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Inhibition of MDM2 Using Idasanutlin (RG-7388) enhances the Chemo-sensitivity of B-ALL Cells to Daunorubicin

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Abstract

Background: Although significant advances have been made in the treatment of cancer, a significant number of patients with acute lymphoblastic leukemia (ALL) show resistance to treatment. Thus, it is necessary to seek novel therapeutic agents to overcome this problem. Studies have indicated that the expression level of mouse double minute 2 (MDM2), a negative regulator of p53, is markedly elevated in patients with refractory or recurrent ALL. Thus, targeting MDM2 using a specific inhibitor, Idasanutlin, can increase the activity of p53. This study evaluated the possible synergistic effect of Idasanutlin and Daunorubicin on the induction of apoptosis in NALM-6 cells.

Materials and methods: In this fundamental study, the anti-proliferative effects of Idasanutlin on NALM-6 cells, either alone or in combination with Daunorubicin, were confirmed by MTT(methyl-thiazol-tetrazolium) assay, Annexin/PI apoptosis assay, and cell cycle analysis. Quantitative reverse transcription-PCR (qRT-PCR) and Western blot analyses were applied to elucidate the molecular mechanisms underlying the anti-leukemic activity of Idasanutlin.

Results: Idasanutlin synergistically enhanced Daunorubicin-induced apoptosis and activated caspase-3, thereby activating programmed cell death in a dose-dependent manner ($P<0.001$). The treatment of NALM-6 cells with Idasanutlin caused cell cycle arrest in the G1 phase by an increase in the expression of p21 ($P<0.001$). Moreover, a significant increase was detected in the expression of pro-apoptotic genes ($P<0.001$), as well as a remarkable decrease in the expression of anti-apoptotic ($P<0.01$) and multidrug resistance1 (MDR1) genes ($P<0.01$).

Conclusions: It seems that Idasanutlin can cooperatively promote daunorubicin-induced apoptosis in NALM-6 cells. These findings open up a new horizon in the application of Idasanutlin in combination with Daunorubicin to overcome drug resistance in patients with ALL.

Keywords: Acute lymphoblastic leukemia; Apoptosis; Daunorubicin; Drug resistance; Idasanutlin

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Introduction

Acute lymphoblastic leukemia (ALL) is considered a malignancy of lymphoid precursors, which are mainly found in the bone marrow and blood circulation (1). ALL is the most frequent type of childhood leukemia, which accounts for more than 80% of children diagnosed with blood cancer (2, 3). Daunorubicin is broadly used as one of the combination chemotherapeutic agents for the treatment of ALL. Studies have indicated that Daunorubicin is capable of intercalating into DNA base pairs, which causes the denaturation of DNA and inhibits the activity of the topoisomerase II enzyme,

leading to a double-strand break in DNA and the inhibition of DNA and RNA synthesis. This phenomenon promotes cells to undergo apoptosis in a p53-dependent manner (4).

The p53 is known as a tumor suppressor protein activated in response to DNA damage. It acts as a transcription factor and increases the expression of the p21 gene, which causes cell cycle arrest in the G1 phase as well as the overexpression of PUMA and NOXA genes (5, 6). These biological events ultimately result in the induction of apoptosis via p53 signaling (7). Studies have demonstrated that the mutation rate in the p53 gene transcription is about 5% in newly diagnosed ALL patients, while such a rate reaches 10% in ALL cases with recurrence (8, 9). However, most patients afflicted with ALL carry wild-type p53. The expression of p53 protein is regulated by mouse double minute 2(MDM2), which is an E3 ligase enzyme and belongs to the ubiquitin-proteasome system (UPS). MDM2 can bind to p53 and initiate the ubiquitination process, leading to the degradation of p53 in the UPS (10, 11).

Resistance to chemotherapeutic drugs usually results in the development of treatment failure, disease recurrence, and poor prognosis of patients with ALL (12). Extensive research has proposed several mechanisms underlying the resistance to anthracyclines (e.g., Daunorubicin). These mechanisms include the overexpression of anti-apoptotic genes, such as the BCL-2 gene, along with an increase in the expression of breast cancer resistance protein (BCRP) and multidrug resistance (MDR1) genes, which are responsible for resistance to Daunorubicin (4, 13). Regarding the undeniable role of MDM2 in the increased expression of the MDR1 gene, the development of resistance to treatment in ALL patients might be associated with MDM2 (14). Idasanutlin is a potent and specific inhibitor of MDM2, causing the inhibition of anti-apoptotic genes through an increase in the activity and function of p53. Besides, Idasanutlin prevents the overexpression of genes involved in drug resistance by inhibiting MDM2, thus simultaneously neutralizing the two mechanisms of chemo-resistance. The idea of MDM2 inhibition in the achievement of better therapeutic outcomes was further supported by the design of the first generation of MDM2 inhibitors, called Nutlin. The inhibitory role of this agent led to the inactivation of some proteins involved in drug resistance (p-gp and BCRP) in rhabdomyosarcoma cancer cells (15). According to the hypothesis suggesting that Idasanutlin can boost the efficiency of Daunorubicin, this study aimed to assess the impact of Idasanutlin (RG-7388) on the induction of apoptosis and expression of drug resistance genes in B-ALL cells treated with Daunorubicin.

