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Article

Profiling The Effect of Targeting Wild Isocitrate Dehydrogenase 1 (IDH1) on the Cellular Metabolome of Leukemic Cells

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Abstract: Leukemia is one of the most common primary malignancies of the hematologic system in both children and adults and remains a largely incurable or relapsing disease. The elucidation of disease subtypes based on mutational profiling has not improved clinical outcomes. IDH1/2 are critical enzymes of the TCA cycle that produces α-ketoglutarate (αKG). However, their mutated version is well reported in various cancer types, including leukemia, which produces D-2 hydroxyglutarate (D-2HG), an oncometabolite. Recently, some studies have shown that wild-type IDH1 is highly expressed in non-small cell lung carcinoma (NSCLC), primary glioblastomas (GBM), and several hematological malignancies and correlates with disease progression. This work shows the treatment of wild-type IDH1 leukemia cells with a specific IDH1 inhibitor switched leukemic cells towards glycolysis from oxidative phosphorylation (OXPHOS) phenotype. We also noticed a reduction in αKG in treated cells, possibly suggesting inhibition of IDH1 enzymatic activity. Further, we found that IDH1 inhibition reduces the metabolites related to one-carbon metabolism, which is essential to maintaining global methylation in leukemic cells. Finally, we observed that metabolic alteration in IDH1 inhibitor-treated leukemic cells promotes reactive oxygen species (ROS) formation and loss of mitochondrial membrane potential, leading to apoptosis in leukemic cells. We showed that wild wild-type IDH1 targeting leukemic cells promotes metabolic alterations that can be exploited for combination therapies for a better outcome.

Keywords: Wild Type IDH1; Metabolomics; Glutamine Metabolism; Reactive Oxygen Species; OXPHOS

1. Introduction

Leukemia is a leading cause of mortality worldwide [**1**]. The frequency of leukemia is recognized by mutation, and chromosomal aberration and translocation result in oncogenic activities. The present treatments continue to be with harmful side effects [**2**,**3**]. Recently, a new small molecular targeted inhibitor has been designed to transform deadly diseases into controllable conditions [**4**]. The impact of *IDH* mutations on AML prognosis persists somewhat debatable, although a generally secondary outcome is seen with *IDH1* mutations and a relatively favorable prognosis may be seen with *IDH2* mutations. Among these new molecular targets, isocitrate dehydrogenase family (IDH1/2) enzymes emerged as a desirable target.

IDH 1/2 family enzymes are metabolic enzymes that catalyze the oxidation of isocitrate to alpha-ketoglutarate in the citric acid cycle and produce crucial reducing equivalent NADPH [5]. Numerous new emerging reports of exomic sequencing identified

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frequent mutations in the IDH1 enzymes, or its homolog IDH2, in leukemia and other cancers. *IDH1* and 2 are important genes in acute myeloid leukemia [6,7,8].

Recently, it has been shown that wild-type IDH1 is highly expressed in a variety of cancer types like non-small cell lung carcinoma (NSCLC), primary glioblastomas (GBM), and several hematological malignancies and correlates well with disease progression [9,10]. Cancer cells are uncontrolled and quick proliferation without induction of external stimuli. To supply the energy needs cancer cells to undergo metabolic reprogramming other than Warburg effects. Leukemic cells are associated with high oxidative phosphorylation and allied with chemoresistance. IDH mutation plays a significant role in leukemogenesis and metabolism. IDH mutants downregulate ROS production and metabolic alteration. In leukemic cells, the TCA cycle plays a prominent role in energy metabolism and glutaminolysis. Glutamine is an important metabolite in the regulation of GSH and maintaining redox potential.

IDH1 inactivation results in decreased NADPH, with consequent exhaustion of GSH and increased levels of ROS, reduction of lipid biosynthesis, and enhanced histone methylation and differentiation marker expression. Similarly, shRNAs targeting IDH1 decreased in vitro and in vivo growth of NSCLC cell lines[11]. Furthermore, IDH1 silencing in a diffuse large B cell lymphoma (DLBCL) cell line decreased αKG and GSH production, with subsequent ROS increase and tumor growth reduction. These results showed that wild-type IDH1 is an essential therapeutic target in cancer.

Since wild-type IDH1 enzyme is a crucial metabolic enzyme, in this study, we aimed to understand the metabolic impact of targeting wild-type IDH1 by using GSK864 (an IDH1 mutant inhibitor, also inhibits wild type IDH1 at high concentrations) [12] in leukemia cell types as an experimental model. Results showed that targeting wild-type IDH1 inhibition shifts leukemia cell metabolism towards oxidative phosphorylation from glycolysis, enhances ROS, and deregulates membrane potential leading to cell death.

2. Results

2.1. Wild type IDH1 inhibition reduces leukemic cell proliferation

Leukemic cell lines Jurkat and MV4-11 were treated with wild-type IDH1 inhibitor, namely GSK864, and examined their cell proliferation assay (Figure 1A). IC50 values of GSK864 for these cell lines were examined after 48-hour treatment. Briefly, GSK864 treatment at the concentration of 2 μmol/mL showed significant s inhibitory effects on proliferation of both Jurkat and MV4-11 cell lines. The physiological examination under a microscope also showed that the number of cells increased towards apoptotic morphology (chromatin condensation)(Figure 1B).

