Thiazolidenediones Mediate Apoptosis in Prostate Cancer Cells in Part through Inhibition of Bcl-xL/Bcl-2 Functions Independently of PPARγ

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Abstract

Certain members of the thiazolidenedione family of the peroxisome proliferator-activated receptor γ (PPARγ) agonists, such as troglitazone and ciglitazone, exhibit antitumor effects; however, the underlying mechanism remains inconclusive. This study shows that the effect of these thiazolidenediones members on apoptosis in prostate cancer cells is independent of PPARγ activation. First, close structural analogues of thiazolidenediones, whereas devoid of PPARγ activity, retain the ability to induce apoptosis with equal potency. Second, both PC-3 (PPARγ-expressing) and LNCaP (PPARγ-deficient) cells are sensitive to apoptosis induction by troglitazone and its PPARγ-inactive analogue irrespective of their PPARγ expression status. Third, rosiglitazone and pioglitazone, potent PPARγ agonists, show marginal effects on apoptosis even at high concentrations. Evidence indicates that the apopotic effect of troglitazone, ciglitazone, and their PPARγ-inactive analogues 5-[4-(6-hydroxy-2,3,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene]-2,4-thiazolidinone-dione (Δ2-TG) and 5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidin-2,4-dione, respectively, is in part attributable to their ability to inhibit the anti-apoptotic functions of Bcl-xL and Bcl-2. Treatment of PC-3 cells with troglitazone or Δ2-TG led to reduced association of Bcl-2 and Bcl-xL with Bak, leading to caspase-dependent apoptosis. Bcl-xL overexpression protects LNCaP cells from apoptosis induction by troglitazone and Δ2-TG in an expression level–dependent manner. Considering the pivotal role of Bcl-xL/Bcl-2 in regulating mitochondrial integrity, this new mode of mechanism provides a framework to account for the PPARγ-independent action of thiazolidenediones in inducing apoptosis in cancer cells. Moreover, dissociation of these two pharmacologic activities provides a molecular basis to develop novel Bcl-xL/Bcl-2 inhibitors, of which the proof of principle is illustrated by a Δ2-TG analogue with potent in vivo antitumor activities. (Cancer Res 2005; 65(4): 1561-9)

Introduction

Thiazolidenediones, including troglitazone, rosiglitazone, pioglitazone, and ciglitazone, are synthetic ligands of the peroxisome proliferator-activated receptor γ (PPARγ; ref. 1). This family of PPARγ agonists improves insulin sensitivity by increasing transcription of certain insulin-sensitive genes involved in the metabolism and transport of lipids, thus representing a new class of p.o. anti-diabetic agents. More recently, certain thiazolidenediones, especially troglitazone and ciglitazone, have also been shown to inhibit the proliferation of many cancer cell lines that express high levels of PPARγ, including but not limited to those of colon, prostate, breast, and liposarcoma (2). As PPARγ-mediated effects of thiazolidenediones promote the differentiation of preadipocytes, one school of thought attributes the same mechanism to the terminal differentiation and cell cycle arrest of tumor cells (3). However, the PPARγ-activated target genes that mediate the antiproliferative effects remain elusive as genomic responses to PPARγ activation in cancer cells are highly complicated (4). Reported causal mechanisms include attenuated expression of protein phosphatase 2A (5), cyclins D1 and E, inflammatory cytokines and transcription factors (2), and increased expression of an array of gene products linked to growth regulation and cell maturation (4). On the other hand, several lines of evidence indicate that the inhibitory effect of thiazolidenediones on tumor cell proliferation was independent of PPARγ activation. For example, the antitumor effects seem to be structure specific, irrespective of potency in PPARγ activation (i.e., troglitazone and ciglitazone are active, whereas rosiglitazone and pioglitazone are not). Also, there exists a 3-order-of-magnitude discrepancy between the concentration required to produce antitumor effects and that to mediate PPARγ activation. To date, an array of non-PPARγ targets have been implicated in the antitumor activities of troglitazone and/or ciglitazone in different cell systems, which include intracellular Ca2+ stores (6); phosphorylating activation of extracellular signal-regulated kinases (7, 8), c-Jun-NH2 kinase, and p38 (9); up-regulation of early growth response-1 (10), p27Kip1 (11), p21WAF1/CIP1 (12), p53, and Gadd45 (13); and altered expression of Bcl-2 family members (9). However, some of these targets seem to be cell type–specific owing to differences in signaling pathways regulating cell growth and survival in different cell systems.

