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Troglitazone suppresses glutamine metabolism and tumor cell growth through a ppar-independent mechanism.

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TROGLITAZONE SUPPRESSES GLUTAMINE METABOLISM AND TUMOR CELL GROWTH THROUGH A PPAR-INDEPENDENT MECHANISM

By

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B.A., Purdue University, 1988

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A Thesis Approved on

November 19, 2014

by the following Thesis Committee:

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Dr. Thomas Geoghegan

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Dr. Russell Prough

DEDICATION

I dedicate this thesis to my grandmothers

Mrs. Esther Travis

and

Mrs. Sarah Trout

for serving as continued sources of inspiration.

ACKNOWLEDGEMENTS

First, I would like to express my appreciation to Dr. Brian Clem, my boss and mentor, for providing me with the opportunity to work in his lab. I am a better scientist with enhanced critical thinking skills as a result of working with Dr. Clem. Next, I would like to thank committee members Dr. Russell Prough and Dr. Thomas Geoghegan for providing continued guidance and expertise in matters associated with the focus of this Master's project. Their insights were most valuable. In addition, I would like to thank Dr. Barbara Clark for her oversight of progress through the Master's program and the Department of Biochemistry and Molecular Biology members for the opportunity to study and complete the Master's program. Lastly, I would like to thank Dr. Clem and Dr. Jason Chesney for their support in the application to the Master's program in BMB.

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ABSTRACT

TROGLITAZONE SUPPRESSES GLUTAMINE METABOLISM AND TUMOR CELL GROWTH THROUGH A PPAR-INDEPENDENT MECHANISM

Miriam R. Reynolds

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In tumor cells, glutamine metabolism provides anaplerotic carbon for the TCA cycle, fatty acid synthesis, and precursors for the production of nucleotides and glutathione. This metabolic alteration is primarily driven by disruptions in oncogenic or tumor suppressor function and results in glutamine dependency for tumor cell survival. Troglitazone, a PPARγ agonist, has been reported to alter glutamine metabolism. This thesis project addresses whether troglitazone treatment could disrupt glutamine metabolism in glutamine-dependent tumor cells. Results obtained with troglitazone treatment include: dose-dependent inhibition of cell proliferation, glutamine uptake, glutaminolytic protein expression, steady-state ATP levels, and glutamine carbon flux into the TCA cycle. Silencing PPARα/γ activity by using siRNA or a PPARγ antagonist did not alter troglitazone's effects on cell proliferation or glutamine uptake. Furthermore, troglitazone treatment caused the suppression of c-Myc protein expression, an oncogene known to regulate glutaminolysis. Thus, troglitazone was shown to disrupt tumor glutamine metabolism through a PPAR-independent manner.

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INTRODUCTION

In an effort to understand the mechanism of action of troglitazone as it relates to glutamine metabolism and cell growth in cancer cells, I embarked on a research project that entailed acquiring an understanding of complex metabolic pathways and transcriptional activators involved in metabolic processes.

Intermediary metabolism

Intermediary metabolism refers to the set of chemical reactions that occur in a living organism, specifically within the context of the cell. Metabolism may be broadly divided into two categories: catabolism, or the breakdown of molecules into components resulting in the eventual release of energy, and anabolism, or the synthesis of molecules from components requiring the input of energy. Within cellular metabolism, catabolic and anabolic processes are coupled in order to coordinate metabolic pathways and maximally conserve energy.

Chemical reactions are catalyzed by specialized proteins called enzymes and multiple enzymes may be associated with the activation of a given metabolic pathway. Regulation of enzymatic activity within a pathway occurs in response to changes within the cellular environment or in response to signals received from other cells. All cells, whether prokaryotic or eukaryotic, utilize similar metabolic pathways in order to ensure survival. A central component of cellular metabolism under aerobic conditions involves those chemical reactions comprising the tricarboxylic acid (TCA) cycle, an enzymatic

pathway first delineated by Hans Krebs (Krebs, 1937). Glucose and glutamine carbons may be channeled into the TCA cycle where their respective carbon skeletons serve as energetic substrates. For example, pyruvate derived from glucose may be oxidized within the mitochondria to form acetyl-CoA which undergoes a condensation reaction with oxaloacetate to form citrate, the first reaction in the TCA cycle. Similarly, the amino acid glutamine may be deamidated to form glutamate followed by a subsequent deamination reaction yielding alpha- ketoglutarate (α-KG). α-KG serves as a central intermediate metabolite in the TCA cycle. The oxidation of carbon subunits within the TCA cycle yields soluble coenzymes possessing reducing power, coenzymes such as dihydronicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_{2)}$. These coenzymes are utilized by the electron transport chain (ETC) and the associated electron carrier proteins within the mitochondrial membrane to generate adenosine triphosphate (ATP) for the cell's use in driving metabolic pathways and processes. Thus, by limiting the flow of carbon subunits into the TCA cycle, co-factor function and the energetics of a cell may be altered.

Regulation of the TCA cycle may occur at the level of substrate availability, product inhibition or competitive feedback inhibition. For each acetyl-CoA molecule oxidized to completion within the TCA cycle, 3 NADH, 1 $FADH_2$ and 1 GTP (1 ATP equivalent) are generated. The TCA cycle is turned "on" in response to high ADP/ATP or high NAD+/NADH ratios in the cell. The NADH electron carrier molecule is generated in the chemical reactions catalyzed by the TCA enzymes isocitrate dehydrogenase, alpha- ketoglutarate dehydrogenase and malate dehydrogenase. The NADH produced in these reactions may in turn serve as a negative allosteric regulator of

the enzymes citrate synthase, a-ketoglutarate dehydrogenase and isocitrate dehydrogenase while ATP may inhibit isocitrate dehydrogenase.

Glutaminolysis

High levels of glutamine utilization have been reported in rapidly dividing cells (Newsholme E.A. et al., 1985). The amino acid glutamine (GLN) is used for the biosynthesis of nucleotides, glutathione and macromolecules including proteins and lipids (Newsholme P. et al., 2013). Even quiescent cells that are not actively proliferating have high demands for TCA substrates including glucose and to a lesser extent glutamine, presumably for maintaining membrane integrity (Lemon et al., 2010). Glutaminolysis refers to the catabolic process within cells whereby the amino acid glutamine is partially oxidized within the TCA cycle (McKeehan, 1982). Glutamine carbons may serve as energetic substrates for the TCA cycle, and in addition, may serve as a nitrogen sink or repository for excess ammonia which is toxic to cells (Nissim , 1999).

The S-phase of the cell cycle, a period during which a cell doubles its DNA content, is dependent upon the presence of glutamine for de novo nucleotide synthesis (DeBerardinis et al., 2007). Glutamine is the substrate for five enzymes involved in the de novo synthesis of both purines and pyrimidines with the rate-limiting step of de novo purine synthesis being catalyzed by 5'-phosphoribosyl-1-pyrophosphate (PRPP) aminotransferase (Cory et al., 2006). Rates of DNA and protein synthesis in human solid tumor cell lines have been shown to be correlated to glutamine concentrations in culture media (Wasa et al., 1996).

Glutaminolysis requires the activity of glutaminase, an amidohydrolase that catalyzes the reaction where glutamine generates glutamate. Two unique glutaminase isoforms have been characterized, GLS1 and GLS2, and each isoform displays tissue specificity (Mates et al., 2013). GLS1, or kidney-type glutaminase, is a phosphate-dependent isoform expressed in periportal hepatocytes and epithelial cells of renal tubules (Mates et al., 2013). GAC is a splice variant of the kidney-type glutaminase and is localized in the mitochondria and associated with tumorigenesis (Cassago et al., 2012). GLS2, or liver-type glutaminase, is a phosphate-independent isoform found in hepatocytes and within the cellular nuclei of neurons within the central nervous system (Mates et al., 2013).

Tumor cells that require glutamine for survival may be described as being glutamine "addicted" (Wise et al., 2008). Previous research findings have shown that glutamine addicted cells are unable to withstand glutamine deprivation and will undergo apoptosis when glutamine is depleted (Yuneva et al., 2007). The ability of cells to synthesize glutamine as opposed to securing the amino acid from extracellular sources requires the activity glutamine synthetase. Reports show that some cancer cell lines with β-catenin mutations in Wnt signaling pathway are able to survive glutamine deprivation by transcriptional activation of glutamine synthetase, an enzyme that catalyzes the amination reaction of glutamate with ammonia to form glutamine (Cadoret et al., 2002). Therefore, mechanisms that may regulate glutaminolysis are of considerable interest in cancer biology.

PPAR nuclear receptors

PPARs, peroxisome proliferation-activated receptors, are nuclear hormone receptors that serve as transcriptional activators. PPARs are bound by ligand, naturally occurring or synthetic: naturally occurring ligands include fatty acids and eicosanoids and synthetic ligands include thiazolidinediones for PPARγ and fibrates for PPARα (Lee et al., 2003). Once PPARγ has bound ligand, as series of activating events ensue including: releasing of co-repressor molecules, PPAR dimerizing with the RXR receptor activated by its ligand 9-cis retinoic acid, and binding of the PPAR:RXR complex to PPAR response elements (PPREs) within promoters of targeted genes followed by transcriptional activation (IJpenberg et al.,1997, Schoonjans et al., 1996). Three isotypes of PPARs have been identified: PPARα, PPARγ and PPARβ/δ and each displays tissue specific expression (Lee et al., 2003). PPARα was the first family member to be identified and cloned and was shown to be responsible for peroxisome proliferation in liver of rodents animals treated with known hepatocarcinogens as well as responsible for the hypolipaedemic effect of the carcinogens (Isseman et al.,1990). PPARγ activity is required for adipocyte differentiation and lipid storage while PPARα activity is associated with fatty acid oxidation, lipid metabolism, and amino acid metabolism (Rakhshandehroo et al., 2010 , Desvergene et al., 1999, Kersten at al., 2001).

Troglitazone

Troglitazone, a synthetic ligand that demonstrates high affinity binding to peroxisome proliferation- activated receptor gamma (PPARγ) and to a much lesser extent PPARα (Lehmann et al., 1995), was initially used for the treatment of Type II diabetes but was removed from the market in 2000 due to reports of idiosyncratic liver toxicity (Saha et al., 2010). Troglitazone is a first-generation thiazolidinedione (TZD) drug

(Hauner H., 2002). Troglitazone reportedly decreases insulin resistance resulting in reduced plasma glucose and insulin levels in Type II diabetic patients via a mechanism involving the up-regulation of GLUT1 and GLUT4 glucose transporter proteins in skeletal muscle cells and adipocytes (Hauner H., 2002) and down-regulating key gluconeogenic genes including PEPCK (Davies et sl., 2001).

Since troglitazone's removal from the drug market for treatment in Type II diabetes patients, researchers have examined a role for troglitazone in the inhibition of tumor cell growth and proliferation both in vivo and in vitro (Kubota et al., 1998). Antineoplastic studies using TZDs, specifically troglitazone, have revealed findings suggesting that TZDs exert their antitumorigenic properties through both PPAR-dependent and PPAR-independent mechanisms (Galli et al., 2006).

Synthetic PPAR antagonists have been developed for experimental purposes in addition to troglitazone and other synthetic agonists. This category includes GW9662, an irreversible suicide inhibitor of PPARγ (Leesnitzer et al., 2002). Although binding to PPARγ with the greatest affinity, GW9662 is capable of binding to PPARα (10 fold less affinity) and PPARβ/δ (600 fold less affinity) (Leesnitzer et al., 2002).

In addition to the reported effects in skeletal muscles and adipocytes, troglitazone has been shown to play a role in modulating glutamine metabolism in different cell types (Coates et al., 2002, Routh et al., 2002, Welbourne et al., 2004, Toturro et al., 2004, Friday et al., 2011, Oliver et al., 2008 and 2010). However, the mechanism of action of troglitazone on glutamine metabolism has not been thoroughly elucidated, including the possible role of PPARs. In addition, troglitazone has been shown to reduce the levels of

c-Myc in prostate cancer cells (Akinyeke et al., 2011) and c-Myc has been shown to play a crucial role in glutamine metabolism.

c-Myc Oncoprotein

The c-Myc oncoprotein serves as a transcription factor and has been demonstrated to regulate apoptosis, cell cycle, cell growth and metabolism and differentiation (Dang et al., 1999). Overexpression of c-Myc in cultured cells or animal transgenic models is associated with a deregulated cell cycle and neoplastic transformation (Gabay et al., 2014). Other oncogenic proteins such as Ras have been shown to stabilize the c-Myc protein (Sears et al., 2000). Downstream effectors of Ras signaling including two protein kinases, ERK and GSK3β, are notable candidates for phosphorylating c-Myc at key conserved residues including the activating phosphorylation event of c-Myc at the serine 62 position within the amino-terminus of the c-Myc protein by the predicted ERK kinase which serves to stabilize the protein (Sears et al., 2000). Phosphorylation of c-Myc at the threonine 58 position within the amino-terminus of the c-Myc protein by GSK3β serves to destabilize the c-Myc protein (Gregory et al., 2003). The sequential and differential phosphorylation of these key conserved sites results in degradation of the c-Myc protein through the proteasome-ubiquitin pathway (Gregory et al., 2003).

