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Peroxisome Proliferator-Activated Receptor γ-Independent Ablation of Cyclin D1 by Thiazolidenediones and Their Derivatives in Breast Cancer Cells

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Running Title: Small-molecule cyclin D1-ablative agents

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Abbreviations: CDK, cyclin-dependent kinase; ERα, estrogen receptor α; PPARγ, peroxisome proliferator-activated receptor γ; PGJ_2, 15-deoxy-Δ12,14-prostaglandin J_2; Δ2-TG, 5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-2,4-thiazolidinedione; Δ2-CG, 5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione; Δ2-TG-6, 5-[4-(6-allyoxy-2,5,7,8-tetramethyl-chroman-2-yl-methoxy)-benzylidene]-2,4-thiazolidinedione; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide.

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Abstract

In light of the clinical relevance of targeting cyclin D1 in breast cancer, we have investigated the mechanism underlying the effect of the peroxisome proliferator-activated receptor-γ (PPARγ) agonists troglitazone and ciglitazone on cyclin D1 repression. We obtain evidence that the ability of high doses of troglitazone and ciglitazone to repress cyclin D1 is independent of PPARγ activation. PPARγ-inactive troglitazone and ciglitazone analogues (Δ2-TG and Δ2-CG) are able to facilitate cyclin D1 ablation with potency similar to that of troglitazone and ciglitazone in MCF-7 cells. Reverse transcription PCR shows that the mRNA level of cyclin D1 remains unaltered in drug-treated cells, indicating the repression is mediated at the posttranscriptional level. Moreover, the ablative effect of these agents is specific to cyclin D1 as the expression levels of many other cyclins and cyclin-dependent kinases examined remain unchanged after drug treatment. Our data indicate that troglitazone- and Δ2-TG-induced cyclin D1 repression is mediated via proteasome-facilitated proteolysis as it is inhibited by different proteasome inhibitors, including MG132, lactacystin, and epoxomicin, and is preceded by increased ubiquitination. The dissociation of these two pharmacological activities, i.e., PPARγ activation and cyclin D1 ablation, provides a molecular basis to use Δ2-TG as a scaffold to develop a novel class of cyclin D1-ablative agents. Accordingly, a series of Δ2-TG derivatives are synthesized. Among them, Δ2-TG-6 represents a structurally optimized agent with potency an-order-of-magnitude higher than that of Δ2-TG in cyclin D1 repression and MCF-7 cell growth inhibition.

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Introduction

Cyclin D1 represents an important downstream effector of diverse proliferative and transforming signaling pathways, including those mediated by β-catenin (Shtutman et al., 1999), ERα (Lukas et al., 1996; Prall et al., 1998; Wilcken et al., 1997), Her-2/Neu (Lee et al., 2000), NFκB (Henry et al., 2000; Joyce et al., 1999), Rac (Westwick et al., 1997), Ras (Albanese et al., 1995), Src (Lee et al., 1999), STATs (Bromberg et al., 1999; Matsumura et al., 1999), and Wnt (D'Amico et al., 2000). In mammary cells, transcriptional activation of cyclin D1 in response to these mitogenic signals leads to G1/S progression and increased proliferation. Cyclin D1 overexpression has been implicated in oncogene-induced mammary tumorigenesis as it is noted in over 50% of primary breast carcinomas correlating with poor prognosis (Kenny et al., 1999; McIntosh et al., 1995). In addition to activating cyclin-dependent kinases (CDKs) and sequestering of CDK inhibitors in the G1/S transition, the function of cyclin D1 as a CDK-independent activator of estrogen receptor α (ERα) is especially noteworthy (Lamb et al., 2000; McMahon et al., 1999; Neuman et al., 1997; Zwijsen et al., 1997). Cyclin D1 overexpression confers resistance to antiestrogens in breast cancer cells (Hui et al., 2002; Musgrove et al., 2001), and represents a negative predictive factor for tamoxifen response (Stendahl et al., 2004). Together, these findings suggest that an anti-cyclin D1 therapy might be highly specific for treating human breast cancer (Yu et al., 2001).

Peroxisome proliferator-activated receptor γ (PPARγ) agonists, including 15-deoxy-Δ12,14-prostaglandin J_2 (PGJ_2) and thiazolidenediones, have been shown to downregulate cyclin D1 expression as part of the mechanism for causing cell cycle arrest and growth inhibition in breast cancer cells (Lapillonne et al., 2003; Qin et al., 2003; Qin

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et al., 2004; Wang et al., 2001; Yin et al., 2001). Two distinct PPARγ-dependent mechanisms have been reported to account for PGJ_2- and ciglitazone-mediated cyclin D1 repression. First, PGJ_2 treatment could lead to the sequestration of p300, a co-activator protein, thereby preventing the transcriptional activation of the cyclin D1 promoter (Wang et al., 2001). Second, PGJ_2 and ciglitazone could activate proteasome-mediated degradation of cyclin D1 (Qin et al., 2003).