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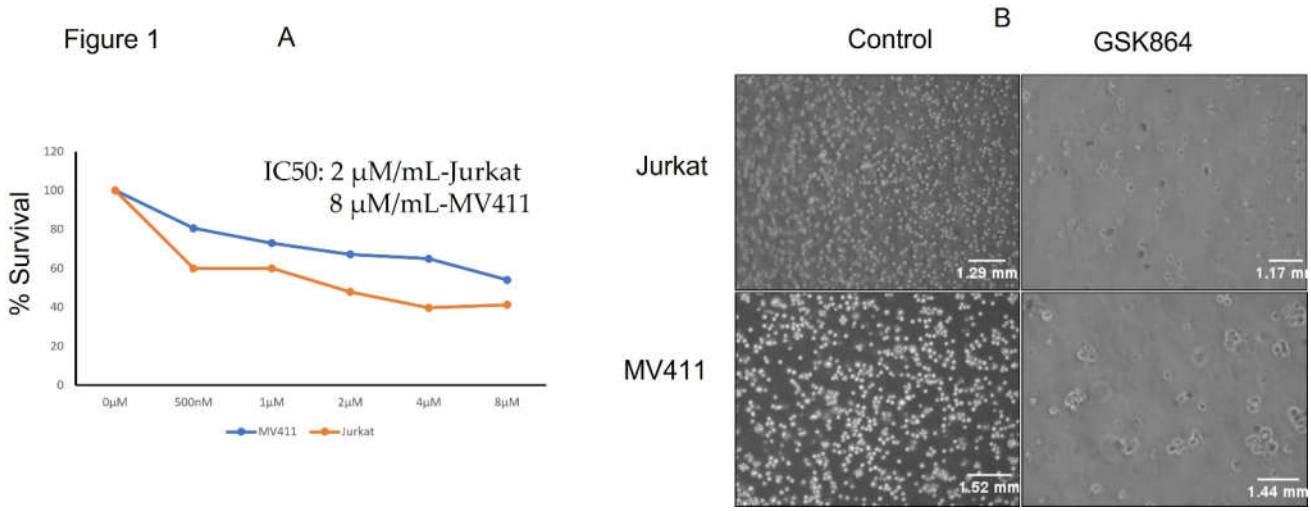

Figure 1. GSK864treatments reduce cell proliferation and alter cellular morphology (A, B). MTT assays were performed to determine cell viability upon treatment with different concentrations of GSK864for 48 h in both Jurkat and MV4-11. All the images are captured using a Nikon phase contrast microscope at 20×. $p < 0.01$.

2.2. Wild type IDH1 inhibition alters global leukemic cell's metabolic landscape

To investigate the metabolic landscape of leukemic cells treated with Wild type IDH1 inhibitor, i.e., GSK864, metabolites were extracted from untreated (mock) and GSK864 treated Jurkat and MV4-11cells. The untargeted metabolomics was performed using our house facility of GC–MS/MS. The spectra of the biological three replicates from the cell lines (Jurkat and MV4-11) were obtained. The intracellular metabolite's GC–MS/MS spectral separation(TIC- Total ion chromatogram) is shown in Supplementary Figure S1. A broadband comprehensive metabolite list with identification, p-value, features, and peak intensity is shown in Table S1. HMDB databases were used to identify the metabolic markers. The metabolomic variation between each sample was demonstrated by (multivariate analysis) using a PLS-DA score plot (Figure 2A), with FDR correction $p < 0.05$ and $q < 0.05$. Comparative representation of identified metabolites between untreated and treated samples was done using a metabolic heat map with an FDR-corrected q-value <0.05 and established by ward clustering (Figure 2B, C). Results GSK864 (IDHi) treatment.

For the pathway enrichment analysis, the differential regulated metabolites have been mapped to the KEGG database using MetaboAnalyst 5.0. A top 25 enriched pathways (Figure 2D), significantly with $p < 0.05$ (Supplementary Table S2). The enriched pathways involved are the Warburg effect, Gluconeogenesis, Urea cycle, Malate-Aspartate Shuttle, Glycolysis, Glycine and serine metabolism, and glutamate metabolism. Top significantly regulated metabolites are shown in (Figure 2E).

