Journal of Trace Elements in Medicine and Biology 48 (2018) 87–96

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Lithium chloride attenuates mitomycin C induced necrotic cell death in MDA-MB-231 breast cancer cells via HMGB1 and Bax signaling

Mahdieh Razmi[a], Azra Rabbani-Chadegani[a,*], Fatemeh Hashemi-Niasari[a], Parinaz Ghadam[b]

[a] Department of Biochemistry, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[b] Department of Biotechnology, Faculty of Biological Science, University of Alzahra, Tehran, Iran

ARTICLE INFO

Keywords:
Lithium chloride
Mitomycin C
HMGB1
Breast cancer cells
Apoptosis

ABSTRACT

The clinical use of potent anticancer drug mitomycin C (MMC) has limited due to side effects and resistance of cancer cells. The aim of this study was to investigate whether lithium chloride (LiCl), as a mood stabilizer, can affect the sensitivity of MDA-MB-231 breast cancer cells to mitomycin C.

The cells were exposed to various concentrations of mitomycin C alone and combined with LiCl and the viability determined by trypan blue and MTT assays. Proteins were analyzed by western blot and mRNA expression of HMGB1 MMP9 and Bcl-2 were analyzed by RT-PCR. Flow cytometry was used to determine the cell cycle arrest and percent of apoptotic and necrotic cells. Concentration of Bax assessed by ELISA.

Exposure of the cells to mitomycin C revealed IC_{50} value of 20 μM, whereas pretreatment of the cells with LiCl induced synergistic cytotoxicity and IC_{50} value declined to 5 μM. LiCl combined with mitomycin C significantly down-regulated HMGB1, MMP9 and Bcl-2 gene expression but significantly increased the level of Bax protein. In addition, the content of HMGB1 in the nuclei decreased and pretreatment with LiCl reduced the content of HMGB1 release induced by MMC. LiCl increased mitomycin C-induced cell shrinkage and PARP fragmentation suggesting induction of apoptosis in these cells. LiCl prevented mitomycin C-induced necrosis and changed the cell death arrest at G2/M-phase. Taking all together, it is suggested that LiCl efficiently enhances mitomycin C-induced apoptosis and HMGB1, Bax and Bcl-2 expression may play a major role in this process, the findings that provide a new therapeutic strategy for LiCl in combination with mitomycin C.

1. Introduction

Breast cancer is the main reason of cancer mortality in females. Triple-negative breast cancer (TNBC) is one subgroup of breast cancer, defined by the absence of progesterone, estrogen, and HER2 receptors that is not sensitive to targeted therapy. Chemotherapy is the only systemic treatment for triple-negative patients. Therefore, it is essential to explore more effective novel treatment strategies for TNBC therapy that attempts focus on the discovery of new combinatorial therapy using drugs with different synergistic effects [1,2].

Mitomycin C (MMC, Fig. 1a), as a potent antitumor drug, is produced by *Streptomyces lavendulae* and used for the treatment of various types of cancers [3,4]. MMC structure is composed of aziridine ring, a carbamoyl moiety and a bridged carbinolamine that binds to DNA after reductive activation [5]. Its cytotoxicity is through the formation of DNA interstrand cross-links that leads to breakage of DNA, inhibition of DNA replication, transcription, and ultimately cell death [6,7]. MMC induces apoptosis through changes in expression of Bim, Bax, caspase 9, and Fas/FasL genes in breast cancer cells [8,9]. However, adverse toxic effects and resistance of cancer cells, have limited the application of MMC, and combination of MMC with novel biological agents such as curcumin, erizotinib and other drugs are commonly proposed [10,11].

Recent studies indicate that lithium, an FDA-approved mood stabilizer drug, possesses antitumor, anti-inflammatory, and anti-metastasis activity in different types of malignancies and in vitro studies have demonstrated that lithium inhibits cell proliferation and induces apoptosis in cancer cells [12–14]. In addition, studies have revealed that lithium can be used as an adjuvant for chemotherapy to enhance the sensitivity of tumors to chemotherapy drugs [15], however, whether lithium enhance MMC-induced cytotoxicity in breast cancer has not been examined.

HMG proteins are the most important group of non-histone

Abbreviations: TNBC, triple negative breast cancer; MMC, mitomycin C; HMGB1, high mobility group B1; AO/EtBr, acridine orange/ethidium bromide; HRP-IgG, horseradish peroxidase conjugated imonoglobin G; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; MTT, 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid; PARP, poly ADP-ribose polymerase; PI, propidium iodide; FBS, fetal bovine serum; MMP-9, matrix metaloprotein-9

* Corresponding author at: Department of Biochemistry, Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384 Tehran, Iran.
E-mail address: arabbani@ut.ac.ir (A. Rabbani-Chadegani).

https://doi.org/10.1016/j.jtemb.2018.03.011
Received 20 December 2017; Received in revised form 19 February 2018; Accepted 12 March 2018

chromatin proteins which are divided into three families according to their DNA-binding domains (HMGA, HMGB, and HMGN). Among them, HMGB proteins are the largest group with three families: HMGB1, HMGB2, and HMGB3 [16,17]. In mammalian cells, HMGB1 functions as a DNA chaperone in replication, transcription, and repair in nucleus. In addition, HMGB1 represents different functions in the cell after translocation into extracellular space acting as a signaling molecule (cytokine) in proliferation, inflammation, invasion, and resistance in tumor cells [18].

In the present study, we aimed to explore the molecular mechanism of MMC combined with LiCl on cytotoxicity, apoptosis, and HMGB1 and MMP9 expression in MDA-MB-231 cells. The results demonstrate that combination of MMC with LiCl attenuates mitomycin C induced necrotic cell death, reduces HMGB1 and MMP9 expression, and ultimately arrest the cells in G_2/M phase.

2. Materials and methods

2.1. Reagents

Mitomycin C (MMC) was purchased from Helale Ahmar Pharmacy, Tehran, Iran (manufactured by Naprod Life Sci India). Appropriate concentration of lithium chloride (Merck) prepared in deionized water and stored at −20 °C. Trypan blue, MTT, cocktail protease inhibitor, anti-rabbit IgG-HRP, ethidium bromide, and trypsin were obtained from Sigma Chemical Company (Becton Dickinson, San, CA, USA). Antibodies were purchased from Abcam (Cambridge, UK). Annexin V-FITC apoptosis detection kit was obtained from Jingmei Biotech Co., Ltd. (China). Fetal bovine serum (FBS) and RPMI-1640 medium were from Gibco (Denmark). RPMI-1640 supplemented with 3.7 g/L $NaHCO_3$, 30 mg/L asparagine, 100 U/mL penicillin, and 10 mg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA) pH 7.2 was prepared and after sterilization by 0.2 µm Millipore filter, kept at 4 °C before use.

2.2. Cytotoxicity assay

Human triple negative breast cancer cell line MDA-MB-231 was obtained from Pasteur Institute (Tehran-Iran) and cultured in RPMI-1640 medium supplemented with 10% FBS in the condition of 5% CO_2, 95% humidity at 37 °C. The cells were then sub-cultured in long phase of growth by trypsinization. For drug treatment, the cells were exposed to various concentrations of mitomycin C (0–50 µM) and LiCl (0–400 µM) for 12, 24 and 48 h. For combination therapy, the cells were first treated with LiCl for 5 h, then exposed to different concentrations of mitomycin C (0–50 µM) and incubation continued for 24 h.

Viability of the cells assessed using trypan blue exclusion and MTT dye assay. For trypan blue exclusion, the dye (0.4%, w/v) was added to drug treated cells and the controls and viability determined using hemocytometer. MTT assay was followed by the method of Mosmann [19] with some modification. The cells (10^4 cells/well) were seeded in 96-well culture plates and treated with different concentrations of the drugs. At the end of treatment, 10 µl of MTT (5 mg/ml in H_2O) was added to each well and the cells incubated as above for 4 h. Finally, 150 µl DMSO was added to each well to dissolve the formosan crystals, and the absorbance measured at 570 nm using a BioTek ELISA micro plate reader (Model Power Wave XS2, Bio Tek, USA). The relative viability of the treated cells was expressed as the percent of the control.

