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Differentiation of Human Neuroblastoma by Phenylacetate Is Mediated by Peroxisome Proliferator-activated Receptor γ[1]

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ABSTRACT

Phenylacetate (PA) is a member of a class of aromatic fatty acids that has demonstrated antitumor activity in experimental models and in humans. Previous reports have shown that PA and its analogues can act as ligands for the peroxisome proliferator-activated receptor (PPAR) and thereby regulate certain gene expression through peroxisome proliferator response elements. The role of this activity in the antitumor activity of PA has not been determined. To address this question, we have used the human neuroblastoma cell line LA-N-5, which expresses PPARγ and can be induced to differentiate with PA and with classical PPARγ ligands. Our results indicated that the PPARγ ligands 15-deoxy-Δ prostaglandin J2 and GW1929 as well as PA induced LA-N-5 cells to differentiate to a similar phenotype as evidenced by inhibition of cell proliferation, neurite outgrowth, increased acetylcholinesterase activity, and decreased N-*myc* gene expression. Furthermore, induction with all of the compounds was accompanied by up-regulation of mRNA levels of the nuclear retinoic acid receptor β (RARβ) and specific activation of a reporter gene construct (ΔSVβRE-CAT) that contains the canonical RA response element located in the RARβ promoter. All of the assessed functional and molecular effects of PA on LA-N-5 cells, as well as those of the classical PPARγ ligands, were inhibited by cotreatment with specific PPARγ antagonists (GW9662 and/or GW0072). Taken together, these studies have confirmed a role for PPARγ in neuroblastoma cell biology and indicated that the PPARγ signaling pathway plays a direct role in the PA-induced differentiation response of this cell type.

INTRODUCTION

Aromatic fatty acids, of which PA[3] is a prototype, constitute a class of low toxicity drugs with demonstrated antitumor activity in experimental models and in humans. PA is a natural metabolite of phenylalanine, which was originally described as a plant growth hormone (1). Normally found in micromolar concentrations in human plasma, PA has a long clinical history as treatment for conditions associated with hyperammonemia such as urea cycle disorders in children (2, 3). This clinical experience has indicated that millimolar blood serum levels can be achieved without significant adverse effects.

As demonstrated in a variety of experimental *in vitro* and *in vivo* models, PA and its analogues can induce selective cytostasis and reduce the malignant potential of various hematological and solid neoplasms at nontoxic millimolar concentrations shown to be readily achievable in humans (4–7). Early clinical trials with PA have now documented activity in high-grade gliomas, hormone-independent prostatic carcinoma, and lymphoid malignancies. PB, which is metabolized to PA in humans, was also recently shown to benefit cancer patients who have failed conventional therapies (8, 9). For both compounds, the treatments were well tolerated, with a dose-limiting toxicity of somnolence. Despite this substantial experimental and clinical experience with PA compounds, their antitumor mechanism of action remains unknown.

In previous studies, we showed that PA can stimulate the differentiation of human nb cells by itself and can impact the differentiation program induced by RA by activating one of the retinoid nuclear receptors, RARβ (6, 10). It has also been shown that PA can activate other members of the steroid receptor superfamily, namely, the PPARs (11). Recently, Samid *et al.* (12) demonstrated that PA derivatives can directly act as ligands to PPARγ and that the cytostatic effects of these drugs on certain cell lines directly correlated with their PPAR-activating ability. In a previous study, we documented that the LA-N-5 human nb cell line and primary nb cells from patient tissue express abundant amounts of PPARγ and that PPARγ ligands can stimulate the differentiation of this cell type (13). These new results have now led us to address the question of whether the PPARγ signaling pathway is involved in the differentiation-inducting activity of PA on nb cells. Our present findings indicate that this is indeed the case.

