Peroxisome proliferator-activated receptor-γ antagonists exhibit potent antiproliferative effects versus many hematopoietic and epithelial cancer cell lines

Jack D. Burton, Mary E. Castillo, David M. Goldenberg and Rosalyn D. Blumenthal

Peroxisome proliferator-activated receptor-γ ligands have preclinical and clinical anticancer activity. Most studies in this area address agonists, with relatively few reports on anticancer effects of peroxisome proliferator-activated receptor-γ antagonists. Thus, we evaluated the two pure peroxisome proliferator-activated receptor-γ antagonists, T0070907 and GW9662, on a panel of hematopoietic and epithelial cell lines. The peroxisome proliferator-activated receptor-γ antagonists and a reference agonist (pioglitazone) were tested in an in-vitro proliferation assay on a panel of seven hematopoietic and nine epithelial cancer cell lines, some of which are chemoresistant. Peroxisome proliferator-activated receptor-γ expression was measured by immunoblotting, as was the effect of treatment with these agents on peroxisome proliferator-activated receptor-γ levels. The effect of exogenous interleukin-6, an antiapoptotic cytokine, on growth inhibition was evaluated as well as the apoptotic effects of these drugs. The peroxisome proliferator-activated receptor-γ antagonists showed significantly greater potency on all cell lines (IC_{50}s of 3.2–29.7 versus 26.5–78.7 μmol/l for pioglitazone) and greater maximum growth inhibition. Peroxisome proliferator-activated receptor-γ levels did not correlate with growth inhibition in this panel of cell lines. Combinations of peroxisome proliferator-activated receptor-γ antagonists and the agonist actually showed schedule-dependent increases in growth inhibition. Exogenous interleukin-6 did not induce resistance to these agents. Both the antagonists and the agonist induced apoptosis, but only the former drugs showed caspase dependence. These two peroxisome proliferator-activated receptor-γ antagonists have significantly more potent in-vitro antiproliferative effects versus hematopoietic and epithelial cancer cell lines. This effect does not correlate with peroxisome proliferator-activated receptor-γ levels, suggesting alternative mechanisms or other targets of action. These findings support further translational studies to explore the mechanism of action and therapeutic potential of this class of agents. *Anti-Cancer Drugs* **18:525–534 © 2007 Lippincott Williams & Wilkins.**

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Introduction

Peroxisome proliferator-activated receptor-γ (PPARγ) is one of the three known peroxisome proliferator-activated receptors and is a member of the nuclear receptor superfamily. As it has a predominantly nuclear location, regardless of whether cognate ligands are present, it is classified as a type II nuclear receptor. It functions as a transcription factor by heterodimerizing with the retinoid-X receptor (RXR), after which this complex binds to specific DNA sequence elements called peroxisome proliferator response elements [1]. To become fully active as a transcription factor, PPARγ must be bound by ligand. RXR can be affected by binding its own cognate ligands, usually resulting in incremental increases in transcriptional activity. After the PPARγ/RXR heterodimer binds to peroxisome proliferator response elements in promoter regions of target genes, coactivator proteins such as p300 (CBP), SRC-1 and Drip205 (or TRAP220) family members are recruited to this complex to modulate gene transcription [2–4]. Different PPARγ ligands appear to be able to recruit different coactivators, which may explain differences in the biological activity between ligands [5].

The cardinal biologic activity of PPARγ is the induction of differentiation of adipocytes, the cell type that expresses the highest levels of PPARγ among normal tissues. Lower levels of PPARγ are, however, found in other normal tissues and cell types such as skeletal muscle, liver, breast, prostate, colon, type 2 alveolar pneumocytes, some endothelial cells as well as monocytes and B-lymphocytes. Two PPARγ isoforms (γ1 and γ2) are derived by alternate promoter usage resulting in an additional 28 amino acids at the N-terminus of PPARγ2.

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Most tissues express the PPARγ1, whereas the PPARγ2 isoform is expressed mostly by adipocytes. The longer N-terminal domain of PPARγ2 may affect function, as this isoform was shown to confer a higher level of ligand-independent transcriptional activity, which was further increased by physiologic concentrations of insulin [6].

High levels of PPARγ expression by fat and its role in adipogenesis led to the recognition that agonistic PPARγ ligands have antidiabetic effects. The chemical class of PPARγ agonists known as thiazolidinediones (TZDs) demonstrated high-affinity binding to PPARγ [7] as well as favorable therapeutic properties and such drugs were eventually registered for the treatment of type II diabetes mellitus. Three TZD drugs have been registered in the US: rosiglitazone (Avandia), pioglitazone (Actos) and troglitazone (Rezulin). Subsequent to its marketing and widespread use, troglitazone was associated with idiosyncratic and, in rare cases, fatal hepatic toxicity, and, thus, was withdrawn from the market. The former two drugs, however, have proven to be well tolerated and effective therapeutic options for the management of diabetes.

Within a few years after reports of the cloning of PPARγ and its expression in normal tissues [8,9], PPARγ expression was observed in an array of primary cancers and derivative cell lines. Its expression was reported initially in liposarcoma [10], and soon thereafter in the colon, breast and prostate carcinoma, followed by additional cancer types [11–14]. In addition to the in-vitro and preclinical in-vivo anticancer effects of TZDs, pilot clinical studies using troglitazone showed antitumor activity in patients with liposarcoma and prostate cancer [15,16]. Compounds from other chemical classes were also shown to bind PPARγ and to have antiproliferative effects in cancer models, such as the naturally occurring eicosanoid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2), the *N*-aryl tyrosine derivative, GW1929 [17], and the triterpenoid, 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid (CDDO) [18]. Although compounds that exhibit PPARγ agonist activity, such as TZDs, have PPARγ-dependent antiproliferative effects, they have also been shown to have antiproliferative effects in cell types that are genetically PPARγ-null [19]. Further uncertainty about mechanisms of anticancer effects of PPARγ ligands has resulted from variability in the classification of some compounds [e.g. bisphenol A diglycidyl ether (BADGE) has been shown to have both agonist and antagonist activity] [20,21].

