• LIVER CANCER •

# Peroxisome proliferator-activated receptor gamma ligands inhibit cell growth and induce apoptosis in human liver cancer BEL-7402 cells

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# Abstract

**AIM:** To investigate the characteristics of PPAR gamma ligands induced apoptosis in liver cancer cells.

**METHODS:** The effects of ligands for each of the PPAR gamma ligands on DNA synthesis and cell viability were examined in BEL-7402 liver cancer cells. Apoptosis was characterized by Hochest33258 staining, DNA fragmentation, TUNEL and ELISA, and cell cycle kinetics by FACS. Modulation of apoptosis related caspases expression by PPAR gamma ligands was examined by Western blot.

**RESULTS:** PPARgamma ligands, 15-deoxy-<sup>12, 14</sup>-prostaglandin J2 (15d-PGJ2) and troglitazone (TGZ), suppressed DNA synthesis of BEL-7402 cells. Both 15d-PGJ2 and TGZ induced BEL-7402 cell death in a dose dependent manner, which was associated with an increase in fragmented DNA and TUNEL-positive cells. At concentrations of 10 and 30  $\mu$ M, 15d-PGJ<sub>2</sub> or troglitazone increased the proportion of cells with G<sub>0</sub>/G<sub>1</sub> phase DNA content and decreased those with S phase DNA content. There was no significant change in the proportion of cells with G<sub>2</sub>/M DNA content. The activities of Caspases-3, -6, -7 and -9 were increased by 15d-PGJ2 and TGZ treatment, while the activity of Caspase 8 had not significantly changed.

**CONCLUSION:** The present results suggest the potential usefulness of PPAR gamma ligands for chemoprevention and treatment of liver cancers.

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# INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are

members of the nuclear hormone receptor family. Three distinct PPARs, termed PPAR- $\alpha$ , PPAR- $\beta$  and PPAR- $\gamma$ , have been identified. PPAR- $\alpha$  is abundant in primary hepatocytes, where it regulates the expression of proteins involved in fatty acid metabolism. PPAR- $\beta$  is the most widely distributed subtype and is often expressed at high levels. PPAR-y is predominantly seen in adipose tissue, where it plays a critical role in regulating adipocyte differentiation. The ability of PPAR- $\gamma$  to regulate cell differentiation and proliferation has inspired a number of researchers to explore the use of PPAR-y agonists as chemotherapeutic agents<sup>[1-7]</sup>. PPAR- $\gamma$  is highly expressed in human lipocarcinomas and various other human tumors including breast, lung, colon, prostate, bladder and gastric cancer<sup>[8-13]</sup>. Furthermore, prostaglandin 15d-PGJ<sub>2</sub> and/or troglitazone induce apoptosis and growth inhibition of human breast, lung, colon, prostate, bladder, gastric and thyroid carcinoma cells in vitro.

In support of the *in vitro* data, there are now many reported examples of tumor growth suppression/arrest in tumor-bearing rodent models treated with PPAR- $\gamma$  agonist therapies. For example, troglitazone treatment of nude mice implanted with papillary thyroid tumors reduced tumor growth and prevented distant metastasis. Both estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines undergo cell cycle arrest when treated with15d-PGJ<sub>2</sub> or troglitazone and similar effects are observed in rodent breast cancer *in vivo* models<sup>[14-18]</sup>. PPAR- $\gamma$ ligands have been shown to inhibit growth and induce terminal differentiation of liposarcoma cells, and to inhibit growth and induce apoptosis of breast cancer cells,

In the field of gastroenterology, many investigators have focused on the role of PPAR- $\gamma$  in colon cancer, since PPAR- $\gamma$  is highly expressed in human colon and colon tumors. The effects of PPAR- $\gamma$  on colon cancer are still unclear and controversial<sup>[19-25]</sup>, since PPAR- $\gamma$  ligands have been reported both to promote the development and to reduce the growth rate of colon tumors. PPAR- $\gamma$  ligands also inhibit the growth of human gastric carcinoma cells through induction of apoptosis<sup>[25]</sup>. However, the effects of PPAR- $\gamma$  ligands on growth of human liver cancer cells have not been examined. In this study, we investigated the effects of PPAR- $\gamma$  ligands 15d-PGJ<sub>2</sub> and troglitazone on growth of human liver cancer BEL-7402 cells and whether 15d-PGJ<sub>2</sub> and troglitazone affected the cell cycle, apoptosis, and Caspases activity of BEL-7402 cells.

