RESEARCH ARTICLE

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Curcumin Inhibits the PPARδ-p-Akt-GLUT1 Pathway and Ameliorates the Antiproliferative Effects of Doxorubicin in MDA-MB-231 Cells

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Abstract

Objective: The aim of the present study was to examine whether *GLUT1* was involved in the antiproliferative activity of curcumin and doxorubicin by understanding mechanistically how curcumin regulated GLUT1. **Methods:** Expression level of GLUT1 in MCF-7 and MDA-MB-231 cells were quantitated using quantitative real-time PCR and western blot. GLUT1 activity was inhibited in MDA-MB-231 cells with the pharmacological inhibitor WZB117 to assess the anti-proliferative effects of doxorubicin using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). To examine cell proliferation, trypan blue assay was used in cells transfected with *GLUT1* siRNA or plasmid overexpressing *GLUT1* with doxorubicin and/or commercially available curcumin. The role of PPARδ and Akt on the regulation of *GLUT1* by curcumin was examined by overexpressing these proteins and western blot was employed to examine their protein expression. **Results:** The data revealed that there was a 1.5 fold increase in *GLUT1* mRNA and protein levels in MDA-MB-231 compared to MCF-7. By inhibiting *GLUT1* in triple negative breast cancer cell line, MDA-MB-231 with either the pharmacological inhibitor WZB117 or with *GLUT1* siRNA, we observed the enhanced antiproliferative effects of doxorubicin. Additional observations indicated these effects can be reversed by the overexpression of *GLUT1*. Treatment of MDA-MB-231 with curcumin also revealed downregulation of GLUT1, with further growth suppressive effects when combined with doxorubicin. Overexpression of *GLUT1* blocked the growth suppressive role of curcumin and doxorubicin ($p< 0.05$). Mechanistically, we also observed that the regulation of *GLUT1* by curcumin was mediated by the Peroxisome proliferator-activated receptor (PPAR) δ/Akt pathway. **Conclusion:** Our study demonstrates that regulation of *GLUT1* by curcumin via the PPARδ/Akt signaling improves the efficacy of doxorubicin by promoting its growth inhibitory effects in MDA-MB-231 cells.

Keywords: metabolism- triple negative breast cancer- cancer signaling

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Introduction

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer that lacks estrogen receptor α (ERα), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2) [1, 2]. Drug resistance or reduced efficacy to chemotherapeutic agents remains an obstacle to curing cancer and preventing tumors from going into long-term remission. To address this issue, combination therapy, along with radiotherapy and surgery are approaches used to control tumor metastasis [3]. However, standard therapy has not completely eliminated the progression of the disease owing to the development of targeted cancer therapies. Due to the absence of biomarkers and the heterogeneity of TNBC, the initial treatment option has been chemotherapy. However, molecular classifications of TNBC have identified targeted therapies to improve the clinical outcomes of patients [1, 4]. Though novel treatments have improved the survival rate of TNBC patients, some patients either do not respond to these regimens or develop resistance. Ongoing research is continuing to identify molecular targets through tumor sequencing, and individualized treatment options through molecular subtyping may improve the outcome of this disease [5].

Altered metabolic processes in cancer cells are crucial to tumor maintenance and therapeutic strategies are being investigated to block the glycolytic pathway [6]. Such changes in cancer metabolism can lead to tumorigenesis and resistance to chemotherapeutic agents as observed in many cancer subtypes [7-9]. To meet energy demands, cancer cells increase the Glucose transporter 1 (*GLUT1*), a protein associated with basal glucose uptake. Elevated expression of *GLUT1* is associated with high histological grade and poor overall survival in several cancers [10-12]. *GLUT1* plays an important role in cancer progression *in*

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vitro [13-15], and is assigned as a prognostic marker in studies demonstrating a correlation between high *GLUT1* and poor survival outcomes [16, 17, 11]. Studies have noted the role of *GLUT1* in chemoresistance [18-20] and how strategies to silence the expression of *GLUT1* re-sensitizes cells to cancer therapeutics [21-29].

The PI3K/Akt pathway affects a variety of biological processes such as cell growth, apoptosis and cancer metabolism. By blocking this pathway, cancer cells become sensitized to chemotherapeutic agents which reverses cellular processes associated with cancer progression [30]. Some studies report that the PI3K/Akt signaling cascade is involved in activating the expression of GLUT1 and mediating multiple cellular responses [31, 32]. Correlation studies have demonstrated high levels of *GLUT1* enhances the activation of the Akt signaling in gastric cancer [13] and in vascular smooth muscle cells, overexpression of GLUT1 increases the PI3K/Akt signaling [33]. In gastrointestinal stromal tumor cells, WZB117 reduces the active form of Akt [27]. Not only is there positive feedback between *GLUT1* expression by the PI3K/Akt pathway, the reverse is also possible, but it is unknown mechanistically how this occurs.

