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Targeting Metabolic Pathways in AML Cell Lines: Impact of Hypoxia-Inducible Factor-1α (HIF-1α) and Lactate Dehydrogenase-A (LDH-A) Inhibition

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ABSTRACT

Introduction: The Warburg effect is considered one of the most important metabolic alterations in tumor cells. While extensively studied in solid tumors, the role of this effect in AML is less defined. HIF-1α and LDH-A are key regulators of glycolysis, promoting lactate production and the expression of lactate transporters. In this study, we examined the impact of HIF-1α and LDH-A inhibition on metabolic pathways, cell viability, and lactate transporter mRNA expression in AML cell lines.

Methods: K-562 and HL-60 cells were treated with silibinin, an HIF-1α inhibitor, and sodium oxamate, a LDH-A inhibitor. Cell viability and apoptosis were evaluated using the MTT assay and flow cytometry, respectively. Acidification rate and LDH-A activity were assessed using lactate assay and LDH-A assay kits, respectively. Relative mRNA expression of *MCT1* and *MCT4* was determined using qRT-PCR.

Results: Treatment with silibinin and sodium oxamate reduced proliferation and increased apoptosis in both K-562 and HL-60 cells. As expected, both agents decreased extracellular lactate release in K-562 cells, and sodium oxamate inhibited the LDH-A activity in both cell lines. Interestingly, the expression of *MCT1*, but not *MCT4*, was downregulated in K-562 cells after treatment.

Conclusion: Our findings show that HIF-1α and LDH-A inhibitors not only serve as cytotoxic drugs but also regulate the expression of lactate transporter and interfere with the metabolism-related mechanisms in AML cells. ***DOI: 10.61882/ibj.5155***

Keywords: Acute myeloid leukemia, Hypoxia, Lactates, Warburg effect

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INTRODUCTION

Acute myeloid leukemia is a complex heterogeneous malignancy of the myeloid lineage with high rate of morbidity and mortality, mostly affecting adults. While immunotherapeutic approaches have been introduced in clinical settings, chemotherapy is still the first-line treatment[1,2]. Cancer cells employ various immune

List of abbreviations
AML: acute myeloid leukemia; **DMSO:** dimethyl sulfoxide; **HIF-1α:** hypoxia inducible factor 1 alpha; **LDH-A:** lactate dehydrogenase A; **MCT:** monocarboxylate transporter; **PI:** propidium iodide; **qRT-PCR:** quantitative reverse-transcription PCR; **TME:** tumor microenvironment

evasion mechanisms to suppress the host immune responses. One of the main immune evasion mechanisms is the Warburg effect (aerobic glycolysis), which leads to a high rate of glucose uptake and lactate production in the presence or absence of oxygen.

LDH-A, as the main enzyme in cancer metabolism, converts pyruvate to lactate and NADH to NAD^+. This process compensates for reduced oxidative phosphorylation, meeting the energy needs for cancer cell survival and proliferation[3]. Lactate is an oncometabolite secreted from cancer cells into TME. It induces extracellular acidification, resulting in adverse effects on immune cell dysfunction[4].

HIF-1α is a transcription factor that targets a broad range of genes involved in metabolic alteration, evasion, metastasis, and angiogenesis in solid tumors. HIF-1α triggers the Warburg effect by transactivating and upregulating the *LDH-A* gene expression[5]. Within cancer cells, excessive lactate produced by LDH-A is exported into TME through MCT4. This export not only helps maintain a physiologic pH but also increases lactate levels in TME[6]. In the TME, the accumulation of MCT-mediated lactate in effector T cells suppresses the anti-tumor response, and the entry of lactate into T cells occurs along the concentration gradient[7]. A noteworthy point is that HIF-1α can upregulate *MCT4* expression and overexpress *MCT1* in cancer cells[8,9].

Studies have shown the role of LDH-A and HIF-1α in the metabolism of solid tumors, but further research is needed to elucidate their roles in hematological malignancies. Therefore, this study aimed to investigate the effects of LDH-A and HIF-1α on metabolic pathways and gene expression in AML cells to shed light on potential mechanisms of disease progression.

MATERIALS AND METHODS

Cell lines and reagents

K-562 and HL-60 cells, as AML models, were purchased from the Pasteur Institute of Iran (Tehran, Iran). Cells were cultured in RPMI-1640 medium (LM-R1640/500, Biosera, France) supplemented with 10% heat-inactivated fetal bovine serum (FB-1093/100, Biosera), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (XC-A4122/100, Biosera). Silibinin, an HIF-1α inhibitor, was purchased from Sigma-Aldrich (S0417, Massachusetts, USA), dissolved in DMSO, and stored frozen in aliquots. Sodium oxamate, an LDH-A inhibitor, was purchased from Santa Cruz Biotechnology (sc-215880, Texas, USA), dissolved in distilled water, and freshly prepared as a working stocks. Doxorubicin, an approved chemotherapy drug was purchased from Sobhan Oncology (Tehran, Iran).

