USM SHORT-TERM PROJECT FINAL REPORT

TRANSCRIPTIONAL AND TRANSLATIONAL EXPRESSION OF PPARγ IN THE HUMAN COLORECTAL CANCER DELL LINE, COLO 205

BY:
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1883

USM J/P-06

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN

CANSELORI

UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: .....Dr. Nik Soriani Yaacob .....

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Nama Penyelidik-penyelidik Lain *(Jika berkaitan)* : .....Prof. Dr. Norazmi Mohd. Nor.....

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2) Pusat Pengajian/Pusat/Unit: .....PP Sains Perubatan.....

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3) Tajuk Projek: .....Transcriptional and translational expression of PPARγ in the human colorectal cancer cell line, COLO 205

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman di antara 100 – 200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris. Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dpatan rojek tuan/puan kepada pihak Univesiti).

The role of PPARγ in tumourigenesis is controversial. The objective of this project was to determine whether the PPARγ ligand, ciglitazone, could modulate cell growth and PPARγ expression in the colonic carcinoma cell line, COLO205. Measurement of LDH (lactate dehydrogenase) release was used to quantify the cytotoxic effects of ciglitazone on COLO205 cells. Ciglitazone induced cell death in a time and concentration-dependent manner with EC_{50} of 25 μM and complete cell death at about 70 μM concentration after 24 hrs treatment. Following treatment of COLO 205 cells with 25 μM ciglitazone, the levels of PPARγ mRNA expressed was measured by real-time PCR whilst its protein level assessed by Western blotting. We observed that both the transcriptional (mRNA) and translational (protein) levels of PPARγ were significantly reduced following ciglitazone treatment. The observation that PPARγ ligands, could affect the growth of COLO205 cells indicate that PPARγ plays an important role in the regulation of cell growth and cell death in colorectal cancer cells.

Peranan PPARγ dalam tumorigenesis masih kontroversial. Objektif projek ini adalah untuk mengenalpasti sama ada ligand PPARγ, ciglitazone, boleh memodulasi pertumbuhan sel dan ekspresi PPARγ dalam sel karsinoma kolon, COLO205. Pengukuran rembesan LDH (laktat dihidrogenase) digunakan untuk mengkuantifikasi tindakbalas sitotoksik ciglitazone ke atas sel COLO 205. Ciglitazone merangsang kematian sel bersandarkan masa dan kepekatan dengan kadar EC_{50} sebanyak 25 μM dan kematian sel sepenuhnya pada kepekatan 70 μM selepas 24 jam. Dengan menggunakan 25 μM ciglitazone ke atas COLO 205, aras mRNA PPARγ diukur dengan "real-time" PCR manakala aras protein diukur melalui kaedah pemblotan Western. Didapati kedua-dua aras transkripsi (mRNA) dan translasi (protein) PPARγ berkurangan selepas dikultur dengan ciglitazone. Keupayaan ciglitazone menjejaskan pertumbuhan COLO205 menunjukkan kemungkinan PPARγ memainkan peranan dalam regulasi pertumbuhan dan kematian sel kanser kolon.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
PPARγ.....	PPARγ.....
kanser kolon	colonic cancer
real-time PCR	real-time PCR
ciglitazone.....	ciglitazone.....
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.....	.....
.....	.....
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5) Output Dan Faedah Projek

(a) Penerbitan *(termasuk laporan/kertas seminar)*
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

Halisa Mohd. Darus, Khoo Boon Yin, Nik Soriani Yaacob and Norazmi Mohd. Nor: Growth inhibition of human colorectal cancer cell lines by peroxisome proliferator-activated receptor-γ ligands. 28th Annual Conference of The Malaysian Society for Biochemistry and Molecular Biology, Putrajaya Marriott Hotel, Putrajaya, Kuala Lumpur, 12 August, 2003.....
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(b) Faedah-faedah Lain Seperti Perkembangan Produk, Prospek Komersial Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

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(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah:

.....Pn. Halisa Mohd. Darus *(Kursus Sarjana)*.....
.....(PP Sains Kesihatan) .....
.....
.....

ii) Pra Siswazah:

.....Cik Yong Kim Far.....
.....(PP Sains Kesihatan) .....
.....
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iii) Lain-lain:

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.....Tiada.....
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6. Peralatan Yang Telah Dibeli:

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UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

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TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENYELIDIKAN
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USM SHORT–TERM PROJECT FINAL REPORT

Transcriptional and translational expression of PPARγ in the human colorectal cancer cell line, COLO 205

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Schools of [1]Medical and [2]Health Sciences, USM Health Campus
USM Short Term Grant (304/PPSP/6131140)

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors of the nuclear hormone receptor superfamily that includes the receptor for steroid, retinoid and thyroid hormone. These nuclear receptors are characterised by their ability to bind to specific DNA sequences and, when activated by a ligand, PPARs regulate the expression of various genes and their functions (Auwerx 1999; Vamecq *et al.*, 1999). PPAR was first cloned from the mouse liver by Isseman and Green in 1990. The PPAR family consists of three distinct isoforms, namely PPARα, PPARδ (or PPARβ) and PPARγ. Each isoform is encoded by a separate gene (Tontonoz *et al.* 1994). PPARγ is further divided into the PPARγ1, PPARγ2, PPARγ3 and PPARγ4 isoforms (Sundvold and Lien, 2001) whereby PPARγ2 has an additional 30 amino acids at its N-terminus but is believed to have similar functions as those of PPARγ1 (Sundvold *et al.* 1995). PPARγ3 is only found in humans and the expressed protein is identical to that of PPARγ1 (Fajas *et al.*, 1998). PPARγ is found mainly in adipocytes and cells of the immune system (Braissant *et al* 1996; Lemberger *et al* 1996) and is involved in the regulation of adipogenesis, glucose metabolism and macrophage development and function (reviewed in Kersten *et al.*, 2000).

