell survival. Therefore, we focused additional studies on the induction of apoptosis by CDDO-Me and on some aspects of the mechanism of its action in human NSCLC cells.

Materials and Methods

Reagents. CDDO and CDDO-Me were synthesized at Dartmouth College (Hanover, NH; Ref. 7). They were dissolved in DMSO at a concentration of 10 mM, and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. The caspase inhibitors CBZ-Val-Ala-Asp-Fluoromethyl Ketone (FMK; Z-VAD-FMK) and CBZ-Asp-Glu-Val-Asp-FMK (Z-DEVD-FMK) were purchased from Enzyme System Products (Livermore, CA).

Cell Lines and Cell Culture. H460, H1944, H596, H157, H1792, H522, H292, and H226 were obtained from Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). Calu-1, SK-MES-1, and A549 were purchased from the American Type Cell Culture (Rockville, MD). H460-Neo (vector control), H460-Bcl2-8 (a transfected clone that expresses a low level of exogenous Bcl-2), and H460-Bcl2-8 (a transfected clone that expresses a high level of exogenous Bcl-2) cell lines were generated as described previously (13). These cells were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air.

Cell Survival Assay. Cells were seeded at densities ranging from 3 × 103 to 6 × 105 cells/well in 96-well tissue culture plates. On day 2, cells were treated with different concentrations of CDDO or CDDO-Me. Control cultures received the same amount of DMSO as did the treated cultures. At certain times after treatment initiation, cell numbers were estimated by the SRB assay as described previously (15). Cell survival was calculated by using the equation: % cell survival = (A/A0) × 100, where A0 and A represent the absorbance in treated and control cultures, respectively. IC50, the drug concentration causing a 50% decrease in cell survival, was determined by interpolation from dose-response curves.

Detection of Apoptosis. Cells were plated on 10-cm diameter dishes 1 day before treatment. After a 24 h-treatment, cells were harvested by trypsinization and counted. One million cells were used for detecting DNA breaks or fragments possessing 3'-hydroxyl ends using an APO-

Results

Effects of CDDO-Me on Cell Survival in Human NSCLC Cells. Lung cancers include small cell lung carcinoma and NSCLC. The latter include three types, adenocarcinoma,
epidermoid (or squamous), and large-cell carcinoma, and accounts for >75% of lung cancers (17). Therefore, we focused our study on determining the effects of CDDO-Me on cell growth in NSCLC cells. We first compared and contrasted the effects of CDDO-Me on survival of 11 of the human NSCLC cells lines in a concentration-dependent fashion with IC_{50} for different cell lines ranging from 0.13 to 0.31 μM after a 3-day treatment. In contrast, CDDO exhibited much weaker effects than CDDO-Me on the survival of these cell lines with IC_{50} ranging from 0.45 to >1 μM. In fact, the IC_{50} of CDDO for 9 of 11 cell lines (except for H460 and H292) were ≥1 μM. The effects of CDDO-Me on cell survival were also time-dependent (Fig. 2B). Its action was very rapid. After a 12- or 24-h treatment, 1 μM of CDDO-Me decreased cell survival by >45% in the three tested cell lines. A549 was apparently less sensitive to CDDO-Me than H460 and H157 were. However, after prolonged treatment (i.e., 3 days), a low dose (i.e., 0.25 μM) of CDDO-Me still decreased cell survival by >50% in this cell line (Fig. 2B). We did not find any correlation between cell sensitivity to CDDO-Me and p53 status (Fig. 6B). For example, H157 cells carry mutant p53 (18) and were more sensitive to CDDO-Me than A549 cells, which have wild-type p53 (Ref. 18; Fig. 2). These results suggest that the effects of CDDO-Me on cell survival are p53-independent.

