RESEARCH ARTICLE

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Thiosemicarbazone Complexes and 6-MP Suppress Acute Lymphoblastic Leukemia via the *NOTCH* Signaling Pathway and Regulation of *LUNAR1* and *NALT1 lncRNA*

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Abstract

Background: Acute Lymphoblastic Leukemia (ALL) is the most common type of leukemia among children. There are several types of drugs that are common in treating and controlling leukemia, including 6-M. Moreover, the anti-cancer effects of the Thiosemicarbazone-Ni complex were surveyed as well as 6-MP. The current study aimed to evaluate the effect of the Thiosemicarbazone-Ni complex in comparison with 6-MP on the expression of *NALT1* and *LUNAR1 LncRNAs* and their target gene, *NOTCH1*, in the Jurkat E6.1 cell line. **Materials and Methods:** Jurkat E6.1 cell was treated with 6-MP with the concentrations of 1, 5, 10, and 25μM and 0.5, 1, 2, and 5μM of Ni-nanoparticles, loaded with Thiosemicarbazone complexes for 24, 48, and 72 h. Subsequently, the RNA extraction and cDNA synthesis were performed and the expression of *NALT1, LUNAR1, NOTCH1*, and *GAPDH* genes was surveyed by Real-time PCR. Finally, the results were analyzed by statistical analysis. **Results:** The highest significant reduction in the expression of LUNAR1 was observed at 6-MP at 10μM for the first 72 h and the Thiosemicarbazone-Ni complex showed its effective dose at 5μM for 48 h. The highest decrease in NOTCH1 expression was observed in 6-MP treatment at 10μM concentration for 24h and Ni-Thiosemicarbazone treatment, the significant decrease was at 0.5μM for 72h. The most significant reduction in NALT1 expression was observed in treatment with Ni-Thiosemicarbazone complex and 6-MP at the concentrations of 2 and 10μM respectively for 24h. **Conclusion:** Given the association between the aforementioned genes and the positive effects of the drugs at achieved concentrations and times, it can be concluded that Thiosemicarbazone-Ni complex and 6-MP can show putative roles in regulating the expression of studied genes.

Keywords: *Nalt1- Notch1- Lunar1-* Acute lymphoblastic leukemia- Mercaptopurine

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Introduction

Acute lymphoblastic leukemia (ALL) is a lethal disease resulted by the alteration and promotion of lymphoid progenitor cells in the bone marrow and blood. Although leukemia is more prevalent among children, adults can suffer from ALL in a devastating manner [1, 2]. T cell transformation to a malignant cell has different stages in genetic alteration contributing to uncontrolled cell growth, increased amplification, survival, and distinction during thymocyte development [3]. T-ALL cells show other specific immunophenotypic characteristics like ALL-related T-cell precursors (ETP). As an example, ALL-related T cells differs from other types of T cells lacking in the expression of *CD1a* and *CD8* and low expression of *CD5* [4].

It has been suggested NOTCH1 signaling activation is one of the main carcinogenic pathways in T cell transformation [3] which occurs in more than 50% of T-ALL cells [5]. Although these signaling pathways is necessary in T cell progression, the genetic alteration in these pathways can lead to the pathogenesis of ALL [6]. Rare chromosomal translocations such as t (7;9) and (q34ؾq34.3) result in mutations in the human *NOCH1* gene in T-ALL cells [5] *NOTCH1* gene is the most prominent oncogene being involved in the pathogenesis of T-ALL [7]. Due to the importance of NOTCH signaling pathway in the generation and differentiation of stem cells, the appropriate function of this pathway is necessary in survival of these cells. In a nutshell, any alteration in Notch signaling pathway can be a key factor in the progression of leukemia [5, 8].

NALT1 expression is associated with increased level of *NOTCH1* gene. Increased level of *NALT1* expression was observed to be correlated with increased risk of T-ALL in children. NALT1 promotes cell proliferation in vitro and in vivo. NALT1 LncRNA can interact with NOTCH1, contributing to activation of this gene. Elevated expression of *NALT1* and *NOTCH1* can be correlated with each other which can result in the pathogenesis of ALL. Clinical reports of ALL in children show the effect of this gene and its regulator LncRNA in the pathogenesis

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of this disorder. Recently, surveying the role of NOCTH signaling pathway in normal development of T cells and targeting it in T-ALL patients have attracted researchers to make treatment more effective [9, 10].