2.3. Morphological changes and colony-formation

Acridine orange/ethidium bromide (AO/EtBr) double staining was used to determine the morphological changes of the cells in the absence and presence of MMC alone or in combination with LiCl employing fluorescence microscope. Control and drug treated cells were trypsinized, washed with cold phosphate buffered saline (PBS) and stained with fluorescence solution containing 100 µg ethidium bromide and 100 µg acridin orange in 1 ml PBS (pH 7.2). The cells were then examined under Zeiss fluorescence microscope connected to a CCD camera. The cells contained organized chromatin structure classified as normal and cells with condensed or fragmented chromatin were classified as apoptotic.

The cells (10^6/ml) were incubated in the absence and presence of mitomycin C alone and combined with LiCl for 24 h, trypsinized, harvested, washed twice with cold PBS and seeded at a density of 10^3 cells in a 60 mm diameter Petri dish for each treatment. The cells were cultured for 10–14 days, and the colonies fixed with formaldehyde (2%), stained with 0.5% crystal violet and counted under inverted microscope using scoring grid. The survival rate was determined by the number of colonies in the treated cells divided by the number of colonies in the untreated cells, the control.

2.4. Flow cytometry

In order to determine cell cycle distribution, the control and drug treated cells (10^6/ml) were washed with cold PBS, fixed in 70% ethanol at 4 °C for 2 h, and stained with 20 µg/ml propidium iodide (PI) in the presence of 20 µg/ml RNase A for 30 min at 37 °C in the dark. The cell cycle analysis performed using a FAC Scan flow cytometry (Becton Dickinson, San, CA, USA) and data were analyzed with WinMDI software.

Apoptosis analysis was also performed using Annexin V-PI kit according to the manufacturer's instruction. In this assay, the control and drug treated cells (10^6/ml) were stained with annexinV/PI for 20 min in the dark at room temperature and apoptosis analysis performed using FAC Scan flow cytometer. Percentage of apoptotic cells was determined by flow max software. Experiments were performed in triplicate.

2.5. Extraction of proteins and western blot analysis

For HMGB1 extraction, the cells were incubated in the absence and presence of mitomycin C alone and combined with LiCl for desired periods of time, then harvested, washed twice with cold PBS, centrifuged for 5 min at 1000*g* and HMGB1 protein extracted by 0.35 NaCl in 10 mM Tris-HCl (pH 7.2) containing 1/40 ratio cocktail protease inhibitor essentially as described by Goodwin [20].

To characterize the amount of HMGB1 released from the cells, the cultures media from the control and drug treated cells were centrifuged and the supernatants extracted with 5% PCA by respect to 60% PCA followed by precipitation with 12% TCA. The proteins were then analyzed on 15% SDS polyacrylamide gel electrophoresis as described by Laemmli [21].

For PARP extraction, the cells were detached and washed twice with ice-cold PBS, then suspended in extraction buffer containing 62 mM Tris-HCl, 2% SDS, 10% glycerol, 4 M urea, 0.3% bromophenol blue, 5% β- mercaptoethanol at 4 °C, and stirred vigorously for 1 h. The samples were centrifuged for 5 min at 4 °C and the cell lysates were subsequently separated on 12% SDS-PAGE and immunoblotted.

For western blot analysis, the cell lysates were separated on SDS-PAGE and transferred onto a nitrocellulose membrane (Whattman). The

membranes were blocked using 1% (w/v) gelatin in Tris–NaCl buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl) for 1 h at 37 °C and washed three times of 5 min with Tris–NaCl buffer. The membranes were incubated with primary antibodies (anti HMGB1 or PARP antibody specific for 89 kDa fragment) at 4 °C overnight. The membranes were rinsed with Tris–NaCl/Tween20 (0.05%) washing buffer and incubation continued with anti-rabbit IgG-HRP (Abcam) at room temperature for 2 h. After three times washing, the blots were detected by an enhanced chemiluminescence (ECL) detection system according to the manufacturer's instruction. Intensity of the bands quantified using Image-J software.

2.6. Real-time polymerase chain reaction (RT-PCR) and ELISA

The cells were harvested after treatment with mitomycin C alone and in combination with LiCl and total RNA extracted using TRIzol reagent (Cinna Gen Co., Iran) according to the manufacturer's instruction. The RNA (2 µg) was then reverse transcribed into cDNA 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 RT-PCR Green-Master (Jena Bioscience, Germany). The reaction conditions consisted of the following steps: 95 °C for 3 min as pre-denaturation step, 40 amplification cycles at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 15 s, and final extension at 72 °C for 10 min. The following primers were employed in these reactions: HMGB1: forward 5′-ACAGCCATTGCAGTACATTGA-3′, reverse 5′ −ATGCTCCT CCCGACAAGTTT-3′ (efficiency = 97%), MMP-9: forward 5′-TCTTCCC TGGAGACCTGAGAAC-3′, reverse 5′-TCTTCCCTGGAGACCTGAGAAC-3′ (efficiency = 95%), Bcl-2: Forward 5′ ACGACTTCTCCCGCCGCTACC 3′, Reverse 5′ ACAATCCTCCCCCAGTTCACCC 3′ (efficiency = 76%), β-Actin: forward 5′ CAAGATCATTGCTCCTCCTG-3′, reverse 5′-ATCCAC ATCTGCTGGAAGG-3′ (efficiency = 95%. All gene sequences were obtained from the NCBI website, Primer3 oligoanalyzer and gene runner program was used to design primers for the specific genes and their specificity analyzed by NCBI Blast Program. β-Actin was used as an endogenous control and quantitation of gene expression determined using $\Delta\Delta C_t$ calculation, where C_t is the threshold cycle. HMGB1, MMP-9 and β-actin mRNA expression levels were analyzed as fold-change by the $2^{-\Delta\Delta Ct}$ method [22].

The concentration of Bax was estimated by Bax ELISA kit (Abcam, Cambridge, UK). Briefly, drug treated and untreated cells were lysed according to the kit instruction. The cell lysates were placed in 96-well microtiter plates coated with monoclonal detective antibody and incubated for 1 h. After removing unbound material by washing buffer, Horseradish peroxidase (HRP) conjugated secondary antibody was added to bind to the antibody. Horseradish peroxidase catalyze the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution with intensity proportional to the amount of protein present in the sample. The absorbance of each well measured at 450 nm using a microplate reader, and concentration of Bax determined by interpolation from standard curves obtained with known concentrations of standard protein.

2.7. Analysis of synergetic effect

The synergetic effect between LiCl and mitomycin C evaluated by the combination index (CI) using CompuSyn software program which is based on the median effect principle. The CI is calculated by the following formula: CI = D1/(Dx)1 + D2/(Dx)2, where (Dx)1 and (Dx)2 are individual doses of LiCl and MMC required to inhibit a given level of cell growth, and D1 and D2 are the doses of LiCl and MMC necessary to produce the same effect in combination, respectively [23]. In general, CI values below 1 suggest synergy, whereas CI values above 1 indicate antagonism between the drugs. CI values in the 0.9-1.1 range mainly indicate additive effects. The mean of CI values at a fraction affected (FA) was used to calculate between four LiCl/MMC combinations. Fraction affect (FA) is defined as the growth inhibition affected by the dose.

2.8. Statistical analysis

The data are presented as mean ± SD. Student's *t*-test was used to determine the significance of differences between the control and treatment groups. P-values < 0.05, < 0.01 or < 0.001 were defined as statistically significant.

3. Results

3.1. LiCl synergistically enhances sensitivity of MDA-MB-231 cells to MMC

To investigate the potential effects of LiCl or mitomycin C on survival rate of MDA-MB-231 cells, both conventional trypan blue exclusion and MTT assays were used. Time course study was done with or without different concentrations of MMC for 12, 24, 48 h and the result is shown in Fig. 1B. As is seen, after 12 h incubation, the cells in the control are 95% viable and slight decrease in the viability of MMC treated cells is observed. Exposure of the cells to mitomycin C for longer periods of time reduce viability in a dose-dependent manner, thus after 24 h, survival rate is decreased from 98% in the control to 87% at 5 µM and less than 49% at 20 µM of MMC. When the cells exposed for longer time (48 h), viability of the cells is diminished to 78% and 26% when 5 and 20 µM of MMC was used, respectively ($P < 0.001$). In contrast, various concentrations of LiCl (0- 400 µM) alone had no cytotoxic effect on MDA-MB-231 cells after 5, 12, 24 and 48 h treatments and 95% of the cells were viable when assayed by MTT and trypan blue (data not shown). In combination test, the cells were treated simultaneously with 200 µM of LiCl plus different concentrations of MMC (0–40 µM) or 5 h pre-incubation with LiCl and then exposure to various concentration of mitomycin C. As is shown in Fig. 1C, in simultaneous treatment, viability is reduced to 80% and 47% when 5 and 20 µM of MMC are used, whereas in combination, viability is decreased to 51% and 25% at 5 and 20 µM of MMC, respectively. The result revealed that 5 h pre-incubation with LiCl is sufficient time, therefore, this incubation time was used throughout the experiments. Fig. 1D shows the results obtained from MTT assay which confirms trypan blue exclusion assay. IC_{50} value of mitomycin C is around 20 µM, but when it is combined with lithium chloride, IC_{50} is decreased to around 5 µM. Fig. 1D also shows the selective effect of the anion (Cl^-) on the viability, representing that after exposure of the cells to sodium chloride for 5 h at the same concentration of LiCl (200 µM) before treatment with MMC, shows similar viability pattern as when MMC is used alone. In addition, using drug combination effect analysis as described in methods section (2.7), the mean value of the combination index (CI) is less than 1, indicating that LiCl–mitomycin C combination has a synergistic effect (Fig. 1E).