In light of the potential use of thiazolidenediones in prostate cancer prevention and treatment (14, 15), signaling mechanisms whereby these PPARγ agonists inhibit the proliferation of prostate cancer cells represent the focus of this investigation. We report here the development of novel thiazolidenedione derivatives that lack activity in PPARγ activation but retain the ability to induce apoptosis in two prostate cancer cell lines with distinct PPARγ expression status, suggesting that these two pharmacologic activities are unrelated. More importantly, we show that thiazolidenedione-mediated apoptosis was attributable in part to the inhibition of the anti-apoptotic functions of Bcl-xL and Bcl-2 by disrupting the BH3 domain-mediated interactions with...
proapoptotic Bcl-2 members. From a translational perspective, dissociation of these two pharmacologic activities (i.e., PPARγ activation and Bcl-xL/Bcl-2 inhibition) provides a molecular basis to use 5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)]benzyliden]-2,4-thiazolidine-dione (Δ2-TG) as a scaffold to generate a novel class of Bcl-xL/Bcl-2 inhibitors. Accordingly, we developed a structurally optimized Δ2-TG derivative (TG-88) with high in vivo potency in inhibiting PC-3 tumor growth.

Materials and Methods

Reagents. Troglitazone and ciglitazone were purchased from Sigma (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Rosiglitazone and pioglitazone were prepared from the respective commercial capsules by solvent extraction followed by recrystallization or chromatographic purification. Δ2-TG, 5-[4-(1-methyl-cyclohexylmethyl)]-benzyliden]-thiazolidine-2,4-dione (Δ2-CG), 5-[4-[2-(methyl-pyridin-2-yl)-amino]-ethoxy]-benzyliden]-thiazolidine-2,4-dione (Δ2-NG), 5-[4-[2-(5-ethyl-pyridin-2-yl)-ethoxy]-benzyliden]-thiazolidine-2,4-dione (Δ2-PG; Fig. L4), and TG-88 are thiazolinedenedione derivatives with attenuated or unappreciable activity in PPAR activation, of which the synthesis will be published elsewhere. The identity and purity (≥99%) of these synthetic derivatives were verified by proton nuclear magnetic resonance, high-resolution mass spectrometry, and elemental analysis. For in vitro experiments, these agents at various concentrations were dissolved in DMSO, and were added to cells in medium with a final DMSO concentration of 0.1%. For the in vivo study, TG-88 was prepared as a suspension by sonication in a vehicle consisting of 0.5% methylcellosolve and polysorbate 80 in sterile water. The pan-caspase inhibitor Z-VAD-FMK was purchased from BD Biosciences (Bedford, MA). The Cell Death Detection ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). The Nuclear Extract kit and PPARγ Transcription Factor Assay kit were obtained from Active Motif (Carlsbad, CA). Rabbit antibodies against Bax, Bak, Bid, and cleaved caspase-9 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit antibodies against Bad, cytochrome c, and mouse anti-Bcl-2, anti-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-actin was from ICN Biomedicals, Inc. (Costa Mesa, CA). Goat anti-rabbit IqG-horseradish peroxidase conjugates and rabbit anti-mouse IqG horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA). Hamster anti-human Bcl-2 antibody for immunoprecipitation was purchased from PharMingen (San Diego, CA).

Cell Culture. LNCaP androgen-dependent (p53+/+) and PC-3 androgen- nonresponsive (p53−) prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% carbon dioxide. Preparation of the stable Bcl-xL-overexpressing LNCaP clones B11, B1, and B3 were previously described (16).

Cell Viability Analysis. The effect of individual test agents on cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 6 to 12 replicates. Cells were seeded and incubated in 96-well, flat-bottomed plates in serum-free media for 24 hours and were exposed to various concentrations of test agents dissolved in DMSO (final concentration, 0.1%) in serum-free RPMI 1640 for different time intervals. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μL of 0.5 mg/ml MTT in 10% fetal bovine serum−containing RPMI 1640, and cells were incubated in the carbon dioxide incubator at 37°C for 2 hours. Supernatants were removed from the wells and the reduced MTT dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

Apoptosis Detection by ELISA. Induction of apoptosis was assessed with a Cell Death Detection ELISA kit (Roche Diagnostics) following the manufacturer’s instruction. This test is based on the quantitative determination of cytoplasmic histone–associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death. In brief, 1 × 10⁶ cells were cultured in a T-25 flask in 10% fetal bovine serum−containing medium for 24 hours, and were treated with the test agents at various concentrations in serum-free medium for 24 hours. Both floating and adherent cells were collected; cell lysates equivalent to 5 × 10⁶ cells were used in the ELISA.

