Acta Biochimica Polonica

Vol. 55 No. 1/2008, 75–84

on-line at: www.actabp.pl

Regular paper

Pioglitazone, a PPAR-gamma ligand, exerts cytostatic/cytotoxic effects against cancer cells, that do not result from inhibition of proteasome

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Received: 07 November, 2007; revised: 19 February, 2008; accepted: 03 March, 2008
available on-line: 10 March, 2008

Thiazolidinediones are oral antidiabetic agents that activate peroxisome proliferator-activated receptor-gamma (PPAR-γ) and exert potent antioxidant and anti-inflammatory properties. It has also been shown that PPAR-γ agonists induce G0/G1 arrest and apoptosis of malignant cells. Some of these effects have been suggested to result from inhibition of proteasome activity in target cells. The aim of our studies was to critically evaluate the cytostatic/cytotoxic effects of one of thiazolidinediones (pioglitazone) and its influence on proteasome activity. Pioglitazone exerted dose-dependent cytostatic/cytotoxic effects in MIA PaCa-2 cells. Incubation of tumor cells with pioglitazone resulted in increased levels of p53 and p27 and decreased levels of cyclin D1. Accumulation of polyubiquitinated proteins within cells incubated with pioglitazone suggested dysfunction of proteasome activity. However, we did not observe any influence of pioglitazone on the activity of isolated proteasome and on the proteolytic activity in lysates of pioglitazone-treated MIA PaCa-2 cells. Further, treatment with pioglitazone did not cause an accumulation of fluorescent proteasome substrates in transfected HeLa cells expressing unstable GFP variants. Our results indicate that pioglitazone does not act as a direct or indirect proteasome inhibitor.

Keywords: pioglitazone, thiazolidinediones, peroxisome proliferator-activated receptor-gamma, proteasome

INTRODUCTION

Pioglitazone belongs to a class of antidiabetic drugs called thiazolidinediones (TZDs). These compounds have been shown to improve insulin sensitivity and inhibit hepatic gluconeogenesis by activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Kahn *et al.*, 2000).

PPAR-γ receptor is a member of the nuclear receptor superfamily (Theocharis *et al.*, 2004). After heterodimerization with retinoic acid-like receptor (RXR) it recognizes a specific sequence — the peroxisome proliferator response element (PPRE) located within promoters of target genes, and acts as a transcription regulator for genes involved in lipid and glucose metabolism and energy homeostasis (Desvergne & Wahli, 1999). Moreover, it also affects expression of genes that regulate proliferation, terminal differentiation, apoptosis, angiogenesis and inflammation. Natural ligands of PPAR-γ are long-

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Abbreviations: 15-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; GADD45, damage-inducible gene 45; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PPAR-γ, peroxisome proliferator-activated receptor-gamma; PPRE, peroxisome proliferator response elements; RXR, retinoic acid-like receptor; TZDs, thiazolidinediones; UFD, ubiquitin-fusion degradation.

chain polyunsaturated fatty acids, arachidonic acid metabolites such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-PGJ_2) and other fatty acid-derived compounds. High levels of PPAR-γ are detected in adipose tissue and its important role in adipocytogenesis is well established (Lecka-Czernik *et al.*, 2002). Lower levels of this receptor are found in intestinal epithelium, skeletal muscle, retina and lymphatic tissue (Michalik *et al.*, 2002). Moreover, PPAR-γ are expressed in a variety of tumor cells (Ray *et al.*, 2004; Strakova *et al.*, 2004; Subbarayan *et al.*, 2004; Park *et al.*, 2005; Cellai *et al.*, 2006; Emery *et al.*, 2006; Nomura *et al.*, 2006; Nam *et al.*, 2007; Occhi *et al.*, 2007).

Recent reports show that PPAR-γ agonists may exert cytostatic and cytotoxic activity against tumor cells *in vitro* (Brockman *et al.*, 1998; Sarraf *et al.*, 1998; Takahashi *et al.*, 1999; Chang & Szabo, 2000; Ray *et al.*, 2004). Although these findings are rather new and need clinical verification, a growing number of observations indicate that PPAR-γ agonists might exert an antitumor activity *in vivo* as well. Synthetic ligands of PPAR-γ receptors include not only anti-diabetic TZDs such as troglitazone, rosiglitazone, ciglitazone and pioglitazone, but also some non-steroidal anti-inflammatory drugs such as indomethacin, ibuprofen or fenoprofen (Wang *et al.*, 2006), and specific PPAR-γ ligands like GW 7845 (Suh *et al.*, 1999). Thiazolidinediones have been shown to induce apoptosis of tumor cells, associated with caspase-3 activation and decreased expression of anti-apoptotic proteins BCL-2 and BCL-X_L (Elstner *et al.*, 1998; Chattopadhyay *et al.*, 2000). Gene expression analysis with DNA microarrays in TZDs-treated tumor cells revealed PPAR-γ targets among proteins involved in growth regulatory pathways (Vignati *et al.*, 2006). It was shown that TZDs induce G0/G1 arrest and apoptosis of malignant cells. Although the mechanism of this action still needs elucidation, several reports indicate that PPAR-γ agonists influence expression of cell cycle regulatory proteins. TZDs can increase the levels of cyclin-dependent kinase inhibitors such as $p21^{Waf1}$ and $p27^{Kip1}$, thereby blocking cell cycle progression (Elstner *et al.*, 1998; Elnemr *et al.*, 2000; Radhakrishnan & Gartel, 2005). Other reports showed TZDs-mediated up-regulation of the tumor suppressor p53, involved in cell cycle regulation, control of DNA repair systems and apoptosis (Liu *et al.*, 2006). One of the suggested mechanisms of this action was inhibition of proteasome-dependent degradation of cell cycle inhibitors (Motomura *et al.*, 2004).

