



Published in final edited form as:

*Cancer Res.* 2010 June 15; 70(12): 4949–4960. doi:10.1158/0008-5472.CAN-09-1962.

## Role of peroxisome proliferator-activated receptor- $\gamma$ and its co-activator DRIP205 in cellular responses to CDDO (RTA-401) in AML

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### Abstract

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is a member of the nuclear receptor family of transcription factors with important regulatory roles in cellular growth, differentiation and apoptosis. Using proteomic analysis, we demonstrated expression of PPAR $\gamma$  protein in a series of 260 newly diagnosed primary AML samples. Forced expression of PPAR $\gamma$  enhanced the sensitivity of myeloid leukemic cells to PPAR $\gamma$  agonists CDDO- or 15d15DPGJ<sub>2</sub>-induced apoptosis, through preferential cleavage of caspase-8. No effects on cell cycle distribution or differentiation were noted, despite prominent induction of p21 in PPAR $\gamma$ -transfected cells. In turn, antagonizing PPAR $\gamma$  function by siRNA or pharmacological PPAR $\gamma$  inhibitor significantly diminished apoptosis induction by CDDO. Overexpression of co-activator protein DRIP205 resulted in enhanced differentiation induction by CDDO in AML cells through PPAR $\gamma$  activation. Studies with DRIP205 deletion constructs demonstrated that the NR boxes of DRIP205 are not required for this co-activation. In a Phase I clinical trial of CDDO (RTA-401) in leukemia, CDDO induced an increase in PPAR $\gamma$  mRNA expression in 6 of 9 patient samples; of those, induction of

differentiation was documented in 4, and of p21 in 3 patients, all expressing DRIP205 protein. In summary, these findings suggest that cellular levels of PPAR $\gamma$  regulate induction of apoptosis via caspase-8 activation, while the co-activator DRIP205 is a determinant of induction of differentiation, in response to PPAR $\gamma$  agonists in leukemic cells.

## Keywords

PPARgamma; DRIP205; AML; CDDO; differentiation; apoptosis

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## Introduction

Peroxisome-proliferator-activated receptors (PPARs) are ligand-activated transcription factors that are members of the nuclear-hormone-receptor gene superfamily. To date, three PPAR subtypes have been identified, namely PPAR $\alpha$ , PPAR $\beta/\delta$  and PPAR $\gamma$  (also termed as NR1C1, NR1C2 and NR1C3 (1)). PPAR $\gamma$  is an important regulator of lipid and glucose homeostasis, of cellular differentiation and inflammation. There are three PPAR $\gamma$  isoforms, which differ at their 5-prime ends, each under the control of its own promoter (2). Most tissues express the PPAR $\gamma$ 1 isoform, whereas the PPAR $\gamma$ 2 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  $\times$  receptors (RXRs). Transcriptional regulation by nuclear receptors (NRs), 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 (TR), Vitamin D receptor (VDR), and PPAR and facilitates NR-mediated transcription. Recent studies have further demonstrated a functional role of TRAP220 in the optimal VDR- and retinoic acid receptor (RAR)-mediated myelomonocytic differentiation processes in hematopoietic cells (3). It was also found to act as a pivotal co-activator for GATA-1 in erythroid development (4) and has been shown to play a role in differentiation and proliferation of keratinocytes in response to VDR stimulation (5, 6).

PPAR $\gamma$  ligands have been extensively investigated and are known to inhibit proliferation, induce differentiation or apoptosis in different cancer cell types including hematologic malignancies (7). Our studies have shown that PPAR $\gamma$  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 PPAR $\gamma$  (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 CLL B cells (12). In breast cancer, CDDO-induced growth inhibition correlates with PPAR $\gamma$  transactivation and is mediated at least in part by up-regulation of p21<sup>cip1/waf1</sup> and down-regulation of cyclin D1 (13, 14). Notably, CDDO and its derivatives also induce differentiation of leukemic cells (15-17), the effects which 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, PPAR $\gamma$ -dependent and -independent mechanisms of action (18). One of the most powerful anti-inflammatory and anti-carcinogenic activities induced by CDDO and related

compounds is activation of Nrf2/ARE signaling pathway, a major regulator of anti-oxidant cellular responses (for review, see Liby K, et al (19)).

