15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced apoptosis does not require PPARγ in breast cancer cells

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Abstract **Naturally occurring derivatives of arachidonic acid are potent agonists for the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ) and block cancer cell proliferation through the induction of apoptosis. We have previously reported that induction of apoptosis using cyclopentenone prostaglandins of the J series, including 15deoxy$\Delta^{12,14}$PGJ$_2$ (15dPGJ$_2$), is associated with a high degree of PPAR-response element (PPRE) activity and requires early de novo gene expression in breast cancer cells. In the current study, we used pharmacologic and genetic approaches to test the hypothesis that PPARγ is required for 15dPGJ$_2$-induced apoptosis. The PPARγ agonists 15dPGJ$_2$, troglitazone (TGZ), and GW7845, a synthetic and highly selective tyrosine-based PPARγ agonist, all increased transcriptional activity of PPARγ, and expression of CD36, a PPARγ-dependent gene. Transcriptional activity and CD36 expression was reduced by GW9662, a selective and irreversible PPARγ antagonist, but GW9662 did not block apoptosis induced by 15dPGJ$_2$. Moreover, dominant negative expression of PPARγ blocked PPRE transcriptional activity, but did not block 15dPGJ$_2$-induced apoptosis. These studies show that while 15dPGJ$_2$ activates PPRE-mediated transcription, PPARγ is not required for 15dPGJ$_2$-induced apoptosis in breast cancer cells. Other likely mechanisms through which cyclopentenone prostaglandins induce apoptosis of cancer cells are discussed.**—Clay, C. E., A. Monjazeb, J. Thorburn, F. H. Chilton, and K. P. High. **15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$-induced apoptosis does not require PPARγ in breast cancer cells.** ***J. Lipid Res.*** **2002.** 43: **1818–1828.**

Supplementary key words cyclopentenone prostaglandins • arachidonic acid metabolism • peroxisome proliferator-activated receptor γ

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand activated transcription factor that induces expression of PPAR-response element (PPRE) containing genes critical to diabetes, obesity, inflammation, and cancer (1). PPARγ is activated by a diverse array of synthetic compounds including thiazolidinediones (TZDs), triterpenoids and tyrosine-based compounds, and naturally occurring lipid compounds including derivatives of fatty acid metabolism and oxidized fractions of LDL. The tyrosine based PPARγ agonists (GW7845 and GW1929) induce neuroblastoma differentiation (2), inhibit mammary carcinogenesis (3), reverse the diabetic phenotype in mouse models (4), and block atherosclerosis (5) in part by inhibiting vascular smooth muscle cell proliferation and neointima formation (6). The synthetic triterpinoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) induces differentiation and apoptosis in human osteosarcoma and myeloid leukemia cells (7, 8). The TZDs, troglitazone (TGZ, Rezulin®), rosiglitazone (Rosi, BRL49653, Avandia®), and pioglitazone (Pio, Actos®) are effective anti-diabetes drugs and reduce the growth of several cancer cell types (9). However, their clinical application as chemotherapeutic drugs has been discouraging to date, due to unpredictable clinical performance and lack of efficacy in human trials (10–12). However, very recent data suggest that some properties of these drugs may not be related to their capacity to activate PPARγ (13), suggesting there may be opportunities to enhance the anti-cancer activity of these compounds by better understanding their mechanism of action while maintaining their relative safety versus conventional chemotherapeutic agents.

Of the naturally occurring PPARγ agonists, the cyclopentenone prostaglandin, 15deoxy$\Delta^{12,14}$PGJ$_2$ (15dPGJ$_2$), is among the most potent for both transactivating PPARγ

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Abbreviations: CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; DN, dominant negative PPARγ; 15dPGJ$_2$, 15deoxy$\Delta^{12,14}$PGJ$_2$; PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, PPAR-response element; ROS, reactive oxygen species; TGZ, troglitazone; TZD, thiazolidinedione; WT, wild type PPARγ.

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(14, 15) and inducing apoptosis (16). However, controversy exists as to the molecular mechanism(s) of $15dPGJ_2$ activity. Clearly, $15dPGJ_2$ is an effective PPARγ agonist, but it also exerts effects that are independent of PPARγ (17). Two electrophilic carbonyls within the ring structure of $15dPGJ_2$ can form covalent Michael adducts with cysteine containing proteins. In this way, $15dPGJ_2$ negatively regulates NFκB activity by covalent inhibition of the IKK, IκBα, and the DNA binding domain of NFκB (18–21). Additionally, the immediate precursor to $15dPGJ_2$ biosynthesis, $\Delta 12PGJ_2$, inhibits ubiquitin isopeptidase activity of the proteosome pathway (22). $15dPGJ_2$ may also induce the formation of reactive oxygen species that lead to cell death (23, 24).

In addition to its diverse mechanisms of action, the concentration of $15dPGJ_2$ dictates opposing biologic outcomes in several types of cancer cells and cell lines (9, 25). Specifically, low concentrations of $15dPGJ_2$, increase cellular proliferation, and moderate concentrations induce cell cycle arrest and cellular differentiation, while higher concentrations induce apoptosis. However, it is clear that $15dPGJ_2$ induces apoptosis only when expression of critical gene products occurs, since inhibition by actinomycin D or cycloheximide blocks $15dPGJ_2$-induced apoptosis (26). Thus, transcriptional activation is required for $15dPGJ_2$-induced apoptosis and it is reasonable to suspect PPRE containing genes are the most likely mediators. It is clear from knockout studies that PPARγ is required for differentiation of adipose tissue (27, 28) and perhaps differentiation of cancer cells. However, in the current study, we show that while PPARγ does account for the PPRE-mediated transcriptional activation of $15dPGJ_2$, it does not mediate $15dPGJ_2$-induced apoptosis in breast cancer cells. Other plausible mechanisms of $15dPGJ_2$-induced apoptosis are discussed.

