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Role of Peroxisome Proliferator-Activated Receptor-γ and Its Coactivator DRIP205 in Cellular Responses to CDDO (RTA-401) in Acute Myelogenous Leukemia

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Abstract

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear receptor (NR) family of transcription factors with important regulatory roles in cellular growth, differentiation, and apoptosis. Using proteomic analysis, we showed expression of PPARγ protein in a series of 260 newly diagnosed primary acute myelogenous leukemia (AML) samples. Forced expression of PPARγ enhanced the sensitivity of myeloid leukemic cells to apoptosis induced by PPARγ agonists 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and 15-deoxy-^{12,14}-15DPGJ$_2$, through preferential cleavage of caspase-8. No effects on cell cycle distribution or differentiation were noted, despite prominent induction of p21 in PPARγ-transfected cells. In turn, antagonizing PPARγ function by small interfering RNA or pharmacologic PPARγ inhibitor significantly diminished apoptosis induction by CDDO. Overexpression of coactivator protein DRIP205 resulted in enhanced differentiation induction by CDDO in AML cells through PPARγ activation. Studies with *DRIP205* deletion constructs showed that the NR boxes of *DRIP205* are not required for this coactivation. In a phase I clinical trial of CDDO (RTA-401) in leukemia, CDDO induced an increase in PPARγ mRNA expression in six of nine patient samples; of those, induction of differentiation was documented in four patients and that of p21 in three patients, all expressing DRIP205 protein. In summary, these findings suggest that cellular levels of PPARγ regulate induction of apoptosis via caspase-8 activation, whereas the coactivator DRIP205 is a determinant of induction of differentiation, in response to PPARγ agonists in leukemic cells. *Cancer Res; 70(12); 4949–60. ©2010 AACR.*

Introduction

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that are members of the nuclear hormone receptor gene superfamily. To date, three PPAR subtypes have been identified, namely, PPARα, PPARβ/δ, and PPARγ (also termed as NR1C1, NR1C2, and NR1C3, respectively; ref. 1). PPARγ is an important regulator of lipid and glucose homeostasis, cellular differentiation, and inflammation. There are three PPARγ isoforms differing at their 5′ ends, each under the control of its own promoter (2). Most tissues express the PPARγ1 isoform, whereas the PPARγ2 isoform is specific to adipocytes. However, the receptor is expressed in many others tissues and cell types such as monocytes and macrophages, skeletal muscle, breast, prostate, colon, and type 2 alveolar pneumocytes.

PPARs form heterodimers with retinoid X receptors (RXR). Transcriptional regulation by nuclear receptors (NR), including PPARs and RXRs, involves the binding and recruitment of coactivators and/or mediators to target gene promoters. One component of the TRAP-Mediator complex, TRAP220 (the thyroid hormone receptor–associated protein, also known as ARC/DRIP205), is directly associated with the thyroid receptor, vitamin D receptor, and PPAR and facilitates NR-mediated transcription. Recent studies have further shown a functional role of TRAP220 in the optimal vitamin D receptor– and retinoic acid receptor–mediated myelomonocytic differentiation processes in hematopoietic cells (3). It was also found to act as a pivotal coactivator for GATA-1 in erythroid development (4) and has been shown to play a role in differentiation and proliferation of keratinocytes in response to vitamin D receptor stimulation (5, 6).

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PPARγ ligands have been extensively investigated and are known to inhibit proliferation and induce differentiation or apoptosis in different cancer cell types, including hematologic malignancies (7). Our studies have shown that PPARγ ligands alone or in combination with RXR-specific activators can inhibit clonal proliferation and induce differentiation of HL-60, U937, and THP1 human myeloid leukemic cell lines (8). 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a synthetic triterpenoid, is a ligand for PPARγ (9) that induces growth arrest, apoptosis, and/or differentiation of a variety of tumor cell types. We have shown that CDDO induces Bcl-2 downregulation, mitochondrial depolarization, and caspase activation in myeloid leukemic cells (10). It also potently enhances apoptosis induced by tumor necrosis factor in myeloid leukemia cells (11) and refractory chronic lymphocytic leukemia B cells (12). In breast cancer, CDDO-induced growth inhibition correlates with PPARγ transactivation and is mediated, at least in part, by upregulation of *P21* and downregulation of *cyclin D1* (13, 14). Notably, CDDO and its derivatives also induce differentiation of leukemic cells (15–17), effects that in some cell types are synergistically enhanced by concomitant ligation of the RXR nuclear receptor. The multiple effects of CDDOs have been attributed to both PPARγ-dependent and PPARγ-independent mechanisms of action (18). One of the most powerful anti-inflammatory and anticarcinogenic activities induced by CDDO and related compounds is activation of the Nrf2/ARE signaling pathway, a major regulator of antioxidant cellular responses (for review, see ref. 19).

In this study, we investigated the role of PPARγ and its coactivator DRIP205 on the apoptosis- and differentiation-inducing properties of CDDO in leukemic cells. The results show that the PPARγ ligands CDDO and 15-deoxy-^{12,14}-15DPGJ$_2$ (15d15DPGJ$_2$) induce higher degrees of apoptosis in leukemic cells modified to overexpress PPARγ. Furthermore, we provide evidence that overexpression and recruitment of the coactivator DRIP205 contributes to the enhanced differentiation by this agent. Finally, we report induction of myeloid differentiation of PPARγ/DRIP205–coexpressing leukemic blasts from patients with acute myelogenous leukemia (AML) treated with CDDO (RTA-401) in a "first-in-man" clinical trial.

Materials and Methods

Reagents and antibodies

CDDO was manufactured under the RAID Program and kindly provided by Drs. E. Sausville (National Cancer Institute, Bethesda, MD) and M. Sporn (Dartmouth Medical College, Hanover, NH). 15d15DPGJ$_2$ was purchased from Cayman Chemical Company. *N*-(4′-Aminopyridyl)-2-chloro-5-nitrobenzamide (T007; ref. 20), a selective PPARγ antagonist, was synthesized as described previously (21). Annexin V-FITC was purchased from Roche Diagnostic Co., and CD14-FITC, CD11b-phycoerythrin (PE), and CD34-PE were from BD Biosciences. Rabbit polyclonal caspase-3 antibody (CPP32, 1:1,000) was from PharMingen; mouse monoclonal anti–caspase-8 (1:1,000) and rabbit polyclonal anti–caspase-9 (1:1,000) were purchased from Cell Signaling Technology; rabbit polyclonal anti-TRAP220, mouse monoclonal anti-PPARγ (1:500), mouse monoclonal poly(ADP-ribose) polymerase (anti-PARP) antibodies (1:200), and mouse monoclonal anti–proliferating cell nuclear antigen (anti-PCNA; 1:500) were from Santa Cruz Biotechnology; mouse monoclonal anti-p21 antibody (1:1,000) was from Calbiochem; mouse monoclonal anti-p27 (1:1,000), mouse monoclonal anti–HO-1 (1:1,000), and mouse monoclonal anti-Bip/GRP78 (1:1,000) antibodies were from BD Biosciences; mouse monoclonal anti-Flag and mouse monoclonal anti–β-actin (AC74) were from Sigma Chemical Co.; and goat anti-mouse and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were from Bio-Rad.

Cell lines and primary AML samples

HL-60, U937, MCF-7, and SW480 were purchased from The American Type Culture Collection. Bone marrow or peripheral blood samples from 260 AML or high-risk myelodysplastic syndrome (MDS) samples were assessed for PPARγ expression by reverse-phase protein array (Supplementary Methods; ref. 22). Samples were collected for the Leukemia Sample Bank at the University of Texas M.D. Anderson Cancer Center between January 15, 1998 and March 9, 2006, on Institutional Review Board (IRB)–approved protocol Lab01-473, and consent was obtained according to the Declaration of Helsinki. Samples were analyzed under an IRB-approved laboratory protocol (Lab05-0654).

