2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolidine (CDDO-Im) Directly Targets Mitochondrial Glutathione to Induce Apoptosis in Pancreatic Cancer*

Received for publication, July 12, 2005, and in revised form, August 18, 2005. Published, JBC Papers in Press, August 23, 2005. DOI 10.1074/jbc.M507518200

Ismael Samudio†, Marina Konopleva‡, Numsen Hall, Jr.†, Yue-Xi Shi†, Teresa McQueen†, Timothy Hsu‡, Randall Evans†, Tadashi Honda†, Gordon W. Gribble†, Michael Sporn‡, Hiram F. Gilbert∗, and Michael Andreeff† 1

From the †Section of Molecular Hematology and Therapy, Department of Blood and Marrow Transplantation, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, the ‡Department of Chemistry, Dartmouth College and the ¶Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755, the ††Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030, and the **Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas 77843

Pancreatic cancer (PC) is a lethal disease characterized by a high metastatic potential and rapid progression with a median survival rate of only 24 weeks in untreated cases (1, 2). Tumor resection is still the most effective treatment for PC. However, because of local invasion and/or metastasis, only ~15–20% of PC patients qualify for surgical intervention. For locally advanced, unresectable, and metastatic disease, treatment is palliative at best and usually consists of 5-fluorouracil or gemcitabine alone or in combination with radiotherapy. Unfortunately, despite the moderate success of gemcitabine (2',2'-difluorodeoxyuridine), median survival rates remain under 6 months for patients with metastatic disease (2–4). Given the poor performance of existing therapies, the development of novel therapies for the treatment of advanced metastatic pancreatic cancer is of utmost importance.

Glutathione (GSH) is a critical intracellular antioxidant that scavenges reactive oxygen species (ROS) directly and indirectly by serving as cofactor for glutathione peroxidases, glutaredoxins, and lipid peroxidases (5,6). Oxidized GSH (GSSG) must be converted back to GSH by the action of glutathione reductase, an enzyme that utilizes the pyridine nucleotide NADPH as a cofactor. The ratio of GSH/GSSG, a direct measure of the redox status of the cells, decreases during cell death due to NADPH oxidation and GSSG extrusion, and the exogenous addition of GSSG has been demonstrated to trigger apoptosis (7–9). In addition, electrophilic agents like diethylmaleate, N-ethylmaleimide, and ethacrynic acid form adducts with GSH and induce apoptosis, suggesting that cell death can also be triggered in the absence of pyridine nucleotide oxidation by directly decreasing GSH levels (10–12). Interestingly, mitochondria are unable to synthesize GSH, thus depending on its import from the cytosol by the action of an unidentified carrier system (13). Maintenance of adequate mitochondrial GSH (GSHm) levels is critical for scavenging ROS produced during mitochondrial metabolism as well as for prevention of apoptosis (14). Accordingly, depletion of GSHm has been shown to sensitize cells to apoptosis induced by tumor necrosis factor α and ceramide, and conversely, artificially increasing GSHm levels abrogates this effect (15,16). Furthermore, GSH has been reported to directly modulate the opening of the mitochondrial permeability transition pore (PTP) and apoptosis by interacting with a dithiol protein site (17,18), suggesting that GSHm also serves to maintain

Surgical resection is the only curative strategy for pancreatic cancer (PC). Unfortunately, >80% of pancreatic cancer patients bear inoperable, locally advanced, chemoresistant tumors demonstrating the urgent need for development of novel therapeutic approaches to treat this disease. Here we report that the synthetic triterpenoid 2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolidine (CDDO-Im) antagonizes PC cell growth by inducing apoptosis at submicromolar concentrations. Notably, we demonstrate for the first time that the cytotoxicity of CDDO-Im is accompanied by the rapid and selective depletion of mitochondrial glutathione that results in accumulation of reactive oxygen species, oxidation of the cellular glutathione pool, loss of mitochondrial membrane potential, and phosphatidylserine externalization. The parent compound CDDO as well as the methyl ester of CDDO also depleted mitochondrial glutathione, demonstrating that this effect is mediated by the triterpenoid nucleus of these agents. Cotreatment with sulfhydryl nucleophiles completely prevented apoptosis and loss of viability induced by CDDO-Im, whereas alklylation of intracellular thiols by diethylmaleate or cotreatment with dithiothreitol decreased the accumulation of a biotinylated derivative of CDDO, TP-301, in PC cells, suggesting that intracellular reduced thiols are functional targets of the electrophilic triterpenoid nucleus of CDDO and its derivatives. In conclusion, our report is the first to identify mitochondrial glutathione as a target of CDDO and its derivatives and demonstrates that depletion of this antioxidant in the mitochondria is an effective strategy to induce cell death in PC cells. These results suggest that CDDO and its derivatives may offer a clinical benefit for the treatment of PC.
critical PTP sulfhydryl groups in the reduced state. Taken together, these findings suggest that targeting GSH and/or GSHm levels may be an effective therapeutic strategy to induce cell death or sensitize cells to apoptotic stimuli.

The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is effective in inducing apoptosis in a variety of tumor cell types including leukemia (19–22), multiple myeloma (23, 24), breast (25), lung (26), osteosarcoma (27), and skin (28). Furthermore, recent reports demonstrate that the C28 imidazolide derivative of CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im), is 5-fold more potent than CDDO as an antitumor agent in vivo and in vitro (29, 30). CDDO and CDDO-Im reportedly disrupt intracellular redox balance in multiple myeloma cells, thereby activating the extrinsic apoptotic pathway, and exhibit some selectivity in apoptosis induction between tumor and normal cells (23, 24). In addition, a recent report demonstrated that CDDO induced cytchrome c release in isolated mitochondria, suggesting that this agent directly targets the mitochondria to activate the intrinsic apoptotic pathway (21).

