

# AMG-232, a New Inhibitor of MDM-2, Enhance Doxorubicin Efficiency in Pre-B Acute Lymphoblastic Leukemia Cells

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## Abstract

**Background:** Doxorubicin (DOX)-induced cardiotoxicity appears to be a growing concern for extensive use in acute lymphoblastic leukemia (ALL). The new combination treatment strategies, therefore might be an effective way of decreasing its side effects as well as improving efficacy. AMG232 (KRT-232) is a potential MDM-2 inhibitor, increasing available p53 through disturbing p53-MDM-2 interaction. In this study, we examined the effects of AMG232 on DOX-induced apoptosis of NALM-6 cells.

**Methods:** The anti-leukemic effects of Doxorubicin on NALM-6 cells, either alone or in combination with AMG232, were confirmed by MTT assay, Annexin/PI apoptosis assay, and cell cycle analysis. Expression of apoptosis and autophagy-related genes were further evaluated by Real time-PCR method. To investigate the effect of AMG232 on NALM-6 cells, the activation of p53, p21, MDM-2, cleaved Caspase-3 proteins was evaluated using western blot analysis.

**Results:** The results showed that AMG232 inhibition of MDM-2 enhances Doxorubicin-induced apoptosis in NALM-6 cells through caspase-3 activation in a time and dose-dependent manner. Furthermore, co-treatment of AMG232 with Doxorubicin hampered the transition of NALM-6 cells from G1 phase through increasing p21 protein. In addition, this combination treatment led to enhanced expression of apoptosis and autophagy-related genes in ALL cell lines.

**Conclusions:** The results declared that AMG232 as an MDM-2 inhibitor could be an effective approach to enhance antitumor effects of Doxorubicin on NALM-6 cells as well as an effective future treatment for ALL patients.

**Keywords:** Acute Lymphoblastic Leukemia, AMG 232, Autophagy, Doxorubicin, p53.

## Introduction

Acute lymphoblastic leukemia (ALL), the most common form of malignant neoplasia affecting children (1). There are different types of treatment for patients with ALL including chemotherapy (2). Despite long-term survival and high recovery rates for patients, relapse or resistance to chemotherapy is the main causative of death in ALL (3). Doxorubicin (DOX) currently used as one of the most effective anticancer drugs for treatment ALL (4-6). The anti-tumor activity of DOX is

primarily through disruption of DNA repair and inducing apoptosis (7). However, the negative point associated with administration of DOX which has limited its clinical application is induction of cardiomyopathy and congestive heart failure (8, 9). Numerous treatment options have been developed to reduce doxorubicin-mediated cardiotoxicity, including lowering the dosage or applying combined therapies (10). Evidence has suggested that p53 significantly enhances

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DOX-induced apoptosis in tumor cells (11). It is a transcription factor that acts as a tumor suppressor and plays a pivotal role in the regulation of cell cycle, apoptosis, and autophagy (12). Murine double minute 2 (MDM-2) can bind to p53 and act as a ubiquitin ligase, resulting in its degradation. MDM-2 regulates p53 stability via proteasomal degradation, thus inhibition of this interaction results in recruiting and enhancing p53 different biological functions in cells with wild-type p53 (13). Apart from relapsed patients, P53 mutations are infrequent in ALL (14). Autophagy, a homeostatic mechanism involved in cell survival has a dual role in cancer. Some studies show that the induction of autophagy results in overcoming resistance to DOX in certain types of cancers, however its role in lymphoid neoplasms remains controversial (15, 16).

AMG 232 (KRT-232) is a compound that binds to MDM-2 and disturbs the interaction between the p53 protein and MDM-2, thus increasing the available activates p53. As an effective treatment strategy, it is currently under clinical trial for the treatment of various solid tumors (17, 18). Thus, the combination of AMG 232 with agents such as Dox to maintain efficacy and minimize cytotoxic effects, as well as enhance its antitumor activity may be a promising therapeutic approach in the years ahead. Since the effects of AMG 232 on apoptosis and autophagy have not yet been clarified in pre-B acute lymphoblastic leukemia cells, the present study was designed to evaluate the capacity of AMG 232 to increase the sensitivity of NALM-6 cells to Dox as a valuable therapeutic approach.

## Materials and Methods

### *Cell Culture and Solution Preparation*

NALM-6 cell line was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute, Tehran, Iran. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, UK), 2 mM L-glutamine (Gibco, UK), and 100 U/ mL

penicillin, 100 U/ mL streptomycin (Gibco, UK), in a controlled atmosphere (37 °C, 5% CO<sub>2</sub> concentration and 95% humidity). The NALM-6 cell line in the logarithmic growth phase with passage number 4 or 5 was used for subsequent experiments. AMG 232 was purchased from Medchemexpress (UK) and prepared by dissolving 5 mg of AMG 232 powder in 1.76 mL dimethyl sulfoxide (DMSO, Merck). Doxorubicin hydrochloride was obtained from Ebewe, Austria, with an initial concentration of 50 mg/25 mL and stored at 4 °C for subsequent use.

### *Cytotoxicity Assay*

To evaluate AMG 232 effect on cell viability and metabolic activity in ALL cell line, NALM-6 cells were exposed to different concentrations of AMG232 (100•300•500 nM) or Doxorubicin (50 nM), either alone or in combined treatment. Briefly, NALM-6 cells were seeded into a 96-well culture plate (20×10<sup>3</sup> cells/well) and incubated with designated concentrations of drugs for 24 h and 48 h. Afterward, the cells were incubated with 10 µl of MTT solution (5 mg/ml, methyl-thiazol-tetrazolium, Sigma-Aldrich, Germany) for 4 h at 37 °C. The resulting formazan crystals were solubilized by the addition of 100 µL DMSO, and the absorbance was measured by ELISA reader. To assess drug combination effects of AMG232 and Doxorubicin, combination index (CI) and isobologram analysis was computed using CompuSyn Ver1.1 software (ComboSyn, Inc., Paramus, NJ, USA) according to the classic isobologram equation. The additive effect (CI= 1), synergism (CI< 1), and antagonism (CI> 1) between drugs are evaluated according to their CI values.

### *Annexin-V Assay*

To explore the MDM-2-mediated inhibitory effect of AMG232 on apoptosis, flow cytometric analysis of annexin-V binding assay was performed. NALM-6 cells were seeded into twelve-well plates at a density of

150×10<sup>3</sup> cells/well. After 24 h and 48 h treatment with different concentrations of AMG 232 (100·300·500·1000·10000 nM) and DOX (50 nM), the cells were collected and resuspended in cold PBS. Cells were pelleted and incubated with 100 µL annexin-V/PI (MabTag, Germany) incubation reagent containing 90 µL of 1X binding buffer, 5 µL PI, 5 µL annexin-V for 20 min in the dark at room temperature and were detected by FACS Calibur (Becton Dickinson). The apoptotic index was calculated by FlowJo version 7.6.1 software (BD Life Sciences, USA), measured in each of three independent experiments.