Given the described role for c-Myc in neoplastic transformation, researchers have focused on elucidating c-Myc's role in regulating tumor cell metabolism (Gabay et al., 2014). C-Myc has recently been shown to play a role in regulating key enzymes and proteins in the glutaminolytic pathway, namely glutaminase (GLS1) (Gao et al., 2009) and the glutamine transporter, ASCT2 (Wise et al., 2008). By down regulating

miR23a/b, a micro RNA that negatively regulates the GLS1 mRNA, c-Myc post-transcriptionally regulates this key enzyme (Gao et al., 2009). Upstream of GLS1 activation, the level of glutamine transporter expression serves as a control point for the glutaminolytic process (Fuchs et al., 2005). Glutamine transporters previously shown to be up-regulated in human cancer cell lines include the neutral -amino acid transporter protein, Slc1A5 (ASCT2) and Slc38A1 (Bode et al., 1995), and c-Myc serves as a transcriptional activator for both genes (Wise et al., 2008).

In this project, I propose to determine the consequence of troglitazone treatment on glutamine metabolism and cell proliferation in glutamine addicted cancer cell types and define whether PPAR activity is required for troglitazone's effects. In addition, I seek to examine the role of c-Myc in potentially mediating troglitazone's alteration on glutaminolysis in three independent cell types.

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 et al, 2013). HeLa 299 and TKO MEF cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Rockford, IL, USA), 25 mM glucose, 4 mM glutamine and 50 μg/ml gentamicin sulfate and the H460 cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Rockford, IL, USA), 25 mM glucose, 4 mM glutamine and 50 μg/ml gentamicin sulfate, respectively. Cells were maintained at 5% CO_2 at 37 °C.

Glutamine uptake

TKO MEF, HeLa 229 and H460 cells were seeded at $8x10^4$ in 6-well plates. Cells were then treated with troglitazone (5-80uM) for 48 hours. Cells were then washed with phosphate-buffered saline and incubated for thirty minutes in glutamine-deficient media. Cells were then supplemented with 0.5 uCi [U-^{14}C]-glutamine (Perkin Elmer, Waltham, MA, USA) 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, Waltham, MA, USA). Counts were normalized to milligram protein as determined by BCA protein assay (Pierce, Rockford, IL, USA).

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, Danvers, MA, USA), GLS1 (Abnova, Taipei City, Taiwan), c-MYC (Cell Signaling, Danvers, MA USA), PPARγ (Santa Cruz, Santa Cruz, CA), PPARα (Santa Cruz, Santa Cruz, CA), and β-Actin (Pierce, Rockford, IL, USA). Protein detection was performed using HRP-conjugated secondary antibodies and ECL Select Western Detection Reagent (GE Life Sciences, Piscataway, NJ, USA).

Stable isotope resolved metabolomics analysis by NMR

H460 cells were seeded at 1×10^5 cells in 6-well plates. H460 cells were then treated for 24 hours with different concentrations of troglitazone(5-80uM) 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 [$^{13}C_5$,$^{15}N_2$]-glutamine (Perkin Elmer, Waltham, MA, USA). 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 were performed as previously described (Reynolds et al., 2013).

Cellular steady-state ATP levels

Exponentially growing TKO MEF, HeLa 229 and H460 cells were treated with different concentrations of troglitazone (5-80uM) for 48 hours. Cells were then lysed in a 5x passive lysis buffer and ATP concentrations were determined using the ATP Determination Kit following manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

Cellular ATP levels were normalized to milligram protein as determined by BCA protein assay (Pierce, Rockford, IL, USA).

Cell proliferation

To determine glutamine sensitivity, TKO MEF, HeLa 229 and H460 cells were seeded at $8x10^4$ in 6-well plates. At 24 hours post-seeding, media was replaced with either complete DMEM containing 10% FBS, 25 mM glucose, 4 mM glutamine and 50 μg/ml gentamicin sulfate or DMEM without glutamine containing 10% FBS, 25 mM glucose, 4 mM glutamine and 50 μg/ml gentamicin sulfate (RPMI 1640 in the case of H460 cells). Cell proliferation was assessed as viability using trypan blue exclusion and enumeration using a hemocytometer 48 hours after media change. For troglitazone, GW9662 and MG132 experiments, reagents were added 24 hours post- seeding and in the case of siRNA experiments, 24 hours post-transfection. Troglitazone (5-80μM), GW9662 (10μM) and MG132 (1mM) (Cayman Chemicals, Ann Arbor, MI, USA) were reconstituted in DMSO.

Cell transfections

siRNA transfections were performed as previously described (Reynolds et al, 2013) using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and the following siRNAs: PPARα: 5' -GGAAAGUCCCUUAUCUGAA- 3' (Ambion #s72003_Mm) and PPARα: 5' -GAUCAAGUGACAUUGCUAA- 3' (Ambion #s10880_Hs); PPARγ: 5' - GGGCGAUCUUGACAGGAAA- 3' (Ambion #s72013_Mm) and PPARγ: 5' -GACAAAUCACCAUUCGUU- 3' (Ambion #s10886_Hs) at a final concentration of 40 nM. siRNA species were specific for mouse (TKO MEF) or human

(HeLa 229, H460) cell lines. Cell viability, protein expression and glutamine uptake were analyzed at 48 hours post-transfection as described above.

Statistics

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

RESULTS

Mouse embryonic fibroblast deficient for Rb pocket proteins and several tumor cancer cell lines are glutamine-dependent

To investigate a role for troglitazone in the inhibition of glutaminolysis, we identified glutamine-dependent cell lines to be used for further experiments. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to treatment media for 24 hours and 48 hours. Cells were incubated in either complete medium (Glc + Gln) or medium depleted of glutamine (Glc-Gln), and viable cell number was determined using trypan blue staining and hemocytometer enumeration. Removal of glutamine from the media resulted in a significant decrease in cell viability in all three cell lines at 24 hours and 48 hours (**Figure 1**).

Incubation with troglitazone results in the dose-dependent inhibition of glutamine uptake

To determine troglitazone's effect on glutamine uptake, all three cell lines were treated with different concentrations of troglitazone (5uM - 80uM). After 24 hours and 48 hours, glutamine uptake was determined using ^{14}C-labeled glutamine. Treatment with troglitazone was found to significantly inhibit glutamine uptake in TKO MEF, HeLa 229 and H460 cells in a dose-responsive manner compared to cells in the vehicle-treated control group (**Figure 2**).

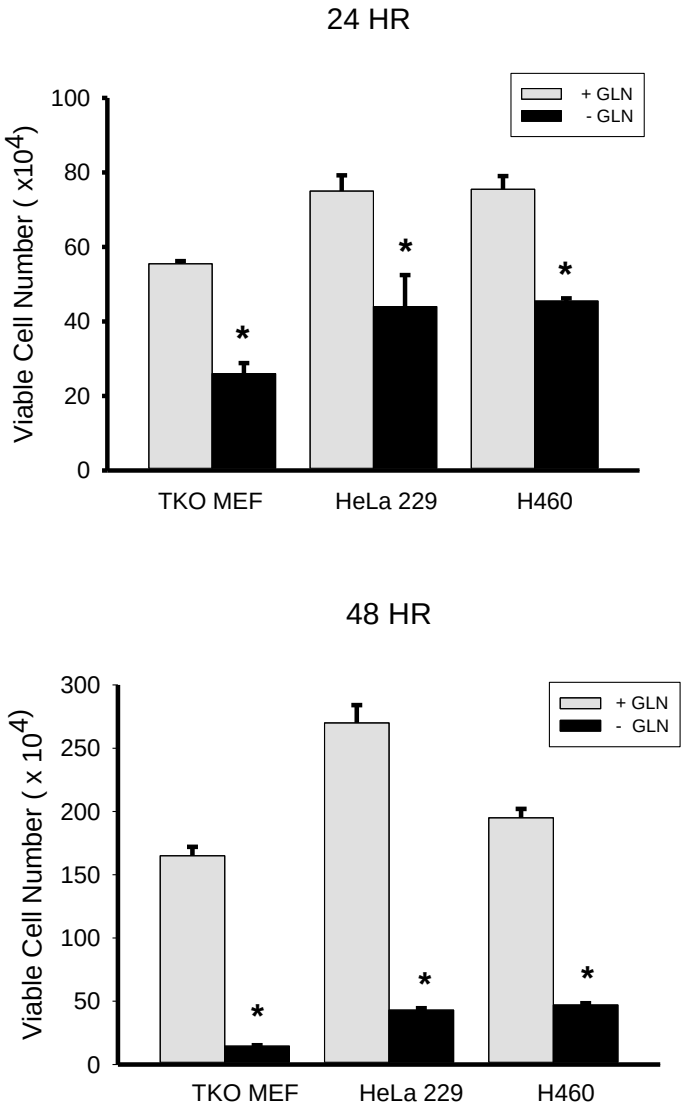


Figure 1 - Mouse embryonic fibroblast deficient for Rb pocket proteins and human tumor cancer cell lines are glutamine-dependent.

Figure 1: TKO MEF Rb pocket protein-deficient mouse embryonic fibroblasts, HeLa 229 human cervical cancer cells and H460 non-small cell human lung cancer cells are glutamine-dependent. Cells were plated at a density of 8.0 x 10^4 in 6-well culture plates and exposed to treatment media with or without glutamine for 24 hours and 48 hours. Cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Data is presented as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

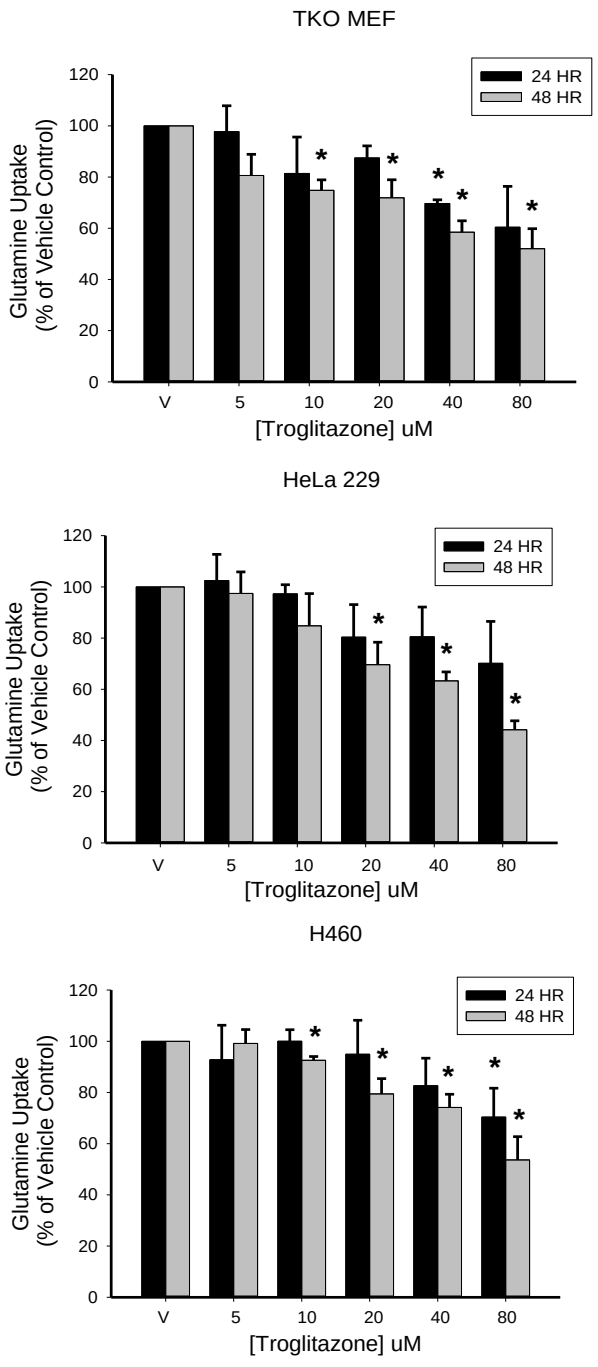


Figure 2 - Incubation with troglitazone results in the dose-dependent inhibition of glutamine uptake.

