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Research Article

Proteasome inhibition overcomes the resistance of renal cell carcinoma cells against the PPARγ ligand troglitazone

K. von Schwarzenberg[a], S. A. E. Held[a], A. Schaub[b], K. M. Brauer[b], A. Bringmann[a] and P. Brossart[a, *]

[a] Department of Hematology and Oncology, University of Bonn, Wilhelmstr. 35 – 37, 53111, Bonn (Germany), Fax: 49-228-287-22635, e-mail: peter.brossart@ukb.uni-bonn.de
[b] Department of Hematology, Oncology, Immunology, Rheumatology and Pulmology, University of Tübingen, Tübingen (Germany)

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Abstract. In order to analyze the effects of peroxisome proliferator-activated receptor-γ (PPARγ) activation on renal cell carcinomas we utilized several cell lines that were treated with the high affinity PPARγ agonist, troglitazone. Incubation of RCC cells with troglitazone resulted in reduced secretion of growth factors that was due to the inhibition of MAP kinase signaling and reduced nuclear localized expression of relB and HIF1alpha. Interestingly, the cell lines used showed a different sensitivity towards apoptosis induction that did not correlate with the inhibition of growth factors or expression of pro- and anti-apoptotic molecules. To overcome this resistance the cells were treated with a combination of troglitazone and the proteasome inhibitor, bortezomib. The combination of both compounds induced apoptosis even in cells resistant to both agents alone, due to increased induction of ER-stress and caspase-3 mediated cell death.

Keywords. Renal cell carcinoma, PPARγ, apoptosis, proteasome inhibition, signal pathways.

Introduction

Renal cell carcinoma (RCC) is the most frequent cancer of the adult kidney and accounts for about 2 % of all tumors [1]. It arises from epithelial cells and appears in different histological subtypes, of which the most common is the clear renal cell carcinoma (75 %) [1, 2]. At an early stage nephrectomy has quite favorable prospects but, by the time of diagnosis, one-third of patients have already developed metastasis [1, 3]. These patients have a very poor prognosis due to the resistance of RCC to radiation and conventional chemotherapy. Recent treatment consists of the administration of cytokines such as IL-2 or IFN-α, yielding response rates of 10 – 20 % in the patient population with favorable risk factors [4].

A common alteration found in RCC is a functional loss of the von-Hippel-Lindau (VHL) tumor suppressor gene. This can be caused by a germline mutation and subsequent inactivation of the second allel [5, 6]. VHL belongs to an E3 ubiquitin ligase complex that promotes the degradation of the Hypoxia-inducible factor (HIF) in the presence of oxygen. Recent work has shown that oxygen-dependent hydroxylation of two proline residues of HIF is essential for HIF-binding to pVHL and targets HIFα for the proteasome-ubiquitin pathway [7].

HIF is an alpha/beta heterodimeric transcription factor with a DNA binding domain that regulates

* Corresponding author.

oxygen-dependent gene expression. HIFβ is constitutively expressed, whereas HIFα is the oxygen regulated component that determines HIF activity and accumulates in hypoxia or in cells with disrupted VHL function. HIF plays a key role in the regulation of glucose metabolism, angiogenesis and erythropoiesis under oxygen-limiting conditions [8]. As a result of frequent intratumoral hypoxia and mutations in the VHL gene, the HIF pathway is activated in many malignancies while it is not expressed in other tissues. This leads to the up-regulation of genes that are involved in angiogenesis and cancer progression [5]. Recently, several compounds that disrupt the VEGF/VEGFR interaction, such as the humanized monoclonal antibody bevacizumab (Avastin) or the tyrosine kinase inhibitors sorafinib and sunitinib, were introduced in the treatment of RCC. Both compounds resulted in improved outcome for metastatic RCC [9, 10, 11].

Another transcription factor that is expressed in RCC is the peroxisome proliferator activated receptor-gamma (PPARγ), a member of the nuclear hormone receptor superfamily. This ligand-activated transcription factor is predominantly expressed in adipose tissue where it regulates glucose metabolism and leads to the transcription of adipocyte-specific genes [12]. In addition to its classic role in adipose metabolism, PPARγ has been shown to be expressed at low levels in other tissues such as breast, colon, kidney or in cells of the immune system [13, 14, 15, 16]. It has been implicated in those tissues in many physiological processes related to different aspects of cellular development, differentiation and apoptosis. After ligand binding, PPARγ heterodimerizes with the retinoid X receptor (RXR) and binds to specific PPAR-respond-elements (PPRE) within the promoter of its target genes. Apart from its function in normal tissues, different studies have shown that the activation of PPARγ leads to growth arrest, differentiation and apoptosis in a variety of tumors such as in breast cancer and prostate cancer [14, 15]. We and others recently showed that PPARγ activation might inhibit the secretion of cytokines growth factors, such as IL-6, by interfering with the NF-κB and MAPK signaling pathways [17, 18, 19].

A number of natural ligands of PPARγ have been identified, including eicosanoid derivates such as prostaglandins (15d-PGJ2) and polyunsaturated fatty acids. Synthetic high affinity agents are the group of thiazolidinediones (TZD), such as troglitazone and rosiglitazone, which are used primarily as insulin-sensitizing antidiabetic agents [20, 21].

Apart from their antidiabetic activity TZDs have potent anti-inflammatory effects and are of special interest as potential anti-cancer agents, since they induce growth arrest and apoptosis in a broad spectrum of tumor cells [14,15].

Ligand activation of PPARγ induces ubiquitination and subsequent degradation of this receptor by the proteasome, indicating that proteasome inhibitors could increase the PPARγ activity in malignant cells. Bortezomib is a synthetic, highly specific inhibitor of the active 20S proteasome. We and others showed that treatment of different tumor cells or dendritic cells with bortezomib or PPARγ ligands interferes with intracellular pathways, such as the NF-κB or MAPK, and leads to inhibition of growth factors including IL-6 [18, 19, 22]. Interestingly, IL-6 was shown to be an autocrine secreted growth factor for RCC. Another common aspect of PPARγ ligands and proteasome inhibition is the induction of endoplasmatic stress (ER stress). ER stress occurs when Ca^{2+} homeostasis is disrupted, protein glycolisation is inhibited or misfolded proteins are accumulated [23]. The PPARγ agonist, prostaglandin J_2, induces ER stress by initiating the unfolded protein response (UPR) which leads to attenuation of protein synthesis and other cytoprotective mechanisms [24]. Bortezomib, however, activates ER stress by accumulation of misfolded proteins [25].

The aim of our study was to analyze the effects of PPARγ agonists such as troglitazone on three different RCC cell lines (A498, CAKI-2, MZ1774). We found that ligand induced activation of PPARγ impedes the secretion of growth and angiogenic factors and affects renal cell carcinoma cells at multiple levels by interfering in different constitutively activated intracellular pathways such as NF-kB, HIF1α, and the mitogen-activated protein (MAP) kinase. We also observed that the combination of troglitazone with the proteasome inhibitor bortezomib increases the effects of troglitazone and sensitizes cell lines resistant to troglitazone to undergo apoptosis by increased induction of ER-stress.

Material and methods

Cell culture and reagents. RCC cell lines A498, CAKI-2 and MZ1774 were cultured in RP10 media (RPMI 1640 supplemented with 10% inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin) all purchased from Gibco-BRL (Grand Island, NY) at 37°C in a 5% CO_2 containing atmosphere. Trypsin was purchased from Gibco-BRL as well. Troglitazone was obtained from Biomol GmbH (Hamburg, Germany). Bortzezomib was obtained from the pharmacy of the University Clinics in Tübingen, and tunicamycin from Sigma

(Steinheim, Germany). GW9662 was purchased from Calbiochem (Darmstadt, Germany).

