

Lack of efficacy of troglitazone at clinically achievable concentrations, with or without 9-*cis* retinoic acid or cytotoxic agents, for hepatocellular carcinoma cell lines

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Although the PPAR γ agonist troglitazone has been shown to induce growth inhibition of hepatocellular carcinoma (HCC) cells at high concentration, this study indicates troglitazone does not significantly inhibit the growth of HCC cells at clinically achievable concentrations (1–10 μ M), and this lack of activity could not be improved by the addition of 9-*cis*-retinoic acid. Furthermore, no synergistic effect was found between troglitazone and cytotoxic anticancer agents.

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The peroxisome proliferator-activated receptor γ (PPAR γ), a member of the nuclear hormone receptor superfamily, functions as a ligand-dependent transcription factor and plays an important role in several signalling pathways, including lipid metabolism, glucose homeostasis, and inflammation (Kersten *et al*, 2000). Heterodimerisation of PPAR γ and retinoid X receptor (RXR) is required for binding to specific DNA response elements of target genes. Binding with either PPAR γ or RXR ligands will elicit transcriptional activation of the target genes (Vanecq and Latruffe, 1999; Corton *et al*, 2000). Synergistic activation of downstream genes may occur when both ligands are present (Kliwer *et al*, 1992; Desvergne and Wahli, 1999). The thiazolidinediones were found to be specific agonists of PPAR γ , with EC50 less than 1 μ M (Lehmann *et al*, 1995). The potency of the thiazolidinediones in activating PPAR γ *in vitro* was found to correlate closely with their lipid- and glucose-lowering activity *in vitro* (Berger *et al*, 1996; Willson *et al*, 2001). Troglitazone, a thiazolidinedione derivative, has been demonstrated to induce adipocyte proliferation and differentiation with the concentrations of 0.5–5 μ M (Tafari, 1996).

The anticancer activity of PPAR γ agonists was first demonstrated in a liposarcoma model. Thiazolidinedione derivatives induced terminal differentiation of liposarcoma cells (Tontonoz *et al*, 1997; Demetri *et al*, 1999; Tsibris *et al*, 1999). Subsequent studies indicated that PPAR γ agonists, at concentrations of 1–10 μ M, may induce growth inhibition in a variety of cancers, including cancers of breast, colon, and prostate (Kubota *et al*, 1998; Sarraf *et al*, 1998; Mehta *et al*, 2000). In addition to promotion of cell differentiation, the PPAR γ agonists-induced growth inhibition may involve various mechanisms such as induction of cell cycle arrest, inhibition of DNA synthesis, and

increase of cancer cell necrosis and apoptosis. Moreover, synergistic or additive effects of growth inhibition between PPAR γ and RXR α agonists have been found in liposarcoma and breast cancer cells. Clinical trials exploring the feasibility of using PPAR γ agonists in the treatment of human cancers are underway (Koeffler, 2003).

Recent studies have demonstrated that PPAR γ agonists may induce growth inhibition in hepatocellular carcinoma (HCC) cells in a dose- and time-dependent manner (Koga *et al*, 2001; Rumi *et al*, 2001; Yoshizawa *et al*, 2002). Troglitazone, a thiazolidinedione derivative, enhanced the expression of the cyclin-dependent kinase inhibitors p21^{WAF1/Cip1} and p27^{Kip1} and resulted in cell cycle arrest at G0/G1 phase. PPAR γ agonists may also augment Fas-mediated apoptosis of HCC cells induced by tumour necrosis factor α (Okano *et al*, 2002). However, these effects were observed at relatively high concentrations (20–50 μ M) of troglitazone, while the clinically achievable concentrations are around 2–5 μ M (Berger *et al*, 1996; Tafari, 1996; Spencer and Markham, 1997; Willson *et al*, 2001). Therefore, the utility of PPAR γ agonists for the treatment of HCC remained undetermined.

