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# Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) ligands do not potentiate growth of human cancer cell lines

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**Ligands for peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) increase skeletal muscle fatty acid catabolism, improve insulin sensitivity, increase serum high-density lipoprotein cholesterol, elicit anti-inflammatory activity and induce terminal differentiation. Contradictory findings are also reported suggesting that PPARβ/δ ligands potentiate tumorigenesis by increasing cell proliferation, by inhibiting apoptosis through phosphorylation of Akt and by increasing cyclooxygenase-2 (COX2) and vascular endothelial growth factor (VEGF) expression. The contradictory findings could be due to differences in the model system (cancer cell line versus *in vivo*), differences in cell culture conditions (with and without serum) or differences in ligands. The present study examined the effect of two different PPARβ/δ ligands (GW0742 and GW501516) in human cancer cell lines (HT29, HCT116, LS–174T, HepG2 and HuH7) cultured in the presence or absence of serum and compared *in vitro* analysis with *in vivo* analysis. Neither PPARβ/δ ligand increased cell growth or phosphorylation of Akt and no increase in the expression of VEGF or COX2 were detected in any cancer cell line in the presence or absence of serum. Similarly, liver, colon and colon polyps from mice administered these PPARβ/δ ligands *in vivo* did not exhibit changes in these markers. Results from these studies demonstrate that serum withdrawal and/or differences in ligands do not underlie the disparity in responses reported in the literature. The quantitative nature of the present findings are inconsistent with the hypothesis that cancer cell lines respond differentially as compared with normal cells, and provide further evidence that PPARβ/δ ligands do not potentiate tumorigenesis.**

## Introduction

There is strong evidence from multiple, independent laboratories that ligands for peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) increase skeletal muscle fatty acid catabolism, improve insulin sensitivity, increase serum cholesterol, possess anti-inflammatory activity and induce terminal differentiation. These features of PPARβ/δ ligands make them attractive candidates for small-molecule therapeutics. An increase in serum high-density lipoprotein cholesterol in insulin-resistant mice was one of the first biological effects described for a PPARβ/δ ligand (1). This observation has since been repeated by a number of laboratories in rodent models, non-human primates and humans (2–5), and explains in part why at least one PPARβ/δ ligand (GW501516) is currently in phase II clinical trials (6). In addition to increasing serum high-density lipoprotein cholesterol concentration, PPARβ/δ ligands are also candidates for treating the metabolic syndrome as they can increase fatty acid catabolism in skeletal muscle, improve insulin resistance and suppress inflammation in macrophages and other cell types [reviewed in (7)]. Despite these positive characteristics of PPARβ/δ ligands, there is considerable controversy regarding the safety of this class of compounds due to contradictory reports in the literature, in particular those describing the effects of PPARβ/δ ligands in cancer models.

There are some reports suggesting that ligand activation of PPARβ/δ potentiates carcinogenesis through a number of mechanisms including increasing cell growth due to inhibition of apoptosis and/or promoting cell proliferation by increasing cyclooxygenase-2 (COX2) and/or vascular endothelial growth factor (VEGF) expression and signaling. For example, there are reports that the potent PPARβ/δ ligand GW501516 (i) leads to inhibition of serum withdrawal-induced apoptosis and increased phosphorylation of Akt in human colon cancer cell lines (8,9); (ii) stimulates proliferation of human liver, breast and prostate cancer cell lines (10,11); (iii) increases COX2 expression in human liver cancer cell lines (11,12) and human cholangiocarcinoma cell lines (13) and (iv) causes increased expression of VEGF in breast and colon cancer cell lines (10,14). Interestingly, whereas GW501516 inhibits serum withdrawal-induced apoptosis and increased phosphorylation of Akt in human colon cancer cell lines (8,14), others have reported that colon cancer cell lines are non-responsive to PPARβ/δ-stimulated cell growth in either the presence or absence of serum (10,15). Whereas these and other reports suggest that PPARβ/δ ligands potentiate tumorigenesis, there are many other findings that are inconsistent with this viewpoint. For example, (i) expression of PPARβ/δ and/or PPARβ/δ ligands are anti-inflammatory in many cell types (colon epithelium, macrophages, cardiomyocytes, immune cells, keratinocytes, myoblasts, endothelial cells and hepatocytes) (16–25); (ii) evidence from *in vitro* and *in vivo* models suggest that PPARβ/δ and its ligands function to promote differentiation in intestinal epithelium, breast and colon cancer cell lines, trophoblasts and primary keratinocytes (19,22,26–32); (iii) a number of independent reports demonstrate inhibition of cell growth by PPARβ/δ ligands in a number of cells (colonocytes, keratinocytes, cardiomyocytes, lung fibroblasts and renal and lung cancer cell lines) (19,27,32–41) and (iv) increased phosphorylation of Akt is not always observed upon specific activation of PPARβ/δ (27,28,42,43). The reason for these discrepancies in the literature is unclear. However, given the potential of PPARβ/δ ligands as therapeutic agents, it is of great interest to determine how these inconsistent findings occur. For example, it is of great interest to understand why the PPARβ/δ ligand GW501516 increases COX2 expression in a human liver cancer cell line (HepG2) (11,12) while GW501516 suppresses IL-6-induced acute phase proteins in the same human liver cancer cell line (44), inhibits hepatic inflammation *in vivo* (20) and the PPARβ/δ ligand GW0742 inhibits COX2 expression in LPS-stimulated macrophages (23).

