Troglitazone exerts metabolic and antitumor effects on T47D breast cancer cells by suppressing mitochondrial pyruvate availability

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Received March 27, 2019; Accepted November 15, 2019

DOI: 10.3892/or.2019.7436

Abstract. The aim of the present study was to investigate the metabolic and anticancer effects of troglitazone (TGZ) with a focus on the potential role of mitochondrial pyruvate utilization. 2-Deoxyglucose (2-DG) was more cytotoxic in CT26 cancer cells compared with T47D cells, despite a smaller suppression of glucose uptake. On the other hand, TGZ caused a more prominent shift to glycolytic metabolism and was more cytotoxic in T47D cells. Both effects of TGZ on T47D cells were dose-dependently reversed by addition of methyl pyruvate (mPyr), indicating suppression of mitochondrial pyruvate availability. Furthermore, UK5099, a specific mitochondrial pyruvate carrier inhibitor, closely simulated the metabolic and antitumor effects of TGZ and their reversal by mPyr. This was accompanied by a substantial reduction of activated p70S6K. In CT26 cells, UK5099 did not reduce activated p70S6K and only modestly decreased cell proliferation. In these cells, combining glutamine restriction with UK5099 further increased glucose uptake and completely suppressed cell proliferation. Thus, TGZ-mediated inhibition of mitochondrial pyruvate utilization is an effective treatment for cancer cells that are more dependent on mitochondrial glucose metabolism. By contrast, cancer cells that are more glycolysis-dependent may require suppression of glutamine utilization in addition to blocking mitochondrial pyruvate availability for a full antitumor effect.

Introduction

Cancer cells consume large amounts of nutrients to promote survival and drive tumor progression (1). Therefore, metabolic

reprogramming with substrate dependence represents an attractive target for cancer treatment (2). The most distinct metabolic cancer hallmark is a heightened dependence on glycolytic flux (2). This feature is widely exploited by positron-emission tomography imaging of tumors using 2-[¹⁸F]fluoro-2-deoxy-D-glucose (¹⁸F-FDG) (3). Furthermore, enhanced glycolysis of cancer cells can be therapeutically targeted by 2-deoxyglucose (2-DG). This glucose analogue is phosphorylated to 2-DG-6-phosphate, which competitively inhibits glucose-6-phosphate from entering the glycolytic pathway (4,5). Thus, cancer cells exposed to high 2-DG doses undergo growth arrest and apoptotic death. However, targeting of glycolysis with 2-DG has exhibited limited clinical efficacy (6), which likely reflects the metabolic plasticity of cancer cells.

When glycolytic flux is blocked, cancer cells can reciprocally increase oxidative metabolism to accrue sufficient energy and biomass for growth and survival (7). In addition to 2-DG, thiazolidinediones comprise a group of energy restriction mimetic agents that can target cancer metabolism (4,8). Recent studies support the repurposing of troglitazone (TGZ) as a promising thiazolidinedione for cancer therapy (9). Our group previously demonstrated that TGZ acutely impairs mitochondrial oxidative respiration in a peroxisome proliferator-activated receptor- γ -independent manner (10). The two pathways for glucose metabolism (i.e., mitochondrial oxidative phosphorylation vs. cytoplasmic lactate production) branch out from pyruvate. In a recent study, pioglitazone was observed to block cytosolic pyruvate from entering the mitochondria for oxidative phosphorylation in hepatocytes (11). Import of pyruvate across the inner mitochondrial membrane requires a transport system known as the mitochondrial pyruvate carrier (MPC). Cells stripped of MPC function have been reported to exhibit a profound suppression of glucose oxidation and cell growth (12). Thus, TGZ may be an alternative candidate to 2-DG in therapeutic targeting of cancer cells that are more dependent on mitochondrial rather than glycolytic glucose metabolism.

