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MODULATORY EFFECTS OF LNM ON LYMPHOCYTIC APOPTOTIC PATHWAY THROUGH P53, BCL2,

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ABSTRACT

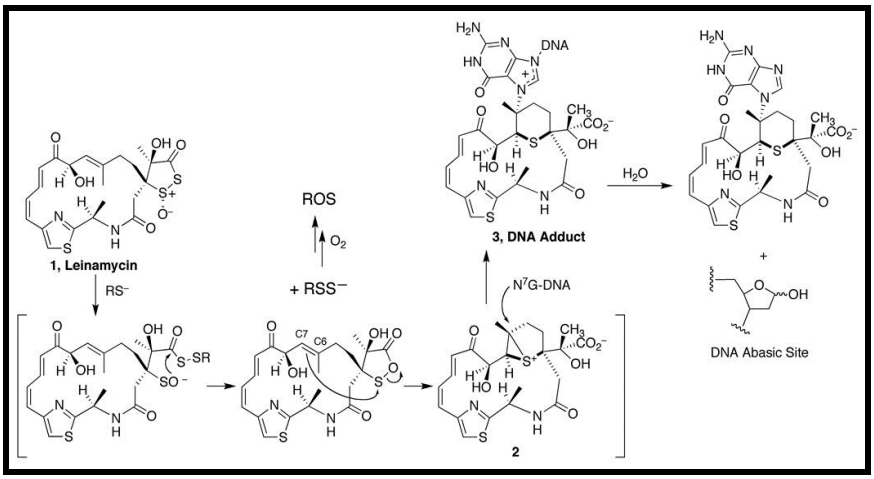
By using immunocytochemistry technique the apoptotic activity of Leinamycin against lymphocytes isolated from Acute Lymphocytic Leukemia patients in comparison with healthy individuals was determined by measuring P53 and Bcl-2, were they play very important role in cell cycle and then programed cell death.Results showed that LNM at concentration 4.5 µg /ml caused significant upregulation of P53 while it decreased the expression level of the anti-apoptotic protein Bcl-2 in lymphocyte. The results showed significantly ($P< 0.001$) improvement of P53 expression level in treated cells (43.363 ±1.893%) when compared with untreated cells (14.371 ± 0.449 %), Whereas, LNM caused dramatic decreasing in Bcl-2 expression pattern when compare with untreated cells (30.08± 1.0095, 63.2± 1.43; $P < 0.001$) respectively.

KEYWORDS: P53, BCL-2, Leukemia, programed cell death, Leinamycin.

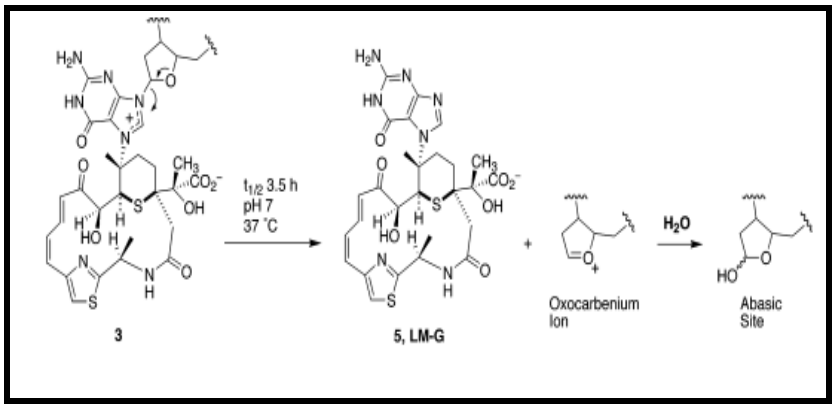
INTRODUCTION

Cytotoxic compounds that are in clinical use as antitumor drugs represent an immense variety of structural types, according to the many different targets focused on in the drug therapy of cancer. Within this group of compounds, natural products still play an important role and have been proven to be valuable tools for the development of new antitumor drugs, In Iraq ,cancer is spreading alarmingly at high rates blamed on the mutagenic and carcinogenic agents (like depleted uranium) employed in the wars of 1991 and 2003, several media reports of apparent excess rates of cancers and birth defects in the town of Fallujah (Busby *et*