LUNAR1 was identified to be required for efficient T-ALL growth due to its role in enhancing its neighbor coding gene expression, the insulin-like growth factor receptor 1 (IGF1R) (Figure 1). This LncRNA has been shown to be downregulated when Notch1 is inhibited, overexpressed in primary T-ALL cells, and expressed considerably higher in T-ALL samples with a Notch mutation than those without mutations. Given Notch1's well-established role as a transcriptional activator, lncRNAs that are negatively impacted by Notch1 signaling might be considered indirect targets. The evaluation of the relationship between NOTCH1 and LUNAR1 reveals direct binding of NOTCH1 to LUNAR1 [11].

Nanotechnology's breakthrough in the production of nano-formulations for reducing adverse effects of medications has various benefits for cancer therapies [12]. The potential for biocompatibility, boosting transport across biological barriers, and improving the anticancer properties of a pharmaceutical drug have all drawn attention to nanoparticles [13]. These nanoparticles might represent a promising alternative to traditional cancer treatments. Novel metal and metal salt nanoparticles, such as platinum, titanium oxide, copper, silver, and gold, have piqued attention in this respect due to their prospective applications in biosensors, biocatalysts, anti-biofilm, antioxidant, and antibacterial properties [14]. Thiosemicarbazone compounds containing transition metals like copper and nickel have also been proven to exhibit intriguing pharmacological effects. Several studies have demonstrated the wide pharmacological application of Thiosemicarbazone complex in clinic [15-17]. The anti-cancer effects of Thiosemicarbazone and their metal-related complexes against a wide range of human hematological malignancies as well as leukemic cells, have been investigated [18]. A valid relationship has been confirmed between tumor growth rate and the function of Ribonucleotide Reductase (RR) enzyme, a necessary enzyme in DNA synthesis. It has been confirmed that Thiosemicarbazone compounds can show inhibition effects on RR enzyme [19].

The other drug used in the current study, termed 6-mercaptopurine (6-MP), can exhibit anticancer properties by interfering with protein, DNA, and RNA synthesis and inducing cell death. Moreover, 6-MP can inhibit de novo purine synthesis and works as an anti-proliferative agent. A study proved that 6-MP can cause an early metabolic stress in growing T cells, which inhibits proliferation and increases apoptosis. The suppression of the metabolic checkpoints mTOR, HIF-1, and Myc by 6-MP was related with total suppression of glycolytic and glutaminolytic fluxes into the TCA (tricarboxylic acid cycle) cycle, according to the findings. As a result, 6-MP could be a promising anti-cancer agent in T-ALL [20].

The first aim of the present study was to investigate the regulatory effects of LUNAR1 and NALT1 LncRNAs by targeting the *NOTCH1* gene. Secondly, the up-regulation effects of NALT1 on *NOTCH1* gene and also the correlation between NOTCH1 and LUNAR1, resulting in the development and progression of lymphoblastic leukemia, were investigated. Finally, the usefulness of Thiosemicarbazone-Ni complexes and 6-MP drug in inhibiting and controlling the Notch signaling pathway as well as, evaluating the effect of the mentioned drugs in preventing lymphoblastoma progression (with the emphasis on the association with the NOTCH1 mutation), by interfering with LUNAR1 and NALT1 LncRNAs, were investigated.