MATERIALS AND METHODS

Cells and reagents

MDA-MB-231 breast cancer epithelial cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine (Life Technologies, Rockville, MD). $15dPGJ_2$ was purchased from Cayman Chemical (Ann Arbor, MI). $15dPGJ_2$ is rapidly inter-converted to a mixture of at least five active isomers (29). Troglitazone was a generous gift from Parke Davis Warner Lambert (Plainsboro, NJ) and GW7647 (30), GW7845 (31), GW0742 (32), and GW9662 (33) were generous gifts from Dr. Timothy M. Willson and Dr. Peter J. Brown (Glaxo Smith Kline, Research Triangle Park, NC). Each compound was used at a concentration where it is selective for the indicated receptor subtype. All tissue culture experiments were done in humid 5% CO_2 atmosphere at 37°C.

Breast cancer cellular responses to $15deoxy\Delta^{12,14}PGJ_2$

$15dPGJ_2$ was submitted to the Developmental Therapeutics Program (National Institutes of Health, National Cancer Institute, Bethesda, MD, http://dtp.nci.nih.gov) for in vitro screening against 60 human tumor cell lines (34–36). Briefly, the human tumor cell lines were grown at 37°C, 5% CO_2 and 100% relative humidity in 100 μl of RPMI 1640 medium containing 5% FBS and 2 mM L-glutamine in 96-well microtiter plates at densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition. One hundred microliters aliquots of $15dPGJ_2$ in growth media was added to the appropriate microtiter wells already containing 100 μl of media, resulting in the indicated final drug concentrations. Plates were incubated for 48 h at 37°C, 5% CO_2 and 100% relative humidity. Adherent cells were fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 10% TCA) and suspension cells were fixed by gently adding 50 μl of 80% TCA (final concentration, 16% TCA). Cells were then incubated for 60 min at 4°C, washed five times with tap water, and air-dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations.

Relative levels of PPARγ mRNA expression in breast cancer cells

Relative PPARγ mRNA in breast cancer cell lines was determined by the Developmental Therapeutics Program (National Institutes of Health, National Cancer Institute, Bethesda, MD, http://dtp.nci.nih.gov). Briefly, mRNA was isolated from logarithmically growing cells and labeled cDNA was prepared by reverse transcription of test cell mRNA in the presence of Cy5-dUTP. Reference probes were made by pooling equal amounts of mRNA from HL-60, K562, NCI-H226, COLO205, SNB-19, LOX-IMVI, OVCAR-3, OVCAR-4, CAKI-1, PC-3, MCF7, and Hs578T cell lines. Labeled cDNA was prepared from the pooled reference cell mRNA by reverse transcription in the presence of Cy3-dUTP. Test and reference probes were combined, denatured, and hybridized overnight to Synteni microarrays (Incyte Genomics, Fremont, CA) containing cDNA from 9,703 human clones, including PPARγ. Arrays were scanned using a laser-scanning microscope, the ScanAlyze program was used to analyze the microarray images and relative RNA level were determined by log (test cell line mRNA levels/reference pool RNA level).

Cell proliferation assays

1×10^4 MDA-MB-231 cells were seeded in 1 ml of medium in each well of a 24-well plate. After 24 h, the indicated concentration of drug was added. After 96 h, medium was removed, cells were washed with PBS, and stained with 0.16% w/v methylene blue in methanol. After 10 min, cells were washed with PBS and digital images were obtained.

Transcriptional activity assays

2×10^5 MDA-MB-231 cells were seeded in 2 ml of media in a 35 mm dish. After 24 h, cells were transfected with 1.0 μg of a 3× PPRE-tk-luciferase vector, which has three copies of PPRE upstream of the TK promoter/luciferase fusion gene (37), a kind gift from Dr. Bruce Spiegelman, and 1 μg of β-galactosidase as an internal control using Fugene 6 (Roche, Indianapolis, IN). After 24 h, cells were incubated for 1 h with or without the PPARγ antagonist, GW9662 (10 μM), and the indicated PPARγ agonists were provided, $15dPGJ_2$ (10 μM), TGZ (100 μM), or GW7845 (10 μM). After 24 h, cells were scraped, transferred to microfuge

tubes, and luciferase activity was measured using a Luciferase Assay Kit (Promega, Madison, WI) according to manufacturer's protocol. Light intensity was measured using a Turner 20E luminometer (Turner Designs, Sunnyvale, CA). All experiments were done in triplicate. Luciferase activity was standardized to β-galactosidase activity and reported as mean fold increase over control with standard deviation.

Apoptosis assays

5×10^5 MDA-MB-231 cells were seeded in 3 ml of media in 60 mm dishes. After 24 h, cells were incubated for 1 h with or without the PPARγ antagonist, GW9662 (10 μM) and the indicated concentration of PPARγ agonist was provided, $15dPGJ_2$ (10 μM), TGZ (100 μM), or GW7845 (10 μM). After 24 h, cells were collected by trypsinization, pelleted, and the percentage of cells undergoing apoptosis was determined by flow cytometry using a TACS Annexin V-FITC Kit (Trevegin, Gaithersburg, MD) according to manufactuer's protocol. Fluorescent intensity was measured using a Coulter Epics XL-MCL flow cytometer (Hileah, FL).

Microinjection

1×10^4 MDA-MB-231 cells were seeded in 35 mm dishes. After 24 h, cells were injected as described previously (38) with 0.25 μg/μl of yellow fluorescent protein and 0.25 μg/μl of either the wild type form of PPARγ (WT) or the dominant negative form of PPARγ (DN), a generous gift of Dr. VKK Chatterjee (39), using a Zeiss Aviovert microscope equipped with an Eppendorf FemtoJet and Injectman (Brinkman Instruments, Westbury, NY). After 24 h, the number of live cells was determined by counting fluorescent cells, and the indicated PPARγ agonists were provided, $15dPGJ_2$ (10 μM), TGZ (100 μM), or GW7845 (10 μM). Twenty-four hours and 48 h after the addition of PPARγ agonist, the number of surviving cells was determined by counting and digital images were obtained using a Hamamatsu C4742-95 digital camera (Bridgewater, NJ) and OpenLab software (Improvision, Warwick, UK).