In this study, we obtain evidence that the ability of troglitazone and ciglitazone to down-regulate cyclin D1 and, to a lesser extent, ERα was independent of PPARγ activation. We demonstrate that PPARγ-inactive troglitazone and ciglitazone analogues could facilitate proteasome-mediated proteolysis of cyclin D1 in a manner similar to that of their parent thiazolidenediones. The dissociation of these two pharmacological activities provides a molecular basis to develop a novel class of cyclin D1-ablative agents, of which the proof of principle is illustrated by a troglitazone analogue with an-order-of-magnitude higher efficacy than troglitazone in cyclin D1 repression and MCF-7 cell growth inhibition.

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Materials and Methods

Reagents. Troglitazone, ciglitazone, MG132, lactacystin, and SB216763 were purchased from Sigma (St. Louis, MO). Rosiglitazone and pioglitazone were prepared from the respective commercial tablets by solvent extraction followed by recrystallization or chromatographic purification. Epoxomicin was a kind gift from Dr. Kyung Bo Kim (University of Kentucky). Δ2-TG {5-[4-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene]-2,4-thiazolidinedione} Δ2-CG {5-[4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione}, and Δ2-TG-6 {5-[4-(6-allyoxy-2,5,7,8-tetramethyl-chroman-2-ylmethoxy)-benzylidene]-2,4-thiazolidinedione} are thiazolidenedione derivatives devoid of activity in PPARγ activation, the synthesis of which 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. These agents at various concentrations were dissolved in DMSO, and added to cells in medium with a final DMSO concentration of 0.1%. Rabbit antibodies against p-GSK and mouse anti-cyclin D1 and anti-ubiquitin were purchased from Cell Signaling Technology Inc. (Beverly, MA). Rabbit antibodies against ER-α (sc-544), CDK2, CDK4, cyclin A, cyclin B, cyclin D2, cyclin D3, cyclin E, and mouse anti-α-tublin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-actin was from ICN Biomedicals Inc (Costa Mesa, CA).

Cell culture. ER-positive MCF7 and ER-negative MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2.

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Cell viability analysis. The effect of individual test agents on cell viability was assessed by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay in six replicates. Cells were seeded and incubated in 96-well, flat-bottomed plates in DMEM-F12 media with 10% FBS for 24 h, and were exposed to various concentrations of test agents dissolved in DMSO (final DMSO concentration, 0.1 %) in 5% FBS-supplemented DMEM-F12 medium. Controls received DMSO vehicle at a concentration equal to that of drug-treated cells. The medium was removed, replaced by 200 μl of 0.5 mg/ml of MTT in 10% FBS-containing RPMI-1640 medium, and cells were incubated in the CO_2 incubator at 37 ^{o}C for 2 h. 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.

Analysis of PPARγ activation. The analysis was carried out by using a PPARγ transcription factor ELISA kit (Active Motif, Carlsbad, CA), in which an oligonucleotide containing the peroxisome proliferator response element (PPRE) 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, MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, and treated with DMSO vehicle or individual test agents, 10 μM each, for 48 h. Cells were collected, and nuclear extracts were prepared with a Nuclear Extract kit (Active Motif, Carlsbad, CA). Nuclear extracts of the same protein concentration from individual treatments were subject to the PPARγ transcription factor ELISA according to the manufacturer's instruction.

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Western Blot Analysis. MCF-7 or MDA-MB-231 cells were seeded in 10% FBS-containing DMEM-F-12 for 24 h and treated with various agents as indicated. After individual treatments for 24 h, both the incubation medium and adherent cells in T-25 or T-75 flasks were scraped and collected by centrifugation at 2,000 x g for 10 min. The pellets were recovered, placed on ice, and triturated with 20 to 50 µl of a chilled lysis buffer (M-PER® Mammalian Protein Extraction Reagent, Pierce, Rockford, IL) to which was added 1% protease inhibitor cocktail (set III, EMD Biosciences, Inc. San Diego, CA). After a 30 min-incubation on ice, the mixture was centrifuge at 16,100 x g for 3 min. Two µl of the suspension was taken for protein analysis using the Bradford assay kit (Bio-Rad, Hercules, CA). To the remaining solution was added the same volume of 2 x SDS-PAGE sample loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol 20% glycerol, and 0.1% bromophenol blue). The mixture was boiled for 10 min. Equal amounts of proteins were loaded onto 10% SDS-PAGE gels. After electrophoresis, protein bands were transferred to nitrocellulose membranes in a semi-dry transfer cell. The transblotted membrane was blocking with TBST [Tris-buffered saline (TBS) containing 0.1% Tween 20] containing 5% nonfat milk for 90 min, and the membrane was incubated with the appropriate primary antibody in TBST-5% nonfat milk at 4°C overnight. After washing three times with TBST for a total of 45 min, the transblotted membrane was incubated with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:1000) for 1 h at room temperature and washed four times with TBST for a total of 1 h. The immunoblots were visualized by enhanced chemiluminescence.

