The triterpenoid CDDO induces apoptosis in refractory CLL B cells

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Chronic lymphocytic leukemia (CLL) cells develop chemo-resistance over time. Most anticancer agents function through induction of apoptosis, and therefore resistance against these agents is likely to be caused by selection for CLL cells with defects in the particular apoptosis pathway that is triggered by these drugs. Anticancer agents that function through alternative apoptotic pathways might therefore be useful in treating chemo-resistant CLL. Triterpenoids represent a class of naturally occurring and synthetic compounds with demonstrated antitumor activity. We examined the effects of CDDO (triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid) on CLL B cells in vitro. CDDO induced apoptosis in a dose-dependent manner in all (n = 30) CLL samples tested, including previously untreated and chemo-resistant CLL specimens. CDDO induced rapid proteolytic processing of caspase-8, but not caspase-9, in CLL B cells, suggesting activation of a mitochondria-independent pathway. CDDO-induced apoptosis of CLL B cells was blocked by cytokine response modifier A (CrmA), a suppressor of caspase-8, but not by X-linked inhibitor of apoptosis protein–baculovirus IAP repeat–3 (XIAP-BIR3), a fragment of XIAP, which selectively inhibits caspase-9. Examination of CDDO effects on expression of several apoptosis-relevant genes demonstrated significant reductions in the levels of caspase-8 homolog Fas-ligand interleukin-1–converting enzyme (FLICE)–inhibitory protein (c-FLIP), an endogenous antagonist of caspase-8. However, reductions of FLIP achieved by FLIP antisense oligonucleotides were insufficient for triggering apoptosis, indicating that CDDO has other targets in CLL B cells besides FLIP. These data suggest that the synthetic triterpenoid CDDO should be further explored as a possible therapeutic agent for treatment of chemo-resistant CLL. (Blood. 2002;100:2965-2972)

Introduction

Susceptibility to apoptosis is an essential prerequisite for successful eradication of tumor cells by cytotoxic T lymphocytes, natural killer cells, radiation, or chemotherapy. Consequently, resistance to apoptosis has been established as one of the mechanisms responsible for the failure of therapeutic approaches in many types of cancers, including hematopoietic malignancies. B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults in the Western Hemisphere, with more than 12 000 new cases diagnosed annually in the United States alone.[1] CLL is considered an incurable disease. Therefore, identifying new agents with novel mechanisms of action that complement conventional cytotoxic therapies and that abrogate chemo-resistance will be necessary if further advances in the therapy of this disease are to be realized.

Apoptosis is caused by the activation in cells of a family of cysteine proteases known as caspases.[2] At least 2 major pathways for caspase activation have been delineated, including a pathway linked to the tumor necrosis factor (TNF) family of death receptors ("extrinsic") and a pathway activated by mitochondria ("intrinsic").[3,4] The mechanisms by which antineoplastic drugs kill leukemia cells may be diverse, but most cytotoxic agents seem to employ the intrinsic pathway for inducing apoptosis.[5] Consequently, when resistance develops, defects in the mitochondria-initiated pathway for cell death are often found. With the extrinsic pathway, ligand binding induces clustering of TNF-family death receptors, causing recruitment of caspase-binding adaptor proteins to their cytosolic domains, and resulting in caspase activation by the "induced-proximity" mechanism.[6] The intrinsic pathway is triggered by cytochrome *c* release from mitochondria, which binds the caspase-activating protein apoptotic protease-activating factor–1 (Apaf-1), forming the apoptosome. The apical proteases in the extrinsic and intrinsic pathways are caspase-8 and caspase-9, respectively. Activation of both extrinsic and intrinsic pathways results in the activation of downstream caspases such as caspase-3, caspase-6, and caspase-7.[7]

CLL B cells are generally known to be resistant to apoptosis induced by TNF-family death ligands, such as TNF-α, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL)[8-11] (also in I.M.P., S.K., unpublished data, April 2001). It is possible, however, that the extrinsic apoptosis pathway is blocked at a proximal, surface level in CLL B cells and that downstream portions of the pathway remain functional. Therefore, it is important to identify anticancer agents that operate through the "alternative" extrinsic apoptosis pathway in CLL B cells, especially for patients who develop resistance to conventional chemotherapeutic agents.

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Triterpenoids represent a class of naturally occurring and synthetic proximal proliferator-activated receptor–γ (PPARγ) ligands with demonstrated antitumor activity. The oleanane CDDO (triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid) and related synthetic triterpenoids have pleiotrophic actions, including modulation of PPARγ transcriptional activity and suppression of nuclear factor–κB (NF-κB) activation.[12-15] CDDO has been demonstrated to activate caspase-8 in cultured leukemia cell lines and osteosarcoma cells.[13,16,17] We therefore explored the effects of CDDO on apoptosis of cultured CLL B cells freshly isolated from patients. We show here that CDDO is a potent apoptosis-inducing agent in CLL B cells in vitro, and provide evidence that CDDO functions through the extrinsic apoptosis pathway. CDDO appears to be effective even in chemo-refractory CLL specimens that have defects in the intrinsic apoptosis pathway.

Patients, materials, and methods

Patient specimens

Heparinized peripheral blood was obtained from patients whose B-CLL was diagnosed according to standard criteria.[18] Procedures were conducted under institutional review board (IRB) approval with all patients signing informed consent.[18] In addition, frozen samples from patients were used for some experiments. Patients were termed fludarabine resistant when they were treated with fludarabine but received a diagnosis with progressive disease after treatment. Lymphocytes were isolated by Ficoll density-gradient centrifugation, and immunofluorescence flow cytometry verified these as being composed of greater than 90% $CD5^+CD19^+CD23^+$ B cells.

Cell culture and apoptosis assay

CLL B cells were cultured at 2×10^6/mL in Iscove modified Dulbecco medium (IMDM) containing 20% fetal calf serum (FCS), 1 mM L-glutamine, and antibiotics. At the initiation of cultures, CLL B cells were supplemented with either CDDO (0.1 to 1.0 μM); CDDO-Me (0.1 to 1.0 μM), synthesized as described[19]; troglitazone (10 μM) (Sankyo Pharma, La Jolla, CA); or fludarabine A (F-Ara-A) (0.1 μM to 1.0 μM) (gift from W Plunket, MD Anderson Cancer Center, Houston, TX). For some experiments, TRAIL (100 nM) (Alexis, San Diego, CA) was added 6 hours after other drugs, and cells were then cultured for an additional 16 to 24 hours. At least 5×10^5 cells were recovered by centrifugation after 24 hours for evaluation of the percentage of apoptotic cells by means of double staining with annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) (from BioVision, Mountain View, CA), followed by fluorescence-activated cell sorter (FACS) analysis with the use of the FL-1 and FL-3 channels (FACSort; Becton Dickinson, San Jose, CA). Alternatively, cells were lysed in radioimmunoprecipitation analysis (RIPA) buffer (10 mM Tris [tris(hydroxymethyl)aminomethane] [pH 7.4], 150 mM NaCl, 1% Triton × 100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 5 mM EDTA (ethylenediaminetetraacetic acid) containing protease inhibitors (complete tablets; Roche, Indianapolis, IN) for immunoblot analysis.