Here we report for the first time that the cytotoxic effects of CDDO-Im in PC cells are mediated by perturbations in GSHm, leading to caspase-independent apoptosis. Our results demonstrate that CDDO-Im rapidly and selectively decreases GSHm and that this occurs prior to loss in mitochondrial membrane potential and phosphatidylserine (PS) externalization. Furthermore, we demonstrate that alkyla-
tion of intracellular sulfhydryls or cotreatment with reducing agents abrogates the accumulation of a biotinylated derivative of CDDO in PC cells, suggesting that reduced sulfhydryls are direct molecular targets of this agent. Thus, we hypothesize that CDDO-Im may be effective in treating PC by triggering apoptosis in PC cells via perturbations in GSHm.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Biochemicals**—CDDO-Im and the biotinylated derivative of CDDO, TP-301, were synthesized as described (31, 32). 2-Mercaptoethanol (BME), glutathione, dithiothreitol (DTT), ascorbic acid, and diethylmaleate (DEM) were purchased from Sigma. The annexin V-fluorescein isothiocyanate conjugate and the WST reagent were obtained from Roche Applied Science. 5-(and-6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), MitoTracker CMXRos, and MitoTracker Green were all obtained from Invitrogen. The calpain inhibitor E64d was obtained from Peptide Institute Inc. (Osaka, Japan), z-VAD-fmk was from Alexis Biochemicals (Axxora LLC, San Diego, CA), antibodies specific for caspase 3, caspase 8, and caspase 9 were from Cell Signaling Technologies (Beverly, MA), and the anti-poly(ADP-ribose) polymerase 1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used were of the highest purity available.

**Cell Culture**—PANC1 and COLO357 cells were a generous gift of Dr. Paul Chiao (The University of Texas M. D. Anderson Cancer Center). Both cell lines were grown in RPMI supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The concentrations of CDDO-Im and other agents used and the treatment intervals are indicated below and under “Results.” Cells were maintained in a humidified 5% CO2 atmosphere at 37 °C.

**Biochemical Assay for GSH and GSSG**—Briefly, 5 × 10^6 to 1.5 × 10^7 cells were cultured under normal conditions and treated as indicated. Cells were then collected, washed twice in ice-cold PBS, and resuspended in 55 μl 10 mM HCl. After incubation on ice for 5 min, insoluble cellular debris was spun down, and protein concentration was measured from 5 μl of supernatant using a commercially available BCA protein kit. The remaining supernatant was mixed with 50 μl of 10% metaphosphoric acid and incubated at room temperature for 5 min. Proteins were precipitated at 12,000 × g for 5 min, and the supernatant was neutralized by the addition of 10 μl of a 4 M triethanolamine solution. 25 μl of the supernatant were derivatized for exclusive measurement of GSSG by the addition of 2.5 μl of a 1 mM ethanolic solution of 2-vinylpyridine and incubated at room temperature, and GSH concentrations were calculated from a standard curve of GSSG. Results are expressed as picomoles of GSH per microgram of protein. The GSH/GSSG ratio is calculated as follows: [(pmol of GSH in untreated supernatant) − (pmol of GSH in 2-vinyl pyridine-treated supernatant)]/(pmol of GSH in untreated supernatant).

For measurement of GSH and GSSG in the mitochondrial fraction, mitochondria were isolated from 1.5 × 10^6 cells by density centrifugation using a commercially available kit (Pierce) and washed twice in isolation buffer (75 mM KCl, 10 mM EDTA, and 250 mM sucrose, pH 7.4), and mitochondrial pellets were resuspended in 50 μl of 10 mM HCl. Protein concentrations and total GSH content were measured as described above. These experiments were conducted in duplicate and repeated at least three times. Results presented are means ± S.D. of representative experiments.

**Flow Cytometric Analysis of Annexin V Positivity**—After appropriate treatments, cells were harvested by trypsinization and washed twice in annexin binding buffer (5 mM CaCl2, 140 mM NaCl, and 1 mM Heps, pH 7.4). Cells were then resuspended in 100 μl of annexin V binding buffer containing a 1:30 dilution of annexin V-fluorescein isothiocyanate conjugate and 1 μg/ml propidium iodide and incubated in the dark for 15 min. Cells were then washed twice with annexin binding buffer and analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences) using a 488-nm argon excitation laser. Results presented are means ± S.E. of three independent experiments.

**Measurement of Mitochondrial Membrane Potential (ΔΨm)**—After appropriate treatments, cells were harvested by trypsinization and washed twice in PBS. Cells were then resuspended in 100 μl of PBS containing 0.5 μg/ml MitoTracker CMXRos and 15 ng/ml MitoTracker Green and incubated at 37 °C for 45 min. Cells were then washed twice in PBS and analyzed by flow cytometry in a FACSCalibur flow cytometer using a 488-nm argon excitation laser. Results presented are means ± S.E. of three independent experiments.