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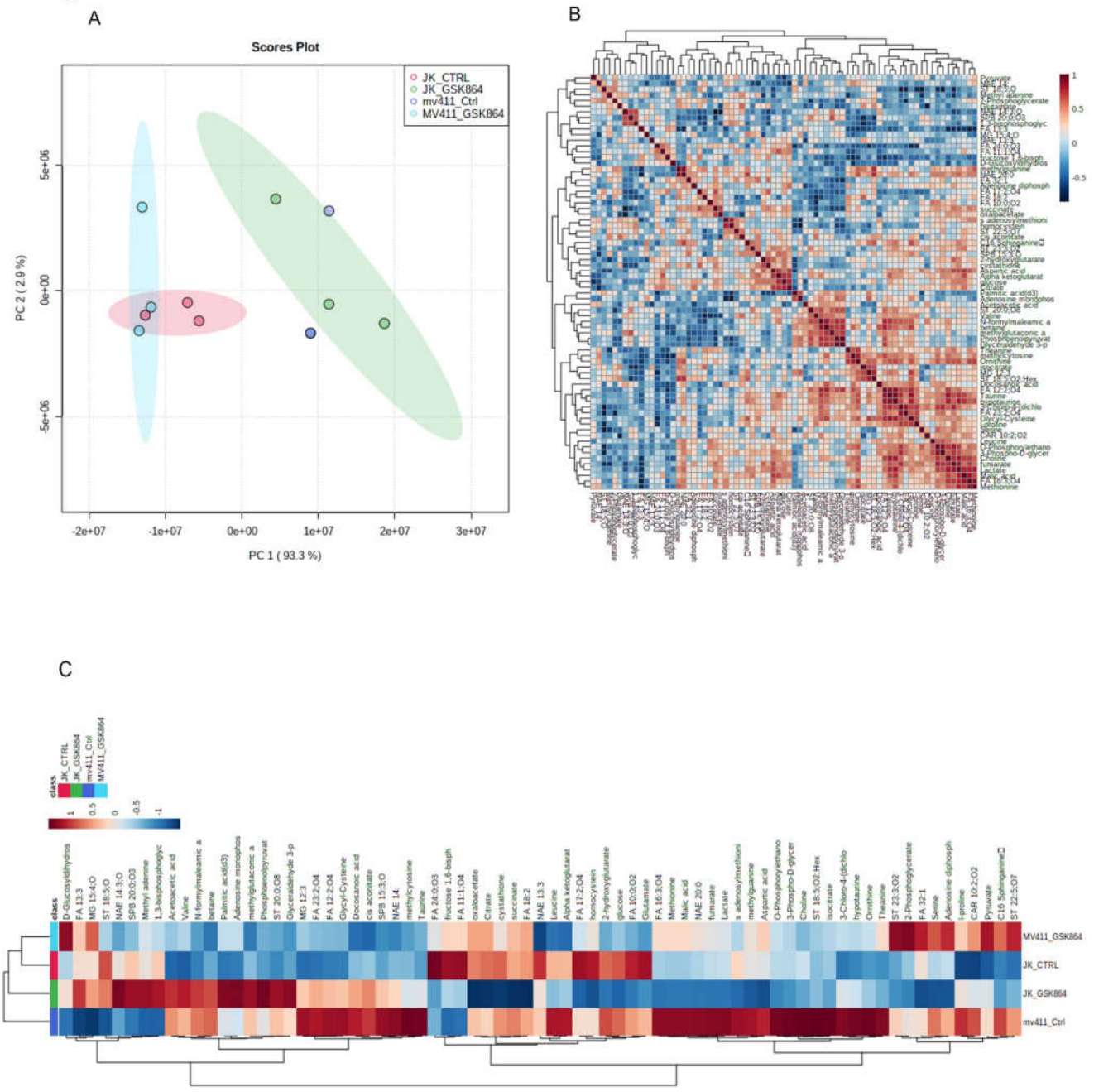
Figure 2



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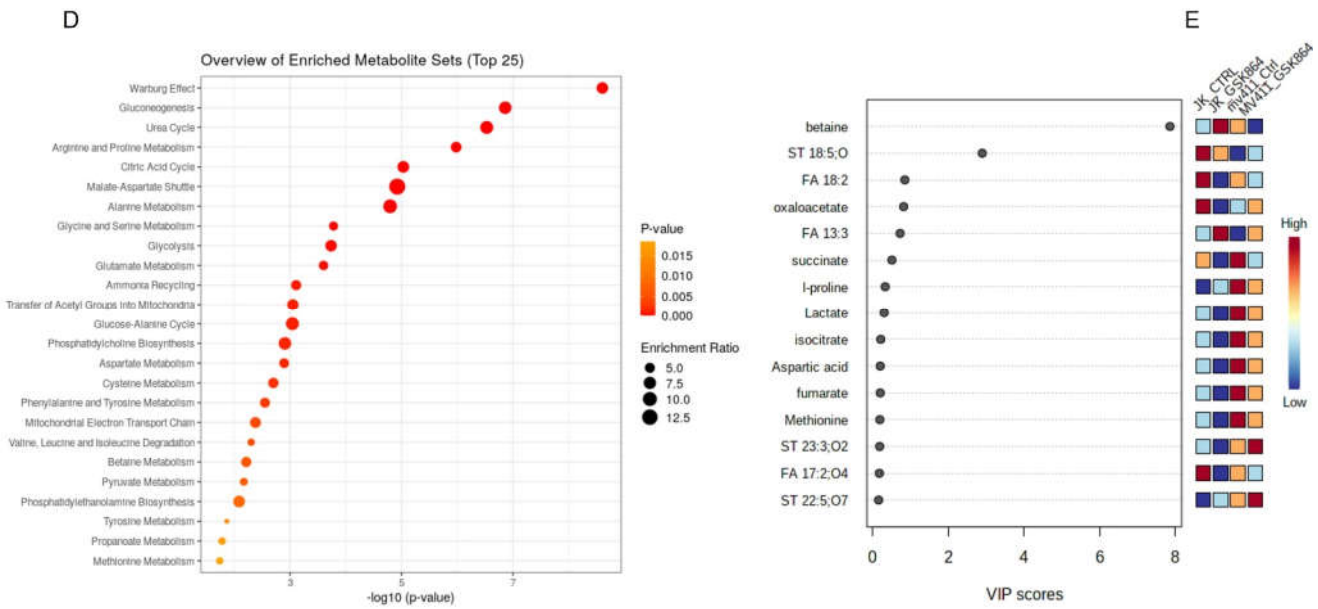