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Coimmunoprecipitation/Western Blot. MCF-7 cells were cultured in 10% FBS-containing DMEM-F-12 in 75-mm plates for 24 h. Cell were treated with DMSO vehicle, 30 μM troglitazone, or 20 μM Δ2-TG in 5% FBS-containing DMEM-F12 medium for another 20 hours. Cells were rinsed with PBS at room temperature, scraped off the flask, transferred into centrifuge tubes, and centrifuged at 2,000 x g for 10 min to pellet the cells. The pellet was resuspended in ice-cold 0.5 ml of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% protease inhibitor cocktail), and gently mixed on an orbital shaker at 4°C for 15 min followed by centrifugation at 14,000 x g for 15 min to yield cell lysates. These cell lysates were treated with 100 μl of protein A-agarose bead slurry, followed by brief centrifugation, to remove nonspecific binding proteins. Equal amounts of proteins from these lysates, as determined by the Bradford assay, were mixed with anti-cyclin D1 in an orbital shaker at 23 ^{o}C for 2 h, followed by 100 μl of protein A-agarose bead slurry at 4 ^{o}C for 12 h. The immunocomplex was collected by brief centrifugation, washed 4 times with 800 μl of ice-cold RIPA buffer, and suspended in 50 μl of the aforementioned 2 x SDS sample loading buffer. The suspension was boiled for 10 min, cooled, and briefly centrifuged to remove the beads. Western blot analysis was performed with anti-cyclin D1 or anti-ubiquitin as described above.

Reverse transcriptase (RT-) PCR analysis of mRNA transcripts of cyclin D1 gene. MCF7 cells were subject to total RNA isolation by using RNeasy mini kit (Qiagen, Valencia, CA). RNA concentrations and quality were assessed spectrophotometrically by measuring absorption at 260 nm. Aliquots of 20 μg of total RNA from each sample was reverse transcribed to cDNA using Omniscript RT Kit (Qiagen) according to

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manufacturer's instructions. The primers used were as follows: cyclin D1, forward, 5'-ATGGAACACCAGCTCCTGTGCTGC-3', reverse, 5'-TCAGATGTCCACGTCCCGCACGT-3'; β-actin, forward, 5'-TCTACAATGAGCTGCGTGTG-3', reverse, 5'-GGTCAGGATCTTCATGAGGT-3'. The reaction conditions were as follows: for cyclin D1 (*a*) initial denaturation at 95°C for 5 min; (*b*) 34 cycles of amplification (95°C for 1 min, 65°C for 1 min 45s, and 72°C for 1 min); and (*c*) a final extension step of 10 min at 72°C; for β-actin (*a*) initial denaturation at 95°C for 3 min; (*b*) 40 cycles of amplification (95°C for 30 s, 58°C for 20 s, and 72°C for 45 s); and (*c*) a final extension step of 10 min at 72°C. The PCR reaction products were separated electrophoretically in a 1.2 % agarose gel and stained with ethidium bromide.

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Results

Effect of thiazolidenediones on cyclin D1 downregulation is independent of PPARγ. Three lines of evidence suggest that thiazolidenedione-mediated cyclin D1 down-regulation in breast cancer cells was independent of PPARγ activation. First, we assessed the effect of troglitazone on cyclin D1 expression in two breast cancer cell lines: ER-positive MCF-7 and ER-negative MDA-MB-231. Among many genotypic differences, these two cell lines exhibit differential PPARγ expression, i.e., PPARγ expression in MDA-MB-231 cells was at least an-order-of-magnitude higher than that of MCF-7 cells (Fig. 1A). Despite this discrepancy, MCF-7 cells showed a higher degree of susceptibility to troglitazone-mediated cyclin D1 down-regulation as compared to the PPARγ-rich MDA-MB-231 cells (panel B).

Second, we assessed the effect of four different thiazolidenediones, i.e., troglitazone, ciglitazone, rosiglitazone and pioglitazone, on intracellular cyclin D1 in MCF-7 cells. Among them, troglitazone and ciglitazone at high doses were effective in reducing cyclin D1 and ERα levels (Fig. 2B and C). In contrast, rosiglitazone and pioglitazone lacked appreciable effects at comparable concentrations (data not shown) even though these two agents are more active than troglitazone and ciglitazone in PPARγ activation.

Third, we examined the effect of GW9962, a potent PPARγ antagonist (Leesnitzer et al., 2002; Seargent et al., 2004) on troglitazone-mediated cyclin D1 repression in MCF-7 cells. Even at concentrations three orders of magnitude higher than the IC_{50} in PPARγ binding, GW9962 had no appreciable effect on cyclin D1 expression, and did not prevent troglitazone-mediated cyclin D1 down-regulation (Fig. 1C).