Thus, the studies reported herein focused on two compounds (GW9662 and T0070907) that are pure PPARγ antagonists, to characterize their anticancer effects across a panel of 16 cancer cell lines of both epithelial and hematopoietic origin, including important cancer types with limited chemosensitivity (colon cancer) or broad-spectrum chemoresistance (kidney cancer). In comparison with a PPARγ agonist drug (pioglitazone) with known antiproliferative effects, our data show that the two antagonist compounds have significantly greater cytotoxic activity across this diverse panel of epithelial and hematopoietic cancer cell lines. Combinations of each of the antagonists with the agonist did not result in the reduction of antiproliferative effects, but rather resulted in schedule-dependent increases in growth inhibition. The mechanism of action of the antagonists differed from the agonist in that the apoptotic effects of the former drugs showed caspase dependence. Furthermore, importantly, the antiproliferative activity of antagonists in multiple myeloma (MM) lines was not reduced by the addition of exogenous IL-6.

Materials and methods

Cell lines

The colon carcinoma (HT29, HCT-15, LS174T, Moser), renal cell carcinoma (RCC; A498), breast carcinoma (MCF7, MDA-MB-231, ZR75–30), (MM U266B1) and nonHodgkin's lymphoma (NHL; Ramos) cell lines were obtained from the American Type Culture Collection (Manassas, Virginia, USA). Other cell lines were generously provided by the following investigators: Dr Takemi Otsuki [KMS12-PE and KMS12-BM (MM)], Dr Joshua Epstein [CAG (MM)], Dr Yuji Matsuzawa [OPM-6 (MM)], Dr Alan Epstein [SU-DHL6 (NHL)] and Dr Claus-Dieter Gerharz [ClearCa-2 (RCC)]. All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and antibiotics, L-glutamine, sodium pyruvate and nonessential amino acids.

Reagents and chemicals

The registered form of pioglitazone (Actos; Takeda, Japan) was the PPARγ agonist used in these studies. The PPARγ antagonist compounds, GW9662 (2-chloro-5-nitrobenzanilide) and T0070907 (2-chloro-5-nitro-*N*-4-pyridinylbenzamide), were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Stock solutions of these agents were prepared using a 50 : 50 mixture of *N,N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO). Equal volumes of this solvent that corresponded to the amounts used for the dilutions of the PPARγ ligands were added to growth medium as negative controls in the in-vitro cellular proliferation experiments, and this amount was always less than 0.1% v/v. Propidium iodide (PI) and etoposide (positive control drug) for the flow cytometric assessment of apoptosis were obtained from Sigma-Aldrich (St Louis, Missouri USA). The pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD), was obtained from R&D Systems (Minneapolis, Minnesota, USA) and was dissolved initially in the DMF : DMSO solvent described above.

In-vitro cellular proliferation assay

The in-vitro effects of the compounds listed above on the panel of cancer cell lines (also listed in Table 1) were assessed in cell growth assays in 96-well, flat-bottom plates, in which $1.5–10 \times 10^3$ cells/well were added to triplicate wells. Varying concentrations of the PPARγ ligands were added in a final volume of 200 µl of growth medium per well. The solvent (DMF : DMSO) was added to control wells at equal volumes to those used for the three test compounds. The plates were then incubated at 37°C in a 5% CO_2-supplemented atmosphere until the control wells showed 60–80% cellular confluence (5–7 days incubation period). At this point, for adherent cells, the media was removed and replaced by 100 µl of 0.5 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) in growth medium and the plates were put back in the 37°C incubator for 4–6 h. For nonadherent cells, 25 µl/well of a 1 : 1 dilution of MTT stock solution (5 mg/ml) : growth medium was added directly to the wells and plates were placed back in the incubator for 4–6 h. Plates were then centrifuged at 400*g* for 10 min. Supernatants were removed from the wells and the reduced MTT dye was solubilized with 100 µl/well of DMSO. Optical density was determined on a Spectramax 250 plate reader (Molecular Devices Co; Sunnyvale, California, USA) at 570 nm. Percent inhibition was calculated using the formula: % inhibition $= 1 - (OD_{test}/OD_{control}) \times 100$. IC_{50} values were calculated by plotting the log of the percent inhibition values versus drug concentrations. All concentrations were carried out in triplicate in each assay and the mean ± SEM was calculated from at least three replicate studies.

Additional MTT assays were performed to evaluate the effects of combination treatment with the agonist (pioglitazone) and each of the PPARγ antagonists (GW9662 and T0070907). The Moser cell line (colon carcinoma) was used in these experiments. Pioglitazone and the two antagonists, T0070907 and GW9662, were used at concentrations (5–15 µmol/l) that gave from 10 to 30% growth inhibition when they were used as single agents. Individual drugs and binary combinations of pioglitazone with each of the antagonist drugs were added to 96-well culture plates and evaluated by the MTT assay described above. Three different schedules were tested: simultaneous addition of pioglitazone plus antagonist; pioglitazone added 24 h before the antagonist and in the reverse order.

In a separate set of assays using two of the interleukin (IL)-6-independent MM cell lines (KMS12-PE and OPM-6), the effect of exogenous IL-6 on the antiproliferative activity of the two PPARγ antagonists was assessed, as this cytokine can induce drug resistance in MM and other cancer types. Cells were added to 96-well plates as described above, to which varying concentrations of the antagonist compounds were added. To half of the wells, 5 ng/ml of recombinant human IL-6 was added at time zero. Plates were incubated and processed, and the resulting IC_{50} values were derived as described above.

