Peroxisome proliferator-activated receptor γ and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias

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Abstract
The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor family that forms heterodimers with retinoid X receptor. These heterodimers bind to DNA and activate the transcription of target genes. Here, we report that the PPARγ receptor protein is expressed in primary myeloid and lymphoid leukemias and in lymphoma and myeloma cell lines. In this study, we compared the activity of several PPARγ; ligands including BRL49653 (rosiglitazone), 15-deoxy-Δ12,14-prostaglandin J₂, and the novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid on leukemia cells. Exposure to these PPARγ ligands induced apoptosis in myeloid (U937 and HL-60) and lymphoid (Su-DHL, Sup-M2, Ramos, Raji, Hodgkin’s cell lines, and primary chronic lymphocytic leukemia) cells. A similar exposure to these PPARγ ligands induced the differentiation of myeloid leukemic cells. A combination of PPARγ ligands with a retinoid X receptor agonist (i.e., LG100268) or a retinoic acid receptor agonist (i.e., all trans-retinoic acid) enhanced differentiating and growth-inhibitory effects. 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid induced differentiation and apoptosis with much greater potency than the other PPARγ ligands in established cell lines and primary chronic lymphocytic leukemia samples. Exposure to 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induced mitochondrial depolarization and caspase activation, which was associated with apoptosis induction. In Bcl-2-overexpressing chronic lymphocytic leukemia cells, the small-molecule Bcl-2 inhibitor HA14-1 sensitized these cells to 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid–induced apoptosis. These results suggest that PPARγ ligation alone and in combination with retinoids holds promise as novel therapy for leukemias by activating the transcriptional activity of target genes that control apoptosis and differentiation in leukemias. [Mol Cancer Ther 2004;3(10):1249–62]

Introduction
Recent progress in the molecular and cellular biology of the nuclear steroid receptor superfamily has identified certain members as nuclear targets for cancer therapy (1). They include estrogen receptors, retinoic acid receptors, retinoid X receptors (RXR; the RXR-specific ligands are termed “rexinoids”), and the vitamin D receptor (the vitamin D–specific ligands are termed “deltanoids”). These nuclear receptors are putative cancer therapy targets because they function as transcription factors that control the expression of many genes related to cell differentiation (1, 2). The strongest evidence for the therapeutic potential of this approach comes from the efficacy of retinoic acid receptor α activation in the treatment of acute promyelocytic leukemia (3). Over 90% of patients with acute promyelocytic leukemia achieve complete remission following treatment with the naturally occurring retinoid all trans-retinoic acid (ATRA; ref. 4).

The peroxisome proliferator-activated receptor γ (PPARγ) is a recent addition to the nuclear steroid receptor superfamily that is believed to be involved in the regulation of lipid metabolism. Like other nuclear steroid receptors, the dysregulation of PPARγ has been implicated in aberrant differentiation. PPARγ is an important mediator of the differentiation of preadipocytes to adipocytes and is a target in the treatment of diabetes, where receptor activation increases sensitivity to endogenous insulin. To activate transcription, PPARγ must form a heterodimer with RXRα (5). The PPARγ/RXR heterodimers can then be activated by the ligation of PPARγ or RXR ligands (6), but the simultaneous ligation of both PPARγ and RXR maximizes their activation. PPARγ can be activated by
naturally occurring ligands such as the long-chain fatty acids 15-deoxy-A12,14-prostaglandin J2 (15-d-PGJ2) and 9- and 13-cis-hydroxyoctadecadienoic acid (7–9). They are also activated by synthetic ligands including the antidiabetic agents troglitazone, BRL49653 (rosiglitazone), and pioglitazone; t-Tyr-based PPARγ ligands (10); Fmoc-l-Leu amino acids (11); and certain triterpenoids (12).

Although PPARγ ligands have mainly been used for the treatment of diabetes, they have also been shown to induce lipogenic differentiation of malignant cells in patients with liposarcomas (13). In hematopoiesis, PPARγ expression is increased during differentiation of monocytes to macrophages and the activation of PPARγ expression is increased during differentiation of monocytes and the ligand-induced activation of PPARγ/RXR-regulated genes induces macrophage differentiation (9). PPARγ expression has been detected in human breast, lung, bladder, colon, prostate, and pancreatic cancer cells and the ligand-induced activation of PPARγ/RXR induced apoptosis in these cell types (14–21). PPARγ/RXR signaling is involved in the induction of apoptosis in T lymphocytes (22) and myeloid leukemias (23). Interestingly, a recent report showed that synthetic PPARγ agonists used at high concentrations inhibited the translation initiation of eukaryotic initiation factor-2 by phosphorylation in PPARγ-deficient embryonic stem cells, suggesting the existence of a receptor-independent antiproliferative mechanism (24).

In this study, we investigated the effects of structurally different PPARγ ligands on a variety of myeloid and lymphoid leukemic cell lines and primary leukemia samples. The PPARγ ligands decreased viability and induced apoptosis, with the novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) being the most potent cytotoxic agent. Concomitant RXRα ligation enhanced the effects of PPARγ ligands and the combination of a PPARγ ligand with ATRA induced maturation of myelomonocytic cells. Furthermore, the proapoptotic effects of CDDO could be further enhanced by inhibition of Bcl-2. Hence, the apoptotic and differentiation-inducing properties of PPARγ ligands and their low toxicity make them promising candidates for antileukemia therapy.