PPARγ and carcinogenesis

Although it has long been known that PPARα ligands such as hypolipidaemic drugs cause hepatocarcinogenesis in rodents (Reddy and Chu 1996), the possible involvement of PPAR in human neoplasms has only recently emerged. PPARγ has been shown to be expressed in human colon cancer (Sarraf *et al.*, 1998), prostate cancer (Mueller *et al.*, 2000) and breast cancer (Clay *et al.*, 1999) cell lines. However the involvement of PPARγ activity in inducing

growth inhibition in such tumours have been contradictory. For example, it has previously been shown that PPARγ can inhibit the proliferation of human colorectal carcinomas (Brockman *et al.,* 1998). In sharp contrast with this, however, was the report that activation of PPARγ promotes the development of colon tumors in C57BL/6J-APCMin/+mice (Lefebvre *et al.,* 1998), a clinically relevant model for both human familial adenomatous polyposis and sporadic colon cancer (Su *et al.,* 1992). Recent evidence suggests that PPARγ ligands could have an anti-tumor effect in humans as these compounds decrease cell growth and induce apoptosis in several malignant human cell types, including colorectal carcinomas (Sarraf *et al.,* 1998). Specific ligands of PPARγ such as the antidiabetic thiazolidinediones (TZDs), natural fatty acid derivatives, non-steroidal anti-inflammatory drugs (NSAIDs) and certain polyunsaturated fatty acids have been identified (Palmer *et al.,* 1998).

In agreement with the potential role of PPARγ ligands for the treatment of cancer, our present study was aimed at observing the growth inhibition of the colorectal cancer cell line, COLO205 by the PPARγ ligand, ciglitazone. This aim was slightly different from the original one proposed under this grant whereby the determination of both PPARα and PPARγ expression by two colorectal cell lines, HT-29 and COLO205 was proposed. The change was necessary because the amount of funds approved was about 45% less than that requested and also based on current developments in this research area including the use of a more advanced technique of gene quantification, namely, using real time PCR. In addition, we propose to correlate these findings with the expression of the corresponding proteins by the cell line.

Objective

The objective of this study was to quantify the mRNA expression of PPARγ1 in the colorectal cancer cell line, COLO 205, at various time points following treatment with a thiazolidinedione, using real-time PCR. For this purpose, an internal standard for PPARγ1 was constructed. The transcriptional expression of this gene was correlated with the corresponding protein expression levels.

Methodology

Maintenance of cells

COLO 205 cells were maintained at 37°C in 5% CO_2 humidified atmosphere in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin and passaged every 2 to 3 days. The amount of FBS was reduced to 2% prior to treatment.

Optimisation of cell concentration

Near confluent COLO 205 cells in 75cm^2 culture flaks were harvested and titrated by 10-fold serial dilution ($10^3 - 10^5$ cells/ml) across a 24-well tissue culture plate to determine the optimum number of cells for seeding. Cell-free supernatants were collected for LDH activity determination as cell growths reached 70% confluence as observed under an inverted microscope. A further 2-fold serial dilution was then performed to check which number of cells would give about 70-80% confluency after 24-48 h incubation. The optimal cell concentration for seeding was determined to be about 1×10^5 cells/ml.

Cytotoxicity assay

The lactate dehydrogenase (LDH) release cytotoxicity assay was performed using the Cytotoxicity Detection Kit (Roche, Germany) to establish the EC_{50} values. Briefly, cells were cultured in 24-well plates in the presence or absence of ciglitazone, at a concentration range of 2.5 to 100 μg/ml, in triplicates, and harvested at various time points (0 to 72 hrs). Maximum LDH release (high control) was determined by solubilising cells with Triton X-100 (1% w/v). Spontaneous LDH release (low control) was also determined from cells incubated with medium alone. Cell-free supernatants were collected into 96-well plates for LDH assay by a reaction in which tetrazolium salt was reduced to a red formazan salt. The absorbance was then read at 492nm using a microtiter plate spectrophotometer. The mean absorbance for each treatment group was calculated and the results were expressed as % cytotoxicity [(experimental value – low control) / (high control – low control) x 100]). EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves.

