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PPAR γ -independent induction of growth arrest and apoptosis in prostate and bladder carcinoma

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Abstract

Background: Although PPAR γ antagonists have shown considerable pre-clinical efficacy, recent studies suggest PPAR γ ligands induce PPAR γ -independent effects. There is a need to better define such effects to permit rational utilization of these agents.

Methods: We have studied the effects of a range of endogenous and synthetic PPAR γ ligands on proliferation, growth arrest (FACS analysis) and apoptosis (caspase-3/7 activation and DNA fragmentation) in multiple prostate carcinoma cell lines (DUI45, PC-3 and LNCaP) and in a series of cell lines modelling metastatic transitional cell carcinoma of the bladder (TSU-Pr1, TSU-Pr1-B1 and TSU-Pr1-B2).

Results: 15-deoxy-prostaglandin J₂ (15dPGJ₂), troglitazone (TGZ) and to a lesser extent ciglitazone exhibited inhibitory effects on cell number; the selective PPAR γ antagonist GW9662 did not reverse these effects. Rosiglitazone and pioglitazone had no effect on proliferation. In addition, TGZ induced G₀/G₁ growth arrest whilst 15dPGJ₂ induced apoptosis.

Conclusion: Troglitazone and 15dPGJ₂ inhibit growth of prostate and bladder carcinoma cell lines through different mechanisms and the effects of both agents are PPAR γ -independent.

Background

The transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ) has sparked significant interest in the cancer field owing to observations of increased expression in several diverse carcinomas [1-4]. Moreover, endogenous and synthetic PPAR γ agonists elicit notable growth inhibitory effects *in vitro* and *in vivo* (colon, breast and prostate carcinomas) and are capable of preventing metastasis [2,5-9]. PPAR γ is a member of the family of

nuclear hormone receptors and exists as two isoforms (PPAR γ 1 and PPAR γ 2) encoded by multiple transcript variants [10]. PPAR γ 1 is the predominant isoform in humans and is highly expressed in adipose tissue and liver [8,10]. Historically, PPAR γ is recognized as a critical transcription factor in the regulation of adipocyte differentiation and genes involved in energy storage and utilisation [11], and PPAR γ agonists (rosiglitazone, pioglitazone) are currently in clinical use for the treatment of Type II diabetes [12].

Several high affinity ligands are routinely used to investigate PPAR γ -mediated effects, including the family of synthetic thiazolidinedione ligands (rosiglitazone, RGZ; pioglitazone, PGZ; ciglitazone, CGZ; troglitazone, TGZ; listed from highest to lowest affinity (K_d 30–750 nM) for PPAR γ) and the endogenous ligand 15-deoxy-prostaglandin J_2 (15dPGJ2), a metabolite of prostaglandin D_2 ($K_d \approx 300$ nM) [13,14]. While previous studies demonstrate that some PPAR γ ligands inhibit growth of multiple carcinoma cell lines [6,15-17], many reports demonstrate that PPAR γ ligand-mediated growth inhibition can vary depending on the cancer type. In astrogloma, colorectal, and hepatocellular carcinoma, as well as in astrocytes and preadipocytes, growth inhibition can be limited to a particular member of the thiazolidinedione family [18-21], and has been shown to be selective for the endogenous PPAR γ ligand, 15dPGJ2, over members of the thiazolidinedione family [22]. Indeed, even within a carcinoma type, growth inhibition induced by PPAR γ ligands can be cell line specific [23]. In addition, studies utilising the irreversible PPAR γ -selective antagonist GW9662, have revealed PPAR γ -dependent and -independent mechanisms of growth inhibition [21,24-26], further highlighting the incongruity of responsiveness between cancer types.

The high incidence of prostate and bladder carcinoma poses a significant health risk for men, including Australian and North American males. Interestingly, the expression of PPAR γ is increased with grade and advancement of disease [1,3]. In clinical trials of patients with advanced prostate cancer, treatment with TGZ has resulted in prolonged stabilisation of prostate specific antigen (PSA) levels [28,29]. Although genetic deficiency of PPAR γ does not alter the development of experimental prostate cancer [30], individual PPAR γ ligands have been shown to inhibit *in vitro* cellular proliferation of both human bladder and prostate carcinoma cell lines [1,2,31-34]. In prostate carcinoma cell lines the antiproliferative effects of these agonists have also been associated with morphological changes indicative of terminal differentiation [34] and a less malignant phenotype [2].

The antineoplastic properties of the thiazolidinedione ligands have offered promise in clinical and pre-clinical studies of prostate carcinoma, but are yet to be fully characterised in bladder carcinoma. In other cancer types, it is becoming evident that growth inhibition can be ligand dependent, and cannot always be attributed to PPAR γ activation. These studies also illustrate that the specific PPAR γ agonist utilised critically determines the outcome. In this study we have utilized a broad range of PPAR γ agonists and the PPAR γ -selective antagonist GW9662, to examine the effect of PPAR γ activation in prostate and bladder carcinoma cell lines.

Methods

Cell lines and reagents

TSU-Pr1, DU145, PC-3 and LNCaP cell lines were obtained from Dr Dan Djakiew, Georgetown University, USA [35]; TSU-Pr1-B1 and TSU-Pr1-B2 showing increasing metastatic potential were generated in our laboratory by *in vivo* selection to bone; HL60 cell line was obtained from the Peter McCallum Cancer Centre, Australia; 3T3-L1 cell line was obtained from the American Type Culture Collection (Rockville, MD). All cell lines were cultured in DMEM (Gibco, Australia) supplemented with 10% foetal bovine serum (FBS, JRH Biosciences, Australia). Rosiglitazone (RGZ), troglitazone (TGZ), pioglitazone (PGZ), ciglitazone (CGZ), 15dPGJ2 and GW9662 were purchased from Sapphire Biosciences (Australia) and were dissolved in dimethylsulfoxide (DMSO) (Sigma, Australia). Doxorubicin (Dox) and Sulforhodamine B (SRB) were purchased from Sigma.

qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Australia) as per the manufacturer's instructions. RNA was reverse transcribed (Superscript II; Invitrogen, Australia) to cDNA using random hexamers (Invitrogen, Australia). Quantitative RT-PCR was performed on an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems; Australia) in 10 mM Tris-HCl pH 8.0, 2.5 mM Mg(C₂H₃O₂)₂, 50 mM KCl, 200 μ M dNTPs, 1/40,000 dilution of SYBR Green I (Molecular Probes; Eugene, Oregon USA), 1 μ g/ml 6-carboxy-X-rhodamine (6-ROX; Molecular Probes), 8% DMSO, 200 nM primers and 0.625 U AmpliTaq Gold polymerase (Applied Biosystems) per 25 μ L reaction. Levels of PPAR γ 1,3 transcript variants (NM_138712 and NM_138711; primers: 5'-CCATTTTCTCAAACGAGAGTCAGCCTTT-3' and 5'-CTCTGTGTCAACCATGGTCATTCGTT-3'), PPAR γ 2 transcript variant (NM_015869; primers: 5'-GATGTCTTGACTCATGGGTGTATTACAAA-3' and 5'-GTTTGCAGACAGTGTATCAGTGAAGGAA-3') and PPAR γ 4 transcript variant (NM_005037; primers: 5'-CGCCGTGGCCGCAGAAAT-3' and 5'-GATCCACG-GAGCTGATCCCAA-3') were measured. Each primer set gave rise to predicted sized amplicons of 55, 202 and 80 bp respectively. L32 (NM_000994; primers: 5'-CAGGGTTCGTAGAAGATTCAAGGG-3' and 5'-CTTGGAGGAA-CATTGTGAGCGATC-3'; amplicon: 190 bp), a ribosome associated protein, was used as a housekeeping gene to normalise all samples as previously described [36]. All reactions were performed in quadruplicate. PCR conditions were 95°C, 10 min followed by 50 cycles of reaction at 95°C, 15 s, 60°C, 1 min. Specificity of PCR products was determined by dissociation curve analysis as recommended by the manufacturer (ABI) and products were confirmed to be single bands by visualisation following gel electrophoresis. The comparative CT method was vali-