Western Blot Analysis of Cytochrome c Release into the Cytoplasm. Cytosolic-specific, mitochondria-free lysates were prepared according to an established procedure (16). In brief, after individual treatments for 24 hours, both the incubation medium and adherent cells in T-75 flasks were collected and centrifuged at 200 × g for 5 minutes. The pellet fraction was recovered, placed on ice, and triturated with 300 μL of a chilled hypotonic lysis solution [50 mmol/L PIPES-KOH (pH 7.4) containing 220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol/L MgCl₂, 1 mmol/L DTT, and a mixture of protease inhibitors including 100 μmol/L 4-(2-aminoethyl)benzenesulfonfluride, 80 μmol/L aprotinin, 5 μmol/L bestatin, 1.5 μmol/L E-64 protease inhibitor, 2 μmol/L leupeptin, and 1 μmol/L pepstatin A]. After a 45-minute incubation on ice, the mixture was centrifuged at 200 × g for 10 minutes. The supernatant was collected in a microcentrifuge tube, and centrifuged at 14,000 rpm for 30 minutes. An equivalent amount of protein (50 μg) from each supernatant was resolved in 10% SDS-polyacrylamide gel. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome c antibodies as described below.

Immunoblotting. Cells in T-75 flasks were collected by scraping and suspended in 60 μL of PBS. Two microliters of the suspension was taken for protein analysis using the Bradford assay kit (Bio-Rad, Hercules, CA). The same volume of 2× SDS-PAGE sample loading buffer [100 mmol/L Tris-HCl (pH 6.8), 4% SDS, 5% [γ-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue] was added to the remaining solution. The mixture was sonicated briefly and then boiled for 5 minutes. Equal amounts of proteins were loaded onto 10% SDS-PAGE gels.

After electrophoresis, protein bands were transferred to nitrocellulose membranes in a semidry transfer cell. The transblotted membrane was washed twice with TBS containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 40 minutes, the membrane was incubated with the appropriate primary antibody in TBST-1% nonfat milk at 4°C overnight. All primary antibodies were diluted 1:1,000 in 1% nonfat milk−containing TBST. After treatment with the primary antibody, the membrane was washed thrice with TBST for a total of 15 minutes followed by incubation with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:5,000) for 1 hour at room temperature and three washes with TBST for a total of 1 hour. The immunoblots were visualized by enhanced chemiluminescence.

Analysis of PPARγ Activation. The analysis was carried out by using a PPARγ transcription factor ELISA kit (Active Motif) in which an oligonucleotide containing the peroxisome proliferator response element was immobilized onto a 96-well plate. PPARs contained in nuclear extracts bind specifically to this oligonucleotide and are detected through an antibody directed against PPARγ. In brief, PC-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and treated with DMSO vehicle or individual test agents, 10 μmol/L each, for 48 hours. Cells were collected and nuclear extracts were prepared with a Nuclear Extract kit (Active Motif). Nuclear extracts of the same protein concentration from individual treatments were subject to the PPARγ transcription factor ELISA according to the manufacturer’s instruction.

Competitive Fluorescence Polarization Assay. The binding affinity of the test agent to Bcl-2 and Bcl-xL was analyzed by a competitive fluorescence polarization assay in which the ability of the agent to displace the binding of a BAK BH3–domain peptide to either Bcl-2 or Bcl-xL was determined. Thu-BakB3A, a BAK-BH3 peptide labeled at the NH₂ terminus with fluorescein, was purchased from Genemed Synthesis (San Francisco, CA). COOH-terminal-truncated, His-tagged Bcl-xL was purchased from EMD Biosciences (San Diego, CA) and soluble glutathione S-transferase-fused Bcl-2 was obtained from Santa Cruz Biotechnology. The Kᵦ determination was carried out in a dual−path length quartz cell with readings taken at λₐₘₜ 480 nm and λₙₜ 530 nm at room temperature using a luminescence spectrometer according to an established procedure (17).
**Troglitazone Induces Apoptosis in Prostate Cancer Cells**

