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Troglitazone suppresses glutamine metabolism through a PPAR-independent mechanism

Miriam R. Reynolds and Brian F. Clem*

Department of Biochemistry and Molecular Genetics, James Graham Brown Cancer Center, University of Louisville, Louisville, KY

Abstract

Enhanced glutamine metabolism is required for tumor cell growth and survival, which suggests that agents targeting glutaminolysis may have utility within anti-cancer therapies. Troglitazone, a PPAR γ agonist, exhibits significant anti-tumor activity and can alter glutamine metabolism in multiple cell types. Therefore, we examined whether troglitazone would disrupt glutamine metabolism in tumor cells and whether its action was reliant on PPAR γ activity. We found that troglitazone treatment suppressed glutamine uptake and the expression of the glutamine transporter, ASCT2, and glutaminase. In addition, troglitazone reduced ^{13}C -glutamine incorporation into the TCA cycle, decreased [ATP], and resulted in an increase in reactive oxygen species (ROS). Further, troglitazone treatment decreased tumor cell growth, which was partially rescued with the addition of the TCA-intermediate, alpha-ketoglutarate, or the anti-oxidant N-acetylcysteine. Importantly, troglitazone's effects on glutamine uptake or viable cell number were found to be PPAR γ -independent. In contrast, troglitazone caused a decrease in c-Myc levels, while the proteasomal inhibitor, MG132, rescued c-Myc, ASCT2 and GLS1 expression, as well as glutamine uptake and cell number. Lastly, combinatorial treatment of troglitazone and metformin resulted in a synergistic decrease in cell number. Therefore, characterizing new anti-tumor properties of previously approved FDA therapies supports the potential for repurposing of these agents.

Keywords

Anaplerosis; Cancer; c-Myc; Glutaminolysis; Thiazolidinediones; Tumor

Introduction

The family of peroxisome proliferator-activated receptor (PPAR) nuclear hormone receptors function as transcriptional regulators of distinct target genes that control many cellular processes including adipocyte differentiation as well as lipid, carbohydrate, and amino acid metabolism. While free fatty acids and eicosanoids are endogenous ligands that mediate PPAR activity, development of a class of small molecule agonists for PPAR γ , termed thiazolidinediones (TZDs), have provided significant clinical benefit for the treatment of

*Corresponding author: 505 S. Hancock St., CTRB, Rm. 422, University of Louisville, Louisville, KY 40202, 1-502-852-8427 (office), 1-502-852-3661 (fax), bfclem01@louisville.edu.

type II diabetes.(Oakes, Kennedy et al. 1994, Young, Cawthorne et al. 1995, Petersen, Krssak et al. 2000, Smith, Lister et al. 2000) Beyond their use as anti-diabetic therapies, administration of these compounds, in particular troglitazone, has yielded significant cytotoxicity both *in vitro* and *in vivo* against various tumor cell types, suggesting that TZDs also possess utility as cancer chemotherapeutic agents.(Kubota, Koshizuka et al. 1998, Galli, Ceni et al. 2004, Galli, Mello et al. 2006, Srivastava, Kollipara et al. 2014) Accordingly, a variety of putative mechanisms have been proposed for troglitazone's anti-proliferative effects, and multiple studies have attributed these effects to both PPAR γ -dependent and – independent processes.

In early studies, troglitazone activation of PPAR γ was observed to induce tumor differentiation and inhibition of cancer growth in liposarcoma patients(Demetri, Fletcher et al. 1999), while Takahashi *et.al.* demonstrated that troglitazone increased apoptosis in gastric cancer through a PPAR γ -dependent mechanism.(Takahashi, Okumura et al. 1999) In contrast, TZDs also have been shown to suppress several pro-oncogenic factors and cell cycle regulators and result in cell cycle arrest independent of PPAR γ expression.(Akinyeke and Stewart 2011, Bolden, Bernard et al. 2012) In addition, inhibition of the Na⁺/H⁺ exchanger (NHE1), which results in cellular acidosis and reduced DNA synthesis, has been described as a PPAR γ -independent mechanism of troglitazone in breast cancer cells. (Turturro, Friday et al. 2004) Lastly, both the Turturro and Welbourne groups have demonstrated that troglitazone was able to alter cellular glutamine metabolism in normal renal-derived cells and specific cancer cell types.(Coates, Nissim et al. 2002, Routh, McCarthy et al. 2002, Friday, Oliver et al. 2011) Given that certain tumor cells exhibit an enhanced dependence on glutaminolysis for growth and survival, we postulated that changes in glutamine metabolism may be another potential mechanism by which troglitazone exerts its anti-cancer activity.