Due to the toxicity associated with chemotherapeutic agents, natural products have become a source of investigation [34]. A component of turmeric, curcumin is known for its anti-invasive properties [35, 36], and in lung cancer and pancreatic cancer, curcumin suppressed *GLUT1* protein expression [37, 38]. Previous studies reported curcumin downregulated the expression of nuclear receptor peroxisome-proliferator-activated receptor delta (PPARδ) in TNBC [2] and that PPARδ was involved in regulating *GLUT1* in colorectal cancer cell line, HeLa cells and MCF-7 breast cancer cells [39]. Biological functions are associated with the overexpression of PPARδ such as its contribution to tumorigenesis [40, 41]. While there is no known clinical inhibitor for *GLUT1*, curcumin has been shown to block metabolic pathways and inhibit PPARδ [2]. Further studies are warranted in other mammary carcinoma cells to understand the regulation of *GLUT1* by curcumin and whether PPARδ is involved in *GLUT1* expression.

In this study, we evaluated the effects of WZB117 and *GLUT1* siRNA on the growth of MDA-MB-231 cells, and its impact on doxorubicin-mediated growth suppression. Inhibition of *GLUT1* with WZB117 or silencing *GLUT1* improved the efficacy of doxorubicin. Treatment with curcumin suppressed the expression of *GLUT1* and was reversed by the overexpression of PPARδ. Furthermore, reduction of *GLUT1* protein expression by curcumin was overcome by the overexpression of Akt. We propose that these studies will contribute to the understanding of how *GLUT1* is involved in reducing the activity of antitumor drugs in TNBC, and encourage the use of nontoxic natural products like curcumin to target cancer metabolism and improve response to cancer therapeutics.

Materials and Methods

Reagents

Antibodies against GLUT1 (D3J3A), PPARδ (E803H), p-Akt (D9E), Akt (C67E7) and GAPDH (D4C6R) were purchased from Cell Signaling (Danvers, MA, USA). Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibodies were from Bio-Rad Laboratories (Hercules, CA, USA). WZB117 (resuspended in DMSO), Doxorubicin (resuspended in water) and the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were obtained from Sigma (St. Louis, MO, USA). Curcumin from *Curcuma longa* was purchased from Sigma (C1386) and prepared as a stock solution of 30 mM in DMSO. DMSO was used as control for treatment with WZB117 or curcumin. H_2O_2 was used as a positive control for cell growth inhibition.

Cell Lines

Highly aggressive TNBC MDA-MB-231 and the less aggressive MCF-7 cells (Estrogen Receptor (ER) positive, Progesterone Receptor (PR) positive and Human Epidermal Growth Factor Receptor 2 (HER2) negative) purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and antibiotics-antimycotics (Atlanta Biologicals) at 37°C in an incubator set at 5% CO_2.

Western Blotting

Collected cells were lysed and protein concentration was determined using the Bradford Assay (Bio-Rad Laboratories, Gaitherburg, MD, USA). Cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), probed with the appropriate antibody, and GAPDH was used as loading control. Bands were detected using Odyssey Fc imaging system. Densitometry was conducted using ImageJ.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from MDA-MB-231 and MCF-7 cells was extracted using Trizol (Life Technologies, Grand Island, NY). 2 µg total RNA was reverse transcribed into cDNA using the protocol from the high capacity cDNA kit (Applied Biosystems, Waltham, MA, USA). Expression of *GLUT1* was determined by qRT-PCR, using commercially available Taqman Chemistry and Assay on Demand Probes (Applied Biosystems). GAPDH was used for normalization. Detection and data analysis were carried out on the ABI Step One Plus Real-Time PCR System. Relative quantity of gene expression was performed using $2^{-\Delta\Delta Ct}$ method [42].

Cell proliferation assay

MDA-MB-23 cells plated in a 96 well plate were treated with varying concentrations of WZB117 with or without 0.5 µM doxorubicin for 48 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. Values are normalized in relation to control cells.

Cell Transfection

MDA-MB-231 cells plated in 6 well plates

were transfected with specific siRNAs (Santa Cruz Biotechnology, Dallas, TX, USA) using Lipofectamine RNAimax (Invitrogen, Waltham, MA). With PPARδ or Akt expression plasmid (Origene, Rockville, MD, USA), cells were transfected using MegaTran 2.0 transfection reagent (Origene), and treated with curcumin 24 h post-transfection.