Total RNA extraction and cDNA synthesis

COLO 205 cells were cultured in an appropriate concentration of ciglitazone (based on the EC_{50} value) in 25cm^2 flasks for up to 72 hrs. The cells were harvested at various time points and lysed in an appropriate buffer. Total RNA was then extracted from each cell sample using RNeasy Minikit (Qiagen, USA) according to the manufacturer's instructions. The RNA integrity was confirmed by gel electrophoresis and RNA purity and concentration were

measured by spectrophotometry (Biophotometer; Eppendorf, Germany). The total RNA (between 1 to 3 μg) was then reverse-transcribed into cDNA using RevertAid™ H Minus First Strand cDNA Synthesis kit (MBI Fermentas, USA) in the presence of oligo(dT)$_{18}$ primer and murine-moloney leukemia virus (M-MuLV) reverse transcriptase in nuclease-free deionized water according to the manufacturer's instructions. The success of cDNA synthesis was initially verified by PCR using primers specific for 18s rRNA (Maxim Biotech).

Real-time PCR

The mRNA expression of PPARγ1 was measured by a more advanced method of quantitation known as real-time PCR, instead of by competitive PCR as originally proposed. This was carried out using specifically designed primers and Taqman probes, employing the ABI7000 Sequence Detection System (Applied Biosystems, USA). A homologous internal standard was constructed for the quantitation of the PPARγ1 gene. For this purpose, a specific fragment of the target gene was first amplified from a cDNA library of human adipose tissue (Maxim Biotech) by PCR with gene specific primers, cloned into a TOPO® vector (Invitrogen, USA) and transformed in *E.coli* competent cells to produce the recombinant plasmid, pNSY HG1 (Figures 1 and 2).

The concentration of the homologous internal standard (pNSY HG1) stock solution was adjusted to 1 pmol/μl. Ten-fold dilution series of the standard was performed to obtain the standard curves. Each amplification reaction was performed in a 96-well optical reaction plate with appropriate controls, in triplicates. The reaction was started at 50°C for 2 min for optimal AmpErase® uracyl-N-glycosylase (UNG) enzyme activity. The temperature was increased to 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of denaturation at 95°C for 15 sec and primer annealing and extension steps at 60°C for 1 min each.

Western blotting

The protein expression of PPARγ in COLO 205 cells following treatment with the ciglitazone was detected by Western blotting. COLO 205 cells were cultured in 75cm^2 flasks and cell lysates were prepared by cell lysis and centrifugation. The protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, USA). The samples were then resuspended in loading buffer and subjected to SDS-PAGE. Following transfer of proteins onto nitrocellulose membranes, immunoblotting was carried out using anti-PPARγ polyclonal antibody (Santa Cruz Biotechnology, USA) and peroxidase-conjugated streptavidin (Dako,

