# Ligands of peroxisome proliferator-activated receptor γ induce apoptosis in multiple myeloma

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**The activation of proliferator-activated receptor γ (PPAR-γ) by its natural and synthetic ligands induces apoptosis in several tumor cell lines, including malignant B-lineage cells. We investigated whether treatment with pioglitazone (PGZ), rosiglitazone (RGZ) or 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) inhibited tumor cell growth in five human multiple myeloma cell lines (LP-1, U-266, RPMI-8226-S, OPM-2 and IM-9) and human bone marrow myeloma cells expressing PPAR-γ protein. MTT assays revealed growth arrest induced by the natural activator of PPAR-γ 15d-$PGJ_2$ and a lower antiproliferative effect with thiazolidinediones (PGZ and RGZ) in a dose-dependent manner. Induction of apoptosis was indicated by Annexin-V staining. At a dose of 50 μM, 15d-$PGJ_2$ led to a high rate of apoptosis in all cell lines (60–92%). Furthermore, induction of apoptosis in sorted bone marrow plasma cells from myeloma patients was detected. Thiazolidinediones comprise anti-myeloma activity *in vitro* and should be explored further for the treatment of multiple myeloma. *Anti-Cancer Drugs* 15:955–960 © 2004 Lippincott Williams & Wilkins.**

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## Introduction

Multiple myeloma represents a rapidly expanding research field with important new insights during recent years [1–4]. Nevertheless, this disease remains incurable with conventional treatment and the median survival is about 42 months [5]. There have been continuous attempts to improve the response rates, overall survival and quality of life in myeloma patients by novel treatment strategies [6–9]. However, more effective treatment options are still urgently needed.

Proliferator-activated receptor γ (PPAR-γ) is a member of the nuclear receptor superfamily, which includes the retinoic acid receptors, the thyroid hormone receptors and steroid receptors [10]. PPAR-γ binds to the promotor region of a target gene as heterodimer with the retinoid X receptor (RXR) and stimulates transcription of target genes [10]. This complex can be activated by PPAR-γ or RXR ligands leading to transcription of target genes. Furthermore, PPAR-γ was shown to interact with other transcription factors such as Jun or NF-κB. In this manner, PPAR-γ may prevent them from binding to their response elements [11]. PPAR-γ is mainly expressed in normal adipocytes, adrenal gland, spleen, liver and activated macrophages [12]. Furthermore, it was discovered that PPAR-γ is expressed in tumor tissue as well. Activation of PPAR-γ by its ligands has been shown to reduce tumor growth, interfere with tumor cell differentiation and induce apoptosis in a variety of human malignancies, including solid tumors like colon, breast, lung, liver, prostate cancer, as well as hematological malignancies like myeloid leukemia [13–19]. Recently, it has been shown that both human B lymphocytes and B lymphomas express PPAR-γ. Although the precise mechanisms underlying growth arrest mediated through PPAR-γ have not been elucidated, PPAR-γ agonists have been shown to induce apoptosis in malignant B-lineage cells, including one mouse myeloma cell line [20,21].

15-Deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) is a natural activator of PPAR-γ. It is the biologically active metabolite of $PGD_2$ which is a major product of cyclooxygenase in bone marrow and in macrophages, suggesting a role in immunological responses [12,22]. Activation was induced by 15d-$PGJ_2$ as well as thiazolidinediones. Thiazolidinediones, including troglitazone, rosiglitazone (RGZ) and pioglitazone (PGZ), comprise a group of synthetic PPAR-γ agonists currently in use for the treatment of type 2 diabetes mellitus and had revealed anti-tumor activity *in vitro* [13,23]. Troglitazone may cause idiosyncratic liver damage and its application has been discontinued.

The influence of PPAR-γ agonists on cell growth of human multiple myeloma cells has not been analyzed so

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far. Our study addressed the proliferation inhibiting potential and the apoptosis induction of two thiazolidinediones, RGZ and PGZ, as well as 15d-$PGJ_2$ in human myeloma cell lines and in sorted human multiple myeloma cells *in vitro*. First, expression of PPAR-γ in human myeloma cells was analyzed by Western blot. Then, proliferation inhibition and induction of apoptosis by the PPAR-γ ligands was investigated.