Proteasomal proteolysis is the major non-lysosomal protein degradation pathway (Grune, 2000; Kloetzel, 2001; Mani & Gelmann, 2005). It is essential for such cellular functions as metabolic adaptation, removal of abnormal proteins, stress response, generation of antigen peptides presented by MHC I, and cell cycle control (Kloetzel, 2001; 2004; 2004). The process is conducted by a large multi-catalytic protease complex — the 26S proteasome – consisting of 20S proteasome, which forms a cylindrical, catalytic core, and two asymmetric 19S regulatory complexes associated on both sides of the 20S complex. The 20S core is built of four rings consisting of seven subunits each. Proteolytic activity is localized at the inner surface of three out of the seven subunits of the inner rings (Peters *et al.*, 1993; Wlodawer, 1995; Coux *et al.*, 1996).

The correct function of proteasomes is indispensable for tumor cells' growth due to their role in ordered and rapid degradation of many key regulatory proteins (Spataro *et al.*, 1998; Mani & Gelmann, 2005). Therefore, proteasomes are potent targets for antitumor therapy and their inhibitor Bortezomib is approved for clinical use in relapsed multiple myeloma (Richardson & Anderson, 2003; Cecchi *et al.*, 2005; Ludwig *et al.*, 2005; Meiners *et al.*, 2007). The consequences of action of proteasome inhibitors include cell cycle arrest (Fan *et al.*, 2001; Zhang *et al.*, 2004; Drakos *et al.*, 2007; Yan *et al.*, 2007) and induction of apoptosis (Pigneux *et al.*, 2007).

Understanding of the processes in which TZDs affect the cell cycle is of great importance from the perspective of type 2 diabetes treatment and potential antitumor applications. The aim of the present study was to determine whether proteasomes are involved in the antitumor activity of pioglitazone.

MATERIALS AND METHODS

Cell lines. Human pancreatic cancer MIA PaCa-2 cell line was obtained from American Type Culture Collection (ATCC). Stable cell lines overexpressing marker substrates HeLa-GV and HeLa-R were kindly provided by Dr. Cezary Wójcik from the Department of Anatomy and Cell Biology, Indiana University, School of Medicine (Evansville, USA). Cells were cultured in Dulbecco's Modified Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (all from Gibco BRL, Paisley, UK), hereafter referred to as culture medium. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Drugs and reagents. Pioglitazone was kindly donated by Takeda Chemical Industries Ltd. (Osaka, Japan). It was diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a final concentration of 50 mM. Stock solution was aliquoted and stored frozen (–20°C).

Cytostatic/cytotoxic assays. The cytostatic and/or cytotoxic effects of combination treatment were measured using a crystal violet staining quantitative assay and XTT assay based on the ability of

mitochondrial dehydrogenase enzymes in living cells to convert the XTT substrate (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyloamino)carboxyl]-2H-tetrazolium hydroxide) into a water-soluble color product — formazan. Although the result of these tests do not give a direct information about condition of cells, the amount of crystal violet bound is proportional to the number of cells and the production of formazans is proportional to the viability of cells, which makes these methods useful in cytostatic/cytotoxic studies (Scudiero *et al.*, 1988). The MIA PaCa-2 cells were dispensed into a 96-well flat-bottomed microtiter plate (Nunc, Rochester, NY, USA) at a concentration 3×10^3 cells/100 μl per well and allowed to attach overnight. Then, the cells were treated for 24, 48 or 72 h with different pioglitazone concentrations. Control cells were incubated with DMSO at a concentration corresponding to the highest dose of pioglitazone (0.2%). DMSO at this concentration did not cause any significant cytostatic/cytotoxic effects as compared with untreated control. For crystal violet staining the medium was removed, wells were washed with PBS and stained with 0.5% crystal violet in 30% ethanol for 15 min at room temperature. The plates were washed four times with tap water. The cells were lysed in 1% SDS solution, and dye uptake was measured at 550 nm using an ELISA reader (SLT Labinstrument GmbH, Salzburg, Austria). The relative number of cells was calculated as follows: relative number of cells = [(experimental absorbance — background absorbance)/(untreated control absorbance — background absorbance)] × 100%. Alternatively, after pioglitazone treatment the culture medium were replaced with medium containing 0.35 mg/ml of XTT (Sigma-Aldrich) and 7.5 μg/ml of phenazine methosulfate (Sigma-Aldrich). After 4 h of incubation, production of formazan was measured at 450 nm using an ELISA reader. The relative viability was calculated as follows: relative viability = [(experimental absorbance — background absorbance)/(untreated control absorbance — background absorbance)] × 100%.