Another issue to consider is that depriving multiple rather than a single bioenergetic pathway may be required to achieve a sufficient cytotoxic effect in cancer cells with

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Key words: ¹⁸F-fluorodeoxyglucose, cancer, troglitazone, mitochondrial pyruvate carrier, glutamine

lower dependency on mitochondrial glucose metabolism. The principal substrate used by cancer cells deprived of glucose to fuel ATP production and biomass formation is glutamine. Indeed, some cancer cells obtain more than half of their ATP through oxidation of α -ketoglutarate derived from glutamine (13). Other cancer cells may become addicted to glutamine for survival (14). This suggests the potential usefulness of combining glutamine deprivation with inhibition of mitochondrial glucose metabolism for tumor treatment (15).

Successful targeting of tumor metabolism for imaging and treatment may emerge from a better understanding of how antimetabolic agents affect cancer cell glucose uptake and survival. The present study investigated the effects of TGZ on cancer cells with divergent sensitivity to 2-DG. T47D cells were investigated, as TGZ was previously observed to exert strong suppressive effects on the mitochondrial function and oxidative phosphorylation of these cells (10). CT26 cells were selected as a cancer cell type exhibiting low TGZ sensitivity based on pilot experiments. Furthermore, the role of mitochondrial pyruvate availability in these effects and the potential synergistic action of glutamine deprivation were further explored.

Materials and methods

Cell culture and materials. The T47D human breast, HT29 human colon and CT26 mouse colon cancer cell lines (American Type Cell Culture), the human colon cancer cell lines HCT15 and HCT116 and the human breast cancer cell line MDA-MB-231 (Korean Cell Line Bank) were maintained in RPMI-1640 medium (Gibco BRL; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Serana Europe GmbH), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin in 5% CO₂ at 37°C. The HT29 cell line was authenticated at our institution by short tandem repeat profiling. For T47D cells, experiments were performed in phenol red-free media supplemented with 5% charcoal-stripped serum to avoid the effects of trace amounts of estrogen.

Cells were treated for the indicated durations with 2-DG (Sigma-Aldrich; Merck KGaA), TGZ (Biorbyt), UK5099 (Santa Cruz Biotechnology, Inc.), and L-glutamic acid Y-(p-nitroanilide) hydrochloride (GPNA; Sigma-Aldrich; Merck KGaA) by addition to the culture media. mPyr (Santa Cruz Biotechnology, Inc.) was added to the culture media 4 h prior to treatment with TGZ or UK5099.

¹⁸F-FDG uptake measurement. Cells in 12-well plates treated for the indicated durations were incubated with 370 kBq ¹⁸F-FDG added to the media. Following incubation for 30 min in 5% CO₂ at 37°C, the cells were rapidly washed twice with cold phosphate-buffered saline (PBS) and lysed with 0.1 N sodium hydroxide. Cell-associated radioactivity was measured on a γ -counter (PerkinElmer, Inc.) and expressed as % uptake relative to the controls.

Lactate production assay. Culture media collected from cells was assayed for L-lactate concentration with a Cobas kit (Roche/Hitachi) in accordance with the manufacturer's instructions. This assay uses an enzymatic reaction that converts lactate

to pyruvate and hydrogen peroxide. The hydrogen peroxide then undergoes an enzymatic reaction to generate a colored dye that is measured by a Roche/Hitachi analyzer.

Sulforhodamine-B (SRB) assay. Cell survival and proliferation were measured using SRB assays. Briefly, cells were fixed with 10% (w/v) trichloroacetic acid and stained with SRB for 30 min. After appropriate dilution, unstained viable cells were counted using a counting chamber and hemocytometer. After excess dye was removed by repeated washing with 1% (v/v) acetic acid, protein-bound dye was dissolved in 10 mM of Tris base solution and measured for optical density at 510 nm on a microplate reader (Versa Max; Molecular Devices) (16). In a pilot experiment, SRB assays were performed to test the survival of several cancer cell lines in response to a 48-h exposure to TGZ. Other SRB experiments were performed after 48 or 72 h of treatment.