**Measurement of ROS Generation**—COLO357 and PANC1 cells were treated with CDDO-Im for 30 min. Cells were then harvested by centrifugation, washed twice with PBS, and loaded with the ROS-sensitive probe CM-H2DCFDA (Molecular Probes, Eugene OR). Cells were incubated at 37 °C for 30 min and washed twice in PBS, and FL1 fluorescence was examined by flow cytometry on a FACSCalibur flow cytometer using a 488-nm argon excitation laser. Results presented are means ± S.E. of three independent experiments.

**DNA Fragmentation Assay**—COLO357 and PANC1 cells were seeded in 100-mm tissue culture dishes at 1.5 × 10^6 cells/plate. 24 h after seeding, cells were treated for an additional 36 h with various concentrations of CDDO-Im. Cells were then harvested by centrifugation, washed in ice-cold PBS twice, and resuspended in 500 μl of sonication buffer (1% SDS, 1 mM EDTA, and 50 mM Tris-Cl, pH 8.0). Cells were homogenized by passing through a 22-gauge needle and incubated in ice for 1 h. Insoluble material was precipitated, and the supernatant
was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) once to remove excess protein. DNA was then purified using the Qiagen PCR purification kit (Valencia, CA) according to the manufacturer’s instructions. DNA was run on a 1.8% agarose gel prestained with ethidium bromide and photographed under a UV light source.

**Western Blotting**—COLO357 and PANC1 cells were treated with 1 and 1.5 μM CDDO-Im, respectively, for 14 h. Cells were then harvested by centrifugation, washed twice with PBS supplemented with 1 mM phenylmethylsulfonyl fluoride, and resuspended in ice cold lysis buffer (1% Triton X-100, 45 mM KCl, and 10 mM Tris, pH 7.5). Cells were homogenized by passing through a 22-gauge needle and incubated on ice for 20 min. Insoluble material was precipitated by centrifugation at 12,000 g for 15 min, and supernatants were assayed for protein concentration using the BCA kit from Pierce. 25 μg of protein were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to Hybond-P membranes (Amersham Biosciences) followed by immunoblotting. Signals were detected using a PhosphorImager (Storm 860, version 4.0; Amersham Biosciences).

**RESULTS**

**CDDO-Im Inhibits PC Cell Growth In Vitro by Inducing Apoptosis**—A previous report suggested that CDDO was effective in decreasing the proliferation of PC cells in culture, but neither the mechanism of action nor the effect of the more potent CDDO derivatives was investigated (33). Therefore, we investigated the effects of CDDO-Im on the growth of PC cells. The results shown in Fig. 1, A and B indicate that CDDO-Im significantly prevents the growth of COLO357 and PANC1 cells at nanomolar doses with 96-h IC50 values of 167 and 396 nM, respectively (p < 0.01). Furthermore, the results in Fig. 1, C and D demonstrate that the observed inhibition of cell growth is accompanied by increased PS exposure (96-h EC50 = 250 and 419 nM, respectively), suggesting that apoptosis mediates the growth inhibitory effects of CDDO-Im. To further investigate the growth inhibitory mechanism of CDDO-Im, we examined the short term viability of PC cells treated with this synthetic triterpenoid by measuring PS exposure and ΔΨm. As the results presented in Fig. 2, A and B illustrate, CDDO-Im effectively decreased the viability of both COLO357 and PANC1 cells in a time-dependent manner and suggest that apoptosis induction by CDDO-Im is accompanied by rapid decreases in ΔΨm, resulting in 96% mitochondrial depolarization by 24 h (p < 0.0001). Finally, we examined the genomic integrity of PC cells treated with CDDO-Im and observed the characteristic apoptotic DNA laddering pattern (Fig. 2 C). These data demonstrate that CDDO-Im prevents the growth of PC cells by inducing mitochondrial dysfunction and apoptosis.

**CDDO-Im Induces a Rapid and Selective Decrease in GSHm Levels in PC Cells**—A previous report suggested that the cytotoxicity of CDDO-Im is mediated by alterations in intracellular redox homeostasis (30). Because GSH levels are critical regulators of redox homeostasis and also because our results indicated that CDDO-Im induces mitochondrial dysfunction in PC cells, we quantitated biochemically the total GSH content (GSH plus GSSG) in whole-cell and mitochondrial fractions from COLO357 cells treated with CDDO-Im. Interestingly, the results presented in Fig. 3, A and B demonstrate that treatment of COLO357 cells with CDDO-Im resulted in a time-dependent and dose-dependent decrease in total GSHm levels (46% loss at 120 min; p < 0.03) without marked effects on total whole-cell GSH (GSHwc) levels, and
similar observations were made in PANC1 cells (not shown). Because adequate GSHm levels are necessary to prevent the accumulation of ROS (34), we investigated further to determine whether treatment of PC cells with CDDO-Im resulted in increased levels of ROS as measured by the oxidation of CM-H2DCFDA acetyl ester. Indeed, as the results in Fig. 3 illustrate, treatment with 1 μM CDDO-Im significantly (p < 0.01) increased accumulation of ROS by 3.2-fold in COLO357 and 2.3-fold in PANC1 cells, suggesting that the decrease in GSHm levels impairs the antioxidant capacity of PC cells. Biochemical measurements in whole-cell acid extracts of COLO357 cells treated with various doses of CDDO-Im for 2 h (Fig. 3D) demonstrated that this agent at 1 μM also induced a significant (p < 0.05) 2.3-fold decrease in the GSH/GSSG ratio, suggesting that the increased accumulation of ROS resulted in oxidation of the GSH pool, and similar results were obtained in PANC1 cells (not shown). Finally, the results in Fig. 3E demonstrate that the parent compound CDDO (5 μM), as well as the methyl ester of CDDO at 1 μM, also induced a profound depletion of GSHm in PANC1 cells within 3 h; similar observations were made in COLO357 cells (not shown), demonstrating that the triterpenoid nucleus of these agents mediate the perturbations in GSHm. Taken together, our observations suggest that the triterpenoid nucleus of CDDO-Im induces perturbations in GSHm that occur prior to the initiation of apoptosis and lead to increased accumulation of ROS and subsequent oxidation of the GSH pool.

