Original article

Exploration of metabolic responses towards hypoxia mimetic DMOG in cancer cells by using untargeted metabolomics

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ABSTRACT

Hypoxia is considered as one of the most crucial elements of tumor microenvironment. The hypoxia inducible transcription factors (HIF-1/2) are used by the cancer cells to adapt hypoxic microenvironment through regulating the expression of various target genes, including metabolic enzymes. Dimethyloxalylglycine (DMOG), a hypoxic mimetic used for HIF stabilisation in cell and animal models, also demonstrates multiple metabolic effects. In past, it was shown that in cancer cells, DMOG treatment alters mitochondrial ATP production, glycolysis, respiration etc. However, a global landscape of metabolic level alteration in cancer cells during DMOG treatment is still not established. In the current work, the metabolic landscape of cancer cells during DMOG treatment is explored by using untargeted metabolomics approach. Results showed that DMOG treatment primarily alters the one carbon and lipid metabolism. The levels of one-carbon metabolism related metabolites like serine, ornithine, and homomethionine levels significantly altered during DMOG treatment. Further, DMOG treatment reduces the global fatty acyls like palmitic acids, stearic acids, and arachidonic acid levels in cancer cell lines. Additionally, we found an alteration in glycolytic metabolites known to be regulated by hypoxia in cancer cell lines. Collectively, the results provided novel insights into the metabolic impact of DMOG on cancer cells and showed that the use of DMOG to induce hypoxia yields similar metabolic features relative to physiological hypoxia.

1. Introduction

Hypoxia-inducible factors (HIF) transcription factors that can endorse the persistence of solid tumors in low oxygen environments. HIF's heterodimeric is HIF-1 and HIF-2, most of the previous studies reported HIF-1 known to regulate an assembly of proteins and genes. HIF-2 can also impact protein and gene expression, HIF-arbitrated on metabolome signaling (Lando et al. 2002; Khan et al. 2018). The functional relationship between phenotypes is characterized less. HIF-1 upregulates glycolytic enzymes (Masson et al. 2001), which control glycolytic flux, including pyruvate kinase, hexokinase, lactate dehydrogenase (LDH), phosphofructokinase, and gene encoding glucose transporters. HIF-1 regulates the expression of monocarboxylate transporter-4 (MCT4); it enables the metabolic relationship between hypoxic and aerobic cells (Ruan et al. 2009; Sonveaux et al. 2008). The cells under hypoxic conditions excrete lactate through induced expression of MCT1 and LDHA. HIF is attributed to phenotypic alteration via metabolism, like the amount of ATP, which can be minimized to 80 % in HIF-1 β deficiency (Roberts et al. 2009; Armitage and Barbas, 2014; Armitage et al. 2015).

Earlier studies on hypoxia and HIF focused on glucose uptake, glycolysis and their metabolic interactions. Only a few studies showed the impact of hypoxia on lipid metabolism through HIF-1 activation of PPARγ and fatty acid binding proteins (FABPs) (Bensaad et al., 2014; Khrisnan et al., 2009). The metabolic association of Prolyl hydroxylases (PHDs) in hypoxia is well regulated. PHD is the commonly investigated dioxygenase, in regulating hypoxia signaling mechanism (Loenarz and Schofield, 2011; Ivan and Kaelin, 2017). The function of PHD is oxygen dependent, hydroxylation of HIF inhibited by hypoxia, and it gets stabilized. Dimethyloxalylglycine (DMOG) is deesterified within cells, thus forming N-oxalylglycine (NOG). NOG is an analogue of αKG, its methylene group of αKG is replaced with an NH moiety. NOG inhibits PHD

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but it shows minimal permeability inside the cellular plasma membrane. So, DMOG is used as a prodrug in animal and cellular studies (Son et al. 2013; Jin et al. 2015; Chan et al. 2016). DMOG mimics the effect of genetic aberration of PHD and its inactivation.

Since NOG is an analogue for αKG, NOG hampers the other metabolic activity and signaling mechanism of αKG involved. MCT2 facilitates DMOG uptake into the cells, yielding NOG upon entry. Increase in the concentration of intracellular NOG levels inhibits most metabolic pathways. Glutamate dehydrogenase (GDH) is a critical enzyme of glutamine metabolism whose binding is affected by NOG leading to the alteration of glutamate metabolism and TCA cycle (Kim et al. 2006; Fukuda et al. 2007; Aragonés et al. 2008; Sun and Denko 2014).