# MATERIALS AND METHODS

# Cell line and reagents

Human liver cancer cell line BEL-7402 was provided by the American Type Culture Collection. Cells were grown in RPMI-1640 medium supplemented with 15 % new born bovine serum, penicillin G (100 kU/L) and kanamycin (0.1 g/L) at 37 °C in a 5 % CO<sub>2</sub>-95 % air atmosphere. Anti-Caspases-3, -6, -7, -8 and -9 antibodies were obtained from Sigma Chemical Co. 15-deoxy- $\Delta$ <sup>12,14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) and troglitazone were

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obtained from Cayman Chemical Co. All other chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA).

#### Determination of cell proliferation rate

BEL-7402 cells ( $1 \times 10^{5}$ ) were seeded in 24 well plates and cultured for 24 h. The cultures were divided into three groups: the first group (control) was cultured in the RPMI1640 medium, the second group was cultured in the continuous presence of 20  $\mu$ M 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), the third was cultured in the continuous presence of 20  $\mu$ M troglitazone. Cells were then harvested every 24 h by trypsinization and cell numbers were counted with a hemocytometer, three cultures were used for experiments at each time point.

# [<sup>3</sup>H] thymidine incorporation

Subconfluent cells were cultured in 24-well plates and incubated for 24 h with 5uCi of [<sup>3</sup>H] thymidine. The cells were then washed 3 times with HBSS, lysed with 1M NaOH, and lysate was counted by liquid scintillation.

#### Hoechst 33258 staining

Cells were fixed with 4 % formaldehyde in phosphate buffered saline (PBS) for 10 min, stained by Hoechst33258 (10 mg/L) for one hour, and subjected to fluorescence microscopy. After treated with 15d-PGJ<sub>2</sub> or troglitazone, the morphologic changes including reduction in volume, nuclear chromatin condensation were observed.

#### Electron microscopy (EM)

Control BEL-7402 cells or those treated with 15-deoxy- $\triangle^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) or troglitazone for 48 h and that remained attached to the surface of the culture dishes were gently washed with serum-free medium, and then fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer. These cells were scraped from the surface of the dishes and pelleted by spinning for 5 min at 10 000×g. The cells were osmicated with 1 % osmium tetroxide, then block was stained, dehydrated in graded ethanol, infiltrated with propylene oxide, and embedded with EMBED overnight and cured in a 60 °C oven for 48 h. Silver sections were cut with an Ultracut E microtome, collected on a formvar and carbon-coated grid, stained with uranyl acetate and Reynold's lead citrate, and viewed under a JEOL100 CX electron microscope.

#### Ladder detection assay

After induction of apoptosis, cells ( $7 \times 10^6$ /sample, both attached and detached cells) were lyzed with 150 µl hypotonic lysis buffer (edetic acid 10 mM, 0.5 % Triton X-100, Tris-HCl, ph7.4) for 15 min on ice and were precipitated with 2.5 % polyethylene glycol and NaCl 1 M for 15 min at 4 °C. After centrifugation at 16 000×g for 10 min at room temperature, the supernatant was incubated in the presence of proteinase K (0.3 g/L) at 37 °C for one hour and precipitated with isopropanol at -20 °C. After centrifugation, each pellet was dissolved in 10 µl of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5 % agarose gel containing ethidium bromide. Ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

# Detection of apoptotic DNA fragmentation

BEL-7402 cells were grown in 96-well culture plates. The cells were incubated with various doses of 15d-PGJ<sub>2</sub> and troglitazone for 6 h. Apoptotic DNA fragmentation was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Roche Co. This assay was based on a quantitative sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200 µl of lysis buffer provided in the kit,

the lysates were centrifuged, and 20  $\mu$ l of the supernatant containing cytoplasmic histone-associated DNA fragments was reacted overnight at 4 °C in streptavidin-coated microtitrator wells with 80  $\mu$ l of the immunoreagent mixture containing biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washed, the immunocomplex-bound peroxidase was probed with 2,2' -azino-di[3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405 nm.