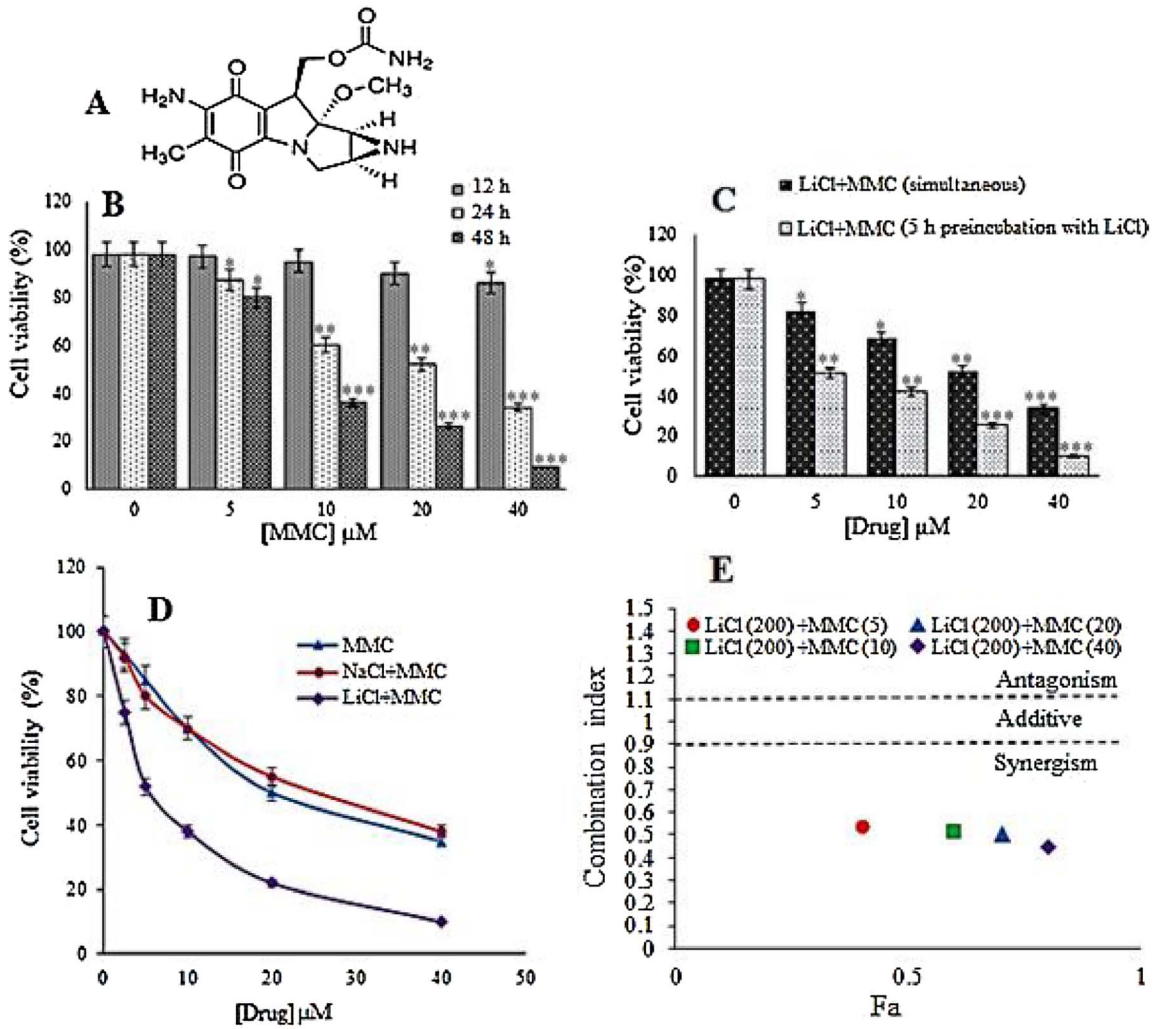

Fig. 1. Cytotoxic effects of MMC alone and combined with LiCl on the viability of MDA-MB-231 breast cancer cells evaluated by trypan blue exclusion and MTT assays. (A) Chemical formula of mitomycin C. (B) Viability of MDA-MB-231 cells after 12, 24 and 48 h in the absence and presence of different concentrations of MMC (0–40 µM) obtained from trypan blue exclusion assay. (C) The effect of 200 µM LiCl plus MMC, simultaneously or 5 h pre-incubation of the cells with LiCl then exposure to MMC (0–40 µM). (D) The cells were treated with MMC, LiCl + MMC, and NaCl + MMC for 24 h in 96-well plate and cytotoxicity determined by MTT assay. (E) Combination index (CI) analysis of growth inhibition in MDA-MB-231 cells after 24 h of incubation with 200 µM of LiCl and different concentrations of MMC (0–40 µM). Data from were converted to fraction affected (Fa) and plotted against CI. Values obtained from three independent experiments. Significant difference versus control *P < 0.05, **p < 0.01, ***p < 0.001.

3.2. The effect of LiCl and MMC combination on HMGB1 and MMP-9 expression and content

Matrix metallopeptidase 9 (MMP-9), a type IV collagenase involved in the degradation of extracellular matrix, promotes cancer progression by increasing cancer cell proliferation, migration, invasion, metastasis and angiogenesis [24]. Since MMP-9 is highly expressed in triple negative breast cancer cells [25], the expression of this protein was assessed. For this purpose, total RNA was extracted from the cells cultured in the absence and presence of various concentrations of drugs, converted to cDNA and analysis of gene expression performed by RT-PCR. β-Actin was used to establish normalized expression ($\Delta\Delta C_t$). As shown in Fig. 2A, the expression of MMP-9 is decreased upon addition of MMC. At low concentration of MMC (5 µM), the level of MMP-9 expression is similar to the control, but upon increasing MMC concentration, mRNA expression is decreased to about 49% of the control. The mRNA level of MMP-9 is significantly down-regulated in the cells that LiCl and MMC are combined, at 5 and 20 µM of the MMC, about 65% and 25% decrease in the expression is observed compared to the control.

High mobility group protein B1 (HMGB1) is the most abundant non-histone protein which play important role in inter and extracellular signaling events. It binds to DNA and nucleosomes, regulating expression of a number of genes specifically in processes such as transcription, recombination, nucleosome remodeling and activate inflammatory pathways. Evidence shows that HMGB1 dysfunction is associated with hallmark of cancer contributes to cancer development and resistance to chemotherapy [18]. Therefore, we aimed to investigate whether mRNA expression of HMGB1 protein is affected by mitomycin C alone and in combination with LiCl. As is seen in Fig. 2B, HMGB1 expression in the presence of MMC, is diminished as the drug concentration raised, thus at 5, 10, 20 and 40 µM of MMC, about 88%, 73%, 57% and 39% decrease in the expression is observed respectively compared to the control. Whereas, pre-incubation of the cells with 200 µM of LiCl and then exposure to mitomycin C, HMGB1 expression is significantly decreased to about 67% and 27% when 5 and 20 µM MMC is used respectively.

