

# Effect of HIF-1 $\alpha$ and LDHA Blockade on Gene Expression of Tumor Immune Evasion in Myeloid Leukemia Cell Lines

Tamana Eskandari<sup>1</sup>, Mehrshad Shams<sup>2</sup>, Ali Khanari<sup>2</sup>, Amir Kahrizi<sup>2</sup>, Hossein Asgarian-Omran<sup>2,3</sup>, Reza Valadan<sup>2,4</sup>, Mohsen Tehrani<sup>2,4</sup>, Saeid Taghiloo<sup>2,4,5\*</sup>

<sup>1</sup>Department of Immunology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran;

<sup>2</sup>Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari,

Iran; <sup>3</sup>Gastrointestinal Cancer Research Center, Mazandaran University of Medical Sciences, Sari, Iran;

<sup>4</sup>Molecular and Cell-Biology Research Center, Mazandaran University of Medical Sciences, Sari, Iran; <sup>5</sup>Thalassemia Research Center, Hemoglobinopathy Institute, Mazandaran University of Medical Sciences, Sari, Iran

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## ABSTRACT

**Background:** Tumor cells utilize metabolic reprogramming to obtain the energy required for rapid proliferation. In this study, we blocked two key factors, hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) and lactate dehydrogenase (LDHA), in the metabolic pathway in acute myeloid leukemia (AML) cell lines to evaluate their effect on the expression of genes involved in tumor evasion.

**Methods:** Silibinin and sodium oxamate, as HIF-1 $\alpha$  and LDHA blockers, respectively, were used to treat K-562 and HL-60 cells. The MTT assay was used to assess cell viability, and quantitative reverse transcription PCR was used to measure the mRNA expression levels of PD-L1, CD47, and CD155.

**Results:** Both K-562 and HL-60 cells showed a significant decrease in CD47 expression. Additionally, HL-60 cells indicated a significant suppression of CD155 expression. In contrast, PD-L1 expression remained unchanged after treatment.

**Conclusion:** Our findings suggest that blocking signaling pathways involved in metabolic reprogramming of cancer cells could be a promising approach for modulating the expression of certain immune checkpoint ligands, warranting further investigation. **DOI: 10.61882/ibj.5324**

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

**Corresponding Author:** Saeid Taghiloo

Department of Immunology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran; Tel.: (+98-11) 33543081;

Fax: (+98-11) 33543249; E-mail: saeid.taghiloo@yahoo.com, s.taghilo@mazums.ac.ir; ORCID ID: 0000-0002-8813-6046

## 1. INTRODUCTION

Acute myeloid leukemia (AML) is a type of blood cancer characterized by the rapid proliferation of immature myeloid cells, leading to the production of cancerous blood cells<sup>[1]</sup>. Despite treatment advances, relapse is common due to the ability of leukemic cells to escape immune surveillance, a process facilitated by complex interactions with the tumor micro-environment<sup>[2]</sup>.

In normal cells, glucose first enters the cell, undergoes glycolysis, and is converted to pyruvate. This pyruvate then enters the mitochondria, where it undergoes the Krebs cycle and oxidative phosphorylation to produce

acetyl-CoA and generate energy for the cell<sup>[3]</sup>. However, the metabolic processes in cancer cells differ significantly from those in normal cells. These variations allow tumor cells to escape the immune system, promote metastasis, and develop resistance to anticancer drugs. The Warburg effect, or aerobic glycolysis, is one of the major metabolic adaptations observed in cancer cells. Cancer cells rely on glycolysis to produce energy, even in hypoxic conditions. These metabolic alterations lead to rapid ATP production and the adoption of an acidic microenvironment within the tumor, thus resulting in the invasiveness of cancer cells<sup>[4]</sup>.

