

# Cellular Responses to the DNA Damaging Natural Compound Leinamycin

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## Abstract

Leinamycin is a thiol dependent DNA alkylating agent which shows very potent activity against various human cancer cell lines (IC<sub>50</sub> values in the low nanomolar range). This natural compound forms guanine adducts (N7) in DNA which are converted into abasic sites and simultaneously generates Reactive Oxygen Species (ROS), to produce DNA strand breaks in human cancer cells. Our present study shows that leinamycin induces a group of DNA repair and transcription factor genes involved in DNA repair in a MDA-MB-231 human breast cancer cell line, which can mediate chemoresistance to leinamycin. In addition, N-acetylcysteine decreases leinamycin-mediated ROS production while increasing leinamycin mediated apoptotic cell death, without affecting the induction of repair genes. These data indicate that ROS is not a crucial player in leinamycin induced DNA damage and that a precursor of glutathione, N-acetylcysteine, can potentiate leinamycin mediated cytotoxicity by increasing the activation of leinamycin into its DNA reactive form.

**Keywords:** Leinamycin; DNA repair; Reactive oxidative species; N-acetylcysteine

## Introduction

Leinamycin is an antitumor antibiotic, isolated from a strain of *Streptomyces sp.* found in soil samples by a Japanese pharmaceutical company, Kyowa Hakko Kogyo Ltd. Leinamycin has many novel structural components including a 1, 2-dithiolan-3-one 1-oxide moiety which is connected in a spiro fashion to an 18-membered lactam macrocycle that is coupled with a thiazole ring [1-4]. Leinamycin has shown to have potent anticancer activity in both *in vivo* and *in vitro* tumor models and is currently under consideration to be developed as an anticancer agent [5]. Previous studies suggest that the 1, 2-dithiolan-3-one 1-oxide in leinamycin is the primary target of thiol attack as described in Figure 1 [6-8]. This reaction leads to the conversion of this heterocycle to a 1, 2-oxathiolan-5-one which undergoes a novel rearrangement reaction to produce a DNA attacking episulfonium ion [6-8]. This episulfonium ion associates noncovalently with the double stranded DNA and alkylates the N7 position of the guanine residue very efficiently [6-10]. The guanine adduct is the only covalent DNA lesion which is formed by the reaction of thiol activated leinamycin with double stranded DNA. *In vitro* studies have demonstrated that the leinamycin-guanine adduct in double stranded DNA undergoes very fast depurination ( $t_{1/2} = 3$  h). This leads to the generation of cytotoxic apurinic (AP) sites in the DNA duplex [11]. It is possible that the rapid depurination of the leinamycin-guanine adduct is the basis of the potent biological activity exhibited by leinamycin [12]. Additionally, AP sites are promptly converted into DNA strand breaks which are also toxic to the cells [13]. Recent studies have also shown that AP sites can form interstrand crosslinks in DNA under physiologically relevant conditions [14]. Although cells have the ability to repair these DNA lesions to a certain extent, a large number of AP sites or strand breaks can overwhelm the repair systems leading to cell death [15].

Development of resistance to DNA damaging agents is manifested by increasing DNA repair capacity which is one major response exhibited by cancer cells following exposure to these agents [16-18]. One way to elucidate the possible mechanism of resistance in leinamycin mediated DNA damage is to study the expression profile of DNA repair genes in human cancer cell lines and to clarify the biological significance of

the induction of these genes. Previous studies have shown that cancer cells exposed to DNA damaging agents or radiation are often known to induce DNA repair genes transiently, thereby increasing the capacity of cells to repair such DNA lesions [19,20]. This transient elevation in repair gene expression can be explained by the observation that a constitutive expression of certain repair proteins may become more detrimental to the cell by causing endogenous DNA damage [20]. Therefore, it is important to investigate which DNA repair genes are induced in response to leinamycin in human cancer cells in order to develop leinamycin as a chemotherapeutic agent in future.