Figure 2: PPARγ agonist troglitazone inhibits glutamine uptake in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone for 24 hours and 48 hours. Glutamine uptake was determined using ^{14}C- labeled glutamine. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

Incubation with troglitazone results in the dose-dependent suppression of glutaminolytic proteins

Both ASCT2 and GLS1 have been demonstrated to be overexpressed in cancer cell lines and expression of these proteins is directly correlated with glutamine utilization in tumor cells. To determine if these glutaminolytic proteins may be contributing to the reduction in glutamine uptake, we examined ASCT2 and GLS1 expression in cells treated with different concentrations of troglitazone (5uM-80uM). Treatment with troglitazone was found to significantly suppress the expression of glutaminolytic proteins, ASCT2 and GLS1, in TKO MEF, HeLa 229 and H460 cells in a dose-responsive manner (**Figure 3**).

Incubation with troglitazone results in the dose-dependent suppression of steady-state ATP levels

Glutamine can supplement ATP production through TCA cycle anaplerosis. To examine whether diminished glutamine uptake in response to treatment would affect ATP levels, cells were incubated with different concentrations of troglitazone (5uM - 80uM). After 48 hours, cells were lysed and cellular ATP levels were determined using a firefly luciferase/luciferin bioluminescence assay. A significant dose-dependent decrease in steady-state ATP levels was observed at 40uM in all three cell lines (**Figure 4).** Although other metabolic processes may affect ATP levels within the cell, the decrease in ATP levels in these cell lines correlates with dose-dependent decreases in glutamine uptake (Figure 2).

Incubation with troglitazone results in reduced incorporation of glutamine carbon into the TCA cycle

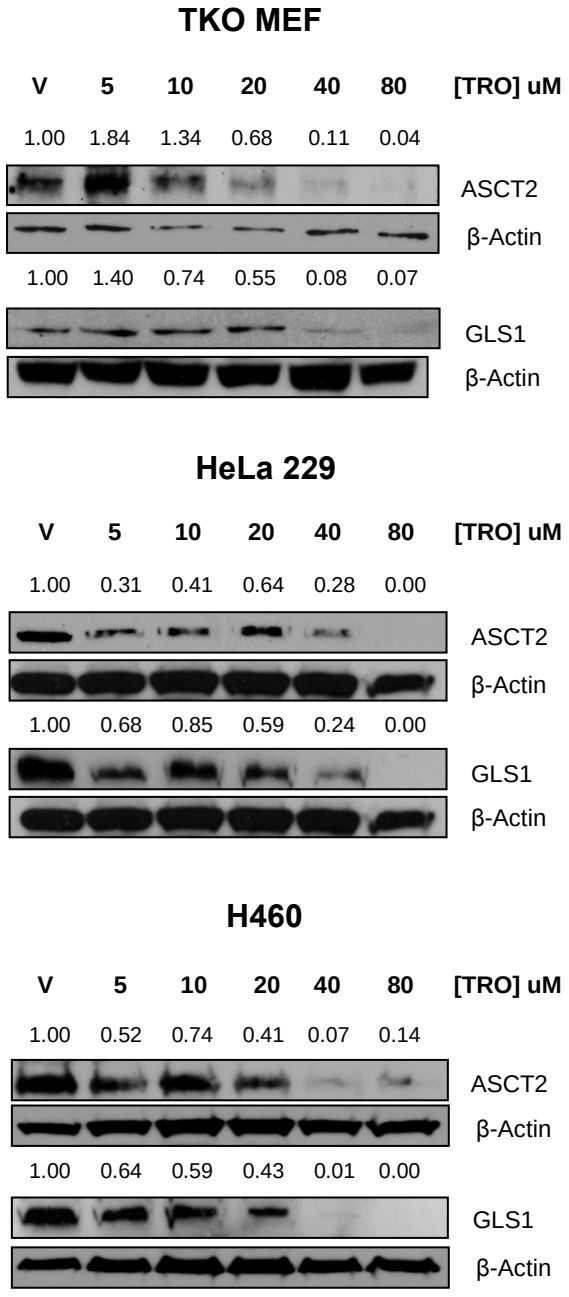


Figure 3 - Incubation with troglitazone results in the dose-dependent suppression of glutaminolytic proteins.

Figure 3: PPARγ agonist troglitazone suppresses glutaminolytic enzymes in TKO MEF, HeLa 229 and H460 cells. Cell lysates were obtained 48 hours after treatment with different concentrations of troglitazone ranging from 5uM-80uM. Protein expression for GLS1, ASCT2 and β-Actin were determined by Western blot analysis. Blots reveal a dose-dependent inhibition on levels of expression of glutaminolytic proteins, GLS1 and ASCT2, in response to troglitazone treatment in all three cell lines.

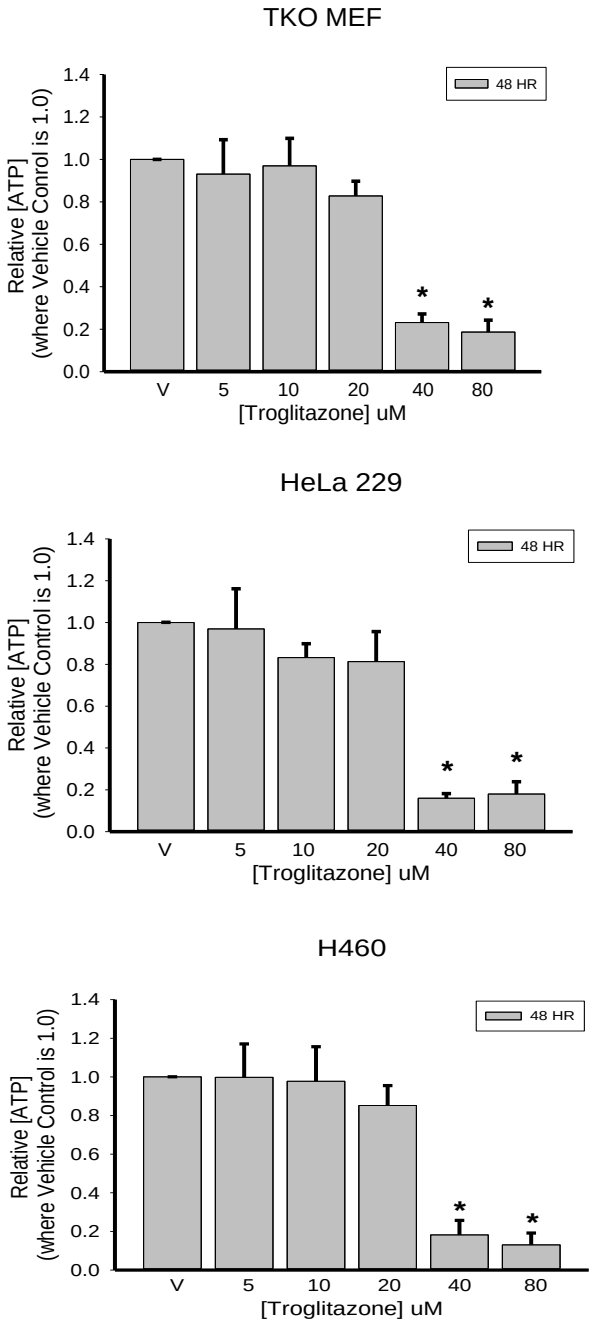


Figure 4 - Incubation with troglitazone results in the dose-dependent suppression of steady-state ATP levels.

Figure 4: PPARγ agonist troglitazone suppresses steady-state ATP levels in TKO MEF, HeLa and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to treatment media for 48 hours. ATP levels were determined using a luciferase reporter kit. Data is presented as relative ATP concentration where vehicle is 1.0, and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

To directly examine downstream glutamine metabolism, 2D-TOCSY NMR analysis using ^{13}C - labeled glutamine was employed to establish whether reduced glutamine uptake results in the concomitant reduction of glutamine anaplerosis into the TCA cycle. Glutamine carbons enter the TCA cycle after deamidation to glutamate followed by conversion to alpha-ketoglutarate. The H460 cell line was incubated with different concentrations of troglitazone (5uM-80uM). At 24 hours, cells were labeled with ^{13}C - glutamine for an additional 24 hours. Troglitazone treatment resulted in a significant dose-dependent decrease in labeled glutamine-derived carbon incorporation into aspartate which is directly labeled from the TCA cycle intermediate oxaloacetate (**Figure 5 A-B**).

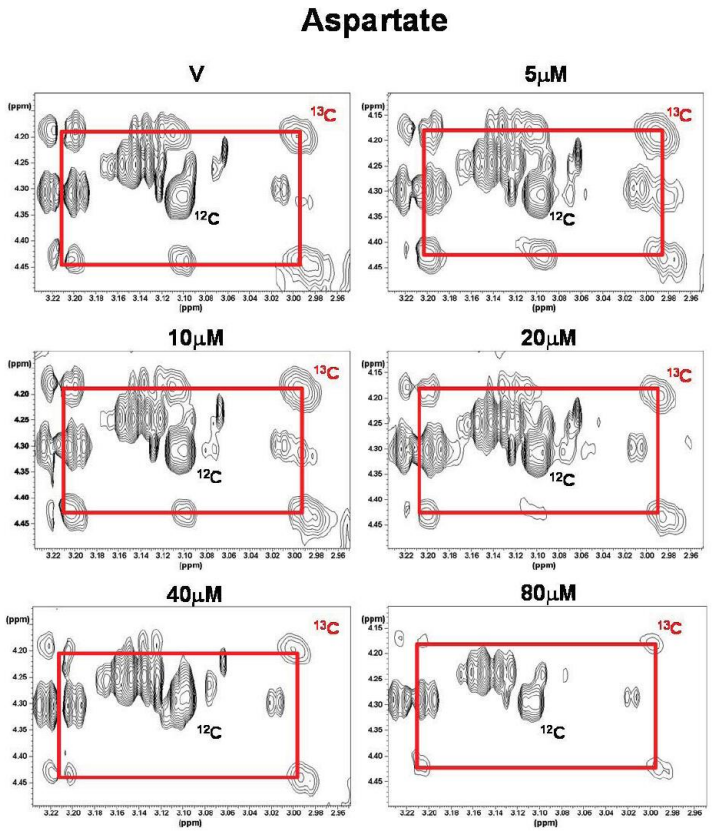
Incubation with troglitazone results in the dose-dependent inhibition of cell proliferation

To determine effects on cell proliferation, all three cell lines were treated with different concentrations of troglitazone (5uM - 80uM). After 24 hours and 48 hours, cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Treatment with troglitazone was found to inhibit cell proliferation in a dose-responsive manner in TKO MEF, HeLa 229 and H460 cells at 24 hours and 48 hours (**Figure 6**).

Incubation with siRNAs directed against PPARα and PPARγ results in the suppression of PPARα and PPARγ protein expression

To determine what role PPARs may be playing in troglitazone's effects on glutaminolysis and cell proliferation, I knocked down expression of PPARγ and PPARα using siRNA species directed against these targets (**Figure 7**). Western blot analysis

A



B

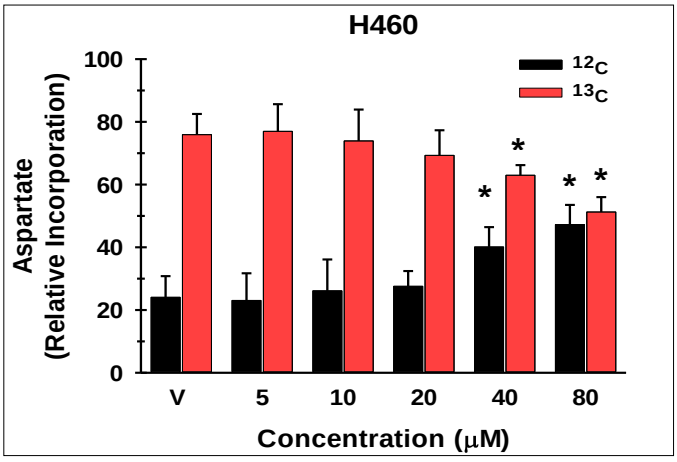


Figure 5A-B - Incubation with troglitazone results in the reduced incorporation of glutamine carbon into the TCA cycle.

Figure 5A-B: Incubation with troglitazone results in the reduced incorporation of glutamine carbons into the TCA cycle in H460 cells. Incorporation of glutamine carbon into aspartate was determined by stable isotope labeling with [$^{13}C_5$, $^{15}N_2$]-glutamine. Cells were treated with different concentrations of troglitazone for 48 hours. (A) Shown are the 2-D NMR spectra of TOSCY cross-peaks corresponding to aspartate C2-C3 and ^{13}C satellites. The pattern indicates a mixture of fully labeled aspartate via the first turn of the Krebs cycle, and aspartate that shows the second turn of the Krebs cycle after condensation of OAA with unlabeled acetyl CoA from unlabeled glucose. The boxes connect the ^{13}C satellite peaks of aspartate, consistent with the direct production of aspartate from glutamine. The central peak denoted ^{12}C is from aspartate that contains no ^{13}C. (B) Graphical representation of the fraction of ^{13}C-aspartate to ^{12}C-aspartate shows a dose-dependent decrease with increasing concentrations of troglitazone. Data are represented as relative incorporation where ^{12}C of ^{13}C is 100. Statistical significance is reported as * $p < 0.05$.