Flow cytometric analysis of apoptosis and cell cycle

Early apoptotic events were detected by the flow cytometric measurement of externalized phosphatidylserine with the Annexin-V-FLUOS Staining Kit from Roche Diagnostics. Cell cycle analysis was conducted using propidium iodide as described (23).

DNA fragmentation assay

Cells were washed twice with PBS and resuspended in lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.025% Triton X-100, pH 8.0) for 10 to 20 minutes on ice. Lysates were digested with RNase A (50 μg/mL) and then with proteinase K (2 mg/mL) for 1 hour each at 37°C. DNA samples were separated on 1.8% agarose gel containing ethidium bromide and visualized by UV light.

Differentiation assay

The differentiation of myeloid leukemic cells was determined by their ability to produce superoxide, as measured by the reduction of nitroblue tetrazolium (NBT) as described (24). The analysis of differentiation-specific cell surface antigens was measured by flow cytometry using the PE-conjugated anti-CD11b and FITC-conjugated anti-CD14 monoclonal antibodies.

Transient transfection and luciferase activity assay

Plasmids. SV40 β-galactosidase was obtained from Promega Corp.; pM vector was purchased from Clontech; and pMD, pMDm5, pMDm6, pMDm7, pMDm7Δ, and plasmids

were generated by PCR (25). FLAG-tagged wild-type (wt) PPARγ construct was kindly provided by Dr. K. Chatterjee (Department of Medicine, University of Cambridge, Cambridge, United Kingdom). pcDNA3-DRIP205 expression plasmid was kindly provided by Dr. Leonard P. Freedman (Merck Research Laboratories, West Point, PA).

For transient transfection assays, SW480 cells were cotransfected with DNA using FuGENE. In coactivation experiments, cells were cotransfected with 250 ng of SV40 β-galactosidase (used as an internal control) and 1 μg of the reporter construct containing three copies of the acyl-CoA oxidase PPARγ response element (PPRE) cloned upstream of the TK-LUC reporter, PPREx3-LUC reporter (kindly provided by Dr. Ronald M. Evans, The Salk Institute, La Jolla, CA), and various amounts of DRIP205 or deletion mutant constructs. After 24 hours, transfected cells were treated with DMSO or 0.75 μmol/L CDDO for another 48 to 72 hours. Relative luciferase activity was calculated by dividing luciferase activity by β-galactosidase activity for each well.

Western blot analysis

Cells were lysed at a density of $1 \times 10^6/50$ μL in protein lysis buffer (0.25 mol/L Tris-HCl, 2% sodium dodecylsulfate, 4% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) supplemented with a protease inhibitor cocktail (Roche Diagnostic). For preparation of nuclear lysates, cells were washed in cold PBS and digitonin-permeabilized for 5 minutes on ice at a density of 20 million cells/mL in extraction buffer (250 mmol/L sucrose, 70 mmol/L KCl; 137 mmol/L NaCl; 4.3 mmol/L Na_2HPO4; 1.4 mmol/L KH_2PO4 (pH 7.2), 200 μg/mL digitonin, and protease inhibitors). Cells were centrifuged at 1,000 × *g* for 5 minutes at 4°C, and pellet containing nuclear fraction was lysed as above. Cell lysates were then loaded onto a 10% to 12% SDS-PAGE gel (Bio-Rad). After electrophoresis, proteins were transferred onto Hybond-P membranes (Amersham Pharmacia Biotech), followed by immunoblotting. Signals were detected using a PhosphorImager (Storm 860, version 4.0, Molecular Dynamics).

Clinical trial of CDDO in patients with AML

Patients with refractory/relapsed AML were treated with CDDO (RTA-401, Reata Pharmaceuticals) from 0.6 to 75 $mg/m^2/h \times 5$ days, in a phase 1 clinical trial, following informed consent according to the University of Texas M.D. Anderson Cancer Center and Princess Margaret Hospital-Ontario Cancer Institute guidelines (Supplementary Table S1). Bone marrow or peripheral blood sample mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical) density-gradient centrifugation. Apoptosis was examined by simultaneous staining with CD34 allophycocyanin (APC; BD) and tetramethylrhodamine methyl ester (TMRM; Invitrogen) to measure mitochondrial inner transmembrane potential ($\Delta\psi_m$). Cell differentiation was examined by simultaneous staining with CD34-APC (BD), CD33-PE-Cy7 (BD), CD14-FITC (BD), and CD11b-PE (BD) at the indicated time points.

Quantitative real-time PCR

Bone marrow and peripheral blood samples obtained from AML patients were lysed with RNA Stat 60 (Tel-Test). The RNA was subjected to purification in an RNeasy ion-exchange column (Qiagen) with on-column DNase treatment. cDNA was prepared from 1.0 μg of total RNA per 20 μL of mix containing 0.07 μg/μL random-sequence hexamer primers, 0.5 mmol/L deoxynucleotide triphosphates, 5 mmol/L DTT, 0.2 units/μL SuperAsin RNase inhibitor (Ambion), and 10 units/μL SuperScript III (Invitrogen). Real-time PCR was carried out using an ABI Prism 7700 instrument as described (26). For primer and probe sets to detect *PPARγ, p21, NQO1*, and housekeeping gene *ABL1*, we used TaqMan Gene Expression Assays Hs00234592_m1, Hs00355782_m1, Hs00168547_m1, and Hs00245445_m1, respectively. The abundance of each transcript of interest relative to that of ABL1 was calculated as follows: relative expression = 100 × 2 exp [−ΔCt], where ΔCt is the mean Ct of the transcript of interest less the mean Ct of the transcript for ABL.

Transfection of PPARγ small interfering RNA

Cells were transfected by the Amaxa electroporator Nucleofector I from Amaxa Biosystems, using the Nucleofector Kit C (program W-001). Small interfering RNA (siRNA) PPARγ transfection was performed using validated Stealth RNAi VHS 40941 for duplex 1 (siRNA1) and VHS 40944 for duplex 2 (siRNA2; Invitrogen). Cells (10^6) were resuspended in 100 μL of Nucleofector Solution C and transfected by electroporation with scramble LO GC Duplex Stealth RNAi Negative Control (12 935-200; Invitrogen) or with PPARγ siRNA. Forty-eight hours after transfection, DMSO or CDDO was added to the cells for 24 hours, and protein expression was monitored by immunoblotting.

Results

PPARγ protein expression in AML

We have previously shown that PPARγ protein is expressed in both myeloid and lymphoid leukemic cell lines (8). Expression of PPARγ protein was analyzed in 260 newly diagnosed leukemia-enriched AML/MDS samples. PPARγ protein was variably expressed in different AML subtypes (Supplementary Fig. S1). There was no difference in overall survival between patients expressing high or low levels PPARγ (not shown).

