

Thiazolidinediones Modulate the Expression of β -Catenin and Other Cell-Cycle Regulatory Proteins by Targeting the F-Box Proteins of SCF E3 Ubiquitin Ligase Independently of PPAR γ *

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Running Title: Small-molecule modulators of the SCF Ubiquitin-Proteasome System

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Abbreviations: PPAR γ , peroxisome proliferator-activated receptor γ ; SCF, Skp1-Cul1-F-box-protein; β -TrCP, β -transducin repeat-containing protein; GSK3 β , glycogen synthase kinase 3 β ; PrECs, normal prostate epithelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; GST, glutathione *S*-transferase; APC, adenomatous polyposis coli

Number of text pages: 31

Tables: 0

Figures: 7

References: 45

Number of words in the Abstract: 250

Number of words in Introduction: 535

Number of words in Discussion: 899

ABSTRACT

Considering the role of aberrant β -catenin signaling in tumorigenesis, we investigated the mechanism by which the peroxisome proliferator-activated receptor γ (PPAR γ) agonist troglitazone facilitated β -catenin downregulation. We demonstrate that troglitazone and its more potent PPAR γ -inactive analogues, Δ 2TG and STG28, mediated the proteasomal degradation of β -catenin in prostate cancer cells by up-regulating the expression of β -TrCP, an F-box component of the SCF E3 ubiquitin ligase. Evidence indicates that while siRNA-mediated β -TrCP knockdown protected cells against STG28-facilitated β -catenin ablation, ectopic β -TrCP expression enhanced the degradation. The involvement of β -TrCP in β -catenin degradation was also corroborated by the pull-down analysis and the concurrent down-regulation of known β -TrCP substrates examined, including Wee1, I κ B α , cdc25A, and NF κ B/p105. Furthermore, GSK3 β represented a key regulator in the effect of these thiazolidinedione derivatives on β -catenin proteolysis even though these agents increased its phosphorylation level. It is noteworthy that this drug-induced β -TrCP upregulation was accompanied by the concomitant downregulation of Skp2 and Fbw7, thereby affecting many of the target proteins of these two F-box proteins (such as p27 and cyclin E). Consequently, the ability of troglitazone to target these F-box proteins provides a molecular basis to account for its reported effect on modulating the expression of aforementioned cell cycle-regulatory proteins. Despite this complicated mode of pharmacological actions, normal prostate epithelial cells, relative to LNCaP cells, were less susceptible to the effects of STG28 on modulating the expression of β -catenin and β -TrCP, suggesting the translation potential of using STG28 as a scaffold to develop more potent chemopreventive agents.

Introduction

Substituted thiazolidinediones such as troglitazone, ciglitazone, and rosiglitazone belong to a family of oral anti-diabetic drugs, which increase insulin sensitivity in adipose tissue, muscle, and liver primarily through the activation of peroxisome proliferator-activated receptor γ (PPAR γ) [review; (Day, 1999)]. It is well documented that ligand activation of PPAR γ can regulate multiple crucial biological processes including growth, differentiation and apoptosis through the transcriptional expression of insulin-sensitive genes involved in glucose metabolism and energy balance. It is noteworthy that these agents at high doses exhibit *in vitro* and *in vivo* antitumor effects against many types of cancers, including colon, breast, and prostate [review: (Koeffler, 2003; Weng et al., 2006)]. However, recent evidence indicates the effect of thiazolidinediones on differentiation, cell cycle, and apoptosis in these cancer cells might involve both PPAR γ -dependent and γ -independent pathways (Bae and Song, 2003; Baek et al., 2003; Gouni-Berthold et al., 2001; Huang et al., 2005; Motomura et al., 2000; Okura et al., 2000; Shiau et al., 2005; Sugimura et al., 1999; Takeda et al., 2001; Yang et al., 2006). In light of the chemopreventive potential of PPAR γ agonists, it is crucial to delineate the mechanism by which these agents mediate antitumor effects.

Among various signaling mechanisms targeted by the thiazolidinedione family of PPAR γ agonists, the ability of troglitazone to facilitate the proteasomal degradation of a series of cell cycle regulatory proteins such as cyclin D1 (Huang et al., 2005; Qin et al., 2003) and β -catenin (Liu and Farmer, 2004; Moldes et al., 2003; Sharma et al., 2004) is especially noteworthy. For example, PPAR γ -facilitated adipocyte differentiation is associated with an extensive down-regulation of β -catenin expression (Moldes et al., 2003), suggesting a crosstalk between these

two signaling pathways. β -Catenin is a dual-function protein that regulates cell adhesion and transcription in different subcellular compartments [Reviews: (Bienz, 2005; Brembeck et al., 2006; Harris and Peifer, 2005)]. In the cytoplasm and nucleus, β -catenin regulates the transcription of target genes crucial to cell proliferation and differentiation through the Wnt signaling cascade, while at the plasma membrane, it controls E-cadherin-mediated cell adhesion. Through the integration of these two signaling networks, β -catenin plays a pivotal role in maintaining the integrity of cellular functions. Substantial evidence indicates that dysregulation of β -catenin signaling contributes to human malignancies as a result of loss of cell-cell adhesion and increased transcription of Wnt target genes (Verras and Sun, 2006; Wong and Pignatelli, 2002). Consequently, targeting β -catenin expression represents a viable strategy for therapeutic and/or preventive intervention.

In this study, we used PPAR γ -inactive analogues of troglitazone, Δ 2TG and STG28, to discern the involvement of PPAR γ in troglitazone-mediated suppression of β -catenin expression in prostate cancer cells. Several lines of evidence indicate that the effect of these thiazolidinediones on GSK3 β -dependent repression of β -catenin was attributable to ubiquitination-dependent proteasomal degradation independently of PPAR γ . Moreover, the enhanced ubiquitination of β -catenin was associated with increased accumulation of the F-box protein β -TrCP (β -transducin repeat-containing protein) (Kitagawa et al., 1999; Latres et al., 1999; Liu et al., 1999; Winston et al., 1999) of the SCF (Skp1-Cul1-F-box-protein) complex through protein stabilization. From a translational perspective, the high potency of STG28 in facilitating β -catenin degradation provides a proof-of-principle that potent β -catenin-ablating agents could be developed through structural optimization of troglitazone.