Our previous study revealed that leinamycin is able to produce reactive oxygen species (ROS) in addition to DNA alkylation with attack of the thiol on leinamycin [21,22]. In this reaction, ROS is generated from the persulfide through an initial reduction of molecular oxygen to superoxide radical, followed by disproportionation to hydrogen peroxide, leading to production of the hydroxyl radical via Fenton reaction chemistry [21]. The ability of leinamycin to produce ROS and unique types of DNA damage represents a new biochemical route that possesses strong cytotoxic activity against human cancer cells [21,22]. ROS is cytotoxic and intracellular generation of ROS can cause DNA strand cleavage and lead to cell death through general oxidative stress [23]. Also, depletion of cellular thiols can cause oxidative stress and can sensitize cells to apoptosis [23-24]. In addition, oxidative stress can potentiate the cell killing capabilities of DNA alkylating agents [24]. Thus, the ROS producing ability of leinamycin can also contribute to its potent biological activity. N-acetylcysteine (NAC) is a thiol (sulfhydryl-containing) compound and the thiol group enables NAC to act as an

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antioxidant by increasing the level of glutathione and reducing free radicals [25-27]. NAC is used as a chemopreventive agent in certain cancers including lung, skin, head, neck, liver and breast cancer [28]. NAC is also known to reduce toxic side effects associated with cancer chemotherapy. For example, NAC attenuates cisplatin induced acute renal failure [29]. Leinamycin is a thiol dependent DNA damaging agent [6-8] and NAC is a thiol compound and precursor of reduced glutathione (GSH) [28]. Therefore, it is important to understand how NAC can modulate the cytotoxic effect of leinamycin in human cancer cells.

## Materials and Methods

### Chemicals

Staurosporine and NAC were obtained from Sigma-Aldrich® (St. Louis, MO) and were dissolved in DMSO and PBS buffer, respectively. KU-55933 (ATM inhibitor) was obtained from Tocris Bioscience and was dissolved in DMSO. Leinamycin was gifted by Dr. Yutaka Kanda of Kyowa Hakko Kogyo Ltd. It was dissolved in DMSO (5mg/ml stock solution) and stored at -80°C.

### Cell culture

The MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and they were cultured according to ATCC's instruction. Briefly, cells were cultured in RPMI 1640 media supplemented with 9% fetal bovine serum. The cell lines were maintained under humid atmosphere and 5% CO<sub>2</sub> at 37°C. The cells were sub-cultured with trypsin - EDTA at a ratio range of 1:3 to 1.5. Trypan blue exclusion assay was used to measure the cell viability prior to experiments.

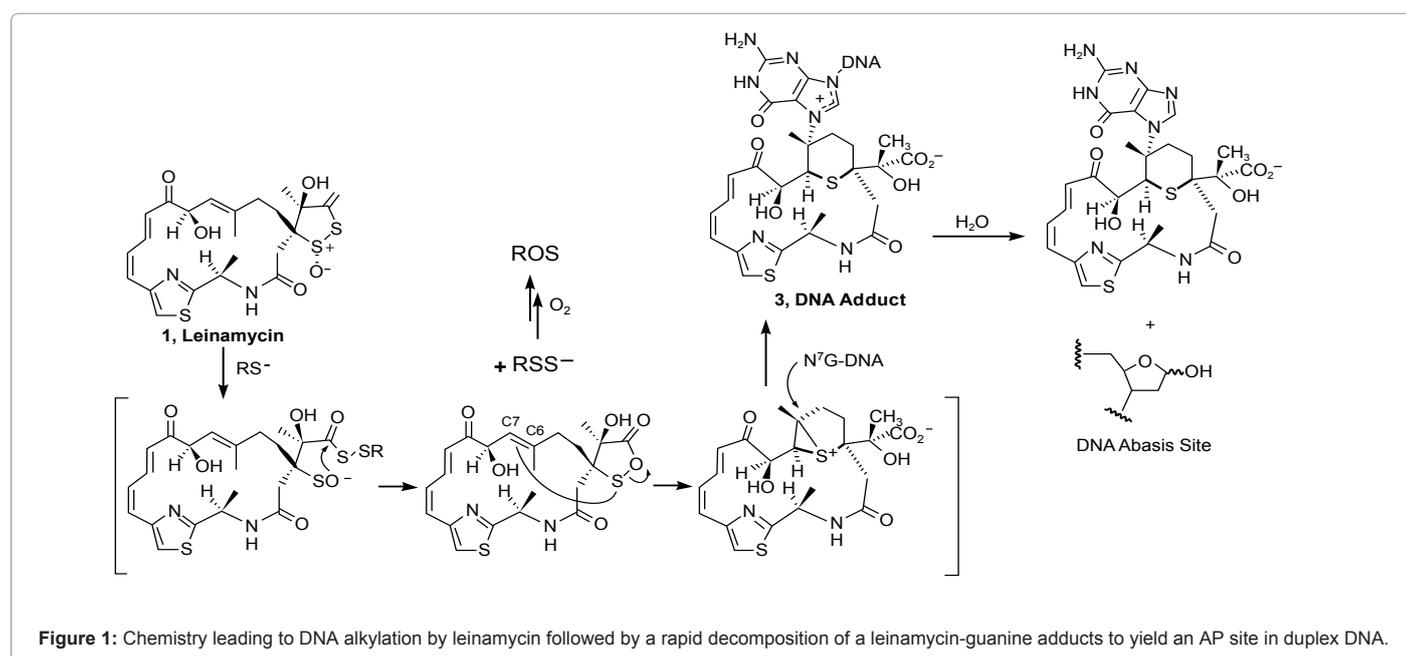
### Semi quantitative reverse transcriptase polymerase chain reaction