**Sulfhydryl Containing Nucleophiles Prevent the Cytotoxicity Induced by CDDO-Im in PC Cells**—Because CDDO-Im displays two potential electrophilic Michael acceptor sites at position 1 and 9 of the triterpenoid nucleus (Fig. 4A), we investigated to determine whether reducing nucleophilic agents would abrogate the cytotoxicity of this agent. As shown in Fig. 4, B–E, BME, DTT, and GSH, but not ascorbic acid, effectively prevented PS exposure and GSHm loss in PC cells treated with CDDO-Im. More importantly, BME cotreatment (2 mM) significantly prevented the loss of GSHm by 37% (p < 0.05) in PANC1 cells treated with 1.5 μM CDDO-Im and by 52% (p < 0.03) in COLO357 cells treated with this agent at 1 μM (Fig. 5A). Similar observations were made using DTT (not shown). The finding that BME prevented the decrease in GSHm levels in PC cells led us to investigate whether this agent would also prevent the accumulation of ROS in COLO357 cells treated with CDDO-Im. Supporting a critical role for GSHm in preventing accumulation of ROS, our experiments demonstrated that BME completely prevented the increase in CM-H2DCFDA fluorescence in COLO357 cells treated with 1 μM CDDO-Im (Fig. 5B). In contrast, BME was ineffective in preventing the increase in CM-H2DCFDA fluorescence induced by treatment of COLO357 cells with 100 μM H2O2 (Fig. 5B).
suggesting that ROS generation is downstream of the redox perturbation induced by CDDO-Im.

Finally, using a C-28 biotinylated derivative of CDDO, TP-301, we explored the possibility that reduced sulfhydryls may be direct molecular targets of the triterpenoid nucleus. Our hypothesis stated that if intracellular sulfhydryls form Michael adducts with the triterpenoid nucleus of CDDO and its derivatives, then chemical alkylation of sulfhydryls would prevent the accumulation of TP-301 in PC cells. Briefly, COLO357 cells were treated with 15 \mu M TP-301 for 6 h alone or in combination with DTT or DEM, an electrophile that forms Michael adducts with reduced sulfhydryls. Cells were then fixed, permeabilized, and stained with avidin conjugated with phycoerythrin (PE) followed by flow cytometry analysis of the cellular PE content (Fig. 5C). Our results indeed revealed that DEM cotreatment significantly (p < 0.005) decreased by 80% the avidin-PE content of COLO357 cells treated with TP-301, indicating that reduced sulfhydryl groups are indeed direct molecular targets of the triterpenoid nucleus of CDDO and its C28 derivatives. DTT had a similar albeit weaker effect (43% reduction; p < 0.005) on the accumulation of avidin-PE in TP-301-treated cells. Notably, microscopy analysis of the intracellular distribution of TP-301 illus-
trated that this agent was almost exclusively localized to the cytoplasm of COLO357 cells (Fig. 5C, inset), and similar observations were made in PANC1 cells (not shown), suggesting that the triterpenoid nucleus of CDDO and its C28 derivatives displays affinity for non-nuclear components of PC cells. In summary, our results demonstrate that the depletion of GSHm, the generation of ROS, and the cytotoxicity induced by CDDO-Im in PC cells can be abrogated by sulfhydryl-containing nucleophiles independent of their ability to directly scavenge ROS. These results are among the first to suggest that reduced sulfhydryls may indeed be direct molecular targets of CDDO and its derivatives, and they demonstrate the cytoplasmic localization of the triterpenoid nucleus of CDDO.

