Induction of redox imbalance and apoptosis in multiple myeloma cells by the novel triterpenoid 2-cyano-3, 12-dioxoolean-1,9-dien-28-oic acid

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Abstract

The synthetic oleanane triterpenoid 2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO) and its chemical derivatives induce differentiation and apoptosis of human leukemia cells. The precise mechanisms responsible for the effects of CDDO, however, remain unclear. In the present study, we examined the effects of CDDO and its C-28 imidazolide ester (CDDO-Im) on apoptosis of multiple myeloma (MM) cells. The results show that both CDDO and CDDO-Im are potent inducers of MM cell apoptosis and that CDDO-Im is more active than CDDO. CDDO-Im treatment was associated with (*a*) depletion of glutathione, (*b*) increases in reactive oxygen species, (*c*) a reduction of the Fas-associated death domain (FADD)-like interleukin-1-converting enzyme (FLICE) inhibitory protein, (*d*) activation of caspase-8, and (*e*) a decrease of the mitochondrial transmembrane potential. The reducing agents, *N*-acetyl-L-cysteine, DTT, and catalase inhibited each of these CDDO-Im-induced proapoptotic signals. Inhibition of caspase-8 with z-IETD-fmk also abrogated CDDO-Im-induced decreases of the mitochondrial transmembrane potential and inhibited apoptosis. These results demonstrate that CDDO-Im disrupts intracellular redox balance and thereby activates the extrinsic caspase-8-dependent apoptotic pathway. We further show that CDDO-Im induces apoptosis of primary MM cells at submicromolar concentrations and that MM cells are more sensitive to this agent than normal bone marrow mononuclear cells. These results suggest that CDDO compounds have potential as new agents for the treatment of MM. [Mol Cancer Ther. 2004;3(1):39–45]

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Introduction

Multiple myeloma (MM) is a B-cell malignancy that is characterized by an excess of monotypic plasma cells in the bone marrow. MM is associated with monoclonal protein in serum and/or urine, decreased normal immunoglobulin levels, and lytic bone disease (1). Conventional chemotherapies, consisting of combinations of alkylating agents, steroids, plant alkaloids, and anthracyclines, can extend patient survival to an average of 3–4 years (1). High-dose chemotherapy with autologous stem cell transplantation can prolong survival to 4–5 years (1). However, MM remains largely incurable because of the emergence of chemoresistance. Increased levels of Bcl-x_L have been found in chemoresistant MM cell lines and samples from refractory MM patients (2, 3). Chemoresistant MM cells have also been reported to inactivate anticancer drugs more efficiently than chemosensitive MM cells (4, 5). In this regard, increases in the expression of glutathione (GSH) or the activity of GSH-related enzymes in MM cells has been associated with resistance to anticancer drugs (5). Novel treatment approaches that overcome such chemorefractory mechanisms may therefore be effective in the treatment of MM.

2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a synthetic oleanane triterpenoid. CDDO inhibits proliferation of various human tumor cell lines and induces monocytic differentiation and apoptosis of human myeloid leukemia cells (6, 7). Two major pathways have been identified for the induction of apoptosis. The intrinsic pathway is activated by release of mitochondrial cytochrome *c* (8–10). Cytochrome *c* forms a complex with Apaf-1 and activates caspase-9 leading activation of caspase-3 (11, 12). The extrinsic pathway is initiated by activation of death receptors and thereby cleavage of procaspase-8 (13, 14). Caspase-8 directly activates caspase-3 (15). Caspase-8 also cleaves Bid and thereby plays a role in the release of cytochrome *c* (16, 17). The available evidence indicates that CDDO-induced apoptosis is mediated by the extrinsic caspase-8 pathway, while conventional cytotoxic anticancer drugs activate the intrinsic pathway (18–21). The precise mechanisms responsible for CDDO-induced activation of the extrinsic pathway are, however, not clear.

In the present study, we analyzed the effects of the CDDO C-28 imidazolide ester (CDDO-Im) on MM cells. CDDO-Im is a potent inducer of apoptosis in MM cell lines and primary MM cells. The results also demonstrate that CDDO-Im induces GSH depletion and activates caspase-8 through disruption of intracellular redox balance.

