Peroxisome proliferator-activated receptors (PPAR) agonists affect cell viability, apoptosis and expression of cell cycle related proteins in cell lines of glial brain tumors*

N. STRAKOVA[1], J. EHRMANN[1**], J. BARTOS[2], J. MALIKOVA[1], J. DOLEZEL[2], Z. KOLAR[1]

[1]Laboratory of Molecular Pathology, e-mail: jiri.ehrmann@tiscali.cz, Department of Pathology, Faculty of Medicine, Palacky University, Olomouc, CZ 77515; [2]Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Academy of Science of the Czech Republic, Olomouc, CZ 77900

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The nuclear receptors PPARs (peroxisome proliferator-activated receptors) are transcription factors activated by specific ligands. PPARs play an important role in carcinogenesis, inflammation, atherosclerosis, lipid metabolism and diabetes. There is evidence that activation of PPARs by specific ligands is able to suppress the growth of different types of human cancer by mechanisms including the growth arrest, apoptosis and induction of differentiation, although the detailed signalling pathways have not been completely elucidated to date. The aim of our study was to determine whether synthetic ligands of PPARα and PPARγ could affect the viability, proliferation, differentiation, apoptosis and expression of some cell cycle related proteins in glial tumor cell lines. The study was performed on human glioblastoma cell lines U-87 MG, T98G, A172 and U-118 MG. Cell lines were treated by ligands of PPARα (bezafibrate, gemfibrozil) and PPARγ (ciglitazone). MTT, flow cytometry, TUNEL assay and immunoblotting were used for detection of changes in cell viability, proliferation, differentiation and apoptosis. Bezafibrate, ciglitazone and gemfibrozil inhibited viability of glioblastoma cell lines. The synthetic ligands significantly reduced or induced the expression of cyclins, $p27^{Kip1}$, $p21^{Waf1/Cip1}$, MDM-2, Bcl-2, Bax, PARP, Caspase 3, androgen receptors, etc. and did not affect the expression of the differentiation marker GFAP. Flow cytometry confirmed arrest of the cell cycle although the detection of apoptosis was controversial. Apart from hypolipidemic and hypoglycaemic effects, PPAR ligands may also have significant cytostatic effects of potential use in anticancer treatment.

Key words: PPAR, glioblastoma, cell cycle, apoptosis, fibrates, thiazolidinediones

Nuclear receptors are transcription factors belonging to the steroid-thyroid-retinoid receptor superfamily which include receptors for steroids, thyroid hormone, vitamin A and D derived hormones and some fatty acids [26]. They are activated by specific ligands and they play an important role during the cell signalling. However, in some nuclear receptors the natural ligand has not been identified and hence the term "orphan" receptors (OR) was suggested a decade ago. To date, five families of OR have been distinguished: 1. Liver X receptor – LXR, 2. Pregnane X receptor – PXR, 3. Constitutive androstane receptor – CAR, 4. Farnesoid X receptor – FXR and lastly 5. Peroxisome proliferator-activated receptors – PPARs [23]. The PPAR was first cloned from mouse liver in 1990 as the nuclear receptor that mediates the effect of synthetic compounds called peroxisome proliferators (PPs) [20]. PPs are able to increase both the size and number of peroxisomes. They have various metabolic functions within the cell such as peroxide derived respiration, β oxidation of fatty acids and cholesterol metabolism [5]. Three PPAR isoforms are known to exist: PPARα, PPARδ (also known as NUC1 or PPARβ) and PPARγ. They are encoded by separate genes, perform separate functions and exhibit different tissue localization [1]. After activation by ligand as is the case with nuclear receptors, PPARs binds to a specific element in the promoter region of target genes. However, dimerization of PPAR with RXR and the presence of coactivators are necessary for the transcriptional activity of PPAR responsive element (PPRE) in DNA [40]. PPARs play an important role

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**corresponding author

during rodent hepatocarcinogenesis, during inflammation, atherosclerosis development, lipid metabolism, diabetes, and they also have important role in cancer [9, 22, 29].

There is evidence that some PPARs are able to suppress the growth of different types of human cancer by distinct mechanisms including growth arrest, apoptosis and induction of differentiation. Mutations of PPARγ in colon carcinoma, by contrast, lead to loss of ligand binding ability and loss of cell growth suppression which may indicate that functional PPARγ is required for normal growth properties of human colon cells [34]. While, the detailed signaling pathways leading to growth arrest and differentiation have not yet been completely elucidated, nevertheless it is evident that PPAR ligands (agonists) may have potent anticancer potential and may serve as a rational basis for therapy of some tumors or their chemoprevention as shown *in vitro* studies focused on liposarcoma [8], ovarian carcinoma [11], breast carcinoma [28, 38], prostate carcinoma [2, 25], urinary bladder carcinoma [12], some types of B-lymphoma [31], erythroleukaemia [16], non small lung cell cancer [17], gastric and colon carcinomas [35, 13]), liver carcinoma [41], renal cell carcinoma [19] and esophageal adenocarcinoma [37]. Since changes in PPARs expression has been demonstrated in various types of cancers and because PPARs have been shown to be expressed in both rodent and human glial cells [7, 30, 4, 6], the aim of this study was to explore the role of these receptors in glial tumors. The effect of PPARs agonists on the viability, apoptosis and expression of some cell cycle regulated proteins on human glial tumor cell lines was analyzed in this paper.

Material and methods

Cell cultures and reagents. Four human glioblastoma cell lines, T98G, U-87 MG, U-118 MG and A172 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 and 50 mg/100 ml gentamycine. U-87 MG and T98G cells were cultured in humidified atmosphere with 5 % CO_2 at 37 °C and U-118 MG and A172 with 10 % CO_2 at 37 °C.

The cell lines were treated by agonists of PPAR (two derivatives from fibrate family and two from thiazolidinedione family).

Gemfibrozil and bezafibrate (PPARγ ligands) and non substituted thiazolidinedione (PPARγ ligand) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ciglitazone (another PPARγ ligand) was obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The bezafibrate was dissolved in DMEM. The solubility of gemfibrozil was better in ethanol (EtOH) than in dimethyl sulfoxide (DMSO) and therefore the gemfibrozil was dissolved in DMEM containing ethanol. By contrast, thiazolidinedione and ciglitazone were dissolved in DMSO. The final concentration of vehicle was lower than 0.3 %. No changes in cell viability were recorded when either EtOH or DMSO were used (the effect of the each solvent on cell viability assay was checked in each experiment).