Cancer cells preferentially utilize aerobic glycolysis for glucose catabolism. This metabolic alteration, first reported by Otto Warburg, is characterized by an increase in glucose flux to lactate at the expense of glucose oxidation within the mitochondria.(Warburg 1956) As a result, tumor cells can compensate for the rerouting of glucose carbon away from the TCA cycle by metabolizing glutamine to replenish critical intermediates, such as alpha-ketoglutarate, through anaplerosis.(DeBerardinis, Mancuso et al. 2007, DeBerardinis and Cheng 2009) In addition, glutamine serves as a necessary precursor for nucleotide biosynthesis and glutathione production, which is required to maintain redox homeostasis and cell viability.(Estrela, Ortega et al. 2006, DeBerardinis, Mancuso et al. 2007, Wise and Thompson 2010) It is well established that certain tumor cells, including HeLa human cervical carcinoma cells, preferentially utilize glutamine as their primary mitochondrial energetic substrate.(Reitzer, Wice et al. 1979) More recently, we demonstrated that glutamine withdrawal results in a rapid decrease in steady-state ATP levels within a glutamine-dependent cell type lacking all three retinoblastoma (RB) pocket proteins. (Reynolds, Lane et al. 2014) Importantly, regulation of glutaminolysis in many tumor cells is accomplished in part through modulating the expression of several key proteins, such as the major glutamine transporter ASCT2 (sodium-coupled neutral amino acid transporter) and glutaminase (GLS1), which catalyzes the deamidation of glutamine to glutamate.(Wise and Thompson 2010, Mates, Segura et al. 2013) Consequently, the identification of

chemotherapeutics that alter the activity or expression of enzymes within glutaminolysis is currently being investigated.(Emadi, Jun et al. 2014, Gross, Demo et al. 2014)

While troglitazone has been reported to either suppress or alter glutamine metabolism in various cells, we examined whether troglitazone would disrupt glutaminolysis through a PPAR γ -dependent or –independent manner within cells reliant on increased glutamine utilization for cell growth and survival. Using three separate cell types, we found that troglitazone inhibited glutamine uptake, incorporation of glutamine carbon into the TCA cycle, and steady-state ATP levels by suppressing key glutaminolytic proteins. In addition, troglitazone treatment led to an increase in reactive oxygen species (ROS). Growth of each cell line was suppressed with troglitazone treatment, and addition of a cell-permeable TCA intermediate analog, dimethyl- α -ketoglutarate, or the antioxidant N-acetylcysteine was found to partially rescue growth in H460 lung cancer cells. Importantly, the effects on glutamine uptake and cell growth were determined to be independent of PPAR γ or PPAR α activity, yet troglitazone resulted in a dose-dependent reduction in c-Myc. Lastly, within H460 cells, inhibition of proteasomal activity rescued c-Myc and ASCT2 protein levels, and partially rescued GLS1 expression, glutamine uptake and viable cell number. Taken together, our results indicate that in specific glutamine-dependent cells, troglitazone causes an overall suppression of glutaminolysis and disruption of cellular bioenergetics and may provide significant anti-tumor clinical benefit, particularly within combinatorial therapies that may sensitize neoplastic cells to alterations in glutamine metabolism.

Results

Troglitazone decreases glutamine uptake and expression of glutaminolytic proteins

To investigate a role for troglitazone in the inhibition of glutaminolysis, we examined glutamine uptake and expression of glutaminolytic proteins in response to treatment in three glutamine-dependent cell types, H460, HeLa 229, and RB-family triple knock-out mouse embryonic fibroblasts (TKO) [Supplemental Figure 1].(Reitzer, Wice et al. 1979, Reynolds, Lane et al. 2014) After 48 hour treatment, we found that troglitazone caused a dose-dependent decrease in glutamine uptake as measured using ^{14}C -labeled glutamine (Figure 1A). Both ASCT2 and GLS1 have been demonstrated to be over-expressed in cancer cell lines and their expression directly correlates with glutamine utilization in tumor cells. (DeBerardinis, Mancuso et al. 2007, Le, Lane et al. 2012) To determine whether these glutaminolytic proteins may be contributing to the reduction in glutamine uptake, we examined ASCT2 and GLS1 expression in cells treated with troglitazone. All three cell lines exhibited a dose-dependent decrease in expression of ASCT2 and GLS1 (Figure 1B). Taken together, troglitazone appears to potentially regulate glutamine metabolism by affecting expression of major glutaminolytic proteins.