Cell viability assay

As shown in Figure 1, viability of leukemic cells was evaluated using the MTT assay. K-562 (5×10^3/well) and HL-60 (1×10^4/well) were seeded in 96-well culture plates and treated with various concentrations of silibinin and sodium oxamate, and then incubated at 37 °C for 48 hours. Silibinin was used at concentrations of 400-1600 µM for both cell lines. Concentrations of

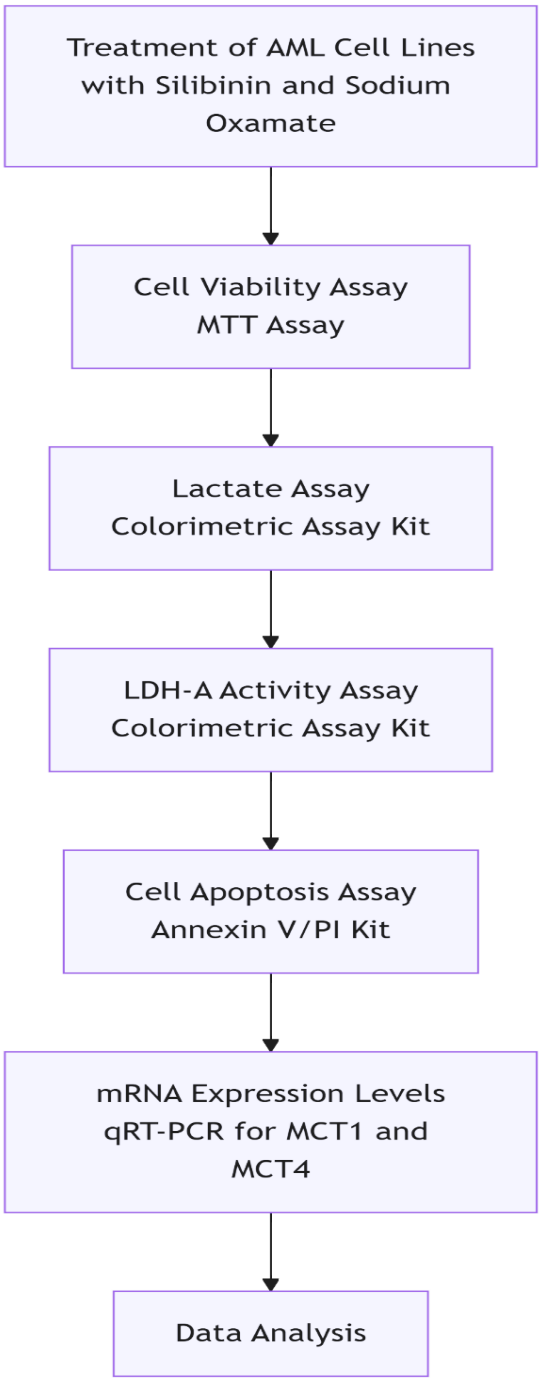

Fig. 1. Schematic overview of the integrated experimental workflow.

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sodium oxamate were 60-140 mM and 5-50 mM for K-562 and HL-60 cells, respectively. Also, doxorubicin was used as a chemotherapy drug at concentrations of 156.2-10,000 nM and 4.68-150 nM for K-562 and HL-60 cells, respectively. Following incubation, MTT reagent (475989, Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/mL, and the plate was incubated at 37 °C for 4 hours. The microplates were centrifuged at 300 ×g for 10 min, the supernatants were discarded, and 150 µL of DMSO (102952, Merck, Darmstadt, Germany) was added to each well. Crystals of formazan were dissolved through shaking of microplates, and absorbance was measured using a microplate spectrophotometer (Synergy H1 BioTek, Winooski, USA) at 570 nm and 720 nm (reference wavelength). Determination of cell viability was calculated as follows: percentage of cell viability = (absorbance sample – absorbance blank)/(absorbance control – absorbance blank) × 100. Culture medium without cells was considered as blank, and HL-60 cells cultured without any drugs (untreated cells) were defined as the control.

Lactate and LDH-A activity assays

K-562 and HL-60 cells were seeded in six-well cell culture plates at 7.5×10^4/well and 1.5×10^5/well, respectively, and treated with IC_{50} concentrations of silibinin (K-562: 1127 µM; HL-60: 811.1 µM), sodium oxamate (K-562: 84.95 mM; HL-60: 18.23 mM), and doxorubicin (K-562: 345.2 nM; HL-60: 76.38 nM) for 48 hours. Following incubation, supernatants were collected, and lactate concentrations were assessed using a lactate assay kit (917-181, Delta Darman Part, Tehran, Iran). Total LDH enzymatic activity was assessed using the LDH Assay Kit (1820424, Delta Darman Part).