Induction of Apoptosis by CDDO-Me in Human NSCLC Cells. To elucidate whether CDDO-Me decreases cell survival through the induction of apoptosis in the NSCLC cells, we then examined the effects of CDDO-Me on apoptosis in three NSCLC cell lines (H157, H460, and A549) using different approaches. Using Annexin V assay, we detected the increase of both early apoptotic population (Annexin V-positive) and late apoptotic population (Annexin V- and PI-positive) by 50–70% after a 24-h treatment with 1 μM of CDDO-Me (Fig. 3A). There was only a small portion of cells undergoing necrosis (PI-positive), which was <5% in all three of the cell lines (Fig. 3A). Moreover, we also detected a concentration-dependent increase in apoptotic (i.e., TdT-FITC-positive) cell population in the three NSCLC cell lines using a TUNEL-flow cytometry analysis. After a 24-h treatment, CDDO-Me at 1 μM caused >50% of the cells to undergo apoptosis in these cell lines, which were consistent with the results generated from Annexin V binding assay, whereas CDDO-Me at 0.25 μM did not induce apoptosis in A549 cells. However, ~30% of H157 and H460 cells still underwent apoptosis by 0.25 μM CDDO-Me (Fig. 3B). CDDO-Me at 0.5 μM induced apoptosis by 68.6%, 44.8%, and 17.4% in H157, H460, and A549 cells, respectively. It appears that A549 cells were less sensitive than H157 and H460 cells to CDDO-Me-induced apoptosis. Taken together, we conclude that CDDO-Me decreases cell survival through the induction of apoptosis in human NSCLC cells.

Induction of Cytochrome C-mediated Caspase Activation by CDDO-Me in Human NSCLC Cells. The cytochrome c release from mitochondria that leads to caspase activation represents an important apoptotic pathway, especially for apoptosis induced by cytotoxic agents (19–22). Therefore, we next examined the effects of CDDO-Me on several important events for apoptosis such as cytochrome c release, activation of caspases, and cleavage of some vital protein substrates in this pathway. We found that CDDO-Me not only triggered a rapid release of cytochrome c from mitochondria (Fig. 4A) but also activated procaspase-9 as evidenced by the appearance of a M, 39,000 caspase-9 band on Western blot gel (Fig. 4B). The release of cytochrome c occurred as early as 4 h after treatment and, therefore, preceded the activation of procaspase-9, which occurred after a 6-h and a 12-h treatment in H460 and A549, respectively (Fig. 4, A and B). It is apparent that procaspase-9 was more rapidly activated in H460 than in A549 cells. We noted that low levels of cytochrome c were detected in untreated control cells. This could be caused either by spontaneous apoptosis or by a contamination of trace amount of mitochondrial protein during preparation of cytosolic fraction. Nevertheless, a large amount of cytochrome c was detected in cytosolic fractions prepared from CDDO-Me-treated cells, which were significantly higher than that from untreated cells (Fig. 4A).

Correspondingly, we observed a time-dependent activation of effector caspases including caspase-3, -6, and -7 in both H460 and A549 cells. Activation of these caspases was detected by Western blot analysis, evidenced by the de-
crease of procaspase forms, the appearance of their cleaved bands, and cleavage of their corresponding substrate proteins. As shown in Fig. 4C, after the treatment with CDDO-Me, the levels of procaspase-3, -6, and -7 were decreased, and these reductions were accompanied by the increased cleavage of their substrate PARP to an M, 89,000 fragment in a time-dependent manner. We noted that the cleavage of PARP occurred after a 6-h treatment in H460 cells and after
a 12-h treatment in A549 cells, respectively, indicating that similar to caspase-9 activation, the activation of caspase-3 or -7 also happened more rapidly in H460 cells than in A549 cells. Moreover, we found that CDDO-Me also exerted a concentration-dependent effect on the activation of these effector caspases. As shown in Fig. 4D, CDDO-Me at a concentration of 0.25 μM was sufficient to activate caspase-3, -6, and -7 in H460 cells. However, a higher concentration (0.5 μM or higher) was needed to activate these caspases in A549 cells. Apparently, H460 cells were more sensitive than A549 cells to CDDO-Me-induced caspase activation, which is consistent with their responses to the effects of CDDO-Me on cell survival and apoptosis. Using different antibodies, we were able to detect not only a concentration-dependent decrease of the levels of pro-caspase-3 and -7 but also a concentration-dependent increase in the amount of cleaved bands (active forms of caspase) of caspase-3 and -7. We detected a decrease of procaspase-6 level but failed to detect cleaved forms of caspase-6. However, we could detect the cleavage of lamin A/C, which is a specific substrate of caspase-6 and a marker of caspase-6 activation (Ref. 23; Fig. 4D). Thus, CDDO-Me activates not only caspase-3 and -7 but also caspase-6 in both tested lung cancer cell lines. Taken together, these results demonstrate that CDDO-Me targets the mitochondria and activates cytochrome c-mediated caspase cascade.