**Determination of IC_{50} Values.** Data from cell viability and fluorescence polarization assays were analyzed by using the CalcuSyn software (Biosoft, Ferguson, MO) to determine IC_{50} values, in which the calculation was based on the medium-effect equation [i.e., \( \log(f_a/f_u) = m \log(D) - m \log(D_{50}) \)], where \( f_a \) and \( f_u \) denote fraction affected and unaffected, respectively; \( m \) represents the Hill-type coefficient signifying the sigmoidicity of the dose-effect curve; and \( D \) and \( D_{50} \) are the dose used and IC_{50}, respectively; ref. 18.

**Co-immunoprecipitation.** PC3 cells treated with 50 \( \mu \)mol/L troglitazone or \( \Delta_2 \)-TG for 12 hours were collected and lysed by NP40 isotonic lysis buffer with freshly added protease inhibitors [142 mmol/L KCl, 5 mmol/L MgCl\(_2\), 10 mmol/L HEPES (pH 7.2), 1 mmol/L EGTA, 0.2% NP40, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 \( \mu \)g/mL each aprotinin, leupeptin, and pepstatin]. After centrifugation at 13,000 \( \times \) g for 15 minutes, the supernatants were collected, preincubated with protein A-Sepharose (Sigma) for 15 minutes, and centrifuged at 1,000 \( \times \) g for 5 minutes. The supernatants were exposed to Bcl-2 or Bcl-xL antibodies in the presence of protein A-Sepharose at 4°C for 2 hours. After brief centrifugation, protein A-Sepharose were collected, washed with the aforementioned lysis buffer twice, suspended in 2 \( \times \) SDS sample buffer, and subjected to Western blot analysis with antibodies against Bak.

**Xenograft Tumor Growth.** Male NCr athymic nude mice (5-7 weeks of age) were obtained from the National Cancer Institute (Frederick, MD). Mice were group-housed under conditions of a constant 12-hour photoperiod with ad libitum access to sterilized food and water. All experimental procedures utilizing these mice were done in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.

Each mouse was inoculated s.c. in the right flank with 5 \( \times \) 10^5 PC-3 cells suspended in 0.1 mL serum-free medium containing 30% Matrigel (BD Biosciences) under isoflurane anesthesia. Forty-eight hours later, mice were randomly divided into three groups (n = 8) and were given daily TG-88 at 100 and 200 mg/kg body weight per day by gavage for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. The volume of drug or vehicle administered to each mouse was 0.02 mL/g of body weight. Tumors were measured weekly. Tumor growth data are expressed as mean tumor volumes \( \pm \) SE. For all data, differences were considered significant at P < 0.05. Statistical procedures were done using SPSS for Windows (SPSS, Inc., Chicago, IL).

**Results**

**Development of Thiazolidenedione Derivatives Lacking PPARγ Ligand Activity.** It was reported that introduction of a double bond adjoining the terminal thiazolidine-2,4-dione ring of rosiglitazone abrogated its PPARγ ligand property (19). As part of our effort to discern the role of PPARγ activation in the antimetastatic effects of thiazolidenediones, we synthesized this rosiglitazone derivative and the counterparts of troglitazone, pioglitazone, and ciglitazone, and examined the ability of the resulting molecules (\( \Delta_2 \)-TG, \( \Delta_2 \)-RG, \( \Delta_2 \)-PG, and \( \Delta_2 \)-CG) vis-à-vis their parent thiazolidenediones to activate PPARγ in PC-3 cells (Fig. 1).

Among these new compounds, \( \Delta_2 \)-RG showed a 77% reduction in the activity in PPARγ activation compared with rosiglitazone, which is in line with that reported in the literature (19). In contrast, \( \Delta_2 \)-TG, \( \Delta_2 \)-PG, and \( \Delta_2 \)-CG were completely devoid of the ligand-binding activity because the respective levels of PPARγ activation were not statistically different from that of the DMSO vehicle (P > 0.01). The loss/attenuation of PPARγ activity in these \( \Delta_2 \) derivatives was presumably attributable to the structural rigidity, as a result of the double bond introduction surrounding the heterocyclic system.