Cell viability assay. Cell survival was determined using a colorimetric MTT assay as described previously [3]. Briefly, assays were performed in quadruple at different concentration of fibrates and ciglitazone (see Results) in 96 well plates. Cells were plated out in 96-well cell culture plates at a density of 2 800–5 000 cells per well. Following attachment overnight, the cells were treated from 12 to 72 hours by the ligands of PPAR. Concentrations of bezafibrate, gemfibrozil, non substituted thiazolidinedione and ciglitazone leading to 50 % inhibition of growth (IC_{50}) was determined by measuring MTT reductase activity (3,[4,4-dimethylthiazol-2-yl] 2,5 diphenyltetrazolium bromide; Serva Electrophoresis, Heidelberg, Germany).

10 µl of 0.5 % MTT was added to each well, and the cells were incubated with substrate for 4 hours at 37 °C. After incubation, blue formazan crystals were solubilized in 100 µl 10 % SDS. The absorbance was read at 540 nm using a microplate reader and this directly correlated with the cell viability. IC_{50} were determined as 50 % decrease of absorbance compare with the absorbance of the cells treated only by DMEM (control cells). In addition, cell viability of the treated cells was compared with both cells in DMEM and cells in DMEM with maximum used concentration of vehicle.

Western blot analysis. Standard immunoblotting techniques were used. The proteins from whole cell lysate were run on 10–12 % polyacrylamide gels. Electrophoresed proteins were transferred onto nitrocellulose membrane by semi-dry electrophoretic transfer. Non-specific binding sites were blocked with blocking buffer (5 % fat-free skimmed milk with 0.1 % Tween 20 in PBS). Subsequently, the membrane was incubated with the primary antibody (Tab. 1) diluted in the blocking buffer overnight at 4 °C. Then the membrane was washed with the washing buffer (PBS – 0.1 % Tween 20) for 1 hour at room temperature. After that the membrane was incubated with diluted goat anti-mouse IgG-horseradish peroxidase conjugated antibody (dilution 1:6000, Santa Cruz, California) or goat anti-rabbit IgG-horseradish peroxidase conjugated antibody (dilution 1:2000, DakoCytomation, Carpinteria, California) for 30 minutes at 4 °C. The proteins were detected by chemiluminiscence reagent – ECL plus (Amersham Biosciences, Vienna, Austria).

The protein expressions (after 12 hours or 24 hours) of cell lysates of treated cells were compared to suitable controls (12 hours and 24 hours).

Flow cytometry. 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 1 000 rpm for 10 minutes at 4 °C, washed in cold PBS twice and fixed with methanol (70 %; v/v) by low-speed vortexing.

Table 1. Primary antibodies used

No.	Antibody	Clone	Poly-mono-clonal	Origin	Dilution	Mass [kDa]	Producer
1	AR	441	monoclonal	mouse	1:100	110	Santa Cruz
2	bax	B-9	monoclonal	mouse	1:100	21	Santa Cruz
3	bcl-2	100	monoclonal	mouse	1:500	29	Biogenex
4	bcl-X_L	H-62	polyclonal	rabbit	1:100	29	Novocastra
5	bid	–	polyclonal	rabbit	1:1000	22+15	Cell Signaling Technology
6	cas-3	CPP32	polyclonal	rabbit	1:3000	32+19+17	Brno*
7	cas-8	P-20	polyclonal	rabbit	1:200	35	Santa Cruz
8	cdk2	D-12	monoclonal	mouse	1:500	36	Santa Cruz
9	c-myc	Ab-2	monoclonal	mouse	1:100	65	Oncogene
10	cyclin B1	7A9	monoclonal	mouse	1:50	50-55	Novocastra
11	cyclin D1	ascites	monoclonal	mouse	1:500	36	Brno*
12	cyclin E	13A3	monoclonal	mouse	1:200	45	Novocastra
13	ER α	6F11	monoclonal	mouse	1:100	50	Novocastra
14	GFAP	H-50	polyclonal	rabbit	1:200	50	Santa Cruz
15	MDM-2	4B2	monoclonal	mouse	ready to use	95	Brno*
16	p16	DCS-50	monoclonal	mouse	1:10	16	Novocastra
17	p21	118	monoclonal	mouse	1:800	21	Brno*
18	p27	SX53G8	monoclonal	mouse	1:250	27	DakoCytomation
19	p53	DOI	monoclonal	mouse	1:10	53	Brno*
20	PARP	F-2	monoclonal	mouse	1:10000	46	Brno*
21	PPAR α	H-98	polyclonal	rabbit	1:200	50	Santa Cruz
22	PPAR γ	E-8	monoclonal	mouse	1:50	50	Santa Cruz
23	PTEN	26H9	monoclonal	mouse	1:1000	50	Cell Signaling Technology
24	RAR α	C-20	polyclonal	rabbit	1:200	45	Santa Cruz
25	RAR β	C-19	polyclonal	rabbit	1:100	52	Santa Cruz
26	Rb total	G4-340	monoclonal	mouse	1:500	100	CCC Copenhagen**
27	Rb-P	Rb-10	monoclonal	mouse	1:500	100	CCC Copenhagen**
28	RXR α	D-20	polyclonal	rabbit	1:200	50	Santa Cruz
29	RXR β	11-13	monoclonal	mouse	1:100	53	Santa Cruz
30	tubulin a	DM 1A	monoclonal	mouse	1:2000	50	Sigma
31	VDR	D-6	monoclonal	mouse	1:100	55	Santa Cruz

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Prior to analysis cells were peleted to remove methanol and resuspended in 0.5 ml OTTO I buffer (Otto 1990). 1 ml OTTO II buffer supplemented with 20 μg/ml DNA-specific fluorescent dye DAPI was added. Finally cells were analyzed using FACS Vantage flow cytometer (Becton Dickinson).