Troglitazone suppresses glutamine incorporation into the TCA cycle, reduces steady-state ATP levels, and decreases cell growth

To establish whether reduced glutamine uptake results in the concomitant reduction of glutamine anaplerosis into the TCA cycle, we employed 2D-TOCSY NMR analysis using ^{13}C -labeled glutamine. Glutamine carbons enter the TCA cycle after deamidation to

glutamate followed by conversion to alpha-ketoglutarate. To test this, H460 cells were incubated with varying concentrations of troglitazone in the presence of ^{13}C -glutamine and cell extracts were examined for carbon enrichment into aspartate, which is directly labeled from the TCA-intermediate, oxaloacetate. Similar to glutamine uptake, we found that troglitazone treatment resulted in a significant dose-dependent decrease in labeled glutamine-derived carbon incorporation into aspartate (Figure 2A, Supplemental Figure 2). We then postulated that suppression of glutamine anaplerosis would result in diminished mitochondrial activity, so we measured steady-state ATP levels in response to troglitazone. Again, we observed a significant dose-dependent decrease in steady-state ATP levels in all three cell lines after 48 hour treatment. (Figure 2B, Supplemental Figure 3). Lastly, troglitazone reduced viable cell number in the TKO, HeLa 229, and H460 cells (Figure 3A, Supplemental Figure 4). Previous studies have demonstrated that a cell permeable analog of the TCA-intermediate alpha-ketoglutarate, dimethyl-alpha-ketoglutarate, was able to increase steady-state ATP levels and cell number in response to loss of glutamine metabolism (Wise, DeBerardinis et al. 2008, Reynolds, Lane et al. 2014). Addition of dimethyl-alpha-ketoglutarate (αKG) was found to partially rescue both steady-state ATP levels as well as viable cell number in H460 cells in response to troglitazone treatment (Figure 3B–C). Glutamine is a significant carbon source for the synthesis of glutathione and glutamine withdrawal results in decreased glutathione levels and increased ROS production. Since troglitazone previously has been demonstrated to increase ROS in certain cancer cells (Srivastava, Kollipara et al. 2014), we examined ROS production in the H460 cells and the effect of co-treatment with the antioxidant N-acetylcysteine (NAC). Indeed, troglitazone elevated ROS levels in the H460 cells, and the addition of NAC was able to partially rescue both ROS levels and viable cell number after 48 hour treatment (Figure 3D–F). Taken together, we postulate that troglitazone can suppress cell number, in part, through decreasing glutamine metabolism, which supports maintenance of steady-state ATP levels and suppression of ROS.

siRNA directed against PPAR γ and PPAR α are unable to rescue glutamine uptake and cell number in response to troglitazone

Troglitazone is a well-established potent agonist of the PPAR γ nuclear receptor and at higher concentrations may also weakly activate other receptor family members, such as PPAR α . (Lehmann, Moore et al. 1995) To investigate whether troglitazone exerts its inhibitory effects on glutamine metabolism and cell growth through a PPAR-dependent mechanism, we decreased expression of both PPAR γ and PPAR α (Supplemental Figure 5) in the presence or absence of troglitazone treatment. Suppression of either PPAR γ or PPAR α failed to demonstrate a significant rescue of either glutamine uptake or viable cell number in all cell lines (Figure 4A–B, Supplemental Figure 6&7). Further confirmation of a lack of a role for the PPAR γ nuclear receptor in modulating troglitazone's inhibitory effects on glutamine uptake and cell number in these cell lines was ascertained by utilizing an irreversible PPAR γ antagonist, GW9662. (Leesnitzer, Parks et al. 2002) Notably, GW9662 alone did not affect glutamine uptake or cell number in either the H460, HeLa229, or TKO MEFs (Supplementary Figure 8A–B), and GW9662 in combination with troglitazone failed to demonstrate a significant rescue of either glutamine uptake or cell number (Supplemental

Figure 8C–H). Taken together, these results indicate that the effects of troglitazone are facilitated through a PPAR-independent mechanism.

