Cancer Chemother Pharmacol (2005) 56: 400–408
DOI 10.1007/s00280-005-1029-9

ORIGINAL ARTICLE

**Jiajun Liu · Huiling Lu · Renwei Huang
Dongjun Lin · Xiangyuan Wu · Qu Lin · Xinyao Wu
Jing Zheng · Xianglin Pan · Jun Peng · Yuqin Song
Maohong Zhang · Ming Hou · Feng Chen**

Peroxisome proliferator activated receptor-γ ligands induced cell growth inhibition and its influence on matrix metalloproteinase activity in human myeloid leukemia cells

Received: 25 October 2004 / Accepted: 7 February 2005 / Published online: 19 April 2005

Abstract Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is one of the best characterized nuclear hormone receptors (NHRs) in the superfamily of ligand-activated transcriptional factors. PPAR-γ ligands have recently been demonstrated to affect proliferation, differentiation and apoptosis of different cell types. The present study was undertaken to investigate PPAR-γ ligands induced cell growth inhibition and its influence on matrix metalloproteinase MMP-9 and MMP-2 activities on leukemia K562 and HL-60 cells in vitro. The results revealed that PPAR-γ expression was detectable in the two kinds of leukemia cells; Both 15-deoxy-delta(12,14)-prostaglandin J2(15d-PGJ2) and troglitazone (TGZ) have significant growth inhibition effects on these two kinds of leukemia cells. These two PPAR-γ ligands could inhibit the leukemic cell adhesion to the extracellular matrix (ECM) proteins and the invasion through matrigel matrix. The expressions of MMP-9 and MMP-2 as well as their gelatinolytic activities in both HL-60 and K562 cells were inhibited by 15d-PGJ2 and TGZ significantly. We therefore conclude that PPAR-γ ligands 15d-PGJ2 and TGZ have significant growth inhibition effects on myeloid leukemia cells in vitro, and that PPAR-γ ligands can inhibit K562 and HL-60 cell adhesion to and invasion through ECM as well as downregulate MMP-9 and MMP-2 expressions. The data suggest that PPAR-γ ligands may serve as potential anti-leukemia reagents.

Keywords MMP · PPAR · Leukemia · ECM

Abbreviations MMP: Matrix metalloproteinase · PPAR-γ: Peroxisome proliferator activated receptor-γ · 15d-PGJ2: 15-deoxy-delta(12,14)-prostaglandin J2 · TGZ: Troglitazone · ECM: Extracellular matrix

J. Liu (✉) · R. Huang · D. Lin · X. Wu · Q. Lin
Department of Haematology and Oncology,
The Third Affiliated Hospital of Sun Yat-sen University,
Guangdong Guangzhou, 510630, P.R. China
E-mail: liujj@gzsums.edu.cn

H. Lu · X. Wu · J. Zheng
Lab of Forensic and Biological Science,
Medical College of Sun Yat-sen University,
Guangdong Guangzhou, 510080, P.R. China
E-mail: luhai@gzsums.edu.cn

X. Pan · J. Peng · M. Zhang · M. Hou · F. Chen
Department of Hematology and Oncology,
Qilu Hospital of Shandong University,
Shandong Jinan, 250012, P.R. China

Y. Song
Department of Oncology,
Health Science Center of Peiking University,
Beijing, 100083, P.R. China

Introduction

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors (NHRs), initially described as molecular targets for compounds which induce peroxisomal proliferation. In common with other members of the nuclear receptor gene family, the PPARs function as ligand-activated transcription factors forming heterodimer with the retinoid X receptor (RXR). Upon ligand binding, the complex of PPAR and RXR binds to specific recognition sites on DNA, the peroxisome proliferator response elements (PPREs) and regulates transcription of specific genes [1]. To date, three PPAR subtypes exist being the products of distinct genes and commonly designated as PPAR-α, PPAR-β and PPAR-γ. PPAR-γ is one of the best characterized NHRs in the superfamily of ligand-activated transcriptional factors which is predominantly expressed in adipose tis-

Table 1 The PCR primers used in this study

PPAR-γ mRNA (474 bp)
Sense primer: 5′-TCTCTCCGTAATGGAAGACC-3′
Anti-sense primer: 5′-GCATTATGAGACATCCCCAC-3′
β-actin mRNA (243 bp)
Sense primer: 5′-CTTCTACAATGAGCTGCGTG-3′
Anti-sense primer: 5′-TCATGAGGTAGTCAGTCAGG −3′
MMP-9 mRNA (480 bp)
Sense primer: 5′-CAACATCACCTATTGGATCC-3′
Anti-sense primer: 5′-CGGGTGTAGAGTCTCTCGCT-3′
MMP-2 mRNA (474 bp)
Sense primer: 5′-GGCCCTGTCACTCCTGAGAT-3′
Anti-sense primer: 5′-GGCATCCAGGTTATCGGGGA-3′

sue and plays an important regulatory role in lipid and glucose metabolism, adipocyte differentiation, and energy homeostasis [2]. PPAR-γ ligands have recently been demonstrated to affect proliferation, differentiation and apoptosis of different cell types. Both in vitro and in vivo studies suggest the importance of specific PPAR-γ ligands as cell cycle modulators, establishing their antineoplastic properties [3]. Recently a number of studies have demonstrated that PPAR-γ is expressed in a variety of types of cancer cells and has crucial roles in suppressing cell growth [3, 4]. Previous studies have shown that PPAR-γ expression can be detected virtually in a large number of hematologic cells such as in bone marrow erythroid, myeloid, and monocytoid progenitors, some T, and B lymphocytes, monocytes, macrophages, and other monocytic derivatives [5], and some human leukemia and lymphoma cells such as U937 and Raji cells have also been reported to express PPAR-γ [6, 7]. PPAR-γ can be activated not only by a naturally occurring arachidonic acid metabolite 15-deoxy-delta(12,14)-prostaglandin J2(15d-PGJ2), but also by synthetic ligands such as those belonging to the antidiabetic thiazolidinedione (TZD) class of compounds [8]. Later laboratory data have shown that activation of PPAR-γ by either TZDs or 15d-PGJ2 leads to either inhibition of cell growth and apoptosis on most of the human acute lymphocytic leukemia (ALL) cell lines in vitro [9].