TUNEL staining. For detection of apoptotic cells the TUNEL (TdT-mediated dUTP nick labeling) method was used. The staining was performed according the protocol recommendation (In Situ Cell Death Detection KIT, Roche, Mannheim, Germany).

Statistical analysis. The data of the MTT experiments are expressed as means ±SE of four independent experiments ($p<0.05$).

Results

Cell viability. Both synthetic ligands of PPARα (bezafibrate, gemfibrozil) and PPARγ (ciglitazone) inhibit cell growth in glial tumor cell lines.

Bezafibrate and gemfibrozil were tested in concentrations ranging from 2.10^{-3} mol/l to 1.10^{-5} mol/l. 50 % inhibition of growth (IC_{50}) after 24 hour treatment of bezafibrate was reached only in U-118 MG cell line in concentrations of 2.10^{-3} mol/l (Fig. 1). In the rest cell lines, the cell viability decreased less than 50 % (data not shown). The gemfibrozil had stronger effect; the IC_{50} after 24 hour treatment was achieved in all cell lines. In U-118 MG at the concentration of $1.8.10^{-3}$ mol/l, in T98G at the concentration of $1.95.10^{-3}$ mol/l, in U-87 MG at the concentration of $1.9.10^{-3}$ mol/l and in A172 at the concentration of 2.10^{-3} mol/l (Fig. 2).

Non substituted thiazolidinedione (synthetic PPARγ ligand) was completely ineffective in all cell lines (12–72 hours; data not shown) and therefore chemically modified (substituted) molecule – ciglitazone was used and its effect was tested in concentrations ranged from 5.10^{-4} mol/l to 5.10^{-8} mol/l. The IC_{50} of ciglitazone after 24 hour was $1.8.10^{-4}$ mol/l in U-118 MG; $2.3.10^{-4}$ mol/l in T98G; $2.2.10^{-4}$ mol/l in U-87 MG and $2.1.10^{-4}$ mol/l in A172 (Fig. 3).

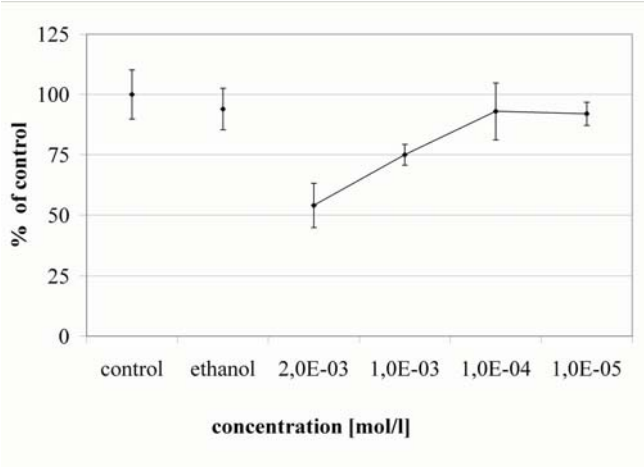

Figure 1. Effect of bezafibrate (ligand of PPARα) on cell viability in U-118 MG cells. Different concentration of bezafibrate ($1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$, $2x10^{-3}$ mol/l) were added to the glioblastoma cell line U-118 MG for 24 hours.

Cell cycle. Since IC_{50} in majority of cell lines after treatment of bezafibrate and non substituted thiazolidinedione was not reached, another experiments were focused only on the effect of gemfibrozil and ciglitazone on the cell cycle.

The treatment of gemfibrozil (24 hours; IC_{50}) led to increase in percentage of cells in G_1 phase (control 69 %, gemfibrozil 81 %) and decrease in percentage of cells in S phase (control 15 %, gemfibrozil 7 %) in U-87 MG line. The similar effect was reached in U-118 MG line after 24 hours of gemfibrozil treatment (G_1 fraction increased from 48 % in controls to 71 % in gemfibrozil; S fraction decreased from 37 % in control to 16 % in gemfibrozil) (Fig. 4). Ciglitazone caused a drop in cell number in the S and G_2/M-phases of the cell cycle in all cell lines. The most significant changes were found in U-118 MG after 24 hours treatment (S-phase: control 37 %, ciglitazone 30 %; G_2/M phase: control 22 %, ciglitazone 3.5 %). The results in other cell lines (T98G and A172) were not significant (data not shown). Also, we did not find any significant subG_1 (apoptotic) fraction in all treated cell lines (data not shown).

The histograms of treated cells were compared with suitable controls (histograms of growing cells in medium). The histograms of the cells treated by pure medium and medium with vehicle were very similar (data not shown).

Protein expression

This section of text was separated into the two parts: the effect of PPARα ligand – gemfibrozil and the effect of PPARγ ligand – ciglitazone.