Immunoblotting of activated p70S6K. Cells were lysed with cold PRO-PREP lysis buffer (Intron Biotechnology, Inc.) with a proteinase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) and a phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.). A total of 10 μ g protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with 5% non-fat milk in Tris-buffered saline with 0.5% Tween-20 for 1 h at room temperature and incubated with a rabbit polyclonal antibody against phospho-p70S6K (9205S; 1:1,000 dilution; Cell Signaling Technology, Inc.) overnight. This was followed by incubation with HRP-conjugated anti-rabbit IgG (cat. no. 7074S; 1:2,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 1 h. Immune reactive proteins were detected with an enhanced chemiluminescence system and measured by a GS-800TM calibrated densitometer and Quantity One software 4.6.3 (Bio-Rad Laboratories, Inc.). After visualizing phosphorylated proteins, the membranes were subjected to a stripping procedure and were re-incubated with rabbit polyclonal antibody against total protein (cat. no. 9202S; 1:1,000 dilution; Cell Signaling Technology, Inc.).

Statistical analysis. All data are presented as mean $\pm$ standard deviation. The level of significance of the differences between groups was analyzed using a Student's t-test for two groups and analysis of variance with Tukey's post-hoc test for ≥ 3 groups. P-values < 0.05 were considered to indicate statistically significant differences.

Results

2-DG suppresses survival of CT26 cells more efficiently compared with T47D cells. In a pilot experiment, 48 h treatment with 40 μ M TGZ reduced the survival of HCT15 and HCT116 human colon cancer cells and MDA-MB-231 human breast cancer cells to 60.5 $\pm$ 5.0, 37.9 $\pm$ 4.8 and 76.8 $\pm$ 4.8% of controls, respectively (Fig. S1). HT29 and CT26 mouse colon cancer cells exhibited survival rates of 77.5 $\pm$ 7.5 and 94.2 $\pm$ 2.5% of controls, respectively, by the same treatment (Fig. S1). Based on these results, CT26 cells were selected as a cell type with low TGZ sensitivity. Treatment with 50 mM 2-DG for 1 h

Figure 1. Effects of 2-DG on cancer cell glucose uptake and survival. (A) ^{18}F -FDG uptake in T47D and CT26 cancer cells exposed for 1 h to 50 mM of 2-DG. (B) SRB assays showing survival of T47D and CT26 cells treated for 72 h with graded concentrations of 2-DG. Data are presented as mean $\pm$ standard deviation of % relative to untreated cells obtained from (A) three or (B) six samples per group. $^{\dagger}P < 0.0001$ compared to CT26 cells with the same treatment. ^{18}F -FDG, 2-[^{18}F]fluoro-2-deoxy-D-glucose; 2-DG, 2-deoxyglucose; SRB, sulforhodamine-B.

only modestly reduced ^{18}F -FDG uptake by CT26 cancer cells to $54.2 \pm 0.7\%$ of controls, whereas it completely suppressed ^{18}F -FDG uptake of T47D cancer cells to $2.0 \pm 0.1\%$ of controls (Fig. 1A). However, 2-DG suppressed the survival of CT26 cells more potently compared with T47D cells. Hence, 72 h of treatment with 64 mM 2-DG decreased the survival of the two cell types to 25.0 ± 0.8 and $52.6 \pm 2.6\%$ of controls, respectively (both $P < 0.0001$; Fig. 1B).

These results revealed a dissociation between 2-DG-mediated glycolysis inhibition, which was more efficient in T47D cells, and suppression of survival, which was more efficient in CT26 cells.

TGZ stimulates glucose uptake and suppresses survival more efficiently in T47D cells. Treatment with 10 μM TGZ for 1 h rapidly increased ^{18}F -FDG uptake of T47D cells to a significantly greater extent ($272.5 \pm 4.1\%$ of controls) compared with that of CT26 cells ($157.8 \pm 4.9\%$; $P < 0.001$; Fig. 2A).

The antitumor effect of TGZ on T47D cells was also more prominent compared with that on CT26 cells. Thus, the surviving fraction after 72 h of treatment with 80 μM TGZ was 37.7 ± 1.9 and $55.2 \pm 1.1\%$ of controls for the respective cell types (both $P < 0.001$; Fig. 2B).