Materials and Methods

Cell culture and Preparation of different concentrations of the drug

The present case-control study was conducted from April to September 2018 at Zanjan Islamic Azad University, Research and Science Center. For this purpose, Jurkat E.6.1 cell line, a human T-lymphoblastic leukemia cells (T-ALL), was purchased from Pasteur Institute of Iran at passage 1 and density of 80% (2×10^5cell / cm^2). First, JurkatE.6.1 cells were cultured in Dulbecco's Modified Eagle's Medium (RPMI 90%) medium containing 10% FBS and kept in an incubator at 37°C with 5% CO_2 for 6 days. Three passages were applied to the cells, once in every two days, and in each passage, cells were transferred into the new flasks with the fresh culture medium. Subsequently, the fourth passage of cells were chosen for the next treatment steps. Cells were counted and colored with Trypan blue. The cell density was calculated to be 3×10^4 cells/cm^2. The cells were divided into control and treated groups. In 10 and 1cc of distilled water, a 6-MP tablet equal to 50 mg and 0.001 gr of Thiosemicarbazone-Ni complex were dissolved, respectively. The MTT assay was then performed to determine the effective dosage of the medicines employed (6-MP and Thiosemicarbazone-Ni complex). For the next step, the doses of 1, 5, 10, and 25 µm for 6-MP and 0.5, 1, 2, and 5 µm for Thiosemicarbazone-Ni complex, were employed for next treatments. The prepared doses were used for 24, 48, 72 h. Non-treated cells were used as the control group.

RNA extraction and real time PCR

RNA was extracted from treated and non-treated cells using high pure viral Nucleic Acid Kit (Roche Diagnostics Gmbh, Manheim, Germany, and Cat #11858874001). Procedures were performed according to the kit manufactures. For cDNA synthesis, the related kit (Takara Bio, Otsu, Japan, Cat # RR037Q) was used for extracting RNA.

The SYBER Green Real-time PCR master mix in the volume of 10µl was prepared as follow: 5X Primer Script Buffer (1X) (2µM) ،Primer script RT enzyme Mix1 (0.5µl), Oligo dT (0.5µM), Random hexamers (0.5µM), Total RNA (3.5µM), RNase free dH_2O (3 µM). The aforementioned substances were mixed and cycled at RT for 15 minutes at 37°C and then 8-16 seconds at 85°C. Using Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany) and applying the kit instruction

(Takara Bio, Otsu, Japan, Cat # RR820Q) the NALT1 and LUNAR1 lncRNAs, *NOTCH1* gene and *GAPDH* as a house keeping gene were inserted and amplified. The primer sequences used for NALT1 as a forward primer: (5'-TGTTTGCTGCCAGAGAAATG-3') and reverse (5'-CCAGCTGTGCTTACCTCTCC-3') and for Notch1 as a forward primer :(5'-ATCAGGTGGTCAGGCAAAAC-3') and reverse :(5'-CTCAGAACCACACCTGCTGA-3'), and a forward Primer for LUNAR1 (5'-CATTCACAGGGTGTGGTCAG-3') and reverse (5'-CATTCTGGGAACCGAAAGAA-3') a forward Primer for GAPDH (5'-ACCACAGTCCATGCCATCAC-3') and reverse (5'-TCCACCACCCTGTTGCTGTA-3'). The FASTA sequence of the cDNA library of genes was achieved from the NCBI website. Primer3 software was used for primer designing procedure. Finally, using the aforementioned software, the results were blasted on the NCBI website to confirm specificity and primer binding to all four genes. The reactions were adjusted at 25 µl volume. 12.5 µl of Cyber Green (1X), 1 µl of forward and reverse primer (0.4 µM), 2 µl of cDNA, and 8.5 µl of deionized water were mixed. The first denaturation schedule of the device was at 95°C for 10 min and DNA fragments were amplified in 40 cycles (denaturation of 95°C for five seconds, 51.8 annealing, 30 seconds for the Notch1, NALT1 and LUNAR1, lncRNAs, and final extension 72°C, 30 seconds).

Gene Expression Confirmation and Statistical Analysis

To confirm the gene presence, the PCR products were electrophoresed on 2% agarose gel. After the reaction, ΔCT value of target gene to reference gene was calculated for total samples. Then $2^{-\Delta\Delta ct}$ (fold change) was obtained for each sample. To evaluate the expression of Notch1 as well as *NALT1* and *LUNAR1 lncRNAs* by Real time PCR, Livak method and Rest program (2002) were used respectively. P. value <0.05 was considered as the level of significance rate.