Western blotting. For Western blotting studies, MIA PaCa-2 cells were cultured with different concentrations of pioglitazone. After 48 h of culture, cells were collected and lysed as described before (Nowis *et al.*, 2006). Protein concentration was measured using Bradford's method (Bio Rad, Rockville NY, USA). Equal amounts of proteins were separated on 12.5% SDS/polyacrylamide gel, transferred onto nitrocellulose membranes, blocked with TBST (Tris-buffered saline (pH 7.4), 0.05% Tween 20) with 5% nonfat milk and 5% fetal bovine serum. Primary antibodies were applied overnight at 4°C, dilution 1:1000. Antibodies recognizing the following antigens were used: p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), ubiquitin (P4D1, Cell Signaling Technology Inc., Beverly, MA, USA), $p27^{Kip1}$ (Cell Signaling Technology, Inc.). Anti-α-tubulin antibody (Santa Cruz Biotechnology Inc.) served as a protein loading control. After extensive washing with TBST, the membranes were incubated for 45 min in corresponding horseradish peroxidase-coupled secondary antibodies (ImmunoPure Antibody goat anti-mouse and donky anti-rabbit, Pierce, Rockford, IL, USA). The chemiluminescent reaction was developed using West Pico (Pierce) reagent.

Isolation of 20S proteasomes. Constitutively expressed 20S proteasomes were isolated from T2 cells. Cells were lysed with DTT (dithiothreitol, 1 mM) and the supernatant was applied to DEAE-Sephacel (Pharmacia Fine Chemicals, Freiburg, Germany). 20S proteasome was eluted with an NaCl gradient in TEAD (20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM sodium azide and 1 mM DTT) from 100 to 350 mM NaCl. 20S proteasome was concentrated by ammonium sulfate precipitation (between 40 and 70% of saturation) and separated in a 10–40% sucrose gradient by centrifugation at 40 000 r.p.m. for 16 h (SW40; L7, Beckman & Coulter, Palo Alto, USA). Finally, 20S proteasome was purified on a MonoQ column and eluted with an NaCl gradient at 280 mM NaCl. The fractions containing purified 20S proteasome were dialyzed against 50 mM NaCl in TEAD and stored on ice. The purity was determined by SDS/PAGE.

Protease assays. Fluorescent proteasome substrates: Suc-LLVY-AMC or with Z-GGL-AMC (BACHEM, Bubendorf, Switzerland and Calbiochem, San Diego, CA, USA) were used to estimate chymotrypsin-like activities of the 20S proteasome. 20S proteasome (100 ng) was pre-incubated for 15 min with 0.1–100 μM pioglitazone, 1 μM MG132 or DMSO (0.2%) used as control. The reaction was started by addition of substrate (50 μM). Substrates were incubated with 20S proteasome at 37°C in assay buffer (20 mM Tris/HCl, pH 7.2, 1 mM EDTA, 1 mM DTT) for 1 h. The released AMC was detected by fluorescence emission at 460 nm (excitation at 390 nm) using a Fluostar Reader (SLT Labinstruments, Crailsheim, Germany). Activity was estimated in fluorescence units and as percentage of control value.

Measurements of proteasomes activity within cells. Cells were harvested after 24, 48 or 72 h of incubation with pioglitazone or DMSO (0.2%) and lysed with 0.1% NP40 in TEAD in the presence of commercial protease inhibitor mixture Complete (Roche). The proteasomal activity was measured in 10 μl of lysates using Suc-LLVY-AMC (BACHEM, Calbiochem) to estimate the chymotrypsin-like activity of the 20S proteasome. The released AMC was detected by fluorescence emission at 460 nm (excitation at 390 nm) using a Fluostar Reader. The results of those measurements were calculated rela-

tive to the number of cells estimated in the crystal violet staining quantitative assay and shown as the percentage of untreated control. Cells treated for 2 h with 10 μM MG132 served as a positive control of proteasome activity inhibition.

Assessment of proteasome function using stable cell lines overexpressing fluorescent proteasome substrates. To further study the influence of pioglitazone on proteasome activity we examined the accumulation of proteins using known fluorescent substrates of the proteasomal degradation. HeLa cells expressing two different unstable cytosolic GFP variants were used. R-GFP has a destabilizing N-terminal Arg residue and is recognized and targeted for degradation by the N-end rule pathway (HeLa-R), while $Ub_{G76V}GFP$ has non-cleavable ubiquitin fused in frame with GFP, targeting the product for degradation through the ubiquitin-fusion degradation (UFD) pathway (HeLa-GV).