Cytokine determination. Cells were incubated at 2 x 10^6 cells in 10 ml medium and treated with different concentrations of troglitazone or bortezomib, as indicated. Supernatants were stored at –20°C and subsequently analyzed for contents of VEGF (R&D Systems) and IL-6 (Beckman Coulter) according to the manufacturer's instructions.

Measurement of apoptosis. Apoptosis was measured by the method of Nicoletti [26]. 1 x 10^5 cells were incubated in a hypotonic buffer containing 1 % sodium citrate, 0.1 % Triton X-100, 50 µg of propidium iodide per ml and subsequently measured by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest analysis software. The hyplodiploid cells to the left of the 2N peak were considered as apoptotic.

PAGE and Western Blotting. Nuclear extracts were prepared from RCC as described previously [27]. For the whole-cell lysates, a buffer containing 1 % Igepal, 0.5 % sodium-deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/mL aprotinin, and 1 mM sodium-orthovanadate was used.
Protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce, Perbio Science, Bonn, Germany). For detection of nuclear localisation of NF-kB family members and HIF1α about 20µg of protein was separated on a polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with TBST containing 0.25 % Slimfast solution for 1 h. Subsequently the blot was probed with a monoclonal antibody against relB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or against HIF1α (BD Biosciences, San Jose, CA, USA). For analysis of the activation status of ERK1/2, PPARγ, Caspase-3, PARP-1, BiP and Caspase-4, 20–40µg whole cell lysates were separated on a polyacrylamide gel, and transferred on a nitrocellulose membrane. The blot was probed with monoclonal antibodies against phosphoERK1/2, ERK, phospho-STAT3, STAT3 (Cell signaling, Danvers, MA, USA), PPARγ, Caspase-3, BiP, PTEN, Bcl-X (BD Biosciences, San Jose, CA, USA), Caspase-4 (Stressgen Bioreagents, Ann Arbor, MI, USA), PARP-1 (Axxora, Lörrach, Germany), p-JAK1 (Upstate, Temecula, CA, USA), survivin (R&D Systems, Minneapolis, MN, USA), GAPDH (HyTest Ltd., Turku, Finland), p-mTOR, Mcl-1 or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. Protein bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Buckinghamshire, UK).

RT-PCR

Total RNA was isolated from cells using the QIAGEN Rneasy mini anion-exchange spin columns (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. 1µg of total RNA was subjected to a 20µl cDNA synthesis reaction using SuperScript RTII (Invitrogen). Oligo(dT) was used as a primer. 1µl cDNA was used in a volume of 15µl to perform PCR amplification. Primers used were HIF1α fw gaagcgcaatcaagg and rv tgggtaggagagatgc. The PCR temperature profiles were as follows: 5 min pretreatment at 94 °C and 35 cycles at 94°C for 30 seconds, annealing at 60°C for 30 seconds and 72°C for 30 seconds. For β_2-microglobulin, primers and conditions were used as described previously [28].

Fluorimetric assay of caspase-3 activity. Caspase activity was determined from cytosolic extracts of 10^5 cells by the cleavage of the fluorogenic caspase substrate DEVD-AMC [29, 30]. In brief, for fluorimetric assay of caspase activity, cytosolic cell extracts were prepared by lysing cells in a buffer containing 0.5 % NP-40, 20 mM HEPES pH 7.4, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin and 1 mM phenylmethyldisulfonil fluoride (PMSF). Cell lysates were incubated with 50 µM of the fluorogenic substrate DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin, Bachem, Heidelberg, Germany) in 200 µl buffer containing 37.5 mM HEPES pH 7.3, 75 mM NaCl, 7.5 % sucrose, 0.075 % CHAPS and 10 mM DTT. The release of aminomethylcoumarin was determined by fluorometry using an excitation length of 360 nm and an emission wave-length of 460 nm.

Results

Activation of PPARγ reduces IL-6 secretion. To determine whether PPARγ is expressed in renal cell carcinoma we performed a gene expression analysis using primary tissue samples of RCC and normal kidney. We found that PPARγ was expressed at high levels in all analyzed tumor samples (data not shown). We recently showed that PPARγ ligands can inhibit the secretion of cytokines such as IL-6 in treated cells [17].

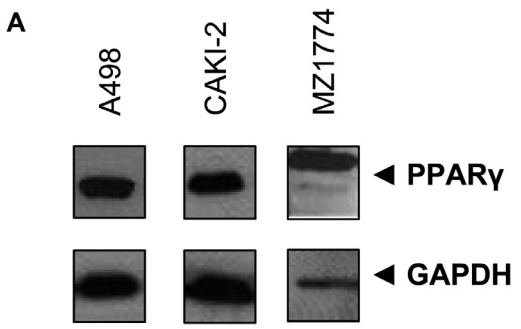

Figure 1. PPARγ is expressed in the renal cell carcinoma cell lines A498, CAKI-2 and MZ1774 (A).Treatment of renal cell carcinoma (RCC) cell lines with troglitazone inhibits secretion of autocrine growth factors. The RCC cell lines A498, CAKI-2 and MZ1774 were treated with increasing concentrations of troglitazone ranging from 1 to 20 μM for 48 hours. The secretion of IL-6 was measured in the culture supernatants using a commercially available ELISA. The mean values of three independent experiments with SD are shown.

Interestingly, IL-6 is a cytokine that acts as an autocrine growth factor in RCC and was shown to be overexpressed in many RCC cell lines [31].
To analyze the effect of PPARγ on the secretion of IL-6 we treated the RCC cell lines with different concentrations of troglitazone, a synthetic PPARγ agonist with a high affinity and specificity for the receptor, and measured the level of the cytokine secreted in the supernatants. The expression of PPARγ in the used RCC cell lines is shown in Figure 1A. Exposure of cells to troglitazone inhibited the production of IL-6 in a concentration-dependent manner (Fig. 1B).

Effect of troglitazone on intracellular signaling cascades. Secretion of growth factors and cytokines by tumor cells is mediated via different signal transduction pathways such as the STAT, MAPK or PI3K. Furthermore, in renal cell carcinomas different intracellular pathways such as the MAP kinase pathway are often constitutively activated due to overexpression of growth factors or mutations in the Raf and Ras kinases. Signaling via the Raf/ERK kinase pathway activates many genes that mediate tumor angiogenesis and survival [32].
We next analyzed the involvement of the MAPK in the inhibitory effects of troglitazone. As shown in Figure 2A, PPARγ activation resulted in reduced phosphorylation of ERK1/2.
This indicates that the activation of PPARγ affects the Raf/ERK pathway in RCC leading to inhibition of growth factor secretion.
To determine if this effect is PPARγ dependent we treated the cells in combination with the PPARγ inhibitor GW9662. We found that GW9662 abolished the reduced phosphorylation of ERK1/2 in CAKI-2 cells, indicating a PPARγ dependent mechanism (Fig. 2B).
In the next series of experiments we addressed the influence of the PPARγ ligand on the expression and phosphorylation of molecules involved in the PI3K and JAK/STAT signaling. There was no difference in the activation status and expression of PTEN or mTOR upon the treatment of cells with different troglitazone concentrations (Fig. 2C). We could only find a slightly reduced phosphorylation of STAT3 and JAK1 in CAKI-2 and MZ1774 cells (Fig. 2D). These results further exclude a possible toxic effect of troglitazone.