Immunofluorescence

Cells were transfected with 1.0 μg of FLAG-tagged WT or DN using Fugene 6 (Roche, Indianapolis, IN) and after 24 h, the expression and localization of PPARγ was determined. Cells were washed with PBS, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature (RT), washed with PBS, permeablized with 0.3% Triton X-100 in PBS for 10 min at RT and washed in PBS-0.1% Tween. Cells were blocked with 10% goat serum in PBS-0.1% Tween for 10 min at RT and incubated with M2-FLAG primary antibody (25 μg/ml) (Sigma, St. Louis, MO) for 1 h at 37°C in humid atmosphere. Cells were washed with PBS-0.1% Tween and incubated with rhodamine red-X-conjugated anti-mouse IgG secondary antibody (1:100, v/v) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37°C in humid atmosphere. Cells were washed with PBS-0.1% Tween and digital images were obtained as described above.

RESULTS

PPARγ expression does not correlate with $15dPGJ_2$-induced apoptosis

The proliferation of breast cancer cell lines exposed to various concentrations of $15dPGJ_2$ for 48 h was determined and these same breast cancer cell lines were screened for relative PPARγ expression (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) (**Fig. 1**). All breast cancer cell lines tested were sensitive to $15dPGJ_2$-induced apoptosis (Fig. 1A) independent of PPARγ mRNA expression level (Fig. 1B). These early data suggested that PPARγ might not play a pivitol role in $15dPGJ_2$-induced apoptosis.

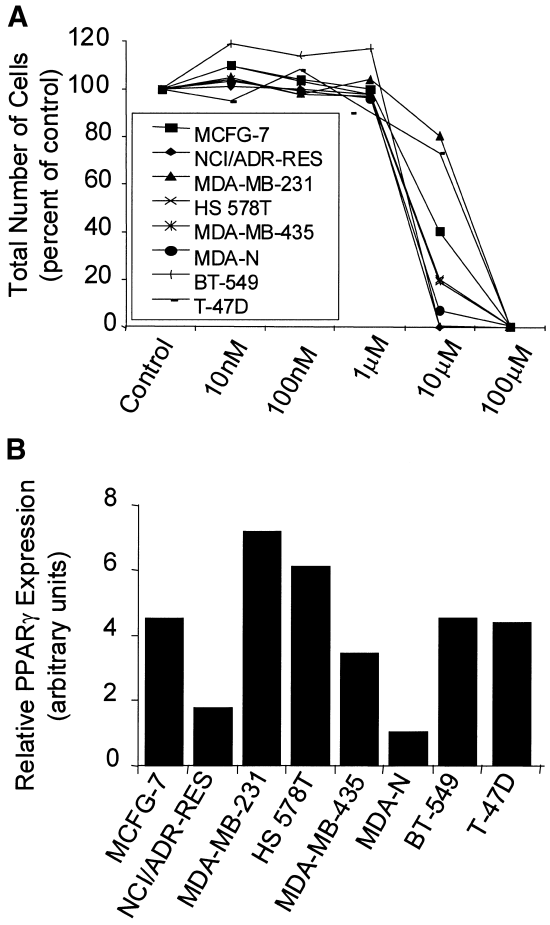


Fig. 1. Peroxisome proliferator-activated receptor gamma (PPARγ) mRNA expression does not correlate with $15deoxy\Delta^{12,14}PGJ_2$ ($15dPGJ_2$)-induced inhibition of cellular proliferation. A: The indicated breast cancer cell lines were incubated with the indicated concentration of $15dPGJ_2$ for 48 h and cell viability was determined using a sulforhodamine B (SRB) assay as described in Materials and Methods. B: Relative RNA level of PPARγ in logarithmically growing breast cancer cells was determined using a microarray experiment as described in Materials and Methods. The cell line MDA-N was used as reference.

Pharmacologic PPARγ antagonism does not block $15dPGJ_2$-induced apoptosis

The structure of the PPARγ agonists and antagonists tested, as well as the structure of WT and DN constructs used is shown in **Fig. 2**. We tested three different classes of PPARγ agonists, $15dPGJ_2$, TGZ, and GW7845, and an irreversible PPARγ antagonist, GW9662, for their capacity to alter cellular proliferation and induce apoptosis of MDA-MB-231 breast cancer epithelial cells. We have previously reported that $15dPGJ_2$ and TGZ induce rapid and irreversible apoptosis in this cell line (16). Here we show that $15dPGJ_2$ and TGZ block cellular proliferation of MDA-MB-231 cells, but the selective PPARα agonist, GW7647,