Depletion of GSHm by CDDO-Im Results in Caspase-Independent Alterations in Cytoplasmic Morphology and Apoptosis—We also investigated the morphological changes induced by CDDO-Im in PC cells by utilizing confocal microscopy of the fluorescent mitochondrial probe CMXRos and the nuclear stain 4',6-diamidino-2-phenylindole. COLO357 cells were grown on chamber slides and treated with CDDO-Im as in Fig. 2A alone or in combination with the pancaspase inhibitor z-VAD-fmk that was added 30 min prior to CDDO-Im. Three
hours after CDDO-Im treatment, samples were loaded with CMXRos and further incubated for 30 min prior to confocal visualization. CDDO-Im treatment of PC cells induced rapid cytoplasmic shrinking that preceded \( \Delta\Psi \text{m} \) loss and was not accompanied by nuclear condensation or membrane blebbing (Fig. 6A). Moreover, the observed cytoplasmic shrinking was insensitive to pharmacological inhibition of caspases, suggesting that this morphological change is part of an “early apoptotic” program that is initiated independent of caspase activation. BME, GSH, and DTT, but not ascorbic acid, completely prevented the alterations in cytoplasmic morphology that were observed in roughly 50% of cells treated with CDDO-Im for 3 h (not shown). Interestingly, although we observed cytochrome \( c \) release we failed to detect the release of apoptosis-inducing factor in either cell line in response to treatment with CDDO-Im (not shown). Because apoptosis may or may not be accompanied by caspase activation (35), we examined the activation of caspases in PC cells treated with CDDO-Im (Fig. 6B). Our immunoblot analysis demonstrates that CDDO-Im induces processing of initiator procaspases 8 and 9 and executioner procaspase 3 as well as caspase-dependent cleavage of poly(ADP-ribose) polymerase 1. Moreover, the activation of initiator and effector caspases was reversed by BME and glutathione, suggesting that redox perturbations can mediate the activation of the caspase cascade in pancreatic cancer cells. Similar results were obtained in PANC1 (not shown). Our experiments also suggest that in both cell lines the proteolytic processing of initiator and executioner caspases is sensitive to pharmacological inhibition of calpains (E64d), ruling out these calcium-activated thiol proteases as mediators of CDDO-Im-induced caspase activation. Interestingly, z-VAD-fmk abrogated CDDO-Im-induced PS externalization in COLO357 cells but not in PANC1 cells and only minimally prevented \( \Delta\Psi \text{m} \) loss in COLO357, suggesting that caspase-dependent apoptosis partially contributes to the cytotoxicity of this synthetic oleic acid derivative in a cell context-dependent manner (Fig. 6, C and D). Altogether, these data demonstrate that even though the perturbations in GSHm and redox homeostasis induced by CDDO-Im lead to the development of apoptotic morphology and caspase activation, the cytotoxicity of this agent in PC cells is mediated, in large part, by caspase-independent mechanisms.

**DISCUSSION**

The synthetic triterpenoid CDDO displays antiproliferative, differentiating, and anti-inflammatory effects in vitro and in vivo (21, 25, 27, 28, 33, 36), and its C-28 imidazole (CDDO-Im) and methyl ester derivatives have been reported to be 5-fold more potent (23, 26, 29, 37, 39).
Interestingly, CDDO and CDDO-Im were identified as ligands for the peroxisome proliferator-activated receptor (PPAR) (40), but, like other PPAR agonists, their antiproliferative effects have PPAR-dependent and PPAR-independent components (41). Underlining receptor-independent events, CDDO and its derivatives have been reported to induce the rapid oxidation of the intracellular GSH pool and the generation of ROS that results in apoptosis of leukemic (30) and multiple myeloma cells (24). Furthermore, a recent report demonstrated that CDDO promoted the release of cytochrome c from isolated mitochondria, indicating that this organelle is a direct target of this agent (21). Nevertheless, the mechanisms by which CDDO and its derivatives induce perturbations in redox homeostasis and mitochondrial dysfunction remain to be elucidated.

This report demonstrates that CDDO-Im effectively abrogates the growth of PC cells at nanomolar doses by inducing apoptosis. Notably, we observe that CDDO-Im is effective against PANC1 cells shown to be chemoresistant to a variety of chemotherapeutic agents including gemcitabine (42–44). More importantly, in an attempt to further elucidate the mechanism by which CDDO-Im induced apoptosis in PC cells, we are the first to identify depletion of total mitochondrial glutathione (GSH plus GSSG; GSHm) as a novel mechanism of toxicity for CDDO-Im as well as to provide evidence that this critical antioxidant may be a direct molecular target for the triterpenoid nucleus of CDDO and its derivatives. The kinetics of GSHm loss induced by CDDO-Im suggest that this effect is not due to a generalized depletion of glutathione because total whole-cell glutathione (GSH plus GSSG; GSHwc) was not affected, and they demonstrate that perturbations in GSHm levels occur prior to the onset of apoptosis. The data also suggest that CDDO-Im-induced depletion of GSHm results in the generation of ROS, mitochondrial dysfunction, and oxidation of intracellular glutathione.

**FIGURE 6.** CDDO-Im induces caspase activation but caspase-independent onset of apoptotic morphology in PC cells. A, COLO357 cells were seeded in chamber slides and treated with CDDO-Im (CDDOim) for 3 h alone or in combination with z-VAD-fmk pretreated for 30 min. Cells were then loaded with CMXRos for 30 min, fixed in 1% paraformaldehyde, and stained with 4',6-diamidino-2-phenylindole (DAPI). Top to bottom, untreated, CDDO-Im, z-VAD-fmk (z-VAD), and CDDO-Im plus z-VAD-fmk. DIC, differential interference contrast. B, COLO357 cells were treated with CDDO-Im (CDDOim) alone and in combination with BME, GSH, a cell-permeable calpain inhibitor (E64d), or the pancaspase inhibitor z-VAD-fmk (Z-VAD), and protein lysates were immunoblotted with poly(ADP-ribose) polymerase 1 (PARP1) and caspase (Casp) antibodies. C and D, COLO357 and PANC1 cells were treated with CDDO-Im (CDDOim) alone and in combination with z-VAD-fmk (z-VAD) as in panel A, and annexin V binding (C) and Ym (D) were measured by flow cytometry as described under “Experimental Procedures.”
thermore, pretreatment with sulphydryl-containing nucleophiles prevented the depletion of GSHm, the generation of ROS, the loss of ΔΨm, and the externalization of PS in PC cells treated with CDDO-Im, suggesting that the electrophilic character of CDDO-Im is important for its cytotoxic activity. Also important, we observed that CDDO-Im induced a rapid alteration of cytoplasmic morphology that was insensitive to pharmacological inhibition of caspases; this early apoptotic event was completely prevented by cotreatment with sulphydryl antioxidants, suggesting that redox stress can mediate rapid caspase-independent changes in cytoplasmic structure. Interestingly, BME was unable to scavenge intracellular ROS in cells treated with H2O2 yet completely prevented the generation of ROS induced by treatment with CDDO-Im. This critical observation indicates that sulphydryl-containing nucleophiles prevent the cytotoxicity of CDDO-Im, not by scavenging excess ROS but by preventing the loss of GSHm induced by CDDO-Im.