Results

After investigation of the expression of *NOTCH1, LUNAR1, NALT1* genes at different times and concentrations of 6-MP and Ni, the results showed the relationship between the studied genes as follows:

Lunar1 expression changes treated with 6-MP at concentrations of 1, 5, 10, 25 and thiosemicarbazone complexes (Ni) at concentrations of 0.5, 1, 2, 5µM in 24, 48, and 72 h: The results showed that in the first 24h after treatment with 6-MP at concentrations of 5µM (0/187), 10µM (0/004) and 25µM, (0/795) a significant decrease (P <0.001) in gene expression was observed. The greatest amount of this reduction in expression is related to the concentration of 10µM drug in 24h. The decrease in expression at 48h was related to the concentration of 25µM (0/001) and at 72h to the concentration of 10µM (0/002). According to the results, in all times, the lowest expression, which indicates the highest effect of the drug, is related to the concentration of 25µM (0/001) in 48h. Moreover, after treatment with Ni the results showed that only at concentration of 5µM (0.948) and at 48h, a significant decrease (P <0.001) in gene expression was observed.

Notch1 expression changes under 6-MP and thiosemicarbazone complexes (Ni) treatment: The results showed that after treatment with 6-MP at concentrations of 5µM (0.298), 10µM (0.001), 25µM (0.155), a significant decrease (P <0.001) in gene expression was observed. The highest amount of this expression was significant in the first 24h at a concentration of 10µM (0.001). The highest significant reduction in expression in 48h was related to the concentration of 5µM (0.119). After 72h at the concentration of 10µM (0.68) we had the highest significant decrease in expression. According to the

Table 1. Expression Levels of Lunar1, Notch1, and Nalt1, with GAPDH as the Control, in Response to Varying Concentrations of Nickel (Ni) over 24, 48, and 72 hours. Each concentration is denoted in the form of 'x Ni y,' where 'x' represents the concentration (in arbitrary units) and 'y' indicates the time in hours. Expression levels are normalized to the control (GAPDH).

Concentration and Time	Lunar1	Notch1	Nalt1
0.5 Ni 24	2.416	3.276	0.052
1 Ni 24	3.111	1.365	0.084
2 Ni 24	1.042	1.575	0.05
5 Ni 24	0.11	3.193	0.744
0.5 Ni 48	1.792	3.893	0.67
1 Ni 48	1.48	1.66	0.903
2 Ni 48	1.391	2.275	0.965
5 Ni 48	0.948	2.261	0.724
0.5 Ni 72	1.491	0.995	4.353
1 Ni 72	1.86	0.955	2.628
2 Ni 72	1.595	0.927	0.081
5 Ni 72	1.689	0.963	0.124

Table 2. Expression Levels of Lunar1, Notch1, and Nalt1, with GAPDH as the Control, in Response to Different Concentrations of 6-mercaptopurine (6-MP) across 24, 48, and 72 hours. Concentrations are represented as 'x 6-mp y,' where 'x' specifies the concentration (in arbitrary units) and 'y' indicates the time in hours. The expression values are normalized relative to the control (GAPDH).

Concentration and Time	Lunar1	Notch1	Nalt1
1 6-mp 24	2/01	1/844	1/848
5 6-mp 24	0/187	0/298	0/618
10 6-mp 24	0/004	0/001	0/003
25 6-mp 24	0/795	0/155	0/159
1 6-mp 48	0/085	0/851	1/238
5 6-mp 48	0/08	0/119	2/04
10 6-mp 48	0/005	0/248	4/481
25 6-mp 48	0/001	1	4/467
1 6-mp 72	0/088	1/444	3/93
5 6-mp 72	0/458	1/862	4/858
10 6-mp 72	0/002	0/68	5/037
25 6-mp 72	0/12	0/313	6/648