Trypan Blue Exclusion Assay

MDA-MB-231 cells were transfected with control or *GLUT1* siRNA overnight using Lipofectamine RNAimax and treated with 0.5 μM doxorubicin for 48 h. Cells were trypsinized, collected and counted using trypan blue from MP Biomedicals (Solon, OH, USA). Cells transfected with control or *GLUT1* expression plasmid (Origene), using MegaTran 2.0 were treated with 0.5 μM doxorubicin in the presence or absence of curcumin for 48 h, and cells were counted. Live cells were represented as number of cells/ml.

Statistical analysis

Statistical significance was determined using two-tailed student t-test and p values were noted. Differences between groups were considered statistically significant at $p < 0.05$.

Results

Inhibition of GLUT1 improved the efficacy of doxorubicin-mediated growth suppression

Expressions of *GLUT1* was examined in MCF-7 cells and MDA-MB-231 cells. Compared to MCF-7 cells, MDA-MB-231 cells expressed approximately 1.5-fold higher levels of GLUT1 protein (Figure 1a) and correspondingly, elevated levels of *GLUT1* mRNA (Figure 1b). Thus, MDA-MB-231 cells were chosen as a model system for further studies.

To study the relationship of *GLUT1* on the growth of MDA-MB-231, WZB117 [29, 27] was used for experimentation. Doxorubicin impeded cell proliferation, however treatment of the cells with either 6 μM or 10 μM WZB117 did not enhance doxorubicin-induced growth suppression (Figure 2a and 2b). Using varying concentrations of WZB117 in the presence of a fixed concentration of doxorubicin (0.5 μM) demonstrated that higher doses of WZB117 (greater than 20 μM) enhanced the efficacy of doxorubicin to block cell growth (Figure 2c). Silencing of *GLUT1* (Figure 2d) reduced growth of MDA-MB-231 with a more pronounced antiproliferative effect in the presence of doxorubicin (Figure 2d). Alternatively, overexpression of *GLUT1* promoted cell growth and reversed the growth inhibitory effects of doxorubicin (Figure 2e). Taken together, in MDA-MB-231, either WZB117 or *GLUT1* siRNA caused the cells to be more sensitive to growth reduction by doxorubicin. Corroborating these findings, overexpression of *GLUT1* reduces the efficacy of doxorubicin-mediated growth suppression.