There are a number of possible reasons to explain some of the reported differences described for PPARβ/δ ligands. Three high-affinity PPARβ/δ ligands have been described (45,46) and there are some structural differences between them (Figure 1A), which could account for these differences. Additionally, some of the reported disparities could be due to differences in model system. For example, whereas PPARβ/δ ligands can induce terminal differentiation *in vivo* and in primary cell cultures [reviewed in (47)], it is possible that cancer cell lines respond differently to PPARβ/δ ligands and increase cell growth. Lastly, inhibition of apoptosis reported to occur in cancer cell lines in response to PPARβ/δ ligands is typically dependent on serum withdrawal from the culture medium. Thus, it remains possible

**Abbreviations:** COX2, cyclooxygenase-2; PARP, poly (ADP-ribose) polymerase; PCR, polymerase chain reaction; PPARβ/δ, peroxisome proliferator-activated receptor-β/δ; VEGF, vascular endothelial growth factor.

that differences in the PPARβ/δ ligand structure, differences in between *in vivo* and cancer cell line models and/or differences observed in the presence or absence of serum account for some of the reported variability in response to treatment. The present studies evaluated the possible influence of these variables using two different PPARβ/δ ligands (GW0742 and GW501516), and comparing the effects of these ligands in human cancer cell lines in the presence or absence of serum to those observed in tissues *in vivo*.

## Materials and methods

### *Chemicals*

GW0742 was synthesized by GlaxoSmithKline (Research Triangle Park, NC). GW501516 was synthesized according to the procedures described previously by others (46,48). It was characterized using $^{1}$H-NMR (dimethyl sulfoxide-$d_6$) and mass spectrometry, and determined to be 99% pure based on high-performance liquid chromatography analysis.

### *Cell culture*

HT29, HCT116, LS-174T and HepG2 cells were obtained from ATCC in 2006. HuH7 cells were kindly provided by Dr Curtis J.Omiecinski. Cells were maintained in either Minimal Essential Medium (MEM) or McCoy's 5A medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum at 37°C and 5% $CO_2$. For proliferation assays, cells were plated on 12 well dishes at a density of 20 000–30 000 cells per well 24 h prior to determining plating efficiency with a Z1 coulter particle counter® at time 0 (Beckman Counter, Hialeah, FL). Cells were then either serum starved for 24 h or not prior to ligand treatment. After this 24 h period, cells were maintained in respective culture medium with or without serum and treated with either GW0742 or GW501516 for 24, 48 and 72 h at concentrations of 0 (dimethyl sulfoxide control), 100 nM, 1 μM or 10 μM. These concentrations of ligand were used because concentrations ranging from 100 nM to 1 μM are known to specifically activate PPARβ/δ, and similar concentrations have been used by others, allowing for comparisons between these studies. Cells were quantified every 24 h with a Z1 coulter particle counter® (Beckman Counter). Triplicate samples for each treatment were used for each time point for every treatment, and each replicate was counted three times. For quantitative western blot analyses, cells were plated at a density of ∼100 000 cells per well in six well dishes and cell number was quantified 24 h later as described above. Cells were then either serum starved for 24 h or not prior to ligand treatment. After this period, cells were maintained in respective culture medium with or without serum and treated with either GW0742 or GW501516 for 24 h at concentrations of 0 (dimethyl sulfoxide control), 100 nM, 1 μM or 10 μM. Twenty-four hours after ligand treatment, protein samples were obtained using MENG buffer with 1% NP40.

### *Quantitative western blot analysis*

Protein samples were obtained from control- and ligand-treated cells as described above. A total of 25–50 μg of protein from each sample was resolved