Materials and Methods

Cells and Reagents

Human U266 and RPMI8226 myeloma cells (American Type Culture Collection, Manassas, VA) were maintained

in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were suspended at a density of 5×10^5/ml and then treated with CDDO or CDDO-Im. In certain experiments, 10 mM *N*-acetyl-L-cysteine (NAC), 0.5 mM DTT, 1 mM cystine (oxidized cysteine; all from Sigma, St. Louis, MO), or caspase-8 inhibitor (z-IETD-fmk; R&D Systems, Inc., Minneapolis, MN) were added to the culture media at 1 h before treatment with CDDO or CDDO-Im. Catalase (1000 units/ml; Sigma) was added to the culture media 3 h before CDDO-Im treatment. Bone marrow samples were obtained from MM patients with informed consent under Institutional Review Board (IRB) approval.

Determination of Cell Viability

Cell viability was assessed using 3-(4,5-dimethyl-2, 5-diphenyl-2*H* tetrazolium bromide (MTT; Sigma) as described (22).

Detection of Apoptotic Cells

Sub-G_1 content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by a FACScan flow cytometer (Becton Dickinson and Co., Lincoln Park, NJ). In studies of clinical samples, bone marrow mononuclear cells were isolated by gradient centrifugation and treated with CDDO or CDDO-Im. After triple staining with phycoerythrin (PE)-conjugated anti-CD38 antibody (Coulter Beckman, Hialeah, FL), allophycocyanin (APC)-conjugated anti-CD45 antibody (Coulter Beckman), and FITC-conjugated Annexin-V (Clontech, Polo Alto, CA), cells were analyzed by flow cytometry. The $CD38^{bright}/CD45^{low}$ population was analyzed as the MM cell fraction (23).

Measurement of Reactive Oxygen Species Levels

Cells were incubated with 25 μM 5- (and -6)-carboxy-2′,7′-dihydrodihydrofluorescein diacetate (c-H_2DCFDA; Molecular Probes, Eugene, OR) for 3 h at 37°C to assess reactive oxygen species (ROS)-mediated oxidation to the fluorescent compound c-H_2DCF (24). After washing twice, the cells were resuspended in PBS and analyzed by flow cytometry (FL1 channel). Ten thousand cells were analyzed by flow cytometry (Becton Dickinson).

Determination of GSH Levels

Cells were stained with 200 μM monochlorobimane (mBCI; Molecular Probes) for 30 min and analyzed by flow cytometry as described (25).

Analysis of Mitochondrial Transmembrane Potential

Cells were incubated with 0.5 nM 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$ [3]; Molecular Probes) for 30 min and analyzed by flow cytometry as described (26).

Immunoblot Analysis

Total cell lysates were prepared as described (27). Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with anti-Fas-associated death domain (FADD)-like interleukin-1-converting enzyme (FLICE) inhibitory protein (FLIP; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), anti-caspase-3 (Santa Cruz Biotechnologies), anti-caspase-8 (BD Biosciences PharMingen, San Diego, CA), or anti-β-actin (Santa Cruz Biotechnologies) antibodies. After incubation with horseradish peroxide-conjugated second antibody, the immune complexes were visualized by an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, United Kingdom).

Results

MM Cells Respond to CDDO and CDDO-Im with Induction of Apoptosis

To assess the sensitivity of MM cells to CDDO and CDDO-Im, we performed MTT assays on the U266 and RPMI8226 human MM cell lines. CDDO and CDDO-Im induced death of both cell lines at submicromolar concentrations (Fig. 1A). The IC_{50}s of CDDO and CDDO-Im for the U266 cells were approximately 0.7 and 0.35 μM, respectively (Fig. 1A). Similar results were obtained with the RPMI8226 cells (Fig. 1A), indicating that the CDDO-Im derivative is more potent than CDDO. The demonstration that CDDO-Im-induced cell death is associated with the induction of sub-G_1 DNA (Fig. 1B) indicated that, as shown for CDDO-induced cell death of other cells (6, 18, 19), CDDO-Im induces apoptosis of MM cells. As determined by MTT assays of cell death, the induction of apoptosis was dose dependent (Fig. 1C).