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Separation of the cyclin D1-ablative effect from the PPARγ agonist activity. To further discern the role of PPARγ in thiazolidenedione-induced cyclin D1 ablation, we synthesized the unsaturated derivatives of troglitazone and ciglitazone, i.e., Δ2-TG and Δ2-CG (Fig. 2A), both of which were inactive in PPARγ activation according to a PPARγ transcription factor ELISA (right panel). The effects of troglitazone, ciglitazone, and their Δ2-counterparts on the expression of cyclin D1 and ERα in MCF-7 cells were analyzed by Western blotting.

<Insert Fig. 2>

As shown, Δ2-TG and Δ2-CG, though devoid of PPARγ activity, were able to reduce the expression levels of cyclin D1 and ERα in MCF-7 cells in a dose-dependent manner with potency higher than that of troglitazone and ciglitazone (Fig. 2B). For example, the minimum concentration required for the complete ablation of cyclin D1 was 30 µM for both Δ2-TG and Δ2-CG, as compared to 40 and 50 µM for troglitazone and ciglitazone, respectively. In contrast, the effect of these agents on ERα lagged behind that of cyclin D1, requiring substantially higher concentrations to achieve the same extent of repression.

Fig. 3A depicts the time course of cyclin D1 down-regulation by 40 µM troglitazone and 30 µM Δ2-TG in MCF-7 cells. Both agents achieved complete ablation at 24 h after treatment. However, semi-quantitative PCR shows that the mRNA level of cyclin D1 remained unaltered after 24 h-exposure (Fig. 3), suggesting that troglitazone- and Δ2-TG-induced cyclin D1 ablation was mediated at the posttranscriptional level.

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<Insert Fig. 3>

To examine whether the ablative effect of troglitazone- and Δ2-TG was unique to cyclin D1, we assessed the expression levels of cyclins D2, D3, A, B, and E, and cyclin-dependent kinases (CDKs) 2 and 4 in MCF cells treated with different doses of troglitazone- and Δ2-TG (Fig. 4). Among these cell cycle-regulating proteins, while cyclin D2 and CDK4 showed a slight decrease in the expression level, no appreciable effect was observed with the other cyclins and CDKs, indicating that the ablative effect was highly specific.

<Insert Fig. 4>

Troglitazone and Δ2-TG facilitate proteasome-mediated proteolysis of cyclin D1. Pursuant to the report that the effect of PGJ_2 and ciglitazone on cyclin D1 repression was attributable to proteasome-mediated degradation (Choi et al., 1997; Qin et al., 2003), we tested the effect of three proteasome inhibitors (MG132, lactacystin, epoxomicin) on troglitazone and Δ2-TG-facilitated cyclin D1 ablation in MCF-7 cells. As shown in Fig. 5, all three proteasome inhibitors were effective in rescuing the drug-induced cyclin D1 repression.

<Insert Fig. 5>

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As proteasome-facilitated proteolysis of cyclin D1 is preceded by ubiquitination (Coqueret, 2002), we examined the formation of ubiquitinated cyclin D1in MCF-7 cells treated with the DMSO vehicle, 30 μM troglitazone or 20 μM Δ2-TG for 20 h. The cell lysates were exposed to cyclin D1 antibodies, followed by protein A-beads. Equivalent amounts of the immunoprecipitated proteins were subject to Western blotting with either cyclin D1 or ubiquitin antibodies (Fig. 6). As shown, while cyclin D1 expression was diminished in troglitazone- and Δ2-TG-treated MCF-7 cells (left panel; IP, anti-cyclin D1; WB, anti-cyclin D1), the extent of ubiquitination of cyclin D1 increased as indicated by a complex ladder of ubiquitinated cyclin D1 bands (right panel; IP, anti-cyclin D1; WB, anti-ubiquitin).

<Insert Fig. 6>

Recent evidence indicates that cyclin D1 ubiquitination could be facilitated by either a glycogen synthase kinase (GSK)- 3β-dependent or –independent pathway. In the GSK-3β-dependent pathway, CDK-bound cyclin D1 undergoes GSK-3β-mediated phosphorylation, followed by translocation to the cytoplasm where it undergoes proteasomal degradation (Diehl et al., 1998; Diehl et al., 1997). Alternatively, free cyclin D1 can be ubiquitinated independently of GSK-3β, though the exact mechanism remains elusive (Germain et al., 2000). Here, we obtained two lines of evidence to exclude the involvement of GSK-3β in troglitazone- and Δ2-TG-facilitated cyclin D1 degradation. First, the GSK-3β phosphorylation level remained unaltered in troglitazone- and Δ2-TG-

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treated MCF-7 cells (Fig. 7A). Second, co-treatment with the selective GSK-3β inhibitor SB216763 could not rescue troglitazone- or Δ2-TG-induced cyclin D1 ablation (panel B).