## Material and methods

### Cell culture

The five multiple myeloma cell lines (LP-1, U-266, RPMI-8226-S, IM-9 and OPM-2) were obtained from the DSMZ (Braunschweig, Germany). Apart from LP-1, all cell lines were maintained in continuous culture in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS. LP-1 was cultured in DMEM (Biochrom). Cell lines were cultured at 37°C in a humidified 5% $CO_2$ atmosphere and were passaged 3 times weekly. The cell density was kept between $2 \times 10^5$ and $1 \times 10^6$/ml.

Myeloma cells obtained from routinely performed bone marrow aspirates of patients with multiple myeloma were sorted immunomagnetically using MACS mouse anti-human CD138 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. All freshly isolated cells tested *in vitro* derived from patients with multiple myeloma in stage III according to Durie and Salmon classification. Four of the five patients were previously treated. Three of them had multiple myeloma refractory to both anthracyclines and alkylating agents.

The investigations have been approved by the Ethics committee of the University Hospital Charité in Berlin, in accordance with the Declaration of Helsinki. An informed consent was obtained from all patients.

### Chemicals

The synthetic ligands of PPAR-γ, RGZ and PGZ, were kindly provided by GlaxoSmithKline Pharmaceuticals (Mayfield, UK) and by Takeda Chemical Industries (Osaka, Japan), respectively. Both were dissolved at appropriate concentrations in DMSO and ethanol and in cell culture medium at a final concentration of less than $10^{-3}$ M. 15d-$PGJ_2$ was obtained from Calbiochem (San Diego, CA) and dissolved in ethanol. The solvents did not exceed 1% in the final solution. Aliquots were stored at −80°C.

### Cell proliferation assay

Myeloma cell lines were seeded in 96-well flat-bottom microtiter plates, at a cell density of $5 \times 10^5$ cells/ml. In dose–response studies PPAR-γ ligands (RGZ, PGZ or 15d-$PGJ_2$) were added in different concentrations ($10^{-4}$, $5 \times 10^{-5}$, $10^{-5}$, $5 \times 10^{-6}$, $10^{-6}$ and $5 \times 10^{-7}$ M). The microtiter plate was incubated for 48 h protected from light at 37°C, 5% $CO_2$ and 100% relative humidity. Proliferation was measured by cell proliferation kit I (MTT) (Roche Diagnostics, Mannheim, Germany). For the last 4 h of culture, cells were pulsed with 10 µl of the MTT labeling reagent at a final concentration of 0.5 mg/ml. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purpure formazan crystals by metabolic active cells [24]. To solubilize the crystals, 100 µl of a solubilization solution containing 10% SDS in 0.01 M HCl was added into each well and the plate was allowed to stand overnight in the incubator in a humidified 37°C/5% $CO_2$ atmosphere. Finally, the absorbance was measured spectrophotometrically using a 550-nm wavelength ELISA reader and Anthos software. Control experiments were performed with solvents of each PPAR-γ ligand alone, as well as in the absence of both the PPAR-γ ligands and their solvents. Solvents had no significant influence on cell proliferation.

### Annexin-V staining assay

Apoptosis was measured by Annexin-V staining using the Annexin-V–FITC kit (Bender MedSystems, Vienna, Austria). Cells ($5 \times 10^5$/ml) were incubated in the presence or absence of PPAR-γ ligands in flat-bottom plates. They were centrifuged and washed 2 times with PBS. The pellet was resuspended in 195 µl binding buffer supplied with the Annexin-V kit, spiked with 5 µl Annexin-V–FITC and incubated for 10 min at room temperature protected from light. After washing, once again a pellet of each sample was resuspended in 190 ml binding buffer and 10 µl propidium iodide was added shortly before samples were analyzed by flow cytometry. All samples were analyzed on a FACS flow cytometer with an argon laser of 488 nm emission wavelength and the CellQuest Pro software (Becton Dickinson, Mountain View, CA). As in the MTT assay, control experiments were performed with solvents, which had no significant influence on cell growth. Similar data were obtained in at least two independent experimental sets. The percentage of specific apoptosis was calculated as follows: [experimental Annexin-V binding (%) – control Annexin-V binding (%)]/[100 – control Annexin-V binding (%)] × 100.