In this study, we investigated the role of PPAR $\gamma$  and its co-activator DRIP205 on the apoptosis- and differentiation-inducing properties of CDDO in leukemic cells. The results demonstrate that the PPAR $\gamma$  ligands CDDO and 15d15DPGJ<sub>2</sub> induce higher degrees of apoptosis in leukemic cells modified to overexpress PPAR $\gamma$ . Furthermore, we provide evidence that overexpression and recruitment of the co-activator DRIP205 contributes to the enhanced differentiation by this agent. Finally, we report induction of myeloid differentiation of PPAR $\gamma$ /DRIP205 co-expressing leukemic blasts from patients with 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 (NCI) and M. Sporn (Dartmouth Medical College). 15-deoxy- $\Delta^{12,14}$ -15DPGJ<sub>2</sub> (15d15DPGJ<sub>2</sub>) was purchased from Cayman Chemical Company (Ann Arbor, MI). N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) (20) a selective PPAR $\gamma$  antagonist, was synthesized as described previously (21). Annexin V FITC was purchased from Roche Diagnostic Co. (Indianapolis, IN), and CD14 FITC, CD11b PE, and CD34 PE from BD Biosciences (San Jose, CA). Rabbit polyclonal Caspase-3 antibody (CPP32, 1:1000; PharMingen); mouse monoclonal anti-caspase-8 (1:1000) and rabbit polyclonal anti-caspase-9 (1:1000) were purchased from Cell Signaling Technology (Beverly, MA); rabbit polyclonal anti-TRAP220; mouse monoclonal anti-PPAR $\gamma$  (1:500), mouse monoclonal poly(adenosinediphosphate [ADP]-ribose) polymerase (anti-PARP) antibodies (1:200) and mouse monoclonal anti-PCNA (1:500) were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-p21 antibody (1:1000) was from Calbiochem (La Jolla, CA); mouse monoclonal anti-p27 (1:1000), mouse monoclonal anti-HO-1(1:1000) and mouse monoclonal anti-Bip/GRP78(1:1000) antibodies were from BD Biosciences (San Jose, CA); mouse monoclonal anti-Flag and mouse monoclonal anti- $\beta$ -actin (AC74) from Sigma Chemical Co (St. Louis, MO); and goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies from Bio-Rad (Hercules, CA).

### Cell lines and primary AML samples

HL-60, U937, MCF7 and SW480 were purchased from The American Type Culture Collection (ATCC, Rockville, MD). Bone marrow or peripheral blood samples from 260 AML or high-risk myelodysplastic syndrome (MDS) samples were assessed for PPAR $\gamma$  expression by **Reverse Phase Protein Array** (RPPA) (Supplemental methods and (22)). Samples were collected for the Leukemia Sample Bank at the University of Texas M.D. Anderson Cancer Center between 1/15/1998 and 3/9/2006, 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 (Indianapolis, IN). Cell cycle analysis was conducted using propidium iodide (PI) as described (23).

### DNA fragmentation assay

Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in lysis buffer (10mM Tris-HCl, 1 mM EDTA [ethylenediaminetetraacetic acid], 0.025% Triton X-100, pH 8.0) for 10 to 20 minutes on ice. Lysates were digested with RNaseA (50 $\mu$ g/ml) and then 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 surface differentiation-specific cell surface antigens was measured by flow cytometry using the phycoerythrin-conjugated anti-CD11b and FITC-conjugated anti-CD14 monoclonal antibodies from Becton Dickinson (San Jose, CA).

### Transient Transfection and Luciferase Activity Assay

*Plasmids*—SV40  $\beta$ -galactosidase was obtained from Promega Corp., Madison, MI; pM vector was purchased from Clontech (Palo Alto, CA); and pMD, pMDm5, pMDm6, pMDm7, pMDm7 $\Delta$ , and plasmids were generated by PCR (25). FLAG-tagged wt-PPARG 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 co-transfected with DNA using the FuGENE. In coactivation experiments, cells were co-transfected with 250 ng of SV40  $\beta$ -galactosidase (used as an internal control), 1  $\mu$ g of the reporter construct containing three copies of the acyl-CoA oxidase PPRE cloned upstream of the TK-LUC reporter, PPREx3-LUC reporter (kindly provided by Dr. Ronald M. Evans, The Salk Institute), and various amounts of DRIP205 or deletion mutant constructs. After 24h, transfected cells were treated with DMSO or CDDO 0.75 $\mu$ M for another 48-72h. Relative luciferase activity was calculated by dividing luciferase activity by  $\beta$ -galactosidase activity for each well.

### Western blot analysis

Cells were lysed at a density of  $1 \times 10^6/50 \mu$ L in protein lysis buffer (0.25 M Tris-HCl, 2% sodium dodecylsulfate, 4%  $\beta$ -mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) supplemented with a protease inhibitor cocktail (Roche Diagnostic Co.). For preparation of nuclear lysates, cells were washed in cold PBS and digitonin-permeabilized for 5 min on ice at a density of 20 million cells/ml in extraction buffer (250mM sucrose, 70mM KCL: 137mM NaCl: 4.3mM Na<sub>2</sub>HPO<sub>4</sub>: 1.4mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.2), 200  $\mu$ g/ml digitonin and protease inhibitors). Cells were centrifuged at 1000g for 5 min at 4°C, and pellet containing nuclear fraction was lysed as above. Cell lysates were then loaded onto a 10-12% SDS-PAGE gel (Bio-Rad). After electrophoresis, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, England), followed by immunoblotting. Signals were detected using a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA).

### Clinical trial of CDDO in patients with AML

Patients with refractory/relapsed AML were treated with CDDO (RTA-401, Reata Pharmaceuticals, Irving, Tx) from 0.6 to 75 mg/m<sup>2</sup>/hr per hour  $\times$  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 (Suppl. Table 1). Bone marrow or peripheral blood samples mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemicals) density-gradient centrifugation. Apoptosis was examined by simultaneous staining with CD34 APC (BD) and TMRM [tetramethylrhodamine methyl ester (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, Friendswood, TX). The RNA was subjected to purification by an RNeasy ion-exchange column (Qiagen, Valencia, CA) with oncolumn DNase treatment. cDNA was prepared from 1.0  $\mu$ g of total RNA per 20  $\mu$ L mix containing 0.07  $\mu$ g/ $\mu$ L random-sequence hexamer primers, 0.5 mM dNTPs, 5 mM DTT, 0.2 u/ $\mu$ L SuperAsin RNase inhibitor (Ambion, Austin, TX), and 10 u/ $\mu$ L 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 PPAR $\gamma$ , 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 ABL-1 was calculated as follows: relative expression (RE) =  $100 \times 2^{-\Delta Ct}$ , where  $\Delta Ct$  is the mean Ct of the transcript of interest less the mean Ct of the transcript for ABL.