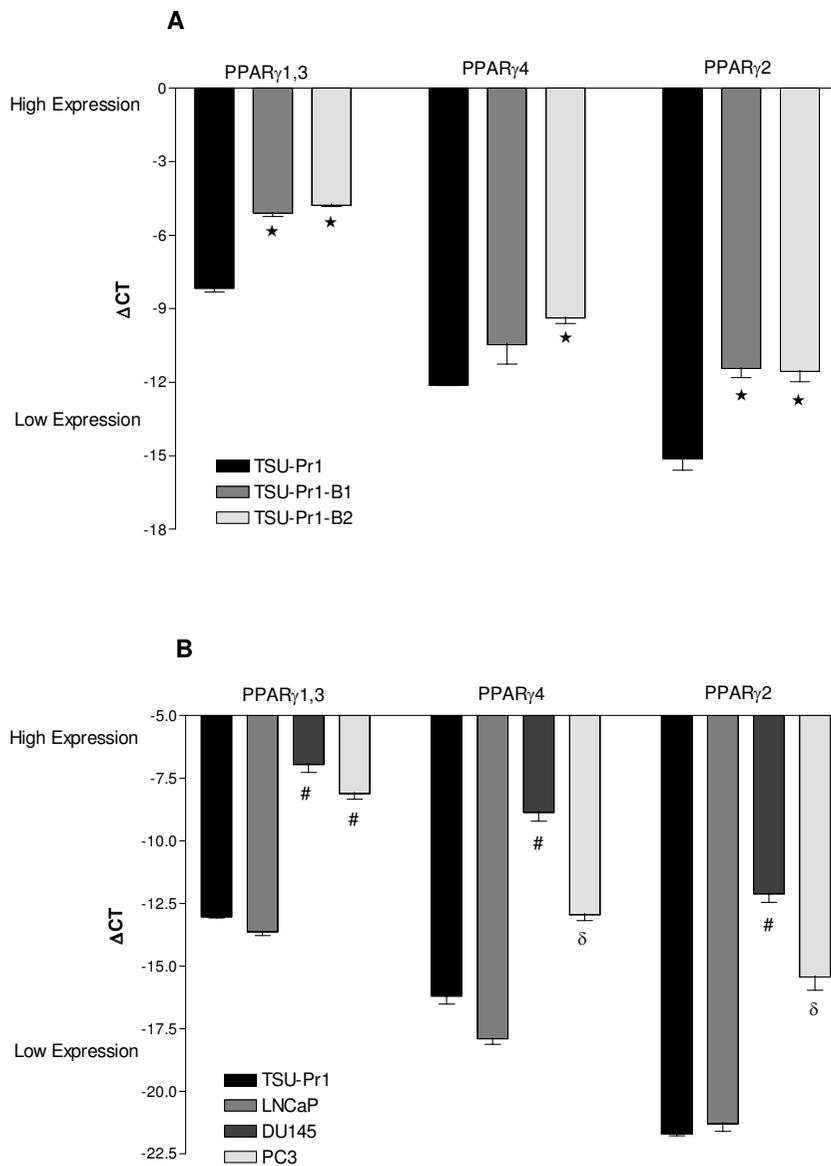


Figure 1
Detection of PPAR γ isoforms in bladder (TSU-Pr1, TSU-Pr1-B1, TSU-Pr1-B2) and prostate (DU145, PC-3 and LNCaP) cell lines. PPAR γ I (encoded by PPAR γ variants 1, 3 and 4) and PPAR γ 2 isoforms were measured by qRT-PCR. Δ CT values were derived by normalisation to levels of the L32 housekeeping gene in each cDNA sample. * Significantly different to TSU-Pr1 ($p < 0.001$), # significantly different to TSU-Pr1 and LNCaP, ($p < 0.01$), δ significantly different to DU145 ($p < 0.03$) (unpaired t test)

dated by assessing the amplification efficiency of each of the primer sets listed above in a single PCR run as per the manufacturer's recommendations (ABI).

Proliferation analysis

Cell number was estimated using the SRB assay. Cells were seeded in 96-well plates at a starting density of 1×10^3 cells/well and treated with PPAR γ ligand or control vehicle (0.2 % DMSO) in the presence or absence of GW9662 (10 μ M) the following day. At days 0, 1, 3, 5, 7 and 9, cells were fixed with 25 μ L cold (4°C) 50% trichloroacetic acid for 1 hr at 4°C. Cells were stained with 100 μ L SRB (0.4 g/100 mL in 1% acetic acid) for 15 minutes at room temperature (RT), washed with 1% acetic acid and left to dry overnight. Bound SRB was resuspended in 10 mM Tris base (pH 10.5) and read at 550 nm on a Labsystems Multiskan RC spectrophotometer.

Cell cycle analysis

Cells were seeded in six 10 cm dishes at varying densities according to ligand treatment (1×10^5 cells/plate; control, RGZ 10 μ M, TGZ 50 μ M and 15dPGJ2 5.6 μ M and 3×10^5 cells/plate; TGZ 100 μ M and 15dPGJ2 10 μ M) and after 24 hrs media was replaced with fresh media containing the specific PPAR γ ligand treatment or control vehicle (0.1% DMSO) for 72 hrs. Floating and adherent cells were combined, pelleted (400 g), washed in phosphate buffered saline (PBS) and resuspended in 200 μ L PBS containing 0.1% FBS. The cell suspension was diluted 10-fold in ice-cold 90% ethanol and placed at -20°C for 15 minutes. Cells were then washed twice with 0.1% FBS in PBS and resuspended in RNase (2 μ g/mL) (Roche, Australia) and 5 mg/mL Propidium Iodide (Sigma). Flow cytometric analysis of DNA content was performed using a FACScalibur (Becton Dickinson) with Cellquest software (Becton Dickinson) and analysed using Modfit cell cycle software (Verity Software House, Maine) as previously described

Determination of DNA fragmentation

DNA was extracted using the Apoptotic DNA Ladder Kit (Roche), according to the manufacturer's instructions. Briefly, cells were seeded in 10 cm dishes and after 24 hrs media was replaced with fresh media containing the specific PPAR γ ligand, doxorubicin (Dox, 1 μ M) or control vehicle (0.1% DMSO) for 72 hrs. Floating and adherent cells were combined, lysed, applied to a column, and the DNA eluted. Samples were treated with RNase (2 μ g/mL, 15 mins, RT). DNA was run on a 1% agarose gel containing ethidium bromide and visualised on a UV transilluminator (Promega, Australia).

Caspase activation

Levels of active caspase-3/7 after ligand treatment were measured using the CaspaTag™ Caspase-3/7 *In situ* Assay

Kit (Chemicon International, Australia), according to the manufacturer's instructions. Briefly, 1×10^6 cells (Control, RGZ 10 μ M, TGZ 50 μ M and 15dPGJ2 5.6 μ M) and 3×10^6 cells (TGZ 100 μ M, 15dPGJ2 10 μ M, Dox 1 μ M) were seeded in 15 cm dishes and treated for 48 hrs to induce apoptosis. Cells were then exposed to a carboxyfluorescein-labeled fluoromethyl ketone inhibitor of caspase-3/7 (FAM-DEVD-FMK) and transferred to a microtiter plate. Levels of active caspase-3/7 were measured by reading the fluorescence of each microwell with excitation (485 nm) and emission (535 nm) wavelengths using a Wallac 1200 spectrophotometer.

Statistical analysis

Results are expressed as mean \pm standard error (SEM) from 2–3 experiments performed in triplicate. Unpaired t test or ANOVA followed by the Dunnett post test were used to determine statistical significance. $P < 0.05$ was considered significant.