MDA-MB-231 cells were seeded in a six well plate for 24 hours. The next day, cells were treated with different concentrations of leinamycin (0 ng/mL, 12.5 ng/mL and 25 ng/mL) for 24 hours. Next, total RNA was

automatically purified from the harvested cells on the QIAcube (QIAGEN) using RNeasy Mini QIAcube Kit (QIAGEN), and cDNA was synthesized using qScript™ cDNA SuperMix. Primer sequences for reference genes were obtained from the literature and were synthesized by Sigma-Aldrich. Primer sequences for reverse transcription-PCR (RT-PCR) were human actin, forward 5' TTCTGGGCATGGAGTCCTGTGG3'/backward 5' CGCCTAGAAGCATTTGCGGTGG3'; human E2F1, forward 5'TGATACCCCAACTCCCTCTA3'/backward 5' AAAGCAGGAGGGAACAGAGC3'; human DNA ligase I (LIG1), forward 5'ACGAAGCAGGAAGTGCAG3'/backward 5'GGCCACAGCCAGGTAAGG3'; human Egr1, forward 5' CTGCGACATCTGTGGAAGAA3'/backward 5'TGTCCTGGGAGAAAAGGTTG3'; human PARP1, forward 5'GAGCGATGCCTATTACTGCAC3'/backward 5'GAACAACCTCCTGAAGGCTCTT3'; human RAD51, forward 5'GTTAGCCTCGAACTCCTAGGCTCAGA3'/backward 5'CTCGGGAAGCGCCGACTCTCCTTAG3'; and BRCA1, forward 5'GGTGGTACATGCACAGTTGC3'/backward 5'TGACTCTGGGCTCTGTCTT3'. PCR was carried out in a Bio-Rad Thermal Cycler. Quantification of RT-PCR was performed using a Kodak gel logic imaging system.

### ROS assay

The cellular production of ROS by leinamycin in the breast cancer cell line MDA-MB-231 was measured using dichlorofluorescein diacetate (DCFDA) and flow cytometry as previously described [22]. Briefly, for the NAC treatment, the MDA-MB-231 cells were seeded with 2 mM NAC. The next day media was removed and the cells were dosed with various concentrations of leinamycin for 24 hours. For leinamycin only treatment, cells were treated with various concentrations of leinamycin for 24 hours. Cells were trypsinized, pelleted, washed, and incubated in 1 mL of PBS/5 mM glucose with 50 μM DCFDA for 60 min at 37°C. The cells were then collected and washed twice with 1 mL of PBS to remove residual DCFDA. Dichlorofluorescein (DCF) fluorescence was quantified at 525 nm, when excited with 488 nm light with the Coulter ELITE ESP Flow Cytometer at the Flow Cytometry Core Facility at the University of Arizona.



### Caspase-3 activity assay

Caspase 3-like activity was determined using the ApoAlert Caspase Fluorescent Assay Kit (Clontech Laboratory, Inc.) according to the manufacturer's protocol. In brief, MDA-MB-231 cells were seeded with 2 mM NAC. The next day, media was removed and the cells were treated with various concentrations of leinamycin for 24 hours. Cells were then harvested, washed with ice-cold PBS and lysed in cell lysis buffer. Cell lysates were mixed with caspase assay buffer containing 20  $\mu$ mol/L Ac-DEVD-AFC as a caspase-3 substrate and incubated for 1 h at 37°C. Enzyme-catalyzed release of the fluorophore 7-amino-4-trifluoromethyl coumarin (AFC) from the labeled substrate, DEVD-AFC, was monitored using a Synergy HT Multi-Detection Microplate Reader (BioTek) with an excitation wavelength of 395 nm and an emission wavelength of 509 nm.