Troglitazone incubation results in a dose-dependent suppression of c-Myc

Given previous reports demonstrating a role for the oncoprotein c-Myc in regulating glutaminolysis through direct or indirect control of both ASCT2 and GLS1 expression (Wise, DeBerardinis et al. 2008, Gao, Tchernyshyov et al. 2009), respectively, we investigated whether troglitazone treatment would affect c-Myc expression in these glutamine-dependent cell lines. Indeed, we observed a dose-dependent decrease in c-Myc expression in response to 48 hour troglitazone treatment (Figure 5). Notably, our results support previous work from Akinyeke *et al.*, which demonstrated inhibition of c-Myc expression in response to troglitazone treatment in prostate cancer cells. (Akinyeke and Stewart 2011)

MG132 prevents proteasomal degradation of c-Myc, ASCT2 and GLS1 in the presence of troglitazone and provides a significant rescue of glutamine uptake and cell number

Work from Stewart's group also had demonstrated that suppression of c-Myc in response to troglitazone treatment occurs via proteasomal-mediated degradation. (Akinyeke and Stewart 2011) To determine whether troglitazone also mediates a proteasomal decrease in c-Myc expression in our study, H460 cells were treated for 24 hours with varying concentrations of troglitazone in the presence or absence of the proteasomal inhibitor, MG132. Co-treatment with MG132 was able to rescue c-Myc expression in H460 cells and its known downstream glutaminolytic targets, ASCT2 and GLS1 (Figure 6A). In addition, we found that c-Myc, ASCT2, and GLS1 rescue by MG132 accompanied an increase in glutamine uptake and cell number in H460 cells treated with troglitazone (Figure 6B&C).

Combinatorial treatment of troglitazone with metformin results in a synergistic decrease in cell number in H460 and HeLa 229 cells

In order to identify additional anti-cancer strategies, on-going studies are examining the potential utility of re-purposing prior FDA approved therapies in combination with other anti-tumor agents. Metformin is a biguanide that is FDA-approved for the treatment of type II diabetes, and like TZDs, have been shown to exert significant anti-tumor activity. (Bost, Sahra et al. 2012) Recently, metformin was demonstrated to sensitize prostate cancer cells to inhibitors of glutamine metabolism. (Fendt, Bell et al. 2013) Therefore, we examined whether co-treatment with metformin would enhance the anti-growth effects of troglitazone in these cell types. Using a concentration of metformin (1mM) that yielded no cellular effects alone, we found that combinatorial treatment with troglitazone (10μM) led to a synergistic decrease in viable cell number in both the H460 and HeLa 229 cells (Figure 7A–B).

Discussion

Thiazolidinediones, a class of PPAR γ agonists, have been demonstrated to exhibit significant anti-tumor activity across a broad spectrum of cancer cell types, and extensive studies have proposed a myriad of putative mechanisms for their inherent tumor inhibitory properties. In addition, these ligands also have been shown to alter nutrient flux through important

metabolic pathways, including glutamine metabolism, within both normal and neoplastic cells.(Petersen, Krssak et al. 2000, Routh, McCarthy et al. 2002, Friday, Oliver et al. 2011) Importantly, as a result of oncogenic activation or loss of tumor suppressor function, certain cancer cells demonstrate an increased demand for glutaminolysis and suppression of this pathway is selectively cytotoxic compared to primary cells.(Wise, DeBerardinis et al. 2008, Son, Lyssiotis et al. 2013) Within the present study, we examined whether PPAR γ agonists, in particular troglitazone, was able to decrease glutamine metabolism, specifically within cell types that utilize glutamine as a major energetic substrate.(Reitzer, Wice et al. 1979, Reynolds, Lane et al. 2014) We found that prolonged troglitazone treatment led to a significant decrease in glutamine uptake in three independent cell types. This decrease was accompanied by diminished expression of a major glutamine transporter ASCT2 as well as glutaminase (GLS1). While glutamine carbon may have several fates, mitochondrial anaplerosis is critical in maintaining TCA intermediates for biosynthetic processes and energy production.(DeBerardinis and Cheng 2009) We also found that troglitazone-mediated loss of glutamine uptake correlated with suppression of glutamine entry into the TCA cycle (suppression of labeled aspartate) and a decrease in steady-state ATP levels, and troglitazone treatment led to significant inhibition of viable cell number in each cell line. Importantly, troglitazone was able to promote these cellular effects even in the absence of PPAR γ expression or activity, which, to our knowledge, is the first direct report that troglitazone alters glutamine metabolism via a PPAR γ -independent mechanism.