Overexpression of PPARδ reverses curcumin-mediated suppression of GLUT1

In lung and pancreatic cancer cells, curcumin inhibits

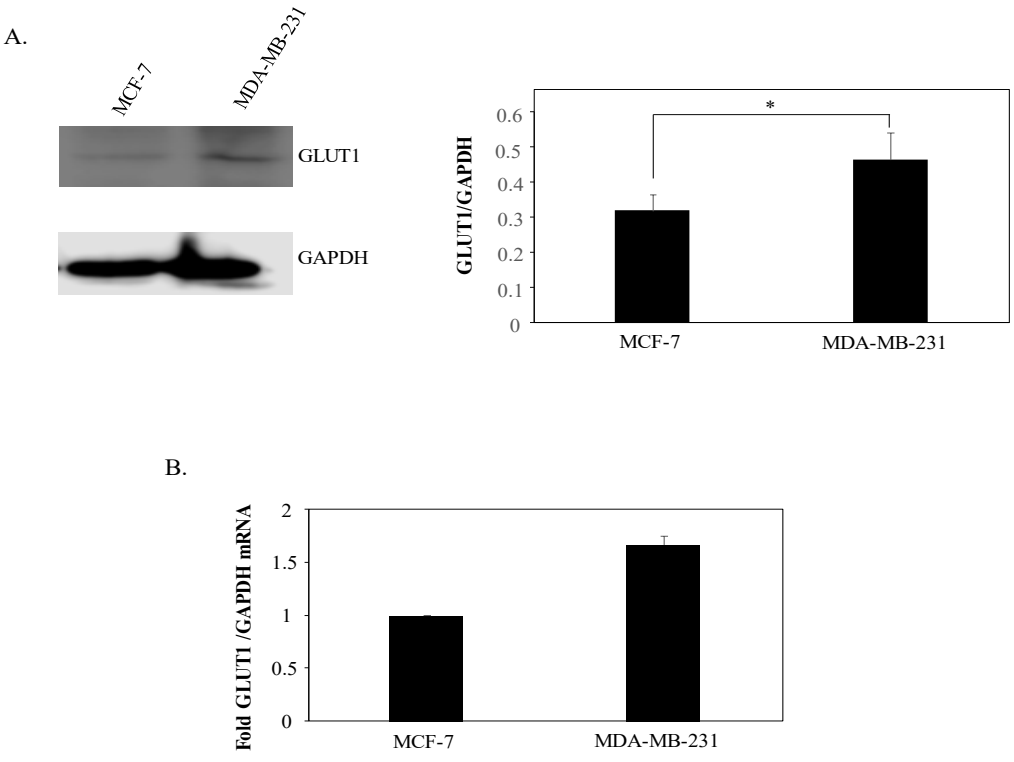

Figure 1. Expression of *GLUT1* in Mammary Carcinoma Cells. (a) Extracts from MCF-7 and MDA-MB-231 cells were resolved with SDS-PAGE and immunoblotted with anti-*GLUT1*. GAPDH was used for normalization. Densitometry analyses provide relative amount of *GLUT1* normalized to *GAPDH*. Data are mean ± SE (n=3). *, $p<0.05$ (b) Total RNA was collected from MCF-7 and MDA-MB-231 cells and GLUT1 mRNA was analyzed by qRT-PCR. *GAPDH* was used for normalization. Data are mean ± SE (n=3).

