ENERGY RESTRICTION AS AN ANTITUMOR TARGET OF THIAZOLIDINEDIONES

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Running head: Novel energy restriction-mimetic agents

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Cancer cells gain growth advantages in the microenvironment by shifting cellular metabolism to aerobic glycolysis, the so-called Warburg effect. There is a growing interest in targeting aerobic glycolysis for cancer therapy by exploiting the differential susceptibility of malignant versus normal cells to glycolytic inhibition, of which the proof-of-concept is provided by the in vivo efficacy of dietary caloric restriction and natural product-based energy restriction-mimetic agents (ERMAs) such as resveratrol and 2-deoxyglucose in suppressing carcinogenesis in animal models. Here, we identified thiazolidinediones (TZDs) as a novel class of ERMAs in that they elicited hallmark cellular responses characteristic of energy restriction, including transient induction of silent information regulator (Sirt)1 expression, activation of the intracellular fuel sensor AMP-activated protein kinase, and endoplasmic reticulum stress, the interplay among which culminated in autophagic and apoptotic death. The translational implications of this finding are multifold. First, the novel function of troglitazone and ciglitazone in targeting energy restriction provides a mechanistic basis to account for their peroxisome proliferator-activated receptor (PPAR)γ-independent effects on a broad spectrum of signaling targets. Second, we demonstrated that Sirt1-mediated upregulation of β-transducin repeat-containing protein-facilitated proteolysis of cell cycle- and apoptosis-regulatory proteins is an energy restriction-elicited signaling event and is critical for the antitumor effects of ERMAs. Third, it provides a molecular rationale for using TZDs as scaffolds to develop potent ERMAs, of which the proof-of-principle is demonstrated by OSU-CG12. OSU-CG12, a PPARγ-inactive ciglitazone derivative, exhibits one- and three-orders-of-magnitude higher potency in eliciting starvation-like cellular responses relative to resveratrol and 2-deoxyglucose, respectively.

Thiazolidinediones (TZDs) are selective ligands for the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)γ (1,2). These TZDs improve insulin sensitivity by transcriptional activation of insulin-sensitive genes involved in glucose homeostasis, fatty acid metabolism, and triacylglycerol storage in adipocytes, and promote the differentiation of preadipocytes by mimicking the genomic effects of insulin (3,4). Moreover, TZD-mediated PPARγ activation has been shown to promote the differentiation of preadipocytes by mimicking the genomic effects of insulin on adipocytes, and to modulate the expression of adiponectin, proinflammatory cytokines like IL-6 and TNFα, and a host of endocrine regulators in adipocytes and macrophages (3,4). Through these beneficial effects, TZDs offer a new type of oral therapy for type II diabetes by reducing insulin resistance and assisting glycemic control.

Like adipocytes, many human cancer cell lines exhibit high levels of PPARγ expression. In vitro exposure of these tumor cells to high doses (>50 μM) of troglitazone and ciglitazone led to cell cycle arrest, apoptosis and redifferentiation (5-9), suggesting a putative link between PPARγ signaling and the antitumor activities of TZDs. Furthermore, the in vivo anticancer efficacy of troglitazone was demonstrated in a few clinical cases that involved patients with liposarcomas or prostate cancer (10,11). While the identities of target genes that contribute to the antiproliferative activities of PPARγ agonists remain elusive (7), accumulating evidence indicates that TZDs mediate PPARγ-independent antitumor effects by targeting diverse signaling pathways governing proliferation and survival of cancer cells (12). Of the various “off-target” mechanisms identified, the effects of TZDs on the repression of diverse cell
cycle- and apoptosis-regulatory proteins are especially noteworthy (13,14). We previously demonstrated that this effect was attributable to the ability of TZDs to activate β-transducin repeat-containing protein (β-TrCP)-mediated proteolysis of target proteins, including β-catenin, cyclin D1, and Sp1, by increasing the expression level of β-TrCP, a versatile F-box protein of the Skp1/Cul1/F-box (SCF) ubiquitin ligase (13,15,16). Furthermore, decreased Sp1 expression leads to the transcriptional repression of a series of genes involved in oncogenic transformation (16), including those encoding androgen receptor (AR), estrogen receptor α (ERα), and epidermal growth factor receptor (EGFR) (Fig. 1A). In contrast, nonmalignant cells are resistant to these PPARγ-independent antitumor effects, underscoring the translational potential of TZDs to develop novel antitumor agents with a unique mode of mechanism. The proof-of-principle of this premise was provided by two PPARγ-inactive derivatives, STG28 and OSU-CG12, which exhibit multifold higher antitumor potencies than the respective parent compounds, troglitazone and ciglitazone, while lacking the ability to transactivate PPARγ (17,18) (Fig. 1A).

In the course of our investigation of the mechanism underlying TZD-induced activation of β-TrCP signaling, we observed that this β-TrCP-mediated proteolysis also occurred under conditions of glucose deprivation (15,16), raising the possibility that TZDs act by mimicking energy restriction. Here, we demonstrate that troglitazone, ciglitazone, STG28, and OSU-CG12 were able to elicit hallmark cellular responses characteristic of energy restriction in LNCaP prostate cancer and MCF-7 breast cancer cells, paralleling those induced by glucose starvation and two known energy restriction-mimetic agents (ERMAs), 2-deoxyglucose (2-DG) and resveratrol (19-21). These changes include reduced glycolytic rate, transient induction of the NAD1-dependent histone deacetylase silent information regulator (Sirt1) (22), and activation of the intracellular fuel sensor AMP-activated protein kinase (AMPK) (23) and endoplasmic reticulum (ER) stress (24,25), the interplay among which culminates in autophagy and apoptosis. This study provides the first evidence that β-TrCP-dependent proteolysis represents a downstream cellular event of transient Sirt1 induction, which underlies the effect of energy restriction on apoptosis induction.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Reagents - LNCaP cells and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained with 10% fetal bovine serum (FBS)-supplemented RPMI 1640 medium and F12/DMEM medium, respectively. Nonmalignant prostate epithelial cells (PrECs) were maintained in prostate epithelial growth medium (Lonza Inc., Walkersville, MD). All cells were cultured at 37°C in a humidified incubator containing 5% CO2.

