Energy Restriction as an Antitumor Target of Thiazolidinediones*

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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 vitro 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 as a novel class of ERMAs in that they elicited hallmark cellular responses characteristic of energy restriction, including transient induction of Sir1 (silent information regulator 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 γ-independent effects on a broad spectrum of signaling targets. Second, we demonstrated that Sir1-mediated up-regulation 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 thiazolidinediones as scaffolds to develop potent ERMAs, of which the proof-of-principle is demonstrated by OSU-CG12. OSU-CG12, a peroxisome proliferator-activated receptor γ-inactive ciglitazone derivative, exhibits 1- and 3-order 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, pro-inflammatory cytokines like interleukin-6 and tumor necrosis factor α 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). Although 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 the 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 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 (AR) poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; mTOR, mammalian homolog of target of rapamycin; TSC, tuberous sclerosis complex; LC, light chain; eIF, eukaryotic translation initiation factor; Atg, autophagy-related gene; HA, hemagglutinin; GADD, growth arrest and DNA damage-inducible gene; siRNA, small interfering RNA; shRNA, small hairpin RNA; WT, wild type; RT, reverse transcription; PBS, phosphate-buffered saline; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); LDH, lactate dehydrogenase.
p70S6K, p70S6K, tuberous sclerosis complex (TSC) 2, microtubule-associated protein light chain 3 (LC3), actin, eukaryotic initiation factor (eIF2) α, eIF2α, and autophagy-related gene (Atg) 5 from Cell Signaling (Beverly, MA); epidermal growth factor receptor, Sp1, mammalian target of rapamycin (mTOR), mTOR, Thr(P)389.

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), respectively, according to the manufacturer’s instructions. The sequences of the PCR primers used were as follows: Sirt1, 5'-GCAAGGTTGGAGGTTCTTGCCGTTCAAGGTGG-3' and 5'-GAACAGGTTCCGGAATC-3'; 5'-AACATGAAGGAGGTGTTGGT-3'; Sp1, 5'-GGCGAGAGGCCATTTATGTG-3' and 5'-AGTGGCATCAACGTCACTGCA-3'; GRP78, 5'-GGTCTTCAATGAGCTGCGTGTG-3' and 5'-GGAGAGGCCATTTATGTG-3'; fatty acid synthase, 5'-TATGTCTTCTTGCTGAGACTG-3' and 5'-TCTGCAACATAGGTTTAAGAT-3'; cyclin D1, 5'-ATGGAACACCACTCCTGTTGCTG-3' and 5'-TCAGATGTCGCCATCTGCCCAGCT-3'; actinδ, 5'-ATTGACCTTCTTCTGTCAGACTG-3' and 5'-ATGCTTCTTCTTGCTGAGACTG-3'; hexokinase 2, 5'-TCAGATGTCGCCATCTGCCCAGCT-3' and 5'-TATGTCTTCTTCTGTCAGACTG-3'; and Sirt1, 5'-ACAACCTGAGACTG-3' and 5'-ACAACCTGAGACTG-3'.

**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-supplemented RPMI 1640 medium and F12/Dulbecco's modified Eagle's medium, respectively. Nonmalignant prostate epithelial cells (PrECs) were maintained in prostate epithelial growth medium (Lonza Inc., Walkersville, MD). All of the cells were cultured at 37 °C in a humidified incubator containing 5% CO₂, 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 (ERMs), 2-deoxyglucose (2-DG), and resveratrol (19–21). These changes include reduced glycolytic rate, transient induction of the NAD⁺-dependent histone deacetylase Sirt1 (silent information regulator 1) (22), and activation of the intracellular fuel sensor AMP-activated protein kinase (AMPK) (23) and 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.
in 500 µl of Krebs buffer (25 mM NaHCO₃, 115 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 0.25% bovine serum albumin, pH 7.4) containing 1 mM nonradioactive glucose and 5 μCi/ml [5-³H]glucose for 1h at 37 °C. Aliquots from each treatment group were added to 0.2N 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 ³H₂O-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 (dpm) / [5-³H]glucose (dpm/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 (PerkinElmer Life Sciences) and 100 mM nonradioactive 2-DG and was terminated after 5 min by extensive washing with PBS. The cells were solubilized in 0.1% SDS buffer, and aliquots were taken for measurement of radioactivity using a scintillation counter.

NADH Assay and Lactate Assay—Determinations of intracellular levels of NADH and lactate were performed using an EnzyChrom NAD+/NADH assay kit and a L-lactate assay kit, respectively (BioAssay Systems, Hayward, CA). Briefly, LNCaP cells were cultured in 24-well plates at the density of 2×10⁵ cells/well for 24 h followed by treatments of 10 mM 2-DG or 10 µM OSU-CG12 for various time intervals. The cells were trypsinized and collected, the intracellular levels of NADH and lactate were determined according to the manufacturer’s instructions.

