JPET #63438

TITLE

The synthetic ligand of PPARγ ciglitazone affects human glioblastoma cell lines

Nicol Strakova, Jiri Ehrmann, Petr Dzubak, Jan Bouchal and Zdenek Kolar

Department of Pathology, Laboratory of Molecular Pathology (N.S., J.E., J.B., Z.K.) and Laboratory of Experimental Medicine (P.D.), Faculty of Medicine, Palacky University Olomouc, Czech Republic

1

JPET #63438

RUNNING TITLE PAGE

a) **Running title**
   PPAR gamma in human glioblastoma cells

b) **The name, address, and telephone and fax numbers of the corresponding author**
   Nicol Strakova, Hnevotinska 3, 775 15 Olomouc, Czech Republic,
   Phone: 00420-585 639 541, Fax: 00420-585 632 966
   Email: Nicol.Strakova@seznam.cz

c) **The number of text pages, number of tables, figures, and references, and the number of words in the *Abstract, Introduction* and *Discussion* (each item should be placed on a separate line)**
   Text pages: 33
   Number of tables: 1
   Number of figures: 8
   Number of references: 40
   Number of words in the Abstract: 249
   Number of words in the Introduction: 669
   Number of words in the Discussion: 1 484

d) **A list of nonstandard abbreviations used in the paper.**
   AR – Androgen Receptor
   C – Control
   CIG - Ciglitazone
   cas3 – Caspase 3
   CDK – Cyclin Dependent Kinase
   DMEM – Dulbecco´s modified Eagle's medium
   DMSO - Dimethylsulfoxide
   EGFR – Epidermal Growth Factor Receptor
   FBS - Foetal Bovine Serum
   GBM - Glioblastoma multiforme
   GFAP – Glial Fibriall Acid Protein
   hTERT - Human Telomerase Reverse Transcriptase
   hTR - Human Telomeric Repeat
   MTT - (3, [4,4-dimethylthiazol-2-yl] 2,5 diphenyltetrazolium bromide)
   PARP – Poly ADP/Ribose-Polymerase
   PDGFR – Platelet-derived Growth Factor Receptor
   PPAR - Peroxisome Proliferator-Activated Receptor
   PPRE - Peroxisome Proliferator Response Elements
   pRb - protein Rb
   pRb-P - phophorylated protein Rb
   PTEN – Phosphatase and Tensin Homolog Deleted on Chromosome Ten
   RXR – Retinoid X Receptor
   TRAP - Telomeric Repeat Amplification Protocol
   TUNEL - TdT-mediated dUTP nick labelling
   TZD – Thiazolidinedione
   VDR – Vitamin D Receptor

e) **A recommended section assignment to guide the listing in the table of contents.**
   Cellular & Molecular

2

ABSTRACT

Glioblastoma multiforme is the most common malignant brain tumour in adults, and it is among the most lethal of all cancers. Recent studies have shown that ligand activation of PPARγ can induce differentiation and inhibit proliferation of several cancer cells. In this study we have investigated whether one PPARγ ligand in particular, ciglitazone, inhibits cell viability and additionally whether it affects the cell cycle and apoptosis of human glioblastoma cell lines, T98G, U-87 MG, A172 and U-118 MG. All glioblastoma cell lines were found to express PPARγ protein and following treatment with ciglitazone, localisation was unchanged. Ciglitazone inhibited viability in a dose-dependent manner in all four tested glioblastoma cells after 24 hour treatment. Analysis of the cell cycle showed arrest in the G1 phase and partial block in G2/M phase of the cell cycle. Cyclin D1 and cyclin B expression was decreased. Phosphorylation of Rb protein dropped, as well. We found that ciglitazone was followed by increased expression of $p27^{Kip1}$, $p21^{Waf1/Cip1}$. It also led to apoptosis induction; bax expression in T98G was elevated. Expression of the anti-apoptotic protein bcl-2 was reduced in U-118 MG and U-87 MG and showed a slight decrease in A172 cells. Flow cytometry confirmed the induction of apoptosis. Moreover, PPARγ ligand decreased telomerase activity in U-87 MG and U-118 MG cell lines. Our results demonstrate that ciglitazone inhibits the viability of human glioblastoma cell lines via induction of apoptosis and a result this ligand may offer potential new therapy for the treatment of CNS neoplasms.

1. Glioblastoma

The incidence of central nervous system neoplasm ranges from 3.8 to 5.1 cases per 100 000 in the population. Glioblastoma multiforme (GBM), the most common malignant brain tumor (34 %) in adults is among the most lethal of all cancers (Gurney and Kadan-Lottick, 2001). GBM composed of poorly differentiated neoplastic astrocytes, typically affects adults and is preferentially located in cerebral hemispheres. These gliomas are notoriously invasive tumors (Jiang *et al.*, 2003) and their prognosis remains very poor despite surgery, radio- and chemotherapy. As most patients with GBM typically survive less than 1 year, new therapeutic strategies are urgently needed (Fan *et al.*, 2002). Genetic analyses suggest that there are two different types of glioblastoma: 1. *de novo* glioblastoma and 2. secondary glioblastoma, which arises from lower grade tumors. The secondary GBMs occur in younger patients (Ishii *et al.*, 1999). GBMs commonly overexpress oncogenes such as EGFR and PDGFR and contain mutation and deletions of tumor suppressor genes such as PTEN, MYCN and TP53 (Mischel *et al.*, 2003).

2. PPARs (Peroxisome Proliferator-Activated Receptors)

The PPARs are a subgroup of ligand activated transcription factors. They belong to the nuclear receptor family. Other members of this family include steroid and thyroid hormone receptors, retinoid receptors, and vitamin D receptors (Mangelsdorf *et al.,* 1995).

Peroxisome proliferators, like fatty acids, modulate tissue specific responses, e.g. they stimulate the expression of enzymes involved in lipid catabolism, namely the peroxisomal β-oxidation system (Cimini *et al.*, 2000) (Heuvel *et al.*, 1999).

The PPAR family comprises three closely related gene products, PPAR α, β/δ and γ. All have a highly conserved structure (Chattopadhyay *et al.*, 2000).

3. PPARγ

PPARγ, like other members of nuclear receptor superfamily, is characterized by three domains: the N-terminal domain (important for functional regulation by phosphorylation), the DNA binding domain (that binds receptor to specific DNA sequences – Peroxisome Proliferator Response Elements, PPRE) and the ligand binding domain in the COOH-terminal region (Ehrmann *et al.,* 2002).

Free PPAR is bound by co-repressors that inactivate the transcription function of the receptor. This receptor is activated by a particular ligand into the ligand binding domain resulting in conformational changes to PPAR while the receptor is released from binding with the co-repressor. PPAR then recruits co-activator proteins and RXRα to form a heterodimeric complex of nuclear receptors. This complex {PPARγ:RXRα} binds to PPRE on DNA (involved in the promoter of different genes) to regulate transcription. PPARγ activation play a role in diverse physiological and pathophysiological events including stimulation of adipocyte differentiation, activation of insulin, regulation of lipid metabolism, inhibition of tumor cell proliferation and diverse effects on inflammatory processes (Houseknecht *et al.*, 2002).

4. PPARγ ligands

Endogenous PPARγ ligands are polyunsaturated fatty acids and eicosanoids, such as 15-deoxy-delta 12,14-prostaglandin J2 and leukotriene B4. A number of synthetic PPARγ ligands have been identified over the past 7 years of which the most well known are the

thiazolidinedione (pioglitazone, ciglitazone, rosiglitazone, etc.). Thiazolidinediones (TZDs) are a class of antidiabetic agents that improve insulin sensitivity and reduce plasma glucose and blood pressure in patients with type 2 diabetes mellitus (Houseknecht *et al.*, 2002).

