Original Article

Restoration of MiR-34a Expression by 5-Azacytidine Augments Alimta -Induced Cell Death in Non-Small Lung Cancer Cells by Downregulation of HMG B1, A2 and Bcl-2 Pathway

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Abstract

Objective: Alimta (Pemetrexed) as an antifolate drug has been approved for the treatment of lung cancer. The aim of the present study was to investigate the combination effect of 5-Azacytidine (5-aza) and Alimta on the miR-34a and its target genes expression and induction of apoptotic cell death in non-small lung cancer A549 cells.

Materials and Methods: In this experimental study, lung cancer A549 cells were treated with various concentrations of Alimta alone and combined with 5-Aza. Then, viability was assessed by trypan blue and MTT assays. mRNA expressions were performed by real time-polymerase chain reaction (PCR) and western blot. Flow cytometry used to detect apoptotic/ necrotic cells and cell cycle arrest.

Results: Alimta alone reduced viability of the cells in a dose dependent manner with the half-maximal inhibitory concentration (IC_{50}) value of 12 μM. Pretreatment of the cells with 5-aza (5 μM) induced a synergistic cytotoxic effect with IC_{50} of 3 μM. Sequential exposure of the cells to 5-aza and Alimta enhanced miR-34a expression and significantly downregulated *HMGB1, HMGA2* and *BCL-2* expressions. Also, it was associated with reduction of nuclear HMGB1 and HMGA2 content. Caspase-3 activation, HMGB1 release into extracellular space and staining of the cells with annexine V/PI suggested that 5-aza reduced late apoptotic/necrotic cell death induced by Alimta. In addition, combination of 5-aza and Alimta arrested the cells at S and sub-G1 phases and inhibited colony formation.

Conclusion: 5-aza synergistically enhances Alimta induced apoptotic cell death through HMG proteins regulation, *MIR34A* gene expression and intrinsic apoptosis mechanism, providing a promising combination therapy in clinical lung cancer therapy.

Keywords: Alimta, Apoptosis, 5-Azacytidine, Lung Cancer Cell, miR-34a

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Introduction

Lung cancer, the leading cause of cancer-associated mortality, is classified into two main histological categories: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC cells comprise approximately 80% of all lung cancers (1). Despite substantial improvements in the diagnosis and treatment of NSCLC, the high mortality rate of this disease has not considerably altered, demanding a novel treatment strategy.

MicroRNAs (miRs) constitute a class of endogenous, small non-coding RNAs consisting of 21-25 nucleotides in length that can negatively regulate gene expression at the post-transcriptional level by influencing stability and/or translation of their target messenger RNA (2). These small RNAs play critical roles in fundamental biological and physiological processes such as cell proliferation, development, differentiation, cell cycle regulation, and apoptosis (3, 4). Considering their broad effects, miRs serve as oncogenes/tumor suppressors as their expression is widely altered in various types of cancers (5). The miR-34 family (miR-34a, b and c) is characterized as tumor suppressor genes which their expression significantly decreased in cancer versus normal cells (6, 7).

Several known oncogenes have been identified as direct targets of miR-34a, such as Bcl-2 (8) and High mobility group proteins (HMGB1 and HMGA2) (9, 10). HMG proteins are the most abundant non-histone chromosomal proteins which are divided into three families (HMGB, HMGA and HMGN) according to their DNA binding motifs. HMGB is the largest group with three sub families, HMGB1, HMGB2, HMGB3, all containing 2 HMG-Box motifs and an acidic tail domain. They act as a DNA chaperone, also, play important role in replication, transcription, recombination and DNA repair (11). In addition, HMGB1 represents different and paradoxical function in the cells and nucleus, it binds to minor groove of

DNA and regulates transcription. In extracellular space, HMGB1 acts as a signal molecule (cytokine) in inflammation, cell proliferation, invasion and tumor progression (12). HMGA family has two members, HMGA1 and HMGA2, with AT-Hook DNA biding motif. HMGA proteins are overexpressed in embryonic development and human malignancies (13).

5-Azacytidine (5-aza, Vidaza) is a nucleoside cytosine analog (Fig.1A) that integrates into DNA of rapidly growing tumor cells during replication. This inhibits DNA methylation which leads to reactivation of tumor suppressors that suppresses tumor cell proliferation and induces cell differentiation and apoptosis sensitivity (14). In addition, several studies have demonstrated that 5-aza, because of its demethylating activity can increase sensitivity of cancer cells to standard chemotherapy drugs such as cytarabine, etoposide, cisplatin, gemcitabine, valproic acid and irinotecan in which lower therapeutic dosage of each individual drug is required in comparison to combination drug therapy (15, 16).

One of the most widely used chemotherapy drug with promising clinical activity for the treatment of NSCLC patients is Alimta (Pemetrexed disodium). Alimta is a novel multi-targeted anti-folate containing the pyrrolopyrimidine-based nucleus chemically similar to folic acid. It is in the list of chemotherapy drugs under folate antimetabolites which exert their antineoplastic activity by inhibiting folate-dependent enzymes used in the de novo biosynthesis of purines and pyrimidines (17). Although Alimta shows antitumor activity against NSCLC cells, its clinical application is limited by its side effects. Therefore, combination of Alimta with chemotherapeutic agents including gemcitabine, platinated drugs, nintedanib and other anticancer drugs has been suggested (18, 19).

In the present study, we aimed to investigate a novel combination regimen of anticancer drug Alimta with 5-aza exploring whether restoration of miR-34a expression by 5-aza can enhance sensitivity of A549 cells to cytotoxic and antitumor activity of Alimta.

Materials and Methods

Alimta (Lilly company, India) and 5-aza (Pharmion company, USA) purchased from 13 Aban pharmacy (Tehran, Iran). Before use, drugs diluted in deionized water to provide a final concentration of 2 mg/ml and stored at -20°C in the dark. Trypan blue, MTT, cocktail protease inhibitor, anti-rabbit IgG-HRP and trypsin obtained from Sigma Chemical Company (Becton Dickinson, San, CA, USA). Antibodies purchased from Abcam (Cambridge, UK). Production of caspase 3 polyclonal antibody described previously (20). Annexin V-FITC apoptosis detection kit obtained from Roche (Karlsruhe, Germany). PBS and RPMI-1640 medium purchased from Gibco company (Gibco, Invitrogen, Denmark). RPMI-1640 supplemented with 3.7 g/l $NaHCO_3$, 30 mg/l asparagine, 1% penicillin and streptomycin pH=7.2 was prepared, sterilized by 0.2 μm Millipore filter and then, kept at 4°C before use.