**Involvement of Activation of Caspase Cascade in CDDO-Me-induced Apoptosis.** To determine whether activation of the caspase cascade is required for CDDO-Me-induced apoptosis, we examined the effect of CDDO-Me on apoptosis induction in the presence of caspase inhibitors. As shown in Fig. 5, the pan-caspase inhibitor Z-VAD-FMK and the caspase-3 inhibitor Z-DEVD-FMK suppressed CDDO-Me-induced apoptosis in both H460 and A549 cells. This indicates that activation of cytochrome c-mediated caspase cascade is required for CDDO-Me-induced apoptosis in human NSCLC cells.

**CDDO-Me Induces Apoptosis Independent of Bcl-2 Expression Level.** The proteins Bcl-2, Bcl-xL, and Bax are important components of the apoptotic pathway and act as either negative or positive regulators of apoptosis (24). However, as shown in Fig. 6A, CDDO-Me did not change the expression level of either Bcl-2 or Bcl-xL in H460 cells. CDDO-Me also did not change the expression level of Bax, but it did increase the level of Bax-α starting from 6 h after CDDO-Me treatment (Fig. 6A). Bcl-2 functions as an anti-apoptotic protein (24). If Bcl-2 plays any role in CDDO-Me-induced apoptosis, its basal expression level may affect cell sensitivity to CDDO-Me, and overexpression of Bcl-2 should protect cells from apoptosis induced by CDDO-Me. By comparing the basal Bcl-2 expression levels of some NSCLC cell lines with the cytotoxic effects of CDDO-Me (IC50), we found that NSCLC cell lines exhibited a similar sensitivity to CDDO-Me treatment regardless of basal Bcl-2 expression level, indicating that there was no simple correlation between basal Bcl-2 expression level and cell sensitivity to CDDO-Me (Fig. 6B). Furthermore, we stably transfected the Bcl-2 gene or control neo gene into H460 cells and isolated two trans-
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Discussion

The triterpenoid CDDO is a potent novel molecule with a wide range of actions, many of which are potentially useful for cancer prevention or treatment (9). More recently, this agent was reported to induce apoptosis in human myeloid leukemia and osteosarcoma cells (10, 11). In the present study, we investigated the effects of another triterpenoid CDDO-Me, a derivative of CDDO, on the cell growth and apoptosis using a panel of human NSCLC cell lines. Although CDDO-Me was found to be as active as CDDO in suppressing the increased production of nitric oxide by IFN-γ in mouse macrophages (7), we have demonstrated that CDDO-Me was much more potent than CDDO in decreasing the survival of human NSCLC cells. Therefore, CDDO-Me may be a better candidate for use as a therapeutic agent for cancer treatment. CDDO-Me decreases the survival of human NSCLC cells through induction of apoptosis. Therefore, our results warrant additional studies on the therapeutic activity of CDDO-Me in vivo. It should be pointed out that CDDO-Me inhibited cell growth or decreases cell survival not only in lung cancer cells but also in other types of cancer including human prostate, head and neck, colon, and breast cancer cells.4