### Materials and Methods

### Reagents

CDDO was synthesized by Dr. Takashi Honda and colleagues (Dartmouth Medical College, Hanover, NH; ref. 25) and kindly provided through the Rapid Access to Intervention Development mechanism by Dr. Edward Sausville (National Cancer Institute/Cancer Therapy Evaluation Program, Bethesda, MD). ATRA was purchased from Sigma Chemical Co. (St. Louis, MO). RXR-specific ligand LG100268 (26) was kindly provided by Dr. Reid Bissonnette (Ligand Pharmaceuticals, San Diego, CA). The agonists for PPARγ included 15-d-PGJ2 (Cayman Chemical Co., Ann Arbor, MI; refs. 7, 8), BRL49653 (provided by SmithKline Beecham Pharmaceuticals, Harlow, United Kingdom), and troglitazone (provided by Drs. W. Johnson and A. Saltei, Parke-Davis/Warner-Lambert, Ann Arbor, MI). LG100268 (26) was kindly provided by Dr. Reid Bissonnette (Ligand Pharmaceuticals, San Diego, CA). The agonists for PPARγ included 15-d-PGJ2 (Cayman Chemical Co., Ann Arbor, MI; refs. 7, 8), BRL49653 (provided by SmithKline Beecham Pharmaceuticals, Harlow, United Kingdom), and troglitazone (provided by Drs. W. Johnson and A. Saltei, Parke-Davis/Warner-Lambert, Ann Arbor, MI). LG100268 (26) was kindly provided by Dr. Reid Bissonnette (Ligand Pharmaceuticals, San Diego, CA).

### Inhibition of DNA Synthesis and Cell Viability

[3H]Thymidine Uptake. Triplicate samples of 1 × 10^5 cells suspended in 200 μL RPMI were cultured in the presence or absence of PPARγ ligands in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) in a humidified atmosphere of 5% CO2 in air at 37°C. After 2 days, 0.1 μCi [3H]thymidine (specific activity, 6.7 Ci/mmol, DuPont, Wilmington, DE) was added to each well and the cultures were incubated overnight. The cells were then deposited onto harvester filters using a semiautomatic cell harvester.
Radioactivity was measured in a scintillation counter and the results are expressed as the counts per minute for the respective samples. The inhibition of DNA synthesis was expressed as the percentage of the counts per minute measured in treated sample divided by the counts per minute in the control (DMSO-treated) sample. Cell viability was determined by counting triplicate samples for trypan blue dye–excluding cells or by the ViaLight proliferation/cytotoxicity kit (LumiTech Ltd., Nottingham, United Kingdom). In this assay, the bioluminescence of ATP is a measure of viability (33). Briefly, ATP was first extracted from cells using nucleotide releasing reagent, after which ATP monitoring reagent was added and light emission was measured using a luminometer.

**Colony Formation in Soft Agar**

Cells were seeded in a two-layer soft agar system as described previously (34). Briefly, the bottom layer consisted of 0.5% agar in which the test substances were mixed; the top layer was 0.3% agar (Difco Laboratories, Detroit, MI) in which 1,000 to 5,000 cells were mixed per plate. All experiments were done in triplicate. After 10 days of incubation at 37°C in a humidified atmosphere containing 5% CO₂ in air, colonies (>40 cells) were counted using an inverted microscope.

**Studies of Induction of Differentiation**

The differentiation of myeloid leukemic cells was determined by their ability to produce superoxide, as measured by the reduction of nitroblue tetrazolium (NBT), which occurs when cells undergo either monocyte or granulocyte differentiation (35). Staining the cells for α-naphthyl acetate esterase (NSE, Sigma Chemical) was also used as a cytosolic indicator of differentiation. Differentiation–specific cell morphology was assessed on cytospin preparations stained with the Diff-Quick Stain Set (Baxter Healthcare Corp, Miami, FL). The analysis of surface differentiation–specific cell surface antigens was measured by flow cytometry. The phycoerythrin-conjugated anti-CD11b and FITC-conjugated anti-CD14 monoclonal antibodies (Becton Dickinson, San Jose, CA) were used at a 1:10 dilution. The percentage of positive cells was calculated by subtracting the percentage of cells with a fluorescence intensity greater than the set marker using the isotype control (background) from the percentage of cells with a fluorescence intensity greater than the same marker using the specific antibody (36).

**Phagocytosis**

After exposure to PPARγ ligands and/or ATRA, leukemic cells were washed twice in PBS and cultured in regular medium for 1 day and their ability to phagocytose yeast was tested as described elsewhere (37). Briefly, *Candida albicans* were opsonized in 10% human AB serum. A 5:1 ratio of *C. albicans* to leukemic cells was incubated at 37°C for 30 minutes. The cells were stained with a Diff-Quick Stain Set, and the percentage of cells containing three or more yeast particles was determined microscopically.

**Flow Cytometric Analysis of Apoptosis**

In primary AML samples, apoptosis was determined by phosphatidylserine/Annexin V flow cytometry. Briefly, cells were washed in PBS and resuspended in 100 μL binding buffer containing Annexin V (Roche Diagnostic Corp., Indianapolis, IN). Cells were analyzed by flow cytometry after the addition of propidium iodide (38). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane. In CLL samples, Annexin V positivity was determined on CD19⁺ B cells after appropriate gating.

For analyzing time-dependent induction of apoptosis, a three-color apoptosis assay combining measurement of phosphatidylserine/Annexin V, analysis of the mitochondrial membrane potential (ΔΨm) using chloromethyl X-rosamine (39), and detection of active caspases by the CaspaTag assay was developed and validated (40). Briefly, cells were washed in PBS and resuspended in 200 μL chloromethyl X-rosamine (Molecular Probes, Eugene, OR) working solution (300 nmol/L in RPMI) followed by the addition of 5 μL CaspaTag (FAM-DEVD-FMK, Intergen, Inc., Purchase, NY) working solution (150× in PBS). After a 1-hour incubation at 37°C, cells were washed in PBS and the pellet was resuspended in 100 μL binding buffer containing Annexin V-biotin (100×, Roche Diagnostic Corp.). Cells were incubated in the dark at room temperature for 15 minutes, washed once with Annexin binding buffer, and stained with 10 μL avenir-phycocerythrin on ice for 30 minutes. After washing with the Annexin binding buffer, the cell pellet was resuspended in binding buffer and analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using appropriate compensation. Untreated cells were stained using the same protocol and served as controls.