### DNA cleavage assay

A supercoiled plasmid (PGL3MT1) was incubated overnight with 1  $\mu$ g/mL leinamycin in the presence of various concentrations of NAC at room temperature, in a buffer containing 20 mM Tris-HCl, pH 7.6, 20 mM NaCl and 1mM DTT. Agarose gel electrophoresis was performed to examine the DNA cleavage activity of leinamycin in the presence of NAC.

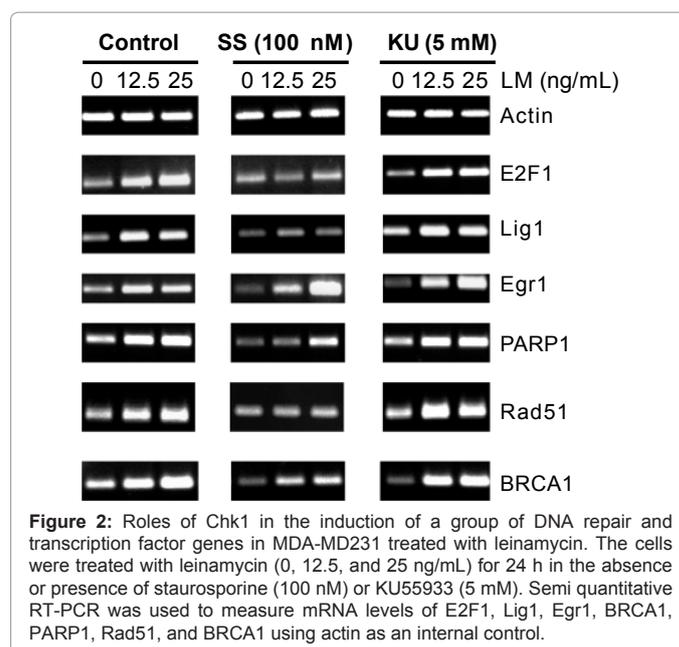
## Results

### Leinamycin induces a group of DNA repair genes and transcription factors

We examined the effect of leinamycin on the expression of a group of DNA repair and transcription factor genes in the MDA-MB-231 breast cancer cell line. The list of genes we examined includes the DNA repair genes BRCA1, RAD51, LIG1 and PARP1 and the transcription factors E2F1 and EGR1 [16,30,31]. As shown in Figure 2, an elevation in the expression of these genes was observed in a dose dependent manner in MDA-MB231 cells treated with leinamycin compared to the untreated cells. Our result suggests that leinamycin is able to induce DNA repair genes that are similarly induced using clinical DNA damaging agents [32,33]. Next, we hypothesized that the induction of DNA repair genes after exposure to leinamycin might be linked to the activation of DNA damage checkpoint mechanisms. To test our hypothesis, we examined whether the known checkpoint kinase inhibitors, staurosporine and KU 55933, could abrogate the induction of these genes after exposure to leinamycin in MDA-MB231 cells. As shown in Figure 2, the induction of DNA repair genes, including LIG1, BRCA1, RAD51, and PARP1, and transcription factor E2F1 gene were abrogated in MDA-MB231 cells treated with leinamycin in the presence of staurosporine at submicromolar concentrations (100 nM). Staurosporine is known to inhibit Chk1, but not Chk2 [30,34]. However, the ATM inhibitor KU 55933 had little effect on the induction of these repair genes by leinamycin in MDA-MB231 cells (see Figure 2), ruling out the possible involvement of ATM and Chk2 in induction of DNA repair genes by leinamycin. Interestingly, the upregulation of Egr1 gene was little affected in the presence of staurosporine or KU 55993, indicating that neither Chk1 nor ATM acts as a key operator for EGR1 upregulation after leinamycin treatment.