#### TUENL assay

TUNEL assay was performed using the apoptosis detection system. Cells were fixed by 4 % paraformaldehyde in PBS overnight at 4 °C. The samples were washed three times with PBS and permeabilized by 0.2 % Triton X-100 in PBS for 15 min on ice. After washed twice, cells were equilibrated at room temperature for 15 to 30 min in equilibration buffer(potassium cacodylate 200 mM, dithiothreitol 0.2 mM, bovine serum albumin 0.25 g/L, and cobalt chloride 2.5 mM in Tris-HCl 25 mM, pH 6.6) and then incubated in the presence of fluorescein-12-dUTP 5  $\mu$ M, dATP 10  $\mu$ M, edetic acid 100  $\mu$ M, and terminal deoxynucleotidyl transferase at 37 °C for 1.5 h in the dark. The tailing reaction was terminated by 2×standard saline citrate (SSC). The samples were washed three times with PBS and analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

#### Flow cytometry

For DNA content analysis, cells were treated with different concentrations of 15-deoxy- $\Delta^{12, 14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) and troglitazone for 24 h. 1×10<sup>6</sup> cells were harvested, pelleted and washed with phosphate-buffered saline (PBS), and resuspended in PBS containing 20 mg/L PI and 1 g/L ribonuclease A. 10<sup>6</sup> fixed cells were examined under each experimental condition by flow cytometry, and percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-G<sub>1</sub>). DNA divided by the total number of cells was examined. Cell cycle analysis was performed under the same experimental conditions and distributions were determined using the CellFit program. All measurements were carried out under the same instrumental settings.

#### Western blot analysis

The cells were lysed in lysis buffer [hepes 25 mM, 1.5 % Triton X-100, 1 % sodium deoxycholate, 0.1 %SDS, NaCl 0.5 M, edetic acid 5 mM, NaF 50 mM, sodium vanadate 0.1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, and leupeptin 0.1 g/L, pH7.8] at 4 °C with sonication. The lysates were centrifuged at 15 000 g for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (Tris-HCl 42 mM, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol and 0.002 % bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated sequentially with anti-Caspases-3, -6, -7, -8 and -9 antibodies and then with peroxidase-conjugated secondary antibodies in the second reaction. Detection was performed with enhanced chemiluminescence reagent. The results on Western blot analysis represented the average of three individual experiments.

#### Statistical analysis

Data were presented as the mean  $\pm$  standard error of the mean, unless otherwise indicated. Multiple comparisons were examined for significant differences using analysis of variance, followed by individual comparisons with the Bonferroni posttest. Comparisons between two groups were made with the Student's *t* test. A *P*<0.05 was considered significant.

#### RESULTS

# Effects of 15d-PGJ<sub>2</sub> and troglitazone on proliferation and cell cycle

Cells were cultured in the presence or absence of  $15d-PGJ_2$  or troglitazone and cell numbers were determined over three days. In the absence of 15d-PGJ<sub>2</sub> or troglitazone, the number of control cells doubled approximately every 24 h in RPMI 1640 medium supplemented with 10 % fetal calf serum. By contrast, in the continuous presence of 20 µM 15d-PGJ<sub>2</sub> or troglitazone' the growth of BEL-7402 cells was significantly inhibited (Figure 1A). We next examined by [<sup>3</sup>H]-thymidine incorporation whether 15d-PGJ<sub>2</sub> or troglitazone affected DNA synthesis of BEL-7402 cells. Cells were treated with various doses of 15d- $PGJ_2$  or troglitazone (10, 20, 30  $\mu$ M). The results showed that 15d-PGJ<sub>2</sub> or troglitazone significantly and dose-dependently inhibited [3H]-thymidine incorporation into BEL-7402 cells (Figures 1B, 1C). Table 1 indicates the effects of  $15d-PGJ_2$  or troglitazone on the cell cycle distribution of BEL-7402 cells. 15d-PGJ<sub>2</sub> or troglitazone at 10  $\mu$ M induced limited or no change in the cell cycle distribution of cells. At concentrations of 20 and 30  $\mu$ M, 15d-PGJ<sub>2</sub> or troglitazone increased the proportion of cells with G<sub>0</sub>/G<sub>1</sub> phase DNA content and decreased those with S phase DNA content. There was no significant change in the proportion of cells with G<sub>2</sub>/M DNA content.