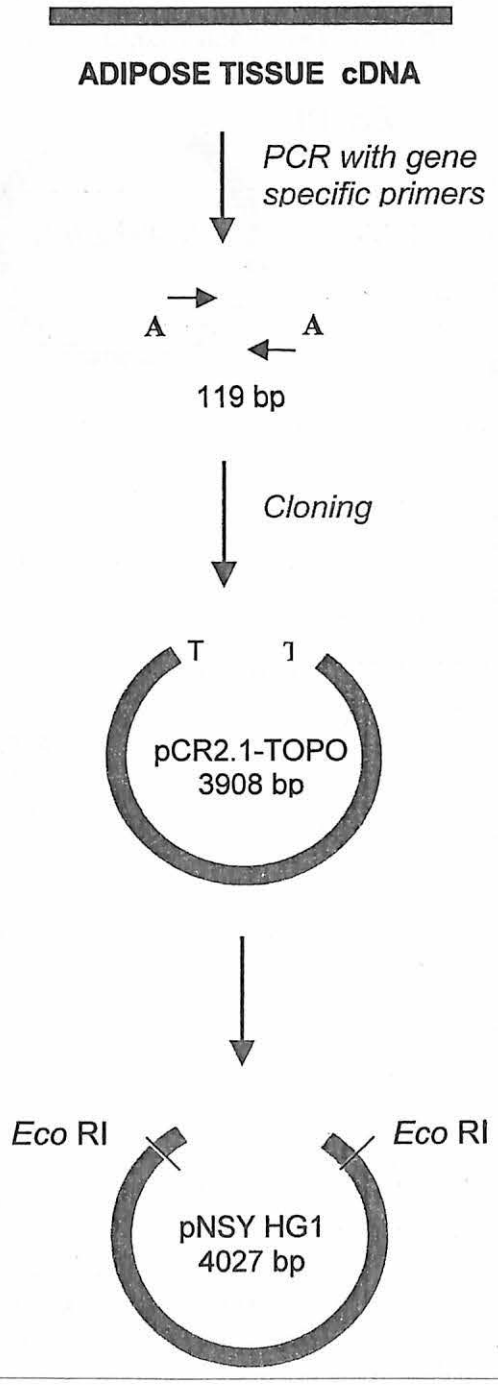


Figure 1: Cloning of pNSY HG1 for the preparation of hPPARγ1 internal standard

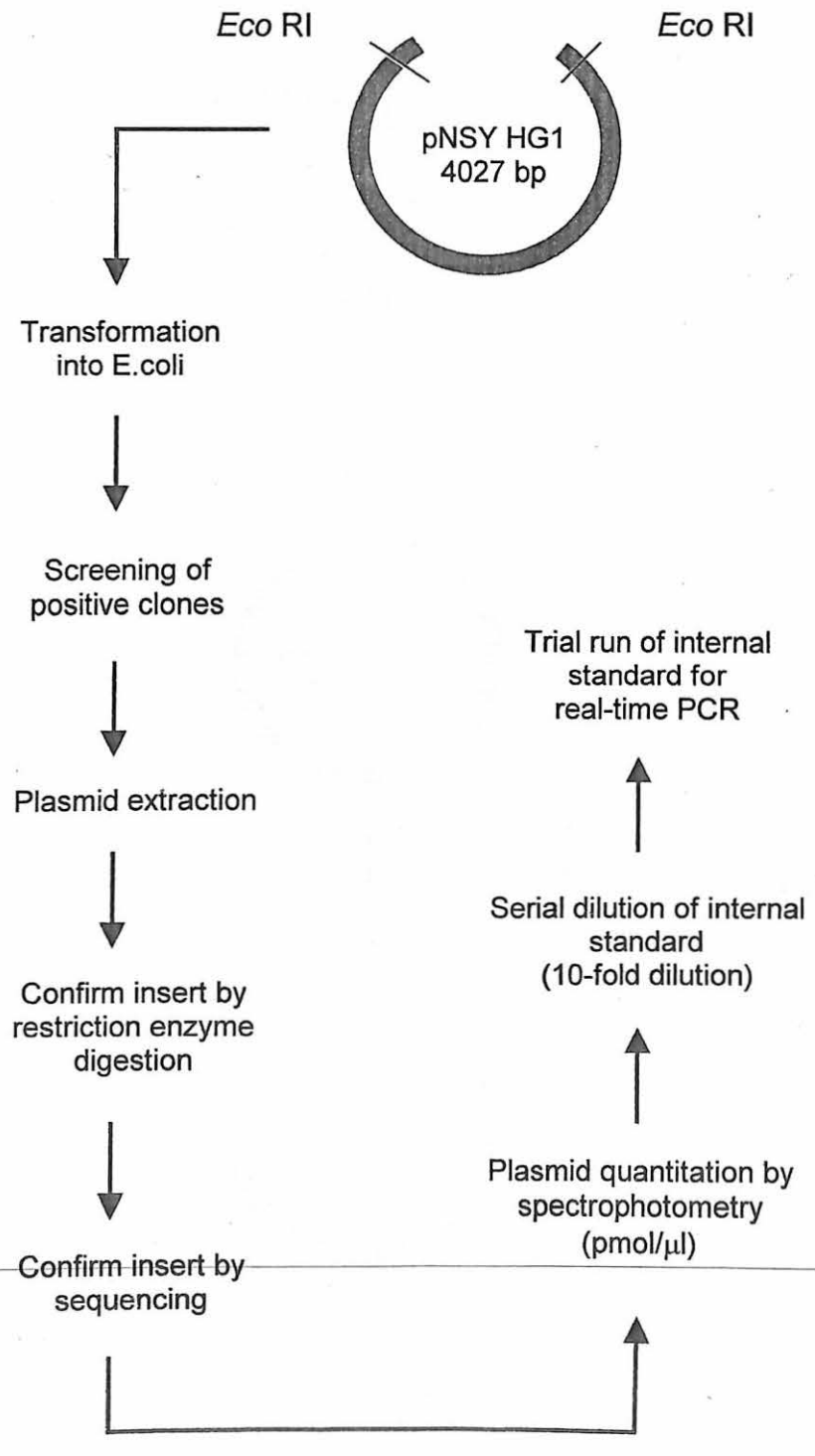


Figure 2: Preparation of PPARγ1 internal standard for real-time PCR

Denmark). Detection of the target protein was then carried out using an enhanced chemiluminescence system (Amersham Biosciences, UK) according to the manufacturer's instructions.

Statistical Analyses

One-way ANOVA Bonferroni-Dunn test was used to determine the difference in mRNA expression levels. The data were presented as mean ± standard error mean (SEM) and $p<0.05$ is considered to be statistically significant.

Results

Figure 3 shows the time course LDH release (0 to 72 hrs) by COLO 205 cells treated with up to 100 μM ciglitazone. The EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves and the mean values are shown in Table 1. An EC_{50} value of 30 μM was obtained following 24 hr exposure of the cancer cells to ciglitazone. Longer exposure times decreased the EC_{50} values. Exposure to ≤10μM ciglitazone caused <10% cytotoxicity to the tumour cells and the drug concentration of 75 μM had resulted in 100% cell death in 24 hrs. The threshold concentration of ciglitazone for COLO 205 cells was estimated at 25 μM (Figure 4). This concentration was hence used to treat the cells for further investigations.

Figure 5 shows the amplification profiles of the PPARγ1 gene in COLO 205 cells treated/untreated with 25μM ciglitazone and 10-fold dilutions of its homologous internal standard, pNSY HG1, using Real-time PCR. Ciglitazone had caused a significant decrease in PPARγ1 mRNA levels in these cells following 6, 12, 18, 24, 36, 48 and 60 hrs incubation with the drug with more than 50% reduction observed as early as after 6 hrs (Figure 6).

Unlike the changes in the mRNA levels, no significant reduction was observed in the protein expression of PPARγ1 in COLO 205 cells after 6 hrs of treatment with 25μM ciglitazone (Figure 7). Significant reduction in the protein levels occurred at least after 24 hrs of drug exposure.