Recent studies have shown that ligand activation of PPARγ can induce differentiation and inhibit proliferation of prostate (Kubota *et al.*, 1998) (Hisatake *et al.*, 2000), breast (Mueller *et al.*, 1998) (Elstner *et al.*, 1998), colon (Sarraf *et al.,* 1998), gastric (Takahashi *et al.,* 1999), lung (Tsubouchi *et al.*, 2000), bladder (Guan *et al.,* 1999), thyroid (Ohta *et al.*, 2001) and renal (Inoue *et al.*, 2001) cancer cells, as well as liposarcomas (Tontonoz *et al.*, 1997) *in vitro* and *in vivo* (Han *et al.*, 2003) (Hisatake *et al.*, 2000) (Houseknecht *et al.*, 2002). These observations suggest that PPARγ might be useful for cancer therapy. However, the role of PPARγ ligands in the cell cycle control of human glioblastoma cells remains unknown.

The aim of our study was to highlight the antidiabetic drug ciglitazone, and show that ciglitazone is able to inhibit cell viability and affect the cell cycle and apoptosis in four human glioblastoma cell lines.

METHODS

1. Cell lines and culture condition

The human glioblastoma cell lines, T98G, U-87 MG, A172 and U-118 MG, were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1.7 mM L-glutamine, penicillin and streptomycin. T98G and U-87 MG were cultured in humidified atmosphere with 5 % CO_2 at 37°C, A172 and U-118 MG 10 % CO_2 at 37°C.

2. Chemicals

Ciglitazone [(±)-5-[4-(1-methylcyclohexylmethoxy) benzyl] –thiazolidine-2,4-dione] was obtained from Alexis Biochemicals (Laufelfingen, Switzerland). Ciglitazone was dissolved in dimethylsulfoxide (DMSO). The final concentration of vehicle in DMEM was 0.1%.

MTT (3,[4,4-dimethylthiazol-2-yl] 2,5 diphenyltetrazolium bromide) was purchased from Serva (Heidelberg, Germany).

3. Immunocytochemistry

Immunostaining was undertaken to establish the expression and localisation of PPARγ using fluorescent microscopy. The cells were seeded on small ring glasses into 60 mm culture dishes and incubated in 5 ml DMEM with or without ciglitazone (IC_{50}) for 24 hours. The dishes were washed in phosphate buffer saline (PBS), fixed in methanol:aceton (1:1, v/v) for 10 minutes and stained with polyclonal rabbit primary antibody recognizing human PPARγ

(Santa Cruz, 1:20) for 1 hour. After washing by PBS, the glasses were incubated for 30 minutes with secondary antibody anti-rabbit labelled with FITC. Finally, DAPI staining was used for detection of the all cellular nucleus.

4. In vitro growth rate - cell viability assay

Cell survival was determined using a colorimetric MTT assay as described previously (Carmichael *et al.*, 1987). Briefly, assay was performed in quadruple in 96 well plates. Cells were plated out at a density 2 800 - 5 000 cells per well. Following attachment for 24 hours, the cells were treated for 12, 24, 48 or 72 hours in presence or absence with different concentration (5.10^{-4} mol/l – 5.10^{-8} mol/l) of ciglitazone. The concentration leading to 50% inhibition of viability (IC_{50}) after 24 hours was determined by measuring MTT reductase activity. The yellow solution 10μl of 0.5 % MTT was added to each well, and incubated with the cells for 4 hours 37°C. Finally the established blue crystals were solubilised in 100 μl 10% sodium dodecyl sulphate (SDS) and the absorbance was read at 540 nm using a microplate reader.

5. FACS

Flow cytometry was used to evaluate the number of cells in the particular phases of the cell cycle. Control and treated cells were washed twice with PBS and scraped from the tissue flask in EDTA, centrifuged at 1 800 rpm for 10 minutes at 4°C, washed in cold PBS twice and fixed with chilled -20°C ethanol (70 %; v/v) by low-speed vortexing. For detection of DNA content analysis we used propidium iodide staining. Finally cells were analysed using FACSCalibur flow cytometer (Becton Dickinson). All experiments were replicated three times.

6. Western blot analysis

The glioblastoma cell lines were seeded in 100 mm dishes. The cells were treated by IC_{50} of ciglitazone. This concentration was evaluated by MTT analysis for 24 hours (see above). In order to monitor changes over the 24 hour treatment, we collected the cells after both 24 hours and 12 hours.

The cell proteins were extracted with lysis buffer (10 mM HEPES pH 7.9, 10mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM aprotinin, 1 mM pepstatin, 14 mM leupeptin, 50 mM NaF, 30 mM β-glycerolphosphate, 1 mM NA_3VO_4, and 20 mM p-nitrophenyl phosphate. Cells were incubated on ice for 1 hour and vortexed every 10 minutes. After 30 minutes of centrifugation at 20,000 x rpm, supernatants were collected and kept at -80ºC. Protein concentrations were measured using Bio-Rad Protein Reagent (Bio-Rad Laboratories, Richmond, CA, USA) following the manufacturer's instruction. Twenty micrograms of total protein was separated on 10 % polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membrane by semi-dry electro-transfer. Non-specific binding site were blocked with blocking buffer (5% fat-free skimmer milk with 0.1% Tween 20 in PBS). Subsequently, the membrane was incubated with the particular primary antibody diluted in the blocking buffer overnight at 4°C. The membrane was then washed with the washing buffer (PBS – 0.1 % Tween 20) for 1 hour at room temperature. Then membrane was subsequently incubated with diluted goat anti-mouse IgG-horseradish peroxidase conjugated antibody (dilution 1:6,000, Santa Cruz, California) or goat anti-rabbit IgG-horseradish peroxidase conjugated antibody (dilution 1:2,000, DAKO, Copenhagen, Denmark) for 30 minutes at 4°C. The protein was detected by chemiluminiscence reagent - ECL plus (Amersham Biosciences, Vienna, Austria).

The protein expression (after 12 hours or 24 hours) of cell lysates in treated cells was compared to appropriate control (12 hours and 24 hours). As an internal control, mouse monoclonal antibodies anti-α-tubulin (Sigma, 1:1,000) were used.

7. Telomerase activity

Telomerase activity was analysed by TRAPeze kit (Intergen) based on the TRAP (Telomeric Repeat Amplification Protocol) method. This kit is a highly sensitive *in vitro* assay system for detection of telomerase activity.

The concentration of proteins in cell lysates was measured by the Bradford method and aliquots of the lysate containing 1μg of protein were loaded for the primer elongation. In that step of the reaction, telomerase adds a number of telomeric repeats (GGTTAG) onto the 3` end of a substrate oligonucleotide. The extended products are amplified by the polymerase chain reaction (PCR) using the substrate oligonucleotide and reverse primer, generating a ladder of products with 6 base increments starting at 50 nucleotides: 50, 56, 62, 68, etc. Each reaction mixture contains a primers and template for amplification of 36 bp internal standard which serves as PCR amplification internal control (IC). After separation on 12% polyacrylamide gel, the telomerase ladder was visualised by Sybr Gold and CCD camera DIANA 2 (Raytest). The analysis was replicated three times.

8. TUNEL (TdT-mediated dUTP nick labelling)

For detection of apoptotic cells the TUNEL method was used. Staining was performed according to the protocol (In Situ Cell Death Detection KIT, Roche, Mannheim, Germany).

9. Statistics

The data of the MTT experiments are expressed as means ± SE for the four independent

experiments (* $p < 0.05$).

RESULTS

1. PPARγ in the glioblastoma cell lines

Expression of PPARγ protein by Western blot analysis and by immunocytochemistry was the first experiments performed in this study. All glioblastoma cell lines expressed PPARγ protein, although the cell line U-118 MG expressed the highest level of proteins. The expression of PPARγ protein was slightly lower in T98G and U-87 MG while the lowest expression of PPARγ protein was noticed in A172 cell lines (Fig.1).

These results were confirmed using immunocytochemistry. PPARγ was located in the cytoplasm of glioblastoma cell lines (Fig 2a).