# Effect of 15d-PGJ<sub>2</sub> or troglitazone on induction of apoptosis in BEL-7402 cells

**Morphological changes** 15d-PGJ<sub>2</sub> or troglitazone treatment of BEL-7402 cells altered their morphology and induced DNA strand breaks in a manner consistent with apoptosis. That the changes were indeed induced by apoptosis and not necrosis was confirmed by EM and Hoechst 33258 staining. 15d-PGJ<sub>2</sub> or troglitazone-treated cells showed compacted nuclear chromatin with fine granular masses marginated against the nuclear enveloped and condensed cytoplasm, the nuclear outline was convoluted and the organelles were preserved.

**TUNEL assay** To determine whether 15d-PGJ<sub>2</sub> or troglitazone has a capacity to induce apoptosis in BEL-7402 cells, exponentially growing cells were exposed to various concentrations of 15d-PGJ<sub>2</sub> or troglitazone. TUNEL assay was performed. 15d-PGJ<sub>2</sub> or troglitazone dramatically increased the number of TUNELpositive cells in a dose-dependent manner.

**DNA fragments** Agarose gel electrophoresis exhibited DNA ladder formation in BEL-7402 cells after exposed to different concentrations of 15d-PGJ<sub>2</sub> or troglitazone for 48 h. Compared with control, the DNA laddering was more clearly observed by the treatment with 15d-PGJ<sub>2</sub> or troglitazone. ELISA assay also showed that 15d-PGJ<sub>2</sub> or troglitazone induced DNA fragment in a dose-dependent manner (Figure 2).



**Figure 1** Concentration and time effect of 15d-PGJ2 or TGZ on growth of BEL-7402 cells. (A) BEL-7402 cells were incubated with 20  $\mu$ M 15d-PGJ2 or TGZ for 12, 24, 48, 72, 96 h. (B) BEL-7402 cells were incubated with various concentrations of 15d-PGJ2 for 48 h; (C) BEL-7402 cells were incubated with various concentrations of TGZ for 48 h. The value was represented as mean ±SEM (*n*=3). <sup>a</sup>*P*<0.05 and <sup>b</sup>*P*<0.01 versus corresponding control group.



**Figure 2** DNA fragmentation by ELISA assay, as measured by absorbance (OD 450 values). Culture of BEL-7402 cells for 48 h in the presence of 15d-PGJ2/TGZ resulted in dose dependent DNA fragmentation. A) 15d-PGJ2; B) TGZ. <sup>b</sup>*P*<0.01 compared to respective control. The value was represented as mean  $\pm$  SEM (*n*=3).

**Flow cytometry** In order to determine the effect of 15d-PGJ<sub>2</sub> or troglitazone on apoptosis in BEL-7402 cells, cells were exposed to 15d-PGJ<sub>2</sub> or troglitazone for 48 h, apoptotic damage of DNA was detected according to the sub-G<sub>1</sub> peak on a flow cytometer. Cells in sub-G<sub>1</sub> phase were increased from  $2.1\pm0.3$  % to  $55.8\pm4.7$  % or  $50.0\pm4.1$  % after 15d-PGJ<sub>2</sub> or troglitazone treatment (Table1).

# Effect of 15d-PGJ<sub>2</sub> or troglitazone on the activities of Caspase-3, -6, -7, -8 and -9

In order to elucidate the pathway leading to apoptosis, we

examined the activation of Caspases-3, -6, -7, -8 and -9, which were reported to initiate apoptosis upon various stimuli. BEL-7402 cells treated with 15d-PGJ<sub>2</sub> or troglitazone for 24 h were analyzed for the enzymatic activity by Western blot. The results showed that Caspases-3, -6, -7, -8 and -9 were activited after 15d-PGJ<sub>2</sub> or troglitazone treatment in BEL-7402 cells, while the activity of Caspase 8 had not significantly changed (Figure 3).