However, our data contrast with prior work demonstrating that TZDs promote a shift in glutamine metabolism away from flux through alanine aminotransferase, towards deamination via mitochondrial glutamate dehydrogenase.(Routh, McCarthy et al. 2002) This was found to be stimulated, in part, by intra-cellular acidosis that occurs in response to acute troglitazone treatment(Coates, Nissim et al. 2002) and would, in essence, sustain glutamine carbon anaplerosis within the mitochondria. Indeed, Friday *et. al.* showed that glutamine deamination in response to troglitazone treatment for 24 hours was able to restore mitochondrial membrane potential in breast cancer cells.(Friday, Oliver et al. 2011) These results conflict with our data in that troglitazone led to a decrease in glutamine metabolism, stemming from suppression of glutamine uptake, as a result of diminished ASCT2 and/or GLS1 expression, as well as a decrease in glutamine carbon incorporation into the TCA cycle metabolite, aspartate. The reasoning for this discrepancy is not clear, but our studies employed both higher troglitazone concentrations (40–80 μ M) and longer exposures (48 hours). In addition, we utilized three separate cell systems that have been established to be dependent on glutamine for cell survival and growth. This differential response to troglitazone treatment may suggest that underlying oncogenic/tumor suppressor alterations that drive the metabolic phenotype within cancer cell types may influence the cellular response to troglitazone, such as the presence of oncogenic K-Ras mutations or enhanced c-Myc activity, both of which have been found to promote glutamine dependency.(Yuneva, Zamboni et al. 2007, Wise, DeBerardinis et al. 2008, Son, Lyssiotis et al. 2013) This is of particular significance since several groups have demonstrated that troglitazone leads to a dose-dependent decrease in c-Myc expression in a PPAR γ -independent manner.(Cerbone, Toaldo et al. 2007, Akinyeke and Stewart 2011) Due to c-Myc's reported role in regulating glutamine metabolism, we investigated whether this phenomenon also occurs in our cell

systems, and found that c-Myc levels were suppressed upon troglitazone treatment in all three cell types. Specifically, c-Myc can induce glutaminolysis by regulation of ASCT2 and GLS1 (Wise, DeBerardinis et al. 2008, Gao, Tchernyshyov et al. 2009), and both of these proteins were down-regulated in a similar manner in response to troglitazone treatment. Taken together, our data suggest that inhibition of glutamine metabolism potentially through direct suppression of c-Myc and other downstream targets, such as ASCT2 and GLS1, may be another mechanism by which troglitazone exerts its anti-tumor activity in certain cell types.

Recently, Srivastava *et al.* demonstrated that PPAR γ agonists, specifically pioglitazone, suppresses non-small cell lung cancer through increased production of ROS via a PPAR γ -dependent mechanism. (Srivastava, Kollipara et al. 2014) Importantly, the inhibitory action of pioglitazone was diminished upon suppression of PPAR γ or within cells lacking endogenous PPAR γ expression. However, while troglitazone was able to induce ROS and decrease cell proliferation, the PPAR γ -dependent requirement for troglitazone was not directly addressed as the studies employing troglitazone were not performed under PPAR γ -suppression or in cells devoid of PPAR γ . This is notable in that others have observed that various TGZs may exert differential downstream effects on mediators of key cellular processes. (Akinyeke and Stewart 2011) Specifically, while troglitazone is able to promote c-Myc degradation in a PPAR-independent manner in prostate cancer cells, other TGZs, including pioglitazone, do not exhibit this c-Myc inhibitory capability (Akinyeke and Stewart 2011), suggesting that troglitazone may have additional mechanisms by which it can exert its anti-proliferative effects. As discussed above, c-Myc directly influences glutamine metabolism, which is integral in the maintenance of glutathione and redox homeostasis. Therefore, troglitazone may increase ROS levels through a decrease in c-Myc levels and glutamine metabolism. Indeed, we observed an increase in ROS levels in H460 cells upon troglitazone treatment (Figure 3D–E). However, in H460 cells, addition of NAC only partially rescued ROS levels and viable cell number (Figure 3E–F). Since glutamine anaplerosis can maintain mitochondrial activity and sustain steady-state ATP levels and we found that troglitazone reduced [ATP] after 48 hour treatment, we determined whether addition of cell permeable dimethyl- α -ketoglutarate (α KG) may reverse these effects of troglitazone. We found that α KG was able to partially rescue steady-state ATP levels and viable cell number (Figure 3B–C). Taken together, we postulate that troglitazone may exert its anti-growth effects, in part, through a c-Myc-dependent regulation of glutamine metabolism.