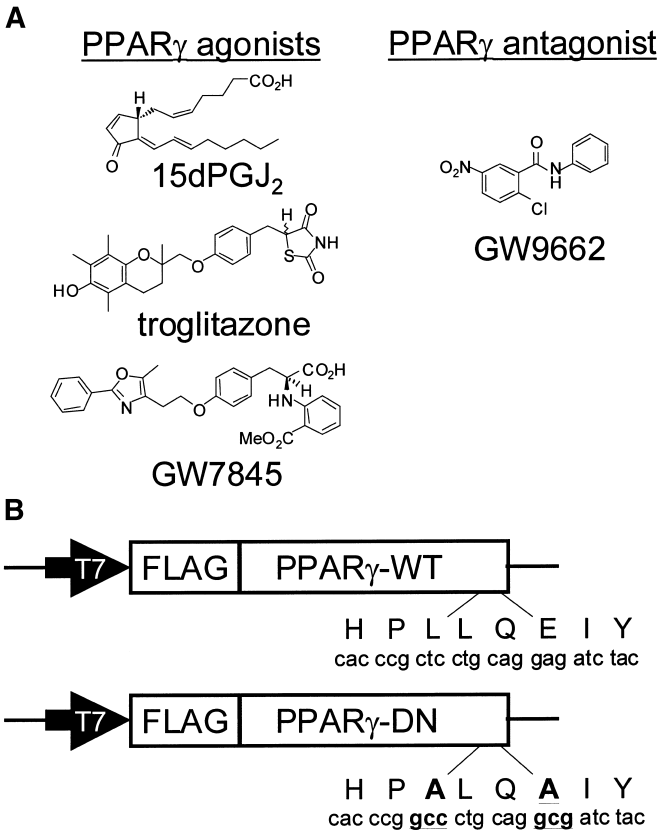


Fig. 2. Structure of PPARγ agonists, an antagonist, and wild type and dominant negative PPARγ constructs. A: The structures of the PPARγ agonists 15dPGJ$_2$, troglitazone (TGZ), and GW7845 and the structure of the irreversible PPARγ antagonist GW9662 are shown. B: The functional structures of the wild type PPARγ (WT) and the dominant negative PPARγ (DN) constructs are shown. The L486A and E471A mutations are underlined.

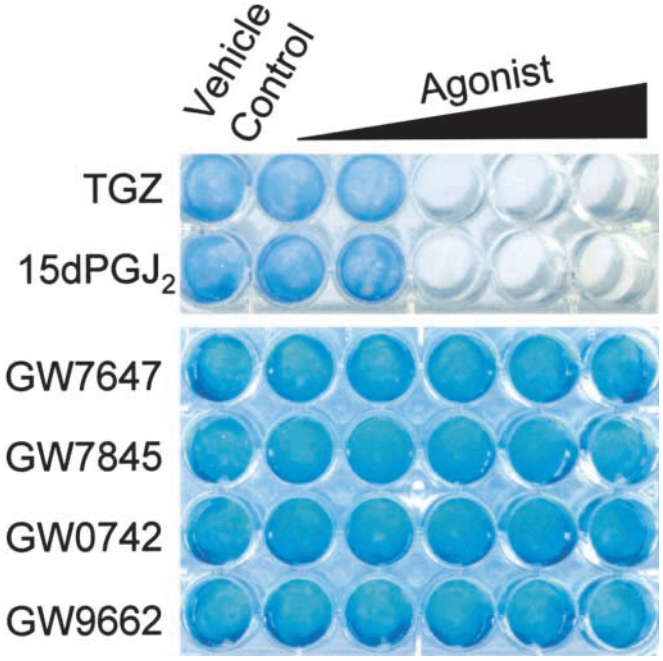
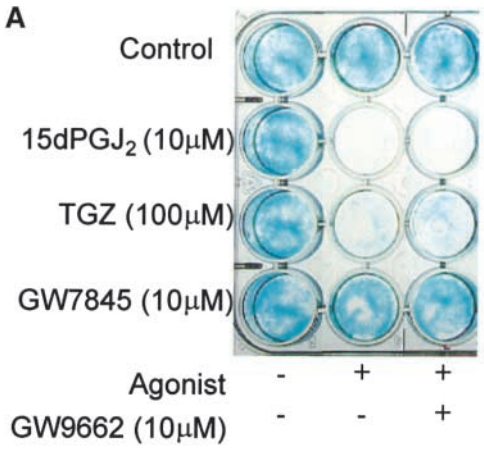


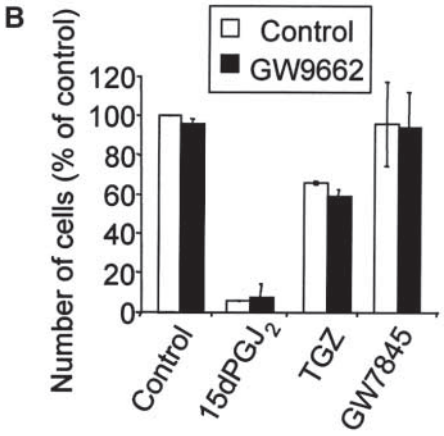
Fig. 3. Effect of PPAR ligands on cellular proliferation. MDA-MB-231 cells were in grown in the presence of various concentrations of the indicated PPAR agonists and cell proliferation was determined by methylene blue staining. PPAR agonist concentration ranges are TGZ (PPARγ agonist), 10, 20, 50, 100, 200 μM; 15dPGJ$_2$ (PPARγ agonist), 0.5, 1, 2.5, 5, 10 μM; GW7647 (PPARα agonist), GW7845 (PPARγ agonist), GW0742 (PPARβ/δ agonist), and GW9662 (PPARγ antagonist), 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M.

the selective PPARγ agonist, GW7845, the selective PPARβ/δ agonist, GW0742, and the selective and irreversible PPARγ antagonist, GW9662, did not alter cellular proliferation compared to control cells in a clonogenic assay (**Fig. 3**). Consistent with this finding, studies by Gupta and colleagues have shown that GW7845 and the PPARγ selective thiazolidinedione rosiglitazone (Rosi, BRL49653, Avandia®) slowed, but did not completely stop proliferation and did not induce apoptosis of colon cancer cells (40). Pretreatment of MDA-MB-231 cells with the irreversible PPARγ antagonist, GW9662, did not block 15dPGJ$_2$ or TGZ-induced inhibition of cellular proliferation (**Fig. 4A, B**). Moreover, 15dPGJ$_2$-induced apoptosis was not significantly reduced ($P = 0.07$) and GW7845 did not induce apoptosis in this cell line. In contrast, TGZ-induced apoptosis was reduced by nearly 50% by GW9662 ($P = 0.04$) (Fig. 4C). Together these data show that selective synthetic activators of different PPARs do not block cellular proliferation and that the anti-cancer effects of less selective PPAR ligands may be independent of PPARs. Furthermore, these data show that pharmacologic inhibition of PPARγ does not rescue cells from apoptosis induced by 15dPGJ$_2$ and that TGZ and 15dPGJ$_2$ may have different mechanisms through which they induce apoptosis, some independent of PPARγ and some potentially involving PPARγ.