MATERIALS AND METHODS

Cell Culture. The LA-N-5 human nb cell line was grown in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/ml penicillin/streptomycin, and 1 μg of amphotericin (complete medium) as described previously (14). 15d-PGJ2 was purchased from Cayman Chemical (Ann Arbor, MI) dissolved in methyl acetate. GW1929, GW9662, and GW0072 were synthesized by the Medicinal Chemistry Department at Glaxo Wellcome Research and Development and were a generous gift from Dr. T. M. Willson of the same institute (Glaxo Wellcome Co., Research Triangle Park, NC). Sodium PA was obtained from Elan Pharmaceutical Research Corp. (Gainesville, GA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

AChE Activity. Specific AChE activity was measured as a biochemical index of the relative state of differentiation of treated and untreated LA-N-5 cells. To measure AChE activity as described previously (14), cells were grown in 12-well plates for 6 days in the presence or absence of the indicated concentrations of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. After washing twice with PBS, cells were collected, ice-cold 10 mM sodium phosphate buffer (pH 7) containing 0.5% Triton X-100 was added, and the suspension sonicated for 20 s. AChE activity was determined photometrically by following the hydrolysis of acetylthiocholine as described previously (14). Protein concentrations were determined with a Sigma Chemical Co. bicinchoninic acid protein assay kit using BSA as the standard. Results (in nmol/h/mg protein) are the means ± SE of triplicate wells in typical experiments and are expressed as a percentage of control. All experiments were repeated at least three times.

Cell Proliferation Assay. LA-N-5 cell proliferation was assessed based on the ability of the cells to stain with SRB (15). Cells were grown in 24-well culture plates for 6 days in the presence or absence of the indicated concentrations of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. The untreated control cells were not less than 90% of confluence before harvest. The culture medium was removed, and the cells were washed thrice with PBS. Trichloroacetic acid (final concentration, 10%) was then added for fixation at 4°C. After

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[3] The abbreviations used are: PA, phenylacetate; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; PPAR, peroxisome proliferator-activated receptor; nb, neuroblastoma; 15d-PGJ2, 15-deoxy-Δ prostaglandin J2; AChE, acetylcholinesterase; SRB, sulfornodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; PB, phenylbutyrate; RXR, retinoid X receptor.

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1 h of fixation, plates were washed five times with tap water. The plates were then air dried, and 0.4% SRB in 1% acetic acid was added for 30 min. Unbound SRB was removed by washing the plates four times with 1% acetic acid. After air drying, SRB dye within cells was dissolved for 5 min with 10 mM unbuffered Tris base (pH 10.5). The absorbance of the extracted SRB dye, which represented protein content, was measured with a spectrophotometer at 540 nm.

Northern Blot. LA-N-5 cells were cultured for the indicated number of days in the presence or absence of PA, 15d-PGJ2, GW1929, GW0072, and/or GW9662. Total RNA (30 μg) was extracted by using TRI reagent (Sigma Chemical Co.) as described previously (16), separated by electrophoresis in a denaturing formaldehyde agarose gel, and blotted onto a nylon transfer membrane (Micron Separations, Inc., Westborough, MA). The RNA was cross-linked to the membranes by irradiation for 1 min under UV light and baked for 5 min at 70°C. A random priming probe kit from Promega (Madison, WI) was used to label RARβ, N-myc, and GAPDH cDNA probes. The blots were hybridized overnight at 42°C with ^{32}P-labeled RARβ, N-myc, or GAPDH probes. After washing twice with 2× SSC/0.1% SDS and twice with 0.1× SSC/0.1% SDS, membranes were exposed for 18–72 h at −70°C to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with intensifying screens.

Cell Transfection and CAT Assay. All transfections were carried out with LA-N-5 cells by the non-liposome-mediated formulation FuGENE 6 transfection method (Roche Molecular Biochemicals, Indianapolis, IN). Cells were maintained in medium without phenol red that contained 10% fetal bovine serum for 24 h before transfection. Transfections (using 6-well plates of 70% confluent cells) contained 3 μl of FuGENE 6 reagent with 2 μg expression plasmid/well in serum-free medium. The ΔSVβRE-CAT reporter construct was used to measure RARβ promoter activation (17, 18); ΔSV-CAT vector served as a control. RA, PA, GW1929, GW0072, and/or GW9662 were added to the cultures immediately after the transfection. Cells were harvested 24 h later and adjusted to 4×10^5 viable cells in 150 μl of PBS (pH 7.4). Cells were lysed by three freeze-thaw cycles. To assay CAT activity, we used the [^{3}H]acetyl-CoA method as described previously (19). The lysate was thawed, and 50 μl of it placed into a glass scintillation vial containing 150 μl of PBS, 7.5 μl of 4× CAT buffer (32 mM chloramphenicol dissolved in ethanol), 22.5 μl of water, and 0.5 μl (0.1 μCi/μl) of [^{3}H]acetyl-CoA per vial. Organic scintillation fluid was then added, and vials were loaded into the scintillation counter for counting at different time periods.