### NAC reduces leinamycin-induced oxidative stress in MDA-MB-231 cells

Previous studies have shown that along with DNA alkylation, leinamycin produces reactive oxygen species (ROS) from the persulfide



**Figure 2:** Roles of Chk1 in the induction of a group of DNA repair and transcription factor genes in MDA-MD231 treated with leinamycin. The cells were treated with leinamycin (0, 12.5, and 25 ng/mL) for 24 h in the absence or presence of staurosporine (100 nM) or KU55933 (5 mM). Semi quantitative RT-PCR was used to measure mRNA levels of E2F1, Lig1, Egr1, BRCA1, PARP1, Rad51, and BRCA1 using actin as an internal control.

species generated in the reaction of thiols with leinamycin [6,8]. A recent study in our lab showed that leinamycin was able to generate oxidative stress in the MiaPaCa human pancreatic cancer cell line [22]. To determine whether leinamycin causes oxidative stress in the MDA-MB-231 human breast cancer cell line and if NAC reduces leinamycin mediated oxidative stress, a fluorescence assay was performed using a DCFDA probe. As shown in Figure 3A, MDA-MB-231 cells were treated with various concentrations of leinamycin (0 ng/mL, 3.125 ng/mL, 6.25 ng/mL, 12.5 ng/mL and 25 ng/mL) in the presence or absence of 2 mM NAC for 24 hours and flow cytometric analysis was performed to measure DCF fluorescence. The signal increased up to 75% in MDA-MB-231 cells pretreated with 25 ng/mL leinamycin compared to untreated cells (see Figure 3B). MDA-MB-231 cells pretreated with 2mM NAC alone did not show any effects in terms of ROS production when compared with untreated cells (Figure 3B). 2mM NAC reduced the production of ROS by 11% and 40% in the cells treated with 12.5 ng/mL and 25 ng/mL leinamycin respectively, compared to the cells treated with leinamycin alone (Figure 3B). Taken together, the data suggests that leinamycin produces ROS in the MDA-MB-231 breast cancer cell line in a dose dependent manner and NAC is able to reduce the leinamycin-induced ROS production in these cells.

### NAC increases leinamycin induced apoptotic activity

Since NAC reduces ROS production in MDA-MB231 treated with leinamycin (Figures 3A and 3B), we determined the effect of NAC in leinamycin-mediated cytotoxicity by measuring caspase-3 activity as an indicator of early apoptosis in MDA-MB-231 cells. As shown in Figure 4, 24 h treatment of the cells with leinamycin does not have a pronounced effect in the activation of caspase-3 in absence of NAC. However, in the presence of NAC, caspase-3 activity is increased up to 80 % and 127 % in the cells exposed to 25 ng/mL and 50 ng/mL leinamycin respectively, compared to the cells treated with leinamycin alone (Figure 4). This data suggests that NAC pretreatment possibly enhances the cytotoxic activity of leinamycin by increasing the DNA damaging activity of leinamycin in MDA-MB-231 cells.

### NAC Potentiates the DNA Damaging Ability of Leinamycin

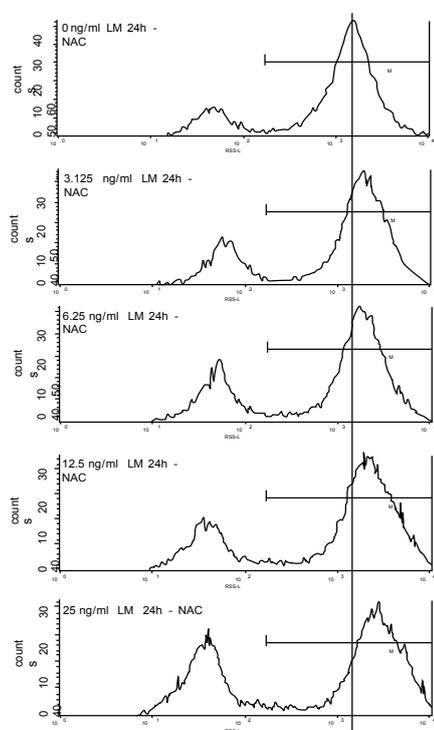
Since leinamycin is a thiol-dependent DNA damaging agent that gets rapidly converted into a DNA alkylating agent when it reaches the thiol rich intracellular environment and because NAC is precursor of GSH, we determined if NAC directly reacts with leinamycin to increase its DNA cleaving activity. To do this we performed a DNA cleavage assay using supercoiled plasmid DNA (e.g., PGL3-MT1) in absence or presence of various concentration of NAC. As shown in Figure 5, a supercoiled form of PGL3MT1 plasmid DNA, which is predominant in the untreated sample, was converted into the relaxed form in the presence of leinamycin suggesting that leinamycin indeed cleaves DNA (compare lane 3 with lane 2 in Figure 5). Importantly, the conversion

of the supercoiled form to its relaxed form is greatly increased in the presence of NAC (see lanes 2-6 in Figure 5). This data indicates that NAC reacts with leinamycin and accentuates the DNA-cleaving ability of leinamycin.