Table 1 Mean IC_{50} values (µmol/l) for the PPARγ ligands

Cell lines	Pioglitazone	T0070907	GW9662
Colon			
Moser[c]	26.5 ± 2.6	15.9 ± 1.0	20.1 ± 0.3
HT29[a]	53.0 ± 4.7	11.2 ± 0.0	14.1 ± 0.5
LS174T[c]	38.7 ± 7.4	7.8 ± 1.9	9.5 ± 0.5
HCT-15[a]	53.1 ± 2.5	13.0 ± 0.5	19.0 ± 0.8
RCC			
A498[c]	38.9 ± 4.9	24.3 ± 0.7	29.1 ± 0.3
ClearCa-2[a]	56.4 ± 3.1	20.8 ± 1.9	21.5 ± 0.7
Breast			
ZR75-30[a]	77.9 ± 7.0	3.9 ± 0.3	10.6 ± 0.9
MCF7[a]	54.8 ± 3.9	10.2 ± 1.9	16.6 ± 2.4
MDA-MB-231[a]	78.7 ± 3.5	20.1 ± 1.1	26.8 ± 1.0
MM			
CAG[b]	62.4 ± 9.9	12.2 ± 1.2	13.8 ± 0.1
KMS12-BM[a]	33.2 ± 5.1	3.2 ± 0.6	11.8 ± 1.6
KMS12-PE[a]	56.4 ± 1.5	4.3 ± 0.3	9.5 ± 0.9
OPM-6[a]	48.9 ± 1.8	4.1 ± 0.3	11.5 ± 0.1
U266B1[a]	56.6 ± 1.3	9.9 ± 0.2	29.7 ± 1.5
NHL			
Ramos[a]	66.5 ± 7.4	12.7 ± 0.7	15.1 ± 0.1
SU-DHL6[a]	53.1 ± 1.4	11.8 ± 0.4	14.8 ± 0.3

Mean IC_{50} values from replicate experiments with this panel of cells for each of the three PPARγ ligands are shown above, expressed in µmol/l ± SEM. Cell lines are grouped according to cancer type.

IC_{50} values from each cell line were compared by single factor analysis of variance with all lines showing significant differences as indicated:

[a]$P < 0.0001$; [b]$P < 0.005$; [c]$P < 0.04$. MM, multiple myeloma; NHL, nonHodgkin's lymphoma; RCC, renal cell carcinoma.

Preparation of cell lysates and immunoblotting

Cell lines were harvested, washed with phosphate-buffered saline and nuclear lysates were prepared by first lysing whole-cell pellets with a solution of 0.05 mol/l Tris–HCl (pH 8)/0.15 mol/l NaCl/1 mmol/l ethylenediaminetetraacetic acid/10 mmol/l NaF/0.4% Zwittergent 3-12 (Calbiochem, San Diego, California, USA)/1 : 200 dilution of protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Nuclei were then pelleted by centrifugation at 8000*g* for 10 min at 4°C and suspended in five volumes of a solution of 20 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid/0.2 mmol/l ethylenediaminetetraacetic acid/0.5 mmol/l 2-mercaptoethanol/10 µg/ml leupeptin/20% glycerol (pH 7.5). To this, 4 mol/l $(NH_4)_2SO_4$ (pH 7.9) was added to achieve a final concentration of 0.3 mol/l, after which the suspension was rocked gently at 4°C for 30 min followed by centrifugation at 16 000*g* for 60 min at 4°C. Protein concentrations of the resulting supernatants were measured by absorbance at 225 nm and estimated using a standard curve generated with bovine serum albumin. From each nuclear lysate, 30 µg of total protein was separated on 10% sodium dodecyl sulfate–polyacrylamide

gel electrophoresis gels (Life Therapeutics, Australia) and electroblotted onto polyvinylidine diflouride membranes (Millipore, Bedford, Massachusetts, USA) at 14 V for 16–18 h. Membranes were preincubated in blocking buffer [Superblock (Pierce, Rockford, Illinois, USA) + 0.05% Tween 20]. Monoclonal anti-PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) at a 1 : 100 dilution in blocking buffer was used to probe the membrane using an incubation period of 22 h at room temperature. After washing, peroxidase-conjugated, affinity-purified rabbit anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) was used at a 1 : 2500 dilution in blocking buffer for 1 h at room temperature. SuperSignal West Pico chemiluminescent substrate (Pierce) was added to the membranes, and blots were exposed to film (Kodak MR, Rochester, New York, USA) for varying time intervals up to 1 h, and were then developed.

In separate experiments, the effect of treatment with each of the three PPARγ ligands on PPARγ expression was assessed. One epithelial (LS174T; colon) and one hematopoietic line (CAG; MM), which showed measurable basal expression of PPARγ by immunoblotting, were used. These lines were treated with the corresponding IC_{50} concentrations of these agents for 48 h, after which cells were harvested, and nuclei were separated and lysed as described above.

Assessment of drug-induced apoptosis

The apoptotic effects of the two antagonists and the reference agonist were assessed in the MM line, KMS12-BM, and in the colon cancer line, Moser. This was performed by flow cytometric analysis to determine the sub-G_1 fraction as assessed by staining with PI. Cells were added to replicate 24-well plates at 4×10^5 cells/well for KMS12-BM and 1.5×10^5 cells/well for Moser in the standard RPMI 1640 medium described above, and incubated at 37°C in a 5% CO_2-supplemented atmosphere. Cells were treated for 42–44 h with two concentrations of the antagonists and the reference agonist (corresponding to the IC_{50} and 2.5 times the IC_{50}). The positive control drug for induction of ZVAD-sensitive apoptosis was etoposide, which was used at its IC_{50} concentration and 2.5 times this level, as with the test drugs. To inhibit caspase activity, ZVAD was used at a final concentration of 40 μmol/l, and was added 3 h before the test and control drugs. At the end of incubation period, cells were transferred to test tubes, washed with phosphate-buffered saline, and resuspended in a hypotonic PI solution (50 μg/ml PI in 0.1% sodium citrate/0.1% Triton X-100) and incubated for 15 min in the dark. Cells were analyzed on a flow cytometer (Becton-Dickinson) to determine percentage of cells in the sub-G_1 fraction using Cell Quest software (Becton-Dickinson).