The metabolic and cytotoxic effects of TGZ are completely reversed by methyl pyruvate (mPyr). The role of mitochondrial pyruvate on the metabolic and antitumor effects of TGZ on T47D and CT26 cells was next investigated. Cells were treated with mPyr, a pyruvate derivative that diffuses freely into the mitochondria independently of carrier-mediated transport. The results revealed that graded doses of mPyr completely abrogated the ability of TGZ to increase ^{18}F -FDG uptake of both T47D and CT26 cells (Fig. 3A and C). Increased lactate production of T47D cells to $150.5 \pm 7.3\%$ of controls by TGZ was also completely reversed by mPyr (Fig. 3A). Although increased lactate production by TGZ was less evident in CT26 cells, mPyr reduced lactate production in both TGZ-treated and control cells (Fig. 3C). Finally, while 40 μM of TGZ significantly reduced the survival of T47D cells to $38.7 \pm 0.7\%$ of controls, the same dose

of TGZ caused only a small reduction in CT26 cell survival. Furthermore, while mPyr completely rescued T47D cells from the anticancer effect of TGZ (Fig. 3B), it further reduced the survival of TGZ-treated CT26 cells (Fig. 3D). This signifies a lack of protective effect of mPyr in these cells.

The metabolic and antitumor effects of TGZ on T47D cells are closely simulated by UK5099. To verify whether blocking MPC function alone was sufficient to increase glucose uptake and decrease the survival of cancer cells, the specific MPC inhibitor UK5099 was used. In T47D cells, the results of UK5099 treatment closely matched those obtained by using TGZ. Hence, ^{18}F -FDG uptake was increased to $136.7 \pm 1.9\%$ of controls following 1 h of exposure to UK5099, and this effect was completely reversed by mPyr (Fig. 4A). Furthermore, treatment with 50 μM UK5099 markedly reduced p70S6K activation to $27.4 \pm 8.7\%$ of controls (Fig. 4B) and completely blocked cell proliferation (Fig. 4C).

Suppression of CT26 cell proliferation requires glutamine restriction combined with UK5099 treatment. Subsequently, the effects of UK5099 on CT26 cells that exhibited lower TGZ sensitivity were examined. The results demonstrated that 50 μM of UK5099 did not decrease p70S6K activation and only modestly suppressed the proliferation of CT26 cells (Fig. 5A).

Therefore, upon combining glutamine restriction with UK5099 treatment in these cells, ^{18}F -FDG uptake, which was modestly stimulated by 50 μM UK5099, was further increased by adding GPNA, a specific inhibitor of glutamine uptake through the alanine-serine-cysteine-preferring transporter 2 (ASCT2) (Fig. 5B). Furthermore, the ability of 50 μM UK5099 to suppress CT26 cell proliferation was significantly potentiated by either adding GPNA or depleting glutamine from the culture media (Fig. 5B).

Discussion

Tumor cells have characteristic metabolic requirements that may represent attractive targets for cancer therapy (1,2). In the

Figure 2. Effects of TGZ on cancer cell glucose uptake and survival. (A) ^{18}F -FDG uptake in T47D breast cancer cells and CT26 colon cancer cells exposed for 1 h to 10 μM of TGZ. (B) Survival of T47D and CT26 cells treated for 72 h with graded TGZ concentrations. Data are presented as mean $\pm$ standard deviation of % relative to untreated cells obtained from (A) three or (B) six samples per group. $^{\dagger}P < 0.001$ compared to CT26 cells with the same treatment. TGZ, troglitazone; ^{18}F -FDG, 2- ^{18}F fluoro-2-deoxy-D-glucose.