### Western blot analysis

Expression of specific proteins was detected by Western blotting of centrifuged cells. First, protein concentrations of cell lysates were measured using the BCA protein assay kit (Perbio Science, Bonn, Germany). Then, 50 µg protein was separated by a 4–12% gradient SDS–PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad, Munich, Germany), blocked in PBS/Tween (0.1%) with 5% non-fat dry milk overnight at room temperature and subsequently incubated with primary antibody for 2 h. As primary antibody, anti-PPAR-γ (sc-7273) was used (Santa Cruz, Heidelberg,

Germany). After thoroughly washing the membrane, it was incubated with peroxidase-conjugated secondary antibody for 90 min. Signal was detected by chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany). As an internal control, β-actin was detected with anti-β-actin antibodies (sc-1616) (Santa Cruz).

### Statistical analysis

The data resulting from MTT assay were presented as mean ± SD. Data from Annexin-V staining assay was confirmed by at least two independent experiments. The Mann–Whitney *U*-test was used for statistical analyses of inter-group comparisons. $p < 0.05$ was considered significant.

## Results

### Expression of PPAR-γ in multiple myeloma cell lines

Expression of PPAR-γ protein was measured by Western blot analysis. We tested the human myeloma cell lines LP-1, U-266, RPMI-8226-S and IM-9 as well as freshly sorted multiple myeloma cells of one patient. In our experiments, Western blot analysis revealed a characteristic band with a molecular mass of 50 kDa representing PPAR-γ protein in all human myeloma cell lines tested, as well as in sorted bone marrow myeloma cells. As positive control, a human adipocyte cell lysate was used (Fig. 1).

### PPAR-γ ligands induced proliferation inhibition

The effect of two thiazolidinediones (RGZ and PGZ) and of a natural PPAR-γ agonist (15d-$PGJ_2$) on the proliferation of human multiple myeloma cell lines was examined by MTT assay. MTT assay was performed with the human multiple myeloma cell lines LP-1, U-266, RPMI-8226-S, IM-9 and OPM-2. All five cell lines were sensitive to the PPAR-γ agonists. The growth inhibition was achieved in a dose-dependent manner.

PGZ induced significant inhibition of proliferation compared to controls in all multiple myeloma cell lines tested. The growth inhibition was dose dependent and revealed as significant in doses higher than 10 μM. At a dose level of 50 μM, cell proliferation was reduced in the MTT assay after 48 h of incubation to 48% in LP-1, 72% in U-266, 77% in RPMI-8226-S, 52% in IM-9 and 56% in OPM-2 (Fig. 2). RGZ inhibited the proliferation of multiple myeloma cell lines significantly and was comparable to the results of PGZ. Cell proliferation was reduced by 50 μM RGZ after 48 h of incubation to 71% in U-266, 69% in IM-9 and 57% in OPM-2. The reduction of proliferation in LP-1 and RPMI-8226-S cells was lower—it was reduced to 84 and 87%, respectively (Fig. 2).

In all cell lines tested, the growth inhibition by 15d-$PGJ_2$ was much more pronounced than the inhibition induced by the synthetic PPAR-γ ligands, PGZ and RGZ. At a dose of 50 μM, cell proliferation was reduced to values between 0 and 26% in all multiple myeloma cell lines tested. In most cell lines the anti-proliferative effect was already detectable at a dose level of 10 μM (Fig. 2).

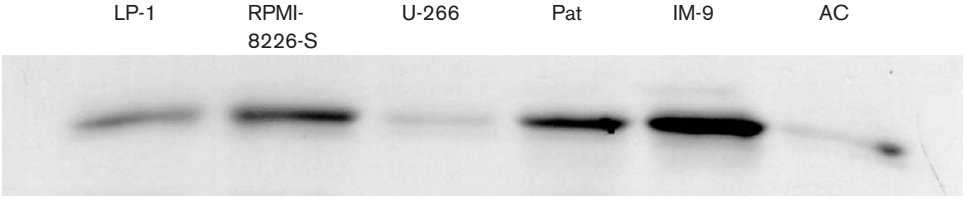
### PPAR-γ agonists induced apoptosis in multiple myeloma cell lines