Figure 2. Metabolomic analysis of leukemic cells treated with IDHi: (A) PCA analysis of total metabolites of Jurkat and MV 4-11; (B and C) correlation and expression heat map of differential metabolites expressed in control and GSK864 treated; (C) top pathways enriched in Jurkat and MV4-11 control and GSK-864 treated cells; (E) VIP score for differentially expressed metabolites during GSK-864 treatment.

2.3. Wild type IDH1 inhibition modulates Warburg phenotype in leukemic cells

To uncover Warburg effect metabolic characteristics underlying the increased forming ability in IDHi. Most cancer cells utilize a high amount of glucose and the production of lactic acid as a fuel source of energy production. Remarkably, IDHi displayed enrichment of metabolites related to glycolytic intermediates, including fructose-1,6-bisphosphate (F1,6 P), 1,3-bisphosphoglycerate (3PG), and compared with untreated leukemic cells (Fig. 3). In addition, the metabolites of other glucose-utilizing pathways, monophosphate (AMP), were significantly accumulated in IDHi (Fig. 3). Consistent with an increase in glycolysis, it was enhanced in IDHi compared to untreated leukemic cells. These identified metabolic profiles were under anaerobic glycolysis, which impacted the Warburg effect. - a well-known hallmark of rapidly proliferating mammalian cells.

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Figure 3

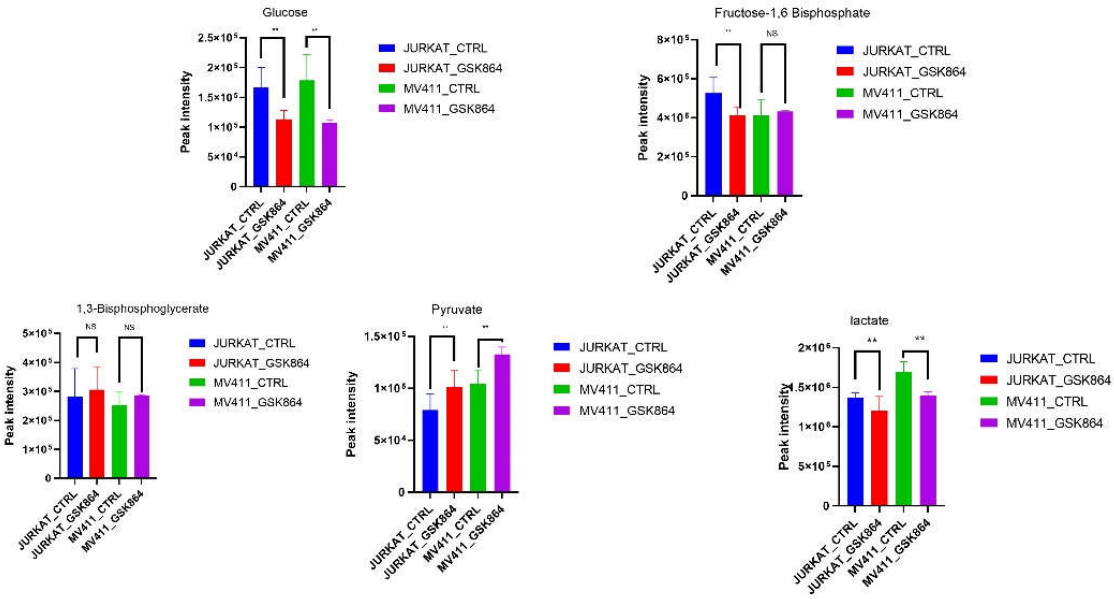

Figure 3. IDHi alters crucial cellular energy pathways in leukemic cells. Quantitative levels of various metabolites involved in glycolysis pathways of control and GSK864 treated cells, * $p < 0.01$. ** $p < 0.00$.

IDHi displayed enrichment of metabolites related to the TCA cycle, OXPHOS intermediates. However, some TCA cycle metabolites, including fumarate, citrate, α-ketoglutarate, acetoacetate, glutamate, oxaloacetate, and pyruvate, were reduced in IDHi (Supplementary Fig. These results suggest that leukemic cells have switched to more from glycolysis from oxidative phosphorylation (OXPHOS) phenotype and regulated by IDHi. (Figure 4).