15dPGJ$_2$-induced PPRE-mediated gene transcription is incompletely blocked by pharmacologic antagonism

PPARγ antagonism using GW9662 had no affect on blocking cellular proliferation of MDA-MB-231 cells, but was able to markedly reduce PPRE-mediated gene transcription. MDA-MB-231 cells were transiently co-transfected with a PPRE driven luciferase construct and a β-galactosidase construct as an internal control. TGZ and GW7845-induced transcriptional activity was completely blocked by GW9662, but 15dPGJ$_2$-induced transcriptional activity was only blocked by half and was still higher than levels achieved by either TGZ or GW7845 in the absence of inhibitor (**Fig. 5A**). However, expression of DN by transient transfection reduced PPRE-mediated gene transcription induced by all three agonists to baseline values (Fig. 5B). While GW9662 is a selective and irreversible inhibitor of PPARγ (33, 41), over-expression of DN more effectively blocked PPRE-mediated gene transcription in the results presented, perhaps by competing with other PPARs or related transcription factors, for binding to PPREs and/or enhanced recruitment of transcriptional co-repressors (39). These findings are consistent with our earlier data that show 15dPGJ$_2$ is the most potent activator of PPRE-mediated gene transcription (16, 25, 26). Surprisingly, the expression of CD36, a reported PPARγ-dependent gene





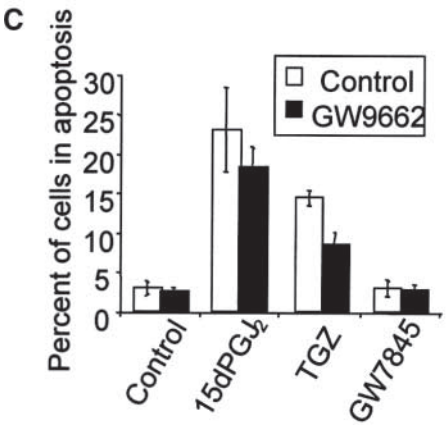


Fig. 4. Pharmacologic antagonism of PPARγ ligands has different effects on $15dPGJ_2$- versus TGZ-induced apoptosis. A: MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPARγ agonist with or without the PPARγ antagonist GW9662. Cellular proliferation was determined by methylene blue staining. Data are representative of three separate experiments. B: 1×10^4 MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPARγ agonists at the same concentrations as in 4A with or without GW9662. Total cell number was determined after 96 h using a hemacytometer. Data are expressed as the mean ± SD of three separate experiments. C: 5×10^5 MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPARγ agonists with or without GW9662 for 36 h and the number of cells undergoing apoptosis was determined by flow cytometry using an annexin V-FTIC kit. Data are expressed as the mean ± SD of three separate experiments. *Apoptosis was significantly reduced by GW9662, $P < 0.05$.

product (17), was inversely correlated with PPARγ activation (Fig. 5C). $15dPGJ_2$ induced only 1.5-fold increase in CD36 expression whereas TGZ and GW7845 increased CD36 expression by nearly 3-fold and the expression of CD36 was blocked by GW9662, no matter which agonist was used. These data suggest that all three PPARγ agonists tested enhance PPRE-mediated gene transcription, but $15dPGJ_2$-mediated PPRE activity cannot be fully blocked by pharmacologic PPARγ antagonism and the protein levels of PPRE-containing genes do not always correlate with luciferase reporter measures of gene expression.

Dominant negative PPARγ localizes to the nucleus but does not rescue cells from $15dPGJ_2$-induced apoptosis

We have shown here that the irreversible antagonist GW9662 reduces transcriptional activation of PPARγ and expression of CD36, but does not rescue cells from $15dPGJ_2$-induced apoptosis. However, since residual PPRE activity could be measured, we could not rule out PPARγ-mediated transcription as a mediator of $15dPGJ_2$-induced apoptosis. Furthermore, since PPARγ must bind DNA for transcriptional activation of PPRE-containing genes, we identified the localization of PPARγ expression in MDA-