#### Flow cytometric investigation of DNA content

To determine whether AMG232 induced inhibitory effects were possibly due to cell cycle arrest the cells were treated with AMG232 and Doxorubicin at the concentrations of 300 nM and 50 nM respectively for 24 h. Briefly, 150×10<sup>3</sup> pretreated cells were harvested, washed twice with cold PBS, and fixed overnight in 70% ethanol at -20 °C. Subsequently, cells were washed twice and resuspended in 100 µg/ml RNaseA (Sinaclon, Iran) and 50 µg/ml propidium iodide (Sigma Aldrich, UK). Finally, the samples were analyzed using BD FACS Calibur Flow Cytometer and Flowjo software.

#### RNA isolation, Reverse transcription and Real-time PCR

To explore the effects of AMG232 and Doxorubicin on p53 signaling pathway, molecular analysis was performed to evaluate apoptosis and autophagy-related genes expression. Total cellular RNA was extracted from the cells after 36 h treatment with AMG 232 (300 nM) and DOX (50 nM) using One Step-RNA Reagent (Bio Basic, Canada) according to the manufacturer's instruction. The quantity of RNA samples was assessed using Thermo Scientific Nanodrop. 1 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 instruction. Generated cDNA was amplified in qPCR using RealQ Plus 2x Master Mix Green, without ROX™ (Amplicon, Denmark) on a light cycler instrument (Roche Diagnostics, Germany). A 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<sup>-ΔΔCt</sup> formula. The housekeeping gene GAPDH was used as an endogenous control to normalize the variability in expression levels. The nucleotide sequences of the primers are given in Table 1.

**Table 1.** Sequences of the primers used for Real-Time RT-PCR.

Gene	Primer	Sequence	Product Size (bp)
GAPDH	Forward	5'-GAAGGTGAAGGTCGGAGTC-3'	226
	Reverse	5'-GAAGATGGTGATGGGATTTC-3'	
P53	Forward	5'- CGCTTCGAGATGTTCCGAGA-3'	102
	Reverse	5' CTTCAGGTGGCTGGAGTGAG-3'	
MDM-2	Forward	5'- CTCCGTGTTTGGTCAGTGGA -3'	81
	Reverse	5'- TGGTCAGGGTAGATGGGTCC -3'	
P21	Forward	5'- ACCTCCTCTAAGGTTGGGCA -3'	110
	Reverse	5'- TGCCTTCACAAGACAGAGGG -3'	
Puma	Forward	5'- CATGCCTGCCTCACCTTCAT -3'	127
	Reverse	5'- GGTCACACGTGCTCTCTCTAA -3'	
Noxa	Forward	5'- GTAGGTTGTAGTCACTTTAGATGGA -3'	127
	Reverse	5'- CCAGATGGTAAAATAGCTGCCT -3'	
Bcl-2	Forward	5'- GTTTTCACGTGGAGCATGGG -3'	132
	Reverse	5'- CCATTGCCTCTCCTCACGTT -3'	
Mcl-1	Forward	5'- GCAGTGAGGGCTTAGGACAC-3'	101
	Reverse	5'- GCCAGTCAGCACTTAGACCA-3'	
Ulk-1	Forward	5'- AGAATGGGGCTTCGCGGAA-3'	125
	Reverse	5'- GCTTCACAGTGGACGACAGG-3'	
DRAM-1	Forward	5'- CCAAGATTTCCAGAGTGTACC-3'	102
	Reverse	5'- AATGGCCTGCGACATTCAC-3'	

**Protein Extraction and Western Blot Analysis**

$5 \times 10^6$  NALM-6 cells were treated with AMG232 (300 nM) and Doxorubicin (50 nM) for 36 h. Then cells were harvested, washed with cold PBS and lysed in RIPA buffer (Sigma, UK). protein concentrations were determined according to the Bradford method. Equivalent amounts of total cellular protein were separated by 10% SDS-PAGE and afterward transferred to nitrocellulose membrane (Hybond-ECL; Amersham Corp., Little Chalfont, UK). Subsequently, membranes were blocked with 5% skim milk and probed with specific primary antibodies. After 3 washes, 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.