Materials and Methods

Cell Culture and Solution Preparation

NALM-6 cell line, prepared from the National Cell Bank of Iran (NCBI) at Pasteur Institute (Tehran), was cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS; ATO CEL), 2 mM L-glutamine (Gibco), 100 U/ mL penicillin, and 100 U/ mL streptomycin (Gibco) in a controlled environment at 37 °C temperature, 5% CO2 concentration, and 95% humidity. The cell culture medium was replaced with a fresh medium when cell confluency reached the desired density of 75%. The NALM-6 cell line in the logarithmic growth phase with passage number 4 or 5 was used for subsequent experiments. Idasanutlin was purchased from Medchemexpress, U.K. A stock solution was prepared by dissolving 5 mg Idasanutlin powder in 1.76 mL dimethyl sulfoxide (DMSO, Merck) with the final concentration of 5 mM. Then, the solution was stored at -80°C. Daunorubicin hydrochloride was obtained from Ebewe Austria with an initial concentration of 50mg/25mL and stored at 4°C.

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Trypan Blue Assay

NALM-6 cells at the density of 1×10^5 cells/well were treated with a single agent either Idasanutlin or in combination with Daunorubicin after 24 and 48 hours. The drug-treated cells were mixed with trypan blue (Invitrogen, Auckland, New Zeeland) and incubated for 1 to 2 minutes at room temperature using a New Bauer hemocytometer. The viable cells were counted, and the percentage of viability was determined.

MTT Assay and Synergism

The MTT method was used to evaluate the effect of Idasa /Don on the apoptosis of NALM-6 cells. The NALM-6 cells were seeded into a 96-well culture plate (25×10^3 cells/well) and incubated with designated concentrations of drugs for 24 h and 48 h. Then, the cells were incubated with 10 µl of MTT solution (5 mg/ml) for 4 h at 37°C. The resulting Formosan crystals were solubilized by adding 100 µL dimethyl sulfoxide (DMSO) to each well, and the absorbance rate was measured at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader.

The combination index (CI) and Isobologram parameters were calculated using Calcusyn Ver1.1 software to determine the dose-effect relationship of Idasanutlin and Daunorubicin alone and in combination.

Annexin V /PI Apoptosis Test

Annexin V-propidium iodide (PI) staining by a flow cytometric analysis was used to determine the induction of apoptosis. The NALM-6-treated cells were seeded into 12-well plates at a density of 150×10^3 cells/well. The cells were collected and resuspended in cold phosphate-buffered saline (PBS) after 36 h treatment with different concentrations of Idasanutlin and Daunorubicin. Cells were pelleted and incubated with 100 µL annexin-V (MabTag) incubation reagent containing 90 µL of 1X binding buffer and 5 µL PI, 5 µL annexin-V conjugate for 20 min in the dark at room temperature. After incubation, the fluorescent signals of the cells were detected by flow cytometry on FACSCalibur (Becton Dickinson). The apoptotic index was calculated by Flowjo Ver 7.6.1 software and measured in each of the three independent experiments.

Cell Cycle Analysis

The distribution of cells in different phases was monitored by flow cytometry in order to reveal the molecular mechanism of Idasanutlin-mediated induction of cell cycle arrest. Following 24 h treatment of NALM-6 cells with 300nM Idasanutlin, either as a single agent or in combination with 90nM Daunorubicin, the DNA content was measured. Briefly, 150×10^3 pretreated cells were harvested, washed twice with cold PBS by centrifugation, and fixed overnight in 70% ethanol at -20˚C. Subsequently, cells were centrifuged, washed twice with cold PBS, and resuspended in 100µg/ml RNaseA (Sinaclon, Iran) and 50µg/ml propidium iodide (Sigma Aldrich, UK). Finally, following 30 min incubation in darkness, samples were analyzed using a BD FACSCalibur Flow Cytometer. The distribution of cells in each stage of the cell cycle was calculated using Flowjo software.

Quantitative Real-time PCR

Total cellular RNA was extracted from the cells after 36 h treatment with Idasanutlin/DON using One Step-RNA Reagent (Bio Basic) according to the manufacturer's recommendations. The quantity of RNA samples was assessed using Thermo Scientific Nanodrop. One µg of total RNA was used for cDNA synthesis. Reverse transcription PCR was performed using the cDNA synthesis kit (Prime Script™ RT reagent Kit, Perfect Real-Time, TaKaRa) following the manufacturer's instructions. Generated cDNA was amplified in qPCR using RealQ Plus 2x Master Mix Green, without ROX™ (Amplicon) on a light cycler

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instrument (Roche Diagnostics, Germany). The qPCR amplification condition included an initial activation/denaturation step for 15 min at 95°C, followed by 40 cycles, including a denaturation step for 15s at 95°C and a combined annealing/extension step for 60s at 60°C. The melting curve analysis was used to confirm the specificity of the products. Each sample was examined in triplicate, and the quantification of mRNA expression levels was determined based on the $2^{-\Delta\Delta Ct}$ formula. The housekeeping gene GAPDH was used as an endogenous control to normalize variability in expression levels. The nucleotide sequences of the primers are given in Table I.