CDDO-Im Decreases Intracellular GSH and Increases ROS Levels

Recent studies have shown that CDDO treatment of U-937 myeloid leukemia cells is associated with substantial decreases of GSH levels (28). To assess the effects of CDDO-Im on GSH levels in MM cells, the U266 and RPMI8226 cells were treated, incubated with mBCI, and analyzed by flow cytometry. CDDO-Im treatment of U266 cells was associated with decreases in GSH levels which were detectable by 3 h and more pronounced at 6 h (Fig. 2A). Similar findings were obtained with CDDO-Im treatment of RPMI8226 cells (Fig. 2A). To determine if CDDO-Im treatment is associated with changes in intracellular ROS levels, we measured oxidation of c-H_2DCFDA. The results obtained with both U266 and RPMI8226 cells demonstrate an increase in c-H_2DCF fluorescence at 1 h, and further increases at 3 h (Fig. 2B). To extend these findings, cells were preincubated with the antioxidant, NAC, for 1 h before CDDO-Im treatment. The results show that NAC blocks CDDO-Im-induced increases in ROS levels (Fig. 2C).

CDDO-Im-Induced Loss of Mitochondrial Transmembrane Potential Is Blocked by Antioxidants

Intracellular GSH status is associated with mitochondrial permeability (29), and decreases in mitochondrial transmembrane potential ($\Delta\Psi_m$) are associated with release of cytochrome *c* and induction of apoptosis (30). We therefore analyzed CDDO-Im-induced changes in $\Delta\Psi_m$ by monitoring $DiOC_6$ [3] fluorescence with flow cytometry. A decrease in $\Delta\Psi_m$ was detectable at 3 h and further decreased at 6 h of CDDO-Im treatment (Fig. 3A). In concert with a prooxidant effect, the

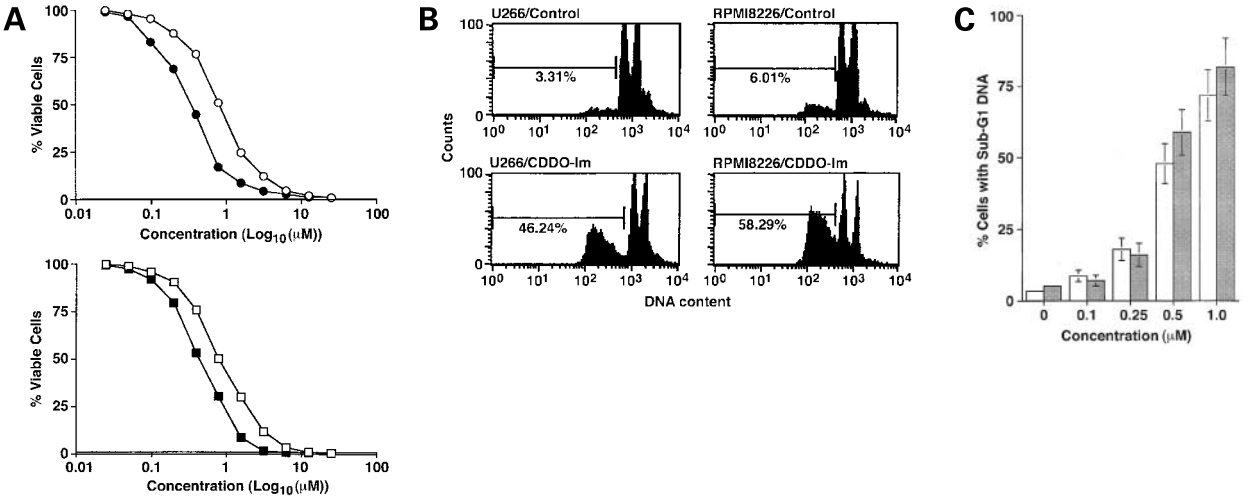

Figure 1. Apoptotic response of MM cells to CDDO and CDDO-Im. **A,** U266 (*upper panel*) and RPMI8226 (*lower panel*) cells were treated with CDDO (○, □) or CDDO-Im (●, ■) for 24 h at the indicated concentrations. The results are presented as the relative $A_{570\ nm}$ compared with non-treated cells. Similar results were obtained in three independent experiments. **B,** U266 and RPMI8226 cells were treated with 0.5 μM CDDO-Im for 24 h. DNA content was analyzed by flow cytometry. The percentage of cells with sub-G_1 DNA is shown for control and CDDO-Im-treated cells. **C,** U266 (*open bars*) and RPMI8226 (*shaded bars*) cells were exposed to CDDO-Im for 24 h at the indicated concentrations. DNA content was analyzed by flow cytometry. *Columns,* percentage apoptosis of three independent experiments; *bars,* SD.

decreases in $\Delta\Psi_m$ induced by CDDO-Im were blocked by pretreatment with NAC (Fig. 3B). The antioxidant, DTT, also blocked CDDO-Im-induced decreases in $\Delta\Psi_m$ (data not shown).