Down-regulation of HMGB1 expression in the drug treated cells encouraged us to assess whether HMGB1 content in the cell is affected by MMC and combined with LiCl. Therefore, HMGB1 was extracted from drug treated cells and the control and subjected to protein analysis on SDS gel and immunobloted against HMGB1 antibody. As is seen in Fig. 2C, in the control (lane 0, absence of MMC) a band in the position of HMGB1 is observed compared to calf thymus HMGB1 protein as a marker (lane M). At low concentration of MMC (5 µM), the band

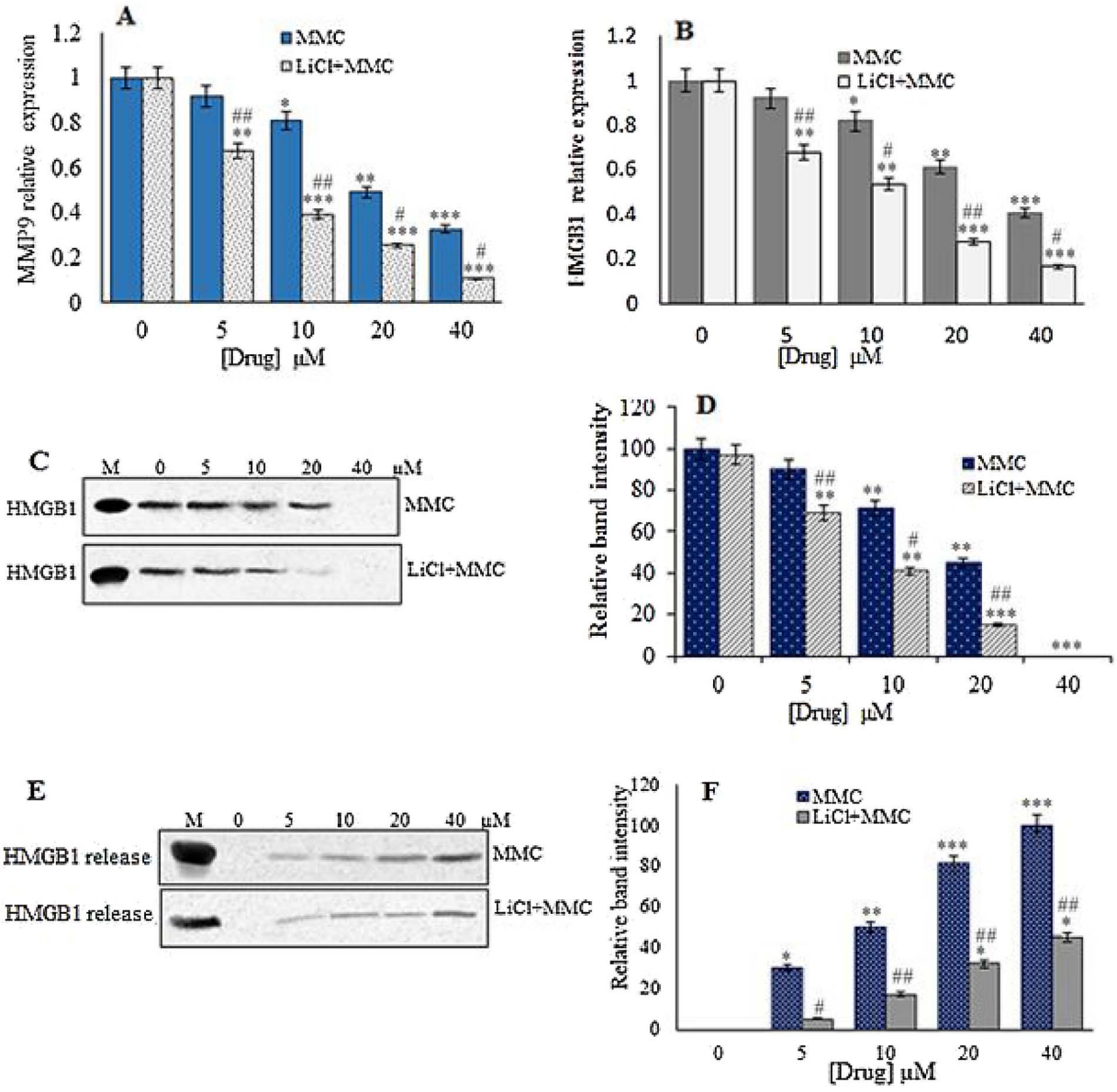

Fig. 2. The effect of MMC alone and combined with LiCl on mRNA expression of MMP-9 (A) and HMGB1 (B) in MDA-MB-231 cells. The cells were treated with various concentrations of the MMC (0–40 µM), or for combination therapy, the cells pre-incubated with 200 µM LiCl and then exposed to different concentration of MMC (0–40 µM), total RNA was extracted, reverse transcribed by RT-PCR. The data were normalized to the housekeeping gene β-Actin. (C) Immunoblotting of HMGB1 extracted from the cells in the absence and presence of MMC alone and plus LiCl and blotted against HMGB1 antibody and Relative bands intensity quantified by Image-J software (D). (E) Extracellular release of HMGB1 assayed by western blot analysis of the supernatants. (F) The protein levels of HMGB1 in control and treated cells quantified by Image-J software. The results are means ± SD of at least four experiments. *P < 0.05, **p < 0.01, ***p < 0.001 significant difference versus control and #P < 0.05, ##P < 0.01 vesus the combination of MMC and LiCl compared to MMC alone.

intensity of HMGB1 protein remain unchanged, but upon increasing MMC concentration, intensity of the bands is decreased thus at 10 and 20 µM of MMC, 71% and 45% reduction is obtained respectively as analyzed by Image-J (Fig. 2D). When the cells treated with 200 µM LiCl plus 5, 10 or 20 µM of MMC, the intensity of HMGB1 protein is significantly diminished to about 69%, 41% and 15% (Fig. 2C and D).

HMGB1, in addition to its role in the nucleus, also functions as a damage-associated molecule when released from necrotic or cells in response to exogenous and endogenous toxicant that has potential to induce re-growth, inflammation, drug resistance, and metastasis [18]. To found out whether reduction in HMGB1 protein content partly is due to its release from the cells, after treatment of the cells with the drugs, culture media were collected, HMGB1 extracted and immunoblotted against HMGB1 antibody. As is seen in Fig. 2E, the control (lanes 0) shows no band at HMGB1 position (compared to calf thymus HMGB1, lane M). Release of HMGB1 occurs when the cells are exposed to different concentrations of mitomycin C as confirmed by densitometry using Image-J analysis. At 5 µM of MMC, the content of HMGB1 release is 30% and at 20 µM of MMC 81% of HMGB1 content is detected. In the samples treated with combination of LiCl and 5 or 20 µM MMC, release of HMGB1 is decreased compared to MMC alone, thus 6% and 32% intensity is observed in the presence of 5 and 20 µM MMC respectively, indicating that LiCl prevents MMC-induced HMGB1 release into the extracellular space.

3.3. The effect of MMC and LiCl combination on PARP cleavage and Bcl-2, Bax expression

HMGB1 release is a specific marker of late apoptosis/necrosis. Since HMGB1 is released to extracellular space upon exposure of the cells to MMC, but pretreatment of the cells with LiCl decreased HMGB1 release, encouraged us to found out whether the cells are preceded into