Cell apoptosis assay

K-562 and HL-60 cells were seeded in six-well cell culture plates at 7.5×10^4/well and 1.5×10^5/well, respectively, and treated with optimized IC_{50} concentrations of silibinin. Apoptosis was then assessed via FITC-Annexin V and PI apoptosis detection kit (IQP-116F, IQ product, Groningen, the Netherlands) based on the manufacturer's protocol. Flow cytometry data were analyzed using FlowJo v10.8.1, which allowed for precise quantification of apoptotic populations. After excluding debris and doublets using forward and side scatter parameters, we gated cells based on Annexin V-FITC and PI staining. Early apoptotic cells were identified as Annexin V-positive and PI-negative, while late apoptotic/necrotic cells were double-positive for Annexin V and PI. Live cells were negative for both markers.

Quantitative reverse-transcription PCR

K-562 and HL-60 cells were seeded in six-well cell culture plates at 5×10^5/well, and then treated with IC_{30} and IC_{40} concentrations of silibinin and sodium oxamate for 48 hours. For K-562 cells, we utilized concentrations of 901.6 µM and 676.2 µM as the IC_{40} and IC_{30} of silibinin, and 67.95 mM and 50.96 mM as the IC_{40} and IC_{30} of sodium oxamate, respectively. For HL-60 cells, the IC_{40} and IC_{30} values of silibinin were 648.8 µM and 486.6 µM, and those of sodium oxamate were 14.58 mM and 10.93 mM, respectively. Total RNA was isolated using RNA Isolation Kit (FATRS 050, Favorgen Biotech Corp., Ping-Tung, Taiwan) based on the manufacturer's protocol. The quantity and quality of extracted RNA were checked and affirmed using spectrophotometry and RNA electrophoresis, respectively. Then, the cDNA was synthesized by reverse transcription of RNA using a cDNA Synthesis Kit (YT4500, Yekta Tajhiz Azma, Tehran, Iran) based on the manufacturer's protocol. To evaluate relative mRNA expression of *MCT*1 and *MCT4*, we designed primers using the CLC Genomics workbench and AlleleID software (Table 1). Their specificity was confirmed by BLAST analysis (NCBI), and their efficiencies were confirmed using the LinregPCR software. The average efficiency values for *HPRT*, *MCT1*, and *MCT4* genes were 1.89, 1.82, and 1.84, respectively. The qRT-PCR was carried out using the

Table 1. Primers used for qRT-PCR

Gene	Directions and sequences	Product size (bp)
SLC16A1 (*MCT1*)	Forward: TTCCATCGGCTTCTCTTATG Reverse: CACCAGGATACTGCTGATAG	157
SLC16A3 (*MCT4*)	Forward: AAGTTCTCCAGTGCCATTG Reverse: CAGAAGAAGTTGCCCAGC	127
HPRT1 (*HPRT*)	Forward: GGACTAATTATGGACAGGACTG Reverse: GCTCTTCAGTCTGATAAAATCTAC	195

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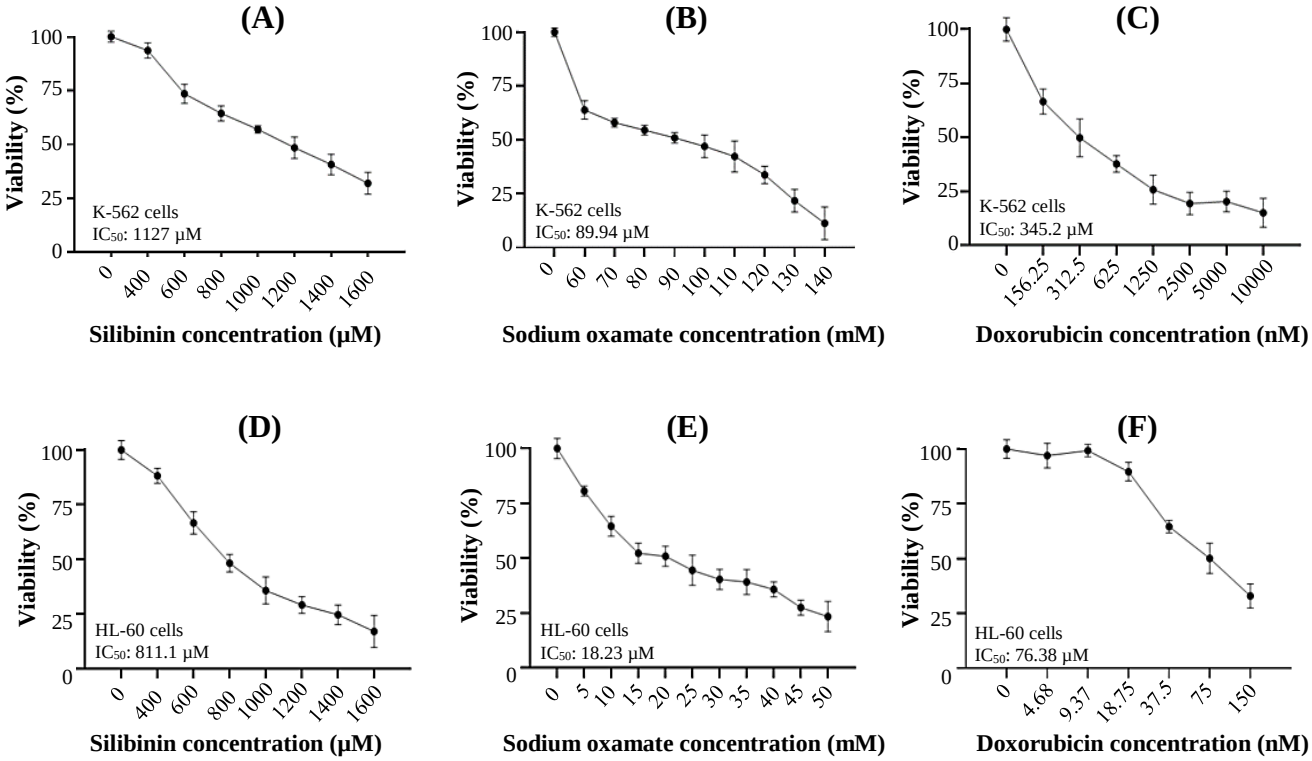