Protein transduction by electroporation

Apoptosis-inducing or apoptosis-inhibiting proteins were introduced into primary CLL B cells by electroporation. Proteins used for these experiments included recombinant purified caspase-8 and cytokine response modifier A (CrmA) (gifts of G. Salvesen, The Burnham Institute, La Jolla, CA), X-linked inhibitor of apoptosis protein (XIAP) (XIAP–baculovirus IAP repeat–3 [XIAP-BIR3]).[21,22] Cytochrome *c* was obtained from Sigma (St Louis, MO). Briefly, cytochrome *c* (60 μM) or caspase-8 (12 nM), in the presence or absence of CrmA (10 μM) or XIAP-BIR3 (20 nM), was added to the cells (2×10^5) in 0.5 mL IMDM medium along with marker protein phycoerythrin–bovine serum albumin (PE-BSA) (4 μg/mL) (used to identify successfully transduced cells). The cell-protein suspensions were then transferred to electroporation cuvettes (0.4-cm diameter) (Biorad, Hercules, CA), kept on ice for 10 minutes, and then subjected to electroporation (Gene Pulser II; Biorad) with the use of 750 to 1250 V/cm and 900 μF capacitance, with voltage adjusted for each patient CLL sample to identify the conditions that gave the best uptake of PE-BSA while still preserving the viability of the majority of the cells. This protocol allowed delivery of proteins in most cells in patients' specimens (58% ± 18%), while preserving viability of the majority of the cells (68% ± 17% of 7-amino-actinomycin D–negative [7-AAD^-]). Cells were maintained on ice for an additional 10 minutes, transferred to 1.5-mL microcentrifuge tubes (Eppendorf, Hamburg, Germany), and cultured at 37°C in a CO_2-air ratio of 5%:95% in humid conditions for 1 hour as caspase activity was assessed. Normalization of total protein concentration included during electroporation of samples was accomplished by addition of unlabeled BSA, without affecting caspase activation.

Caspase activity assays

Cytosolic extracts were prepared from primary CLL cells as previously described.[23,24] Briefly, cells were washed in phosphate-buffered saline (PBS) and then resuspended in an equal volume of hypotonic lysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 10 mM KCl, 1.5 mM $MgCL_2$, 1 mM EDTA, and 1 mM dithiothreitol [DTT]). Cells were incubated on ice for 20 minutes and then disrupted by 15 passages through a 30-gauge needle. Cell extracts were clarified by centrifugation at 16 000*g* for 30 minutes. To initiate caspase activation, 10 μM horse heart cytochrome *c* (Sigma) with 1 mM deoxyadenosine triphosphate (dATP) or purified recombinant caspase-8 (10 nM) was added to cell extracts (5 mg/mL total protein), and mixtures were incubated at 30°C for 30 minutes. Hydrolysis of fluorogenic peptide, acetyl-Asp-Glu-Val-Asp–7-amino-4-trifluoromethylcoumarin (Ac–Asp-Glu-Val-Asp–AFC), was measured as an indicator of caspase activation, as previously described.[23,24] Activated extracts were added to Ac–Asp-Glu-Val-Asp–AFC in caspase buffer (50 mM HEPES [pH 7.4], 10% sucrose, 1 mM EDTA, 0.1% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid}, 100 mM NaCl, and 10 mM DTT). Hydrolysis was measured for 20 minutes by release of AFC (excitation at 405 nm; emission at 510 nm) by means of a spectrofluorometer (fMax; Molecular Devices, Sunnyvale, CA) in the kinetic mode.

Caspase-3–like effector protease activity in intact CLL B cells was measured with CaspaTag (Invitrogen, New York, NY) according to the manufacturer's instructions. CaspaTag is a cell-permeable FITC-labeled peptide that selectively and irreversibly binds active caspase 3. The CaspaTag assay is based on the well-described observation that caspase activation occurs prior to loss of outer membrane integrity and positive staining with viability dyes. CLL B cells (5×10^5/mL) were incubated with CaspaTag for 1 hour, after introducing various apoptosis activators and/or inhibitors by electroporation, followed by analysis by flow cytometry.

Flow cytometry

CLL B cells were treated with CaspaTag and stained with the viability dye 7-AAD at a final concentration of 2 ng/μL (Sigma) for 5 minutes at room temperature just prior to flow cytometry analysis (FACScan; Becton Dickinson). FITC (channel 1), PE (channel 2), and 7-AAD (channel 3) fluorescence was measured, and the Cell Quest program was used to analyze the data. PE^+ (uptake of PE-BSA marker) and 7-AAD^- (viable) cells were gated, and the percentage of gated cells expressing FITC was determined. Cells to which the PE marker was added but that were not electroporated were used to determine the positive and negative cutoffs for the various fluorescent labels.

Immunoblot analysis

Cell lysates were prepared by means of RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton × 100, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA) containing protease inhibitors (complete tablets; Roche). Lysates were normalized for total protein (12.5 μg) and subjected to SDS–polyacrylamide gel electrophrosis (SDS-PAGE) (4% to 20% gradient gels; ISC BioExpress, Kaysville, UT) and immunoblot analysis.[25] Primary

antibodies included anti-DR4 (Millennium, Boston, MA), anti-DR5 (Alexis), anti–Fas-associated death domain protein (anti-FADD) (Pharmingen, Franklin Lakes, NJ), anti–death-associated protein 3 (anti-DAP3) (Transduction Laboratories, San Diego, CA), anti–Fas-ligand interleukin-1 (FLICE)–converting enzyme inhibitory protein (anti-FLIP) (NF-6) (generously provided by M. Peter and J. Tschopp),[26,27] and β-actin (Sigma). Immunodetection was accomplished with the use of horseradish peroxidase (HRPase)–conjugated secondary antibodies and an enhanced chemiluminescence (ECL) method (Amersham, Arlington Heights, IL) involving exposure to x-ray film (XAR; Kodak, Rochester, NY).

Flow cytometry analysis of surface antigens

Cells were collected and washed twice in PBS. Cells were incubated with 50 μg/mL nonspecific human immunoglobulin G (IgG) (Cappel, New York, NY) on ice for 10 minutes to block Fc receptors. Cells were then incubated with PE-conjugated purified monoclonal antibodies against death receptor 3 (DR3), DR4, DR5, decoy receptor 1 (DcR1), and decoy receptor 2 (DcR2) (BioScience, San Diego, CA) on ice for 1 hour. After incubation, cells were washed once with PBS, and the relative level of surface antigens was assessed by FACS analysis, by means of the FL-1 or FL-2 channels of a flow cytometer (FACSort; Becton Dickinson).