Western Blot Analysis

5×10^6 NALM-6 cells were pre-treated with Idasanutlin/Daunorubicin for 36 h. Cells were centrifuged, and cellular pellets were washed with cold PBS and lysed in RIPA buffer (Sigma, UK). After centrifugation of the cell lysate at 13000 RPM for 20 min at 4°C, protein concentrations were determined by the Bradford method. Equivalent amounts of total cellular protein were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Corp., Little Chalfont, UK). Subsequently, the membranes were blocked with 5% skim milk in tris-buffered saline (TBS) containing 0.1% (v/v) Tween- 20 and probed with specific primary antibodies overnight at 4°C. After 3 washes in TBS-T, the proteins were detected using secondary antibodies conjugated with horseradish peroxidase (HRP) and the enhanced chemiluminescence detection system (Amersham ECL Advance Kit; GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol.

Statistical Analysis

All data were analyzed using Excel software Ver. 2019 and Graph Pad Prism Ver. 8 and were reported as mean ± standard deviation of three independent tests. Statistical significance was calculated using ANOVA and two-tailed student's t-test. A p-value ≤ 0.05 was considered statistically significant.

Ethical Consideration: Ethical code number: IR.IUMS.REC.1397.206

Results

The Inhibitory Role of Idasanutlin in the Survival of NALM-6 Cells and its Synergistic Effects when Combined with Daunorubicin

In order to assess the anti-proliferative effect of Idasanutlin on NALM-6 cells, first, the cells were treated with various doses of Idasanutlin alone or in combination with Daunorubicin (90 nM) for 24 and 48 hours. Afterward, the results obtained from Trypan blue staining and MTT assay showed a statistically significant reduction in the survival of NALM-6 cells in a dose-dependent manner.

As depicted in Figures 1A and 1B, the maximum cytotoxicity was observed after 48 hours when Idasanutlin was treated with NALM-6 cells at a concentration of 500 nM. The MTT assay also demonstrated the synergistic effect of Idasanutlin and Daunorubicin on the growth inhibition of NALM-6 cells in comparison with the use of Daunorubicin alone (Figures 1C and 2D).

Maximum cell survival decrease was found in the treatment of NALM-6 cells with a combination of 500 nM Idasanutlin and 90 nM Daunorubicin for 48 hours. Next, using isobologram analysis and the calculation of combination index (CI), the nature of the interaction of the two drugs was determined by means of CompuSyn Ver1.1 software. The CI value lower than 1 indicates synergism, equal to 1 denotes additive effects, and higher than 1 shows antagonism. Regarding the obtained CI value, the combination of Idasanutlin and Daunorubicin indicates synergism when

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employed to induce cell death in NALM-6 cells. A significant synergistic effect was detected when the combination of Idasanutlin and Daunorubicin was used against NALM-6 cells for 24 hours. Besides, the analysis of fraction effect (FE) vs. CI values revealed that the synergistic role of the two drugs was substantially higher than that of the single use of each in the growth suppression of NALM-6 cells.

Idasanutlin Inhibits the Growth of NALM-6 Cells by the Cell Cycle Arrest at the G1 Phase

PI staining was carried out to assess whether the anti-cancer effects of Idasanutlin were correlated with cell cycle arrest. To this aim, NALM-6 cells were treated with 300 nM Idasanutlin and 90 nM Daunorubicin for 24 hours. After that, cells were harvested and subjected to PI staining, and the cell cycle arrest was monitored by flow cytometry analysis. As displayed in Figure 2, a significant cell cycle arrest was detected in cells treated with Idasanutlin. The results demonstrated that the anti-proliferative effect of Idasanutlin mediated via the induction of cell cycle arrest in the G1 phase as well as a marked reduction in the number of the cell at the S phase. further, a significant increment was found in the percentage of cells in the G0/G1 and G2/M phases when treated with the combination of Idasanutlin and Daunorubicin. Besides, a significant increase was observed in the frequency of cells in the subG1 phase.

Idasanutlin Enhances Daunorubicin-induced Apoptosis in NALM-6 Cells through Alteration of p53 Downstream Target Genes and Caspase-3 Cleavage

In order to analyze the inhibitory effect of MDM-2 on the induction of apoptosis by Idasanutlin, Annexin-V binding assay and flow cytometry were conducted. For this purpose, NALM-6 cells were treated with different concentrations of Idasanutlin (100، 300، 500 nM) alone or in combination with 90 nM Daunorubicin for 36 hours. As shown in Figure 3A, apoptosis induction is more pronounced in cells treated with higher concentrations of Idasanutlin than in the control cells. Moreover, the cell death rate is significantly higher in cells treated with the combination of Idasanutlin and Daunorubicin. The number of necrotic/apoptotic cells was remarkably higher in cells treated with the combination of the two drugs (Figure 3B).

To dissect the molecular mechanisms by which Idasanutlin boosted the anti-leukemic effect of Daunorubicin in NALM-6 cells, quantitative real-time PCR was performed to study the effect of these compounds on the transcriptional activity of p53 and its downstream target genes.

As depicted in Figure 3C, the combination of Idasanutlin and Daunorubicin resulted in the upregulation of p53, which in turn increased the transcriptional levels of PUMA, NOXA, and p21. Since p21 is known as an essential factor for the regulation of cell cycle through the inhibition of cyclin-dependent kinases, its expression analysis confirmed the previous results obtained from cell cycle and Annexin-V apoptosis assay. Moreover, the expression rates of Bcl-2 and MCL-1 genes, as anti-apoptotic factors, were assessed using the real-time PCR method. The results indicated that the expression of these two genes was significantly diminished in cells treated with the combination of Idasanutlin and Daunorubicin. These findings were in line with the results obtained from the Annexin-V apoptosis assay (Figure 3C).