The study was approved by the Ethical Committee of Faculty of Science, University of Tehran, Tehran, Iran (IR.UT.SCIENCE.REC.1400.016).

Cell culture and cytotoxicity assay

In this experimental study, human lung adenocarcinoma cancer cell line A549 obtained from Pasteur Institute (Tehran, Iran). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS by incubation in a fully humidified atmosphere containing 5% CO_2 in air at 37°C. The cells were seeded overnight to reach exponential growth phase and then treated with 0-24 μM of Alimta for different time intervals (24, 48, and 72 hours) to obtain optimal dose and time. In addition, cultures were exposed to different doses of 5-aza (0.5-10 μM) for 24 and 48 hours to determine the concentration by which it can affect 10-25% of cultured cells. Combination therapy carried out sequentially and simultaneously. In sequential study, the cells (10^6 cells/ml) were first treated with 5-aza (5 μM) for 24 hours, the media replaced and then followed by 48 hours treatment with various concentrations of Alimta and in simultaneous treatment, the cells were exposed to both drugs at the same time for 72 hours. For trypan blue exclusion assay, an isotonic solution of trypan blue (0.4%, w/v) was added to drug treated cells and the controls. Then viable cells were counted using hemocytometer under a light microscope. MTT assay carried out according to the method of Mosmann (21). The cells (10^4 cells/well) were seeded into 96-well flat bottom cell culture plates (Nunclon, Denmark) overnight, then, the media replaced and the cells treated with various concentrations of drugs. After incubation, 10 μl of MTT (5 mg/ml in H_2O) was added to each well and incubated in the condition of 95% humidity and 5% CO_2 for 4 hours in the dark. Finally, the medium was removed, the resulting purple formazan crystals dissolved in 150 μl dimethyl sulfoxide (DMSO, Sigma, Becton Dickinson, San, CA, USA) and the optical density (OD) recorded at 570 nm using BioTek ELISA microplate reader (Model Power Wave XS2, Bio Tek, USA). The percentage of surviving cells was estimated as the ratio of absorbance of the treated cells versus the control value.

The pharmacologic combinations between 5-aza and Alimta was assessed using the median-drug effect analysis method by Compusyn software. The combination index (CI) was calculated by the following equation: CI=D1/ (Dx) 1+D2/ (Dx) 2, where D1 and D2 are the concentrations of 5-aza and Alimta used in combination to achieve x % drug effect, respectively. Also, (Dx) 1 and (Dx) 2 are single agent doses to achieve the same effect. The CI value less than 1 (<0.9) represents synergism

while, CI value above 1 (>1.1) suggests antagonism, and CI between 0.9 and 1.1 corresponds to additive effects.

RNA isolation and real time polymerase chain reaction

In order to assess the miR expression, Hybrid-RTM miRNA kit (GeneAll, Korea) was used to extract RNA from the treated cells, according to the manufacturer's instruction. The treatment included various concentrations of 5-Aza (2-10 µM) and different doses of Alimta (1-12 µM) alone and combined with 5-Aza. Detecting miR-34a, miR was amplified by adding poly A tail and then reverse-transcribed into first-strand cDNA using Parsgenome MiR-Amp kit (Parsgenome, Tehran, Iran). Real time quantitative polymerase chain reaction (RT-qPCR) was carried out in final volume of 20 µl using SYBR Green Master Mix (Parsgenome, Tehran, Iran) in a Rotor-Gene Q real-time PCR cycler (Qiagen, German). Since reverse primer provided in the kit was the universal primer, specificity of PCR reaction was dependent on the miR-specific forward primer. Also, forward primer of miR-34a was prepared from Parsgenome Corporation (Tehran, Iran) and the PCR was performed following steps: pre-denaturation step: 95°C for 5 minutes, 40 amplification cycles at 95°C for 5 seconds, annealing: 62°C for 20 seconds, extension: 72°C for 30 seconds, and final extension: 72°C for 10 minutes. The *MIR34A* expression level was determined by the comparative cycle threshold (C_t) method, corresponded to the $2^{-\Delta\Delta Ct}$, and normalized to that of U6 snRNA as the internal control.

Detecting expression level of the interest genes, total cellular RNA extracted from cultured cells following treatment with 5-aza (5 µM), Alimta and sequential treatment with 5-aza and Alimta. In this aim RNX-Plus solution (Sinaclon Bioscience, Iran) was used following supplier's instruction. The total RNA concentration was measured using a Nanodrop UV spectrophotometer at 260 nm. Also, cDNA was synthesized from 2 µg of RNA using 2-steps RT-PCR Kit (Vivantis, Malaysia) according to the manufacturer's protocol. RT- PCR was performed in final volume of 20 µl using SYBR Green Master Mix (Jena Bioscience, Germany). The PCR conditions were an initial denaturation at 95°C for 3 minutes followed by 40 amplification cycles at 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 15 seconds, and final extension at 72°C for 10 minutes. In this study, all primers were synthesized by Sinaclon Bioscience (Iran) which their sequences have listed in Table 1. All gene sequences obtained from the NCBI database. Different online tools like Primer 3, oligo analyzer and oligo 7 were used to design primers of interest genes. Further, NCBI Blast Program was employed to search primers specificity. *β-ACTIN*, a housekeeping gene, was used as an endogenous control for normalization of gene expression data. The gene expression quantitation calculated using the comparative C_t method, where C_t is the threshold cycle. The *HMGB1, HMGA2,* and *BCL-2* mRNA expression levels were determined as fold-change by the $2^{-\Delta\Delta Ct}$ formula (22).