Figure 3. Effects of mPyr on metabolism and survival of T47D and CT26 cells exposed to TGZ. (A and C) ^{18}F -FDG uptake (left) and lactate production (right) after 1 h of treatment with 10 μM TGZ in the absence or presence of mPyr. Data are presented as mean $\pm$ standard deviation of triplicate samples expressed as % of controls. $^{\dagger}P < 0.001$; $^{\ddagger}P < 0.005$ compared to cells treated with TGZ without mPyr. (B and D) Survival of cells following 48 h of treatment with 0, 20 or 40 μM TGZ in the absence or presence of mPyr. Data are presented as mean $\pm$ standard deviation of quadruple samples expressed as % relative to controls. $^{\ddagger}P < 0.005$; $^{\dagger}P < 0.001$; $^{**}P < 0.01$ compared to cells treated with the same TGZ dose without mPyr. mPyr, methyl pyruvate; TGZ, troglitazone; ^{18}F -FDG, 2- ^{18}F fluoro-2-deoxy-D-glucose.

present study, it was observed that 2-DG inhibited glycolysis in T47D cells more prominently compared with CT26 cells, but its cytotoxic effect was potent in CT26 cells whereas it was only modest in T47D cells. This indicates that T47D cell survival is less dependent on glycolytic metabolism and suggests the possibility of a greater dependence on oxidative phosphorylation.

The selection of T47D cells for our experiments was based on our previous observation that oxidative phosphorylation in T47D cells is strongly suppressed by TGZ treatment (10). This was accompanied by reduced mitochondrial membrane potential

and increased reactive oxygen species generation, indicating a perturbation of mitochondrial function. The present study was performed as a follow-up to investigate the therapeutic potential of TGZ on these cells. CT26 cells were selected based on a pilot experiment, where various cancer cell lines were tested, and CT26 cells exhibited the smallest reduction in survival by TGZ treatment. Therefore, the CT26 cell line selected to investigate the reason why its response to TGZ differed from that of T47D cells. Although identification of a human cancer cell line with a similar response to TGZ as CT26 cells would have been of interest, this was not attempted in the present study.

Figure 4. MPC inhibition closely simulates the metabolic and antitumor effects of TGZ on T47D cells. (A) MPC inhibition with UK5099 stimulates ^{18}F -FDG uptake in a manner that is dose-dependently reversed by mPyr. Data are presented as mean $\pm$ standard deviation of triplicate samples expressed as % of controls. $^{\#}P < 0.001$ compared with cells treated with TGZ without mPyr. (B) Western blots of activated p70S6K and quantified band intensities corrected by total p70S6K protein. The composite image shows blots of p-p70S6K, t-p70S6K and β -actin protein. (C) Proliferation of cells with or without UK5099 treatment. Data are presented as mean $\pm$ standard deviation of six samples per group expressed as % of controls. $^{\#}P < 0.001$ compared with control cells at the same time point. MPC, mitochondrial pyruvate carrier; mPyr, methyl pyruvate; TGZ, troglitazone; ^{18}F -FDG, 2- ^{18}F fluoro-2-deoxy-D-glucose.

The present study demonstrated that TGZ treatment resulted in greater glucose uptake and cytotoxicity in T47D cells compared with CT26 cells. These findings support the hypothesis that T47D cells rely more on mitochondrial glucose metabolism for survival.

When T47D cells were treated with mPyr, the metabolic and antitumor effects of TGZ were completely reversed. mPyr is a form of pyruvate that freely enters the mitochondria without the need for carrier-mediated transport. A recent study using hepatocytes demonstrated that pioglitazone inhibits pyruvate import into the mitochondria (11). Pyruvate is a key metabolite at the major junction of carbohydrate metabolism between cytosolic glycolysis and the mitochondrial Krebs cycle (17). Transport of pyruvate through the inner mitochondrial membrane occurs through the recently identified MPC (12,17).

Given the importance of oxidative pyruvate metabolism for adequate ATP production and biomass conversion, suppressing mitochondrial pyruvate availability may underlie the antitumor effects exerted by TGZ. To compare the effects of blocking mitochondrial pyruvate import, T47D cells were treated with the specific MPC inhibitor UK5099. Similar to TGZ, UK5099 alone sufficiently augmented glucose uptake and glycolysis, and this metabolic effect was completely abrogated by mPyr.