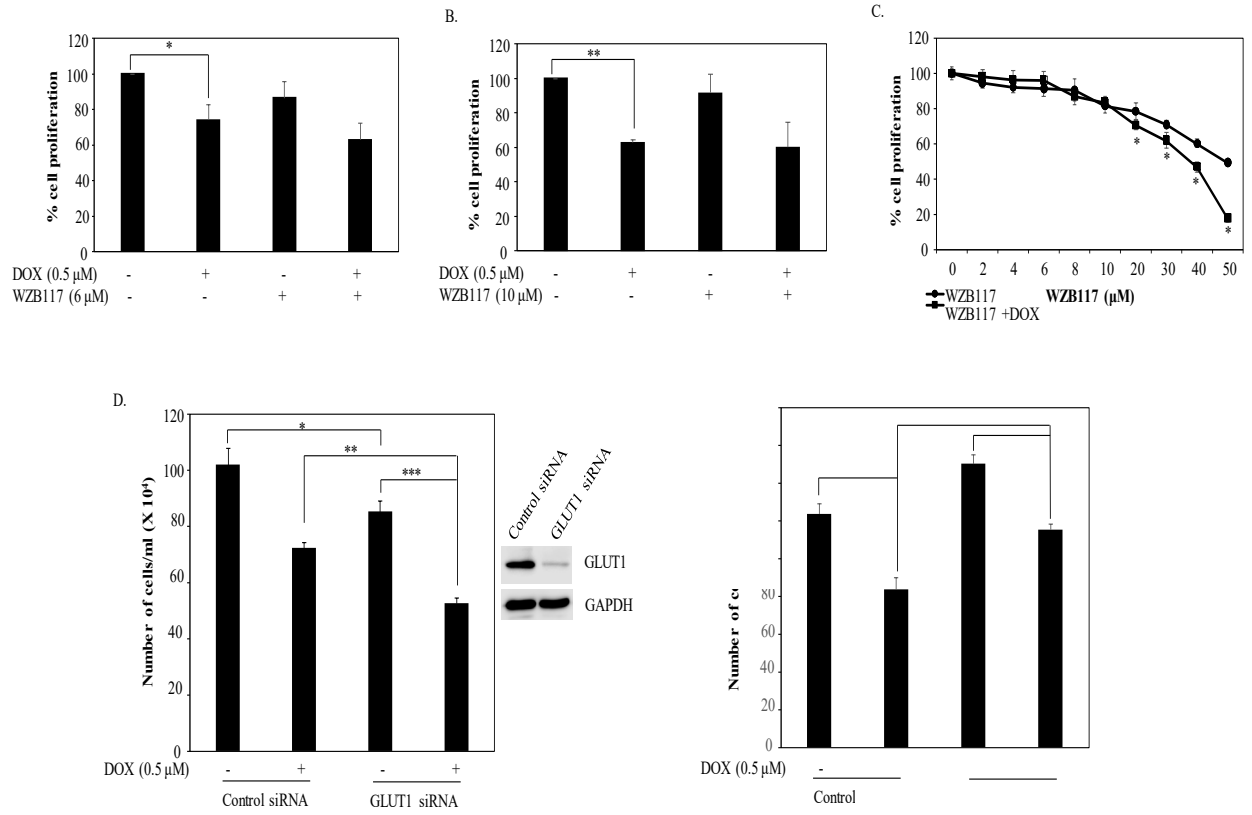

Figure 2. Inhibition of *GLUT1* Improves the Efficiency of Doxorubicin-mediated Growth Suppression in MDA-MB-231. MDA-MB-231 cells were treated with 0.5 μM doxorubicin (DOX) with or without WZB117 at 6 μM (a) or 10 μM (b) for 48 h, after which growth was assessed. Data are mean ± SE of five independent experiments for panel a and four independent experiments for panel b. *: p=0.04, **: p=0.0004. (c) MDA-MB-231 cells were treated with varying concentrations of WZB117 with or without 0.5 μM DOX for 48 h. Data are mean ± SE of three independent experiments *: < p,0.05. (d) MDA-MB-231 cells were transfected with control or *GLUT1* siRNA for 48 h, protein extracted, cell lysate resolved in SDS-PAGE and immunoblotted with GLUT1. Loading control: GAPDH. Transfected MDA-MB-231 cells with control or *GLUT1* siRNA were treated with 0.5 μM DOX for 48 h and live cells were counted using trypan blue. Data are mean of ± SE (n=3). * p= 0.02 (versus control siRNA), ** p=0.03 (versus control siRNA + DOX), *** p=0.02 (versus *GLUT1* siRNA). (e) MDA-MB-231 cells were transfected with *GLUT 1* expression plasmid or control vector, treated with 0.5 μM DOX for 48 h and live cells were counted using trypan blue. Data are mean of ± SE (n=3). * p= 0.04 (versus control vector), ** p= 0.03 (versus *GLUT1* OE), *** p= 0.02 (versus control vestor + Dox).

the expression of GLUT1 [37, 38]. To investigate the effects of curcumin on *GLUT1* protein expression in MDA-MB-231 cells, the cells were treated with curcumin and probed for GLUT1. Compared with control, curcumin (20 μM and 30 μM) reduced GLUT1 protein expression, suggesting that curcumin regulates *GLUT1* in MDA-MB-231 cells (Figure 3a). In order to investigate the functional relevance of *GLUT1* regulation by curcumin on growth inhibition by doxorubicin, *GLUT1* was overexpressed in MDA-MB-231 cells, treated with doxorubicin with or without curcumin, and cell viability was assessed. Cells transfected with *GLUT1* enhanced cell growth and also reversed the suppressive effects of doxorubicin (Figure 3b). Similarly, 30 μM of curcumin treatment on MDA-MB-231 transfected with empty vector suppressed growth, unlike normal epithelial MCF10A cell (data not shown), but was overcome by the introduction of *GLUT1* expression plasmid. As expected, the combination of doxorubicin with curcumin inhibited cells transfected with empty vector, but more importantly, the results showed *GLUT1* transfected MDA-MB-231 cells protected the cells against the cytotoxic effects of doxorubicin and curcumin. These studies show curcumin downregulation of *GLUT1* in MDA-MB-231 is associated with improving the growth inhibitory effects of doxorubicin and the ectopic overexpression of *GLUT1* repressed the effects of doxorubicin and/or curcumin on cell viability.

Since curcumin downregulates PPARδ in MDA-MB-231 cells [2], and curcumin suppresses GLUT1 (Figure 3a), we tested whether silencing PPARδ impacts *GLUT1* expression. Furthermore, in colon cancer cells, upregulation of PPARδ induced GLUT1 [39]. In this study, knockdown of PPARδ diminished *GLUT1* expression in MDA-MB-231 (Figure 4a), whereas overexpression of PPARδ induced GLUT1 protein expression (Figure 4b). In addition, overexpression of PPARδ reversed the downregulation of GLUT1 by curcumin (Figure 4c). These results indicate PPARδ is a regulator in curcumin-mediated downregulation of *GLUT1* in MDA-MB-231 cells.