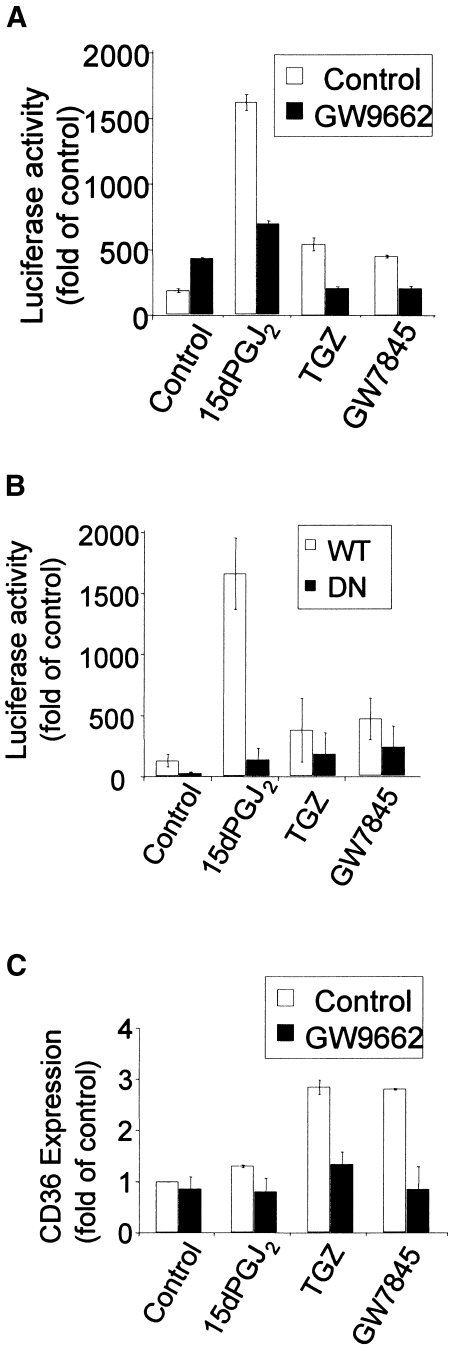


Fig. 5. PPARγ antagonism blocks PPAR-response element (PPRE)-mediated gene transcription and expression of CD36. A: MDA-MB-231 cells were transiently transfected with a PPRE-driven luciferase reporter construct and the degree of PPRE-mediated gene transcription was determined after 24 h exposure to 15dPGJ$_2$ (10 μM), TGZ (100 μM), and GW7845 (10 μM) with or without GW9662 (10 μM). Data are the mean ± SD of three separate experiments. *Luciferase activity was significantly higher versus TGZ or GW7845 without GW9662, $P < 0.05$. B: WT or DN was co-transfected with a PPRE-driven luciferase reporter construct and the degree of PPRE-mediated gene transcription was determined after 24-h exposure to 15dPGJ$_2$ (10 μM), TGZ (100 μM), or GW7845 (10 μM). Data are the mean ± SD of three separate experiments. C: MDA-MB-231 cells were treated for 1 h with or with out GW9662, then grown in the presence of the indicated PPARγ agonists for 12 h and the expression of CD36 was determined by flow cytometry. PPARγ agonist and antagonist concentrations are 15dPGJ$_2$, 1 μM; TGZ, 25 μM; GW7845, .5 μM; GW9662, 1 μM. Data are the mean ± SD of three separate experiments.

MB-231 cells. WT or DN was co-injected by single cell microinjection with YFP and visualized by immunofluorescence. Both WT and DN localized to the nucleus of MDA-MB-231 cells suggesting that WT and DN are functionally active in these cells (**Fig. 6A**). However, consistent with the data from clonogenic and apoptosis assays using the selective antagonist GW9662, dominant negative expression of PPARγ by single-cell microinjection does not rescue cells from 15dPGJ$_2$ or TGZ-induced apoptosis. MDA-MB-231 cells expressing either WT or DN showed morphologic characteristic of apoptosis 24 and 48 h after cells were exposed to 15dPGJ$_2$ or TGZ (Fig. 6B) and the total number of surviving 15dPGJ$_2$ or TGZ-treated cells was decreased at both 24 h and 48 h (Fig. 6C). However, the selective PPARγ agonist, GW7845, did not induce apoptosis in these cells. Taken together, these data suggest that 15dPGJ$_2$-induced apoptosis is not mediated by PPARγ in breast cancer cells.

DISCUSSION

PPARγ dependent gene expression and PPARγ agonist-induced apoptosis using fatty acid derivatives, thiazolidinediones and tyrosine-based agonists has been reported in several cancer cell types (42–52). This is the first report, however, that specifically addresses the role of PPARγ in apoptosis of breast cancer cells. Using three classes of PPARγ agonists and an irreversible antagonist, we show that selective activation of PPARγ (via GW7845) does activate PPRE-driven gene transcription but does not induce apoptosis. Furthermore, inhibition of PPARγ (via GW9662 or a DN) in the presence of 15dPGJ$_2$ or TGZ reduces PPRE-mediated transcription but does not rescue cells from apoptosis. These results show that 15dPGJ$_2$ and TGZ have PPARγ-independent effects in breast cancer cells and suggest that PPARγ does not mediate 15dPGJ2-induced apoptosis. Moreover, these data highlight the need for both a better understanding of cyclopentenone-induced apoptosis and the role of PPARγ in cancer.

Most PPARγ agonists, including 15dPGJ$_2$, 15(s)-HETE, TGZ, and BRL49653, have PPARγ-dependent and PPARγ-independent effects that result in variable biologic effects (9, 25). In mouse models of colon cancer, TGZ was reported to increase aberrant crypt foci and colon polyp number in one model (53, 54), but induce differentiation and a reversal of the malignant phenotype in another model (55). In humans, TGZ was reported to increase, rather than decrease, the size of liposarcomas (12). These disparate results may be due, in part, to PPARγ-dependent and -independent pathways. We have previously shown that 15dPGJ$_2$-blocks the progression of breast tumors in a mouse model (16), and that 15dPGJ$_2$-induced apoptosis requires early de novo gene transcription (26). However, here we report that PPARγ is not required for, and thus not the mediator of, 15dPGJ$_2$-induced apoptosis in breast cancer cells. Alternate proposed mechanisms are represented in **Fig. 7**. One possible mechanism is inhibition of NFκB-mediated survival pathways. The exocyclic electoro-