Myeloid leukemia is a heterogeneous group of diseases in which the malignant clone arises from the uncontrolled proliferation of myelocytic progenitors in the bone marrow. Acute myeloid leukemia (AML) is the main kind of leukemia happening to adults, though a great deal of AML patients can reach complete remission with current treatment protocols, many individuals eventually relapse and the overall outcome has not improved in recent years [10]. Chronic myeloid leukemia (CML), of which the median duration of "chronic" phase is 3–4 years, is found to have a higher morbidity in eastern countries especially in China than that in western countries, even with a lot of modern treatment protocols, many CML patients have eventually died of the subsequent blast crisis. Therefore, it is a permanent requirement to find new anti-leukemia drugs and effective therapies for the clinical treatment of myeloid leukemia [11].

Matrix metalloproteinases are a family of endopeptidases excreted by a number of cell types, capable of cleaving several macromolecules of the extracellular matrix (ECM). The main role of MMPs in angiogenesis, tumor growth and metastasis is degradation of ECM and release and/or activation of growth factors through their degradative activity, and the degradative activity finally results in cancer progression [12]. MMP-9 and MMP-2 is known to play an important role in angiogenesis, tumor growth and metastasis mainly through their degradation of ECM that may result in tumor cell as well as endothelial cell migration due to loss of cell–matrix contacts and cell–cell contacts [13, 14]. In the past decade, a number of articles have investigated MMP-9 and MMP-2 expressions of myeloid cells. Variable expression levels of MMP-9 and MMP-2 could be detected in conditioned media of myeloid cells, and these data suggest that the variation of MMP-9 and MMP-2 may be one of the important indexes in prognosis of leukemia patients [15, 16]. Therefore, MMPs are now being used as new target molecules in the treatment of a large variety of cancers [17, 18].

Though some of recent data suggest that PPAR-γ ligands may represent a promising, novel therapeutic approach for certain human acute leukemias due to its antiproliferation effects, many of its anti-leukemia mechanisms have not been demonstrated in detail. To date, no detailed data are available about the roles and mechanisms of PPAR-γ ligands on MMPs in myeloid leukemia cells. In order to clarify PPAR-γ ligands induced cell growth inhibition and its influence on some matrix metalloproteinase activity on myeloid leukemia cells in vitro and the possible clinical application of PPAR-γ ligands in the treatment of acute and/or CML, we investigated PPAR-γ ligands 15d-PGJ2 and TGZ induced cell growth inhibition and its influences on MMP-9 and MMP-2 expressions as well as their gelatinolytic activities in human leukemia K562 and HL-60 cell lines.

Materials and methods

Main reagents

PPAR-γ ligands 15-deoxy-delta(12,14)-prostaglandin J2(l5d-PGJ2) and Troglitazone (TGZ), a kind of thiazolidinedione (TZD) were purchased from Cayman chemical company (USA), which were dissolved in DMSO, and stored at −20°C. The anti-PPAR-γ antibody was purchased from Santa Cruz Company (Germany). TRIZOL reagent was from GIBCO (USA), and reverse transcriptional kit was from MBI (USA); The PCR primers were purchased from Shanghai Shenggong Company (China).

Detection of PPAR-γ expression using RT-PCR and western blot analysis

The expression of PPAR-γ was first detected before the cells were treated with TGZ and l5d-PGJ2. The total

RNA was extracted by using TRIZOL reagent according to the procedural instructions described on the kit, which was certified to be suitable for RT-PCR by using agarose gel electrophoresis. First-stranded cDNA was synthesized using 5 μg total RNA by RT-PCR kit. The related PCR primers listed in Table 1 were used to produce the correlated products respectively.

The PCR reactions for PPAR-γ and β-actin cDNAs were performed with 30 amplification cycles and the reaction conditions were: denaturation at 94 for 1 min, annealing at 53 for 2 min, and extension at 72°C for 3 min. The PCR products were run on 1% agarose gel in TAE buffer for 20–30 min respectively and then visualized by ethidium bomide staining.

For Western blotting, cells were washed with ice-cold PBS twice and lysed for 30 min at 4°C, then debris was removed by centrifugation for 15 min at 15,000 *g* at 4°C,and equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose filter. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 h at room temperature. The filters were reacted firstly with anti-PPAR-γ antibody at a dilution of 1:1,000 for 2 h, followed by extensive washes with PBS twice and TBST twice. Filters were then incubated with horseradish peroxidase-conjugated secondary antibodies of 1:1,000 for 1 h, washed with TBST and developed using the Super Signal West Pico Kit. As an internal control, β-actin was detected with anti-β-actin antibodies.

Cell culture and cell viability

U937 as well as K562 (derived from CML patients) and HL-60 (derived from AML patients) cells were kindly provided by central laboratory of Sun Yat-sen University cancer center. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS), 100 U/ml penicillin and l00 μg/ml streptomycin, in a humidified 5% CO_2 incubator at 37°C. All the cells were passaged twice weekly and routinely examined for mycoplasma contamination. Cells in logarithmic growth phase were used for further experiments.

The viability of the cells was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, cells at 1×10^5/ml were treated with various concentrations of PPAR-γ ligands (TGZ: 5, 10, 20, 40, 60, 80 and 100 μmol/l, l5d-PGJ2: 1, 5, 10, 20, 30, 40 and 50 μmol/l) in 96-well plates for 72 h. MTT working solution was prepared as follows: 5 mg MTT/ml PBS was sterile by being filtered with 0.45 μm filter units. After incubation, MTT working solution was added to each well and cells were incubated for 4 h. The water insoluble formazan was formed during incubation and it was solublized by adding solublization agent to each well. Amount of formazan was determined measuring the absorbance at 540 nm using an ELISA plate reader.

[^{3}H]-thymidine uptake

For cell proliferation assay, cells at 1×10^5/ml were treated with various concentrations of TGZ and l5d-PGJ2 in 96-well plates. After 72 h of incubation, 1 μCi [^{3}H]-thymidine (Beijing Atomic Energy Research Institute, Beijing, China)/well was added to the 96-well plates, the plates were incubated for an additional 12 h at 37°C, and the cells were harvested and counted for incorporated radioactivity.

Immunocytochemistry

PPARγ protein expression was also deleted by immunocytochemistry. Briefly, After HL-60 and K562 cells were treated with 80 μmol/l TGZ and 40 μmol/l l5d-PGJ2 for 48 h, cells were harvested and evaluated by immunostaining. The primary antibody was a rabbit polyclonal anti-PPARγ (1:1,000; Santa Cruz, CA, USA), and the secondary antibody an ALEXA-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene).