Fig. 2. Dose-concentration-dependent inhibition of AML cell viability by silibinin, sodium oxamate, and doxorubicin. Proliferation of the AML cell line was suppressed following 48 hours of treatment with silibinin and sodium oxamate. IC_{50} values were assessed using the MTT assay after exposure of K-562 (A, B, C) and HL-60 (D, E, F) cells to the indicated concentrations of silibinin, sodium oxamate, and doxorubicin. All experiments were performed in triplicate, and quantitative data are expressed as mean ± SEM.

real-time PCR master mix reagent (A315402-25, Amplicon, Copenhagen, Denmark) on a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA). After normalization to HPRT[10], relative expression levels of *MCT1* and *MCT4* were determined using the Pfaffl method[11].

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 software. Quantitative data are expressed as mean ± SEM. Analysis was performed using the Shapiro-Wilk test to determine the normal distribution of the obtained data. One-way ANOVA, followed by Dunnett's post hoc test, was also applied for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

HIF-1α and LDH-A inhibitors cause inhibitory effects on the proliferation of K-562 and HL-60 cells

The effects of HIF-1α and LDH-A inhibition on the proliferation of AML cells were investigated. Cell viability was assessed using the MTT assay after 48 hours of treatment with various concentrations of silibinin, sodium oxamate, and doxorubicin. As illustrated in Figure 2, treatment with these agents resulted in a concentration-dependent reduction in cell viability. Inhibiting the HIF-1α and LDH-A could decrease the viability and proliferation of AML cell lines, as these molecules play an important role in cancer cell proliferation.

Silibinin and sodium oxamate decrease the extracellular acidification rate in K-562 and HL-60 cells

To determine whether silibinin and sodium oxamate treatment reduce extracellular acidification rates by inhibiting HIF-1α and lactate production, we examined the effects of these agents on lactate release in the culture supernatants of AML cells. In the K-562 cells, both inhibitors significantly decreased extracellular acidification. Extracellular lactate release was significantly reduced by 29.5% in the silibinin-treated K-562 culture supernatant compared to the control group ($p < 0.0001$). Furthermore, in the sodium oxamate-treated K-562 culture supernatant,

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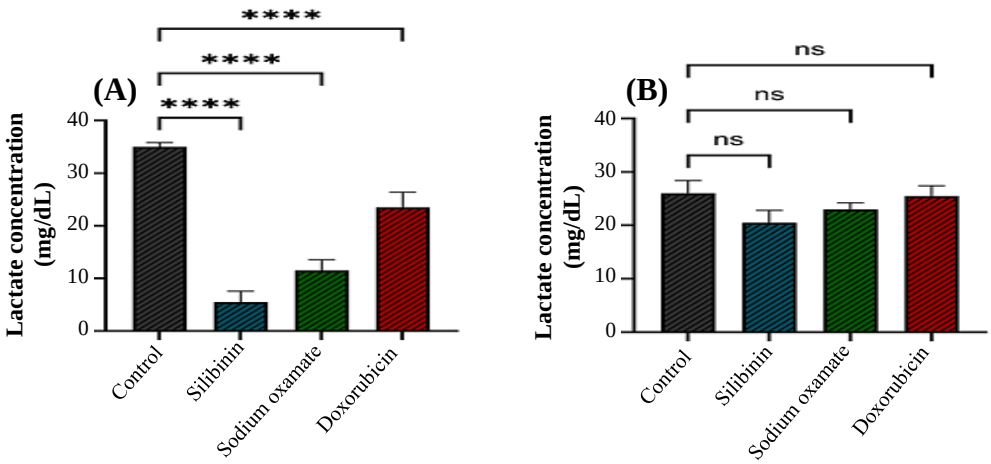

Fig. 3. Effect of silibinin, sodium oxamate, and doxorubicin on extracellular lactate release in AML cells. K-562 (A) and HL-60 (B) cells were treated with IC_{50} concentrations of silibinin, sodium oxamate, and doxorubicin for 48 hours. Production of lactate was assessed using a lactate assay kit. All measurements were performed in triplicate, and quantitative data are expressed as mean ± SEM ($^{****}p <$ 0.0001); ns: non-significant.

extracellular lactate release decreased by 21.5% compared to the control group ($p < 0.0001$; Fig. 3A). Surprisingly, there was no significant drop in extracellular lactate release in both the silibinin ($p = 0.1813$) and sodium oxamate-treated HL-60 culture supernatants compared to the control group ($p = 0.6046$; Fig. 3B).