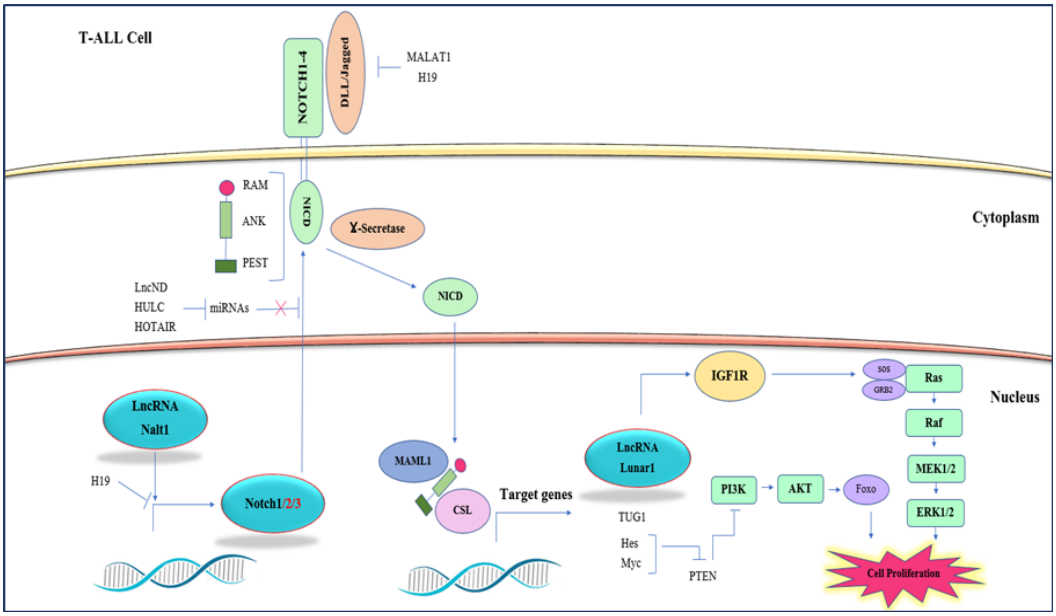

Figure 1. Relationship of LncRNAs Nalt1, Lunar1 and Notch1in NOTCH Signaling Pathways

results, the lowest expression in all the mentioned times, which shows the highest effect of the drug, is related to the concentration of 10µM (0.001) in 24h. Moreover, after treatment with Ni the results showed that only at a concentration of 0.5µM (0.995) in 72h we had a significant decrease in expression (P <0.001).

Nalt1 expression changes treated with 6-MP and thiosemicarbazone complexes (Ni) According to the results, after treatment with 6-MP we had a significant decrease in expression in only the first 24h including concentrations of 5µM (0.618), 10µM (0.003), 25µM (0.159) the lowest of which was the expression of 10µM (0.003) at 24h. after treatment with Ni the results showed that in the first 24h in concentrations of 0.5µM (0.052), 1µM (0.084), 2µM (0.05), decreased expression was significant. The highest decrease in expression at 48h was related to the concentration of 1µM (0.903) and at 72h at the concentration of 2µM (0.081). According to the results, in all the mentioned times, the lowest expression, which shows the highest effect of the drug, is related to the concentration of 2µM(0.05) in 24 h.

Relationship between LUNAR1 and NOTCH1 treated with 6-MP and thiosemicarbazone complex Ni drug

The results of investigation of the relationship between NOTCH1 and LUNAR1 treated with 6-MP at concentrations of 1, 5, 10, and 25µM showed a decrease at three concentrations of 1, 5, 10 at constant times, which was meaningless. the *LUNARE1* and *NOTCH1* gene expression changes at 6-MP treatment at the different time points had the lowest expression at 48h. The results of investigation of the relationship between *NOTCH1* and *LUNAR1* treated with thiosemicarbazone complex Ni showed that there was no significant decrease in expression at constant times. The *LUNARE1* and *NOTCH1* gene expression changes under Ni treatment at different times had the lowest expression at time 48h.

Relationship between *NOTCH1* and *NALT1* treated with 6-MP and thiosemicarbazone complex Ni: The result of investigation of the relationship between *NOTCH1* and *NALT1* treated with 6-MP at concentrations of 1, 5, 10, 25µM showed a significant decrease in expression at constant times. The highest decrease in expression was related to the concentration of 5µM. The *NALT1* and *NOTCH1* gene expression changes treated with 6-MP at the different times had the lowest expression at 72 h. The results of Investigation of the relationship between *NOTCH1* and *NALT1* treated with Ni at concentrations of 0.5, 1, 2, 5µM at constant times showed a meaningless decrease in expression. The highest rate of expression decrease is related to the concentration of 0.5µM. The *NOTCH1* gene and NALT1 expression changes under Ni treatment at different times had the highest expression decrease at 72h (Tables 1 and 2).