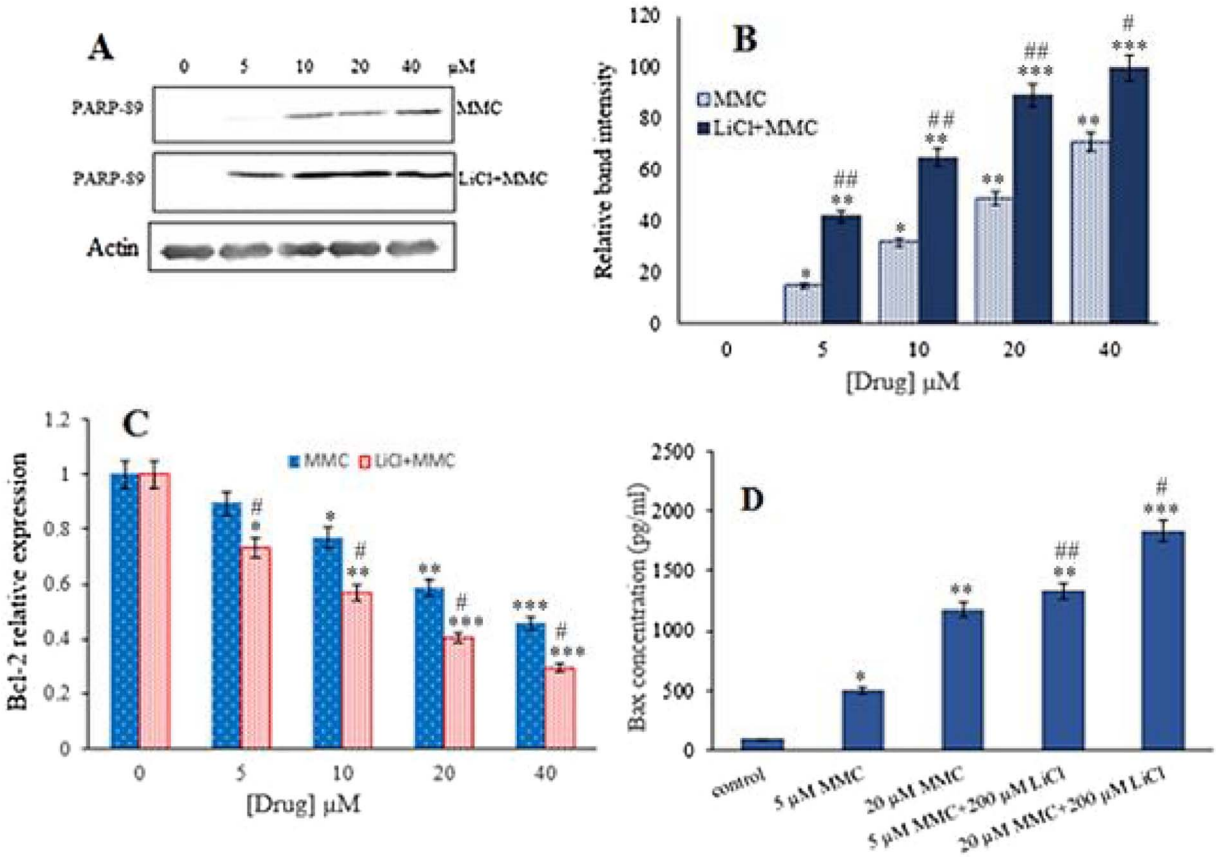

Fig. 3. The effect of MMC alone and combined with LiCl on PARP cleavage (A) detected by immunoblot using antibody recognizing 89 kDa fragment of PARP and quantified by Image-J software (B). (C) Expression of Bcl-2 mRNA in MMC alone and in combination with 200 μM LiCl and analyzed by RT-PCR. D) Determination of Bax content using ELISA kit. *P < 0.05, **p < 0.01, ***p < 0.001 significant difference versus control and #P < 0.05, ##P < 0.01 versus the combination of MMC and LiCl compared with MMC alone. Values are mean ± S.D of three independent experiments.

apoptosis under exposure to MMC and LiCl and the results is presented below:

PARP cleavage is one of the most important biomarker of apoptosis [26]. PARP (116 kDa) is a nuclear DNA-binding protein which is degraded into fragments of 89 and 24 kDa during apoptosis by caspase-mediated proteolysis. To found out whether MMC alone and combined with LiCl has any effect on PARP cleavage in MDA-MB-231 cells, it was extracted from the control and drug treated cells and subjected to immunoblot analysis against PARP antibody specific to 89 kDa fragment and quantified using Image-J. As shown in Fig. 3A and B, MMC induces a concentration-dependent increase in cleaved 89 kDa fragment of PARP, as its content at 5, 10, 20 and 40 μM of MMC is 15%, 32%, 53%, and 71%, respectively. Whereas when the cells are exposed to LiCl and MMC, PARP cleavage is significantly induced to about 42%, 65%, 88% intensity at 5, 10 or 20 μM of MMC, respectively (Fig. 3B).

Apoptosis is usually regulated in part by Bcl-2 genes and pro-apoptotic protein Bax [27], and the balance between expression of these two cell death regulatory genes, decline in Bcl-2 expression and overexpression of Bax, enhance apoptosis by toxicants [28] Therefore we examined Bcl-2 expression and Bax content in the cells treated with MMC and MMC + LiCl and the result is shown in Fig. 3C and D. As shown, Bcl-2 expression is down regulated in both MMC and MMC + LiCl treated cells but with different extents. In MMC treated cells nearly 50% inhibition of Bcl-2 expression is obtained and in combination, inhibition is increased to 30%. Interestingly, the content of Bax protein is increased as MMC concentrations increased thus the content of Bax for 20 μM MMC alone and combined with LiCl is 1200 pmol and 1850 pmol respectively.

3.4. LiCl reduces necrotic and enhances apoptotic cell death induced by MMC

To assess morphological changes of the cells induced by MMC alone and combine with LiCl, double staining with ethidium bromide/acridin orange was applied. Fluorescent acridin orange penetrates the live and dead cells and emits green fluorescence. Ethidium bromide only penetrates into dead cells and emits red fluorescence as a result of intercalation into double-stranded DNA. As is seen in Fig. 4Aa, the cells in the control appear intact and uniformly green with an organized structure. Upon addition of MMC, the cells are deformed thus ethidium bromide penetrates the cells converting them to mixed color, green and orange or red which are an indication of apoptosis and necrosis, respectively (Fig. 4Ab and c). Also, apoptotic morphological features, such as cell shrinkage and chromatin condensation, are evident in the cells treated with different concentration of MMC. In the presence of LiCl alone, the cells appear intact and uniformly green as the control (Fig. 4Ad). But when the cells are treated with LiCl and MMC, the number of apoptotic cells are increased and the content of necrotic cells is diminished (Fig. 4Ae and f).

Flow cytometry is one of the best and potent technique to analyze apoptosis and necrosis, hence the control and drug-treated cells were stained with annexin V/PI and analyzed. As is seen in Fig. 3D, the cells in the upper left quadrant (Q1) represent necrotic cells, cells in the upper right quadrant (Q2) represent late apoptotic/necrotic cells, cells in the lower left quadrant (Q3) represent viable cells and cells in the lower right quadrant (Q4) represent early apoptotic cells. In the control, the cells are 98% vital and the cell populations are concentrated in region Q3 (Fig. 4Ba). Following treatment with MMC (5 and 20 μM) (Fig. 4Bb and c), the percentage of apoptotic and necrotic cells are