**Apoptosis-Inducing Effects of Thiazolidenediones on Prostate Cancer Cells Are Independent of PPARγ Activation.** We first assessed the dose-dependent growth inhibitory effect of troglitazone and \( \Delta_2 \)-TG in two prostate cancer cell lines, androgen-independent PC-3 (p53+/−), and androgen-dependent LNCaP (p53+/+). Among many genotypic differences, these two cell lines exhibit distinct PPARγ expression status (i.e., PPARγ was highly expressed in PC-3 cells but was deficient in LNCaP cells, P < 0.01; Fig. 2A; ref. 15). Nevertheless, despite deficiency in PPARγ, LNCaP cells exhibited a higher degree of susceptibility to troglitazone-mediated **in vitro** antimetastatic effects compared with the PPARγ-rich PC-3 cells (Fig. 2B). In addition, \( \Delta_2 \)-TG, although devoid of PPARγ-activating activity, was more potent than troglitazone in suppressing cell proliferation in both cell lines. The respective IC_{50} values for troglitazone and \( \Delta_2 \)-TG were 30 ± 2 and 20 ± 2 \( \mu \)mol/L in PC-3 cells. The results are shown in Figure 1. (A) Development of PPARγ-inactive thiazolidenedione derivatives. A, chemical structures of troglitazone, rosiglitazone, pioglitazone, ciglitazone, and their respective \( \Delta_2 \) derivatives. B, \( \Delta_2 \)-thiazolidenedione derivatives lack activity in PPARγ activation. Analysis of PPARγ activation was carried out as described in Materials and Methods. In brief, PC-3 cells were exposed to individual test agents (10 \( \mu \)mol/L) or DMSO vehicle in 10% fetal bovine serum-supplemented RPMI 1640 for 48 hours. Amounts of activated PPARγ in the resulting nuclear extracts were analyzed by PPARγ transcription factor ELISA kit. Columns, mean; bars, SD (n = 3, *P < 0.01).
cells, and 22 ± 3 and 14 ± 1 μmol/L in LNCaP cells. This growth inhibition was attributable to apoptotic cell death, as evidenced by mitochondrial cytochrome c release and DNA fragmentation in PC-3 cells (Fig. 2C).

Similar results were obtained with ciglitazone and Δ2-CG with respect to cytochrome c–dependent apoptotic death in PC-3 cells. The relative potency paralleled that of troglitazone and Δ2-TG (Fig. 3A). In contrast, rosiglitazone, pioglitazone, and their Δ2 counterparts showed marginal effects, even at 50 μmol/L, on apoptotic death in PC-3 cells (Fig. 3B).

Together, these data suggest that thiazolidinediones mediated apoptosis induction in prostate cancer cell systems irrespective of PPARγ activation.

**Apoptosis-Active Thiazolidinediones Are Inhibitors of Bcl-xL and Bel-2 Functions.** Our mechanistic study indicated that troglitazone and Δ2-TG were able to sensitize PC-3 cells to the apoptosis-inducing effect of the phosphoinositide 3-kinase inhibitor LY294002.1 This finding, together with our recent report that attributed the resistance of PC-3 cells to LY294002-induced apoptosis to Bcl-xL overexpression (16), suggests a plausible link between thiazolidinedione-induced apoptosis and modulation of the functions of Bcl-xL and/or other Bel-2 members.

Accordingly, we examined this putative link by two distinct approaches at both transcriptional and posttranslational levels. First, we assessed the time-dependent effect of troglitazone (30 μmol/L) on the expression of different Bel-2 family members.

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1 Unpublished data.
in PC-3 cells, including Bcl-xL, Bcl-2, Bax, Bak, Bad, and Bid. This analysis was based on recent reports that treatment of MCF-7 breast cancer and HepG2 hepatoma cells with high doses of troglitazone altered the expression levels of certain Bcl-2 members (9, 14). Second, in light of the recent discovery of small-molecule Bcl-2 or Bcl-xL inhibitors that disrupt BH3 domain–mediated interactions with proapoptotic Bcl-2 members (20–26), we investigated the in vitro effects of thiazolidenediones and their Δ2 counterparts on the anti-apoptotic function of Bcl-xL and Bcl-2. It is well understood that the ability of Bcl-xL and Bcl-2 to form heterodimers with proapoptotic Bcl-2 members, via BH3 domain binding, plays a key role in their anti-apoptotic functions. Therefore, a well-established competitive fluorescence polarization analysis was used to examine the effects of thiazolidenediones on the binding of a Bak BH3 domain peptide to Bcl-xL and Bcl-2.