Electroporation of oligonucleotides

Antisense (FLIP) or control oligonucleotides (300 nM) (antisense oligonucleotide, ISIS 23296 [ACTTGTCCCTGCTCCTTGAA] or control oligonucleotide, ISIS 132383 [AGTTCTCTCTGCCCCTAGAT]) were introduced into CLL B cells by electroporation. Briefly, 0.5 mL CLL B cells in IMDM medium (2×10^6/mL) were added to electroporation cuvettes (0.4 cm) and placed on ice for 10 minutes. As a control for assessing the percentage of uptake, FITC-labeled oligonucleotides (300 nM) were added to another cuvette with cells. Following electroporation (750 to 1250 V/cm and 900 μF), the cells were placed on ice for an additional 10 minutes and then analyzed for percentage of uptake of FITC-oligonucleotides among the viable 7-amino-actinomycin D–negative (7-AAD$^-$) cells. As an additional control, the labeled oligonucleotides were added to a cuvette containing cells without electroporation to determine the basal uptake. The cells were washed once in IMDM medium containing 20% FCS, 1 mM L-glutamine, and antibiotics, and were then resuspended and cultured at 2×10^6 cells per milliliter in the same medium for 24 hours. Cells were harvested, and analysis for apoptosis was assessed by annexin V–FITC/PI labeling (5×10^5 cells per assay), or cell lysates were prepared for immunoblot analysis, as described in "Immunoblot analysis."

Protein microarrays

CLL B cells (2×10^6) were resuspended in 30 μL lysis buffer containing 2 × SDS electrophoresis buffer (125 mM Tris [pH 6.8], 4% SDS, 10% glycerol, 2% β-mercaptoethanol) for 2 hours at 70°C. After cell lysis, samples were boiled for 3 to 5 minutes each, and 3 nL lysate was arrayed onto nitrocellulose slides with a glass backing (Schleicher and Schuell, Keene, NH), with a pin and ring GMSE 470 microarrayer (Affymetrix, Santa Clara, CA) with the use of a 375-μm pin. Spatial densities of 980 spots per slides and greater can easily be accommodated on a 20 × 30 mm slide.

Staining was carried out on an automated slide stainer (Dako, Carpinteria, CA) by means of the catalyzed signal-amplification system per the manufacturer's recommendation (Dako). Briefly, after microapplication of cellular lysates, the slides were treated for 15 minutes with Reblot (Chemicon, Temecular, CA) and subsequently washed 3 times for 10 minutes each with a Tris-buffered saline (TBS) washing buffer (300 mM NaCl, 0.1% Tween20, 50 mM Tris [pH 7.6]). After treatment, the arrays were blocked in a 0.5% casein solution for 30 minutes under constant rocking. Before each of the following steps, arrayed slides were washed 3 times 5 minutes each in TBS washing buffer. Endogenous biotin was blocked with the use of the biotin-blocking kit (Dako) for 5 minutes, followed by application of protein block (Dako) for 5 minutes. The incubation with primary antibody was diluted in antibody diluent (Dako) at a concentration of 1:1000 for 30 minutes and, finally, secondary link-antibody (Dako) for 30 minutes, at a concentration of 1:100 for antimouse (diluted in antibody diluent) and neat for antirabbit.

Amplification and staining was carried out as follows: the labeled microarray was treated with a streptavidin-biotin complex (Dako) solution for 15 minutes, amplification reagent (biotinyl tyramide and hydrogen peroxidase) for 15 minutes, and streptavidin-peroxidase for 15 minutes, and was finally stained with the use of 3,3′-diaminobenzidine tetrahydrochloride as chromogen. Specificity of each antibody was tested by immunoblotting. Antibodies (anti–cleaved caspase-3, anti–cleaved caspase-8, anti–cleaved caspase-9) (Cell Signaling Technology, Beverly, MA) and anti–β-actin (Sigma) were used at 1:500.

Results

Sensitive and drug-resistant CLL patient specimens respond to CDDO

To assess the effect of the triterpenoid CDDO on apoptosis of cultured CLL B cells, we obtained specimens from 5 newly diagnosed, previously untreated CLL patients and 8 fludarabine-refractory CLL patients. These CLL B cells were cultured for 24 hours, in the presence or absence of F-Ara-A (0.1 to 1.0 μM) or with CDDO (0.1 to 1.0 μM) (Figure 1). The induction of apoptosis was determined by annexin V–FITC/PI double staining with the use of flow cytometry analysis. Six of the 8 cases diagnosed with progressive disease after fludarabine treatment demonstrated resistance in vitro to F-Ara-A with respect to apoptosis induction. Two of the previously untreated CLL cases showed intrinsic resistance to F-Ara-A in vitro, although these patients had never been treated with this drug in the clinic. The remaining newly diagnosed CLL specimens responded to F-Ara-A in vitro, with robust induction of apoptosis (Figure 1B). Among the CLL B-cell specimens that were resistant to F-Ara-A in vitro, all 8 cases displayed sensitivity to CDDO, undergoing apoptosis in response to this drug at concentrations below 1 μM (Figure 1A). All 5 F-Ara-A–sensitive CLL B cell specimens also responded to CDDO in vitro by undergoing apoptosis at even lower concentrations of the drug (Figure 1B).

In contrast to CLL B cells, normal B lymphocytes were largely resistant to CDDO (Figure 1C). For these experiments, normal B lymphocytes were purified from human tonsils and cultured in the presence of CDDO. Although relatively high rates of spontaneous

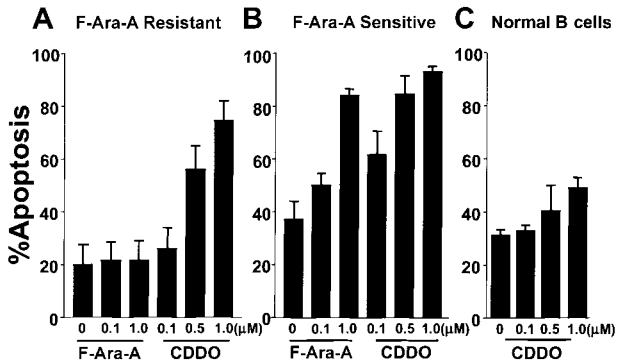

Figure 1. CDDO induction of apoptosis of fludarabine-refractory CLL patient samples. CLL B cells from patients with a new diagnosis of fludarabine-A sensitivity (F-Ara-A sensitive) (n = 5) (panel B), from F-Ara-A–resistant patients (n = 8) (panel A), or normal B cells isolated from tonsil (n = 2) (panel C) were cultured in the presence or absence of F-Ara-A (0.1/1.0 μM) or CDDO (0.1/0.5/1.0 μM). CLL B cells were recovered from cultures, and the percentage of apoptosis was determined by annexin V–FITC/PI double staining with the use of flow cytometry analysis (data represent means ± SE).

apoptosis were observed in cultures of tonsillar B cells, the addition of CDDO at 0.1 to 1.0 μM induced little additional apoptosis (Figure 1C). Similar results were obtained when testing B lymphocytes isolated from peripheral blood of healthy individuals (data not shown). These results are in agreement with unpublished studies (M.B.S.) suggesting little toxic effect of CDDO on normal tissues.

Since CDDO, being a weak PPARγ agonist, is known to bind and regulate PPARγ, we also examined the effects of other PPARγ modulators on apoptosis of CLL B cells in vitro (n = 10), including CDDO-Me, a PPARγ antagonist,[12,28] and troglitazone, a potent PPARγ agonist (Figure 2). CDDO-Me induced increases in the percentage of apoptotic cells in cultures of CLL B cells but not as potently as CDDO. In contrast, troglitazone failed to induce apoptosis of CLL B cells. These results suggest that the proapoptotic activity of CDDO in CLL B cells is unlikely to be due to its effects on PPARγ.