Most interesting, CDDO and its derivatives have been reported to preferentially induce cell death in cancer cells but not in their normal counterparts (23, 24), suggesting one of the following possibilities: (a) that normal cells can adapt to GSHm depletion; (b) that these agents do not deplete GSHm in normal cells; or (c) that both the extent of GSHm depletion and cell death are differentially modulated in normal cells. Current studies are aimed at understanding the molecular basis for the reported tumor selectivity of these synthetic triterpenoids and at further characterizing the therapeutic window for their clinical use.

A previous report from our group demonstrated that CDDO induced caspase activation but caspase-independent cell death in leukemic cells (21). Similarly, our results demonstrate that the loss of viability induced by CDDO-Im is caspase-independent in both COLO357 and Panc1 cells. Interestingly, in COLO357 cells pharmacological inhibition of caspases prevented annexin V positivity but not ΔΨm loss, demonstrating that CDDO-Im-induced cell death can occur in the absence of caspase activation but that caspases contribute to the cytotoxicity of this agent in a cell context-dependent manner. Nevertheless, our Western blot analysis indicated that CDDO-Im induced caspase activation in PC cells and that this process was completely inhibited by sulphydryl-containing nucleophiles, demonstrating that perturbations in GSHm lead to caspase activation in PC cells. Consistent with our observations, Latham et al. (45) showed that perturbations in intracellular redox tone could promote caspase 3 activation. In addition, other reports have suggested that intracellular thiol depletion can directly activate caspase 3 (10) and that epigallocatechin-3-gallate induces an α-acetyl cysteine-sensitive oxidative event that activates apoptotic cell death in a manner distinct from that of chemically induced or receptor-mediated programmed cell death (46). Thus, CDDO-Im induced depletion of GSHm results in a lethal injury to tumor cells that activates caspase-dependent and caspase-independent cell death.

Notably, the chemical structures of CDDO and CDDO-Im display two Michael acceptor moieties at positions 1 and 9 of the triterpenoid nucleus (Fig. 4A). Michael electrophiles have been used for the selective inactivation of cysteine-containing enzymes (47), and a report by Myers et al. (48) demonstrated that an avrainvillamide derivative could efficiently incorporate oxygen and sulfur-based nucleophiles through a Michael acceptor site. In addition, the novel chemotherapeutic irfoliven, derived from the cytoxic fungal metabolite illudin S, displays biologically active Michael acceptors and has been demonstrated to induce caspase-independent cell death with a high selectivity for cancer cells (49, 50). These observations thus demonstrate that Michael electrophiles can target specific nucleophiles and can exert selective biological functions. Accordingly, our experiments using TP-301, a biotinylated derivative of CDDO, demonstrated that accumulation of this compound in the cytoplasm of COLO357 cells was abrogated by cotreatment with the sulphydryl alkylating agent DEM, suggesting that the triterpenoid nucleus of TP-301 competes with DEM to alkylate reduced sulphydryls. Moreover, DTT similarly prevented TP-301 accumulation, indicating that neutralizing the electrophilicity of the triterpenoid nucleus also affects its intracellular accumulation. In support of this hypothesis, a series of elegant experiments recently demonstrated that the Michael acceptor groups in the triterpenoid nucleus of CDDO chemically react with sulphydryl groups in the cysteine-rich cytosolic protein Keap1 and mediate the induction of the antioxidant response in macrophages (51). Taken together, our findings and published reports demonstrate that the Michael acceptor groups in CDDO and its derivatives are indeed biologically active and can target non-nuclear components.

We have not yet identified the mechanism by which CDDO-Im selectively targets GSHm in PC cells. However, published reports indicate that cholesterol can localize to the mitochondrial membrane and prevent GSH import (14, 38, 52). We hypothesize that, like cholesterol, the triterpenoid structure of CDDO and CDDO-Im may facilitate localization of these compounds to the mitochondria, resulting in targeted effects on GSHm. Currently, we are developing methodologies to identify and quantitate intracellular Michael adducts of CDDO and CDDO-Im formed inside of PC cells and are also investigating the effects of these agents on mitochondrial GSH flux.

In conclusion, these results suggest that CDDO-Im is a potent anti-tumor agent that induces cell death in a manner that is apparently distinct from that of other agents currently used for the treatment of PC. More importantly, our observations support the targeting of GSHm as an effective chemotherapeutic strategy to induce cell death in PC and indicate that CDDO-Im may be beneficial in a clinical setting against PC.

Acknowledgments—We thank Dr. James Abbruzzese for a critical review of this report and Rose Lauzon for administrative assistance.