Results

Levels of PPAR γ mRNA in bladder and prostate cell lines

We examined the expression of PPAR γ in TSU-Pr1 and sublines with increasing metastatic potential (Figure 1A). PPAR γ 1,3, PPAR γ 2 and PPAR γ 4 transcript variants were detected in all cell lines. PPAR γ 1,3 was expressed at the highest levels in all three cell lines, followed by PPAR γ 4 (these transcript variants detect PPAR γ isoform 1). PPAR γ 2 (PPAR γ isoform 2) was expressed at the lowest level in all three cell lines. Interestingly, the levels of both PPAR γ isoforms were significantly up-regulated in the more metastatic sublines TSU-Pr1-B1 and TSU-Pr1-B2 compared to TSU-Pr1 (with the exception of the PPAR γ 4 levels in TSU-Pr1-B1 compared to TSU-Pr1).

The prostate cell lines DU145, PC-3 and LNCaP were also examined for PPAR γ expression and compared to expression levels in TSU-Pr1 (Figure 1B). The levels of all transcript variants were similar in TSU-Pr1 and LNCaP cell lines with PPAR γ 1,3 expressed at the highest level and PPAR γ 2 at the lowest level. All transcript variants were expressed at significantly higher levels in DU145 and PC-3 cell lines compared to TSU-Pr1 and LNCaP cell lines. In addition, the expression level of PPAR γ 4 and PPAR γ 2 were significantly higher in DU145 cells compared to the PC-3 cell line.

Thiazolidinedione ligands and 15dPGJ2 decrease cell number in bladder cell line TSU-Pr1

The effects of TGZ (50 and 100 μ M), CGZ (40 μ M), RGZ (10 μ M), PGZ (20 μ M) and 15dPGJ2 (5.6 and 10 μ M) on the TSU-Pr1 bladder carcinoma cell line were examined in the presence or absence of the highly selective PPAR γ antagonist GW9662 (10 μ M). This concentration of GW9662 inhibited TGZ- and RGZ-induced lipid accumu-

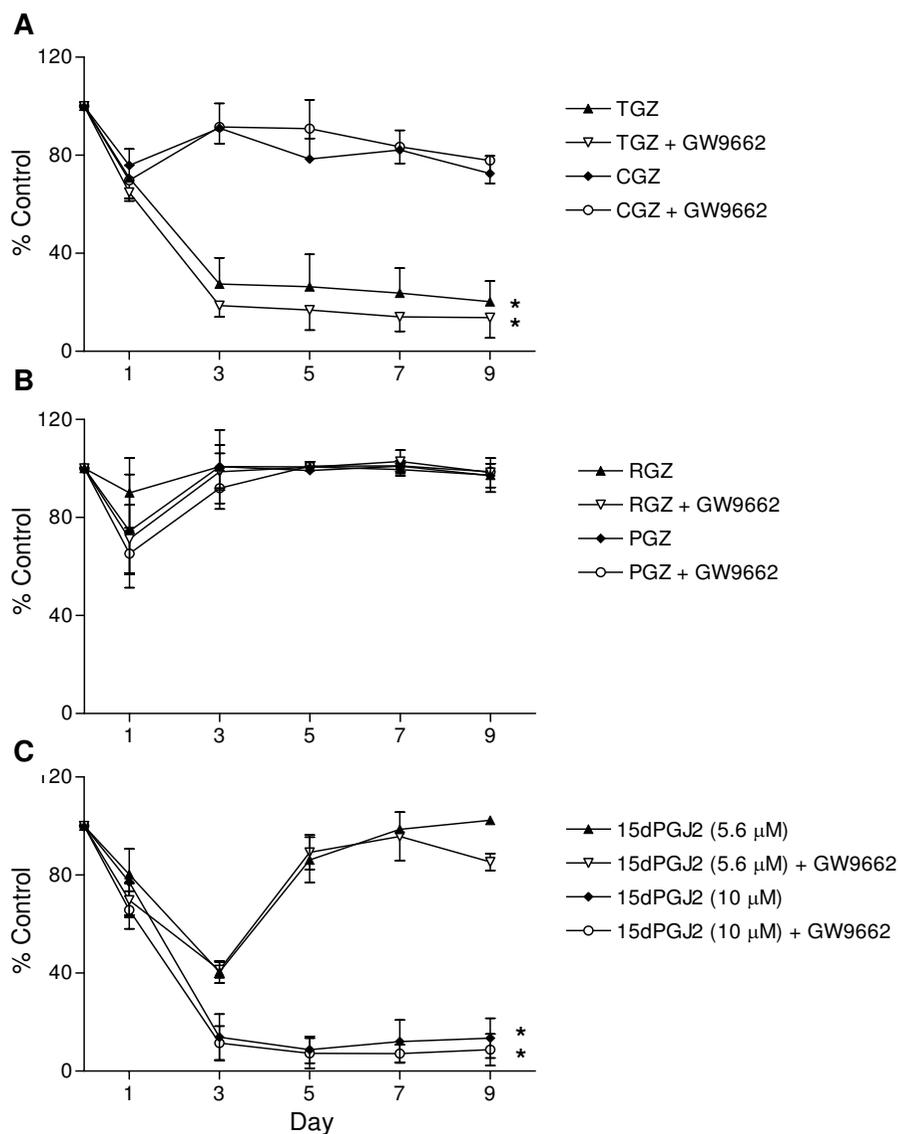


Figure 2
Effect of PPAR γ ligands on TSU-Pr1 cell number. Cells were treated with ligand either alone or in the presence of 10 μ M GW9662 at Day 0. A, Troglitazone (100 μ M; TGZ) and ciglitazone (40 μ M; CGZ). B, Rosiglitazone (10 μ M; RGZ) and pioglitazone (20 μ M; PGZ). C, 15-deoxy-prostaglandin J₂ (15dPGJ₂) 5.6 μ M and 10 μ M. Values are mean \pm SEM of 2–3 experiments performed in triplicate expressed as percent control. Statistical significance * P < 0.01 (ANOVA followed by Dunnett post test).

