LIU et al. TZD18 ON Ph⁺-ALL CELLS

NEOPLASIA

Growth inhibition and apoptosis in human Philadelphia chromosome positive lymphoblastic leukemia cell lines by treatment with the dual PPARα/γ ligand TZD18

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Abstract

Treatment of adult Philadelphia chromosome-positive lymphocytic leukemia is rarely successful. We reported here the effects of TZD18 (MERCK, NJ, USA), a novel dual ligand specific for peroxisome proliferator-activated receptor $\alpha$ and $\gamma$ (PPAR$\alpha$/$\gamma$), on Ph$^+$-lymphocytic leukemia cell lines BV173, SD1 and SupB-15. Exposure of these cells to TZD18 resulted in growth inhibition in a dose- and time-dependent manner which was associated with a G1 cell cycle arrest. This effect was much stronger than that mediated by the PPAR$\gamma$ ligand Pioglitazone (PGZ) which belongs also to the Thiazolidinediones (TZD) class of ligands. However, this effect may not be mediated through PPAR$\gamma$ and PPAR$\alpha$ activation, since antagonists of PPAR$\gamma$ and/or PPAR$\alpha$ could not reverse this effect. Study of the key regulators of cell cycle progression by Western blot showed that the expression of the cyclin dependent kinase inhibitor (CDKI) p27$^{kip1}$, but not that of p21$^{cip1}$, was enhanced, whereas the expression of c-Myc, cyclin E, cyclin D2, cyclin dependent kinase 2 and 4 (CDK-2, and CDK-4) were decreased when these cells were treated with TZD18 (10 or 20 $\mu$M). Therefore, upregulation of p27$^{kip1}$ and downregulation of cyclin Ds, CDK-2 and 4 may, at least in part, account for the G1 cell cycle arrest. Furthermore, a remarkable induction of apoptosis was observed in the cells treated with this dual ligand. No obvious alteration of bcl-2 protein level occurred, but bax was upregulated in these TZD18-treated cells. An activation of both caspase-8 and -9 by TZD18 was also noticed. Importantly, NF-$\kappa$B DNA binding activity was markedly decreased by the TZD18 treatment. In addition, TZD18 enhanced the growth inhibitory effect of Imatinib, a specific tyrosine kinase inhibitor therapeutically used in the treatment of Ph$^+$-leukemia. Overall, our findings strongly suggest that TZD18 may offer a new therapeutic approach to aid in the treatment of Ph$^+$-lymphocytic leukemia.
**Abbreviations**: ALL, acute lymphocytic leukemia; CDKs, cyclin dependent kinases; CDKIs, cyclin dependent kinase inhibitors; CI, combination index; EMSA, electrophoretic mobility shift assay; 15d-PGJ$_2$, 15-deoxy-delta (12,14)-prostaglandin J$_2$; OD, optical density; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; PGZ, Pioglitazone; Ph$,+$, Philadelphia chromosome-positive; PPAR$\gamma$, peroxisome proliferator-activated receptor gamma; TUNEL, terminal deoxynucleotidyl-transferase-mediated UTP nick end labelling; TZD, Thiazolidinedione; Z-VAD-FMK, Benzylocarbonylvalylalanylaspartyl fluoromethylketone.
Introduction

Although significant progress has been made in the treatment of acute lymphocytic leukemia (ALL), Philadelphia chromosome positive (Ph+)/Bcr-Abl+ adult ALL still has a very poor prognosis. Allogeneic stem-cell transplantation is considered as the treatment of choice in this group of patients after their first remission. However, most patients are not eligible for this therapy because of advanced age or lack of a suitable stem-cell donor (reviewed in 1,2). Imatinib (Gleevec, previous known as STI571 and CGP57148), the selective tyrosine kinase inhibitor, displayed pronounced anti-leukemic activity in Ph+-CML and –ALL. It is used in further treatment after allogeneic stem-cell transplantation, and also recommended in the relapsed or refractory Ph+-ALL as salvage therapy to facilitate subsequent transplantation 3. However, quick emergence of resistance to this agent is a major problem in the treatment of Ph+ leukaemia. Another major obstacle to Imatinib-based therapies is the persistence of Ph+-cells despite of application of Imatinib. Based on these arguments, development of novel therapeutic agents for human ALL, especially for Ph+-ALL is necessary.

Peroxisome proliferator-activated receptors (PPARs) belong to the family of nuclear hormone receptors that include receptors for estrogen, thyroid hormone, retinoic acid, 1,25- dihydroxy vitamin D3 as well as retinoid X. To date, three subtypes of PPARs (α, β/δ and γ) have been identified which exhibit distinct tissue distribution and are associated with selective ligands. PPARγ can be activated by synthetic ligands such as those belonging to the antidiabetic thiazolidinedione (TZD) class of compounds 4,5. A naturally occurring arachidonic acid metabolite 15-deoxy-delta (12,14)-prostaglandin J2 (15d-PGJ2) 6 and certain non-steroidal anti-inflammatory drugs 5 are also identified as its ligands.

PPARγ was initially noted to be highly expressed in adipose tissue and was found to have a key function in adipocyte differentiation, insulin sensitization and lipid metabolism 7,8. We and other groups have demonstrated that PPARγ is also widely expressed in a variety of types of tumor cells and has crucial roles in suppressing cell growth and/or invasion, promoting differentiation and/or apoptosis 9-13. Interestingly, Braissant et al. found that the expression of this receptor in immune system was just
as high as its expression in the adipose tissue. We demonstrated that PPAR\(\gamma\) was expressed in ALL cells and its ligands, e.g. the synthetic TZD-class ligand Pioglitazone (PGZ) and 15d-PGJ\(_2\), also potently inhibited growth and induced apoptosis of human ALL cells, including Ph\(^+\)-ALL cells.

PPAR\(\alpha\) is another subtype of the PPAR family. The important physiological role of PPAR\(\alpha\) is to modulate lipid metabolism such as lowering the serum triglycerides and raising high-density lipoprotein cholesterol through increased clearance and decreased synthesis of triglyceride-rich very low-density lipoprotein. Fibrates are PPAR\(\alpha\) synthetic ligands, which have been used clinically for several metabolic syndromes such as dyslipidemia. This class of drugs inhibited the IL-1 stimulated release of IL-6 and inflammatory prostaglandins in vascular smooth muscle cells possibly through negative regulation of NF-κB and AP-1, thus contributing to the treatment of coronary diseases.

The role of PPAR\(\alpha\) in tumor cell growth is unclear. PPAR\(\alpha\) ligand fibrates could lead to peroxisome proliferation in the liver of rodents which could ultimately result in the hepatocellular carcinoma. However, some evidence also suggests that fibrates can inhibit hepatoma cells growth. Furthermore, fibrates caused growth arrest by regulating cell cycle related factors, and induced monocytic differentiation of HL-60 cells. These data indicated that this subtype of the PPAR family could also possess anti-cancer activities.