Results

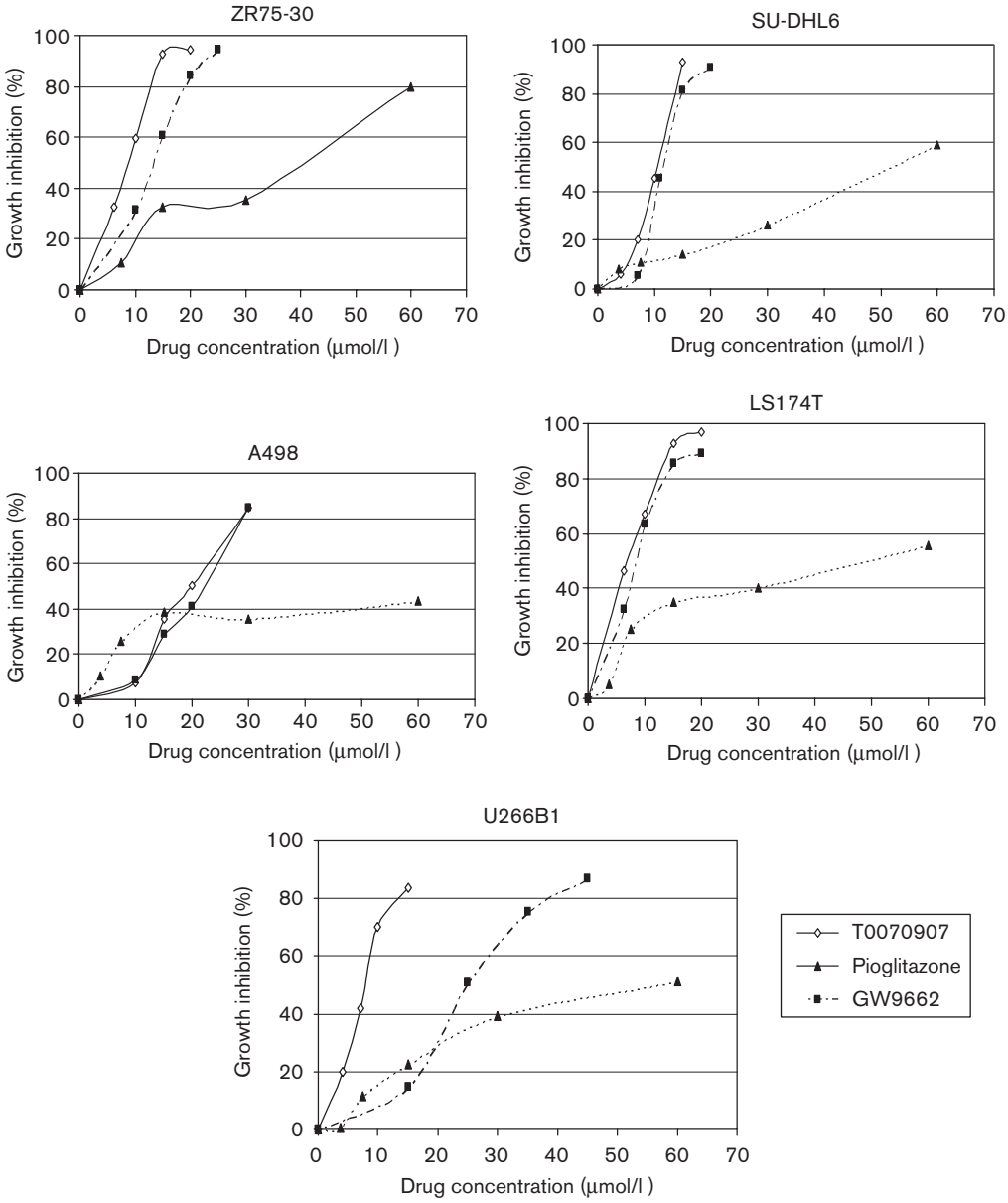
Peroxisome proliferator-activated receptor-γ antagonist compounds have significantly more potent in-vitro growth inhibitory activity than the agonist drug, pioglitazone

A panel of 16 cell lines consisting of several types of epithelial cancer cell lines (colon, renal cell and breast) as well as hematopoietic cell lines (MM and NHL) was used to compare the in-vitro effects of the two PPARγ antagonists versus the agonist drug. The IC_{50} values for the agonist, pioglitazone, ranged from 26.5 to 78.7 μmol/l for all 16 cell lines. In contrast, the antagonist compound, GW9662, exhibited IC_{50} values ranging from 9.5 to 29.7 μmol/l, whereas the other antagonist, T0070907, showed somewhat lower mean values than GW9662 in these cell lines, with a range of 3.2–24.3 μmol/l. In addition to the greater potency of both antagonist compounds in terms of IC_{50} values, these agents also showed greater maximum growth inhibition that consistently exceeded 85%, compared with 50–80% for pioglitazone. The IC_{50} values for the three PPARγ ligands for this panel of cell lines are shown in Table 1. Comparison of these values by single-factor analysis of variance showed that in all 16 cell lines tested, there was a significant difference between the IC_{50}s of the agonist and antagonist compounds (all P values < 0.04), and in 12 of 16 lines, the differences were highly significant ($P < 0.0001$). Dose–response analysis showed that the antagonists also consistently conferred greater maximum growth inhibition (> 85%) at concentrations that were only 2–3-fold greater than the corresponding IC_{50} values. Representative examples of dose–response curves for one cell line from each of the five cancer types in the panel are shown in Fig. 1. As discussed below, the majority of the cell lines tested showed detectable levels of PPARγ, but the threshold level required for antiproliferative responses is not known. Thus, the relative contributions of PPARγ versus other possible drug targets to the observed effects are not currently known.