CDDO induces PPARγ activation in AML cells

We next characterized the relationship between PPARγ expression and the ability of pharmacologic PPARγ ligands to affect the growth and survival of leukemic cells. To this end, we established stable transfectants of U937 cells expressing empty vector (pcDNA3) or Flag-tagged wt-PPARγ (Fig. 1A–C). Overexpression of PPARγ facilitated growth of cells but the differences did not reach statistical significance ($P = 0.1$; Supplementary Fig. S2A). Exposure of cells to the PPARγ ligand CDDO further enhanced PPARγ protein expression in both vector control and transfected cells (Fig. 1C). As shown in Fig. 1D, CDDO induced PPARγ mRNA expression wt-PPARγ–overexpressing

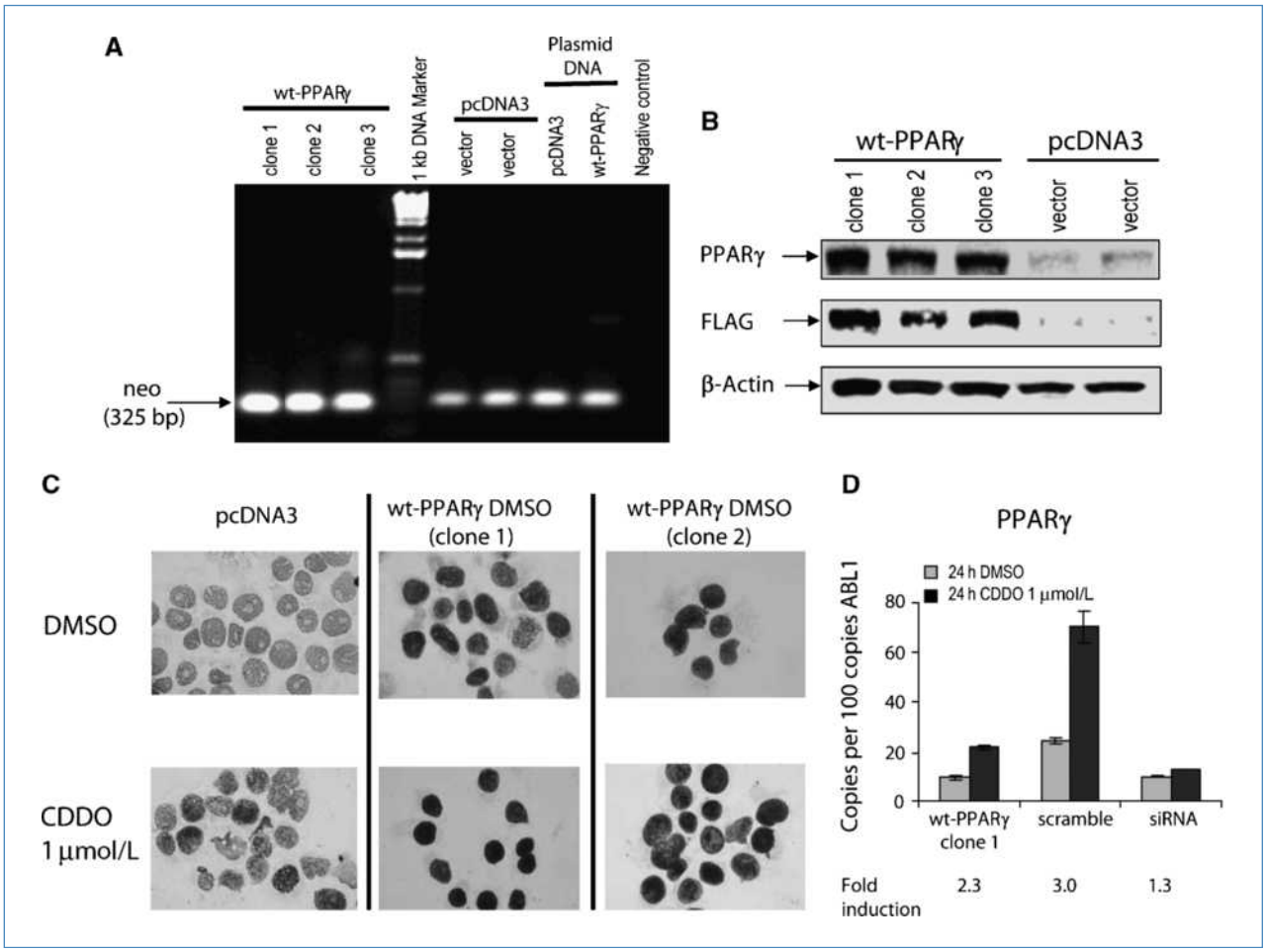

Figure 1. A, reverse transcription-PCR of neomycin gene expression in pcDNA3- or wt-PPARγ–transfected clones. B, PPARγ and FLAG protein expression in transfected cells was detected by immunoblotting. C, leukemic cells transfected with pcDNA3 or wt-PPARγ were treated with 1 μmol/L CDDO for 5 h, and PPARγ protein levels were examined by immunocytochemistry. D, *PPARγ* transcript was measured in wt-PPARγ–transfected cells (clone 1) or in the same cells transfected with scrambled or PPARγ siRNA by real-time PCR. Columns, mean number of transcripts per hundred transcripts of ABL1; bars, SEM.

cells or in the same cells transfected with scrambled siRNA (2.3- and 3-fold, respectively), and this induction was largely abrogated in PPARγ siRNA–transfected cells. These data suggest that PPARγ ligation with CDDO induces expression of its cognate receptor in a PPARγ-dependent fashion.

Relationship between PPARγ expression and growth inhibitory responses to PPARγ agonists

Next, we examined the functional consequences of PPARγ induction and overexpression in response to the receptor activation by CDDO or by the structurally different PPARγ agonist $15dPGJ_2$. Vector control (pcDNA3) or wt-PPARγ–transfected U937 cells were treated with 1 μmol/L CDDO for 24 hours. Overexpression of PPARγ significantly enhanced the sensitivity of leukemic cells to apoptosis induced by CDDO or $15dPGJ_2$ (Fig. 2A and Supplementary Fig. S2B). We next analyzed modulation of apoptosis and cell cycle–regulating proteins in PPARγ-transfected clones. Following treatment with CDDO for 24 hours, cells overexpressing wt-PPARγ exhibited significantly decreased procaspase-3 and increased cleavage of caspase-3 and its substrate PARP (Fig. 2B). In accordance with these results, CDDO induced a higher degree of endonucleolytic DNA cleavage in PPARγ-overexpressing cells. Analysis of the upstream (initiator) caspases showed the appearance of the active (p18) fragment of caspase-8 in these cells, whereas caspase-9 was similarly cleaved in both vector control and PPARγ-overexpressing cells (Fig. 2B). In a time-course experiment, no caspase-8 cleavage and DNA fragmentation were observed in vector-treated cells, whereas both ligands induced caspase-8 cleavage and DNA fragmentation at 5 and 24 hours in PPARγ-overexpressing cells (Fig. 2C). In contrast, caspase-9 cleavage did not substantially differ between control and PPARγ-transfected cells. Activation of caspase-8 may proceed through CHOP-dependent transcriptional upregulation of death receptor 5 (DR5)/tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) triggered by endoplasmic reticulum stress and unfolded protein response (27), and CDDO slightly increased the level of the endoplasmic reticulum stress marker protein Bip/GRP78 in PPARγ-overexpressing cells (Fig. 2B). Knocking

down exogenously transfected PPARγ by two different siRNA constructs abolished apoptosis induced by lower CDDO concentration (0.5 μmol/L) but did not significantly affect it at higher CDDO concentration (1 μmol/L; Fig. 2D), indicating the contribution of PPARγ-dependent and PPARγ-independent mechanisms of cell death. Likewise, the pharmacologic PPARγ inhibitor T007 partially protected PPARγ-overexpressing cells, but not control cells, from CDDO-induced apoptosis (Supplementary Fig. S2C). CDDO potently induced expression of the stress-responsive inducible enzyme hemeoxygenase-1 (HO-1; Fig. 2B) and of one of the critical Nrf2 target genes, NADPH quinine oxidoreductase-1 (*NQO1*; Supplementary Fig. S3A), in both control and PPARγ-overexpressing cells.