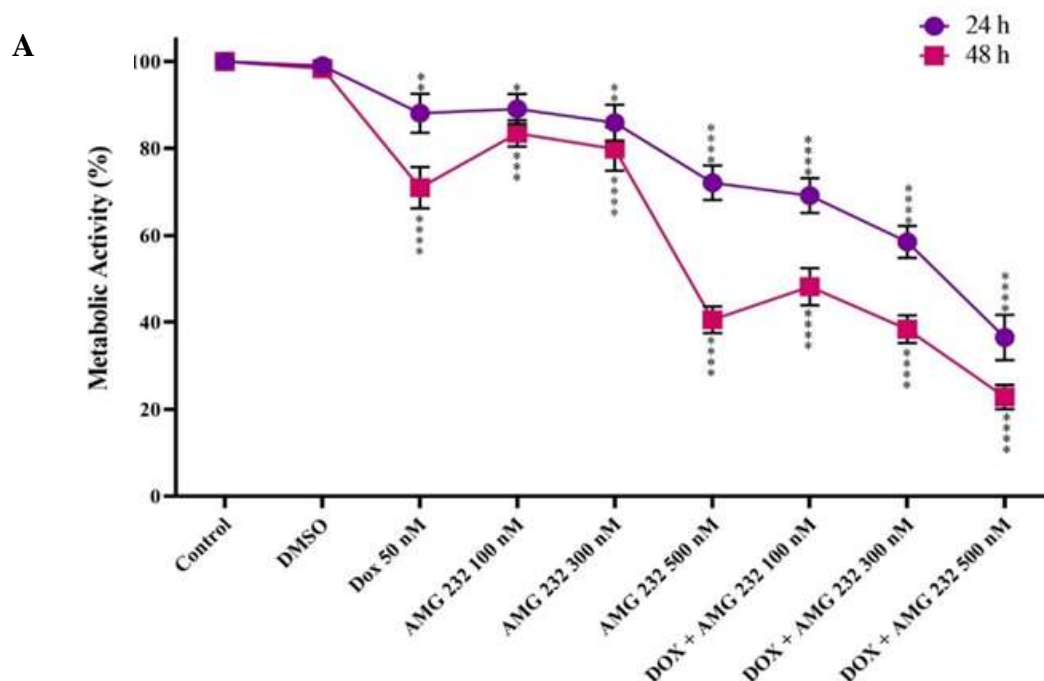
**Statistical Analysis**

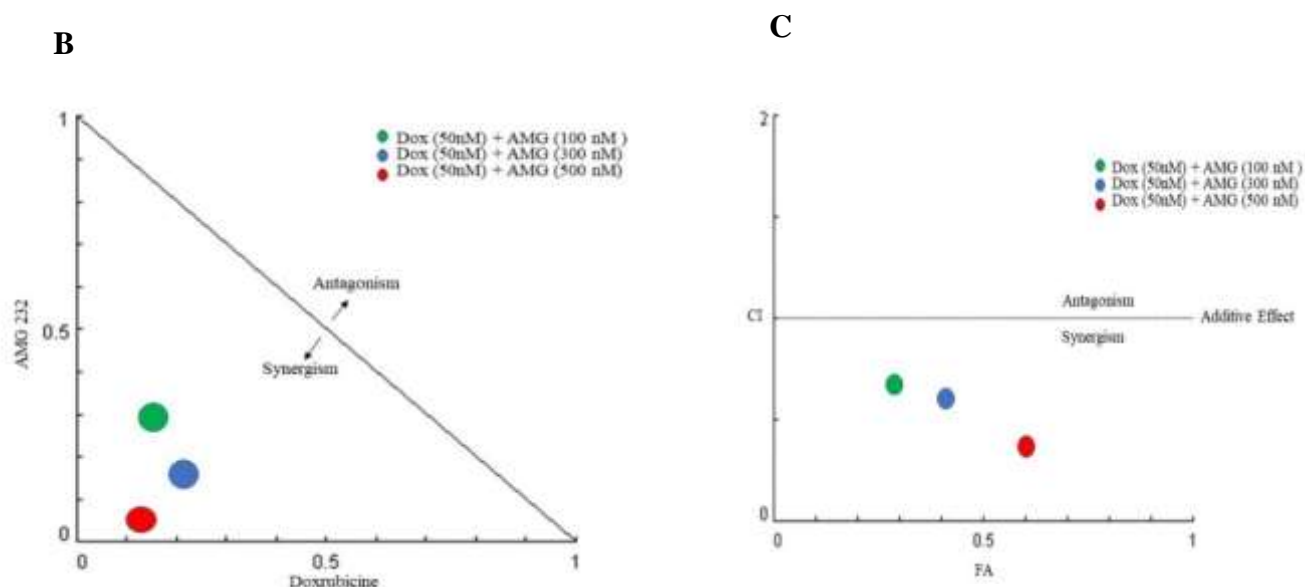
All data were analyzed using Excel software Ver 2019 and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) and are reported by the mean  $\pm$  standard deviation of three independent tests.

The significance of differences between mean values of the groups was analyzed by One-way Analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A p-value < 0.05 was considered statistically significant.

**Results****AMG232 interacted synergistically with Doxorubicin to decrease cell survival and metabolic activity of NALM-6 cells**

MTT assay demonstrated a significant decrease in viability and metabolic activity of NALM-6 cells in a concentration and time-dependent manner. As illustrated in Fig. 1A, the most significant reduction in cell viability was proportional to 48h treatment with 500 nM of AMG232 combined with 50 nM of Doxorubicin. Moreover, it was demonstrated that AMG 232 synergizes with Doxorubicin to potentiate its cytotoxicity. The results were obtained after 24 h treatment of NALM-6 cells and revealed a synergistic correlation between AMG232 and Doxorubicin (Fig. 1B). Moreover, Fraction effect (FA) versus CI analysis showed that the combination of AMG232 and Doxorubicin was more effective in inhibition of cell survival than each agent alone (Fig. 1C).





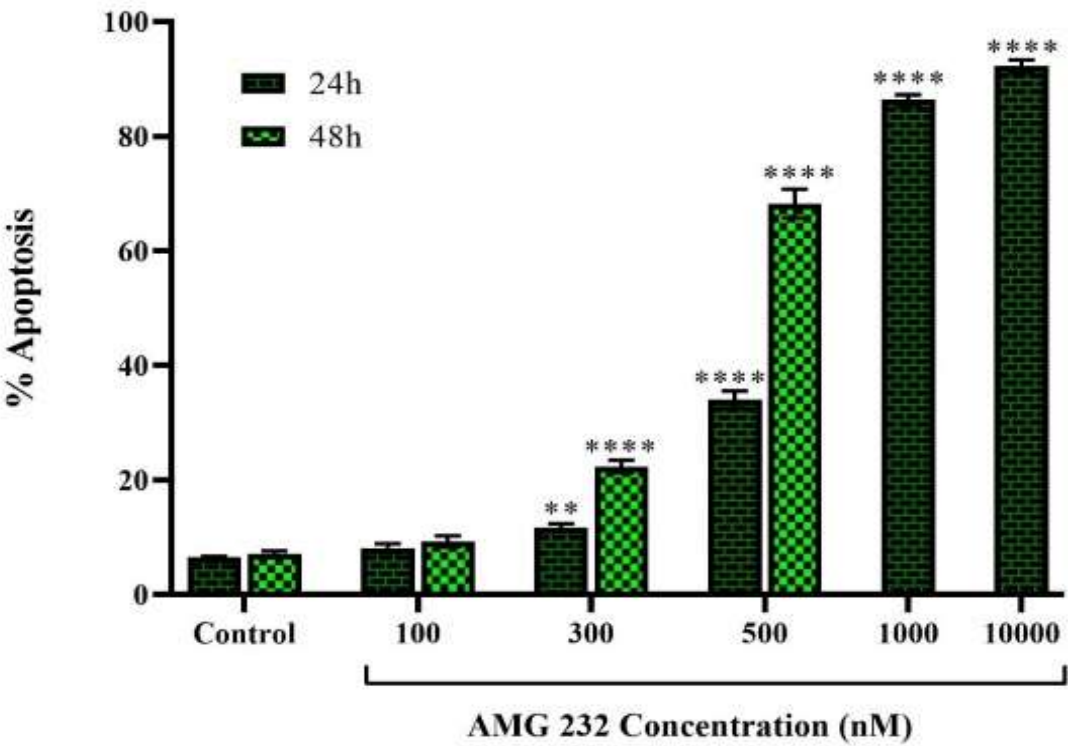
**Fig. 1.** The effects of AMG232 and Doxorubicin on cell viability and metabolic activity. A) MTT results showed that AMG232 sensitized and decreased cell viability of NALM-6 cells treated with Doxorubicin in a concentration- and time-dependent manner. B) Isobologram analysis (Chou- Chou Plot), (Dx)1 and (Dx)2 are related to a single dose, while (D)1 and (D)2 demonstrate combinational effects of AMG232 and Doxorubicin. Points above and below the isoeffect line correlate with antagonism and synergism, respectively. C) Fraction effect (FA) versus CI analysis (Chou-Talalay Plot) revealed a synergistic effect for AMG232 and Doxorubicin.  $CI < 1$ ,  $CI = 1$ ,  $CI > 1$  indicate synergism, additive effect, and antagonism of drugs, respectively. Values of three independent experiments are given as mean $\pm$ SD. The statistical significance of differences between experimental variables was evaluated for either agent alone compared to the control group and combinational doses compared to Doxorubicin. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$  represent significant changes.