**Western Blot Analysis**

Cell lysates derived from ~2 × 10⁵ cells were subjected to SDS-PAGE in 12% polyacrylamide gels followed by protein transfer to a Hybond-P membrane (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and immunoblotting. Antibody against PPARγ was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Bcl-2 from DAKO Corp. (Carpinteria, CA), and caspase-8 and -9 and a specific antibody recognizing only the p20-processed caspase-3 band were from Cell Signaling Technology Inc. (Beverly, MA). Anti-β-actin blots were run in parallel as loading controls. Signals were detected by a PhosphorImager (Storm 860, version 4.0, Molecular Dynamics, Sunnyvale, CA).

**Transfection Assays**

HL-60-CDM-1 cells were transfected with empty expression vector (pcDNA3), FLAG-tagged wild-type PPARγ, or FLAG-tagged L466A/E469A dominant-negative (DN) PPARγ mutant together with a selectable marker (neo). Wild-type human PPARγ and the DN-PPARγ mutant were kindly provided by Dr. Krishna K. Chatterjee (University of Cambridge, Cambridge, United Kingdom; ref. 41). Stable transfectants of the leukemic cells were obtained using the calcium phosphate method (42) and neomycin-resistant clones were selected by treatment with G418. Lysates from the selected clones were evaluated for transgene expression by immunoblot analyses using...
anti-FLAG antibodies. The clones expressing high levels of the specific proteins were further subcloned by limiting dilution. Representative subclones of each of the HL-60-CDM-1 transfectants were passaged twice per week and used for the studies. Data are representative of those derived from at least two independent clonal transfectants of CDM-1/PPARγ cells.

For luciferase reporter assays, 1 µg TK-PPRE-Luc plasmid (a gift from Ron M. Evans, Salk Institute, La Jolla, CA) was transfected into MCF-7 cells using Fugene 6 (Boehringer-Mannheim, Mannheim, Germany). Cells were then cultured for 24 hours in complete medium and treated with CDDO or 15-d-PGJ2 alone or in combination with T007. Luciferase activity was assayed with luciferase assay system (Promega, Madison, WI).

Statistics

Results are expressed as the means ± SD of triplicate samples. Statistical significance was determined by two-tailed, paired, Student’s t test with a P < 0.05 confidence interval. The combination index (CI) for experimental treatment combinations was calculated to determine the synergistic, additive, or antagonistic effects of the combinations using the Chou-Talalay method (43) and CalcuSyn software (Biosoft, Ferguson, MO). When CI is 1, the equation represents the conservation isobologram and indicates additive effects. CI values of <1.0 indicate a more than expected additive effect (i.e., synergism).

Results

PPARγ is Expressed in Lymphoid and Myeloid Leukemic Cells

The expression of PPARγ was examined by Western blot analysis in myeloid and lymphoid leukemic cell lines and in primary samples from leukemia patients. To confirm the specificity of the reaction, a monoclonal antibody to PPARγ or antibody preadsorbed with blocking peptide was used for competition studies. PPARγ was found to be expressed in B and T lymphoid leukemias, lymphomas, Hodgkin’s disease, myeloma cell lines, and primary AML and CLL cells (Fig. 1). PPARγ was expressed at higher levels in myelomonocytic U937 than in myeloid HL-60 cells, confirming data published previously (44). PPARγ protein was also expressed in 9 of 11 primary AML samples with high blast count (≥50%; see examples in Fig. 1). Low expression was noted in two of four samples from patients with advanced myelodysplastic syndrome (refractory anemia with excess blasts). We then compared PPARγ mRNA expression in normal cells and in primary AML samples. In normal peripheral blood (n = 3) and in CD34+ cells separated by apheresis (n = 2), PPARγ was expressed at low levels (peripheral blood, mean 5.2 ± 3.3 compared with a normal calibrator sample; CD34+ cells, 1.7 ± 1.2). In 15 of 20 primary AML samples, PPARγ expression was increased (>2-fold) compared with normal CD34+ cells. In particular, PPARγ was highly expressed in six of seven AML-M4 specimens, with 10,000-fold increased expression in two cases (Table 1).

PPARγ Ligands Decrease Viability of Myeloid and Lymphoid Leukemic Cells

The effects of several PPARγ ligands on the viability of leukemic cells are shown in Fig. 2. In U937 cells with increased expression of PPARγ, BRL49653 decreased viability in a dose-dependent fashion (Fig. 2A). However, a concentration of 50 µmol/L BRL49653 was required for a 50% inhibition of growth in these cells. BRL49653 at 25 µmol/L decreased viability by ~50% in Raji, Su-DHL, Sup-M2 cells and Hodgkin’s cells (Fig. 2B and C), but no effect was seen in HL-60 and Ramos cells (Fig. 2A and B). BRL49653 at 5 µmol/L consistently killed both lymphoid and myeloid cell lines. CDDO was the most potent agent in this group and decreased viability markedly at 1 µmol/L and almost completely eliminated the viable cell population at 2 µmol/L.