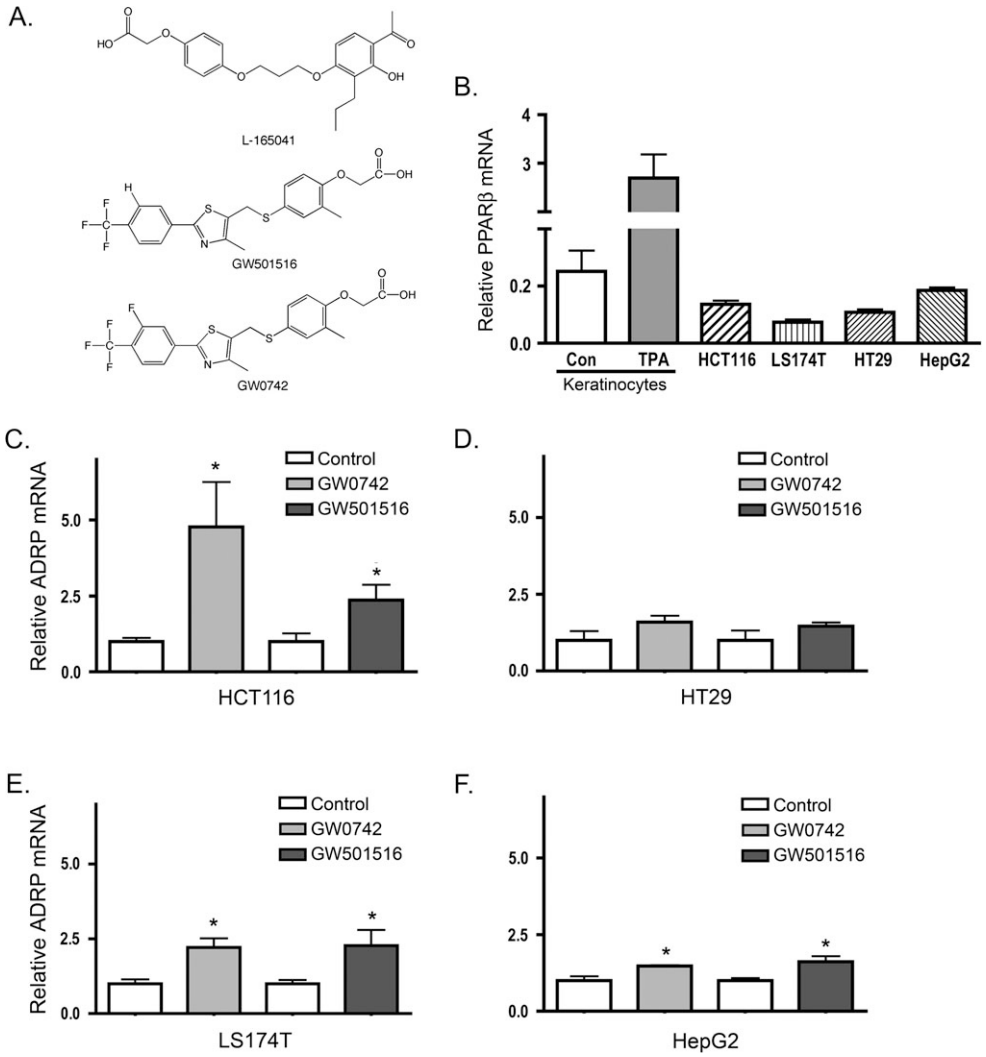


**Fig. 1.** Expression of PPARβ/δ and ligand activation of target genes in human cancer cell lines. (**A**) Structure of three high-affinity PPARβ/δ ligands. (**B**) Expression of mRNA encoding PPARβ/δ was quantified in HCT116, HT29, LS174T and HepG2 cells by real-time PCR. For comparison, expression of mRNA encoding PPARβ/δ was quantified in control and phorbol ester (tetradecanoylphorbol-13 acetate)-treated mouse primary keratinocytes. Expression of mRNA encoding the known PPARβ/δ target gene adipocyte differentiation-related protein was quantified in (**C**) HCT116, (**D**) HT29, (**E**) LS174T and (**F**) HepG2 cells by real-time PCR. *, Significantly different from control, $P \leq 0.05$.

using sodium dodecyl sulfate–polyacrylamide gel. The samples were transferred onto a polyvinylidene fluoride membrane using an electroblotting method. After blocking in 5% milk in Tris Buffered Saline Tween 20 (TBST), the membrane was incubated overnight at 4°C with primary antibody followed by incubation with a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive proteins were detected after incubation in $^{125}$I-labeled streptavidin (Amersham Biosciences, Piscataway, NJ) using phosphorimaging analysis. Hybridization signals for specific proteins of interest were normalized to the hybridization signal of the housekeeping protein, lactate dehydrogenase. Independent triplicate samples were used for analysis of each treatment group. The following primary antibodies were used: anti-VEGF and anti-COX2 (Santa Cruz, Santa Cruz, CA), anti-phospho-Akt (S473; Cell Signaling Technology, Beverly, MA), anti-Akt (BD Transduction Laboratories, San Jose, CA), anti-AngPTL4 (Zymed Laboratories, South San Francisco, CA) and anti-lactate dehydrogenase (Rockland, Gilbertsville, PA).

*RNA analysis*

Total RNA was isolated from cells using Trizol reagent and the manufacturer's recommended procedures. The mRNA encoding PPARβ/δ, adipocyte differentiation-related protein or glyceraldehyde-3-phosphate dehydrogenase was quantified using real-time polymerase chain reaction (PCR) analysis. The cDNA was generated using 2.5 μg total RNA with MultiScribe Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Primers were designed for real-time PCR using the Primer Express software (Applied Biosystems, Foster City, CA). Real-time PCRs were carried out using SYBR green PCR master mix (Finnzymes, Espoo, Finland) in the PTC-200 DNA Engine™ Cycler and detected using the CFD-3200 Opticon™ Detector (MJ Research, Waltham, MA). The following conditions were used for PCR: 95°C for 15 s, 94°C for 10 s, 60°C for 30 s and 72°C for 30 s, and repeated for 45 cycles. The PCR included a no template control reaction to control for contamination and/or genomic amplification. All reactions had >90% efficiency. Relative expression levels of mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase and analysed for statistical significance using one-way analysis of variance (Prism 4.0).

*Effect of GW0742 on expression of VEGF, COX2 and phospho-Akt in colon polyps*

Independent samples of colon polyps were obtained from APC$^{min}$ mice treated with vehicle or GW0742 (10 mg/kg/day) from an experiment previously performed (28). Definitive evidence of ligand activation for these samples was provided from the previously published study, as induction of the known PPARβ/δ target gene fatty acid-binding protein occurred in response to ligand treatment and this effect was absent in mice lacking PPARβ/δ expression (28). Thus, the use of these colon polyps served as an excellent model to determine the effect of ligand activation of PPARβ/δ on VEGF, COX2 and phospho-Akt. Cytosol from individual polyps from six independent mice from each treatment group was used for quantitative western blotting as described above.

*Effect of GW0742 and GW501516 on VEGF, COX2 and phospho-Akt in colon and liver*

Wild-type mice (C57BL6/N) were gavaged once per day for 5 days with either GW0742 or GW501516 at a dose of 10 mg/kg. This dose of PPARβ/δ ligand was chosen because it has been shown to activate PPARβ/δ in both liver and colon (8,28,49). Eight hours after the last dose of ligand, mice were euthanized and liver and colon were removed and snap frozen. Protein was isolated from representative mice from both tissues and used for quantitative western blotting as described above.

## Results

*Expression of PPARβ/δ and activation by GW0742 and GW501516 in human cancer cell lines*

To verify that the cell lines used for these studies expressed a functional PPARβ/δ, mRNA encoding PPARβ/δ and the known PPARβ/δ target gene (28,50), adipocyte differentiation-related protein was quantified. As compared with mouse primary keratinocytes, expression of mRNA encoding PPARβ/δ was significantly less, but detectable in all of the four human cancer cell lines (Figure 1B). In response to either PPARβ/δ ligand, expression of adipocyte differentiation-related protein was increased in all four human cancer cell lines (Figure 1C–F). These results demonstrate that the four cell lines express a functional PPARβ/δ and respond to both GW0742 and GW501516.