Table 1: List of reverse transcription polymerase chain reaction (RT-PCR) primers of evaluation of genes expression levels

Gene	Primer sequence (5´-3´)
BCL-2	F: ACGACTTCTCCCGCCGCTTACC
	R: ACAATCCTCCCCCAGTTCACCC
HMGA2	F: GGCAGCAAAAACAAGAGTCCC
	R: CTCTTCAGTTTCCTCCTGAGC
HMGB1	F: ACAGCCATTGCAGTACATTGA
	R: ATGCTCCTCCCGACAAGTTT
β-ACTIN	F: CAAGATCATTGCTCCTCCTG
	R: ATCCACATCTGCTGGAAGG

Extraction of proteins

The lung cancer A549 cells incubated in the absence and presence of 5-aza (5 µM) alone, Alimta alone and combined with 5-aza for desired periods. At the end of incubation, the cells were harvested by trypsinization, washed twice with ice cold phosphate-buffered saline (PBS, Gibco, Invitrogen, Denmark), and centrifuged for 5 minutes at 10000 g before adding lysis buffer [15 mM NaCl, 25 mM EDTA and 10 mM Tris-HCl (pH=7.2)]. HMGB1 and HMGA2 proteins were extracted following the method of Goodwin et al. (23). This was done by using 0.35 M NaCl in 10 mM Tris-HCl (pH=7.2) that containing 1/40 ratio cocktail protease inhibitor (Sigma, St. Louis, MO, USA).

To define the release of HMGB1 protein due to drugs action, the culture media from the control and drug treated cells, as described above, was collected, centrifuged at 10000 g for 5 minutes Then HMGB1 protein in the supernatants was solubilized in 5% perchloric acid (PCA, Merck, German). The proteins then were precipitated by 12% trichloroacetic acid (TCA, Merck, German), solubilized in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Sigma, Becton Dickinson, San, CA, USA), sample buffer, heated for 3 minutes in boiling water and analyzed on 15% SDS-PAGE.

For caspase and Bcl-2 proteins extraction, the cells after treatment with the drugs were detached, washed with ice-cold PBS and suspended in extraction buffer containing 62 mM Tris-HCl (pH=6.8), 2% SDS, 10% glycerol, 4 M urea, 0.3% bromophenol blue, 5% β- mercaptoethanol at 4°C, and severely vortexed for 1 hour. The samples then were centrifuged for 10 minutes at 4°C and the supernatants subsequently analyzed on SDS-PAGE and immunoblotted.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot

The extracted proteins were run on SDS-PAGE at

100 V for 1.5 hours as described by Laemmli (24). The proteins were then transferred onto a nitrocellulose membrane (Whatman), pre-soaked in double distilled water. Electroblotting was performed at 4°C for 4 hours, followed by blocking with 1% (w/v) gelatin in Tris-NaCl buffer (50 mM Tris-HCl pH=7.4, 150 mM NaCl), for 1 hour at 37°C and washed three times each 5 minutes with Tris-NaCl buffer. The membranes subsequently were incubated with primary antibodies (HMGB1, HMGA2, Bcl-2 and caspase-3) at 4°C overnight. After a series of washes with Tris-NaCl/Tween 20 (0.05%) buffer, the membranes were incubated with anti-rabbit IgG-HRP (Abcam, Cambridge, UK) as secondary antibody, for 2 hours at room temperature. The membranes were rinsed three times with Tris-NaCl/Tween 20 buffer and the immunoreactive signals visualized with the enhanced chemiluminescence (ECL) according to the manufacturer's instruction. Quantification of the bands was carried out using ImageJ software.

Flow cytometry analysis

Using flow cytometry, apoptotic/ necrotic cells were detected by an Annexin V- FITC and propidium iodide (PI) apoptosis assessment kit according to the manufacturer's protocol. In this assay, the cells, which were treated with 5-aza (5 μM), different doses of Alimta (3 and 12 μM) alone and combined with 5-aza, were trypsinized, washed with PBS and suspended in 100 μl 1x buffer. The cells were then stained with AnnexinV/PI, incubated for 15 min in the dark at room temperature and analyzed using a FACScan flow cytometer (Becton Dickinson, San, CA, USA). The percentage of apoptotic cells was determined by flow max software.

In order to evaluate cell cycle distribution, the control and drug treated cells (10^6/ml), as described above, were collected, fixed in 70% ice-cold Ethanol and stored at 4°C for 2 hours. Then, cells were stained with DNA staining solution (PBS containing 20 μg/ml RNase A and 20 μg/ml PI) by incubating at 37°C for 30 minutes in the dark. The cell cycle distribution was analyzed using a FACScan flow cytometry and data analyzed with FlowJo software.

Colony formation assay

Clonogenic survival of Alimta exposed cells, in the absence and presence of 5-Aza, was determined using colony formation assay. The cells, at low density (3×10^3 cells per plate), were seeded in 60 mm diameter plates for each treatment, allowed to adhere overnight and then treated with various concentrations of Alimta alone for 48 hours and combined with 5-aza for 72 hours. At the end of the treatment period, the media was removed, the cells gently rinsed with PBS, and incubated for 8 days in the fresh media. The colonies were fixed in formaldehyde (2%), stained with 0.5% crystal violet solution. Then, colonies containing more than 50 cells, were scored under an inverted microscope. The percentage of colony formation was estimated from the number of colonies in the treated cells divided by the number of colonies in the control.

Statistical analysis

The data was expressed as mean ± standard deviation. The significance of differences between two groups was measured by Student's t test with version 2.0.0.0. $P<0.05$ and 0.001 were considered as significant and very significant differences, respectively.

Results

Low doses of 5-aza synergistically enhances sensitivity of A549 cells to Alimta

Followed by culture of the cells in the absence and presence of different concentrations of Alimta (0-24 μM) for 24, 48 and 72 hours, we observed that 48 hours exposure significantly reduced viability in a dose-dependent manner. As shown in Figure 1B, after 24 hours treatment of the cells with Alimta, the cells in the control, were 96% viable and a small reduction in the viability of the treated cells was observed. Also, cells exposure to various concentrations of Alimta for 48 hours significantly reduced viability in a dose- dependent manner, 96%, 69% and 52% respectively control group, 3 μM and 12 μM doses. Treatment of the cells for a longer period of time (72 hours) decreased survival rate to 68% at 3 μM and 49% at 12 μM of Alimta. As illustrated in Figure 1C, exposure of the cells to 5-aza (≤10 μM) slightly inhibited growth of A549 cells after 24 and 48 hours, in compared with control group (97%), the cell viability was reduced after 24 hours ~86% at 2 μM and 78% at 5 μM of 5-aza concentration. Therefore, 48 hours of incubation in the presence of Alimta and 24 hours pre-incubation with 5-aza (5 μM) was used throughout the experiments.