Antiproliferative effects of peroxisome proliferator-activated receptor-γ antagonists do not correlate with peroxisome proliferator-activated receptor-γ protein expression

Immunoblotting was initially performed on a subset of cell lines that included the four MM lines and three of the colon carcinoma lines. This was carried out to assess the relationship between PPARγ expression and responsiveness to PPARγ ligands. The MM cell lines in the panel were selected to include one line (U266B1) that is IL-6-dependent and three others that are IL-6-independent. This was carried out to follow up on a prior report, which showed that PPARγ expression and sensitivity to a TZD agonist drug (troglitazone) were observed only in IL-6-dependent MM lines [22]. We also analyzed colon carcinoma lines, as this cancer type tends to express high

Fig. 1



Dose–response curves from representative in-vitro proliferation assays from one cell line from each of the five major cancer types in the overall panel of cell lines. ZR75-30, A498 and LS174T are derived from breast, renal cell and colon carcinoma, respectively. U266B1 is a MM cell line and SU-DHL6 is a NHL cell line.

levels of PPARγ; yet, despite their high expression levels, differences in sensitivity to the agonist drug, pioglitazone, amongs the colon lines were observed (Table 1). The three colon carcinoma lines analyzed showed high levels of PPARγ expression and there was also visibly detectable expression by one of the MM lines, CAG (Fig. 2). To quantitate expression levels, densitometric scanning of the gel region containing the PPARγ bands and a control gel region outside of the active lanes of the gel was performed and normalized against an internal standard (β-actin) that was quantitated in the same fashion. A PPARγ/β-actin ratio was calculated and is also provided in Fig. 2. One of the breast cancer lines, MCF7, and the IL-6-dependent MM line, U266B1, had ratios that were not above background, although two other MM lines, which were IL-6-independent, had higher signal intensities that were somewhat above background. This initial assessment of the panel showed no apparent correlation between PPARγ expression and sensitivity to any of the three PPARγ ligands.

Fig. 2

Lanes	1	2	3	4	5	6	7	8
Intensity (norm)	0.04	0.04	0.21	1.09	0.85	1.07	0.02	0.03

Immunoblot in which the expression of peroxisome proliferator-activated receptor-γ (PPARγ) by some cell lines from the overall panel was analyzed. Aliquots of nuclear lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto membranes as described. PPARγ and the control, β-actin were detected using corresponding cognate antibodies and a chemiluminescent technique. The film images were scanned for both of the target bands (PPARγ and β-actin) and they were quantitated, from which normalized intensity values were calculated (PPARγ/β-actin ratios; displayed below each lane). PPARγ bands are shown for the following lanes: 1, KMS12-PE; 2, OPM-6; 3; CAG; 4, HT29; 5, LS174T; 6, Moser; 7, MCF7; 8, U266B1.

Fig. 3

Lanes	1	2	3	4	5	6	7	8
Intensity (norm)	0.47	0.54	0.63	0.20	0.75	0.94	0.94	0.74

Immunoblot that measures peroxisome proliferator-activated receptor-γ (PPARγ) levels after treatment with each of the three cognate ligands. Cell lines were cultured in the presence or absence of these agents at their corresponding IC_{50} concentrations for 48 h and nuclear lysates were prepared. Aliquots of these were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto membranes for immunodetection of PPARγ, with β-actin as the control. Film images were scanned and PPARγ (shown in gel image) and β-actin bands were quantitated. Normalized intensity values were derived (PPARγ/β-actin ratios; displayed below each lane). Lanes 1–4 are from the CAG cell line and lanes 5–8 are from LS174T; lanes 1 and 5, CAG and LS174T cells, respectively, cultured in medium only; lanes 2 and 6, CAG and LS174T, respectively, after pioglitazone treatment; lanes 3 and 7, same lines treated with T0070907; lanes 4 and 8, same lines treated with GW9662.

Treatment with either Peroxisome proliferator-activated receptor-γ agonist or antagonists did not significantly upregulate Peroxisome proliferator-activated receptor-γ protein expression

One epithelial line (LS174T; colon) and one hematopoietic line (CAG; MM), which showed constitutive PPARγ expression, were selected to assess the effects of the three PPARγ ligands on the expression of PPARγ itself, as some reports indicate that agonists have an upregulatory effect [23,24]. These cell lines were treated for 48 h with IC_{50} concentrations of each of the three PPARγ ligands, after which nuclear lysates were prepared for measurement of PPARγ by immunoblotting. In both of these cell lines, PPARγ expression was not upregulated to a significant degree after treatment with either the agonist, pioglitazone, or the antagonist, T0070907. In the LS174T line, the other antagonist, GW9662, also had no effect on PPARγ expression by this cell line, whereas in the CAG line there was an around 2.4-fold decrease in PPARγ after treatment with GW9662 (Fig. 3). The significance of this latter finding is unclear, as the CAG line had similar sensitivity to both T0070907 and GW9662 (IC_{50} values of 12.2 and 13.8 μmol/l, respectively). Overall, no upregulation of PPARγ was observed in these cell lines.

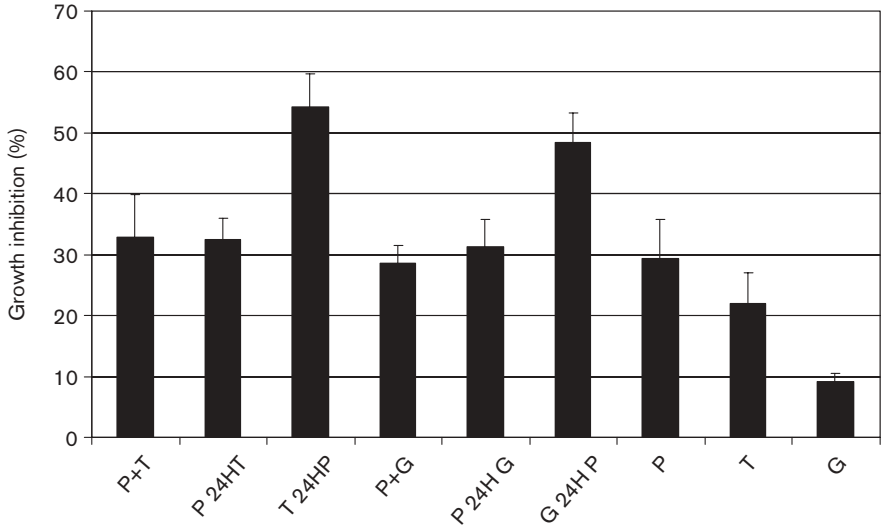
The combination of pioglitazone with each of the Peroxisome proliferator-activated receptor-γ antagonists results in schedule-dependent, augmented growth inhibition instead of antagonism