Statistical Analysis. Data are expressed as the mean ± SE. ANOVA and Student's *t* test (two-tailed) were used for statistical determinations (compared with control group). A level of $P \leq 0.05$ was considered statistically significant. For analysis of PPARγ antagonist effects on PPARγ agonist-induced functional activity, differences between the combination treatment values (agonist + antagonist) minus untreated controls were compared with the differences between single treatment values (agonist alone) minus untreated controls. Because untreated control values were set to 100%, the ability of a compound to antagonize the functional activity of a PPARγ agonist is given by the following formula:

$$\% \text{ Antagonism} = \frac{\text{Activity}_{\text{agonist}} - \text{Activity}_{\text{agonist+antagonist}}}{\text{Activity}_{\text{agonist}} - 100}$$

where activity is expressed as a percentage of the control.

RESULTS

Effects of PA and PPARγ Ligands on the Growth of LA-N-5 Cells. Previous studies have demonstrated dose-dependent growth inhibition of LA-N-5 cells induced by both PA and PPARγ ligands (6, 13). In those studies, maximal growth-inhibitory effects were obtained at approximately 5 mM PA, 10 μM 15d-PGJ2, and 20 μM GW1929, respectively. Fig. 1 demonstrates that in the present studies, these concentrations of the compounds caused growth inhibition of 37% in the case of PA, 41% in the case of 15d-PGJ2, and 53% in the case of GW1929. As shown, the combination of PPARγ antagonist GW0072 with either PA, 15d-PGJ2, or GW1929 reduced the growth-inhibitory effects of treatments with the latter compounds alone. Thus, GW0072 antagonized the antiproliferative effects of PA, 15d-PGJ2, and

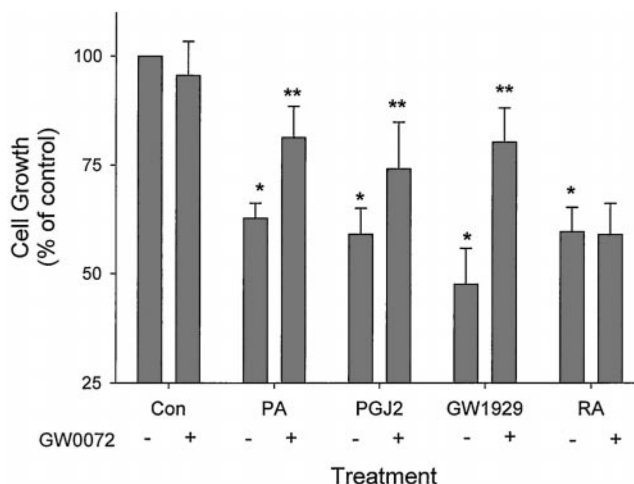

Fig. 1. Effect of PA and PPARγ ligands on the growth of LA-N-5 cells. Cells were treated as indicated with 5 mM PA, 10 μM PGJ2, 20 μM GW1929, 5 μM RA (as a positive control for cell inhibition), or solvent control (*Con*) in either the absence (−) or presence (+) of 20 μM GW0072 for 6 days. Cells in the control group without GW0072 were set at 100%. *Bars* represent the mean ± SE of triplicate samples in at least three independent experiments. For cells cultured in the absence of GW0072, * indicates a significant difference as compared with the control ($P \leq 0.05$). For cells cotreated with GW0072, ** indicates significance as compared with the corresponding single treatment values ($P \leq 0.05$).

GW1929 by 53%, 37%, and 63%, respectively. GW0072 alone had no significant effects on the growth of LA-N-5 cells. As a specificity control in these experiments, RA was used as a known differentiation/antiproliferative agent for these cells (17). Fig. 1 shows that RA inhibited the growth of the cells by 40%, but GW0072 did not significantly affect this growth-inhibitory activity.

Our previous studies have shown that PA and PPARγ ligands can induce neurite outgrowth from LA-N-5 cells at concentrations similar to those found to inhibit cell growth (6, 13). However, because neurite outgrowth is purely a visual qualitative phenomenon, experiments to determine whether GW0072 can antagonize this effect are difficult to interpret and therefore were not addressed at this time. GW0072 alone had no apparent morphological effects on LA-N-5 cells (data not shown).