To determine if PPARγ ligands decrease viability by inducing apoptosis, we conducted a time course experiment in U937 cells with CDDO (2 µmol/L). Discrete events of apoptosis were determined by multiparametric flow cytometry. After 4-hour exposure to CDDO, 21% of cells had lost their MMP (green cell population) and a small proportion (~12%) was Annexin V positive. There was no evidence of caspase activation at this time. After 12 hours, 68% cells were MMP-low and ~14% of these cells were CaspaseTag positive (blue cell population) and 12% were Annexin V positive, respectively. After 24 hours, half of the MMP-low cells became CaspaseTag positive and Annexin V positive. The other half of this population lost MMP but showed no caspase activation and did not express
phosphatidylserine/Annexin V (Fig. 3A). These data suggested that CDDO induces mitochondrial depolarization followed by caspase activation in leukemic cells. Western blot analysis revealed that CDDO induced the cleavage of caspase-8, -9, and -3 after 24-hour exposure (Fig. 3B). Similar data were obtained in lymphoid Ramos cells (data not shown).

Examining the Relationship between PPARγ Expression and Apoptosis Induction by PPARγ Ligands

To characterize the correlation between PPARγ expression and antileukemic activity of certain PPARγ ligands, we tested the sensitivity of PPARγ-overexpressing leukemic cells to CDDO-induced killing. We employed HL-60-CDM-1 cells for these studies because of their low endogenous levels of PPARγ protein (23). Vector control (pcDNA3) or wild-type PPARγ-transfected HL-60-CDM-1 cells (two different clones) were treated with 0.3 and 0.5 μmol/L CDDO for 48 hours. Apoptosis was determined by Annexin V staining using flow cytometry. Whereas CDDO induced apoptosis in the vector control cells, forced overexpression of PPARγ doubled the sensitivity of HL-60-CDM-1 cells to CDDO-induced killing (Fig. 4A). HL-60-CDM-1 cells transfected with a DN-PPARγ mutant that functions as an inhibitor (41) were much less sensitive than the wild-type transfected cells (Fig. 4A). However, low levels of apoptosis were still observed suggesting PPARγ-independent mechanism(s). This effect was more pronounced at higher concentrations where CDDO (≥1 μmol/L) killed 84% of vector controls, 95% and 96% of PPARγ-overexpressing cells, and 74% of DN transfectants.

Table 1. Relative PPARγ mRNA expression levels in primary AML samples

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<td>AML (FAB ND)</td>
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NOTE: PPARγ expression was analyzed by quantitative Taqman reverse transcription-PCR. A comparative CT method was used to calculate the relative amount of PPARγ in the sample (X) normalized to endogenous reference (β2-microglobulin) and relative to the amount of PPARγ in calibrator sample (Y): 2^ΔΔCT, where ΔCT is the difference in threshold cycles for PPARγ and β2-microglobulin and ΔΔCT for sample X = ΔCTX - ΔCTY. One of the normal peripheral blood samples was designated as calibrator or the 1X sample. FAB, French-American-British classification; PB, peripheral blood; BM, bone marrow; Ph, pheresis; ND, not determined.
In an alternative approach, we used the novel selective PPARγ antagonist T007 (27). T007 completely abrogated the CDDO- and 15-d-PGJ2-induced transactivation of PPRE-Luc in MCF-7 cells transfected with PPRE-Luc expression plasmid (Fig. 4B). Pretreatment of U937 cells with 2 μmol/L T007 partially inhibited myeloid differentiation induced by CDDO or 15-d-PGJ2. CD11b was expressed on >90% of control cells. However, CDDO and 15-d-PGJ2 increased the mean fluorescence intensity of CD11b staining (mean fluorescence intensity, CDDO 73 versus DMSO 23, mean fluorescence intensity = 80 for 15-d-PGJ2) and T007 diminished it (mean fluorescence intensity = 53). 15-d-PGJ2, but not CDDO, increased the percentage of cells expressing CD14 differentiation marker (55% compared with 0.1% in DMSO) and this effect was abrogated by T007 (5% CD14+ cells).

Although T007 significantly diminished apoptosis induced by low concentrations of CDDO and 15-d-PGJ2 (Fig. 4C), it only marginally decreased cell death when PPAR ligands were used at higher concentrations, suggesting again the existence of a potential PPAR-independent mechanism of apoptosis.

Figure 3. CDDO induces caspase activation and mitochondrial depolarization in U937 cells. Apoptosis was determined in U937 cells after 4, 12, and 24 hours of treatment with 2 μmol/L CDDO. A, in a multicolor flow cytometry assay, chloromethyl X-rosamine (CMXRos) measures changes in MMP; Annexin V staining determines changes in phosphatidylserine expression on the plasma membrane; and CaspaTag examines caspase activation. Y axis, chloromethyl X-rosamine; X axis, CaspaTag (top) and Annexin V (bottom). Red, live cells preserving their MMP; they are negative for CaspaTag and Annexin V. Green, cells that lose their MMP; at 4 hours, they are CaspaTag negative, but a proportion are Annexin V positive (in vehicle-containing cultures, 5.5% cells were Annexin V positive and 4.1% CaspaTag positive). At 12 hours, the activation of caspases is detected by the CaspaTag assay (blue, 14%). These cells have low MMP and are Annexin V positive. At 24 hours, massive caspase activation and Annexin positivity is noted, but a proportion of chloromethyl X-rosamine-low CaspaTag/Annexin-negative cells exist. B, in the same experiment, the cleavage of caspase-8, -9, and -3 was studied by Western blot analysis at 24 hours. Actin was used as loading control.

PPARγ and RXR Ligand Combinations Are More Potent than PPARγ Ligands Alone in Inhibiting Proliferation and Inducing Differentiation and Apoptosis in Leukemic Cells

PPARγ forms heterodimers with RXR and the ligation of both receptors enhances their antidiabetic effect in vivo (45). RXR is expressed at ubiquitously high levels in all hematopoietic cells examined in this study (data not shown). We therefore evaluated the effects of combined PPARγ and RXR ligation. First, we examined the ability of synthetic PPARγ ligands alone to induce differentiation in HL-60 cells. CDDO, 15-d-PGJ2, and BRL49653 induced myelomonocytic differentiation in HL-60 cells as shown by morphology, NBT reduction assay, and induction of the cell surface CD11b and CD14 differentiation markers. In this respect, CDDO was much more potent on a molar basis than 15-d-PGJ2 and BRL49653 (Fig. 5).