Prooxidant Effects of CDDO-Im Induce Caspase Activation

Recent work has demonstrated that CDDO treatment is associated with caspase-8 activation (18–21). The present studies demonstrate that treatment of MM cells with CDDO-Im also induces caspase-8 cleavage (Fig. 4A). Activation of caspase-8 was detectable at 3 h of CDDO-Im treatment in both cell lines (Fig. 4A). Moreover, the kinetics of caspase-3 activation were similar to those obtained for caspase-8 (Fig. 4A). To determine whether CDDO-Im-induced caspase activation is related to the prooxidant effects, cells were pretreated with NAC or DTT. The results demonstrate that NAC and DTT block CDDO-Im-induced activation of caspase-8 (Fig. 4B). The results also show that NAC and DTT abolish CDDO-Im-induced activation of caspase-3 (Fig. 4B). These findings demonstrate that CDDO-Im activates caspase-8 by redox-related mechanisms.

CDDO-Im-Induced FLIP Down-Regulation Is Mediated by Disruption of Redox Balance

Caspase-8 activation is regulated by FLIP (31). In this context, down-regulation of FLIP has been proposed as a potential mechanism for caspase-8 activation in CDDO treatment (20). We therefore asked if CDDO-Im treatment of MM cells is also associated with decreases in FLIP expression. Decreases in both the long and short forms of FLIP were detectable at 1.5 h after exposure to CDDO-Im and continued to decline over time (Fig. 5A). Importantly, preincubation with NAC or DTT blocked CDDO-Im-induced FLIP reduction (Fig. 5B). These findings demonstrate that CDDO-Im-induced FLIP reduction is in response to the disruption of intracellular redox balance.

Antioxidants Inhibit CDDO-Im-Induced Apoptosis

On the basis of these findings, we next analyzed the effects of antioxidants on CDDO-Im-induced MM cell apoptosis. Pretreatment of U266 cells with NAC or DTT blocked CDDO-Im-induced apoptosis (Fig. 6A). Addition

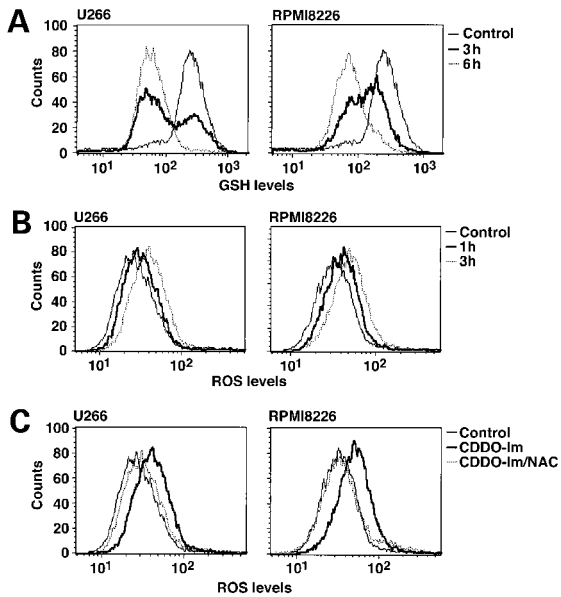

Figure 2. CDDO-Im depletes intracellular GSH and increases ROS production. **A,** GSH levels were analyzed by flow cytometry after incubation with 0.5 μM CDDO-Im for the indicated times and staining with mBCI. The data shown are representative of three independent experiments. **B,** cells were treated with CDDO-Im for 1 and 3 h. The fluorescence of oxidized c-H_2DCF was determined by flow cytometry. Similar results were obtained from three independent experiments. **C,** cells were preincubated with NAC and then exposed to CDDO-Im for 3 h. The data shown are representative of three independent experiments.