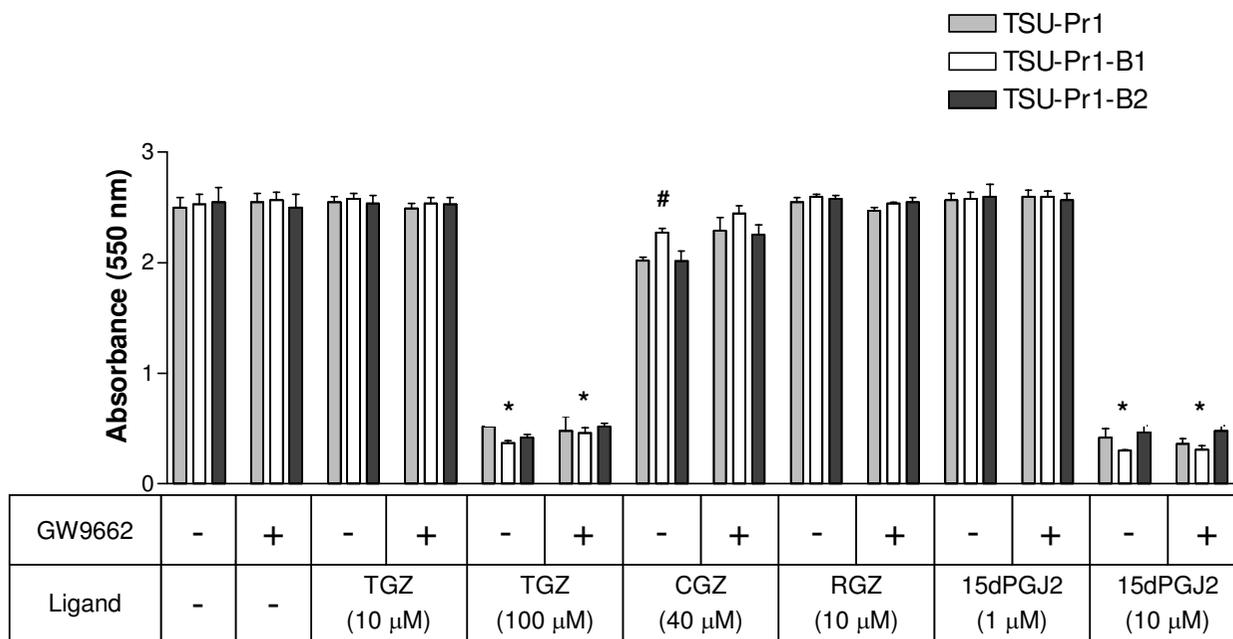


Figure 3
Effect of PPAR γ ligands on cell number of TSU-Pr1/TSU-Pr1-B1/TSU-Pr1-B2 series day 5. TSU-Pr1, TSU-Pr1-B1 and TSU-Pr1-B2 cells were treated with control (0.2% DMSO), GW9662 (10 μ M), troglitazone (10 and 100 μ M; TGZ), ciglitazone (40 μ M; CGZ), rosiglitazone (10 μ M; RGZ) and 15-deoxy-prostaglandin J2 (1 and 10 μ M; 15dPGJ2) in the presence or absence of antagonist GW9662 (10 μ M) at day 0. Values are mean \pm SEM from Day 5. * All cell lines significantly different to the respective controls, # TSU-Pr1 and TSU-Pr1-B2 significantly different to the respective controls (unpaired t test).

lation in differentiating NIH3T3-L1 cells (data not shown). The effects of ligand treatment on cell number were determined at days 0, 1, 3, 5, 7 and 9. TGZ (100 μ M) induced a marked inhibition of cell number (75% inhibition at day 3), which was sustained throughout the time course of the experiment (Figure 2A). TGZ had no effect on cell number at a lower dose (50 μ M) (data not shown). CGZ caused a small decrease (approximately 25%) in cell number at day 1, which subsided to a modest 10–15 % inhibition over days 3 to 9 (Figure 2A). The more potent PPAR γ ligands RGZ and PGZ did not affect TSU-Pr1 cell number (Figure 2B). The endogenous PPAR γ ligand 15dPGJ2 exhibited a potent and sustained effect on cell number at 10 μ M (92% inhibition at day 3). At a lower dose (5.6 μ M), 15dPGJ2 initially inhibited cell number (60% at day 3), however this effect was no longer evident by day 5 (Figure 2). It is possible that 15dPGJ2 is either breaking down or being metabolised such that the 5.6 μ M

treatment is unable to maintain suppression of proliferation over the course of the experiment. The growth inhibitory effects of TGZ, CGZ and 15dPGJ2 on cell number were not reversed by 10 μ M GW9662 (Figure 2A–C). Furthermore, at doses ranging from 1 to 100 μ M, GW9662 had no effect on TGZ-induced growth inhibition of TSU-Pr1 cells (data not shown).

Thiazolidinedione ligands and 15dPGJ2 decrease cell number in TSU-Pr1-B1 and TSU-Pr1-B2 sublines

The effects of thiazolidinedione agonists, 15dPGJ2 and GW9662 were examined in the TSU-Pr1 sublines that have significantly increased expression of PPAR γ . TGZ (10 and 100 μ M), CGZ (40 μ M), RGZ (10 μ M), 15dPGJ2 (1 and 10 μ M) were applied to the cells in the presence or absence of GW9662 (10 μ M). Cell number was determined over a 9 day period and the results from Day 5 have been represented (Figure 3). As was observed in TSU-Pr1

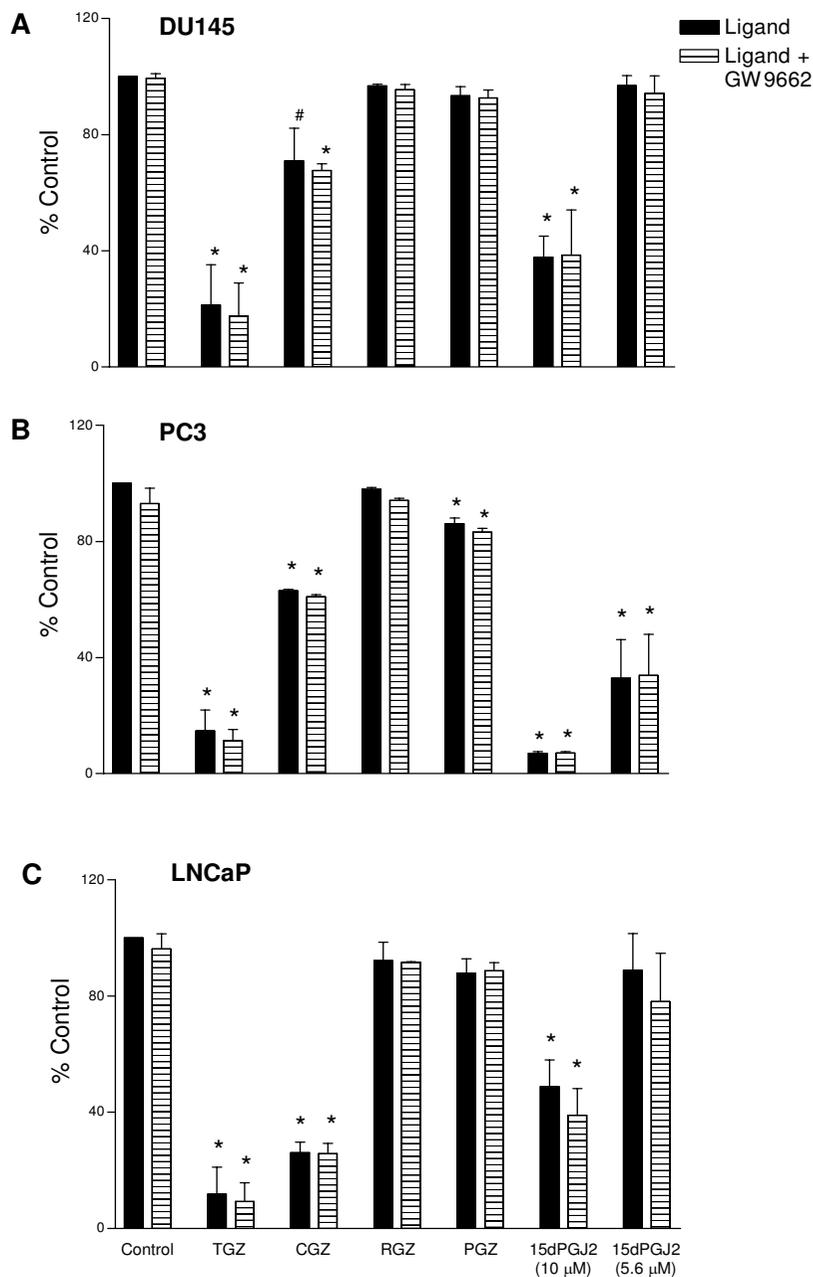
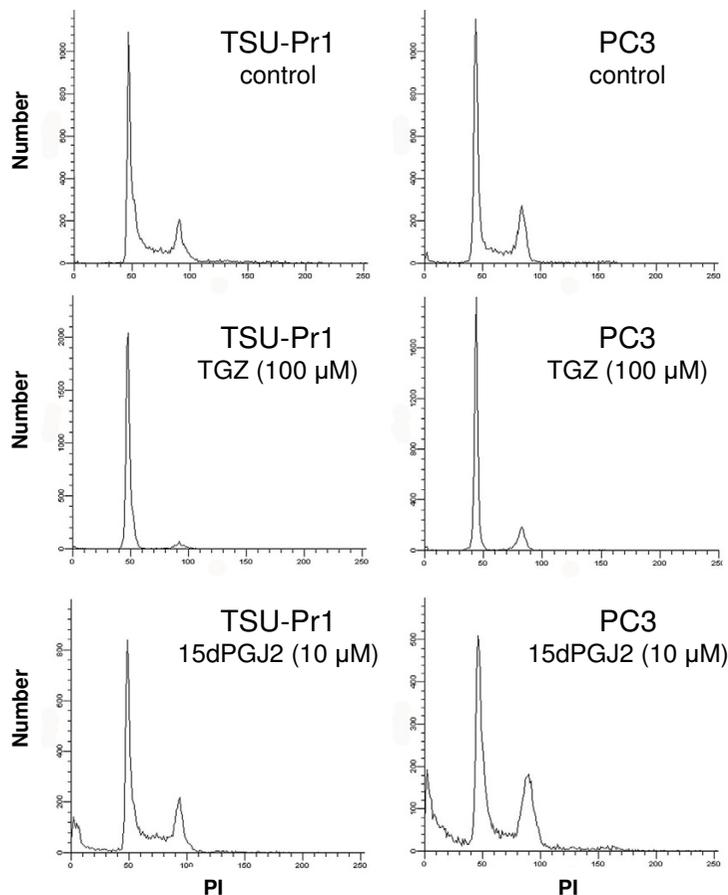