<Insert Fig. 7>

Development of novel Δ2-TG-derived cyclin D1-ablative agents. The findings described above prompted a notion that Δ2-TG could be used as a scaffold to develop novel cyclin D1 ablative agents. Accordingly, a series of Δ2-TG derivatives were synthesized, and their respective activities in ablating cyclin D1 in MCF-7 cells were examined. Among more than 20 derivatives tested, Δ2-TG-6 represented a structurally optimized agent with potency an-order-of-magnitude higher than that of Δ2-TG. Structurally, this increase was attributed to an additional allyl moiety on the terminal hydroxyl function of Δ2-TG (Fig. 8A). As shown, Δ2-TG-6 reduced cyclin D1 levels at concentrations as low as 2.5 μM vis-à-vis ≥ 20 μM for Δ2-TG (panel B). Like its parent molecule, the effect of Δ2-TG-6 on cyclin D1 ablation could be blocked by the proteasome inhibitor MG-132 (panel C). In line with its enhanced ability in cyclin D1 ablation, Δ2-TG-6 exhibited significantly higher potency than Δ2-TG in inhibiting MCF-7 cell proliferation (IC_{50}, 8 μM versus 55 μM) (panel D).

<Fig. 8>

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Discussion

A variety of mechanisms have been proposed to account for the ability of various antiproliferative agents to ablate cyclin D1 expression. These include transcriptional repression of the cyclin D1 promoter (flavopiridol and PGJ_2) (Carlson et al., 1999; Wang et al., 2001), calpain-mediated proteolytic degradation (lovastatin and actinomycin D) (Choi et al., 1997), and proteasome-facilitated proteolysis (retinoic acid and various PPARγ agonists) (Langenfeld et al., 1997; Lapillonne et al., 2003; Wang et al., 2001)]. From a clinical perspective, this drug-induced cyclin D1 repression not only contributes to the inhibition of breast cancer cell proliferation, but can also overcome drug resistance by sensitizing breast cancer cells to apoptotic signals emanating from Akt inhibition (Wu et al., 2002). Thus, an urgent need exists to develop potent cyclin D1-ablative agents that are effective in the therapeutically attainable range ($\leq$ 5 μM) for the treatment and/or prevention of breast cancer.

Of the aforementioned agents, the PPARγ agonists troglitazone and ciglitazone represent attractive molecules for this drug discovery effort. Thus, we first investigated the mechanism underlying troglitazone- and ciglitazone-mediated cyclin D1 down-regulation. Several lines of evidence suggest that the effect of troglitazone and ciglitazone on cyclin D1 is independent of PPARγ activation. First, this cyclin D1-ablative effect was not noted with the more potent PPARγ agonists rosiglitazone and pioglitazone at comparable concentrations, and could not be rescued by the PPARγ antagonist GW9662. Second, despite significantly higher PPARγ expression, MDA-MB-231 cells were less susceptible to troglitazone-mediated cyclin D1 ablation. Third, Δ2-TG and Δ2-CG, through devoid of PPARγ activity, were able to mediate cyclin D1

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ablation with slightly higher potency than that of troglitazone and ciglitazone. Furthermore, troglitazone and Δ2-TG share the mechanism in down-regulating cyclin D1 in MCF-7 cells. Our data indicate that both agents facilitated proteasomal proteolysis via a GSK-3β-independent mechanism. Two lines of evidence suggest that ERα might play a role in the thiazolidenedione-promoted degradation of cyclin D1. First, the cyclin D1 ablation was accompanied by a decrease in ERα expression in MCF-7 cells (Fig. 2). Second, the ERα-negative MDA-MB-231 cells were more resistant to the cyclin D1-ablative effect of troglitazone (Fig. 1). This thiazolidenedione-mediated down-regulation of cyclin D1 and ERα is reminiscent of that of the histone deacetylase inhibitor trichostatin A (TSA)(Alao et al., 2004). TSA has been shown to repress cyclin D1 and ERα expression, in part, through the up-regulation of Skp2/p45, a regulatory component of the Skp1/Cullin/F-box complex implicated in the ubiquitination of cyclin D1 (Alao et al., 2004). Involvement of Skp2 in thiazolidenedione-mediated cyclin D1 ablation is currently under investigation.

The separation of cyclin D1 ablation from PPARγ provides a rationale to use the structure of Δ2-TG as a platform to carry out lead optimization. The proof of principle for this premise was Δ2-TG-6, a close structural analogue that exhibited an-order-of-magnitude higher potency than troglitazone and Δ2-TG in facilitating cyclin D1 repression and inhibiting MCF-7 cell proliferation. The clinical impetus of these small-molecule cyclin D1 ablative agents in breast cancer therapy/prevention is multifold. First, cyclin D1 ablation provides specific protection against breast carcinogenesis (Yu et al., 2001). Second, in light of the role of cyclin D1 overexpression in antiestrogen resistance, cyclin D1 ablation may help overcome the resistance. Third, the synergistic

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interaction between flavopiridol and trastuzumab in inhibiting breast cancer cell proliferation was attributable, in part, to the reduction of cyclin D1 expression (Wu et al., 2002). These agents may sensitize cells to the antiproliferative action of either CDK inhibition or Her-2/Akt inhibition. Consequently, structural modifications of Δ2-TG-6 to further enhance its cyclin D1-ablative potency constitute the current focus of this investigation.