### Transfection of PPAR $\gamma$ small interfering RNA

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

## Results

### PPAR $\gamma$ protein expression in AML

We have previously demonstrated that PPAR $\gamma$  protein is expressed in both myeloid and lymphoid leukemic cell lines (8). Expression of PPAR $\gamma$  protein was analyzed in 260 newly diagnosed leukemia-enriched AML/MDS samples. PPAR $\gamma$  protein was variably expressed in different AML subtypes (Suppl. Fig. 1). There was no difference in overall survival among patients expressing high or low levels PPAR $\gamma$  (not shown).

### CDDO induces PPAR $\gamma$ activation in AML cells

We next characterized the relationship between the PPAR $\gamma$  expression and the ability of pharmacological PPAR $\gamma$  ligands to affect growth and survival of leukemic cells. To this end, we established stable transfectants of U937 cells expressing empty vector (pcDNA3) or Flag-tagged wt-PPAR $\gamma$  (Fig. 1A-C) Overexpression of PPAR $\gamma$  facilitated growth of cells but the differences did not reach statistical significance ( $p=0.1$ , Suppl. Fig. 2A). Exposure of cells to the PPAR $\gamma$  ligand CDDO further enhanced PPAR $\gamma$  protein expression in both, vector control and transfected cells (Fig. 1C). As shown in Fig. 1D, CDDO induced PPAR $\gamma$  mRNA expression in wt-PPAR $\gamma$ -overexpressing cells or in the same cells transfected with scrambled siRNA (2.3- and 3-fold, respectively), and this induction was largely abrogated in

PPAR $\gamma$ -siRNA-transfected cells. This data suggest that PPAR $\gamma$  ligation with CDDO induces expression of its cognate receptor in PPAR $\gamma$ -dependent fashion.

### Relationship between PPAR $\gamma$ expression and growth-inhibitory responses to PPAR $\gamma$ agonists

Next, we examined the functional consequences of PPAR $\gamma$  induction and overexpression in response to the receptor activation by CDDO or by the structurally different PPAR $\gamma$  agonist 15dPGJ<sub>2</sub>. Vector control (pcDNA3) or wt-PPAR $\gamma$  transfected U937 cells were treated with 1 $\mu$  M CDDO for 24 hrs. Overexpression of PPAR $\gamma$  significantly enhanced the sensitivity of leukemic cells to apoptosis induced by CDDO or 15dPGJ<sub>2</sub> (Fig. 2A and Suppl. Fig. 2B). We next analyzed modulation of apoptosis and cell cycle-regulating proteins in PPAR $\gamma$ -transfected clones. Following treatment with CDDO for 24 hours, cells overexpressing wt-PPAR $\gamma$  exhibited significantly decreased pro-caspase 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 PPAR $\gamma$ -overexpressing cells. Analysis of the upstream (initiator) caspases demonstrated appearance of the active (p18) fragment of caspase-8 in these cells, while caspase-9 was similarly cleaved in both, vector control and PPAR $\gamma$ -overexpressing cells (Fig. 2B). In a time-course experiment no caspase-8 cleavage and DNA fragmentation was observed in vector-treated cells, while both ligands induced caspase-8 cleavage and DNA fragmentation at 5 and 24hrs in PPAR $\gamma$ -overexpressing cells (Fig. 2C). In contrast, caspase-9 cleavage did not substantially differ between control and PPAR $\gamma$ -transfected cells. Activation of caspase-8 may proceed through CHOP-dependent transcriptional upregulation of DR5/TRAIL-R2 triggered by ER stress and unfolded protein response (UPR) (27), and CDDO slightly increased the level of ER stress marker protein Bip/GRP78 in PPAR $\gamma$ -overexpressing cells (Fig. 2B). Knocking down exogenously transfected PPAR $\gamma$  by two different siRNA constructs abolished apoptosis induced by the lower CDDO (0.5 $\mu$ M) but did not significantly affect it at higher (1 $\mu$ M) concentrations (Fig. 2D), indicating the contribution of PPAR $\gamma$ -dependent and - independent mechanisms of cell death. Likewise, the pharmacological PPAR $\gamma$  inhibitor T007 partially protected PPAR $\gamma$ -overexpressing, but not control cells, from CDDO-induced apoptosis (Suppl. Fig. 2C). CDDO potentially induced expression of stress responsive inducible enzyme hemoxygenase-1 (HO-1) (Fig. 2B) and of one of the critical Nrf2 target genes NADPH quinone oxidoreductase-1 (NQO-1) (Suppl. Fig. 3A) in both, control and PPAR $\gamma$ -overexpressing cells.