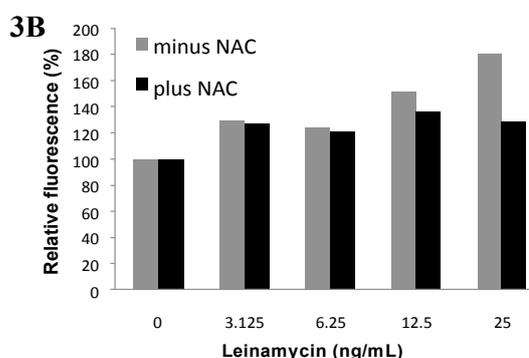
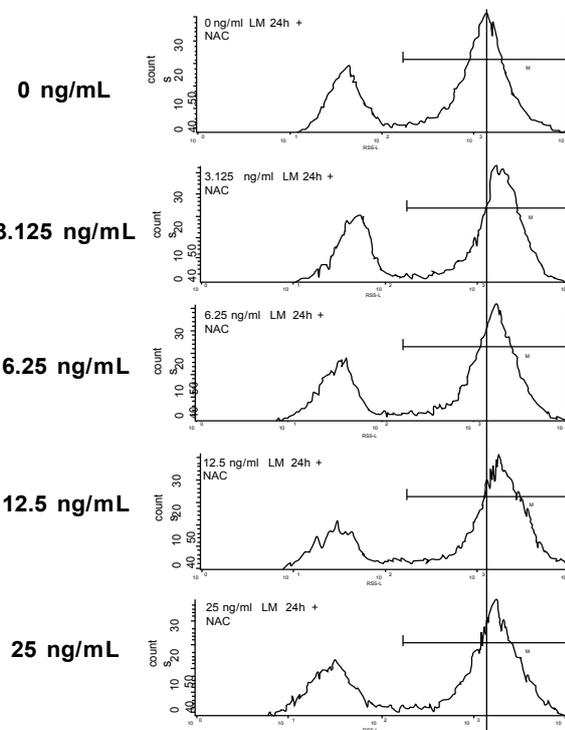
### Effect of NAC on transcriptional induction of DNA repair and transcription factor genes

Leinamycin can cause DNA damage in two ways- leinamycin can act as a DNA alkylating agent, forming leinamycin- guanine adducts leading to DNA strand breaks and by increasing oxidative stress in cells. Since NAC was found to reduce the production of ROS in leinamycin treated MDA-MB-231 cells (see Figure 3), we determined whether the ROS producing ability of leinamycin is as critical as its alkylating

### 3A No pretreatment with NAC



### Pretreatment with NAC

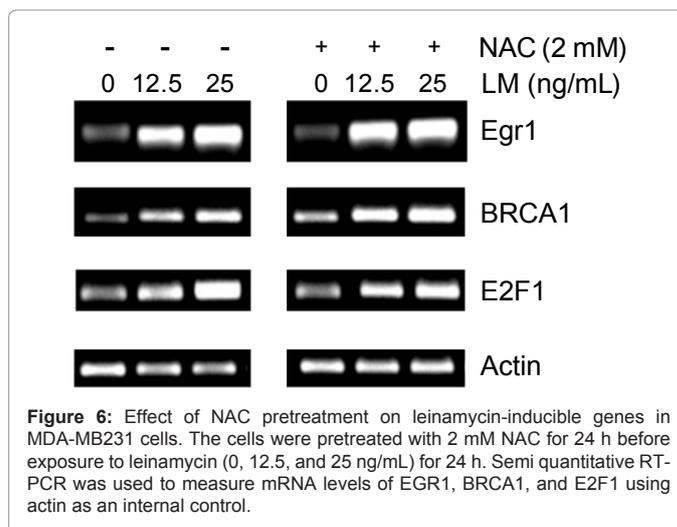


**Figure 3:** Effect of NAC on ROS production in MDA-MB231 cells treated with leinamycin. The cells were pretreated with 2 mM NAC for 24 h before exposure to various concentrations of leinamycin (0, 3.125, 6.25, 12.5, and 25 ng/mL) for 24 h. The cellular production of ROS was measured using DCFDA and flow cytometry. Figures shown are representative of at least two independent experiments. (B) Graphical representation of data presented in Figure 3A.