AChE Activity. In LA-N-5 cells, AChE has been used extensively as a quantitative biochemical marker of differentiation after treatment with a variety of inducing agents including PA, 15d-PGJ2, and GW1929 (6, 13). As reported previously, Fig. 2 shows a 1.5–1.7-fold increase in AChE activity when LA-N-5 cells were treated with 5 mM PA, 10 μM 15d-PGJ2, or 20 μM GW1929 for 6 days. In contrast, the PPARγ antagonist GW0072 did not alter specific AChE activity. However, when added together, GW0072 antagonized the stimulatory effects of PA, 15d-PGJ2, and GW1929 on AChE by 67%, 54%, and 58%, respectively. GW0072 did not significantly alter RA-induced AChE activity.

N-myc Expression. N-myc expression is considered to be a molecular marker of differentiation of LA-N-5 cells and other nb cell lines (17, 20, 21). Previous work has shown that PA induces a moderate (15–35%) decrease of N-myc mRNA levels after long term-term treatment (>8 days; Ref. 6). In contrast, shorter treatments with PA (3–6 days) demonstrated no apparent effects on N-myc mRNA levels, although N-myc protein was found to be decreased (6). In our present studies, we have seen that the modest down-regulation of N-myc mRNA is largely reversed when the 8-day PA treatment is combined with PPARγ antagonists GW9662 or GW0072 (data not shown).

Nuclear RARβ Expression. We have demonstrated previously that PA can impact the RA differentiation program by up-regulating

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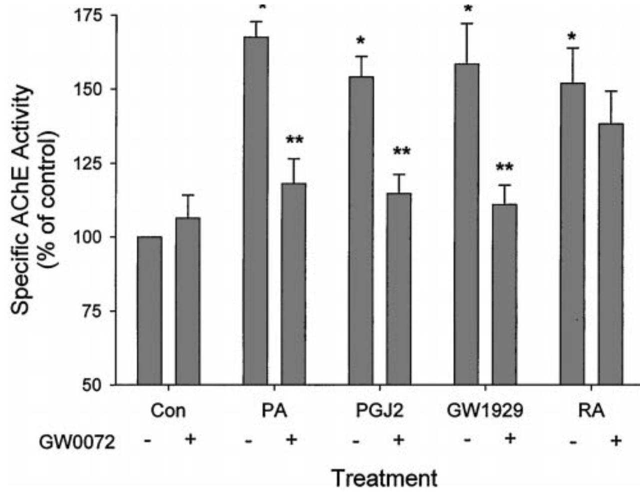

Fig. 2. Effect of PA and PPARγ ligands on AChE activity in LA-N-5 cells. Cells were treated as indicated with 5 mM PA, 10 μM PGJ2, 20 μM GW1929, 5 μM RA (as a positive control for AChE activity), or solvent control (*Con*) in either the absence (−) or presence (+) of 20 μM GW0072 for 6 days. Cells in the control group without GW0072 were set at 100%. *Bars* represent the mean ± SE of triplicate samples in at least three independent experiments. For cells cultured in the absence of GW0072, * indicates a significant difference as compared with the control ($P \leq 0.05$). For cells cotreated with GW0072, ** indicates significance as compared with the corresponding single treatment values ($P \leq 0.05$).

RARβ expression (6). Our studies showed that this effect is regulated at the level of transcription and mediated through the canonical RARE in the RARβ promoter (RAREβ; Ref. 10). To determine whether this activity of PA involves the PPARγ signaling pathway, we first assessed whether "classical" PPARγ agonists can also augment RARβ expression. Fig. 3*a* shows that this was indeed the case; densitometric scanning indicated a 5- to >25-fold increase (normalized to GAPDH) in RARβ mRNA levels induced by 15d-PGJ2 compared with controls and a 10–40-fold increase induced by GW1929 after 2 days of culturing. To confirm a role for PPARγ in this activity, we determined whether PPARγ antagonists could inhibit the PA- and 15d-PGJ2-induced increases in RARβ. Fig. 3*b* demonstrates that the addition of either GW9662 or GW0072 to the cultures completely blocked induction of RARβ expression in LA-N-5 cells. Culturing the cells in the PPARγ antagonists alone resulted in no differences in the expression of RARβ compared with that in control cells (data not shown).