Metabolic reprogramming is a hallmark of cancer cells that allows for their survival under hypoxia. The hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) pathway is central to this adaptation, as it upregulates anaerobic glycolysis-related genes, including lactate dehydrogenase A (LDHA), to stimulate the Warburg effect<sup>[5]</sup>. During glycolysis, pyruvate is converted to lactate by the enzyme lactate dehydrogenase and bypasses the Krebs cycle. Enhanced LDHA activity leads to lactate accumulation and acidification of the tumor microenvironment, which further enhances immune evasion<sup>[3,4]</sup>. HIF-1 $\alpha$  and LDHA have also been shown to regulate immune checkpoint receptors (ICRs), which not only affect immune cell metabolism but also promote immune escape.

*CD47*, an “don’t eat me” signal that is overexpressed in AML, blocks macrophage phagocytosis and is associated with poor prognosis<sup>[6-9]</sup>. Similarly, *PD-L1* inhibits T-cell response through its interaction with the *PD-1* co-receptor, promoting immune tolerance<sup>[10]</sup>. Another mechanism of evasion involves the *CD155* axis, which is part of the TIGIT/DNAM-1 pathway that regulates natural killer and T-cell responses<sup>[11,12]</sup>. Increasing evidence highlights the interplay between HIF-1 $\alpha$ /LDHA and ICRs, with hypoxia and lactate accumulation leading to the upregulation of *CD47* and *PD-L1*. In contrast, the effect of metabolic stress on *CD155* expression appears to be more variable and context-dependent, suggesting a distinct regulatory mechanism<sup>[8,13-19]</sup>.

In this study, we hypothesized that targeting HIF-1 $\alpha$  and LDHA could impair metabolic adaptation and immune evasion in AML. We used silibinin, a natural flavonoid known for its antitumor activity by modulating metabolic pathways<sup>[20]</sup>, and sodium oxamate, an LDHA blocker, which inhibits glycolysis<sup>[21]</sup>. Both sodium oxamate and silibinin block HIF-1 $\alpha$  stabilization and LDHA activity, rewire glycolytic flux, and promote apoptosis in leukemic cells<sup>[22,23]</sup>. Our findings showed the effects of blocking HIF-1 $\alpha$  and LDHA with the above-mentioned two agents on the expression of *CD47*, *CD155*, and *PD-L1*, providing preliminary evidence that metabolic reprogramming may influence the expression of immune checkpoint ligands. These results reveal that this therapeutic strategy could improve the immunogenicity of AML and help overcome resistance to anticancer treatments.

## 2. MATERIALS AND METHODS

### 2.1. Cell lines and reagents

K-562, a chronic myeloid leukemia cell line in blast crisis, and the HL-60, an AML cell line, were obtained from the Pasteur Institute of Iran (Tehran). The HIF-1 $\alpha$  inhibitor, silibinin (Sigma-Aldrich, USA), was

dissolved in dimethyl sulfoxide (DMSO), aliquoted, and kept at -18°C. The LDHA inhibitor, sodium oxamate (Santa Cruz Biotechnology, USA), was prepared by dissolving it in distilled water, with all working solutions being made fresh immediately before use.

### 2.2. Cell culture and treatment

K562 and HL-60 cell lines were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. The cells were maintained under standard conditions in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The half-maximal inhibitory concentration (IC<sub>50</sub>) values were determined on K-562 and HL-60 in our previous study<sup>[24]</sup>. For subsequent experiments, cells were seeded in six-well plates at a density of  $5 \times 10^5$  cells per well and treated with their respective IC<sub>50</sub> concentrations of either silibinin or sodium oxamate for 48 hours. Under these treatment conditions, cell viability was assessed and remained above 50%, ensuring that cytotoxic stress did not interfere with subsequent gene expression analyses.