Defect in the intrinsic apoptosis pathway in fludarabine-refractory CLL samples

The demonstration that CLL B cells that were resistant to a standard chemotherapeutic agent undergo apoptosis after culture with CDDO suggests that the cell death pathways triggered by CDDO and F-Ara-A are different. We therefore used a protein transduction method to compare the functional status of 2 caspase-activation pathways in CLL B cells: the mitochondria-dependent (intrinsic) and the death receptor–mediated (extrinsic) pathways. BSA, caspase-8, or cytochrome *c* was introduced into cells from an F-Ara-A–sensitive CLL patient by electroporation, and PE-BSA was used to determine the protein uptake efficiency in every condition. As shown in Figure 3A, electroporation efficiently delivered proteins into CLL cells. After electroporation, the induction of downstream effector caspase activity was measured by flow cytometry analysis with the use of the cell-permeable, caspase-3–specific, fluorogenic substrate CaspaTag, and the viability of the cells was determined by 7-AAD exclusion. Because each patient specimen differed in terms of optimal electroporation conditions, electroporation parameters were first optimized for each sample before proceeding to caspase activity assays. It is interesting to note that only a small percentage of the CLL cells that were successfully transduced with protein (gate R1 in Figure 3A) were positive for 7-AAD staining 1 hour after electroporation, although this percentage varied among CLL samples. This result further indicates that the majority of CLL cells remain viable after electroporation. As shown in Figure 3B, caspase-8 (Figure Bii) and cytochrome *c* (panel Biii), but not BSA (panel Bi), induced significant caspase-3 activity. This caspase-3 activity was observed as early as 1 hour after electroporation, whereas the CaspaTag-positive cells were still negative for 7-AAD staining, as is typical of cells in early stages of apoptosis. In contrast, a population of CaspaTag-positive and 7-AAD$^+$ CLL cells was observed at 3 hours after electroporation, suggesting that these cells had progressed to the final stages of apoptosis. To further validate this method for assessing apoptosis pathways in CLL, cells from an F-Ara-A–sensitive CLL patient were electroporated with selective inhibitors of the intrinsic or extrinsic pathways, along with cytochrome *c* or caspase-8, respectively. Specifically, the caspase-8 inhibitor CrmA and the caspase-9 inhibitor XIAP-BIR3 were employed for these studies.[20-22] As shown in Figure 3C, electroporation of CLL B cells with either cytochrome *c* or caspase-8 induced activation of effector caspases 1 hour after electroporation, as determined by an increase in the percentage of cells demonstrating CaspaTag positivity. Coelectroporation of CrmA blocked caspase activation induced by caspase-8 (Figure 3Ciii), but had comparatively less effect on cytochrome *c* (Figure 3Cvi). Conversely, coelectroporation of XIAP-BIR3 reduced caspase activation induced by cytochrome *c* (Figure 3Cv), but had relatively little effect on caspase-8 (Figure 3Cii). Introducing CrmA or XIAP-BIR alone did not induce CaspaTag activity (data not shown). These data therefore demonstrate the utility of the electroporation method of protein transduction for assessing integrity of caspase-activation pathways downstream of caspase-8 (extrinsic) and cytochrome *c* (intrinsic).

Next, we used the electroporation method to contrast the status of caspase-activation pathways in F-Ara-A–resistant CLL B cells. In CLL B cells from patients with fludarabine-sensitive disease, cytochrome *c* and caspase-8 induced activation of effector caspases to comparable extents (Figure 4A). Furthermore, CrmA and XIAP-BIR3 partially suppressed caspase activation induced by caspase-8 and cytochrome *c*, respectively. In contrast, cytochrome *c* was considerably less effective than caspase-8 at inducing caspase activation in CLL B cells from fludarabine-refractory patients (Figure 4B). This observation was confirmed by measuring caspase activity in cell lysates in the 2 samples in which enough cells were obtained (data not shown). These findings indicate the presence of a blockage of the intrinsic apoptosis pathway in F-Ara-A–resistant leukemia cells. All together, these findings suggest that resistance to chemotherapy can be associated with a defect in the intrinsic apoptosis pathway, whereas the extrinsic pathway remains functional in CLL B cells.

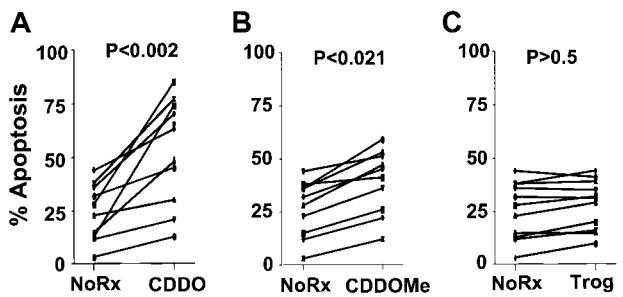

Figure 2. Effects of PPARγ modulators on CLL B cells. Freshly isolated CLL B cells were cultured in the presence or absence of (A) 1 μM CDDO, (B) 0.5 μM CDDO-Me, or (C) 10 μM troglitazone (Trog) for 24 hours. The percentage of apoptosis was determined by double staining with annexin V–FITC and PI by means of flow cytometric analysis. Statistical significance was determined by paired *t* test analysis.

CDDO activates the extrinsic apoptosis pathway in CLL

To determine if CDDO induces apoptosis in CLL through activation of the extrinsic pathway, we cultured CLL B cells with CDDO after introducing the caspase-8 inhibitor CrmA or the caspase-9 inhibitor XIAP-BIR3 by electroporation. For comparisons, CLL B cells were also electroporated with caspase-8. CDDO induced activation of effector caspases in the CLL B cells, as determined by CaspaTag-substrate positivity (Figure 5). Furthermore, CDDO-mediated activation of caspases was inhibited by CrmA but not by XIAP-BIR3. Similar results were obtained with electroporation of caspase-8 (used here as a comparative control), showing suppression of downstream caspases activated by CrmA but not XIAP-BIR3 (Figure 5).

To further explore the apoptosis pathways activated by CDDO in CLL B cells, we also contrasted the kinetics of proteolytic processing ("activation") of procaspase-3, procaspase-8, and procaspase-9 using a semiquantitative protein dot-blot method in conjugation with antibodies specific for the cleaved forms of these