Figure 4 indicates that with the exception of a slight decrease in Bad expression at 24 hours, the exposure of PC-3 cells to 30 μmol/L troglitazone did not cause appreciable change in the expression level of any of these Bcl-2 members throughout the course of investigation.

Nevertheless, data from the competitive fluorescence polarization analysis suggest that troglitazone, ciglitazone, and their Δ2 counterparts inhibited the anti-apoptotic functions of Bcl-xL and Bcl-2 by disrupting the BH3 domain–mediated interactions with proapoptotic Bcl-2 members. Figure 5A depicts the ability of troglitazone and Δ2-TG to displace the binding of a fluorescein-labeled Bak BH3 domain peptide to Bcl-xL and Bcl-2. It is noteworthy that both compounds inhibited the BH3 peptide binding to Bcl-xL and Bcl-2 with equal potency, a distinct difference from many reported small-molecule inhibitors that showed discriminative affinity between these two anti-apoptotic Bcl-2 members. The IC_{50} values for the inhibition of Bak BH3 peptide binding to either Bcl-xL or Bcl-2 were 22 ± 1 and 18 ± 1 μmol/L for troglitazone and Δ2-TG, respectively (Fig. 5A). Ciglitazone and Δ2-CG showed similar effects on the protein-protein interactions with comparable IC_{50} values (Fig. 5B). On the other hand, rosiglitazone, Δ2-RG, pioglitazone, and Δ2-PG, which were ineffective in inducing apoptotic death even at high doses, showed poor inhibitory activities with IC_{50} significantly greater than 50 μmol/L.
In light of the integral role of Bcl-2 members in the modulation of mitochondrial integrity, these *in vitro* binding data suggest that interference of the ability of Bcl-2 and Bcl-xL to bind with their proapoptotic Bcl-2 partners represented a major pathway for troglitazone, Δ2-TG, and ciglitazone counterparts to exert their apoptotic action. To corroborate this premise, we obtained two lines of evidence: (a) troglitazone and Δ2-TG attenuated the binding of intracellular Bcl-2 and Bcl-xL to proapoptotic Bcl-2 members and (b) overexpression of Bcl-xL provided protection against the drug-induced apoptosis.

**Effect of Troglitazone and Δ2-TG on Intracellular Bcl-2 and Bcl-xL Binding to Bak.** The functional relationship among different types of Bcl-2 family members in regulating the apoptosis machinery has been the focus of many recent investigations (27). One school of thought is that Bcl-2 and Bcl-xL sequester Bax, Bak, and other proapoptotic Bcl-2 members through BH3 domain-mediated heterodimerization, thereby abrogating their proapoptotic effects (28–32). For example, electrophoretic introduction of Bak or Bax BH3 domain peptides into PC-3 cells disrupted Bcl-2-Bak heterodimer formation, which resulted in the liberation of Bak and Bak to mediate apoptotic death via a caspase-dependent pathway (32). Consequently, to validate the mode of action of troglitazone and Δ2-TG, we assessed the effects on the dynamics of Bcl-2/Bak and Bcl-xL/Bak interactions in PC-3 cells. Lysates from PC-3 cells treated with troglitazone or Δ2-TG (50 μmol/L) vis-à-vis DMSO for 12 hours were immunoprecipitated with antibodies against Bcl-2 or Bcl-xL. Probing of the immunoprecipitates with anti-Bak antibodies by Western blotting indicates that the level of Bak associated with Bcl-2 and Bcl-xL was significantly reduced compared with the DMSO control (Fig. 6A; *P < 0.01*). This decrease in intracellular associations bore out the *in vitro* binding data that troglitazone and Δ2-TG inhibited the interactions of Bcl-xL and Bcl-2 with the Bak BH3-domain peptide. We further showed that treatment of PC-3 cells with troglitazone or Δ2-TG led to caspase-9 activation in a dose-dependent manner (Fig. 6B) similar to that of cytochrome c release (Fig. 2A). Furthermore, pretreatment of PC-3 cells with the pan-caspase inhibitor Z-VAD-FMK protected cells from troglitazone- and Δ2-TG-induced apoptosis (*P < 0.01*; Fig. 6C), confirming the involvement of caspase activation in apoptotic death.