The cleavage of caspase-3, as a confirmatory assay of Annexin-V staining, was analyzed by the Western blot technique. The results showed a significant increment in the activation of caspase-3 following the treatment of NALM-6 cells with the combination of 300 nM of Idasanutlin and 90 nM of Daunorubicin. The obtained data exhibited that Daunorubicin enhanced the cell death process in which Idasanutlin inhibits the

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MDM-2 activity in NALM-6 cells (Figure 3D).

It appears that the treatment of NALM-6 cells with Idasanutlin stimulates the cleavage of caspase-3 and subsequently the induction of programmed cell death. This event might be due to the disturbed balance between the expression of death repressor and death promoter genes.

Downregulation of MDR1 as well as Alteration in the Expression of P53 and MDM2 Genes in NALM-6 cells Treated with Idasanutlin

The relative expression of p53, MDM2, MDR1, and BCRP genes were analyzed to unravel the mechanism by which the inhibition of MDM2 influences the activity of the p53 pathway. To this aim, the NALM-6 cells were treated with 300nM Idasanutlin and 90 nM Daunorubicin for 36 h. Then, they were harvested, and RNA extraction and cDNA synthesis was carried out to analyze the expression of the above-mentioned genes by real-time PCR. As shown in Figure 5, co-treatment of NALM-6 cells with Daunorubicin and Idasanutlin led to an increase in the expression levels of p53 downstream target genes, namely p53, MDM2, and p21 (Figure 3D).

These results were in agreement with the findings obtained from Annexin-V/PI staining, which demonstrated that co-treatment of NALM-6 cells with Daunorubicin and Idasanutlin stimulated the caspase-3-dependent apoptosis pathway. In order to examine whether Idasanutlin intensifies the cytotoxic effect of Daunorubicin, as a conventional therapeutic agent used for the treatment of ALL, it was treated with NALM-6 cells alone and in combination with Daunorubicin. The results indicated a significantly higher expression rate of apoptosis-related genes in cancer cells treated with the combination of the two drugs compared with those treated with Daunorubicin alone. Thus, the concomitant use of these two agents was more effective in the activation of the p53 signaling pathway than the single use of each agent (Figure 4A). Afterward, the expression of MDR1 and BCRP genes involved in resistance to Daunorubicin was analyzed to evaluate the possible effect of Idasanutlin on the expression of these genes. The findings showed a significant decline in the expression of MDR1 but not BCRP in cells treated with Idasanutlin alone and in combination with Daunorubicin (Figure 4B). The results also indicated that the treatment of NALM-6 cells with Idasanutlin not only activates the p53 signaling pathway but also induces cytotoxicity in which the MDR1 gene is downregulated.

Idasanutlin Induces Programmed Cell Death via the p53 Signaling Pathway Activation

In order to assess whether Idasanutlin is capable of inducing apoptosis via the activation of p53 signaling pathway, the expression of MDM2, p53, and p21 was analyzed. For this purpose, NALM-6 cells were treated with 300 nM Idasanutlin and 90 nM Daunorubicin for 36 h. Afterward, the cells were harvested, and the Western blot analysis was performed. The results showed a significant increase in the expression of p53, p21, and MDM-2 in cells treated with the combination of Idasanutlin and Daunorubicin compared with those treated with Idasanutlin alone (Figure 5). Moreover, the increased expression of p53 was associated with the upregulation of some p53 downstream genes, such as NOXA and PUMA, as well as the downregulation of anti-apoptotic genes, such as BCL2 and MCL1. The increased expression of p21 is accompanied by the activation of p53, while the upregulation of MDM2 is a negative feedback regulator of p53 activity. These results imply that synergism between Idasanutlin and Daunorubicin leads to the activation of the p53-p21 signaling pathway and consequently increases the cytotoxic effect of Daunorubicin.

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Table I: Sequences of the primers used for Real-Time RT-PCR

Gene	Primer	Sequence	Product Size (bp)
GAPDH	Forward Reverse	5'-GAAGGTGAAGGTCGGAGTC-3′ 5'-GAAGATGGTGATGGGATTTC-3′	226
P53	Forward Reverse	5'- CGCTTCGAGATGTTCCGAGA-3′ 5'- CTTCAGGTGGCTGGAGTGAG-3′	102
MDM-2	Forward Reverse	5'- CTCCGTGTTTGGTCAGTGGA -3′ 5'- TGGTCAGGGTAGATGGGTCC -3′	81
P21	Forward Reverse	5'- ACCTCCTCTAAGGTTGGGCA -3′ 5'- TGCCTTCACAAGACAGAGGG -3′	110
Puma	Forward Reverse	5'- CATGCCTGCCTCACCTTCAT -3′ 5'- GGTCACACGTGCTCTCTCTAA -3′	127
Noxa	Forward Reverse	5'- GTAGGTTGTAGTCACTTTAGATGGA -3′ 5'- CCAGATGGTAAAATAGCTGCCT -3′	127
Bcl-2	Forward Reverse	5′- GTTTTCACGTGGAGCATGGG -3′ 5′- CCATTGCCTCTCCTCACGTT -3′	132
Mcl-1	Forward Reverse	5′- GCAGTGAGGGCTTAGGACAC-3′ 5′- GCCAGTCAGCACTTAGACCA-3′	101
MDR1	Forward Reverse	5′- GCGCGAGGTCGGAATGGAT -3′ 5′- CCATGGATGATGGCAGCCAAAGTT -3′	198
BCRP	Forward Reverse	5′- CCCAGGCCTCTATAGCTCAGATCATT -3′ 5′- CACGGCTGAAACACTGCTGAAACA -3′	161