Sodium oxamate inhibits LDH-A enzymatic activity in K-562 and HL-60 cells

To investigate whether sodium oxamate treatment reduces glycolysis by inhibiting LDH-A enzymatic activity, we assessed LDH-A inhibition using sodium oxamate. We observed that LDH-A activity was significantly reduced by 31.5% in the sodium oxamate-treated K-562 culture supernatant compared to the control group ($p < 0.0001$; Fig. 4A). Similarly, in the sodium oxamate-treated HL-60 culture supernatant, there was a remarkable decline in LDH-A activity of 55% compared to the control group ($p < 0.0001$; Fig. 4B). Results showed that sodium oxamate could potentially inhibit LDH-A enzymatic activity in both cell lines.

Silibinin and sodium oxamate induce apoptosis in K-562 and HL-60 cells

Targeting apoptosis plays an important role in cancer treatment. To investigate whether silibinin and sodium oxamate induce apoptosis in leukemic cells, we quantified apoptotic cell populations using Annexin V-

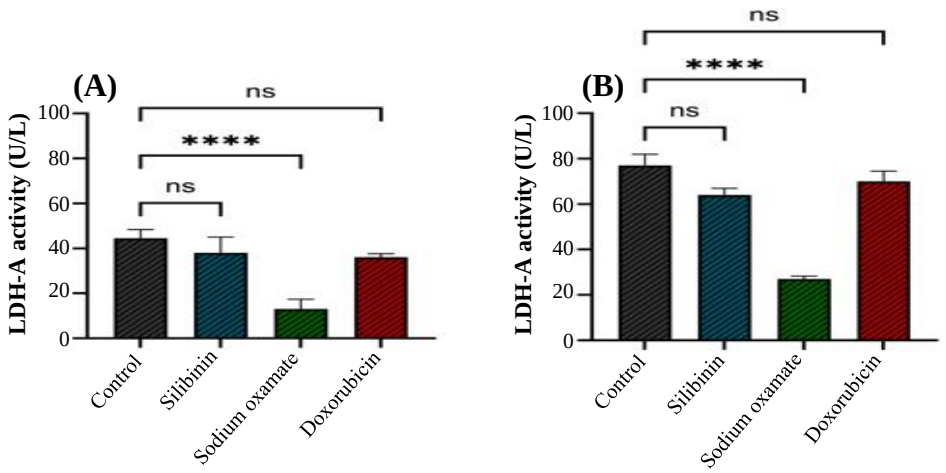

Fig. 4. Effect of silibinin, sodium oxamate, and doxorubicin on LDH-A enzymatic activity in AML cells. K-562 (A) and HL-60 (B) cells were treated with IC_{50} concentrations of silibinin, sodium oxamate, and doxorubicin for 48 hours. LDH-A enzymatic activity was assessed using an LDH assay kit. All measurements were performed in triplicate, and quantitative data are expressed as mean ± SEM ($^{****}p <$0.0001); ns: non-significant.