Discussion

T-ALL (T-cell acute lymphoblastic leukemia) is an aggressive hematologic neoplasm that mostly affects children, teenagers, and young adults [21]. Multiagent high-dose chemotherapy is now used to treat it, although it is commonly linked with acute and chronic life-threatening and devastating side effects. Understanding of the disease's molecular physiopathology has led to the identification of a subgroup of individuals with a unique genetic profile, known as "early T-cell precursors" lymphoblastic leukemia, which is defined by a poor prognosis [22]. The most prevalent mutations (60 percent of adult patients) are in the NOTCH1/FBXW7 pathway, which has a good prognosis [23]. As a result, investigating the expression of this gene and regulatory non-coding genes under treatment of different antic-cancer agents is necessary for the treatment of this aggressive neoplasm [24]. *LUNAR1*, a *NOTCH1* regulatory lncRNA, was shown to be upregulated in cortical and mature T-ALL cells. Moreover, this LncRNA was shown to be

the responsible for T-ALL proliferation by promoting *IGF1R* expression [11]. In addition, a lncRNA is known as "Notch1-associated lncRNA in T-ALL (NALT1)" appears to control *Notch1* expression. *NALT1*, a nuclear lncRNA, is located approximately 100 nucleotides from the Notch1 gene [25]. Wang and colleagues suggested that *NALT1* functions in cis mechanism and controls its nearby gene as a result of its close proximity. ShRNA against *NALT1* reduced Notch1 levels and hampered T cell proliferation in the mentioned investigation, while ectopic *NALT1* overexpression failed to restore *Notch1* expression [10]. These investigations demonstrated that *NALT1* regulates *Notch1* via a cis-regulatory mechanism, which suggests that *NALT1* is a regulatory factor for the expression of *Notch1*. 6-Mercaptopurine (6-MP), an anti-cancer agent and primary drug being used in chemotherapy, has been shown to be an effective drug during the treatment of maintenance phase of acute lymphoblastic leukemia [26]. In the maintenance phase, it is taken alone or in combination with other medicines. In addition to 6-MP, Thiosemicarbazone-Ni Complex was used to investigate its anti-cancer role against T-ALL cells. As chelating agents, Thiosemicarbazones are well-documented in the biology world [27]. Thiosemicarbazones have anti-proliferative effects through inhibiting topoisomerase II, matrix metalloproteinase, kinases, and anti-apoptotic Bcl-2 family members, as well as inhibiting DNA synthesis [28]. Several nickel-based compounds have recently been studied for their anticancer properties. Nickel complexes containing Thiosemicarbazones have also been used in biological employments [29].

The results of the expression alteration of *LncRNAs* termed *LUNAR1* and *NALT1* and *NOTCH1* gene treated with 6-MP and Thiosemicarbazone-Ni complex showed that LncRNA LUNAR1 treated with 6-MP at 25μM (0.001) for 48h had the highest expression reduction. Moreover, the cell lines treated with Thiosemicarbazone-Ni complex at the concentration of 5μM (0.948) for 48h showed the greatest decrease in the expression of *LUNAR1*. Furthermore, the greatest *NOTCH1* gene down-expression was seen with following treatment with 6-MP at a concentration of 10M for 24 h. This number for Thiosemicarbazone-Ni complex treatment was observed at the concentration of 0.5μM (0.995) for 72h. For NALT1 LncRNA, the highest decrease was seen after treatment with 6-MP and Thiosemicarbazone-Ni complex at the concentrations of 10μM (0.003) and 2μM (0.05) respectively for 24h.