In combination assay, the cells sequentially or simultaneously treated with 5-aza plus Alimta and cell survival rate determined by MTT assay. The results revealed the advantage of the sequential over the simultaneous application (Fig.1D). In the simultaneous treatment, viability decreased to 69% and 52% in 3 and 12 μM of Alimta, whereas in sequential combination assay, we observed 54% and 35% of viability at the same concentration. Considering these results, the sequential treatment was selected for further experiments. The inhibitory concentration (IC_{50}) value of Alimta in the A549 cells was 12 μM, while the sequential combination of 5-aza and Alimta reduced IC_{50} value to 3 μM. Using Compusyn software, the combination index (CI) value was less than 1 (Fig.1E), denoting synergism for 5-aza (5 μM) combined with various concentrations of Alimta (1- 24 μM).

In order to explore survival rate and colony forming after treatment with Alimta alone and combined with 5-aza, the clonogenic assay was performed. The results indicated that treatment of the A549 cells with 5 μM of 5-aza had no detectable effect on colony formation and

colony pattern was same as control group (Fig.1F). In the presence of Alimta (3 and 12 µM), a considerable reduction in the number and diameter of colonies was observed, although 5-aza pretreatment before exposure to Alimta was caused significant reduction. In comparison with control (100%), exposure to 3 and 12 µM of Alimta decreased the colony-forming ability to 53% and 24%, respectively (Fig.1G). Whereas, colony formation in the 5-aza pretreated cells was synergistically reduced to 28% and 9% by adding different amount of Alimta, 3 and 12 µM, respectively.

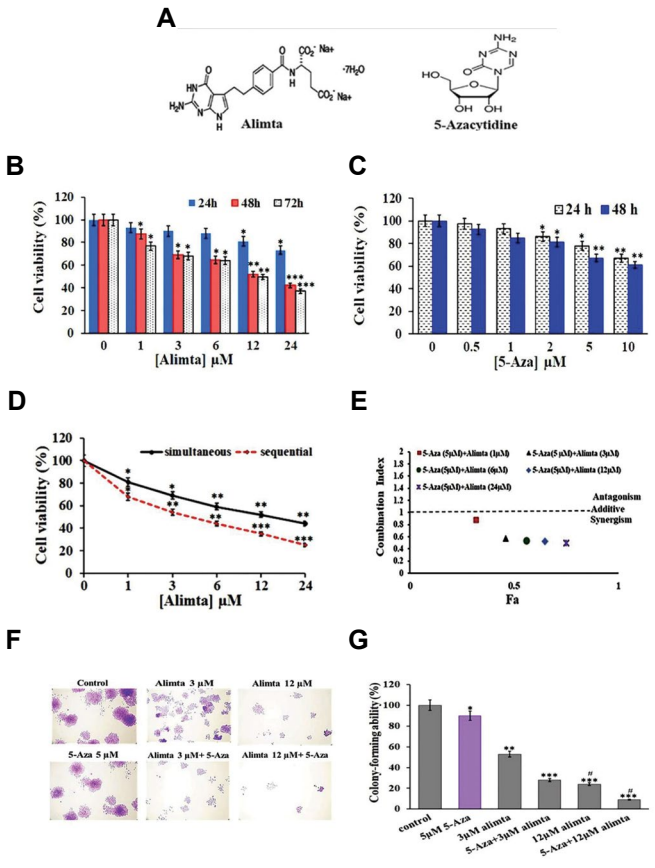

Fig.1: 5-Aza increased sensitivity of A549 cells to Alimta. **A.** Chemical formula of Alimta ($C_{20}H_{19}N_5Na_2O_6.7H_2O$) and 5-Aza ($C_8H_{12}N_4O_5$). **B.** The viability of A549 cells after 24, 48 and 72 hours incubation with varying doses of Alimta (0-24 µM) obtained from MTT assay. **C.** The effect of different concentrations of 5-Aza (0-10 µM) on the survival rate of A549 cells evaluated by MTT assay after 24 and 48 hours exposure. **D.** The viability of the cells treated with 5-Aza and Alimta simultaneously or sequentially determined by MTT assays. **E.** Estimation of synergism: Combination index (CI)<0.9=synergism, CI>1.1=antagonisms. **F.** Colonies were formed by A549 cells after treatment with 0, 3 and 12 µM of Alimta and the cells were pretreated with 5-Aza (5 µM) for 24 hours and then received 0, 3 and 12 µM of Alimta (scale bar: 100 µm). **G.** Histogram displays the percentage of colony-forming ability in the control and drug treated cells. The results are as mean ± SD of three experiments. h; Hour, *; P<0.05, **; P<0.01, ***; P<0.001 significant difference compared to control, #; P<0.05 versus the sequential combination of 5-Aza and Alimta compared to Alimta alone, and 5-Aza; 5-Azacytidine.

Combination of 5-aza and Alimta remarkably enhances miR-34a expression by regulating its targets in the A549 cells

To investigate the effect of low doses of 5-aza (≤10 µM) alone and combined with Alimta on the *MIR34A* expression, the cells were treated for 24 hours with 5-aza (≤10 µM) alone. Using real time PCR, the *MIR34A* gene expression was quantified. The results showed that 5-aza at 2 µM had minimal effect on the *MIR34A* expression, whereas at 5 µM and 10 µM significantly enhance *MIR34A* level by ~ 4 and ~5.9 folds respectively versus the control (Fig.2A). Treatment of cells with various concentrations of Alimta alone caused a very negligible increase in the *MIR34A* expression, however, in the cells that were pretreated with 5-aza and then exposed to Alimta remarkable increase (4-5 fold) in the *MIR34A* expression was detected in comparison with the control (Fig.2B).