### AMG232 induced apoptotic cell death through an increase in p53 protein and caspase 3 cleavage in NALM-6 cells

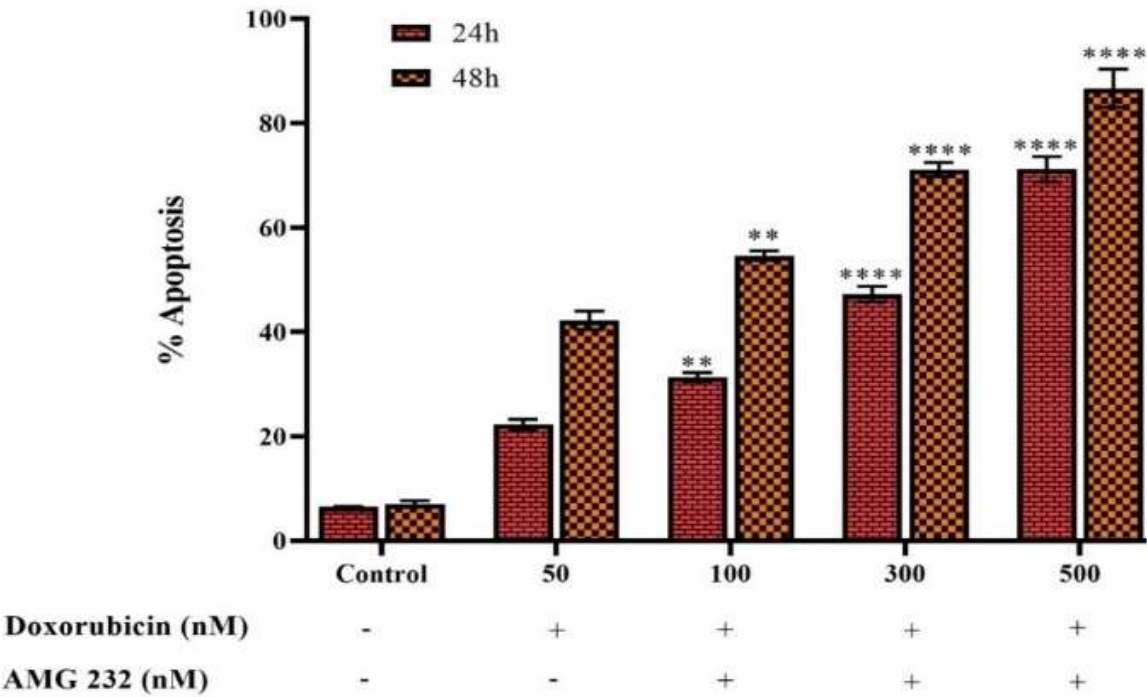
The resulting data indicated that AMG232 enhanced apoptosis of NALM-6 cells in a dose and time-dependent manner (Fig. 2A). Moreover, exposure of NALM -6 cells with increasing concentrations of AMG232 in combination with Doxorubicin resulted in a significant increase in the proportion of apoptotic cells as compared with Doxorubicin alone (Fig. 2B). The highest percentage of apoptotic cells in the early stage was observed

at a concentration of 10  $\mu$ M AMG232 after 24h treatment (Fig. 2C). The highest percentage of apoptotic cells in the early and late stages was proportional to 48h treatment with 500 nM of AMG232 in combination with 50 nM of Doxorubicin (Fig. 2D). Subsequently, Western blot analysis confirmed caspase 3 induced apoptosis in NALM-6 cells. As illustrated in Fig. 2E, combinational treatment of NALM-6 cells with AMG232 and Doxorubicin, induced caspase-3-dependent apoptosis.

A

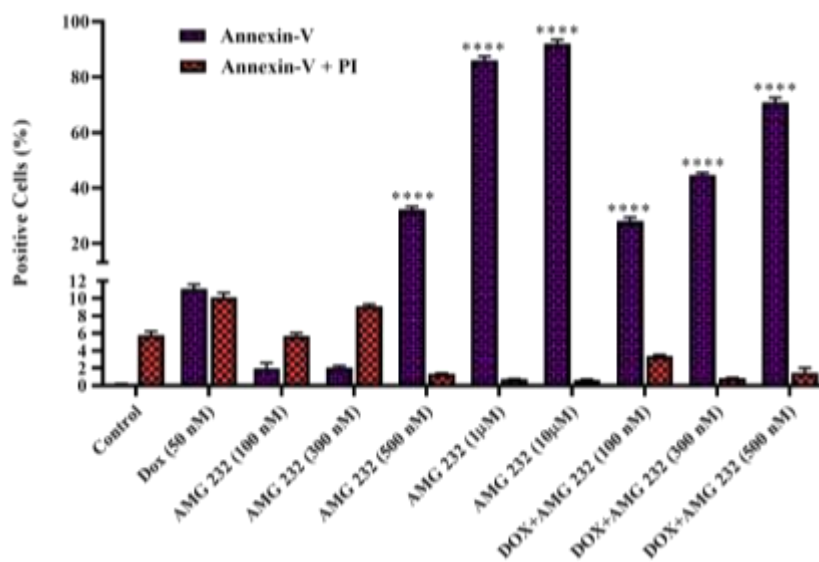
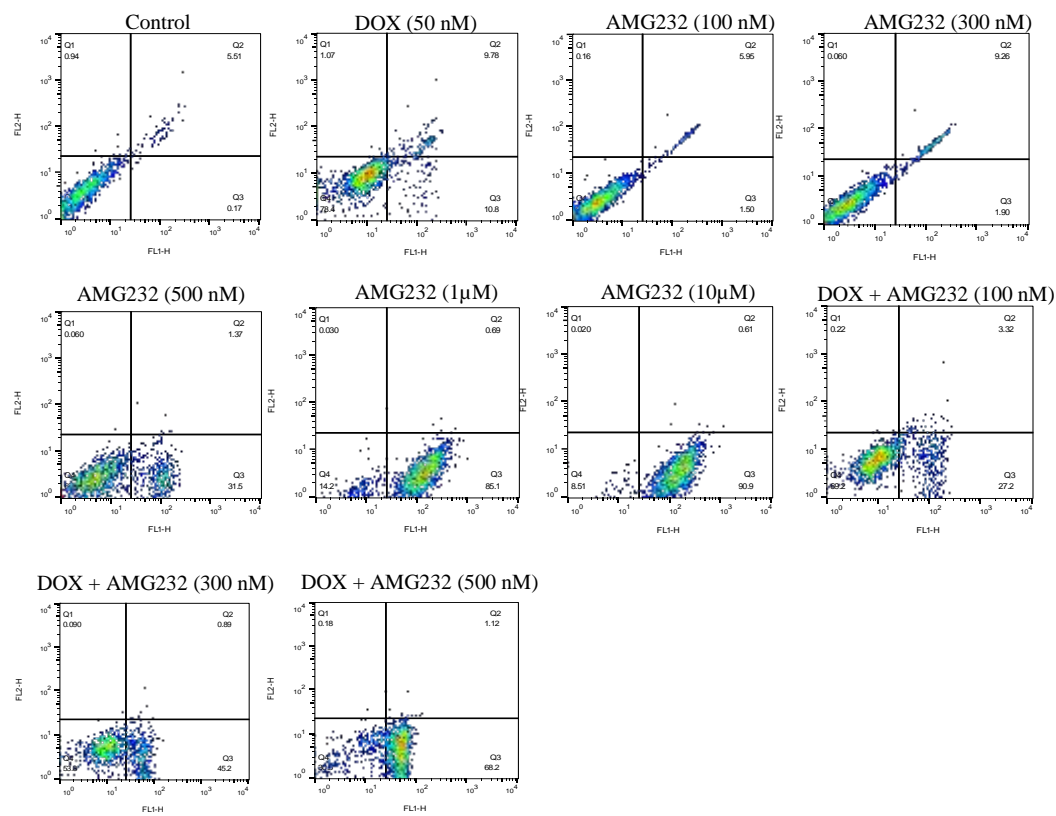