The current study was designed to address the following questions: (1) whether troglitazone alone, at clinically achievable concentrations, may be active against HCC cells; (2) whether troglitazone has a synergistic effect with RXR α agonists on growth inhibition of HCC cells; (3) whether the effects of troglitazone and RXR α agonists correlate with the expression of PPAR γ and RXR α in HCC cells; and (4) whether troglitazone, at clinically achievable concentrations, may enhance the cytotoxic effects of major chemotherapeutic agents.

MATERIALS AND METHODS

Cell culture and reagents

A panel of HCC cell lines was tested in this study: Hep3B, HepG2, SNU-449 (Park *et al*, 1995), (purchased from ATCC), PLC-5, Huh-7

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(Nakabayashi *et al*, 1982), SK-hep1 (Fogh *et al*, 1977), HA-59T (Wuu *et al*, 1990) (gifts of Professor Ming-Yang Lai, Graduate Institute of Medicine, College of Medicine, National Taiwan University). MCF-7 (purchased from ATCC), a breast cancer cell line characterised by expression of RXR α and 9-*cis* retinoic acid (9-*cis* RA) growth inhibition, was used as positive control for RXR α expression and 9-*cis*-RA-induced cytotoxicity in this study (Gottardis *et al*, 1996). The HCC cells and MCF-7 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and maintained in a humidified incubator of 5% CO₂ in air at 37°C.

Troglitazone was purchased from Cayman Chemical (Ann Arbor, MI, USA) and 9-*cis*-RA was purchased from Sigma (St Louis, MO, USA). We used 9-*cis* retinoic acid in our study because in earlier studies it has been demonstrated to have synergistic activity with PPAR γ agonists in regulation of lipid metabolism and in a leiomyosarcoma model. Both troglitazone and 9-*cis*-RA were prepared in dimethyl sulphoxide (DMSO) for cytotoxicity assay. The final concentration of DMSO was controlled at below 0.5%. The cytotoxic agents were provided by the following companies: gemcitabine, Eli Lilly; irinotecan, Rhone-Poulenc-Rorer; cisplatin, David Bull Laboratories; paclitaxel, Bristol-Myers-Squibb. Polyclonal mouse antibodies detecting PPAR γ and rabbit antibodies detecting RXR α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody detecting proliferating cell nuclear antigen (PCNA) was purchased from Chemicon (Temecula, CA, USA). The anti-PPAR γ antibody we used in this study was mapped to the N-terminal of PPAR γ (sc-7196, Santa Cruz, CA, USA). No cross reaction with other PPAR isoforms has been reported by the manufacturer. The dilution of antibodies was 1:1000 for primary antibodies and 1:2500 for secondary antibodies.

Extraction of nuclear proteins and Western blot analysis

The methods of nuclear protein extraction and Western blot analysis have been described previously (Chuang *et al*, 2002). Briefly, cells (3–5 × 10⁶) were scraped from culture dishes, washed with iced phosphate-buffered saline, resuspended in 1 ml of buffer A (1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1% Nonidet P-40, 10 mM HEPES, pH 7.9), and incubated for 10 min at 4°C. The cells were then centrifuged at 1500 rpm for 2 min at 4°C and the pellet was resuspended in 0.1 ml of buffer B (25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 20 mM HEPES, pH 7.9) and incubated for 20 min at 4°C. The cell suspension was then centrifuged at 14 000 rpm for 2 min at 4°C, and the supernatant was collected for protein quantification using a modified Lowry's method. The supernatant was stored in aliquots at –70°C. A measure of 30 μ g of protein was deposited in each lane on the blot. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence agent (Santa Cruz). The proteins were then detected by roentgenography.

For each study at least three independent experiments, including independent cultures, protein collection and quantification, were done and separate blots were done to detect the expression of PPAR γ and RXR α . The most representative figures were shown.