2. Ciglitazone reduced the cell viability of glioblastoma cells

To evaluate the effect of PPARγ ligand, ciglitazone on the viability of human glioblastoma cell lines, we analyzed cell viability by MTT analysis. As shown in Fig.3, ciglitazone inhibited viability in a dose-dependent manner in all four glioblastoma cells. IC_{50} was reached in T98G in concentration $1.8 \cdot 10^{-4}$ mol/l, in U-87 MG $1.7 \cdot 10^{-4}$ mol/l, in A172 $1.5 \cdot 10^{-4}$ mol/l and in U-118 MG $1.5 \cdot 10^{-4}$ mol/l after 24 hours. We found that ciglitazone inhibited cell viability after 48 and 72 hours, as well (Fig. 3).

3. Expression of PPARγ following ciglitazone treatment

Immunocytochemistry showed that after treatment of the cells by ciglitazone, the localisation remained unchanged but we observed stronger expression of PPARγ in our cell

lines (Fig.2). Subsequently, we failed to observe any changes of protein expression by Western blot analysis (Fig.1).

4. Cell cycle arrest

The flow cytometry analysis showed a drop in cell number in S phase and G2/M phases of the cell cycle in glioblastoma cell lines after treatment by ciglitazone. Ciglitazone caused a decrease in percentage of the cell line T98G in S phase of the cell cycle from 37 % in control, untreated cells to 19 % in treated cells. A similar decrease of the cells in the S phase of the cell cycle was observed in other cell lines: a reduction in the S phase in U-87 MG from 29 % in control cell line to 4 % in treated cells, in A172 from 22 % to 8 % in treated cells, in U-118 MG from 20 % to 9 % in treated cells (Fig.4). These results indicate that ciglitazone suppressed viability of T98G, U-87 MG, A172 and U-118 MG by inducing a block in G1 phase and partial block in G2/M phases of the cell cycle. The arrest of the cell cycle in phase G2/M in U-118 MG is debatable.

The effect of ciglitazone on the spectrum of the cell cycle after treatment by ciglitazone IC_{50} for 24 hours was compared using histograms of the cell cycle for control cells (cells cultured in DMEM) and with histograms of the cell cycle of cells cultured in an appropriate concentration of vehicle in DMEM. The two control histograms were basically equal in all glioblastoma cell lines (Fig.4).

5. Expression of cell cycle related proteins

Western blot analysis was used to detect changes in expression of cell cycle regulatory proteins. The cell lines differ from each other in basic level of cell cycle related proteins. For example, the cell line U-87 MG had mutated tumor suppressors p53 and PTEN. The

expression of GFAP (marker of differentiation for astrocytoma cells) was very low in this line. In contrast, in the cell line U-118 MG we observed only mutated PTEN tumor suppressor not p53. T98G had mutated p53 and unexpressed p21$^{Waf1/Cip1}$. It is suspected that T98G cell line had mutation of several pathways cell cycle and apoptosis. We failed to detect p27^{Kip1} in A172 cell line. In addition, PTEN was completely deleted in A172. U-118 MG failed to express androgen receptor.

Ciglitazone had different effects on the expression of the proteins in each cell line (Fig.5). After treatment of glioblastoma cell lines T98G, U-87 MG, A172 and U-118 MG by ciglitazone the expression of cyclin D1 was decreased. This effect was observed after both 12 hours and 24 hours. The phosphorylation of Rb protein was diminished after 12 and 24 hours in all cell lines. The level of total Rb was not markedly reduced as phosphorylation of Rb protein. In addition, the expression of inhibitors of cyclin dependent kinase, p21$^{Waf1/Cip1}$ and p27^{Kip1} was increased after 12 and 24 hours, as well. Apart from A172 we failed to observe the expression of p27^{Kip1} after treatment of the cells by ciglitazone. The expression of protein p16^{Ink4} was very low. This protein was downregulated after treatment of U-118 MG cell line by ciglitazone. In another cell lines we were unable to detect any changes in the level of this protein owing to its low level. Analysis of the expression of mdm-2 protein revealed the lowest level of this protein in T98G cell line. Ciglitazone caused an increase in level of mdm-2 protein in U-87 MG and in A172. The level of mdm-2 in T98G and U-118 MG was unchanged. Ciglitazone resulted in a decrease in level cyclin B1 in U-87 MG, A172 and U-118 MG. The expression of cyclin B1 was unaltered in T98G cell line.

Interestingly, ciglitazone led to reduced expression of androgen receptor in T98G, U-87 MG and A172. U-118 MG showed no expression of androgen receptor.

6. Apoptosis

To determine whether ciglitazone was able to induce apoptosis, we focused on small particles in the flow cytometry analysis. The analysis showed that ciglitazone increased the proportion of subG1 fraction in treated cells in comparison with untreated control cells. The number of apoptotic cells was increased from 8 % in the control to about 25 % in cells treated with ciglitazone in U-87 MG. Ciglitazone induced elevation of the subG1 fraction in all four human glioblastoma cell lines (Tab.1).

In addition, apoptosis was evaluated using expression of apoptosis related proteins. The level of pro-caspase 3 remained almost unchanged. We found no activation of pro-caspase 3. No cleavage of PARP (poly ADP/ribose-polymerase) was observed. Expression of anti-apoptotic protein bcl-2 was significantly decreased in U-118 MG and U-87 MG. Expression of this protein was slightly diminished in A172, as well. In T98G the level of bcl-2 was unchanged. The apoptotic protein bax was increased after 12 hours in T98G (Fig.6). The expression of PTEN was decreased in U-87 MG and U-118 MG after 24 hours. The level of c-myc was increased after both 12 hours and 24 hours in A172 and U-87 MG. The expression of this protein was decreased in U-118 MG cell lines after treatment of the cells by ciglitazone for 24 hours. The expression of c-myc was not changed in T98G.

TUNEL staining failed to confirm apoptosis. Ciglitazone slightly but not significantly increased the number of TUNEL positive cells (data not shown).

7. Differentiation

The glioblastoma cell line U-87 MG expressed very low levels of GFAP (Glial Fibriall Acid Protein). GFAP is a marker for the differentiation of astrocytoma cells. The level of GFAP was increased after treatment of U-87 MG and A172 for both 12 hours and 24 hours. The level of GFAP was decreased after 12 hours in U-118 MG.

We were able to observe a slight decrease of vitamin D receptor (VDR) after 12 hours in U-87 MG. The drop was much higher after 24 hours. In A172 cell line the expression of VDR after 12 hours was decreased and after 24 hours it increased. A similar situation was found for U-118 MG. The protein expression of VDR in T98G was unchanged (Fig.7).

8. Decrease in telomerase activity

Telomerase activity was decreased after treatment of the glioblastoma cell lines U-87 MG and U-118 MG treated by ciglitazone (CIG) for 24 hours (IC_{50}) compared with activity of control cells (C) (Fig.8).

DISCUSSION

Novel therapies for the treatment of central nervous system neoplasm are needed. The present study focused on the cell cycle, differentiation, telomerase activity and apoptosis in human glioblastoma cell lines. Cell lines were characterized using protein expression. Two cell lines (U-87 MG and A172) had mutated p53. Another two (T98G and U-118 MG) had wild type p53. One cell line (A172) had a completely deleted gene of the tumor suppressor PTEN. One cell line had intact inhibitor of CDK, $p27^{Kip1}$. T98G had high resistance to different drugs (Mohri *et al.,* 2000). In sum, we found a spectrum of signalling pathways that are involved in regulation of the cell cycle, cell survival and apoptosis in the cells derived from human astrocytoma.

Studies of humans, animals, and cultured cells support the suggestion that the modulation of PPARγ activation may have therapeutic use in future (Bull *et al.* 2003). PPARs are versatile and potent regulators of cellular function. They play an important role in rodent hepatocarcinogenesis, inflammation, atherosclerosis development, lipid metabolism, diabetes, and in cancer (Everett *et al.*, 2000) (Kersten *et al.*, 2000) (Miller *et al.*, 2000) (Murphy *et al.*, 2000) (Peters *et al.*, 2000). There are several lines of evidence to show that ligands of PPARγ are able to reduce growth of several carcinoma cell lines (Kato EJ, *et al.*, 2002) (James S.Y., *et al.*, 2003) (Tsubouchi *et al.*, 2000) (Yang *et al.* 2001).