PPARγ agonists are known to induce expression of the cell cycle inhibitory protein $p21^{Waf1/CIP1}$, and we have previously

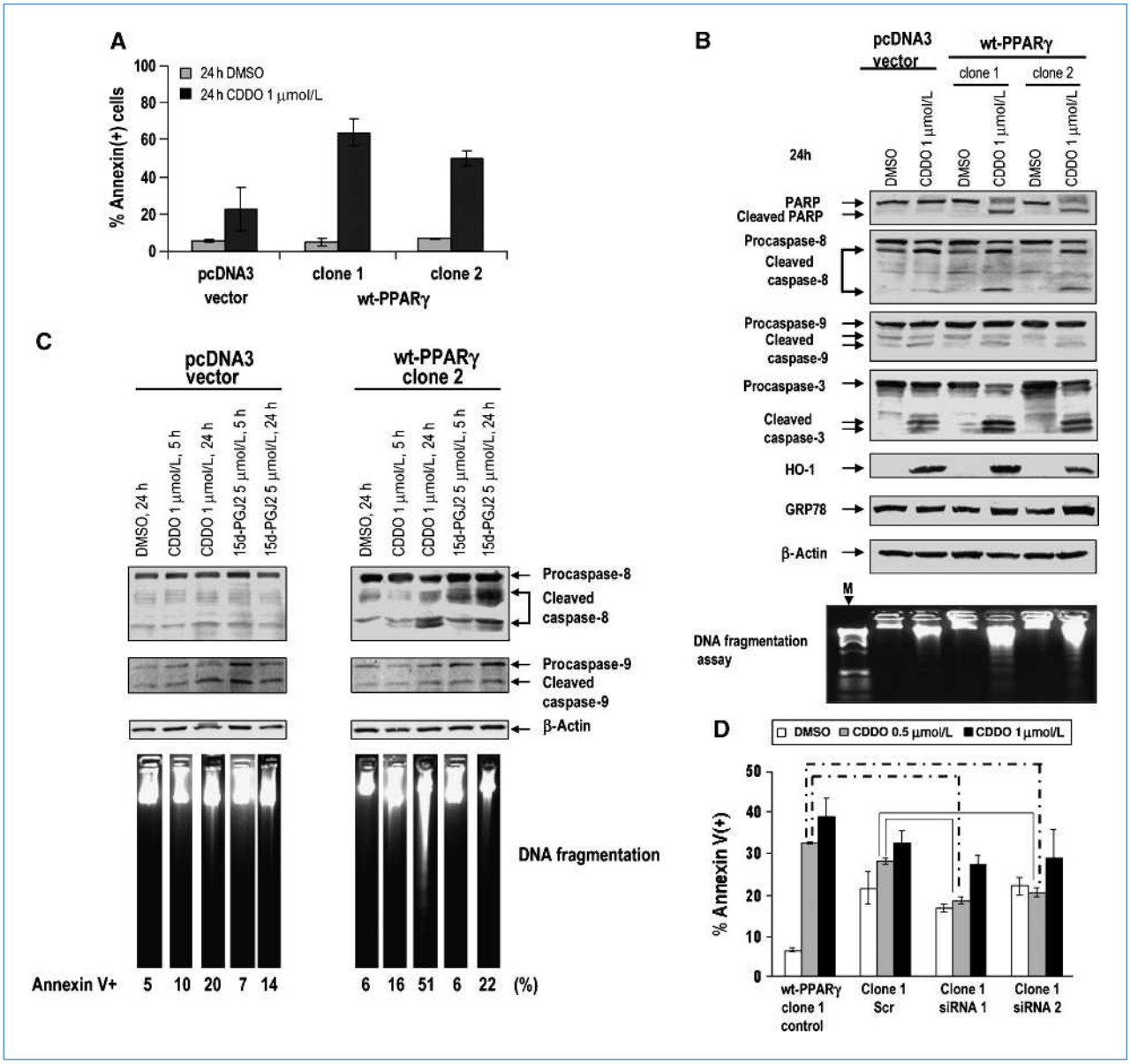

Figure 2. A, U937 (pcDNA3) and U937 (pcDNA3-wt-PPARγ) cells were cultured in the presence of CDDO or vehicle for 24 h, and apoptosis induction was analyzed by Annexin V flow cytometry at 24 h. B, U937 (pcDNA3) and U937 (pcDNA3-wt-PPARγ) cells were grown in the presence of 1 μmol/L CDDO or vehicle for 24 h. Expression of PARP, caspase-3, caspase-8, caspase-9, HO-1, and GRP78 was analyzed by immunoblotting, and effects of CDDO on DNA fragmentation were determined. C, caspase-8 and caspase-9 processing and DNA fragmentation at 5 and 24 h were measured. D, U937-wt-PPARγ cells were transiently transfected with scrambled or PPARγ siRNA (siRNA1 and siRNA2) at a final concentration of 67 nmol/L. Seventy-two hours after transfection, cells were exposed to indicated concentrations of CDDO for 24 h. Induction of apoptosis was measured by Annexin V flow cytometry. Columns, average from three independent experiments. Solid lines, paired *t* test comparing apoptosis in scrambled siRNA–transfected cells with apoptosis in PPARγ siRNA transfectants ($P < 0.01$); dashed lines, paired *t* test comparing apoptosis in untransfected cells with that in PPARγ siRNA transfectants ($P < 0.001$).