PPARγ-mediated Activation through RAREβ. We have previously demonstrated that PA activation of RARβ occurs through the canonical RARE designated RAREβ located in the gene's promoter (10). We now tested whether PA activation of this response element is mediated by PPARγ by first evaluating the effects of the PPARγ agonists on the RAREβ-containing CAT reporter construct ΔSVβRE-CAT in transient transfection experiments with LA-N-5 cells. This construct contains a single copy of RAREβ cloned at the unique *Hin*III site present in a basal promoter CAT construct, ΔSV-CAT. This vector, which served as a control for ΔSVβRE-CAT, was originally constructed by replacing the TK promoter in TK-CAT (22) with the *Sph*I-*Hin*dIII fragment of the SV40 early promoter (18). As can be seen in Fig. 4, in the presence of PA and GW1929, CAT activity from ΔSVβRE-CAT was 1.8- and 1.6-fold above untreated levels, respectively. As an additional positive control for this system, RA caused a 1.75-fold increase in CAT activity.

Next we determined whether the PPARγ antagonist GW9662 would specifically retard the PA-mediated activation of ΔSVβRE-CAT. Fig. 4 shows that this was indeed the case; whereas GW9662 had no significant activity on the reporter itself, it reduced both PA- and GW1929-mediated induction by 54% and 60%, respectively. In contrast, GW9662 had no significant effect on RA induction of CAT activity, confirming that the specificity of the inhibitory activity of GW9662 was mediated by PPARγ. Under the same treatment conditions, activity of the basal construct ΔSV-CAT in the presence of any of the compounds never differed significantly from that of control cultures.

DISCUSSION

Although there have been scores of published laboratory studies and a few clinical trials, the antitumor effects of PA and its analogues remain unclear. Recently, PB combined with RA was shown to induce remission in a patient with acute promyelocytic leukemia whose disease was resistant to RA as a single agent (23). However, this effect of PB was speculated to be due to the ability of the butyrate moiety of this compound to function as a histone deacetylase inhibitor rather than to the metabolic conversion of PB to PA. Early studies suggested that the ability of PA to deplete serum of glutamine due to the formation of phenylacetylglutamine might serve to selectively inhibit the growth of certain types of tumor cells (24). However, because many tumor cells shown to be affected by PA are not particularly