We have shown that ciglitazone is able to reduce viability of four human glioblastoma cell lines in a dose-dependent manner. This is in agreement with the results of James S.Y., *et al.* (2003), who showed that ciglitazone and RXR ligand SR11237 inhibited the growth of breast and lung cancer cells. Kim E.J., *et al.* (2003), reported similar results in neuroblastoma cell lines.

17

Contrary to Kato EJ, *et al.* (2002), we detected expression of PPARγ in T98G, U-87 MG, A172 and U-118 MG. However the localisation of PPARγ in human glioblastoma cell lines treated by ciglitazone remained unchanged. The greater expression of PPARγ that was detected by immunocytochemistry, may suggest that the receptor was activated by its own specific ligand.

We have found that ciglitazone affects the cell cycle in the case of glioblastoma cells. It caused a decrease in the expression of cyclin D1 in all human glioblastoma cell lines. Cyclin D1 is responsible for passage of the cell through the G1 phase of the cell cycle. Cyclin D1 together with cyclin dependent kinase (Cdk4, Cdk6) phosphorylates Rb protein that binds transcriptional factors E2F and blocks their function. Following phosphorylation the E2F are released from their complex with Rb protein. Transcriptional factors E2F are necessary for entrance of the cell to the S phase of cell cycle. Thus cyclin D1 governs phase G1. Cyclin D1 overexpression is required for oncogene-induced tumorigenesis. There is a signal transduction cross talk between PPARγ ligands and mitogenic signals that induce cyclin D1. Reduction of cyclin D1 abundance in vivo using antisense transgenic mice, increased expression of PPARγ in vivo (Wang *et al.*, 2003).

Ciglitazone caused increase in the cyclin-dependent kinase inhibitors p21$^{\text{Waf1/Cip1}}$ and p27$^{\text{Kip1}}$, together with decreased expression of cyclin D1 with consequent decrease in phosphorylation of Rb protein. This leads to arrest in the G1 of the cell cycle. According to our results from the Western blot analysis together with those from flow cytometry analysis we presume that ciglitazone blocked the cell cycle of the glioblastoma cell lines in the G1 phase.

This is supported by the observation of Yang *et al.* (2001), who demonstrated that ciglitazone inhibited growth in colon cancer cells by means of G1 cell cycle arrest. Takashima *et al.* (2001), showed that PPARγ ligands inhibited growth of oesophageal adenocarcinoma

18

cells and this may thus be due to G1 arrest and induction of apoptosis. Wakino *et al.* (2002),

confirmed G1 arrest. They showed that TZDs block events critical for re-entry of quiescent

vascular smooth muscle cells into cell cycle, significantly the G0/G1 to the S phase. In

addition, they found that PPARγ ligands attenuated the mitogen-induced degradation of

p27^{Kip1}. Fuse *et al.* (2000), found a relationship between increasing malignancy of human

gliomas and lowered level of p27^{Kip1}. Their results demonstrate that low-grade astrocytoma

were p27-positive, whereas p27^{Kip1} protein was rarely detected in high-grade astrocytoma. An

increasing mortality risk was significantly associated with low level of p27^{Kip1} expression,

suggesting that a decreased p27^{Kip1} level might be a useful prognostic indicator of the clinical

behaviour of a malignant glioma (Alleyne *et al.*, 1999). In the present study, we demonstrated

that ciglitazone is able to induce level of p27^{Kip1} in T98G, U-87 MG and U-118 MG. This

could influence the malignancy of tumors and may have therapeutic benefit. Decreased levels

of cyclin B1 and analysis of cell cycle by flow cytometry revealed the partial block of G2/M

phases of the cell cycle in ciglitazone treated cells. Arrest of the cell cycle in G2/M phase in

U-118 MG is speculative. We suspect that massive induction of apoptosis in U-118 MG may

affect the proportion of the cells in different phases of the cell cycle and therefore the

percentage of the cells did not change in G2/M phase. We concluded that ciglitazone is able to

arrest the cell cycle in G2/M because of decrease S phase and decrease of level of cyclin B. It

seems that the block in phase G1 is larger than the block in phases G2/M.

According to Hisatake *et al.* (2000), PPARγ ligand suppressed expression of androgen

receptor and this caused suppression of androgen-responsive genes in human prostate cancer

cell lines. We confirmed that ciglitazone decreased expression of androgen receptor in

glioblastoma cell lines, as well, although the consequences of this downregulation are

unknown in brain cells as yet.

We showed that ciglitazone inhibited cell viability and caused cell cycle arrest. The crucial question is whether this PPARγ ligand is able to stimulate apoptosis. We demonstrated that the expression of pro-apoptotic protein bax was increased after treatment of the cell line T98G by ciglitazone. In this cell line expression of antiapoptotic protein bcl-2 was not changed. In contrast, the rest of our cell lines, U-87 MG, A172 and U-118 MG showed decreased levels of anti-apoptotic protein bcl-2, while the level of bax was unchanged. In addition, flow cytometry revealed that the number of the cells in the subG1 fraction rose in treated cells compared with control cell lines. These results confirm that ciglitazone supports apoptosis despite negative TUNEL staining. The principle of TUNEL staining is detection of later apoptosis changes may explain the TUNEL negative results.

Moreover we found increased level of the differentiation related protein GFAP, in U-87 MG and A172 and this may be in accord with the suggested effects of ciglitazone in the differentiation process in various tumors (Sarraf et al, Kubota et al).

Another aspect of our work was focused on analysis of telomerase activity in human glioblastoma cell lines. Telomerase is RNA-dependent DNA-polymerase. It comprises RNA unit (hTR) and catalytic unit hTERT. Most somatic cells in adults have very low telomerase activity or hTERT transcript. The telomerase is constitutively active in most tumor cells. The rise in telomerase activity is a marker of tumor progression.

Telomerase is fine marker of cancer since it is observed in almost all tumor tissue with a high degree of positivity. Telomerase activity is a useful marker of tumor grade in astrocytoma, where it is impossible to distinguish benign from malignant tumors, e.g. brain tumors, according to morphology.

Telomerase activity has been detected in over 85 % of all tumors tested, spanning more than 20 different types of cancers. Cancer cells are therefore characterized by high telomerase activity. GBM in particular express an active form of telomerase and this leads to the

uncontrolled proliferation characteristic of GBM cells. We showed that all studied cell lines, T98G, U-87 MG, A172 and U-118 MG, possess telomerase active. Our human glioblastoma cell lines U-87 MG and U-118 MG treated by IC_{50} ciglitazone showed a reduction in telomerase activity. This provides us with new possibilities for control of cell proliferation in the case of glioblastoma cells.

In conclusion, the thiazolidinedione class of drug are powerful PPARγ ligands capable of not only modifying the energy metabolism of cells but also of causing arrest of the cell cycle resulting in apoptotic changes in cancer cells origin GBM. This study reveals a new way for modifying cell viability of brain tumor cells.

The treatment of cancer is undergoing a shift. Traditionally, cancer patients are treated with radiation therapy and cytotoxic agents that aim to have a greater effect on proliferating cancer cells than they do on non-cancerous cells. Unfortunately their effects on GBM patients have been modest, and median survival has remained largely unchanged over the past decade. Major advances in molecular and cellular biology have substantially improved our understanding of the genetic and proteomic changes involved in tumorigenesis. Our finding has potential application to new treatment of cancer patients.

ACKNOWLEDGEMENTS

REFERENCES

Alleyne Ch, He J, Yang J, Hunter SB, Cotsonis G, James CD and Olson JJ (1999) Analysis of cyclin dependent kinase inhibitors in malignant astrocytomas. *Int J Oncol* **14:**1111-1116.