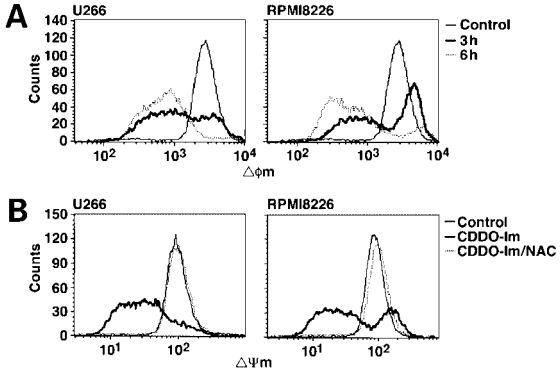

Figure 3. CDDO-Im-induced loss of mitochondrial transmembrane potential is blocked by NAC. **A,** cells were treated with 0.5 μM CDDO-Im for the indicated times and then stained with $DiOC_6$ [3]. The fluorescence of $DiOC_6$ [3] was measured by flow cytometry. The data shown are representative of three independent experiments. **B,** cells were preincubated with NAC and then treated with CDDO-Im for 6 h. The data shown are representative of three independent experiments.

of catalase to the culture medium also inhibited CDDO-Im-induced apoptosis (Fig. 6A). Similar results were obtained with the RPMI8226 cells (Fig. 6A). As a control, pretreatment with cystine (oxidized cysteine) had no effect (Fig. 6A). To confirm the association of caspase-8 activation with CDDO-Im-induced apoptosis, cells were treated with CDDO-Im in the presence of a caspase-8 inhibitor. z-IETD-fmk inhibited CDDO-Im-induced apoptosis of both U266 and RPMI8226 cells in a dose-dependent manner (Fig. 6B). Moreover, z-IETD-fmk blocked CDDO-Im-induced decreases of $\Delta\Psi_m$ (Fig. 6C), demonstrating that caspase-8 activation is responsible for CDDO-Im-induced loss of $\Delta\Psi_m$. These findings indicate that CDDO-Im induces apoptosis by a prooxidant-mediated activation of caspase-8.

CDDO and CDDO-Im Selectively Induce Apoptosis of Primary MM Cells

To assess the effectiveness of CDDO and CDDO-Im in the induction of primary MM cell apoptosis, mononuclear cells obtained from patients with MM were treated with CDDO or CDDO-Im. Apoptosis of the MM cell ($CD38^{bright}$/$CD45^{low}$) and non-MM mononuclear cell (CD38-negative) populations was assessed by Annexin V staining. The results demonstrate that CDDO-Im treatment of MM cells is associated with over 40% apoptosis (Fig. 7). Similar results were obtained with CDDO-Im (Fig. 7A, *left panel*). By contrast, apoptosis of the non-MM cell population was less than 20% (Fig. 7A, *right panel*). Similar results were obtained when cells from five MM patients were treated with CDDO or CDDO-Im (Fig. 7B). To determine if CDDO and CDDO-Im induce apoptosis of primary MM cells by disruption of redox balance, exposure to these agents was performed after preincubation with NAC. As shown for the MM cell lines, NAC blocked the induction of apoptosis (Fig. 7B). These findings demonstrate that CDDO and CDDO-Im selectively induce primary MM cell apoptosis by a redox-mediated mechanism.

Discussion

CDDO-Im Disrupts Redox Balance in MM Cells

Our results demonstrate that CDDO-Im is a potent inducer of MM cell apoptosis and that ROS production is of importance for this response. CDDO-Im-induced increases in ROS were detectable at 1 h and continued to increase for 3 h of treatment. Antioxidants blocked (*a*) activation of caspase-8 and caspase-3, (*b*) decreases of $\Delta\Psi_m$, and (*c*) induction of apoptosis. Intracellular ROS generation has been associated with various apoptotic processes (32). As protection against ROS-induced toxicity, ROS are scavenged by the redox-related enzymes, such as GSH, catalase, superoxide dismutase, and thioredoxin (33). Disruption of these enzymatic antioxidant defense systems can thus contribute to increases in ROS (33, 34). The present study demonstrates that CDDO-Im both decreases intracellular GSH levels and increases ROS levels. Although further studies are needed, GSH depletion could be the major mechanism responsible for CDDO-Im-induced ROS generation.