NF-κB activation is inhibited by troglitazone. It has been proposed that NF-κB is constitutively active in various RCC cell lines and that its activation is especially increased in metastatic RCC. It is involved in the signaling downstream of the MAP and PI3 kinases and induces many genes important for secretion of growth factors, tumor proliferation, survival and angiogenesis. [33, 34]. We and others recently demonstrated that PPARγ ligands might interfere with the NF-κB signaling pathways by reducing the nuclear expression of its family members such as relB [17, 35, 36, 37].
Treatment of RCC cells with troglitazone resulted in a reduced nuclear expression of relB, a NF-κB family

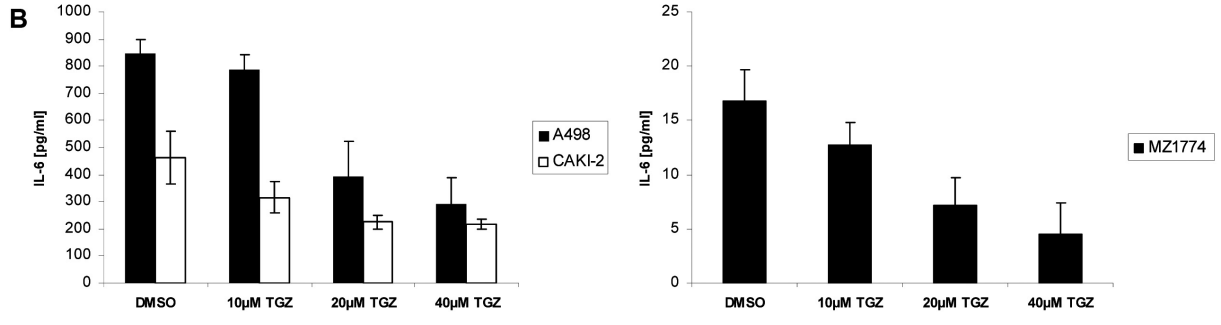

Figure 1. *(continued)*

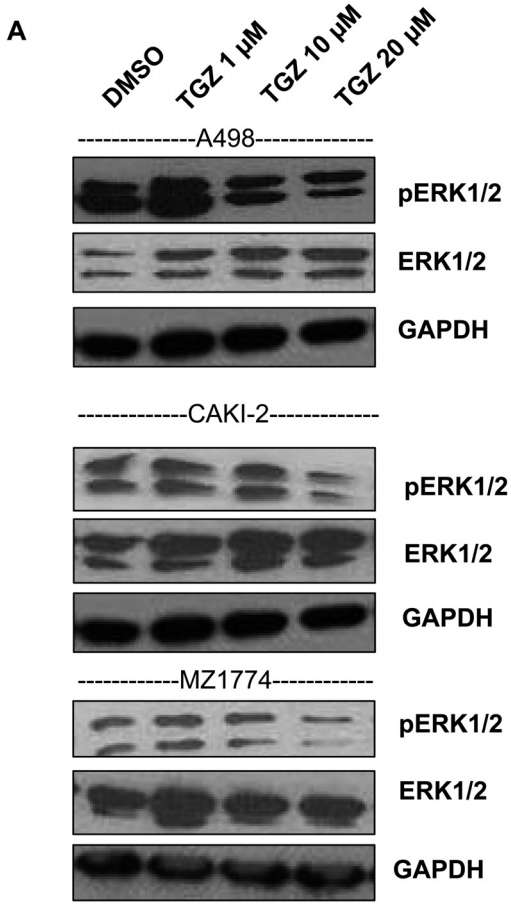

Figure 2. Activation of PPARγ inhibits intracellular signaling casacades. The RCC cell lines were treated with increasing concentrations of troglitazone for 48 hours. A Western Blot analysis of cell lysates shows the reduced phosphorylation of the MAPK ERK1/2. As a loading control, ERK1/2 and GAPDH were used (*A*).

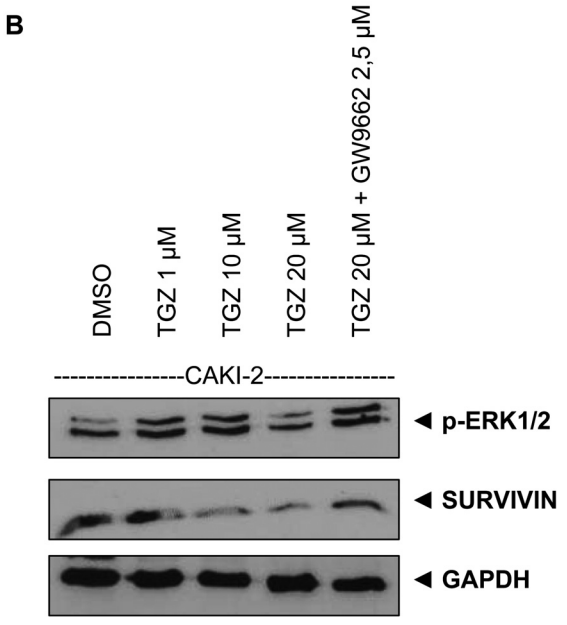

Figure 2. *(continued)* Effects of troglitazone were inhibited by the use of PPARγ inhibitor GW9662 (*B*).

member, with the strongest inhibition found in CAKI-2 cells (Fig. 3A). In order to exclude differences in protein loading Ponceau S staining was performed as described previously.

Treatment with troglitazone leads to reduced expression of HIF1α in the nucleus. As mentioned above, HIF1α is an important transcription factor that plays a crucial role in tumor proliferation and angiogenesis due to upregulation of growth factors such as IL-6, TGFβ, PDGF, and VEGF. HIF1α is constitutively activated in many RCC cell lines due to a mutation of the E3 ubiquitin ligase VHL. To analyze the expression of HIF1α in nuclear extracts we conducted a Western Blot analysis of the A498, CAKI-2 and MZ1774 RCC cell lines treated with different concentrations of troglitazone.

In CAKI-2 and MZ1774 cells we observed a reduced expression of HIF1α in nuclear extracts with increasing concentrations of troglitazone. This represents a novel mechanism in the action of PPARγ ligands. Especially in CAKI-2 cells, the expression of HIF1α was profoundly inhibited when the cells were treated with 20 µM of troglitazone (Fig. 3B). This inhibition is not a result of a lower transcription rate, as there was no downregulation of HIF1α on mRNA level as analyzed by RT-PCR (data not shown).

As already reported by others we found no expression of HIF1α protein in A498 cells at all [38]. On the transcriptional level, however, we detected HIF1α mRNA, which showed no regulation when exposed to troglitazone (Fig. 3C).

Troglitazone induces apoptosis in RCC cell lines. To investigate whether the observed effects of troglitazone are due to apoptosis induction we analyzed the apoptotic rate of cells exposed to troglitazone.