*GW501516 and GW0742 do not increase growth of human cancer cell lines*

To examine the effect of PPARβ/δ ligands on cell growth of human cancer cell lines, cell proliferation was quantified in three different colon cancer cell lines and two liver cancer cell lines in the presence of either GW0742 or GW501516, in the presence or absence of serum. No significant increase in cell proliferation was observed in any of the human cancer cell lines with either potent PPARβ/δ ligand (Figures 2 and 3; supplementary Figures 1, 2 and 3 are available at *Carcinogenesis* Online). In the presence of serum, cell growth was faster in all cell

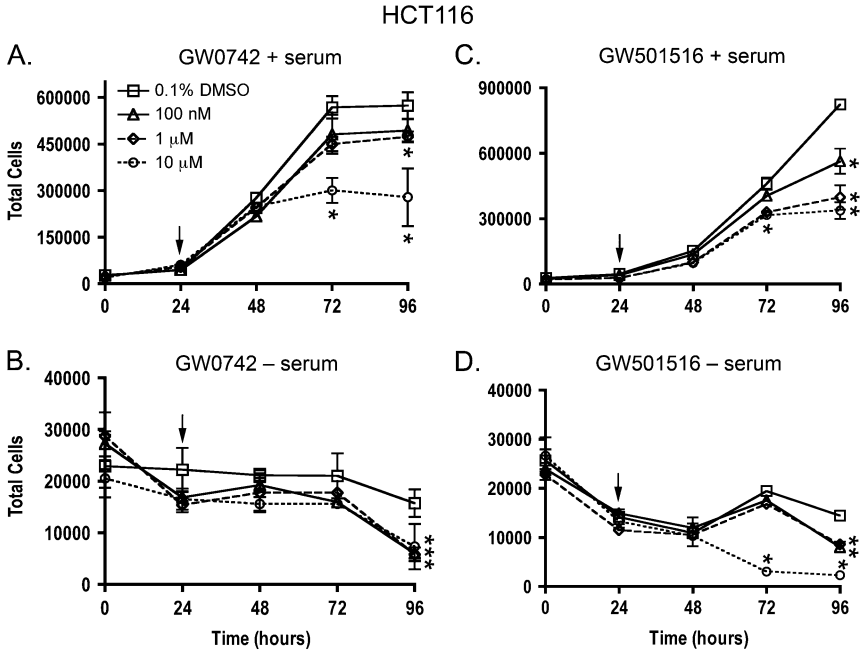


**Fig. 2.** Effect of GW0742 and GW501516 on cell proliferation in the HCT116 colon cancer cell line in the presence or absence of culture medium serum. Cells were treated with the indicated concentration of ligand (arrow) and cell number quantified using Coulter counting as described in Materials and Methods. Values represent the mean ± standard error of the mean. *, Significantly different than dimethyl sulfoxide control, $P \leq 0.05$.

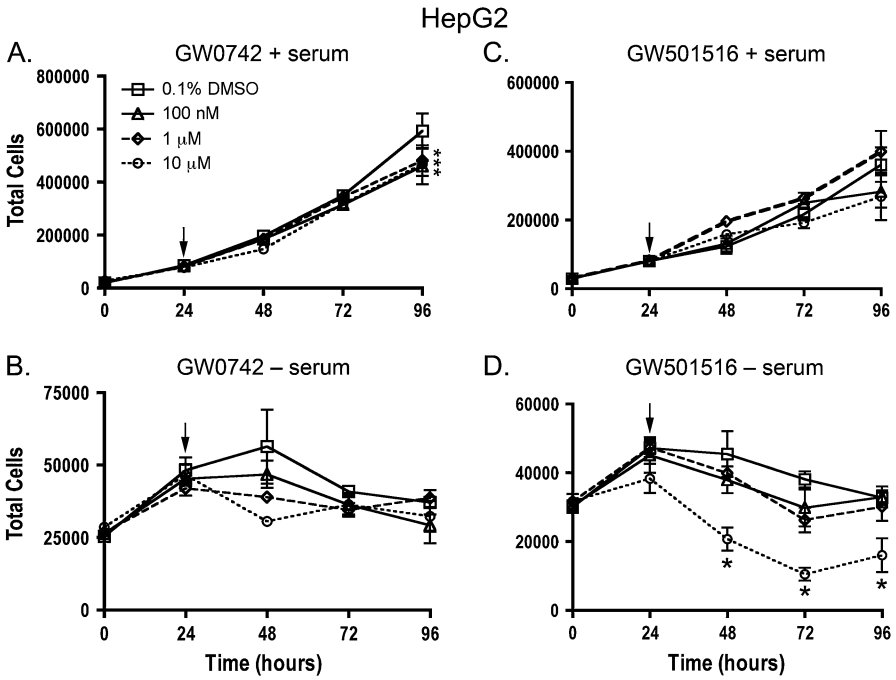


**Fig. 3.** Effect of GW0742 and GW501516 on cell proliferation in the HepG2 liver cancer cell line in the presence (**A** and **C**) or absence (**B** and **D**) of culture medium serum. Cells were treated with the indicated concentration of ligand (arrow) and cell number quantified using Coulter counting as described in Materials and Methods. Values represent the mean ± standard error of the mean. *, Significantly different than dimethyl sulfoxide control, $P \leq 0.05$.

lines as compared with that found in the absence of serum (Figures 2 and 3; supplementary Figures 1, 2 and 3 are available at *Carcinogenesis* Online). Inhibition of cell growth was observed by both GW0742 and GW501516, and this effect was typically observed only in the cells cultured in the presence of 1–10 μM ligand (Figures 2 and 3; supplementary Figures 1, 2 and 3 are available at *Carcinogenesis* Online).