Considering the specific role of anti-apoptotic proteins in the chemo-resistance situation, the expression level of *BCL-2*, a downstream target of miR-34a, was measured in the A549 cells treated with Alimta alone and combined with 5-aza investigated. As is shown in Figure 2C, in the presence of Alimta, the *BCL-2* expression level remarkably decreases, and also, at 12 µM of Alimta, we observed about 65% reduction in compared to the control. Using 12 µM of Alimta in the sequential combination treatment (5-aza and Alimta), the expression level of *BCL-2* was reduced to 27%. *BCL-2* Downregulation in the cells treated with Alimta alone and combined with 5-aza encouraged us to investigate the possible effect of these drugs on Bcl-2 protein content by western blot analysis. Therefore, Bcl-2 protein was extracted and examined against its specific antibody. Then, bands relative intensity was interpreted by ImageJ software (Fig.2D, E). We observed that Bcl-2 protein content in the presence of 3 and 12 µM Alimta was 68% and 27%, respectively, while this amount remarkably decreased when 5-aza pretreated cells that had exposed to 3 and 12 µM of Alimta, 33% (3 µM) and 18% (12 µM).

Because of miR-34a influences the expression of its downstream oncogenic targets such as HMGB1, we considered our hypothesis: miR-34a may play a critical role in the increase A549 cells sensitivity to Alimta. For this propose, the HMGB1 content and its expression level were measured in the A549 cells after treated with Alimta alone and combined with 5-aza. Upon treatment of A549 cells with Alimta, there is a decrease in the *HMGB1* expression level in comparison with the control group. Here, we observed a 62% inhibition at 12 µM of Alimta (Fig.3A). Using 12 µM of Alimta in the sequential combination treatment (5-aza and Alimta), the expression level of *HMGB1* was reduced to 30%.

Downregulation of *HMGB1* gene expression encouraged us to see whether these drugs have any effect on *HMGB1* intracellular content. For this purpose, HMGB1 protein was extracted from treated and control cells. Then, immunoblot against HMGB1 antibody was done (Fig.3C). In the control and Alimta (1 µM) treated cells, a thick band in the position of HMGB1 was observed, also, calf thymus HMGB1 protein used as a marker. HMGB1 content reduced to 60%, 44% and 29% when different concentration of Alimta was used (3, 6 and 12 µM, respectively). In combination assay, when the cells

were pretreated with 5-aza and then exposed to Alimta, the content of HMGB1 protein significantly decreased to 51%, 21% and 10% (3, 6 and 12 µM, respectively, Fig.3D).

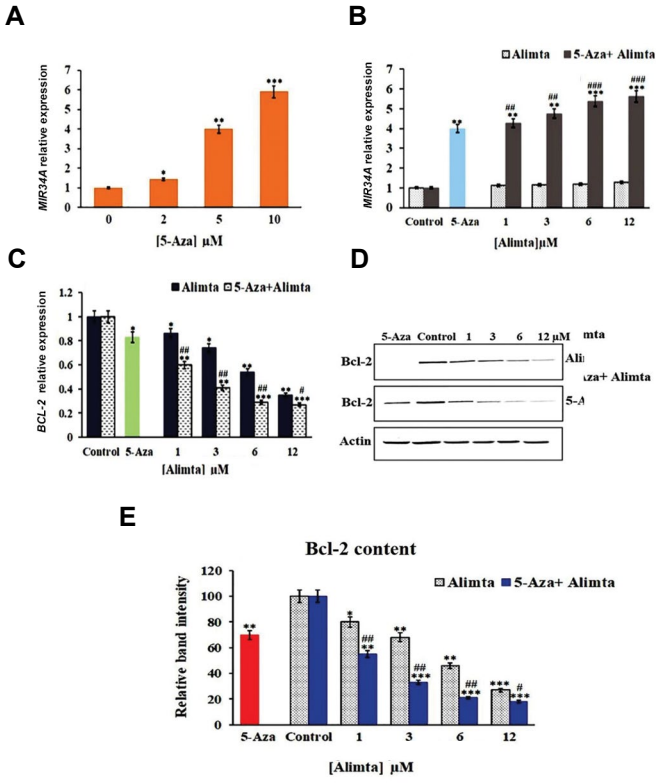

Fig.2: Evaluation of *MIR34A* and *BCL-2* genes expression in A549 cells treated with various concentrations of Alimta for 48 hours alone and pretreated with 5-aza for 24 hours and then exposed to Alimta for 48 hours using real time-PCR and western blot analysis. **A**. *MIR34A* expression level in the A549 cells following treatment with low toxic concentrations of 5-Aza (≤10 µM). **B**. The effect of various concentrations of Alimta (0-12 µM) alone and combined with 5-Aza (5 µM) on *MIR34A* expression. **C.** The effect of Alimta alone and combined with 5-Aza on mRNA expression of *BCL-2* in the A549 cells. **D**. Immunoblot of Bcl-2 protein content, that was extracted from the cells in the absence and presence of Alimta alone or combined with 5-Aza. **E.** Also, intensity of relative bands was quantified by ImageJ software. The column numbers of western blots 1, 3, 6, 12 shows Alimta concentrations (µM). *; P<0.05, **; P<0.01, ***; P<0.001 significant difference compared to control, #; P<0.05, ##; P<0.01, ###; P<0.001 versus the sequential combination of 5-Aza and Alimta compared to Alimta alone, and 5-Aza; 5-Azacytidine.