The data of the present study indicated the positive effect of 6-MP and Thiosemicarbazone-Ni complex at different times and concentrations on the expression of the aforementioned genes. In addition, the obtained results demonstrated that the effect of drugs is highly related to time and their concentrations. Among the studied groups, the group treated with 6-MP for 24h and concentration of 10μM showed the most significant results. The mentioned dose and treatment time showed the most significant effect on expression reduction of *NOTCH1* gene and *NALT1* LncRNA, which indicated the optimal concentration and time for 6-MP. On the other hand, the results proved that 6-MP required high dose and time to decrease *LUNAR1* expression. Most effects of Thiosemicarbazone-Ni complex were different at distinct time and concentrations. Expression analysis showed that *NALT1* had the highest decrease in expression in comparison with NOTCH1 and *LUNAR1*, which was observed at 2μM concentration for 24h. The results confirmed that the reduction effect of Thiosemicarbazone-Ni complex on the expression of target genes and LncRNAs were based on different times. In the other words, in the most of Thiosemicarbazone-Ni complex-treated groups, target gene and lncRNA expression reduction was observed at lower concentrations confirming the fact that Thiosemicarbazone-Ni complex requires low dosages and more time to exert its function in comparison with 6-MP. On the other hand, it was shown that 6-MP functions at higher doses and short time.

The result of the present and related studies can prove the fact that *LUNAR1* and *NALT1 lncRNAs* and *NOTCH1* gene could regulate each other in acute lymphoblastic leukemia. In this regard, it is shown that NALT1 lncRNA is located in the upstream of *NOTCH1* gene and its expression alteration can affect the *NOTCH1* expression. The mechanism may be involved in *NOTCH1* regulation by *NALT1* in T-ALL cells is cis regulation resulting in NOTCH signaling pathway modulation. Moreover, *NOTCH1* overexpression can increase cell proliferation rate by its oncogenic functions. As a result, it has been hypothesized that *NALT1* expression controlling, can modulate the genes of aforementioned pathway (Figure 1). The data of the present study represented that the *NALT1* expression was decreased by 6-MP and Thiosemicarbazone-Ni complex treatment at the mentioned times and concentrations. IGF1R-1 is an insulin receptor that regulates blood sugar levels, and LUNAR1 LncRNA can influence its expression. As a result, IGF1R upregulation can promote cell proliferation rate, contributing to neoplasm creation. This clinical data can be important in the diagnosis, leukemia researching and leukemia biological marker discovering. We also observed that expression reduction of NALT1 can be observed after Thiosemicarbazone-Ni complex and 6-MP treatment at certain concentrations and effective times. Moreover, this result can also be the consequence of IGF1R-1 downregulation. In a nutshell, it could block the NOTCH signal pathway leading to inhibition of uncontrolled amplification (Table 1).

In 2009, FENG GU et al. found that high levels of Stat3 and Notch1 expression were linked to cisplatin resistance. Furthermore, suppression of Stat3 or Notch signaling pathway in vitro, reduced HNSCC cisplatin resistance. Their findings show for the first time that high Stat3 and Notch1 expression are both linked to cisplatin resistance in HNSCC patients, defending the theory that Stat3 and Notch1 interaction generates altered survival pathways in HNSCC patients responding to treatment [30]. In addition, AT-I can suppress cancer cell proliferation and induce apoptosis via inactivating the Notch signaling pathway, according to Li Ma and coworkers in 2014. Furthermore, their data showed that Notch1, Jagged1, and its downstream Hes1/ Hey1 expressions were reduced by AT-I treatment. AT-I decreased the self-renewal potential of gastric stem-like cells (GCSLCs) by suppressing their

sphere forming capability and cell survival, according to their findings. In vitro, AT-I suppressed gastric cancer stem cell (GCSC) characteristics in part by inactivating Notch1, which resulted in lower expressions of Notch1's downstream targets Hes1, Hey1, and CD44 [31]. Notch1 as a major oncogene in NOTCH signaling plays a significant role in targeting the molecular components of this signaling pathway and many other cellular processes such as amplification and differentiation in acute lymphoblastic leukemia, according to our findings and comparisons with earlier research. Similarly, our results confirmed the down-regulation of this gene treated with both 6-MP and Thiosemicarbazone-Ni Complex.