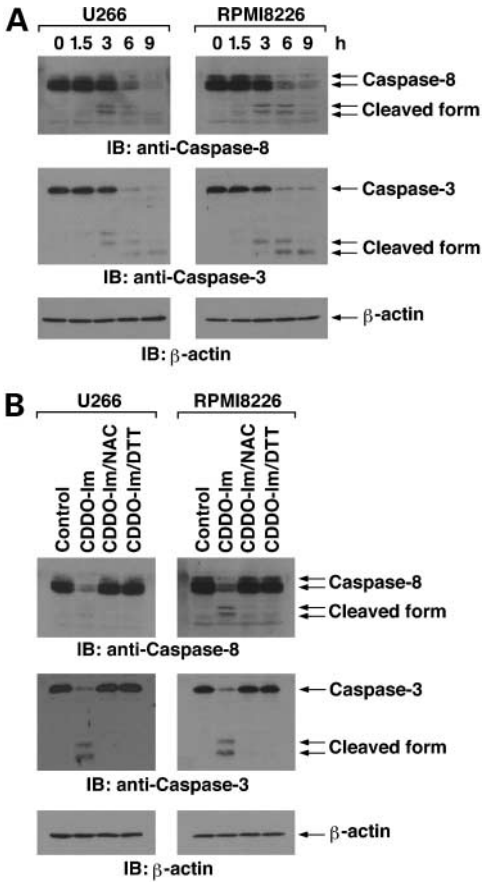

Figure 4. Prooxidant effects of CDDO-Im induce caspase-8 and caspase-3 activation. **A,** cells were treated with 0.5 μM CDDO-Im for the indicated times. **B,** cells were preincubated with NAC or DTT and then treated with CDDO-Im for 6 h. Total cell lysates were subjected to immunoblotting with the anti-caspase-8, anti-caspase-3, and anti-β-actin antibodies. The results are representative of three independent experiments.

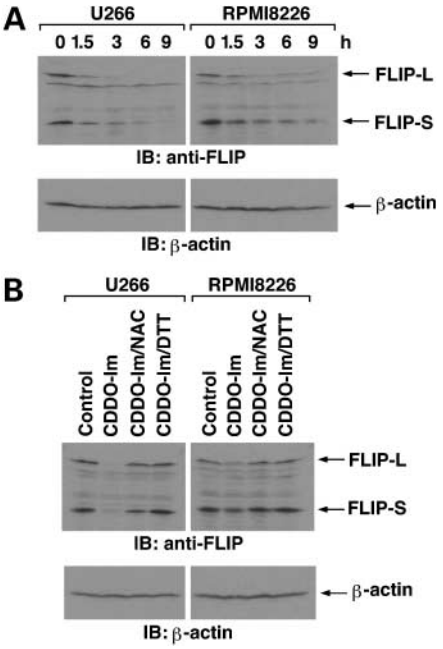

Figure 5. CDDO-Im-induced FLIP reduction is mediated by disruption of redox balance. **A,** cells were treated with 0.5 μM CDDO-Im for indicated times. **B,** cells were preincubated with NAC or DTT and then treated with CDDO-Im for 6 h. Total cell lysates were subjected to immunoblotting with the anti-FLIP and anti-β-actin antibodies. The results are representative of three independent experiments.

GSH as a Target in Anticancer Treatment

Depletion of GSH is an early event in the apoptotic process, and itself can lead to or increase sensitivity to apoptosis (25, 35, 36). GSH conjugates and thereby inactivates agents, such as anticancer drugs, that generate ROS. In this regard, the toxicity of antitumor drugs has been shown to be dependent on intracellular GSH levels. For example, depletion of GSH by buthionine sulfoximine (BSO) increases the lethality of etoposide in different cell types (37, 38). GSH peroxidase is expressed at constitutively lower levels in many malignant cells that are sensitive to arsenic, and lowering of GSH increases sensitivity to arsenite in cells (39). The present results demonstrate that CDDO-Im induces rapid and substantial GSH depletion in MM cells. The results also indicate that the effects of CDDO-Im on GSH levels contribute to CDDO-Im-induced cytotoxicity.