We then combined 15-d-PGJ2 or CDDO with the RXR-specific ligand LG100268 (100 nmol/L). In HL-60 cells, the combination of LG100268 with 15-d-PGJ2 or CDDO induced pronounced myelomonocytic differentiation (Table 2), with CDDO being much more potent than
cell growth minimally (13-20%), whereas in combination with LG100268 (100 nmol/L) inhibition increased to 50%. The induction of cell differentiation also resulted in decreased viability. The trypan blue exclusion assay showed no decrease in cell viability when BRL49653 or LG100268 were used alone, whereas a decrease of >50% was noted when both ligands were combined. Similarly, RXR ligation potentiated the growth-inhibitory effect of 15-d-PGJ2 and CDDO. The RXR-specific ligand LG100268 also enhanced CDDO-induced apoptosis in five primary AML samples (DMSO, 24.7 ± 6.3%; Annexin V positivity: LG100268, 32.4 ± 6.7%; 2 μmol/L CDDO, 57.4 ± 6.1%; CDDO plus LG100268, 73 ± 9%; P < 0.02 compared with CDDO alone).

Combined PPARγ and Retinoic Acid Receptor α Lig-ation (Troglitazone and ATRA) Induces Differentiation and Inhibits Clonogenic Growth of Myeloid Leukemic Cells

Because ATRA has been shown to enhance the growth-inhibitory effects of PPARγ ligands (44, 46, 47), we examined the combined effects of ATRA and the PPARγ ligand troglitazone in myeloid leukemias. A 3-day exposure of U937 cells to either ATRA (1 μmol/L) or troglitazone (10 μmol/L) resulted in 28% and <5% NBT-positive cells, respectively. In contrast, the combination of troglitazone and ATRA induced 78% NBT-positive cells. Whereas CD14 expression did not change, CD11b was induced by ATRA (57%) and more so by ATRA plus troglitazone (95%; data not shown). These phenotypical changes were associated with morphologic evidence of differentiation (Fig. 7A). Controls and troglitazone-exposed U937 cells displayed the characteristic morphology of myelomonocytic leukemic cells, with round nuclei containing condensed chromatin surrounded by a rim of basophilic cytoplasm. Cells cultured with ATRA alone exhibited morphologic signs of differentiation in that these cells had irregularly shaped nuclei, limited chromatin condensation, and an apparent decrease in the nuclear to cytoplasmic ratio. In contrast, cells cultured with both ATRA and troglitazone developed condensed, lobulated nuclear characteristic of cells undergoing granulocyte-like differentiation. In addition, the nuclei were surrounded by the abundant foamy cytoplasm frequently observed in macrophage-differentiated monocytes. Similar changes were observed in THP-1 leukemic cells (data not shown).

NSE is a monocyte-specific enzyme expressed in ~3% of U937 and 13% of THP-1 cells. No significant change in enzyme activity was observed after incubation of cells with either ATRA or troglitazone. However, the combination of both compounds greatly increased the percentage of NSE-positive cells (Table 3). Likewise, the ability U937 and THP-1 cells to phagocytose C. albicans particles increased significantly after a combined treatment of ATRA and troglitazone (Fig. 7B, Table 3) as compared with the effects of either compound alone. The two-layer soft agar system was used to study the effects of troglitazone and ATRA on clonogenic growth of leukemic cells. Whereas troglitazone alone used at 10⁻⁵ to 10⁻⁶ mol/L did not

15-d-PGJ2, at much lower concentrations. Accordingly, when PPARγ ligands were combined with LG100268, the proliferation of HL-60 cells was more inhibited than by any of the ligands alone (Fig. 6). As an example, BRL49653 (rosiglitazone) alone, used at 10 and 25 μmol/L, decreased
significantly affect clonal proliferation of U937 and THP-1 cells, colony formation was significantly diminished by the combination of troglitazone with ATRA (Table 3; troglitazone plus ATRA versus troglitazone or ATRA, \( P < 0.02 \)).

We then analyzed the interactions between ATRA and CDDO in U937 cells using isobologram CalcuSyn software. CDDO and ATRA were used at a fixed 1:1 ratio at 0.5, 0.75, and 1 \( \mu \text{mol/L} \) concentrations for 48 hours. The effects on cell proliferation and apoptosis were assessed by cell

<table>
<thead>
<tr>
<th>CD11b+</th>
<th>CD14+</th>
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<td>NBT (+)</td>
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<td>DMSO</td>
<td>14%</td>
</tr>
<tr>
<td>PGJ2 3 ( \mu \text{mol/L} )</td>
<td>37%</td>
</tr>
<tr>
<td>BRL 10 ( \mu \text{mol/L} )</td>
<td>25%</td>
</tr>
<tr>
<td>CDDO 0.1 ( \mu \text{mol/L} )</td>
<td>23%</td>
</tr>
<tr>
<td>CDDO 0.5 ( \mu \text{mol/L} )</td>
<td>51%</td>
</tr>
</tbody>
</table>

Table 2. Induction of differentiation in HL-60 cells by PPARγ and RXR ligation

<table>
<thead>
<tr>
<th>CD11b/CD14+</th>
<th>Vehicle</th>
<th>15-d-PGJ2 (1 ( \mu \text{mol/L} ))</th>
<th>15-d-PGJ2 (3 ( \mu \text{mol/L} ))</th>
<th>15-d-PGJ2 (5 ( \mu \text{mol/L} ))</th>
<th>CDDO (0.3 ( \mu \text{mol/L} ))</th>
<th>CDDO (0.6 ( \mu \text{mol/L} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>2.7 ± 0.1</td>
<td>3.6 ± 0.4</td>
<td>15.3 ± 0.1</td>
<td>21.5 ± 5.1</td>
<td>15.4 ± 2.6</td>
<td>25.3 ± 2.6</td>
</tr>
<tr>
<td>+ LG 100268</td>
<td>4.9 ± 1.3</td>
<td>10.3 ± 4.7</td>
<td>19.3 ± 2.9</td>
<td>24.8 ± 5.9</td>
<td>55.1 ± 3.4</td>
<td>96.2 ± 1.6</td>
</tr>
</tbody>
</table>