The variation of MMPs mRNA expression detected by RT-PCR

After HL-60 and K562 cells were treated with 80 μmol/l TGZ and 40 μmol/l l5d-PGJ2 for 48 h, RT-PCR was used to detect the variation of MMP-9 and MMP-2 mRNA expression. The total RNA was extracted by TRIZOL reagent according to the procedure instructions described on the kit. The first-stranded cDNA was synthesized using 5 μg total RNA by RT-PCR kit. The related PCR primers listed in Table 1 were used to produce the correlated products. For PCR reactions, 30 cycles consisted of 94°C for 30 s, 55°C for 50 s, 72°C for 1 min, preceded by denaturation for 5 min at 94°C. After the last cycle, the extension step was at 72°C for 10 min. The above PCR products were then run on 1% agarose gel in TAE buffer for 30–40 min and then visualized by ethidium bomide staining.

Adhesion assay

In vitro adhesion assays were performed to evaluate the effects of 15d-PGJ2 and TGZ on the adhesive properties of leukemic cells. The plates for the adhesion assays were precoated with the ECM proteins laminin, fibronectin, vitronectin, or type IV collagen (each at a final concentration of 1 mg/ml in PBS) overnight at 4°C and dried. To study the effects of 15d-PGJ2 and TGZ on leukemia cell adhesion, exponentially growing cells were incubated with different concentration of 15d-PGJ2 and TGZ for 24 h in a humidified 5% CO_2 atmosphere. The cells were centrifuged, washed twice with serum-free medium, counted, and resuspended in serum-free medium to a final concentration of 5×10^5 cells/ml.

1×10^5 cells were added to each well, and cells were allowed to adhere for 1 h at 37°C in a humidified 5% CO_2 atmosphere. The nonadherent cells were removed by gently washing the cells with PBS, and then the adherent fraction was quantitated using MTT assays as described above. In order to observe PPAR-γ ligands inhibited adhesive property of leukemia cells, we first detected cell adhesion after HL-60 and K562 cells treated with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ, then different concentrations of both 15d-PGJ2 and TGZ caused cell adhesive inhibition were analyzed respectively to observe whether PPAR-γ ligands inhibit adhesive property of leukemia cells in a dose dependent manner.

In vitro invasion assay

The in vitro invasiveness of leukemic cells was assayed using a previously published method that uses Matrigel-coated Costar 24-well transwell cell culture chambers (''Boyden chambers'') with 8.0-mm pore polycarbonate filter inserts. The chamber filters were coated with 50 mg/ml of Matrigel matrix, incubated overnight at room temperature under a laminar flow hood, and stored at 4°C. To study the effects of PPAR-γ ligands on the invasiveness of HL-60 and K562 cells, exponentially growing cells were incubated with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ in 0.1% DMSO overnight. The cells were washed twice with serum-free RPMI 1640 containing 0.1% BSA, counted, and resuspended at 1×10^5 cells/ml. An 0.5-ml cell suspension containing 1×10^5 cells in a serum-free RPMI 1640 containing PPAR-γ ligands or vehicle was added to the Matrigel-coated and rehydrated filter inserts. Next, 750 ml of NIH fibroblast-conditioned medium was placed as a chemoattractant in 24-well plates, and the inserts were placed in wells and incubated at 37°C for 48 h. After the incubation period, the invasive cells that migrated into the lower chamber were counted under a light microscope. The invasive fractions of cells treated with PPAR-γ ligands were compared with those of DMSO (0.1%)-treated control cells, and the percentage inhibition of invasiveness was determined.

Zymographic analysis

After treatment with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 48 h, the cell-conditioned media (supernatants) were collected, centrifuged to remove debris, and analyzed by zymography. Serum-free media conditioned by U937 cells, which secrete MMP-9 and MMP-2, was used as a positive control for zymographic analysis. The experiments were performed on 8.5% polyacrylamide gel copolymerized with 2 mg/ml gelatin type I. Fifteen microliters of samples mixed with 5 μm of loading buffer were run under non-reducing conditions without prior boiling. After electrophoresis, gels were washed three times in 2.5% Triton X-100 to remove SDS and allowed proteins to renature and then immersed in buffer contained 50 mmol/l Tris pH 7.5, 5 mmol/l $CaCl_2$, 1 μmol/l $ZnCl_2$ and 0.01% NaN_3 for 24 h at room temperature. The gels were stained with 0.4% (w/v) Coomassie Blue and destained in 35% ethanol/10% acetic acid. Clear zones of gelatin lysis against a blue background stain indicated enzyme activity. Enzyme activity determined by zymographic analysis actually represents the total amount of secreted gelatinolytic protein. Protein markers were prestained to determine the molecular weights of gelatinases. Images of gels were acquired with a digital Kodak camera (Eastman Kodak Company, USA) and the intensity of the bands in zymography was quantified by imaging analysis software from Kodak.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed with a Student's t-test using SAS 6.12 software. Statistical significance was accepted at the level of $P < 0.05$.

Results

PPAR-γ expression in the two cell lines

In the two myeloidleukemia cell lines, PPAR-γ expressions were detected by RT-PCR and Western blot. As shown in Fig. 1a, the fragment of PPAR-γ mRNA was 474 bp in length, and as an internal control, β-actin mRNA was detected in its length of 243 bp (Fig. 1b). Likely, as shown in Fig. 1c, PPAR-γ protein expressions were observed very clearly in the two kinds of cells using western blot analysis (molecular mass of PPAR-γ protein was about 55 kD).

Cell viability

To investigate the growth inhibition effects of PPAR-γ ligands on K562 and HL-60 cells, the cells were treated with various concentrations of TGZ and 15d-PGJ2 for 72 h. As shown in Fig. 2, both TGZ and 15d-PGJ2 had significant growth inhibition effects on the two kinds of cells in a dose-dependent manner. The two kinds of leukemia cells showed different sensitivity to TGZ and 15d-PGJ2 measured by the MTT test; both K562 and HL-60 cells were more sensitive to 15d-PGJ2 than to TGZ.