Materials and Methods

Reagents. Troglitazone, MG132, epoxomicin, lithium chloride, and SB216763 were purchased from Sigma-Aldrich (St. Louis, MO). ZVAD-fmk was obtained from Calbiochem (San Diego, CA). The PPAR γ -inactive thiazolidinedione derivatives Δ 2TG and STG28 (Huang et al., 2005; Yang et al., 2006) were synthesized according to a published procedure (Huang et al., 2006). These agents were added to medium with a final DMSO concentration of 0.1%. Antibodies against various proteins were obtained from the following sources. Mouse monoclonal antibodies: PPAR γ (sc-7273), β -catenin (sc-7963), Wee1 (sc-5285), nucleolin (sc-8031), and Na⁺/K⁺ ATPase α (sc-28800), Santa Cruz Biotechnology (Santa Cruz, CA); HA and Myc, Roche (Indianapolis, IN); β -TrCP, Skp2, and Fbw7, Invitrogen (Carlsbad, CA); β -actin, MP Biomedicals (Irvine, CA). Rabbit antibodies: p-Ser33/Ser37/Thr41 β -catenin, p-Ser9-GSK3 β , GSK3 β , Cdc25A, NF κ Bp105, PARP, and caspase 3, Cell Signaling Technology (Beverly, MA); I κ B α (sc-203), p21 (sc-756), p27 (sc-528), and cyclin E (sc-481), Santa Cruz.

Cell Culture. LNCaP, DU145 and PC-3 prostate cancer cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in T-75 flasks with RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO₂. Normal prostate epithelial cells (PrECs) were obtained from Cambrex Bioscienc-Walkersville (Walkersville, MD), and maintained in defined prostate epithelial growth medium recommended by the vendor.

RNA Isolation and RT-PCR Analysis. After vehicle or drug treatments, LNCaP cells were subject to total RNA isolation by using an RNeasy mini kit (Qiagen, Valencia, CA). RNA concentrations were determined by measuring absorption at 260 nm in a spectrophotometer.

Aliquots of 6 μ g of total RNA from each sample were reverse transcribed to cDNA using an Omniscript RT Kit (Qiagen) according to manufacturer's instructions. PCR primers used in this study included:

β -catenin: 5'-CTGATTTGATGGAGTTGGAC-3' and 5'-TATCAGCTACTTGTCTTGAG-3';

β TrCP: 5'-CACTTAGACACATACAACA-3' and 5'-TCTGCAACATAGGTTTAAGAT-3';

β -actin: 5'-TCTACAATGAGCTGCGTGTG-3' and 5'-GGTCAGGATCTTCATGAGGT-3'.

PCR reaction products were separated electrophoretically in 1.5 % agarose gels and visualized by ethidium bromide staining.

Cell Viability Assay. 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 (96-well format). PrECs and LNCaP cells were seeded at 8,000 and 3,000 cells/well, respectively, in 96-well flat-bottomed plates, and incubated for 24 h in 10% FBS-prostate epithelial growth medium and 10% FBS-supplemented RPMI 1640 medium, respectively. Cells were then treated with DMSO, troglitazone or STG28 at various concentrations in the presence of 10 μ M MG132 or 100 μ M ZVAD-FMK. Controls received DMSO at a concentration equal to that in drug-treated cells. After 48 h, 1/10 volume of 10X MTT (5 mg/ml) was added to each well and cells were incubated at 37°C for 2 h. Medium was removed and the reduced MTT dye was solubilized in 200 μ l/well DMSO. Absorbance was determined at 570 nm.

Immunoblotting. Cells in T-75 flasks were harvested by scraping, and cell lysates were prepared using a commercial lysis buffer (M-PER Mammalian Protein Extraction Reagent; Pierce, Rockford, IL) in the presence of a 1% protease inhibitor cocktail (Calbiochem). After centrifugation for 20 min, 2 μ l of the suspension were taken for protein determination using a Bradford assay kit (Bio-Rad, Hercules, CA). To the remaining solution was added the same

volume of 2X SDS polyacrylamide gel electrophoresis sample loading buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 5% β -mercaptoethanol, 20% glycerol, and 0.1% bromphenol blue), and boiled for 10 min. Equal amounts of proteins were resolved in 10% SDS-polyacrylamide gels. After electrophoresis, gel was transferred to nitrocellulose membranes using a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 1 h, the membrane was incubated with the appropriate primary antibody (diluted 1:1000) in 1% TBST nonfat milk at 4°C overnight. After incubation with the primary antibody, the membrane was washed three times with TBST for a total of 30 min, followed by incubation with horseradish peroxidase conjugated goat anti-rabbit or anti-mouse IgG (diluted 1:2500) for 1 h at room temperature. After three times of extensive wash with TBST for a total of 30 min. The immunoblots were visualized by enhanced chemiluminescence.

Flow Cytometry Analysis of Apoptosis. Cells were seeded in 6-well plates at a density of 5×10^5 cells per well, and after overnight incubation, and were exposed to 10 μ M STG28 for different time intervals. Both floating and adherent cells were collected, and stained with annexin V-Alexa Fluor 488 and propidium iodide (PI) according to the vendor's protocols (Molecular Probes, Eugene, OR). The analysis of annexin V/PI staining was performed using a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson Immunocytometry System, San Jose, CA) equipped with CellQuest software.