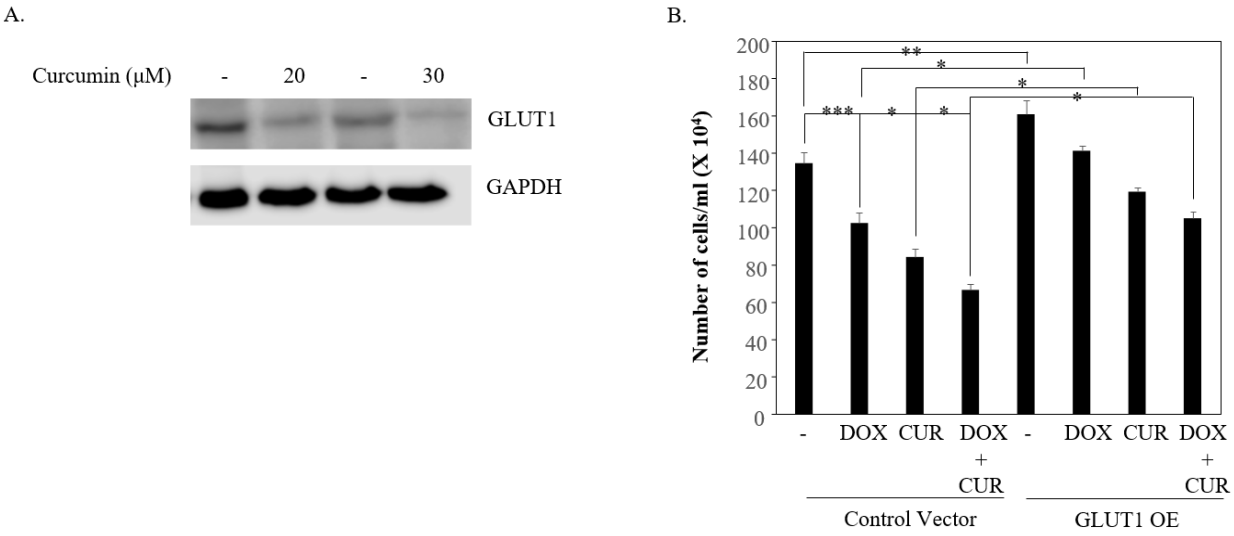

Figure 3. Downregulation of *GLUT1* by Curcumin Promotes the Antiproliferative Effects of Doxorubicin. (a) MDA-MB-231 cells were treated with 20 μM and 30 μM of curcumin for 24 h, cell lysate resolved with SDS-PAGE and immunoblotted against GLUT1. (b) MDA-MB-231 cells were transfected with *GLUT1* expression plasmid or control vector, treated with 0.5 μM DOX and/or 30 μM Curcumin (CUR) for 48 h and live cells were counted using trypan blue. Data are mean of ± SE (n=3). *p<0.05, **, p<0.01, ***, p<0.001.

Overexpression of Akt reverses curcumin-mediated suppression of GLUT1

PPARδ overexpression promotes breast cancer survival through numerous mechanisms, one of which is the activation of the Akt pathway [41, 43]. We sought to investigate whether overexpression of PPARδ enhances the phosphorylation of Akt, which in turn may upregulate *GLUT1* expression. First, we analyzed the expression of activated Akt in the presence of curcumin and as observed in Figure 5a, curcumin reduced the activation of Akt. Transfected MDA-MB-231 cells with either control vector or PPARδ overexpressor, were probed for p-Akt. Our observations demonstrated overexpression of PPARδ enhanced p-Akt compared to control cells. Furthermore, in PPARδ overexpressing cells, activation of Akt was not altered in the presence of curcumin (Figure 5b). This finding suggests the direct involvement PPARδ to activate Akt in MDA-MB-231 cells, and its role to prevent the reduction of activated Akt by curcumin. In addition, this data indicates PPARδ acts upstream of the Akt pathway.

To determine whether the activation of Akt is directly involved in *GLUT1* expression, MDA-MB-231 cells were transfected with control vector or Akt overexpressor and treated with curcumin. Cells transfected with Akt increased the expression of GLUT1 (Figure 5c), but curcumin-mediated suppression of GLUT1 was reversed in cells overexpressing Akt. Based on these results, the PPARδ/Akt signaling axis plays a role in curcumin-mediated suppression of GLUT1 (Figure 5d).

Discussion

Altered metabolic activity promotes cells to become cancerous prompting research to identify pathways associated with cancer metabolism as means for targeted therapy [44]. Studies have documented *GLUT1* as a

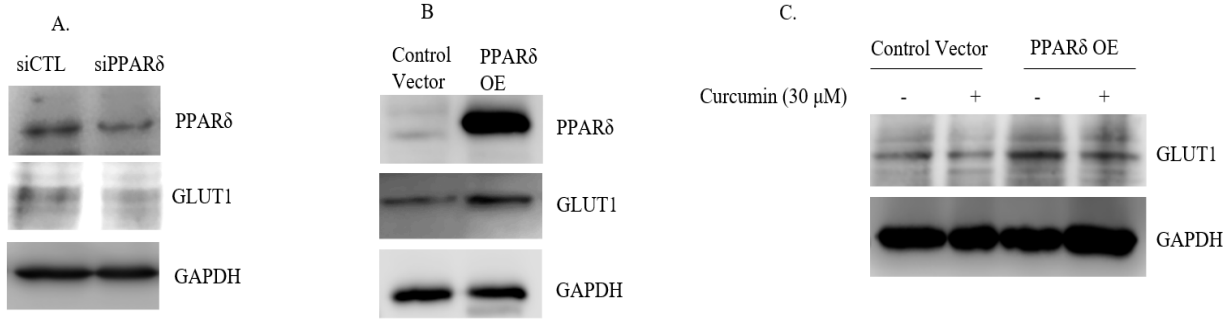

Figure 4. PPARδ Reverses the Downregulation of GLUT1 by Curcumin. (a) MDA-MB-231 cells transfected with control or PPARδ siRNA for 48 h were probed for PPARδ and *GLUT1*. (b) Control or PPARδ expression plasmid were transfected in MDA-MB-231 cells for 48 h and probed for the protein expression of PPARδ and GLUT1 (c) MDA-MB-231 cells transfected with control or PPARδ expression plasmid overnight were treated with or without 30 μM of curcumin 24 h post-transfection, and probed for GLUT1 protein. Data depicts a representation of a Western blot from three independent experiments (a-c).