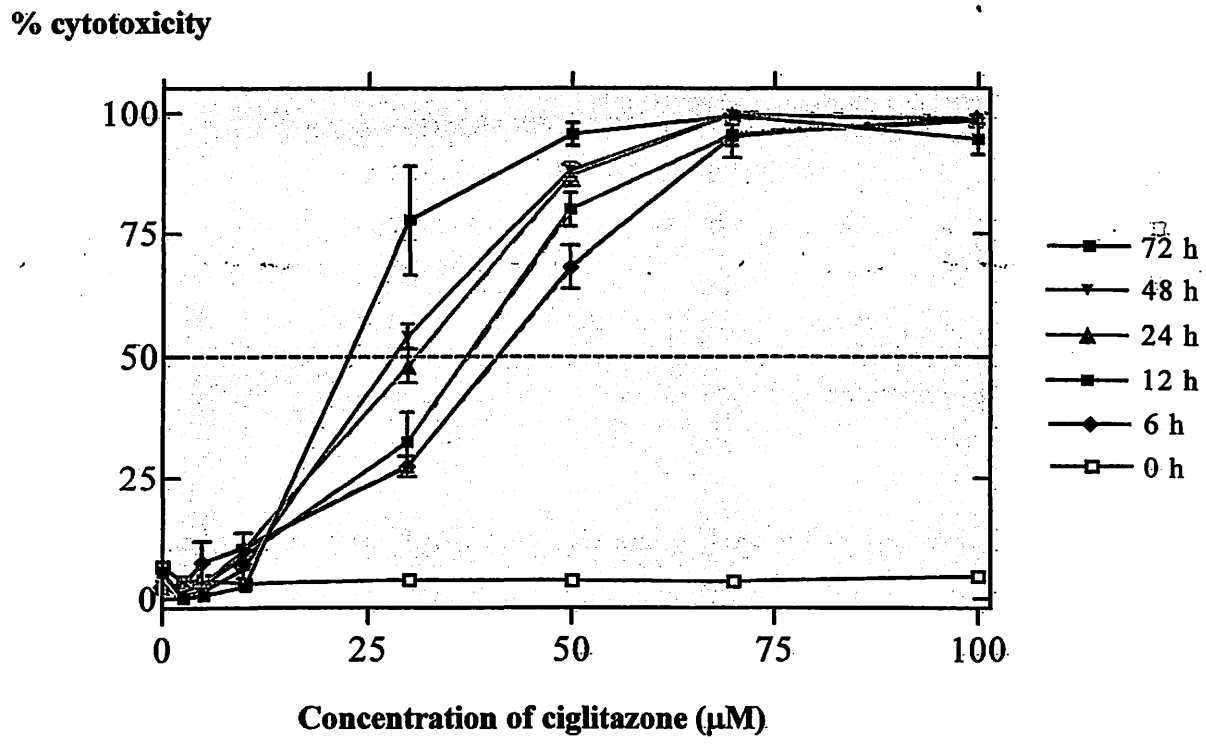


Figure 3: Cytotoxic potential of ciglitazone on COLO 205 cell line

Cells were cultured in the presence of ciglitazone (2.5 to 100 μM) in triplicates. Time course LDH release (0 to 72 hrs) by COLO 205 cells (1×10^5 cells/well) was measured in the culture supernatants using the Cytotoxicity Detection Kit to establish the EC_{50} values. Maximum LDH release (high control) was determined by solubilising cells with Triton X-100 (1% w/v) and spontaneous LDH release (low control) was determined from untreated cells. The absorbance was read at 492nm. The results are expressed as % cytotoxicity [(experimental value – low control) / (high control – low control) x 100]). Data points represent the mean ± SD of three independent experiments performed in triplicates each time. EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves.

Table 1: EC_{50} values (μM) for COLO 205 cell lines treated with ciglitazone

	TIME/hour				
	6	12	24	48	72
COLO205	45 ± 4.78	38 ± 3.27	30 ± 1.70	26 ± 1.25	20 ± 1.63