Figure 4

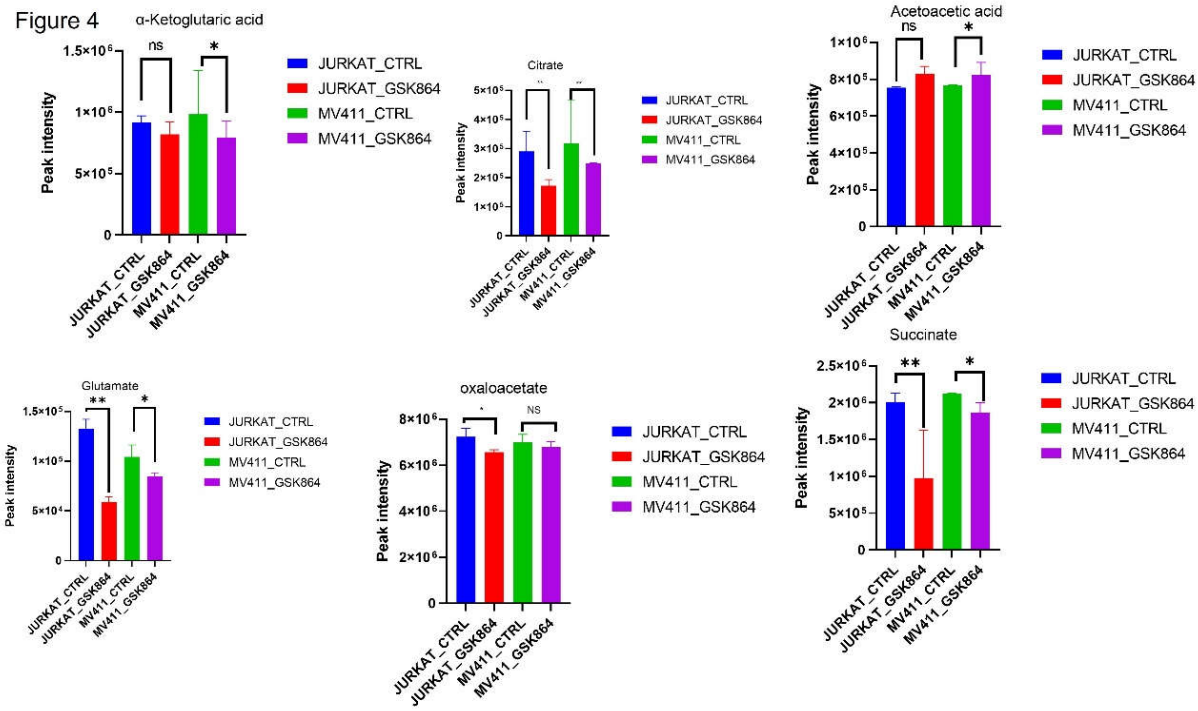

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Figure 4. IDHi alters crucial TCA cycle pathways in leukemic cells. Quantitative levels of various metabolites involved in TCA cycle pathways of control and GSK864 treated cells, * $p < 0.01$. ** $p < 0.00$.

2.4. Wild type IDH1 regulates One-Carbon Metabolism in leukemic cells

Methionine, taurine, and hypotaurine are essential in one-carbon metabolism since methionine is chronologically converted into SAM- S-adenosylmethionine. The methyl group will use it post modification of Histones, RNA, and DNA methylation. Taurine and hypotaurine are cysteine derivatives and crucial metabolites implicated in one-carbon metabolism. The results have shown that IDHi perturbation metabolites involved in one-Carbone metabolisms like methionine, taurine, SAM, Methyl cytosine, and ornithine levels (Figure 5). This result indicates that IDHi alters the methylation shape in leukemic cells.

Figure 5. IDHi alters one-carbon metabolism in leukemic cells. Quantitative levels of various metabolites involved in control and GSK864 treated cells involved in one-carbon metabolism,

2.5. Wild type IDH1 inhibition promotes mitochondrial depolarization in leukemic cell

The IDHi in leukemic cells perturbation TCA cycle intermediatory metabolites in the mitochondria, resulting in respiration inhibition and inducing mitochondrial depolarization. The IDHi reduces polarization in a mitochondrial membrane in MV4-11 and JURKAT, as evident by JC-1 dye (Figure 6). Results showed an approximately 15% reduction in mitochondrial polarization and a 17% increase in mitochondrial depolarization in MV4-11 cells treated with GSK864 compared to control. Still, JURKAT cells showed about a 30% reduction in mitochondrial polarization and a 40% increase in mitochondrial depolarization IDHi Both decreases in polarization and increase in depolarization of mitochondria membrane potential are characteristic features of mitochondria-mediated apoptosis (Figure 6).