**Bcl-xL Overexpression Protects Prostate Cancer Cells from Troglitazone- and Δ2-TG-Induced Apoptosis.** We previously reported that LNCaP cells exhibited lower Bcl-xL expression levels compared with PC-3 cells (16), which underscored differences between these two cell lines in the susceptibility to the apoptotic effects of troglitazone, ciglitazone, and their Δ2 counterparts. To confirm that the inhibition of Bcl-xL functions plays a key role in the apoptosis induction, we examined the impact of Bcl-xL overexpression on the susceptibility to troglitazone- and Δ2-TG-induced cell death in LNCaP cells. Three transfected clones (B1, B3, and B11) that displayed similar expression levels of Bcl-xL were tested *in vitro* parental LNCaP cells (Fig. 7A). Figure 7B depicts the differential protective effects of ectopic Bcl-xL on troglitazone- and Δ2-TG-induced apoptotic death among the three Bcl-xL clones, in which the extent of cytoprotection correlated with the Bcl-xL expression levels. Overexpression of ectopic Bcl-xL...
activation provided a molecular rationale to structurally optimize dissociation of the effect of troglitazone on apoptosis from PPAR in suppressing PC-3 xenograft tumor growth at 500 mg/kg/d (33). Dissociation of the effect of troglitazone on apoptosis from PPAR activation was correlated with the inhibition of troglitazone-and Δ2-TG-induced cytochrome c release (P < 0.01; Fig. 7C).

Development of Potent Δ2-TG-Derived Bcl-xL/Bcl-2 Binding Inhibitors. Troglitazone has previously been shown to be effective in suppressing PC-3 xenograft tumor growth at 500 mg/kg/d (33). Several thiazolidinediones currently in clinical use for the treatment of diabetes, lacked appreciable activity in PPARγ activation. First, several lines of evidence that the effects of these thiazolidinediones on apoptosis in prostate cancer cells were attributable in part to differences in PPARγ activity, exhibiting slightly higher potency than troglitazone and ciglitazone. Second, a correlation exists between the potency in inhibiting BH3 peptide binding to Bcl-xL or Bcl-2 and the effectiveness in inducing apoptotic death irrespective of differences in PPARγ expression levels between LNCaP and PC-3 cells. In contrast, rosiglitazone and pioglitazone, two thiazolidenediones currently in clinical use for the treatment of diabetes, lacked appreciable effects on apoptosis despite their higher potency in PPARγ activation.

Effect of p.o. TG-88 at 100 and 200 mg/kg on the growth of PC-3 tumors in nude mice. Each mouse was inoculated s.c. in the right flank with 5 × 105 PC-3 cells suspended in 0.1 ml serum-free medium containing 30% Matrigel under isoflurane anesthesia. Forty-eight hours later, mice were randomly divided into three groups (n = 8) and were given daily TG-88 at 100 and 200 mg/kg body weight per day by gavage for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Points, mean; bars, SE (n = 8; *P < 0.05 compared with the control group).

Discussion

Although accumulating evidence suggests that troglitazone and ciglitazone mediate PPARγ-independent antitumor effects, the underlying mechanism remains undefined. Here, we obtained several lines of evidence that the effects of these thiazolidinediones on apoptosis in prostate cancer cells were attributable in part to the inhibition of Bcl-xL/Bcl-2 functions independently of PPARγ activation. First, Δ2-TG and Δ2-CG, although devoid of PPARγ activity, exhibited slightly higher potency than troglitazone and ciglitazone, respectively, in inducing apoptotic death irrespective of differences in PPARγ expression levels between LNCaP and PC-3 cells. In contrast, rosiglitazone and pioglitazone, two thiazolidinediones currently in clinical use for the treatment of diabetes, lacked appreciable effects on apoptosis despite their higher potency in PPARγ activation than troglitazone and ciglitazone. Second, a correlation exists between the potency in inhibiting BH3 peptide binding to Bcl-xL or Bcl-2 and the effectiveness in inducing apoptosis in prostate cancer cells. For example, the inability of

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Figure 7. Ectopic Bcl-xL protects LNCaP cells from troglitazone- and Δ2-TG-induced apoptosis by attenuating cytochrome c release in an expression level–dependent manner. A, left, ascending expression levels of ectopic Bcl-xL in B11, B1, and B3 clones. Columns, mean; bars, SD (n = 3, *P < 0.01). Right, Western blot analysis. The band for ectopic Bcl-xL contained a cytotoxic effect of troglitazone and Δ2-TG on Bcl-xL functions (P < 0.01). This protective effect was correlated with the inhibition of troglitazone- and Δ2-TG-induced cytochrome c release (P < 0.01; Fig. 7C).