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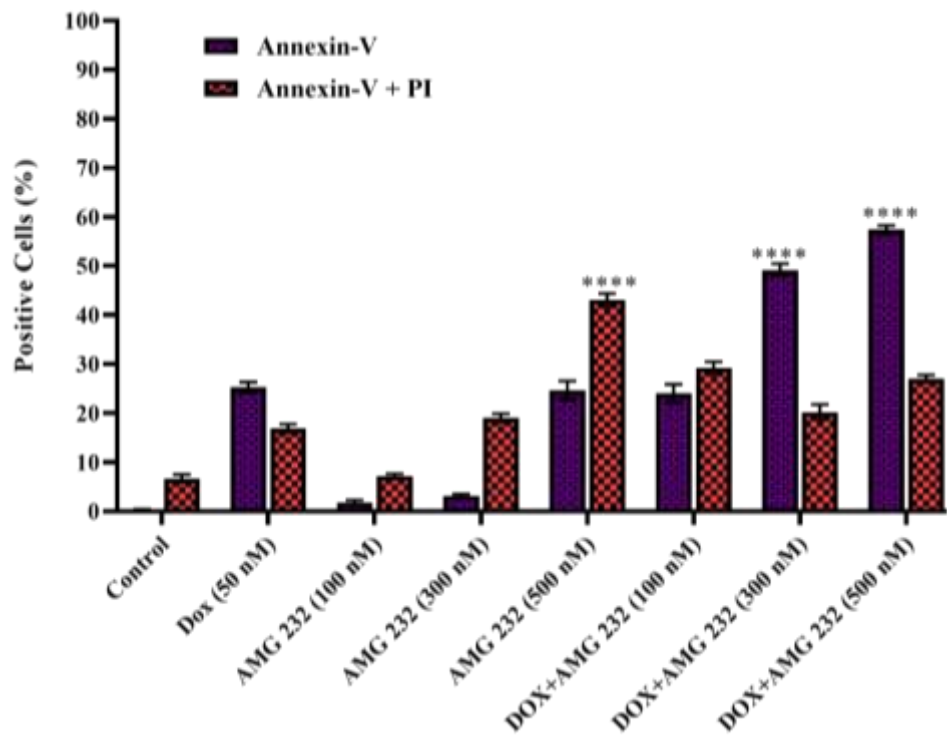
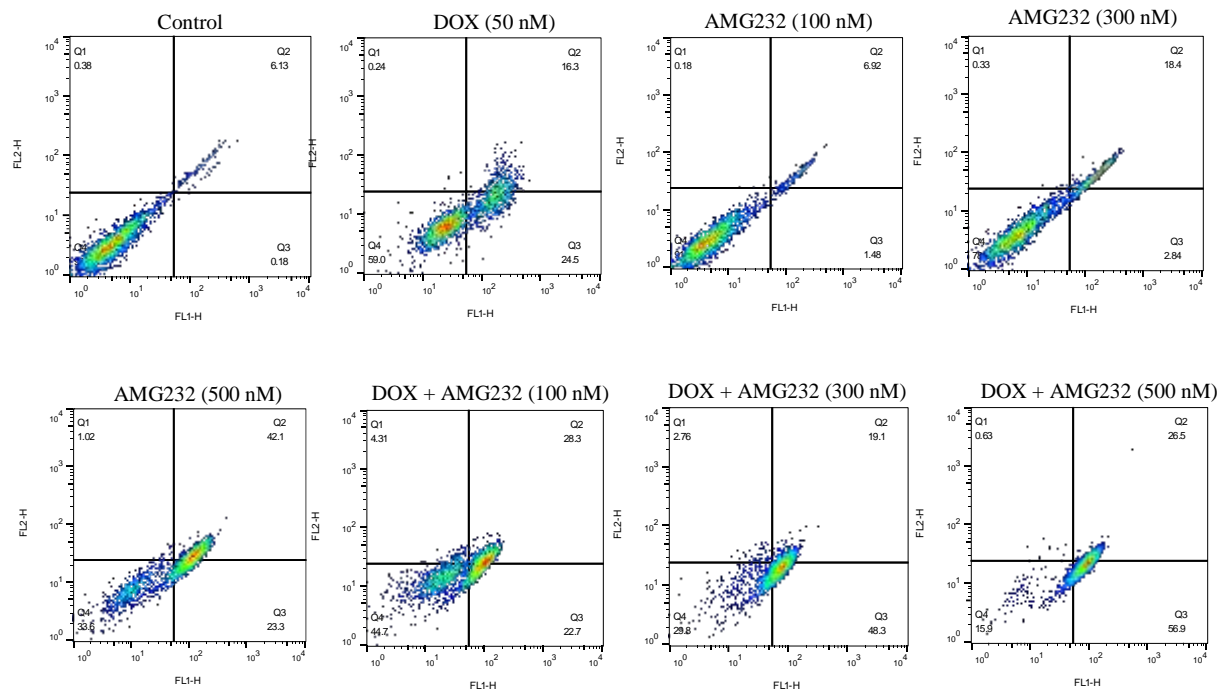