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Footnotes

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Figure legends

Fig. 1 Effect of troglitazone on cyclin D1 down-regulation in breast cancer cells is irrespective of PPARγ expression levels. *A*, differential expression levels of PPARγ in MDA-MB-231 and MCF-7 cells. *B*, dose-dependent effect of troglitazone on cyclin D1 repression in MDA-MB-231 and MCF-7 cells. Cells were treated with troglitazone at the indicated concentrations in 5% FBS-supplemented DMEM-F12 medium for 24 h. These Western blots are representative of three independent experiments. *C*. High doses of the PPARγ antagonist GW9662 have no effect on cyclin D1 expression (left panel) or troglitazone-mediated cyclin D1 ablation (right panel) in MCF-7 cells.

Fig. 2 Pharmacological evidence that the effect of troglitazone and ciglitazone on cyclin D1 down-regulation is dissociated from PPARγ activation. *A*, chemical structures of troglitazone, ciglitazone, and the respective Δ2-derivatives and evidence that Δ2-TG and Δ2-CG are devoid of activity in PPARγ activation (right panel; TG, troglitazone; CG, ciglitazone). Analysis of PPARγ activation was carried out as described under the Materials and Methods. In brief, MCF-7 cells were exposed to individual test agents (10 μM) or DMSO vehicle in 10% FBS-supplemented RPMI 1640 medium for 48 h. Amounts of PPARγ in the resulting nuclear extracts were analyzed by PPARγ transcript factor ELISA kit. Each data point represents mean + S.D. (n = 3). *B*, dose-dependent effect of troglitazone, Δ2-TG on cyclin D1 and ERα expression in MCF-7 cells. MCF-7 cells were exposed to the individual agents at the indicated concentrations in 5% FBS-supplemented

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medium for 24 h, and the expression of cyclin D1 and ERα was analyzed by Western blot analysis (upper panel). Signals were quantitated by densitometry and normalized against β-actin measurements (lower panel). Each data point represents mean ± S.D. (n = 3). *C*, dose-dependent effect of ciglitazone, Δ2-CG on cyclin D1 and ERα expression in MCF-7 cells.

Fig. 3 Troglitazone- and Δ2-TG-mediated cyclin D1 ablation is mediated at the post-transcriptional level. *A*, time-dependent effect of 40 μM troglitazone and 30 μM Δ2-TG on cyclin D1 expression in MCF-7 cells. B, RT-PCR analysis of the mRNA transcripts of cyclin D1 gene in MCF-7 cells after exposure to 40 μM troglitazone (TG) or 30 μM Δ2-TG for 24 h. Signals were quantitated by densitometry and normalized against β-actin measurements (lower panel). Each data point represents mean ± S.D. (n = 3).

Fig. 4 Dose-dependent effects of troglitazone and Δ2-TG on the expression of cyclins and CDKs. MCF-7 cells were exposed to the individual agents at the indicated concentrations in 5% FBS-supplemented medium for 24 h, and the expression of various cell cycle-regulating proteins was analyzed by Western blot analysis.

Fig. 5 Dose-dependent effects of the proteasome inhibitors MG132, lactacystin, and epoxomicin on troglitazone- and Δ2-TG-mediated cyclin D1 ablation. MCF-7 cells were exposed to 40 μM troglitazone or 30 μM Δ2-TG in the presence of various concentrations of the proteasome inhibitor in 5% FBS-supplemented

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medium for 24 h, and the expression of cyclin D1 was analyzed by Western blot analysis.

Fig. 6 Cyclin D1 ubiquitination in troglitazone (TG)- and Δ2-TG-treated MCF-7 cells. Cell were treated with DMSO vehicle, 30 μM troglitazone, or 20 μM Δ2-TG in 5% FBS-containing medium for 20 h. Cell lysates were immunoprecipitated with anti-cyclin D1, and the immunoprecipitates were analyzed by Western blotting with anti-cyclin D1 or anti-ubiquitin as described in the Materials and Methods.

Fig. 7 Evidence that troglitazone and Δ2-TG-induced cyclin D1 down-regulation is independent of GSK-3β activation. *A*, the phosphorylation levels of GSK-3β remained unaltered in MCF-7 cells treated with different doses of troglitazone and Δ2-TG. *B*, the GSK-3β inhibitor SB216763 could not rescue troglitazone- and Δ2-TG-induced cyclin D1 ablation.