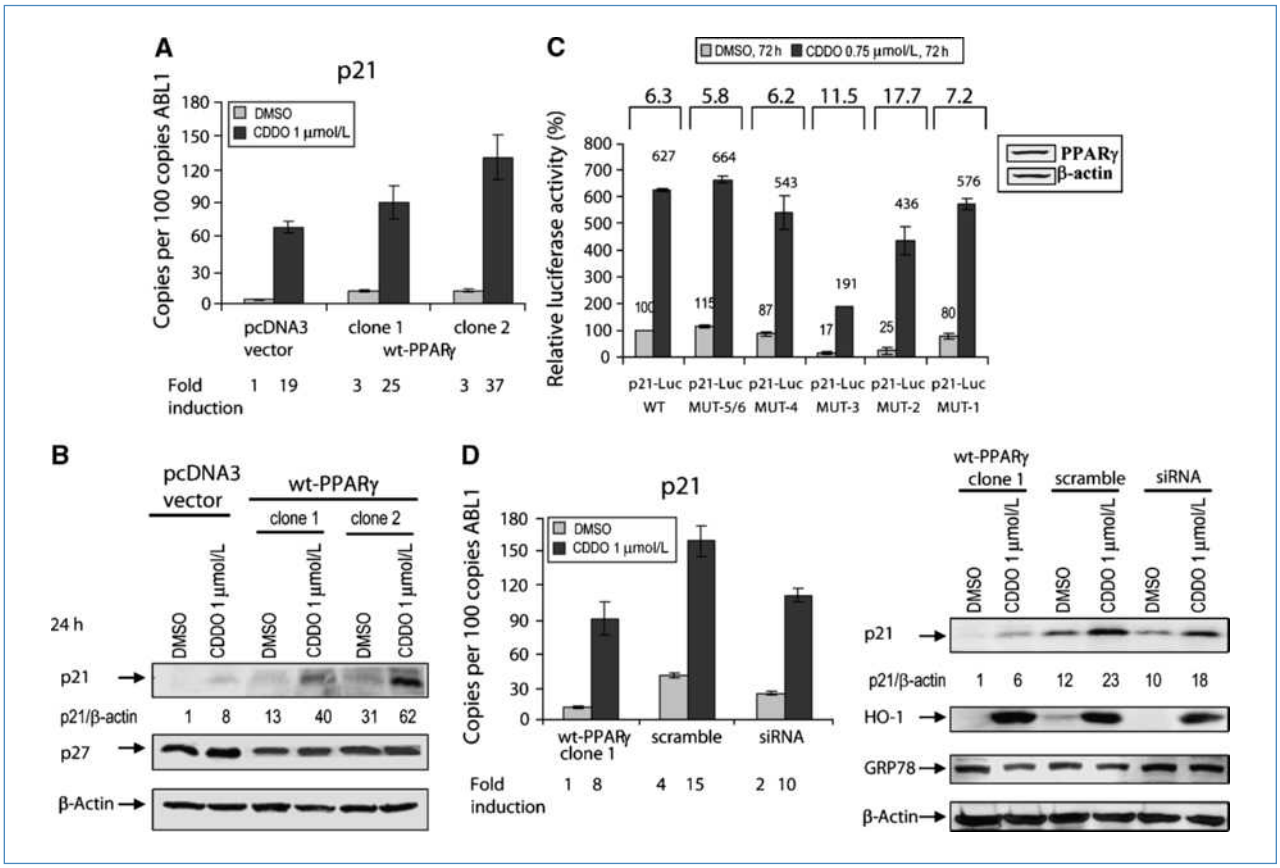

Figure 3. A, U937 (pcDNA3) and U937 (pcDNA3-wt-PPARγ) cells were cultured in the presence of 1 μmol/L CDDO or vehicle for 24 h, and *P21* transcript was measured by real-time PCR. B, cells were harvested at 24 h, and p21/p27 levels were determined by immunoblotting. C, fold transactivation of the *P21* promoter by CDDO (0.75 μmol/L, 72 h). SW480 colon cancer cells were cotransfected with the wt or mutant –2,325/+8 *P21* promoter-luciferase reporter vectors. Cells were cotransfected with β-galactosidase for normalization of the transfection variability. The fold transactivation of each *P21* reporter construct is shown on top of the graph. D, analysis of p21 levels in wt-PPARγ clone 1 electroporated with scrambled or PPARγ siRNA, 48 h after transfection. After 48 h, DMSO or CDDO was added for the next 24 h, and after 24 h, p21, HO-1, GRP78, and β-actin levels were determined by quantitative TaqMan PCR and Western blot analyses.

shown that CDDO induced p21 mRNA and protein in breast cancer cells (14). Consistent with these findings, CDDO induced p21 mRNA in leukemic cells (Fig. 3A). This increase in *P21* transcription was evident both in control cells and in cells overexpressing PPARγ (Fig. 3A and B). The expression of $p27^{KIP1}$ was unchanged, and no significant differences in cell cycle distribution were noted between PPARγ-overexpressing and control cells (Supplementary Fig. S2D). Although *P21* promoter contains a potential conserved consensus PPRE, CDDO may also increase transcription of *P21* indirectly through increased binding of Sp1, Sp3, and Sp4 transcription factors to the GC-rich regions of the *P21* promoter. To determine the mechanism of transcriptional activation of *P21* by CDDO, we conducted promoter assays by transient transfection of SW480 cells expressing endogenous PPARγ (Fig. 3C, inset) with full-length *P21* luciferase promoter plasmid and plasmids containing point mutations in GC-rich elements 1 to 6 (Mut1–Mut6) of the proximal *P21* promoter (28). CDDO induced an ~6-fold increase in *P21*-wt promoter activity (Fig. 3C, p21-Luc wt). Interestingly, CDDO was capable of transactivating all constructs containing point mutations. To determine if CDDO induces p21 expression via PPARγ, we measured p21 levels following depletion of PPARγ by siRNA. As shown in Fig. 3D, inhibition of PPARγ using siRNA failed to block CDDO-induced levels of p21 mRNA or protein expression. These data indicate that CDDO causes induction of p21 levels in a PPARγ-independent fashion. Consistent with documented properties of triterpenoids and other electrophilic compounds to activate the Keap1/Nrf2 antioxidant pathway, CDDO robustly induced HO-1 (Fig. 3D) and *NQO1* (Supplementary Fig. S3B) in cells with functional or silenced PPARγ, indicating that these responses are likewise PPARγ independent. No appreciable change in Bip/GRP78 expression was noted in control or transfected cells (Fig. 3D), indicating no significant contribution of endoplasmic reticulum stress to the proapoptotic effects of CDDO in this cell system.

PPARγ ligands enhance DRIP205 coactivator binding to PPARγ

PPARγ ligands recruit the coactivator DRIP205 to PPARγ. Furthermore, nuclear factor coactivators are known to

mediate tissue-specific effects (29). To ascertain whether DRIP205 overexpression will affect the transcriptional activity of PPARγ, the ability of CDDO and 15dPGJ2 to induce (PPRE) 3-tk-luc reporter was examined in MCF-7 cells transfected with full-length DRIP205 plasmid. Transient cotransfection with (PPRE)3-tk-luc reporter and full-length pcDNA3-DRIP205 plasmid resulted in higher levels of PPARγ transactivation induced by 15dPGJ$_2$ and CDDO (1.9- and 2.7-fold, respectively; Fig. 4A).

Previous studies have shown that the NR boxes in DRIP205/TRAP220 contribute to the physical and functional interactions of these coactivators with NRs (30, 31). Their role in coactivation of PPARγ was further investigated in SW480 cotransfected with (PPRE)3-tk-luc reporter and NH$_2$- or COOH-terminal GAL4-*DRIP205* deletion constructs (Fig. 4B). The full-length *DRIP205* expression plasmid encodes for 1,566 amino acids, which are identical to amino acids 16–1,581 of the TRAP220 coding sequence. CDDO significantly induced activity in cells transfected with (PPRE)3-tk-luc and GAL4-DRIP205 (wild-type). In addition, significant coactivation, albeit to a lesser degree, was observed for several GAL4-DRIP205 chimeras (pMDm5, pMDm7, pMDm7Δ, and pMDm6), two of which express NH$_2$-terminal (pMDm5) or COOH-terminal (pMDm6) regions of *DRIP205* but do not contain the central NR box sequences (Fig. 4C). These results confirm that the NR boxes of *DRIP205* may contribute to, but are not required for, coactivation and indicate that multiple domains of DRIP205 are involved in interactions with PPARγ.

DRIP205 contributes to myelomonocytic differentiation of leukemic cells in response to CDDO

To determine the functional role of DRIP205 in the context of PPARγ ligation in leukemic cells, we investigated the effects of CDDO in HL-60 cells stably transfected with DRIP205 plasmid (three separate clones; Fig. 5A and Supplementary Fig. S4A). No difference in cell growth (Supplementary Fig. S4B), cell cycle progression (Supplementary Fig. S4C), or apoptosis (Supplementary Fig. S4D) was found in cells overexpressing DRIP205, cultured alone or exposed to CDDO. In contrast, CDDO induced a higher degree of myelomonocytic differentiation in DRIP205-overexpressing cells as shown by increased expression of CD11b ($P < 0.01$; Fig. 5B) and by NBT assay (36.7 ± 6.1% versus 79.7 ± 4.7%, $P < 0.001$; Fig. 5C).