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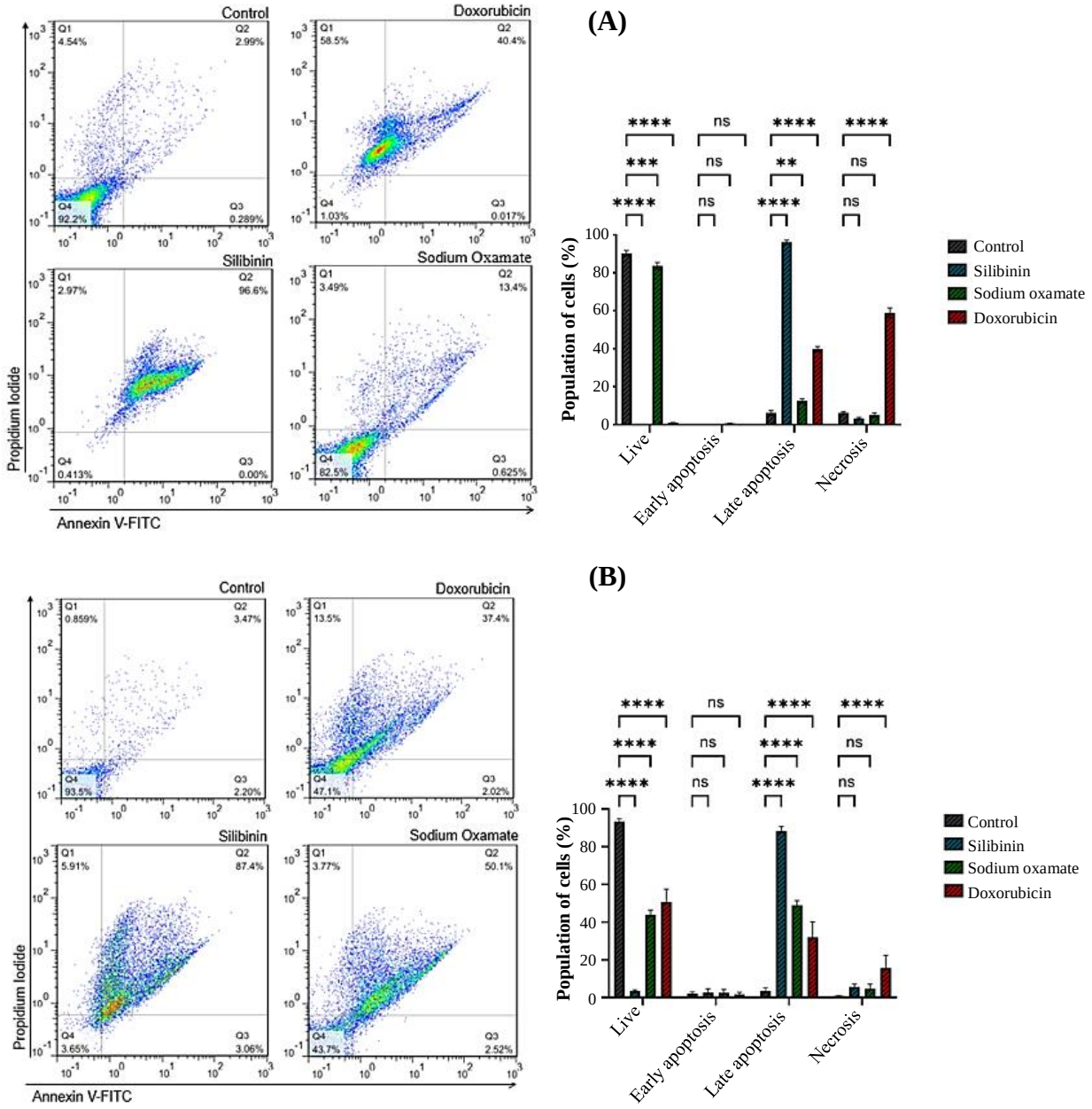

Fig. 5. Effect of silibinin, sodium oxamate, and doxorubicin on apoptosis induction in AML cells. K-562 (A) and HL-60 (B) cells were treated with IC_{50} concentrations of silibinin, sodium oxamate, and doxorubicin for 48 hours and stained with Annexin V-FITC/PI. Apoptosis was analyzed using flow cytometry. All measurements were performed in triplicate, and quantitative data are expressed as mean ± SEM ($^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$); ns: non-significant.

FITC/PI staining after treatments. As shown in Figure 5, silibinin significantly induced the apoptosis pathway compared to the control groups in both K-562 and HL-60 cells ($p < 0.0001$ for both). In silibinin-treated K-562 cells, greater sensitivity to apoptosis was observed compared to HL-60 cells. Additionally, sodium oxamate could significantly trigger the apoptosis pathway in both K-562 ($p < 0.0008$) and HL-60 ($p < 0.0001$) cells compared to the control groups, with HL-60 cells appearing more vulnerable to apoptosis induced by sodium oxamate compared to K-562 cells. A notable point was that both silibinin and sodium oxamate primarily induced apoptosis compared to necrosis in both K-562 and HL-60 cells. This finding indicates that HIF-1α and LDH-A play significant roles in cancer cell proliferation, and cancer cells are more likely to undergo apoptosis rather than necrosis by using the small molecule inhibitors. Cells were also treated with

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doxorubicin as a chemotherapy drug, which effectively induced cancer cell necrosis in both K-562 and HL-60 cells ($p < 0.0001$ for both).

Silibinin and sodium oxamate could not significantly reduce both *MCT1* and *MCT4* mRNA expression in K-562 and HL-60 cells

As *MCT1* and *MCT4* play a crucial role in extracellular acidification through lactate release, we assessed their mRNA expression alterations. To understand the effects of silibinin and sodium oxamate on relative mRNA expression of *MCT1* and *MCT4*, we conducted RT-qPCR at two different concentrations (IC_{30} and IC_{40}). As shown in Figure 6A, both IC_{30} ($p = 0.0416$) and IC_{40} ($p = 0.0046$) of silibinin significantly decreased *MCT1* mRNA expression in K-562 cells. Although IC_{40} ($p = 0.0185$) of sodium oxamate decreased *MCT1* mRNA expression, IC_{30} ($p = 0.1510$) did not significantly affect *MCT1* mRNA expression. A non-significant decrease in *MCT4* mRNA expression was observed in K-562 cells treated with IC_{40} ($p = 0.2488$) of silibinin compared to IC_{30} ($p = 0.6845$) and the control group, while there were no changes with either IC_{30} or IC_{40} of sodium oxamate. As illustrated in Figure 6B, IC_{40} of silibinin reduced *MCT1* and *MCT4* mRNA expression in HL-60 cells compared to IC_{30} and the control group, although this reduction was non-significant. In contrast, *MCT1* and *MCT4* mRNA expression levels remained constant in HL-60 cells treated with both IC_{30} and IC_{40} of sodium oxamate.