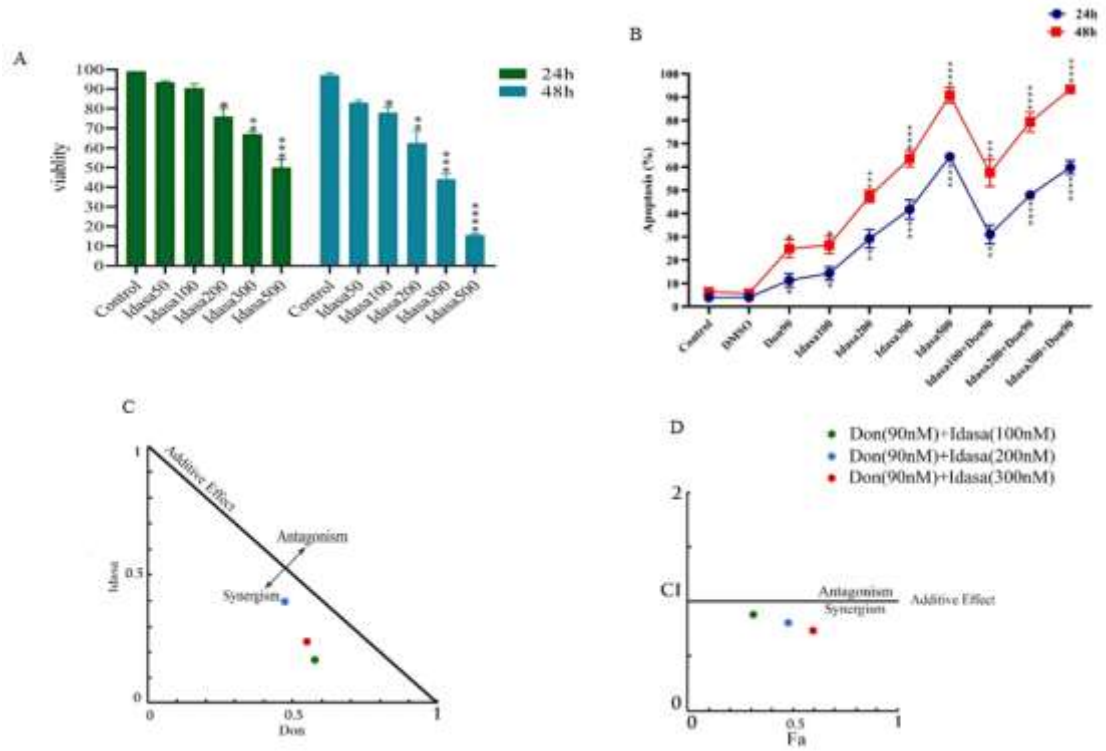

Figure 1. Concomitant treatment of NALM-6 cells with Idasanutlin and Daunorubicin showed a synergistic effect on the viability of cells. A) The cell viability rate was evaluated by Trypan blue staining. B) Various concentrations of Idasanutlin (100, 200, 300, and 500 nM) along with a constant concentration of Daunorubicin (90 nM) were added to the cell culture medium of NALM-6 cells for 24 and 48 hours, and cell death was measured by the MTT assay. C) The Isobologram analysis (Chou-Talalay plot) indicated synergism between Daunorubicin and Idasanutlin. The points below and above the effect line are associated with synergism and antagonism, respectively.D) The analysis of the fraction effect (FE) and CI values showed the presence of synergism between Idasanutlin and Daunorubicin. The CI value above 1 indicates antagonism, equal to 1 denotes additive effects, and below 1 implies synergism. The values are expressed as means±standard deviation (mean ± SD). The statistical analysis was performed on variables obtained from cells treated with Idasanutlin alone or in combination with Daunorubicin, and the results were compared with the control cells. *p <0.05, **p<0.01, and *** p<0.001.