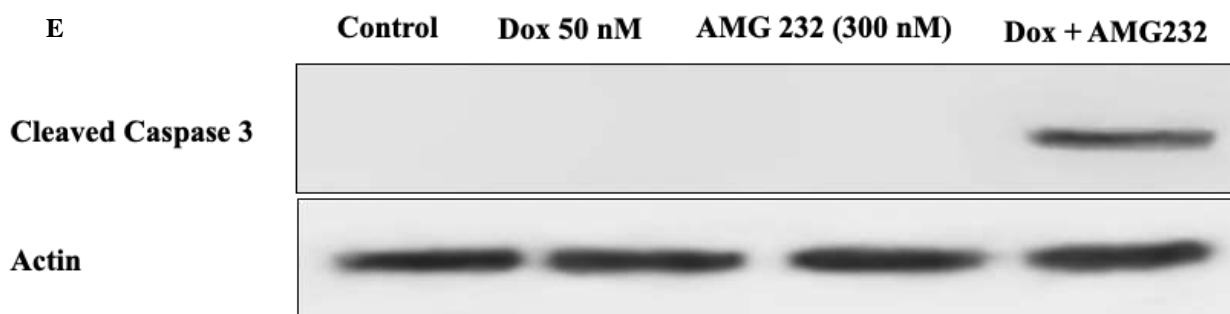
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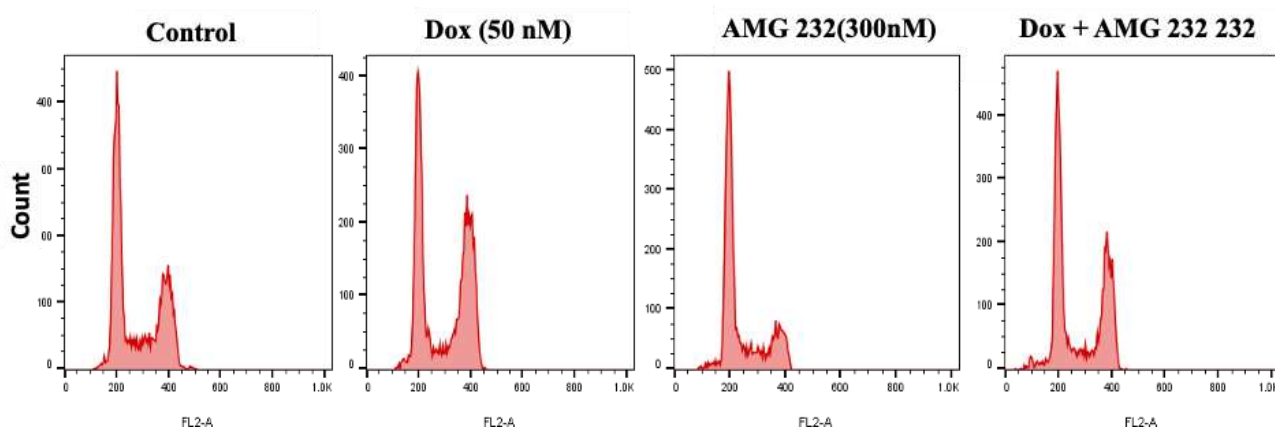
**Fig. 2.** The combination of AMG232 and Doxorubicin resulted in caspase-dependent apoptosis in NALM-6 cells. The effect of AMG232 on NALM-6 cell apoptosis in the absence (A) or presence (B) of Doxorubicin showed a concentration- and time-dependent apoptosis in these cells. Annexin-V/PI apoptosis analysis of NALM-6 cells after 24h (C) and 48h (D) of treatment with indicated agents demonstrated an elevated apoptotic cell population in the early and late stages. E) Western blot results showed caspase-activated apoptosis in response to AMG232 and Doxorubicin combination treatment after 36 h. Values are given as mean $\pm$ SD of three independent experiments. Beta-Actin was amplified as an internal control. The statistical significance of differences between each agent alone was determined in comparison to the untreated group and combination doses in comparison to the Doxorubicin group. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  represent significant changes.

### AMG232-induced G0/G1 cell cycle arrest was mediated through p21 protein increase in NALM-6 cells

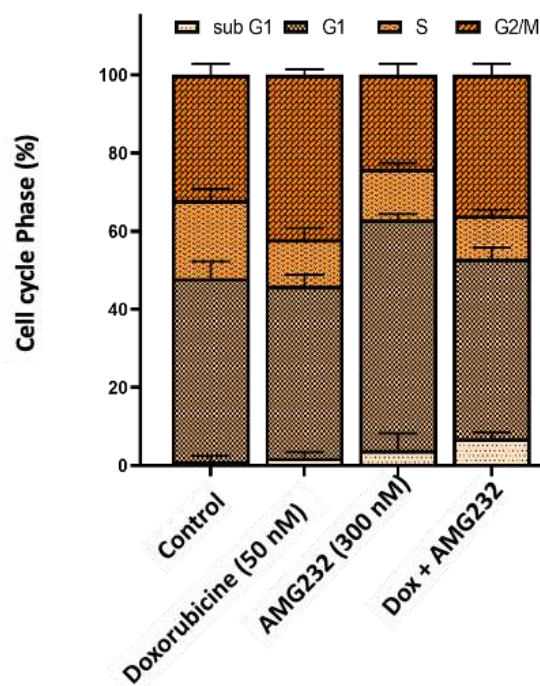
The data indicated that exposure to AMG232 and Doxorubicin elevated percentage of cell populations in G0/G1 and G2/M phases respectively (Fig. 3A). These results revealed that AMG232 induced its anti-cancer effects through a G1 phase arrest while sub-G1 population increased. Besides, the anti-proliferative effects of AMG232 on leukemic cells was further confirmed by a decrease in

the percentage of cell population in S phase. Moreover, combinational treatments led to an increased cell population in sub-G1 phase. These results confirms that AMG 232, in synergy with doxorubicin, increases apoptosis in NALM-6 cells. In addition, to explore cell cycle arrest mechanisms expression of p21 was evaluated through Western blot analysis. These results showed that AMG232 in combination with Doxorubicin activated p53-p21 signaling pathway by a remarkable increase in p21 expression.