Fig. 8 Δ2-TG-6, a structurally optimized cyclin D1-ablative agent. A, structure of Δ2-TG-6. B, dose-dependent effect of Δ2-TG-6 on cyclin D1 down-regulation in MCF-7 cells. C, Δ2-TG-6-mediated cyclin D1 ablation is facilitated by proteasomal proteolysis. D, dose-dependent effects of Δ2-TG-6 versus troglitazone (TG) and Δ2-TG on MCF-7 cell viability. MCF-7 cells were exposed to Δ2-TG-6, troglitazone or Δ2-TG at the indicated concentrations in 5% FBS-supplemented DMEM-F12 medium in 96-well plates for 24 h, and cell viability

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was assessed by MTT assay. Each data point represents the means of six replicates.

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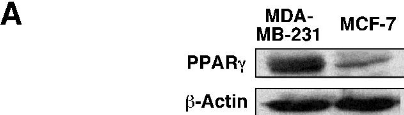
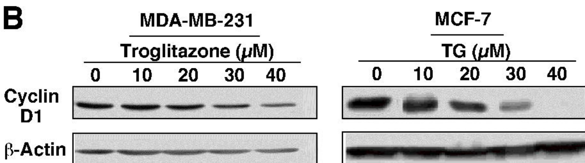
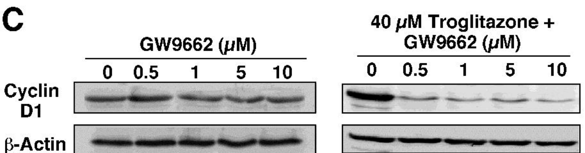
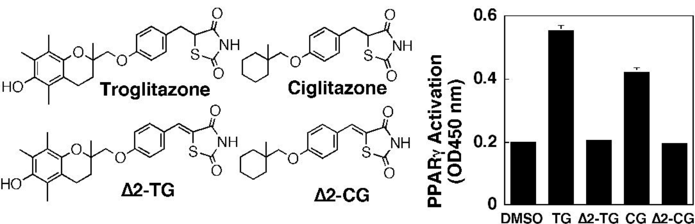
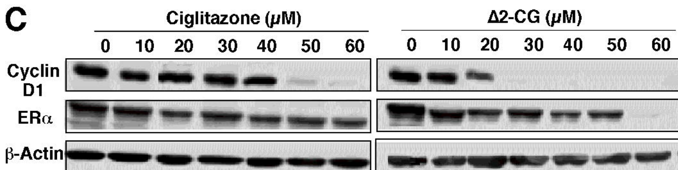

A
MDA-
MB-231
MCF-7
PPARγ
β-Actin


B
MDA-MB-231
Troglitazone (μM)
0
10
20
30
40
Cyclin
D1
β-Actin
MCF-7
TG (μM)
0
10
20
30
40


C
GW9662 (μM)
0
0.5
1
5
10
Cyclin
D1
β-Actin
40 μM Troglitazone +
GW9662 (μM)
0
0.5
1
5
10


A
Troglitazone
Ciglitazone
Δ2-TG
Δ2-CG
PPARγ Activation (OD450 nm)
0.6
0.4
0.2
0
DMSO
TG
Δ2-TG
CG
Δ2-CG

B
Troglitazone (μM)
0 10 20 30 40 50 60
Δ2-TG (μM)
0 10 20 30 40 50 60
Cyclin D1
ERα
β-Actin
% Relative Expression Level
100
75
50
25
0
Cyclin D1
ERα
0 10 20 30 40 50 60
Troglitazone (μM)
Δ2-TG (μM)


C
Ciglitazone (μM)
0 10 20 30 40 50 60
Δ2-CG (μM)
0 10 20 30 40 50 60
Cyclin D1
ERα
β-Actin