Previous results from the Stewart lab have suggested that the troglitazone-mediated decrease in c-Myc expression in prostate cancer cells is, in part, caused through increased c-Myc phosphorylation and proteasomal degradation, which can be rescued by the addition of a proteasome inhibitor or expression of a phosphorylation deficient form of c-Myc (T58A). (Akinyeke and Stewart 2011) Our data are consistent in that the presence of MG132 was able to rescue c-Myc and ASCT2 expression, and result in a partial rescue in GLS1 levels, glutamine uptake and viable cell number. However, while troglitazone treatment of H460 cells harboring stable expression of Sham vector or c-Myc (T58A) exhibited increased phosphorylation of endogenous c-Myc at T58 (Supplementary Figure 9), unexpectedly, we also observed a dose-dependent decrease in total c-Myc expression in the c-Myc (T58A) cells similar to that observed in Sham transduced cells. In addition, phosphorylation of S62

on c-Myc is maintained in both cell types upon troglitazone treatment. In agreement with a loss of c-Myc expression in the c-Myc (T58A) cell line, no significant rescue in glutamine uptake, steady-state ATP levels, or viable cell number was observed in response to troglitazone treatment within the mutant c-Myc expressing cells (Supplementary Figure 10). Additional work is required in order to understand the mechanism by which troglitazone may mediate suppression of phosphorylation deficient c-Myc (T58A) in the H460 cells. Interestingly, a recent study identified a Smad7-Skp2 dependent pathway, which mediates c-Myc proteasomal degradation independent of T58 phosphorylation (Kim, Kang et al. 2014). While Akinyeke *et. al.* demonstrated that troglitazone-mediated suppression of c-Myc resulted in decreased expression of cell cycle regulators and loss of cell proliferation in prostate cancer cells (Akinyeke and Stewart 2011), our data may extend the regulatory role of c-Myc in response to troglitazone treatment to direct regulation of a metabolic pathway critical for growth in glutamine-dependent cell types and suggest that addition of troglitazone to existing therapies that sensitize neoplastic cells to glutamine disruption may yield increased clinical benefit in the treatment of cancer patients.

Based on standard of care for type II diabetes, individuals administered 600mg/day of troglitazone present with average plasma AUC values of 22.1 mg-hr/L and peak concentrations (C_{max}) of 2.8 mg/L or approximately 6 μ M (Loi, Young et al. 1999). While this is below the concentration (~20 μ M) that produced anti-neoplastic effects in these studies, previous oncology trials were able to administer a higher dose of troglitazone (800mg/day) and observe significant clinical responses with no severe adverse events (Demetri, Fletcher et al. 1999, Mueller, Smith et al. 2000), suggesting that higher concentrations of troglitazone may be necessary to promote its anti-tumor properties in cancer patients. Importantly, while troglitazone alone may produce modest anti-tumor effects, its inclusion into combinatorial therapies would be expected to increase its effectiveness at similar or lower doses compared to that observed in monotherapy. In support of this, a synergistic decrease in viable cell number of H460 and HeLa 229 cells was observed upon exposure to a combination of troglitazone and metformin (troglitazone: 10 μ M; metformin: 1mM), (Figure 7A–B). Notably, this concentration of troglitazone may be achievable within patients at the dose (800mg/day) used in previous cancer clinical trials (Demetri, Fletcher et al. 1999, Mueller, Smith et al. 2000). Lastly, co-administration of troglitazone and metformin was found to yield no significant toxicities (normal liver function and plasma lactate levels) within type II diabetic patients (Inzucchi, Maggs et al. 1998), and our data now lends support to the combinatorial repurposing of these compounds as additional anti-tumor strategies.

Materials and Methods

Cell culture

H460 lung carcinoma and HeLa 229 cervical adenocarcinoma were obtained from ATCC and Rb protein family triple knock-out (TKO) MEFs have been previously described. (Reynolds, Lane et al. 2014) HeLa 229 and TKO MEF cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 25 mM glucose, 4 mM glutamine and 50 μ g/ml gentamicin sulfate and the H460 cells were cultured in RPMI 1640

(Gibco) supplemented with 10% fetal bovine serum (Hyclone), 25 mM glucose, 4 mM glutamine and 50 µg/ml gentamicin sulfate, respectively. Cells were maintained at 5% CO₂ at 37 °C.