DISCUSSION

Metabolic regulation has become one of the most prominent strategies in cancer treatment. Targeting metabolic pathways can be conducted through various mechanisms. First, inhibition of glycolytic enzymes such as pyruvate kinase and LDH-A significantly promotes cancer cell death[12-14]. Second, suppression of pyruvate dehydrogenase kinase activates pyruvate dehydrogenase, which reduces ATP generation and ultimately contributes to cancer cell death[15]. Finally, HIF-1α functions as an important regulator of metabolic gene expression in cancer cells[16].

In this study, we assessed the impacts of HIF-1α and LDH-A inhibition on AML cells to determine the therapeutic potential of these two metabolic factors, given the importance of the Warburg effect. In cancer metabolism, the Warburg effect is recognized as a critical target for inducing cancer cell death. HIF-1α has been demonstrated to regulate a wide range of genes involved in tumor metabolism[17], angiogenesis[18], metastasis[19], and chemotherapy resistance[20]. Recent studies by Sellam et al. and Zhang et al. have displayed the pro-tumor activities of HIF-1α and LDH-A in promoting cancer cell proliferation[21,22]. Consistent with their results, our observations revealed that the inhibition of HIF-1α and LDH-A by silibinin and sodium oxamate significantly decreased the proliferation rate of AML cells. This finding indicates that silibinin and sodium oxamate could specifically target HIF-1α and LDH-A.

Incorporating approved chemotherapy drugs with experimental drugs may enhance therapeutic efficacy and validity, particularly in apoptosis assays. Chemotherapy drugs often trigger necrosis, while experimental agents tend to induce late apoptosis. Our apoptosis assay results indicated that doxorubicin significantly induced necrosis in AML cell lines, likely due to the severe cellular stress. In contrast, silibinin and sodium oxamate primarily induced late apoptosis, indicating the more targeted mechanisms of action.

The observed association between reduced acidification, decreased LDH-A activity, and lower cancer cell viability prompted us to examine this relationship. Silibinin-treated cells showed significantly reduced acidification rates, with most undergoing late apoptosis. Additionally, sodium oxamate-treated cells exhibited remarkably decreased LDH-A activity in the culture supernatant, accompanied by late apoptosis in some of the cells. These findings suggest that silibinin and sodium oxamate could potentially inhibit HIF-1α and LDH-A, reducing lactate release into the supernatant. HIF-1α may contribute to this effect by downregulating *LDH-A* and *MCT1* expression levels. Reduced LDH-A activity limits the conversion of pyruvate to lactate, while decreasing *MCT1* expression impairs lactate transport. In both conditions, cancer cells undergo late apoptosis due to intracellular lactate accumulation and decreased pH. These results align with findings from other studies reporting that silibinin reduces lactate release in S2-013 and T3M4 cell lines, and sodium oxamate lowers LDH-A activity in the A-549 cell line[23,24].

Our data showed that although extracellular lactate release was significantly reduced in K-562 cells treated with silibinin, sodium oxamate, and doxorubicin, no remarkable changes were observed in HL-60 cells. This discrepancy may reflect cell line-specific differences in genetic background and inherent metabolic characteristics. Moreover, extracellular lactate release was correlated positively with *MCT1* gene expression in cells, due to the role of MCT1 in releasing lactate to the extracellular space. The more lactate was released, the more the *MCT1* gene was expressed. qRT-PCR analysis depicted reduced *MCT1* gene expression in K-562 cells following treatment with silibinin and sodium oxamate,