TZD18 (Fig 1A) is one of a series of compounds synthesized by Merck (USA) which can specifically bind and activate both PPAR\(\alpha\) and PPAR\(\gamma\). In addition to its specificity to both of these receptors, this compound has superior pharmacokinetic parameters than other members of this series: a high bioavailability, high dose normalized AUC and relative low clearance. It exhibited a favorable effects on lipid homeostasis.

Based on the previously observed anti-leukemic activity of both PPAR\(\alpha\) and PPAR\(\gamma\) ligands in human leukemia cell lines, we hypothesized that a dual ligand specific for these two subtypes of PPAR might be even more effective than ligands for either PPAR\(\gamma\) or PPAR\(\alpha\) alone. Therefore, we investigated the antiproliferative and
proapoptotic activity of TZD18 against human Ph+ -lymphocytic leukemia cells in anticipation that it may have potential as a therapeutic agent for this poorly responsive disease.
Materials and Methods

Compounds, reagents and plasmids: TZD18 was kindly provided by Merck (USA) and was dissolved in dimethyl sulfoxide (DMSO) at $10^{-2}$ M as a stock solution. Imatinib was kindly provided by Novartis (Basel, Switzerland) and dissolved at $10^{-3}$ M in DMSO. PPARγ antagonist GW9662 was purchased from GlaxoSmithKline (Hertfordshire, UK). The PPARα ligand WY14,643 and antagonist MK886 were from Alexis Biochemicals (Berlin, Germany). The pan-caspase inhibitor benzylcarbonylvalylalanylaspartyl fluoromethylketone (Z-VAD-FMK) was obtained from R&D (Wiesbaden-Nordenstadt, Germany). All stock solutions were stored at –70°C and were further diluted to appropriate concentrations with medium before use.

The luciferase reporter vector PPREx3-tk-luc with 3x human PPRE consensus sequence was kindly provided by R. M. Evans, the Salk Institute for Biological Studies, USA; the full-length human PPARγ1 expression plasmid pcDNAFlag-PPARγ1 by Prof. K. K. Chatterjee, Department of Medicine, University of Cambridge, UK and the PPARα expression vector pCMX-mPPARα by Dr. P. Tontonoz, Howard Hughes Medical Institute, University of California, USA. The Renilla luciferase reporter vector was a gift from Dr. C. Mueller-Tidow from Division of Hematology and Oncology, University of Muenster, Germany.

Cell lines: Human Ph⁺-lymphocytic leukemia cell lines BV173 and SD1 used in this study were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen and maintained in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS). Sup B-15 cell line, which was established from the bone marrow of a 10-year-old boy with Ph⁺ ALL (B cell precursor ALL) in second relapse, was also purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen and cultured under the same conditions but with 20 % FCS. Human mesenchymal stem cells were obtained from Dr. Markus Rojewski, Institut fuer klinische Transfusionsmedizin und Immungenetik, University Hospital Ulm, Germany. Cells in logarithmic growth phase were used for further experiments.

Assessment of cell proliferation: A previously described method was used to measure cell proliferation. Briefly, cells at $2 \times 10^5$ per ml were treated with various
agents in 96 well plates. After incubation, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazoliumbromide (MTT, Sigma-Aldrich, Taufkirchen, Germany) solution
was added to each well and cells were incubated further for 4 h. The water insoluble
formazan was formed during incubation, and it was solubilized by adding 100 µl
solubilization solution to each well. The formazan dye was quantified using an Anthos
HTII ELISA reader (Anthos Mikrosysteme, Krefeld, Germany).

**Cell cycle analysis:** Cells were treated with different concentrations of TZD18 for
different durations, washed with PBS and fixed with ice-cold 70 % ethanol. These
samples were treated with RNase, stained with propidium iodide and analysed with
the FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

**RT-PCR:** RT-PCR reactions were carried out with GeneAmp PCR system 2700
(Applied Biosystem, Weiterstadt, Germany). RNA extraction and cDNA preparation
were described previously. Following reverse transcription, 1 µl cDNA was used for
further PCR analysis. The sequences of primers for PPARα are: Forward: 5`-
acttatcctgtggtccccgg; Reverse: 5`-ccgacagaaaggcacttgtga. A touch-down PCR
protocol was used as described by Suchanek KM et al. with the following
modification: samples were amplified for 32 cycles with the annealing temperature
ranged between 65 ºC and 49 ºC, decreasing 0.5 ºC after each cycle, then amplified
for a further 10 cycles at the annealing temperature of 49 ºC.

**Western blot analysis:** Protein concentrations of whole cell lysates as well as either
cytoplasmic or nuclear extracts were measured using a BCA protein assay kit
(Pierce, Bonn, Germany). Western blot analysis was performed as described
previously. The following antibodies from Santa Cruz (Heidelberg, Germany) were
used in this study: anti-PPARα (sc-9000), anti-bax (sc-493), anti-p27 (sc-528), anti-
cyclin D2 (sc-754), anti-cyclin E (sc-247), anti-cyclin dependent kinase (CDK)-2 (sc-
6248), anti-CDK-4 (sc-260), anti-c-Myc (sc-40), anti-NF-κB p65 (sc-372), anti-IκBα
(sc-371), and anti-β-Actin (sc-1616). Anti-Phospho-IκBα (Ser32) (#9241) was from
Cell Signalling (Beverly, MA, USA). Primary antibodies were diluted 1:200 to1:500.

**Transient transfection assay:** Transient transfections were carried out in SD1 cells
by using electroporation with EPI2500 electroporator (Dr. L. Fischer Lab Instruments,
Heidelberg, Germany). Transfection mixes include 7.5 µg of PPReX3-tk-luc, 7.5 µg wild type of human PPARγ expression vector (pcDNAFlag-PPARγ1) or wild type of PPARα expression vector (pCMX-mPPARα), and 100 ng Renilla luciferase reporter plasmid (served as an internal transfection efficiency control). After transfection, cells were cultured and exposed to various agents. Luciferase activities were determined using dual-luciferase reporter assay system (Promega, Madison, USA) according to the instruction of manufacturer.

**Measurement of apoptosis by cell-death ELISA:** Cells were cultured either in the presence or absence of TZD18. Apoptotic cell death was determined with the cell death detection ELISA plus (Roche Diagnostics, Heidelberg, Germany) according to the manufacturer’s recommendations with modifications. Briefly, after lysis and centrifugation, cell lysates were incubated with the biotin-labelled anti-histone and peroxidase-labelled anti-DNA antibodies in a streptavidin-coated microtiter plate for 2 h at room temperature. After incubation, the peroxidase substrate ABTS was added, the plate was incubated at room temperature for about 10 min and the peroxidase activity was determined by an Anthos HTII ELISA reader at 405 nm. Data were presented as fold increase of optical density (OD) as compared to untreated control.