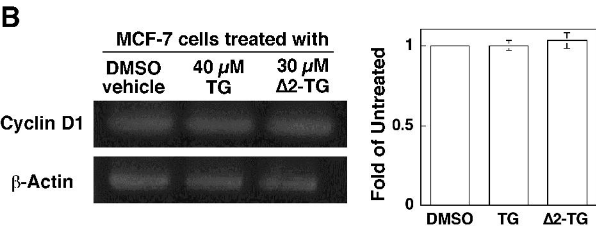
A

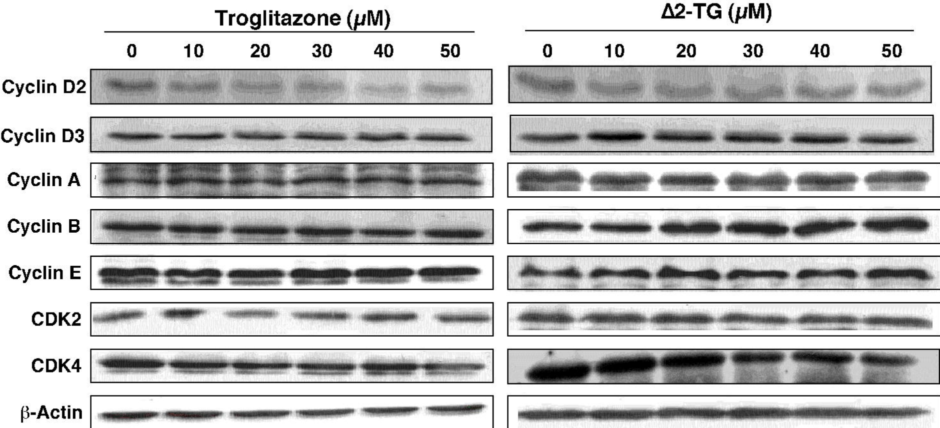
40 μM Troglitazone
Exposure Time (h)
0 6 12 18 24
Cyclin D1
β-Actin

30 μM Δ2-TG
Exposure Time (h)
0 6 12 18 24
Cyclin D1
β-Actin

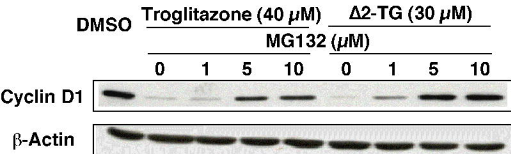
% Relative Expression
100
75
50
25
0
40 μM Troglitazone
0 6 12 18 24
Exposure Time (h)

% Relative Expression
100
75
50
25
0
30 μM Δ2-TG
0 6 12 18 24
Exposure Time (h)





Troglitazone (μM)
0
10
20
30
40
50
Δ2-TG (μM)
0
10
20
30
40
50
Cyclin D2
Cyclin D3
Cyclin A
Cyclin B
Cyclin E
CDK2
CDK4
β-Actin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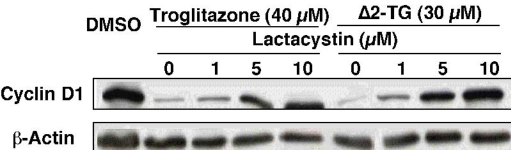
Troglitazone (40 μM)
Δ2-TG (30 μM)
MG132 (μM)
0 1 5 10 0 1 5 10
Cyclin D1
β-Actin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Troglitazone (40 μM)
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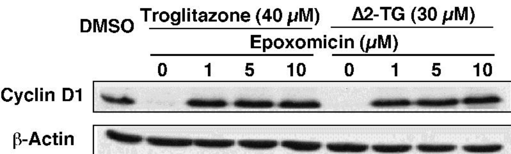
Lactacystin (μM)
0 1 5 10 0 1 5 10
Cyclin D1
β-Actin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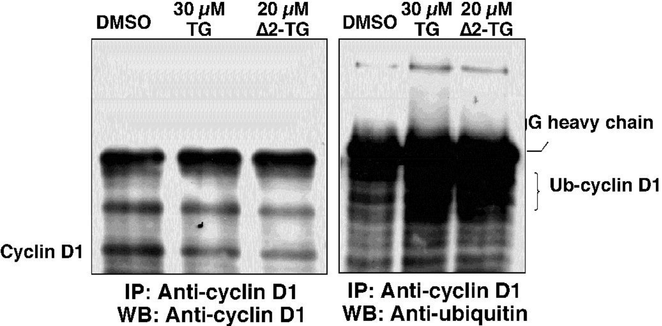
Δ2-TG (30 μM)
Epoxomicin (μM)
0 1 5 10 0 1 5 10
Cyclin D1
β-Actin


DMSO
30 μM TG
20 μM Δ2-TG
DMSO
30 μM TG
20 μM Δ2-TG
IgG heavy chain
Ub-cyclin D1
Cyclin D1
IP: Anti-cyclin D1
WB: Anti-cyclin D1
IP: Anti-cyclin D1
WB: Anti-ubiquitin

A

Troglitazone (μM)

0 10 20 30 40 50

p-GSK-3β

β-Actin

Δ2-TG (μM)

0 10 20 30 40 50

p-GSK-3β

β-Actin

B

Vehicle

30 μM Troglitazone

SB216763 (μM)

0 1 5 10

20 μM Δ2-TG

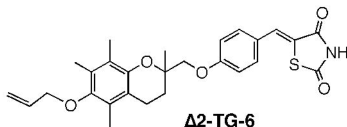
SB216763 (μM)

0 1 5 10

Cyclin D1

β-Actin

A



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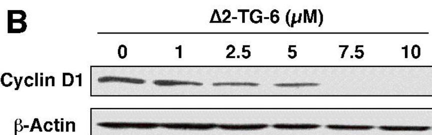
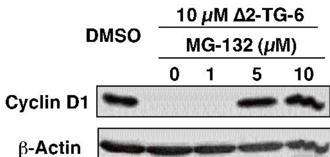


C



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