We found that A498 cells were highly sensitive and showed an apoptotic rate of 63 % after treatment with 20 µM/ml troglitazone. In contrast, CAKI-2 and MZ1774 cells were hardly affected and the viability of the cells was reduced by only 5 % as compared to DMSO treated cells (Fig. 4A). To confirm these results we determined the caspase-3 activity in cells treated with the PPARγ agonist. Activation of caspase-3 is a distinct characteristic of cells undergoing apoptosis. As shown in Figure 4B, only in A498 cells could a considerable increase of caspase-3 activity be detected. To further analyze the different apoptotic effects of TGZ on RCC cell lines we monitored the

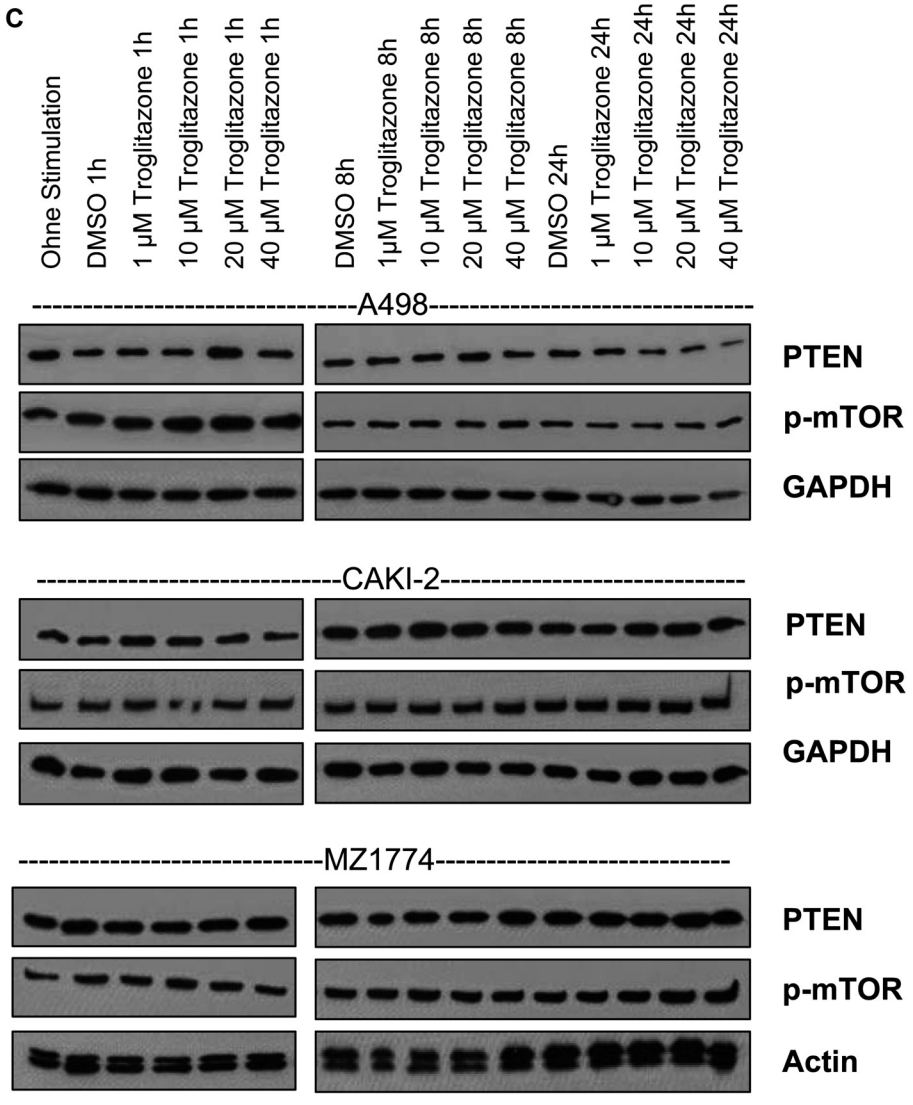

Figure 2. *(continued)* A time course experiment shows the effect on the PI3K pathway (*C*).

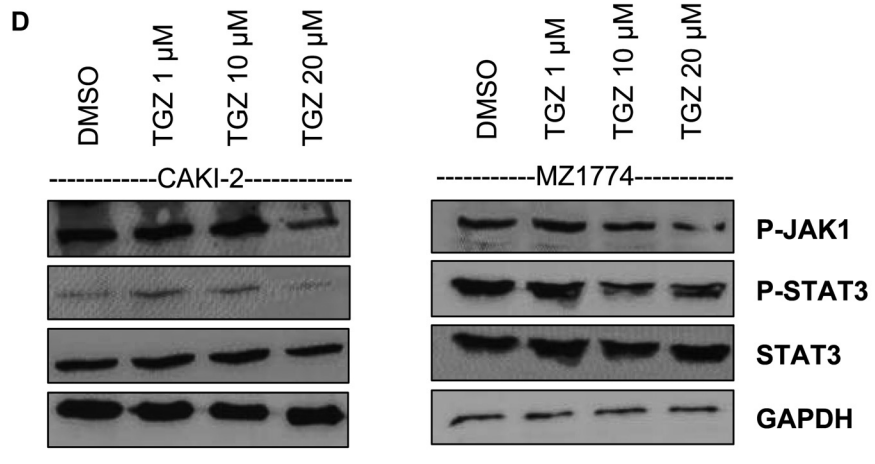

Figure 2. *(continued)* Troglitazone treatment had no effect on the JAK/STAT pathway (*D*).

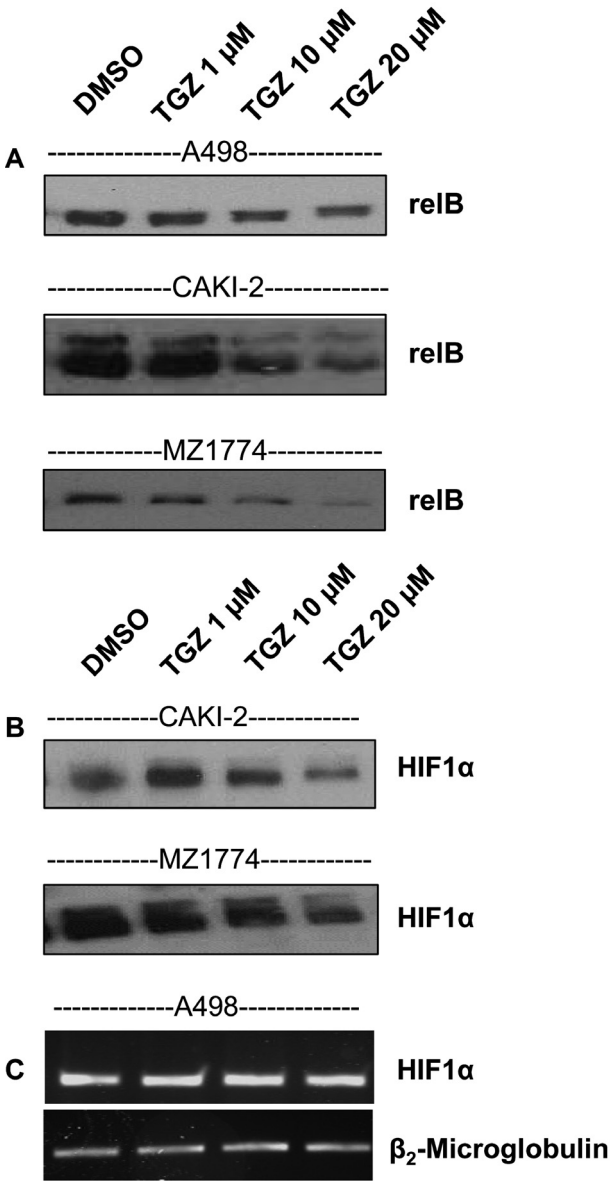

Figure 3. Troglitazone reduces nuclear expression of transcription factors. The RCC cell lines A498, CAKI-2 and MZ1774 were treated with increasing concentrations of troglitazone ranging from 1 to 20 µM for 48 hours. The nuclear localization of NF-κB (A) and HIF1α (*B*) was analyzed in nuclear extract using a Western Blot analysis. Ponceau S staining was used to ensure equal protein loading. The transcription of HIF1α in A498 cells was analyzed via RT-PCR (*C*). β_2-microglobulin was used as a loading control. One representative experiment out of three is shown.