The morphology of both cell lines, HeLa-R and HeLa-GV, was examined after 48 h of treatment with different concentrations of pioglitazone or MG132 directly on 24-well plates by phase contrast microscopy (Nikon Eclipse TE2000-U connected to Nikon Digital Sight DS-U1 camera). Accumulation of marker substrates was assessed by fluorescence intensity and was examined by fluorescence microscopy (Nikon Eclipse TE2000-U connected to Nikon Digital Sight DS-U1 camera). Cells treated with 1 μM MG132 served as a positive control of proteasome activity inhibition.

Statistical analyses. Data were calculated using GraphPad Prism 5 Demo. One-way ANOVA was used to compare tested groups in respect of viability of cells, number of cells and proteasome activity. When the analysis revealed a significant effect, Dunnett's test was used to compare groups treated with the reagents tested *versus* controls. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

MIA PaCa-2 cells were incubated with serial dilutions of pioglitazone for 24, 48 or 72 h. Crystal violet staining and XTT assay revealed

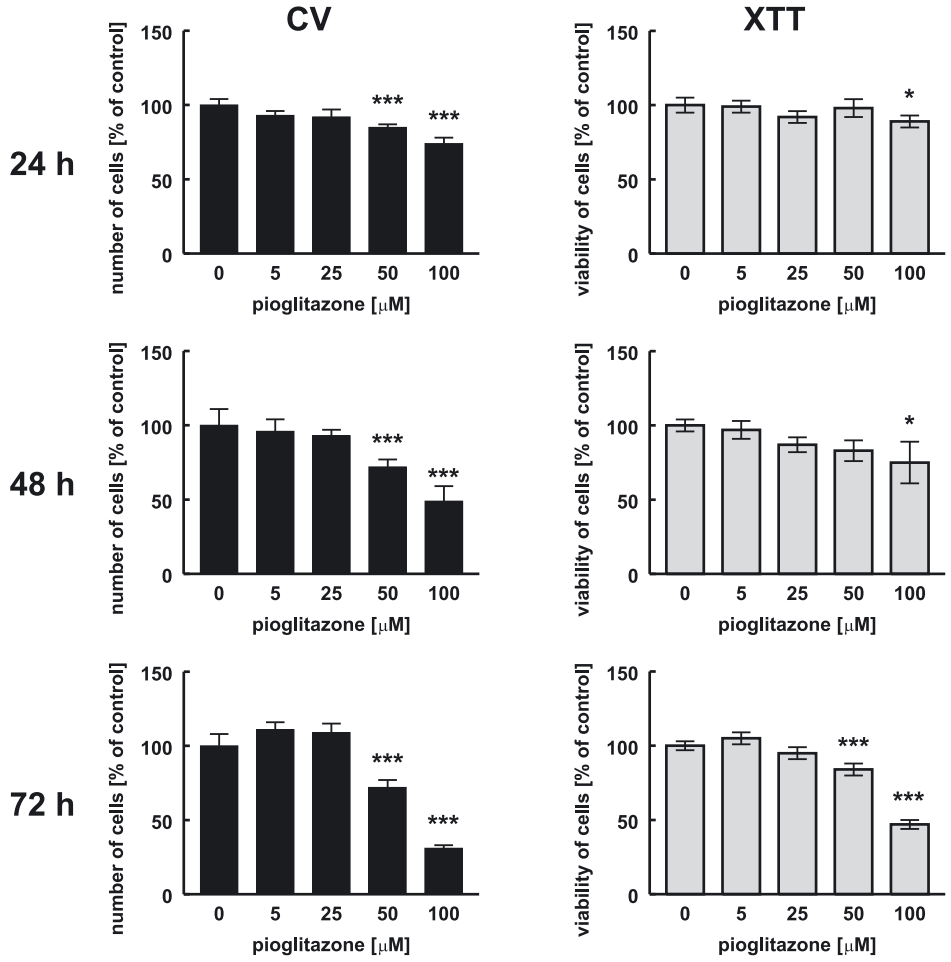

Figure 1. Pioglitazone exerts cytostatic/cytotoxic effects against MIA PaCa-2 cells.
Mia PaCa-2 cells were incubated with pioglitazone at indicated concentrations for 24, 48 and 72 h. Diluent-treated cells served as controls. The cytostatic/cytotoxic effects were measured with crystal violet staining and XTT assay. Results are expressed as means of control ± S.E. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way ANOVA and Dunnett's test).