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Figure 6

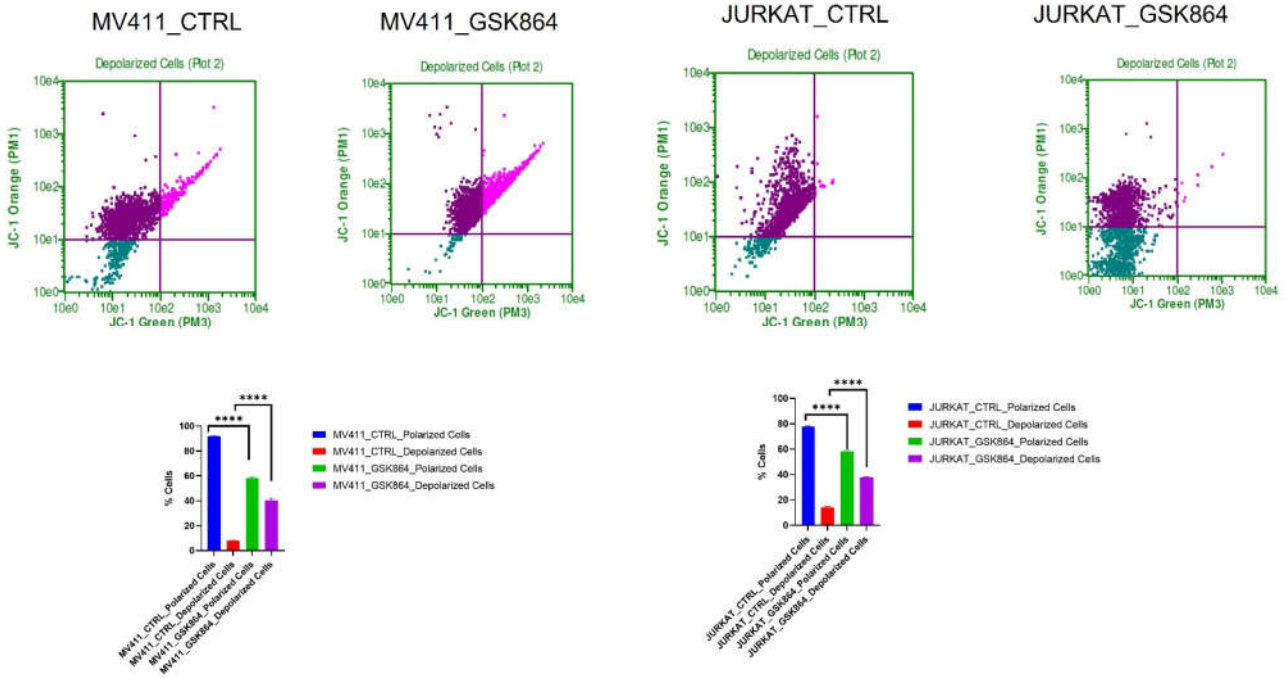

Figure 6. IDHi alters MMP in leukemic cells. Quantitative levels of mitochondrial membrane potential altered in control-treated leukemic cells, * $p < 0.01$. ** $p < 0.00$, $p^{****} < 0.0000$.

2.6. Wild type IDH1 inhibition promotes the accumulation of intracellular ROS and induces apoptosis

The ROS- reactive oxygen species are involved in the loss of growth control, genomic instability, and invasiveness.[13-14]-Disproportionate ROS is harmful to cells, resulting in oxidative damage to RNA, DNA, and proteins.20. the leukemic cells IDHi elevate the ROS level in MV4-11 and Jurkat cells. We observe IDHi induce Intracellular ROS levels of 6 % in MV4-11 and JURKAT shows 2 % induction in intracellular ROS levels(Figure 7).

Figure 7

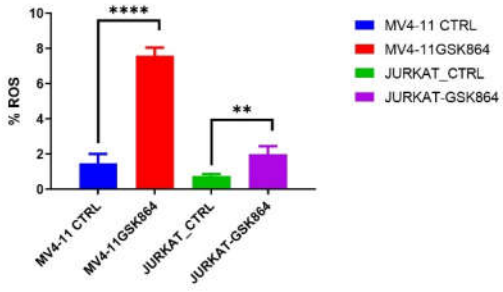

Figure 7. IDHi induces ROS leukemic cells. Quantitative levels of ROS in control and treated leukemic cells, ** $p < 0.00$, $p^{****} < 0.0000$.

Wild-type IDH1 is a potential therapeutic target; we treated cells leukemic cells with GSK864. Cytoxan Red was used to differentiate live from apoptotic cells. We observed

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that IDHi induces 17% of apoptosis in MV411 JURKAT. IDHi could prevent cell proliferation in leukemic cells (figure 8).