*GW501516 and GW0742 do not increase expression of VEGF, COX2 or phosphorylation of Akt in human cancer cell lines*

Quantitative western blotting was performed using protein from three different colon cancer cell lines and one liver cancer cell line in the presence of either GW0742 or GW501516, in the presence or absence of serum. No changes in the phosphorylation of Akt or expression of VEGF were detected in any of the four different cell lines in response to either PPARβ/δ ligand, and this lack of effect was observed in both the presence and the absence of serum (Figures 4 and 5; supplementary Figures 4 and 5 are available at *Carcinogenesis* Online). Indeed, phosphorylated Akt was not detected in any of the colon cancer cell lines. Similarly, COX2 was not detected in any of the three different colon cancer cell lines (data not shown). Real-time PCR analysis of mRNA encoding COX2 in the colon cancer cell lines revealed no changes in COX2 mRNA expression (data not shown). COX2 expression was detected in HepG2 cells, but there was no change in expression observed in response to either PPARβ/δ ligand, in both the presence and the absence of serum (Figure 5). Interestingly, the only consistent change observed in these experiments was the increase in cleaved poly (ADP-ribose) polymerase (PARP) observed in response to serum withdrawal, which occurred in all four cell lines (Figures 4 and 5; supplementary Figures 4 and 5 are available at *Carcinogenesis* Online). The presence of cleaved PARP was unchanged by the presence of either PPARβ/δ ligand in either the presence or the absence of serum (Figures 4 and 5; supplementary Figures 4 and 5 are available at *Carcinogenesis* Online).

*GW0742 does not increase expression of VEGF, COX2 or phosphorylation of Akt in colon polyps from APC$^{min}$ mice*

To examine the effect of ligand activation of PPARβ/δ in colon polyps *in vivo*, colon polyp protein samples from mice used for previous analysis were examined for quantitative western blotting (28). Despite specifically activating PPARβ/δ with GW0742 administration as shown previously by PPARβ/δ-dependent activation of known PPARβ/δ target genes (28), no changes in the expression of VEGF, COX2 or phosphorylated Akt were found in independent colon polyp samples (Figure 6A).

*GW501516 and GW0742 do not increase expression of VEGF, COX2 or phosphorylation of Akt in mouse liver or colon*

Quantitative western blotting was performed using colon or liver protein from mice treated with either GW0742 or GW501516 for 5 days at a concentration known to specifically activate PPARβ/δ. Increased expression of AngPTL4 was observed in colon in response to both GW0742 and GW501516, demonstrating that PPARβ/δ was activated using this protocol (Figure 6B). VEGF was below the level of detection colon, and no differences in the expression of either COX2 or phosphorylated Akt were found in response to either PPARβ/δ ligand (Figure 6B). Similarly, in the liver, no differences in the expression of VEGF, COX2 or phosphorylated Akt were found after administration of either GW0742 or GW501516 (Figure 6C).

## Discussion

The present studies were undertaken to examine the hypothesis that some of the reported disparities in the literature describing the effects of potent PPARβ/δ ligands on growth and expression of proteins that regulate cell growth in human cancer cell lines are due to differences in the model system (*in vivo* versus cancer cell lines), differences in the ligand examined (GW0742 versus GW501516) and/or differences resulting from serum deprivation. Results from the present studies essentially rule out these variables as confounding factors, and raise serious questions regarding a number of previous studies.

The effect of GW0742 and GW501516 on cell growth of three different colon cancer cell lines and two liver cancer cell lines was examined using Coulter counting to quantify cell proliferation. Cell growth was quantified over a 3 day culture period in the presence or absence of serum and in the presence or absence of either GW0742 or GW501516 over a broad concentration range. Previous work by other