It was shown that 15d-$PGJ_2$ as well as thiazolidinediones induced proliferation inhibition of B cell lineage in an apoptotic fashion [22]. To determine an apoptotic effect of the thiazolidinediones, RGZ and PGZ, and of 15d-$PGJ_2$ on human multiple myeloma cell lines, the Annexin-V staining assay was performed. Phosphatidylserine externalization, a hallmark of early apoptosis, was quantified using Annexin-V binding and FACS analysis. Cell lines were incubated with 50 μM of PPAR-γ agonists, a concentration which had been previously proven to be effective for growth inhibition in the MTT assay. Again, 15-d$PGJ_2$ was more effective than PGZ and RGZ. All of the 15d-$PGJ_2$-treated cell lines revealed specific apoptosis ranging between 60.3 and 92.1%. Apoptosis induced by PGZ in U-266, RPMI-8226-S, IM-9 and OPM-2 cell lines ranged between 16.8 and 43.0%; for RGZ, it ranged between 19.6 and 50.0%. Thiazolidinediones weakly induced apoptosis in LP-1 cells (Table 1).

### PPAR-γ agonists induced apoptosis in immunomagnetically sorted multiple myeloma cells from patients

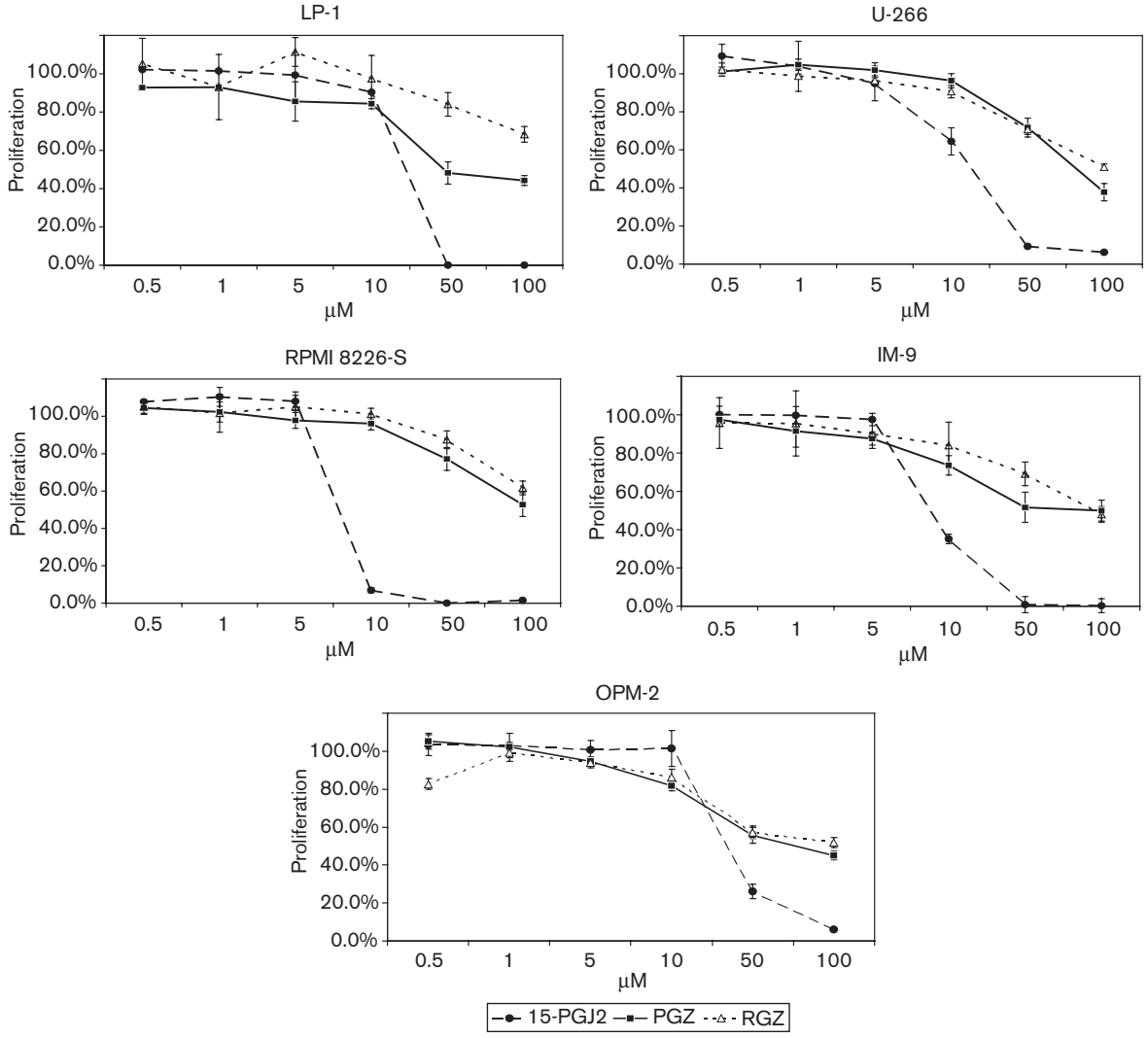
The induction of apoptosis was also investigated in multiple myeloma cells freshly isolated from bone