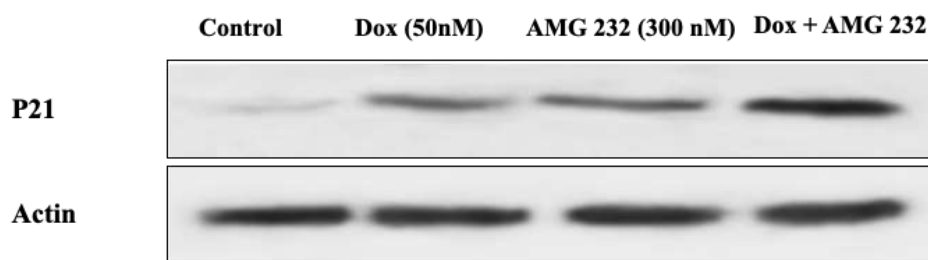
A



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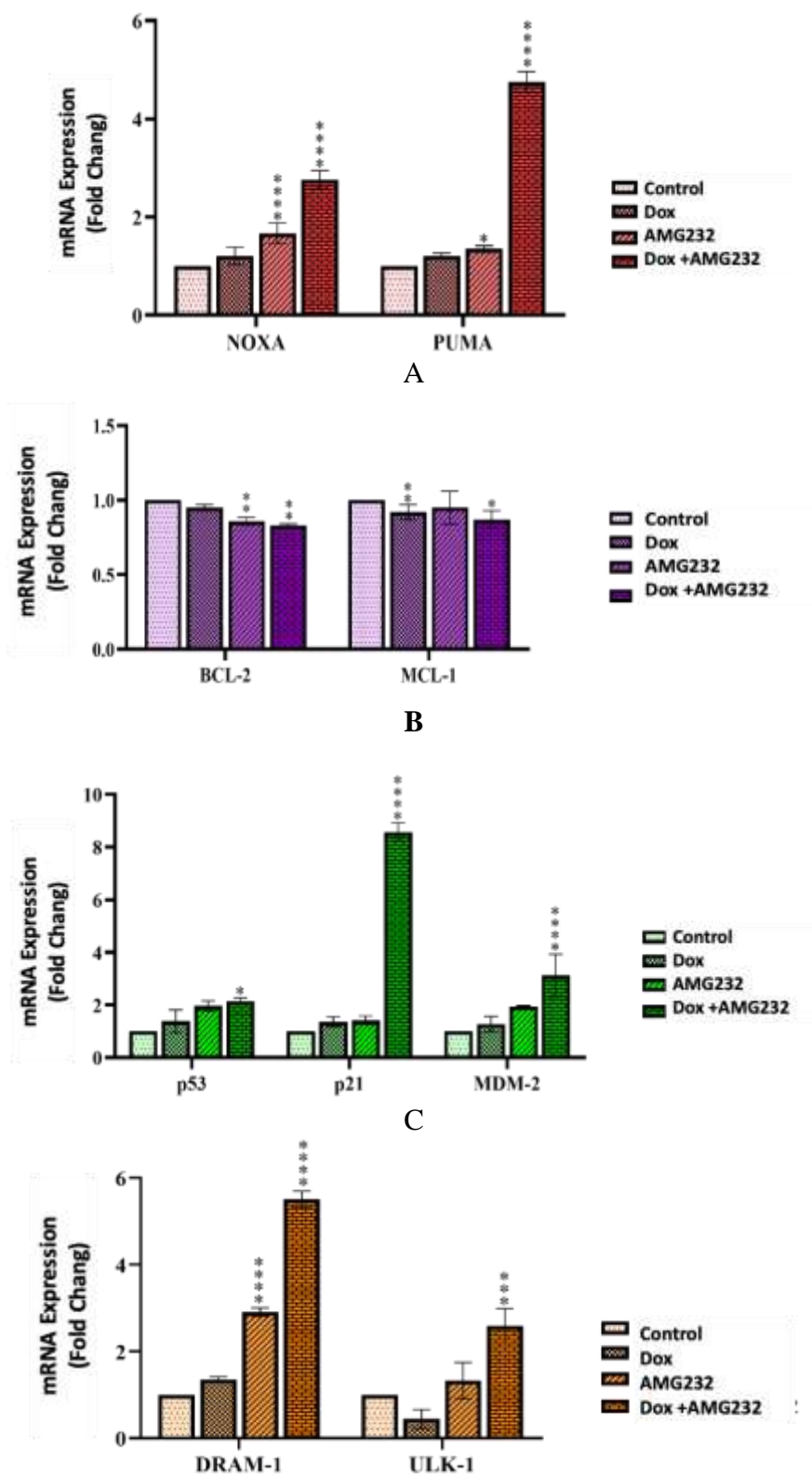


**Fig. 3.** The effects of AMG232 and Doxorubicin on the distribution of NALM-6 cells in different phases of the cell cycle. A) Treatment with AMG232 increased G0/G1 cell percentage. B) The percentage of cell populations in different phases of cell cycle after 24h treatment. Values of three independent experiments are given as mean $\pm$ SD. C) Western blot analysis results demonstrated a significant increment in the expression of p21, one of the main cell cycle inhibitors.  $\beta$ -Actin was used as an internal control.

### ***AMG232 induced apoptotic cell death in NALM-6 cells through the alteration of apoptosis- and autophagy-related genes and proteins***

The data of Real-time PCR indicated an increase in the mRNA levels of pro-apoptotic genes, including PUMA and NOXA, while BCL-2 and MCL-1 mRNA expression levels were decreased (Fig. 4A and B). These findings were consistent with annexin-V/PI flow cytometric analysis, which revealed that treatment of NALM-6 cells with these agents exerts caspase-3-dependent apoptosis. Moreover, the expression of genes involved in p53 signaling pathways (p53, p21, MDM-2) was upregulated (Fig. 4C). The experiments showed a greater increase in mRNA levels of apoptosis related genes in combination therapy

compared with AMG232 or Doxorubicin alone. Thus, it was evident that this drug combination was more effective in, activation of the p53 signaling pathway than either agent alone. Next, to further investigation of apoptosis mechanism induced by AMG232, autophagy-related gene were analyzed. The results demonstrated a significant increase in the expression levels of ULK-1 and DRAM-, two genes involved in autophagy and the p53 signaling pathway (Fig. 4D). Our results confirmed that AMG232 in combination with doxorubicin increased apoptosis and autophagy-related genes which induce apoptosis in NALM-6 cells.