In a study published in 2019, Zhang et al. discovered significantly higher expression of LUNAR1 in clinical CRC samples as compared to normal tissues. *LUNAR1* expression has been linked to tumor aggressiveness, disease-free survival, and overall survival in patients with colorectal cancer (CRC). It was shown that downregulation of *LUNAR1* could decrease cell proliferation, migration, invasion, and tumor development in SW620 cells while triggering apoptosis. Furthermore, inhibiting LUNAR1 has been shown to drastically reduce IGF1 signaling in CRC. These findings suggested that *LUNAR1* was upregulated in CRC and can promote tumor development [32]. Diffuse large B-cell lymphoma is a diverse category of B-cell lymphomas, according to the findings of Peng W and colleagues. Further research found that silencing *LUNAR1* inhibited *DLBCL* cell growth through modulating *E2F1*, *cyclin D1*, and *p21*. Finally, their findings suggest that *LUNAR1* may be used as a possible predictive biomarker in *DLBCL* due to its role in growth control [33]. Trimarchi and coworkers showed in 2014 that the *LUNAR1 lncRNA* is required for T-cell ALL (T-ALL) development and maintains high insulin-like growth factor 1 receptor expression levels via a cis-activation mechanism. They also discovered that Notch activation in T-ALL can control a number of lncRNAs [11]. When we compared our findings to those of prior studies on the function and significance of *LUNAR1* expression variations, we discovered that these changes are controlled by the alterations in the expression of *NALT1* and *Notch1*. We were able to lower the expression of this gene using 6-MP and Thiosemicarbazone-Ni complex at the effective concentrations, as well as indirectly lower the expression of *LUNAR1* by reducing the expression of *NALT1* and *Notch1* genes while using same compounds. The findings support the hypothesis that 6-MP and the Thiosemicarbazone-Ni combination have a favorable influence on the lowering trend of *LUNAR1* expression. This might support the effectiveness of 6-MP and Thiosemicarbazone-Ni complex in treating acute lymphoblastic leukemia.

In 2015, Wang et al. discovered that *NALT1 lncRNA* is only 100bp away from *NOTCH1*. In human samples, the upregulation of *NALT1* associated with *NOTCH1* was confirmed in their investigation. In their cell line, increased level of *NALT1* expression promoted cell proliferation significantly [10]. According to Yan et al., *NALT1* is overexpressed and promotes gastric cancer cell invasion and metastasis [34]. When our findings are compared to those of previous studies, it becomes clear that *NALT1* plays an important role in the notch and acute lymphoblastic leukemia signaling pathways. Our findings confirmed the beneficial effects of 6-MP and the Thiosemicarbazone-Ni complex in regulating *NALT1* expression as well as other notch signaling pathway components. As a result of changes in *NALT1* expression, 6-MP and Thiosemicarbazone-Ni complex may have the potential role to inhibit the progression of acute lymphoblastic leukemia.

As a conclusion, *NOTCH1*, one of the important proteins of *NOTCH* signaling pathway, can be an oncogene when it is dysregulated in the expression level in T-ALL cells. We demonstrated in the present study that cellular expression of *NALT1* and *LUNAR1*, important LncRNAs in the progression of acute lymphoblastic leukemia, will be decreased after treatment with 6-MP and Thiosemicarbazone-Ni complex. Several studies have shown that the effective dose and optimal time to decrease the expression level of aforementioned lncRNAs and gene were different. Briefly, we proved that the mentioned drugs have a good potential in reducing the expression of *NALT1, LUNAR1*, and *NOTCH1* and can reduce the expression level of *IGF1R-1* and block the *NOTCH* signaling pathway due to its anti-cancer effect at lower concentrations. This effect can prevent the overexpression of abovementioned genes and can help to control, treat and reduce the progression of acute lymphoblastic leukemia.

Author Contribution Statement

The first author was responsible for conceptualizing and designing the study and conducting experiments, the Second author conducted the experiments, and the Third and fourth authors supervised the experiments and manuscript. All authors read and approved the final manuscript.

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Approval and Thesis Statement

The study is part of an approved student thesis conducted at the master's level at the Islamic Azad University of Zanjan in 2018.

Ethical Considerations

None.

Conflict of Interest Statement

The authors declare no conflicts of interest in connection with this research.

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