### 2.3. Quantitative reverse transcription PCR (qRT-PCR)

K-562 and HL-60 cells were cultivated at a density of  $5 \times 10^5$  cells per well in six-well plates. Silibinin and sodium oxamate were then added to the cells at optimal concentrations for 48 hours. Control cells were treated with an equivalent volume of vehicle (0.1% DMSO for silibinin experiments or distilled water for sodium oxamate experiments). Total RNA was extracted using an RNA isolation kit (Favorgen Biotech Corp., Ping-Tung, Taiwan) according to the manufacturer’s protocol. Complementary DNA was produced from the isolated RNA using a reverse transcription kit (Yekta Tajhiz Azma, Tehran, Iran). Specific primers were created using the CLC Genomics Workbench and AlleleID software to assess the relative mRNA expression levels of *PD-L1*, *CD47*, and *CD155* (Table 1). The efficacy of the primers was verified using the LinRegPCR program. The qRT-PCR analysis was carried out on an Applied Biosystems StepOne RT-PCR system, utilizing RT-PCR Master Mix. The thermal cycling protocol consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, primer-specific annealing at 58-60°C for 30 seconds, and extension at 72°C for 30 seconds. To verify the specificity of the amplified products, a melting curve analysis was performed following the amplification cycles. Gene expression levels were calculated relative to the internal control gene,  *$\beta$ -actin* (Table 1). Quantification was performed using the Pfaffl method<sup>[25]</sup>, which incorporates the amplification efficiencies of the respective primer pairs.

**Table 1.** Primers used for qRT-PCR

| Gene           | Primer sequences (5'→3')   | Product size (bp) |
|----------------|--|-------------------|
| <i>PD-L1</i>   | 5'-CTATGGTGGTGCCGACTACAA-3'<br>3'-CTGCTTGCCAGATGACTTCG-5'        | 159               |
| <i>CD47</i>    | 5'-GTCATTGTCATTGTTGGAGCCATT-3'<br>3'-GGAGGTAAATCCAATCGCTGTACT-5' | 147               |
| <i>CD155</i>   | 5'-GGACGGCAAGAATGTGAC-3'<br>3'-CCAGTTGTTATCATAGCCAGA G-5'        | 124               |
| $\beta$ -actin | 5'-CCTTCCTGGGCATGGAGTCCT-3'<br>3'-TGG GTG CCA GGG CAG TGA T-5'   | 174               |

#### 2.4. Statistical analysis

Data are presented as mean  $\pm$  SEM from six independent biological experiments ( $n = 6$ ). For each cell line and treatment, gene expression in the treated group was compared to that of its corresponding vehicle-treated control group. Pairwise comparisons were performed using an unpaired, two-tailed Student's *t*-test. The normality of data distribution was confirmed using the Shapiro-Wilk test. A *p* value  $<0.05$  was considered statistically significant.

### 3. RESULTS

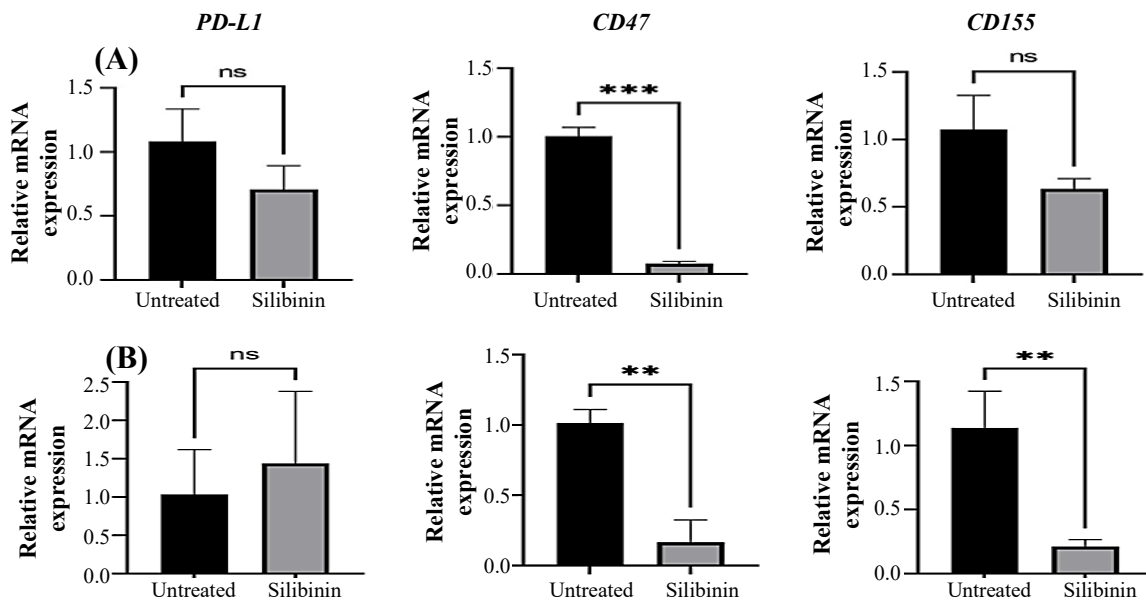
#### 3.1. Effect of HIF-1 $\alpha$ blockade on mRNA expression level of immune checkpoint ligands in AML cells