Figure 4

Effect of PPAR γ ligands on cell number of prostate cell lines at day 5. Cells were treated with control (0.2% DMSO), troglitazone (100 μ M; TGZ), ciglitazone (40 μ M; CGZ), rosiglitazone (10 μ M; RGZ), pioglitazone (20 μ M; PGZ) and 15-deoxy-prostaglandin J2 (5.6 and 10 μ M; 15dPGJ2) in the presence or absence of antagonist GW9662 (10 μ M) at day 0. A, DU145 cells; B, PC-3 cells; C, LNCaP cells. Values are mean \pm SEM of two experiments performed in triplicate expressed as percent control. No significant differences were detected following GW9662 treatment for any agent. Statistical significance compared to control * $P < 0.01$, # $P < 0.05$ (ANOVA followed by Dunnett post test).



		Proportion of cells			
		G ₁	G ₂	S	Apoptotic
Tsu-Pr1	Control	47.64	3.41	48.94	0.43
	RGZ 10 μ M	48.57	6.41	45.01	5.66
	TGZ 50 μ M	85.88	0.00	14.12	1.44
	TGZ 100 μ M	95.68	0.00	4.32	1.63
	15dPGJ2 5.6 μ M	52.09	1.15	46.75	1.54
	15dPGJ2 10 μ M	46.68	5.08	48.24	11.00
PC3	Control	60.12	3.84	36.04	2.04
	RGZ 10 μ M	65.68	3.06	31.26	1.05
	TGZ 50 μ M	87.27	3.02	9.71	2.29
	TGZ 100 μ M	91.19	1.58	7.23	1.21
	15dPGJ2 5.6 μ M	74.62	3.97	21.41	7.17
	15dPGJ2 10 μ M	53.01	14.06	32.93	22.59

Figure 5

Effect of PPAR γ ligands on cell cycle in TSU-Pr1 and PC-3 cells. Cells were cultured for 3 days with control (0.1% DMSO), troglitazone (50 and 100 μ M; TGZ), 15-deoxy-prostaglandin J₂ (5.6 and 10 μ M; 15dPGJ2) or rosiglitazone (10 μ M; RGZ). The effect of these ligands on the proportion of cells in each phase of the cell cycle was determined. Data shown is representative of two experiments.

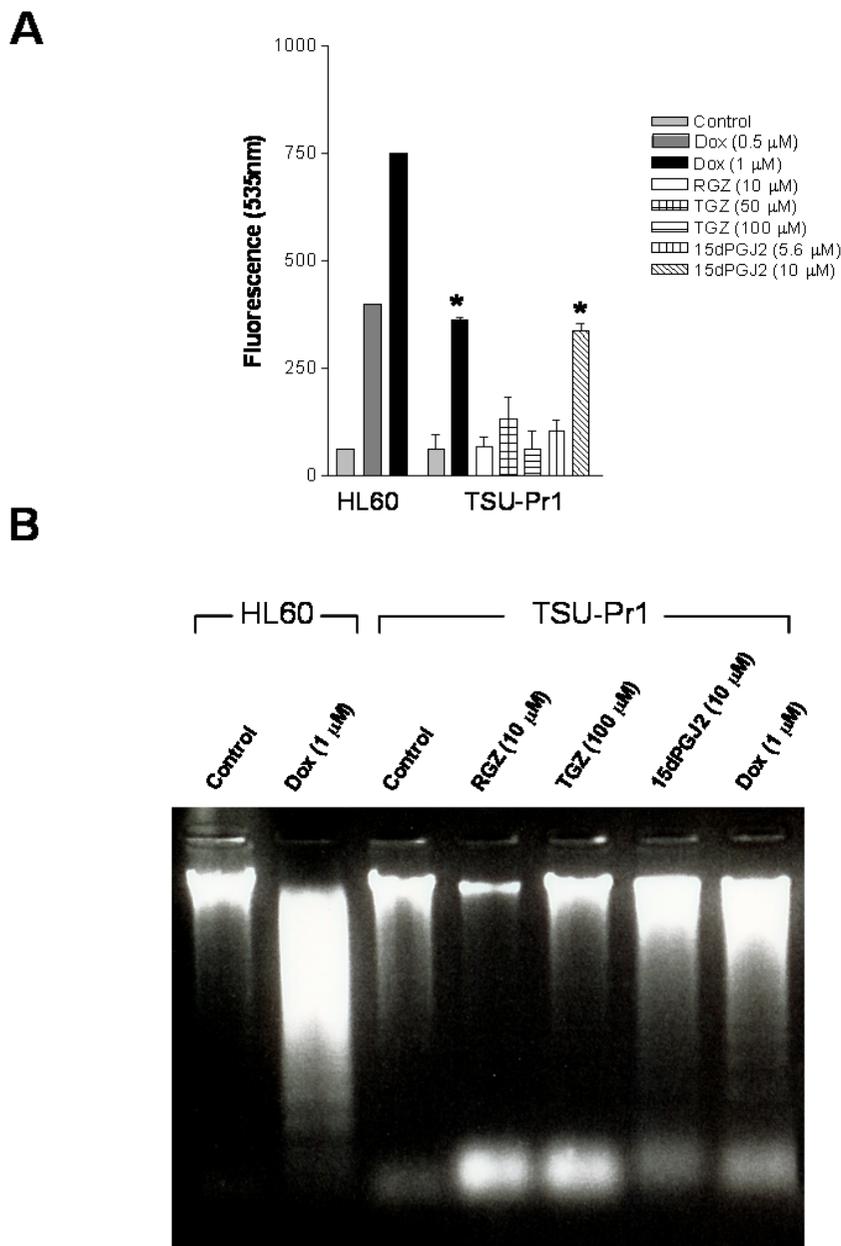


Figure 6
Effect of PPAR γ ligands on apoptosis. The effect of PPAR γ ligands on apoptosis was measured using caspase-3/7 activation (A) and DNA fragmentation (B). A, HL60 and TSU-Pr1 cells were treated with either control (0.1% DMSO), Doxorubicin (0.5 or 1 μ M; Dox). TSU-Pr1 cells were also treated with troglitazone (50 and 100 μ M; TGZ), 15-deoxy-prostaglandin J₂ (5.6 and 10 μ M; 15dPGJ2) or rosiglitazone (10 μ M; RGZ) for 48 hrs. Levels of activated caspase-3/7 were determined by fluorescence measured at 535 nm. Values are mean \pm SEM of 2 experiments performed in triplicate. B, HL60 and TSU-Pr1 cells were treated with Doxorubicin (1 μ M; Dox), troglitazone (100 μ M; TGZ), 15-deoxy-prostaglandin J₂ (10 μ M; 15dPGJ2) or rosiglitazone (10 μ M; RGZ) for 72 hrs. DNA was extracted and run on a 1% agarose gel. Statistical significance * $P < 0.01$, different to TSU-Pr1 control (ANOVA followed by Dunnett post test).

cells (Figure 2 and Figure 3), TGZ (100 μ M) and 15dPGJ2 (10 μ M) dramatically inhibited growth in TSU-Pr1-B1 and TSU-Pr1-B2 cells. There was no difference in the extent of growth inhibition caused by these ligands between the three cell lines despite varying PPAR γ expression levels. Again these effects were not reversed by GW9662.