Since HMGA2 overexpression is correlated with protection of cancer cells against different genotoxic agents, we investigated the possible function of miR-34a in sensitize A549 cells to Alimta through targeting of HMGA2. For this purpose, *HMGA2* gene expression and its content were evaluated in the A549 cells, that were treated with Alimta alone and combined with 5-aza. As is seen in Figure 3B, the control represents high level of *HMGA2* expression but, in the presence of Alimta, the expression level of *HMGA2* remarkably decreases and at 12 µM of Alimta reaches to only 14% of the control amount. Using12-µM of Alimta, in the cells treated with 5-Aza and Alimta, *HMGA2* expression significantly reduced to about 11% of the control. In addition, the effect of drugs on HMGA2 content was also examined and the result shown in Figure 3E. In the presence of low concentration of Alimta (1 µM), a thick band, similar to the control, was observed, however, HMGA2 content decreased following Alimta concentration increment. Here, we observed nearly 45% and 10% of HMGA2 expression following use of 3 and 12 µM of Alimta, respectively. Cells treatment with 5-aza (5 µM) plus 3 and 12 µM of Alimta, was led to a significant reduction of HMGA2 protein content, about 26% and 6%, respectively (Fig.3F).

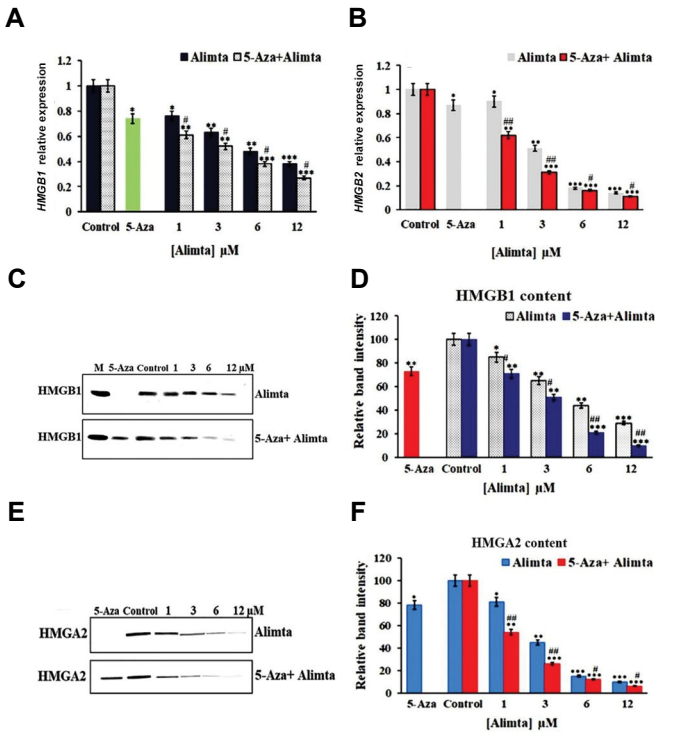

Fig.3: Evaluation of *HMGB1* and *HMGA2* genes expression in the A549 cells treated with various concentrations of Alimta alone and combined with 5-Aza using real time-polymerase chain reaction (PCR). **A, B**. The effect of various concentrations of Alimta (0-12 µM) alone and combined with 5-Aza (5 µM) on *HMGB1* and *HMGA2* genes expression. **C**. Western blot of HMGB1 protein extracted from A549 cells following treatment with Alimta alone and the sequential treatment with 5-Aza and Alimta. M; Calf thymus HMGB1 used as a marker. **D**. Here, intensity of relative bands was quantified by ImageJ software. **E.** Immunoblot of HMGA2 protein. HMGA2 protein was extracted from the cells in the absence and presence of Alimta alone or combined with 5-Aza. **F.** Also, intensity of relative bands was quantified by ImageJ software. The numbers on the blots column (1, 3, 6 and 12) show Alimta concentrations (µM). *; P<0.05, **; P<0.01, ***; P<0.001 significant difference compared to control, #; P<0.05, ##; P<0.01 versus the sequential combination of 5-Aza and Alimta compared to Alimta alone, and 5-Aza; 5-Azacytidine.

5-aza synergistically increases Alimta-induced apoptotic cell death

miR-34a overexpression and its targets downregulation, upon exposure to 5-aza and Alimta, possibly make an anti-survival environment that can lead to apoptosis. Although HMGB1 is a nuclear protein, it also functions as an extracellular signaling molecule that implicates in the inflammatory signaling pathways. To find out whether reduction of HMGB1 protein content is due to its release from the treated cells, the supernatants from the cell cultures was collected and used for HMGB1 western

blot analysis. As is shown in Figure 4A, the control and cells treated with low dose of Alimta (1 μM) did not show any bands in the HMGB1 position, although, by raising Alimta concentration, the content of released HMGB1 enhanced gradually. In the 5-aza pretreated cells, the amount of released HMGB1 protein was diminished (56%) as compared to Alimta alone (Fig.4B).

Caspase-3 plays a central role in apoptotic process and is primarily responsible for the cleavage of special proteins during cell death. Caspase-3 activation assesses in the A549 cells treated with Alimta alone and combined with 5-aza. Western blot analysis showed that the content of caspase-3 increases upon enhancement of Alimta concentration, also, at 3, 6 and 12 μM of Alimta, it was 35%, 48% and 75%, respectively (Fig.4C, D). Sequentially treated with 5-aza and Alimta, in comparison with Alimta alone, a significant increase in the amount of cleaved caspase-3 was observed, that this enhancement, 60%, 85% and 98%, was accompanied with different concentration of Alimta, 3, 6 and 12 μM of, respectively (Fig.4D).