**Measurement of apoptosis by TUNEL:** Cells were treated with either PGZ (20 µM) or TZD18 (20 µM). After incubation, apoptosis was determined by terminal deoxynucleotidyl-transferase-mediated UTP nick end labelling (TUNEL) technique using an in situ cell death detection kit (Roche Applied Biosystems, Heidelberg, Germany) by following the instructions of the manufacturer.

**Measurement of caspase-8 and caspase-9 activities:** Apoptosis was induced by incubating cells with TZD18. The activities of caspase-8 and caspase-9 were determined with caspase-8 and caspase-9 colorimetric activity assay kits (Chemicon, Temecula, CA, USA) according to the manufacturer’s recommendations with modifications. Briefly, 2x10⁶ cells were lysed in lysis buffer, and the protein concentration for each sample was measured using the BCA protein assay kit. Incubation of cell lysates with caspase-8 specific substrate IETD-p-nitroaniline or caspase-9 specific substrate LEHD-p-nitroaniline leads to cleavage of these substrates by caspase-8 or caspase-9, respectively, and release of chromophore p-
nitroaniline. The caspase activities were detected by measuring this chromophore using the Anthos HTII ELISA reader at 405 nm. OD values were then normalized to protein concentrations of the samples. Fold-increase in either caspase-8 or caspase-9 activities before and after TZD18 treatment was determined by comparing the OD value of the apoptotic sample with that of untreated control.

**Measurement of NF-κB activity by electrophoretic mobility shift assay (EMSA) and ELISA:** SD1 cells were treated with either vehicle or 20 µM of TZD18 for 1 to 4 days, respectively. Cells were harvested, nuclear and cytoplasmic protein was extracted by the use of NE-PER® nuclear and cytoplasmic extraction kit (Pierce). For determination of NF-κB binding activities, a double-stranded oligonucleotide containing the NF-κB binding site (5´-AGT TGA GGG GAC TTT CCC AGG C-3´) was used. This oligonucleotide was synthesized and 3´-end-labelled with biotin by TIB Molbiol (Berlin, Germany). EMSA was performed using a LightShift™ chemiluminescent EMSA kit (Pierce) according to the instruction of the manufacturer with modifications. Equal amounts of nuclear protein (5 µg per sample) were incubated with biotin-labelled NF-κB DNA probe in binding buffer for 30 min at room temperature. DNA-protein complexes were separated by nondenaturing 6% polyacrylamide gel electrophoresis. The specificity of the shifted bands was verified by including 100-fold unlabeled double-stranded oligonucleotide in the reaction complexes. The identities of the NF-κB subunits were determined by adding antibodies specific for p50 (sc-114), p65 (sc-109), p52 (sc-7386), c-Rel (sc-71), and RelB (sc-226) (Santa Cruz) to the reactions and observing the super-shifted bands. Detection of biotin-labeled DNA by chemiluminescence was performed according to the instructions of manufacturer. The NF-κB DNA binding activities of cells before after treatment with TZD18 were also quantitatively analyzed by using a TransAM™ NFκB Family Transcription Factor ELISA Assay Kit (Active Motif, Rixensart, Belgium) according to the instruction of the manufacturer.

**Statistical analysis:** The results were presented as a mean ± SD of at least three independent experiments. Synergistic effects of the combination of TZD18 and Imatinib on cell proliferation were assessed using the Chou-Talalay method and CalcuSyn software (Biosoft, Ferguson, MO, USA). Briefly, the dose-effect curve for each drug alone was determined based on the experimental observations using the
median-effect principle; the combination index (CI) for each experimental combination was then calculated according to the following equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D_x)_1(D_x)_2}$$

where $(D)_1$ and $(D)_2$ are the doses of drug 1 and drug 2 that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. CI=1 indicates additive effects; CI values less than 1.0 indicate a more than expected additive effect (synergism).
Results

**PPARα and PPARγ were expressed in lymphocytic leukemia cells.** PPARγ has previously been shown to be expressed in human Ph^+-lymphocytic leukemia cell lines. Direct evidence for the expression of PPARα in ALL cell lines is still lacking. We showed here the expression of PPARα in all three lymphocytic leukemia cell lines by RT-PCR ([Fig. 1B](#)). RNA isolated from a breast cancer cell line MDA-MB-231 was used as a positive control. Western blot analysis confirmed these results ([Fig. 1C](#)). Total protein isolated from the breast cancer cell line SKBR-3 served as a positive control, since PPARα protein was previously identified in this cell line. Next, we tested whether all the agonists and antagonists used in this study were functional in our cell system by cotransfection of either a WT-PPARα or WT-PPARγ expression vector and a PPRE-luciferase reporter vector into SD1 cells. Either PPARα ligand WY14,643 or PPARγ ligand PGZ resulted in a about 3-fold increase of luciferase activity, whereas PPARα antagonist MK886 or PPARγ antagonist GW9662 significantly reversed this effect, respectively. As a dual ligand for these two subtypes of PPARs, the increased luciferase activities by TZD18 could be reversed by addition of appropriate antagonists ([Fig. 1D](#)). These results clearly indicated that all of the ligands used in this study were able to activate PPARγ or PPARα, whereas the antagonists were able to inhibit the activation of PPARγ or PPARα under our conditions.

**TZD18 caused growth inhibition of Ph^+-lymphocytic leukemia cells.** We studied the effect of TZD18 on cell growth in Ph^+-lymphocytic leukemia cell lines BV173, SD1 and Sup B-15. This dual ligand inhibited the proliferation of all of these cell lines in a dose- and time-dependent manner ([Fig. 2A and Fig. 2B](#)). The rank order of sensitivity of these cell lines to TZD18 was: SD1>BV173>Sup B-15. BV173 and SD1 were more sensitive to the treatment with growth inhibition of about 70 % and 80 % at the concentration of 20 µM after four days, respectively, while Sup B-15 was relatively resistant.

The growth inhibitory effect of TZD18 was much stronger than that of PGZ. PGZ showed only 10-20 % of growth inhibition at the concentration of 20 µM in cell lines BV173 and SD1 (data not shown). We have tested TZD18 in different types of tumors.
and normal cells and found that different types of cells reacted to the drug differently: in human glioblastoma cell line GMS-10, up to 40 µM TZD18 resulted in even about 10% growth increase after four days treatment (data not shown), while human mesenchymal stem cells isolated from human bone marrow and normal stromal cells from breast cancer samples had no significant proliferation change after culture with up to 50 µM of TZD18 for 4 days (data not shown). These results suggest that the inhibitory effects on human lymphocytic leukemia cells might not be due to the non-specific cytotoxicity of TZD18.