Certain drugs can bind intracellular GSH and thereby decrease GSH levels. For example, the trivalent arsenic compound complexes with GSH, forming transient $As(GS)_3$ molecules in arsenic trioxide treatment (40). Thus, CDDO-Im could also bind to intracellular GSH and thereby decrease its levels. However, the demonstration that both NAC and DTT protect cells from CDDO-Im-induced cytotoxicity supports another mechanism. Both NAC and DTT contain thiol groups; therefore, CDDO-Im may bind to thiols and thiol-containing molecules which are recognized as key components involved in the maintenance of redox balance (34). Importantly, catalase, which acts as an antioxidant by converting H_2O_2 to H_2O and O_2, also inhibited CDDO-Im-induced apoptosis. These results indicate that ROS production and thereby GSH depletion contributes to CDDO-Im-induced MM cell apoptosis.

CDDO-Im Induces Caspase-8 Activation

CDDO-induced apoptosis is associated with activation of caspase-8 (18–21). Caspase-8-mediated cleavage of Bid has also been proposed as a mechanism for CDDO-induced mitochondrial disruption (18, 19, 21). Activation of Bax can also contribute to CDDO-induced decreases of $\Delta\Psi_m$ (21, 41). In the present study, z-IETD-fmk remarkably inhibited CDDO-Im-induced decreases of $\Delta\Psi_m$ and apoptosis. These results suggest that activation of caspase-8 is an important event in CDDO-Im-induced apoptosis of MM cells. It is therefore possible that CDDO-Im activates the extrinsic pathway and circumvents resistance associated with defects in the intrinsic pathway. Importantly, antioxidants blocked CDDO-Im-induced caspase-8 activation and apoptosis. These findings suggest that disruption of intracellular redox balance is responsible for CDDO-Im-induced activation of caspase-8.

Down-regulation of FLIP has recently been proposed as one of the mechanisms responsible for activation of caspase-8 in response to CDDO treatment (20). The down-regulation of FLIP is regulated by ubiquitination and proteosomal degradation (42). In the present study, we show that CDDO-Im decreases FLIP expression. We

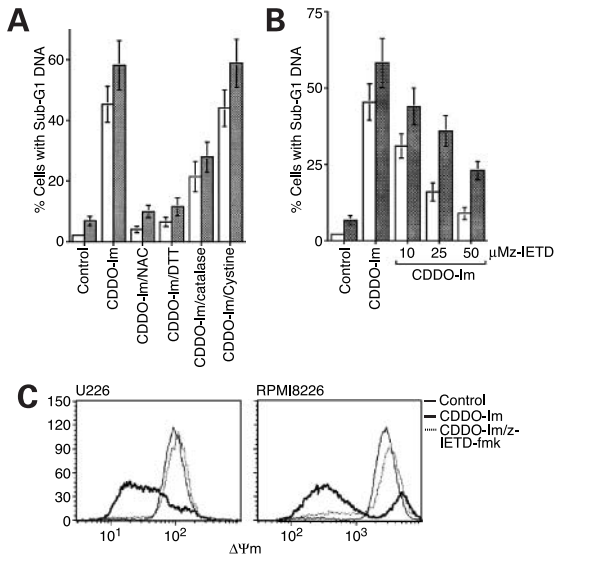

Figure 6. Antioxidants inhibit CDDO-Im-induced apoptosis. **A,** U266 (*open bars*) and RPMI8226 (*shaded bars*) cells were preincubated with NAC, DTT, catalase, or cystine and then treated with 0.5 μM CDDO-Im for 24 h. **B,** cells were preincubated with z-IETD-fmk at indicated concentrations and then treated with CDDO-Im for 24 h. DNA content was analyzed by flow cytometry. *Columns,* percentage apoptosis of three independent experiments; *bars,* SD. **C,** cells were preincubated with 50 μM z-IETD-fmk and then treated with CDDO-Im for 6 h. After staining with $DiOC_6$ [3], fluorescence was measured by flow cytometry. The data shown are representative of three independent experiments.