al., 2010) as well as Increases in childhood leukemia in Basrah have recently been investigated (Hagopian *et al.*, 2010). Antitumor drugs that derived from Streptomyces acts through several mechanisms, to inducing apoptosis through DNA cleavage as Leinamycin initially named DC107 (Hara, 1988; Hara *et al*., 1989). It is displays potent activity against cancer *in vitro* and *in vivo* tumor models, it showed antitumor activity against mouse sarcoma180. The LD50 value of DC 107 was 2.8mg/kg (iv) in mouse, additionally, it has antitumor activity against HeLa S3 tumor cells. Leinamycin increases the life span of mice with murine leukemia P388 by ~60% at a dosage of 0.38 mg/kg (Kanda *et al*., 2003) Early studies by the discoverers of leinamycin indicated that the natural product causes thiol-triggered DNA strand breaks. Subsequent studies showed that attack of a thiol on the 1,2-dithiolan-3-one 1-oxide in leinamycin initiates conversion of this heterocycle to a 1,2-oxathiolan-5-one (Fig.1) that undergoes further rearrangement to an episulfonium ion (Fig. 2) (Keerthi and Gates, 2007). This episulfonium ion associates non-covalently with duplex DNA and alkylates the N7-position of guanine residues in double-stranded DNA with very high efficiency. The guanine adduct is the only covalent DNA lesion resulting from the reaction of thiol-activated leinamycin with duplex DNA. In addition to the DNA-alkylation chemistry described above, attack of a thiol on leinamycin releases a persulfide intermediate (RSSH), that can mediate the generation of reactive oxygen species (ROS) *in vitro* (Sivaramakrishnan and Gates, 2008). Persulfide generats ROS via an initial reduction of molecular oxygen to superoxide radical, followed by disproportionation to hydrogen peroxide, and finally production of the hydroxyl radical via the Fenton reaction. Such chemistry could contribute to the biological activity of leinamycin because intracellular generation of ROS can cause DNA strand cleavage and lead to cell death via general oxidative stress (Ozben, 2007).



In vitro studies revealed that leinamycin-guanine adducts in double-stranded DNA undergo unusually rapid depurination ($t_{1/2}$ = 3 h) to generate AP sites (Shipova and Gates, 2005). In general, the depurination of N7-alkylguanine residues in double-stranded DNA occurs with a half-life in the range of 50–100 h under physiological conditions (Gates et al., 2004). While the chemical basis for the rapid loss of the leinamycin-guanine adduct from duplex DNA remains uncertain, this rapid depurination reaction might be biologically important because AP sites generated in cellular DNA are known to be cytotoxic lesions. In addition, AP sites are readily converted into DNA strand breaks, which are also potentially toxic lesions, recent evidence shows that AP sites can generate inter strand crosslinks in DNA under physiologically relevant conditions rapid generation of large numbers of AP sites by leinamycin has the potential to overwhelm the capacity of these repair enzymes (Fig.2) (Dutta *et al.*, 2007; Viswesh *et al*, 2010).



DNA is an important biological target of the natural product leinamycin caused DNA strand breaks in both time- and dose-dependent manners, with strand cleavage occurring as early as 3 h after MiaPaCa cells were exposed to the drug. Most DNA-damaging agents are known to activate DNA checkpoint mechanisms irrespective of their mode of actions. The checkpoint kinase Chk2 is known to be activated in response to direct DNA cleaving agents such as ionizing radiation (IR), while replication arresting agents, such as the alkylating agents, aphidicolin and hydroxyurea, activate the checkpoint kinase Chk1.Thus, the observed activation of Chk2, but not Chk1, in MiaPaCa cells treated with leinamycin is consistent with a cellular response to DNA strand break formation rather than DNA adduct formation (Barkley *et al.*, 2007).

MATERIAL AND METHODS

Subjects

The study included 50 subjects; 35 patients, venous blood samples were obtained from patients at Baghdad Medical City, they were diagnosed as ALL in the early stage of diagnosis where they didn't take any therapeutic substance, and 15 samples from healthy individuals as a negative controls. Peripheral blood (5 ml) were aspirated from each subject. Blood samples were immediately transferred to sterile heprenized vacutainer tubes for lymphocyte separation depending on (Boyum, 1968).