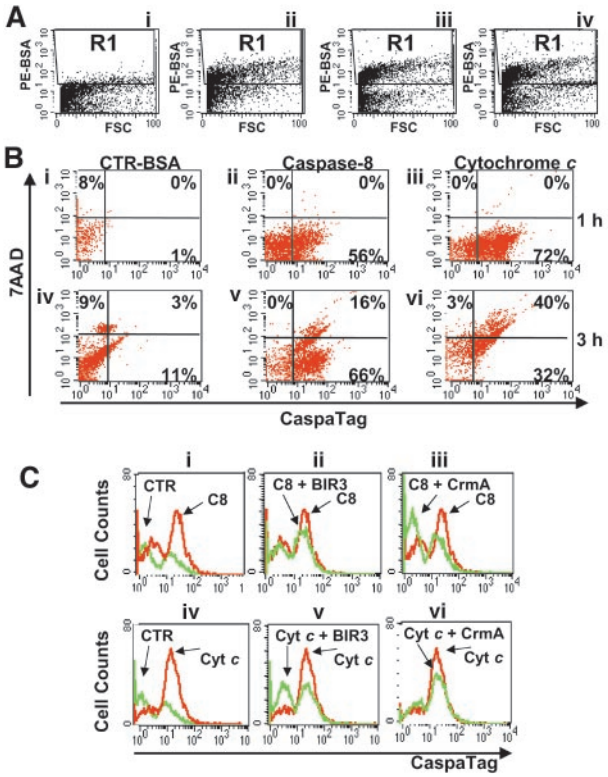

Figure 3. Transduction of apoptosis-inducing proteins into CLL B cells by electroporation. (A) CLL B cells from fludarabine-sensitive patients were incubated with BSA (panels Ai-Aii), caspase-8 (12 nM) (panel Aiii), or cytochrome *c* (60 μM) (panel Aiv), in the presence of PE-BSA (4 μg/mL) for 10 minutes on ice and were then left untreated (panel Ai) or electroporated with the use of 1125 V/cm and 900 μF. The percentage of cells that took up PE-BSA was determined by FACS analysis (FL-2 channel versus forward scatter [FSC]). R1 represents the selected gate containing the successfully transduced cells (PE^+ population). (B) After electroporation, the cells transduced with BSA (panels Bi, Biv), caspase-8 (panels Bii, Bv), and cytochrome *c* (panels Biii, Bvi) (described in "Immunoblot analysis") were cultured for 1 hour (panels Bi-Biii) or 3 hours (panels Biv-Bvi) at 37°C before being incubated with the fluorogenic caspase substrate CaspaTag for an additional hour at 37°C. Then, 7-AAD was added to the cells, and the percentage of CaspaTag–positive (FL-1 channel) and 7-AAD^+ (FL-3 channel) cells contained in the R1 gate (PE^+) was determined by FACS. (C) To analyze the status of the 2 apoptosis pathways (the death receptor–mediated [extrinsic] or the mitochondria-mediated [intrinsic] pathway), CLL B cells from another fludarabine-sensitive patient were analyzed with regard to caspase activation. BSA, caspase-8 (12 nM), or cytochrome *c* (60 μM) was transduced into CLL cells with PE-BSA (4 μg/mL), in the presence or absence of specific inhibitors of caspase-8 (CrmA) (10 μM) or caspase-9 (XIAP-BIR3) (20 nM), by electroporation (1125 V/cm and 900 μF). Panel (i) shows CLL B cells transduced with BSA-CTR (CTR), or caspase-8 (C8); (ii) shows caspase-8 (C8), and caspase-8 + XIAP-BIR3 (C8 + BIR3); (iii) shows caspase-8 (C8), and caspase-8 + CrmA (C8 + CrmA); (iv) shows BSA-CTR (CTR), and cytochrome *c* (Cyt *c*); (v) shows cytochrome *c* (Cyt *c*), and cytochrome *c* + XIAP-BIR3 (Cyt *c* + BIR3); and (vi) shows cytochrome *c* (Cyt *c*), and cytochrome *c* + CrmA (Cyt *c* + CrmA). Effector caspase activity was measured 1 hour after electroporation by means of the cell-permeable fluorogenic caspase-3 substrate CaspaTag, flow cytometric cell analysis, and gating on the viable cell population (7-AAD^-) that had taken up protein ($PE\text{-BSA}^+$). A representative FACS histogram is presented, showing CaspaTag fluorescence (y-axis) versus forward scatter (x-axis) for the gated 7-AAD^-, $PE\text{-BSA}^+$ population. (Data shown are representative of 3 independent experiments.)

individual proteases. As shown in Figure 6, CDDO induced rapid cleavage of procaspase-8, with maximal accumulation of cleaved caspase-8 occurring within 1 hour after CDDO treatment. Cleavage of caspase-3 peaked at approximately 2 hours of culture, whereas cleaved caspase-9 slowly accumulated over time in CDDO-treated CLL B cells. These data are thus consistent with the hypothesis that CDDO triggers apoptosis of CLL B cells primarily through mechanisms that engage the extrinsic pathway at or above the level of caspase-8.

CDDO down-regulates the antiapoptotic protein FLIP in CLL cells

We examined the effects of CDDO and various PPARγ modulators on the expression in CLL B cells of apoptosis-regulatory proteins known to operate within the extrinsic pathway, including DR4, DR5, FADD, DAP3, and FLIP. CDDO potently reduced levels of the antiapoptotic FLIP protein in all CLL B-cell samples analyzed (n = 15), while having no effect on the levels of other proteins involved in the extrinsic pathway (Figure 7A). CDDO-Me reduced FLIP levels in some CLL B-cell samples (3 of 15), though it was less potent than CDDO (Figure 7A; also data not shown). Troglitazone had no effect on levels of FLIP or other extrinsic proteins tested (Figure 7A). Dose-response experiments showed a correlation between CDDO concentration, down-regulation of FLIP protein levels, and the extent of apoptosis induction in cultured CLL B cells (Figure 7B).

FLIP reductions are inadequate to explain proapoptotic effect of CDDO

To determine whether down-regulation of FLIP is sufficient to account for the apoptotic effect of CDDO in CLL B cells, we knocked down FLIP expression using antisense oligonucleotides. To this end, FLIP antisense or randomized-sequence control oligonucleotides were introduced into CLL B cells by electroporation. Immunoblot analysis of the cells performed 1 day later demonstrated antisense-specific reductions in the steady-state levels of FLIP protein (Figure 8A, top). Although potently reducing levels of FLIP protein, antisense FLIP oligonucleotides did not induce apoptosis of CLL B cells, implying that this alone is inadequate to explain the proapoptotic effect of CDDO (Figure 8A).

CDDO sensitizes some CLL B cells to TRAIL-induced apoptosis

The modulation of FLIP in CLL B cells by CDDO raised the question of whether CDDO could be used to sensitize CLL B cells to TNF-family death receptor agents such as TRAIL. To explore this idea, we cultured CLL B cells in the presence or absence of 1 μM CDDO for 6 hours (which was determined to be adequate for reducing FLIP protein levels [not shown]) and then added 100 ng/mL

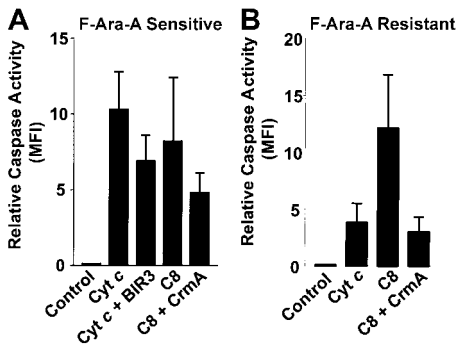

Figure 4. Defects in the intrinsic apoptosis pathway in CLL samples. CLL cells from patients sensitive to fludarabine (n = 3) (panel A) or resistant to fludarabine (n = 8) (panel B) were analyzed with regard to caspase activation. Caspase-8 (12 nM) or cytochrome *c* (60 μM) were transduced into CLL cells with PE-BSA (4 μg/mL), in the presence or absence of the specific caspase-8 inhibitor CrmA (10 μM) or the specific caspase-9 inhibitor XIAP-BIR3 (20 nM) by electroporation (1200 V/cm and 900 μF). The control represents BSA-transduced cells. Effector caspase activity was measured by means of the fluorogenic cell-permeable substrate, CaspaTag, analysis of cells by flow cytometry, and gating on the viable cell population (7-AAD^-) that had taken up protein ($PE\text{-BSA}^+$). Data represent mean CaspaTag fluorescence (mean fluorescence intensity [MFI] ± SE).