DMOG treatment to cancer cells results in dramatic modulation of mitochondrial energy production. Primarily DMOG reduces the rate of mitochondrial respiration and ATP production in cancer cells, which was coupled with compensatory increase in glycolysis (Zhdanov et al. 2013; Peng et al. 2014; Zhdanov et al. 2015). This shows DMOG clearly suppresses PHD-dependent processes regulated by HIFs. Based on these observations, it was clear that DMOG exerts metabolic alterations in cancer cells; however, a global landscape of metabolic alteration in cancer cells during DMOG treatment is still lacking. In the current work, we investigated the cellular metabolism of cancer cells during DMOG treatment by using untargeted metabolomics.

2. Materials and methods

2.1. Cell culture and treatment

HCT116 and HeLa were obtained from ATCC, (Manassas) USA. Cell lines were cultured and maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) culture medium supplemented with 10 % fetal bovine serum (FBS; Gibco one-shot, USA), 50 U/mL pen strep solution (Gibco, USA) at 5 % CO_2 at 37 °C. For metabolic analysis both cell lines were treated with either vehicle control or 1 mM DMOG (CAS No: 89464–63-1, Abcam) for 24 h as described in earlier studies (Shait Mohammed et al. 2021).

2.2. Gene set pathway analysis

The gene profile of GSE3188 containing RNA-seq data of hypoxia and DMOG-induced hypoxia were retrieved from GEO datasets (https://www.ncbi.nlm.nih.gov/geo/). Overlapping significantly upregulated genes from the dataset were then subject to DAVID (https://david.ncifcrf.gov/) for KEGG analysis.

2.3. Metabolites extraction

Metabolites were extracted from cells treated with DMOG. Treated cells were lysed immediately using tissue homogenizer using a combination of ice-cold methanol: acetonitrile: water at a ratio of (2:1:1 v/v) and vortexed for the 30 s and incubated for 24 h at − 20 °C and spun for 15 min at 13,000 rpm at 4 °C. The supernatant was collected, and the samples were taken for LC-MS/MS analysis (Hassan et al. 2020; AlGhamdi et al. 2020; Nur et al. 2022).

2.4. Mass spectrometry

Samples were analyzed using an LC-MS/MS LTQ XL™ linear ion trap instrument (Thermo Fisher Scientific, Waltham, MA, USA). Msn parameters, full scanning mode range from 100 to 1000 m/z. Helium was used as a buffer gas, and nitrogen was used as sheath gas; for run 40, arbitrary units were established as flow rate. The capillary temperature was set at 270 °C and voltage 4.0 V; spray voltage was set at − 3.0 kV (Alzahrani et al. 2021).

2.5. Metabolomics data analysis

The raw data were processed using open access to the online XCMS online database. Peaks were searched against human metabolites in the Human Metabolome Database (HMDB) version 5.0. Pathway analysis and statics were performed using Metaboanalyst (Shait Mohammed et al. 2022).

2.6. Statistical analysis

Differences between control and DMOG treated groups were determined by one-way analysis of variance (ANOVA) for multiple groups, using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Test results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. DMOG treatment alters the global metabolic landscape of cancer cells

To confirm whether DMOG induce hypoxia in cancer cells; overlapping upregulated genes of physiological hypoxia and DMOG induced hypoxia were subjected to KEGG analysis. Surprisingly, the HIF-1 signaling pathway is the top enriched pathway suggesting the DMOG is evident to induce hypoxia in cancer cells (Fig. 1A).

To study metabolic alteration of cancer cells treated with DMOG, the metabolites were extracted from DMOG treated HeLa and HCT116 cells and studied in LC-MS/MS. The global cellular metabolites were examined between DMOG treated and untreated (control) cells. The LC-MS/MS spectrum of three biological replicates was obtained. Total ion chromatogram (TIC) of LC-MS/MS spectrum of the intracellular metabolites is shown in Fig. 1B, and detailed identified features (metabolite) with p-value, and peak intensities is shown in Table S1. Metabolites were identified using the Human Metabolome Data Base (HMDB) version 5.0. The principal component analysis (PCA) plot differentiates the metabolomic segregation between each sample as shown in Fig. 1C; with FDR correction $p < 0.05$ and $q < 0.05$. The accumulation of differentially modulated metabolites between DMOG treated and control are shown in Fig. 2A-B by using Ward clustering.