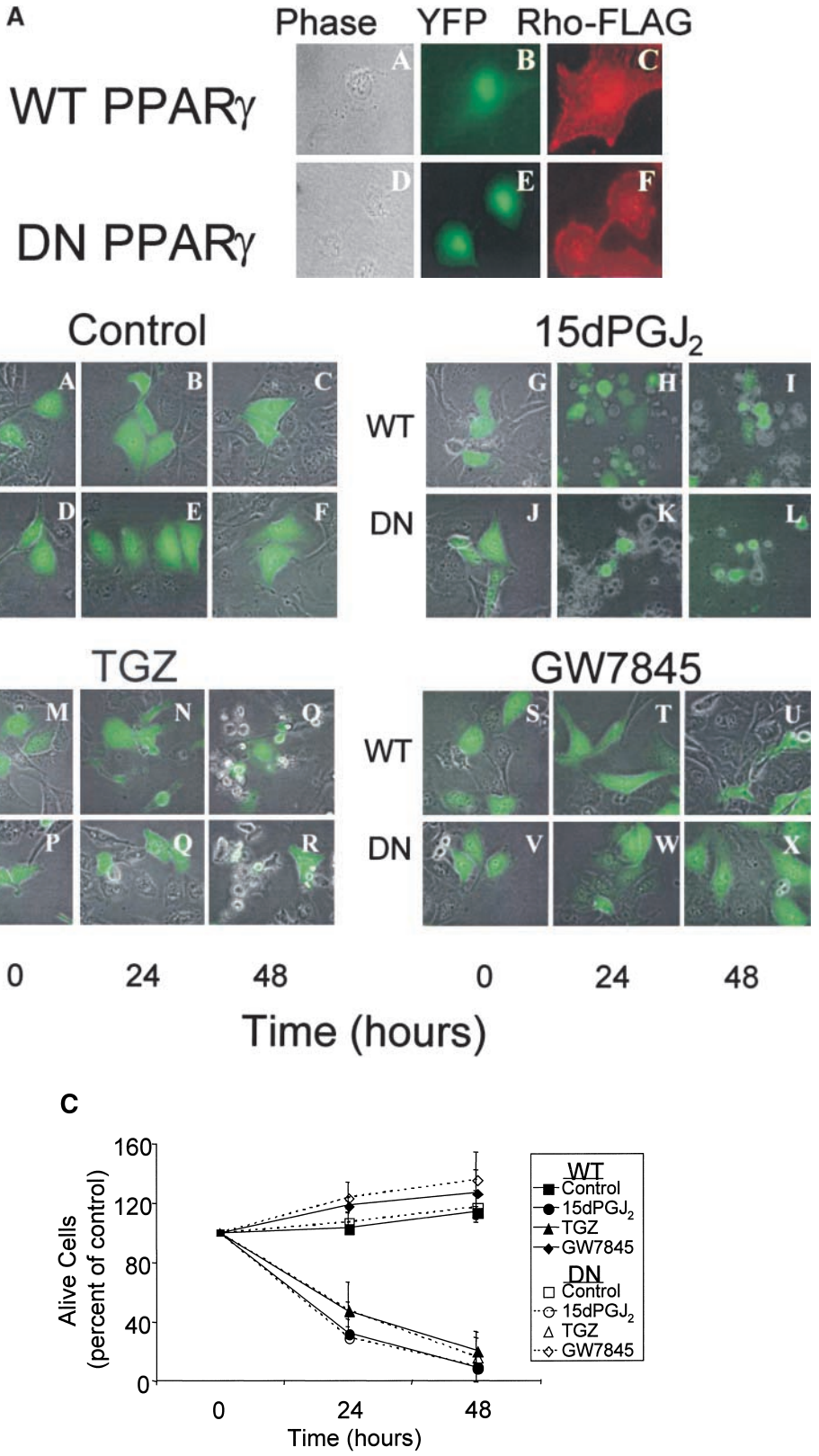


Fig. 6. DN localizes to the nucleus but fails to rescue cells from $15dPGJ_2$ and TGZ-induced apoptosis. A: WT or DN and YFP were co-injected into MDA-MB-231 cells and the expression and localization of PPARγ was determined by immunofluorescent staining. Images are representative of three separate experiments. B: WT or DN and YFP were co-injected into MDA-MB-231 cells. Digital images of the morphology of successfully injected cells were obtained 12 h after injection at which time cells were provided the indicated PPARγ agonist (T = 0). Digital images were obtained 24 and 48 h after addition of the PPARγ agonists. Images are representative of three separate experiments. C: The number of surviving cells was determined 12 h after injection (T = 0) and 24 and 48 h after addition of the indicated PPARγ agonists. Data are the mean ± SD of three separate experiments.