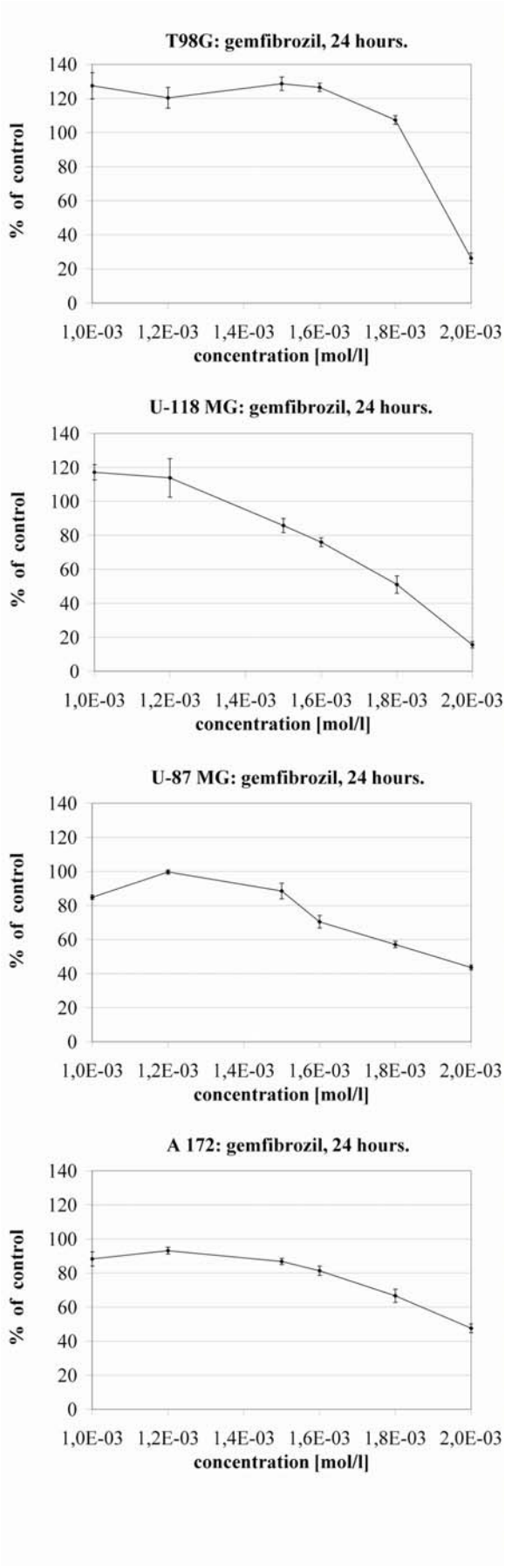

Figure 2. Effect of gemfibrozil (ligand of PPARα) on cell viability in four human glioblastoma cell lines. The concentrations of gemfibrozil ($1.0\ 10^{-3}$, $1.2\ 10^{-3}$, $1.4\ 10^{-3}$, $1.6\ 10^{-3}$, $1.8\ 10^{-3}$ and $2.0\ 10^{-3}$ mol/l) were applied to T98G, U-87 MG, A172 and U-118 MG cell lines for 24 hours to find the concentration which is able to reduce to viability of human glioblastoma cell lines in 50 %.

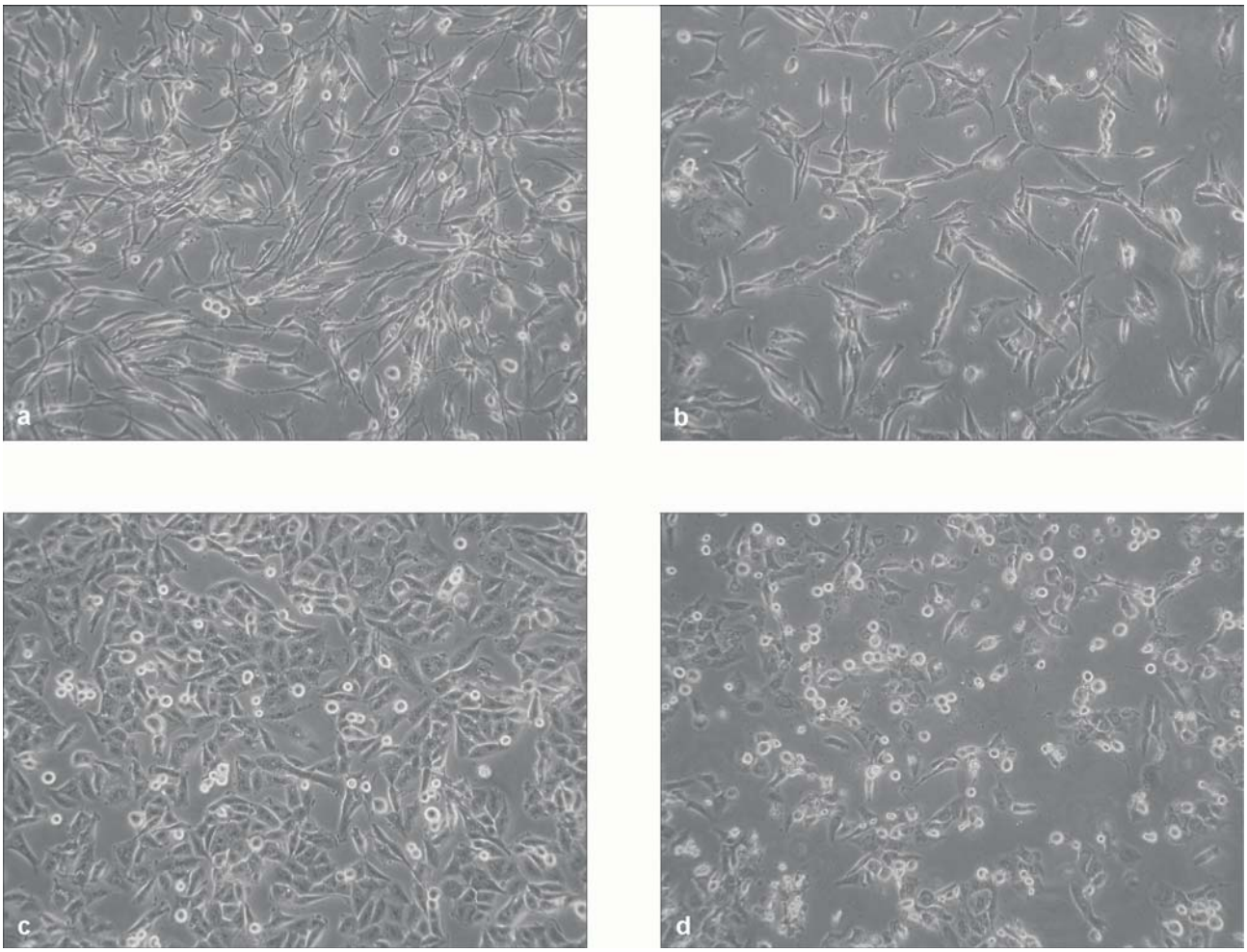

Figure 3. Effect of ciglitazone (ligand of PPARγ) on cell morphology in U-118 MG and T98G human glioblastoma cell lines. The cells were treated by ciglitazone (IC_{50}) for 24 hours (b+d). The results were compared with control cells (a+c). a) U-118 MG Control 24 hours; b) U-118 Ciglitazone 1.8 10^{-4} mol/l 24 hours; c) T98G Control 24 hours; d) T98G Ciglitazone 2.30 10^{-4} mol/l 24 hours. Original magnification 10x100.

The studied proteins were divided into the groups of: steroid receptor related proteins; cell cycle related proteins; group of apoptosis related proteins; and other proteins.