The differential regulated metabolites from DMOG treated cells were mapped for pathway enrichment analysis using the KEGG database using MetaboAnalyst 5.0. KEGG-based identification of top 25 metabolic pathways showed enrichment in urea cycle, gluconeogenesis, Warburg effect, glutamate metabolism, alanine metabolism, TCA cycle, and betaine (one-carbon metabolism) along with other metabolic pathways (Fig. 3A). Further, using the Variable important for projection (VIP) score analysis, we found crucial and highly significant enriched metabolites (Fig. 3B). Overall, these results showed that DMOG treatment exerts strong metabolic effect on treated cells.

3.2. DMOG treatment modulates glycolysis and TCA cycle related metabolites of cancer cells

To investigate the energy metabolism characteristics that are changed during DMOG treatment in the exposed cells we preoptimized the metabolomics data. We noticed that DMOG treatment enriched metabolites related to OXPHOS intermediates, includes pyruvate, and lactate, both were significantly modulated in DMOG treated cells when compared with untreated cells (Fig. 4A). Inter-
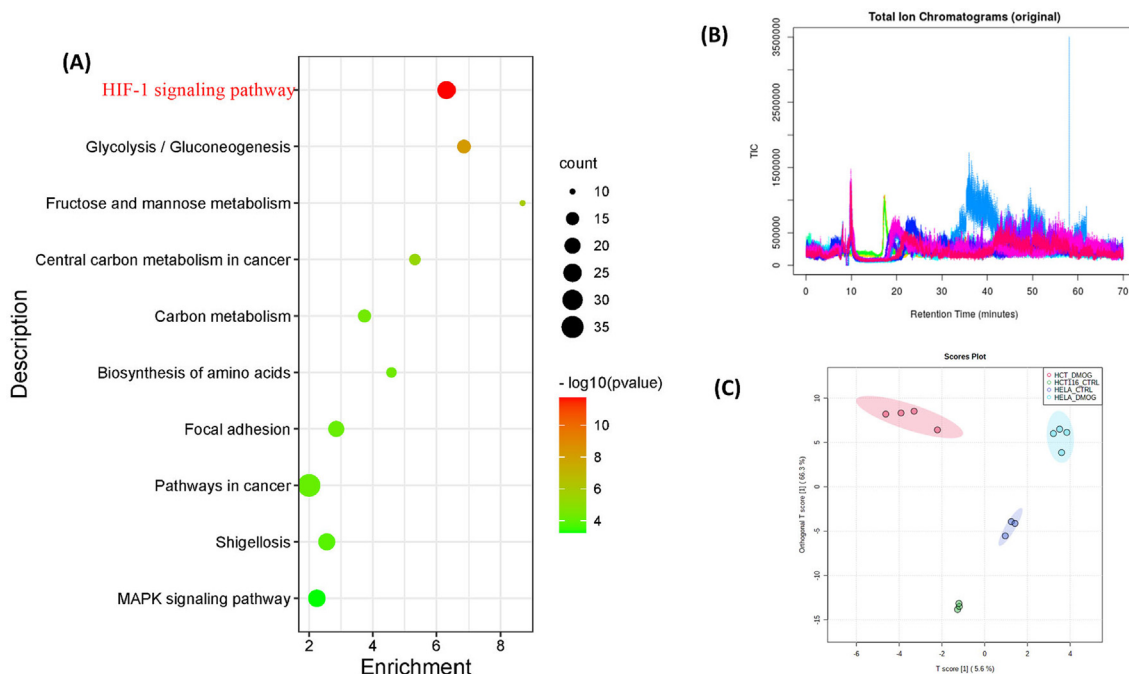
Fig. 1. Metabolomic analysis of cancer cells treated with DMOG. (A) Pathway analysis of overlapping upregulated genes from RNA-seq of physiological hypoxia and DMOG induced hypoxia (B) Total ion chromatogram of cell treated cells with DMOG (C) PCA analysis of total metabolites of HeLa and HCT116 cancer cells.
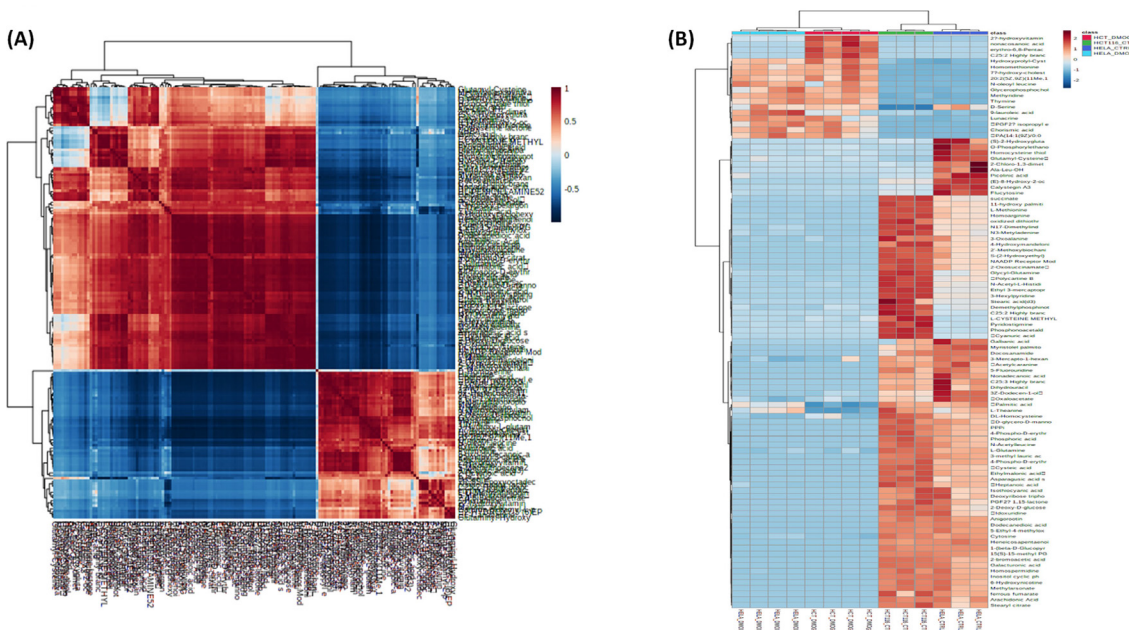