Troglitazone, ciglitazone, STG28, and OSU-CG12 were synthesized according to published procedures (17,18). Glucose-free RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA). 2-DG, resveratrol, 3-methyladenine (3-MA), thapsigargin, nicotinamide, and splitomicin were purchased from Sigma-Aldrich (St. Louis, MO). Compound C and cycloheximide were obtained from Calbiochem (San Diego, CA). 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: β-catenin, cyclin D1, Wee1, p53 and GFP, Santa Cruz (Santa Cruz, CA); β-TrCP, Invitrogen; β-actin, MP Biomedicals (Irvine, CA). Rabbit antibodies: Myc, PARP, NFκB/p105, AR, ERα, p-Ser9-GSK3β, GSK3β, p-Ser473-Akt, Akt, p-Thr202/Tyr204-ERK, ERK, p-Thr180/Tyr182-p38, p38, p-Ser176/180-IκB kinase α (IKKα), IKKα, p-Thr172-AMPK, AMPK, glucose-regulated protein (GRP)78, Sirt1, Ac-K382-p53, inositol requiring enzyme (IRE)1α, pSer2448-mammalian target of rapamycin (mTOR), mTOR, p-Thr389-p70S6K, p70S6K, tuberous sclerosis complex (TSC)2, microtubule-associated protein light chain 3 (LC3), acetyl-lysine (AcK), p-Ser51-eukaryotic initiation factor (eIF2)α, eIF2α, and autophagy-related gene (Atg)5, Cell Signaling (Beverly, MA); EGFR, Sp1, hemagglutinin (HA), and growth-arrest and DNA damage-inducible gene (GADD)153, Santa Cruz; Atg7, Abgent (San Diego, CA) Human DDIT3 SMARTpool siRNA was obtained from Dharmacon (Lafayette, Co). Atg5 and Atg7 shRNA plasmids were purchased from Origene.
(Rockville, MD). The Flag-tagged Sirt1 (wild-type [WT] and H363Y dominant-negative), HA-tagged Sirt1, Myc-tagged AMPK (WT and K45R kinase-dead) and TSC2 shRNA plasmids were purchased from Addgene (Cambridge, MA). WT- and F-box-deleted(AF)-β-TrCP-Myc plasmids were prepared as previously described (16). The GFP-microtubule-associated-protein-1 light chain (LC3) plasmid was kindly provided by Dr. Tamotsu Yoshimori (National Institute for Basic Biology, Okazaki, Japan) (26).

**RNA Isolation and Semiquantitative PCR Analysis**

Total RNA was isolated and reverse transcribed to cDNA using the RNeasy mini-kit and the Omniscript RT kit (Qiagen, Valencia, CA), respectively, according to manufacturer's instructions. The sequences of the PCR primers used were as follows:

- Sirt1, 5'-GAACAGGTTCGGGAATC-3'/5'-AACATGAAAGGTGGTGTC-3';
- Sp1, 5'-GGCGAGGACATTTATGTG-3'/5'-AGTG-GCATCAACGTCTATGCA-3';
- GRP78, 5'-GGTCCCTTCAAGGTG-3'/5'-TGGTACAGTAACACTGCACT-3';
- β-TrCP, 5'-CACTTACACACATACCA-3'/5'-TCTGCAACATAGGTTAAG-3';
- cyclin D1, 5'-ATGGGACACAGCTCTGTGCTGC-3'/5'-TCAGATGTCCACGTCCCGACGT-3';
- fatty acid synthase (FAS), 5'-TATGCTTCTGTCAGCAGT-3'/5'GTGGATGATGCTGATGGA; high kinase (HK), 5'-ACGAGGCACTCCTCCTCAAGTTG-3'/5'-GGTCTTCAAAGGCCACGTTCATC-3';
- phosphofructokinase (PFK)-1, 5'-GGGAA-CCTGAAACCTAACAAGC-3'/5'-CGAAGCCGTCAAGCCATCATAG;
- β-actin, 5'-TCTCAATGAGCTCGTGTTG-3'/5'-GGTCAGGATCTTATGAGGT-3'.

PCR products were separated electrophoretically in 1% agarose gels and visualized by ethidium bromide staining.

**Transient Transfection, Immunoblotting and Fluorescent Microscopic Analysis**

Transfections were performed by electroporation using Nucleofector kit R of the Amaxa Nucleofector system (Amaxa Biosystems, Cologne, Germany). Immunoblotting was performed on cell lysates harvested with M-PER lysis buffer (Pierce, Rockford, IL) (16). For the fluorescent microscopic analysis, drug-treated GFP-LC3-expressing LNCaP cells were fixed with 3.7% formaldehyde at room temperature for 20 min, followed by nuclear counterstaining using a 4,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlingame, CA) before examination.

**Determination of Glycolytic Rate**

Glycolytic rate was determined by measuring the conversion of [5-3H]glucose (GE Healthcare, Piscataway, NJ) to 1H2O in LNCaP cells according to a published procedure (27). Briefly, LNCaP cells were seeded in six-well plates (4 x 10^5 cells/well) and then treated 24 h later with 10 mM 2-DG or 10 µM OSU-CG12 for various intervals. After washing with PBS, cells were trypsinized and resuspended in 500 µL of Krebs buffer [25 mM NaHCO3, 115 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 0.25% BSA (pH 7.4)] containing 1 mM nonradioactive glucose and 5 µCi/mL [5-3H]glucose for 1 h at 37°C. Aliquots from each treatment group were added to 0.2 N HCl in open tubes that were placed upright in scintillation vials containing 1 mL of H2O. The vials were sealed and then incubated for a minimum of 24 h at room temperature to allow H2O produced by glucose consumption to equilibrate with H2O in the outer vial. The amount of 3H retained in the tube and the amount that had diffused into the surrounding H2O by evaporation and condensation were determined separately by using a scintillation counter LS6500 (Beckman). [5-3H]glucose-only and 1H2O-only standards were included in each experiment for calculation of the rate of conversion of [5-3H]glucose to H2O using the following equation: glucose utilized (pmol) = [3H] water formed (d.p.m) / [5-3H]glucose (d.p.m/pmol)] (27).