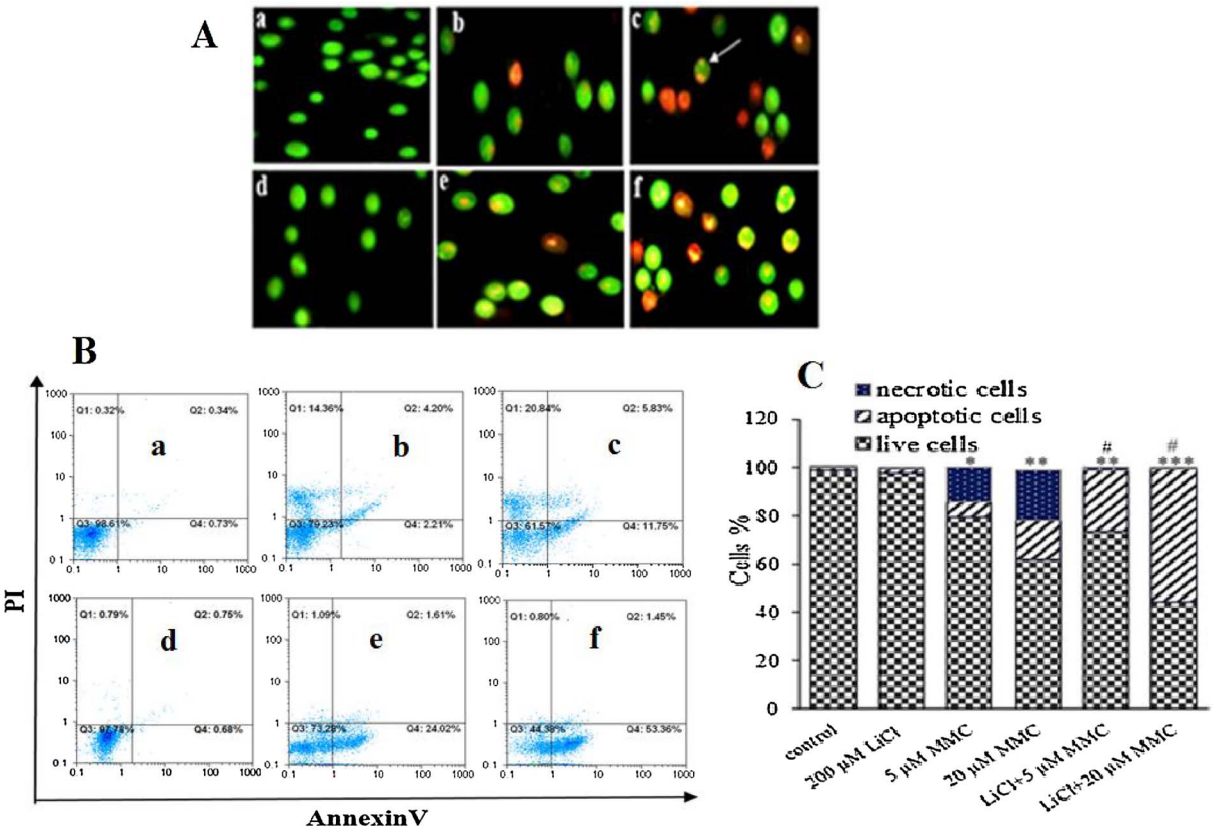

Fig. 4. A) Morphological changes of the cells staining of the cells treated with 0, 5 and 20 µM of MMC (a–c) and when the cells were exposed to 200 µM LiCl for 5 h and then received 0, 5 and 20 µM of MMC (d-f) stained with acridine orang/etidium bromide. B) Flow cytometric assay of the cells 24 h after exposure to MMC and combined with LiCl, stained with Annexin V/PI. (a–c) are 0, 5 and 20 µM MMC and (d–f) the samples exposed to 200 µM LiCl for 5 h and then received 0, 5 and 20 µM MMC, respectively. C) Histogram showing percentages of apoptotic and necrotic cells in the control and drug treated cells. Significant difference versus control *P < 0.05, **p < 0.01, ***p < 0.001 and #P < 0.05 represents the combination of MMC and LiCl compared with MMC alone. The results are means ± SD of at least two experiments.

found to be increased compared to the control, and the treated cells are spread to the regions Q4, Q1 and Q2 regions. Percent of apoptotic cells is increased to 6.41% and 16.58% after exposure to 5 and 20 µM of MMC, whereas necrosis is increased to 14.36% and 20.84% respectively. LiCl alone does not influence the cell population compared to the control (Fig. 4Bd), however, in the cells treated with 200 µM of LiCl plus 5 or 20 µM of MMC, the apoptotic cell fractions increased to 25.63% and 54.81% ($P > 0.001$), respectively, whereas necrotic cells are decreased and does not exceed 1% (Fig. 3De,f). The result is also summarized in Fig. 4C which compares the percentage of apoptotic and necrotic cells in drug treated cells and the controls.

3.5. LiCl and MMC combination inhibit proliferation of MDA-MB-231 cells

To find out whether mitomycin C alone and combined with LiCl inhibit the tumorigenic potential of MDA-MB-231 cells, colony-forming assay was performed. The survival rate determined by the ratio of the number of colonies in treated samples to the number of colonies in the control. Colonies with higher that 50 cells were scored and less than 50 cells were defined as clusters. Fig. 5A shows that exposure of the cells to mitomycin C significantly diminished colony-forming ability from 95% in the control (absence of MMC) to 28% at 5 µM and less than 7% at 20 µM of MMC ($P > 0.001$) as shown in Fig. 5B. Fig. 5Ad-f represent that the cells treated with 200 µM LiCl exhibit nearly the same colonies pattern as the control, while, reduction in colony number was more evident when the cells are pre-incubated with LiCl before exposure to MMC (Fig. 5Ae and f), thus the cell colony forming ability is reduced to less than 5% and zero when 5 or 20 µM of MMC were used.

To determine whether the cell proliferation inhibition mediated by MMC alone and in combination with LiCl was because of cell cycle arrest at a specific phase, cell-cycle analysis performed using PI staining and flow cytometry analysis. As is seen in Fig. 5C and D, in the control, 72.07% of the cells are in Go/G1 phase, 11.42% in S phase and 16.51% in G2/M phase. Upon treatment of the cells with mitomycin C, the cells are accumulated in S and G2 phases, with 42% of the cells in Go/G1 phase, 22% in S phase and 35% in G2 M when 20 µM of mitomycin C is used. When the cells are treated with LiCl alone (Fig. 5Cd) the cell cycle arrest was the same as in the control and no changes is observed. Whereas in the cells treated with 200 µM of LiCl plus 5 or 20 µM of MMC, the fraction of cells in the G2/M phase is significantly increased to 42% and 70%, respectively, indicating that a significant number of the cells are arrested in G2/M phase (Fig. 5Ce,f). The result summarized in Fig. 5D also compares the percentage of cells in different cell cycle phases in drug treated cells and the controls.

4. Discussion

Mitomycin C is a potent anticancer drug in the treatment of breast cancer, but drug resistance and adverse toxic effects caused by mitomycin C have limited the clinical use of this drug that new combination therapies are needed in order to obtain enhanced efficacy and improved selectively along with low adverse toxicity [29]. Since LiCl has therapeutic benefits either as an anticancer agent [13,14] or an adjuvant to chemotherapy [15], we have explored the combined effects of LiCl and MMC in MDA-MB-231 breast cancer cells to found out whether LiCl can increase the sensitivity of cells to MMC.

The results obtained from viability tests reveal that LiCl at its subtoxic concentration, increases sensitivity of MDA-MB-231 cells to chemotherapeutic drug mitomycin C and combined treatment can induce cytotoxicity in a synergistic manner. In the presence of mitomycin C