Fig. 2. (A-B). Heat map of differentially modulated metabolites between DMOG treated and control using Ward based clustering.

estingly, the metabolites related to reductive carboxylation pathway like glutamine and oxaloacetate were significantly reduced during DMOG treatment in cancer cells when compared to untreated cells (Fig. 4B).

3.3. DMOG treatment alters metabolite levels of one-carbon (1C) metabolism in cancer cells

The one-carbon metabolism plays a key role in donating the methyl group to DNA, RNA, histones, for facilitating the post-translational modifications within the adenine and cytosine moieties. Important metabolites in one-carbon metabolism such as

serine, ornithine, and methionine were found to be significantly modulated in DMOG treated cancer cells compared to control (untreated). Additionally, we found that two other supplementary metabolites of one carbon metabolism i.e., homospermidine and homoarginine were significantly decreased in DMOG treated cancer cells when compared with control (Fig. 5).

3.4. DMOG treatment modulates the lipidome profile of cancer cells

Despite extensive information available about the general metabolic changes that might have occurred during hypoxia on cancer cells. The information on the impact of DMOG on cellular
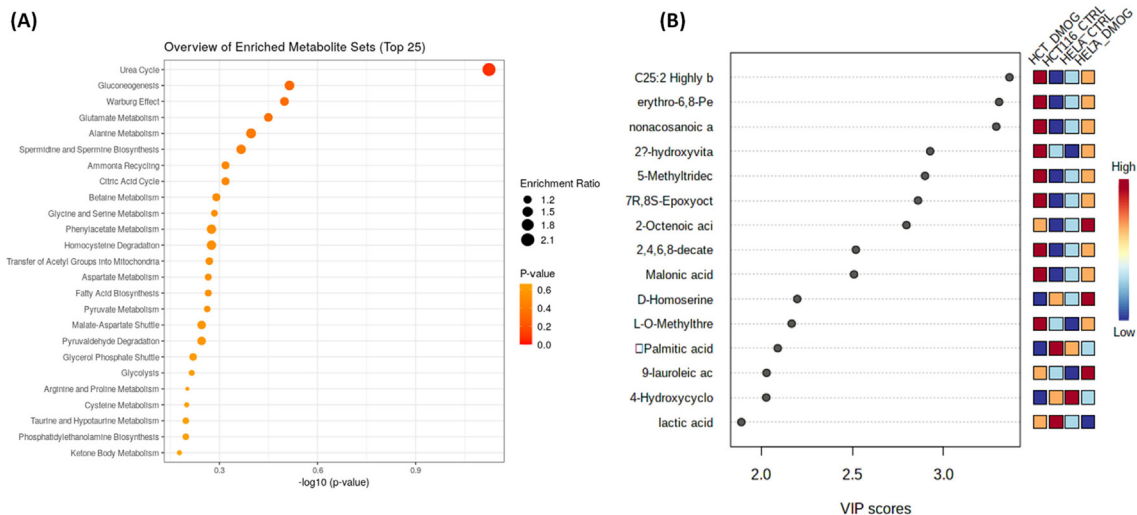
Fig. 3. Top pathways and metabolite enrichment analysis in DMOG treated cancer cells. (A) Metabolic pathway enriched in DMOG treated HeLa and HCT116 cells *versus* control; (B) Variable important for projection (VIP) scores of top 15 important metabolite features identified in DMOG treated cancer cells.
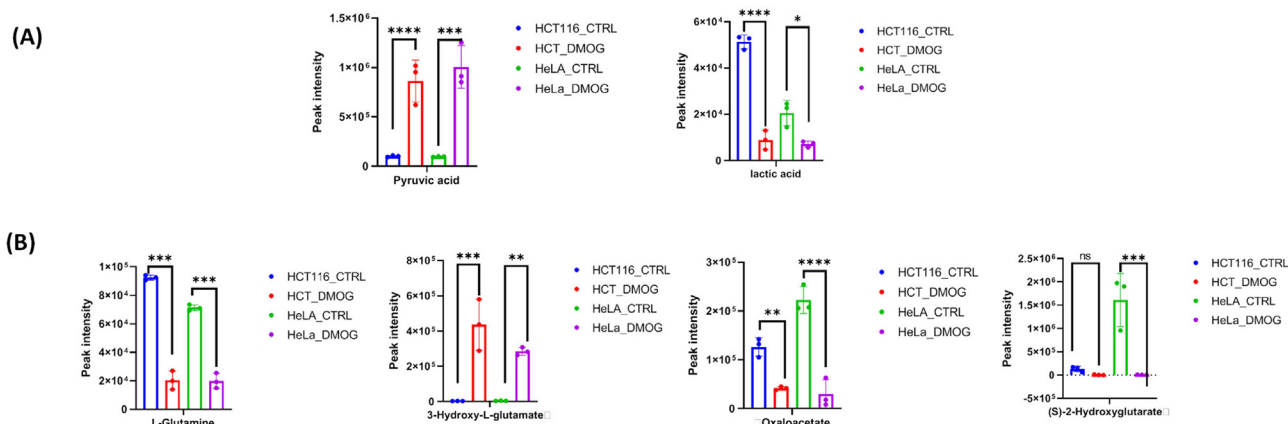

Fig. 4. DMOG alter crucial energy metabolic pathways in cancer cells. Quantitative peak intensities of various metabolites involved in TCA cycle in DMOG treated HeLa and HCT116 cells *versus* control, * $p < 0.01$. ** $p < 0.00$.
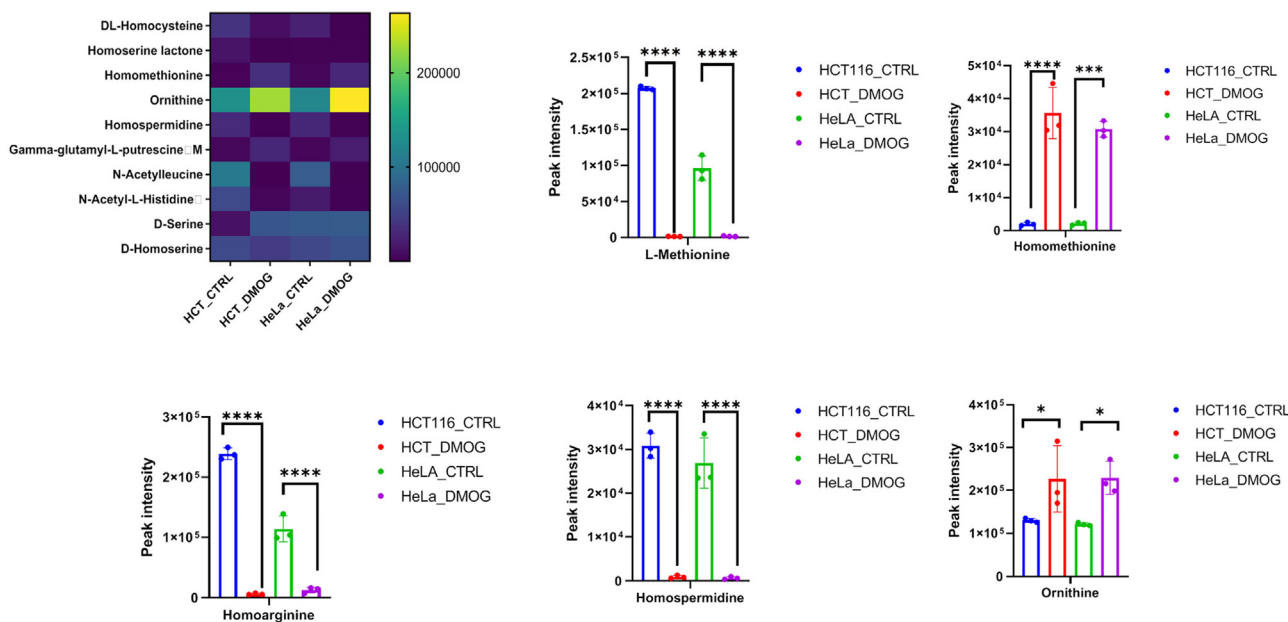