We have previously shown that the anti-proliferative effect of PPARγ ligands was attributable to their inhibition of cell cycle progression. Therefore, we investigated the possible effect of TZD18 on the cell cycle distribution of the lymphocytic cell lines. As indicated in Figure 2C, the cell cycle progression after exposure to TZD18 (10 and 20 µM) for three days was inhibited in a dose-dependent fashion for all of the cell lines. In all cases, a G0/G1 phase arrest was observed. The S-phase in BV173 and SD1 after treatment with 20 µM TZD18 was decreased from 52% and 31% to 29% and 13%, respectively. The data clearly indicate that cell cycle arrest, at least in part, may contribute to the inhibitory effects of this ligand on the growth of lymphocytic leukemia cell lines.

**TZD18 induced apoptosis of Ph⁺-lymphocytic leukemia cells.** Similar to the PPARγ ligands PGZ and 15d-PGJ₂, TZD18 also caused a significant apoptosis of each of the cell lines as determined by TUNEL and cell-death ELISA. The Sup B-15 cells were least sensitive to the TZD18 induced apoptosis which was consistent with the data from proliferation assay. In contrast, a great part of BV173 and SD1 cells underwent apoptosis when they were exposed to TZD18 (10 µM or 20 µM) (Fig. 3A and Table 1). In comparison, PGZ (20 µM, 4 days) induced only 2-fold increase of apoptosis in BV173, and none in SD1 cells (Table 1). Similar to the growth inhibitory effects, apoptosis was also induced by TZD18 (10 µM and 20 µM) in a time-dependent fashion in SD1 cells (Fig. 3B). In addition, TZD18 remarkably activated caspase-9 and, to a less extent, caspase-8 in SD1 and BV173 cells (Fig. 3C). Again, only a minimal activation of caspase-8 and caspase-9 was observed in Sup B-15 after their exposure to TZD18 (data not shown). Furthermore, the ligand-induced apoptosis was totally reversed by addition of the pan-caspase inhibitor Z-VAD-FMK.
to both the BV173 and SD1 cell lines (Fig. 3D) indicating that the apoptosis induced by TZD18 was caspase-dependent.

**TZD18 regulated cell cycle- and apoptosis-related proteins.** Since cyclin Ds and cyclin E, as well as CDK-2 and -4 and their inhibitors, $p21^{cip1}$ and $p27^{kip1}$, are key regulators of G1 to S phase cell cycle progression, we investigated the effects of TZD18 on the expression of these molecules. The pro-growth molecules cyclin D2, CDK-2, CDK-4 and cyclin E were downregulated, whereas anti-growth molecule CDK inhibitor (CDKI) $p27^{kip1}$, but not $p21$ (data not shown), was upregulated by TZD18 (Fig. 4A). We found previously that alteration of the bcl-2 related apoptotic pathways is associated with PPAR$\gamma$ induced apoptosis in human breast cancer cell lines. Therefore, we assessed the regulation of bcl-2 and bax after exposure to TZD18. The anti-apoptotic factor bcl-2 was unchanged (data not shown), while the pro-apoptotic factor bax was dramatically upregulated (Fig. 4A). In addition, the oncoprotein c-Myc, which is a master regulator of the G1/S transition cascade and apoptosis, was prominently downregulated in all three cell lines (Fig. 4A). Furthermore, we also analyzed the changes of these cell cycle and apoptosis related factors at different time points after exposure to TZD 18 (20 µM) in SD1 cells. As shown in Figure 4B, expression change could be observed as early as 24 hrs after culture with TZD18.

**TZD18 induced cell growth inhibition was independent of PPAR$\alpha$ and PPAR$\gamma$.** We have previously demonstrated that the PPAR$\gamma$ antagonists could not reverse the PPAR$\gamma$ agonists induced cell growth inhibition of human lymphocytic leukemia. Similarly, although all the antagonists used in this study are able to inhibit PPAR$\alpha$ or $\gamma$ activities (Fig 1D), the PPAR$\gamma$ antagonist GW9662 and the PPAR$\alpha$ antagonist MK886, either alone or in combination, could not reverse the growth inhibitory effect of TZD18 (Fig. 5). This suggests that this dual ligand may exert its effect on proliferation and apoptosis of lymphocytic leukemia cells independent of its activation of PPAR$\alpha$ and PPAR$\gamma$.

**NF-$\kappa$B binding activities were decreased by TZD18.** Increasing data suggest that PPAR$\gamma$ ligands exert their effects through interaction with the transcription factor NF-$\kappa$B. Therefore, the influence of TZD18 on the NF-$\kappa$B-DNA binding activities was first
investigated with EMSA. Treatment with TZD18 resulted in a time-dependent decrease in the NF-κB-DNA binding activities in cell line SD1. The specificity of the NF-κB bands was confirmed by competition assay and supershift analysis (Fig. 6A). We have then quantitatively measured the NF-κB activities in the same cell nuclear protein as used in EMSA (Fig. 6B). The obtained results confirmed the EMSA data. For further analysis of the mechanism responsible for the decreased NF-κB activities, Western blotting was performed with nuclear and cytoplasmic extracts prepared from SD1 cells at various time points after treatment with TZD18. Decreased nuclear p65 level occurred after TZD18-treatment (Fig. 6C), whereas the p65 band in the whole cell lysates was similar (data not shown) suggesting that p65 translocation to the nucleus was blocked. Because IκBα phosphorylation on serine residues 32 and 36 with subsequent ubiquitination and degradation plays an important role in NF-κB translocation and activation, serine phosphorylation of IκBα (Ser P-IκBα) was also analyzed. Decreased Ser P-IκBα was detected in cytoplasmic lysate isolated from TZD18-treated SD1 cells, while the total IκBα level was increased (Fig. 6C). These findings indicate that increased expression and decreased degradation of IκBα may inhibit the translocation of NF-κB to the nucleus and, subsequently, contribute to the decrease of NF-κB DNA binding activities.