Treatment with UK5099 alone also completely blocked T47D cell proliferation. These findings are reminiscent of a recent study wherein a specific MPC inhibitor reduced tumor cell oxidative glucose metabolism and extracellular lactate uptake, thereby leading to cytotoxicity (18). Taken together, the findings of the present study indicate that suppression of mitochondrial pyruvate availability is the major mechanism through which TGZ exerts metabolic and cytotoxic effects on T47D cells that exhibit a greater dependency on mitochondrial metabolism.

In comparison, CT26 cells, which exhibited a greater dependency on glycolysis and lower sensitivity to TGZ, displayed only a modest reduction of proliferation in response to UK5099. It was also observed that UK5099 suppressed the activation of p70S6K in T47D cells, but not in CT26 cells. This serine/threonine kinase is a downstream target of mechanistic target of rapamycin (mTOR) signaling that is used as a marker of activation of the mTOR pathway. Activation of p70S6K is important in mRNA translation that is required for cell cycle progression, proliferation, and survival pathways in tumors (6,19). A recent study demonstrated that the level of activated p70S6K can predict the response to glycolysis inhibition by identifying cancer cells that use mTOR signaling

Figure 5. Effects of MPC inhibition and glutamine (Gln) deprivation on CT26 cells. (A) Western blots of activated p70S6K and quantified band intensities corrected by total p70S6K protein. The composite image shows blots of p-p70S6K, t-p70S6K and β -actin protein. NS, non-significant. (B) Proliferation of cells with or without UK5099 treatment. Data are presented as mean $\pm$ standard deviation of six samples per group expressed as % of controls. $^{\dagger}P<0.001$; $^{**}P<0.01$ compared with control cells at the same time point. (C) UK5099-stimulated ^{18}F -FDG uptake and effect of additional Gln transporter inhibition with GPNA. Data are presented as mean $\pm$ standard deviation of triplicate samples expressed as % of controls. $^{\dagger}P<0.001$; $^{\ddagger}P<0.005$; $^{**}P<0.01$ compared to cells at 0 h with or without UK5099. (D) Proliferation of untreated cells and cells treated with UK5099 alone, UK5099 plus GPNA, or UK5099 plus Gln deprivation. Data are presented as mean $\pm$ standard deviation of six samples per group expressed as % of controls. $^{\dagger}P<0.001$; $^{\ddagger}P<0.005$ compared to controls at the same time point. MPC, mitochondrial pyruvate carrier; ^{18}F -FDG, 2- ^{18}F fluoro-2-deoxy-D-glucose.

to reprogram energy metabolism in order to survive (20). Therefore, maintenance of p70S6K activation may have contributed to the ability of CT26 cells to survive UK5099 treatment.

Depriving multiple key substrates of cancer bioenergetics, rather than a single substrate, may be more effective for tumor treatment (5). It is well-known that cancer cells become more dependent on glutamine metabolism when pyruvate is restricted or MPC function is impaired (21). Glutamine metabolism generates acetyl CoA and oxaloacetate, thereby supporting tricarboxylic acid cycle activity. In CT26 cells, UK5099 alone stimulated glucose uptake and suppressed proliferation to only a moderate extent, but these effects were significantly enhanced by additional restriction of glutamine utilization. This indicates that, in cells that manage to survive and grow under conditions of low mitochondrial pyruvate availability, additional glutamine restriction may achieve a more efficient antitumor effect.

In conclusion, TGZ was effective against T47D cancer cells with lower 2-DG sensitivity by suppressing mitochondrial

pyruvate availability. Although glycolysis-dependent CT26 cancer cells were less responsive to TGZ or UK5099, anti-tumor efficacy was enhanced by the combined suppression of glutamine utilization.

Acknowledgements

Not applicable.

Funding

The present study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF-2018R1D1A1B07043260).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

Conceptualization: KHJ and KHL. Formal analysis: KHJ, SHM, YSC and KHL. Funding acquisition: KHL. Methodology: JHL, JWP and KHJ. Writing of the original draft: KHJ. Manuscript review and editing: KHL. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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