Figure 8. Effect of p.o. TG-88 at 100 and 200 mg/kg on the growth of PC-3 tumors in nude mice. Each mouse was inoculated s.c. in the right flank with 5 × 105 PC-3 cells suspended in 0.1 ml serum-free medium containing 30% Matrigel under isoflurane anesthesia. Forty-eight hours later, mice were randomly divided into three groups (n = 8) and were given daily TG-88 at 100 and 200 mg/kg body weight per day by gavage for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Points, mean; bars, SE (n = 8; *P < 0.05 compared with the control group).

Discussion

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rosiglitazone and pioglitazone to trigger apoptotic death was reflected in their weak potency in displacing BH3 domain-mediated interactions. It is interesting that introduction of a double bond adjoining the terminal thiazoline-2,4-dione ring in troglitazone and ciglitazone enhanced the Bcl-xL/Bcl-2 inhibitory activity, while abrogating the ability to activate PPARγ. Presumably, this change in pharmacologic profiles was attributable to the structural rigidity surrounding the heterocyclic system as a result of the double-bond introduction. Third, the immunoprecipitation study indicates that the level of Bak associated with Bcl-2 and Bcl-xL was greatly reduced in troglitazone- and Δ2-TG-treated cells compared with DMSO control. Disruption of the BH3 domain-mediated interactions led to the liberation of proapoptotic Bcl-2 members, which caused cells to undergo apoptosis by facilitating cytochrome c release and caspase-9 activation. This premise was borne out by the ability of Z-VAD-FMK to protect cells from troglitazone- and Δ2-TG-induced apoptosis. Fourth, overexpression of Bcl-xL provided LNCaP cells protection against troglitazone- and Δ2-TG-induced apoptosis.

Considering the pivotal role of Bcl-xL and Bcl-2 in regulating mitochondrial integrity, this new mode of action provides a molecular framework to account for the PPARY-independent effects of thiazolidinediones on apoptotic death in cancer cells. It is also noteworthy that troglitazone, ciglitazone, and their Δ2 derivatives lack specificity in recognizing Bcl-xL and Bcl-2. This relaxed specificity might prove advantageous in light of the importance of both Bcl-2 members in regulating apoptosis thresholds to chemotherapeutic agents.

In summary, the impetus of the dissociation of the in vitro antitumor activities of thiazolidinediones from PPARY activation is multifold. First, although troglitazone has been shown to reduce the growth of xenograft tumors in nude mice (33, 34), this PPARY agonist has also been reported to promote the development of colon tumors and enhance colon polyp formation in APCMin mice that are genetically predisposed to intestinal neoplasia (35, 36). Thus, a crucial issue that warrants investigation is the role of PPARY activation in tumorigenic promotion vis-à-vis antitumor effects in these animal model studies. Conceivably, thiazolidinediones and their PPARY-inactive Δ2 derivatives provide useful tools to shed light onto the link between PPARY activation and increased cancer risk. Second, from a translational perspective, separation of these two pharmacologic activities provides molecular underpinnings to use thiazolidinediones, especially Δ2-TG and Δ2-CG, as molecular platforms to design Bcl-xL/Bcl-2 inhibitors with greater in vitro and in vivo antitumor potency. Third, owing to the heterogeneous nature of prostate cancer, different prostate tumor cell lines display differential sensitivity to various apoptotic signals. For example, PC-3 cells are able to resist apoptotic signals emanating from withdrawal of trophic factors, and exposure to cytokines and chemotherapeutic agents, in part owing to elevated levels of Akt activation and Bcl-xL overexpression. Consequently, these molecules have translational relevance to be developed into antitumor agents for the prevention and/or therapy of cancers alone or in combination with other treatments. The proof of principle for this premise was TG-88, a close structural analogue of Δ2-TG, with an order-of-magnitude higher potency than Δ2-TG in blocking Bcl-xL binding and inhibiting PC-3 cell proliferation. Oral TG-88 at 100 and 200 mg/kg/d was effective in suppressing PC-3 xenograft tumor growth without causing weight loss or apparent toxicity, indicating its p.o. bioavailability and potential clinical use. Further development of these novel agents for the prevention and/or treatment of prostate cancer is currently under way.

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