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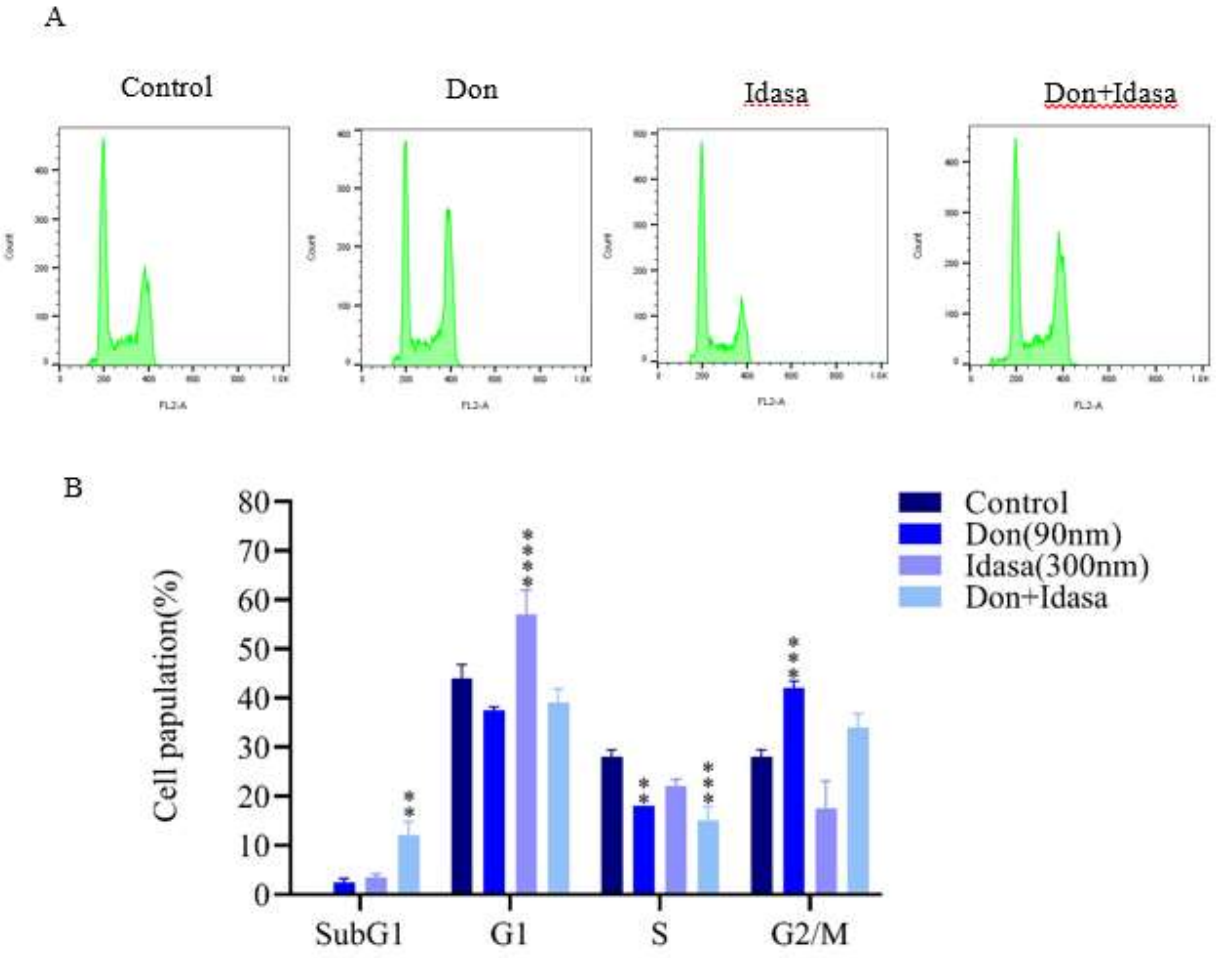

Figure 2. The impact of Idasanutlin and Daunorubicin on the induction of cell cycle arrest in NALM-6 cells. A) The results of flow cytometry analysis of NALM-6 cells treated with Daunorubicin (90 nM) and Idasanutlin (300 nM) for 24 hours, B) The cell population percentage in various phases of the cell cycle after 24 hours; a substantial increase was found in the number of cell population in the G1 phase-treated Idasanutlin. This phenomenon implies that Idasanutlin is capable of inducing the cell cycle arrest in the G1 phase and causing a significant reduction in the frequency of cells in the S phase. The obtained values are represented as mean ±standard deviation (mean ± SD). The statistical analysis was performed on variables obtained from cells treated with Idasanutlin alone or in combination with Daunorubicin, and the results were compared with the control cells. *p <0.05, **p<0.01, and *** p<0.001.

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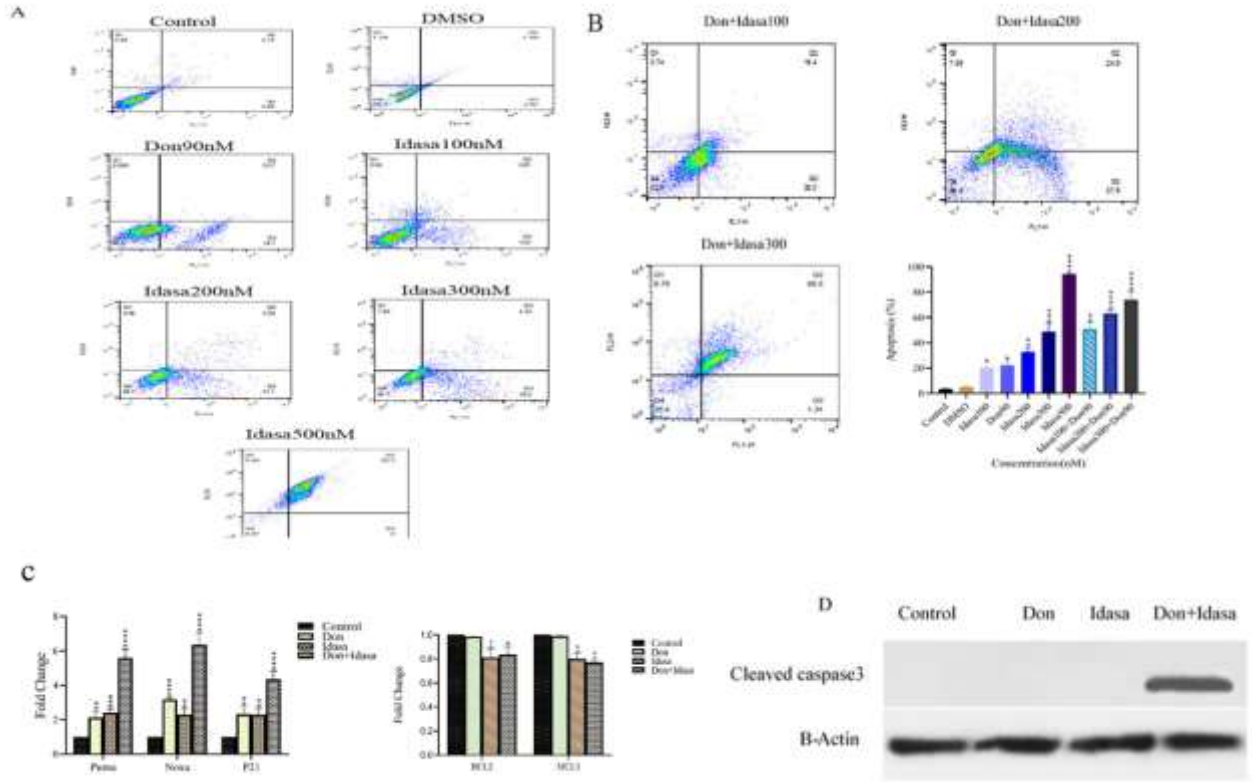