PPAR-γ activation inhibits DNA synthesis of leukemia cells

In order to observe the anti-proliferation effects caused by PPAR-γ ligands, we tested HL-60 and K562 cells for

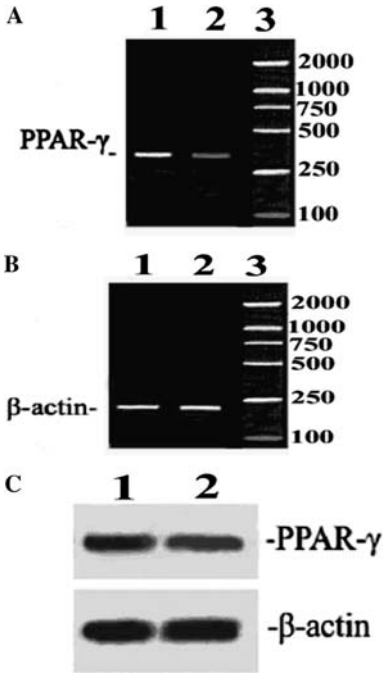

Fig. 1 PPAR-γ expression in HL-60 and K562 cells detected by RT-PCR and Western blot. Total RNA was isolated and RT-PCR was performed for PPAR-γ (**a**) and β-actin (**b**) as a loading control. Equal amounts of total cell lysate were loaded and a Western blot for PPAR-γ was performed (**c**). *Lane 1*: HL-60 cell. *Lane 2*: K562 cell. *Lane 3*: 2,000 bp DNA marker

their [^{3}H]-thymidine uptakes after the cells were treated with different concentrations of TGZ and 15d-PGJ2 for 72 h. As shown in Fig. 3, [^{3}H]-thymidine uptake in these two cell lines was inhibited remarkably by both TGZ

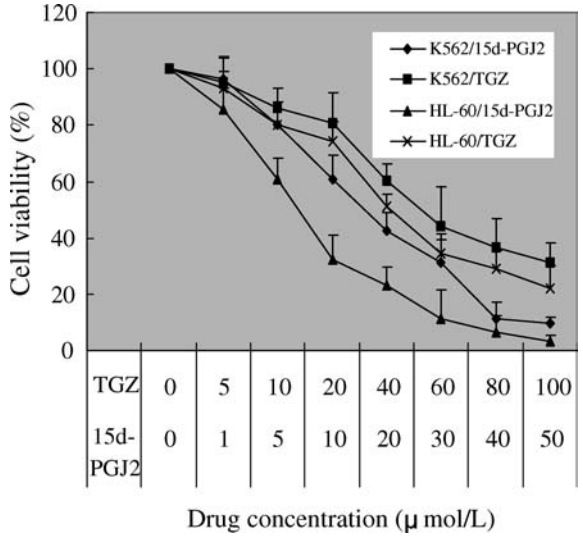

Fig. 2 Cell viability caused by TGZ and 15d-PGJ2. K562 and HL-60 cells were plated on 96 well plates and on the second day, cells were treated with different concentrations of TGZ and 15d-PGJ2 for 72 h. Experiments were done in triplicate. The cell viability was then determined by MTT assay, as described in the"Methods". Values represent mean (±SD) cell viability as a percentage of untreated control samples

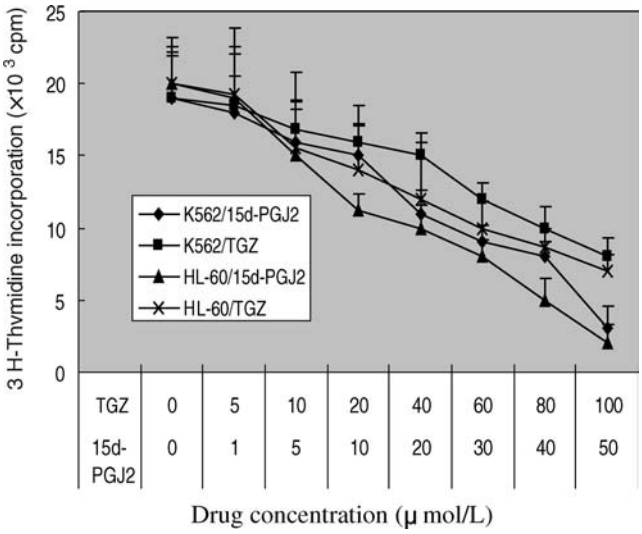

Fig. 3 [^{3}H]-thymidine uptake caused by TGZ and 15d-PGJ2. After HL-60 and K562 cells were treated with different concentrations of TGZ and 15d-PGJ2 for 72 h, their [^{3}H]-thymidine uptakes were measured as described in the"Methods". Results are expressed as the mean c.p.m of triplicate samples. Data shown are representative of three separate experiments

and 15d-PGJ2 in a dose-dependent manner. The data showed that 15d-PGJ2-caused [^{3}H]-thymidine uptake inhibition in both HL-60 and K562 cells was more obviously than that of TGZ. These results suggest that PPAR-γ ligands exert a profound inhibitory effect on the proliferation of HL-60 and K562 cells.

Cell immunostaining

Immunocytochemistry showed that PPAR-γ protein was expressed in the two cell lines. By fluorescence microscopic analysis, immunoreactive PPAR-γ protein (green) in untreated cells was localized predominantly in the cell nuclear, and only less amount of PPAR-γ protein was found in the perinuclear region and the cytoplasm (Fig. 4a). The subcellular distribution of PPAR-γ protein was found after HL-60 and K562 cells were treated with 80 μmol/l TGZ (Fig. 4b) and 40 μmol/l l5d-PGJ2 (Fig. 4c) for 48 h. PPAR-γ protein was observed clearly in the cytoplasm in both HL-60 (Fig. 4b1) and K562 (Fig. 4b2) cells after treatment by TGZ. After treatment by l5d-PGJ2, PPAR-γ protein of both HL-60 (Fig. 4c1) and K562 (Fig. 4c2) cells was decreased remarkably along with the enhancement of the dead cells. The data indicated that it is possible for treatment conditions to affect the subcellular distribution and transportation of PPAR-γ.

mRNA expressions of MMP-9 and MMP-2

As shown in Fig. 5, after treatment with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 48 h, the mRNA expres-