To determine if the enhanced differentiation was mediated through PPARγ, we assessed CD11b expression in cells pretreated with the pharmacologic PPARγ antagonist T007. As shown in Fig. 5D, blocking PPARγ transactivation

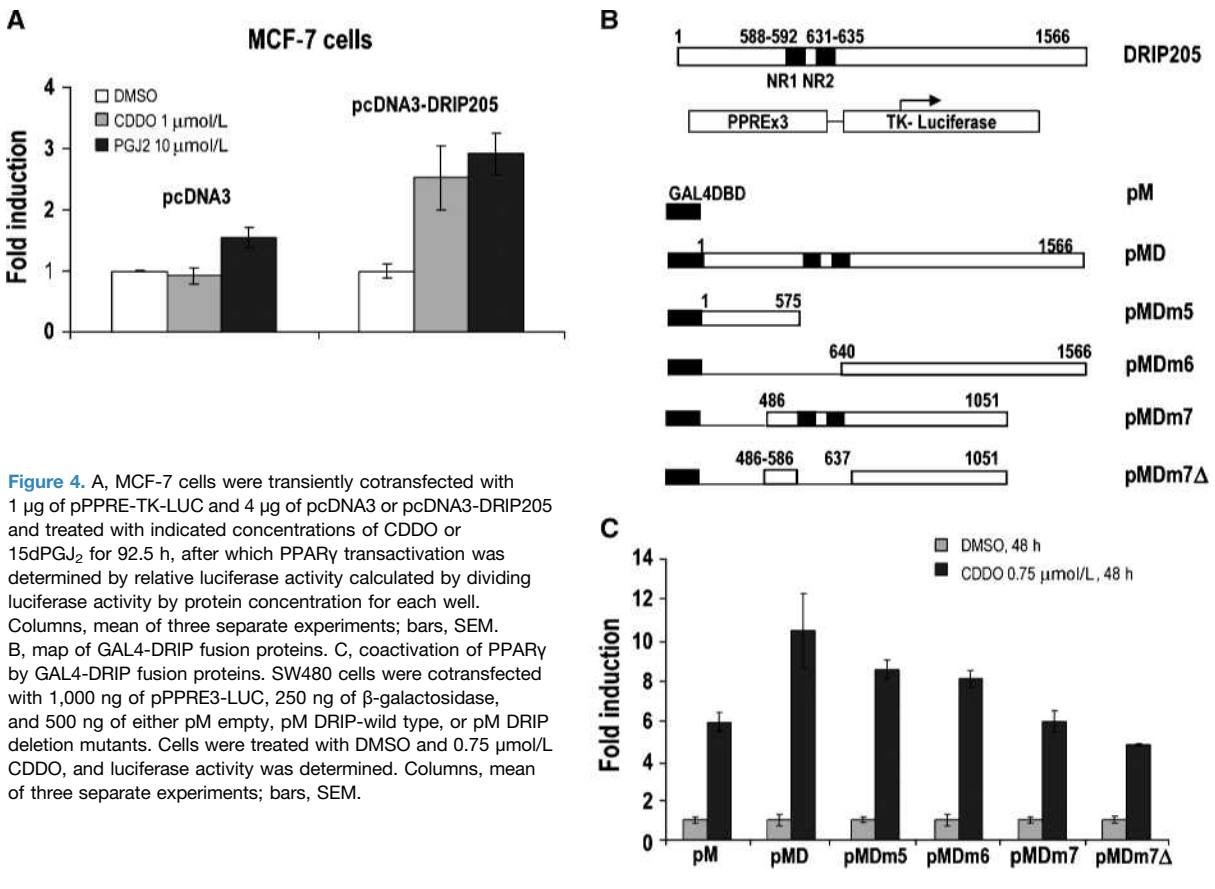

Figure 4. A, MCF-7 cells were transiently cotransfected with 1 μg of pPPRE-TK-LUC and 4 μg of pcDNA3 or pcDNA3-DRIP205 and treated with indicated concentrations of CDDO or 15dPGJ$_2$ for 92.5 h, after which PPARγ transactivation was determined by relative luciferase activity calculated by dividing luciferase activity by protein concentration for each well. Columns, mean of three separate experiments; bars, SEM. B, map of GAL4-DRIP fusion proteins. C, coactivation of PPARγ by GAL4-DRIP fusion proteins. SW480 cells were cotransfected with 1,000 ng of pPPRE3-LUC, 250 ng of β-galactosidase, and 500 ng of either pM empty, pM DRIP-wild type, or pM DRIP deletion mutants. Cells were treated with DMSO and 0.75 μmol/L CDDO, and luciferase activity was determined. Columns, mean of three separate experiments; bars, SEM.

significantly diminished CDDO-induced myelomonocytic differentiation in DRIP205-overexpressing cells but not in vector-transduced control cells.

CDDO induces expression of markers of differentiation and apoptosis in leukemic blasts of patients treated in phase I clinical trial

In a first-in-man clinical phase I trial of increasing doses of CDDO (RTA-401; escalated from 0.6 to 75 mg/m^2/h) in patients with relapsed/refractory AML, we investigated the *in vivo* differentiating and proapoptotic effects of CDDO and correlated these changes with PPARγ and DRIP205 expression in cells from nine patients by quantitative TaqMan PCR (PPARγ) and immunoblotting (PPARγ and DRIP205). Clinical characteristics of the patients are summarized in Supplementary Table S1. PPARγ mRNA was expressed in all samples at baseline albeit at different levels. PPARγ and DRIP205 proteins were expressed in samples from seven of nine patients studied; no expression of either protein was detected in samples from patients #307 and #309 (Fig. 6A). After 6 days of continuous CDDO administration, PPARγ mRNA was induced >2-fold in four patient samples (Supplementary