Figure 3. Co-treatment of NALM-6 cells with Daunorubicin and Idasanutlin increased the cell death rate through a change in the expression levels of p53 downstream target genes as well as the cleavage of caspase-3. The effect of Idasanutlin on the induction of apoptosis in NALM-6 cells after 36h of incubation alone (A) or in combination with Daunorubicin (B). C) The relative expression of p53 downstream genes as well as BCL2 and MCL1 were analyzed in cells treated with 300 nM Idasanutlin and 90 nM Daunorubicin for 36 hours by real-time PCR method when their relative expression levels were normalized against the internal control gene GAPDH. D) The western blot analysis detected the cleavage of caspase-3 in NALM-6 cells treated with 300 nM Idasanutlin and 90 nM Daunorubicin. Equal loading of samples was confirmed by the visualization of beta-actin bands. The obtained values are expressed as the mean ±standard deviation (mean ± SD) of three independent experiments. The statistical analysis was performed on variables obtained from cells treated with Daunorubicin alone or in combination with Idasanutlin, and the values were compared with those of the control group. *p <0.05**p<0.01; *** p<0.001.

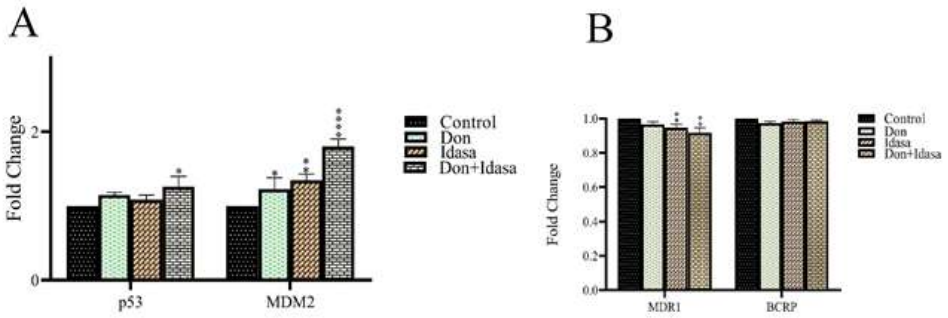

Figure 4. The impact of Daunorubicin and Idasanutlin on the expression of MDM2, p53, and multidrug-resistance genes using the real-time PCR method. NALM-6 cells were treated with 300nM Idasanutlin and 90 nM Daunorubicin for 36 h; A) The treatment of NALM-6 cells with Idasanutlin enhances the effect of Daunorubicin on the activation of genes involved in the p53 signaling pathway; B) Idasanutlin decrease MDR1 gene but not BCRP, either alone or in combination with Daunorubicin. The obtained values are presented as mean ±standard deviation (mean ± SD) of three independent experiments. The GAPDH was employed as an internal control. *p <0.05, **p<0.01, and *** p<0.001.

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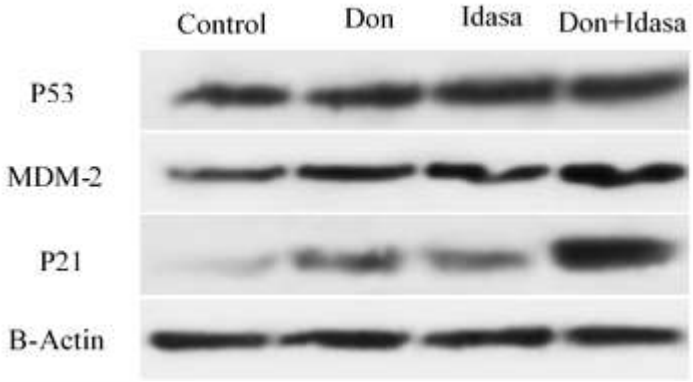

Figure 5. The cotreatment effect of Idasanutlin and Daunorubicin on the activation of p53 signaling pathway; NALM-6 cells were co-treated with 300 nM Idasanutlin and 90 nM Daunorubicin for 36 h. Then, the protein content was extracted from the harvested cells, and the expression of p53 and MDM-2 proteins was evaluated using Western blot analysis. The results showed that the concurrent use of the two drugs caused a significant increase in the expression of p53 and MDM-2 proteins in NALM-6 cells. Beta-actin was used as an internal control.