Bull AW (2003) The role of Peroxisome proliferator-activated receptor γ in colon cancer and inflammatory bowel disease. *Arch Pathol Lab Med* **127:**1121-1123.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* **47:**936–942.

Chattopadhyay N, Singh DP, Heese O, Godbole MM, Sinohara T, Black PM and Brown EM (2000) Expression of Peroxisome Proliferator-Activated Receptors (PPARs) in human astrocytic cells: PPARγ agonists as inducers of apoptosis. *J Neurosci Res* **61:**67-74.

Cimini A, Cristiano L, Bernardo A, Farioli-Vecchioli S, Stefanini S and Ceru MP (2000) Presence and inducibility of peroxisomes in human glioblastoma cell line. *Biochim Biophys Acta* **1474:**397-409.

Ehrmann J, Vavrusova N, Collan Y and Kolar Z (2002) PPARs in health and disease. *Biomed Papers* **146:**11-14.

Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H and Shintaku P (1998) 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* **95:**8806-8811.

Everett L, Galli A and Crabb D (2000) The role of hepatic peroxisome proliferator-activated receptors (PPARs) in health and disease. *Liver* **20:**191-199.

Fan X, Aalto Y, Sanko SG, Knuutila S, Klatzmann D and Castresana JS (2002) Genetic profile, PTEN mutation and therapeutic role of PTEN in glioblastomas. *Int J Oncol* **21:**1141-1150.

Fuse T, Tanikawa M, Nakanishi M, Ikeda K, Tada T, Inagaki H, Asai K, Kato T and Yamada K (2000) p27^{Kip1} expression by contact inhibition as a prognostic index of human glioma. *J Neurochem* **74:**1393-1399.

Guan YF, Zhang YH, Breyer RM, Davis L and Breyer MD (1999) Expression of peroxisome proliferator-activated receptor gamma (PPARgamma) in human transitional bladder cancer and its role in inducing cell death. *Neoplasia* **1:**330-339.

Gurney JG, Kadan-Lottick N (2001) Brain and other central nervous system tumors: rates, trends, and epidemiology. *Curr Opin Oncol* **13:**160-166.

Han C, Demetris AJ, Michalopoulos GK, Zhan Q, Shelhamer JH and Wu T (2003) PPARγ ligands inhibit cholangiocarcinoma cell growth through p53-dependet GADD45 and p21$^{Waf1/Cip1}$ pathway. *Hepatology* **38:**167-177.

Heuvel JP (1999) Peroxisome proliferator-activated receptors: a critical link among fatty acids, gene expression and carcinogenesis. *J Nutr* **129:**575-580.

Hisatake JI, Ikezoe T, Holden S, Tomayasu S and Koeffler PH (2000) Down-regulation of prostate-specific antigen expression by ligands for peroxisome Proliferator-activated receptor γ in human. *Cancer Res* **60:**5494-5498.

Houseknecht KL, Cole BM and Steele PJ (2002) PPAR γ and its ligands: A review. *Domest Anim Endocrinol* **22:**1-23.

Inoue K, Kawahito Y, Tsubouchi Y, Kohno M, Yoshimura R, Yoshikawa T and Sano H (2001) Expression of peroxisome proliferator-activated receptor γ in renal cell carcinoma and growth inhibition by its agonists. *Biochem Biophys Res Commun* **287:**727-732.

Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC and Meir EGV (1999) Frequent co-alteration of TP53, p16/CDKN 2S, p14[ARF], PTEN tumor suppressor genes in human glioblastoma cell lines. *Brain Pathol* **9:**469-479.

James SY, Lin F, Kolluri SK, Dawson MI, Zhang XK Sharon (2003) Regulation of retinoic acid receptor beta expression by peroxisome proliferator-activated receptor gamma ligands in cancer cells. *Cancer Res* **63:**3531-3538.

Jiang H, Conrad C, Fueyo J, Gomez-Manzano C and Liu TJ (2003) Oncolytic adenoviruses for malignant glioma therapy. *Front Biosci* **8:**577-588.

Kato M, Nagaya T, Fujieda M, Saito K, Yoshida J and Seo H (2002) Expression of PPAR γ and its ligand-dependent growth inhibition in human brain tumor cell lines. *Jpn J Cancer Res* **93:**660-666.

Kersten S, Desvergne B and Wahli W (2000) Roles of PPARs in health and disease. *Nature* **405:**421-424.

Kim EJ, Park KS, Jung SY, Sheen YY, Moon DC, Song YS, Kim KS, Song S, Yun YP, Lee MK, Oh KW, Yoon DY and Hong JT (2003) Peroxisome proliferator-activated receptor-gamma activator 15-deoxy-{delta}12,14-prostaglandin J2 inhibits neuroblastoma cell growth through induction of apoptosis: association with ERK signal pathway. *J Pharmacol Exp Ther* **307:**505-517.

Kubota T, Koshizuka K, Williamson EA, Asou H, Said JW, Holden S, Miyoshi I and Koeffler HP (1998) Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res* **58:**3344-3352.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B,Kastner P, Mark M and Chambon P (1995) The nuclear receptor superfamily: the second decade. *Cell* **83:**835-839.

Miller RT, Anderson SP, Corton JC and Cattley RC (2000) Apoptosis, mitosis and cyclophilin-40 expression in regressing peroxisome proliferator-induced adenomas. *Carcinogenesis* **21:**647-652.

Mohri M, Nitta H, Yamashita J (2000) Expression of multidrug resistance-associated protein (MRP) in human gliomas. *J Neurooncol* **49:**105-115.

Mischel PS and Cloughesy TF (2003) Targeted molecular therapy of GBM. *Brain Pathol* **13:**52-61.

Mueller E, Sarraf P, Tontonoz P, Evans RM, Martin KJ, Zhang M, Fletcher C, Singer S and Spiegelman BM (1998) Terminal differentiation of human breast cancer through PPAR gamma. *Mol Cell* **1:**465-470.

Murphy GJ and Holder JC (2000) PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. *Trends Pharmacol Sci* **21:**469-474.

Ohta K, Endo T, Haraguchi K, Hershman JM and Onaya T (2001) Ligands for peroxisome proliferator-activated receptor gamma inhibit growth and induce apoptosis of human papillary thyroid carcinoma cells. *J Clin Endocrinol Metab* **86:**2170-2177.

Peters JM, Rusyn I, Rose ML, Gonzales FJ and Thurman RG (2000) Peroxisome proliferator activated receptor alpha is restricted to hepatic parenchymal cells, not Kupffer cells: implications for the mechanism of action of peroxisome proliferators in hepatocarcinogenesis. *Carcinogenesis* **21:**823-826.

Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C and Spiegelman BM (1998) Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med* **4:**1046-1052.

Takahashi N, Okumura T, Motomura W, Fujimoto Y, Kawabata I and Kohgo Y (1999) Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells. *FEBS Lett* **455:**135-139.

Takashima T, Fujiwara Y, Higuchi K, Arakawa T, Yano Y, Hasuma T and Otani S (2001) PPAR-gamma ligands inhibit growth of human esophageal adenocarcinoma cells through induction of apoptosis, cell cycle arrest and reduction of ornithine decarboxylase activity. *Int J Oncol* **19:**465-471.

Tontonoz P, Singer S, Forman BM, Sarraf P, Fletcher JA, Fletcher CD, Brun RP, Mueller E, Altiok S, Oppenheim H, Evans RM and Spiegelman BM (1997) Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. *Proc Natl Acad Sci U S A* **94:**237-241.

Tsubouchi Y, Sano H, Kawahito Y, Mukai S, Yamada R, Kohno M, Inoue K, Hla T and Kondo M (2000) Inhibition of human lung cancer cell growth by the peroxisome proliferator-activated receptor-gamma agonists through induction of apoptosis. *Biochem Biophys Res Commun* **270:**400-405.