Effect of PPARα ligand gemfibrozil on steroid receptor related proteins. All studied cell lines constitutively expressed PPARα receptor as well as RAR (α and β), RXRα, AR (androgen receptor) and ER (estrogen receptor) proteins. PPARα expression remained unchanged after 12/24 hours exposure to IC_{50} of gemfibrozil in the majority of cell lines (with the exception of T98G – increase in PPARα expression was found after 24 hours). RAR expression (both isoforms) and RXRα were very variable in all cell lines – there was found an increase in RARs and RXR in U-118 MG after 24 hours, a decrease in RARs and RXR in T98G after 24 hours and variable levels of RARs and RXR protein expression in U-87 MG and A172 (both cell lines are characterized by the presence of wild type p53 protein). For details see Table 2. AR expression decreased in all studied cell lines after 12/24 hours incubation with IC_{50} of gemfibrozil. ER expression remained unchanged in this experiment.

Effect of PPARα ligand gemfibrozil on cell cycle related proteins. A decrease was found in p53 expression after 12/24 hours application of gemfibrozil in U-118 MG cell line (with mutated p53). However, p53 expression in T98G was unchanged (Fig. 5). The expression of p53 was negative in U-87 MG and A172 (wild type of p53). To test whether p53 in these cell lines is functional, these lines were irradiated (10 Gy) for 1–6 hours. A significant consequent increase was found in p53 expression (Fig. 6). Variable results in MDM-2 expression after gemfibrozil treatment were noted: it was unchanged in T98G and U-118 MG, decreased in A172 line and increased in U-87 MG cell line. The expression of both phosphorylated and dephosphorylated forms of Rb protein was decreased after 12/24 hours of IC_{50} of gemfibrozil (Fig. 5) in all cell lines.

Significant decrease in cyclin D1 after 12/24 hours was

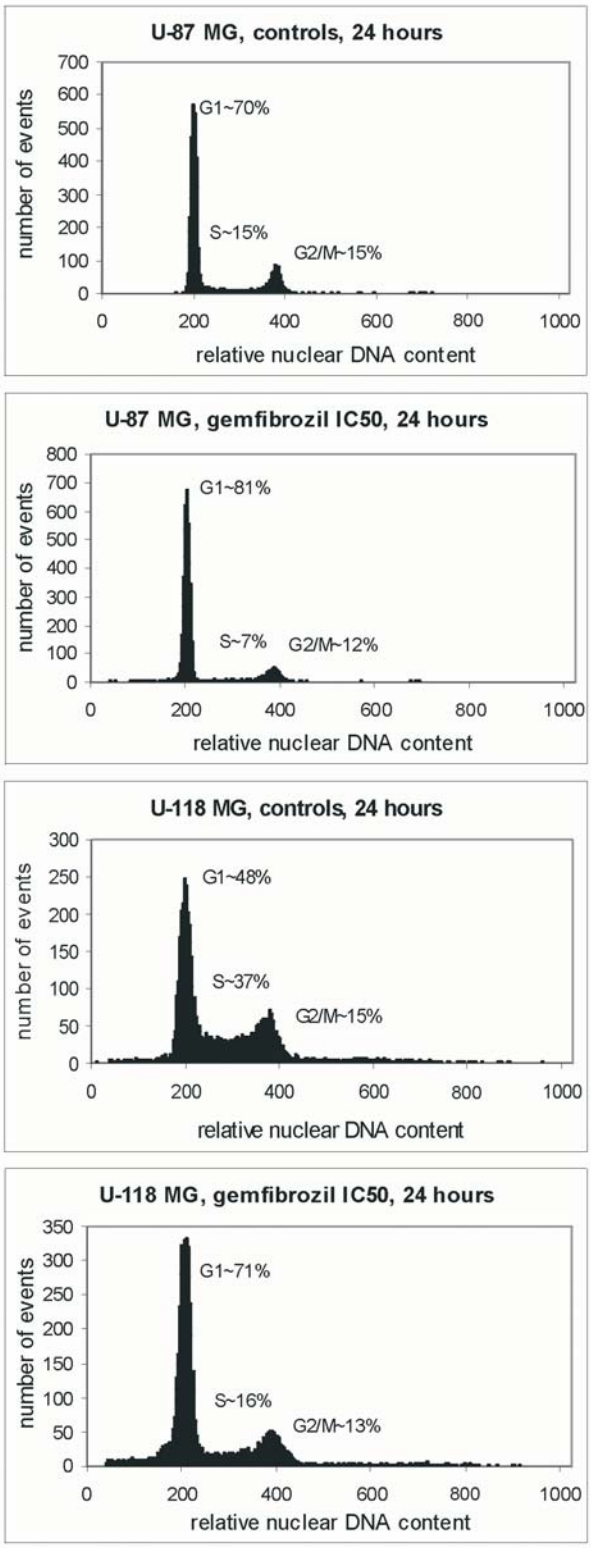

Figure 4. Cell cycle distribution of U-87 MG and U-118 MG cells after flow cytometry analysis. The cell lines were treated by gemfibrozil (IC_{50}) for 24 hours, harvested, stained with DAPI, and analyzed using the flow cytometry. Histograms of the treated cells were compared with control cells. Horizontal and vertical axes indicate relative nuclear DNA content and number of events (cells), respectively.

found in all cell lines (Fig. 5), the expression of cyclin B and E and cdk2 was also invariably decreased although mostly unchanged.

The expression of $p16^{Ink4a}$ was negative in all cell lines, the expression of $p27^{Kip1}$ increase after 12/24 hours in U-87 MG, T98G, U-118 MG (Fig. 5) and expression of $p21^{Waf1/Cip1}$ was negative in control T98G and U-118 MG (mutated p53) and positive in control U-87 MG and A-172. However, the effect of IC_{50} of gemfibrozil led to variable results in $p21^{Waf1/Cip1}$ expression – it was unchanged in T98G and U-87 MG, decreased in A172 line and increased in U-118 MG cell line. As in the case of p53 it was tested whether both $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ are functional by irradiation (10 Gy) for 1–6 hours with consequent significant increase in their expression (Fig. 6).

With the exception of T98G, PTEN expression was negative and no effect of gemfibrozil on PTEN expression was found on studied cell lines. The effect of gemfibrozil on c-myc leads to its degradation after 24 hours.