PPAR $\gamma$  agonists are known to induce expression of cell cycle inhibitory protein p21<sup>Waf1/CIP1</sup>, and we have previously 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 in both, control cells and in cells overexpressing PPAR $\gamma$  (Fig. 3A, B). The expression of p27<sup>KIP1</sup> was unchanged, and no significant differences in cell cycle distribution were noted between PPAR $\gamma$ -overexpressing compared to control cells (Suppl. Fig. 2D). Although p21 promoter contains a potential conserved consensus PPAR $\gamma$  response element (PPRE), CDDO may also increase transcription of p21 indirectly, via 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 PPAR $\gamma$  (insert Fig. 3C) with full-length p21 luciferase promoter plasmid and plasmids containing point mutations in GC-rich elements 1-6 (Mut1-Mut6) of the proximal p21 promoter (28). CDDO induced an approximately 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 PPAR $\gamma$ , we measured p21

levels following depletion of PPAR $\gamma$  by siRNA. As demonstrated in Fig. 3D, inhibition of PPAR $\gamma$  using siRNA failed to block CDDO-induced levels of p21 mRNA or protein expression. This data indicate that CDDO causes induction of p21 levels in PPAR $\gamma$ -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 (Suppl. Fig. 3B) in cells with functional or silenced PPAR $\gamma$ , indicating that these responses are likewise PPAR $\gamma$ -independent. No appreciable change in Bip/GRP78 expression was noted in control or transfected cells (Fig. 3D), indicating no significant contribution of ER stress to pro-apoptotic effects of CDDO in this cell system.

### PPAR $\gamma$ ligands enhance DRIP205 co-activator binding to PPAR $\gamma$

PPAR $\gamma$  ligands recruit the co-activator DRIP205 to PPAR $\gamma$ . Furthermore (29) nuclear factor coactivators are known to mediate tissue-specific effects. To ascertain whether DRIP205 overexpression will affect transcriptional activity of PPAR $\gamma$ , the ability of CDDO and 15dPGJ<sub>2</sub> to induce (PPRE)<sub>3</sub>-tk-luc reporter was examined in MCF-7 cells transfected with full-length DRIP205 plasmid. Transient co-transfection with (PPRE)<sub>3</sub>-tk-luc reporter and full-length pcDNA3-DRIP205 plasmid resulted in higher levels of PPAR $\gamma$  transactivation induced by 15dPGJ<sub>2</sub> 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 co-activators with NRs (30;31). Their role in co-activation of PPAR $\gamma$  was further investigated in SW-480 co-transfected with (PPRE)<sub>3</sub>-tk-luc reporter and N- or C-terminal GAL4-DRIP205 deletion constructs (Fig. 4B). The full-length DRIP205 expression plasmid encodes for 1566 amino acids, which is identical to amino acids 16–1581 of the TRAP220 coding sequence. CDDO significantly induced activity in cells transfected with (PPRE)<sub>3</sub>-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, pMDm7, and pMDm6), two of which express N-terminal (pMDm5) or C-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 co-activation, and indicate that multiple domains of DRIP205 are involved in interactions with PPAR $\gamma$ .

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

To determine the functional role of DRIP205 in the context of PPAR $\gamma$  ligation in leukemic cells, we investigated effects of CDDO in HL-60 cells stably transfected with DRIP205 plasmid (three separate clones, Fig. 5A and Suppl. Fig. 4A). No difference in cell growth (Suppl. Fig. 4B), cell cycle progression (Suppl. Fig. 4C) or apoptosis (Suppl. Fig. 4D) was found in cells overexpressing DRIP205, cultured alone or exposed to CDDO. In contrast, CDDO induced a higher degree of myelo-monocytic differentiation in DRIP205-overexpressing cells as shown by increased expression of CD11b ( $p < 0.01$ , Fig. 5B) and by NBT assay ( $36.7 \pm 6.1$  vs  $79.7 \pm 4.7\%$ ,  $p < 0.001$ , Fig. 5C).

To determine if the enhanced differentiation was mediated through PPAR $\gamma$ , we assessed CD11b expression in cells pre-treated with pharmacological antagonist PPAR $\gamma$  T007. As shown in Fig. 5D, blocking PPAR $\gamma$  transactivation significantly diminished CDDO-induced myelomonocytic differentiation in DRIP205-overexpressing 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 with increasing doses of CDDO (RTA-401, escalated from 0.6 mg/m<sup>2</sup>/hr to 75 mg/m<sup>2</sup>/hr) in patients with relapsed/refractory AML, we investigated the *in vivo* differentiating and pro-apoptotic effects of CDDO and correlated these changes with PPAR $\gamma$  and DRIP205 expression, in cells from 9 patients by quantitative TaqMan PCR (PPAR $\gamma$ ) and immunoblotting (PPAR $\gamma$ , DRIP205). Clinical characteristics of the patients are summarized in Suppl. Table 1. PPAR $\gamma$  mRNA was expressed in all samples at baseline albeit at different levels. PPAR $\gamma$  and DRIP205 proteins were expressed in samples from 7 out of 9 patients studied; no expression of either protein was detected in samples from patients #307 and #309 (Fig. 6A). Following 6 days of continuous CDDO administration, PPAR $\gamma$  mRNA was induced >2-fold in 4 patient samples (Suppl. Table 2). In 4 of the 9 patients, an increase in CD11b<sup>+</sup> and CD14<sup>+</sup> cells and a concomitant reduction of immature cells expressing CD34 or CD33 was observed (#301, 304, 305 and 306, Fig. 6B). Examples of flow cytometric profiles are shown in the Suppl. Fig. 5. Baseline expression of PPAR $\gamma$  protein was highest in samples from patients #301 and #304 (Fig. 6A), and in all four patients increase of PPAR $\gamma$  mRNA was demonstrated (1.5, 2.4, 1.8 and 2.2-fold, respectively, Suppl. Table 2). 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 PPAR $\gamma$  or DRIP205 proteins. Moderate induction of apoptosis documented as loss of mitochondrial membrane potential in circulating CD34<sup>+</sup> cells was observed in samples from 3 patients (#301, #303 and #305); in sample#303 corresponding apoptosis induction was seen in Day 6 bone marrow CD34<sup>+</sup> cells (Fig. 6C). Examples of flow cytometric profiles are shown in the Suppl. Fig. 6. Clinically, patients did not fulfill protocol response criteria, differential counts did not change significantly and MTD was not reached, at the low dose levels in this Phase I study.