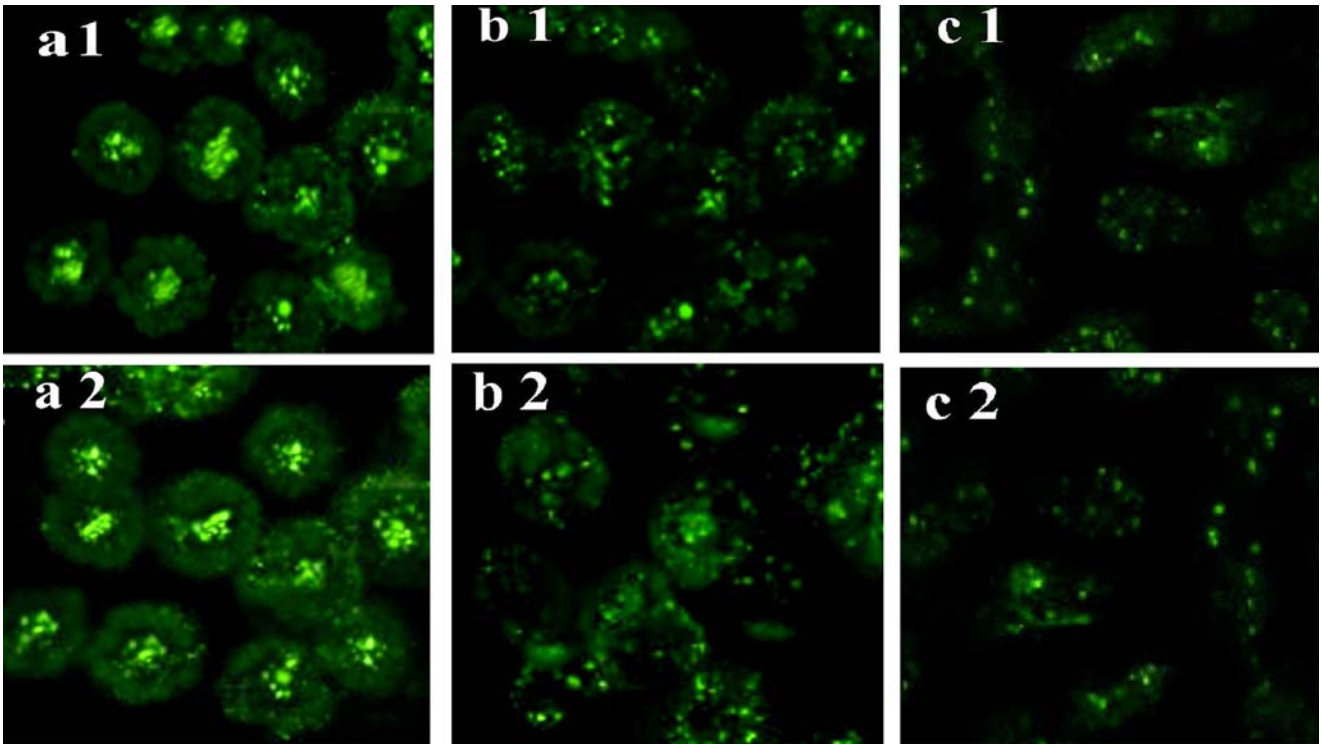

Fig. 4 Immunostaining of PPAR-γ protein. After HL-60 and K562 cells were treated with 80 μmol/l TGZ and 40 μmol/l 15d-PGJ2 for 48 h, immunocytochemistry was used to observe the distribution of PPAR-γ protein as described in the"Methods". Images were captured at ×400 magnification. 1: HL-60 cell. 2: K562 cell. **a** PPAR-γ protein (green) in untreated cells was localized predominantly in the cell nuclear. **b** PPAR-γ protein was observed clearly in the cytoplasm in both HL-60 and K562 cells after treatment by TGZ. **c** After treatment by 15d-PGJ2, PPAR-γ protein of both HL-60 and K562 cells was decreased remarkably along with the enhancement of the dead cells

sions of MMP-9 and MMP-2 in both HL-60 and K562 cells were down-regulated significantly. It was also observed that 15d-PGJ2 induced downregulation of MMP-9 and MMP-2 in both HL-60 and K562 cells was much more obviously than that of TGZ

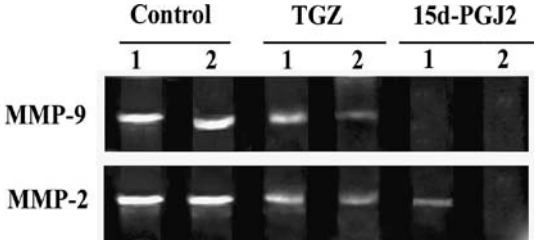

Fig. 5 mRNA expression of MMP-9 and MMP-2. After treatment with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 48 h, the mRNA expressions of MMP-9 and MMP-2 in both HL-60 and K562 cells were down-regulated significantly especially after treatment by15d-PGJ2. *Lane 1*: HL-60 cell *Lane 2*: K562 cell

PPAR-γ ligands inhibit leukemic cell adhesion and invasion

Expansion of leukemic cell populations residing within the bone marrow microenvironment involves adhesion of leukemic cells to bone marrow ECM proteins via cell surface integrin receptors and migration into the surrounding tissues. These ECM proteins to which tumor cells initially attach include laminin, fibronectin, type IV collagen, and vitronectin. Laminin, fibronectin, vitronectin, and collagen have been found in the basal lamina that promote the adhesion and invasion of tumor cells. To determine whether PPAR-γ ligands affects the integrin-mediated leukemic cell adhesion to ECM, HL-60 and K562 cells were incubated with 15d-PGJ2 and TGZ for 24 h, and then the integrin-mediated cell adhesion was examined. As shown in Fig. 6, pretreatment of HL-60 and K562 cells with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ inhibited their adhesion to laminin-, fibronectin- collagen-, and vitronectin-coated plates. Furthermore, both 15d-PGJ2 and TGZ could inhibit leukemic cell adhesion in a dose dependent manner (Figs. 7, 8). Matrigel matrix-coated Boyden chambers were used to examine the ability of PPAR-γ ligands to inhibit the invasiveness of HL-60 and K562 cells. The cells were treated with PPAR-γ ligands for 24 h and then placed in Matrigel matrix-coated Boyden chambers and allowed to invade for 48 h. We observed that pretreatment with different concentrations of PPAR-γ ligands inhibited the invasiveness of K562 and HL-60 cells in a concentration-dependent manner (Fig. 9).