Cell Fractionation. LNCaP cells were incubated with 10% FBS-supplemented RPMI 1640 medium for 24 h, and exposed to 10 μ M STG28 treatment for different time intervals. Collected cells were lysed and fractionated by using a Qproteome Cell Compartment kit (Qiagen., Valencia, CA) according to the manufacturer's instructions. In brief, treated cells were washed

twice with PBS and incubated with extraction buffer CE1 for 10 min at 4°C. After 10 min centrifugation at 1000 xg at 4°C, supernatants were collected as cytosolic fraction, and pellets were subject to extraction buffer CE2 at 4°C for 30 min. The lysates were centrifuged at 6000 xg for 10 min at 4°C to obtain supernatants as membrane fraction. Pellets were further incubated with nuclease at room temperature for 15 min followed by extraction buffer CE3 for 10 min at 4°C. After nuclear membrane was solubilized, nuclear fraction were obtained from the supernatant by centrifugation at 6800 xg for 10 min at 4°C. Subcellular fractions were quantitated and subject to immunoblotting for β -catenin detection. Na⁺/K⁺ ATPase α , nucleolin and β -actin were served as markers and internal controls for membrane, nuclear or cytosolic compartments, respectively.

Transient Transfection and RNA Interference. Transfection of LNCaP cells was performed by electroporation using an Amaxa Nucleofector with a cell line-specific nucleofector kit according to the manufacturer's protocol (Amaxa Biosystems, Cologne, Germany) with the HA-tag ubiquitin plasmid or the pMyc4-h β TrCP-CMV14 plasmid encoding Myc-tagged full-length β TrCP (Yang et al., 2005). The nucleofected cells were seeded in 6-well plates at 5×10^5 cells per well, and incubated in 10% FBS-containing medium for 24 h before drug treatment. The transfection efficiency was determined to be 70-80% by electroporating cells with 2 μ g of pmaxGFP plasmids, followed by fluorescence microscopy to measure GFP expression. For small interfering RNA (siRNA) experiments, LNCaP cells were electroporated with scrambled or β TrCP siRNA (Santa Cruz), seeded in 6-well plates (5×10^5 cells/well), and incubated for 24 h before drug treatment. For small interfering RNA (siRNA) experiments, PC3 and LNCaP cells were electroporated with siRNA against PPAR γ (Upstate) and or β TrCP siRNA (Santa Cruz), respectively, with the respective scrambled siRNA as control. Cells were seeded in 6-well plates

(5×10^5 cells/well), and incubated for 24 h. After 10 μ M STG28 treatment for additional 24 or 48 h, cells were lysed and protein extracts were prepared for Western blot analysis.

Immunoprecipitation. LNCaP cells were nucleofected with 5 μ g of HA-ubiquitin plasmids, incubated in 6-well plates for 24 h, and treated with 60 μ M troglitazone, 40 μ M Δ 2TG, or 10 μ M STG28 for 20 or 44 h, followed by co-treatment with the proteasome inhibitor MG132 for additional 4 h. After harvest, cells were lysed by RIPA lysis buffer (Santa Cruz) with freshly added phosphatase and protease inhibitors consisting of 100 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride, 80 nM aprotinin, 5 μ M bestatin, 1.5 μ M E-64 protease inhibitor, 2 μ M leupeptin, 1 μ M pepstatin A, 2 mM imidazole, 1 mM sodium fluoride, 1 mM sodium molybdate, 1 mM sodium orthovanadate, and 4 mM sodium tartrate dihydrate. After centrifugation at 13,000 $\times g$ for 20 min, the supernatant was collected, preincubated with protein A-Agarose (Santa Cruz) for 15 min, and centrifuged at 1,000 $\times g$ for 5 min. One tenth of the supernatant was stored away at 4°C to be used as input, and the remainder was exposed to anti- β -catenin antibody and protein-A agarose at 4°C for 12 hours. After brief centrifugation, immunoprecipitates were collected, washed with the aforementioned lysis buffer twice, suspended in 2x SDS sample buffer, and subject to Western blot analysis with antibodies against HA and β -catenin.

Preparation of Glutathione S-Transferase (GST)- β -TrCP Fusion Protein. The pMyc4-h β TrCP-CMV14 plasmid encoding Myc-tagged full-length β TrCP was subcloned into BamHI/EcoRI sites of pGEX vector for GST- β -TrCP fusion protein expression. The resulting pGEX- β -TrCP plasmid was verified by DNA sequencing. The GST- β -TrCP recombinant protein was expressed in *Escherichia coli* by isopropyl-1-thio- β -D-galactopyranoside induction. After overnight incubation, bacteria were lysed in PBS containing 1mg/ml lysozyme (Pierce) and sonicated for 5min. The bacterial lysates were subject to Western blot analysis for fusion protein

identification. Recombinant GST- β -TrCP fusion protein was purified by using Glutathione-Sepharose beads (Amersham Biosciences) with gentle rocking at 4 °C for 2 h according to the manufacturer's protocol. Equal amounts of GST protein were prepared as control in a similar manner. The fusion protein immobilized onto glutathione beads was washed three times with cold PBS buffer and used directly for the following GST pull-down assay.

GST Pull-Down Assay. LNCaP cells were seeded and incubated in 10% FBS-containing RPMI 1640 medium in T-25 flasks for 48 h, and then exposed to 10 μ M STG28 for 24 h with or without 10 μ M epoxomicin. Cells were lysed by PBS buffer containing 1% TritonX-100 and a protease inhibitor cocktail (Calbiochem), incubated with glutathione beads for 2 h, and then incubated with equal amounts of GST- or GST- β -TrCP-immobilized glutathione beads at 4 °C for 4 h. The incubation mixtures were washed three times with PBS containing 1% TritonX-100, and the resulting precipitates were subjected to Western blot analysis with mouse antibodies against β -catenin and β TrCP, and goat antibody against GST.