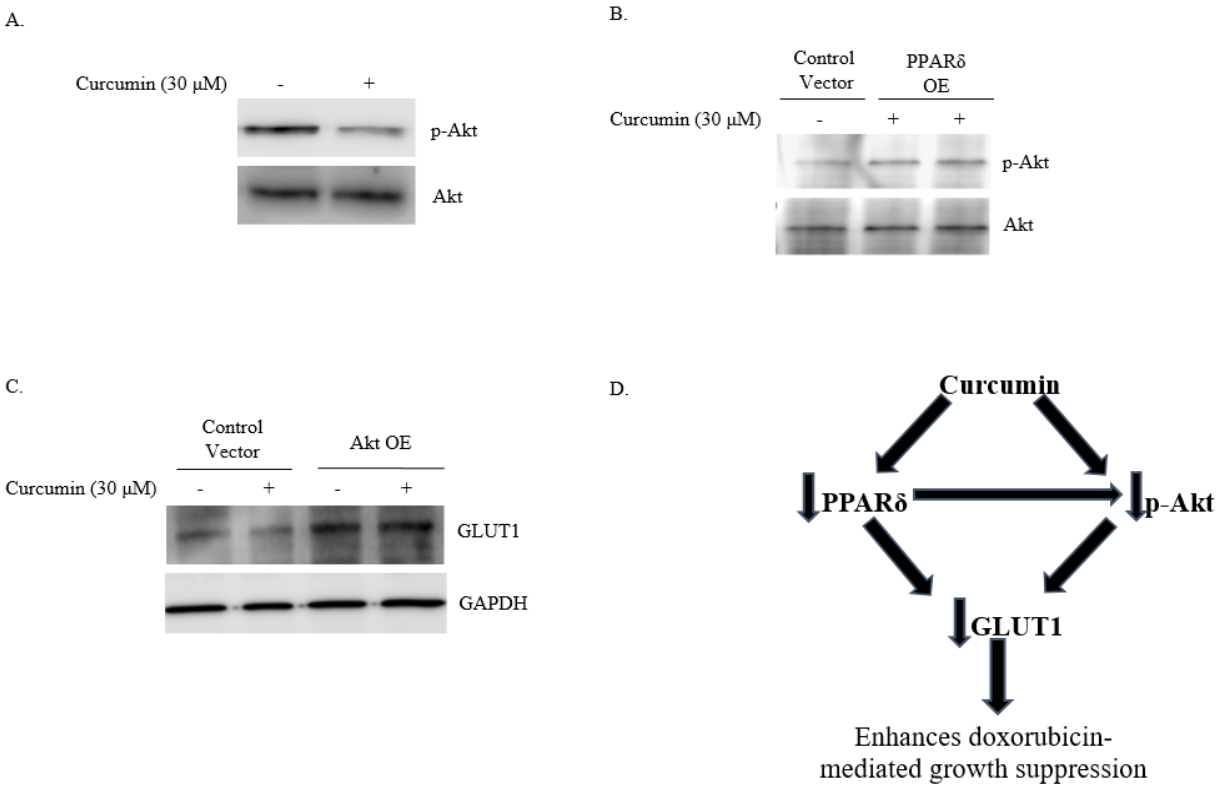

Figure 5. PPARδ and Akt are Involved in the Regulation of Curcumin-mediated Downregulation of GLUT1 (a) MDA-MB-231 cells were treated with 30 μM of curcumin or control for 24 h to probe for p-Akt and Akt. MDA-MB-231 cells were transfected with PPARδ (b) or Akt (c) expression plasmids, treated with 30 μM of curcumin and probed for p-Akt (b) or GLUT1 (c). Loading control: GAPDH. Data depicts a representation of a Western blot from three independent experiments (a-c) (d) A model outlining the effects of curcumin on the interplay between PPARδ, Akt and *GLUT1*.

biomarker, connecting upregulated levels of *GLUT1* during oncogenesis with poor overall survival [11, 16, 17]. Moreover, *GLUT1* is linked to chemoresistance which leads to reduced survival rate [18-20]. Mechanisms on how *GLUT1* contributes to chemoresistance include increased HIF1α [45], regulating the PI3K/Akt pathway [13, 33], and downregulating P-glycoprotein [46]. While there is no known clinical inhibitor against *GLUT1*, our studies propose the use of curcumin in targeting the expression of *GLUT1* in TNBC.