Fig. 5. DMOG alter one carbon metabolism of cancer cells. Quantitative levels of various metabolites involved in methionine pathway of control and DMOG treated cells, * $p < 0.01$. ** $p < 0.00$.
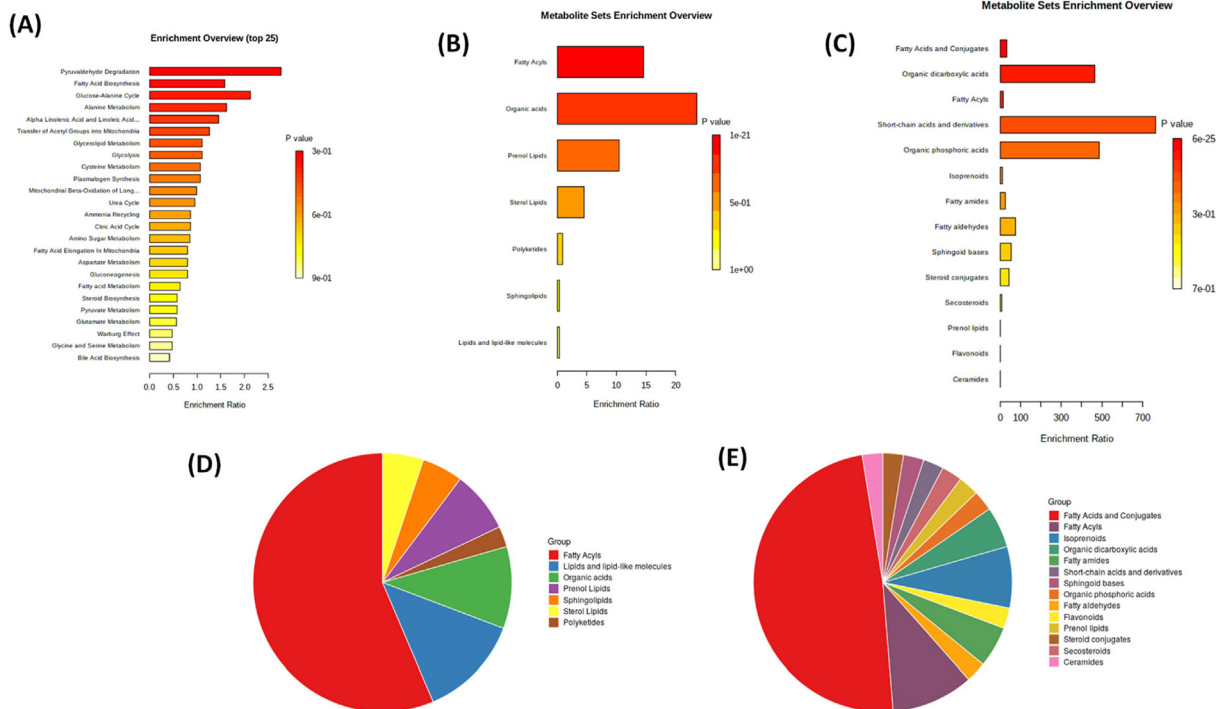
Fig. 6. DMOG alter lipid metabolism in cancer cells. (A) lipids involved in metabolic