Cytotoxicity assay

Cytotoxicity was determined by a tetrazolium-based semiautomated colorimetric assay (MTT assay) (Mosmann, 1983). Three independent experiments of each cytotoxicity test have been done and the results were the mean of the three experiments. For each

experiment at least three replicates were done. Cells were plated in 96-well plates (3–4 × 10³ well⁻¹) and incubated overnight. The drugs were added to the wells and the percentage of surviving cells was measured by MTT assay after continuous drug exposure for 72 h. Concurrent drug exposure was used to evaluate the combination effect of troglitazone with 9-*cis*-RA and troglitazone with four cytotoxic agents (gemcitabine, cisplatin, paclitaxel and irinotecan) that have different mechanisms of cytotoxicity. The IC50 of individual cell lines was calculated by using a linear regression based on the MTT assay results. The drug concentration corresponding to 50% inhibition of control was designated as IC50.

RESULTS

Expression of PPAR γ and RXR α and single-agent activity of troglitazone

The nuclear expression of PPAR γ and RXR α and the growth inhibitory effect of troglitazone on HCC cells are shown in Figure 1. All tested HCC cell lines expressed various levels of PPAR γ and RXR α constitutively (Figure 1A). Troglitazone, up to 10 μ M, had no significant growth inhibitory activity on any of the HCC cell lines. Growth inhibition was noted at concentration more than 20 μ M, and the degree of inhibition did not correlate with the expression levels of PPAR γ or RXR α (Figure 1B).

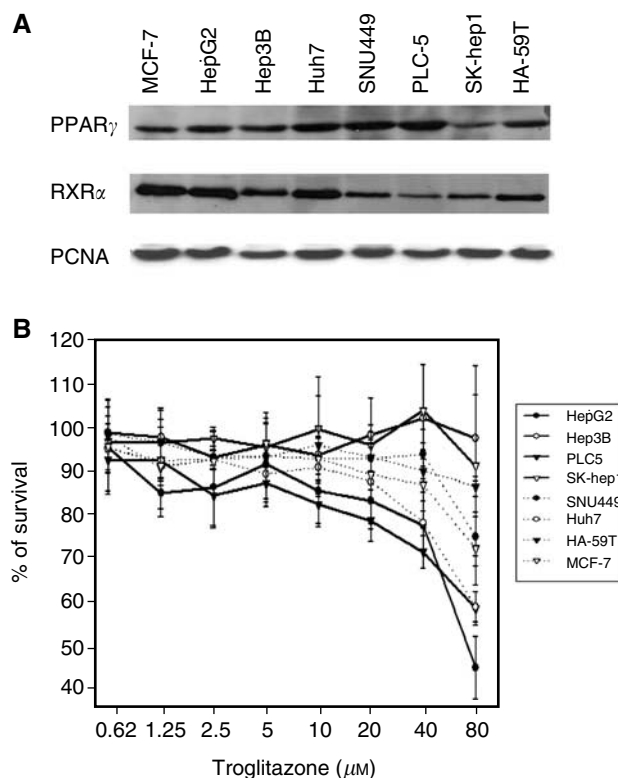


Figure 1 (A) Western blot analysis of PPAR γ and RXR α expression in nuclear protein lysate of HCC cells. All tested HCC cell lines expressed various levels of PPAR γ and RXR α constitutively. PCNA staining was used as loading control. (B) Growth inhibition of HCC cells induced by troglitazone determined by MTT assay. Growth inhibition was noted at concentration more than 20 μ M, and the degree of inhibition did not correlate with the expression levels of PPAR γ or RXR α .