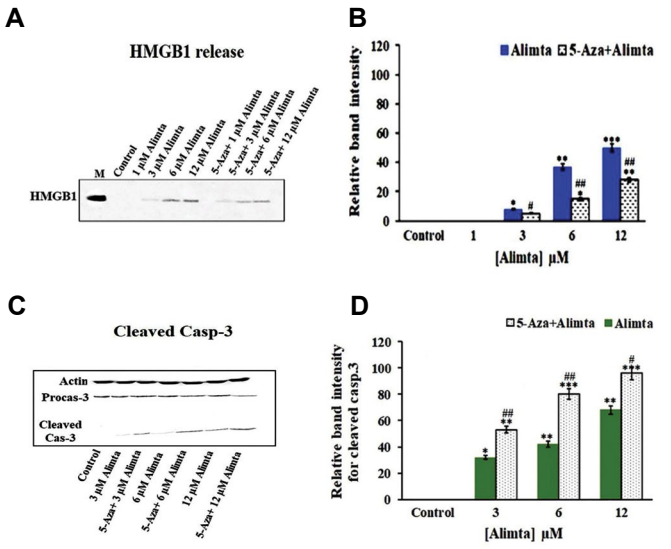

Fig.4: 5-Aza at low toxic dose (5 μM) enhances Alimta- induced apoptotic cell death. **A.** HMGB1 extracellular release was assayed by western blot analysis of the supernatants. M; Calf thymus as a HMGB1 marker. **B.** The protein levels of HMGB1 in control and treated cells quantified by ImageJ software. **C.** Cleavage of pro-caspase-3 to caspase-3 in the A549 cells exposed to Alimta alone and in combination with 5-aza. The β-actin was served as a loading control. **D.** The intensity of relative bands was quantified by ImageJ software. *; P<0.05, **; P<0.01, ***; P<0.001 significant difference compared to control, #; P<0.05, ##; P<0.01 versus the sequential combination of 5-Aza and Alimta compared to Alimta alone, and 5-Aza; 5-Azacytidine.

To obtain further insights into cell death, the percentage of apoptotic and necrotic cells assessed using annexin V/PI dual staining and analyzed by flow cytometry (Fig.5A, B). In the control, alive cells were about 98% that were localized in the region Q3. Upon treatment of the cells with Alimta, 3 and 12 μM, the percentage of apoptotic cells increased to 27.09% and 46.26%, respectively, while most of the treated cells were located in Q2 (late apoptosis) and Q4 (early apoptosis) regions (Fig.5A) whereas necrosis was negligible and did not exceed 6.4% (Q1). As seen, treatment with 5-aza (5 μM) alone had no considerable effect on the apoptosis in the A549 cells and induced apoptosis rate (19.63%) in comparison with the control group. However, in the 5-aza pretreated cells, the content of apoptotic cells significantly increased, 40.36% and 59.47% by adding 3 and 12 μM of Alimta, respectively. The apoptotic/necrotic cells percentage in the both groups, drug treated cells and the control, was compared in the Figure 5B.

To determine whether enhancement of apoptotic cell death in the 5-aza plus Alimta treated cells, distribution of cell cycle phases examined by PI staining and flow cytometry analysis. Although, this was partially due to cell cycle arrest at a specific phase. As illustrated in Figure 5C, the control and the 5-aza alone treated cells represented cell cycle arrest at Go/G1 phase. We observed that Alimta treated cells resulted in an elevation in S-phase cell fraction: 55.58% and 65.21% (3 and 12 μM, respectively) in comparison with the control group (24.28%). Moreover, a slight increase in sub-G1 cell population (12.8%) was observed compared to control. The combination of 5-Aza with Alimta caused a remarkable increase in the sub-G1 phase population, while the population of the cells in the S-phase reduced (Fig.5C). Also, Figure 5D summarizes and compares the percentage of the cells in different cell cycle phases in drug treated cells and the control.

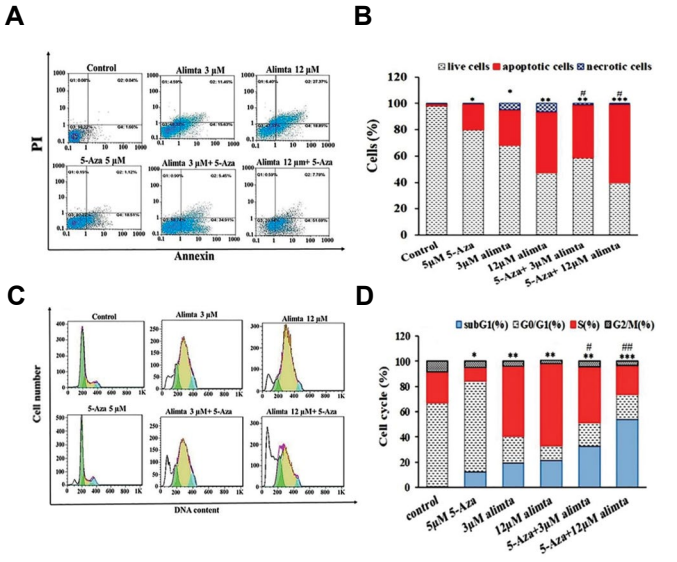

Fig.5: Quantitative analysis of apoptotic cells induced by Alimta alone and combined with 5-Aza using annexin V/PI double staining and flow cytometry assay. **A.** Represent profiles of the cells exposed to Alimta for 48 hours and the cells that first treated with 5-Aza (5 μM) for 24 hours and then exposed to various concentration of Alimta. **B.** Histogram displaying percentages of live, apoptotic and necrotic cells in the control and treated cells. **C.** Cell cycle distribution of the cells treated with Alimta alone and combined with 5-aza after staining with PI and analysis with flow cytometry. **D.** The percentage of the cells in sub-G1, G0/G1, S and G2/M phases of the cell cycle analyzed using Flow JO software. *; P<0.05, **; P<0.01, ***; P<0.001 significant difference compared to control, #; P<0.05, ##; P<0.01 versus the sequential combination of 5-Aza and Alimta compared to Alimta alone, and 5-Aza; 5-Azacytidine.

Discussion

Alimta as an antifolate cytotoxic agent exhibits antitumor activity in the various cancers especially non-small lung cancer. Due to adverse toxic effects, therapeutic potential of this drug has been limited (18). It seems new combination therapy is needed to enhance this plan efficacy. It has been suggested that restoration of miR-34a expression by 5-aza can be a powerful therapeutic strategy against lung cancer (7). In the present study, we conducted *in vitro* experiments to explore a novel combination treatment against A549 lung cancer cells using anticancer drug and epigenetic drug, Alimta and 5-Aza, respectively. Here, we investigated whether restoration of miR-34a expression induced by 5-Aza could increase the cells sensitivity to Alimta.