Effect of PPARα ligand gemfibrozil on apoptosis related proteins. All unaffected cell lines expressed BAX and BID proteins; Bcl-2 protein was expressed in T98G, U-87 MG and U-118 MG. However, the A172 cell line was negative for Bcl-2 protein. Bcl-X_L protein was expressed in T98G, U-87 MG and A172 cell lines although it was negative in U-118 MG. The effect of gemfibrozil on these proteins was very variable. The expression was mostly unchanged although it irregularly decreased or increased (see Tab. 3).

As in the case of the Bcl-2 family, the result was variable. However, degradation of caspase 3 into cleaved fragments (part of apoptotic cascade) after 12/24 hours in T98G, U-87 MG and U-118 MG was noticed (Fig. 5). The A172 line was unchanged. PARP positive expression in controls of all studied lines was observed but gemfibrozil had no effect – neither change in its expression nor in its degradation (sign of apoptosis) was found.

Effect of PPARα ligand gemfibrozil on other proteins. The GFAP (Glial Fibrillary Acidic Protein) was expressed in all studied cell lines; however, the gemfibrozil had no effect on its expression.

Effect of PPARγ ligand ciglitazone on steroid receptor related proteins. All studied cell lines constitutively expressed the PPARγ receptor as well as RXRα, AR (androgen receptor) and VDR (vitamin D receptor) proteins. The expression of PPARγ remained unchanged after 12/24 hour exposition to IC_{50} of ciglitazone in all lines. The expression RXRα decreased in U-118 MG, T98G and U-87 MG and was unchanged in A172. The expression of AR was unchanged in all lines. Expression of VDR decreased in T98G and U-87 MG, whereas in A172 and U-118 MG it remained unchanged (Fig. 7).

Effect of PPARγ ligand ciglitazone on cell cycle related proteins. In contrast to the effect of gemfibrozil, there was observed a drop in p53 expression after 12/24 hour application of ciglitazone only in the T98G cell line (with mutated

Table 2. Effect of PPARα ligand gemfibrozil on steroid receptor related proteins

	T98G				U-118 MG				U-87 MG				A172			
	C12	G12	C24	G24	C12	G12	C24	G24	C12	G12	C24	G24	C12	G12	C24	G24
PPAR	+	↔	+	↑	+	↔	+	↔	+	↔	+	↔	+	↔	+	↔
RXR	+	↓	+	↓	+	↔	+	↑	+	↓	+	↓	+	↓	↓	↓
RAR	+	↔	+	↓	+	↔	+	↑	+	↓	+	↓	+	↓	+	↓
RAR	+	↔	+	↓	+	↓	+	↑	+	↔	↓	↑	+	↓	↓	↔
AR	+	↓	+	↓	+	↓	+	↓	+	↓	+	↓	+	↓	↓	↓
ER	+	↔	+	↔	+	↔	+	↔	+	↔	+	↔	+	↔	+	↔

T98G, U-118 MG – cell lines bearing mutated p53;
U-87 MG, A172 – cell lines bearing wild type p53;
C12, C24 – control experiments, 12 and 24 hour;
G12, G24 – experiments with IC_{50} of gemfibrozil, 12 and 24 hours;
+ positive protein expression; ↑ increased expression; ↓ decreased expression; ↔ unchanged expression.

Table 3. Effect of PPARα ligand gemfibrozil on apoptosis related proteins

	T98G				U-118 MG				U-87 MG				A172			
	C12	G12	C24	G24	C12	G12	C24	G24	C12	G12	C24	G24	C12	G12	C24	G24
Bcl-2	+	↔	+	↔	+	↔	+	↔	+	↔	+	↓	–		–	
BAX	+	↔	↓	↔	+	↑	↑	↔	+	↑	+	↑	+	↔	+	↔
BID	+	↔	+	↔	+	↔	+	↔	+	↔	+	↔	+	↓	+	↓
Bcl-X_L	+	↔	+	↔	–	↔	–		+	↔	+	↔	+	↔	+	↔

T98G, U-118 MG – cell lines bearing mutated p53;
U-87 MG, A172 – cell lines bearing wild type p53;
C12, C24 – control experiments, 12 and 24 hour;
G12, G24 – experiments with IC_{50} of gemfibrozil, 12 and 24 hours;
+ positive protein expression; – negative protein expression; ↑ increased expression; ↓ decreased expression; ↔ unchanged expression.

p53). The other cell lines remained unchanged. Similarly, as it was observed in the case of gemfibrozil, the expression of phosphorylated form of Rb protein decreased after 12/24 hours of IC_{50} of ciglizatone (Fig. 7) in all cell lines.

A significant decrease in cyclin D1 after 12/24 hours in U-87 MG, U-118 MG and A172 was noted; the expression of cyclin D1 remained unchanged in T98G.

The effect of IC_{50} of ciglitazone led to variable results in $p21^{Waf1/Cip1}$, as in gemfibrozil expression – it was unchanged in A172, decreased in U-87 MG and increased in U-118 MG and T98G cell lines. With the exception of increased expression of $p27^{Kip1}$ in U-118 MG, no changes in the expression of this protein in other cell lines after application of ciglitazone were noticed.

The effect of ciglitazone on c-myc led to its increase in all lines after 24 hours.

Effect of PPARγ ligand ciglitazone on apoptosis related proteins. All unaffected cells expressed BAX and Bcl-2. The effect of ciglitazone on these proteins was, as with gemfibrozil, very variable: the expression was mostly unchanged in both pro-

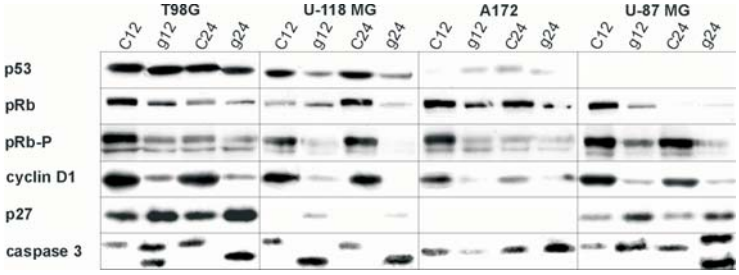

Figure 5. Western blot analysis of p53, pRb, pRb-P, cyclin D1, p27 and caspase 3 in T98G, U-118 MG, A 172 and U-87 MG cells treated by gemfibrozil. Cells were incubated in medium with gemfibrozil (IC_{50}) for 24 hours. The effect of gemfibrozil (IC_{50}) on the protein level after 12 hours was checked. The expressions of proteins of treated cells (g12 – gemfibrozil 12 hours, IC_{50}; g24 – gemfibrozil 24 hours, IC_{50}) were confronted with the protein expression of control, untreated cells (C12 – control 12 hours; C24 – control 24 hours).