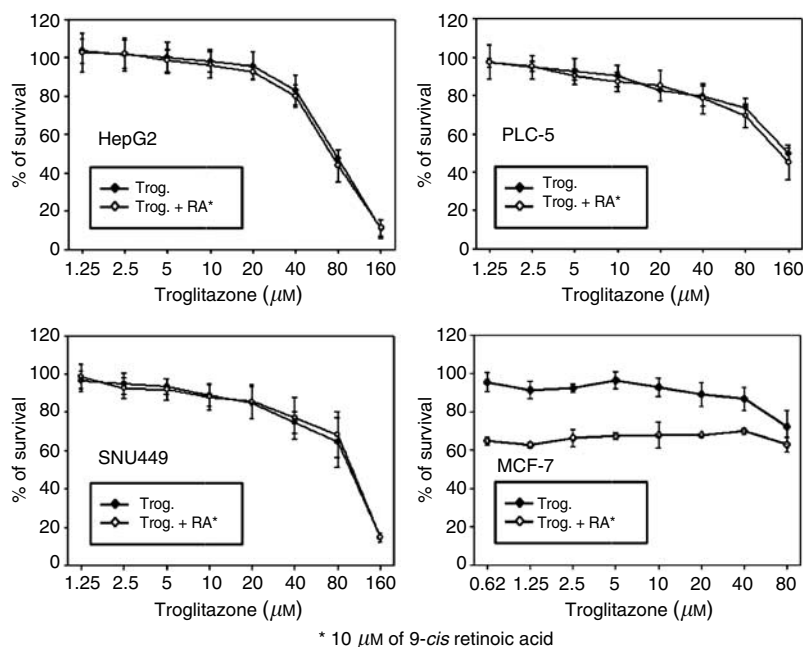


Figure 2 Growth inhibition of HCC cells induced by troglitazone with or without the addition of 10 μM of 9-*cis*-RA. Addition of 9-*cis*-RA did not affect the sensitivity of HCC cells to troglitazone.

Table 1 IC₅₀ (μM) of cytotoxic agents with or without troglitazone in HepG2 and PLC cell lines

	HepG2	PLC-5
Cisplatin	5.1 ± 0.6	52.2 ± 1.5
Cisplatin+T	5.0 ± 1.4	55.5 ± 1.3
Gemcitabine	0.65 ± 0.40	47.3 ± 4.8
Gemcitabine+T	0.87 ± 0.31	43.3 ± 8.4
Paclitaxel	0.95 ± 0.19	6.9 ± 1.5
Paclitaxel+T	0.79 ± 0.21	5.7 ± 1.2
Irinotecan	37.7 ± 8.2	427.5 ± 66.8
Irinotecan+T	36.4 ± 7.3	452.2 ± 122.2

T = troglitazone 10 μM . The IC₅₀ of individual cell lines was calculated by using a linear regression based on the MTT assay results. The drug concentration corresponding to 50% inhibition of control was designated as IC₅₀.

Combination activity of troglitazone and 9-*cis* RA

Addition of 10 μM of 9-*cis*-RA caused 60% growth inhibition of MCF-7 cells but had no significant growth inhibitory effects on any of the HCC cells and did not affect the sensitivity of HCC cells to troglitazone (Figure 2).

Combination activity of troglitazone and anticancer agents

The IC₅₀ of the four cytotoxic agents in three HCC cell lines, with and without the addition of 10 μM of troglitazone, is listed in Table 1. The addition of troglitazone did not change the sensitivity of HCC cells toward any of the cytotoxic drugs.

DISCUSSION

This study indicates that troglitazone does not significantly inhibit the growth of HCC cells at clinically achievable concentrations, and this lack of activity could not be improved by the addition of 9-*cis*-RA. Furthermore, no synergistic effect was found between troglitazone and four representative anticancer agents.

There was no apparent correlation between the degree of growth inhibition by troglitazone and the level of PPAR γ or RXR α expression in our study. No correlation of the sensitivity of HCC cells to troglitazone with expression levels of PPAR γ has been noted (Koga *et al*, 2001; Rumi *et al*, 2001; Okano *et al*, 2002). Similarly, previous studies for the sensitivity of cancer cells to retinoids have demonstrated that the growth inhibitory effects of retinoids did not correlate to the expression levels of different isoforms of retinoic acid receptors and RXRs (van der Leede *et al*, 1993). Other factors, including the levels of 'free' RXR α that is available for PPAR γ -RXR α interaction, the content of other nuclear receptors, and the possible interaction among nuclear coactivators and corepressors, may play an important role in determining the sensitivity of cancer cells to the nuclear receptor agonists (Torchia *et al*, 1998).