**Glucose Uptake Assay**

LNCaP cells in six-well plates were exposed to resveratrol or OSU-CG12 at different concentrations and then incubated with Krebs-Ringer phosphate buffer at 37°C for 30 min. After washing cells with PBS, glucose uptake was initiated by addition of 1 mL of PBS containing 1 µCi/mL [3H]-2-DG (Perkin Elmer, Waltham, MA) and 100 mM nonradioactive 2-DG, and was terminated after 5 min by extensive washing with PBS. Cells were solubilized in 0.1% SDS buffer, and aliquots were taken for measurements of radioactivity using a scintillation counter.
NADH Assay and Lactate Assay - Determinations of intracellular levels of NADH and lactate were performed using the EnzyChrom NAD+/NADH Assay Kit and L-Lactate Assay Kit, respectively (BioAssay Systems, Hayward, CA). Briefly, LNCaP cells were cultured in 24-well plates at the density of 2 x 10^4 cells/well for 24 h followed by treatments of 10 mM 2-DG or 10 µM OSU-CG12 for various time intervals. After cells were trypsinized and collected, intracellular levels of NADH and lactate were determined according to manufacturer’s instructions.

Cell Viability Assay - Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Cells were seeded in 96-well plates (5000 cells/well) and incubated in 10% FBS-supplemented medium for 24 h, and were then treated with individual agents for 72 h. Drug-containing medium was then replaced with MTT (0.5 mg/mL in RPMI 1640), followed by incubation at 37°C for 2 h. After removal of medium, the reduced MTT dye was solubilized in 200 µL/well DMSO, and absorbance at 570 nm was measured.

Cytotoxicity Assay – The cytotoxic effect of OSU-CG12 in LNCaP cells was assessed by use of the CytoTox 96 non-radioactive cytotoxicity assay (Promega Corp.), which quantitates the activity of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Briefly, LNCaP cells were seeded into 96-well plates at the density of 5000 cells/well and treated with individual agents for 72 h. The medium was then collected from each well and assayed for LDH activity according to the manufacturer’s instructions. The data were expressed as percentages of the total LDH activity in the lysate of vehicle-treated control cells.

Statistical Analysis - Western blot and RT-PCR analyses were performed in triplicate. Data were analyzed by the Student’s t test. Differences were considered significant at p < 0.05.

RESULTS

TZDs induce autophagy in cancer cells

We have shown that TZDs and glucose deprivation share the ability to induce β-TrCP-mediated proteolysis (15,16), leading to our contention that TZDs act as ERMAs. In the literature, many small-molecule agents have been reported to target tumor metabolism, among which 2-DG and resveratrol are especially noteworthy (19-21). Both agents, however, exhibit low antiproliferative potency. The IC50 values for 2-DG in inhibiting the viability of LNCaP and MCF-7 cells were 5.5 and 4.2 mM, respectively, while those of resveratrol were 110 and 60 µM, respectively (Fig. 1B). Although the antiproliferative potencies of troglitazone (70 µM and 70 µM) and ciglitazone (70 µM and 42 µM) were comparable to that of resveratrol, their PPARγ-inactive derivatives, STG28 (12 µM and 11 µM) and OSU-CG12 (5.7 µM and 5.0 µM), showed one- and three-orders-of-magnitude higher potencies than resveratrol and 2-DG, respectively. Moreover, these TZDs displayed low cytotoxicity to PrECs, which might be attributable to their inability to induce β-TrCP-mediated proteolysis, as evidenced by the unaltered expression levels of β-TrCP, Sp1, and AR in OSU-CG12-treated PrECs (Fig. 1C).

We previously demonstrated that the antiproliferative effect of these TZDs was, in part, attributable to apoptosis (14,17,18). As autophagy represents a characteristic cellular response to ERMAs (28-30), we assessed the ability of OSU-CG12 to facilitate the conversion of LC3-II from LC3-I, an essential step for autophagosome formation (26), in GFP-LC3-expressing LNCaP cells. Western blotting and fluorescence microscopy demonstrate OSU-CG12-induced conversion of LC3-II, as indicated by parallel changes in the turnover of both GFP-tagged and endogenous LC3, and its accumulation into autophagic vacuoles, respectively, both of which could be blocked by the autophagy inhibitor 3-MA (Fig. 1D and E).

β-TrCP-mediated proteolysis represents an energy restriction-elicted signaling event

To establish β-TrCP-mediated proteolysis as an energy restriction-elicted signaling event, we assessed the effects of troglitazone, ciglitazone, STG28 and OSU-CG12 vis-à-vis glucose starvation, 2-DG and resveratrol on the expression
levels of β-TrCP signaling-associated proteins in LNCaP and MCF-7 cells, including β-TrCP, the β-TrCP substrates Sp1, β-catenin, cyclin D1, Wee1, NF-κB/p105, and the Sp1 target gene products AR, ERα, and EGFR. As shown, the ability of TZDs to modulate the expression of these proteins was shared by glucose starvation, 2-DG, and resveratrol, of which the relative potencies paralleled those observed for growth inhibition (Fig. 2).

As phosphorylation of serine residues within the DSG motif of target proteins is a prerequisite for recognition by β-TrCP (31), we compared the effects of TZDs vis-à-vis glucose starvation, 2-DG, and resveratrol on the activation status of kinases potentially involved in β-TrCP substrate phosphorylation, including GSK3β (β-catenin and Sp1), ERKs (Sp1), IKKα (cyclin D1), Akt, and p38. Consistent with the shared ability of TZDs and energy restriction to promote β-TrCP-mediated proteolysis, exposure to these agents or to glucose-free medium led to similar changes in the phosphorylation levels of these kinases (Fig. 2). Specifically, decreases in the phosphorylation of Akt were accompanied by increases in that of GSK3β, ERKs, p38, and IKKα. Similar effects on these signaling biomarkers were also noted in MCF-7 cells treated with 5 μM OSU-CG12, 5 mM 2-DG, or glucose starvation, suggesting that β-TrCP-mediated proteolysis represents a downstream signaling event of energy restriction.