REFERENCES

1. Abbruzzese, J. L. (2003) Int. J. Gastrointest. Cancer 33, 1–2
2. National Cancer Institute (2002) Surveillance, Epidemiology, and End Results Fast Stat: Pancreatic Cancer http://seer.cancer.gov
3. Li, D., Xie, K., Wolff, R., and Abbruzzese, J. L. (2004) Lancet 363, 1049–1057
4. Abbruzzese, J. L. (2002) Semin. Oncol. 29, 2–8
5. Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., and Turner, N. D. (2004) J. Nutr. 134, 489–492
6. Imai, H., and Nakagawa, Y. (2003) Free Radic. Biol. Med. 34, 145–169
7. Filomeni, G., Rotilio, G., and Cirio, M. R. (2003) FASEB J. 17, 64–66
8. Hancock, J. T., Desikan, R., and Neill, S. J. (2003) Ann. N. Y. Acad. Sci. 1010, 446–448
9. Schäfer, F. Q., and Buettner, G. R. (2001) Free Radic. Biol. Med. 30, 1191–1212
10. Caffrey, R. N., Watson, R. W., Hegarty, N. J., O’Neill, A., Gibbons, N., Brady, H. R., and Fitzpatrick, J. M. (2000) Cancer 88, 2092–2104
11. Rizzardini, M., Lepi, M., Bensasconi, S., Mangolini, A., and Cantoni, L. (2003) J. Neurol. Sci. 207, 51–58
12. Dhanbhoocha, C. M., and Babson, J. R. (1992) Arch. Biochem. Biophys. 293, 130–139
13. Fernandez-Checa, J. C., Kaplowitz, N., Garcia-Ruiz, C., and Colell, A. (1998) Semin. Liver Dis. 18, 389–401
14. Fernandez-Checa, J. C. (2003) Biochem. Biophys. Res. Commun. 304, 471–479
15. Colell, A., Garcia-Ruiz, C., Miranda, A., Ardite, E., Mari, M., Morales, A., Corrales, F., Kaplowitz, N., and Fernandez-Checa, J. C. (1998) Gastroenterology 115, 1541–1551
16. Colell, A., Coll, O., Garcia-Ruiz, C., Paris, R., Tiribelli, C., Kaplowitz, N., and Fernandez-Checa, J. C. (2001) Hepatology 34, 964–971
17. Chernyak, B. V. (1997) Biol. Reprod. 57, 197–302
18. Graziantini, P., Chernyak, B. V., Petronilli, V., and Bernardi, P. (1996) J. Biol. Chem. 271, 6746–6751
19. Inoue, S., Snowden, R. T., Dyer, M. J., and Cohen, G. M. (2004) Leukemia 18, 948–952
20. Ito, Y., Pandey, P., Place, A., Sporn, M. B., Gribble, G. W., Honda, T., Kharbanda, S., and Kufe, D. (2000) Cell Growth & Differ. 11, 261–267
21. Konopleva, M., Tsaio, T., Estrov, Z., Lee, R. M., Wang, R. Y., Jackson, C. E., McQueen, O.
CDDO-Im Antagonizes the Growth of Pancreatic Cancer Cells

T., Monaco, G., Munsell, M., Belmont, J., Kantarjian, H., Sporn, M. B., and Andreeff, M. (2004) Cancer Res. 64, 7927–7935

22. Suh, W. S., Kim, Y. S., Schimner, A. D., Kitada, S., Minden, M., Andreeff, M., Suh, N., Sporn, M., and Reed, J. C. (2003) Gastroenterology 127, 97–105

23. Chauhan, D., Li, G., Podar, K., Hideshima, T., Shingarpure, R., Catley, L., Mitsiades, C., Munshi, N., Tai, Y. T., Suh, N., Gribble, G. W., Honda, T., Schlossman, R., Richardsson, P., Sporn, M. B., and Anderson, K. C. (2004) Blood 103, 3158–3166

24. Ikeda, T., Nakaya, K., Kimura, F., Sato, K., Anderson, A. C., Sporn, M. B., and Gribble, G. W. (2004) Cancer Res. 64, 7570–7578

25. Lapillonne, H., Konopleva, M., Tsao, T., Gold, D., McQueen, T., Sutherland, R. L., Madd, T., and Andreeff, M. (2003) Mol. Cancer Ther. 2, 177–184

26. Kim, K. B., Lotan, R., Yue, P., Sporn, M. B., Suh, N., Gribble, G. W., Honda, T., Wu, G. S., Hong, W. K., and Sun, S. Y. (2002) Mol. Cancer Ther. 1, 177–184

27. Ito, Y., Pandey, P., Sporn, M. B., Datta, R., Kharbanda, S., and Kufe, D. (2001) J. Biol. Chem. 276, 2806–2818

28. Hail, N., Jr., Konopleva, M., Sporn, M., Lotan, R., and Andreeff, M. (2004) J. Biol. Chem. 279, 11179–11187

29. Place, A. E., Suh, N., Williams, C. R., Risingsong, R., Honda, T., Honda, Y., Favaloro, F. G., Jr., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., and Sporn, M. B. (2002) Bioorg. Med. Chem. Lett. 12, 1027–1030

30. Ikeda, T., Sporn, M., Honda, T., Gribble, G. W., and Kufe, D. (2003) Mol. Endocrinol. 17, 261–269

31. Honda, T., Janosik, T., Honda, Y., Han, J., Liby, K. T., Williams, C. R., Couch, R. D., Anderson, A. C., Sporn, M. B., and Gribble, G. W. (2004) J. Med. Chem. 47, 4923–4932