Thiazolidinedione ligands and 15dPGJ2 decrease cell number in prostate cell lines

The effects of thiazolidinedione agonists, 15dPGJ2 and PPAR γ antagonist GW9662 were further examined in a series of prostate cell lines (DU145, PC-3 and LNCaP). LNCaP cells were plated at a higher density (3×10^3 cells/well) due to their slower mitotic rate. The effects of these ligands have been shown at day 5, when maximal inhibition was observed (Figure 4). Patterns of growth inhibition similar to those seen in TSU-Pr1 cells (Figure 2 and Figure 3) were observed in all prostate carcinoma cell lines. TGZ (100 μ M) and 15dPGJ2 (10 μ M) treatment had a marked inhibitory effect on cell number (Figure 4A-C). PC-3 cells were more sensitive to the 15dPGJ2 treatment as evidenced by a significant inhibition at the lower dose of 15dPGJ2 (5.6 μ M) (Figure 4B), an effect which was not observed in either DU145 (Figure 4A) or LNCaP cell lines (Figure 4C) and only partially seen with TSU-Pr1 (Figure 2 and Figure 3). All prostate carcinoma cell lines showed a significant inhibition of cell number in response to CGZ treatment at day 5, an effect that was most pronounced in LNCaP cells (Figure 4) whereas only a slight effect of CGZ was noted in TSU-Pr1 cells (Figure 2 and Figure 3). As observed in TSU-Pr1 cells, the inhibitory effects exerted by these agonists were not reversed by the addition of the PPAR γ antagonist GW9662 (Figure 4) and the thiazolidinedione compounds with the highest affinity for PPAR γ (RGZ and PGZ) showed no effect (data not shown).

TGZ and 15dPGJ2 exert differential effects on cell cycle and apoptosis

Given the similar response profiles to the different PPAR γ ligands in all bladder and prostate carcinoma cell lines examined, the effect of TGZ, 15dPGJ2 and RGZ on cell cycle regulation was examined in more detail in TSU-Pr1 and PC-3 cell lines (Figure 5). TGZ produced a dose-dependent cell cycle arrest in G₁ phase both TSU-Pr1 and PC-3 cell lines. In contrast to this 15dPGJ2 dose dependently increased the proportion of subG₁ cells, accompanied by an arrest in G₂ phase in TSU-Pr1 and PC-3 cell lines. The effect of 5.6 μ M 15dPGJ2 on the proportion of cells in the subG₁ peak was more marked in PC-3 cells. A G₁ arrest was observed in response to RGZ treatment in both cell lines. In addition a small increase in the proportion of cells in the subG₁ peak was observed in response to RGZ treatment in TSU-Pr1 cells.

TGZ and 15dPGJ2 alter levels of caspase-3/7 activation and DNA fragmentation

Doxorubicin (Dox) induces apoptosis in the human myeloid leukaemia cell line HL60 [37] and was used as a positive control for both caspase-3/7 activation and DNA fragmentation (Figure 6A and 6B). Induction of apoptosis was investigated by measurement of levels of caspase-3/7 activation after PPAR γ ligand treatment in TSU-Pr1 (Figure 6A) and PC-3 (data not shown) cell lines. Both Dox (1 μ M) and 15dPGJ2 (10 μ M) induced significant caspase-3/7 activation in TSU-Pr1 (Figure 6A) and PC-3 cells, while TGZ (100 μ M) induced a small but significant increase in caspase-3/7 activation in PC-3 cells. DNA fragmentation was examined to further investigate the induction of apoptosis. Dox induced DNA fragmentation in the HL60 and TSU-Pr1 cell lines (Figure 6B) after 72 hours of ligand treatment. DNA laddering was induced by 15dPGJ2 (10 μ M) treatment in TSU-Pr1 (Figure 6B) and PC-3 (data not shown) cell lines. TGZ (100 μ M) did not induce DNA laddering in any sample examined. RGZ (10 μ M) did not induce measurable caspase 3/7 activation or DNA laddering in either TSU-Pr1 (Fig 6) or PC-3 (data not shown) cell lines suggesting that the increase in the proportion of cells in the subG₁ peak of RGZ (10 μ M) in TSU-Pr1 cells observed in Fig 5 may have been due to cells undergoing necrosis.

Discussion

Thiazolidinedione ligands have been shown to inhibit the growth of multiple cancer cell lines [2,6,17,38], however it is becoming apparent that these antineoplastic effects cannot always be attributed to PPAR γ activation. Reports of PPAR γ ligand induced growth inhibition demonstrate three important points: i) Growth inhibition can be specific to members of the thiazolidinedione family [18,19] ii) Growth inhibition can be selective for an endogenous PPAR γ ligand compared to synthetic ligand [22], iii) PPAR γ ligand mediated growth inhibition is PPAR γ -independent in some cancer types [24,39]. Clearly the response to PPAR γ ligands and the involvement of PPAR γ in these effects differs widely amongst carcinoma types and thus each carcinoma type must be specifically examined. The current study investigated the response to an array of thiazolidinedione agonists and an endogenous PPAR γ agonist, 15dPGJ2, in bladder and prostate carcinoma cell lines of differing metastatic potential. In addition we have utilised the selective PPAR γ antagonist GW9662 to elucidate the involvement of this receptor in the growth inhibitory effects observed.

We detected profound growth inhibition in response to TGZ and 15dPGJ2 in all bladder and prostate carcinoma cell lines. These results are consistent with the growth inhibitory effects reported by Yoshimura et al. albeit that in our hands 15dPGJ2 generally exerted more potent

effects on prostate and bladder cell lines while TGZ was comparatively less potent. The different assays or vehicle utilised may account for these observations. We also found that RGZ and PGZ did not inhibit cell number in the bladder or prostate carcinoma cell lines used in this study. We confirmed that at the dose used in this study, RGZ was capable of inducing adipogenesis in NIH3T3-L1 cells. These findings are similar to the little or no effect on cellular growth exerted by RGZ and PGZ in other cancer systems [18,40]. Our results demonstrate that the known affinities of PPAR γ ligands for PPAR γ did not predict their relative potency. In other studies using prostate cancer cell lines, RGZ has been shown to inhibit cell number in PC-3 cells [2,34,41]. From our study it is clear that the thiazolidinedione family are not acting through PPAR γ . When the mechanism is elucidated it will be important to investigate the activity of the relevant pathway in the various PC-3 cell lines held in different laboratories to determine if this will explain the variation in response to RGZ.

In breast cancer, the PPAR γ antagonist GW9662 has recently been used to definitively exclude PPAR γ activation in apoptotic and proliferative responses to selected PPAR γ agonists [24,39]. At a concentration which inhibits RGZ and TGZ induced adipogenesis in 3T3-L1 cells in our hands, GW9662 did not reverse the growth inhibitory effects of TGZ or 15dPGJ2 in any prostate or bladder cell line examined. Our findings demonstrate that the effects induced by TGZ and 15dPGJ2 in prostate and bladder carcinoma are PPAR γ -independent. Whilst these findings are consistent with PPAR γ -independent growth inhibition observed in some cancer types, it has been clearly demonstrated in other cases that thiazolidinedione agonists and 15dPGJ2-induced growth inhibition is PPAR γ -dependent [21,25,26,42,43]. In further support of a PPAR γ -independent mechanism, the TZD ligands and 15dPGJ2 produced comparable growth inhibition in the TSU-Pr1/TSU-Pr1-B1/TSU-Pr1-B2 bladder carcinoma series despite increasing PPAR γ expression. Likewise, the levels of PPAR γ expression varied significantly amongst the prostate cell lines examined and these differing levels did not associate with the observed growth inhibition.