NOTE: Induction of differentiation was analyzed using the phycoerythrin-conjugated anti-CD11b and FITC-conjugated anti-CD14 monoclonal antibodies. Data represent means ± SD of the percentage of double-positive cells compared with isotype control antibodies from three independent experiments.
counts and Annexin V flow cytometry, respectively. Isobologram analysis (43) showed that the proapoptotic interaction between CDDO and ATRA was synergistic (ED\textsubscript{50}, CI = 0.861; ED\textsubscript{75}, CI = 0.588; ED\textsubscript{90}, CI = 0.683).

**PPAR\textsubscript{\gamma} Ligands Induce Apoptosis in Primary Leukemic but not in Normal Cells**

We used samples from CLL patients to examine the sensitivity of primary leukemic cells to the growth-inhibitory and apoptogenic effects of PPAR\textsubscript{\gamma} ligands. The PPAR\textsubscript{\gamma} ligands 15-d-PGJ\textsubscript{2} (5 \textmu mol/L) and BRL49653 (25 \textmu mol/L) significantly decreased the viability of CLL cell samples by inducing apoptosis (Fig. 8A and B) as shown by Annexin V flow cytometry.

We then tested the effects of CDDO on primary CLL. CDDO reportedly induced apoptosis in CLL cells, including samples from patients with fludarabine-resistant CLL, by activating the caspase-8 and -3 (48). In our study, a similar response was observed in a total of seven primary CLL samples. CDDO induced dose-dependent inhibition of proliferation in CLL cells (data not shown), which was accompanied by the concomitant induction of apoptosis (Fig. 8C).

In contrast, viability of normal CD34\textsuperscript{+} cells in the presence of 15-d-PGJ\textsubscript{2} (5 \textmu mol/L) was only moderately decreased, whereas BRL49653 at pharmacologic concentration (25 \textmu mol/L) was ineffective (data not shown). These data were confirmed by Annexin V flow cytometry (Fig. 8D). CDDO induced significantly less apoptosis in CD34\textsuperscript{+} cells compared with CLL, suggesting differential killing of leukemic versus normal progenitor cells (Fig. 8D).

**CDDO-Induced Apoptosis in CLL Samples Is Enhanced by Simultaneous RXR Ligation or Bcl-2 Blockade**

To further delineate the mechanism(s) by which cells are sensitized to CDDO-induced apoptosis, we treated nine additional CLL samples with the combination of CDDO and a Fas agonistic antibody (CH11) or TRAIL, which induce apoptosis through the extrinsic pathway (49, 50), and the small-molecule Bcl-2 inhibitor HA14-1 that is believed to regulate the intrinsic pathway (51–53). Six of the primary CLL samples were from patients that relapsed after treatment with fludarabine combined with other agents, and sample 8 was derived from a patient with refractory T-cell CLL. CDDO alone at 1 \textmu mol/L induced apoptosis in five of nine samples tested, including samples from two fludarabine-resistant patients and one patient with refractory T-cell CLL (Table 4). The RXR-selective ligand LG100268, being nontoxic alone, enhanced the induction of apoptosis by CDDO in five of nine CLL samples, including two samples in which CDDO alone did not induce cell death.

Activation of the extrinsic apoptotic pathway by CH11 or TRAIL induced moderate cell death in only two of nine CLL samples. When combined with CDDO, CH11 did not affect cytotoxicity, but TRAIL enhanced CDDO-induced killing in two of nine samples. In contrast, HA14-1 was cytotoxic in four of nine samples and potentiated the CDDO-induced apoptosis in all samples tested (Fig. 9A). Furthermore, the combined treatment induced pronounced cell death in the four samples in which CDDO alone was ineffective. In all CLL samples, we found expression of Bcl-2 protein, as shown in Fig. 9B, and there was no change in Bcl-2 expression with any of the treatments (Fig. 9B). To further define the mechanisms by which cells are sensitized to the proapoptotic effects of CDDO, we compared the cleavage of caspase-8 and -9 in all CLL samples treated with the CDDO/HA14-1 combination. In all four samples, CDDO efficiently cleaved caspase-8. Nevertheless, CDDO alone did not induce apoptosis in two of these samples, suggesting that the additional cleavage of caspase-9, facilitated by functional Bcl-2 blockade, was required for full proapoptotic effect of the drug. Examples of CDDO-sensitive (CLL1) and CDDO-resistant (CLL2) samples are shown in Fig. 9B. This observation in CLL suggests that CDDO induces apoptosis via the activation of components of both extrinsic and intrinsic apoptotic pathways.

**Discussion**

This study was conducted to characterize the activity of PPAR\textsubscript{\gamma} nuclear receptor signaling pathway in leukemia cells. Leukemic cell lines showed high expression of PPAR\textsubscript{\gamma} protein in both myeloid and lymphoid cells including Hodgkin’s disease and multiple myeloma cells. Although the presence of PPAR\textsubscript{\gamma} has been reported in leukemic cell lines (44, 54, 55), we report here the frequent expression of PPAR\textsubscript{\gamma} in primary leukemia samples including AML and CLL. PPAR\textsubscript{\gamma} mRNA expression in primary AML was highest in myelomonocytic AML (M4) subtypes. These data suggest that CDDO and other PPAR\textsubscript{\gamma} ligands might be particularly useful in the treatment of myelomonocytic leukemia.
subtypes of AML/myelodysplastic syndrome. This was also noted in a recent clinical trial in which PPARγ ligands exerted antileukemia activity in patients with chronic myelomonocytic leukemia (56).