A PPARγ-expressing colon carcinoma line, Moser, was selected to evaluate the effect of combined treatment with agonist and antagonist, as it was the most sensitive of the cell lines to growth inhibition by pioglitazone and showed moderate sensitivity to both T0070907 and GW9662. Canonical PPARγ-dependent biologic responses would show antagonism with these combinations. On the contrary, increases in growth inhibition were observed with the combinations of pioglitazone and each of the antagonist compounds. This effect was greatest when the antagonist was added 24 h before pioglitazone as compared with the reverse sequence or simultaneous addition as shown in Fig. 4.

Peroxisome proliferator-activated receptor-γ is expressed in interleukin-6-independent multiple myeloma lines, which were more sensitive to the Peroxisome proliferator-activated receptor-γ antagonists than an interleukin-6-dependent multiple myeloma line

A previous report that analyzed cellular responses of three MM lines to the PPARγ agonists, 15-d-PGJ_2 and troglitazone, showed that growth inhibition and certain

Fig. 4



The in-vitro effects of treatment with a peroxisome proliferator-activated receptor-γ (PPARγ) agonist [pioglitazone ('P')] in combination with each of the antagonists [T0070907 ('T') and GW9662 ('G')] using the Moser (colon) cell line is shown as determined by the MTT proliferation assay. Percent growth inhibition is plotted on the *y*-axis and the treatment combinations are shown along the *x*-axis. This cell line was treated with concentrations (5–15 μmol/l) of pioglitazone, T0070907 and GW9662, either alone or in combination. Treatment was carried out concurrently as well as sequentially with 24 h spacing (both sequences).

downstream signaling events were PPARγ-dependent; it also showed that two IL-6-dependent MM lines expressed PPARγ, whereas an IL-6-independent line did not [22]. Furthermore, GW9662 was reported to block the effects of the agonists, and had no antiproliferative activity on its own. We utilized five different MM lines, of which four are IL-6 independent (CAG, KMS12-PE and OPM-6) as well as a fourth that is dependent on an IL-6 autocrine loop (U266B1). In contrast to the prior report cited above, the four IL-6-independent MM lines were as or more sensitive to the growth inhibitory effects of the two PPARγ antagonists as compared with the IL-6-dependent line, U266B1 (Table 1). All five MM lines had similar and significantly higher mean IC_{50} values for the agonist, pioglitazone (33.2–62.2 μmol/l), which were also similar to those observed with pioglitazone on the NHL and epithelial cell lines (Table 1). To examine the impact of exogenous IL-6 on the responses of two of the MM lines (KMS12-PE and OPM-6), MTT assays were performed in the presence and absence of exogenous IL-6 (5 ng/ml). For both of these MM lines, addition of IL-6 did not induce resistance, but instead appeared to increase the sensitivity of these lines to T0070907, with a similar trend observed with GW9662 (Fig. 5).

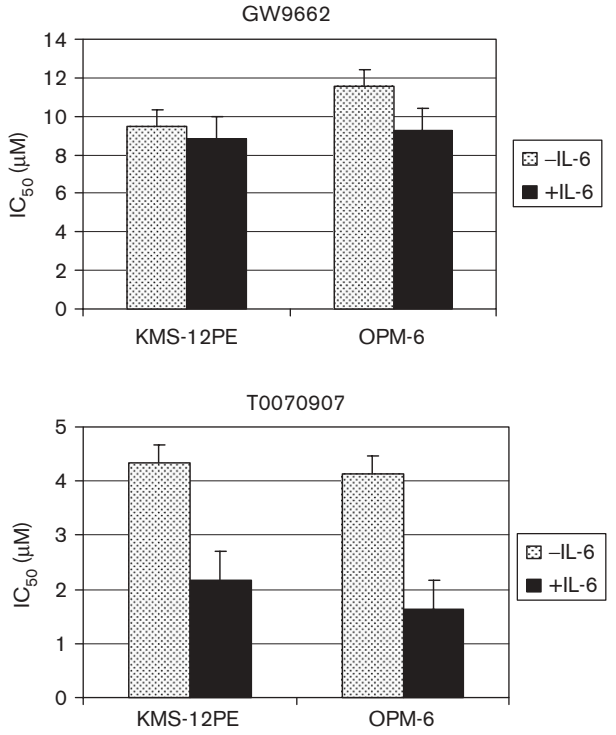
Peroxisome proliferator-activated receptor-γ antagonists induce apoptosis that is partially caspase-dependent while the agonist induces caspase-independent apoptosis

The MM line, KMS12-BM, was initially used to determine whether the growth inhibitory effects of the three PPARγ ligands were associated with stigmata of apoptotic cell death and whether apoptotic effects were caspase-dependent. Cell cycle analysis to determine the sub-G_1 fraction using flow cytometry was performed after treatment with each of the PPARγ ligands and the control drug, etoposide. The role of caspases was assessed using the pan-caspase inhibitor, ZVAD. All three PPARγ ligands induced similar levels of apoptosis above the background level of control cells that were cultured in the absence of drug (Fig. 6). The two antagonists differed, however, from the agonist, pioglitazone, in that ZVAD pretreatment reduced the sub-G_1 fraction by more than 50% with both antagonists, whereas there was no detectable effect of ZVAD on the apoptotic effect of the agonist. Similar results were obtained with an epithelial cell line (Moser; data not shown).