Results

Troglitazone-Mediated Repression of β -Catenin Is Attributable to an “Off-Target”

Mechanism. We obtained four lines of evidence that the effect of troglitazone on suppressing β -catenin expression was dissociated from PPAR γ activation. First, we investigated the effect of troglitazone on β -catenin repression in three prostate cancer cell lines with differential PPAR γ expression: DU-145, LNCaP, and PC-3, in 10% FBS-supplemented medium. Although LNCaP cells expressed substantially lower levels of PPAR γ relative to DU-145 and PC-3 cells, these three cell lines were equally susceptible to troglitazone-mediated β -catenin down-regulation (Fig. 1A). Second, we examined the dose-dependent effect of a potent PPAR γ antagonist, GW9662 (Leesnitzer et al., 2002; Seargent et al., 2004), on troglitazone-facilitated β -catenin repression in PC-3 cells. Even at concentrations three orders of magnitude higher than the IC₅₀ in PPAR γ binding, GW9662 had no appreciable effect on β -catenin expression, and did not prevent troglitazone-mediated β -catenin repression (Fig. 1B). Third, we assessed the effect of two PPAR γ -inactive analogues of troglitazone, Δ 2TG and STG28 (Huang et al., 2006; Huang et al., 2005; Yang et al., 2006), vis-à-vis troglitazone on intracellular β -catenin in LNCaP cells. As evidenced by PPRE-luciferase reporter assay, Δ 2TG and STG28 lacked appreciable activity in PPAR γ transactivation (Fig. 1C, upper panel). Both agents, however, were effective in suppressing β -catenin expression. Especially, the PPAR γ -inactive analogue STG28 exhibited multifold higher potency than troglitazone (lower panel), providing a proof-of-principle that the ability of troglitazone to repress β -catenin could be pharmacologically exploited to develop a

novel class of β -catenin-ablative agents. Fourth, siRNA-mediated knockdown of PPAR γ in PC-3 cells exhibited no appreciable effect on STG28-induced suppression of β -catenin (Fig. 1D).

STG28 Stimulates Tumor Cell-Specific Repression of β -Catenin via Proteasomal Degradation. Fig. 2A depicts the time-dependent effect of troglitazone (60 μ M), Δ 2TG (40 μ M), and STG28 (10 μ M) on β -catenin expression in LNCaP cells at both protein and mRNA levels. Although treatment with these agents led to a progressive decline in β -catenin protein levels, the mRNA level remained largely unaltered throughout the course of study, suggesting that the drug-induced β -catenin repression was mediated at the post-transcriptional stage. We further investigated the temporal and spatial effect of 10 μ M STG28 on β -catenin in different cellular compartments of LNCaP cells via cellular fractionation (Fig. 2B). Western blot analysis indicates that β -Catenin was mainly localized to the membrane and cytoplasm, and that in response to STG28, cytoplasmic β -catenin disappeared at much faster rates than the membrane-bound counterpart. After 48 h of drug treatment, while cytoplasmic β -catenin was eliminated to a great extent, significant amounts of β -catenin still remained on cytoplasmic membranes.

Relative to LNCaP cells, normal prostate epithelial cells (PrECs) were resistant to STG28's effect on suppressing β -catenin expression and cell viability (Fig. 2C). As shown, exposure of PrECs to 10 μ M STG28 did not cause a significant reduction in β -catenin expression (left panel). This discriminatory effect underscored the differential susceptibility of LNCaP cells versus PrECs to the inhibitory effect of STG28 on cell viability via MTT assay (right panel; 48h-treatment).

From a mechanistic perspective, the effect of STG28 on suppressing β -catenin expression might be facilitated by two distinct mechanisms: proteasomal degradation and apoptosis-induced cleavage by caspase-3 (Steinhusen et al., 2000). We obtained two lines of evidence to refute the

possible involvement of caspase-3 in the drug action. First, STG28-mediated β -catenin repression preceded apoptosis. As evidenced by Annexin V/PI staining (Fig. 2D), STG28-induced apoptosis in LNCaP cells occurred predominantly 36 h after drug treatment vis-à-vis 24 h for β -catenin repression (panel C). Second, the proteasome inhibitor MG132, but not the caspase inhibitor ZVAD-FMK, could protect LNCaP cells from troglitazone and STG28-mediated suppression of β -catenin expression (Fig. 3A, left panel). As shown, while MG132 exhibited a complete protection, blockade of caspase-3 activation by the pan caspase inhibitor had no apparent effect on counteracting troglitazone- or STG28-facilitated ablation. In contrast, PrECs were not susceptible to the repressing effect of either agent irrespective of the presence of MG132 or ZVAD-FMK (right panel).

Because proteasome-facilitated proteolysis is preceded by ubiquitination, we examined the formation of ubiquitinated β -catenin in ectopic HA-ubiquitin-expressing LNCaP cells in response to troglitazone (60 μ M), Δ 2TG (40 μ M), and STG28 (10 μ M) (Fig. 3B). Cells were exposed to individual agents for 20 or 44 h, co-treated with 10 μ M MG132 for additional 4 h, and cell lysates were immunoblotted with β -catenin antibodies (input) or immunoprecipitated by anti- β -catenin antibody-agarose conjugates. Equivalent amounts of the immunoprecipitated proteins were subject to Western blotting with HA or β -catenin antibodies. As shown, treatment with individual agents led to reduced β -catenin levels (input), accompanied by increased β -catenin ubiquitination as indicated by a complex ladder of ubiquitinated β -catenin bands.

Troglitazone and Derivatives Suppress β -Catenin Expression via a Glycogen Synthase Kinase-3 β (GSK3 β)-Dependent Mechanism. It is well documented that intracellular β -catenin is tightly controlled by two ubiquitination-dependent proteasomal degradation pathways: adenomatous polyposis coli (APC)-axin/GSK3 β / β -TrCP (Hart et al., 1999; Liu et al., 2002) and

APC/Siah-1 (Liu et al., 2001; Matsuzawa and Reed, 2001). To discern the role of these two pathways, we assessed the effect of troglitazone, Δ 2TG, and STG28 on GSK3 β phosphorylation, and the effect of two GSK3 β kinase inhibitors, LiCl and SB216763, on rescuing STG28-mediated β -catenin degradation in LNCaP cells. Our data indicate that exposure of LNCaP cells to troglitazone, Δ 2TG, and STG28 caused a dose-dependent increase in GSK3 β ^{Ser9} phosphorylation (Fig. 4A). However, inhibition of GSK3 β by LiCl or SB216763 could protect against STG28-facilitated β -catenin repression in a dose-dependent manner (Fig. 4B), suggesting that GSK3 β played a key role in the drug-induced β -catenin degradation despite its phosphorylating deactivation. In addition, we examined the effect of this combination treatment on the expression level of β -TrCP, a putative F-box protein for catenin recognition (please see the next section for more discussion). Although LiCl or SB216763, by itself, could slightly decrease β -TrCP expression, the GSK3 β inhibitor exhibited no appreciable effect on suppressing the ability of STG28 to upregulate β -TrCP expression.