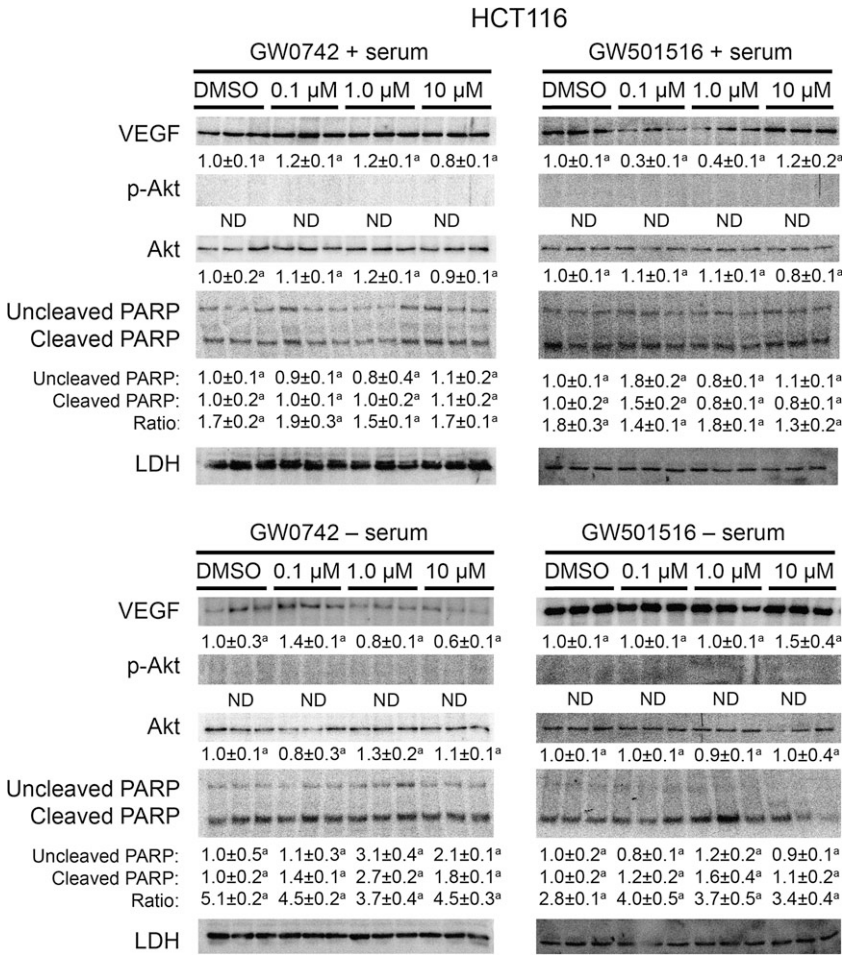


**Fig. 4.** Quantitative western blot analysis of VEGF, phosphorylation of Akt and PARP in the HCT116 colon cancer cell line in the presence (upper panels) or absence (lower panels) of culture medium serum. Cells were treated with the indicated concentration of GW0742 or GW501516 in the presence or absence of culture medium serum. Protein was isolated, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and incubated with a specific primary antibody. After conjugation with a biotinylated secondary antibody, membranes were incubated with $^{125}$I-labeled streptavidin. Hybridization signals were normalized to lactate dehydrogenase and are presented as the mean fold change as compared with the dimethyl sulfoxide control. The cleaved PARP to uncleaved PARP was used as an indicator of apoptosis and is presented as the mean ratio for each respective treatment group. Values represent the mean ± standard error of the mean. Values within the same row with different superscripted letters are statistically significant at $P \leq 0.05$.

laboratories has suggested that GW501516 increases cell growth in serum-deprived HepG2 cells using colorimetric assays that are dependent on mitochondrial dehydrogenase activity (11,12). Similarly, increased cell growth of other human cancer cell lines is also reported in both the presence and the absence of serum in cells cultured in GW501516 (10,13), but in some cell types, this effect is not found (10). In contrast, previous work by others has suggested that PPARβ/δ ligands have no effect on cell growth of HT-29 cells in the presence of serum using either bromodeoxyuridine incorporation (15) or hexosaminidase activity (10) as a marker of cell growth. Combined, this suggests that the presence of serum could influence the effect of ligand activation of PPARβ/δ on cell growth. However, results from the present study demonstrate that modulation of cell growth by either PPARβ/δ ligand was not influenced by the presence or absence of serum in colon cancer cell lines (HT-29, HCT116 or LS 174T) or liver cancer cell lines (HepG2 or HuH7). In general, in the absence of serum, cell proliferation was inhibited as compared with cells grown in serum, and the only statistically significant difference detected resulting from PPARβ/δ ligand treatment was inhibition of cell growth. Inhibition of cell growth is consistent with a number of other studies in a number of different model systems (19,27,32–41). Although more time consuming, assessment of cell proliferation using a Coulter counter is the gold standard as it determines the actual number of cells present rather than relying on enzyme activity to estimate the number of cells. This is important because it is well known that PPARβ/δ ligands can increase fatty acid oxidation by increasing expression of mitochondrial dehydrogenases (51); enzymes whose activity is coupled with cleavage of tetrazolium salts to indirectly determine cell growth. Additionally, PPAR ligands can also increase lysozomal enzyme activity (52), which could potentially influence cell proliferation assays that couple hexosaminidase activity with cell growth. Thus, it is likely that the increased cell growth reported in human cancer cell lines that utilize enzyme-linked assays [e.g. (10–13)] could have been influenced by induced enzyme activity of the mitochondria, since more definitive cell counting was not performed. Collectively, since the present studies utilized the most objective measure of cell proliferation (Coulter counting), there is stronger evidence that neither GW0742 nor GW501516 causes an increase in cell proliferation in the cancer cell lines examined.

In addition to examining cell proliferation over time in human cancer cell lines cultured with and without serum, in the presence or absence of GW0742 or GW501516, relative apoptosis was indirectly assessed by quantitative measurement of cleaved PARP and phosphorylation of Akt. Consistent with the cell proliferation kinetics determined from Coulter counting, the only significant change in PARP cleavage was found in serum-deprived cells where an increase in cleaved PARP was found, which is predictable. However, treating the cells with either GW0742 or GW501516 did not influence PARP