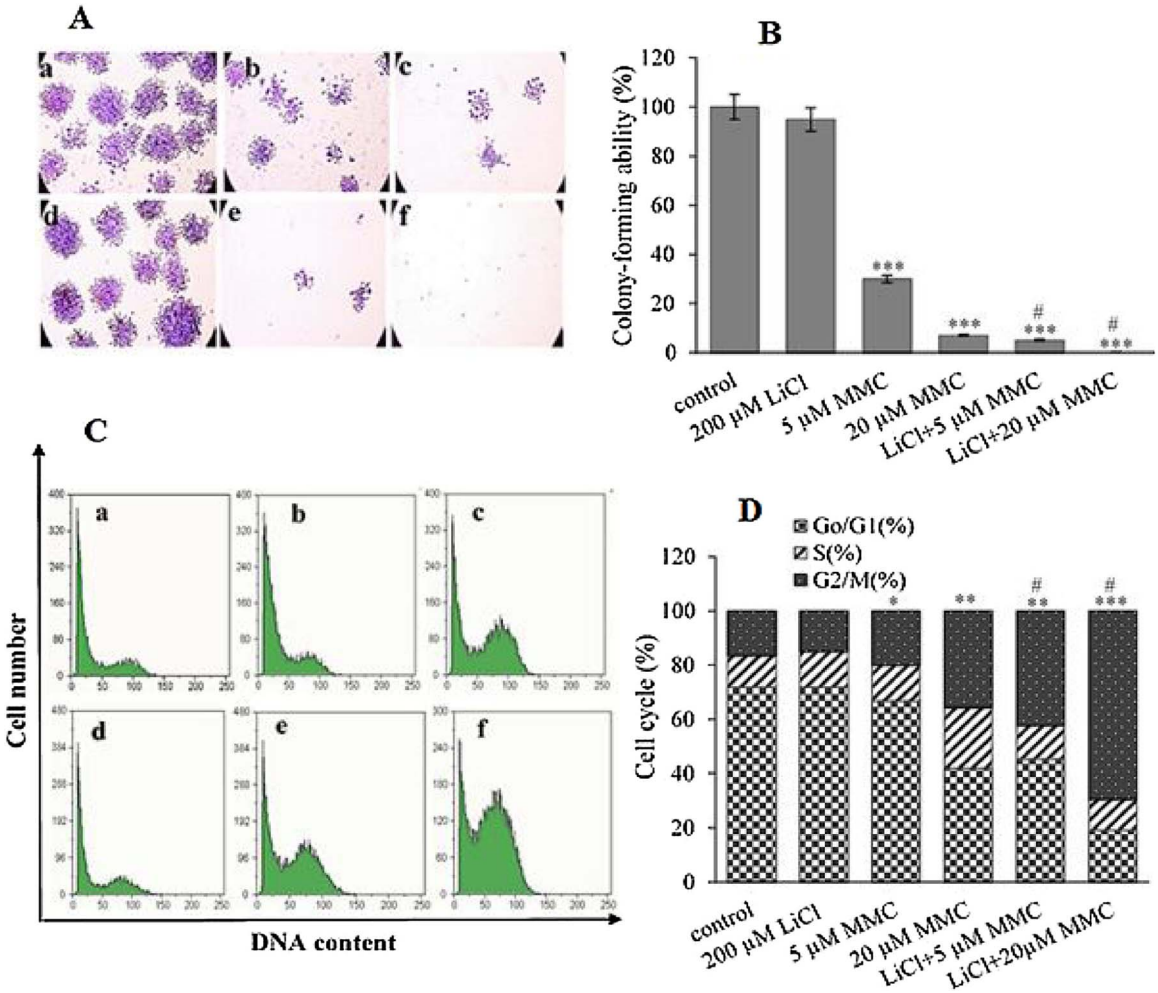

Fig. 5. The effect of MMC alone and combined with LiCl on Colony-forming ability and cell cycle arrest of MDA-MB-231 cells. (A) Colony-forming ability after 10–14 days in the presence of 0, 5 and 20 μM of MMC (a–c, respectively) and when the cells were exposed to 200 μM LiCl for 5 h and then received 0, 5 and 25 μM of MMC (d-f, respectively). (B) Histogram showing percentages of colony formation in the control and drug treated cells. (C) Cell cycle analysis of the cells fixed in 70% ethanol, stained with PI and then cell cycle profile determined by flow cytometry The percentages of the cells in Go/G1, S, and G2 M phase of the cell cycle arrest were analyzed by WinMDI software. (D) Histogram showing percentages of Go/G1, S, and G2/M cell-cycle distribution in the control and treated cells. Significant difference versus control *P < 0.05, **p < 0.01, ***p < 0.001 and #P < 0.05 represents the combination of MMC and LiCl compared with MMC alone. Values are means ± SD of four experiments.

alone, IC_{50} value is 20 μM after 24 h exposure. Different IC_{50} values have been reported for mitomycin C depending on cell type and exposure time. The IC_{50} value obtained for MMC in gastric cancer cells is about 7 μM and MCF-7 breast cancer cells at 5 μM after 48 h exposure [30,31], suggesting that triple negative breast cancer cells are slightly resistance to MMC. LiCl alone has no considerable effect on cytotoxicity of MDA-MB-231 cells, this is possibly due to the application of very low doses of LiCl in this study. This is in agreement with the report that LiCl at low dose (1 mM) has no effect on cytotoxicity of ovarian cancer cells, while at high doses (10 mM) decrease proliferation of these cells [13]. Treatment of the cells with MMC combined with LiCl, significantly reduces cell viability as IC_{50} value is reduced to about 5 μM after 24 h exposure, demonstrating 4-fold reduction in the IC_{50} value of MMC, therefore it is suggested that LiCl may overcome the MMC resistance and reduce the doses needed for effective treatment. Combination of MMC with other anticancer drugs or plant compounds has been reported indicating that in all cases combination reduce MMC anti- proliferation effect, however some of them such as curcumin is toxic itself [11,32]. This effect of LiCl is in consistent with several studies revealing that LiCl sensitizes cancer cells or reverses resistance to cisplatin, vincristine, vinorelbine, temozolomide, and TRAIL in different cancer cells and leads to the reduce doses of these drugs needed for cell growth inhibition [13,15,33,34].

Reduction of the number and size of MDA-MB-231 colonies in the presence of MMC alone and in combination with LiCl suggest reduction in the tumorigenic potential of the cells.

Reduction in HMGB1 content may be partly attributed to its release into the extracellular space in which HMGB1 is clearly detected in the cell culture media under exposure to MMC alone, the state that occurs during late apoptosis/necrosis but combination with LiCl slightly reduce HMGB1 release. This together with the result obtained from flow cytometry confirms that LiCl reduce necrosis induced by MMC. In support of our findings, extracellular HMGB1 has been shown by Amornsupak et al. [35] and Luo et al. [36] to stimulate the resistance, autophagy, necrosis, regrowth and inflammation of cancer cells that survive prior chemotherapy with linoleic acid or doxorubicin. Interestingly in the presence of LiCl and MMC, HMGB1 is significantly down-regulated whereas the release of HMGB1 stimulated by MMC to extracellular space is decreased remarkably. The only explanation for the reduction of HMGB1release in the cells treated with MMC and LiCl is that LiCl attenuates MMC induced necrotic cell death. This is in agreement with the reports suggesting that HMGB1 release occurs at late apoptosis/necrosis [18]. Also PARP cleavage, as well as annexin/PI staining of the cells confirmed the result. Flow cytometry indicates that in the cells exposed to MMC and LiCl, the percent of necrosis is negligible compared to MMC alone, which confirms that LiCl prevents

release of HMGB1 stimulated by MMC to the cell culture medium as a specific marker of necrosis and switches the cell death mode to apoptosis. In agreement with our study, Ke et al. have shown that down regulation of HMGB1 increases radio-sensitivity of human breast cancer cells and also inhibition of HMGB1 release significantly enhances vincristine-induced apoptosis of gastric cancer cells [37]. Consistent with our results, the apoptotic effect of LiCl on anticancer drugs has been widely investigated and shown that LiCl significantly induces chemotherapeutic drugs induced apoptosis in several human cancer cells [15,33].

MMP-9 is also down-regulated in the presence of MMC and LiCl remarkably decreases MMP-9 expression, suggesting that combination of MMC and LiCl reduce metastasis of breast cancer cells. Our result is in agreement with a report indicating that LiCl is useful in the treatment of glioma and colon cancer metastasis [12,38] which may be partly mediated by suppression of HMGB1 expression and its release [36,39]. Moreover, LiCl enhances MMC-induced apoptosis through arrest of MDA-MB-231 cells at G2/M phase, whereas MMC treated cells exhibit arrest of the cells at G1 phase. Our result is in consistent with the finding that LiCl can induce G2/M arrest in various carcinoma cell lines such as ovarian and lung cancer cells [13,15].

Apart from HMGB1 release and PARP cleavage, induction of apoptosis also depends on the intracellular level and balance of two apoptotic markers, Bax and Bcl-2. Overexpression of Bax protein and down regulation of Bcl-2 in the cells treated with LiCl and MMC is consistent with the observation that overexpression of Bax enhances apoptosis induced by MMC and other anticancer drugs in colon cancer [40] providing that the ratio of Bax/Bcl-2 is a key factor to initiate apoptosis through mitochondrial pathway.

Taking all together, these findings demonstrate that LiCl promotes apoptosis induced by mitomycin C, improves cytotoxicity of MMC and represents protective effect on MDA-MB-231 breast cancer cell. Combination of LiCl with MMC represents potential anti-metastatic and synergistic effect. LiCl reduce HMGB1, Bcl-2 and MMP-9 expression but increases Bax level. LiCl inhibits colony formation and arrest the cells at G2/M phase. Therefore the results provide new insights into the mechanism of LiCl action in cell death suggesting a potentially new therapeutic strategy for lithium and a cost-effective approach to minimize destructive effects of MMC in breast cancer cells. However further studies are required to elucidate the real mechanism of action of lithium chloride when combined with mitomycin C, especially signal transduction pathway such as glycogen synthase kinase-3β (GSK-3β) which is regulated by LiCl and suppress proliferation and induce apoptosis of breast cancer cells [28], remains to be explored.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgment

The financial support of University of Tehran -Iran for this research under grant # 30/6/6401017 is acknowledged.