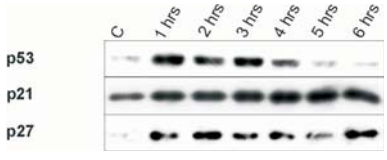

Figure 6. Western blot analysis of p53, p21 and p27 in A172 cells after irradiation. The cells A172 were collected after 1, 2, 3, 4, 5 or 6 hours after irradiation by 10 Gy. The expression of proteins was compared with the expression in the control cells (C).

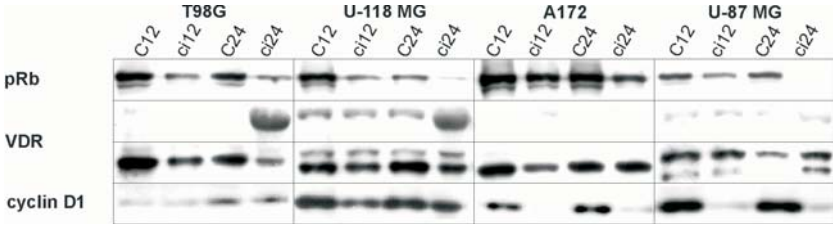

Figure 7. Western blot analysis of pRb, VDR and cyclin D1 in T98G, U-118 MG, A 172 and U-87 MG cells treated by ciglitazone. Cells were incubated in medium with ciglitazone (IC_{50}) for 24 hours. The effect of ciglitazone (IC_{50}) on the protein level after 12 hours was checked. The expressions of proteins of treated cells (ci12 – ciglitazone 12 hours, IC_{50}; ci24 – ciglitazone 24 hours, IC_{50}) were confronted with the protein expression of control, untreated cells (C12 – control 12 hours; C24 – control 24 hours).

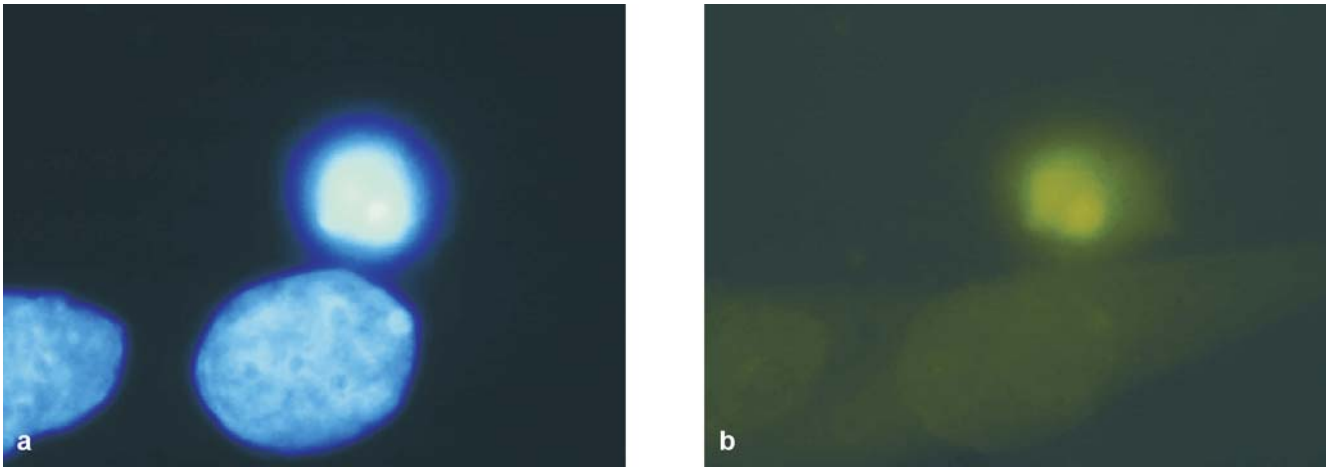

Figure 8. Detection of apoptosis in U-118 MG cell line by TUNEL staining. U-118 MG cells treated by gemfibrozil (IC_{50}) for 24 hours showed morphological features of apoptosis. a) Hoechst 33258 staining of all cell nuclear in the cell line U-118 MG treated by gemfibrozil for 24 hours. b) TUNEL positive cells in the cell line U-118 MG treated by gemfibrozil for 24 hours. Original magnification 10x100.