**TZDs induce energy restriction-associated responses**

To further investigate the link between TZD-induced β-TrCP-mediated proteolysis and disruption of energy metabolism, we examined the ability of TZDs to elicit three hallmark cellular responses to energy restriction: Sirt1 gene expression (22), AMPK activation (23), and ER stress (24,25). Time course of changes in biomarkers representative of each of these responses, i.e., Sirt1 induction and deacetylation of its substrate p53, phosphorylation of AMPK, and expression of GRP78, were assessed in LNCaP cells treated with 10 μM OSU-CG12, vis-à-vis 10 mM 2-DG and glucose starvation. OSU-CG12 exhibited a high degree of similarity relative to 2-DG and glucose starvation in mediating these cellular responses (Fig. 3A). These treatments led to immediate, robust increases in Sirt1 expression and AMPK phosphorylation after 10 min, followed by rises in GRP78 expression at about 1 h post-treatment. In contrast, increases in β-TrCP expression and the consequent degradation of Sp1 and cyclin D1 lagged behind these signature cellular responses by more than 10 h, suggesting that β-TrCP upregulation represents a downstream event of at least one of these energy restriction-induced pathways. It is noteworthy that the induction of Sirt1 expression was transient with a short duration of 1 h for OSU-CG12 and 4 h for 2-DG and glucose deprivation, which was paralleled by changes in p53 acetylation levels. Moreover, RT-PCR analysis indicates that increases in Sirt1 and GRP78 expression in TZD- and 2-DG-treated cells were mediated through changes in mRNA levels, while that of β-TrCP was at the protein level (Fig. 3B).

Additional evidence for the targeting of energy restriction by TZDs is evident in the parallel effects of TZDs and energy restriction on ER stress and AMPK signaling. Treatment of LNCaP cells with any of these TZDs induced ER stress, as manifested by the dose-dependent upregulation of the expression of GRP78, GADD153, and the ER-associated transducer IRE1α, which has been shown to upregulate the expression of ER stress-response proteins (32) (Fig. 3C). The ability of TZDs to activate AMPK signaling was corroborated by the concomitant dephosphorylation of mTOR and p70S6K, both of which are major effectors of cell growth and proliferation via the regulation of protein synthesis (33,34). This modulation of ER stress and AMPK signaling was paralleled by that observed in cells treated with 2-DG, resveratrol or glucose starvation.

In addition, we showed that OSU-CG12 shares with 2-DG the ability to induce the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) (Fig. 3D). This is noteworthy as evidence suggests a mechanistic link between energy restriction and eIF2α phosphorylation (35-37). As part of the metabolic stress response, eIF2α is phosphorylated by a series of stress-responsive protein kinases (38), such as PRK-like ER kinase (PERK), to reduce global translation for the
conservation of cellular resources and to induce the translation of specific mRNAs involved in the regulation of genes associated with metabolism, cellular redox potential, and apoptosis.

**OSU-CG12 inhibits glucose metabolism**

To corroborate the hypothesis that TZDs target glucose utilization in cancer cells, we examined the suppressive effects of OSU-CG12 versus 2-DG on glycolysis in LNCaP cells. As shown, OSU-CG12 mediated a dose- and time-dependent inhibition of the glycolytic rate in a manner similar to that of 2-DG (Fig. 4A). Within 20 min of drug treatment, OSU-CG12, even at 1 µM, significantly inhibited the glycolytic rate \((P < 0.05)\), and at 12 h, the extents of inhibition by 2.5, 5 and 10 µM OSU-CG12 were 45%, 49% and 59%, respectively, which were comparable to that observed after treatment with 1 - 2.5 mM 2-DG. Moreover, the suppressive effects of 2-DG (5 mM) and OSU-CG12 (5 µM) on glycolysis were paralleled by reductions in NADH production and lactate formation (Fig. 4B). Treatment with these two agents for 24 h reduced intracellular NADH and lactate levels by 50-70%.

The impact of OSU-CG12 on glucose metabolism was further supported by the protective effect of high levels of supplemental glucose on drug-induced cell death. Different amounts of glucose were added to glucose-deficient medium to achieve final concentrations ranging from 0.5 to 20 mg/ml. Relative to 2 mg/ml, the content in unmodified 10% FBS-containing medium, 10 and 20 mg/ml glucose provided significant protection against OSU-CG12-induced cell death \((P < 0.05)\) for all time points), while 0.5 mg/ml increased drug sensitivity (Fig. 4C, left panel). This protective effect was also noted in cells treated with 2-DG, but not with thapsigargin, an ER stress-inducing agent used as a negative control (Fig. 4C, central and right panels), suggesting the selectivity of this protection against ERMAs. In addition, this protection correlated with the ability of exogenous glucose to block ERMA-induced starvation-associated responses, including Sirt1 upregulation (Fig. 5A), PARP cleavage, AMPK activation, and induction of the ER stress markers GRP78 and GADD153 (Fig. 5B). Similarly, the inability of supplemental glucose to protect against the antiproliferative effects of thapsigargin was reflected in its ineffectiveness to reverse the drug-induced changes to markers associated with apoptosis, AMPK activation, and ER stress (Fig. 5B).

**Multiple mechanisms might be involved in OSU-CG12-mediated inhibition of glucose metabolism**

Evidence suggests that OSU-CG12-mediated inhibition of glycolysis might result from the concerted action of a series of biochemical responses that occurred at different time points during drug exposure. First, in less than 20 min of drug treatment, OSU-CG12 induced a modest suppression of glucose uptake in a manner similar to resveratrol, a known inhibitor of glucose uptake \((30)\) (Fig. 5C, left panel). As shown, the level of inhibition of \([^3]H\)-2-DG uptake by 2.5 and 5 µM OSU-CG12 was comparable to that of 50 and 100 µM resveratrol, respectively. Structurally, OSU-CG12 and resveratrol exhibit some degree of similarity regarding the spatial arrangement of the hydrophilic functionalities (right panel), which might underlie a shared mode of action in inhibiting glucose uptake. To test this premise, we are currently investigating whether OSU-CG12 shares the reported activity of resveratrol in blocking Glut1 and Glut3 transporters (39).

Second, as AMPK acts as a negative regulator of mammalian homolog of target of rapamycin (mTOR), OSU-CG12-induced AMPK activation led to a concomitant downregulation in the phosphorylation of mTOR and 70-kDa ribosomal protein S6 kinase (p70S6K), a marker for mTOR activation status, shortly after drug exposure (Fig. 5D). The mTOR-p70S6K signaling pathway is well recognized to play a key role in governing cell metabolism through the transcriptional regulation of genes associated with glycolysis and energy metabolism (40-42). This premise was supported by the dose-dependent, suppressive effects of OSU-CG12 and resveratrol on the mRNA levels of hexokinase (HK)2 and phosphofructokinase (PFK)-1, the first two enzymes of the glycolytic pathway, and fatty acid synthase (FAS) (Fig. 5E). Third, OSU-CG12 facilitated the dephosphorylation of Akt, which occurred after 6 h of treatment (Fig. 5E). As Akt is known to stimulate aerobic glycolysis through different mechanisms (43-46), this Akt inactivation might enhance the inhibitory effects
of OSU-CG12 on glucose utilization. Fourth, as aforementioned, OSU-CG12 induced the phosphorylation of eIF2α (Fig. 3D), a well characterized metabolic stress response to a decreased energy status (38,47). Recent evidence indicates that glucose deprivation-induced eIF2α phosphorylation plays a key role in glucose-regulated protein synthesis in pancreatic cells (37).