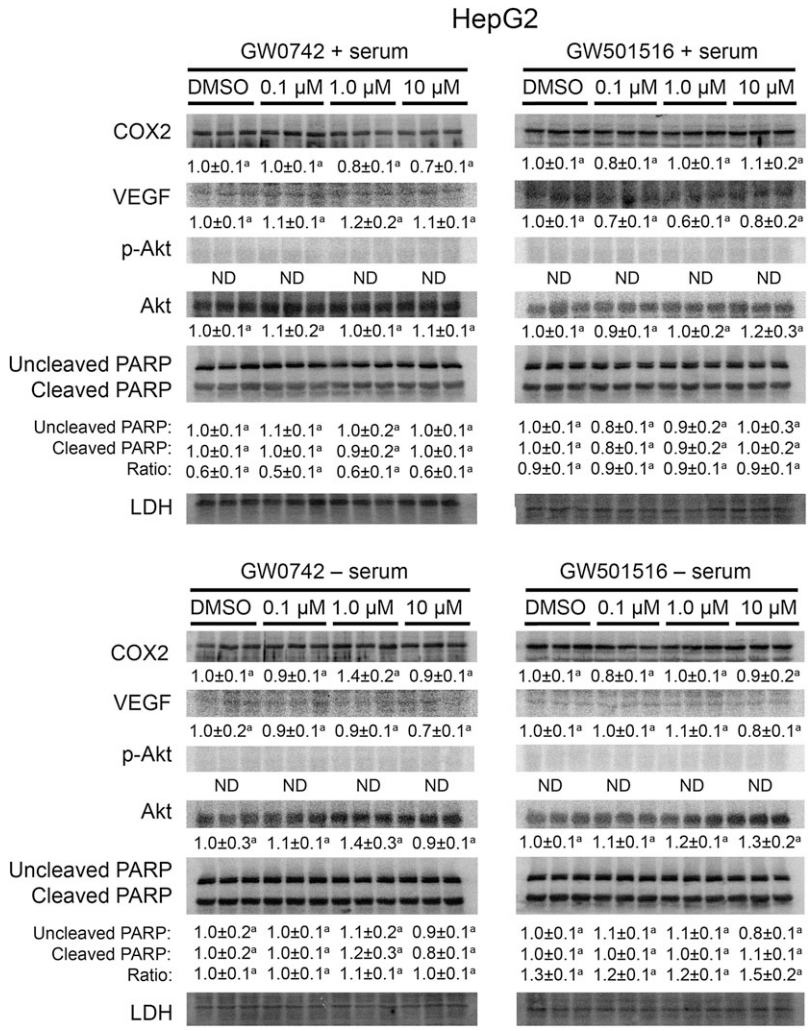


**Fig. 5.** Quantitative western blot analysis of COX2, VEGF, phosphorylation of Akt and PARP in the HepG2 liver cancer cell line in the presence (upper panels) or absence (lower panels) of culture medium serum. Cells were treated with the indicated concentration of GW0742 or GW501516 in the presence or absence of culture medium serum. Protein was isolated, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and incubated with a specific primary antibody. After conjugation with a biotinylated secondary antibody, membranes were incubated with $^{125}$I-labeled streptavidin. Hybridization signals were normalized to lactate dehydrogenase and are presented as the mean fold change as compared with the dimethyl sulfoxide control. The cleaved PARP to uncleaved PARP was used as an indicator of apoptosis and is presented as the mean ratio for each respective treatment group. Values represent the mean ± standard error of the mean. Values within the same row with different superscripted letters are statistically significant at $P \leq 0.05$.

cleavage in any of the human cancer cell lines in the presence or absence of serum. Phosphorylated Akt was not detected in any of the human cancer cell lines, and no differences in the levels of phosphorylated Akt were found in liver, colon or colon polyps of mice treated with either GW0742 or GW501516. The latter is consistent with a number of other studies, showing that PPARβ/δ ligands do not alter phosphorylated Akt levels in a number of different cell types (27,28,42,43). This is in contrast to other studies showing inhibition of serum deprivation-induced apoptosis by GW501516 in HCT116 or LS-174T colon cancer cell lines (8,14). The reason for the difference in these observations with the present studies is unclear. However, results from the present study suggest that this difference is probably not due to structural differences in the ligand, differences between *in vivo* and *in vitro* models, differences between normal and cancerous cells and/or due to differences resulting from the presence or absence of serum in the culture medium. Importantly, in serum-deprived cells, no difference in the actual number of cells in response to ligand treatment consistent with inhibition of apoptosis (e.g. increased cell number) was observed. Thus, it remains possible that previously described changes in TUNEL-positive cells resulting in serum-deprived colon cancer cell lines treated with GW501516 (8,14) do not reflect functional differences in actual cell number, as determined from the present study.

Closely related changes in the expression of COX2 have also recently been described to occur in human liver cancer cell lines following treatment with GW501516 (11). The association between increased activity of COX2 and increased cell growth of many tumor types is well documented. Previous work by others reported increased expression of COX2 in response to GW501516 in serum-deprived human cancer cell lines (11,13). This led to the postulation that PPARβ/δ ligand induction of COX2 leads to increased COX-derived prostaglandins and coordinated increased cell growth and represents a novel feedback loop (11,13). However, results from the present studies are inconsistent with this idea on several levels. No changes in cell proliferation by GW501516 or GW0742 were observed in five different cancer lines [including HepG2 and HuH7 cells which were used previously by others (11)], and no changes in the expression of COX2 were found in three different human colon cancer cell lines or HepG2 cells in response to either GW0742 or GW501516 in the presence or absence of serum. Further, no changes in the expression of COX2 were found in liver, colon or colon polyps after treatment with the PPARβ/δ ligands. Thus, results from the present studies show