Data are given as *mean ± SD* (N) *n*=3

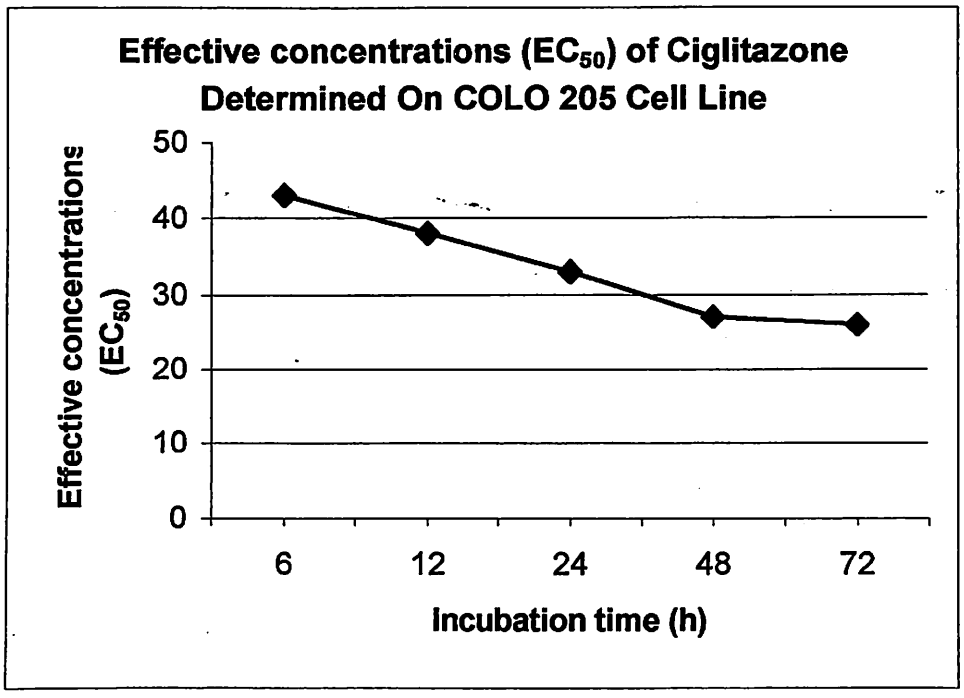
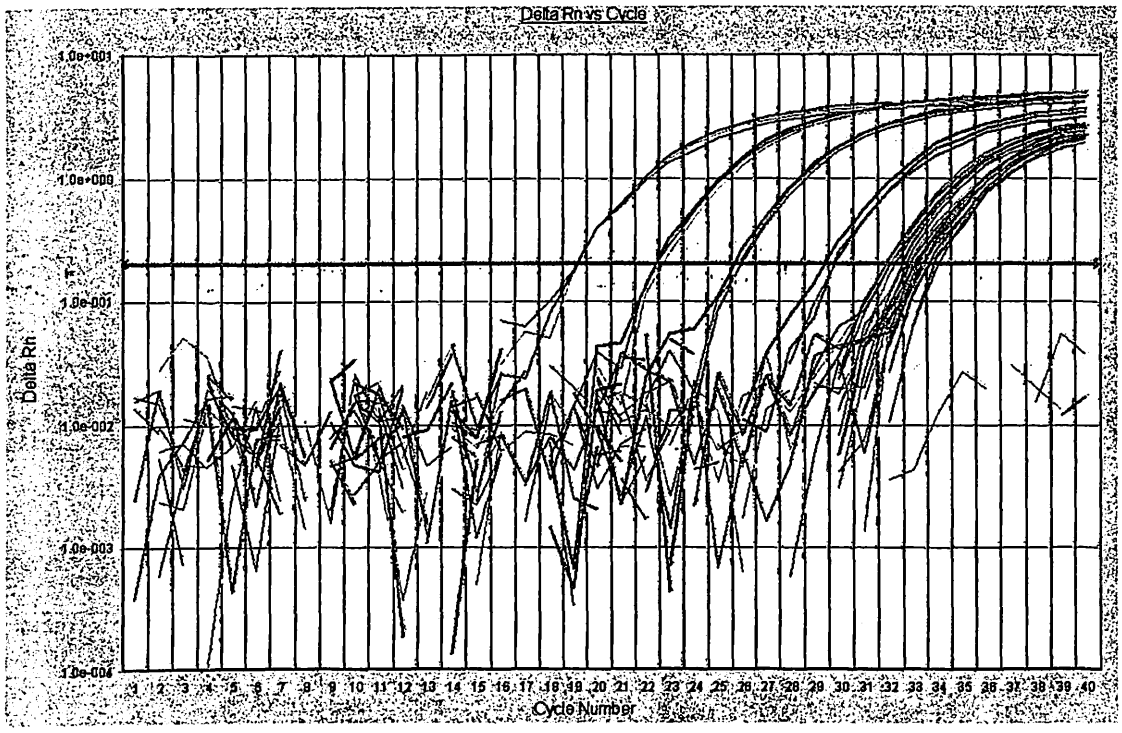


Figure 4: **Time-dependent changes of EC_{50} value for ciglitazone in COLO 205 cells**

EC_{50} values are plotted against the incubation times to estimate the threshold concentration for ciglitazone in the COLO 205 cells. The threshold EC_{50} value gives the drug concentration at which the rate of cell death equals the rate of cell growth.

(a)



(b)