Glutamine uptake

H460, HeLa 229, and TKO MEF cells were treated for 48 hours with varying concentrations of troglitazone, then washed with phosphate-buffered saline and incubated for thirty minutes in glutamine-deficient media. Cells were then supplemented with 0.5 µCi [U-¹⁴C]-glutamine (Perkin Elmer) for 5 minutes. Medium was aspirated, and cells were washed three times with glutamine-free DMEM or glutamine-free RPMI. Cells were then lysed in 0.1% SDS, and sample c.p.m. was acquired on a liquid scintillation counter (Perkin Elmer). Counts were normalized to milligram protein as determined by BCA protein assay (Pierce).

Protein expression

Protein lysates from troglitazone-treated TKO MEF, HeLa 229 and H460 cells were resolved by 12% SDS-PAGE electrophoresis, transferred to PVDF membrane, and blocked in 5% non-fat dry milk, and 0.1% TBS-Tween 20. Blots were then probed for ASCT2 (Cell Signaling), GLS1 (Abnova), p-c-Myc (T58) (Abnova), p-c-Myc (S62) (Abnova), c-MYC (Cell Signaling), PPARγ (Santa Cruz Biotechnology), PPARα (Santa Cruz Biotechnology), and β-Actin (Pierce). Protein detection was performed using HRP-conjugated secondary antibodies and ECL Select Western Detection Reagent (GE Life Sciences).

Stable isotope resolved metabolomics analysis by NMR

H460 cells were seeded at 1×10^5 cells in 6-well plates. Cells were treated for 24-hours with varying concentrations of troglitazone and then for an additional 24 hours in the presence of RPMI 1640 supplemented with 25 mM glucose, 10% dialyzed fetal bovine serum and 4 mM [¹³C₅, ¹⁵N₂]-glutamine (Perkin Elmer). Cell pellets were washed three times using phosphate- buffered saline, and metabolites were extracted twice with 10% trichloroacetic acid. 2D-TOCSY NMR analysis and determination of labeled aspartate incorporation was performed as previously described.(Reynolds, Lane et al. 2014)

Cellular steady-state ATP levels

Exponentially growing H460, HeLa 229, and TKO MEF cells were treated with varying concentrations of troglitazone for 48 hours. Cells were lysed in passive lysis buffer (Promega) and steady-state ATP concentrations were determined using the ATP Determination Kit following manufacturer's protocol (Invitrogen). Cellular ATP levels were normalized to milligram protein as determined by BCA protein assay (Pierce). For rescue studies, H460 cells were treated with 40µM troglitazone and 3.5mM dimethyl-alpha-ketoglutarate for forty-eight hours.

Reactive oxygen species

ROS was determined in H460 cells with or without troglitazone (40µM) and/or N-acetylcysteine (NAC: 5mM) treatment for forty-eight hours by DCF-DA (Invitrogen) fluorescence by flow cytometry.

Viable cell number

To determine the effects of troglitazone on cell number in H460, HeLa 229, and TKO MEF cells, viable cell number was determined after troglitazone treatment by trypan blue exclusion and enumeration using a hemocytometer. For troglitazone, GW9662 and MG132 experiments, reagents were added 24 hours post-seeding or 24 hour post-transfection for the siRNA experiments and then incubated for the indicated time points. Troglitazone (5–80 μ M), GW9662 (10 μ M), dimethyl- α -ketoglutarate (3.5mM) and MG132 (1mM) (Cayman Chemicals) were reconstituted in DMSO. N-acetylcysteine (5mM) was reconstituted in cell culture grade water and pH was adjusted to 7.

Cell transfections

siRNA transfections were performed as previously described (Reynolds, Lane et al. 2014) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) and the following siRNA species. Murine and human specific siRNAs were used for the TKO MEFs and human cell lines (H460 and HeLa 229), respectively. PPAR α : 5'-GGAAAGUCCCUUAUCUGAA- 3' (Ambion #s72003_Mm) and PPAR α : 5'-GAUCAAGUGACAUUGCUGAA- 3' (Ambion #s10880_Hs); PPAR γ : 5'-GGGCGAUCUUGACAGGAAA- 3' (Ambion #s72013_Mm) and PPAR γ : 5'-GACAAAUCACCAUUCGUU- 3' (Ambion #s10886_Hs) at a final concentration of 40 nM. Protein expression, glutamine uptake, and viable cell number were determined 48 hours post-transfection as described above.