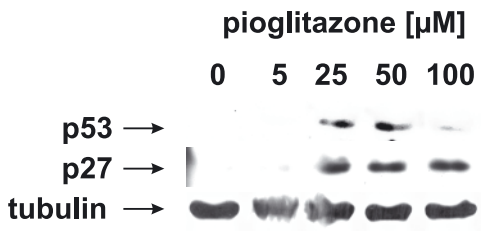

Figure 2. Incubation of MIA PaCa-2 cells with pioglitazone increases levels of $p27^{Kip1}$ and p53.
MIA PaCa-2 cells were incubated with pioglitazone at indicated concentrations. After 48 h of incubation cells were harvested and cell lysates were subjected to Western blot analysis: 50 μg of protein extracts was separated by electrophoresis. Blots were sequentially probed (after stripping) with different antibodies. Appropriate bands were scanned and compared.

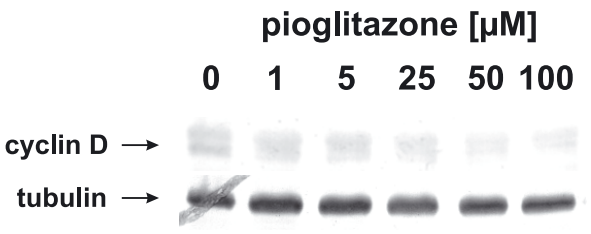

Figure 4. Incubation of Mia PaCa-2 cells with pioglitazone results in decreased levels of cyclin D1.
MIA PaCa-2 cells were incubated with pioglitazone at indicated concentrations. After 48 h of incubation cells were harvested and total cell lysates (50 μg of protein) were electrophoresed. The blots were sequentially probed (after stripping) with antibodies against cyclin D1 and α-tubulin.

slight, albeit statistically significant and dose-dependent, cytostatic/cytotoxic effects against these tumor cells (Fig. 1). Similar effects were also observed with other tumor cell lines tested (not shown).

Incubation of Mia PaCa-2 cells with pioglitazone for 48 h resulted in a dose-dependent induction of p53 and p27 levels (Fig. 2). At a highest pioglitazone concentration tested (100 μM) this effect on p53 was slightly less pronounced. These effects were associated with a slight increase in the levels of polyubiquitinated proteins in total cell lysates (Fig. 3), which might indicate that the levels of these proteins might have increased due to pioglitazone-induced inhibition of proteasome activity. However, under identical conditions of pioglitazone-exposure the level of cyclin D, which is degraded by the proteasome system, decreased.

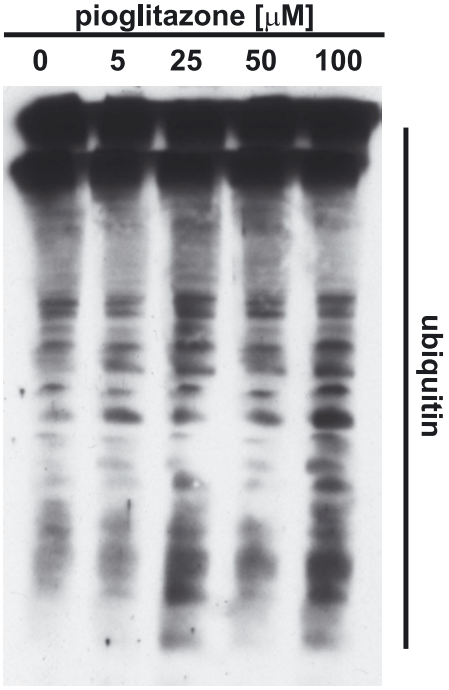

Figure 3. Pioglitazone induces accumulation of ubiquitinated proteins in MIA PaCa-2 cells.
MIA PaCa-2 cells were incubated with pioglitazone at indicated concentrations. After 48 h of incubation cells were harvested and cell lysates were subjected to Western blot analysis: 50 μg of protein extracts was separated by electrophoresis. Blots were sequentially probed (after stripping) with different antibodies. Appropriate bands were scanned and compared.

In order to investigate the influence of pioglitazone on the activity of ubiquitin-proteasome system, the proteolytic chymotrypsin-like activity of proteasomes in MIA PaCa-2 cells incubated for 24, 48 or 72 h with 5–100 μM pioglitazone was studied by measuring degradation of fluorogenic peptide substrates. These measurements revealed no inhibition of proteasomal activity in MIA PaCa-2 cells (Fig. 5). A 24–72 h incubation of Mia PaCa-2 cells with 10 μM MG132, a selective proteasome inhibitor, resulted in a significant, over 90% inhibition of proteasome activity. Also no inhibition was observed when pure 20S proteasomes isolated from T2 cells were incubated with 0.1–100 μM pioglitazone (Fig. 6). Again, 10 μM MG132 used as a positive control produced an over 90% inhibition of proteasome activity.