The premise regarding the central role of GSK3 β was corroborated by the correlation between the time-dependent suppression of β -catenin expression by troglitazone, Δ 2TG, and STG28 (Fig. 2A) and the increased phosphorylation of β -catenin at Ser33/Ser37/Thr41 (Fig. 4C), a known GSK3 β phosphorylation site recognized by β -TrCP (Orford et al., 1997; Sadot et al., 2002). The level of p- β -catenin was hardly detectable in the absence of drug treatment, but increased in a time-dependent manner parallel that of decrease in β -catenin expression when cells were exposed to individual agents.

Together, these findings are in contrast to a previous report that troglitazone mediated β -catenin degradation via a GSK3 β -independent pathway in hepatocytes (Sharma et al., 2004).

However, this discrepancy might be attributable to differences in the regulation of β -catenin degradation between LNCaP cells and hepatocytes.

Troglitazone and Derivatives Exhibit Dichotomous Effects on Modulating the Expression of Different SCF F-Box Proteins. Pursuant to the above findings, we examined the effect of these small-molecule agents on the expression levels of three SCF F-box proteins: β -TrCP, Skp2 and Fbw7 (Fig. 5A). Our data indicate that troglitazone, Δ 2TG, and STG28 increased the expression level of β -TrCP in a dose-dependent manner parallel that of β -catenin repression, providing a mechanistic link between these two pharmacological responses. It is noteworthy that STG28-mediated increase in β -TrCP expression was accompanied by decreased expression of Fbw7 and, to a greater extent, Skp2. This dichotomous effect led to divergent changes in the expression levels of β -TrCP-targeted versus Fbw7/Skp2-targeted proteins. For example, a series of known β -TrCP substrates, including Wee1, I κ B α , Cdc25A, and NF κ B p105, showed dose-dependent decreases in their expression levels as a result of β -TrCP upregulation. In contrast, the drug-induced downregulation of Skp2 and Fbw7 led to accumulation of their substrates p27 and cyclin E.

RT-PCR analysis indicates that exposure of LNCaP cells to different doses of STG28 did not affect the mRNA level of β -TrCP despite a robust increase in its protein level. This finding suggests that the upregulation of β -TrCP by STG28 was mediated by prolonging its protein stability. To test this premise, we assessed the effect of STG28 on the half-life of β -TrCP in LNCaP cells treated with the protein synthesis inhibitor cycloheximide (Fig. 5C). Cells were exposed to DMSO or 10 μ M STG28 for 12 h, followed by the treatment of 100 μ g/ml of cycloheximide for different time intervals. In vehicle-pretreated cells, β -TrCP had a half-life of approximately 6 h, and was almost completely eliminated at 24 h (left panel). In contrast, in

STG28-pretreated cells, the protein level of β -TrCP remained unaltered throughout the course of cycloheximide treatment. Based on this finding, we rationalized that STG28 treatment suppressed the turnover of β -TrCP, resulting in its accumulation in LNCaP cells. Relative to LNCaP cells, normal prostate epithelial cells (PrECs) were resistant to STG28-mediated β -TrCP upregulation (Fig. 5D), which reflected the differential effect of STG28 on suppressing β -catenin expression and cell viability between malignant and nonmalignant cells.

Evidence that β -TrCP Is Involved in STG28-Mediated β -Catenin Proteolysis. Three lines of evidence were obtained to substantiate the involvement of β -TrCP in STG28-mediated β -catenin degradation. First, we used an *in vitro* pull-down assay to assess the binding of bacterially expressed recombinant GST- β -TrCP with endogenous β -catenin in the lysates of STG28-treated LNCaP cells (Fig. 6A). LNCaP cells were treated with DMSO or STG28 (10 μ M) with or without epoxomicin (10 μ M) for 24 h, and the cell lysates were immunoblotted with β -catenin antibodies (input) or treated with agarose beads coated with GST- β -TrCP or GST. Equivalent amounts of the affinity precipitates were subject to Western blotting with antibodies against β -catenin, β -TrCP, or GST antibodies. As shown, co-treatment of cells with STG28 and epoxomicin significantly increased the amount of β -catenin associated with the pull-down.

Second, LNCaP cells were transfected with scrambled siRNA or increasing doses of siRNA against β -TrCP, and the effect β -TrCP knockdown on STG28-induced β -catenin repression was examined (Fig. 6B). As shown, reduced β -TrCP expression was accompanied by a complete protection against STG28-facilitated β -catenin ablation. Third, LNCaP cells were nucleofected with β -TrCP-Myc plasmids, and exposed to different doses of STG28. Fig. 6C indicates that

ectopic β -TrCP expression enhanced the ability of STG28 to facilitate the proteasomal degradation of β -catenin.

Pleiotropic Effects of Troglitazone and Derivatives on the Repression of Multiple β -Catenin Signaling Components. Evidence indicates that β -catenin exerts the dual function in adhesion and transcription by forming complexes with various components of the E-cadherin and Wnt signaling pathways, respectively [Reviews: (Bienz, 2005; Brembeck et al., 2006; Harris and Peifer, 2005)]. To assess the specificity of this drug-mediated proteolysis in the context of the these two signaling networks, we examined the effect of troglitazone, Δ 2TG, and STG28 on the expression of several β -catenin's binding partners, including α -catenin, plakoglobin (a.k.a., γ -catenin), E-cadherin, and axin (Fig. 7). As shown, these small-molecule agents caused differential repression of these proteins, in the order of plakoglobin > α -catenin, E-cadherin > axin. Especially noteworthy is the drug-induced down-regulation of plakoglobin expression, which has been reported to be resistant to APC/axin-dependent proteolytic degradation (Williams et al., 2000).