Further to the PPAR γ -independent effects of TGZ and 15dPGJ2 discovered in prostate and bladder carcinoma, our study also demonstrates that TGZ and 15dPGJ2 induce growth inhibition via different mechanisms. TGZ induced cell cycle arrest, whilst 15dPGJ2 treatment resulted in apoptosis. This provides further evidence that these PPAR γ ligands implement unique intracellular signalling pathways. Recently, TGZ-induced apoptosis in PC-3 cell lines was shown to involve reduced association of Bcl-2 and Bcl-xL with Bak, leading to caspase-dependent apoptosis [15]. The capability of TGZ to induce apoptosis in this system may be reflective of the larger percentage of

control cells in G2/M phase of the cell cycle. Thus, TGZ may be able to inhibit the growth of both actively proliferating tumours and tumours with a lower mitotic rate more typically observed in prostate cancer. The growth arrest and DNA damage-inducible gene 45 (GADD45) has been implicated in TGZ-induced apoptosis in breast carcinoma cell lines [40]. Other potential PPAR γ -independent mechanisms that may induce apoptosis include the generation of reactive oxygen species [21] and induction of cellular acidosis [39]. Recently, Chintharlapalli et al. [44] demonstrated that PPAR γ agonists induce apoptosis in colon cancer through PPAR γ receptor independent actions involving early growth response-1 and NSAID-activated-gene-1.

While treatment with thiazolidinedione ligands has elicited both preventative and deleterious effects in colon and breast cancer [7,45,46], in prostate cancer certain thiazolidinedione ligands exhibit promising antineoplastic properties. TGZ inhibits primary prostatic tumour growth in immunodeficient mice, and in short term culture of human prostate tissue induces selective necrosis of cancer cells while sparing the adjacent normal tissue [2]. In addition, TGZ has been shown to stabilise and reduce PSA levels in clinical trials of patients with advanced prostate cancer [5,28,29]. The potential clinical utility of TGZ reinforces the need to understand the mechanism of action of various thiazolidinedione and other PPAR γ ligands. New evidence suggests PPAR γ may play a role in the differentiation of epithelia [47,48], which is an exciting prospect in the field of cancer research. Our data indicates RGZ and PGZ would be useful candidates to study the role of up-regulated PPAR γ in prostate and bladder carcinoma as these ligands do not exhibit PPAR γ -independent growth inhibitory effects. Our current findings demonstrate that 15dPGJ2 and TGZ induce growth arrest in bladder and prostate carcinoma cell lines in a PPAR γ -independent manner, and via distinct mechanisms. Further characterisation and elucidation of the molecular mechanisms underlying the antineoplastic effects of TGZ and 15dPGJ2 will be important for clinical development and utilization of these agents.

Conclusion

PPAR γ is overexpressed in multiple cancer types and the role of this receptor in carcinoma progression has been widely studied using the family of high affinity thiazolidinedione PPAR γ agonists. However, it has recently been demonstrated that PPAR γ ligands can exert PPAR γ -independent biological responses including growth arrest and apoptosis in selected carcinoma types. In this paper we demonstrate not only reduced survival in response to certain PPAR γ ligands, but that these ligands differentially induce growth arrest or apoptosis in bladder and prostate cell lines in a PPAR γ -independent manner. This study

highlights the need to elucidate the distinct PPAR γ -independent mechanisms of action of individual PPAR γ ligands prior to clinical exploration. Our data also suggests that in studying the activation of PPAR γ , careful consideration should be given to the ligand employed.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

CLC conducted all experiments in this paper and drafted the manuscript. DMT designed and aided in the FACS experiments. CLC, DMT, EWT and EDW provided intellectual input and critically reviewed the manuscript.

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References

- Yoshimura R, Matsuyama M, Segawa Y, Hase T, Mitsuhashi M, Tsuchida K, Wada S, Kawahito Y, Sano H, Nakatani T: **Expression of peroxisome proliferator-activated receptors (PPARs) in human urinary bladder carcinoma and growth inhibition by its agonists.** *Int J Cancer* 2003, **104**:597-602.
- Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koeffler HP: **Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo.** *Cancer Res* 1998, **58**:3344-3352.
- Segawa Y, Yoshimura R, Hase T, Nakatani T, Wada S, Kawahito Y, Kishimoto T, Sano H: **Expression of peroxisome proliferator-activated receptor (PPAR) in human prostate cancer.** *Prostate* 2002, **51**:108-116.
- DuBois RN, Gupta R, Brockman J, Reddy BS, Krakow SL, Lazar MA: **The nuclear eicosanoid receptor, PPARgamma, is aberrantly expressed in colonic cancers.** *Carcinogenesis* 1998, **19**:49-53.
- Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA, Hlatky L, Hahnfeldt P, Folkman J, Kaipainen A: **PPAR gamma ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis.** *J Clin Invest* 2002, **110**:923-932.
- Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP: **Ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice.** *Proc Natl Acad Sci U S A* 1998, **95**:8806-8811.
- Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM: **Differentiation and reversal of malignant changes in colon cancer through PPARgamma.** *Nat Med* 1998, **4**:1046-1052.
- Han S, Roman J: **Suppression of prostaglandin E2 receptor subtype EP2 by PPARgamma ligands inhibits human lung carcinoma cell growth.** *Biochem Biophys Res Commun* 2004, **314**:1093-1099.
- Koeffler HP: **Peroxisome proliferator-activated receptor gamma and cancers.** *Clin Cancer Res* 2003, **9**:1-9.
- Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J: **The organization, promoter analysis, and expression of the human PPARgamma gene.** *J Biol Chem* 1997, **272**:18779-18789.
- Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM: **PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro.** *Mol Cell* 1999, **4**:611-617.
- Saltiel AR, Olefsky JM: **Thiazolidinediones in the treatment of insulin resistance and type II diabetes.** *Diabetes* 1996, **45**:1661-1669.
- Kliwer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM: **A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation.** *Cell* 1995, **83**:813-819.
- Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM: **15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma.** *Cell* 1995, **83**:803-812.
- Shiau CW, Yang CC, Kulp SK, Chen KF, Chen CS, Huang JW: **Thiazolidinediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARgamma.** *Cancer Res* 2005, **65**:1561-1569.
- Sato H, Ishihara S, Kawashima K, Moriyama N, Suetsugu H, Kazumori H, Okuyama T, Rumi MA, Fukuda R, Nagasue N, Kinoshita Y: **Expression of peroxisome proliferator-activated receptor (PPAR)gamma in gastric cancer and inhibitory effects of PPARgamma agonists.** *Br J Cancer* 2000, **83**:1394-1400.
- Guan YF, Zhang YH, Breyer RM, Davis L, Breyer MD: **Expression of peroxisome proliferator-activated receptor gamma (PPAR-gamma) in human transitional bladder cancer and its role in inducing cell death.** *Neoplasia* 1999, **1**:330-339.
- Bae MA, Rhee H, Song BJ: **Troglitazone but not rosiglitazone induces G1 cell cycle arrest and apoptosis in human and rat hepatoma cell lines.** *Toxicol Lett* 2003, **139**:67-75.
- Baek SJ, Wilson LC, Hsi LC, Eling TE: **Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity.** *J Biol Chem* 2003, **278**:5845-5853.
- Lennon AM, Ramauge M, Dessouroux A, Pierre M: **MAP kinase cascades are activated in astrocytes and preadipocytes by 15-deoxy-Delta(12-14)-prostaglandin J(2) and the thiazolidinedione ciglitazone through peroxisome proliferator activator receptor gamma-independent mechanisms involving reactive oxygenated species.** *J Biol Chem* 2002, **277**:29681-29685.
- Perez-Ortiz JM, Tranque P, Vaquero CF, Domingo B, Molina F, Calvo S, Jordan J, Cena V, Llopis J: **Glitazones differentially regulate primary astrocyte and glioma cell survival. Involvement of reactive oxygen species and peroxisome proliferator-activated receptor-gamma.** *J Biol Chem* 2004, **279**:8976-8985.
- Nikitakis NG, Siavash H, Hebert C, Reynolds MA, Hamburger AV, Saik JJ: **15-PGJ2, but not thiazolidinediones, inhibits cell growth, induces apoptosis, and causes downregulation of Stat3 in human oral SCCa cells.** *Br J Cancer* 2002, **87**:1396-1403.
- Okano H, Shiraki K, Inoue H, Yamanaka Y, Kawakita T, Saitou Y, Yamaguchi Y, Enokimura N, Yamamoto N, Sugimoto K, Murata K, Nakano T: **15-deoxy-delta-12-14-PGJ2 regulates apoptosis induction and nuclear factor-kappaB activation via a peroxisome proliferator-activated receptor-gamma-independent mechanism in hepatocellular carcinoma.** *Lab Invest* 2003, **83**:1529-1539.
- Clay CE, Monjazebe A, Thorburn J, Chilton FH, High KP: **15-Deoxy-delta12,14-prostaglandin J2-induced apoptosis does not require PPARgamma in breast cancer cells.** *J Lipid Res* 2002, **43**:1818-1828.
- Kim EJ, Park KS, Chung SY, Sheen YY, Moon DC, Song YS, Kim KS, Song S, Yun YP, Lee MK, Oh KW, Yoon do Y, Hong JT: **Peroxisome proliferator-activated receptor-gamma activator 15-deoxy-Delta12,14-prostaglandin J2 inhibits neuroblastoma cell growth through induction of apoptosis: association with extracellular signal-regulated kinase signal pathway.** *J Pharmacol Exp Ther* 2003, **307**:505-517.
- Bruemmer D, Yin F, Liu J, Berger JP, Sakai T, Blaschke F, Fleck E, Van Herle AJ, Forman BM, Law RE: **Regulation of the growth arrest and DNA damage-inducible gene 45 (GADD45) by peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells.** *Circ Res* 2003, **93**:e38-47.