**Fig. 1**




Western blot analysis of the human multiple myeloma cell lines (LP-1, RPMI-8226-S, U-266 and IM-9) and of freshly isolated myeloma cells of a patient (Pat), all expressing PPAR-γ protein, a protein of 50 kDa. A human adipocyte (AC) cell lysate was used a positive control.

**Fig. 2**




Effect of the PPAR-γ ligands PGZ, RGZ or 15d-$PGJ_2$ on the proliferation of multiple myeloma cell lines. Cells were incubated with either PGZ or RGZ or 15d-$PGJ_2$. Cell proliferation was measured by MTT assay. Data are presented as percent of proliferation relative to untreated cells.

marrow of patients. After 12 h of incubation, the apoptotic effects of PPAR-γ agonists were measured. Multiple myeloma cells of five different patients were tested (Table 1). As with myeloma cell lines, 15d-$PGJ_2$ induced apoptosis more effectively than thiazolidinediones in sorted human multiple myeloma cells. The specific apoptosis rates lay between 29.0 and 96.7%. Apoptosis induced by PGZ showed interindividual differences. In the myeloma cells from four patients, the rate of specific apoptosis ranged between 8.5 and 28.4%, but in one patient induction of apoptosis was not observed either with PGZ or with RGZ. For RGZ, the rate of apoptosis induced in the myeloma cells from the other four patients ranged between 6.5 and 25.5% (Table 1).

**Table 1 Apoptosis *in vitro* in multiple myeloma cells induced by the PPAR-γ ligands PGZ, RGZ or 15d-$PGJ_2$**

| Cell line/patient | Specific apoptosis after treatment (%) | | |
|---|---|---|---|
| | PGZ | RGZ | 15d-$PGJ_2$ |
| LP-1 | 0 | 11.7 | 79.0 |
| U-266 | 43 | 50 | 71.1 |
| RPMI-8226-S | 31.6 | 31.0 | 63.7 |
| IM-9 | 20.8 | 22.9 | 92.1 |
| OPM-2 | 16.8 | 19.6 | 60.3 |
| Patient 1 | 8.5 | 13.0 | 96.7 |
| Patient 2 | 25.7 | 17.7 | 43.4 |
| Patient 3 | 28.4 | 6.5 | 29.0 |
| Patient 4 | 25.8 | 25.2 | 52.3 |
| Patient 5 | 1.7 | 0 | 44.2 |

Cells were incubated with either PGZ or RGZ or 15d-$PGJ_2$. Rate of specific apoptosis in different cell lines and in freshly sorted multiple myeloma cells from five patients is presented.

Interestingly, the rate of specific apoptosis induced by 15D-$PGJ_2$ was not statistically different for sorted human bone marrow myeloma cells sensitive versus refractory to conventional chemotherapy with anthracyclines and alkylating agents ($p = 0.8$).

## Discussion

Recently, it was revealed that normal B cells and lymphoma cell lines express PPAR-γ protein [20,22]. Among these cell lines, one mouse myeloma cell line was tested so far, expressing PPAR-γ protein as well, but the role of PPAR-γ ligands in human multiple myeloma has not been evaluated [21]. In the present study, we found that PPAR-γ protein is strongly expressed in human multiple myeloma cells, including multiple myeloma cell lines as well as freshly isolated bone marrow myeloma cells from patients. These data are in line with those obtained in B cell lines using RT-PCR, Western blot analysis and immunohistochemistry [21]. Theoretically, the response to PPAR-γ ligands might be influenced by the quantitative expression of PPAR-γ in different tissues. However, in B cells of various differentiation status, the relative amount of PPAR-γ protein did not vary significantly [11,21].