Discussion

In light of the pivotal role of aberrant β -catenin signaling in tumorigenesis (Morin et al., 1997; Verras and Sun, 2006; Wong and Pignatelli, 2002), it has been proposed that the ability of troglitazone to suppress β -catenin expression could be pharmacologically exploited in cancer prevention (Girnun et al., 2002; Girnun and Spiegelman, 2003). Thus, this study was aimed at elucidating the mechanism by which troglitazone suppresses β -catenin signaling in cancer cells.

A crucial issue pertinent to troglitazone-mediated β -catenin degradation was the dependence of this pathway on PPAR γ activation. Previous studies in preadipocytes and hepatocytes suggested that the pharmacological action of troglitazone involved a PPAR γ -dependent targeting of β -catenin to proteasomal degradation (Liu and Farmer, 2004; Moldes et al., 2003; Sharma et al., 2004). Accordingly, it was proposed that the crosstalk between PPAR γ and β -catenin signaling underlay the effect of troglitazone and other PPAR γ agonists on cell differentiation and growth inhibition during adipogenesis. In this study, several lines of evidence refuted the involvement of PPAR γ in troglitazone-facilitated β -catenin degradation in prostate cancer cells. First, the ablation of β -catenin was only observed at supra-pharmacological doses of troglitazone ($\geq 40 \mu\text{M}$), which were several orders of magnitude higher than that required for PPAR γ activation. In addition, this ablation was not affected by the PPAR γ antagonist GW9662. Second, different prostate cancer cell lines were equally susceptible to troglitazone-mediated repression of β -catenin irrespective of their PPAR γ expression levels. Third, $\Delta 2\text{TG}$ and STG28,

albeit lacking PPAR γ activity, were able to mediate β -catenin repression with higher potency than troglitazone.

In addition to PPAR γ , the role of GSK3 β in troglitazone-mediated β -catenin degradation warranted investigation in light of contradictory data in the literature (Liu and Farmer, 2004; Sharma et al., 2004). This study indicates that although GSK3 β was subject to phosphorylation and deactivation in response to STG28, it represented a key regulator in the pharmacological effect of STG28 on β -catenin degradation in prostate cancer cells. Our data suggest that GSK3 β , albeit with diminished activity, is involved in β -catenin phosphorylation, which is required for recognition by β -TrCP to facilitate subsequent ubiquitination-dependent proteasomal degradation (Liu et al., 1999). This mechanistic link is demonstrated by the findings that pharmacological inhibition of GSK3 β or siRNA-mediated knockdown of β -TrCP could protect against STG28-mediated β -catenin repression.

Overall, the discrepancy regarding the involvement of PPAR γ and GSK3 β between this and other studies might be attributed to differences in signaling pathways regulating the turnover of β -catenin between prostate cancer cells versus adipocytes and hepatocytes. This premise was corroborated by the resistance of normal prostate cancer cells to STG28-mediated repression of β -catenin, and the consequent effect on cell viability.

This study demonstrates that troglitazone and its PPAR γ -inactive derivatives facilitated ubiquitin-dependent proteasomal degradation of β -catenin by increasing β -TrCP expression levels through protein stabilization. Consequently, many known β -TrCP substrates, including Wee1, I κ B α , Cdc25A, and NF κ B p105 (Nakayama and Nakayama, 2005; Nakayama and Nakayama, 2006), were also susceptible to the pharmacological effect of these agents.

It is also noteworthy that the drug-induced β -TrCP upregulation was accompanied by the downregulation of Skp2 and Fbw7, thereby affecting many of the target proteins of these two F-box proteins such as p27 and cyclin E. Consequently, the ability of troglitazone to repress Skp2 and Fbw7 provides a molecular basis to account for its reported effect on up-regulating p27 expression in cancer cells (Koga et al., 2003). Further analysis of protein expression in these drug-treated LNCaP cells showed a dose-dependent reduction in the expression levels of many proteins involved in the β -catenin signaling network, including α -catenin, plakoglobin, E-cadherin, and to a lesser extent, axin. Although the mechanism underlying the down-regulation of these proteins remained to be elucidated, the pleiotropic effect of troglitazone and its derivatives on suppressing the expression of multiple effector proteins ensured the complete blockade of β -catenin signaling mechanisms.

Moreover, the modulation of the expression of SCF F-box proteins might also underlies the ability of troglitazone, Δ 2TG, and STG28 to repress a series of proteins, including cyclin D1 (Huang et al., 2005), prostate-specific antigen (PSA) (Yang et al., 2006), and androgen receptor (AR) (Yang et al., 2007). For instance, we recently reported that these thiazolidinedione derivatives mediated the repression of AR and its downstream target PSA by facilitating the proteasomal degradation of the transcription factor Sp1 (Yang et al., 2007). We hypothesize that cyclin D1 and Sp1 are targeted by SCF E3 ligase-facilitated proteasomal degradation, which is currently under investigation.

In summary, this study provides evidence that troglitazone and its PPAR γ -inactive derivatives modulated the expression of β -catenin and many cell-cycle regulatory proteins by targeting the F-box proteins of SCF E3 ligase. These small-molecule agents increased the expression of β -TrCP, while reducing the expression of Skp2 and Fbw7, resulting in changes in

the expression profiles of a multitude of proteins involved in the regulation of cell cycle progression and apoptosis. From a mechanistic perspective, how these small-molecule agents simultaneously modulate the expression of β -TrCP, Skp2, and Fbw7 warrants investigation in light of the importance of the SCF ubiquitin-proteasome system in the regulation of cell cycle progression and apoptosis.

However, despite this complicated mode of pharmacological mechanism, normal prostate epithelial cells were resistant to the effect of STG28 on proteasomal degradation and reducing cell viability. This discriminative effect between normal versus malignant cells underlies the impetus of using STG28 as a scaffold to develop a novel class of antitumor agents targeting the ubiquitin-proteasome system. This drug discovery effort is currently under way in this laboratory.

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Footnotes

This work was supported by NIH Grant CA-112250, Department of Defense Prostate Cancer Research Program Award W81XWH-05-1-0089, and a Prostate Cancer Foundation Competitive Award.