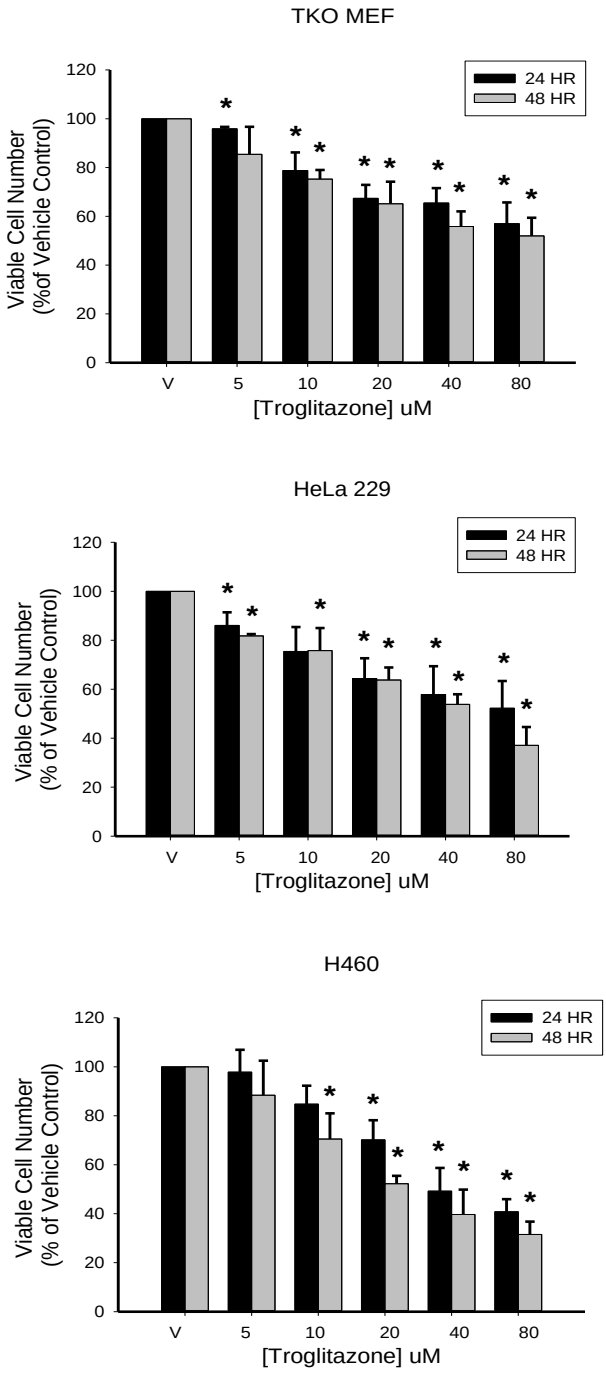


Figure 6 - Incubation with troglitazone results in the dose-dependent inhibition of cell proliferation.

Figure 6: PPARγ agonist troglitazone inhibits cell proliferation in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone for 24 hours and 48 hours. Cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

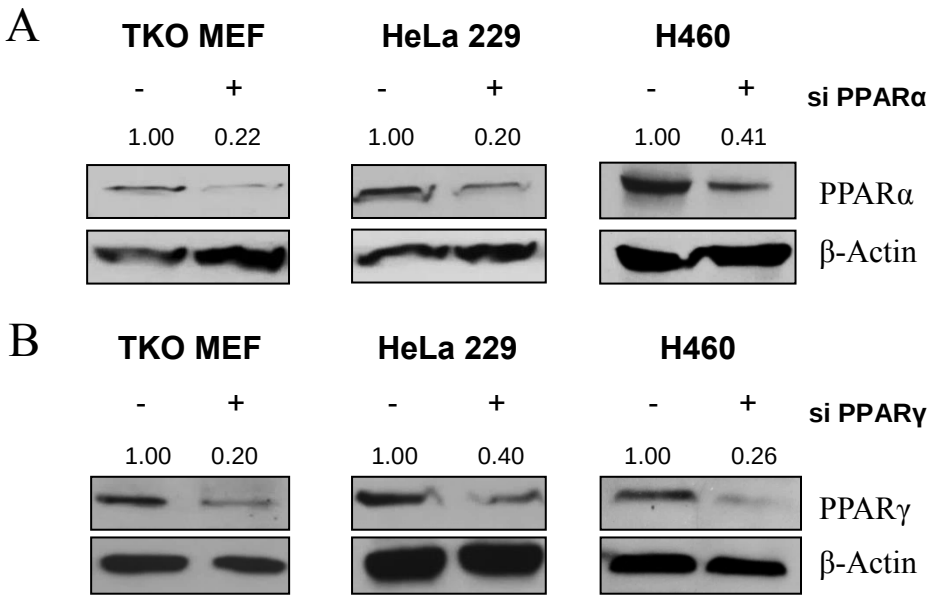


Figure 7A-B - Incubation with siRNAs directed against PPARα and PPARγ results in the suppression of PPARα and PPARγ protein expression.

Figure 7A-B: Incubation with siRNAs directed against PPARγ and PPARα results in the suppression of PPARγ and PPARα protein expression. TKO MEF, HeLa 229 and H460 cells were transfected with siRNAs directed against PPARα (A) and PPARγ (B) and protein expression for PPARα and PPARγ and β-Actin were determined by Western blot analysis.

revealed that expression of both PPARγ (**Figure 7B**) and PPARα (**Figure 7A**) were greatly diminished 48 hours post-transfection.

Incubation with siRNA directed against PPARγ in the presence of troglitazone fails to rescue glutamine uptake

Cells were transfected with siRNA directed against PPARγ (**Figure 7B**) and then treated with different concentrations of troglitazone (5uM - 80uM). After 48 hours, glutamine uptake was determined using ^{14}C -labeled glutamine. Knockdown of PPARγ in the presence of troglitazone failed to result in a significant rescue of glutamine uptake within any of the three cell lines (**Figure 8**).

Incubation with siRNA directed against PPARγ in the presence of troglitazone fails to rescue cell proliferation

Cells were transfected with siRNA directed against PPARγ (**Figure 7B**) and then treated with different concentrations of troglitazone (5uM - 80uM). After 48 hours, cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Knockdown of PPARγ in the presence of troglitazone failed to result in a significant rescue of cell proliferation within any of the three cell lines (**Figure 9**).

Incubation with siRNA directed against PPARα in the presence of troglitazone fails to rescue glutamine uptake

Cells were transfected with siRNA directed against PPARα (**Figure 7A**) and then treated with different concentrations of troglitazone (5uM - 80uM). After 48 hours, glutamine uptake was determined using ^{14}C -labeled glutamine. Knockdown of PPARα in the presence of troglitazone failed to result in a significant rescue of glutamine uptake within any of the three cell lines (**Figure 10**).

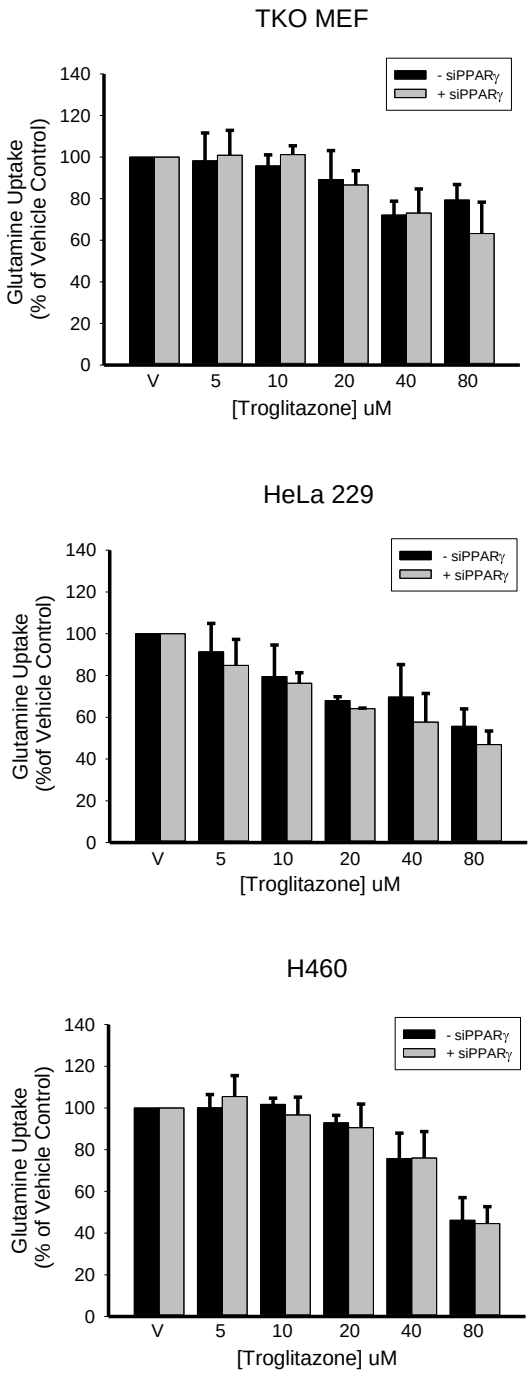


Figure 8 - Incubation with siRNA directed against PPARγ in the presence of troglitazone fails to rescue glutamine uptake.

Figure 8: Incubation with troglitazone and siRNA directed against PPARγ fails to rescue glutamine uptake in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without siRNA for 48 hours. Glutamine uptake was determined using ^{14}C- labeled glutamine. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

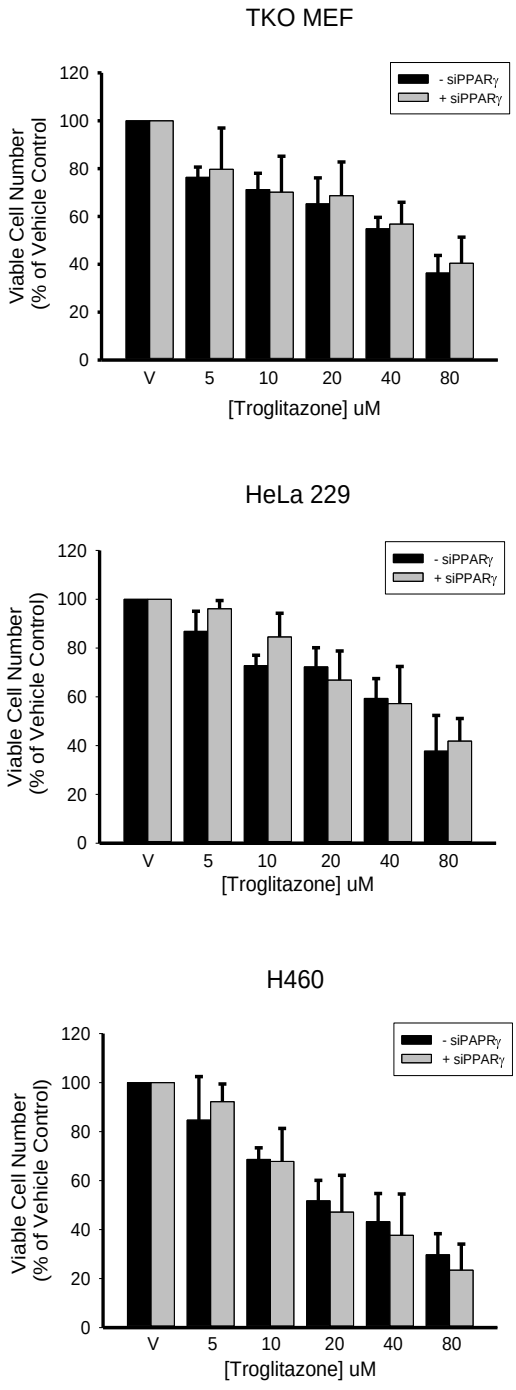


Figure 9 - Incubation with siRNA directed against PPARγ in the presence of troglitazone fails to rescue cell proliferation.