It has been reported that PPARγ ligands can induce leukemic cells to differentiate toward macrophages (44, 57). In our study, 15-d-PGJ₂, BRL49653, and CDDO induced myelomonocytic differentiation of HL-60 cells, with CDDO being the most potent. CDDO and 15-d-PGJ₂ also induced apoptosis in leukemic cells, whereas rosiglitazone only seemed to inhibit proliferation.

The expression of PPARγ in lymphoid malignancies provides a potential target for novel therapeutic strategies for these malignancies as well. Although the proapoptotic
properties of 15-d-PGJ2 have been described previously (54, 55, 58, 59), here we report that CDDO is more potent than 15-d-PGJ2 in triggering apoptosis in lymphoid cells including Hodgkin’s and myeloma cells. Moreover, CDDO was effective in primary refractory T-cell CLL, confirming recently published data showing that PPARγ agonists exhibit selectivity in apoptosis induction in transformed, but not normal, T-lineage cells (59). Apoptosis seems the primary mechanism of CDDO. Whereas activation of certain components of the extrinsic pathway (i.e., caspase-8), which is dysregulated in leukemias, provides an attractive target of CDDO-mediated cytotoxicity, the simultaneous inhibitions of Bcl-2 expression or function resulted in highly increased induction of programmed cell death in lymphoid cells with high Bcl-2 levels.

Because CDDO reportedly binds to and transactivates the nuclear receptor PPARγ (12), we explored the correlation between PPARγ expression levels and sensitivity to CDDO. Our experiments using genetically modified HL-60-CDM-I cells showed sensitization to the cytotoxic effects of CDDO in cells transfected with wild-type PPARγ. This effect was significantly diminished in DN-PPARγ transfectants. The DN-PPARγ receptor reportedly retains ligand and DNA binding but exhibits reduced transactivation and activity due to impaired coactivator recruitment (41). Alternatively, pretreatment of U937 cells with the specific PPARγ antagonist, T007, diminished CDDO- and 15-d-PGJ2-induced apoptosis and differentiation, suggesting that these effects are PPARγ dependent. However, at higher concentrations, CDDO was capable of inducing apoptosis in cells carrying the DN mutant, and blockade of PPARγ did not prevent cytotoxic effects of the compound, suggesting existence of other, yet unidentified, cellular targets. The induction of apoptosis by PPARγ agonists may therefore be independent of PPARγ modulation as was reported for some other biological effects of PPARγ ligands (60, 61). For example, a recent study showed that, at concentrations that induce optimal transcriptional activation of PPARγ, thiazolidinedione protected T cells from

Table 3. Combination of troglitazone and ATRA induces differentiation and inhibits clonogenic growth of myeloid leukemic cells

<table>
<thead>
<tr>
<th></th>
<th>NSE+ Phagocytosis %</th>
<th>Colony Formation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937 Control</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Troglitazone 10−5 mol/L</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>ATRA 10−8 mol/L</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Troglitazone + ATRA</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>THP-1 Control</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Troglitazone 10−5 mol/L</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>ATRA 10−8 mol/L</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>Troglitazone + ATRA</td>
<td>40</td>
<td>80</td>
</tr>
</tbody>
</table>

NOTE: Results represent the mean of three independent experiments.

*Percentage of NSE-positive cells.
1Percentage of cells phagocytozing C. albicans.
3Percentage of leukemic colonies compared with vector control (100%).

Figure 8. PPARγ ligands decrease viability of primary CLL cells. A, cells from six primary CLL samples were cultured in the presence of BRL49653 (25 μmol/L) or 15-d-PGJ2 (5 μmol/L) for 5 days. Viability was determined by ViaLight assay as described in Materials and Methods. Columns, mean percentage compared with DMSO-treated control cultures; bars, SD. B, induction of apoptosis was determined by Annexin V flow cytometry as described in Materials and Methods. Columns, mean of cell numbers or the percentage of apoptotic cells in seven different samples compared with untreated control cultures; bars, SD. D, effects of PPARγ ligands on normal cells. For BRL49653 and 15-d-PGJ2 experiments, purified CD34+ cells (n = 4) were used. For CDDO experiments, purified CD34+ cells from six apheresis samples were examined. Induction of apoptosis was determined by Annexin V flow cytometry.
growth factor deprivation, whereas it induced apoptosis when used at higher concentrations (62). Similarly, 15-d-PGJ2 or thiazolidinedione were capable of decreasing the release of inflammatory cytokines (60) or causing G1 cell cycle arrest (24) in PPARγ-null embryonic stem cells. Conclusive proof of PPARγ dependence will come from testing PPARγ ligands in vivo in a PPAR-deficient background or in vitro by silencing expression of PPARγ using small interfering RNA.

The combination of the PPARγ ligand troglitazone and certain retinoids reportedly suppressed colony formation of leukemic cells in an additive fashion (44). Because PPARγ and RXR are known to function as heterodimers, we hypothesized that combined treatment with PPARγ and RXR-specific ligands would significantly potentiate the antileukemic effect of PPARγ ligands alone. Indeed, the combination of PPARγ and RXR activation (using the specific ligand LG100268) profoundly induced differentiation in HL-60 cells. Furthermore, LG100268 enhanced CDDO-induced apoptosis in an additive fashion in primary AML and CLL samples. Of note, the RXR agonist was used at only 100 nmol/L, a concentration at which the compound induced apoptosis in an additive fashion in primary AML samples as shown by a variety of functional assays (NBT, NSE, CD11b, and the ability to phagocytose C. albicans). Trogli-tazone and ATRA combined synergistically decreased the clonogenic capacity of myelomonocytic leukemia lines as shown by a variety of functional assays (NBT, NSE, CD11b, and the ability to phagocytose C. albicans). Troglitazone and ATRA combined synergistically decreased the clonogenic capacity of myelomonocytic leukemia lines.