**Combination of TZD18 and Imatinib markedly inhibited cell growth of Ph⁺-ALL cell lines.** BV173 cells were extremely sensitive to Imatinib. For example, 0.5 µM of Imatinib inhibited cell proliferation of BV173 cells by 68 % and 2.5 µM of Imatinib resulted in 98 % inhibition (data not shown). The Sup B-15 cells were less sensitive; nevertheless, 2.5 µM of Imatinib caused more than 60 % growth inhibition of these cells. In contrast to these two lymphocytic leukemia cell lines, SD1 cells were relatively resistant to Imatinib with 2.5 µM of this drug producing only about 12 % growth inhibition. Interestingly, TZD18 (10 µM) enhanced the inhibitory effect of Imatinib on cell proliferation in all three cell lines significantly (Fig. 7A). We further analyzed the pharmacologic interactions between TZD18 and Imatinib using a non-constant ratio combination design with CalcuSyn software. The obtained sobologram suggested that the combination of the two drugs resulted in the synergistic inhibition (CI<1.0) of cell proliferation in all three cell lines (Fig. 7B).
Discussion

Previously, we have shown that the PPARγ ligands PGZ and 15d-PGJ2 inhibited cell proliferation of lymphocytic leukemia cell lines, which was associated with a G1-cell cycle arrest and apoptosis. Furthermore, cell cycle- and apoptosis-related genes, such as cyclin D1, cyclin D2, and p27kip1 were altered by these ligands. In this study, we demonstrated that a novel PPARα and PPARγ dual ligand, TZD18, which belongs structurally to the TZD class, also showed anti-growth effects on Ph+-lymphocytic leukemia cell lines, which was much stronger that that of PGZ. Our studies further revealed that activation of PPARα and PPARγ seemed not to be required for TZD18 to exert its effects. Of more clinical relevance, this compound enhanced the ability of Imatinib to inhibit cell proliferation of Imatinib-sensitive and -resistant Ph+-lymphocytic leukemia cell lines.

PPARγ is expressed in human lymphocytic leukemia cell lines as observed by us and other groups. However, to date, reports of PPARα expression in hematopoietic cells are only limited to human myeloblastic leukemia cell line HL-60 and murine lymphocytes. We have now shown that human Ph+-ALL cell lines also express PPARα.

In comparison to PGZ, TZD18 more potently inhibited the growth of all Ph+-lymphocytic leukemia cell lines tested through a G0/G1 cell cycle arrest. In contrast, the percentage of cells in G2/M-phase changed only minimally. This result suggests that the effect of the PPARα/γ ligand on cell cycle progression was similar to what we have previously reported for the PPARγ ligands. Interestingly, we have found that several tumor and normal cell lines did not response to the exposure to this substance strongly suggesting that the effects of TZD18 observed on human Ph+-ALL cell lines is not sue to the non-specific cytotoxicity of this agent.

PPARγ ligands have been shown previously to inhibit the G1-S phase progression of colon, pancreatic, and breast cancers as well as glioblastomas and leukemias. Targeting the key regulators of the G1/S transition is probably the
cause of the observed cell cycle arrest. For example, PPARγ ligands result in a downregulation of D type cyclins \(^{47-50}\) and CDKs \(^{50}\), and/or an upregulation of CDKIs such as p21\(^{\text{cip1}}\) \(^{44,47,49,51}\), p27\(^{\text{kip1}}\) \(^{12}\) and p16\(^{\text{ink4a}}\) \(^{48}\). A similar effect was also observed with the PPARα ligand clofibrate using the human leukemia cell line HL-60. This hypolipidemia drug caused growth inhibition and cell cycle blockade which was related to downregulation of cyclin D2 \(^{52}\). Congruent with these reports, we showed here that TZD18 also modulated the key regulators of G1/S progression, in a manner that these alterations might lead to a cell blockage in G0/G1, i.e. it reduced the expression of cyclin D2, cyclin E, CDK-2 and CDK-4 and enhanced the expression of CDKI p27\(^{\text{kip1}}\). We have further noted that c-Myc protein was markedly downregulated by TZD18. This oncoprotein stimulates G1 progression through multiple mechanisms such as repressing, directly or indirectly, p21\(^{\text{cip1}}\) and p27\(^{\text{kip1}}\) expression, increasing CDK-4 and inducing cyclin D1 and cyclin D2 synthesis (reviewed in \(^{53}\)). Although our studies did not show whether the alterations of these cell cycle regulators are either a direct or indirect consequence of downregulation of c-Myc, we believe that these alterations may account for the observed cell cycle arrest induced by TZD18.

Downregulation of cyclin D1 by PPARγ ligands has been described in human vascular smooth muscle cells \(^{54}\), non-small lung cancer cells \(^{47}\), as well as our previous studies with human lymphocytic leukemia cells \(^{12}\). The reduced expression of cyclin D1 was demonstrated to be responsible for Troglitazone (a PPARγ ligand of TZD class) mediated cell cycle arrest in MCF-7 cells \(^{50}\). However, we did not observe changes of cyclin D1 in Ph\(^{+}\)-lymphocytic leukemia cell lines after their exposure to TZD18. On the contrary, we have found in this study that cyclin D2 was highly expressed in these cells, and its expression was markedly reduced by TZD18 treatment. Cyclin D2 has been reported to be constitutively expressed at high levels in lymphocytic malignancies, especially in BCR-ABL-transformed cells, whereas expression of this protein was either low or absent in Ph-negative lymphocytic leukemia cell lines \(^{55-57}\). The link between BCR-ABL–transformation and cyclin D2 overexpression was further elucidated by experiments showing that ectopic overexpression of cyclin D2 rescued the inhibition of Bcr-Abl protein tyrosine kinase activities caused by Imatinib \(^{56}\). Furthermore, bone marrow cells from cyclin D2-deficient strains of mice, failed to proliferate in response to transfection of a BCR-ABL expression vectors \(^{57}\). We have also observed that growth inhibition caused by the
PPARγ ligands PGZ and 15d-PGJ₂ was associated with downregulation of cyclin D2 in lymphocytic leukemia cells, including Ph⁺-lymphocytic leukemia. Taken together, cyclin D2 may be one of the key regulators of the growth and maintenance of Ph⁺-lymphocytic leukemia cells. Downregulation of cyclin D2 by the PPARα ligands clofibrate and ciprofibrate has also been described in HL-60 cells. Therefore, we speculate that repression of cyclin D2 by PPAR ligands may represent a crucial mechanism underlying the PPAR ligand-induced growth inhibition of Ph⁺-lymphocytic leukemia cells.