References

[1] F.L. Du, B.L. Eckhardt, B. Lim, J.K. Litton, S. Moulder, F. Meric-Bernstam, et al., Is the future of personalized therapy in triple-negative breast cancer based on molecular subtype, Oncotarget 6 (2015) 12890–12908.
[2] B.S. Yadav, S.C. Sharma, P. Chanana, S. Jhamb, Systemic treatment strategies for triple-negative breast cancer, World J. Clin. Oncol. 5 (2014) 125–133.
[3] H. Cheng, B. Hong, L. Zhou, J.E. Allen, G. Tai, R. Humphreys, et al., Mitomycin C potentiates TRAIL-induced apoptosis through p53-independent upregulation of death receptors, ABBV Cell Cycle 11 (2012) 3312–3323.
[4] J.C. Ko, J.C. Chen, T.J. Wang, H.Y. Zheng, W.C. Chen, P.Y. Chang, et al., Astaxanthin down-regulates Rad51 expression via inactivation of AKT kinase to enhance mitomycin C-induced cytotoxicity in human non-small cell lung cancer cells, Biochem. Pharmacol. 105 (2016) 91–100.
[5] J.C. Andrez, Mitomycins syntheses: a recent update, Beilstein J. Org. Chem. 5 (2009) 33.
[6] W.A. Remers, R.T. Dorr, Chemistry, biology and therapeutics of mitomycins, Alkaloids: Chem. Biol. Prospect. 6 (1988) 1–74.
[7] J. Blasiak, DNA-damaging anticancer drugs-A prespective for DNA repair-oriented therapy, Curr. Med. Chem. 24 (2017) 1488–1503.
[8] K.Y. Wu, H.Z. Wang, S.J. Hong, Mechanism of mitomycin-induced apoptosis in cultured corneal endothelial cells, Mol. Vis. 14 (2008) 1705–1712.
[9] Y.H. Kang, K.A. Lee, C.J. Ryu, H.G. Lee, J.S. Lim, S.N. Park, et al., Mitomycin C induces apoptosis via Fas/FasL dependent pathway and suppression of IL-18 in cervical carcinoma cells, Cancer Lett. 237 (2006) 33–44.
[10] J.C. Ko, M.S. Tsai, S.H. Weng, Y.H. Kuo, Y.F. Chiu, Y.W. Lin, Curcumin enhances the mitomycin C-induced cytotoxicity via downregulation of MKK1/2–ERK1/2-mediated Rad51 expression in non-small cell lung cancer cells, Toxicol. Appl. Pharmacol. 255 (2011) 327–338.
[11] A. Lev, S. Deihimi, E. Shagisultanova, J. Xiu, A.R. Lulla, D.T. Dicker, et al., Preclinical rationale for combination of crizotinib with mitomycin C for the treatment of advanced colorectal cancer, Cancer Biol. Ther. 18 (2017) 694–704.
[12] Y.S. Maeng, R. Lee, B. Lee, S.I. Choi, E.K. Kim, Lithium inhibits tumor lymphangiogenesis and metastasis through the inhibition of TGFBIp expression in cancer cells, Sci. Rep. 6 (2016) 20739.
[13] A.P. Novetsky, D.M. Thompson, I. Zighelboim, P.H. Thaker, M.A. Powell, D.G. Mutch, et al., Lithium and inhibition of GSK3β as a potential therapy for serous ovarian cancer, Int. J. Gynecol. Cancer 23 (2013) 361–366.
[14] H. Li, K. Huang, X. Liu, J. Liu, X. Lu, K. Tao, et al., Lithium chloride suppresses colorectal cancer cell survival and proliferation through ROS/GSK-3β/NF-κB signaling pathway, Oxid. Med. Cell Longev. 2014 (2014) 241864.
[15] Y. Lan, X. Liu, R. Zhang, K. Wang, Y. Wang, Z. Hua, Lithium enhances TRAIL-induced apoptosis in human lung carcinoma A549 cells, Biometals 26 (2013) 241–254.
[16] T. Chen, S.Y.R. Dent, Chromatin modifiers: regulators of cellular differentiation, Nat. Rev. Genet. 15 (2014) 93–106.
[17] M. Štros, HMGB proteins: interactions with DNA and chromatin, Biochim. Biophys. Acta 1799 (2010) 101–113.
[18] S. Martinotti, M. Patrone, E. Ranzato, Emerging roles for HMGB1 protein in immunity, inflammation, and cancer, Immunotargets Ther. 4 (2015) 101–109.
[19] G.H. Goodwin, C. Sanders, E.W. Johns, A new group of chromatin-associated proteins with a high content of acidic and basic amino acids, Eur. J. Biochem. 38 (1973) 14–19.
[20] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the $2_{\Gamma}^{-\Delta\Delta C}$ method, Methods 25 (2001) 402–4088.
[23] X. Wang, C. Luo, X. Cheng, M. Lu, Lithium and an EPAC-specific inhibitor ESI-09 synergistically suppress pancreatic cancer cell proliferation and survival, Acta Biochim. Biophys. Sin. 49 (2017) 573–580.
[24] J. Song, H. Su, Y.Y. Zhou, L.L. Guo, Prognostic value of matrix metalloproteinase 9 expression in breast cancer patients: a metaanalysis, Asian Pac. J. Cancer Prev. 14 (2013) 1615–1621.
[25] C. Mehner, A. Hockla, E. Miller, S. Ran, D.C. Radisky, E.S. Radisky, Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer, Oncotarget 5 (2014) 2736–2749.
[26] M. Isabelle, X. Moreel, J.P. Gagné, M. Rouleau, C. Ethier, P. Gagné, et al., Research investigation of PARP-1, PARP-2, and PARG interactomes by affinity-purification mass spectrometry, Proteome Sci. 8 (2010) 22.
[27] S. Cory, J.M. Adams, The Bcl-2 family: regulates the cellular life or death switch, Nat. Rev. Cancer 2 (2002) 647–656.
[28] T. Kobayashi, H. Sawa, J. Morikawa, W. Zhang, H. Shiku, Bax induction activates apoptotic cascade via mitochondrial cytochrome c release and Bax overexpression enhances apoptosis induced by chemotherapeutic agents in DLD-1 colon cancer cells, Jpn. J. Cancer Res. 91 (2000) 1264–1268.
[29] M. Ragonese, M. Racioppi, P.F. Bassi, L. Di-Gianfrancesco, N. Lenci, A. Filianoti, et al., Mitomycin C: new strategies to improve efficacy of a well-known therapy, Urologia 83 (2016) 24–28.
[30] C.W. Whited, S.H. Dailey, Is mitomycin C useful as an adjuant therapy in endoscopic treatment of laryngotracheal stenosis, Laryngoscope 125 (2015) 2243–2244.
[31] N. Sagara, M. Katoh, Mitomycin C resistance induced by *TCF-3* overexpression in gastric cancer cell line MKN28 is associated with DT-diaphorase down-regulation, Cancer Res. 60 (2000) 5959–5962.
[32] Q.M. Zhou, X.F. Wang, X.J. Liu, H. Zhang, Y.Y. Lu, S.B. Su, Curcumin enhanced antiproferative effect of mitomycin C in human breast cancer MCF-7 cells in vitro and in Vivo, Acta Pharmacol. Sin. 32 (2011) 1402–1410.
[33] A. Bilir, M. Erguven, N. Yazihan, E. Aktas, G. Oktem, A. Sabanci, Enhancement of vinorelbine-induced cytotoxicity and apoptosis by clomipramine and lithium chloride in human neuroblastoma cancer cell line SH-SY5Y, J. Neurooncol. 100 (2010) 385–395.
[34] S. Han, L. Meng, Y. Jiang, W. Cheng, X. Tie, J. Xia, et al., Lithium enhances the antitumour effect of temozolomide against TP53 wild-type glioblastoma cells via NFAT1/FasL signaling, Br. J. Cancer 116 (2017) 1302–1311.
[35] K. Amornsupak, T. Insawang, P. Thuwajit, P. O-Charoenrat, S.A. Eccles, C. Thuwajit, Cancer-associated fibroblasts induce high mobility group Box 1 and contribute to resistance to doxorubicin in breast cancer cells, BMC Cancer 14 (2014) 955.

[36] Y. Luo, Y. Chihara, K. Fujimoto, T. Sasahira, M. Kuwada, R. Fujiwara, et al., High mobility group Box 1 released from necrotic cells enhances regrowth and metastasis of cancer cells that have survived chemotherapy, Eur. J. Cancer 49 (2013) 741–751.
[37] S. Ke, F. Zhou, H. Yang, Y. Wei, J. Gong, Z. Mei, et al., Downregulation of high mobility group Box 1 modulates telomere homeostasis and increases the radiosensitivity of human breast cancer cells, Int. J. Oncol. 46 (2015) 1051–1058.
[38] M.O. Nowicki, N. Dmitrieva, A.M. Stein, J.L. Cutter, J. Godlewski, Y. Saeki, et al., Lithium inhibits invasion of glioma cells; possible involvement of glycogen synthase kinase-3, Neuro Oncol. 10 (2008) 690–699.
[39] P. Ni, Y. Zhang, Y. Liu, X. Lin, X. Su, H. Lu, et al., HMGB1 silence could promote MCF-7 cell apoptosis and inhibit invasion and metastasis, Int. J. Clin. Exp. Pathol. 8 (2015) 15940–15946.
[40] M. Suganthi, G. Sangeetha, G. Gayathri, B.R. Sankar, Biphasic dose-dependent effect of lithium chloride on survival of human hormone-dependent breast cancer cells (MCF-7), Biol. Trace Elem. Res. 150 (2012) 477–486.