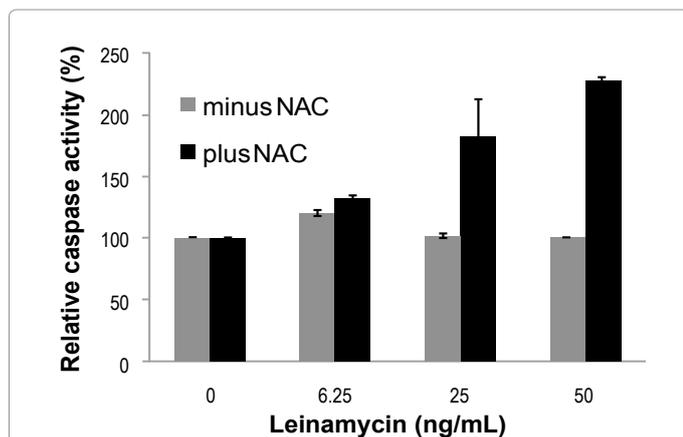
property for the induction of DNA repair genes. In this experiment, mRNA levels of EGR1, BRCA1 and E2F1 genes were analyzed using sqRT-PCR in MDA-MB-231 cells treated with leinamycin in the presence or absence of NAC. As shown Figure 6, MDA-MB-231 cells pretreated with 2 mM NAC did not show any effect in terms of increased mRNA expression of EGR1, BRCA1 and E2F1, compared to the cells treated with leinamycin alone. Regardless of the decrease in ROS production by NAC treatment, MDA-MB-231 cells still induce these genes following leinamycin induced DNA damage, suggesting that ROS may not be a crucial player in leinamycin induced DNA damage.

## Discussion

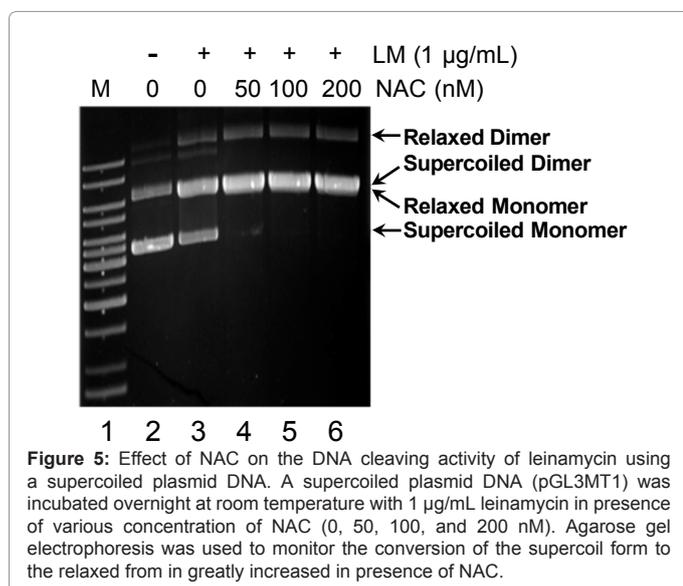
The biological relevance of the DNA damaging properties of leinamycin has recently been established in human cancer cell lines [22]. Recent studies have also shown that MiaPaCa cells treated with leinamycin effectively forms DNA strand breaks in both a time and dose dependent manner and DNA strand breaks have been shown to appear as early as 3 hours after leinamycin exposure [22]. This is



**Figure 6:** Effect of NAC pretreatment on leinamycin-inducible genes in MDA-MB231 cells. The cells were pretreated with 2 mM NAC for 24 h before exposure to leinamycin (0, 12.5, and 25 ng/mL) for 24 h. Semi quantitative RT-PCR was used to measure mRNA levels of EGR1, BRCA1, and E2F1 using actin as an internal control.



**Figure 4:** Effect of NAC pretreatment on apoptosis in MDA-MB231 cells treated with leinamycin. The cells were pretreated with 2 mM NAC for 24 h before exposure to various concentrations of leinamycin (0, 6.25, 25, and 50 ng/mL) for 24 h. Data are means  $\pm$  SEMs of three separate experiments.