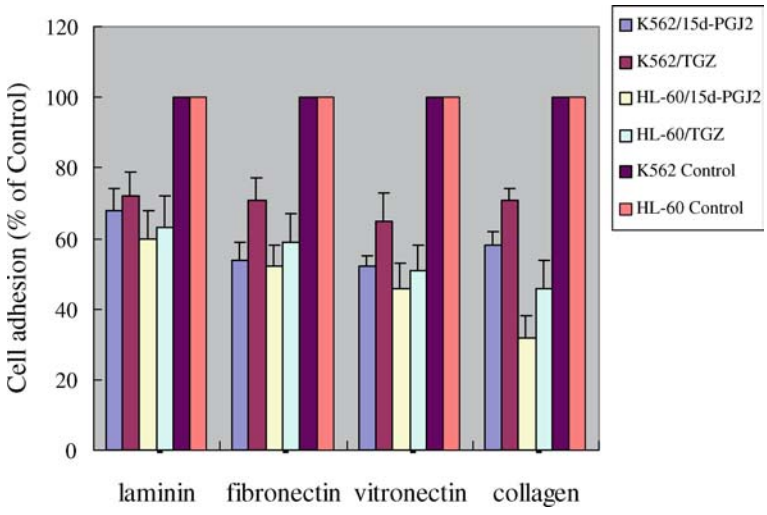

Fig. 6 PPAR-γ ligands inhibited adhesive property of leukemia cells. HL-60 and K562 cells were incubated with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 24 h, and then processed for adhesion assays using laminin-, fibronectin-, collagen type IV, or vitronectin-coated 96-well plates as described in "Materials and Methods"

Zymographic analysis

Gelatin zymography analysis revealed that MMP-9 and MMP-2 were secreted into serum-free media by HL-60 and K562 cells. As shown in Fig. 10, after treatment with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 48 h, the activities of these two MMPs in both HL-60 and K562 cells were significantly inhibited by 15d-PGJ2, while there was less considerable variation in TGZ treated cells.

Discussion

Recently, a number of laboratory studies have shown that PPAR-γ is actually expressed in many kinds of tumor cells, including lung cancer [19], breast cancer [20], prostate cancer [21], gastric cancer [22] and so on. As a highly effective, highly selective, nontoxic new therapy target for human cancers, PPAR-γ ligands have been used and found to have remarkable growth inhibition effects on PPAR-γ expressing cancer cells [3, 4, 7, 19, 20, 21, 22]. Previous studies have demonstrated that PPAR-γ are also expressed in many leukemia cells and activation of PPAR-γ by either TZDs or 15d-PGJ2 leads to inhibition of cell growth on some leukemia cell lines in vitro [10, 23, 24]. These data suggest that PPARγ ligands may represent a promising, novel therapeutic approach for certain human leukemia.

Recent studies have revealed that PPAR-γ ligands such as 15d-PGJ2 and TGZ have significant inhibition effects on tumor angiogenesis and may serve as potent anti-angiogenesis reagent both in vitro and in vivo [25]. VEGF is now proved to be the most powerful angiogenic factor in human cancer and appears to be essential for the growth of cancer cells and their metastasis. Therefore, it is a permanent requirement to find novel VEGF inhibitors for the treatment of cancer [26], and

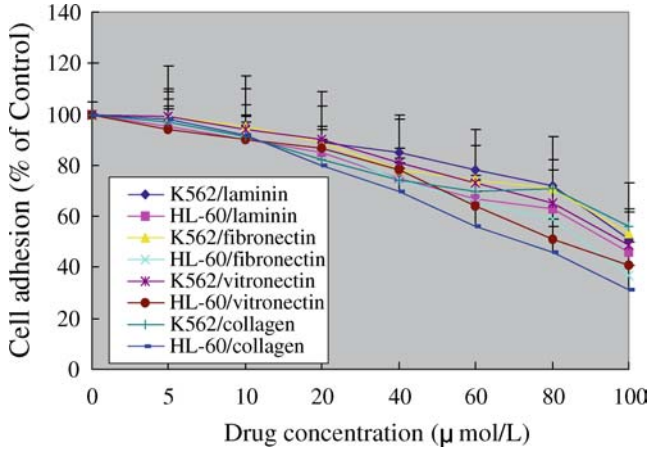

Fig. 7 TGZ inhibited leukemic cell adhesion. Cells were treated with different concentrations of TGZ for 24 h. The data showed that TGZ could inhibit cell adhesion in a dose-dependent manner

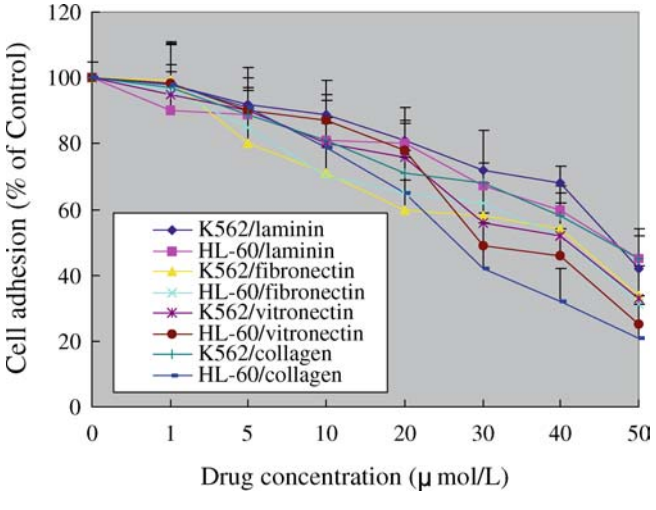

Fig. 8 15d-PGJ2 inhibited leukemic cell adhesion. Cells were treated with different concentrations of 15d-PGJ2 for 24 h. A dose-dependent manner was found remarkably in 15d-PGJ2 caused cell adhesive inhibition

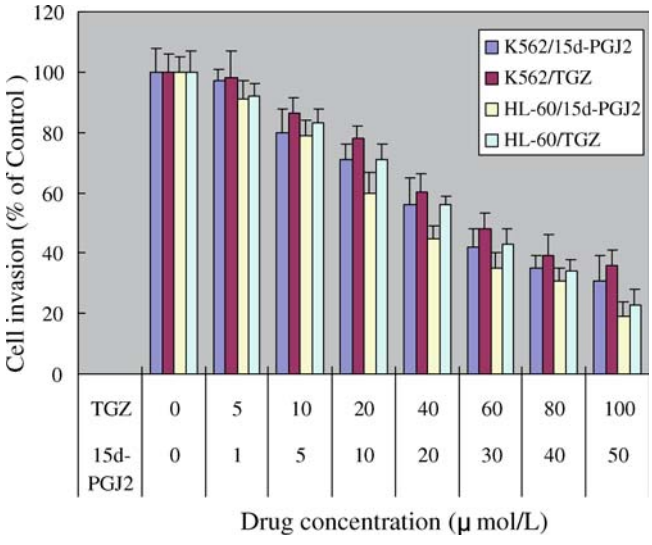

Fig. 9 PPAR-γ ligands inhibited invasive property of leukemic cells. HL-60 and K562 cells were incubated with various concentrations of 15d-PGJ2 and TGZ for 24 h and then processed for invasion assays using Matrigel matrix-coated Boyden chambers as described in "Materials and Methods". Both 15d-PGJ2 and TGZ inhibited the invasiveness of K562 and HL-60 cells in a dose-dependent manner

PPAR-γ ligands are known to have potent inhibition effects on VEGF-induced angiogenesis [25]. New data have shown that VEGF may trigger growth, survival, and migration of leukemia cells. Dysregulation of VEGF expression and signaling pathways therefore plays an important role in the pathogenesis and clinical features of haematological malignancies [27].