Figure 9: Incubation with troglitazone and siRNA directed against PPARγ fails to rescue cell proliferation in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without siRNA for 48 hours. Cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

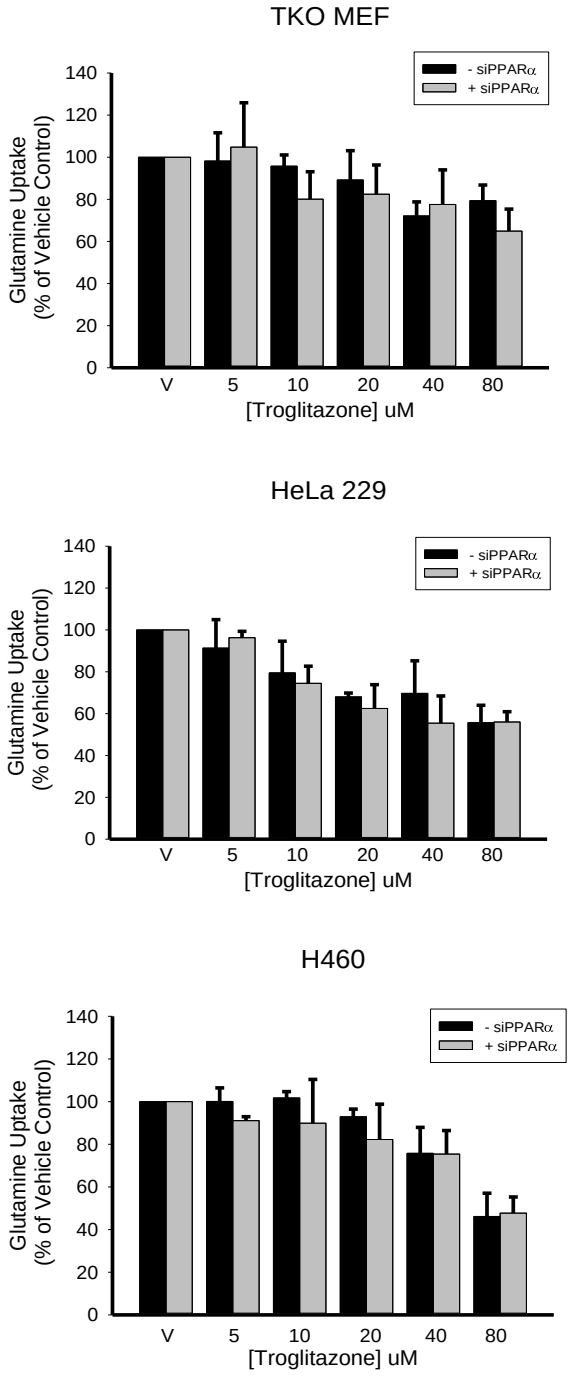


Figure 10 - Incubation with siRNA directed against PPARα in the presence of troglitazone fails to rescue glutamine uptake.

Figure 10: Incubation with troglitazone and siRNA directed against PPARα fails to rescue glutamine uptake in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without siRNA for 48 hours. Glutamine uptake was determined using ^{14}C- labeled glutamine. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

Incubation with siRNA directed against PPARα in the presence of troglitazone fails to rescue cell proliferation

Cells were transfected with siRNA directed against PPARα (**Figure 7A**) and then treated with increasing concentrations of troglitazone (5uM-80uM). After 48 hours, cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Knockdown of PPARα failed to demonstrate a significant rescue of cell proliferation (**Figure 11)**.

Incubation with PPARγ antagonist, GW9662, in the presence of troglitazone fails to rescue glutamine uptake

Cells were treated with GW9662, a PPARγ antagonist, at a concentration of 10uM and in the presence of increasing concentrations of troglitazone (5uM-80uM). After 48 hours, glutamine uptake was determined using ^{14}C-labeled glutamine. Inhibition of PPARγ by GW9662 failed to demonstrate a significant rescue of glutamine uptake within any of the three cell lines (**Figure 12**).

Incubation with PPARγ antagonist, GW9662, in the presence of troglitazone fails to rescue cell proliferation

Cells were treated with GW9662, a PPARγ antagonist, at a concentration of 10uM and in the presence of increasing concentrations of troglitazone (5uM-80uM). After 48 hours, cell proliferation was assessed by viability using trypan blue staining and hemocytometer enumeration. Inhibition of PPARγ by GW9662 failed to demonstrate a significant rescue of cell proliferation within any of the three cell lines (**Figure 13**).

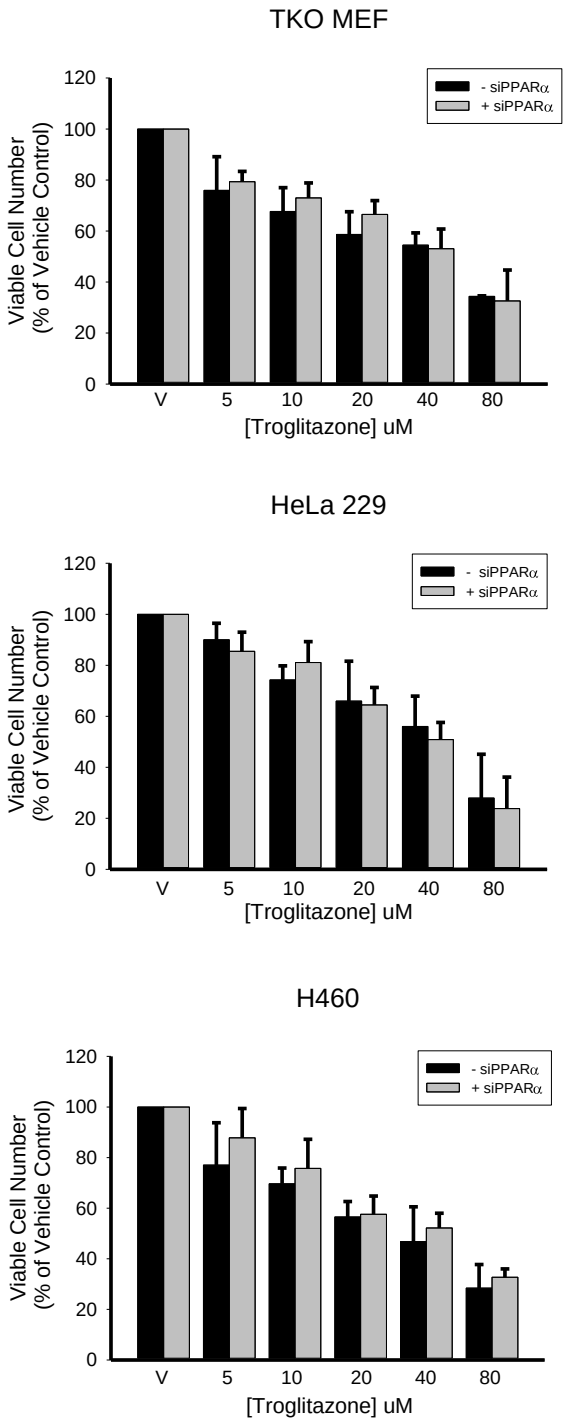


Figure 11 - Incubation with siRNA directed against PPARα in the presence of troglitazone fails to rescue cell proliferation.

Figure 11: Incubation with troglitazone and siRNA directed against PPARα fails to rescue cell proliferation in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without siRNA for 48 hours. Cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

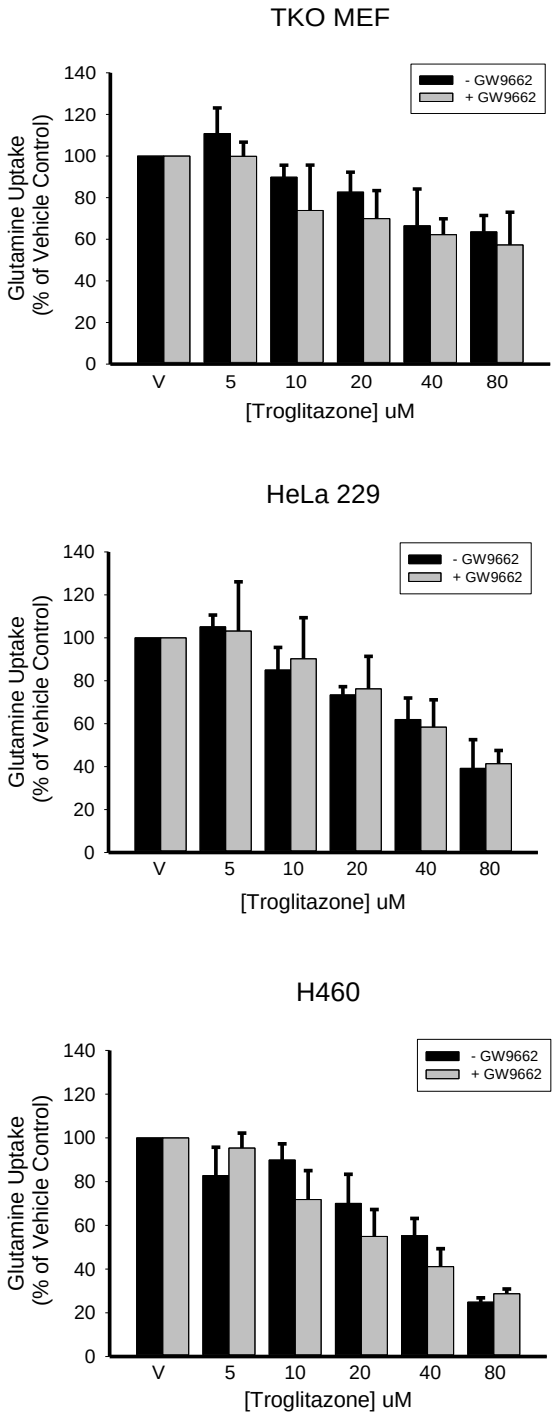


Figure 12 - Incubation with PPARγ antagonist, GW9662, in the presence of troglitazone fails to rescue glutamine uptake.

Figure 12: Incubation with troglitazone and PPARγ antagonist, GW9662, fails to rescue glutamine uptake in TKO MEF, HeLa 229 and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without GW9662 for 48 hours. After 48 hours, glutamine uptake was determined using ^{14}C-labeled glutamine. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

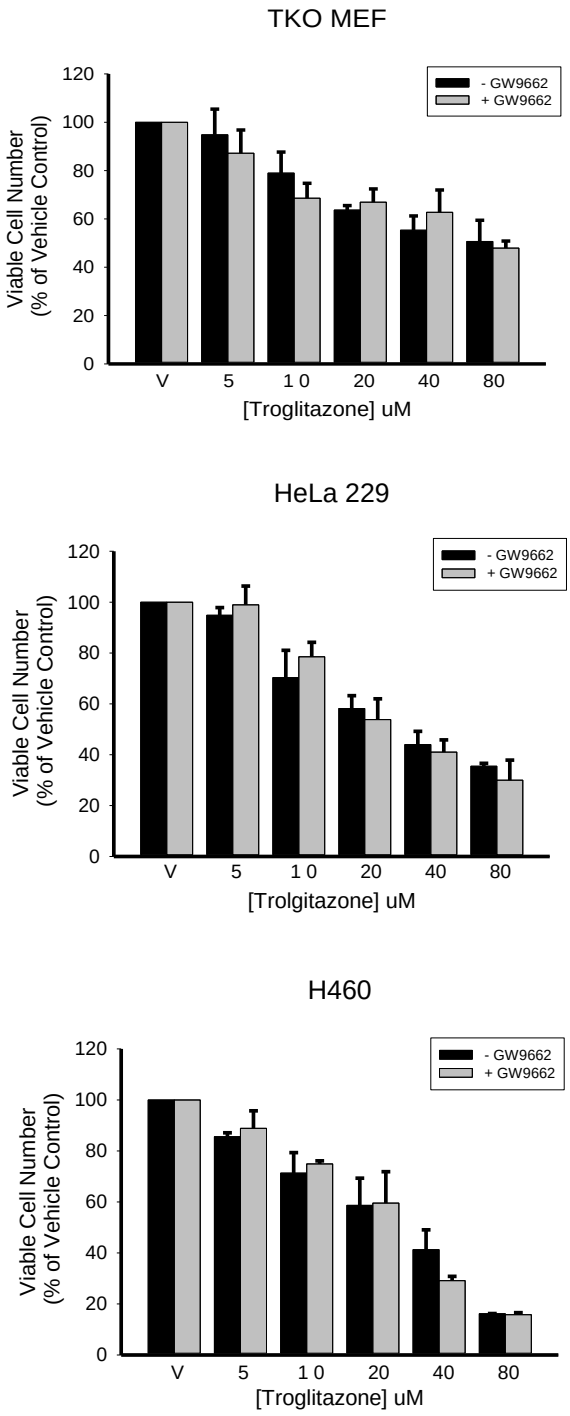


Figure 13 - Incubation with PPARγ antagonist, GW9662, in the presence of troglitazone fails to rescue cell proliferation.