Figure 8

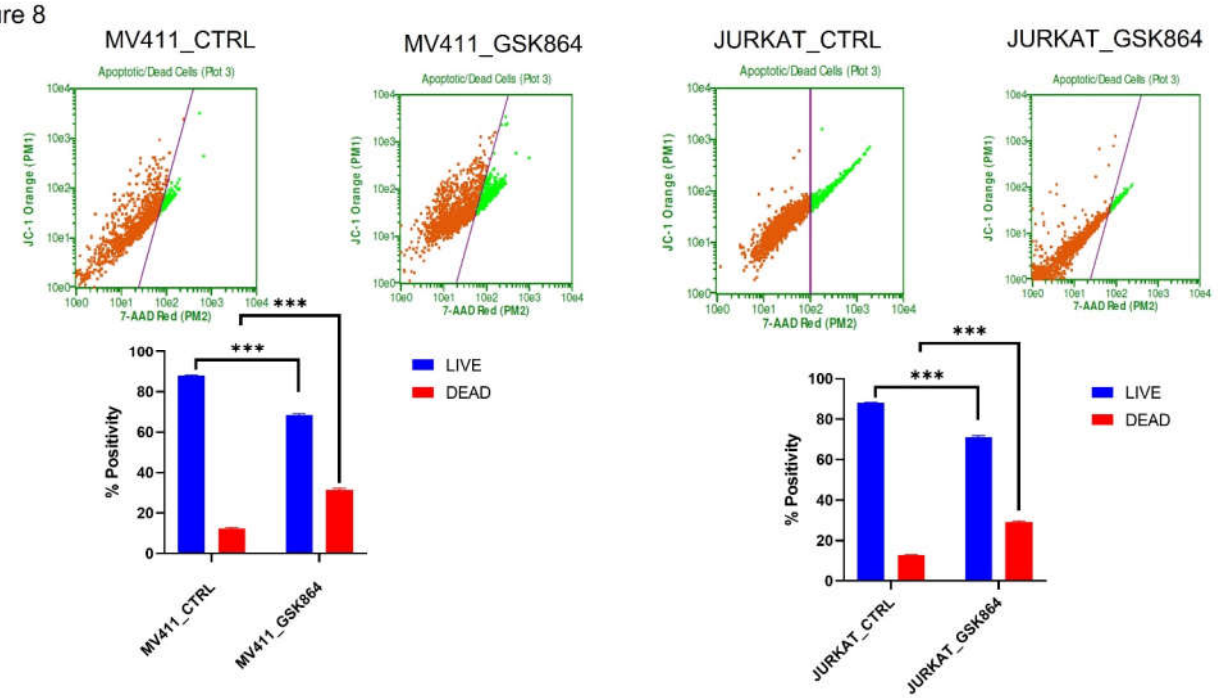

Figure 8. IDHi induces cell death in leukemic cells. Live and the dead assay was performed in control, and GSK864 treated leukemic cells, *** $p < 0.000$, $p^{****} < 0.0000$.

3. Discussion

In leukemia, the *IDH1*/*IDH2* gene restructures the enzyme for isocitrate while the increased affinity for α-ketoglutarate (αKG) with a production of 2HG [15]. Under functional conditions, the IDH isoforms produce metabolites product of the Krebs cycle. In contrast, IDH2 (the isoform localized in mitochondria) and IDH1 (the isoform localized in the cytoplasm and peroxisomes) are involved in oxidative decarboxylation and generate αKG from isocitrate and yield reduced NADPH from NADP+. Mutants of IDH1/2 mutants' catalyst the conversion of α-KG α-ketoglutarate to D2HG- D-2-hydroxyglutarate and promote glutaminolysis pathway to support carbon requirement for anaplerosis to fuel the Krebs cycle [15]. And they constrain αKG synthesis due to reduced glycolytic influx and diminishing decreasing kreb cycle [16]). Most of the observed IDH1 mutations result in an amino acid substitution at R132 in the enzyme. Mutational IDH1 creates a neomorphic activity of the enzyme that substitutes the product from alpha-ketoglutarate (αKG) to D-2-hydroxyglutarate (D-2HG), which accumulates to extremely high levels in tumors with IDH1 mutations (~100-fold increase) [6]. D-2 HG inhibits αKG-dependent dioxygenase enzymes activity, including Jumonji C domain-containing histone demethylases and Tet 5-methylcytosine (5mC) hydroxylases, resulting in epigenetic alterations and perturbed cellular differentiation that may contribute to tumorigenesis [9]

. First, we explored the global metabolic landscape of both cell lines treated with IDHi using untargeted metabolomics. Further, using this approach we identified , novel metabolites , and critical regulatory pathways pretentious by IDHi in the leukemic cells. Data showed that s the IDHi results in modifying the overall cellular metabolic rate of leukemic cells. The most perilous pathways regulated by IDHi are the Warburg effect, glycolysis, TCA cycle, one-carbon metabolism (glycine-serine metabolism), and lipid metabolism.

We observed that IDHi in leukemic cells switches cell metabolism toward glycolytic phenotype as suggested by , increase pyruvate levels, and decreases in lactate levels. This

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metabolic adaptation is well known to play a substantial role in energy metabolism. Glutamate–glutamine metabolism promotes glutathione biosynthesis [17]. The significant part of glutamine metabolism was to reduce ROS by increasing antioxidants and switching energy production from glutamine via the TCA cycle in cancer cells [18]. Our results showed that IDHi reduced glutamate levels and there by limits intracellular uptake, resulting in increased oxidative stress and OXPHOS in leukemic cells.