expression of the anti-apoptotic molecules such as Bcl-x, Mcl-1 and survivin. We found that Mcl-1 and survivin were downregulated in a dose dependent manner in all tested cell lines. Bcl-x expression was reduced only in A498 and CAKI-2 cells (Fig. 4C). In order to determine if these effects occur due to PPARγ activation we analyzed the expression of survivin in CAKI-2 cells. We found that treatment of cells with GW9662 inhibited the effects of troglitazone on survivin expression (Fig. 2B). These effects are not due to a modified expression of the PPARγ molecule itself, as neither in the cytosol nor in the nucleus could differences in the expression be found after exposure to troglitazone (Fig. 4D). Interestingly, the highest downregulation of Mcl-1 and survivin was observed in both cell lines (CAKI-2 and MZ1774) that were almost completely resistant to apoptosis induction. According to our results there was no correlation between apoptosis induction, the expression of anti-apoptotic molecules or the inhibition of MAPK and NF-κB pathways.

Synergistic effects of troglitazone and bortezomib. In order to analyze a possible effect of proteasome inhibition on the action of PPARγ agonists we treated CAKI-2 and MZ1774 cells that were resistant to apoptosis induction by troglitazone with the proteasome inhibitor bortezomib in addition to troglitazone. Bortezomib is a specific inhibitor of the 20S proteasome and it was shown that regulation via ubiquitination is essential for the function of many proteins involved in tumorgenesis, such as the cyclins, NF-κB or p53. We presumed that proteasome inhibition can increase the effect of PPARγ ligand, as both compounds were shown to interfere with the NF-κB and MAPK signaling and to induce endoplasmatic stress [17, 24, 25, 35].

Therefore, we pretreated the cells with bortezomib before adding troglitazone to the culture medium. This sequence of compounds was important because, when the cells were treated with troglitazone first, we could not detect any synergistic effect (data not shown).

As shown in Figure 5A, incubation with troglitazone and bortezomib profoundly increases the apoptotic rate compared to treatment with the two compounds alone. This effect could also be seen in downregulation of the expression of survivin and PARP-1, especially in CAKI-2 cells (Fig. 5B).

Moreover, we observed that the ability of troglitazone to decrease phosphorylation of ERK1/2 was clearly increased by pretreatment with bortezomib (Fig. 5C). Furthermore, we analyzed the effect of treatment with the two compounds on the expression of HIF1α. Here we found that the nuclear expression of HIF1α in MZ1774 was profoundly decreased when incubated with troglitazone and bortezomib (Fig. 5D). Finally, in line with these results as shown in Figure 5D, we found a 3 to 4-fold decreased secretion of IL-6 in CAKI-2 cells treated with the combination of both compounds compared to cells treated with troglitazone alone (Fig. 5E).

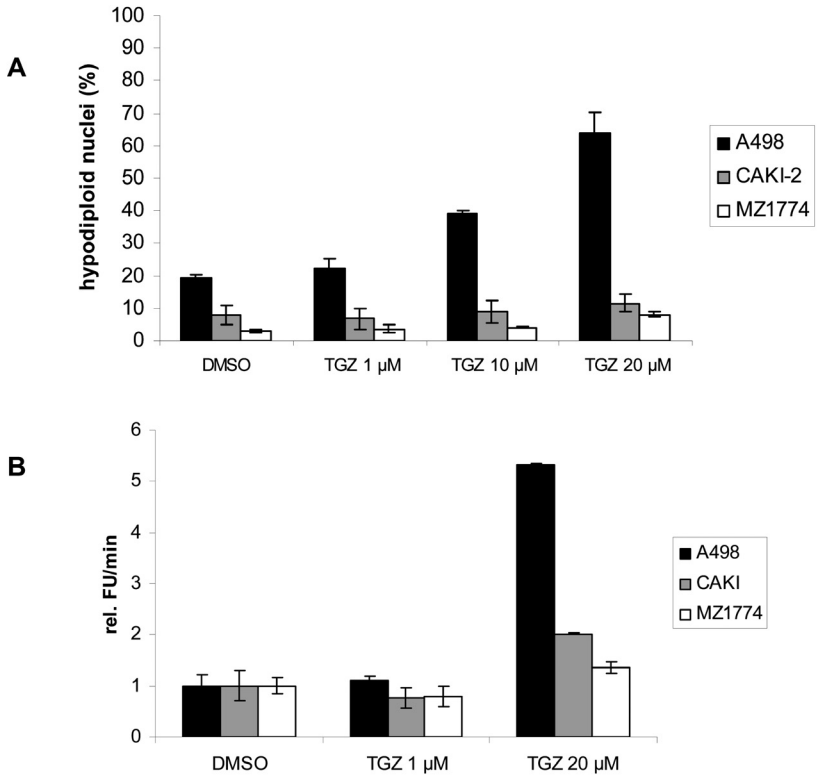

Figure 4. RCC cell lines demonstrate different sensitivity to apoptosis induction by PPAR-gamma ligands. RCC cell lines were incubated with varying concentrations of troglitazone for 48 hours and apoptosis was determined by flow-cytometric assessment of apoptotic hypodiploid nuclei (*A*). In addition, caspase-3 activity (*B*) was analyzed from cytosolic extracts of cells by the cleavage of the fluorogenic caspase substrate DEVD-AMC.

Bortezomib potentiates the ER stress induced by PPARγ ligand

Disruption of Ca^{2+}-homeostasis, inhibition of protein glycolisation or accumulation of unfolded proteins can challenge the function of the endoplasmatic reticulum (ER), which results in ER stress [39]. It is known that PPARγ ligands induce ER stress by inducing the unfolded protein response (UPR) in β-cells [24]. Bortezomib also induces ER stress by accumulation of misfolded proteins [25, 40]. The UPR leads to different cytoprotective mechanisms or to ER-induced apoptosis.

Therefore, we analyzed the effects PPARγ activation and proteasome inhibition on the stress induced ER chaperone BiP and Caspase-4, which is localized in the ER and mediates ER-induced apoptosis. As positive control we used tunicamycin, which inhibits glycolisation of proteins. We found that BiP, which is induced during ER-stress, was strongly upregulated by the combined treatment. Caspase-4 was cleaved into its active form when treated with bortezomib or troglitazone and the cleavage was increased when incubated with both compounds (Fig. 6), thus confirming that activation of PPARγ and inhibition of the proteasomal degradation can increase the effects of PPARγ agonists on the inhibition of tumor growth and induction of ER-stress mediated apoptosis.

Discussion

Our present study indicates that the activation of PPARγ has an impact on multiple cellular functions in renal cell carcinoma cell lines. We found that troglitazone interferes with several intracellular signal cascades including MAPK, NF-κB and HIF1α, resulting in reduced secretion of growth factors such as IL-6. Furthermore, we show that the activity of PPARγ agonists was increased by the proteasome inhibitor bortezomib that can overcome the resistance of RCC cells to these drugs. According to our results, a combination of these agents might represent a novel and interesting approach to treat patients with RCC that should be addressed in Phase I clinical trials.