## Discussion

PPAR $\gamma$  ligands inhibit cancer cell proliferation, induce apoptosis and/or differentiation in multiple tumor types, and these effects have been attributed to both, PPAR $\gamma$ -dependent and - independent mechanisms. In this study, we evaluated the role of PPAR $\gamma$  and one of its cellular co-activators, DRIP205, in the pro-apoptotic and differentiating properties of PPAR $\gamma$  agonists CDDO and 15dPGJ<sub>2</sub>. A high-throughput RPPA technique demonstrated high levels of PPAR $\gamma$  expression in 260 primary AML samples. To functionally characterize the relationship between baseline PPAR $\gamma$  levels and cellular effects of PPAR $\gamma$  agonists in leukemic cells, we generated stably transfected myeloid leukemic cells overexpressing the receptor. U937 cells induced to overexpress wt-PPAR $\gamma$  were more sensitive to the pro-apoptotic effects of PPAR $\gamma$  ligands CDDO and 15dPGJ<sub>2</sub> compared to vector-transduced cells. These pro-apoptotic effects were significantly inhibited by silencing PPAR $\gamma$  with siRNA or by blocking PPAR $\gamma$  activation with the pharmacological antagonist T007, consistent with previously published findings of PPAR $\gamma$ -dependent and -independent mechanisms of action of this class of agents. Time-course analysis demonstrated that high PPAR $\gamma$  levels facilitated cleavage of caspase-8 and -3 (but not of caspase-9) which resulted 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), JNK-mediated induction of TRAIL receptor expression (33) and inhibition of NF $\kappa$ -B-dependent anti-apoptotic proteins (11). Conversely, data reported by us and others demonstrate 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 PPAR $\gamma$ , whereas direct mitochondrial effects of CDDO observed at higher concentrations are likely PPAR $\gamma$ -independent, possibly by modifying the mitochondrial proteins through nucleophilic attack and Michaelis addition (36). Unlike in non-small lung cancer cells (27), CDDO did not induce significant ER stress response, hence making the upregulation of DR5 unlikely mechanism of caspase-8 activation. The exact mechanistic link between PPAR $\gamma$  transactivation and activation of the extrinsic apoptotic pathway in AML remains to be determined.

It has recently been demonstrated that synthetic triterpenoids are potent activators of Nrf2/ARE signaling in a variety of cell types, resulting in marked induction of a variety of anti-oxidative 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 PPAR $\gamma$ . These observations are consistent with the notion that these responses are likely mediated by chemical structure of CDDO and other electrophilic compounds capable to modify cysteine residues on KEAP1 protein (37), and represent important PPAR $\gamma$ -independent activities of this class of compounds.

PPAR $\gamma$  agonists including CDDO modulate cell cycle progression in multiple tumor types (13, 14). Our present data show that CDDO induced expression of p21<sup>waf1/CIP</sup> protein in leukemic cells. This induction was observed in parental cells, in cells overexpressing PPAR $\gamma$ , or in cells transfected with PPAR $\gamma$  siRNA. These findings indicate that the ability of CDDO to activate p21 promoter is likely mediated via PPAR $\gamma$ -independent mechanisms. Since 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 utilized 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 PPAR $\gamma$ -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 to 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 3 of the 9 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 co-activators and co-repressors, in the biological responses to nuclear receptor agonists. PPAR $\gamma$  is known to interact with both the p160/SRC-1 family of coactivators and the multisubunit DRIP/Mediator co-activator complex. Our results show that CDDO induced significant activity in cells transfected with (PPRE)<sub>3</sub>-tk-luc and full-length DRIP205. In addition, significant coactivation was observed utilizing several N- and C-terminal domain mutants of 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 PPAR $\gamma$ , similar to findings reported for ER $\alpha$  coactivation by DRIP205 (25). Interestingly, recent structural and functional analyses indicate that a direct interaction of PPAR $\gamma$  with DRIP/Mediator complex through the NR motifs of DRIP205 is not required for PPAR $\gamma$ -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 shown to induce differentiation in myeloid leukemia cells (16, 17, 44) and in this study CDDO induced a higher degree of myelo-monocytic differentiation in DRIP205-overexpressing HL-60 cells, a process mediated through PPAR $\gamma$ . While we recently reported that one of the mechanisms of differentiation induction by CDDO involves modulation of CEBP $\alpha$  expression and function (45), our data shown here provide first evidence that high cellular levels of the co-activator DRIP205 can enhance the differentiation induced by PPAR $\gamma$  ligation and is therefore an important determinant of tissue-specific effects of PPAR $\gamma$  agonists. We here report that leukemic blasts from patients treated in a phase I clinical trial of CDDO (RTA-401) express DRIP205 in 7/9 samples, all of which expressed PPAR $\gamma$  mRNA and protein. Further, sequential studies demonstrated increased expression of the differentiation markers CD11b and/or CD14 in 4 patients. In these patients, CDDO induced PPAR $\gamma$  transcription. Even though the numbers are too small to draw definitive conclusions, the data suggest that CDDO activates PPAR $\gamma$  in a subset of patients with AML *in vivo*, whose cells express DRIP205. We did not observe correlation between PPAR $\gamma$  levels and apoptosis induction, possibly due to very low levels of CDDO in this Phase I study. Alternatively, this finding may indicate that PPAR $\gamma$ -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-APL patients with AML who were treated with this agent in a Phase I trial (46). Taking into consideration multiple studies that demonstrate that addition of RXR ligands synergistically enhance the differentiating and growth-suppressive effects of PPAR $\gamma$  ligands (8, 47), the combined use of these agents appears worth testing in the therapy of AML. The ongoing efforts by the Nuclear Receptor Signaling Atlas (NURSA) consortium to profile co-activator/co-repressors in primary AML may assist in identifying patients who are likely to benefit from PPAR $\gamma$ /RXR ligation strategies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