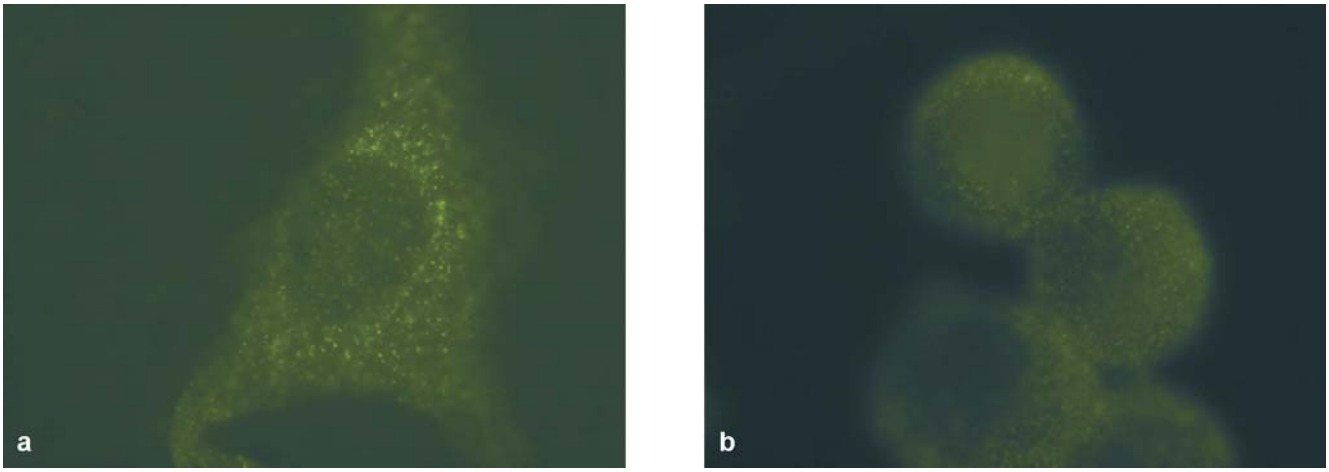

Figure 9. Immunofluorescent localization of PPARγ in U-118 MG cell line. The cells were incubated in presence or absence of gemfibrozil for 24 hours, then harvested and finally stained by FITC. a) control, untreated U-118 MG after 24 hours, showing PPARγ expression located in cytoplasma. b) U-118 MG cell line treated by gemfibrozil 24 hours, showing translocation of PPARγ expression into the nucleus. Original magnification 10x100.

teins. However, there was detected a decrease in Bcl-2 in U-118 MG after 12 and 24 hours of incubation.

Effect of PPARγ ligand ciglitazone on other proteins. The GFAP was expressed in all studied cell lines; ciglitazone had slight effect on its expression.

TUNEL staining. Gemfibrozil (IC_{50}, 24 hours) led to an increase in the number of apoptotic, TUNEL positive cells in U-118 MG, T98G and U-87 MG. A172 cells remained unaffected (Tab. 4). These results were supported by Hoechst 33258 staining for detection of apoptotic cells (Fig. 8).

Table 4. The results of TUNEL staining (gemfibrozil IC_{50}/24 hours)

	T98G		U-118 MG		U-87 MG		A172	
	C24	G24	C24	G24	C24	G24	C24	G24
TUNEL (%)	0	15	0	17	0	11	0	0

C24…control experiments; 24 hours
G24…experiments with IC_{50} of gemfibrozil; 24 hours
TUNEL (%)… percentage of TUNEL positive cells

Discussion

Currently, there is accumulating evidence that various synthetic ligands of PPARs are potent inhibitors of the growth of several malignant tumors (see Introduction). In this study it was found that both synthetic ligands of PPARα (bezafibrate, gemfibrozil) and PPARγ (ciglitazone) inhibit cell growth in human glial tumor cell lines. This is in agreement with the results of two recent studies which were performed both on human and rat glioma cell lines [18, 43]. As we anticipated on the basis of the results of other studies [30, 4, 6], our Western blot analysis showed that all studied cell lines constitutively expressed PPARα, PPARγ and RXRα. Evidently, activation of these receptors depends on their intact function. Our finding of translocation of PPARγ and PPARα proteins from cytoplasm to nuclear membrane after treatment by studied molecules may suggest that these proteins are functional (Fig. 9). The inhibition of cell growth is also strongly supported by our finding of decreased expression of Rb protein and cyclin D1 and increased expression of $p27^{Kip1}$ after 12/24 hours of treatment by IC_{50} of both molecules. This is also supported by the findings of others [27, 42]. Moreover, consequent flow cytometry analysis showed block of cells in transition to the G1-S checkpoint of the cell cycle. These results may suggest that the main "targets" of synthetic ligands of PPARs are proteins regulating the G_1 phase of the cell cycle. Our results also showed that p53 status and MDM-2 protein probably do not significantly influence the effects of the molecules examined which may be in accordance with other studies focused on the role of p53 in GBM [24, 36]. On the other hand, an important role in glioblastoma development may be played by the PTEN protein [10]. With the exception of T98G no constitutive PTEN expression in untreated cell lines and no effect of synthetic ligands of PPARs on PTEN expression in these lines was detected. This suggests that PTEN is inactivated (mutated) in some glioma cell lines and therefore it would be interesting to study the effect of synthetic ligands of PPARs in cell lines transfected with functional, wild type PTEN [39].

The marker of astrocytic differentiation, GFAP (Glial Fibrillary Acidic Protein), was expressed in all studied cell lines but neither gemfibrozil nor ciglitazone had any strong effect on its expression. This result reflects another study performed on oligodendrocytes in which PPARγ agonist had no effect on oligodendrocyte differentiation [33]. In the same study it was found that PPARδ agonists accelerated oligodendrocyte differentiation. Interestingly, there is also a paper reporting the suppression of adipocyte differentiation via RAR up-regulation and PPARγ suppression [21]. Therefore, it seems that the PPAR signalling pathway is only part of more complex process of differentiation.

In many studies cell growth inhibition was associated with increased apoptosis (see Introduction). For this reason the presence of apoptosis after treatment of synthetic ligands of PPAR was studied in this paper. Flow cytometry failed to reveal any significant $subG_1$ (apoptotic) fraction in any treated cell line. In contrast, TUNEL staining showed increase in the number of apoptotic, TUNEL positive cells in U-118 MG, T98G and U-87 MG after treatment of IC_{50} 24 hours of gemfibrozil. These results are also supported by the results of the Hoechst 33258 staining. The expression of apoptosis related proteins was examined, as well. No significant changes in expression of either the Bcl-2 family proteins or the PARP protein were found although there was noticed a degradation of procaspase 3 into cleaved fragments (part of apoptotic cascade) after 12/24 hours in T98G, U-87 MG and U-118 MG. It has been found that aside from expected pro-apoptotic effects, the PPARs may also have antiapoptotic effect (via inhibition of nitric oxide synthase) [15] and these may be associated with nonapoptotic cell death [2]. Therefore it seems, that as in the case of the process of differentiation, apoptosis is a more complex event and PPARs probably play a dual role (pro- and anti-apoptotic) in these regulatory networks.

Finally, there are very recent studies proposing the use of PPARγ ligands in the treatment of pituitary adenomas [14] and various cancers by inhibiting of angiogenesis [32]. Since the effect of treatment of bezafibrate and ciglitazone in our study was achieved by concentrations which allow potential practical use, we also propose that PPAR ligands may be potentially used in anticancer treatment.

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