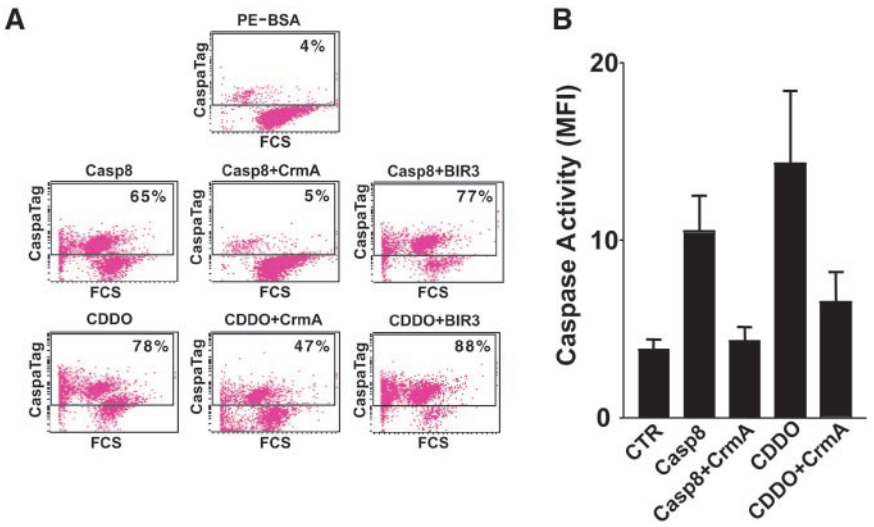

Figure 5. CDDO activation of the extrinsic apoptosis pathway in CLL B cells. CLL B cells were cultured in the presence or absence of 1 μM CDDO, or they were electroporated with caspase-8, with or without CrmA (10 μM) or XIAP-BIR3 (20 nM), along with PE-BSA (4 μg/mL) fluorescent marker protein. Effector caspase activity was determined by CaspaTag assay (n = 5). (A) Representative FACS histogram is presented, showing CaspaTag fluorescence (y-axis) versus forward scatter (x-axis) for the gated 7-AAD$^-$, PE-BSA$^+$ population. The horizontal line indicates the cutoff of cells with activated effector caspases. (B) Data from 5 experiments were averaged (± SE), showing relative levels of caspase activity (MFI).

TRAIL to the cultures. Apoptosis of the CLL B cells was then assessed 24 hours later by double staining with annexin V–FITC/PI, by means of flow cytometry analysis. The majority of CLL B cells (25 of 30) responded strongly to CDDO by itself, and in this group of patient samples no significant sensitization to TRAIL was observed, nor did TRAIL alone induce apoptosis (data not shown). However, for the few CLL samples in which CDDO induced less than 50% apoptosis, 2 of 5 patient specimens demonstrated sensitization to TRAIL-induced apoptosis (Figure 8B). In parallel experiments, we determined that CDDO did not change the levels of TRAIL surface receptors DR4, DR5, DcR1, and DcR2 as determined by flow cytometric analysis with the use of PE-labeled antibodies recognizing these receptors (data not shown). Taken together, these findings suggest that some leukemia cells from CLL patient specimens can be sensitized to TRAIL-induced apoptosis by treatment with CDDO.

Discussion

CLL represents a quintessential example of a malignancy caused by defects in the regulation of apoptosis, as opposed to uncontrolled cell proliferation.[29] Progression to chemoresistance in these patients is thought to be a consequence, at least in part, of an apoptosis-resistant phenotype of their CLL cells, given that cross-resistance to several cytotoxic agents is typically observed. Certainly, however, classical mechanisms can also make contributions to the problem of chemoresistance, such as altered drug uptake or metabolism.[30] Identifying new agents with alternative mechanisms of action that complement conventional therapies and that can overcome CLL drug resistance will be necessary if further advances in the therapy of this disease are to be accomplished.

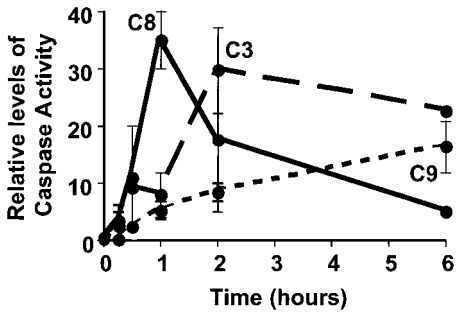

Figure 6. CDDO induction of cleavage of caspases in a time-dependent manner. CLL B cells were cultured in the presence or absence of CDDO (1 μM), and cell lysates were prepared at various times. Levels of cleaved caspase-3, caspase-8, and caspase-9 were determined by a protein micro–dot-blot method, with the use of primary antibodies recognizing the cleaved forms of these proteases. Relative levels of cleaved caspases were first normalized relative to β-actin, and then normalized relative to nontreated CLL B-cell cultured at the same time points (means ± SD; n = 3).

In this study, we have analyzed the effect on CLLs of PPARγ modulators known to have antitumor activity in cancer cell lines.[31-34] We report that the triterpenoid CDDO induces a significant apoptosis response in CLL cells in culture. Furthermore, CDDO retains its proapoptotic potential against CLL specimens that are resistant to the conventional therapeutic agent fludarabine. In contrast, CDDO was evidently less toxic to normal B cells. In this regard, animal studies have suggested that CDDO is well tolerated in vivo, lacking substantial toxicity (M.B.S., unpublished data).

The demonstration that CLL resistant to standard anticancer agents undergoes apoptosis after culture with CDDO indicates that CDDO induces apoptosis through different mechanisms than those triggered by cytotoxic chemotherapeutic drugs. To address this issue, we analyzed the functional status of the 2 caspase-activation pathways—the mitochondria-mediated (intrinsic) pathway and the death receptor–mediated (extrinsic) pathway—in CLL cells.[3,4] Our results indicate that the majority of previously untreated CLL patient specimens have functional intrinsic and extrinsic apoptosis pathways. This group of patients was sensitive to conventional chemotherapeutic agents, as well as CDDO. In contrast, analysis of a group of patients with resistance to fludarabine revealed a correlation with defects in the intrinsic apoptosis pathway. Interestingly, CLL B cells from this group of patients were still sensitive to CDDO treatment in vitro. These results indicate that F-Ara-A and CDDO function through the activation of different apoptosis pathways. Indeed, we found that CDDO induced activation of caspase-8 within the first hour of treatment, followed by activation of caspase-3, consistent with the idea that CDDO triggers the same pathway activated by TNF/Fas-family cytokine receptors. In addition, the apoptosis-inducing activity of CDDO was suppressed by the caspase-8 inhibitor CrmA but not by the caspase-9 inhibitor XIAP-BIR3. In contradistinction, previous studies have demonstrated that fludarabine triggers CLL B-cell apoptosis via the intrinsic mitochondrial pathway, involving caspase-9 cleavage and