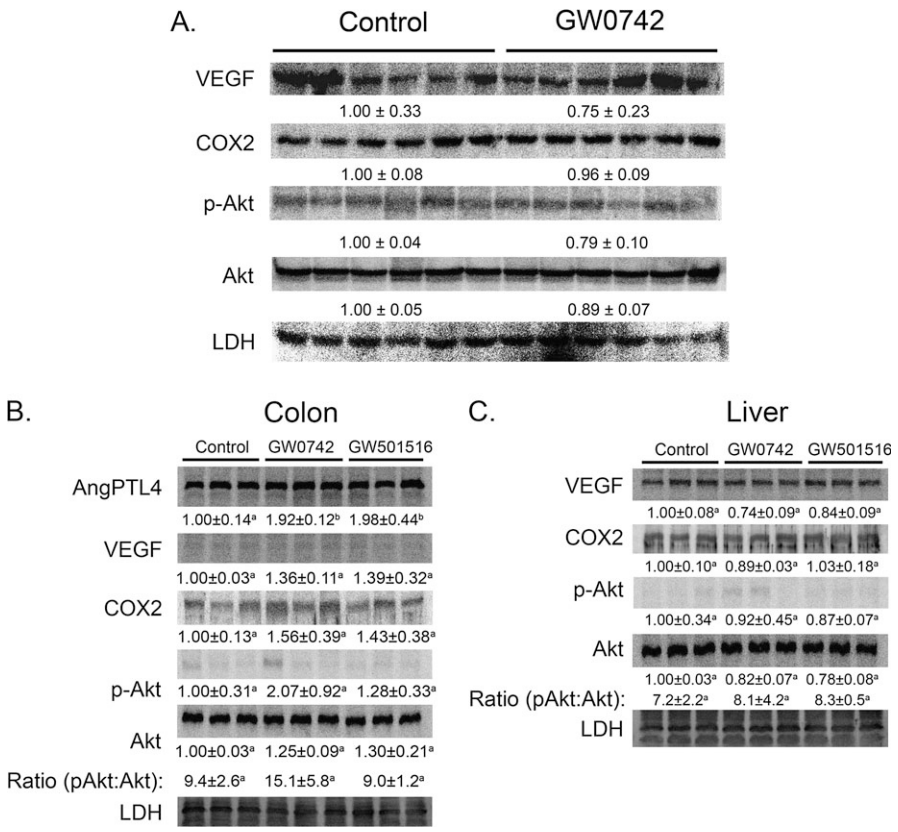


**Fig. 6.** Quantitative western blot analysis of VEGF, COX2 and phosphorylation of Akt in colon polyps from APC$^{min}$ mice treated with GW0742 (10 mg/kg/day) and liver and colon from mice treated with either GW0742 or GW501516 (10 mg/kg/day for 5 days). (**A**) Protein was isolated from six independent colon polyps from six different mice separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and incubated with a specific primary antibody. After conjugation with a biotinylated secondary antibody, membranes were incubated with $^{125}$I-labeled streptavidin. Hybridization signals were normalized to lactate dehydrogenase and are presented as the mean fold change as compared with the dimethyl sulfoxide control. Values represent the mean ± standard error of the mean. (**B**) Protein was isolated from colon (B) or liver (**C**) from three different mice, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and incubated with a specific primary antibody. After conjugation with a biotinylated secondary antibody, membranes were incubated with $^{125}$I-labeled streptavidin. Hybridization signals were normalized to lactate dehydrogenase and are presented as the mean fold change as compared with the dimethyl sulfoxide control. Values represent the mean ± standard error of the mean. Values within the same row with different superscripted letters are statistically significant at $P \leq 0.05$

that there is no evidence that ligand activation of PPARβ/δ causes increased expression of COX2 in human cancer cell lines, or in liver, colon or colon polyps *in vivo*. This disparity raises serious concerns regarding the hypothesis that PPARβ/δ ligands induce COX2 leading to coordinate increased cell growth of cancer cell lines (11,13). One possible explanation for this disparity is that the studies showing increased expression of COX2 in human cancer cell lines used enhanced chemiluminescence detection of proteins, which is not quantitative unless performed within the linear working range. In contrast, the present study utilized radioactive detection, which is the gold standard for quantifying protein expression. It is also worth noting that a number of laboratories have shown that PPARβ/δ and/or PPARβ/δ ligands have potent anti-inflammatory activity in a number of different cells types and can down-regulate expression of a number of inflammatory mediators (16–25,44) in addition to down-regulating expression of COX2 in activated macrophages (23). Further research is required to delineate why, or if, PPARβ/δ ligands do in fact lead to increased or decreased COX2 expression in specific model system and the mechanisms underlying these changes.

The last controversial issue examined in the present studies was the effect of PPARβ/δ ligands on VEGF expression, which could also influence cell proliferation. Several recent reports suggest that PPARβ/δ ligand increases expression of VEGF in both cancer cell lines and endothelial cells (10,14,53). In particular, a putative PPARβ/δ-dependent increase in VEGF expression was reported to occur in LS-174T cells treated with GW501516 and this increase in VEGF expression led to increased phosphorylation of Akt and inhibition of apoptosis (14). In contrast, no changes in VEGF expression, phosphorylation of Akt or cleaved PARP were detected in three different colon cancer cell lines, including LS-174T cells, in response to either GW0742 or GW501516 in the presence or absence of serum. Thus, the differences between these studies are unlikely due to presence or absence of serum. No changes in VEGF expression or phosphorylation of Akt were observed in colon or liver of mice treated with ether GW0742 or GW501516 at concentrations known to activate PPARβ/δ. VEGF expression and phosphorylation of Akt were also unchanged in colon polyps from APC$^{min}$ mice treated with GW0742. These findings suggest that the reported differences are also not due to differences between *in vivo* and *in vitro* models. It is also worth noting that work by others used enhanced chemiluminescence for detecting VEGF and phosphorylated Akt, whereas the present studies utilized radioactive detection to more accurately quantify the same proteins. Interestingly, the lack of change in VEGF expression by either GW0742 or GW501516 is consistent with another report showing no change in serum VEGF expression in mice following administration of GW0742 (6 or 60 mg/kg/day for up to 16 weeks) (17). Additionally, the more reliable evaluation of cell number by Coulter counting showed no increases in cell growth by either PPARβ/δ ligand in a number of cell lines under a variety of conditions. These combined observations are highly inconsistent with the idea that ligand activation of PPARβ/δ up-regulates VEGF leading to increased phosphorylation of Akt and inhibits apoptosis as proposed by others (14).

Collectively, results from these studies clearly demonstrate that GW0742 and GW501516 fail to increase cell proliferation, inhibit apoptosis and modulate COX2, phosphorylated Akt or VEGF expression in human colon and liver cancer cell lines, in contrast to recent reports (11,13,14). It remains possible that there are other intra-laboratory variables that account for some of these differences, and a number of important questions remain. How can compounds that induce terminal differentiation, inhibit cell growth and possess strong anti-inflammatory activity also promote cell growth, inhibit apoptosis, increase COX2 and/or VEGF expression? The observations presented in this report do not support the hypothesis that PPARβ/δ ligands potentiate cell growth in human cancer cell lines. In particular, the hypotheses suggested by others that PPARβ/δ promotes tumorigenic events through putative PPARβ/δ-dependent modulation of apoptosis/VEGF signaling (14,54) and/or through PPARβ/δ-dependent modulation of COX2 (11,13) are inconsistent with the present findings. Further work by independent laboratories will be necessary to build up a stronger body of evidence before a clear understanding of how PPARβ/δ ligands function will be obtained.

## Supplementary material

Supplementary material can be found at http://carcin.oxfordjournals.org/

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