Figure Legends

Fig. 1 Pharmacological evidence that thiazolidinedione-mediated down-regulation of β -catenin is independent of PPAR γ activation. A, troglitazone mediates β -catenin repression in DU-145, LNCaP, and PC-3 cells with similar potency irrespective of their PPAR γ expression levels. Upper panel: expression levels of PPAR γ in DU-145, LNCaP, and PC-3 cells. Lower panel: dose-dependent effect of troglitazone on suppressing β -catenin expression in these three cell lines. Cells were exposed to different doses of troglitazone in 10% FBS-supplemented RPMI 1640 medium for 72 h. B, the PPAR γ antagonist GW9662, at a dose range of 0 – 20 μ M, could not rescue troglitazone-mediated β -catenin repression in PC-3 cells. C, the PPAR γ -inactive analogues Δ 2TG and STG28 are effective in repressing β -catenin. *Upper panel*, chemical structures of troglitazone, Δ 2TG, and STG28 and evidence that Δ 2TG and STG28 are devoid of activity in PPAR γ activation using a luciferase reporter assay in PC-3 cells as described in Materials and Methods. Columns, means; bars, SD (n = 6). *Lower panel*, dose-dependent effect of troglitazone, Δ 2TG, and STG28 on repressing β -catenin in LNCaP cells 10% FBS-supplemented medium after 72-h exposure. D, siRNA-mediated knockdown of PPAR γ does not interfere with the ability of STG28 on β -catenin repression.

Fig. 2 Troglitazone, Δ 2TG and STG28 repress β -catenin at the post-transcriptional level in LNCaP cells, and differential inhibitory effect of STG28 on β -catenin expression and cell viability in LNCaP versus PrECs. A, time-dependent effect of troglitazone (TG), Δ 2TG and STG28 on suppressing β -catenin protein (*upper panel*) and mRNA (*lower panel*) levels in LNCaP cells in 10% FBS-supplemented medium. B, time-dependent effect of 10 μ M STG28 on suppressing β -catenin expression in different cellular compartments of LNCaP cells. C, differential effects of STG28 on suppressing β -catenin protein expression (*left panel*) and cell viability (*right panel*) in LNCaP cells versus PrECs. Cell viability was determined at 48 h of drug treatment. D, flow cytometric analysis of the time-dependent effect of 10 μ M STG28 on inducing apoptosis in LNCaP cells via annexin V staining.

Fig. 3 Evidence that the effect of troglitazone and derivatives on β -catenin repression is mediated through proteasomal degradation in LNCaP cells. A, effects of the proteasome inhibitor MG132 and the caspase inhibitor ZVAD-FMK on rescuing the drug-induced β -catenin repression. Cells were exposed to 60 μ M TG or 10 μ M STG28 in 10% FBS-supplemented medium for 48 hr in the presence of 10 μ M MG132 or 100 μ M ZVAD-FMK for 48 h. B, time-dependent effects troglitazone (60 μ M), Δ 2TG (40 μ M), and STG28 (10 μ M) on increased β -catenin ubiquitination. Cells were electroporated with 5 μ g HA-ubiquitin plasmids, and incubated in 10% FBS-containing medium for 24 h, followed by treatment with individual agents for 24 or 48 h. Equal amounts of cell lysates were immunoblotted with anti- β -catenin antibodies (input; lower panel) or

immunoprecipitated with anti- β -catenin antibodies, followed by Western blot analysis with anti-HA and anti- β -catenin antibodies.

Fig. 4 GSK3 β plays a key role in troglitazone-, Δ 2TG-, and STG28-mediated β -catenin repression despite phosphorylating deactivation. A, LNCaP cells were exposed to different doses of troglitazone (TG), Δ 2TG or STG28 in 10% FBS-supplemented medium for 72 h, and the phosphorylation status of Ser9-GSK3 β was detected by Western blot analysis. B, dose-dependent effects of the GSK3 β inhibitors LiCl and SB216763 on the expression levels of β -catenin and β -TrCP in STG28-treated cells. Cells were treated with 10 μ M STG28 alone or in combination with different doses of LiCl or SB216763 in 10% FBS-supplemented medium for 72 h, followed by Western blot analysis. C, time-dependent effect of troglitazone, Δ 2TG, and STG28 on β -catenin phosphorylation at Ser33/Ser37/Thr41, known phosphorylation sites for GSK3 β . LNCaP cells were treated with individual agents at the indicated concentration for different time intervals, and cell lysates were immunoblotted with anti-p-S33/S37/T41- β -catenin antibodies after β -catenin was normalized.

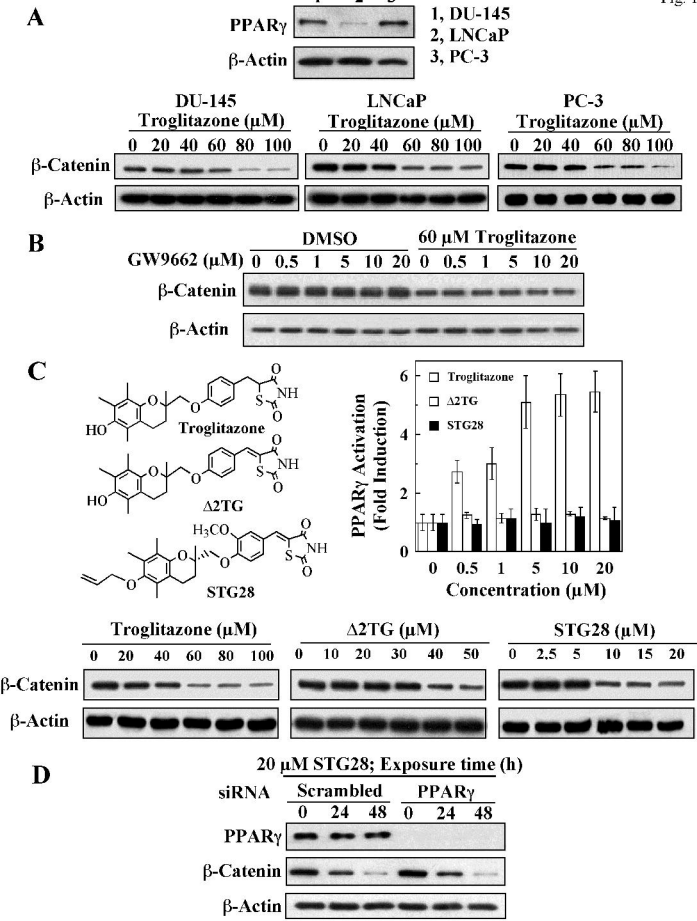
Fig. 5 A mechanistic link between thiazolidinedione-facilitated β -catenin repression and β -TrCP up-regulation in LNCaP cells. A, dose-dependent effects of troglitazone, Δ 2TG, and STG28 on modulating the expression of the F-box proteins β -TrCP, Skp2, and Fbw7, and their respective substrates, including Wee1, I κ B α , Cdc25A, NF κ B p105, p27, and cyclin E. Cells were exposed to different doses of individual agents in 10% FBS-supplemented medium for 72 h. B, STG28 has no effect on β -TrCP mRNA levels.