Discussion

The use of anthracycline in cancer therapy has been widely accepted owing to its ability to induce DNA damage and stimulate apoptosis pathways in the p53-dependent pathway (16). Although the optimization of combination therapies has been successful in achieving 80% complete cure in ALL patients, drug resistance and overall clinical outcomes have been lower in patients with T-ALL than those diagnosed with B-ALL (17-19). Since the p53 mutation rate is not considerable in patients diagnosed with ALL, the inhibition of this protein by MDM2 reduces its activity in several types of cancer in which p53 mutation is not common (20). Elevated levels of MDM2 expression in pediatric ALL have been ascribed to the lower therapeutic response rate (21). A number of studies have shown that both the sensitivity of ALL cancer cells and the duration of recovery prior to relapse are dependent upon the expression of MDM2. In addition, increased expression of MDM2 is correlated with poor treatment response and prognosis in ALL patients (8, 22). Cells exhibiting high expression of MDM2 are resistant to topoisomerase II inhibitors and other chemo-therapeutic drugs (8, 23). Several lines of evidence indicate that MDM2 increases the expression of MDR1, which is involved in the development of chemo resistance (24, 25); thus, targeting the MDM2 protein may enhance the efficiency of Daunorubicin. Idasanutlin, also known as RG-7388, is classified into a new generation of MDM2 inhibitors introduced in 2013 (26). Currently, RG-7388 is being analyzed in clinical trials designed for the treatment of chronic myeloid leukemia (CML), acute myeloid leukemia (AML), multiple myeloma, and myelodysplastic syndrome (MDS) (27). In the present study, the impact of Idasanutlin (RG-7388) on apoptosis and expression of drug-resistant genes was evaluated in B-ALL cells treated with Daunorubicin. The findings showed that the cytotoxicity of Idasanutlin was dose-dependent regardless of being used alone or in combination with Daunorubicin. Previous studies conducted on the designed inhibitors of MDM2 have shown similar results to the current findings on ALL. In 2015, a study performed by Lakoma et al. indicated that the treatment of neuroblastoma cell lines and its xenograft models with RG7388 caused an increment in the apoptosis of cells carrying the wild-type p53 by activation of p53-dependent pathway as well as a reduction in tumor proliferation in xenograft samples of a murine model of

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neuroblastoma (28). In this context, Lehman et al. evaluated the simultaneous effect of Idasanutlin and a specific inhibitor of Bcl-2, Venetoclax, on various types of AML cell lines as well as their xenograft models. They found that the use of two different inhibitors caused a synergistic response in cancer cells (29). Chen et al. analyzed the single or combined use of RG7388 with other chemotherapeutic agents, such as cisplatin, doxorubicin, temozolomide, and topotecan against neuroblastoma cells, expressing the wild-type p53. They found that the use of RG7388 had a synergistic role in the induction of apoptosis in these cell lines (30). In 2016, Zanjirband et al. assessed the impact of two different types of MDM2 inhibitors, namely RG7388, and nutlin-3, in combination with cisplatin on different ovarian cancer cell lines. They revealed that the concomitant use of the two inhibitors of MDM2 and cisplatin resulted in the promotion of apoptosis by activation of the p53-dependent pathway and caused cell death in these cell lines (31). In another study, they evaluated the effect of combinational treatment with Idasanutlin, nulin-3, and rucaparib on ovarian cancer cell lines. Their results indicated that Idasanutlin had a synergistic effect on the induction of apoptosis in these cell lines (32). In another recent pre-clinical study in 2020, Bell et al. examined the effect of Idasanutlin as a single agent in 17 high-risk adults with ALL, both B-ALL (n=15) and T-ALL (n=2) and patient-derived xenograft samples (n=6). They showed that Idasanutlin has a potential anti-tumor effect and suggested further investigations to assess the precise effect of Idasanutlin on the treatment of ALL (33). In the present research, the treatment of NALM-6 cells with Daunorubicin and Idasanutlin led to the upregulation of PUMA and NOXA genes, which act as pro-apoptotic genes. Inversely, the combination of the two compounds resulted in the downregulation of BCl-2 and MCL1 genes, which act as anti-apoptotic genes, ultimately stimulating the apoptosis process. These results were in agreement with the findings of Lehmann et al. (29) and those of a recent study carried out by Vernooij et al., which revealed treatment with Idasanutlin could downregulate MCL1 (34). The expression of drug-resistant genes (MDR1 and BCRP) was also investigated in this study. The results showed that combinational treatment with Idasanutlin and Daunorubicin compared to Daunorubicin alone caused a substantial reduction in the expression of MDR1 but not BCRP, These results were in accordance with the hypothesis that MDM2 may increase the expression of MDR1 as well as the results obtained by Grigoreva et al., who applied computational modeling to design MDM2 inhibitors that potentially act as p-gp inhibitors (MDM2 and p-gp). However, the precise mechanism underlying the role of MDM2 inhibitors in the elimination of drug resistance is not clear yet, and further studies are warranted.

Conclusion

Idasanutlin had an anti-leukemic activity against NALM-6 cells. Idasanutlin can also overcome several mechanisms involved in drug resistance. Since some ALL patients are resistant to conventional treatment regimens, the combination of this drug with Daunorubicin can highly boost the anti-proliferative effect of Daunorubicin. However, further in vivo investigations are recommended to determine the safety and efficacy of this combination therapy in the treatment of B-ALL patients.

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Conflict of interest

The authors declare no conflict of interest.

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