27. **Cancer in Australia 2000.** In: *AIHW & Australasian Association of Cancer Registries 2003.*
28. Mueller E, Smith M, Sarraf P, Kroll T, Aiyer A, Kaufman DS, Oh W, Demetri G, Figg WD, Zhou XP, Eng C, Spiegelman BM, Kantoff PW: **Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer.** *Proc Natl Acad Sci U S A* 2000, **97**:10990-10995.
29. Hisatake JJ, Ikezoe T, Carey M, Holden S, Tomoyasu S, Koeffler HP: **Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer.** *Cancer Res* 2000, **60**:5494-5498.
30. Saez E, Olson P, Evans RM: **Genetic deficiency in Pparg does not alter development of experimental prostate cancer.** *Nat Med* 2003, **9**:1265-1266.
31. Haslmayer P, Thalhammer T, Jager W, Aust S, Steiner G, Ensinger C, Obrist P: **The peroxisome proliferator-activated receptor gamma ligand 15-deoxy-Delta 12,14-prostaglandin J2 induces vascular endothelial growth factor in the hormone-independent prostate cancer cell line PC 3 and the urinary bladder carcinoma cell line 5637.** *Int J Oncol* 2002, **21**:915-920.
32. Nakashiro KI, Hayashi Y, Kita A, Tamatani T, Chlenski A, Usuda N, Hattori K, Reddy JK, Oyasu R: **Role of peroxisome proliferator-activated receptor gamma and its ligands in non-neoplastic and neoplastic human urothelial cells.** *Am J Pathol* 2001, **159**:591-597.
33. Butler R, Mitchell SH, Tindall DJ, Young CY: **Nonapoptotic cell death associated with S-phase arrest of prostate cancer cells via the peroxisome proliferator-activated receptor gamma ligand, 15-deoxy-delta 12,14-prostaglandin J2.** *Cell Growth Differ* 2000, **11**:49-61.
34. Xu Y, Iyengar S, Roberts RL, Shappell SB, Peehl DM: **Primary culture model of peroxisome proliferator-activated receptor gamma activity in prostate cancer cells.** *J Cell Physiol* 2003, **196**:131-143.
35. Djakiew D, Pflug BR, Delsite R, Onoda M, Lynch JH, Arand G, Thompson EW: **Chemotaxis and chemokinesis of human prostate tumor cell lines in response to human prostate stromal cell secretory proteins containing a nerve growth factor-like protein.** *Cancer Res* 1993, **53**:1416-1420.
36. Sharp JA, Waltham M, Williams ED, Henderson MA, Thompson EW: **Transfection of MDA-MB-231 human breast carcinoma cells with bone sialoprotein (BSP) stimulates migration and invasion in vitro and growth of primary and secondary tumors in nude mice.** *Clin Exp Metastasis* 2004, **21**:19-29.
37. McGahon AJ, Costa Pereira AP, Daly L, Cotter TG: **Chemotherapeutic drug-induced apoptosis in human leukaemic cells is independent of the Fas (APO-1/CD95) receptor/ligand system.** *Br J Haematol* 1998, **101**:539-547.
38. Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, Fletcher C, Singer S, Spiegelman BM: **Terminal differentiation of human breast cancer through PPAR gamma.** *Mol Cell* 1998, **1**:465-470.
39. Turturro F, Friday E, Fowler R, Surie D, Welbourne T: **Troglitazone acts on cellular pH and DNA synthesis through a peroxisome proliferator-activated receptor gamma-independent mechanism in breast cancer-derived cell lines.** *Clin Cancer Res* 2004, **10**:7022-7030.
40. Yin F, Bruemmer D, Blaschke F, Hsueh WA, Law RE, Herle AJ: **Signaling pathways involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells.** *Oncogene* 2004, **23**:4614-4623.
41. Shappell SB, Gupta RA, Manning S, Whitehead R, Boeglin WE, Schneider C, Case T, Price J, Jack GS, Wheeler TM, Matusik RJ, Brash AR, Dubois RN: **15S-Hydroxyeicosatetraenoic acid activates peroxisome proliferator-activated receptor gamma and inhibits proliferation in PC3 prostate carcinoma cells.** *Cancer Res* 2001, **61**:497-503.
42. Roth AD, Leisewitz AV, Jung JE, Cassina P, Barbeito L, Inestrosa NC, Bronfman M: **PPAR gamma activators induce growth arrest and process extension in B12 oligodendrocyte-like cells and terminal differentiation of cultured oligodendrocytes.** *J Neurosci Res* 2003, **72**:425-435.
43. Farrow B, Evers BM: **Activation of PPARgamma increases PTEN expression in pancreatic cancer cells.** *Biochem Biophys Res Commun* 2003, **301**:50-53.
44. Chintharlapalli S, Papineni S, Baek SJ, Liu S, Safe SH: **1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes Are Peroxisome Proliferator-Activated Receptor {gamma} Agonists But Decrease HCT-116 Colon Cancer Cell Survival Through Receptor-independent Activation of Early Growth Response-1 and NAG-1.** *Mol Pharmacol* 2005.
45. Saez E, Tontonoz P, Nelson MC, Alvarez JG, Ming UT, Baird SM, Thomazy VA, Evans RM: **Activators of the nuclear receptor PPARgamma enhance colon polyp formation.** *Nat Med* 1998, **4**:1058-1061.
46. Saez E: **PPAR[gamma] signalling exacerbates mammary gland tumor development.** *Genes Dev* 2004, **18**:528-540.
47. Varley CL, Stahlschmidt J, Lee WC, Holder J, Diggle C, Selby PJ, Trejdosiewicz LK, Southgate J: **Role of PPARgamma and EGFR signalling in the urothelial terminal differentiation programme.** *J Cell Sci* 2004, **117**:2029-2036.
48. Drori S, Girmun GD, Tou L, Szwajca JD, Mueller E, Kia X, Shivdasani RA, Spiegelman BM: **Hic-5 regulates an epithelial program mediated by PPARgamma.** *Genes Dev* 2005, **19**:362-375.

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