To further characterize the potential influence of pioglitazone on the activity of the ubiquitin-proteasome system an *in vivo* model was used. To this end HeLa cells stably transfected with two different plasmid vectors encoding cytosolic green fluorescent protein (GFP) variants rapidly degraded in proteasomes were used. HeLa-GV cells contain a GFP variant with a Gly/Val substitution that results in constitutive ubiquitination and degradation in the ubiquitin-fusion degradation (UFD) pathway. HeLa-R cells produce an unstable GFP variant that is rapidly processed in the N-end rule degradation pathway. Fluorescence analyses showed that upon pioglitazone treatment (at a concentration range of 5

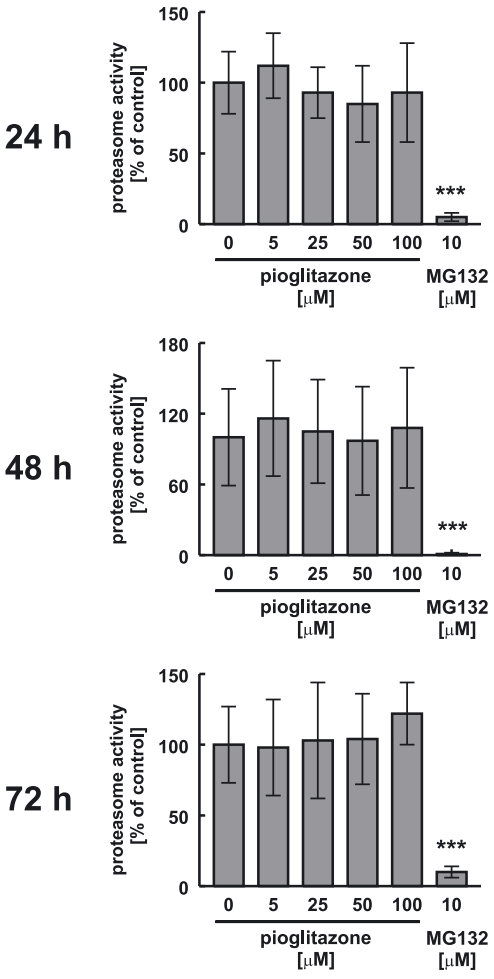

Figure 5. Pioglitazone does not affect chymotrypsin-like activity of proteasomes in MIA PaCa-2 cells.
MIA PaCa-2 cells were treated with pioglitazone at indicated concentrations. After 24, 48 and 72 h of incubation cells were harvested and chymotrypsin-like activity of proteasomes in cell lysates was determined by measuring the fluorescence intensity of processed substrate Suc-LLVY-AMC. Results are expressed as percentage of controls ± S. E. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way ANOVA and Dunnett's test). MIA PaCa-2 cells treated for 2 h with 10 μM MG132 served as a positive control.

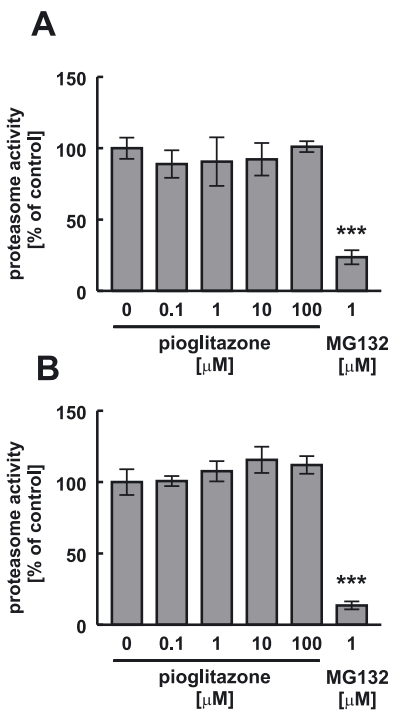

Figure 6. Pioglitazone does not affect chymotrypsin-like activity of isolated T2 proteasomes.
Isolated 20S subunits of T2 proteasomes were pre-incubated with pioglitazone at indicated concentrations or with 1 μM MG132. One hour after substrate addition chymotrypsin-like activity was determined by measuring the fluorescence intensity of processed substrates: Suc-LLVY-AMC (A) and Z-GGL-AMC (B). Results are expressed as percentage of controls ± S.E. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (One-way ANOVA and Dunnett's test).

to 100 μM, for 48 h) neither of the GFP variants accumulated in the cytoplasm. MG132 at 1 μM inhibited both pathways of protein degradation (Fig. 7).

DISCUSSION

It has recently been demonstrated that members of the TZD family exert inhibitory effects against a number of tumor cells (Brockman *et al.*, 1998; Sarraf *et al.*, 1998; Takahashi *et al.*, 1999; Chang & Szabo, 2000). However, the underlying mechanisms of their action remain poorly understood. It has been shown that the antitumor effects of TZDs treatment result from induction of cell cycle arrest and/or apoptosis (Eibl *et al.*, 2001; Morosetti *et al.*, 2004; Yang *et al.*, 2007). Intriguingly, TZDs may exert these effects in cells either having or lacking PPAR-γ expression (Rumi *et al.*, 2004; Shiau *et al.*, 2005; Ray *et al.*, 2006). Thus, upon TZDs treatment PPAR-γ-dependent and independent effects can be observed (Akasaki *et al.*, 2006; Han & Roman, 2006; Ray *et al.*, 2006; Hazra *et al.*, 2007). Since the human pancreatic tumor cell line MIA PaCa-2 used in this study expresses PPAR-γ (Eibl *et al.*, 2001), both types of action may participate in the pioglitazone-mediated cytostatic/cytotoxic activity (Han & Roman, 2006). One of the candidate targets for PPAR-γ-independent action of TZDs are proteasomes (Motomura *et al.*, 2004; Chen & Harrison, 2005; Yang *et al.*, 2007).