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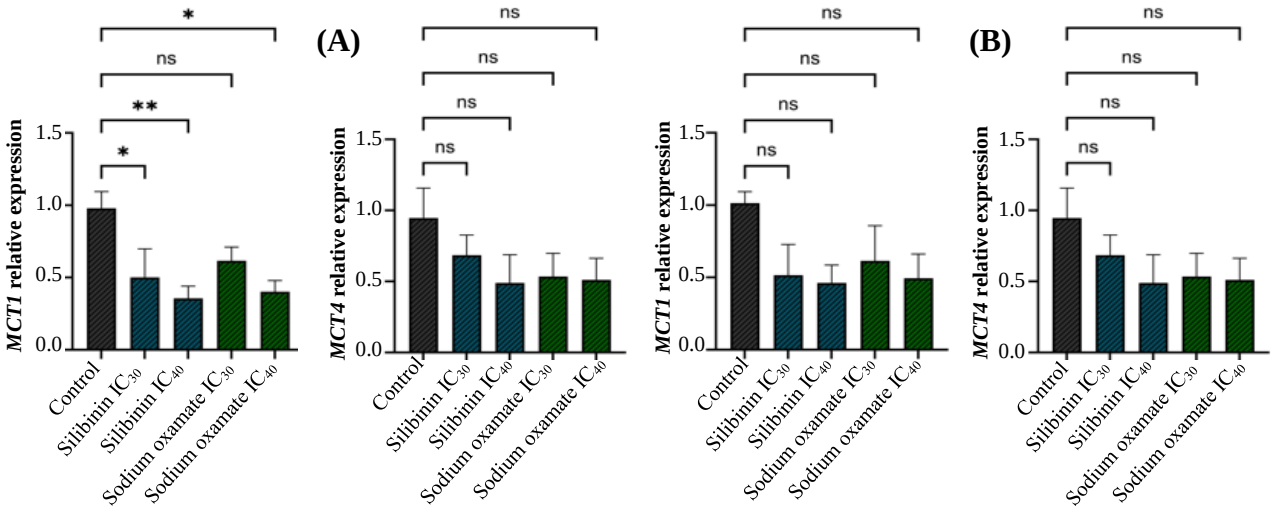

Fig. 6. Effects of silibinin and sodium oxamate on *MCT1* and *MCT4* mRNA expression levels in treated AML cells. K-562 (A) and HL-60 (B) cells were treated with IC_{30} and IC_{40} concentrations of silibinin and sodium oxamate for 48 hours. Relative mRNA expression of *MCT1* and *MCT4* was measured using qRT-PCR. Treatments with IC_{30} and IC_{40} concentrations were performed in triplicate, while qRT-PCR reactions were conducted in duplicate. Quantitative data are expressed as mean ± SEM ($^{*}p < 0.05$; $^{**}p < 0.01$). ns: non-significant.

whereas HL-60 cells showed no change. Therefore, AML cell lines differ in *MCT1* and *MCT4* mRNA expression as well as in lactate release via MCTs.

Regarding the hypothesis that HIF-1α increases *MCT1* and *MCT4* gene expression, our data demonstrated that the inhibition of HIF-1α via silibinin significantly reduced mRNA expression levels of *MCT1* but did not affect *MCT4*. This finding supports the findings of Liu et al., who reported a reduced *MCT1* expression level under hyperoxia[9], indicating that reduced hypoxia and HIF-1α activity could decrease *MCT1* mRNA expression. Although we did not observe significant changes in *MCT4* expression following silibinin treatment, Choi et al. have shown that *MCT4* expression is reduced when HIF-1α is inhibited[25]. They have also reported that HIF-1α binds directly to the *MCT4* gene promoter and regulates its expression. Sodium oxamate, as a specific LDH-A inhibitor, reduces cytosolic enzymatic activity, limiting the conversion of pyruvate to lactate and decreasing lactate concentrations. Current evidence does not support a direct link between reduced lactate levels and changes in *MCT* mRNA expression. This is consistent with the fact that lactate, while recognized as an oncometabolite, remains confined to the cytosol and lacks the capacity to act as a transcription factor capable of binding to the *MCT* gene promoter.

This study presents certain limitations, such as the use of only two cell lines, reliance on in vitro data, and the absence of functional protein assays. Future research is encouraged to validate these findings in primary AML samples, conduct in vivo experiments, and explore co-culture assays involving T cells.

CONCLUSION

Inhibition of HIF-1α and LDH-A by silibinin and sodium oxamate exerted significant anti-leukemic effects in AML cells by reducing proliferation, metabolic activity, and overall viability. These findings highlight the therapeutic potential of targeting key glycolytic regulators in AML and warrant further in vivo and mechanistic investigations. The selective inhibition of proteins overexpressed in cancer cells suggests a promising therapeutic strategy with minimal impact on normal hematopoietic cells. However, further preclinical studies are needed to confirm their efficacy.

DECLARATION

Acknowledgments

Copilot was used to improve clarity and correct grammar in some sentences. The authors retained full control over the content, ensuring that the original ideas and arguments were preserved without alteration.

Ethical approval

All the experimental procedures in this study were approved by the Research Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (ethical code: IR.MAZUMS.IMAMHOSPITAL.REC. 1401.043.

Consent to participate

Applicable.

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Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AK: conceptualization, data management, formal analysis, research, methodology, project management, resources, software, writing, original draft, writing, editing, and review; HAO: methodology, project management, and resources; ST: formal analysis and methodology; RV: methodology and project management; AN: conceptualization, research, and methodology; AA: conceptualization, research, methodology, and resources; EZ: resources; RS: resources; MEJ: resources; MT: conceptualization, data management, formal analysis, research, methodology, project management, resources, software, supervision, validation, and visualization, writing, editing, and review.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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