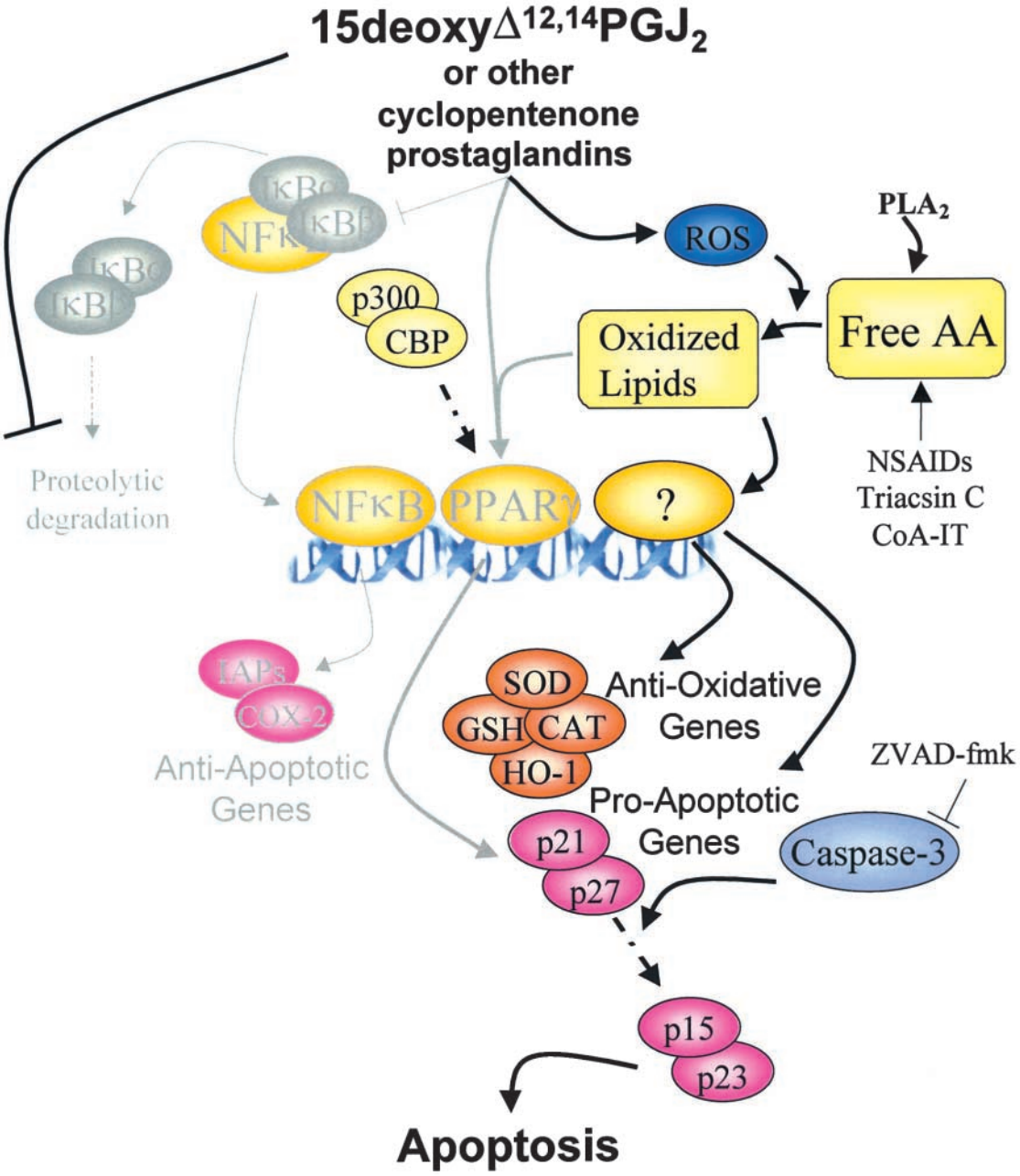


Fig. 7. Potential mechanisms for cyclopentenone prostaglandin-induced apoptosis. The exocyclic electrophilic carbonyl of J series cyclopentenone prostaglandins confers unique pro-apoptotic activity in part, perhaps, by inhibition of isopeptidase activity of the ubiquitin proteosome and covalent inactivation of NFκB-mediated survival pathways. 15dPGJ$_2$ and other cyclopentenone prostaglandins mediate transcriptional inhibition of COX-2 and induce reactive oxygen species (ROS). COX-2 inhibition may lead to increased intracellular levels of free arachidonic acid and increased intracellular oxidative stress may lead to the production of oxidized lipids and lipid modified proteins could account for some of the 15dPGJ$_2$-induced PPRE-mediated gene transcription and/or result in cell death. 15dPGJ$_2$ and other cyclopentenone prostaglandins, increase expression of glutamate-cysteine ligase, GSH reductase, superoxide dismutase, heme oxygenase-1, and catalase, which may be cyto-protective at low levels, but higher expression levels are cytotoxic. Black lines represent likely pathways for 15dPGJ$_2$-induced apoptosis. Light gray lines represent less likely pathways based on current data.

philic carbonyl of 15dPGJ$_2$ covalently inactivates IKK, IκBα, and IκBβ NFκB (18–21). However, if this were a major initiator of apoptosis, inhibition of new RNA and protein would be expected to enhance apoptosis. We found the opposite to be true (26). A second mechanism could be inhibition of the ubiquitin proteosome, which would lead to accumulation of unmodified proteins and signal cell death. The immediate precursor to 15dPGJ$_2$ synthesis, Δ12-PGJ$_2$, blocks polyubiquitin disassembly by inhibition of isopeptidase activity (22); however these events occurred at very high concentrations of Δ12-PGJ$_2$. Nonetheless, inhibition of the proteosome is a focus of novel drug design and cancer therapy (56, 57). Third, 15dPGJ$_2$ inhibits transcriptional activation of COX-2, and perhaps other

arachidonic acid metabolizing enzymes (58, 59) that may lead to increased intracellular levels of free arachidonic acid, an event know to induce apoptosis (60–62).

Finally, 15dPGJ$_2$ and other cyclopentenone prostaglandins induce reactive oxygen species (ROS), in part by the generation of superoxide anion (63), leading to the production of oxidized lipids and lipid modified proteins resulting in cell death (23, 24). ROS could oxidize LDL, a potent anti-inflammatory PPARγ agonist (64, 65) or oxidize fatty acid derivatives that activate PPRE-mediated gene transcription and exert potent anti-neoplastic activity (Chilton, unpublished observations) (66). Lipid oxidation induced by cyclopentenone prostaglandins can lead to increased expression of cyto-protective enzymes in normal cells, but may represent a novel approach to the treatment of cancer cells. For example, Levonen and colleagues showed that low concentration of 15dPGJ$_2$, and other cyclopentenone prostaglandins, increase expression of glutamate-cysteine ligase as well as GSH reductase independent of PPARγ (67). However, the cyto-protection was overwhelmed by higher concentrations of 15dPGJ$_2$. Robbins and colleagues showed that γ-linolenic acid and 15dPGJ$_2$ increase the expression of catalase and other antioxidant enzymes in normal astrocytes, but not in glioma cells (68) (unpublished observations). In addition, 15dPGJ$_2$ induces expression of heme oxygenase-1 (22), which is cyto-protective at low levels while higher expression levels are cyto-toxic (69–71).

PPARγ is clearly involved in lipid metabolism and is essential for cellular differentiation (27, 28). However, the current study shows that PPARγ is not required for 15dPGJ$_2$-induced apoptosis in breast cancer cells. Furthermore, these studies show that PPARγ specific agonists, and likely the endogenous PPARγ ligand(s), may not be pro-apoptotic, but may be anti-angiogenic (58) and protective against ischemia/reperfusion injury (72), inflammatory diseases (73–75), and the complications associated with diabetes (76–78). The synthesis and activity of endogenous PPARγ ligands such as 15dPGJ$_2$ has been a matter of debate. However, the identification of increased in vivo production of 15dPGJ$_2$ in lipopolysaccharide-stimulated RAW264.7 macrophages and in macrophages of human atherosclerotic plaques (79) provides better clues to the site-specific production and biologic activity of 15dPGJ$_2$. Clearly, the pleiotropic nature of PPARγ signaling and the mechanisms by which fatty acid derivatives, particularly the cyclopentenone prostaglandins, exert anti-inflammatory and anti-neoplastic activity warrants additional investigation.

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