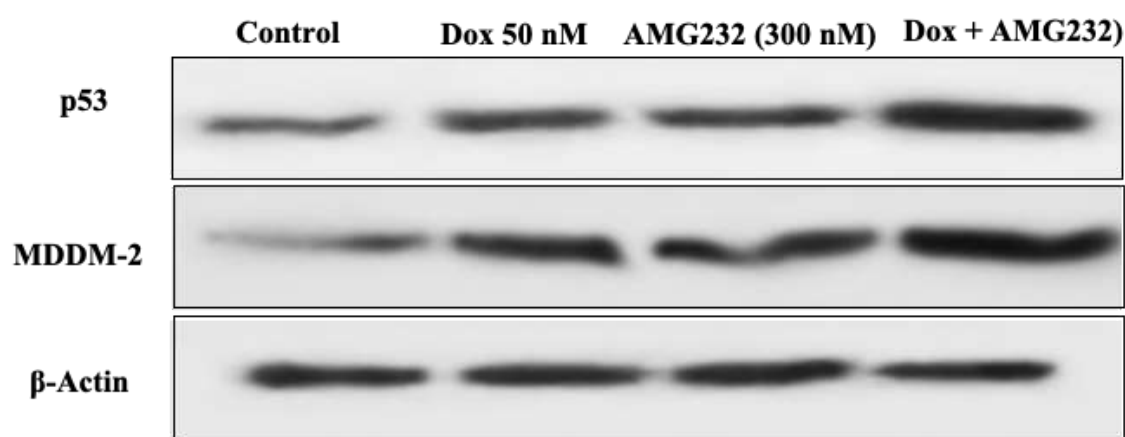


**Fig. 4.** The effects of AMG232 and Doxorubicin on apoptosis and autophagy-related genes. AMG232 synergized with Doxorubicin resulted in A) an elevation of the expression levels of pro-apoptotic genes B) a reduction in the expression levels of anti-apoptotic genes C) an increment in genes involved in the p53 signaling pathway D) an increase in autophagy-related genes. Values of three independent experiments are given as mean±SD.  $\beta$ -Actin was used as an internal control. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$  represent significant changes from untreated controls.

### AMG 232 induced apoptotic cell death through the activation of the p53 signaling pathway

The results of western blot analysis demonstrated a remarkable increase in expression p53, p21, and MDM-2 which was more evident in combined modality, as compared with either agent alone (Fig. 4E). Moreover, increased p53 level was accompanied by upregulation of some target genes including NOXA, PUMA, and

autophagy-related genes. Upregulation of p21 is an alternative indicator of p53 activation (Fig. 3C). MDM2 upregulation is a negative feedback regulator of p53 activity. These results indicated that the combinational treatment of AMG232 with Doxorubicin is associated with activation of the p53-p21 signaling pathway and consequently increased expression of apoptosis-related genes and cytotoxic effects.



**Fig. 5.** The effects of AMG232 with Doxorubicin in, activation of the p53-p21 signaling pathway. The results revealed that combinational treatment could induce an increased expression of p53 and MDM-2 in NALM-6 cells. The experiments were repeated three time separately, one of which is shown.  $\beta$ -Actin was used as an internal control.

## Discussion

In the present study, we investigated the effect of the MDM-2 inhibitor, AMG232, on NALM-6 cells in combination with Doxorubicin. The results revealed that treatment with AMG232 decreased metabolic activity of NALM-6 cells and induced apoptotic cell death, either alone or in combination with Doxorubicin (Fig. 1). The mechanism of cell death triggered by DNA damaging agents such as Dox is mainly mediated through p53 activation. However, Doxorubicin dose reduction may result in the reduction of p53-induced tumor suppression. Therefore, MDM2-targeted therapy may be a potential new strategy to improve the efficacy of chemotherapy regimens. According to this, phase 1 study of AMG232 was evaluated in relapsed/refractory multiple myeloma, AML, and solid tumors with wild-type p53 (17, 18). Our findings are in accordance with the following studies that suggest AMG232 has a significant impact on various tumor cells. It is

reported that AMG232 has a remarkable effect on glioblastoma and patient-derived glioblastoma stem cells (19). A recent study demonstrated that ovarian cancer cell lines with high expression levels of MDM2, are resistant to T-cell-mediated killing, and targeting MDM2 with AMG232 results in T-cell-mediated killing sensitivity of tumor cells (20). The potential anticancer effects of AMG232 in combination with trametinib in non-small cell lung cancer (NSCLC) and Patient-derived xenografts (PDXs) models were shown in a study conducted by Zhang et al (21). In another study the combination therapy of AMG232 with radiotherapy induced tumor response in adenoid cystic carcinoma (22). Besides, treatment of metastatic Melanoma with AMG232 in PDXs hindered tumor growth, either alone or in combination with BRAF and/or MEK inhibitors (23). It is proven that antitumor agents' activity is mediated through activation of apoptosis or suppression cell cycle

progression. p21, promotes cyclin-dependent cell cycle arrest which prevents further G1/S transition (24). Based on the results of our synergistic experiments, treatment of NALM-6 cells with AMG232 in combination with Doxorubicin led to the induction of growth-suppressive and apoptotic cell death in a time and dose-dependent manner (Figs. 2 and 3). A recent study published by Canon et al, highlighting that AMG232 potentially suppresses cell proliferation and induces apoptosis in p53 wild-type tumor cells. G0/G1 phase cell cycle arrest and S phase cell accumulation were associated with induction of p21 protein. Moreover, AMG232 had no effect on p53-mutant tumor cell growth; suggesting a p53-dependent anti-proliferative effect or possibly the contribution of other p53-family proteins such as p73, which needs to be supported by further experiments. In addition, AMG232 alone or in multidrug chemotherapy regimens with Doxorubicin, Cisplatin or Carboplatin, reduced tumor size of *in vivo* tumor models (25). In accordance with our findings, combination treatment strategies result in overexpression of two members of pro-apoptotic genes, PUMA and NOXA, coupled with suppression of anti-apoptotic genes, BCL-2 and MCL-1. Furthermore, stabilization of p53 and overexpression of p21, MDM-2, and p53 occurred after AMG232 treatment. PUMA and

NOXA, as pro-apoptotic mediators and p53 targets, are members of BCL-2 family proteins leading to mitochondrial outer membrane permeabilization via decreased expression levels of BCL-2 and MCL-1 (26). Our experiments declared that incubation of NALM-6 cells with AMG232 resulted in an increased expression of DRAM-1 and ULK-1 autophagy genes and consequently apoptosis induction in leukemia cells. Similarly, Werner et al. reported that treatment of tumor cells with AMG232 and radiation synergistically contributes to the activation of p53-p21 signaling pathway followed by cell cycle halting in G1 phase and overexpression of autophagy-related genes as well as an increase in acidic vacuolar organelles (AVO) cells for apoptosis induction (27). Our study showed for the first time that AMG232, as the newest generation of MDM2 inhibitors, leads to apoptosis of NALM-6 cells. The results of this study showed that the AMG232 induces p53 dependent apoptosis and autophagy. However, further investigations are needed to explore the pathways involved in apoptosis, such as p63, p73, and to evaluate the effect of the AMG232 *in vivo*.

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