Figure 13: Incubation with troglitazone and PPARγ antagonist, GW9662, fails to rescue proliferation in TKO MEF, HeLa and H460 cells. Cells were plated at a density of 8.0×10^4 in 6-well culture plates and exposed to different concentrations of troglitazone with or without GW9662 for 48 hours. Cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Data is presented as percent of vehicle and shown as the mean ± s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$.

Incubation with troglitazone results in the suppression of c-Myc protein expression

Given previous reports demonstrating a role for c-Myc in regulating glutaminolysis through direct or indirect control of both ASCT2 and GLS1, I investigated whether troglitazone would affect c-Myc expression. Cell extracts were obtained 48 hours after incubation using concentrations of 5uM-80uM of troglitazone. Protein expression for c-Myc was determined by Western blot using anti-c-Myc antibody, and a dose-dependent inhibition was observed in all three cell lines (**Figure 14**).

Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone rescues expression of the c-Myc protein

To determine whether the decrease in c-Myc levels is proteasomal mediated, H460 cell extracts were obtained 24 hours after troglitazone incubation using concentrations 5uM-80uM in the presence or absence of the proteasome inhibitor, MG132 (1mM). Protein expression for c-Myc was determined by Western blot using anti-c-Myc antibody, and co-treatment with MG132 was able to rescue c-Myc expression in H460 cells (**Figure 15**).

Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone results in the partial rescue of cell proliferation and glutamine uptake

To examine whether this rescue phenotype extends to glutamine uptake and cell proliferation, H460 cells were treated with different concentrations of troglitazone (5uM-80uM) with or without the proteasome inhibitor MG132 (1mM). Co-treatment with MG132in the presence of troglitazone results in a partial rescue of cell proliferation (**Figure 16A**). After 24 hours, glutamine uptake was determined using ^{14}C -labeled

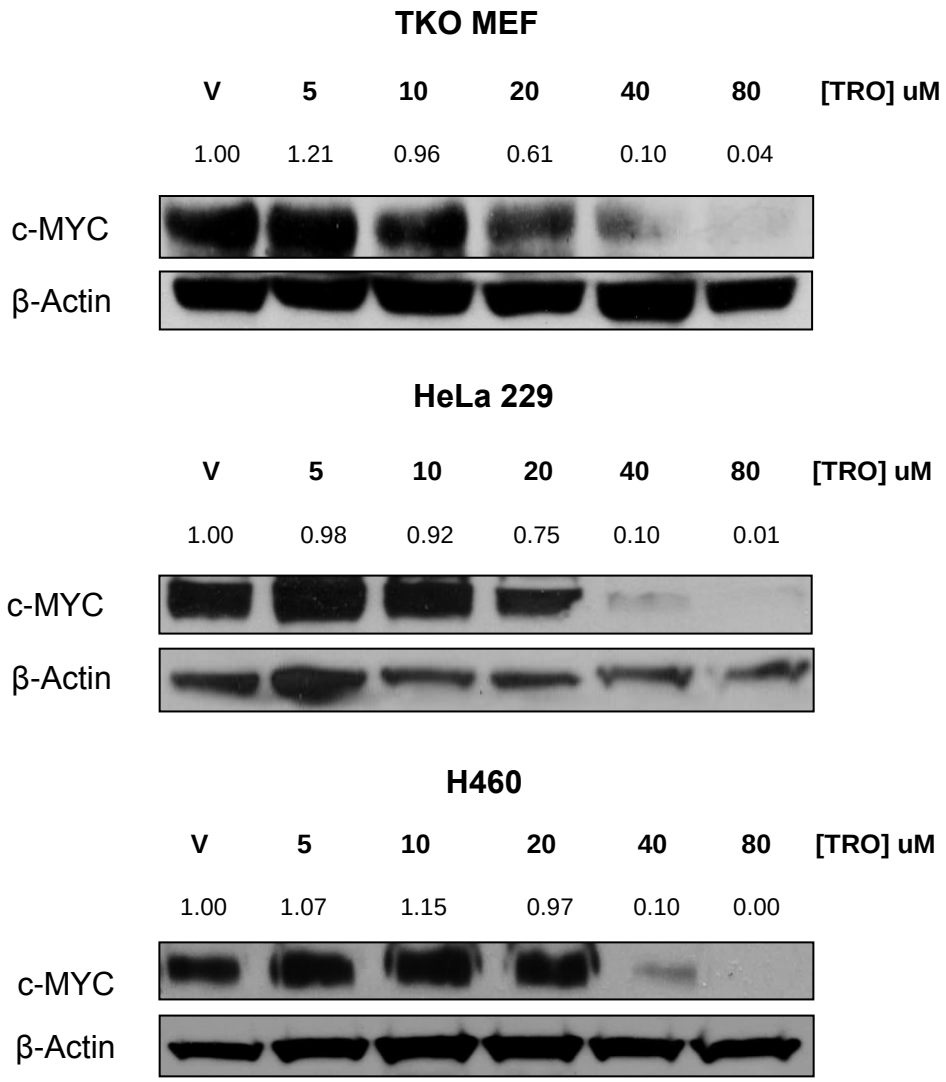


Figure 14 - Incubation with troglitazone results in the suppression of c-Myc protein expression.

Figure 14: Incubation with troglitazone results in the suppression of c-Myc protein expression in TKO MEF, HeLa 229 and H460 cells. Cell lysates were obtained 48 hours after treatment with different concentrations of troglitazone. Protein expression for c-Myc and β-Actin were determined by Western blot analysis. Blots reveal a dose-dependent inhibition on levels of expression of c-Myc in response to troglitazone treatment in all three cell lines.

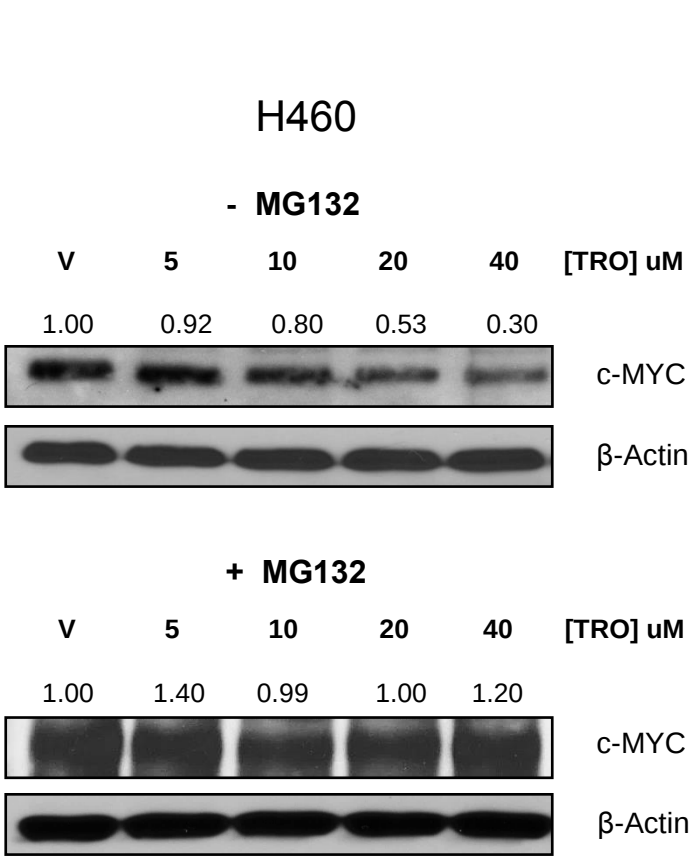


Figure 15 - Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone results in the rescue of c-Myc protein expression.

Figure 15: Incubation with MG132 in the presence of troglitazone prevents proteasomal degradation of the c-Myc protein at 24 hours. Protein expression for c-Myc protein and β-actin in H460 cells in response to different concentrations of troglitazone at 24 hours with or without the proteasome inhibitor, MG132, was determined by Western blot analysis. Shown is a representative image from two separate experiments. Rescue of the expression of the c-Myc protein was observed in response to troglitazone treatment with MG132 in the H460 cells.

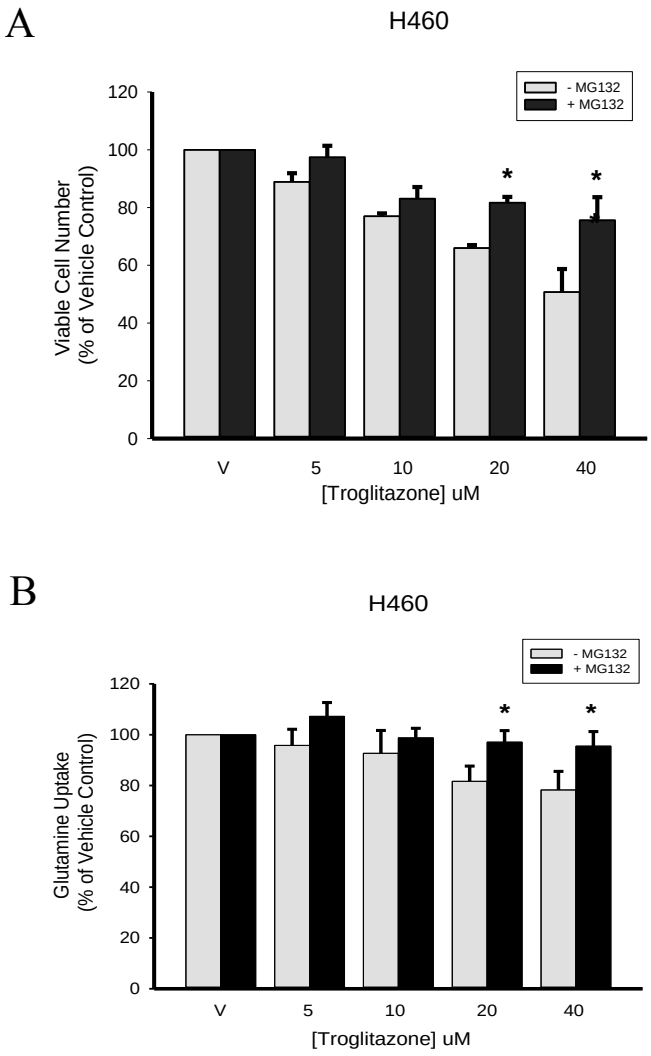


Figure 16 A-B - Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone results in the partial rescue of cell proliferation and glutamine uptake.

Figure 16 A-B: Incubation with MG132 in the presence of troglitazone in the H460 cells at 24 hours provides a partial rescue of cell proliferation and glutamine uptake. (A) After 24 hours of troglitazone treatment with or without MG132, cell proliferation in the H460 cells was assessed by counting viable cell number using trypan blue staining and hemocytometer enumeration. A partial rescue of cell proliferation with MG132 was observed at 20uM and 40uM. Data are represented as percent of vehicle. Mean $\pm$ s.d. from three independent experiments. Statistical significance is reported as * $p < 0.05$. (B) After 24 hours of troglitazone treatment with or without MG132, glutamine uptake in the H460 cells was assayed by ^{14}C-glutamine labeling. A partial rescue of glutamine uptake with MG132 was observed at 20 uM and 40uM. Data are represented as percent of vehicle. Mean $\pm$ s.d. from three independent experiments. Statistical significance is reported as * $p <$ 0.05

glutamine, and co-treatment with MG132 in the presence of troglitazone results in a partial rescue of glutamine uptake (**Figure 16B**).

Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone results in the partial rescue of glutaminolytic protein expression

Since MG132 was able to rescue c-Myc expression, I determined whether MG132 also rescued expression of c-Myc downstream targets, ASCT2 and GLS1. H460 cell extracts were obtained 24 hours after troglitazone incubation using concentrations 5uM-80uM in the presence or absence of the proteasome inhibitor, MG132 (1mM). Protein expression for ASCT2 and GLS1 was determined by Western blot, and co-treatment with MG132 was able to substantially rescue ASCT2 and GLS1 expression in H460 cells (**Figure 17**).

Overexpression of mutant c-Myc protein in the presence of troglitazone fails to rescue cell proliferation and glutamine uptake

Mutation of T58A in c-Myc has been reported to suppress proteasomal degradation. To determine if overexpression of the c-Myc mutant is able to rescue cellular processes from troglitazone treatment, H460 cells that stably express Sham or T58A mutant c-Myc were treated with different concentrations of troglitazone (5uM-80uM). After 48 hours, cell lysates were obtained, and protein expression for c-Myc was determined by Western blot using anti-c-Myc antibody. Overexpression of T58A mutant c-Myc was unable to rescue c-Myc expression in H460 cells (**Figure 18A**). In these same cells, after 48 hours, cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. Overexpression of T58A mutant c-Myc in the presence of troglitazone fails to result in the rescue of cell proliferation (**Figure 18B**).