Wakino S, Kintscher U, Liu Z, Kim S, Yin F, Ohba M, Kuroki T, Schonthal AH, Hsueh WA and Law RE (2002) Peroxisome proliferator-activated receptor gamma ligands inhibit

mitogenic induction of p21$^{Waf1/Cip1}$ by modulating the protein kinase C delta pathway in vascular smooth muscle cells. *J Biol Chem* **276:**47650-47657.

Wang C, Pattabiraman N, Zhou JN, Fu M, Sakamaki T, Albanese C, Li Z, Wu K, Hulit J, Neumeister P, Novikoff PM, Brownlee M, Scherer PE, Jones JG, Whitney KD, Donehower LA, Harris EL, Rohan T, Johns DC, Pestell RG and Wang C (2003) Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. *Mol Cell Biol* **23:**6159-73.

Yang WL and Frucht H (2001) Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. *Carcinogenesis* **22:**1379-83.

29

FOOTNOTES

Citation of meeting abstracts where the work was previously presented:

Vavrusova N., Ehrmann J. jr., Kolar Z. (2003) The synthetic ligand of PPAR gamma ciglitazone affects viability, proliferation and apoptosis in human glioblastoma cell lines, 19th European congress of Pathology, Ljubljana 9/2003.

Person to receive reprint requests:

Nicol Strakova

Department of Pathology & Laboratory of Molecular Pathology

Faculty of Medicine, Palacky University

Hnevotinska 3

775 15 Olomouc

Czech Republic

Phone: 00420-585 639 541

Fax: 00420-585 632 966

Email: **Nicol.Strakova@seznam.cz**

LEGENDS TO FIGURES

Figure 1: Expression of PPARγ. Human glioblastoma cell lines were treated by ciglitazone (IC_{50}) for 24 hours (CIG24) and compared with control cells (C24). The cell proteins were analyzed by Western blot analysis. For detection of expression of PPARγ the primary antibody mouse monoclonal anti-PPARγ (clone E-8, Santa Cruz Biotechnology, Inc.) was used. The analysis has confirmed that all human glioblastoma cell lines expressed PPARγ. In addition, the effect of ciglitazone was analyzed after 12 hours treatment (CIG12) and again compared with control untreated cells (C12). Western blot analysis showed that PPARγ expression was unchanged.

Figure 2: Localisation of PPARγ following ciglitazone treatment. Glioblastoma cell line U-87 MG was treated by ciglitazone (IC_{50}) for 24 hours and compared with control cells. **(a) (b) (c) (d)** cells were incubated with primary antibody monoclonal mouse anti-PPARγ antibody (clone E-8, Santa Cruz Biotechnology, Inc.) for 2 hours. After that the cells were stained by anti-mouse FITC secondary antibody **(a) (c) (e)** and by DAPI **(b) (d) (f).** DAPI staining was used for detection of all cellular nuclear. **(a) (b)** untreated control cells; **(c) (d)** cells treated by ciglitazone and **(e) (f)** control staining without primary antibody anti-PPARγ. Immunocytochemistry staining showed higher positivity of PPARγ in treated cells than in control cells. The localisation of PPARγ was unchanged. The original magnification was 100x10.

Figure 3: Effect of ciglitazone on cell viability in four human glioblastoma cell lines. The following concentrations of ciglitazone (1.0×10^{-5}, 1.0×10^{-4}, 1.2×10^{-4}, 1.4×10^{-4}, 1.6×10^{-4}, 1.8×10^{-4},

2.0 10^{-4} and 2.2 10^{-4} mol/l) were applied to T98G, U-87 MG, A172 and U-118 MG cell lines for 24, 48 and 72 hours. The concentration capable of reducing the viability of human glioblastoma cell lines by 50% after 24 hours (IC_{50}) was used for following the effect of ciglitazone on cell cycle, apoptosis, differentiation and telomerase activity on glioblastoma cell lines in all experiments.

Figure 4: Cell cycle distribution of T98G, U-87 MG, A172 and U-118 MG cells after flow cytometry analysis. The cell lines were treated by ciglitazone (IC_{50}) for 24 hours, harvested, stained with propidium iodide, and analyzed using the flow cytometer. Histograms of the treated cells were compared with control untreated cells and with cells grown with adequate concentration of vehicle (DMSO). Horizontal and vertical axes indicate relative nuclear DNA content and number of events (cells), respectively.

Figure 5: Western blot analysis of p53, mdm-2, p21, p27, p16, cyclin D1, pRb-P, pRb, cyclin B1 and androgen receptor (AR) in U-87 MG, A172, U-118 MG and T98G cells treated by ciglitazone. Cells were incubated in medium with ciglitazone (IC_{50}) for 24 hours. The expressions of proteins of treated cells (CIG12 - Ciglitazone 12 hours, IC_{50}; CIG24 - Ciglitazone 24 hours, IC_{50}) were confronted with the protein expression of control, untreated cells. (C12 - Control 12 hours; C24 - Control 24 hours). mcm-7 expression was used as a loading marker.

Figure 6: Western blot analysis of bcl-2, bax, bid, caspase-3, PARP, c-myc and PTEN in U-87 MG, A172, U-118 MG and T98G cells treated by ciglitazone. Cells were incubated in medium with ciglitazone (IC_{50}) for 24 hours. We checked the effect of ciglitazone (IC_{50}) on the protein level after 12 hours. The protein expressions of treated cells (Cig12 - Ciglitazone

12 hours, IC50; Cig24 - Ciglitazone 24 hours, IC50) were compared with the protein expression of control, untreated cells. (C12 - Control 12 hours; C24 - Control 24 hours). The expression of mcm-7 was used as a loading marker.

Figure 7: Western blot analysis of GFAP and VDR in U-87 MG, A172, U-118 MG and T98G cells treated by ciglitazone. Cells were incubated in medium with ciglitazone (IC_{50}) for 24 hours. We checked the effect of ciglitazone (IC_{50}) on the protein level after 12 hours. The protein expressions of treated cells (CIG12 - Ciglitazone 12 hours, IC_{50}; CIG24 - Ciglitazone 24 hours, IC_{50}) were compared with the protein expression of control, untreated cells (C12 - Control 12 hours; C24 - Control 24 hours). The expression of mcm-7 was used as a loading marker.

Figure 8: Analysis of telomerase activity. Telomerase activity was analysed by TRAPeze kit in U-87 MG, U-118 MG, A172 and T98G cells treated by ciglitazone (IC_{50}) for 24 hours (IC - internal control for PCR amplification). Telomerase activity in control samples did not change over time. We detected decreased telomerase activity in U-87 MG and U-118 MG treated by ciglitazone (CIG) for 24 hours (IC_{50}) compared with control cell activity (C).

Table1: Analysis of the subG1, G1, S and G2/M phases of the cell cycle of the human glioblastoma cell lines treated by ciglitazone for 24 hours. Analysis of flow cytometry was used for detection of apoptotic cells and for distribution of the cells in the cell cycle. The cell lines were treated by ciglitazone (IC_{50}) for 24 hours, harvested, stained with propidium iodide, and analyzed using flow cytometer. Histograms of the treated cells were compared with untreated controls. Horizontal and vertical axes indicate relative nuclear DNA content and number of events (cells), respectively.

cell line	treatment	*% apoptotis*	*% cell cycle distribution*		
		sub G1	**G1**	**S**	**G2/M**
U-87 MG	Control	8	60	28	12
	Ciglitazone	25	89	4	7
A172	Control	34	66	22	12
	Ciglitazone	38	83	8	9
U-118 MG	Control	32	67	20	13
	Ciglitazone	88	75	9	16
T98G	Control	24	54	37	9
	Ciglitazone	4	75	19	6