**Table 1** Effect of 15d-PGJ2 and TGZ on cell cycle distribution and apoptosis in BEL-7402 cells

Treatment (µm)	%Cell cycle distribution			o/ · · ·
	$G_0/G_1$	S	$G_2/M$	%apoptosis
Control 15d-PGJ2	49.7±1.5	34.8±2.1	15.5±1.1	2.1±0.3
10	50.5±2.6	$34.2 \pm 1.7$	15.3±0.5	$13.9 \pm 1.1^{b}$
20	$64.8{\pm}2.9^{\mathrm{b}}$	$19.5{\pm}1.5^{\mathrm{b}}$	15.7±0.2	$33.5{\pm}2.3^{\mathrm{b}}$
30	$71.6 \pm 4.2^{\mathrm{b}}$	$14.1{\pm}0.6^{\rm b}$	$14.4 \pm 1.3$	$55.8 \pm 4.7^{\mathrm{b}}$
TGZ				
10	$54.2 \pm 2.1$	29.4±1.3	$16.6 \pm 0.9$	$10.3 \pm 1.1^{b}$
20	$61.5 \pm 3.1^{b}$	$28.2 \pm 1.7^{b}$	$14.9 \pm 1.7$	$25.5 \pm 1.8^{b}$
30	$68.9{\pm}4.8^{\rm b}$	$14.2{\pm}0.8^{\rm b}$	$16.9 \pm 1.4$	$50.0{\pm}4.1^{\mathrm{b}}$

Cell cycle distribution was determined after 24 h of treatment in one group and apoptosis was determined after 48 h of treatment in the other group. The tabulated percentages were an average calculated on the results of three separate experiments. The value was represented as mean±SEM (n=3). <sup>b</sup>P<0.01 versus corresponding control group.



Figure 3 Western blot analysis of the activities of Caspases-3, -6, -7, -8 and -9 in human liver cancer cell line BEL-7402 cells: lane 1: control, lane 2:  $30 \mu$ M TGZ treated BEL-7402 cells; lane 3:  $30 \mu$ M 15d-PGJ2 treated BEL-7402 cells.

#### DISCUSSION

Several members of the family of nuclear hormone receptors (NHR) play crucial roles in the control of cellular homeostasis, and administration of their cognate ligands has successfully been used in cancer treatment<sup>[26-33]</sup>. The nuclear receptor superfamily includes members such as the estrogen, thyroid and glucocorticoid receptors as well as the subfamily of peroxisome proliferator-activated receptors. The PPAR family comprises PPAR- $\alpha$ , PPAR- $\beta$  and PPAR- $\gamma$ . The PPARs bind as heterodimers with retinoic-x acid receptor (RXR) to a subset of DR-1 elements, peroxisome proliferator response elements and have been shown to regulate expression of genes involved in the transport, metabolism and storage of fatty acids. Transcriptional activation of the PPAR-RXR heterodimers is enhanced upon binding of a large variety of ligands including saturated and unsaturated fatty acids, arachidonic acid derivatives and a wide range of synthetic drugs with different subtype specificities.

For the last 10 years, administration of peroxisome

proliferators, PPAR- $\alpha$  agonists, has been known to induce hepatocarcinogenesis in rodents. However, cancer development is probably induced by mechanisms secondary to PPAR- $\alpha$  transcriptional activation. A role in growth regulation for the two remaining PPAR subtypes has also been suggested. Whichever, upregulaties PPAR- $\beta$  expression has been associated with colon cancer and activation of PPAR- $\beta$ stimulates post-confluent proliferation of pre-adipocytes. Opposite effects on cell proliferation are mediated by activation of PPAR- $\gamma$ . PPAR- $\gamma$  agonists could be promising therapeutic or chemopreventive agents in oncology, since they can induce apoptosis or differentiation in several tumors, by acting as inhibitors of malignancy progression. PPAR- $\gamma$  activation inhibits the growth of several tumors as shown by in intro and in vivo studies performed on liposarcoma, breast cancer and leukemia. However, conflicting evidence exists on the role of PPAR-y activation in colon cancer, where different studies have shown that PPAR-y activation promotes tumor development or, in contrast, protects against colon cancer, depending on the cell model<sup>[34-42]</sup>. In the present study, in order to examine the effects of PPAR- $\gamma$  ligands 15d-PGJ<sub>2</sub> or troglitazone on the BEL-7402 cell growth, we employed cell counting and [<sup>3</sup>H]thymidine incorporation assay. The results showed 15d-PGJ<sub>2</sub> or troglitazone significantly and concentration-dependently inhibited the growth of BEL-7402 cells. To examine whether growth inhibition of BEL-7402 cells by 15d-PGJ<sub>2</sub> or troglitazonem was a result of cell cycle arrest, BEL-7402 cells treated with either vehicle or 15d-PGJ<sub>2</sub>/troglitazone were analyzed by FACScan. BEL-7402 cells treated with 15d-PGJ<sub>2</sub> and troglitazone exhibited decreased fractions of S phase cells from 34.8±2.1 % in controls to14.4±1.3 % and 16.9±1.4 %, respectively, resulting in a remarkable increase in accumulation if cells at  $G_1$  phase, increased from a control level of 49.7±1.5 % to 71.6±1.5 % and 68.9±4.8 %. Therefore, the inhibitory effect of 15d-PGJ<sub>2</sub> or troglitazone on growth of BEL-7402 cells may thus be due in part to PPAR- $\gamma$ -mediated G<sub>1</sub> cell cycle arrest. Similar findings of  $G_1$  cell cycle arrest by PPAR- $\gamma$  ligands have been reported for colon cancer cells and prostate cancer cells. Several reports suggested that PPAR-y ligands affected cell cyclerelated genes and proteins. Others demonstrated that PPAR- $\gamma$ activation caused G1 cell cycle arrest of fibroblasts and SV40transformed adipogenic HIB1B cells, and that this arrest was strongly associated with loss of E2F/DP DNA binding through modulation of phosphorylation by phosphatase 2A<sup>[43-47]</sup>.