To explore how blocking HIF-1 $\alpha$  affects the expression of immune checkpoint ligands in AML cell lines, we treated the cells with silibinin, a blocker of HIF-1 $\alpha$ . After treatment, the relative mRNA levels of

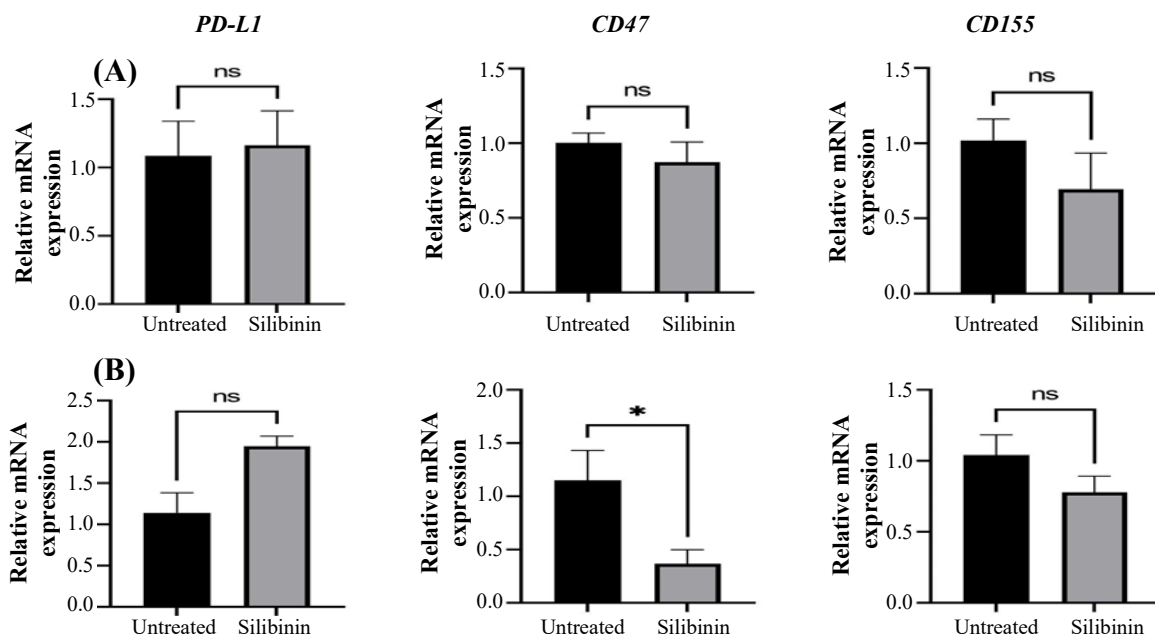
*PD-L1*, *CD47*, and *CD155* were measured using qRT-PCR. As shown in Figure 1A, silibinin significantly lowered *CD47* mRNA levels ( $p < 0.001$ ) in K-562 cells compared to untreated controls, while it did not have a notable effect on the expression of *PD-L1* and *CD155*. In HL-60 cells, silibinin treatment significantly reduced the expression of both *CD47* and *CD155* ( $p < 0.01$ ) but did not alter *PD-L1* levels (Fig. 1B).