Later studies suggest that PPAR gamma ligations such as 15dPGJ2 alone or in combination with retinoids have significant anti-proliferations effects on leukemia cell lines as well as primary leukemia samples [28]. Another studies demonstrate that 15dPGJ2 and its precursors such as PGD2, PGJ2 and Delta12-PGJ2 can stimulate the proliferation on leukemia cell line U937 at lower concentrations, whereas they have shown anti-proliferative effects at high concentrations [29]. In this study, we found that the PPAR-γ ligands 15d-PGJ2 and TGZ could induce cell growth inhibition on myeloid leukemia K562 and HL-60 cells in a dose-dependent manner, and no proliferation effects were found throughout the culture in the two cell lines. RT-PCR revealed that mRNA expression of MMP-9 in both HL-60 and K562 cells was down-regulated significantly, and it was also found that the downregulation of MMP-9 in 15d-PGJ2 treated cells was much more obviously than that of TGZ treated cells. The pretreatment of HL-60 and K562 cells with PPAR-γ ligands inhibited their adhesion to laminin-, fibronectin- collagen, and vitronectin-coated plates. Furthermore, PPAR-γ ligands could significantly inhibit the invasiveness of K562 and HL-60 cells in a concentration-dependent manner. Zymographic analysis indicated that the activity of MMP-9 and MMP-2 in both HL-60 and K562 cells was significantly inhibited by 15d-PGJ2, while there was less considerable variation in TGZ treated cells.

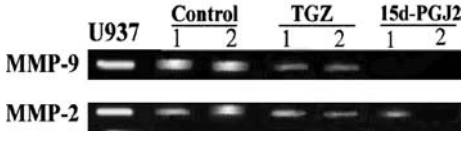

Fig. 10 Zymographic analysis. Zymography was performed using 10% polyacrylamide gels containing 2 mg/ml gelatin. Media conditioned by U937 cells was used as standard showing the position of MMP-9 (92 kDa) and MMP-2 (72 kDa) activities. After treatment with 40 μmol/l 15d-PGJ2 and 80 μmol/l TGZ for 48 h, the activities of MMP-9 and MMP-2 in both HL-60 and K562 cells were significantly inhibited by 15d-PGJ2, while there was less significant variation of TGZ treated cells. *Lane 1*: HL-60 cell *Lane 2*: K562 cell

Among the MMPs, MMP-9 and MMP-2 are the most studied in the processes of cancer, including the process of metastasis [11]. Though the possible roles for MMPs in leukemia cells have been hypothesized due to the important role of MMP-2 and MMP-9 in tumor angiogenesis, to date, the precise role of MMP-expression in acute leukemia is still not very clear. The role of MMP-9 and MMP-2 in acute leukemia is most likely dependent on its degradative capacity by facilitating endothelial cell migration in vitro [12, 13]. Recent laboratory data have shown that in acute leukemia, increased vessel density is found at diagnosis, while disappearing when complete remission is achieved [30, 31]. MMP-2 expression has been shown to play a possible role in the increased vessel density found in bone marrow of AML-patients at diagnosis by facilitating in vitro endothelial cell migration [32]. Some articles have suggested that MMP-9 expression might be involved in physiological processes such as the differentiation process in hematopoiesis [33]. Many studies show a reflection of MMP-2 and MMP-9 with invasive behavior in AML cell lines, and different levels of MMP-2 and MMP-9 expressions are reported in these cells [34–36]. Our results agree with the previous studies that the expressions of both MMP-9 and MMP-2 were detectable in HL-60 and K562 cells [37]. In the present study, we found that the activities of both MMP-9 and MMP-2 were inhibited by PPAR-γ ligands. Furthermore, The downregulation of MMP-9 and MMP-2 as well as their activity in 15d-PGJ2 treated cells was much more considerable than that of TGZ treated cells. To our knowledge, this is the first report about the roles of PPAR-γ ligands on MMPs in myeloid leukemia cells.

Taken together, the results of this study demonstrate that PPAR-γ ligands have significant antiproliferation effects on myeloid leukemia cells in vitro, and that PPAR-γ ligands can inhibit K562 and HL-60 cell adhesion to and invasion through ECM as well as downregulate MMP-9 and MMP-2 expressions. The data suggest that PPAR-γ ligands may serve as potential antileukemia reagents, and in vivo anti-leukemia effects of PPAR-γ ligands as well as its potential clinical effectiveness needs further and profound investigation.

Acknowledgements We thank professor Jiang WQ for presenting microculture tetrazolium. We also thank the members of our laboratories for their insight and technical support. This work is supported by the grants from National Natural Science Foundation of China (No. 30471933).