Culture stage

LNM at concentration 4.5 µg /ml were adding to the cell culture tubes which contained completed RPMI-1640 medium, the final volume of the mixture must be taken into account. About 0.3ml of PHA was added to each tube.0.5ml of cell suspension was added to each tube, the contents were mixed gradually by inversion and incubated at 37° C for 24hrs), tubes mixed each 24h (for normal and affected individuals).

Cultured cell groups were harvested after completed of incubation periods by centrifugation at 1500 rpm for 10 min, the supernatant was removed and the cell pellet was re- suspended by adding buffer. The cells were adjusted to 1×10^6 cell/ml in PBS. Then, 10µl of cells suspension was spotted over positive charged slide (3 spots in each slide) and left to dry at room temperature. From fixative (absolute acetone), 20µl was added over each spot and left to dry. The slides were foiled with Parafoil and kept at – 20ºC until used.

Determination of P53 and Bcl-2 family proteins in blood samples

The commercial Immunocytochemistry kit (USBiological, USA) used to determine p53 and Bcl-2 proteins levels in patients with ALL cancer type as well as healthy individuals depending on (Huang et al., 2002). Primary antibody was diluted in dilution: 1:100 forp53, bcl-2, 1:25 , sample fixed by absolute aceton on positive charged slides as in were allowed to reach room temperature, the Parafoil was removed and the slides were dipped into PBS jar for about 5min.Slides were placed on a flat surface, enough drops of 0.6% hydrogen peroxide in methanol were dropped to cover each smear and slides were incubated at 25ºC for 5min.Then, slides were washed gently with DW and place in TBS for 5 min.

Slides were taped (but not washed) to remove excess blocking reagent, then, (20µl) of diluted rabbit mAb (primary Ab) specific for human p53 and Bcl2 proteins were added (the primary Ab was replaced by PBS in negative controls) and slides were incubated at 37ºC for 30 min. Unreached mAb were removed by dipping slides sequentially in three PBS jars, each for 5min and then dried gently. Incubated with prepared Biotinylated goat Ab working solution from bottle 1(20µl), and incubated at 37ºC for 30 min. Then, slides were washed in PBS, bathed in PBS jar for 5min and wiped around smear. Incubation with the prepared streptABComplex working solution from bottle 2 and incubated for 30min at 37ºC. Then, slides were washed and bathed as in step 5.Incubate with a prepared chromogenic substrate solution for peroxidase (20µL) and incubated for 5-15 min at 37ºC. Slides were rinsed gently with dH2O using washing bottle, and dipped in a bath of hematoxline counter stain for about 15sec at 25ºC.

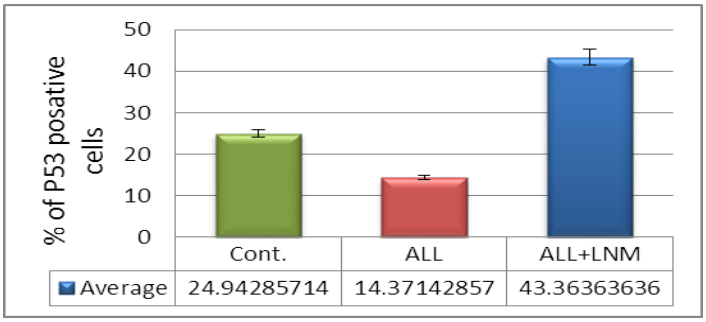
Slides were rinsed with dH_2O and then dipped in distal water for 5min. A drop of mounting (DPX) was placed onto the wet smear and quickly covered with a cover slid and left to dry.