A frequent event in RCC is the constant activation of intracellular pathways that induce the expression of genes important for tumor proliferation, survival and angiogenesis. Signal cascades that are known to play a central role in RCC development and metastatic spread are the NF-κB, the MAP kinase and the HIF pathways [32, 34]. NF-κB is normally inactivated in the cytoplasma bound to its inhibitory protein I-κB. It is activated when growth factors like IL-6 or TGF-β bind to specific receptors, which leads to proteasomal degradation of I-κB and nuclear translocalisation of the NF-κB complex. In the renal cell carcinoma, as in many other tumors, the inactivation of NF-κB is abolished due to mutations in proteins downstream of

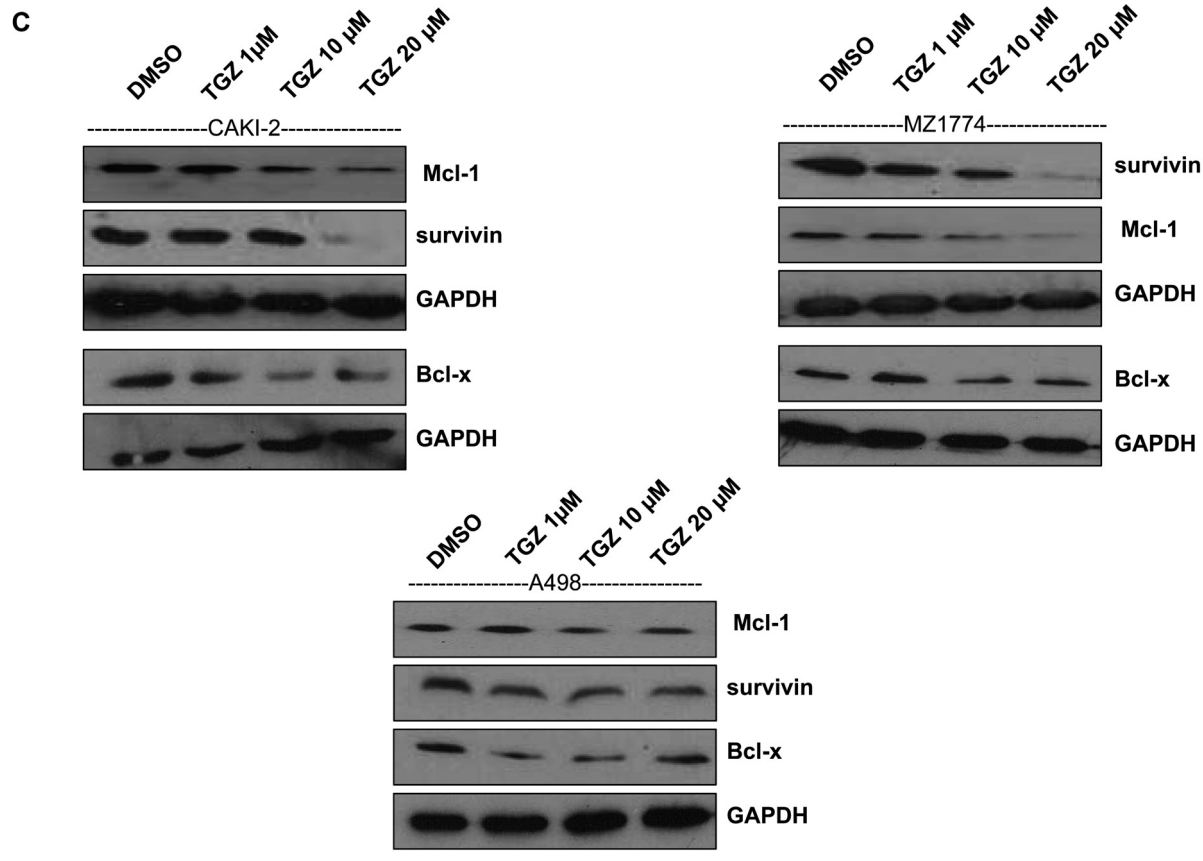

Figure 4. *(continued)* The expression of the anti-apoptotic proteins survivin, Mcl-1 and Bcl-X_L were analyzed in cell lysates via Western Blot analysis (*C*).

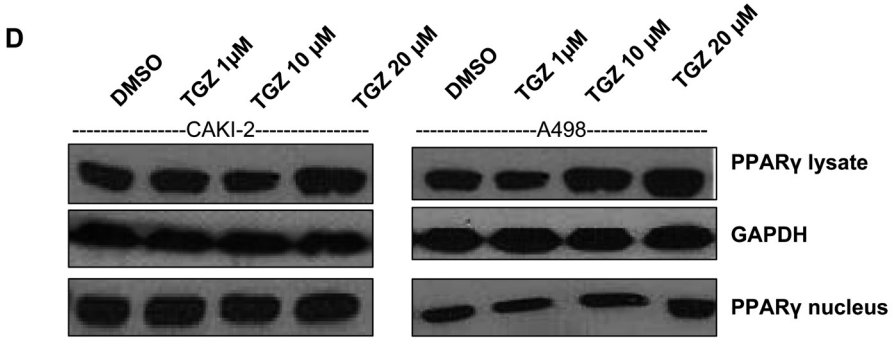

Figure 4. *(continued)* Furthermore, the expression of PPARγ was measured in cytosolic lysates and the nucleus, shown for CAKI-2 and A498 cells (*D*). The mean values of triplicate cultures with SD are shown.

the pathway. Several reports have shown that ligand induced activation of PPARγ inhibits the expression of the NF-κB family members relA and relB, and that the activation of PPARγ downregulates NF-κB in epithelial cells [13, 41]. Furthermore it was demonstrated that PPARγ physically interacts with the NF-κB members p50 and p75 [35, 36, 37]. We found that troglitazone inhibited the nuclear translocalisation of relB, a key component of the anti-apoptotic signaling pathway.

We subsequently analyzed the effect of troglitazone on the MAP kinase pathway. This cascade consists of a protein kinase cascade that is activated in response to many growth factors or hormones that influence cell proliferation and differentiation. In the renal cell carcinoma the MAP kinase pathway is constitutively activated due to mutations in kinases downstream of ERK1/2, such as MEK or the Raf and Ras kinases [32]. We observed that the phosphorylation of ERK1/2 is profoundly reduced in RCC cells upon treatment with PPARγ ligand troglitazone. The decreased phos-