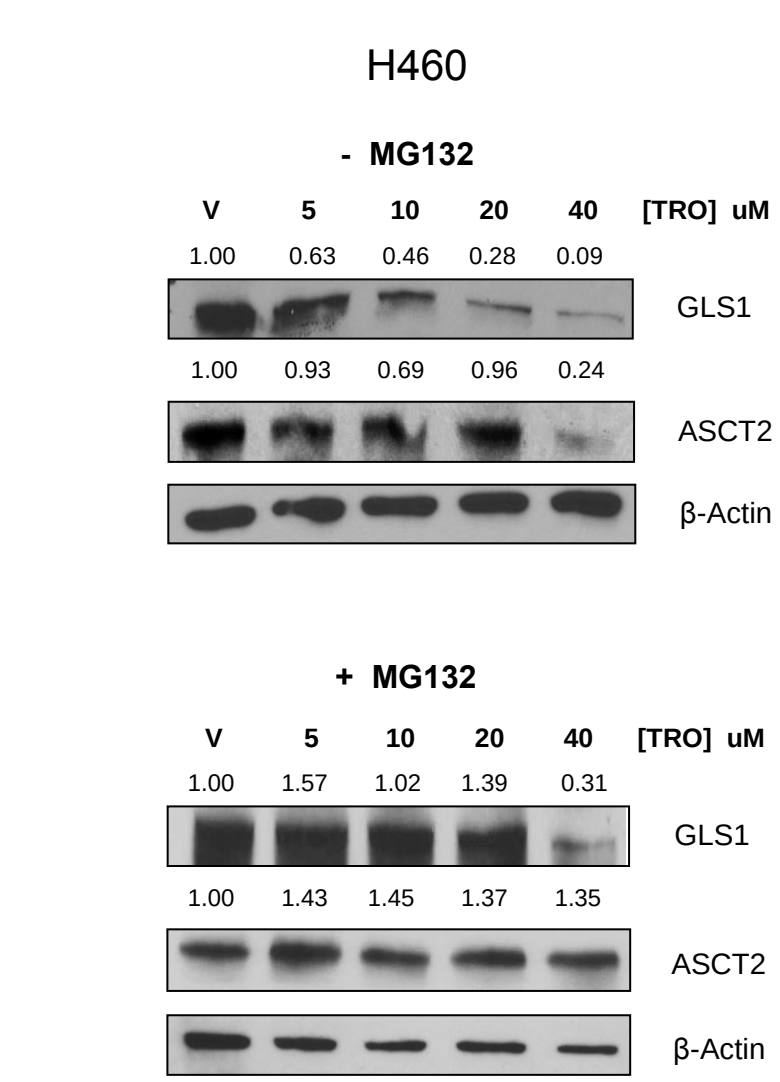
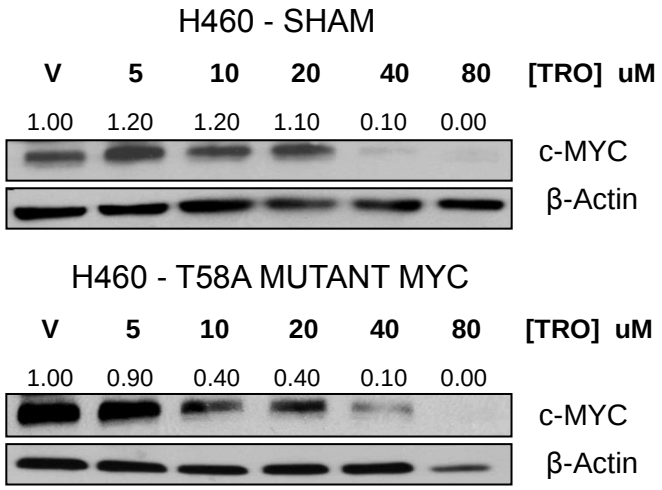


Figure 17 - Incubation with proteasomal inhibitor, MG132, in the presence of troglitazone results in the partial rescue of glutaminolytic protein expression.

Figure 17: Incubation with MG132 in the presence of troglitazone results in the partial rescue of expression of the glutaminolytic proteins, ASCT2 and GLS1. Protein expression for ASCT2, GLS1 and β-actin in H460 cells in response to different concentrations of troglitazone at 24 hours with or without the proteasome inhibitor, MG132, was determined by Western blot analysis. Shown is a representative image from two separate experiments. Partial rescue of the expression of the glutaminolytic proteins, ASCT2 and GLS1, was observed in response to troglitazone treatment with MG132 in the H460 cells.

A



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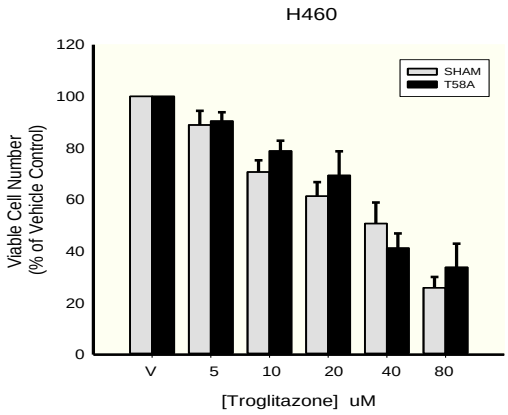

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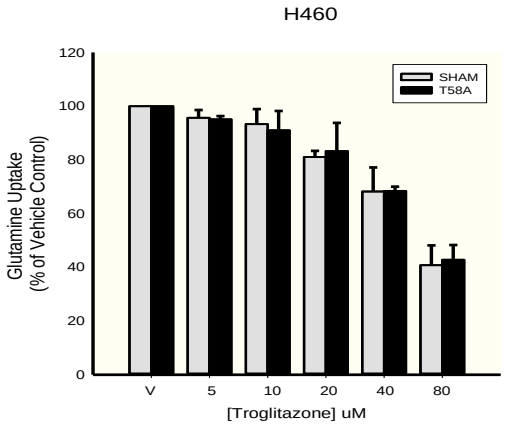

Figure 18 A-C - Overexpression of the T58A mutant c-Myc in the presence of troglitazone fails to rescue cell proliferation or glutamine uptake.

Figure 18 A-C: Overexpression of the T58A mutant c-Myc in the presence of troglitazone fails to rescue cell proliferation or glutamine uptake. (A) Protein expression for c-Myc and β-actin in H460 cells stably transfected with Sham or T58A mutant c-Myc in response to different concentrations of troglitazone at 48 hours was determined by Western blot analysis. Shown is a representative image from two separate experiments. Partial rescue of the expression of the c-Myc protein at 40uM troglitazone was observed in the presence of T58A mutant c-Myc. (B) In these same cells, after 48 hours of troglitazone treatment, cell proliferation was assessed by counting viable cell number using trypan blue staining and hemocytometer enumeration. No rescue of cell proliferation with T58A mutant c-Myc was observed. Data are represented as percent of vehicle. Mean $\pm$ s.d. from three independent experiments. (C) In these same cells, after 48 hours of troglitazone treatment, glutamine uptake was assayed by ^{14}C-glutamine labeling. No rescue of glutamine uptake with T58A mutant c-Myc was observed. Data are represented as percent of vehicle. Mean $\pm$ s.d. from three independent experiments.

Lastly, after 48 hours, glutamine uptake was determined using ^{14}C -labeled glutamine, and overexpression of T58A mutant c-Myc in the presence of troglitazone also fails to rescue glutamine uptake (**Figure 18C**).

Incubation with troglitazone and metformin results in a synergistic inhibition of cell proliferation

To examine whether troglitazone's inhibitory effect on cell proliferation could be augmented, we combined troglitazone with another therapeutic drug, metformin, which has been previously shown to sensitize cells to glutamine disruption. After 48 hour and 72 hour incubation with either troglitazone (10uM) or metformin (1mM), or a combination of troglitazone (10uM) plus metformin (1mM), cell proliferation was assessed as viability using trypan blue staining and hemocytometer enumeration. A statistically significant and synergistic inhibitory effect was observed for the combination troglitazone plus metformin treatment for both HeLa 229 cells at 48 hours and H460 cells at 48 hours and 72 hours **(Figure 19).**

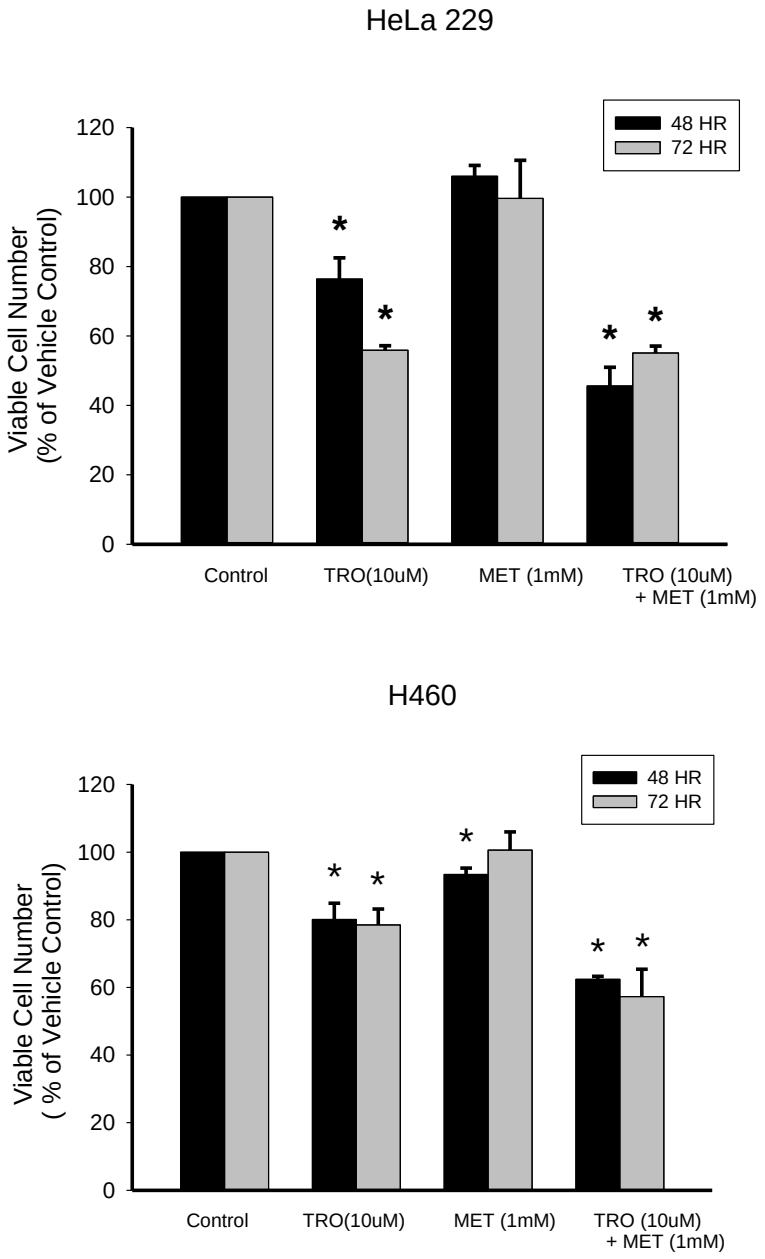

Figure 19 - Incubation with troglitazone and metformin results in an additive inhibitory effect on cell viability.

Figure 19: H460 and HeLa 229 cells were incubated with 10uM troglitazone or 1mM metformin or 10uM troglitazone with 1mM metformin for 48 hours and 72 hours. Cell proliferation in the cells was assessed by counting viable cell number using trypan blue staining and hemocytometer enumeration . Data are represented as percent of vehicle. Mean $\pm$ s.d. from three independent experiments. Statistical significance is represented as * $p < 0.05$. H460 cells at 48 hours and 72 hours showed statistically significant and synergistic inhibition of cell proliferation with the combinatorial troglitazone and metformin treatment while the HeLa 229 cells at 48 hours showed statistically significant and synergistic inhibition of cell proliferation with the combinatorial troglitazone and metformin treatment.

DISCUSSION

The goal of this research project was to determine the mechanism by which troglitazone regulates glutamine metabolism and cell proliferation in select neoplastic cell types.

Troglitazone inhibits glutaminolysis

I began this project by examining the effects of troglitazone on three cell lines deemed to be dependent on glutamine for survival, TKO MEF, HeLa 229, and H460 (Figure 1). Troglitazone, a previously FDA approved drug for the treatment of Type II diabetes, has been shown to possess anti-tumorigenic properties (Colmers et al., 2012). I have shown through a series of experiments that troglitazone treatment in glutamine-dependent cell lines resulted in diminished glutaminolysis as evidenced by the inhibited expression of key glutaminolytic proteins, GLS1 and ASCT2 (Figure 3), glutamine uptake (Figure 2) and reduced steady-state ATP levels (Figure 4). Using the H460 cell line, glutamine carbon flux into the TCA cycle as measured by NMR analysis using ^{13}C-labeled glutamine was shown to be suppressed with increased troglitazone concentrations (Figure 5A-B). In addition, diminished glutaminolysis was accompanied by a concomitant inhibition of cell proliferation (Figure 6).