**Grant support:** Leukemia and Lymphoma Society grant R64149-07 (MK)

Leukemia Spore (NCI: (1 P50 CA100632-01) (MA)

Leukemia and Lymphoma Society grant 6089 and NIH PO1 grant CA-55164 (SMK)

Cancer Center Support Grant P30 CA016672 34 (KRC)

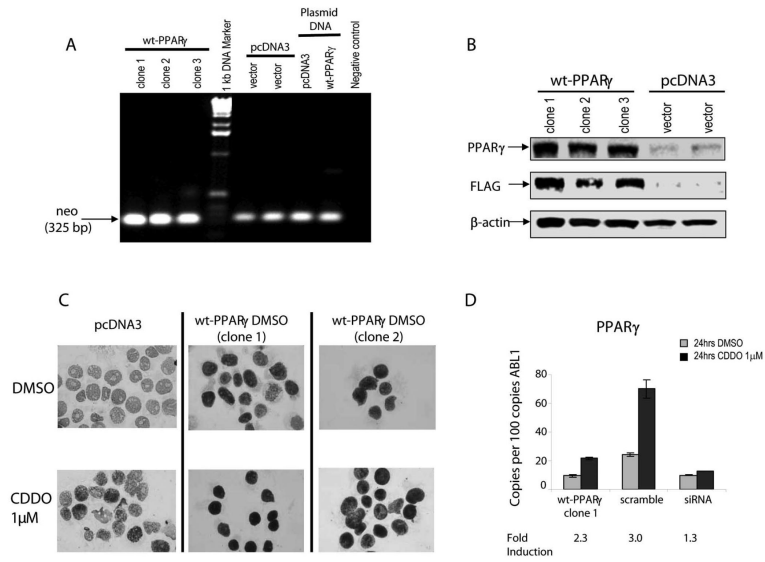
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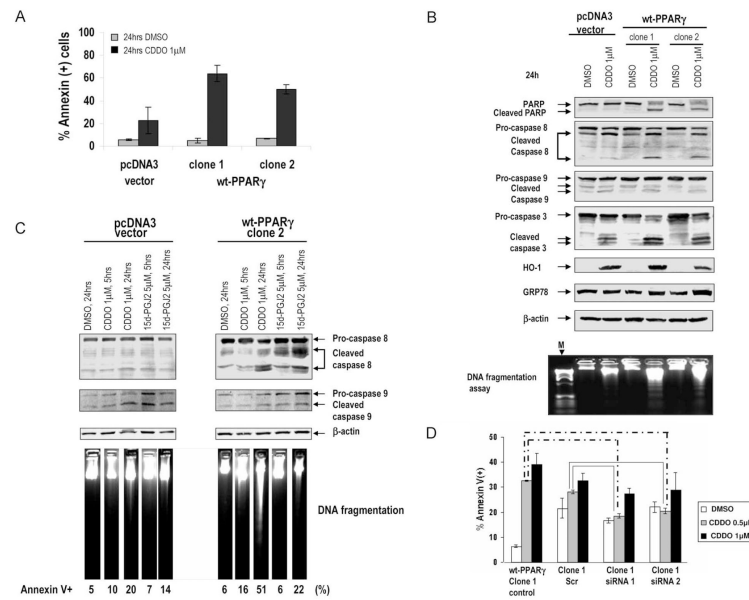
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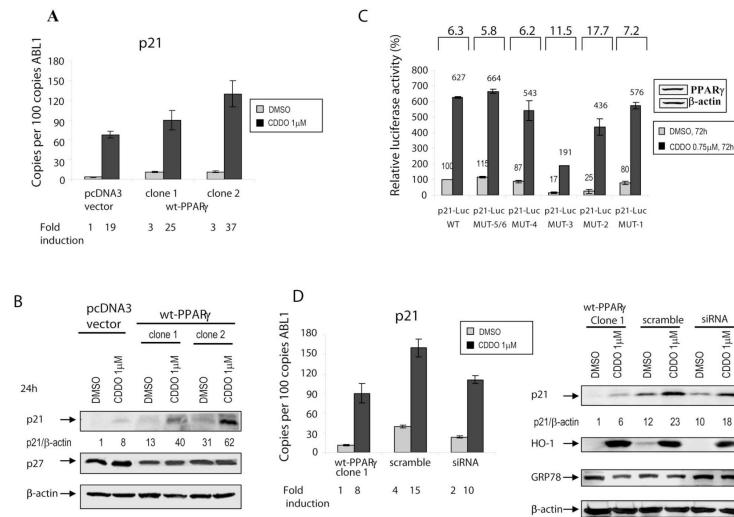
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**Fig. 1.** A. RT-PCR of neomycin gene expression in pcDNA3 or wt-PPAR $\gamma$  transfected clones. **B.** PPAR $\gamma$  and FLAG protein expression in transfected cells was detected by immunoblotting. **C.** Leukemic cells transfected with pcDNA3 or wt-PPAR $\gamma$  were treated with 1  $\mu$ M CDDO for 5 hours, and PPAR $\gamma$  protein levels were examined by immunocytochemistry. **D.** PPAR $\gamma$  transcript was measured in wt-PPAR $\gamma$  transfected cells (clone 1) or in the same cells transfected with scramble or PPAR $\gamma$  siRNA, by real-time PCR. Results are reported as the mean number of transcripts per hundred transcripts of ABL1. Error bars denote the standard error of the mean.