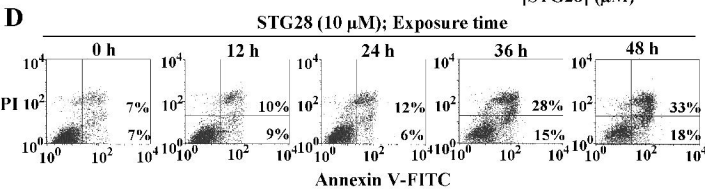
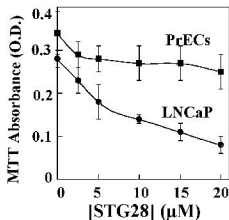
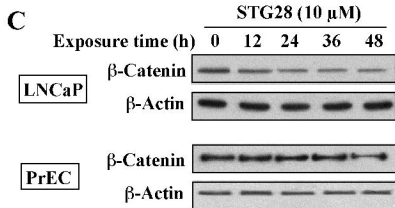
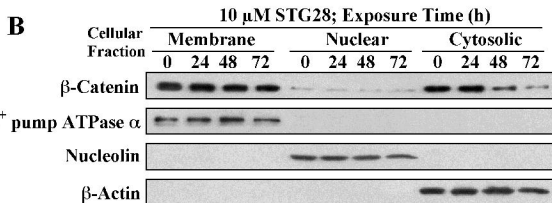
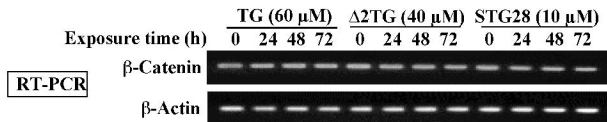
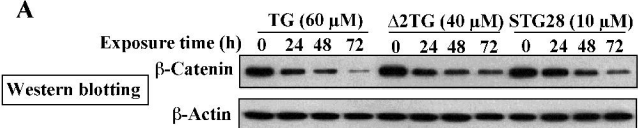
C, STG28 prolongs β -TrCP protein half-life. Cells were pretreated with either DMSO or 10 μ M STG28 in 10% FBS-supplemented medium for 12 h, and β TrCP protein half-life was examined by exposing to 100 μ g/ml cycloheximide for different time intervals, followed by Western blot analysis. D, differential effects of STG28 (10 μ M) on the upregulation of β -TrCP expression between LNCaP cells and PrECs.

Fig. 6 Evidence that β -TrCP is involved in STG28-mediated β -catenin proteolysis in LNCaP cells. (A) *In vitro* pull-down of β -catenin by bacterially expressed GST- β -TrCP from lysates of drug-treated versus vehicle-treated LNCaP cells. Cells were exposed DMSO or 10 μ M STG28, alone or in the presence of epoxomicin, in 10% FBS-supplemented medium for 24 h, and equal amounts of cell lysates were incubated with recombinant GST- β -TrCP or GST immobilized onto glutathione beads. The resulting complexes were washed, centrifuged, and subject to Western blot analysis with anti- β -catenin, β TrCP or GST antibodies (upper panel). One-tenth of cell lysates were collected as Input (lower panel), and β -catenin expression was detected. B, siRNA-mediated knockdown of β TrCP rescues STG28-induced β -catenin repression. LNCaP cells were nucleofected with scramble or increasing doses of β TrCP siRNA followed by treatments with DMSO or 10 μ M STG28 for additional 72 h. Western blot was performed to detect expressions of β -catenin and β TrCP. C, ectopic expression of β -TrCP enhances STG28-mediated degradation of β -catenin. Cells were nucleofected with 5 μ g of pCMV or β -TrCP-Myc plasmids, followed by exposure to different doses of STG28 for 72 h. The values in percentage denote the relative intensity of protein bands of drug-treated samples to that

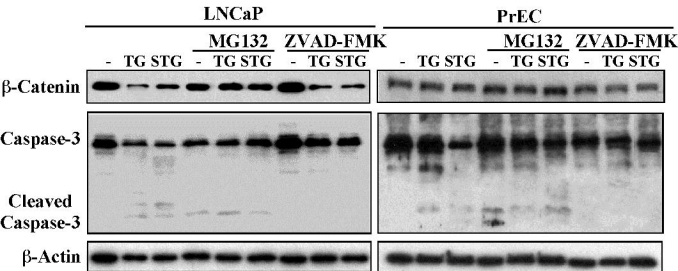
of the respective DMSO vehicle-treated control after being normalized to the respective internal reference β -actin.

Fig. 7 Effects of troglitazone, Δ 2TG, and STG28 on down-regulating multiple components of β -catenin signaling pathways. LNCaP cells were exposed to different doses of individual agents in 10% FBS-supplemented medium for 72 h, and equal amounts of cell lysates were subject to Western blot analysis with antibodies against α -catenin, plakoglobin (γ -catenin), E-cadherin, and axin.





A



B