### Table 4. Induction of apoptosis by CDDO is enhanced by Bcl-2 inhibitor HA14-1 in primary CLL samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Vehicle (DMSO)</th>
<th>CDDO (1 μmol/L)</th>
<th>LG 100268 (1 μmol/L)</th>
<th>CH11 (50 ng/mL)</th>
<th>TRAIL (50 ng/mL)</th>
<th>HA14-1 (15 μmol/L)</th>
<th>CDDO+LG1002 68</th>
<th>CDDO+CH11</th>
<th>CDDO+HA14-1</th>
<th>CDDO+TRAIL</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>14.8</td>
<td>20.1</td>
<td>14.9</td>
<td>8.8</td>
<td>13</td>
<td>26.7</td>
<td>21.6</td>
<td>12.9</td>
<td>88.1</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>6.1</td>
<td>4.9</td>
<td>2.6</td>
<td>2.8</td>
<td>7.7</td>
<td>15.1</td>
<td>5.4</td>
<td>35</td>
<td>54.6</td>
</tr>
<tr>
<td>3</td>
<td>7.2</td>
<td>8.0</td>
<td>8.6</td>
<td>ND</td>
<td>ND</td>
<td>32.8</td>
<td>10.5</td>
<td>14.3</td>
<td>42.2</td>
<td>13.8</td>
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<tr>
<td>4</td>
<td>5.4</td>
<td>5.5</td>
<td>4.5</td>
<td>5</td>
<td>5</td>
<td>6.7</td>
<td>12.8</td>
<td>9.9</td>
<td>53.5</td>
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</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>5.2</td>
<td>5.5</td>
<td>5.9</td>
<td>5.9</td>
<td>75.7</td>
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<td>93.6</td>
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<tr>
<td>6</td>
<td>3.1</td>
<td>7.4</td>
<td>6.4</td>
<td>2.5</td>
<td>0.8</td>
<td>12.7</td>
<td>21.8</td>
<td>4.8</td>
<td>99.3</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>5.6</td>
<td>10.5</td>
<td>10.3</td>
<td>11.7</td>
<td>6.5</td>
<td>58.4</td>
<td>22.2</td>
<td>15.1</td>
<td>83.5</td>
<td>12.9</td>
</tr>
<tr>
<td>8</td>
<td>18.2</td>
<td>53.5</td>
<td>28.2</td>
<td>28.3</td>
<td>30.9</td>
<td>19.6</td>
<td>63.2</td>
<td>59.2</td>
<td>59.9</td>
<td>59.1</td>
</tr>
<tr>
<td>9</td>
<td>2.3</td>
<td>69</td>
<td>4.4</td>
<td>2.8</td>
<td>2</td>
<td>6.9</td>
<td>80.9</td>
<td>64.6</td>
<td>95.7</td>
<td>59.4</td>
</tr>
</tbody>
</table>

Mean ± SEM: 7.2 ± 1.8, 32.1 ± 9.3, 9.8 ± 2.6, 8.3 ± 3.1, 9.7 ± 3.3, 27.5 ± 8.2, 32.9 ± 8.4, 25.1 ± 7.8, 72.3 ± 8.3, 33.8 ± 7.4

**Figure 9.** Blockade of Bcl-2 with HA14-1 synergistically enhances CDDO-induced apoptosis in primary CLL cells via caspase activation. A. Cells from nine primary CLL samples were cultured with 50 ng/mL CH11, 50 ng/mL TRAIL, 15 μmol/L HA14-1 (HA), 1 μmol/L CDDO, either alone or in combination, for 72 hours. Apoptosis was determined by Annexin V flow cytometry after gating on CD19+ CLL cells. B. Western blot analysis of caspase activation in two CLL samples treated with CDDO, HA14-1, or their combination. Cells from patient 1 were sensitive, whereas cells from patient 2 were resistant to CDDO-induced apoptosis. **Bottom**, percentage of CD19/Annexin V-positive cells. The cleavage of caspase-8, -9, and -3 was determined as described in Materials and Methods. Bcl-2 protein levels showed no change with either treatment. Actin was used as loading control.
ATRA binds to all three retinoic acid receptors and directly activates them (69); ATRA does not bind to RXRs but shows RXR-stimulating activity in transactivation assays perhaps due to its isomerization to 9-cis-retinoic acid (70, 71). This may explain the observed synergism between troglitazone and ATRA. Alternatively, “activated” PPARγ/RXR heterodimers are able to cross-bind and activate a retinoic acid response element (72, 73). Whereas ATRA alone did not show activity in clinical trials in patients with AML (other than acute promyelocytic leukemia; refs. 74, 75), the combination of retinoids with PPARγ ligands may be beneficial. In summary, our results suggest that PPARγ ligands combined with clinically available RXR ligands have potential utility for the treatment of a broad spectrum of hematologic malignancies. Of importance, significantly less toxicity against normal progenitor cells compared with leukemias was observed, suggesting differential killing of leukemic cells. A clinical phase I trial combining BRL49653 (rosiglitazone) with the RXR-ligand boxareton (Targretin) was recently initiated at University of Texas M.D. Anderson Cancer Center and will test the safety and biological and clinical effects of this concept in hematopoietic malignancies.

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Molecular Cancer Therapeutics

Peroxisome proliferator-activated receptor γ and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias

Marina Konopleva, Elena Elstner, Teresa J. McQueen, et al.


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