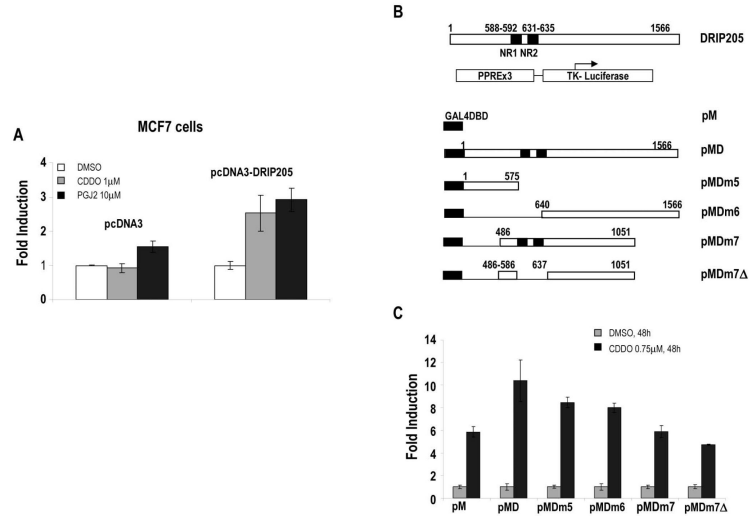
**Fig. 2.**

**A.** U937( pcDNA3) and U937 (pcDNA3-wt-PPAR $\gamma$ ) cells were cultured in the presence of CDDO or vehicle for 24h, and apoptosis induction was analyzed by Annexin V flow cytometry at 24 hours. **B.** U937( pcDNA3) and U937 (pcDNA3-wt-PPAR $\gamma$ ) cells were grown in the presence of 1 $\mu$ M CDDO or vehicle for 24h. Expression of PARP, caspase-3,-8, -9, **HO-1** and **GRP78** was analyzed by immunoblotting, and effects of CDDO on DNA fragmentation were determined. **C.** Caspase-8, and -9 processing and DNA fragmentation at 5 hours and 24 hours was measured. **D.** U937-wt-PPAR $\gamma$  cells were transiently transfected with scramble or PPAR $\gamma$  siRNA (siRNA1 and siRNA2), at a final concentration of 67nM. Seventy-two hours after transfection, cells were exposed to indicated concentrations of CDDO for 24 hrs. Induction of apoptosis was measured by annexinV flow cytometry. The data represent average results from three independent experiments. Solid lines, paired t-test comparing apoptosis in scrambled siRNA-transfected cells with apoptosis in PPAR $\gamma$  siRNA transfectants ( $p < 0.01$ ); dashed lines, paired t-test comparing apoptosis in untransfected cells with PPAR $\gamma$  siRNA transfectants ( $p < 0.001$ ).