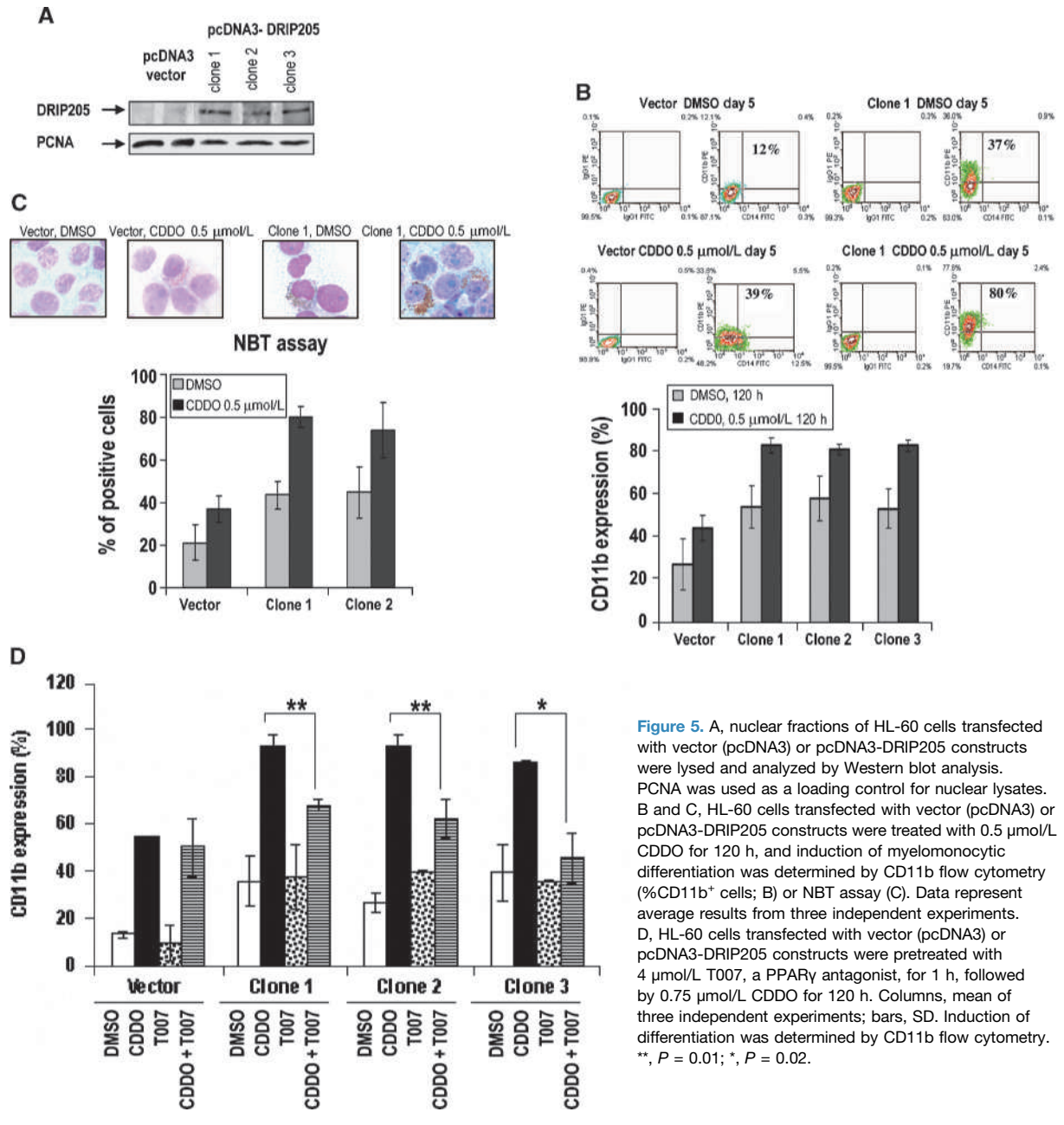

Figure 5. A, nuclear fractions of HL-60 cells transfected with vector (pcDNA3) or pcDNA3-DRIP205 constructs were lysed and analyzed by Western blot analysis. PCNA was used as a loading control for nuclear lysates. B and C, HL-60 cells transfected with vector (pcDNA3) or pcDNA3-DRIP205 constructs were treated with 0.5 μmol/L CDDO for 120 h, and induction of myelomonocytic differentiation was determined by CD11b flow cytometry (%CD11b$^+$ cells; B) or NBT assay (C). Data represent average results from three independent experiments. D, HL-60 cells transfected with vector (pcDNA3) or pcDNA3-DRIP205 constructs were pretreated with 4 μmol/L T007, a PPARγ antagonist, for 1 h, followed by 0.75 μmol/L CDDO for 120 h. Columns, mean of three independent experiments; bars, SD. Induction of differentiation was determined by CD11b flow cytometry. **, $P = 0.01$; *, $P = 0.02$.

Table S2). In four of the nine patients, an increase in $CD11b^+$ and $CD14^+$ cells and a concomitant reduction of immature cells expressing CD34 or CD33 were observed (#301, #304, #305, and #306, Fig. 6B). Examples of flow cytometric profiles are shown in Supplementary Fig. S5. Baseline expression of PPARγ protein was highest in samples from patients #301 and #304 (Fig. 6A), and in all four patients increase in PPARγ mRNA was shown (1.5-, 2.4-, 1.8-, and 2.2-fold, respectively; Supplementary Table S2). In these, p21 mRNA was induced >2-fold in samples #304, #305, and #306. No change in differentiation markers was observed in patients #307 and #309 with no detectable baseline PPARγ or DRIP205 proteins. Moderate induction of apoptosis, documented as loss of mitochondrial membrane potential in circulating $CD34^+$ cells, was observed in samples from three patients (#301, #303, and #305); in sample #303, corresponding apoptosis induction was seen in day 6 bone marrow $CD34^+$ cells (Fig. 6C). Examples of flow cytometric profiles are shown in Supplementary Fig. S6. Clinically, patients did not fulfill protocol response criteria: differential counts did not change significantly and maximum tolerated dose was not reached at the low dose levels in this phase I study.

Discussion

PPARγ ligands inhibit cancer cell proliferation and induce apoptosis and/or differentiation in multiple tumor types, and these effects have been attributed to both PPARγ-dependent and PPARγ-independent mechanisms. In this study, we evaluated the role of PPARγ and one of its cellular coactivators, DRIP205, in the proapoptotic and differentiating properties of PPARγ agonists CDDO and $15dPGJ_2$. A high-throughput reverse-phase protein array technique showed high levels of PPARγ expression in 260 primary AML samples. To functionally characterize the relationship between baseline PPARγ levels and cellular effects of PPARγ agonists in leukemic cells, we generated stably transfected myeloid leukemic cells overexpressing the receptor. U937 cells induced to overexpress wt-PPARγ were more sensitive to the proapoptotic effects of PPARγ ligands CDDO and $15dPGJ_2$ compared with vector-transduced cells. These proapoptotic effects were significantly inhibited by silencing PPARγ with siRNA or by blocking PPARγ activation with the pharmacologic antagonist T007, consistent with previously published findings of PPARγ-dependent and PPARγ-independent mechanisms of action of this class of agents. Time-course analysis showed that high PPARγ levels facilitated cleavage of caspase-8 and caspase-3 (but not of caspase-9), resulting in accelerated PARP cleavage, DNA fragmentation, and apoptosis. Of note, several reports indicated the ability of CDDOs to activate the extrinsic apoptotic pathway and sensitize to TRAIL via diverse molecular mechanisms including FLIP downregulation (32), c-jun NH_2-terminal kinase–mediated induction of TRAIL receptor expression (33), and inhibition of NF-κB–dependent antiapoptotic proteins (11). Conversely, data reported by us and others show that CDDO and its more potent derivative CDDO-Me promoted the release of cytochrome *c* from isolated mitochondria, suggesting that CDDOs directly target the mitochondria to trigger the intrinsic pathway of cell death (34, 35). Data presented here suggest a proximal role for caspase-8 downstream of ligand-activated PPARγ, whereas direct mitochondrial effects of CDDO observed at higher concentrations are likely PPARγ independent, possibly by modifying the mitochondrial proteins through nucleophilic attack and Michaels addition (36). Unlike in non–small lung cancer cells (27). CDDO did not induce significant endoplasmic reticulum stress response, hence making upregulation of DR5 an unlikely mechanism of caspase-8 activation. The exact mechanistic link between PPARγ transactivation and activation of the extrinsic apoptotic pathway in AML remains to be determined.

It has recently been shown that synthetic triterpenoids are potent activators of Nrf2/ARE signaling in a variety of cell types, resulting in marked induction of a variety of antioxidative genes and detoxifying enzymes (37–39). In our studies, CDDO promptly upregulated expression of HO-1 and NQO1 in leukemia cells engineered to overexpress or silence PPARγ. These observations are consistent with the notion that these responses are likely mediated by the chemical structure of CDDO and other electrophilic compounds capable of modifying cysteine residues on KEAP1 protein (37) and represent important PPARγ-independent activities of this class of compounds.

PPARγ agonists including CDDO modulate cell cycle progression in multiple tumor types (13, 14). Our present data show that CDDO induced expression of p21waf1/CIP protein in leukemic cells. This induction was observed in parental cells, in cells overexpressing PPARγ, or in cells transfected with PPARγ siRNA. These findings indicate that the ability of CDDO to activate *P21* promoter is likely mediated via PPARγ-independent mechanisms. Because induction of p21 expression is frequently mediated via increased binding of Sp1, Sp3, and Sp4 transcription factors to the GC-rich regions of the *P21* promoter, we used the constructs containing point mutations in the GC elements. In contrast to our previous study in pancreatic cells (40), we were unable to identify the specific site required for PPARγ-dependent activation of *P21*. Of note, p21 is regulated by many different pathways and transcription factors, and CDDO could conceivably mediate its effects on p21 expression through an alternate pathway. Surprisingly, induction of p21 protein expression did not translate into a discernible cell cycle arrest in leukemic cells. The observation that CDDO preferentially induces apoptosis rather than cell cycle arrest in AML cells attests to the cell type–dependent properties of these agents, likely related to the distinct mitochondrial architecture of leukemic cells compared with solid tumor cells. Whether functional consequences of p21 overexpression other than control of cell cycle, such as regulation of apoptosis, differentiation, or transcriptional activation (41), are operational in leukemic cells remains to be investigated. Notably, p21 mRNA induction was observed in samples from three of the nine patients treated with very low doses of CDDO (RTA-401) in a phase I clinical trial.