Previous studies have shown that PPAR- $\gamma$  activation generally promotes apoptosis and/or differentiation in several normal and tumor cells such as human breast cancer cells, human gastric cancer cells, human non-small cell lung carcinoma, human glioblastoma cells, macrophages, endothelial cells and liposarcoma. In the present study, to determine the underlying mechanisms of the growth inhibitory effect of PPAR- $\gamma$ ligands, we investigated whether 15d-PGJ<sub>2</sub> or troglitazone acted by inducing apoptosis of liver cancer BEL-7402 cells. We performed DNA fragments and morphological changes assay EM or Hoechst 33258. The results showed that 15d-PGJ<sub>2</sub> or troglitazone induced apoptosis in a dose-dependent manner, indicating that growth inhibition of BEL-7402 by PPAR- $\gamma$ ligands was, in part, associated with apoptosis.

Recent evidence indicates that increased expression and activation of some Caspase zymogens in tumor cells can lead to efficient inhibition of tumor cell growth, invasion and metastasis and tumor regression<sup>[48-56]</sup>. Such a Caspase-dependent cessation of tumor cell proliferation and dissemination is accomplished via an active process of tumor cells death collectively named as apoptosis. It has been demonstrated that a high level of activity of effector Caspases-3, -6, -7 and -8, in tumor cells plays a decisive role in their commitment to apoptosis<sup>[57-66]</sup>. To-date studies on zymogens of the effector

Caspases in primary human tumors showed an increased expression of procasp-3 and -6 in breast carcinoma, pancreatic carcinoma and non-small cell lung carcinoma compared to normal tissue and benign or premalignant lesions. This suggests that tumor cells of some epithelial neoplasms may acquire an increased apoptotic potential during progression at the levels of primary tumor. The zymogens of casp-3 and -7 in tumor cells can be activated by the initiator Caspases, such as casp-8 and casp-9, and by the aspartyl-specific serine proteinase granzyme B upon its perforin-assisted entry into the cytoplasm of tumor cells. Procasp-6 can be activated by casp-3 while the generated casp-6 can activate in turn the zymogen of casp-3. In the present study, in order to elucidate the pathway leading to apoptosis, we examined activation of Caspases-3, -6, -7, -8 and -9, which have been reported to initiate apoptosis upon various stimuli. BEL-7402 cells treated with15d-PGJ<sub>2</sub> or troglitazone for 24 h were analyzed for the enzymatic activity by Western blot. The results showed that Caspases-3, -6, -7, -8 and -9 were activited after 15d-PGJ<sub>2</sub> or troglitazone treatment in BEL-7402 cells, while the activity of Caspase 8 had not significantly changed, indicating that activation of Caspases plays an important role in the apoptosis induced by 15d-PGJ<sub>2</sub> or troglitazone.

In conclusion, the present results, together with reports by other investigators, suggest a potential usefulness of PPAR gamma ligands for chemoprevention and treatment of liver cancers. Further basic as well as clinical studies are required to develop new strategies to fight liver cancers using PPAR gamma ligands.

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