Cell Viability Assay—Cell viability was determined using the MTT assay. The cells were seeded in 96-well plates (5000 cells/well) and incubated in 10% fetal bovine serum-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 Me2SO, 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 nonradioactive 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. The 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 ERMs. 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, whereas those of resveratrol were 110 and 60 μM, respectively (Fig. 1B). Although the antiproliferative potencies of troglitazone (70 and 70 μM) and ciglitazone (70 and 42 μM) were comparable with that of resveratrol, their PPAR γ-inactive derivatives, STG28 (12 μM and 11 μM) and OSU-CG12 (5.7 μM and 5.0 μM), showed 1- and 3-order 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).

*ERα

We previously demonstrated that the antiproliferative effect of these TZDs was, in part, attributable to apoptosis (14, 17, 18). Because autophagy represents a characteristic cellular response to ERMs (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-methyladenine (Fig. 1, D and E).

β-TrCP-mediated Proteolysis Represents an Energy Restriction-elicited Signaling Event—To establish β-TrCP-mediated proteolysis as an energy restriction-elicited 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 epidermal growth factor receptor. 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).

Because 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), IκB kinase (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 IκB kinase. Similar effects on these signaling biomarkers were also noted in MCF-7 cells treated with 5 mM 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 ~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 up-regulation 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, whereas 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 up-regulation of the expression of GRP78, GADD153, and the ER-associated transducer inositol-requiring enzyme 1α, which has been shown to up-regulate 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 70-kDa ribosomal protein S6 kinase (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 α-subunit of eIF2α (Fig. 3D). This is noteworthy because 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, 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, values that are comparable with 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% fetal bovine serum-containing medium, 10 and 20 mg/ml glucose provided significant protection against OSU-CG12-induced cell death (p < 0.05 for all time points), whereas 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, middle and right panels), suggesting the selectivity of this protection against ERMs. 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 [3H]2-DG uptake by 2.5 and 5 μM OSU-CG12 was comparable with 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 (Fig. 5C, 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).
CG12 and resveratrol on the mRNA levels of hexokinase 2 and phosphofructokinase-1, the first two enzymes of the glycolytic pathway, and fatty acid synthase (Fig. 5E). Third, OSU-CG12 facilitated the dephosphorylation of Akt, which occurred after 6 h of treatment (Fig. 5D). Because 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 elf2α (Fig. 3D), a well-characterized metabolic stress response to a decreased energy status (38, 47). Recent evidence indicates that glucose deprivation-induced elf2α phosphorylation plays a key role in glucose-regulated protein synthesis in pancreatic cells (37).

Inhibition of β-TrCP Protects against Apoptosis—Because 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 because of the 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 up-regulation, 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 up-regulate β-TrCP expression.

Enforced expression of dominant-negative Sirt1 (H363YSirt1) reversed the effect of OSU-CG12 on inducing β-TrCP expression and PARP cleavage (Fig. 6C, left panel), whereas 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 up-regulation of β-TrCP expression in OSU-CG12-treated cells was mediated through deacetylation-dependent protein stabilization. First, using cycloheximide to assess protein stability, we showed that, in Me2SO-pretreated cells, β-TrCP remained unchanged after treatment with OSU-CG12 alone or in combination with either inhibitor (Fig. 6D, left panel). In contrast, OSU-CG12 at 5 μM increased the stability of β-TrCP because 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 (Fig. 6D, 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, whereas 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 E3 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 up-regulation 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 up-regulation 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. Because energy restriction induces autophagy through 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 because no effects on the expression of β-TrCP or GADD153 were observed. To confirm that β-TrCP up-regulation 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 (Fig. 8C, 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 restric-

![FIGURE 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 panel) or immunoprecipitated (IP) with anti-Myc antibodies, followed by Western blot analysis (WB) with anti-Myc, anti-HA, and anti-acetyl (Ac)-lysine antibodies.](image-url)
tion-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 LDH 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) (Fig. 9A, middle 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. Because 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 in vitro potencies of these agents limit their therapeutic applications.

Here, we demonstrate that TZDs represent a novel class of ERMs 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, and ER stress, each...
Novel Energy Restriction-mimetic Agents

![Graph](image)

**FIGURE 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 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 ERMs 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 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 inositol-requiring enzyme 1α (IRE1α), which might also play a role in down-regulating cell growth.

of which mediates a distinct signaling pathway culminating in the antiproliferative effects of OSU-CG12 (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 ERMs from the molecular scaffold of TZDs is feasible. From a translational perspective, the development of novel ERMs with greater antitumor potencies may provide advantages in clinical development.

Several lines of evidence suggest 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 ERMs through the activation of β-TrCPs. The α-B-crystallin-mediated induction of α-B-crystallin expression 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 that 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 ERMs, our data suggest that the interplay between autophagy and apoptosis plays a key role in mediating their antiproliferative activities.

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