Cell cycle arrest can be followed by cell differentiation and/or apoptosis. TZD class of PPARγ ligands have been reported to induce apoptosis in a variety of human solid tumors and hematological malignancies (reviewed in). By using TUNEL and cell death ELISA, we have demonstrated that TZD18 induced apoptosis in all three Ph⁺-lymphocytic leukemia cell lines in a time- and dose-dependent manner. We showed that a pan-caspase inhibitor Z-VAD-FMK completely abolished the TZD18 induced apoptosis indicating that apoptosis caused by this substance is caspase-dependent. Normally, caspase-dependent apoptosis is initiated through two partly interdependent routes. One is the extrinsic pathway which is triggered by ligation of cell death receptors leading to activation of caspase 8. The other is the intrinsic pathway in which the bcl-2 superfamily and mitochondria are involved. In this case, changes of expression of anti- and pro-apoptotic proteins of the bcl-2 family disrupt the mitochondrial membranes and result in the release of pro-apoptotic proteins and the activation of caspase 9. Both caspase 8 and 9 in turn activate apoptosis executers such as the caspase 3 launching apoptosis program. Although we did not observe any change of the anti-apoptotic protein bcl-2, we noticed that the pro-apoptotic protein bax was significantly upregulated after the Ph⁺-lymphocytic leukemia cells were cultured with TZD18. This resulted in a reduced bcl-2/bax ratio which may result in increased mitochondrial permeability and apoptosis. Numerous reports demonstrated that the bcl-2/bax ratio rather than level of bcl-2 alone was important for drug-induced apoptosis of leukemic cells. At the same time, we observed a dramatic increase of activities of caspase 9 by TZD18 treatment. These data strongly suggest apoptosis induced by TZD 18 may occur through the intrinsic pathway. This is congruent with recent reports that 15d-PGJ₂ and the PPARγ ligand CDDO induced apoptosis of lymphocytes by activating the mitochondrial pathway.
Interestingly, a modest increase of caspase 8 was also observed in this study. However, we did not observe any changes in the expression of cell death receptors (fas, DR4) or their ligands such as Fas ligands and TRAIL. Neither TNFα, activating antibodies of Fas nor recombinant TRAIL enhance the apoptosis produced by TZD18 (data not shown). We hypothesized that the cell-death receptor pathway is not important for apoptosis triggered by TZD18. Activation of caspase 8 is most probably by a mechanism independent of cell death receptor.

We previously showed that the anti-proliferative effect of PGZ was independent of activation of PPARγ in lymphocytic leukemia cells. In this study, we explored the ability of PPARγ antagonist GW9662 and PPARα antagonist MK886 to reverse the effects of TZD18. Although both antagonists could inhibit the activities of PPARα and γ under our conditions as shown by PPRE-luciferase assay, GW9662 and MK886, either alone or in combination, failed to reverse the anti-proliferative effects of TZD18, indicating that this PPAR ligand may act via a PPARα- and γ-independent mechanism. Furthermore, the fact that a classic PPARα ligand WY14,643 did not inhibit but slightly enhanced, proliferation of the three Ph+-lymphocytic leukemia cell lines (data not shown) further supports this speculation.

NF-κB is a collective term that refers to a small class of closely related dimeric transcription factors for a number of target genes, including growth factors, modulators of angiogenesis, cell-adhesion molecules, and antiapoptotic factors. Activation of NF-κB is tightly controlled by inhibitory IκB proteins. Increased IκB degradation by phosphorylation and subsequent ubiquitination leads to increased nuclear translocation of NF-κB and its DNA binding activities. It is believed that NF-κB is a positive regulator of cell cycle progression and activates target genes such as cyclin-D1, -D2, -D3 and -E. It is also an important regulatory factor for c-Myc protein. Furthermore, activated NF-κB in tumor cells has been shown to increase transcription of target genes whose products block apoptosis. These genes included members of TNF-family (TRAF1 and TRAF2), members of bcl-2 family, cellular inhibitors of apoptosis (cIAPs) and others. Recently, activation of NF-κB/Rel by Bcr-Abl was demonstrated in Ph+ leukemia both in vitro and in vivo. NF-κB/Rel has been shown to be required for Bcr-Abl mediated tumorigenicity and
transformation of Bcr-Abl positive myeloid cells in vitro and in a murine model. Numerous reports have shown that PPARγ and PPARα ligands exert their anti-tumor or anti-inflammation effects through inhibiting multiple steps of the NF-κB signaling pathway. We have shown in this study that TZD18 exerted an inhibitory effect on NF-κB-DNA binding activities similar to other PPARγ and PPARα ligands. Furthermore, we have shown that expression of NF-κB inhibitor IκBα was increased and phosphorylation of IκBα decreased after TZD18 exposure. All these changes may result in an increase of level of IκBα and, subsequently, a blockage of translocation of NF-κB to nucleus. Indeed, we showed in this study that the level of p65, one partner of NF-κB dimer, was decreased in nucleus after TZD18 treatment. It is most likely that TZD18 exerted its inhibitory effect on NF-κB-DNA binding activities through increasing the level of NF-κB inhibitors and blocking the nuclear translocations NF-κB. It is not surprised that expression of several NF-κB target genes, e.g. c-myc, cyclin Ds and bax, were also regulated by TZD18. Therefore, Interference of NF-κB signaling pathway by TZD18 may represent a major mechanism for observed proliferation inhibition and apoptosis in Ph⁺-lymphocytic leukemia cells.

The tyrosine kinase inhibitor Imatinib binds competitively to the ATP binding site of Abl tyrosine kinase of the Bcr-Abl fusion protein, thereby inhibiting signaling by this oncogenic protein and resulting in significant growth inhibition of Ph⁺-ALL cells. This agent frequently induces remissions of individuals with Ph⁺-ALL (reviewed in 81,82), but unfortunately, resistance to the drug and relapses occur after several months. Alternative strategies include combination therapies of Imatinib with cytotoxic agents, such as either cytarabine, daunorubicin, or in combination with other novel signal transduction modulators, such as farnesyl transferase inhibitors. In this study, TZD18 enhanced, synergistically, the growth inhibitory activities of Imatinib in these Ph⁺-lymphocytic leukemia cell lines. More interestingly, TZD18 either alone or in combination with Imatinib, is extremely effective in Ph⁺-ALL cell line SD1, which was relatively resistant to Imatinib therapy. Although the combination therapy of TZD18 and Imatinib for Ph⁺-ALL cells requires further evaluation, our data suggest that TZD18 may represent a very promising therapeutic agent to aid in the treatment of this extremely aggressive disease.
Taken together, we have evaluated the anti-cancer effects of the novel PPARα/γ dual agonist TZD18, for the first time, in three Ph⁺-ALL cell lines. This compound was able to inhibit the proliferation and induce apoptosis of these cells more effectively than that of conventional TZD PPAR ligand PGZ. Of potential clinical importance is that this compound enhanced the cytotoxic effects of Imatinib. Hence, our data indicate the potential usefulness of TZD18 in the treatment of Ph⁺-lymphocytic leukemia.