**Inhibition of β-TrCP protects against apoptosis**

As β-TrCP facilitates the degradation of a series of cell cycle- and apoptosis-regulatory proteins, we hypothesized that β-TrCP might play a crucial role in mediating the antiproliferative effects of ERMAs. Thus, we examined the effect of ectopic expression of WT-β-TrCP versus ΔF-β-TrCP, which acts as a dominant-negative mutant due to lack of the F-box motif (48), on OSU-CG12 and 2-DG-induced growth inhibition. Relative to the pCMV control, ectopic expression of WT-β-TrCP and ΔF-β-TrCP enhanced and protected against, respectively, the suppressive activities of OSU-CG12 and 2-DG to LNCaP cell viability (Fig. 6A). These effects correlated with the abilities of WT-β-TrCP and ΔF-β-TrCP to promote and suppress, respectively, drug-induced apoptosis, as manifested by PARP cleavage and degradation of the β-TrCP substrates cyclin D1 and Sp1 (Fig. 6B).

**Increased β-TrCP expression is consequent to transient Sirt1 induction**

Considering the temporal relationship between increased β-TrCP expression and induction of key energy restriction-associated responses, i.e., Sirt1 upregulation, AMPK activation, and ER stress (Fig. 3), we hypothesized that β-TrCP activation might be consequent to one of these hallmark responses. Thus, we examined the effects of inhibiting the function and/or expression of Sirt1, AMPK, and GADD153 on the ability of OSU-CG12 to upregulate β-TrCP expression.

Enforced expression of dominant-negative Sirt1 (H363Y/Sirt1) reversed the effect of OSU-CG12 on inducing β-TrCP expression and PARP cleavage (Fig. 6C, left panel), while ectopic expression of HA-tagged WT-Sirt1 mimicked the effect of OSU-CG12 on increasing β-TrCP levels in conjunction with reduced expression of cyclin D1 and Sp1 (right panel). Furthermore, using the Sirt1 deacetylase inhibitors nicotinamide and splitomicin, we obtained evidence that Sirt1-induced upregulation of β-TrCP expression in OSU-CG12-treated cells was mediated through deacetylase-dependent protein stabilization. First, using cycloheximide to assess protein stability, we showed that, in DMSO-pretreated cells, β-TrCP exhibited a half-life of less than 12 h (Fig. 6D, left panel). In contrast, OSU-CG12 at 5 μM increased the stability of β-TrCP as its protein level remained unaltered for up to 24 h. This protein stabilizing effect, however, was reversed when cells were co-treated with nicotinamide or splitomicin. Second, RT-PCR analysis confirmed that the mRNA level of β-TrCP remained unchanged after treatment with OSU-CG12 alone or in combination with either inhibitor (right panel). Moreover, pharmacological inhibition of Sirt1 activity protected LNCaP cells from OSU-CG12-induced cell death in a manner similar to that of the dominant-negative inhibition by ΔF-β-TrCP (not shown). Together, these findings suggest a causal relationship between the transient Sirt1 induction and increased β-TrCP expression through protein stabilization.

From a mechanistic perspective, this β-TrCP accumulation might be attributable to the protective effect of Sirt1 against ubiquitin-dependent degradation of β-TrCP through an acetylation-dependent or -independent mechanism. To discern these two possibilities, we examined the time-dependent effect of OSU-CG12 (10 μM) on the levels of ubiquitination versus acetylation on β-TrCP in LNCaP cells ectopically expressing Myc-tagged β-TrCP and HA-tagged ubiquitin. As shown, OSU-CG12-mediated increases in β-TrCP expression were accompanied by a gradual reduction in the level of ubiquitinated β-TrCP, while no appreciable acetylation was noted (Fig. 7). This finding suggests that the transient induction of Sirt1 might block the expression of a β-TrCP-specific E3 ligase, resulting in decreases in ubiquitin-dependent degradation of β-TrCP. The identity of this E-3 ligase is currently under investigation.

In contrast to the effects of Sirt1 inhibition on β-TrCP expression, the dominant-negative or pharmacological inhibition of AMPK activation
via the ectopic expression of a kinase-dead mutant or treatment with Compound C, respectively, failed to affect the upregulation of β-TrCP expression in OSU-CG12-treated cells (Fig. 8A, left and right panels, respectively). Similarly, siRNA-mediated silencing of GADD153 had no influence on OSU-CG12-induced β-TrCP expression (Fig. 9B, left panel). These data refute the involvement of AMPK and ER stress in the TZD-induced upregulation of β-TrCP expression.

**Autophagy plays a role in the antiproliferative effects of energy restriction**

The above findings indicate an important role for the Sirt1-β-TrCP pathway in 2-DG and OSU-CG12-induced apoptosis. Subsequently, we assessed the potential involvement of AMPK and ER stress signaling in the antitumor effects of these agents. As energy restriction induces autophagy via the AMPK-TSC1/2-mTOR pathway (49), we rationalized that blocking AMPK function would prevent LNCaP cells from undergoing autophagy in response to ERMs. Dominant-negative or pharmacological inhibition of OSU-CG12-induced AMPK activation (Fig. 8A, left and right panels, respectively), as evidenced by the unchanged phosphorylation level of its downstream targets mTOR and p70S6K, prevented the conversion of GFP-tagged LC3-I to LC3-II. This inhibition of autophagy was independent of drug-induced changes to β-TrCP and ER stress as no effect on the expression of β-TrCP or GADD153 were observed. To confirm that β-TrCP upregulation was not consequent to OSU-CG12-induced autophagy, we examined the effect of shRNA-mediated silencing of Atg5 and Atg7, both essential modulators of the autophagy machinery, on β-TrCP expression in drug-treated cells. The data show that inhibition of autophagy through the knockdown of Atg5 or Atg7 had no apparent effect on OSU-CG12-induced β-TrCP accumulation (Fig. 8B).