**Fig. 3.**

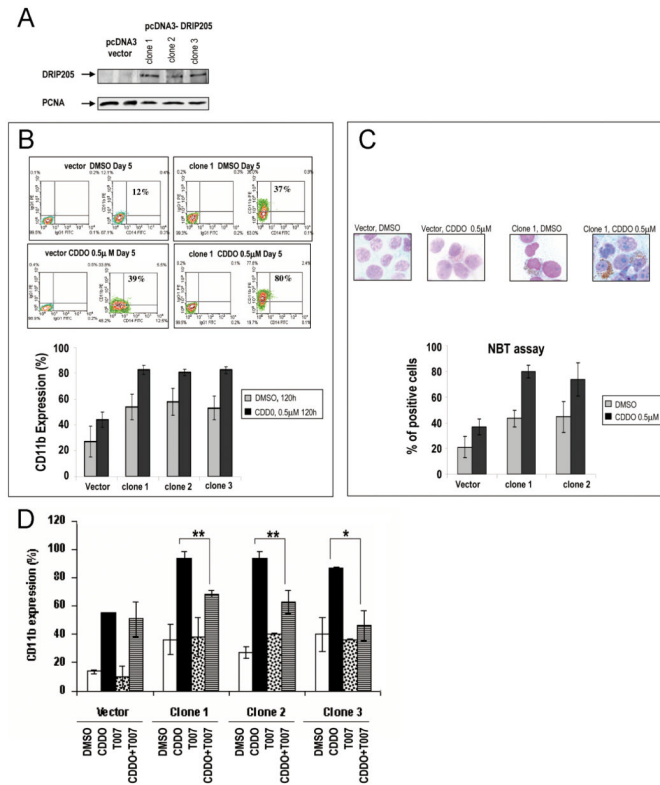
**A.** U937 (pcDNA3) and U937 (pcDNA3-wt-PPAR $\gamma$ ) cells were cultured in the presence of 1 $\mu$ M CDDO or vehicle for 24h, and p21 transcript was measured by real-time PCR. **B.** Cells were harvested at 24h, and p21/p27 levels were determined by immunoblotting. **C.** The fold transactivation of the p21 promoter by CDDO (0.75 $\mu$ M, 72 hrs). SW480 colon cancer cells were co-transfected with the wt- or mutant -2325/+8 p21 promoter-luciferase reporter vectors. Cells were co-transfected with  $\beta$ -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-PPAR $\gamma$  clone 1 electroporated with scrambled or PPAR $\gamma$  siRNA, 48h after transfection. Following 48 hrs, DMSO or CDDO were added for the next 24 hrs, and after 24hrs p21, **HO-1**, **GRP78** and  $\beta$ -actin levels were determined by quantitative TaqMan PCR and Western blot analyses.





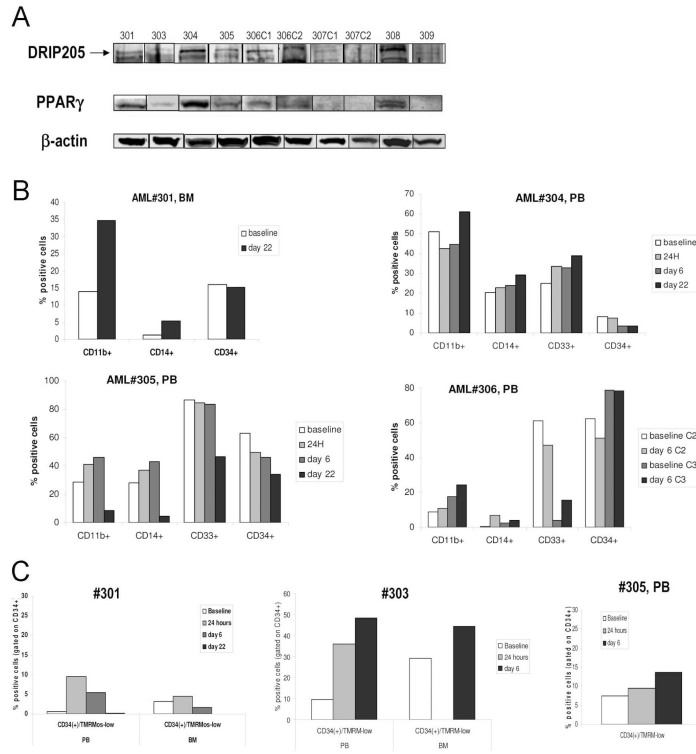
**Fig. 4.**

**A.** MCF-7 cells were transiently co-transfected with 1 $\mu$ g of pPPRE-TK-LUC and 4 $\mu$ g of pcDNA3 or pcDNA3-DRIP205, treated with indicated concentrations of CDDO or 15dPGJ<sub>2</sub> for 92.5 hours, after which PPAR $\gamma$  transactivation was determined by relative luciferase activity calculated by dividing luciferase activity by protein concentration for each well. Results are shown as mean  $\pm$  SEM for three separate experiments. **B.** Map of GAL4-DRIP fusion proteins. **C.** Coactivation of PPAR $\gamma$  by GAL4-DRIP fusion proteins. SW480 cells were co-transfected with 1000 ng of pPPRE3-LUC, 250 ng of  $\beta$ -galactosidase, 500 ng of pM empty or pM DRIP deletion mutants, treated with DMSO and 0.75 $\mu$ M CDDO, and luciferase activity was determined. Results are shown as mean  $\pm$  SEM for three separate experiments.



**Fig. 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, C.** HL-60 cells transfected with vector (pcDNA3) or pcDNA3-DRIP205 constructs were treated with 0.5  $\mu$  M CDDO for 120 hours, 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  $\mu$  M T007, a PPAR $\gamma$  antagonist, for 1 hour, followed by 0.75  $\mu$  M CDDO for 120 hours. Data represent average results (mean $\pm$ SD) of three independent experiments. Induction of differentiation was determined by CD11b flow cytometry. \*\* denotes  $p=0.01$ , and \* $p=0.02$ .



**Fig. 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 PPAR $\gamma$  by Western blot.  $\beta$ -actin was used as a loading control. In baseline sample from patient#2, not enough material was available for immunblotting. **B.** Patients were treated with CDDO (RTA-401) during a Phase I clinical trial, and cells were collected from the peripheral blood (PB) or bone marrow (BM) and assessed for expression of surface markers CD11b, CD14, CD33 and CD34 by flow cytometry at the indicated times (see also suppl. Table 2). Four of nine patients (patients #301, 304, 305, 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, 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.