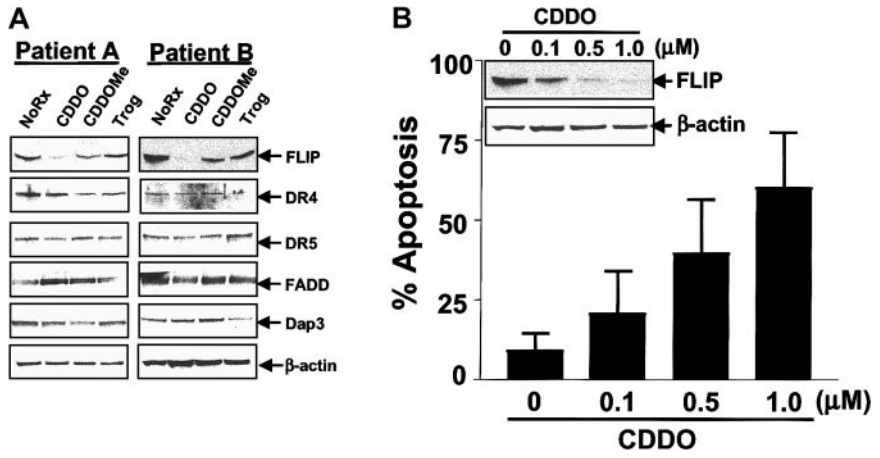

Figure 7. CDDO down-regulation of the antiapoptotic protein FLIP in CLL. CLL B cells were cultured for 24 hours in the presence or absence of 1 μM CDDO, 0.5 μM CDDO-Me, or 10 μM troglitazone. CLL B cells were recovered from cultures, and the percentage of apoptosis was determined by annexin V–FITC/PI double staining with the use of flow cytometry analysis. Alternatively, protein-containing lysates were prepared, normalized for total protein content (12.5 μg per lane), and analyzed by SDS-PAGE/immunoblot assay with the use of antibodies specific for DR4, DR5, FADD, DAP3, FLIP, or β-actin. (A) Two representative patient samples are shown of a total of 12 analyzed. (B) A dose response is shown for CDDO at 0.1 to 1.0 μM (means ± SD; n = 3).

activation as an early event.[35] Therefore, the sensitivity of CLL B cells to CDDO presumably depends on an intact extrinsic apoptosis pathway, whereas resistance to fludarabine and related cytotoxic agents may be due to blocks in the intrinsic pathway. These results are in agreement with Ito and coworkers,[16] who reported that CDDO exerts its proapoptotic effect through activating caspase-8 in established myeloid leukemia cell lines in vitro. In that study, caspase-8 was additionally found to induce Bid cleavage, resulting in cytochrome *c* release from mitochondria, thus amplifying the apoptosis induction.[16] This secondary activation of the mitochondrial pathway may explain why we observed a late induction of caspase-9 activation in CDDO-treated CLL B cells.

It is thought that most anticancer drugs induce cytochrome *c* release from mitochondria, thereby exerting their cell death effects through the intrinsic apoptosis pathway.[5,36] This hypothesis is supported by several findings. Caspase $9^{-/-}$ embryonic stem cells are sensitive to death receptor–mediated apoptosis, but exhibit marked decreases in drug-induced apoptosis.[37,38] Likewise, Apaf-$1^{-/-}$ thymocytes are sensitive to CD95, but resistant to cellular stress-induced apoptosis.[39,40] However, other studies indicate that cell death induced by anticancer drugs may involve the extrinsic apoptosis pathway. In this regard, Jones and coworkers[10] recently reported that Fas-independent caspase-8 activation might mediate the proapoptotic effects of fludarabine, chlorambucile, and γ-radiation in B-CLL cells. However, as the authors indicated, kinetic studies revealed that caspase-8 activation occurred after cytochrome *c* release, and it therefore may reflect a secondary caspase-amplification mechanism, as has been previously reported for caspase-8 activation in epithelial tumor cells.[41]

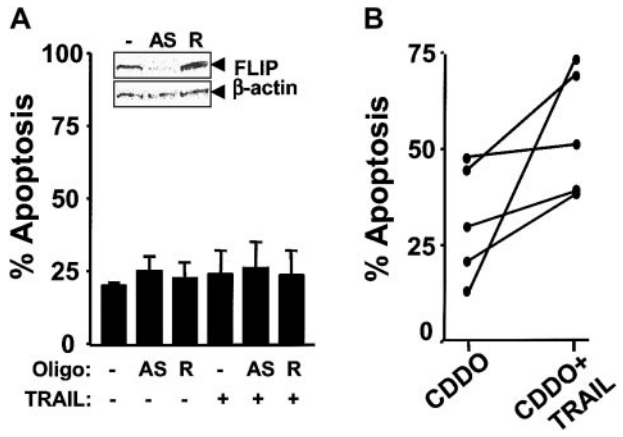

Figure 8. Effects of FLIP antisense and TRAIL on CLL B cells. (A) Antisense (AS) FLIP or random (R) control oligonucleotides (300 nM) were introduced into CLL B cells by electroporation. CLL B cells were then cultured overnight in the presence or absence of 100 nM TRAIL. The CLL B cells were recovered from cultures, and the percentage of apoptosis was determined by annexin V–FITC/PI double staining by means of flow cytometric analysis (bottom). Simultaneously, protein-containing lysates were prepared, normalized for total protein content (12.5 μg per lane), and analyzed by SDS-PAGE/immunoblot assay with the use of antibodies specific for FLIP or β-actin (top). (Data are representative of 3 independent experiments.) (B) Freshly isolated CLL B cells were cultured in the presence or absence of 1 μM CDDO. After 6 hours, 100 nM TRAIL was added, and the CLL B cells were cultured overnight. The percentage of apoptosis was determined by double staining with annexin V–FITC and PI with the use of flow cytometric analysis. Shown are the CLL patient specimens in which CDDO by itself induced less than 50% apoptosis (n = 5, of a total of 30).

To explore the mechanism by which CDDO triggers the extrinsic apoptosis pathway in CLL B cells, we analyzed the effects of this compound on the levels of various apoptosis-regulatory proteins. The levels of the antiapoptotic protein FLIP were decreased by CDDO in a dose-dependent manner, in contrast to other apoptosis-relevant proteins examined, including FADD, DAP3, DR3, DR4, DR5, DcR1, and DcR2. The FLIP protein is structurally very similar to procaspase-8 and procaspase-10, having 2 tandem death effector domains (DEDs) followed by a caspase-protease–like domain. Unlike procaspase-8 and procaspase-10, however, the caspaselike domain of FLIP is defective in protease activity, and thus FLIP functions as a *trans*-dominant inhibitor of caspase-8 and caspase-10 in a variety of contexts.[42] Overexpression of FLIP has been associated with resistance to Fas, TNF-α, and TRAIL, members of the TNF family of death ligands.[42,43] The modulation of FLIP in CLL B cells by CDDO raised the question of whether CDDO could be used to sensitize CLL B cells to TNF-family ligands, particularly given that these leukemia cells are known to be generally resistant to death receptor–induced apoptosis.[8] Indeed, we found that among CLL B-cell specimens that exhibited modest responses to CDDO in vitro (less than 50% apoptosis), some of these patient specimens could be triggered to undergo superior apoptotic responses when cultured with the combination of TRAIL and CDDO, compared with either agent alone. Thus, while not effective for all CLL B-cell specimens tested, CDDO may be useful for sensitizing some patients to immune-mediated pathways for apoptosis. However, antisense oligonucleotide experiments indicated that down-regulation of FLIP by itself was insufficient to mimic CDDO, failing to induce apoptosis or to sensitize CLL B cells to TRAIL. These observations thus suggest that FLIP is only one of the apoptosis-regulatory targets that CDDO modulates in CLL cells. Future analysis of the effects of CDDO on CLL B cells using DNA microarrays or

proteomic methods may shed light on the proapoptotic mechanisms of this interesting agent.