Furthermore, we assessed the effect of TSC2 knockdown on OSU-CG12-mediated autophagy by fluorescence microscopy. Transient transfection with TSC2 shRNA led to complete suppression of TSC2 expression without affecting the ability of OSU-CG12 to activate AMPK (Fig. 8C, left panel). This knockdown also prevented OSU-CG12-induced formation of GFP-LC3-positive puncta (right panel), suggesting the pivotal role of the AMPK/TSC1/2 pathway in this autophagy induction.

Although it is well recognized that autophagy parallels apoptosis in governing cancer cell homeostasis in response to therapy, its function in modulating drug-induced cell death by either promoting or inhibiting it varies in different cellular contexts (50,51). To assess the role of autophagy in energy restriction-induced cell death, we examined the effect of ectopic expression of dominant-negative AMPK on OSU-CG12-mediated apoptosis and suppression of cell viability. Although inhibition of the AMPK-autophagy pathway could not protect against OSU-CG12-induced apoptosis, as manifested by lack of PARP cleavage (Fig. 9A, left panel), MTT and lactate dehydrogenase release assays indicate that it substantially reduced the abilities of OSU-CG12 to suppress cell viability and to mediate cell death, respectively, relative to the pCMV control (P < 0.01 for all data points) (central and right panels), which was also noted in 2-DG- and resveratrol-treated cells (not shown). In contrast, siRNA-mediated silencing of GADD153 had no effect on PARP cleavage in OSU-CG12-treated cells (Fig. 9B, left panel); nor did it affect the cellular susceptibility to OSU-CG12’s antiproliferative activity, as indicated by both MTT and lactate dehydrogenase release assays (central and right panels). Together, these findings reveal that, in addition to the Sirt1-β-TrCP pathway, AMPK activation-induced autophagy plays an important role in mediating the antiproliferative effects of ERMs in cancer cells.

**DISCUSSION**

Cancer cells gain growth advantages in the microenvironment by shifting cellular metabolism to aerobic glycolysis, the so-called Warburg effect (52-54). There is a growing interest in targeting aerobic glycolysis for cancer therapy by exploiting the differential susceptibility of malignant versus normal cells to glycolytic inhibition (55), of which the proof-of-concept is provided by the in vivo efficacy of dietary caloric restriction (23,56-58), resveratrol (19,20), and 2-DG (59) in suppressing carcinogenesis in various spontaneous or chemical-induced tumor animal models. As chronic energy restriction proves to be difficult to
implement as a chemopreventive strategy, 2-DG and resveratrol have received wide attention because of their abilities to mimic the beneficial effects of energy restriction by inhibiting glucose metabolism and uptake, respectively (19,20,59). However, as indicated by our data, 2-DG and resveratrol require at least 1 mM and 100 µM, respectively, to attain antitumor activities. Thus, the relatively weak \textit{in vitro} potencies of these agents limit their therapeutic applications.

Here, we demonstrate that TZDs represent a novel class of ERMAs in that they elicit hallmark cellular responses characteristic of energy restriction in a manner reminiscent of that of resveratrol and 2-DG. OSU-CG12 mimicked the effect of energy restriction, as manifested by a reduced glycolytic rate and decreased NADH and lactate production. This drug-induced metabolic deficiency signaled the induction of key starvation-associated responses, including transient Sirt1 induction, AMPK activation, ER stress, each of which mediates a distinct signaling pathway culminating in OSU-CG12's antiproliferative effects (Fig. 9C). Moreover, these energy restriction-associated responses could be achieved at concentrations in the 5-µM range relative to 100 µM and 5 mM for resveratrol and 2-DG, respectively, indicating that the development of potent ERMAs from the molecular scaffold of TZDs is feasible. From a translational perspective, the development of novel ERMAs with greater antitumor potencies may provide advantages in clinical development.

Several lines of evidence suggests that OSU-CG12-mediated inhibition of glucose metabolism might be attributable to the cumulative effect of a series of biochemical events at different stages of drug action, including the immediate responses of reduced glucose uptake and inhibition of mTOR-p70S6K signaling, followed by Akt inactivation and eIF2α phosphorylation. Thus, OSU-CG12 inhibits glucose metabolism through effects at different molecular levels, including the cellular uptake of glucose and the transcription of genes associated with glycolysis and energy metabolism. Further investigation of additional mechanisms is currently underway.

Previous studies have implicated AMPK activation and ER stress as targets for selective cancer cell killing during calorie restriction (23,60). However, the role of Sirt1 in regulating cell death response is less well defined considering its controversial role as a tumor promoter or tumor suppressor (61). Sirt1 is able to regulate epigenetic changes as well as the functions of a broad spectrum of nonhistone signaling proteins via deacetylation (62). Here, we provide the first evidence that the transient increase in Sirt1 expression plays a crucial role in mediating the induction of apoptosis by ERMAs through the activation of β-TrCP-facilitated proteolysis. It is noteworthy that the Sirt1-mediated upregulation of β-TrCP expression was achieved through protein stabilization, for which Sirt1’s deacetylase activity was critical. This stabilization of β-TrCP protein might be attributable to the ability of Sirt1 to suppress the expression/activity of a specific E3 ligase that targets β-TrCP for proteasome-mediated proteolysis, which is currently under investigation. Moreover, although AMPK has been reported to enhance Sirt1 activity by increasing intracellular NAD$^+$ levels (63), our data indicate neither genetic nor pharmacological inhibition of AMPK had any effect on β-TrCP expression in TZD-treated cancer cells, suggesting that AMPK activation did not play a role in β-TrCP protein stabilization. Although substantial evidence indicates the importance of autophagy in cancer, its role in modulating therapeutic response, by either enhancing or protecting cells from drug-induced cell death, remains unclear (50,51). In the case of ERMAs, our data suggest that the interplay between autophagy and apoptosis plays a key role in mediating their antiproliferative activities.