32. Honda, T., Honda, Y., Faraloro, F. G., Jr., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., and Sporn, M. B. (2002) Bioorg. Med. Chem. Lett. 12, 1027–1030

33. Suh, N., Wang, Y., Honda, T., Gribble, G. W., Dmitrovsky, E., Hickey, W. F., Mau, R. A., Place, A. E., Porter, D. M., Spinella, M. J., Williams, C. R., Wu, G., Dannenberg, A. J., Flanders, K. C., Letterio, J. J., Mangelsdorf, D. J., Nathan, C. F., Nguyen, L., Porter, W. W., Ren, R. F., Roberts, A. B., Roche, N. S., Subbaraoaiah, K., and Sporn, M. B. (1999) Cancer Res. 59, 336–341

34. Fernandez-Checa, J. C., Garcia-Ruiz, C., Colell, A., Morales, A., Mari, M., Miranda, M., and Ardite, E. (1998) Biofactors 8, 7–11

35. Ravagnan, L., Bournier, T., and Kroemer, G. (2002) J. Cell. Physiol. 192, 131–137

36. Minns, L. A., Ruthon-Gatell, D., Ely, K. H., Rabinot, N., Luangsay, S., and Kasper, L. H. (2004) J. Am. Chem. Soc. 126, 119–126

37. Konopleva, M., Tsao, T., Rivolo, P., Stouf, I., Estrov, Z., Leysath, C. E., Zhao, S., Harris, D., Chang, S., Jackson, C. E., Munsell, M., Suh, N., Gribble, G., Honda, T., May, W. S., Sporn, M. B., and Andreeff, M. (2002) Blood 99, 326–335

38. Yao, P. M., and Tabas, I. (2001) J. Biol. Chem. 276, 42468–42476

39. Zou, W., Liu, X., Yue, P., Zhou, Z., Sporn, M. B., Lotan, R., Khuri, F. R., and Sun, S. Y. (2004) Cancer Res. 64, 5926–5939

40. Wang, Y., Porter, W. W., Suh, N., Honda, T., Gribble, G. W., Leesnitzer, L. M., Plunket, K. D., Mangelsdorf, D. J., Blanchard, S. G., Willson, T. M., and Sporn, M. B. (2000) Mol. Endocrinol. 14, 1550–1556

41. Melchior, B., Konopleva, M., Hu, W., Melchiorova, K., Andreeff, M., and Freedman, R. S. (2004) Gynecol. Oncol. 93, 149–154

42. Duxbury, M. S., Ito, H., Benoit, E., Zinner, M. J., Ashley, S. W., and Whang, E. E. (2004) Surgery 136, 261–269

43. Duxbury, M. S., Ito, H., Zinner, M. J., Ashley, S. W., and Whang, E. E. (2004) Clin. Cancer Res. 10, 2307–2318

44. Anderson, K. M., Seed, T., Meng, J., Ou, D., Alrefai, W. A., and Harris, J. E. (1998) Anticancer Res. 18, 791–800

45. Latham, P., Lund, E. K., Brown, J. C., and Johnson, L. T. (2001) Gut 49, 97–105

46. Saeki, K., Kobayashi, N., Inazawa, Y., Zhang, H., Nishitoh, H., Ichijo, H., Saeki, K., Isemura, M., and Yuo, A. (2002) Biochem. J. 368, 705–720

47. Dragovich, P. S., Webber, S. E., Babine, R. E., Fuhrman, S. A., Patick, A. K., Matthews, D. A., Lee, C. A., Reich, S. H., Prins, T. J., Marakovi, J. T., Littlefield, E. S., Zhou, R., Tikhe, J., Ford, C. E., Wallace, M. B., Meador, J. W., III, Ferre, R. A., Brown, E. L., Binford, S. L., Harr, J. E., Delisle, D. M., and Worland, S. T. (1998) J. Med. Chem. 41, 2806–2818

48. Myers, A. G., and Herzon, S. D. (2003) J. Am. Chem. Soc. 125, 12080–12081

49. Woynarowska, B. A., and Woynarowska, J. M. (2002) Biochim. Biophys. Acta 1587, 309–317

50. Herzeg, M. C., Liang, H., Johnson, A. E., Woynarowska, B., and Woynarowska, J. M. (2002) Breast Cancer Res. Treat. 71, 133–143

51. Dinkova-Kostova, A. T., Liby, K. T., Stephenson, K. K., Holtzclaw, W. D., Gao, X., Suh, N., Williams, C., Risingsong, R., Honda, T., Gribble, G. W., Sporn, M. B., and Talalay, P. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4584–4589

52. Coll, O., Colell, A., Garcia-Ruiz, C., Kaplowitz, N., and Fernandez-Checa, J. C. (2003) Hepatology 38, 692–702
2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) Directly Targets Mitochondrial Glutathione to Induce Apoptosis in Pancreatic Cancer
Ismael Samudio, Marina Konopleva, Numsen Hail, Jr., Yue-Xi Shi, Teresa McQueen, Timothy Hsu, Randall Evans, Tadashi Honda, Gordon W. Gribble, Michael Sporn, Hiram F. Gilbert, Stephen Safe and Michael Andreeff

J. Biol. Chem. 2005, 280:36273-36282.
doi: 10.1074/jbc.M507518200 originally published online August 23, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M507518200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 20 of which can be accessed free at http://www.jbc.org/content/280/43/36273.full.html#ref-list-1