It was widely demonstrated that the natural PPAR-γ ligand, 15d-$PGJ_2$, exerts proliferation inhibitory effects on different tumor cells via PPAR-γ [13–19,25,26]. Furthermore, there is evidence that not only for 15d-$PGJ_2$, but also for thiazolidinediones, PPAR-γ activation is the main mechanism by which apoptosis is induced in tumor cells [15,17–19,26,27]. Our results show that proliferation inhibition of human multiple myeloma cells, which express PPAR-γ, is influenced via PPAR-γ ligands. MTT assay revealed a high anti-proliferative activity of 15d-$PGJ_2$, and a somewhat lower activity of PGZ and RGZ in human multiple myeloma cell lines. PGZ and RGZ revealed similar proliferation inhibitory effects in each multiple myeloma cell line tested. Anti-proliferative effects have been demonstrated to be dose dependent. However, 15d-$PGJ_2$ revealed a higher activity in myeloma cells than thiazolidinediones. Different anti-proliferative activities of 15d-$PGJ_2$, on the one hand, and thiazolidinediones, on the other hand, were reported in other tumor cells with high PPAR-γ protein expression as well [20,21,25,26]. Thiazolidinediones, e.g. Troglitazone and Ciglitazone, were 10-fold less potent than 15d-$PGJ_2$ in their anti-proliferative effect on other B cell lineages [22]. This may be due to the increased ability of 15d-$PGJ_2$ to bind PPAR-γ [12,28]. We could confirm that 15d-$PGJ_2$ had substantially stronger inhibitory effects than RGZ and PGZ, with $1-5 \times 10^{-5}$ M concentrations rendering strong proliferation inhibition in all myeloma cell lines tested. Padilla *et al.* reported a significant growth-inhibiting effect of 15d-$PGJ_2$ at a dosage of $1 \times 10^{-6}$ M in mouse myeloma cells, while we observed a significant inhibition at a dosage of at least $1 \times 10^{-5}$ M [21]. Thus, higher concentrations are needed for the inhibition of proliferation in human myeloma cells.

In several studies it was postulated that tumor cells treated with PPAR-γ agonists were killed in an apoptotic fashion even though the precise pathway by which PPAR-γ agonists lead to apoptosis remains unknown so far [22]. In macrophages, the treatment with PPAR-γ agonists inhibited their activation and differentiation, and, furthermore, PPAR-γ agonists inhibited STAT and NF-κB activities, both members of transcription factor families, which are also involved in cell proliferation and cell survival of tumor cells [12]. This may be one of the mechanisms underlying anti-myeloma activity of PPAR-γ agonists, as STAT and NF-κB belong to important cellular pathways involved in cytokine-dependent cell proliferation of multiple myeloma cells [29,30]. Moreover, it should be taken into account that 15d-$PGJ_2$ and thiazolidinediones can inhibit cytokine production in monocytes *in vitro* including IL-6, which is a known paracrine and autocrine growth factor for multiple myeloma [31].

To confirm that proliferation inhibition of human multiple myeloma cells observed in the MTT assay was due to apoptosis, we performed the Annexin-V staining assay. Both 15d-$PGJ_2$ and thiazolidinediones induced apoptosis in myeloma cell lines. Again, 15d-$PGJ_2$ was revealed to be more effective than PGZ and RGZ at similar doses ($5 \times 10^{-5}$ M). Since cell lines contain multiple chromosomal aberrations and may have a higher proliferation rate than human bone marrow myeloma cells, we conducted the Annexin-V assay with freshly isolated multiple myeloma cells from patients and could show that apoptosis occurred in these cells as well. As PPAR-γ agonists induced apoptosis in multiple myeloma cells and these ligands may have a synergistic effect in combination with other agents with anti-myeloma activity, this should be investigated in further studies.

## Conclusion

This paper presents the findings that human multiple myeloma cells express PPAR-γ protein, representing a potential target for anti-cancer treatment. A natural PPAR-γ ligand, 15d-$PGJ_2$, as well as the synthetic ligands, PGZ and RGZ, which are already being used in anti-diabetic treatment, induce apoptosis *in vitro* in human multiple myeloma cells. Therefore, PPAR-γ ligands might contribute to the treatment of multiple myeloma.

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