\textbf{Acknowledgements} – The authors thank Mr. En-Chi Hsu for valuable technical assistance. Public Health Service Grant CA112250 from the National Cancer Institute and Department of Defense Prostate Cancer Research Program grant W81XWH-09-0198
REFERENCES

FIGURE LEGENDS

Fig. 1. PPARγ-independent antiproliferative effects of TZDs. (A) Upper, structures of troglitazone, ciglitazone, and their respective PPARγ-inactive derivatives STG28 and OSU-CG12. Lower, a schematic diagram depicting the mode of action of these TZDs in activating β-TrCP-mediated proteolysis and the consequent transcriptional repression of Sp1’s target genes. (B) Dose-dependent effects of troglitazone (TG), ciglitazone (CG), STG28, and OSU-CG12 (CG12) vis-à-vis resveratrol (Resv) and 2-DG on the viability of LNCaP and MCF-7 cells versus PrECs in 10% FBS-supplemental medium for 72 h. MTT data are expressed as means ± S.D. (n=6). (C) Lack of effect of OSU-CG12 on the expression levels of β-TrCP, Sp1, and AR in PrECs. (D) Evidence that TZDs induce autophagy. Time-dependent effect of 5 μM OSU-CG12 on the conversion of both GFP-tagged and endogenous LC3-I to LC3-II, which could be blocked by 3-MA (1 mM). (E) Microscopic analysis of the effect of 5 μM OSU-CG12, alone or in the presence of 1 mM 3-MA, on the pattern of GFP-LC3 fluorescence.

Fig. 2. Energy restriction and TZDs share the ability to facilitate β-TrCP-mediated proteolysis. Endpoints included the expression of β-TrCP, β-TrCP substrates, and Sp1 target gene products, and the phosphorylation status of kinases involved in facilitating β-TrCP-substrate recognition.

Fig. 3. TZDs share the ability of 2-DG and glucose starvation to elicit energy restriction-associated cellular responses in LNCaP cells. (A) Western blot analysis of the time-dependent effects of 10 μM OSU-CG12 vis-à-vis 10 mM 2-DG and glucose starvation on various markers associated with energy restriction (induction of Sirt1 expression, p53 deacetylation, AMPK phosphorylation, and the expression of ER stress indicator GRP78) and with β-TrCP-dependent proteolysis (expression of β-TrCP, Sp1, and cyclin D1). (B) Parallel analysis of the mRNA expression levels of Sirt1, GRP78, β-TrCP, Sp1, and cyclin D1 by RT-PCR in cells treated with CG12 and 2-DG as described above. (C) Western blot analysis of the effect of TZDs at different doses, relative to 2-DG, resveratrol, and glucose starvation, on ER stress and AMPK/mTOR/p70S6K signaling. (D) Western blot analysis of the time-dependent effect of 10 μM OSU-CG12 versus 10 mM 2-DG on the phosphorylation of eIF-2α, a marker associated with energy restriction.

Fig. 4. Evidence that OSU-CG12 targets glucose metabolism in LNCaP cells. (A) Time- and dose-dependent effect of OSU-CG12 (right) vis-à-vis 2-DG (left) on glycolytic rate. Data are expressed as the means ± S.D. (n=3). (B) Time-dependent effect of 5 μM OSU-CG12 vis-à-vis 5 mM 2-DG on the intracellular levels of NADH (left) and lactate (right). Data are expressed as the means ± S.D. (n=3). (C) Supplemental glucose provides dose-dependent protection against the antiproliferative activity of OSU-CG12 (left) and 2-DG (central), but not the ER stress inducer thapsigargin (right). Data are expressed as means ± S.D. (n=6).

Fig. 5. Mechanisms underlying OSU-CG12-mediated inhibition of glucose metabolism in LNCaP cells. (A) Supplemental glucose (20 mg/ml) reversed the transient induction of Sirt1 by OSU-CG12 (CG12) and 2-DG. (B) Dose-dependent protective effects of supplemental glucose against the induction of PARP cleavage, AMPK activation, and the expression of GRP78 and GADD153 in cells treated with different doses of OSU-CG12 or 2-DG. The ER stress-inducing agent thapsigargin was used as a negative control. (C) Left, dose- and time-dependent effects of OSU-CG12 and resveratrol on [3H]-2DG uptake. Data are expressed as the means ± S.D. (n=3). Right, ball-and-stick structures of OSU-CG12 and resveratrol. (D) Western blot analysis of the time-dependent suppressive effects of 5 μM OSU-CG12 on the phosphorylation of mTOR, p70S6K, and Akt. (E) RT-PCR analysis of the dose-dependent effects of OSU-CG12 vis-à-vis resveratrol
on the mRNA levels of hexokinase (HK)2 and phosphofructokinase (PFK)-1, the first two enzymes in the glycolytic pathway, and fatty acid synthase (FAS) after 24 h of exposure.

Fig. 6. **β-TrCP expression is important for the antiproliferative effects of ERMAs and is upregulated by Sirt1-mediated stabilization of β-TrCP protein in LNCaP cells.** (A) Effects of ectopic expression of WT-β-TrCP-Myc and ΔF-β-TrCP-Myc versus pCMV control on the dose-dependent inhibition of cell viability by OSU-CG12 (left) and 2-DG (right). Data are expressed as means ± S.D. (n=6). (B) Effects of ectopic expression of WT-β-TrCP-Myc and ΔF-β-TrCP-Myc on the ability of OSU-CG12 (5 μM) and 2-DG (5 mM) to facilitate PARP cleavage and the degradation of the β-TrCP substrates cyclin D1 and Sp1. (C) Sirt1 upregulation elevates β-TrCP expression levels in OSU-CG12-treated LNCaP cells. Left, dominant-negative inhibition of Sirt1 blocked OSU-CG12-mediated β-TrCP induction and PARP cleavage. Right, ectopic expression of HA-Sirt1 increased β-TrCP expression in a dose-dependent manner with corresponding decreases in the expression of cyclin D1 and Sp1. (D) Sirt1 increased β-TrCP expression via protein stabilization. Left, inhibition of Sirt1 deacetylase activity by nicotinamide or splitomicin reversed the ability of OSU-CG12 to enhance β-TrCP protein stability. Right, RT-PCR analysis showed that β-TrCP mRNA levels in LNCaP cells treated as described above remained unchanged.

Fig. 7. **OSU-CG12-mediated β-TrCP accumulation results from reduced ubiquitination via an acetylation-independent mechanism.** LNCaP cells transiently transfected with plasmids encoding β-TrCP-Myc and ubiquitin-HA were exposed to 10 μM OSU-CG12 for different time intervals. Equal amounts of cell lysates were immunoblotted with anti-Myc, anti-β-TrCP, and anti-Sirt1 antibodies (input, left) or immunoprecipitated (IP) with anti-Myc antibodies, followed by Western blot analysis (WB) with anti-Myc, anti-HA, and anti-acetyl (Ac)-lysine antibodies.