References

1. Theocharis S, Margeli A, Vielh P, Kouraklis G (2004) Peroxisome proliferator-activated receptor-gamma ligands as cell-cycle modulators. Cancer Treat Rev 30:545
2. Evans RM, Barish GD, Wang YX (2004) PPARs and the complex journey to obesity. Nat Med 10:355
3. Panigrahy D, Shen LQ, Kieran MW, Kaipainen A (2003) Therapeutic potential of thiazolidinediones as anticancer agents. Expert Opin Investig Drugs 12:1925
4. Theocharis S, Margeli A, Kouraklis G (2003) Peroxisome proliferator activated receptor-gamma ligands as potent antineoplastic agents. Curr Med Chem Anti-Canc Agents 3:239
5. Greene ME, Pitts J, McCarville MA, Wang XS, Newport JA, Edelstein C, Lee F, Ghosh S, Chu S (2000) PPARγ: observations in the hematopoietic system. Prostaglandins Other Lipid Mediat 62:45
6. Asou H, Verbeek W, Williamson E (1999) Growth inhibition of myeloid leukemia cells by troglitazone, a ligand for peroxisome proliferator activated receptor, and retinoids. Int J Oncol 15:1027
7. Padilla J, Leung E, Phipps RP (2002) Human B lymphocytes and B lymphomas express PPARγ and are killed by PPARγ ligands. Clin Immunol 103:22
8. Zhang X, Young HA (2002) PPAR and immune system—what do we know? Int Immunopharmacol 2:1029
9. Berger J, Moller DE (2002) The mechanisms of action of PPARS. Annu Rev Med 53:409
10. Schiffer CA (2001) Acute myeloid leukemia in adults: where do we go from here? Cancer Chemother Pharmacol 48(Suppl 1):S45
11. Sievers EL (2000) Targeted therapy of acute myeloid leukemia with monoclonal antibodies and immunoconjugates. Cancer Chemother Pharmacol 46(Suppl):S18
12. Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463
13. John A, Tuszynski G (2001) The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathol Oncol Res 7:14
14. Stetler-Stevenson WG (1999) Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest 103:1237
15. Kuittinen O, Savolainen ER, Koistinen P, Turpeenniemi-Hujanen T (1999) Gelatinase A and B (MMP-2, MMP-9) in leukaemia MMP-2 may indicate a good prognosis in AML. Anticancer Res 19:4395
16. Ismair MG, Ries C, Lottspeich F (1998) Autocrine regulation of matrix metalloproteinase-9 gene expression and secretion by tumor necrosis factor-alpha (TNF-alpha) in NB4 leukemic cells: specific involvement of TNF receptor type 1. Leukemia 12:1136
17. Zhang XM, Huang SP, Xu Q (2004) Quercetin inhibits the invasion of murine melanoma B16-BL6 cells by decreasing pro-MMP-9 via the PKC pathway. Cancer Chemother Pharmacol 53:82
18. Heath EI, O'Reilly S, Humphrey R, Sundaresan P, Donehower RC, Sartorius S, Kennedy MJ, Armstrong DK, Carducci MA, Sorensen JM, Kumor K, Kennedy S, Grochow LB (2001) Phase I trial of the matrix metalloproteinase inhibitor BAY12-9566 in patients with advanced solid tumors. Cancer Chemother Pharmacol 48:269
19. Keshamouni VG, Reddy RC, Arenberg DA, Joel B, Thannickal VJ, Kalemkerian GP (2004) Peroxisome proliferator-activated receptor-gamma activation inhibits tumor progression in non-small-cell lung cancer. Oncogene 23:1000
20. Saez E, Rosenfeld J, Livolsi A, Olson P, Lombardo E, Nelson M (2004) PPAR gamma signaling exacerbates mammary gland tumor development. Genes Dev 18:528–540
21. Pham H, Banerjee T, Nalbandian GM, Ziboh VA (2003) Activation of peroxisome proliferator-activated receptor (PPAR)-gamma by 15S-hydroxyeicosatrienoic acid parallels growth suppression of androgen-dependent prostatic adenocarcinoma cells. Cancer Lett 189:17
22. Sato H, Ishihara S, Kawashima K, Moriyama N, Suetsugu H, Kazumori H (2000) Expression of peroxisome proliferator-activated receptor (PPAR)gamma in gastric cancer and inhibitory effects of PPARgamma ligands. Br J Cancer 83:1394
23. Abe A, Kiriyama Y, Hirano M, Miura T, Kamiya H, Harashima H (2002) Troglitazone suppresses cell growth of KU812 cells independently of PPARgamma. Eur J Pharmacol 436:7
24. Yamakawa-Karakida N, Sugita K, Inukai T, Goi K, Nakamura M, Uno K (2002) Ligand activation of peroxisome proliferator-activated receptor gamma induces apoptosis of leukemia cells by down-regulating the c-myc gene expression via blockade of the Tcf-4 activity. Cell Death Differ 9:513
25. Margeli1 A, Kouraklis G, Theocharis S (2003) Peroxisome proliferator activated receptor-c (PPAR-c) ligands and angiogenesis. Angiogenesis 6:165
26. Ueda Y, Yamagishi T, Samata K, Ikeya H, Hirayama N, Okazaki T, Nishihara S, Arai K, Yamaguchi S, Shibuya M, Nakaike S, Tanaka M (2004) A novel low molecular weight VEGF receptor-binding antagonist, VGA1102, inhibits the function of VEGF and in vivo tumor growth. Cancer Chemother Pharmacol 54:16
27. Frankel AE, Gill PS (2004) VEGF and myeloid leukemias. Leuk Res 28:675
28. Konopleva M, Elstner E, McQueen TJ, Tsao T, Sudarikov A, Hu W, Schober WD, Wang RY, Chism D, Kornblau SM, Younes A, Collins SJ, Koeffler HP, Andreeff M (2004) Peroxisome proliferator-activated receptor gamma and retinoid X receptor ligands are potent inducers of differentiation and apoptosis in leukemias. Mol Cancer Ther 3:1249
29. Azuma Y, Watanabe K, Date M, Daito M, Ohura K (2004) Induction of proliferation by 15-deoxy-delta12,14-prostaglandin J2 and the precursors in monocytic leukemia U937. Pharmacology 71:181
30. Moehler TM, Ho AD, Goldschmidt H, Barlogie B (2003) Angiogenesis in hematologic malignancies. Crit Rev Oncol Hematol 45:227
31. Padró T, Berdel WE, Büchner T, Mesters RM (2000) Angiogenesis and anemia in acute myeloid leukemia. Blood 96:3656
32. de Bont ESJM, Rosati S, Jacobs S, Kamps WA, Vellenga E (2001) Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. Br J Haematol 113:296
33. Devy L, Hollender P, Munaut C (2002) Matrix and serine protease expression during leukemic cell differentiation induced by aclacinomycin and all-trans-retinoic acid. Biochem Pharmacol 63:179
34. Zhang B, Wu K-F, Cao Z-Y, Rao Q, Ma X-T, Zheng G-G, Li G (2004) IL-18 increases invasiveness of HL-60 myeloid leukemia cells: up-regulation of matrix metalloproteinases-9 (MMP-9) expression. Leuk Res 28:91
35. Narla RK, Dong Y, Klis D, Uckun FM (2001) Bis(4,7-dimethyl-1,10-phenanthroline) Sulfatooxovanadium(IV) as a novel antileukemic agent with matrix metalloproteinase inhibitory activity. Clin Cancer Res 7:1094
36. Hayashibara T, Yamada Y, Onimaru Y, Tsutsumi C, Nakayama S, Mori N (2002) Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukaemia cell invasion. Br J Haematol 116:94
37. Lynch CC, McDonnell S (2000) The role of matrilysin (MMP-7) in leukaemia cell invasion. Clin Exp Metastasis 18:401