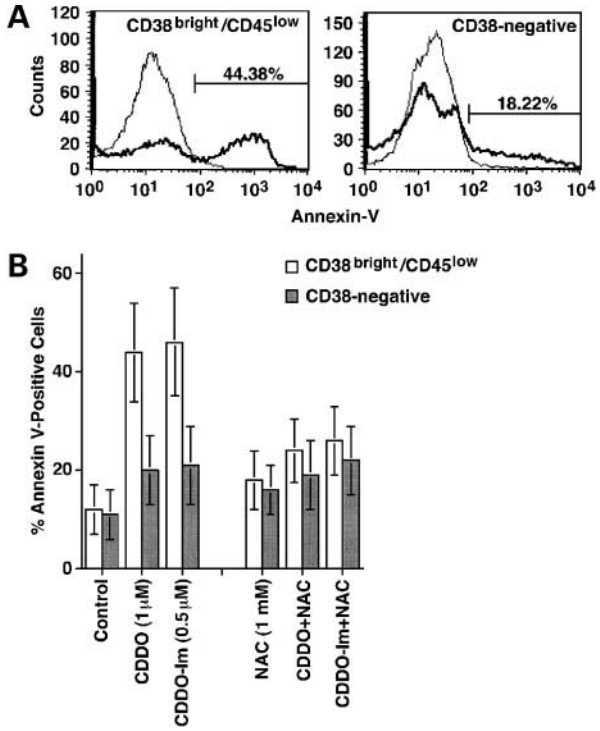

Figure 7. CDDO and CDDO-Im selectively induce apoptosis of primary MM cells. **A,** mononuclear cells were obtained from patients with MM and treated with 0.5 μM CDDO-Im for 6 h. Cells were triple stained with APC-conjugated CD45 antibody, PE-conjugated CD38 antibody, and FITC-conjugated Annexin V, and analyzed by flow cytometry. Annexin V staining patterns are shown for control (untreated; *thin lines*) or CDDO-Im-treated (*thick lines*) populations of MM (*CD38^{bright}/CD45^{low}*; *left panel*) or non-MM (*CD38-negative*; *right panel*) cells. **B,** mononuclear cells from five patients with MM were treated with 1 μM CDDO or 0.5 μM CDDO-Im for 6 h and then analyzed as above for Annexin V staining. The mononuclear cells were also preincubated with 1 mM NAC for 1 h and then treated with CDDO or CDDO-Im. *Columns,* percentage (mean of five patient samples) of Annexin V-positive cells in the MM (*CD38^{bright}/CD45^{low}*; *open bars*) or non-MM (*CD38-negative*; *shaded bars*) cell populations; *bars,* SD.

also demonstrate that ROS production is responsible for down-regulation of FLIP. The demonstration that ROS regulate FLIP in Fas-induced apoptosis of cardiac myocytes lends support to our findings (43). However, down-regulation of FLIP with an antisense oligonucleotide is insufficient to induce apoptosis in chronic leukemia cells (20). Thus, additional redox-mediated mechanisms are probably responsible for CDDO-Im-induced activation of caspase-8.

CDDO and CDDO-Im Induce Apoptosis of Primary MM Cells

On the basis of the data from MM cell lines, we asked whether CDDO-Im induces cell death in primary MM cells. Both CDDO and CDDO-Im induced apoptosis of primary MM cells at submicromolar concentrations. In addition, MM cells were more sensitive to the cytotoxic effects of CDDO and CDDO-Im as compared to that for non-plasma cells. The demonstration that NAC blocks CDDO- and CDDO-Im-induced apoptosis indicates that, like MM cell lines, primary MM cells respond to these agents with disruption of redox balance. These findings indicate that CDDO and CDDO-Im selectively induce MM cell apoptosis by a ROS-dependent mechanism, and support the study of these compounds in MM treatment.

Most MM patients ultimately progress to a refractory state and succumb to their disease. High levels of Bcl-x_L expression have been reported in chemoresistant MM cell lines and samples from refractory MM patients (2, 3). In addition, increases in GSH or activity of GSH-related enzymes confer resistance of MM cells to anticancer drugs (5). The results of the present study indicate that CDDO and CDDO-Im could overcome Bcl-x_L-mediated resistance by the activation of extrinsic caspase-8 pathway. Moreover, our results indicate that CDDO and CDDO-Im could reverse resistance mechanisms mediated through GSH by down-regulating GSH levels.

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