Slides were examined under 40X- magnification power of light microscope for the assessment of immunostaining. A total of 100cells were counted to determine the percentage of reactivity of p53and Bcl_2 mAb. In this study, cells considered as positive when the nucleus was being stained with dark brown color. Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007.The percentage of positive cell calculated as following:

$$\textbf{Percentage of positive cells} = \frac{\textbf{No.of positive cells}}{\textbf{Total No. of cells}} \times 100$$

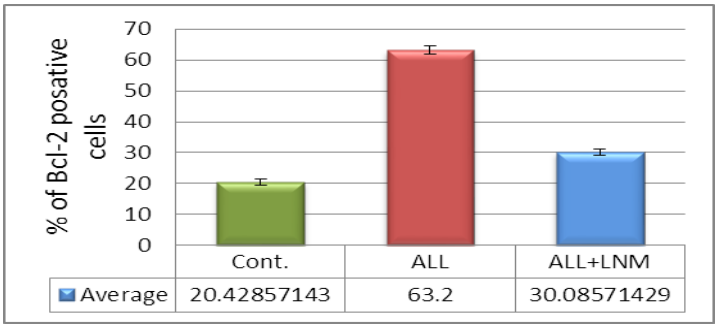
RESULTS AND DISCUSSION

According to immunocytochemical staining technique, the activity of P53 was measured in treated and untreated cells with 4.5 µg /ml LNM for 24 hrs. The results showed significantly ($P < 0.001$) improvement of P53 expression level in treated cells (43.363 ±1.893%) when compared with untreated cells (14.371 ± 0.449 %).

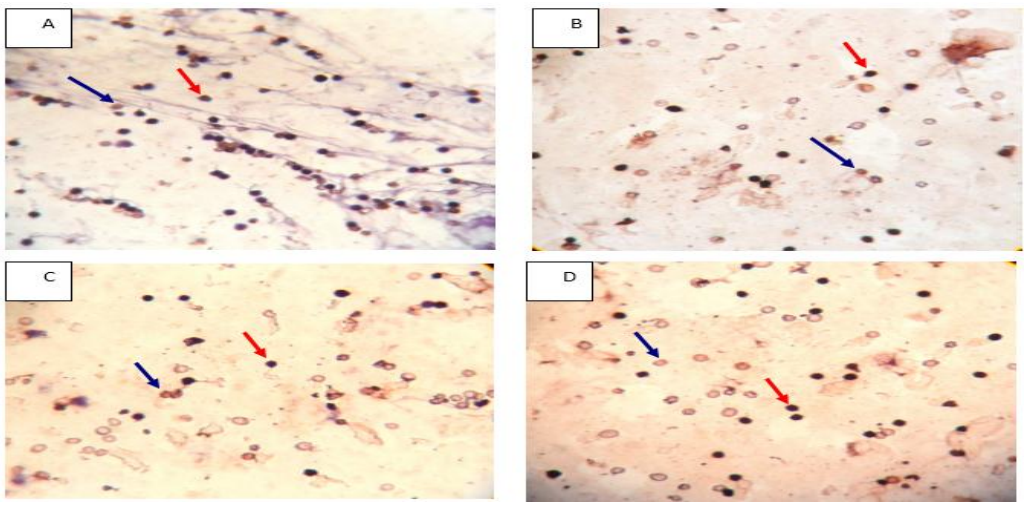


P53 expression in acute leukemia lymphocytes treated with LNM. Value is meaning ± SEM. Asterisks indicate statistically significant difference from All group, *P*< 0.001

Moreover, immunocytochemistry technique was used to evaluate the contribution of pro- and anti-apoptotic factor (Bcl-2) in the reponse of ALL to LNM treatment. The results revealed that LNM lead to dramatic decreasing in Bcl-2 expressin pattern when compare with untreated cells (30.08± 1.0095, 63.2± 1.43; $P < 0.001$) respectively.



BCL_2 expression in acute leukemia lymphocytes treated with LNM. value is meaning ± SEM. Asterisks indicate statistically significant difference from All group, *P*< 0.001.



Immunocytochemical staining of lymphocytes of ALL patient with P53 and Bcl2 mAbs, before (A, C), and after exposure (B, D) to LNM respectively. visualization by

peroxidase /DAB (brown) and counter stained with Meyer`s hematoxylin. *Note*: Blue arrow refer to +ve cell, while red arrow refer to –ve cell.