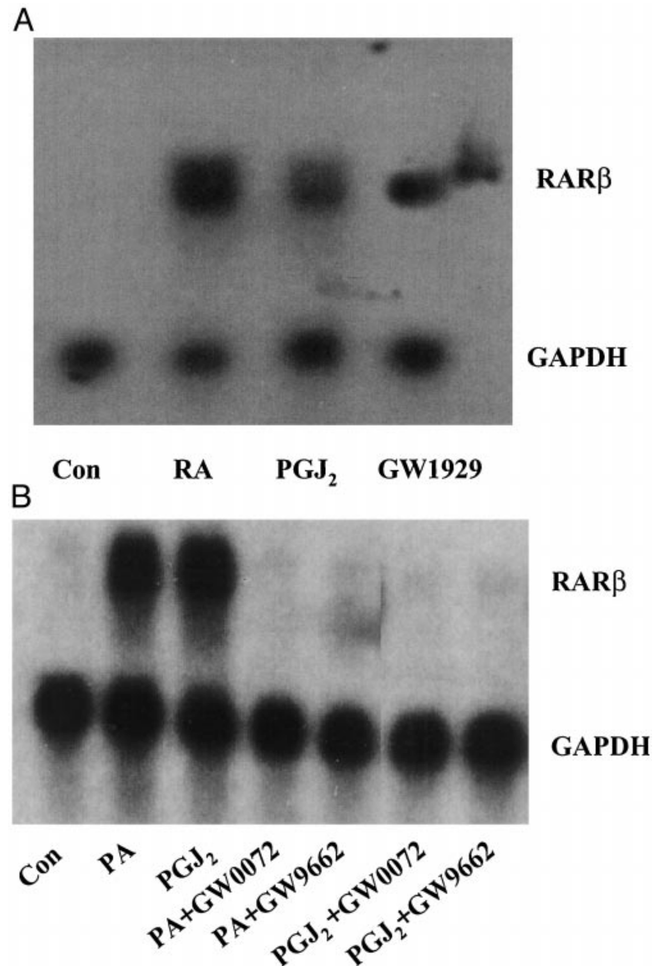

Fig. 3. Effect of PA and PPARγ ligands on RARβ mRNA levels in LA-N-5 cells (mean ± SE). Cells were cultured for 2 days in the absence (*Con*) or presence of 5 mM PA, 10 μM PGJ2, or 10 μM GW1929 (*A*) or 5 mM PA + 20 μM GW0072, 5 mM PA + 20 μM GW9662, 10 μM PGJ2 + 20 μM GW0072, or 10 μM PGJ2 + 20 μM GW9662 (*B*), as indicated. RARβ and GAPDH mRNA levels were then measured by Northern blotting using ^{32}P-labeled cDNA probes. Results of a representative Northern blot are presented. Significant differences of PA or PPARγ ligand treatments compared with the control (*A*) or significant differences of combination treatments compared with PA or 15d-PGJ2 (*PGJ_2*) alone (*B*) were observed in at least three independent experiments ($P \leq 0.05$).

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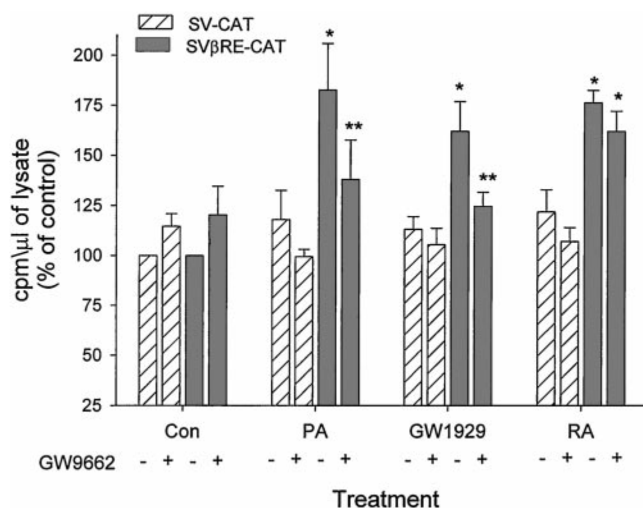

Fig. 4. Effect of PA and PPARγ ligands on ΔSVβRE-CAT expression. LA-N-5 cells were transfected with the ΔSV-CAT (▨) or ΔSVβRE-CAT (▤) constructs by the FuGENE 6 nonliposomal formulation method and immediately treated as indicated with 5 μM RA, 5 mM PA, 10 μM GW1929, or solvent control (*Con*) in either the absence (−) or presence (+) of 20 μM GW9662. Cell extracts from normalized cell number were assayed for CAT activity and expressed as cpm/μl lysate. Solvent controls without GW9662 were set at 100%. *Bars* represent the mean ± SE of three independent experiments. When using the ΔSV-CAT construct, no significant differences in CAT activity were noted between any treatment conditions. When using the ΔSVβRE-CAT construct, * indicates a significant difference between treated cells in the absence of GW9662 and solvent control ($P \leq 0.05$); ** indicates significance between treated cells in the presence of GW9662 and the corresponding single treatment values ($P \leq 0.05$).

sensitive to glutamine depletion, this mechanism alone could not explain the antitumor activity of this compound. Other investigations demonstrated that PA, due to its ability to block β-hydroxy-β-methylglutaryl CoA reductase, inhibits cholesterol synthesis and protein prenylation in glioma, melanoma, prostatic carcinoma, and nb cells (5, 6). These cells, like most cancer cells, are dependent on intracellular synthesis of isoprenoids for growth and survival. The possibility that inhibition of protein prenylation may play a role in the antitumor effects of PA has been supported by studies demonstrating that the prenylation-inhibitory activity of different PA derivatives correlated with their cytostatic activity (5). In particular, PA inhibition of $p21^{ras}$ isoprenylation has been linked to its ability to induce phenotypic reversion of ras-transformed cells (25, 26). Farnesyl transferase inhibitors, which also block ras isoprenylation, have demonstrated similar antitumor effects (27, 28). What this posttranslational mechanism cannot explain, however, is the ability of the PA derivatives to alter transcription of certain genes such as RARβ, which is known to be involved in cell growth and differentiation (6, 10), in a protein synthesis-independent fashion.