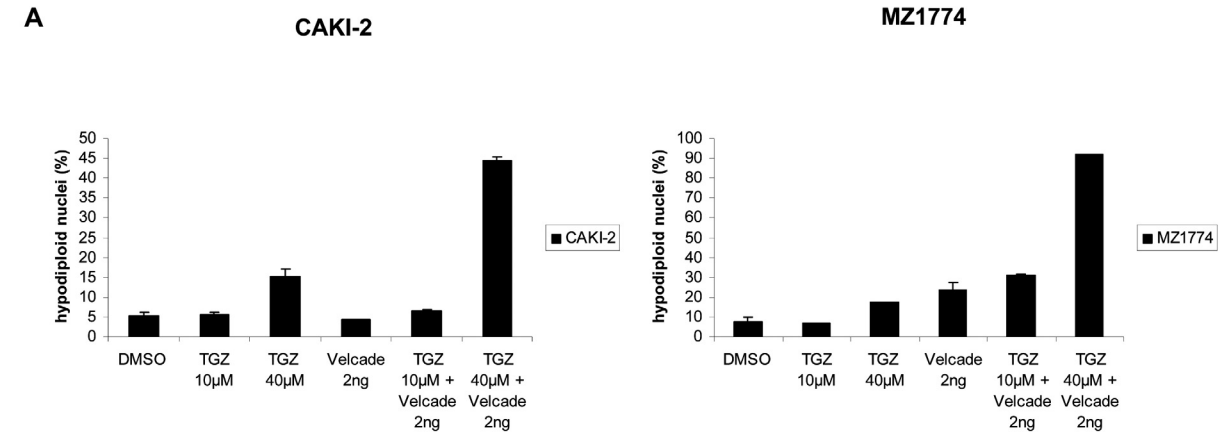

Figure 5. Synergistic effects of PPARγ activation and proteasome inhibition. The RCC cell lines were either treated with increasing concentrations of troglitazone or with 2 ng bortezomib alone. In addition, cells were first pretreated with the proteasome inhibitor bortezomib for 24 hours and then incubated with troglitazone for 48 hours. Apoptosis was determined by flow-cytometric assessment of apoptotic hypodiploid nuclei (*A*).

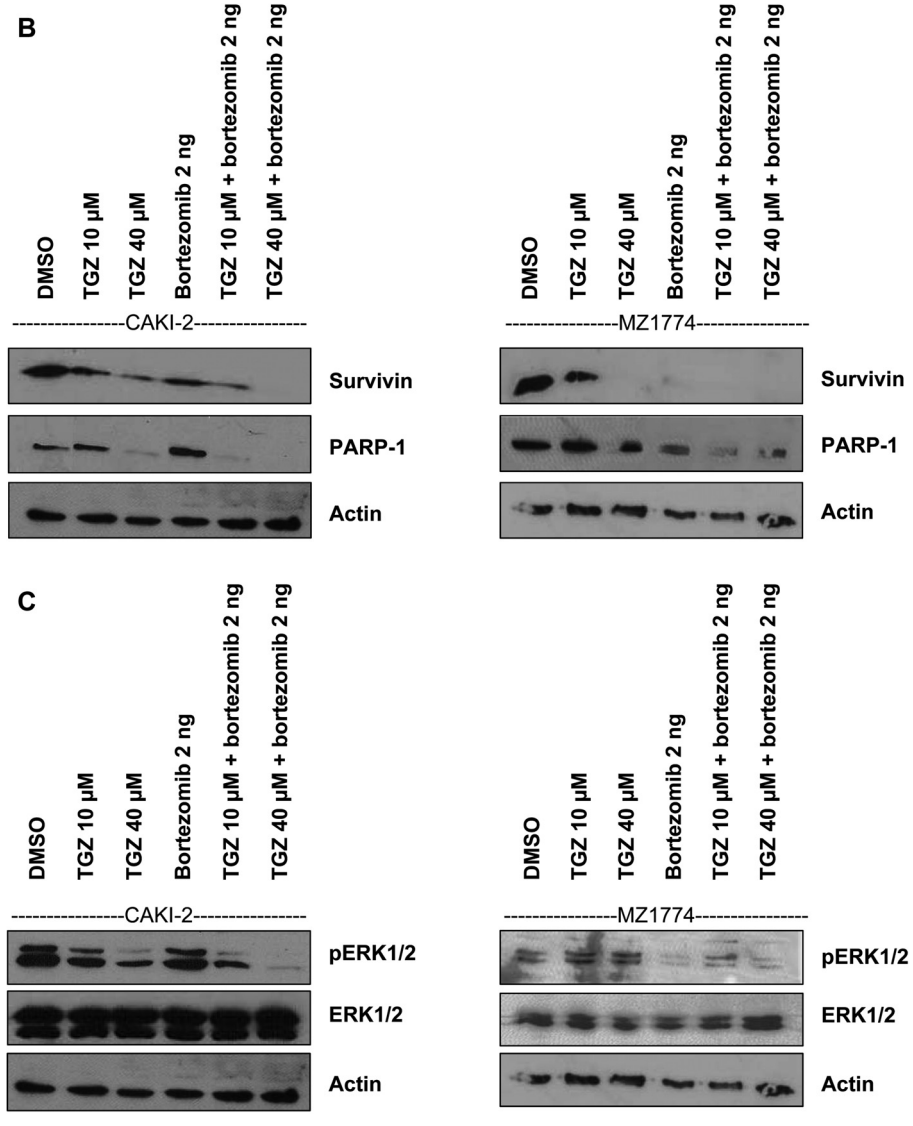

Figure 5. *(continued)* The expression of the anti-apoptotic protein survivin and PARP-1 (*B*), the phosphorylation of the MAPK ERK1/2 (*C*) and the nuclear expression of HIF1α (*D*) were analyzed in cell-lysates or nuclear extracts. Additionally the secretion of the growth-factor IL-6 was measured in the culture supernatants (*E*) using an ELISA assay.