It has recently been demonstrated that the TZDs family member troglitazone exerts a proteasome-inhibitory activity and by blocking the cell cycle inhibitor $p27^{Kip1}$ degradation induces cell-cycle arrest (Motomura *et al.*, 2004). Here, we studied another PPAR-γ agonist pioglitazone. Our data demonstrate that this TZDs family member is not capable of acting as a direct or indirect proteasome inhibitor. Previously, differences in the mechanisms of action of troglitazone and other

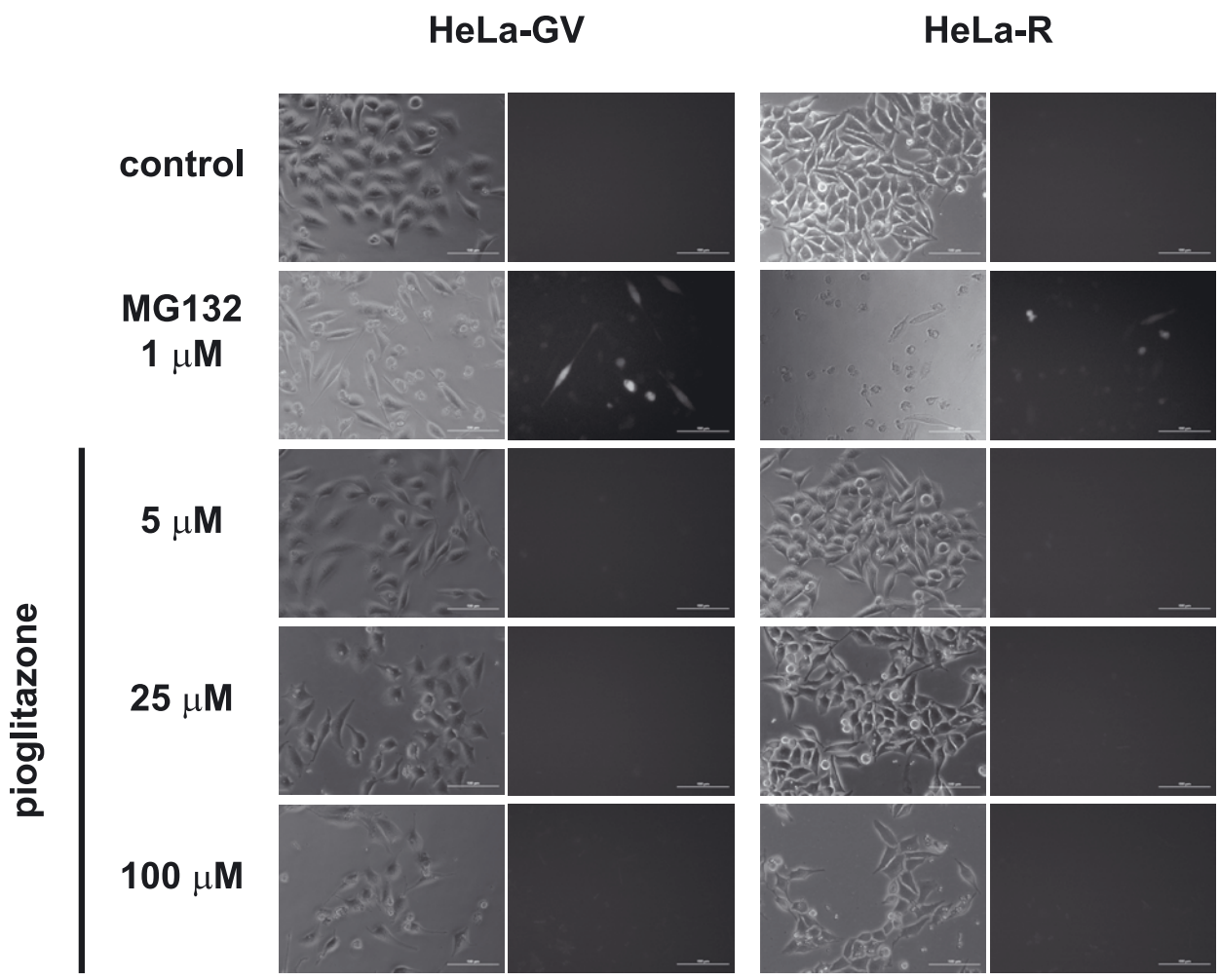

Figure 7. Pioglitazone does not affect degradation of proteasome substrates in tumor cells.
HeLa-GV and HeLa-R cells were treated with pioglitazone at indicated concentrations or with 1 μM MG132. After 48 h of incubation accumulation of marker substrates was examined with fluorescence microscopy. Representative photographs from one of three independent experiments are shown.