Robust inhibition of the glutamine transporter, ASCT2, was evident at 40uM troglitazone in all three cell lines at 48 hours (Figure 3). Corresponding values reported for glutamine uptake were approximately 60% to 70% of those values reported for

respective controls (Figure 2) suggesting that alternative glutamine transporters are active. The expression of the Slc38A2 (SNAT2), a sodium-coupled neutral amino acid transporter, has been shown to be regulated transcriptionally by activating protein complexes bound to amino acid response elements within the SNAT2 promoter during amino acid starvation (Palii et al., 2006). HeLa cells have been shown to be able to up-regulate SNAT2 in response to amino acid deprivation (Gaccioli et al., 2006).

Previously, researchers have shown that troglitazone is capable of modulating glutamine metabolism by generating cellular acidosis which in turn regulates anaplerosis via the driving of glutamate flux through glutamate dehydrogenase (GDH), a pH-dependent enzyme located within the mitochondrial matrix, as opposed to the routing of glutamate through alanine aminotransferase (ALT) (Coates et al., 2002, Routh et al., 2002, Welbourne et al., 2004, Toturro et al., 2004, Friday et al., 2011, Oliver et al., 2008 and 2010). Troglitazone's effect on modulating glutamate flux occurs in response to troglitazone's ability to inhibit the sodium-dependent proton exchanger, NHE1, thus preventing extrusion of protons into the extracellular space, causing the shift towards a more acidic cytosolic pHi (Coates et al., 2002, Routh et al., 2002, Welbourne et al., 2004, Toturro et al., 2004, Friday et al., 2011, Oliver et al., 2008 and 2010). This cytosolic acidification favors the activation of GDH which is a pH sensitive enzyme (Moreadith et al., 1984). The deamination reaction of glutamate by GDH produces alpha-ketoglutarate (a-KG), an intermediary metabolite for the TCA cycle, which, in turn, enhances the production of GTP. Ultimately, the GTP produced serves as a potent allosteric inhibitor of GDH (Fang et al., 2002). A role for the NHE1 in cancer development and progression has been a topic of debate for years (Reshken et al., 2000).

Researchers looking at growth factor signaling and breast cancer cell growth in the MCF-7 cell line showed that troglitazone's inhibitory effect on breast cancer cell growth as it relates to DNA synthesis was associated with NHE1 inhibition and cellular acidosis and not disruption of the growth factor signaling pathway (Friday et al., 2011)

Flux away from ALT and towards GDH may be associated with a reduction in pyruvate levels as well as a reduction in cytosolic pH. Pyruvate levels diminished as a result of reduced glycolysis and enhanced flux of glutamine through the GDH pathway into the TCA cycle has been documented to occur in various tumor cell lines (Yang et al., 2009). A role for the oncoprotein c-Myc within this metabolic profile may be evident since c-Myc has been shown to transcriptionally regulate many proteins involved in glycolysis including hexokinase II (HK2) and the glucose transporter GLUT1 (Dang et al., 2009). In the SNB-19 glioma cell line, binding of the predominant hexokinase isoform to mitochondrial membranes is enhanced under alkaline cytosolic pH (Miccoli et al., 1996) and hexokinase association with the mitochondrial membrane has long been argued to be a primary marker for a tumorigenic phenotype (Ros et al., 2013, Nakashima et al., 1986, Pederson et al., 2002). Troglitazone treatment resulting in altered cytosolic pH through NHE1 inhibition may be suppressing hexokinase association with the mitochondrial membrane.

C-Myc has been shown to regulate the expression of GLS1 and ASCT2 (Yuneva et al., 2007, Wise et al. 2008), thus I examined c-Myc expression in the glutamine-dependent cell lines in response to troglitazone treatment. In previous reports, researchers using prostate cancer cell lines showed that troglitazone treatment resulted in reduced c-Myc expression (Akinyeke et al., 2011). Expectedly, I observed that

troglitazone treatment resulted in diminished expression of c-Myc protein in the three cell lines (Figure 14). When H460 cells were treated with troglitazone in the presence of the proteasome inhibitor, MG132, c-Myc protein expression was rescued (Figure 15). Cell proliferation (Figure 16A), glutamine uptake (Figure 16B) and glutaminolytic protein expression (Figure 17) were partially rescued suggesting that troglitazone's inhibitory effect on glutaminolysis within the three cell lines is regulated, in part, through a proteasomal-mediated process. To firmly establish a role for c-Myc in troglitazone's inhibitory effect on glutaminolysis, I used a lentiviral delivery system to overexpress c-Myc in the H460 cell line (Figure 18A). The c-Myc mutant, referred to as T58A c-Myc, codes for an alanine at the threonine 58 site thus rendering the c-Myc protein resistant to inhibitory phosphorylation at this residue (Gupta et al., 1993). However, I was unable to rescue either cell proliferation (Figure 18B) or glutamine uptake (Figure 18C), suggesting that other potential mechanisms regulate c-Myc expression in response to troglitazone in these cell types.

The observation that certain types of tumor cells preferred glutamine as an energetic substrate over other amino acids and even glucose has been documented (Eagle et al., 1955, Reitzer et al., 1979). Troglitazone treatment at concentrations of 40uM and 80uM significantly inhibited steady-state ATP levels in all three cell lines (Figure 4). However, a limited inhibitory effect on glutamine uptake in the H460 cells at these same concentrations was evident (Figure 2) indicating that there are excess moles of ATP produced per mole of glutamine metabolized, or troglitazone treatment in the H460 cells may be causing a simultaneous inhibitory effect on another energy producing metabolic pathway such as the glycolytic pathway. Previously, H460 cells have also been described

as possessing a glycolytic phenotype (Amoedo et al., 2011). Interestingly, 40uM troglitazone treatment in the H460 cells resulted in an altered cellular morphology, changing the cells from a more rounded and clustered appearance to a sparser and more elongated appearance (data not shown), demonstrating that the abrupt reduction in steady-state ATP levels coincides with the cellular rearrangement of cytoskeleton proteins. Other researchers using the H460 cell line have reported similar findings when cells were treated with the histone deacetylase (HDAC) inhibitor, sodium butyrate (Ameodo et al., 2011). Of note, troglitazone has been shown to inhibit HDAC activity in breast cancer cell lines leading to the inhibition of PI3/AKT signaling (Davies at al., 2010).

Troglitazone exerts effects through a PPAR-independent mechanism

Because troglitazone is a specific ligand for the nuclear hormone receptor PPARγ (Lehman et al., 1995), I needed to assess the possible role of PPARs in altering glutamine metabolism in our cell lines in response to troglitazone treatment. For this purpose, I elected to use siRNAs directed against PPARα and PPARγ targets (Figure 7A-B). PPARα is known to regulate enzymes involved in amino acid metabolism such as the aminotransferases ALT and AST (Kersten et al., 2001) and troglitazone may bind to PPARα though with a greatly reduced affinity than PPARγ (Lehamn et al., 1995). In addition, I utilized a potent irreversible PPARγ selective antagonist 2-chloro-5-nitrobenzanilide (GW9662) at a concentration of 10uM, a concentration that is predicted to be sufficient to antagonize PPARα which displays a lesser affinity for the antagonist. (Leesnitzer et al., 2002). The results obtained from these studies confirm that troglitazone's inhibitory effects on cell growth are not PPAR-dependent since knockdown of either PPARγ or PPARα concomitant with troglitazone treatment was unable to rescue

glutamine uptake (Figure 8, Figure 10) and cell proliferation (Figure 9, Figure 11). Additional evidence for a PPAR-independent mechanism was obtained using the PPARγ antagonist, GW9662. Again, treatment with troglitazone concomitant with GW9662 treatment failed to result in the rescue of cell proliferation (Figure 13) or glutamine uptake (Figure 12) in any of the three cell lines.

Troglitazone and metformin: synergistic effect

Previously, researchers have shown that the drug metformin is able to sensitize tumor cells to glutamine withdrawal (Fendt et al., 2013). Troglitazone treatment coupled with metformin treatment resulted in a synergistic inhibitory effect on cell proliferation in two cell lines (Figure 19). Cell proliferation at 48 hours and 72 hours with the combination drug treatment resulted in a synergistic inhibitory effect in the H460 cells. In addition, cell proliferation at 48 hours with the combination drug treatment also resulted in a synergistic inhibitory effect in the HeLa 229 cells. Previous research using troglitazone in combination with other drugs such as lovestatin resulted in synergistic inhibition of proliferation in cancer cells with the researchers citing that the inhibition occurred as a result of E2F1 protein inhibition by lovestatin and p27 protein (cell cycle inhibitor) accumulation in response to troglitazone (Yao et al., 2006) While the clinical safety profile of co-administration of metformin and troglitazone is currently being examined within Type II diabetic patients, our data lends support to the combinatorial repurposing of these compounds as additional anti-tumor strategies.

SUMMARY AND CONCLUSIONS

The search for agents capable of modulating the metabolic phenotypes of tumor cells is a focus of cancer research. Previously, FDA approved drugs for the treatment of Type II diabetes including the thiazolidinedione drug, troglitazone , have shown some promise in inhibiting proliferation of tumor cells *in vitro* and *in vivo* (Galli et al., 2006, He et al., 2006, Kubota et al., 1998). Furthermore, troglitazone has been shown to modulate glutaminolysis in certain cell lines (Routh et al., 2002, Coates et al., 2002, Welborne et al., 2004, Turturro et al., 2004, Friday et al., 2011, Oliver et al., 2008, Oliver et al,. 2010). Here, I reported that within glutamine -dependent transformed and tumor cell lines, including mouse embryonic fibroblasts depleted of RB pocket proteins (TKO MEF), human cervical adenocarcinoma cells (HeLa 229) and human non-small cell lung carcinoma cells (H460), troglitazone treatment inhibits glutamine uptake, glutaminolytic protein expression, steady-state ATP levels, and glutamine carbon flux into the TCA cycle, concomitantly with inhibiting proliferation. Taken together, these results indicate an overall suppression of glutaminolysis and cell proliferation by troglitazone.

Knockdown of the expression of PPARγ or PPARα by siRNA species or inhibition of PPAR activation by PPARγ antagonist, GW9662, failed to restore glutamine uptake and cell proliferation in cells that were co-treated with troglitazone. These results suggest that suppression of glutaminolysis and cell proliferation by troglitazone occurs through a PPAR-independent mechanism.

Transcription factors other than PPARs have been shown to play a role in the regulation of glutaminolytic enzymes. The transcription factor c-Myc is able to alter the cellular transcriptome and stimulate mitochondrial glutaminolysis, ultimately leading to glutamine dependency (Yuneva et al., 2007, Wise et al., 2008). The c-Myc protein has been shown to regulate the glutaminase (GLS1) protein expression through a post-transcriptional mechanism (Gao et al., 2009). Recently, researchers demonstrated that c-Myc expression was inhibited in prostate cancer cell lines treated with troglitazone (Akinyeke et al. ,2011). Results from this thesis project support Akinyeke's findings. Troglitazone treatment inhibited expression of c-Myc protein levels in a dose-dependent manner in three glutamine-dependent cell lines. Treatment of the H460 cell line with a proteasomal inhibitor, MG132, in conjunction with troglitazone resulted in the rescue of the c-Myc protein and partial rescue of cell proliferation and glutamine uptake, indicating troglitazone's inhibitory effect on cell growth and c-Myc expression are proteosomal-mediated. However, overexpression of a T58A mutant c-Myc, which is resistant to phosphorylation that triggers proteasomal degradation, was unable to rescue cell proliferation and glutamine uptake in H460 cells that were treated with troglitazone, suggesting that additional layers of regulation affect c-Myc stability in these cells.

The potential for drug synergy encourages the clinical use of drug combinations for the treatment of many diseases, including cancer. Metformin, a biguanide drug used for the treatment of Type II diabetes which gained FDA approval for use in the U.S.A. in 1995 (Wysowski et al., 2003) has shown promise as an anti-tumorigenic agent (Kourelis et al., 2012). Furthermore, metformin has been shown to sensitize cancer cells to glutamine disruption (Fendt et al., 2013). Using the glutamine-dependent cell lines, I

observed a synergistic inhibitory effect on cell proliferation with combined treatment of 10uM troglitazone and 1mM metformin, thus providing evidence for the possible repurposing of these agents, especially in combination with other targeted treatment strategies.

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