A hallmark feature of leukemia and other cancer cells is the ability to escape apoptosis that correlates with chemotherapy resistance. Therefore, there have been enormous efforts in developing new molecules that could reactivate the apoptotic program in tumor cells (Lu and Chen, 2011). Regarding to mechanisms whereby LNM exert its chemotherapeutic effects (Gates, 2009), the P53 pathway was involved. The results showed that LNM caused significant an upregulation in P53 expression level in lymphocyte after exposure to 4.5 µg /24 h of LNM ($P< 0.001$). There is a scientific evidence to suggest that cytoplasmic retention of p53 is more pronounced at lower levels of DNA damage and is saturable at severe levels of damage, leading to translocation of p53 to the nucleus followed by wild-type function (Buckbinder *et al*., 1995; Liontas and Yeger, 2004). It is possible that this agent, particularly at this dose, may cause sufficient DNA damage in lymphocyte to induce nuclear translocation and activation of wild-type p53. Phosphorylation of p53 has been associated with cellular damage occurring during anticancer therapy (Komarova et *al*., 1997). P53 phosphorylation increases its half-life and thus increases the accumulation and functional activation of p53 in response to DNA damage, thereby stabilizing it (Tibbetts *et al*., 1999; Mirzayans *et al*., 2012). This information provides evidence that increase in p53 protein expression after LNM treatment may due to increased stability. These results provide definitive evidence for the requirement of p53 in ALL induced apoptosis. Transcriptional activation of p53 might lead to activation of pro-apoptotic Bax which in turn enhance the permeability of the mitochondrial membrane, resultant in releasing of apoptogenic factors. (Roy *et al*., 2005). In relation to Bcl-2 protein expression, previous work mentioned that enforced Bcl-2 expression delays apoptosis of cell lines deprived of essential survival factors and prolongs the life-span of lymphocytes in Bcl-2 transgenic mice (Coustan-Smith *et al*., 1996; Antonsson, 2001). Thus, Overexpression of Bcl-2 may also confer resistance to cytotoxic agents. For example, enforced BCL-2 expression increases the resistance of thymocytes to glucocorticoids (Reed, 1995; McCarthy *et al*., 2008).

Concurrent with above findings, it found that LNM decreased the expression level of the anti-apoptotic protein Bcl-2 in lymphocyte from ALL patients ($P < 0.001$). This molecule controls the mitochondrial membrane permeability, a crucial event in the apoptotic processes (Roman *et al*., 2002). The Bcl-2 family has been shown to form a heterodimer with members of the

pro-apoptotic Bax family and might thereby neutralize its pro-apoptotic effects (Srinivas *et al*., 2000; Klobusicka *et al*., 2001; Tzifi *et al*., 2012). Thus, it has been suggested that the ratio between the level of pro-apoptotic Bax and that of the anti-apoptotic factor Bcl-2 determines whether a cell responds to an apoptotic signal. In this study, 4.5 µg/ml LNM treatment caused a modest decrease in Bcl-2 protein expression (Fig. 4-24). Keeping in mind, the expression of P53 was significantly increased. Taken together, these data suggest that LNM could modulate the Bcl-2/Bax ratio that is critical for in vitro apoptosis and in vivo chemoresistance in leukemic lymphocyte.

Alteration in the Bax/Bcl-2 ratio causes the release of cytochrome c from mitochondria into cytosol. Cytosolic cytochrome c can bind to Apaf-1 and activate caspase 9 in the apoptosomes in response to diverse inducers of cell death. Activation of caspase 9 leads finally to the activation of caspase 3, which is one of the key mediators of p53-induced apoptosis (Roy *et al*., 2005; Communal *et al*., 2002). At this time, there is no previous data explain the effect of LNM on P-53, or Bcl-2 protein expression. Thus, results in this study represents a step forward toward understanding the possible mechanisms of apoptotic induction and antitumor activity of LNM.

CONCLUSIONS

Leinamycin produced by *Streptomyces atroolivaceous* THS44, isolated from Iraqi soil, exhibited potential immunomodulatory effects as cancer therapeutic agent against lymphocytes isolated from ALL patients by increasing proapoptotic p53 as well as decreasing antiapoptotic Bcl2 protein level.

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