Statistics

Statistical significance for glutamine uptake, fractional aspartate enrichment, steady-state ATP levels, and viable cell number was determined by a two-sample, nonparametric, two-tailed student *t*-test. $p < 0.05$ was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Troglitazone decreases glutamine uptake and expression of glutaminolytic proteins
A. Glutamine uptake in H460, HeLa 229, and TKO MEF cells was assayed by ^{14}C -glutamine labeling in response to varying concentrations of troglitazone for 48 hours. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$, compared to vehicle for each cell type. **B.** Protein expression of the glutamine transporter, ASCT2, glutaminase, GLS1, and β -actin within H460, HeLa 229 and TKO MEF cells in response to varying concentrations of troglitazone for 48 hours. Shown are representative images from three independent experiments.

Figure 2. Troglitazone suppresses glutamine incorporation into the TCA cycle and reduces steady-state ATP levels

A. Incorporation of glutamine carbon into aspartate was determined by stable isotope labeling of H460 cells with [$^{13}\text{C}_5$, $^{15}\text{N}_2$]-glutamine, as described in the *Materials and Methods*. Data are presented as relative incorporation, which corresponds to the fractional enrichment of either ^{12}C or ^{13}C -containing species.* $p < 0.05$, compared to respective vehicle. **B.** Steady-state ATP levels were determined for H460 cells in response to varying concentrations of troglitazone for 48 hours as described in the *Materials and Methods*. Data are presented as relative ATP concentrations where vehicle is 1.0, and shown as the mean $\pm$ s.d. from three independent experiments.* $p < 0.05$, compared to vehicle control.

Figure 3. Alpha-ketoglutarate or N-acetylcysteine partially rescue the troglitazone-mediated decrease in H460 cell growth

A. After 48 hours of treatment with varying concentrations of troglitazone, H460 viable cell number was determined using trypan blue staining and hemocytometer enumeration. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$, compared to vehicle control. **B.** Steady-state ATP levels or **(C)** viable cell number were determined in H460 cells treated in the presence or absence of troglitazone (40 μ M) with or without dimethyl-alpha-ketoglutarate (α KG: 3.5mM) for 48 hours. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$ for indicated comparisons. **D–E.** ROS levels or **(F)** viable cell number were determined in H460 cells treated in the presence or absence of troglitazone (40 μ M) with or without N-acetylcysteine (NAC: 5mM) for 48 hours. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$ for indicated comparisons.

Figure 4. siRNAs directed against PPAR γ and PPAR α are unable to rescue glutamine uptake and cell number in H460 cells treated with troglitazone

H460 cells transfected with siRNAs directed against either PPAR γ (A) or PPAR α (B) were incubated with varying concentrations of troglitazone for 48 hours. Glutamine uptake in the H460 cells was assayed by ^{14}C -glutamine labeling. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. Viable cell number was determined using trypan blue staining and hemocytometer enumeration. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. Statistical analysis on both glutamine uptake and viable cell number was performed comparing each group within a specific treatment concentration. The lack of asterisks indicates no significant difference (* $p < 0.05$).

Figure 5. Troglitazone treatment results in a dose-dependent suppression of c-Myc
Expression of c-Myc and β -actin within H460, HeLa 229 and TKO MEF cells in response to 48 hour incubation with varying troglitazone concentrations was determined by Western blot analysis as described in the *Materials and Methods*. Shown are representative images from three independent experiments.

Figure 6. MG132 prevents proteasomal degradation of c-Myc and ASCT2 in the presence of troglitazone and promotes a significant rescue of GLS1 expression, glutamine uptake and cell number

A. Expression of c-Myc and the glutaminolytic proteins, ASCT2 and GLS1, was examined in H460 cells in response to varying concentrations of troglitazone for 24 hours in the presence or absence of the proteasome inhibitor, MG132. Shown are representative images from two independent experiments. After 24 hours of troglitazone treatment in the absence or presence of MG132, **(B)** glutamine uptake and **(C)** viable cell number determined in H460 cells as described in the *Materials and Methods*. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$ for indicated comparisons.

Figure 7. Combinatorial treatment with troglitazone and metformin results in decreased cell number

Viable cell number in H460 and HeLa229 incubated with the indicated treatments was determined using trypan blue staining and hemocytometer enumeration. Data are presented as percent of vehicle and shown as the mean $\pm$ s.d. from three independent experiments. * $p < 0.05$ compared to vehicle control. TRO: troglitazone, MET: metformin