TZDs have been shown. For example, troglitazone, but not other TZDs up-regulated the expression of damage-inducible gene 45 (*GADD45*) (Yin *et al*., 2004). Since troglitazone seems to exert the strongest antitumor effects among TZDs, it is possible that suppression of proteasome activity is at least partially responsible for its enhanced anticancer activity. It is also possible that the discrepant effects observed in our study result from the use of a different tumor cell line to that used by Motomura *et al*. (2004).

The daily dose of pioglitazone in diabetes type 2 treatment is 45–60 mg and the corresponding clinical peak plasma concentration of pioglitazone is 2.0–4.8 μM (Gillies & Dunn, 2000). In our study we used pioglitazone at concentrations from 5 to 100 μM. Although only the lowest concentration used in this study is clinically achievable at the pioglitazone dose recommended for diabetes treatment, it can be anticipated that the drug might be used at higher doses in oncology.

The cytostatic/cytotoxic effects in MIA PaCa-2 cells cultured in the presence of pioglitazone are associated with an increase in the expression levels of the cell cycle inhibitors $p27^{Kip1}$ and p53 (Fig. 2). Accumulation of these proteins has been previously described to result from TZDs action (Leung *et al*., 2004; Liu *et al*., 2006). Moreover, numerous studies have demonstrated that both $p27^{Kip1}$ and p53 are effectively degraded by proteasomes (Kudo *et al*., 2000; Yu *et al*., 2003; Saville *et al*., 2004; Mani & Gelmann, 2005; Zavrski *et al*., 2007; Zhu *et al*., 2007). An additional argument for the potential engagement of this pathway in pioglitazone-mediated effects was the accumulation of ubiquitinated proteins in Mia PaCa-2 cells observed after incubation with pioglitazone (Fig. 3). However, we also observed that in pioglitazone-treated MIA PaCa-2 cells cyclin D1 level was decreased. This observation was unexpected as regulation of cyclin D1 is partly controlled by

proteasomal degradation (Dragnev *et al*., 2001; Feng *et al*., 2007). Also relatively lower p53 level observed at the highest concentration of pioglitazone (100 μM) could result either from an enhanced activity of the ubiquitin-proteasome system or from an unspecific drug cytotoxicity. The decline in the level of cyclin D1 may have resulted from down-regulation of its gene expression at the transcriptional or translational level or from enhanced degradation. Indeed, enhanced proteasome-dependent degradation of cyclin D1 induced by PPAR-γ agonists has been described (Qin *et al*., 2003; Huang *et al*., 2005). Analysis of gene expression profiles in TZDs-treated tumor cells revealed a decrease in the levels of other proteins such as the anti-apoptotic proteins BCL-2 and BCL-X_L (Elstner *et al*., 1998; Chattopadhyay *et al*., 2000; Lee *et al*., 2006) or stress proteins such as HSP70 (Davies *et al*., 2003). This suggests that modulation of protein expression under the influence of TZDs is more complex and proteasomal processing is neither the only nor the most important factor affecting cell proliferation and survival.

The mechanism of pioglitazone-mediated increase in the levels of polyubiquitinated proteins is unknown. As we did not observe any proteasome inhibitory effect of pioglitazone on isolated 20S proteasome or in cell lysates, it can be concluded that this effect is independent of the blockade of proteasome processing and may be caused by cellular stress (Hermann *et al*., 2003; Caraglia *et al*., 2005). For instance, up-regulation of ubiquitination enzymes is an essential mechanism by which the endoplasmic reticulum (ER) stress enhances ER-associated degradation (Shen *et al*., 2007). Indeed, recent evidence has demonstrated that TZDs induce ER stress (Weber *et al*., 2004). Moreover, we demonstrated that both the ubiquitin-dependent and N-terminus-dependent proteasome degradation pathways are not affected by pioglitazone, as we did not observe accumulation of fluorescent proteasome substrates in HeLa-GV and HeLa-R cells.

To conclude, our results indicate that pioglitazone demonstrates weak cytostatic/cytotoxic effects that are not due to inhibition of proteasome activity. Elucidation of the underlying mechanisms of action and of the differences between TZD family members is necessary for their potential use in oncology.

Acknowledgements

This work was supported by a UICC International Cancer Technology Transfer Fellowship and by a grant from the Ministry of Science and Higher Education No. NN401 3240 33, Poland.

We would like to thank Anna Czerepińska, Elżbieta Gutowska and Ilse Drung for their excellent technical assistance.

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