pathway. (B) Major structural class of lipids were mapped using MetaboAnalyst (C) Main structural class of lipids were mapped using MetaboAnalyst (D) The total accumulation of lipid in major structural class (E) The total accumulation of lipid in main structural class and their quantitative levels.
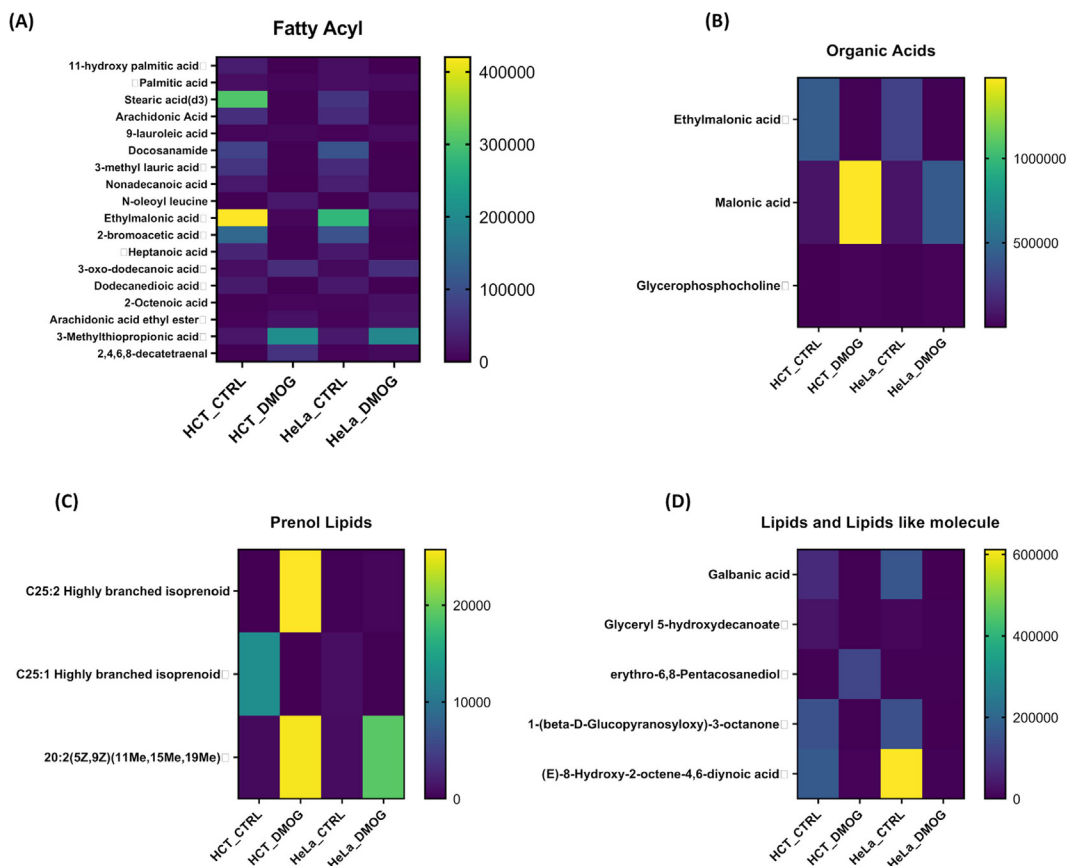