Fig. 8. **OSU-CG12 induces autophagy by targeting the AMPK/TSC2/mTOR/p70S6K pathway.** (A) Dominant-negative or pharmacological inhibition of AMPK blocked OSU-CG12-mediated autophagy, but had no effect on ER stress. Left, effects of the ectopic expression of the wild type (WT) versus the K45R kinase-dead, dominant-negative (DN) form of AMPK on the ability of OSU-CG12 to modulate the expression levels of p-mTOR, p-p70S6K, β-TrCP, and GADD153, and the conversion of GFP-LC3 in GFP-LC3-expressing LNCaP cells. Right, parallel analysis of the effects of the AMPK inhibitor Compound C. (B) shRNA-mediated silencing of Atg5 and Atg7 was used as a positive control to confirm the protective effect of AMPK inhibition on OSU-CG12-induced GFP-LC3 II conversion. (C) shRNA-mediated knockdown of TSC2 protected cells from OSU-CG12-induced autophagy. Left, validation of the effectiveness of shRNA-mediated knockdown of TSC2 by western blot analysis of TSC2 and p-AMPK expression in OSU-CG12-treated cells. Right, GFP-LC3-expressing LNCaP cells transfected with scrambled or TSC2 shRNA were exposed to DMSO or OSU-CG12 for 36 h, and then examined by fluorescence microscopy to assess patterns of GFP-LC3 fluorescence.

Fig. 9. **Relative roles of AMPK activation and GADD153 in mediating the effect of OSU-CG12 on apoptosis and viability in LNCaP cells.** (A) Dominant-negative (DN) inhibition of AMPK had no effect on OSU-CG12-induced PARP cleavage (left), but partially protected LNCaP cells from OSU-CG12-mediated suppression of cell viability (central) and release of lactate dehydrogenase (LDH) (right). Data are expressed as means ± S.D. (n=6). (B) siRNA-mediated knockdown of GADD153 had no effect on OSU-CG12-induced PARP cleavage or β-TrCP induction (left), OSU-CG12-mediated antiproliferative activity (central), or OSU-CG12-mediated LDH release (right). Viability and LDH data are expressed as means ± S.D. (n=6). (C) Schematic diagram depicting the mechanism by which TZDs act as ERMAs by perturbing glucose homeostasis,
resulting in the hallmark cellular responses, including transient Sirt1 induction, AMPK activation, and ER stress. Our data indicate a mechanistic link between Sirt1 induction and β-TrCP protein accumulation, culminating in apoptosis through the proteasomal degradation and transcriptional repression of a series of apoptosis-regulatory proteins. The AMPK activation results in the autophagy via the conventional AMPK-TSCs-mTOR-p70S6K pathway. The ER stress signal triggers the up-regulation of sensor proteins, such as GRP78, GADD153 and IRE1α, which, might also play a role in down-regulating cell growth.
A. Thiazolidinediones (TZDs) and PPARγ-inactive TZDs

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Proteasomal Degradation

TZDs → β-TrCP

β-Catenin → Cyclin D1

IKKα

ERKs

GSK3β

Transcriptional repression

Androgen receptor

Estrogen receptor

EGFR

Other Sp1 targets

Other β-TrCP substrates

Sp1

NF-κB/p105, Wee1, and other β-TrCP substrates

B. Graphs showing cell viability:

- LNCaP
- MCF-7
- PrECs

C. Table showing concentrations of CG12

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E. Fluorescence images:

- DMSO
- 3-MA
- CG12
- CG12+3-MA

β-Actin
### LNCaP

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*ERα
**Fig. 6**

### B

#### 5 μM CG12 (h) in LNCaP cells transfected with

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#### LNCaP cells; 5 μM CG12 (h)

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### D

#### Western blotting

- **LNCaP cells; pretreatment for 12 h with**
  - 5 μM CG12
  - DMSO
  - Nicot.
  - Splito.
- **CHX (h)**
  - 0 12 24 36
- **β-TrCP**
  - 0 12 24 36
- **β-Actin**
  - 0 12 24 36

#### RT-PCR

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<thead>
<tr>
<th></th>
<th>5 μM</th>
<th>Nicot.</th>
<th>Splito.</th>
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</thead>
<tbody>
<tr>
<td><strong>CG12</strong></td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
</tr>
<tr>
<td><strong>β-TrCP</strong></td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
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<tr>
<td><strong>β-Actin</strong></td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
<td>0 12 24 36</td>
</tr>
</tbody>
</table>
10 μM CG12 exposure time; LNCaP cells ectopically expressing β-TrCP-Myc + Ubiquitin-HA

<table>
<thead>
<tr>
<th></th>
<th>Input (cell lysates)</th>
<th></th>
<th>IP: anti-Myc</th>
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<tbody>
<tr>
<td>WB</td>
<td>min</td>
<td>h</td>
<td>IgG</td>
</tr>
<tr>
<td>Myc</td>
<td>0 10 20 30 40 50 1 2 4 6 12 24</td>
<td></td>
<td>Myc</td>
</tr>
<tr>
<td>β-TrCP</td>
<td></td>
<td></td>
<td>HA</td>
</tr>
<tr>
<td>Sirt1</td>
<td></td>
<td></td>
<td>Ac-lysine</td>
</tr>
<tr>
<td>β-Actin</td>
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</tbody>
</table>
A  LNCaP cells; 5 μM CG12 (h)

<table>
<thead>
<tr>
<th></th>
<th>pCMV</th>
<th>DN-AMPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP</td>
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<tr>
<td>β-Actin</td>
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B  5 μM CG12 (h); siRNA for

<table>
<thead>
<tr>
<th></th>
<th>Scrm.</th>
<th>GADD153</th>
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</thead>
<tbody>
<tr>
<td>GADD153</td>
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<tr>
<td>PARP</td>
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<tr>
<td>β-TrCP</td>
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<tr>
<td>β-Actin</td>
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</tbody>
</table>

C  Energy restriction

TZDs — Glycolytic rate
NADH production

Sirt1 ↑ — β-TrCP ↑ — Proteasomal degradation & transcriptional repression — Apoptosis

p-AMPK ↑ — p-mTOR ↓ — p-p70S6K ↓ — Autophagy

ER stress ↑ — GRP78 ↑ — GADD153 ↑ — IRE1α ↑
Energy restriction as an antitumor target of thiazolidinediones
Shuo Wei, Samuel K. Kulp and Ching-Shih Chen

J. Biol. Chem. published online January 21, 2010

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