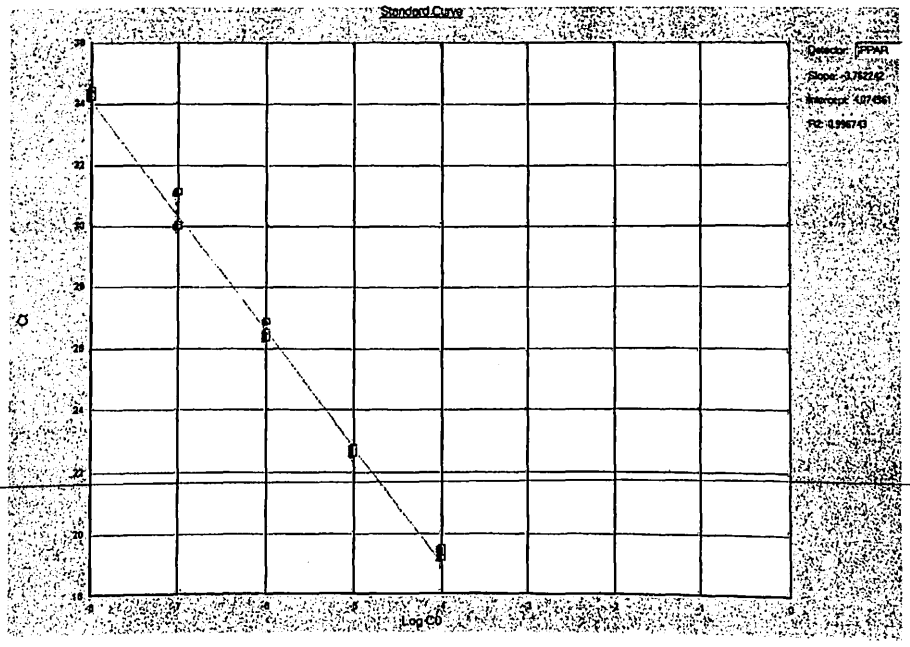


Figure 5: Real-time PCR profiles (a) and standard curve of pNSY HG1 (b)

Real-time PCR was performed on the cDNA samples obtained from cultured cells together with 10-fold dilutions of the pNSY HG1 (internal standard) for quantitation of PPARγ1 mRNA levels in COLO 205 cells treated/untreated with 25 μM ciglitazone for 6 to 72 hrs. All samples were run in triplicates

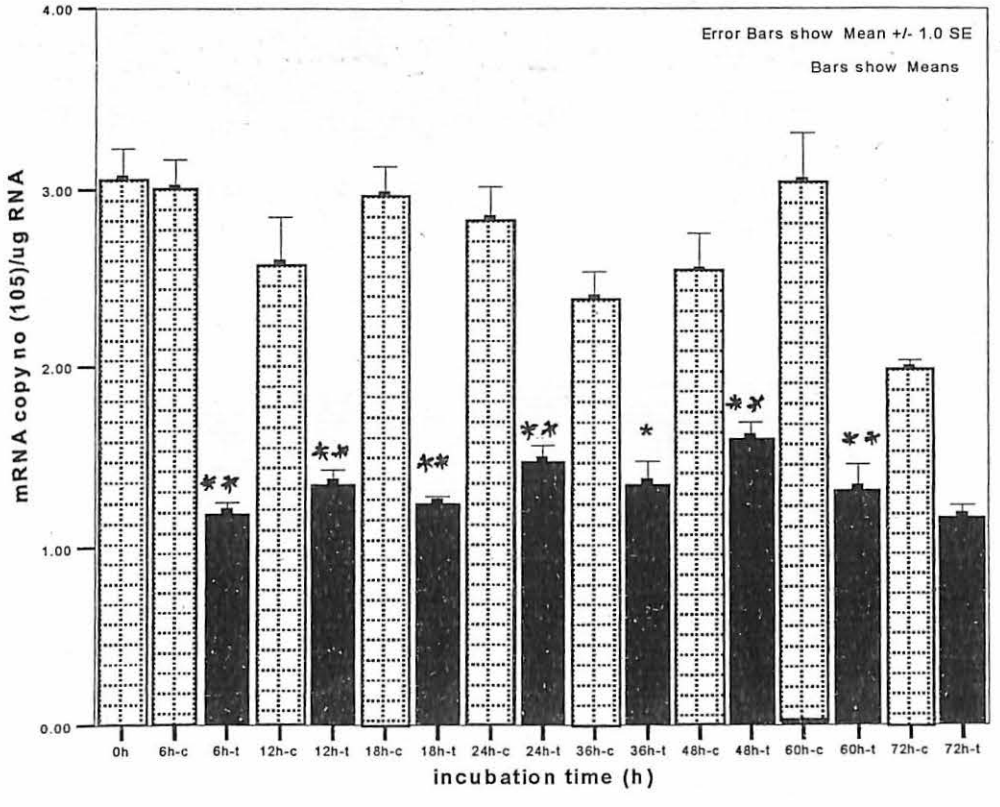


Figure 6: Effect of ciglitazone on PPARγ1 mRNA expression in COLO 205 cells

Cells were treated with 25 μM ciglitazone for 6, 12, 18, 24, 36, 48, 60 and 72 hrs. Gene expression was measured by quantitative real-time PCR using a homologous internal standard. Data are expressed as mean±SE (n = 3 to 6). One way-ANOVA Bonferroni-Dunn test was performed comparing the treated cultures (t) with the corresponding control cultures. p values of < 0.05 (*) and < 0.01 (**) are considered significantly different.