#### 3.2. Effect of LDHA blockade on mRNA expression levels of immune checkpoint ligands in AML cells

We treated AML cell lines with sodium oxamate, a blocker of LDHA, to investigate how blocking LDHA affects the expression of immune escape molecules. qRT-PCR was used to assess the mRNA levels of *PD-L1*, *CD47*, and *CD155*. In K-562 cells, there were no significant differences in the expression of *PD-L1*, *CD47*, or *CD155* compared to untreated cells (Fig. 2A).



**Fig. 1.** Effects of silibinin on *PD-L1*, *CD47*, and *CD155* mRNA expression levels in treated AML cell lines. (A) K-562 and (B) HL-60 cells were treated with silibinin for 48 hours. Relative mRNA expression of *PD-L1*, *CD47*, and *CD155* were then measured using qRT-PCR. Treatments were performed in triplicate, and qRT-PCR analyses were conducted in duplicate ( $n = 6$ ). Data are presented as mean  $\pm$  SEM. Statistical significance was determined using an unpaired *t*-test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ); ns: not significant.



**Fig. 2.** Effects of sodium oxamate on *PD-L1*, *CD47*, and *CD155* mRNA expression levels in treated AML cell lines. (A) K-562 and (B) HL-60 cells were treated with sodium oxamate for 48 hours. Relative mRNA expression of *PD-L1*, *CD47*, and *CD155* was then measured using qRT-PCR. Treatments were performed in triplicate, and qRT-PCR analyses were conducted in duplicate ( $n = 6$ ). Data are presented as mean  $\pm$  SEM. Statistical significance was determined using an unpaired t-test ( $*p < 0.05$ ); ns: not significant.

In contrast, HL-60 cells exhibited a significant reduction in CD47 mRNA expression ( $p < 0.05$ ) after LDHA blockade, while the levels of *PD-L1* and *CD155* remained stable (Fig. 2B).

#### 4. Discussion

ICRs and their ligands are critical for self-tolerance but are often exploited by cancers to evade immunity. Therefore, targeting ICRs such as *PD-L1*, *CD47*, and *CD155* has become an effective immunotherapeutic strategy<sup>[26-30]</sup>. According to the classical Warburg effect, cancer cells primarily depend on anaerobic metabolism and glycolysis. This metabolic shift is regulated by transcription factors like HIF-1 $\alpha$ , which activates genes such as LDHA that catalyze lactate production, aiding tumor survival, growth, and adaptation to low-oxygen conditions<sup>[31-35]</sup>. Blocking this metabolic pathway may alter the expression of ICRs and boost antitumor immune responses.

Silibinin, a natural flavonoid extracted from *Silybum marianum*, exhibited significant anticancer properties across various tumors, including skin, lung, breast, and prostate cancers. Its mechanism involves blocking HIF-1 $\alpha$  and LDHA, which disrupts the metabolic adaptation of cancer cells to hypoxia and reduces the expression of ICR ligands, including *PD-L1*<sup>[36-38]</sup>. Sodium oxamate, a competitive LDHA blocker, decreases lactic acid production in tumor cells, reshaping the immune-

suppressive tumor microenvironment into an immune-active one. Research conducted on non-small cell lung cancer models has demonstrated that sodium oxamate suppresses LDHA activity, reduces tumor viability, and enhances the infiltration of immune cells, immune checkpoint blockers, like anti-PD-1 antibodies, by alleviating lactic acid-induced immunosuppression and promoting antitumor immunity<sup>[39]</sup>.

Studies across different cancer types, including nasopharyngeal carcinoma and lung cancer, support the simultaneous targeting of HIF-1 $\alpha$  and LDHA as a promising strategy to inhibit tumor growth and modulate ICR expression, potentially overcoming immune resistance<sup>[38,39]</sup>. This approach provides a strong rationale for using silibinin and sodium oxamate to suppress HIF-1 $\alpha$  and LDHA in leukemia cell lines. We hypothesized that blocking HIF-1 $\alpha$  and LDHA would reduce the expression of critical immune checkpoint ligands—namely *PD-L1*, *CD47*, and *CD155*—thereby countering a key mechanism of tumor immune escape.