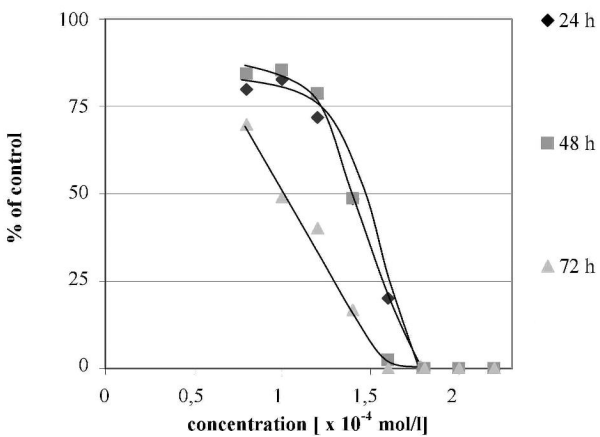|	U-87 MG	A172	U-118 MG	T98G
	C12 CIG12 C24 CIG24	C12 CIG12 C24 CIG24	C12 CIG12 C24 CIG24	C12 CIG12 C24 CIG24

PPAR -γ

mcm-7
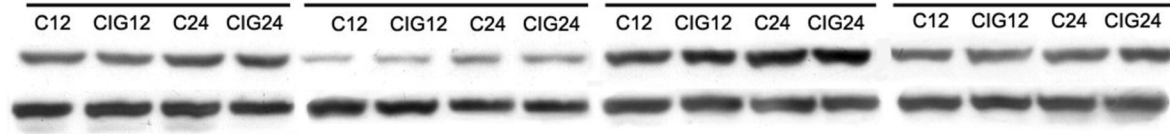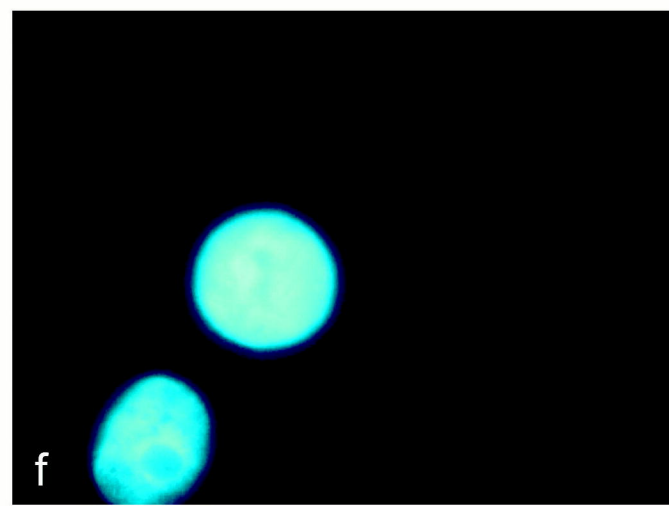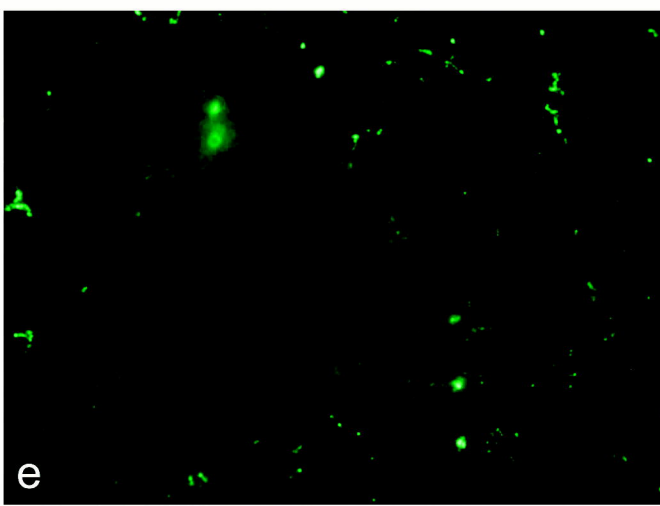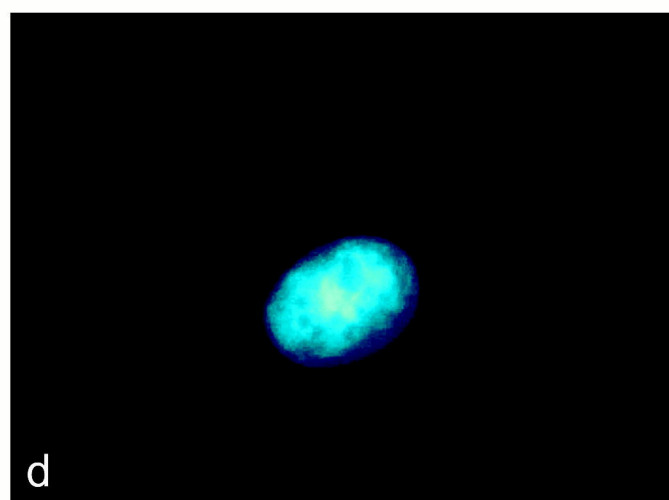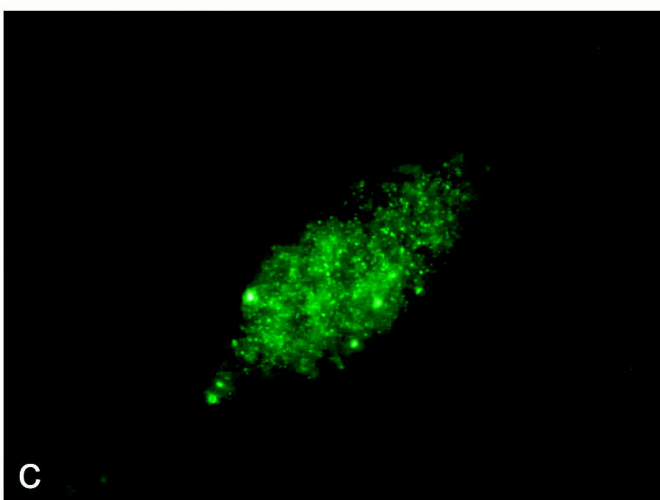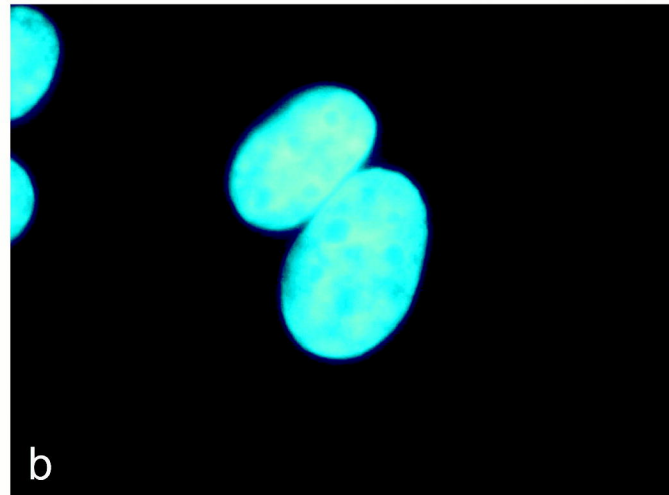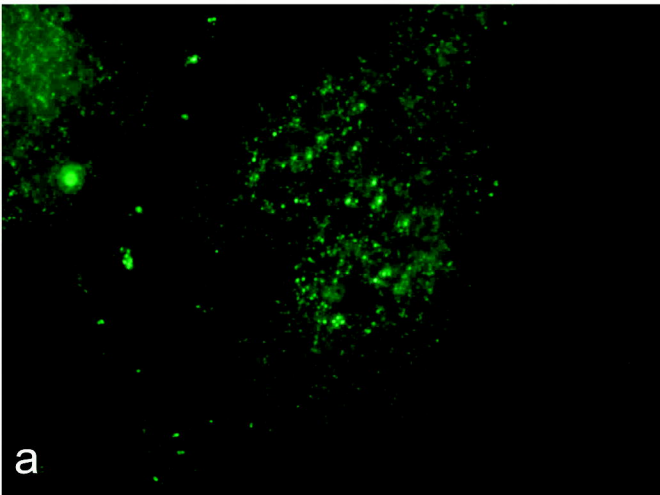
T98G: Ciglitazone 24, 48 and 72 hours.
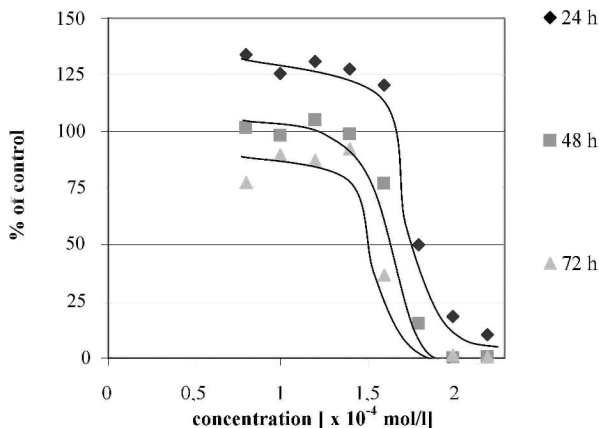
U-87 MG: Ciglitazone 24, 48 and 72 hours.
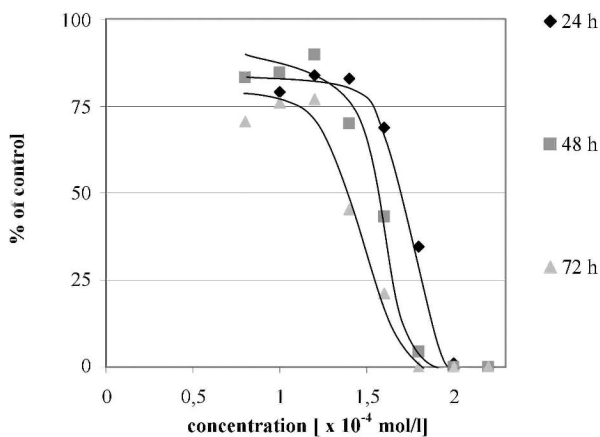
A172: Ciglitazone 24, 48 and 72 hours.
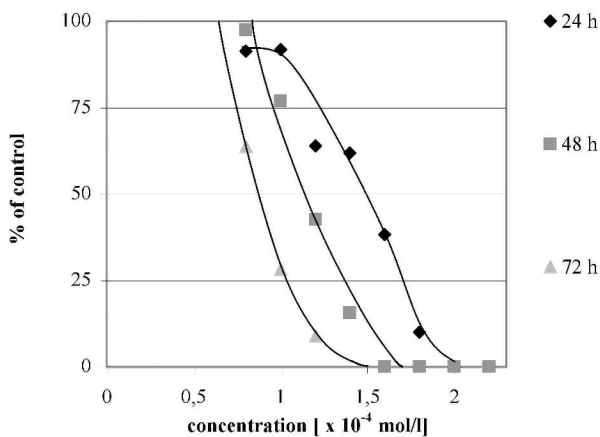
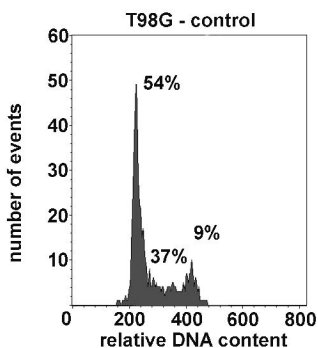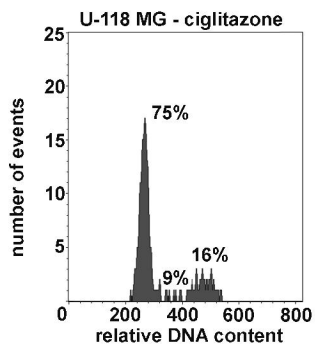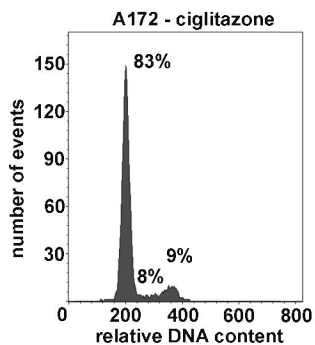**U-118 MG: Ciglitazone 24, 48 and 72 hours.**