Although troglitazone and other thiazolidinediones are considered specific PPAR γ agonists, several lines of evidence suggest that the growth inhibition of HCC cells induced by high concentrations of troglitazone may occur through PPAR γ -independent mechanisms. First, thiazolidinediones induce differentiation of adipose tissue and insulin sensitization at submicromolar levels and these effects are closely related to their binding affinity to PPAR γ receptor (Lehmann *et al*, 1995; Goldstein, 2002). On the other hand, concentrations of troglitazone and other thiazolidinediones required to induce significant anticancer effect are usually 10 μM or higher. Besides, no evident relationship between anticancer efficacy and the expression of PPAR γ in cancer cells or the binding affinity of thiazolidinediones to PPAR γ has been established. Second, addition of RXR α agonist may also improve the insulin-sensitising effect of thiazolidinediones by enhancing the formation of PPAR γ -RXR α heterodimer (Mukherjee *et al*, 1997). This synergistic effect has also been found in liposarcoma and breast cancer cell lines but not in the present study, in spite of the constitutive expression of PPAR γ and RXR α in the nuclei of HCC cells. Third, inhibition of cell growth by troglitazone through PPAR γ -independent mechanisms has also been demonstrated in PPAR γ ^{-/-} embryonal stem cells (Palakurthi *et al*, 2001). Troglitazone may inactivate the eukaryotic initiation factor 2 (eIF2) and abrogate the expression of G1 cyclins, thus resulting in cell cycle arrest at G1-S transition. Because the concentration of

troglitazone needed to induce cell cycle arrest (10 μM or higher) is significantly higher than the clinically achievable serum concentration (2–5 μM), the clinical usefulness of troglitazone alone as an anticancer agent for HCC appears to be limited.

Troglitazone has also been demonstrated to induce cell cycle arrest through increased expression of the cyclin-dependent kinase inhibitors p21^{WAF1/Cip1}, p27^{Kip1}, and p18^{INK4c}. Cell cycle modulation has been intensely investigated as a novel way to induce sensitisation to chemotherapeutic drugs as well as to inhibit cancer cell growth (Senderowicz and Sausville, 2000; Sherr, 2000). However, the concentration of troglitazone necessary to induce this effect is much higher than that achievable clinically. Four major anticancer agents were tested in this study for their effect in combination with troglitazone. The lack of synergistic activity between troglitazone and cytotoxic drugs in this study suggests that troglitazone may not be effective as a biochemical modulator for HCC.

The reason for the lack of efficacy of troglitazone as a biochemical modulator for HCC cells remained undetermined. Most of the HCC cell lines came from patients with surgically resected tumours. Some cell lines, such as PLC5, have been found to have integration of hepatitis B viral DNA into the host genome. The implication of viral DNA integration into host cells on drug sensitivity is not known, although *in vitro* data suggested that expression of hepatitis B viral proteins may increase apoptosis threshold and resistance to cytotoxic agents of cancer cells (Doong et al, 1998; Shih et al, 2000).

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Nevertheless, the PPAR γ agonists may have other anticancer effects. It has been demonstrated that PPAR γ agonists are potent inhibitors of angiogenesis (Xin et al, 1999). Rosiglitazone, another thiazolidinedione derivative, has been shown to inhibit both primary tumour growth and metastasis (Panigrahy et al, 2002). The potential mechanisms of action include direct inhibition of endothelial cell proliferation, decrease of vascular endothelial growth factor production by tumour cells, and increased activity of the tissue inhibitor of matrix metalloproteinase (TIMP). Notably, the concentrations of rosiglitazone that had the strongest antiproliferative effect on endothelial cells (0.01–1 μM) are clinically achievable. Therefore, it remains a possibility that new PPAR γ agonists with novel antitumour mechanisms that are effective for the treatment of HCC can be developed.

In conclusion, troglitazone, at clinically achievable concentrations, does not appear to be active against HCC cells, either alone or in combination with 9-*cis*-RA or chemotherapeutic agents. Further exploration for new derivatives of PPAR γ agonists with better antitumour activity in HCC is needed.

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