Acknowledgments

We thank Dr. M. Ruthardt for the cell line Sup B-15, Ms. Q. Guo from Merck (USA) for the compound TZD18, Prof. R. M. Evans for PPRE vector, Prof. K. K. Chatterjee for PPARγ vector, Dr. P. Tontonoz for PPARα vector, Mr. H. Krebbel for phosphorylated IκBα antibody, Dr. C. Mueller-Tidow for Renilla vector and advice in transfection assays, Ms. S. Liu and T. Tian for excellent technical assistance, Mr. G. Adie for helping in the preparation of this manuscript, and Dr. A. Hocker for help in microscopic imaging. EE thanks the generous support of grants from Deutsche J. Carreras Leukämie-Stiftung and Deutsche Forschungsgemeinschaft.
Reference List


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Figure legends

Figure 1: Analysis of expression and function of PPARα and PPARγ in Ph⁺-ALL cells. A: Structure of compound TZD18. B: RT-PCR analysis of the expression of PPARα. Total RNA was isolated, 0.2 µg of each RNA sample was applied to RT-PCR, and the specific amplification products for PPARα were visualized by electrophoresis and subsequent ethidium bromide staining. Total RNA extracted from the breast cancer cell line MDA-MB-231 was used as a positive control. C: PPARα expression was determined by Western blot as described in the Materials and Methods. Total protein isolated from the breast cancer cell line SKBR-3 served as a positive control for PPARα expression. D: Functional analysis of several PPAR agonists and antagonists. SD1 cells were cotransfected with either a PPARα or a PPARγ expression vector (wt-PPARα or wt-PPARγ), a PPRE reporter vector (PPREx3-tk-luciferase), and a Renilla luciferase vector as an internal control for the transfection efficiency. 18 h after transfection, the cells were incubated with PPARα ligand (WY14,643), PPARγ ligand (PGZ), TZD18, PPARα antagonist (MK886), PPARγ antagonist (GW9662), or their combinations for further 24 h. All drugs were used at 10 µM. After incubation, firefly and Renilla luciferase activities were determined. The firefly luciferase activity was normalized with Renilla luciferase activity and is shown as the fold increase over vehicle-treated controls. The result is a representative of three independent experiments.

Figure 2: Effect of the PPARα/γ ligand TZD18 on the proliferation of tested cell lines. TZD18 inhibits proliferation of tested cell lines in a dose (A) and time (B) dependent manner. 2x10⁵ /ml cells were incubated in the presence of TZD18 at various concentrations for different days. Cell proliferation was measured by MTT test; results were expressed as percentage of control (without treatment). Values are mean ± SD of six individual experiments. C: TZD18 alters cell cycle progression. Cells (2x10⁵/ml) were incubated either in the presence or absence of TZD18 (10 or 20 µM, 3 days), fixed, treated with RNAse and stained for DNA with PI. Cell cycle distribution was determined by FACS analysis. Results represent the percentage of the total cell population. Figure is representative of three independent experiments.
Figure 3: Effect of TZD 18 on apoptosis of tested cell lines. A: Apoptotic cells in BV173 after treatment with PGZ (20 µM) or TZD18 (20 µM) were measured by TUNEL assay as described in Materials and Methods. B: SD1 cells were cultured in the presence of TZD18 (10 or 20 µM) for different hrs, washed and lysed. Apoptosis was measured by cell-death ELISA as described in Materials and Methods. Results are expressed as fold-change of enrichment of nucleosomes in the cytoplasm of cells treated with drugs compared to controls (without treatment). Data represent the mean ± SD of triplicate experiments. C: Cells were cultured in the presence of TZD18 (20 µM) for four days, washed and lysed in lysis buffer. Caspase-8 and caspase-9 activities in the cell lysates were measured as described in Materials and Methods. Results were expressed as fold increase of OD values compared to control (without treatment). Figure is representative result of three independent experiments. D: Cells were incubated in the presence of either TZD18 (20 µM), Z-VAD-FMK (20 µM) or both for four days. Cell apoptosis was examined as described in Materials and Methods. Apoptosis was expressed as fold-change of enrichment of nucleosomes in the cytoplasm of cells treated with drugs compared to sample with the lowest value (cells treated with Z-VAD-FMK). Results are the mean ± SD of three individual experiments.

Figure 4: Expression of cell cycle- and apoptosis-related proteins in tested cell lines cultured with TZD18. A: BV173, SD1 and Sup B-15 cells were incubated either with or without TZD18 (10 or 20 µM, 4 days), and cell lysates were examined for cell cycle- and apoptosis-related proteins by Western blot as described in the Materials and Methods. A representative blot of three independent experiments is shown. The relative intensity of each band was quantified using Phoretix™ ID Quantifier software and expressed as percent of control. B: Equal amounts of protein prepared from SD1 cells exposed to TZD18 (20µM) for different durations were examined for the same cell cycle- and apoptosis-related proteins as in panel A.

Figure 5: TZD18 effect on cell proliferation is independent of PPARγ and PPARα. Cells (2x10^5 /ml) were incubated with antagonists for either PPARγ (GW9662, 2 µM) or PPARα (MK886, 2 µM) in the absence or presence of TZD18 (20 µM) for four days. Cell proliferation was measured by the MTT assay as described in
Materials and Methods. Results are expressed as percentage of control (without treatment). Values are mean ± SD of at least three experiments.

Figure 6: TZD18 inhibits NF-κB DNA binding activities. Nuclear and cytoplasmic extracts were prepared from untreated and TZD18 treated (20 µm) SD1 cells after 24, 48, 72, and 96 hrs cultures. Equal amounts of protein were used for all assays. A: EMSA analysis was performed to determine the DNA-binding capacity of NF-κB by using nuclear extracts. Competition analysis was done by using a 100-fold excess of unlabelled NF-κB consensus binding site. Antibodies specific for p50, p65, p52, cRel and RelB were used in supershift analysis. The arrows indicate the positions of the super-shifted bands. The result is representative of three independent experiments. B: The quantitative measurement of the NF-κB activity was performed with nuclear proteins as described in Materials and Methods. NF-κB activity of TZD18-treated cells was expressed as percentage of untreated control. Data represented mean ± SD of triplicate experiments. C: NF-κB expression in nuclear extracts and phosphorylated I-κBα expression in cytoplasmic extracts were analyzed by western blot. Membranes were blotted with antibodies specific for NF-κB subunit p65 and I-κBα phosphorylated at serine 32.