Emerging evidence suggests the critical importance of the cellular context, in particular the composition of

tissue-specific coactivators and corepressors, in the biological responses to NR agonists. PPARγ is known to interact with both the p160/SRC-1 family of coactivators and the multisubunit DRIP/Mediator coactivator complex. Our results show that CDDO induced significant activity in cells transfected with (PPRE)3-tk-luc and full-length DRIP205. In addition, significant coactivation was observed using several NH_2- and COOH-terminal domain mutants of

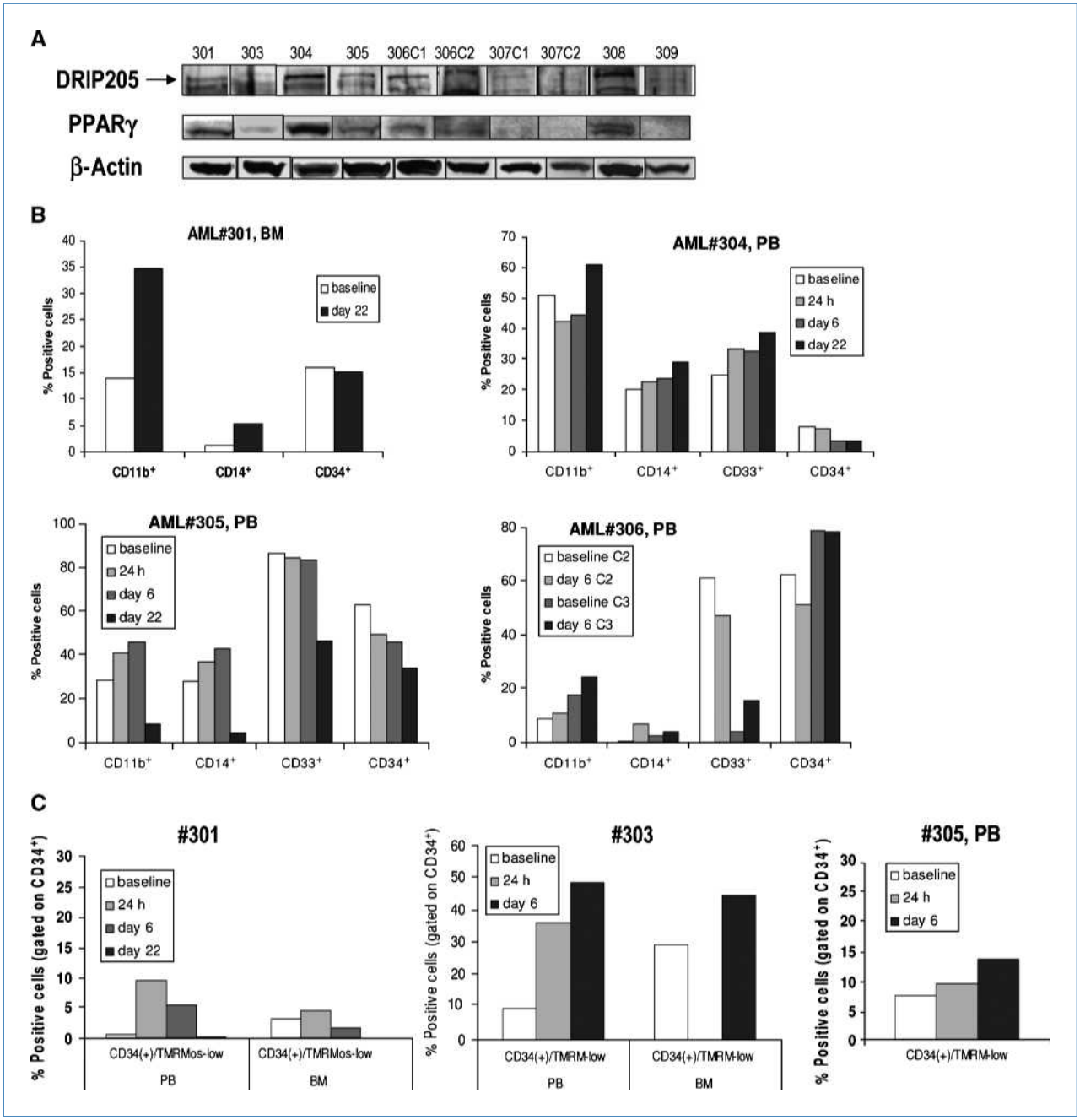

Figure 6. A, peripheral blood (PB) or bone marrow (BM) samples from patients enrolled in the phase 1 clinical trial were lysed and probed with DRIP205 and PPARγ by Western blot. β-Actin was used as a loading control. In baseline sample from patient #2, not enough material was available for immunoblotting. B, patients were treated with CDDO (RTA-401) during a phase I clinical trial, and cells were collected from the PB or BM and assessed for expression of surface markers CD11b, CD14, CD33, and CD34 by flow cytometry at the indicated time points (see also Supplementary Table S2). Four of nine patients (patients #301, #304, #305, and #306) showed alterations of these parameters during the observed period. PB baseline percentages are not available from patient #301; therefore, BM percentages are provided. C, cells from the PB or BM were counterstained with CD34-APC and TMRM. Three of five patients (patients #301, #303, and #305) showed alterations of these parameters during the observed period. Data are presented as percentage of $CD34^+$ cells that have lost mitochondrial membrane potential (TMRM-low). BM baseline percentages are not available from patient #305.

DRIP205, and this coactivation did not require the NR boxes (Fig. 4B). These results suggest that multiple domains of DRIP205 are involved in interactions with PPARγ, similar to findings reported for estrogen receptor-α coactivation by DRIP205 (25). Interestingly, recent structural and functional analyses indicate that a direct interaction of PPARγ with DRIP/Mediator complex through the NR motifs of DRIP205 is not required for PPARγ-stimulated adipogenesis (42).

DRIP205 is involved in the vitamin D–triggered regulation of gene transcription during keratinocyte differentiation (6), and overexpression of DRIP205 was observed in some cancer cell lines (43). CDDOs have been shown to induce differentiation in myeloid leukemia cells (16, 17, 44), and in this study, CDDO induced a higher degree of myelomonocytic differentiation in DRIP205-overexpressing HL-60 cells, a process mediated through PPARγ. Whereas we recently reported that one of the mechanisms of differentiation induction by CDDO involves modulation of CEBPα expression and function (45), our data shown here provide first evidence that high cellular levels of the coactivator DRIP205 can enhance the differentiation induced by PPARγ ligation and is therefore an important determinant of tissue-specific effects of PPARγ agonists. We here report that leukemic blasts from patients treated in a phase I clinical trial of CDDO (RTA-401) express DRIP205 in seven of nine samples, all of which expressed PPARγ mRNA and protein. Further, sequential studies showed increased expression of the differentiation markers CD11b and/or CD14 in four patients. In these patients, CDDO induced *PPARγ* transcription. Although the numbers are too small to draw definitive conclusions, the data suggest that CDDO activates PPARγ in a subset of patients with AML *in vivo*, whose cells express DRIP205. We did not observe correlation between PPARγ levels and apoptosis induction, possibly due to very low levels of CDDO in this phase I study. Alternatively, this finding may indicate that PPARγ-dependent functions of CDDO may manifest primarily through differentiation induction rather than apoptotic responses in primary AML cells. Recently, the RXR agonist bexarotene was shown to induce differentiation in non–acute promyelocytic leukemia patients with AML who were treated with this agent in a phase I trial (46). Taking into consideration multiple studies showing that addition of RXR ligands synergistically enhances the differentiating and growth-suppressive effects of PPARγ ligands (8, 47), the combined use of these agents seems to be worth testing in the therapy of AML. The ongoing efforts by the Nuclear Receptor Signaling Atlas consortium to profile coactivators/corepressors in primary AML may assist in identifying patients who are likely to benefit from PPARγ/RXR ligation strategies.

Disclosure of Potential Conflicts of Interest

M. Andreeff and M. Konopleva: ownership interest and consultants, Reata Disc. The other authors disclosed no potential conflicts of interest.

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