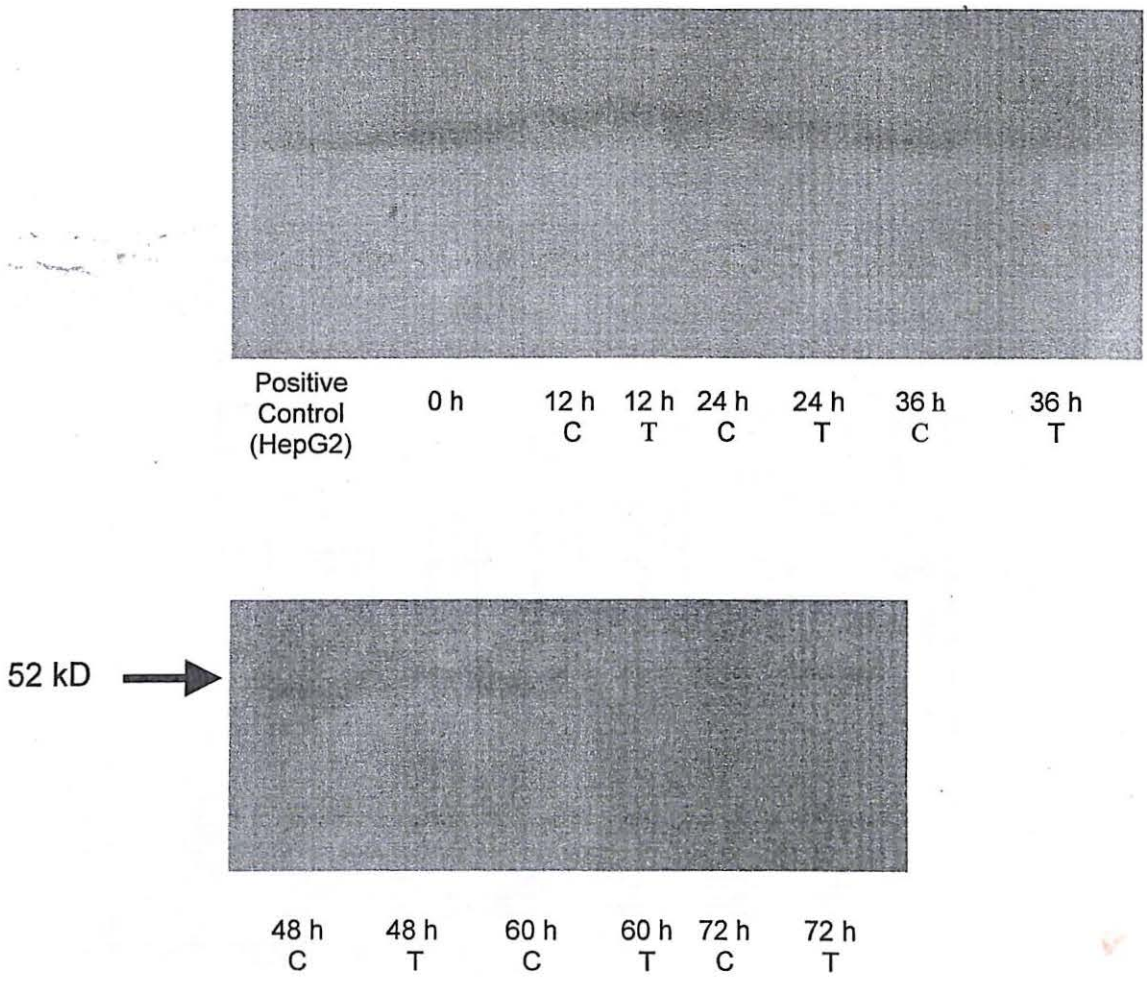


Figure 7: Effect of ciglitazone on PPARγ protein expression in COLO 205 cells

Cells were treated with 25 μM ciglitazone for 6, 12, 18, 24, 36, 48, 60 and 72 hrs. Cell lysates were prepared and electrophoresed on 10% SDS-PAGE (100 μg/lane). Proteins from gels were transferred to PVDF membrane, and PPARγ was detected with goat anti-human PPARγ polyclonal antibody, and visualised with enhanced chemiluminescence.

Discussion

A homologous internal standard for real-time PCR quantitation of hPPARγ1 was successfully produced and confirmed by DNA sequence analysis. The resulting recombinant plasmid, known as pNSY HG1, was used to prepare the standard curves for quantitative analyses of target cDNA samples.

Measurement of LDH release from cells into the medium has been established as a useful parameter for the measurement of cellular cytoxicity (Korzeniewski & Callewaert, 1983). This parameter was thus used in the current study to quantify the cytotoxic effect of the PPARγ ligand (a thiazolidinedione class of drug), ciglitazone on COLO 205 cells that were established from a metastatic colonic adenocarcinoma. Ciglitazone had induced the colorectal cancer cell death in a time and concentration-dependent manner with EC_{50} of 25 μM and complete cell death at about 70 μM concentration after 24 hrs treatment. This slowing or inhibition of tumour cell growth indicates the potential use of ciglitazone or other PPARγ ligands as potential anti-tumour drugs.

Conclusion

The observation that PPARγ ligands, could affect the growth of COLO205 cells indicate that PPARγ plays an important role in the regulation of cell growth and cell death in colorectal cancer cells. This study may help to examine the therapeutic potential of PPARγ ligands (natural or synthetic) for colorectal cancer. Elucidating the role of PPARs in the mechanism of tumourigenesis will pave the way for future establishment of an *in vitro* system that can be used for identification of specific agonists or antagonists of these receptors among the local natural products that are currently used for the purported treatment of various conditions. Future studies should therefore include comparison of inhibitory effects of PPARγ ligands on other types of colorectal cell lines, such as HT-29 cells that originate from a well-differentiated adenocarcinoma, compared to COLO 205 cells which are established from a metastatic colonic adenocarcinoma.

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