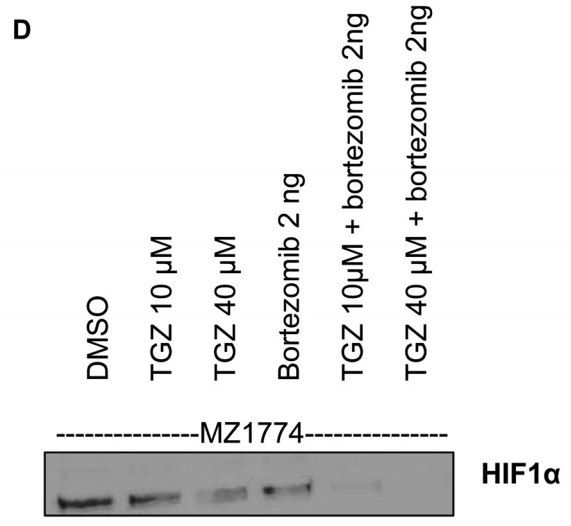

Figure 5. *(continued)*

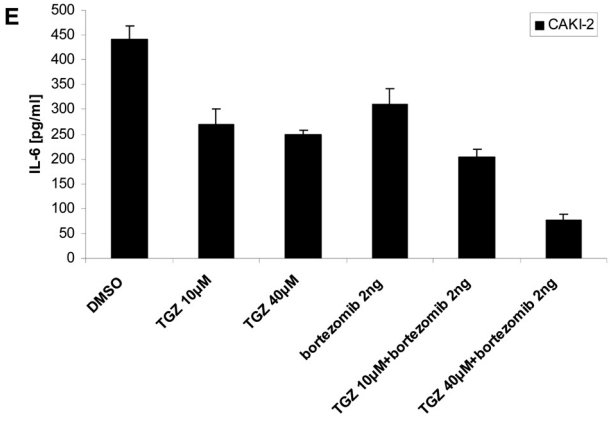

Figure 5. *(continued)*

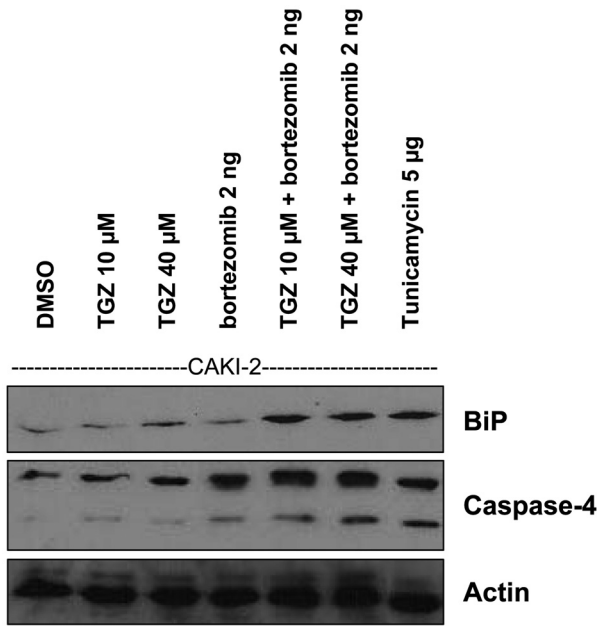

Figure 6. Coincubation of troglitazone and bortezomib increases ER-stress induction. RCC cell lines were treated with varying concentrations of troglitazone or with 2 ng bortezomib alone for 48 hours. Additionally, the cells were pretreated with 2 ng bortezomib and then with troglitazone for 48 hours. The expression of the ER chaperone BiP and the activation of caspase-4 were determined in cell lysates using a Western Blot analysis.

phorylation of ERK is different depending on the cell line and concentration used. It was already shown that PPARγ activation has a different effect on MAPK signaling, depending on the cell type and the ligand used. PPARγ ligands activate ERK in rat liver epithelial cells and neuroblastoma cells and downregulate ERK phosphorylation in pancreatic cancer cells [42, 43, 44, 45]. The important role of MAPK in apoptosis and cell survival and the different sensitivity of the RCC cell lines towards troglitazone could explain the distinct downregulation of phospho-ERK. We also showed that this effect is PPARγ dependent, as there was no reduction of ERK phosphorylation when the PPARγ inhibitor GW9662 was used. In many cancers, including RCC, PTEN is mutated and loses its phosphatase activity [46]. In normal cells it regulates the activation of Akt/PI3K by dephosphorylating the adaptor protein SHC. Recent research has identified two putative binding sites for PPARγ in the promoter of PTEN and showed that PPARγ ligands may increase PTEN expression in murine and in human inflammatory cells and tumor cell lines [47, 48]. In order to analyze this effect we conducted time course experiments over 24 hours. However, we could not find a regulation of PTEN in RCC cells at any point. (Fig. 2C). The constitutive or defective activation of the JAK/STAT pathway often occurs in primary tumors and is important for tumor proliferation and survival. JAK1 is a receptor for the autocrine growth factor IL-6 [49, 50, 51].We, however, only found a slight reduction of JAK1 or STAT3 phosphorylation after troglitazone treatment. Interestingly, CAKI-2 cells were not phosphorylated effectively at all (Fig. 2D).

HIF1α is a major trigger for survival and proliferation of renal cell carcinoma cells. Due to a mutation of the VHL tumor suppressor gene that can be found in more than 70 % of RCC, HIF1α accumulates in tumor cells. This leads to an increased secretion of growth and angiogenic factors such as IL-6, VEGF and PDGF, resulting in tumor growth, blood vessel formation and hematogenic spread [8]. We observed that PPARγ ligand TGZ profoundly reduces the nuclear expression of HIF1α in CAKI-2 and MZ1774 cells. The accumulation seems not to depend only on a mutation in the VHL tumor suppressor gene, since CAKI-2 cells

have a wild-type VHL gene [52]. Blocking HIF1α function by PPARγ agonists is a new and important observation and represents a novel mechanism by which these compounds can inhibit tumor growth and establish metastatic disease. One of the growth factors that was shown to be secreted by RCC and promote the expansion and proliferation of malignant cells is IL-6. IL-6 is a multipotent cytokine which is involved in numerous biological activities such as regulation of proliferation and differentiation and inducing the expression of acute-phase proteins [48]. But it is also involved in the pathophysiology of various neoplasias such as Non-Hodgkin Lymphomas, multiple myeloma, and renal cell carcinoma [53, 54]. Therefore, we analyzed the expression of IL-6 upon treatment of cells with troglitazone. We found a reduced secretion of IL-6 after activation of PPARγ in all treated cell lines. This is probably due to the observed inhibition of the MAPK and NF-κB signaling as well as the reduced expression of HIF1α.

We next wanted to determine the role of apoptosis in the observed effects by troglitazone. Activation of PPARγ is known to induce apoptosis in many different cancers such as colon cancer [55], lung cancer and breast cancer [14]. There are conflicting reports as to how apoptosis is induced by troglitazone. Chinetti et al. found that PPARγ activation inhibits the transcriptional activity of the nuclear factor-κB p65/relA subunit [13]. Shimada et al. observed that activation of PPARγ leads to a downregulation of anti-apoptotic genes [16]. We found that troglitazone induced apoptosis in a dose-dependent manner in A498 cells while CAKI-2 and MZ1774 cells were not affected. They show however, a similar downregulation of HIF1α, IL-6, pERK1/2 and relB. Furthermore, we found that the anti-apoptotic factors, such as Mcl-1 or surviving, are downregulated after exposure to troglitazone in these cells. Mcl-1 is an anti-apoptotic Bcl-2 family member and its elimination has been proposed to be required for apoptosis induction by genotoxic stimuli [56]. Survivin belongs to the family of inhibitors of apoptosis proteins (IAP) and high-level expression of the protein characterizes an aggressive form of RCC [57].

In order to overcome the resistance to induction of apoptosis in CAKI-2 and MZ1774 cells we coincubated the cells with the proteasome inhibitor bortezomib. Bortezomib is a specific inhibitor of the chymotryptic-like site of the 20S proteasome and was shown to induce apoptosis and growth arrest in a broad variety of tumors. Proteasomal degradation is essential for many proteins involved in tumorgenesis, such as the cyclins, NF-κB or p53. Interestingly, PPARγ is also degraded via the proteasome after ligand-activation. It was shown that coincubation of PPARγ agonists and proteasome inhibitors increase PPRE transactivity and RXR-α expression and therefore could increase the activity of PPARγ [58]. We demonstrated that pretreatment of the RCC cell lines with bortezomib resulted in increased induction of apoptosis by PPARγ ligands even in cells resistant to PPARγ activation alone. This effect was only observed when cells were pretreated with bortezomib. This indicates that a synergistic effect can only be obtained when the proteasome is inhibited prior to PPARγ activation. We furthermore found that coincubation of bortezomib and troglitazone leads to a stronger inhibition of MAPK signaling and HIF1α nuclear expression than treatment with each compound alone. In line with these results, coincubation of the cells with bortezomib and troglitazone results in an increased reduction of IL-6 secretion as compared to treatment with both drugs alone.

Previous reports showed that PPARγ agonists can activate the unfolded protein response (UPR) and thus initiate a variety of cytoprotective mechanisms of the ER that can ultimately lead to ER mediated apoptosis [24]. It was also demonstrated that bortezomib induces ER stress by accumulation of misfolded proteins. Bortezomib does not activate the UPR but leads to the expression of ER chaperones, efflux of calcium and activation of caspase-4, which are typical characteristics of ER-stress [25]. In our experiments we found that combined treatment of RCC cells results in an increased induction of ER stress as shown for the upregulation of ER chaperone BiP and activation of ER localized caspase-4.

Our work demonstrates that PPARγ activation might represent an interesting approach for treatment of renal cell carcinoma patients when combined with the proteasome inhibitor bortezomib.

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