*PD-L1*, a key ligand for PD-1, is often overexpressed in tumors to inhibit T-cell function, facilitating immune evasion<sup>[26]</sup>. Our findings showed that silibinin not only did not significantly alter *PD-L1* expression but also did not reduce it as expected. This observation implies that *PD-L1* expression in these cells may be regulated independently of HIF-1 $\alpha$  or LDHA activity, possibly

reflecting compensatory activation of alternative transcription factors, such as *c-Myc*<sup>[40]</sup>, or the involvement of other transcriptional regulators<sup>[41]</sup>, or signaling pathways<sup>[40,42,43]</sup>. Conversely, silibinin significantly reduced *CD47* expression, a molecule that sends “don’t eat me” signals to macrophages, preventing phagocytosis<sup>[6]</sup>. This downregulation could enhance the immune system ability to eliminate leukemic cells, underscoring silibinin therapeutic potential. Sodium oxamate decreased *CD47* expression only in HL-60 cells, reflecting possible differences in cellular biology and metabolism between the two cell lines<sup>[44]</sup>.

Regarding *CD155*, which modulates immune inhibition through interactions with natural killer and T-cell receptors<sup>[45]</sup>, silibinin reduced its expression only in HL-60 cells, with no effect on K-562 cells. Sodium oxamate did not significantly change *CD155* expression. These results suggest cell type-specific regulation and distinct drug impacts on pathways controlling *CD155*<sup>[46]</sup>.

The variable responses to silibinin and sodium oxamate likely stem from their unique molecular targets and mechanisms. The broader range of actions for i antioxidant and anti-inflammatory effects, along with its modulation of multiple signaling pathways, may explain its more substantial influence on gene expression<sup>[47]</sup>. Additionally, the contrasting behaviors of HL-60 and K-562 cells highlight the importance of cellular context in determining the efficacy of targeted therapies.

This study has limitations. First, we measured mRNA expression, but future studies should assess protein levels and provide functional validation. Second, pharmacological inhibitors may have off-target effects; genetic approaches could provide more specific insights. Third, our findings are based on two leukemic cell lines; hence, using primary AML samples or in vivo models would strengthen clinical relevance. Finally, while using fixed IC<sub>50</sub>-based dosing regimen is a standard approach, it may not account for potential stress-related effects that occur at sub-IC<sub>50</sub> concentrations, while standardized, may induce stress responses. Conducting full dose-response studies in future work could help dissect whether the observed regulatory mechanisms are a direct pharmacological response or an indirect consequence of cellular stress.

## 5. CONCLUSION

Our findings indicate that pharmacological inhibition of HIF-1 $\alpha$  and LDHA can modulate the mRNA expression of certain immune checkpoint ligands, including *CD47* and *CD155*, in leukemic cell lines. These preliminary observations suggest a potential link between tumor metabolism and immune

regulation; however, they should be interpreted as pharmacologically induced associations rather than direct evidence of HIF-1 $\alpha$  or LDHA pathway blockade. Future studies should validate these findings at the protein and functional levels and explore the combination of metabolic inhibitors with existing immunotherapies to assess potential synergistic effects.

## DECLARATION

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### Generative AI and AI-assisted technologies

Copilot was used to improve clarity and correct grammar in some sentences.

### 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.REC.1404.112).

### Consent to participate

Not applicable.

### Consent for publication

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

### Authors' contributions

TE and MS: carried out the assays and contributed to data collection and analysis; AKh: helped in performing assays; AK: helped in performing assays; HAO: helped in PCR optimization and analysis of PCR data; RV: helped in PCR optimization and analysis of PCR data; MT and ST: designed and conducted the research.

### Data availability

The dataset presented in the study is available upon request from the corresponding author during submission or after its publication.

### 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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