Fig. 7. DMOG alters fatty acid and prenol lipid class of cancer cells. Quantitative levels of various lipid class and their classification in control and DMOG treated cells, * $p < 0.01$. ** $p < 0.00$.

lipidome of cancer cells is not well established. In this regard, we have explored and identified differentially modulated lipid species in DMOG treated cancer cells. The lipid features from the untargeted metabolomics were mapped to the KEGG database platform of MetaboAnalyst 5.0 to identify differential lipids classes and various pathways of the DMOG treated cancer cells (Fig. 6). The list includes fatty acids as shown to be a major lipids class modulated by DMOG treatment in cancer cells compared to control (Fig. 6A). The super chemical class lipids (Fig. 6. B & D) showed that fatty acyls are one of the top enriched lipid sets and it occupies more than 50 % of total lipids identified and modulated by DMOG. In the main chemical class lipid analysis showed that fatty acid conjugates followed by organic dicarboxylic acids are top enriched lipid sets and fatty acid conjugates shared 48 % of total lipids (Fig. 6C & E).

Most fatty acyl lipids like palmitic acids, stearic acids, and arachidonic acid etc. were significantly reduced except 2,4,6,8-decatetraenal and 3-Methylthiopropionic acid (Fig. 7. A-B). Prenol lipids like isoprenoid-C25:2 observed to have higher expression level; conversely, isoprenoid-C25:1 lower expression level in DMOG treated cells compared to control. In other classes of lipids and lipid-like molecule, DMOG treatment resulted in a significant decrease in galbanic acid, glyceryl 5-hydroxydecanoate, *erythro*-6,8-pentacosanediol and (E)-8-hydroxy-2-octene-4,6-diynoic acid in both cell types. Similar to other classes of lipid, the organic acid lipid class also showed significant reduction in DMOG treated cells except malonic acid (Fig. 7. C-D).

Overall, we showed that DMOG treatment modulates not only the general metabolism but also modifies the cellular lipidome of cancer cells.

4. Discussion

The hydrolysed product of DMOG, called methyl oxoacetate ester NOG enters the cell through the monocarboxylate transporter 2 (MCT2). Increased concentration of NOG inhibits glutamine metabolism as a result implicating in cytotoxicity and ATP depletion. DMOG is reported to influence αKG function affecting αKG-dependent metabolism and signaling (Gong et al. 2012; Pértega-Gomes et al. 2013). The surface membrane of many cancer cell types was shown to have high MCT2 expression which aid in facilitating methyloxalylglycine (MOG) uptake (Kell. 2013). The pharmacodynamics of MOG is determined by the tumor microenvironment, a feature found in all cancer tissues (Gong et al. 2012; Pértega-Gomes et al. 2013; Gan et al. 2016). In this study, we have shown the impact of DMOG on cancer cells, through its modulation of cellular metabolism. First, we investigated the untargeted metabolomics and showed the metabolic identification and major regulatory pathways affected by DMOG. DMOG binds with αKG and affects multiple cellular metabolisms of cancer cells. The top enriched pathways identified in DMOG treated cancer cells include urea cycle, gluconeogenesis, Warburg effect, glutamate metabolism, alanine metabolism, TCA cycle, and betaine (one-carbon metabolism).

We observed that DMOG treated cancer cells switches cell metabolism toward glycolysis and increase pyruvate levels and reduction in lactate. This metabolic implementation shows a significant role in energy metabolism, and glutamate metabolism endorses glutathione biosynthesis (Christen et al. 2016). The role of glutamine metabolism is to decrease ROS by inducing antioxidants and shift energy production from glutamine via reductive carboxylation in cancer cells (Hamada et al. 2009) Our results showed that DMOG energy metabolism modulates glutamate uptake, thereby shifting metabolic gears towards OXPHOS in DMOG treated cancer cells.

We have identified one-carbon metabolism from our metabolomic data as an important pathway in DMOG treated cancer cells. Previous study has also results have also illustrated impact of one-carbon metabolism in extracellular matrix (ECM) detached cancer cells treated with vitamin C (Nur et al. 2021). Mechanistically, the one-carbon metabolism produces S-adenosylmethionine (SAM), which is the source of methyl group used for DNA methylation, and DNA methylation regulates gene expression (Nur et al. 2021). Alteration of one-carbon metabolism in DMOG treated cancer cells could be due to competitive binding of DMOG to α-KG. The α-KG is a known cofactor for certain demethylases, whose absence could lead to hypermethylation in the genomic CpG, protein methylation, and histone methylation. Since α-KG is also an intermediate in TCA cycle; therefore, reduction in α-KG will cause energy deprivation thereby compelling DMOG treated cells to shift towards Warburg effect and promoting hypoxia. Overall, our data suggests that in DMOG treated cancer cells alteration in one-carbon metabolism is due to increased homomethionine, ornithine and serine whose impact could alter the transfer of methyl group(s) to protein, DNA and RNA.

5. Conclusion

Collectively, the results provided novel insights of the metabolic impact of DMOG on cancer cells. Additionally, the use of DMOG to induce hypoxia yield similar metabolic features relative to physiological hypoxia (induced due to deprived oxygen condition). These metabolic features include TCA cycle, one-carbon metabolism, glutamate metabolism, Warburg effect and gluconeogenesis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.103426.

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