Discussion

The results of these studies show that two pure PPARγ antagonists, GW9662 and T0070907, exhibit greater antiproliferative activity in all of the epithelial and hematopoietic cell lines tested ($n = 16$) than the agonist drug, pioglitazone. The IC_{50} values for T0070907 were slightly lower than those obtained with GW9662, with 14 of the 16 lines showing values ≤ 20 μmol/l for the former drug. Also of note was that near-maximal inhibition could be consistently achieved by concentrations of both of the antagonists that were only 2–3-fold higher than the IC_{50}, whereas the dose–response with pioglitazone was more gradual and did not reach the same levels of maximum

Fig. 5



The IC_{50} values of two multiple myeloma lines (KMS12-PE and OPM-6) for the two Peroxisome proliferator-activated receptor-γ (PPARγ) antagonists are shown for GW9662 (top panel) and T0070907 (bottom panel). Cell lines were treated with the same concentration range of these agents in the presence and absence of 5 ng/ml of recombinant human interleukin-6, which was added at the start of the assay.

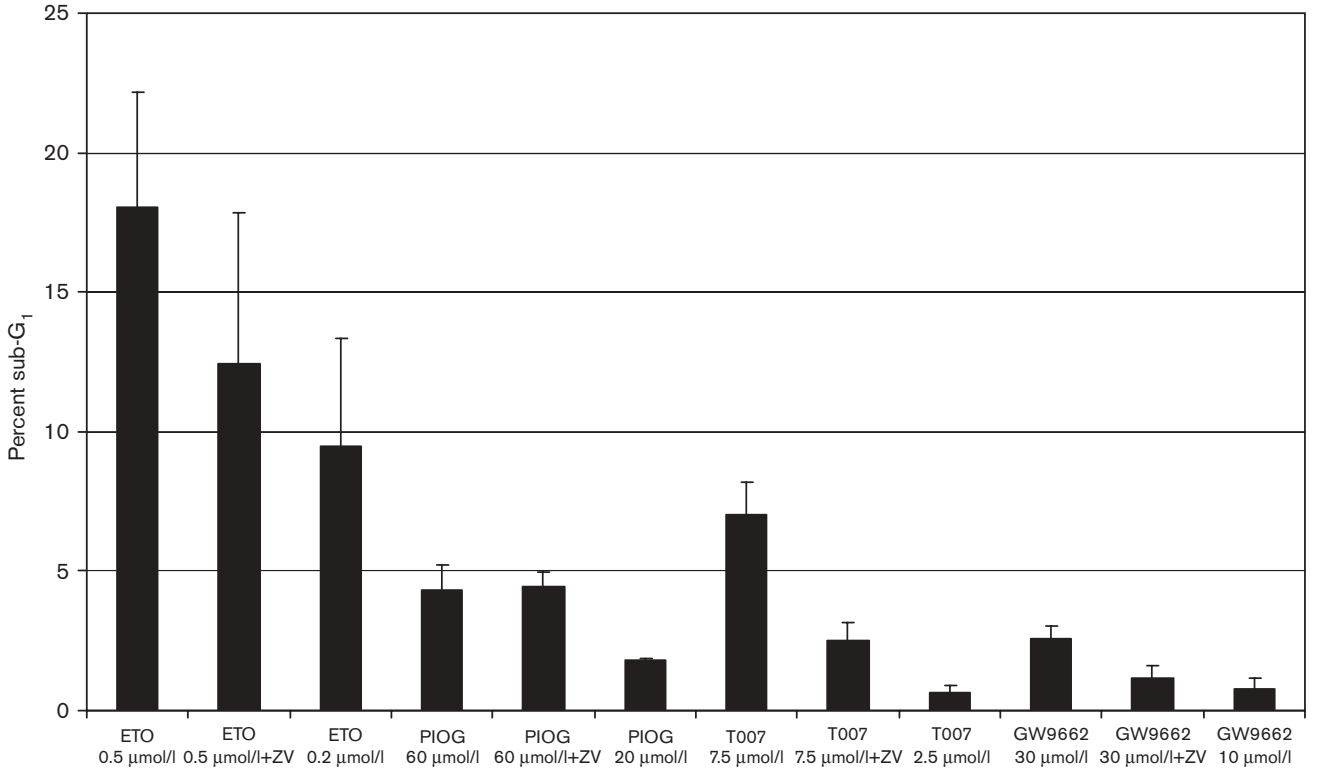
inhibition. This indicates therapeutic potential for these antagonist drugs, as serum levels in the low micromolar range are commonly observed with many drugs in routine clinical use. These effects of the antagonists were not related to the levels of PPARγ expressed by the cell lines. In addition, the antiproliferative effect of the agonist was not blunted by either of the antagonists, but instead was either unchanged or augmented, particularly when the antagonist was added 24 h before the agonist. We also show that the PPARγ agonist and the two antagonists induced similar levels of apoptosis, but differed in that the antagonists showed a substantial level of caspase dependence that was not observed with the agonist in the two cell lines tested.