In this study, we have addressed the possibility that at least part of the differentiation-inducing activity of PA on human nb cells is mediated by its ability to act as a ligand for PPARγ. In the studies by Samid *et al.* (12), it was demonstrated that PA and it analogues can bind directly to PPARγ with K_d values in the range of 0.2–6.0 mM, in contrast to the reported K_d values of 15d-PGJ2 and GW1929 of about 20 μM (29) and <40 nM (30), respectively. Furthermore, the low affinity of PA-PPARγ binding corresponded to the potency of the PA derivatives to act as inhibitors of the proliferation of breast and glioma cancer cells (12). Thus, PA compounds can be considered ligands of PPARγ, but they bind with low affinity. Our results have shown that in human nb cells, which express abundant levels of PPARγ (13), PPARγ antagonists GW9662 and GW0072 can suppress the differentiation-inducing activity of PA. GW9662, an irreversible antagonist of PPARγ, was reported to inhibit the induction of CD36 by interleukin 4 and to block the action of BRL49653, a PPARγ agonist (31). GW0072 was identified as a high affinity PPARγ ligand that was a weak partial agonist of PPARγ transactivation. However, this compound has been shown to be a potent antagonist of adipocyte differentiation (32). When added at micromolar concentrations, GW9662 and GW0072 inhibited the antiproliferative activity, neurite outgrowth, increased AChE activity, and reduction of N-myc expression in LA-N-5 cells caused by millimolar concentrations of PA. On the other hand, differentiation effects induced by RA were not affected by cotreatment with the PPARγ antagonists, confirming that the specificity of their activity is mediated through PPARγ. These findings are consistent with the contention that low affinity binding of PA to PPARγ is at least partially responsible for the differentiation-inducing activity of PA in nb cells. The observation that high affinity PPARγ agonists at lower (micromolar) concentrations produce effects similar to those of PA provides more evidence to support this contention and indicates that functional PPARγ binding is sufficient for inducing certain aspects of nb cell differentiation

Up-regulation of RARβ has been shown to be an important early event in the RA-induced differentiation response of many types of cancer cells, including nb (33, 34). We have shown previously that PA can also induce RARβ mRNA expression in human nb cells and that this effect is regulated at the level of transcription and mediated through RAREβ (10). Our data have now demonstrated that the PPARγ ligands 15d-PGJ2 and GW1929, which is a high affinity synthetic PPARγ ligand (35), also up-regulate RARβ expression. Furthermore, we have shown that RARβ induction by PA, as well as that by 15d-PGJ2, was blocked by the PPARγ antagonists.

To interpret our findings of increased ΔSVβRE-CAT activation by PA and GW1929, it should be remembered that this construct does *not* contain a significant portion of the RARβ gene promoter (36) but only contains the small regulatory element RAREβ (a tandem repeat of the AGGTCA half-site) that has been shown to directly bind and be activated by all of the RA nuclear receptors (α, β, and γ), either in the form of homodimers or complexed with RXR as a heterodimer (37). Thus, specific activation of ΔSVβRE-CAT by PPARγ ligands must be mediated directly through RAREβ. PPAR/RXR heterodimers have been shown to bind weakly to the RAREβ in DNA gel shift assays (38), and, along with the present data, this observation suggests the occurrence of functionally significant cross-binding of this response element with "activated" PPARγ/RXR heterodimers. This type of cross-talk has now been confirmed between other members of the steroid hormone receptor superfamily. For example, glucocorticoid and progesterone receptors share common binding sequences in the promoters of the uteroglobin and metallothionein genes (39, 40), RARs can bind and activate genes through certain thyroid hormone response elements (41), and a novel class of common *cis*-acting response elements for retinoid, vitamin D, and estrogen receptors has been described previously (42). Future studies will test the hypothesis that liganded PPARγ complexed with other cellular cofactors (*e.g.,* RXR) can directly bind and activate RAREβ.

In conclusion, we have demonstrated that high affinity PPARγ ligands can mimic the differentiation-inducing activity of PA on human nb cells using a variety of functional and molecular criteria. The contention that signaling through PPARγ plays a direct role in PA-induced differentiation was supported by the ability of specific PPARγ antagonists to inhibit this activity. Recently, RA has been shown to be an effective compound for prolonging the remission time, increasing the survival, and reducing the recurrence of nb when used in the setting of minimal residual disease (43). Our demonstration of an apparent cross-talk between the PPARγ and RA signaling pathways that results in up-regulation of RARβ suggests a novel approach using PPARγ ligands to enhance the retinoid sensitivity of nb cells or to overcome the retinoid resistance that is sometimes seen in the clinic.

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