Caspases play important roles in apoptosis triggered by various proapoptotic signals (19, 20). In general, activation of the caspase cascade requires both initiator caspases such as caspase-8, -9, and -10 and effector caspases such as caspase-3, -6, and -7. The effector caspases cleave several vital substrates such as PARP and DFF45 leading to apoptosis (19, 20). It has been well documented recently that the cytochrome c release from mitochondria and its activation of caspase-9 through binding to the protein Apaf-1 is thought to mediate apoptosis triggered by signals such as chemotherapeutic agents (19–22). In this study, CDDO-Me triggered a rapid release of cytochrome c from mitochondria to cytosol, activated procaspase-9 and its downstream pro-caspases including procaspase-3, -6, and -7, followed by the cleavage of their substrate PARP. Moreover, the pan-caspase inhibitor Z-VAD-FMK and the caspase-3 inhibitor Z-DEVD-FMK suppressed CDDO-ME-induced apoptosis. Therefore, we conclude that CDDO-Me induces a cytochrome c-mediated, caspase-dependent apoptosis in human NSCLC cells.

Bcl-2, Bcl-xL, and Bax have been implicated as major players in the control of apoptosis (19, 21). Bcl-2 and Bcl-xL promote cell survival, whereas Bax promotes cell death (24, 29). More recently, these proteins have been found to regulate apoptosis by controlling cytochrome c release from mitochondria and activation of caspase-9 (24, 30). CDDO-Me did not alter the expression levels of Bcl-2 and Bcl-xL, but it did elevate Bax-a level. Whether Bax-a increase plays a role in CDDO-Me-induced apoptosis in human NSCLC cells remains to be elucidated. By comparing the basal Bcl-2 expression levels with the cytotoxic effects of CDDO-Me in these cell lines, we did not find any correlation between the basal Bcl-2 expression level and cell sensitivity to CDDO-Me. Moreover, we found that overexpression of exogenous Bcl-2 failed to protect cells from CDDO-Me-induced apoptosis. Taken together, we conclude that CDDO-Me induces apoptosis independent of Bcl-2 level.

p53 is another important factor that affects the cell response to drug effects on growth inhibition and apoptosis induction (31, 32). The majority of evidence supports the notion that cells with wild-type p53 exhibit increased sensitivity to radiation or chemotherapeutic agents, whereas cells lacking wild-type p53 expression still undergo apoptosis but need a relatively high dose of radiation or chemotherapeutic drugs (31–35). In this study, we found that CDDO-Me decreased cell survival and induced apoptosis regardless of p53 status in human lung cancer cells. Therefore, we conclude that CDDO-Me-induced apoptosis in human cancer cells is p53-independent.

Recent studies have shown that PPARγ ligands induce apoptosis in several types of cancers including human lung cancer cells (36–39). CDDO-Me was reported recently to be a PPARγ antagonist, whereas CDDO is a PPARγ agonist (12). To determine whether CDDO-Me-induced apoptosis is

4. P. Yue, R. Lotan, and S-Y. Sun, unpublished observations.
linked to its antagonistic activity on PPARγ, we examined the apoptosis-inducing activity of CDDO-Me in the presence of several PPARγ agonists including CDDO, ciglitizone, and 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2). We speculated that the effect of CDDO-Me on apoptosis induction would be abrogated in the presence of PPARγ agonists if it induces apoptosis through binding to and antagonizing PPARγ. In fact, we found that cotreatment of cells with these PPARγ agonists either did not affect or even enhanced the effect of CDDO-Me on apoptosis depending on used cell lines. These results suggest that the effect of CDDO-Me on apoptosis induction is unlikely to be mediated by antagonism of PPARγ pathway.

In conclusion, we have demonstrated that the novel synthetic triterpenoid CDDO-Me is a potent apoptosis-inducing agent in human NSCLC cells, which acts through a pathway involving cytochrome c release from mitochondria and subsequent activation of caspases. Moreover, CDDO-Me induces apoptosis independent of both p53 status and Bcl-2 level. Based on these findings, we suggest that CDDO-Me may be a good candidate for additional evaluation as a cancer therapeutic agent for human lung cancer as well as other types of cancer.

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References

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