Our results are consistent with two recent reports of in-vitro antiproliferative effects of one of the two PPARγ antagonists we tested, GW9662, in substantially smaller groups of mostly epithelial cancer cell lines (excluding renal cell and with one colon cancer line), which also showed increased growth inhibition by combinations of agonists and GW9662 [25,26]. One of these reports evaluated another agonist, rosiglitazone, and GW9662 on a small group of only breast cancer cell lines [25]. The other report tested a group of PPARγ ligands including GW9662 on a small panel of human epithelial cancer cell lines ($n = 3$; one each of prostate, colon and breast) with similar results [26]. These initial reports did not address mechanism. The possibility that PPARγ antagonists possess anticancer activity was suggested by prior studies using the compound, BADGE [27]. This, however, is a relatively low-affinity ligand for PPARγ and has been classified as both an agonist and antagonist depending on the cellular assay system that is used [20,21]. Schaefer *et al.* [28] have provided the only data on antiproliferative effects of the antagonist, T0070907, as compared with two agonists in one hepatocellular carcinoma cell line, HepG2. Mechanistic studies showed that the effect of this antagonist was in part dependent upon PPARγ levels, as small interfering RNA-mediated knockdown of PPARγ expression inhibited cell–substrate adhesion of this line, which was enhanced by cotreatment with PPARγ–siRNA and T0070907. Our results are consistent with this mechanism, but would also be consistent with the mechanism of ligand-dependent transrepression, which was recently described with PPARγ in an in-vitro model of inflammatory signaling [29]. In this model, transrepression was mediated by a pure agonist, rosiglitazone, as well as by a mixed agonist/antagonist, GW0072. This PPARγ-dependent mechanism reduced the expression of an inflammatory, NF-κB-regulated gene (*iNOS*), which suggests the general possibility that PPARγ ligands (agonists, mixed agonists or antagonists) might also inhibit proliferative signaling in cancer cells via this mechanism.

Our results extend observations on the antiproliferative effects of PPARγ antagonists to renal cell carcinoma, four additional colon carcinoma lines as well as to human hematopoietic cells lines (NHL and MM). In addition, our results address the antiproliferative effects of T0070907 and GW9662 in comparison with the agonist, pioglitazone across this entire panel of 16 lines. We also provide data showing that there is an apoptotic effect of both of the pure antagonist drugs that was of a similar magnitude as the agonist, but at lower concentrations corresponding to their lower IC_{50} values. Previously, only the variable and lower affinity agonist/antagonist, BADGE, has been evaluated for its apoptotic effects. The data presented here show a difference between the antagonist drugs and the agonist in that the former showed caspase-dependent apoptotic effects.

Another goal of our studies was to assess the activity of PPARγ ligands on hematopoietic cancer cell lines, particularly those derived from MM. A recent report, which used a group of three MM lines, showed that antiproliferative responses to the agonists, troglitazone and 15-d-PGJ_2, were observed only with the two IL-6-dependent MM lines [22], with no effect of GW9662 in

Fig. 6



The apoptotic effects of the three Proxisome proliferator-activated receptor-γ (PPARγ) ligands were evaluated by flow cytometric measurement of the sub-G_1 fraction as described above. The KMS12-BM cell line was cultured in the presence or absence of PPARγ ligand or the control drug, etoposide (ETO), for a period of 42–44 h. To some of the wells, 40 μmol/l ZVAD was added 3 h before addition of drug (bars labeled '+ZV'). After the incubation period, cells were permeabilized and stained with propidium iodide as described. Results are shown with the background levels from corresponding controls already subtracted. PIOG, pioglitazone.

this group of MM lines. We evaluated a completely different group of five MM lines, one of which is dependent upon autocrine IL-6 (U266B1), and found somewhat higher IC_{50} values for all five of these MM lines with pioglitazone than that what was reported for troglitazone [22], but no significant differences between the autocrine IL-6-dependent U266B1 and the four IL-6-independent lines. As with the epithelial cancer and NHL cell lines, all five MM lines had significantly lower IC_{50} values with both GW9662 and T0070907 compared with the agonist. These values were fairly similar across all five MM lines with T0070907, whereas the U266B1 line was somewhat less sensitive to the other antagonist, GW9662. We also found that three of the four IL-6-independent MM lines tested expressed higher levels of PPARγ than the IL-6-dependent line. In addition, the effect of exogenous IL-6 on the sensitivity of two of the IL-6-independent MM lines to the PPARγ antagonists was assessed. Exogenous IL-6 did not decrease the sensitivity of these MM lines to the growth inhibitory effects of these agents. There was even a trend towards lower IC_{50} values in the presence of IL-6 with T0070907. When considered alongside the previous report with other MM lines [22], our results indicate greater heterogeneity in PPARγ expression and responses to PPARγ ligands in MM lines and also greater potential for clinical translation. Overall, the greater sensitivity of these MM lines (four of which are IL-6 independent) to the antagonists as well as the lack of effect of exogenous IL-6, in contrast to the cytoprotection typically conferred by this cytokine, makes agents in this class promising therapeutic candidates for clinical translation in MM as well as other cancer types.

Preclinical in-vivo data suggest that drugs of the PPARγ antagonist class are safe and have predictable and potentially beneficial metabolic effects. Very high doses of another PPARγ antagonist (SR-202; 400 mg/kg) have been safely administered to wild-type mice as well as two other mouse strains with alterations in metabolic function (PPARγ +/– hemizygous and ob/ob mice) for a period of 20 days. In addition to being safe, beneficial effects of treatment in terms of reducing adipogenesis and insulin resistance were observed [30]. Thus, further evaluation of this drug class should also be carried out in animal models of cancer. The additive, sequence-dependent

increase in growth inhibition observed in the studies reported herein suggests that the combinations of an antagonist with an agonist should be explored for the treatment of cancer.

In summary, our results clearly demonstrate that the pure PPARγ antagonists, GW9662 and T0070907, have significantly greater antiproliferative potency for a broad array of cancer cell lines (including epithelial and hematopoietic lines) than an agonist drug, pioglitazone. Not only are the corresponding IC_{50} values for the antagonists significantly lower than the agonist, pioglitazone, but a greater degree of growth inhibition (> 85% versus 50–80%) was observed with these drugs at concentrations only 2–3-fold greater than the IC_{50} in panel of cell lines that included cell lines with relative and near-total chemoresistance (colon and renal cell, respectively). The lack of a cytoprotective effect of IL-6 against the cytotoxic effects of this class of agents also indicates their potential clinical utility, given the importance of this cytokine in the growth of MM and multiple other cancer types. Thus, members of this novel drug class are strong candidates for further development for therapy of a wide range of malignancies.

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