In this study, we examined the inhibition of *GLUT1* on enhancing the cytotoxic effects of doxorubicin and its link to the PPARδ/Akt signaling pathway. Comparing MCF-7 cells with MDA-MB-231 cells, the expression of *GLUT1* was higher in MDA-MB-231 cells. Since numerous articles have used WZB117 to inhibit *GLUT1* activity [29, 27], our study showed that through a dose-dependent response, WZB117 improved the efficacy of doxorubicin. Corroborating these findings, silencing *GLUT1* using siRNA improved the sensitivity of these cells to the antiproliferative actions of doxorubicin.

While curcumin does not impact normal epithelial breast cells, it does however, have a negative effect on cancer cell's metabolism [47, 48], supporting its use in therapy. Chemotherapeutic agents like doxorubicin are toxic and we explored the possibility of using curcumin to regulate *GLUT1* in order to improve the efficacy of doxorubicin. Results of the study indicated curcumin downregulated GLUT1 protein expression in MDA-MB-231, and even though curcumin has low bioavailability, future studies with analogs of curcumin with improved bioavailability may be studied for its effect on *GLUT1* and improving the efficacy of doxorubicin for clinical usage. Furthermore, we demonstrated while curcumin and doxorubicin reduced growth of MDA-MB-231 cells, *GLUT1* over-expressing cells overcame cell growth repression by these agents. Therefore, abrogation of *GLUT1* by curcumin enhanced the sensitivity of doxorubicin to promote growth suppression, which suggest the importance of identifying natural products like curcumin to target regulators of breast cancer metabolism.

Accumulating evidence show that PPARδ is expressed in highly aggressive cancers [40] implicating the nuclear receptor to poor prognosis in patients [49, 50]. In our previous work, MDA-MB-231 cells expressed higher levels of PPARδ mRNA and protein compared to MCF-7 cells [2]. PPARδ is being considered as a target for metabolic syndromes and our results pointed to the involvement of PPARδ as a direct upstream target of GLUT1. The negative effects of PPARδ were observed in curcumin-treated MDA-MB-231 cells overexpressing PPARδ. Under these conditions, PPARδ reversed curcumin's effects on inhibiting GLUT1. These findings are in accordance with previous studies which documented that the effects of PPARδ overexpression in colon cancer cell lines and breast cancer MCF-7 cells translated to the

upregulation of *GLUT1* [39]. Based on these observations, it is likely the regulation of *GLUT1* by PPARδ is a global effect among mammary carcinoma cells. Moreover, as evidenced in this study, *GLUT1* is highly expressed in MDA-MB-231 cells compared to MCF-7 cells which may be a direct correlation to the higher expression levels of PPARδ in MDA-MB-231 cells [2], suggesting PPARδ is a regulator of *GLUT1*. Additional studies using other cancer models will be necessary to examine the correlation of *GLUT1* and PPARδ expression, and this may be expanded to tumor specimens.

Not only is PPARδ a mediator of *GLUT1* in MDA-MB-231, we also observed the role the Akt pathway plays in regulating *GLUT1*. Overexpression of Akt induced *GLUT1* expression, which may be independent or dependent on PPARδ. The inhibitory effects of curcumin on *GLUT1* expression may be mediated in part by PPARδ and/or the Akt signaling pathway, which contribute to enhancing the growth inhibitory effects of doxorubicin. From these results, it seems reasonable to suggest the existence of an interplay between *GLUT1* and the PPARδ/Akt axis, and targeting these proteins by curcumin may be combined with doxorubicin in breast tumors with high *GLUT1* expression. The present study has been limited to MDA-MB-231 cells and future experiments are required to clarify the relationship of the *GLUT1* to the PPARδ-Akt axis using experimental breast cancer models with a different classification. Further investigation is necessary to understand the association between the *GLUT1* and PPARδ/Akt pathway, providing potential combined targeted therapies to improve sensitivity of chemotherapeutics drugs in TNBC.

Author Contribution Statement

PT, MF, RP and KM collected and analyzed data. PT wrote the manuscript. MF, RP and KM reviewed and revised the manuscript. All authors read and approved the final manuscript. Some data were part of student (MF and RP) approved thesis work.

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Availability of data

The data are available from the corresponding author upon reasonable request.

Ethics

This study was approved by Institutional Biosafety Committee at the University of South Alabama

Competing Interests

The authors have no relevant financial or non-financial interests to disclose. The authors declare that they have no conflict of interest. This study follows the AIMRDA standard reporting recommendation.

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