**Figure 5:** Effect of NAC on the DNA cleaving activity of leinamycin using a supercoiled plasmid DNA. A supercoiled plasmid DNA (pGL3MT1) was incubated overnight at room temperature with 1  $\mu$ g/mL leinamycin in presence of various concentration of NAC (0, 50, 100, and 200 nM). Agarose gel electrophoresis was used to monitor the conversion of the supercoil form to the relaxed form in greatly increased in presence of NAC.

an important aspect of leinamycin's DNA damaging property, as many clinically used anticancer agents including ionizing radiation, bleomycin and the enediynes can cause extensive DNA strand breaks in similar fashion [35]. We proposed that a group of DNA repair genes could be transcriptionally induced to mediate chemoresistance in human cancer cells treated with leinamycin. As we speculated, in the present study, leinamycin was found to induce a group of DNA repair (e.g., LIG1, PARP1, RAD51, and BRCA1) and transcription factor (e.g., E2F1 and EGR1) genes whose functions involve various DNA repair process [16,30,31]. BRCA1 is known to play an important role in DNA double strand break repair [36], DNA ligase 1 is involved in base excision repair [37] and PARP1 helps recruit DNA repair proteins to the site of damaged single stranded DNA in addition to helping in base excision repair [38]. RAD51 is a human gene that encodes the RAD51 protein [16]. The RAD51 protein is a member of the RAD51 protein family which assists in repair of DNA double strand breaks [39]. Egr1 and E2F1 are transcription factors that have been implicated in DNA repair along with cell cycle regulation, cell proliferation and apoptosis [30,31].

DNA Damage Response (DDR) is a complex signaling network consisting of sensors, transducers and effectors, which together execute the damage response by halting the cell cycle and allowing the damage to be repaired [40,41]. The sensors include a multiple protein complex such as the Rad9-Rad1-Hus1 (9-1-1) complex, and the Rad17-RFC complex which senses the DNA damage [40]. The signal transducers include a set of conserved proteins with known motifs including the phospho-inositide kinase (PIK)-related proteins; ATM and ATM-Rad3-related (ATR), Checkpoint Kinases (CHK); Chk1 and Chk2, and the BRCT-repeat containing protein ; There is also a potential to develop small molecule inhibitors which could block the activity of a core component of this signaling pathway [34,42], thereby reducing the repair capacity of cancer cells and making them more vulnerable to the attack of leinamycin. On the basis of our findings, further studies could help elucidate a signaling pathway or identify core components involved in DNA repair followed by leinamycin induced DNA damage. Further characterization of these cellular responses to leinamycin could serve as a template for development of novel anticancer agents by investigating the biological significance of the induction of a few selected genes with respect to cell survival, DNA repair, and apoptosis following leinamycin exposure.

NAC is an important antioxidant that increases the level of GSH. Our data indicates that NAC plays a dual role in mediating leinamycin-induced cytotoxicity in MDA-MB-231 breast cancer cells. NAC reduces leinamycin-induced generation of ROS but sensitizes MDA-MB-231 cells to undergo apoptosis. NAC also potentiates leinamycin induced DNA cleavage as shown in our DNA damage experiment indicating that leinamycin becomes more active in the presence of NAC. In addition, NAC doesn't exert any affect on leinamycin-induced induction of DNA repair genes and transcription factors. ROS is known to damage DNA and induce apoptosis [2,23], but there is an increase in apoptosis in MDA-MB-231 cells even after treatment with NAC which is a known ROS quencher. Taken together, these studies indicate that ROS might not be a crucial player in leinamycin induced DNA damage. As NAC pretreatment increases apoptotic activity in MDA-MB-231 cells, NAC can confer potential selectivity to leinamycin against cancer cells. More molecules of leinamycin can get activated if leinamycin and NAC are administered together, thus making leinamycin a more potent DNA damaging agent.

Our findings confirm that a group of genes and transcription factors involved in DNA repair is overexpressed in MDA-MB-231 cells in response to leinamycin, which can mediate chemoresistance to leinamycin in human cancer cells. Our data also indicates that ROS is not a crucial player in leinamycin induced DNA damage and NAC, a major precursor of glutathione, can potentiate leinamycin mediated cytotoxicity by promoting the activation of leinamycin into its DNA reactive form.

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