Figure 7: TZD18 enhances growth inhibition of Imatinib. A: Cells (2x10^5 /ml) were incubated with different concentrations of Imatinib as indicated in the figure in the presence or absence of TZD18 (10 µM, 4 days). Cell proliferation was measured by the MTT assay as described in the Materials and Methods. Results are expressed as proliferation percentage of control (without treatment). Values are mean ± SD of six parallel experiments. B: Statistical analysis of the effects of the combination of TZD18 and Imatinib. BV173, SD1, and Sup B-15 cells were cultured in the presence of escalating doses of Imatinib (0.25, 0.5, 1, 2.5 µM). or TZD18 (5, 10, 15, 20 µM), and the combination of 0.5 µM Imatinib with varied concentration of TZD18 (5, 10, 15, 20 µM), or the combination of 10 µM TZD18 with varied concentration of Imatinib (0.25, 0.5, 1, 2.5 µM). After four days, cell proliferation was measured with MTT assay. CI values for each data point of this non-constant ratio design was calculated using CalcuSyn software. All CI values were <1. Reprehensive Dx-normalized isobolograms obtained from SD1 cells are shown (left: TZD18 concentration fixed at 10 µM; right: Imatinib concentration fixed at 0.5 µM).
Table 1: Induction of apoptosis by PGZ and TZD18 in Ph⁺-ALL cell lines. BV173, SD1 and Sup B-15 cells were treated with PGZ (20 µM) or TZD18 (10 µM and 20 µM) for 4 days. Apoptosis was determined by TUNEL assay and cell-death ELISAPLUS kit as described in Materials and Methods. Results for TUNEL assay are expressed as percentage of the positive cells. Results for cell-death ELISA are expressed as fold-change of enrichment of nucleosomes in the cytoplasm of cells treated with drugs compared to controls (without treatment). Data represent the mean ± SD of triplicate experiments.
Fig. 1

**Fig. 1A**

**Fig. 1B**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water</th>
<th>MDA-MB-231</th>
<th>BV173</th>
<th>SD1</th>
<th>Sup B-15</th>
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<tbody>
<tr>
<td><strong>PPARα</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><strong>β-Actin</strong></td>
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**Fig. 1C**

<table>
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<tr>
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<th>Sup B-15</th>
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<td><strong>β-Actin</strong></td>
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</table>
Fig. 2

Fig. 2A

![Graph showing proliferation (% of control) vs. concentration of TZD18 (µM). The graph includes data for BV173, SupB-15, and SD1 at various concentrations.]

Fig. 2B

![Graph showing proliferation (% of control) vs. incubation time (hrs). The graph includes data for BV173 and SD1 at different concentrations.]

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Fig. 2C

Control | TZD18 (10 μM) | TZD18 (20 μM)

**BV173**

G0/G1: 36%  
S: 53%  
G2: 11%

G0/G1: 39%  
S: 50%  
G2: 11%

G0/G1: 64%  
S: 29%  
G2: 7%

**SD1**

G0/G1: 57%  
S: 31%  
G2: 12%

G0/G1: 73%  
S: 20%  
G2: 7%

G0/G1: 83%  
S: 12%  
G2: 5%

**Sup B-15**

G0/G1: 39%  
S: 45%  
G2: 16%

G0/G1: 46%  
S: 39%  
G2: 15%

G0/G1: 48%  
S: 30%  
G2: 22%

FL-2 Area
Fig. 3

Fig. 3A

Fig. 3B

Incubation Time (hrs)

Apoptosis (fold over control)

TZD18 10µM
TZD18 20µM

Control
PGZ (20 µM)
TZD18 (20 µM)
Fig. 3C

![Bar chart showing activity (Fold over control) for BV173, SD1, and Sup B-15. The chart displays data for Caspase-8 and Caspase-9, with Control as a baseline.]

Fig. 3D

![Bar chart showing apoptosis for Control, Z-VAD-FMK (20 µM), TZD18 (20 µM), Z-VAD-FMK (20 µM) + TZD18, SD1, and BV173. The chart compares apoptosis levels across different treatments.]

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Fig. 4A

<table>
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<th>Control</th>
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<th>TZD18 20µM</th>
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<td>1.9</td>
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<tr>
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<td>0.3</td>
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<td>CDK-4</td>
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<tr>
<td>c-Myc</td>
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</tr>
<tr>
<td>cyclin E</td>
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<td>0.5</td>
</tr>
<tr>
<td>cyclin D2</td>
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<td>0.7</td>
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<tr>
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<td>0.4</td>
</tr>
<tr>
<td>Sup B-15</td>
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<td>0.3</td>
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β-Actin
Fig. 4B

<table>
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<th>48hrs</th>
<th>72hrs</th>
<th>96hrs</th>
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<td>Control</td>
<td>TZD18 20μM</td>
<td>Control</td>
<td>TZD18 20μM</td>
</tr>
</tbody>
</table>

- bax
- p27
- CDK-2
- CDK-4
- c-Myc
- cyclin E
- cyclin D2
- β-Actin
Fig. 5

![Graph showing proliferation (% of control) for different treatments: MK886, GW9662, MK886+GW9662, TZD18, TZD18+MK886, TZD18+GW9662, TZD18+MK886+GW9662. The graph compares BV173, SD1, and Sup B-15 conditions.](image-url)
Fig. 6

Fig. 6A

Fig. 6B

Incubation hours

NFkB Binding Activity (% of control)
Fig. 6C

<table>
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<tr>
<th></th>
<th>24hrs</th>
<th>48hrs</th>
<th>72hrs</th>
<th>96hrs</th>
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<td>P-(\text{IκB}_\alpha) (Ser32)</td>
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<tr>
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Fig. 7A:

![Graph showing proliferation (% of control) for BV173, SD1, and Sup B-15 cells treated with various concentrations of imatinib and TZD18.](image-url)
Fig. 7B:
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatments</th>
<th>TUNEL (percentage of positive)</th>
<th>Enrichment factor a (fold over control)</th>
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<tr>
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<td>1.00±0.05</td>
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<td>PGZ (20 µM)</td>
<td>10.2±0.47</td>
<td>1.96±0.11</td>
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<tr>
<td></td>
<td>TZD18 (10 µM)</td>
<td>7.6±0.76</td>
<td>2.15±0.06</td>
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<tr>
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<td>TZD18 (20 µM)</td>
<td>29.2±6.5</td>
<td>9.46±0.27</td>
</tr>
<tr>
<td>SD1</td>
<td>Control</td>
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<td>1.00±0.06</td>
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<td>PGZ (20 µM)</td>
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<td>n.d.</td>
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<td>TZD18 (30 µM)</td>
<td>n.d.</td>
<td>2.36±0.07</td>
</tr>
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a Enrichment factor is the enrichment of nucleosomes in the cytoplasm measured by cell death ELISA and is an indicator of apoptosis. n.d.: not done.
Growth inhibition and apoptosis in human Philadelphia chromosome positive lymphoblastic leukemia cell lines by treatment with the dual PPAR α/γ ligand TZD18

Hongyu Liu, Chuanbing Zang, Martin H Fenner, Dachuan Liu, Kurt Possinger, H P Koeffler and Elena Elstner