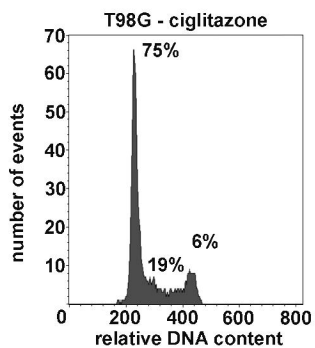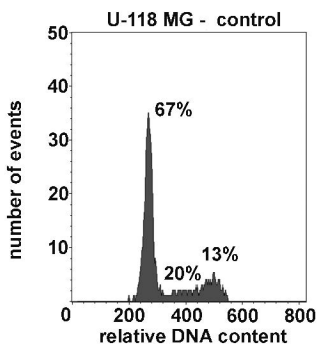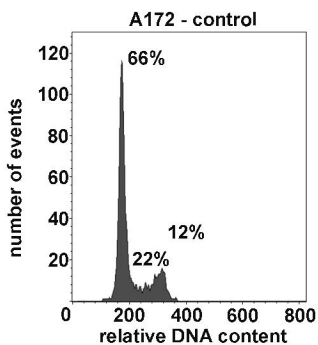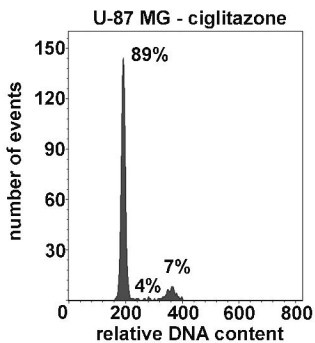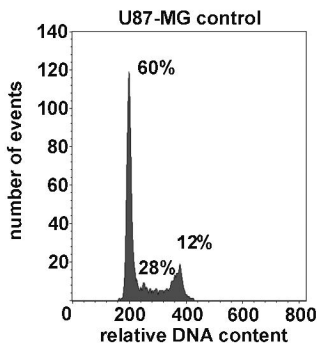
	U-87 MG				A172				U-118 MG				T98G			
	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24
p53																
mdm-2																
p21																
p27																
p16																
cyc D1																
pRb-P																
pRb																
cyc B1																
AR																
mcm-7																
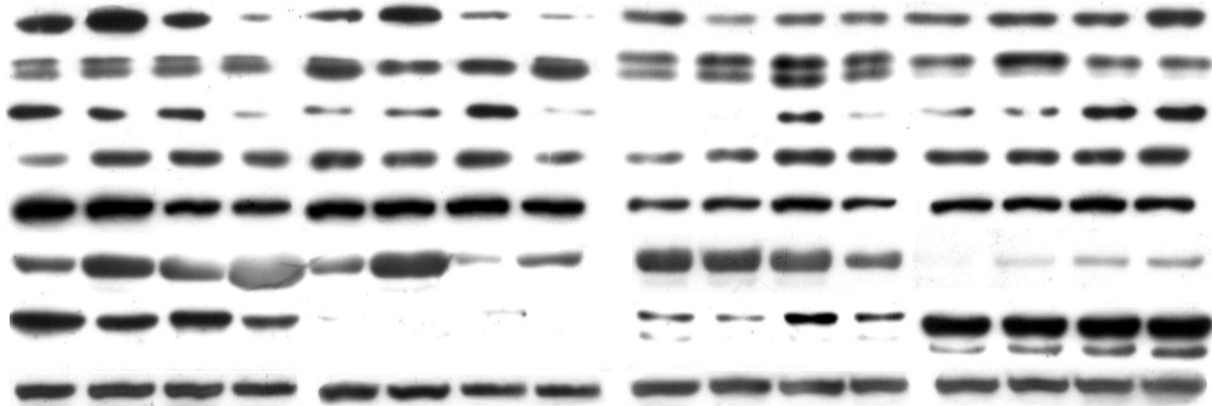
	U-87 MG				A172				U-118 MG				T98G			
	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24	C12	CIG12	C24	CIG24

GFAP

VDR

mcm-7