We found that while CDDO induced apoptosis of CLL B cells, the thiazoladine-dione troglitazone did not, and the CDDO analog, CDDO-Me, was less potent than CDDO. In this regard, troglitazone is a more potent PPARγ agonist than CDDO, which has only weak agonist activity.[12] Furthermore, CDDO-Me is a PPARγ antagonist.[12,44] Thus, our findings suggest that the proapoptotic activity of CDDO is unlikely to be linked to its functions as a PPARγ modulator. Interestingly, CDDO has been reported to have other PPARγ-independent activities, including suppression of pathways involving NF-κB.[31] Future medicinal chemistry efforts therefore may reveal the relevant protein target of this synthetic triterpenoid in the context of apoptosis induction of CLL B cells. Moreover, on the basis of the data presented here, we propose that CDDO or active analogs of this compound should be considered for experimental clinical trials for patients with fludarabine-refractory CLL.

Acknowledgments

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References

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics. CA Cancer J Clin. 1997;47:5-27.
2. Reed JC. Apoptosis-based therapies. Nat Drug Disc. 2002;1:111-121.
3. Green D. Apoptotic pathways: the roads to ruin. Cell. 1998;94:695-698.
4. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell. 1997;91:443-446.
5. Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med. 2000;6:513-519.
6. Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. Proc Natl Acad Sci U S A. 1999;96:10964-10967.
7. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998;281:1312-1316.
8. Panayiotidis P, Ganeshaguru K, Foroni L, Hoffbrand AV. Expression and function of the FAS antigen in B chronic lymphocytic leukemia and hairy cell leukemia. Leukemia. 1995;9:1227-1232.
9. Wang D, Freeman GJ, Levine H, Ritz J, Robertson MJ. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. Br J Haematol. 1997;97:409-417.
10. Jones DT, Ganeshaguru K, Virchis AE, et al. Caspase 8 activation independent of Fas (CD95/APO-1) signaling may mediate killing of B-chronic lymphocytic leukemia cells by cytotoxic drugs or γ radiation. Blood. 2001;98:2800-2807.
11. Snell V, Clodi K, Zhao S, et al. Activity of TNF-related apoptosis-inducing ligand (TRAIL) in haematological malignancies. Br J Haematol. 1997;99:618-624.
12. Wang Y, Porter WW, Suh N, et al. A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor γ. Mol Endocrinol. 2000;14:1550-1556.
13. Stadheim TA, Suh N, Ganju N, Sporn M, Eastman A. The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. J Biol Chem. 2002;277:16448-16455.
14. Honda T, Gribble GW, Suh N, et al. Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. J Med Chem. 2000;43:1866-1877.
15. Suh N, Honda T, Finlay HJ, et al. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res. 1998;58:717-723.
16. Ito Y, Pandey P, Place A, et al. The novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. Cell Growth Differ. 2000;11:261-267.
17. Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S, Kufe D. The novel triterpenoid CDDO induced apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. Mol Pharmacol. 2001;59:1094-1099.
18. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood. 1996;87:4990-4997.
19. Honda T, Rounds BA, Gribble GW, Suh N, Wang N, Sporn MB. Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide producion in mouse macrophages. Bioorg Med Chem Lett. 1998;8:2711-2714.
20. Zhou Q, Salvesen GS. Viral caspase inhibitors CrmA and p53. Methods Enzymol. 2000;322:143-154.
21. Takahashi R, Deveraux QL, Tamm I, et al. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem. 1998;273:7787-7790.
22. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 1999;18:5242-5251.
23. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 1996;86:147-157.
24. Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell death proteases. Nature. 1997;388:300-304.
25. Krajewski S, Zapata JM, Reed JC. Detection of multiple antigens on Western blots. Anal Biochem. 1996;236:221-228.
26. Scaffidi C, Medema JP, Krammer PH, Peter ME. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J Biol Chem. 1997;272:26953-26958.
27. Irmler M, Thome M, Hahne M, et al. Inhibition of death receptor signals by cellular FLIP. Nature. 1997;388:190-195.
28. Sporn MB, Suh N, Mangelsdorf DJ. Prospects for prevention and treatment of cancer with selective PPARgamma modulators (SPARMs). Trends Mol Med. 2001;7:395-400.
29. Reed JC. Molecular biology of chronic lymphocytic leukemia: implications for therapy. Semin Hematol. 1998;35:3-13.
30. Plunkett W, Gandhi V. Pharmacology of purine nucleoside analogues. Hematol Cell Ther. 1996;38(suppl2):S67-S74.
31. Suh N, Wang Y, Honda T, et al. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. Cancer Res. 1999;59:336-341.
32. Sarraf P, Mueller E, Jones D, et al. Differentiation and reversal of malignant changes in colon cancer through PPARγ. Nat Med. 1998;4:1046-1052.
33. Elstner E, Muller C, Koshizuka K, et al. Ligands for peroxisome proliferator-activated receptorγ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. Proc Natl Acad Sci U S A. 1998;95:8806-8811.
34. Kim Y, Suh N, Sporn M, Reed JC. An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. J Biol Chem. 2002;277:22320-22329.
35. Genini D, Adachi S, Chao Q, et al. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood. 2000;96:3537-3543.
36. Herr I, Debatin K-M. Cellular stress response and apoptosis in cancer therapy. Blood. 2001;98:2603-2614.
37. Hakem R, Hakem A, Duncan GS, et al. Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell. 1998;94:339-352.
38. Kuida K, Haydar TF, Kuan C-Y, et al. Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell. 1998;94:325-337.
39. Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell. 1998;94:727-737.
40. Yoshida H, Kong YY, Yoshida R, et al. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell. 1998;94:739-750.
41. Tang D, Lahti JM, Kidd VJ. Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporine-mediated apoptosis. J Biol Chem. 2000;275:9303-9307.
42. Tschopp J, Irmler M, Thome M. Inhibition of Fas death signals by FLIPs. Curr Opin Immunol. 1998;10:552-558.
43. Schneider P, Tschopp J. Apoptosis induced by death receptors. Pharm Acta Helv. 2000;74:281-286.
44. Konopleva M, Tsao T, Ruvolo P, et al. Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia. Blood. 2002;99:326-335.