The viability results demonstrated dose and time dependent cytotoxicity of Alimta on A549 cells with IC_{50} value of 12 μM after 48 hours exposure. Different IC_{50} values has reported for Alimta in various cancer cells. Different IC_{50} values have been reported for Alimta in various cancer cells, for example about 11 μM in the SNU-5 gastric cancer cells after 72 hours and 22 μM in the malignant pleural mesothelioma cells after 24 hours exposure (25, 26), that implies that IC_{50} values depend on different parameters: the cell type, number of cells and exposure time. The sequential combination of 5-Aza, at a low toxic dose (5 μM), and Alimta exerts strong synergistic cytotoxicity in A549 cells with IC_{50} value of 3 μM, implying that at least 4 fold less Alimta is needed to achieve successful treatment against lung cancer. The synergistic inhibition of 5-Aza combined with cytarabine, etoposide, cisplatin, gemcitabine and docetaxel in different cancer cells also represents that the 5-Aza increases sensitivity of cancer cells to these chemotherapeutic drugs (18, 19, 27).

In this study, the clonal growth of A549 cells treated with Alimta alone and in combination with 5-Aza is remarkably decreased, suggesting that the combination therapy significantly reduces the tumorigenic potential of A549 cells. Our result is in accordance with the finding that combination of 5-Aza and cisplatin significantly inhibit colony- forming ability of HTB56 NSCLC cells (28). In the present study, the synergistic cytotoxic and anti-proliferative effects were observed in response to the sequential combination of 5-Aza and Alimta in A549 cells, that is explained by restoring expression of tumor suppressor genes such as *miR-34a*.

Our result indicates that 5-aza; can restore the expression of miR-34a in the A549 lung cancer cells. This finding is in accordance with earlier studies that showing restoration of miR-34a expression using 5-Aza in pancreatic and prostate cancer cells (29, 30). In contrast, the cells exposure to different doses of Alimta, as a DNA-damaging agent, causes only a negligible increase in the miR-34a expression, which probably related DNA induced damage and possibly miR-34a hyper-methylation. Significant overexpression of miR-34a in the 5-Aza pretreated cells is possibly due to Alimta, that induced activation of p53 (31).

The sequential combination of 5-Aza and Alimta remarkably decreased miR-34a target genes expression, *BCL-2, HMGB1* and *HMGA2*. In agreement with our finding, Kojia et al. (32) have reported that dysregulation expression of miR-34a plays an important role in paclitaxel resistance of prostate cancer cells via upregulating *BCL-2* gene expression. These findings together with our data suggest that miR-34a makes cancer cells more susceptible to cytotoxic drugs through modulating its target genes expression and also, decrease in *BCL-2* expression level may induce intrinsic mitochondrial apoptosis pathway.

The HMGA2 expression is limited to proliferating cells such as cancer cells. Our results indicated that in the presence of Alimta alone or combined with 5-aza, HMGA2 level declines that suggesting that the HMGA2 level decrease is due to its mRNA expression inhibition by the drugs action. Moreover, downregulation of HMGB1, as a novel downstream target of miR-34a (12), was observed at both mRNA and protein level in the cells treated with 5-Aza and Alimta. Also, HMGB1 plays different roles. Moreover, its role in the nucleus, can serve as a damage-associated molecule. It seems its reduced level might be due to its passive release from the late apoptotic/necrotic cells into the extracellular space. The results of this study indicate that exposure of the cells to Alimta stimulates release of HMGB1, whereas when the cells are treated sequentially with 5-Aza and Alimta, the release of HMGB1 is remarkably decreased, demonstrating that 5-Aza significantly reduces late apoptosis / necrosis mediated by Alimta. This finding is in consistent with the result of flow cytometry analysis that reveals in the cells pretreated with 5-Aza the percentage of late apoptotic/ necrotic cells are declined but the percent of early apoptotic cells increases. Unlike HMGA2, there is not an exact correlation among *HMGB1* expression, its nuclear content and release, therefore more investigation of probable direct interaction between Alimta and HMGB1 provides further insights.

The present study also demonstrated that restoration of miR-34a and downregulation of its target genes favors apoptotic cell death through caspase-3 activation and subsequently PARP cleavage induction. This is in agreement with the finding of Nalls et al. (29) who have reported that miR-34a upregulation by chromatin-modulating agents induces apoptosis via activating caspases-3 and 7 in human pancreatic cancer stem cells. Under our experimental condition, 5-aza alone treatment, we observed Go/G1 cell cycle arrest is in accordance with the finding that 5-Aza inhibits proliferation of bladder cancer cells by inducing G1-phase cell cycle arrest (33). In contrast, Hu et al. (34) have described endometrial cancer cells arrest at G2/M phase. The Alimta alone displayed S-phase cell cycle arrest, which is in agreement with the reports showing that Alimta induces S-phase cell cycle arrest in various human cancer cells (35). But, the result is in contrast to Li et al. (36) that Alimta exerts anti-cancer effects by inducing the cell cycle arrest at Go/G1-

phase in carcinoma ESCC cells. Sequential treatment of 5-aza and Alimta reveals decrease in S-phase cell cycle arrest preceding most population of the cells into sub-G1 arrest. Although, Feng et al. (37) have reported opposite results, that was a reduction in G1-phase and increase in S-phase cell cycle arrest in lung adenocarcinoma cell.

Conclusion

The present study demonstrates that 5-Aza enhances sensitivity of A549 cells to Alimta induced apoptosis through restoration of miR-34a expression. Restoration of miR-34a, as a tumor-suppressor, downregulates numerous oncogenic targets such as *BCL-2, HMGA2* and *HMGB1* that establishes a pro-apoptotic environment in the cells. It is suggested that downregulation of Bcl-2 stimulates mitochondrial cytochrome c release, which in turn, leads to sequentially, caspase-3 activation and induction of apoptosis through the intrinsic (mitochondria mediated) apoptosis pathway. Taken together, our results suggest that combination of an epigenetic drug, 5-aza, and Alimta can be a novel and beneficial therapeutic approach in order to improve treatment outcome of lung cancer. Although, the results presented here is an *in vitro* study and further *in vivo* experiments is requested to broad the therapeutic application, and warrant its clinical use.

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Authors' Contributions

S.A.; Experiments design and performance, data analysis and manuscript drafting. A.R.-C.; Experiments design, data analysis, manuscript writing and finalizing. J.D.; Caspase antibody provider and manuscript reviewer. H.G.F.; Participation and help in part of the experiments and contributed in data analysis. All authors read and approved the final manuscript.

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