The mechanisms by which methylation arises, and its effects on cancer, are becoming better understood. *IDH1* encodes enzymes that convert isocitrate into α-ketoglutarate, and it is a cofactor for certain DNA demethylases, leading to hypermethylation in genomic CpG histone methylation. IDHi also competitively inhibited the formation of isocitrate into α-ketoglutarate, which inhibits the TCA cycle and metabolite involved in one Carbon-carbon metabolism. Our data suggest that IDHi results in reduced methionine and serine metabolism. However, these claims require additional confirmatiry experiments. Overall, we showed that targeting wild type IDH1 in leukemic cells invoke redox linked stress by modulating pathways associated with glutamine pathway.

4. Materials and Methods

4.1. Leukemic Cell Culture

Wild type IDH1 containing leukemic cell lines Jurkat and MV411 cells were obtained from ATCC, USA. Leukemic cell lines were cultured and maintained in RPMI (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco one-shot, USA), 50 U/mL pen strep (Gibco), 5% CO2 at 37 °C. The cells were treated with either vehicle control and GSK864 (Sigma SML1767) for 48 h [19].

4.2. MTT Assay

Briefly, one × 10^4 cells were seeded in 96-well plates, and cells were treated with different concentrations of GSK864. After 48 h of treatment, 10 µL of MTT (5 mg/mL) in the ratio of 1:10 was added. Then, the plate was incubated for another three h at 37 °C in a CO_2 incubator. After incubation, media was removed, and DMSO 100 µL was added and incubated at RT for 10 min. The OD was measured at 540 nm [1].

4.3. Metabolites Extraction

Metabolites were extracted from cells treated with GSK864. Treated cells were lysed immediately using a 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 hours at −20°C and spin for 15 min at 13,000 rpm at four °C[20-22,]. The supernatant was dried entirely in a speed vac. The derivatization was carried out in two steps, step1, protection of carbonyl function by adding 20 µL of 40mg/mL solution of methoxyamine hydrochloride in pyridine and heated at 30°C /90 min. Step 2 increased the volatile of the small molecules; the samples were treated with derivatizing agent 180 µL of N-Methyl-N-trimethylsilytrifluroroacetamide with 1% trimethylchlorosilane at 37°C /30 min[17].

4.4. GC-Mass Spectrometry

An Agilent 7890 GC oven was ramped by eight °C/min from 60°C (1 min initial time) to 315°C, resulting in a 31.875 min run time with cooling down to 60°C. 1 µL was injected into the Agilent split/splitless injector at 250°C by a 10µL syringe with 4 sample pumps, one pre-injection wash, and two post-injection washes using both solvent A and solvent B. The raw .D files were converted into a data format.

4.5. Data Processing and Analysis

The raw data were processed using open access to the XCMS online database. Peaks were searched against human metabolites in the Human Metabolome Database. Pathway analysis and statics were performed using Metaboanalyst [20-22].

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4.6. ROS Assay

1 X 10^6 cells were plated on six-well plates. The cells were treated with the GSK864 compound for 48 hours. The CELLROX (Invitrogen) was used to measure the level of intracellular reactive oxygen species (ROS) in live cells. GSK864 treated cells were incubated in a culture medium with 500nM CELLROX for a 60 min plate at 37 °C and 5% CO_2. Flow cytometry immediately analyzed the samples, using 488-nm excitation for the CellROX® Green [23,24].

4.7. Mitochondria Membrane Potential Assay

1. X 10^6 cells were plated on six-well plates. The cells were treated with the GSK864 compound for 48 hours treated with GSK864, and a 1 μg/mL JC-1 Solution reagent was added. After one hour of incubation,37 °C and 5% CO_2 and PI were added before 5 min and analyzed by flow cytometry. Values were calculated based on the control group [24].

4.8. Live and Dead Assay

1 X 10^6 cells were plated on six-well plates. The cells were treated with the GSK864 compound for 48 hours treated with GSK864. Cytoxan RED Solution reagent was added. After one hour of incubation at 37 °C at 5% CO_2 and analysis, the samples were immediately by flow cytometry. Values were calculated based on the control group [24]).

4.9. Statistical Analysis

Differences between control and urolithin A- and urolithin B-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.

5. Conclusions

In conclusion, we showed that inhibition of wild type of IDH modulates the overall inter-cellular metabolism in leukemic cells. Inhibition of wild type IDH1 facilitates the oxidative stress by modulating pathways associated with glutamine pathway, which ultimately results in apoptosis. Our results have provided clues for identifying novel metabolic targets that are crucial for maintaining viability in wild type IDH1 containing leukemic cells

Limitation and future work: we will compare our results with in vivo and IDH1/2 knockout model.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, M.I.K.; methodology, M.R.S., F.A.; software, H.C, and M.R.S.; validation, M.R.S., F.A., and M.I.K.; formal analysis S.H., M.R.S., F.A. and M.I.K; investigation, H.A.A.; resources, M.R.S and M.I.K; data curation, M.R.S., and F.A. and M.I.K.; writing—original draft preparation M.R.S.; writing—review and editing, F.A. and M.I.K.; visualization, H.C. and M.R.S.; supervision F.A. and M.I.K.; project administration, M.I.K.; funding acquisition F.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest

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