Indian Journal of Experimental Biology
Vol. 62, October 2024, pp. 779-787
DOI: 10.56042/ijeb.v62i10.10032

Nootkatone debilitate bleomycin-induced pulmonary toxicity in lung cancer A549 cells: *In silico* and genomic evolution

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Received 31 March 2024; revised 15 May 2024

The biggest issue with bleomycin (BLM) chemotherapy is pulmonary damage. BLM manifests oxidative stress via an uncontrolled progression of reactive oxygen species in pneumocystis, a relative deficit of the deactivation enzyme BLM hydrolase and the formation of inflammatory cytokines leading to apoptosis. There have been several attempts to treat patients for this adverse effect by giving them antioxidant rich supplements to minimise free radicals. Compounds that are extracted from plants or that are based on plants emphasise more on curing such patient issues in order to support treatment, rejuvenate, or manage normal metabolism. Hence, in this study we concentrated on pretreatment of lung cancer derived A549 cells with phytocompound nootkatone (NKT) to prevent BLM-mediated oxidative stress. We find in our study, the BLM-exposed cells have displayed morphological anomalies such as shrinkage, blabbing, and chromatin condensation, among others. Yet, even after exposure to BLM, no such abnormalities were seen in NKT pretreated cells. In NKT pretreatment cells, there was a significant surge in endogenous antioxidants and a decrease in lipid peroxidation, in contrast, cells exposed to BLM had lower levels of antioxidants and greater levels of lipid peroxidation. In gene expression analysis, the pro-apoptotic gene *Bcl-2/Bax* and the apoptotic executor caspase 3 were significantly suppressed in the NKT pretreated cells, which attenuated BLM-mediated apoptosis. The current investigation, showed that the NKT pretreatment has displayed merit for pulmonary protective activity against BLM-induced oxidative stress by boosting the intracellular antioxidant defence.

Keyword: Gene expression, Molecular docking, Oxidative stress, Phytocompound

Progressive lung disease Pulmonary fibrosis has been regarded as a common issue brought on by radiation, environmental contaminants, cancer, chemotherapy or other medical conditions, even as a result of chronic inflammatory disorders[1]. Overall, the occurrence of pulmonary toxicity was modest, but recently, prevalence and incidence rates have risen due to the general aging population., environmental hazards, the COVID-19 pandemic, and a lack of general awareness. Most frequently, lung diseases, both acute and chronic, are attributed to drug induced pulmonary toxicity. Such toxicity has been associated with both cytotoxic and noncytotoxic medicines. In contrast to other cytotoxic medications, bleomycin does not significantly impair myelosuppression or immune function, making it a main chemotherapeutic agent [2]. Bithiazole, a substance that intercalates into the DNA helix and separates the strands, is a structural component of bleomycin that causes DNA damage and the production of reactive oxygen species (ROS). Beyond its effects on DNA, bleomycin can cause damage to cells by causing lipid peroxidation, a process that can exacerbate the drug's harmful effects, especially in lung tissues. This method could account for some of bleomycin's ability to damage alveolar cells and cause pulmonary inflammation in the process[3]. While there is no sauthorised standard treatment for bleomycin-induced pulmonary damage, some of the most widely used strategies include skipping bleomycin from future chemotherapy, maintaining a non bleomycin based treatment in pertinent individuals, or corticosteroid therapy[3,4]. Sesamol, a type of lignan, and empagliflozin have been demonstrated to mitigate pulmonary toxicity and fibrosis induced by bleomycin in rodent models[5,6]. Similarly, polyphosphate has been shown to lessen bleomycin's toxicity in A549 lung cancer cells[7]. Nootkatone (NKT), a sesquiterpene ketone (+)

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nootkatone, has a grapefruit like flavor in orange and grapefruit juice, is used commercially as a spice or flavour ingredient, and has numerous biological activities[8]. NKT was first isolated from *Cupressus nootkatensis* (Alaska cedar), is found in orange, grapefruit, lemon, mandarin, and so on[9]. NKT is safely used in products such as juices and cosmetics to enhance flavour and fragrance. It is known to be non-toxic and non-carcinogenic, and it is approved for use in and on people by FDA[10]. Research has indicated that NKT possesses several pharmacological attributes, including antibacterial, antioxidant, antifibrotic, neuroprotective, anticancer, and anti-allergic effects[11].

Investigating the potential protective roles of NKT cells against the early manifestations of BLM-induced pulmonary toxicity is pertinent. This importance arises because the onset of pulmonary toxicity from BLM involves initial oxidative stress and apoptosis, which progress to fibrosis and impaired lung function. Additionally, acknowledging NKT cells' antioxidant capabilities in the early stages, along with their beneficial effects in experimental conditions characterised by inflammation and oxidative stress, is crucial.

Materials and Methods

Reagents

Bleomycin, Nootkatone, Dulbecco's Modified Eagle's Medium, and MTT were sourced from Sigma (St. Louis, MO). ELISA, cytokine kits and Catalase activity kits were acquired from Abcam (United Kingdom) The Malondialdehyde (MDA) assay kit was obtained from Sigma-Aldrich, St. Louis, MO, USA, and the Glutathione peroxidase (GPx) activity kit was acquired from ZellBio GmbH, Germany. For RNA extraction, TRIzol reagent was obtained from Invitrogen, while the cDNA kit and SYBR® Green PCR were procured from Applied Biosystems.

Cell culture

50 µg/mL of gentamicin and 10% fetal bovine serum (FBS) were added to DMEM to support the growth of A549 lung cancer cells. These cells were maintained at 37°C in a humidified environment with 5% CO2 and 95% air in a CO2 incubator. Subculturing in tissue culture flasks measuring 25 cm^2 allowed for the maintenance of the cells. The cell viability tests were limited to cells that were in the exponential growth phase.

Pulmonary protective activity of NKT

To assess BLM and NKT's cytotoxicity, an MTT assay was conducted with cells treated at different concentrations, followed by a 24 h incubation at 37°C. Afterward, the medium was swapped for DMEM without FBS and MTT, incubated in darkness for 4 h. Formazan crystals formed were dissolved in DMSO, and cell viability was measured at 570 nm[12].

Morphological examination of cellular abnormality

After trypsinisation, cells were placed on glass coverslips in 6 well plates and treated with NKT (15 and 30 µM) and BLM (10 µM) for 24 h. BLM at a concentration of 10 µM causes a decrease in cell viability (approximately 70%), accompanied by an increased expression of inflammatory and pro-apoptotic genes. This dosage was selected also based on previous work by Muller *et al.,* 2021 along with details on our preliminary dose response experiments that helped establish the optimal concentrations for observing significant effects while minimising cytotoxicity[7]. Subsequent observation of A549 cells under an inverted microscope (Olympus BX53, Tokyo, Japan) revealed morphological changes such as membrane blebbing, shrinkage, and necrosis.

Estimation of total protein

For the protein estimation cells treated with NKT (15 and 30 µM) and BLM (10 µM), 200 µL of RIPA Lysis Buffer (UFC Biotechnology) also having protease inhibitor cocktail was added to each well cell pellet. Whole cellular proteins were extracted by centrifugation of cell lysates using a procedure described earlier[13]. Protein quantification was achieved by the Lowry method technique[14].

Lipid peroxidation inhibitory activity

Lipid peroxidation was determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product, proportional to the MDA present. The intensity of the colour was measured spectrophotometrically at 532 nm[15]. A549 cells in culture were pretreated with NKT (15 and 30µM) and exposed with BLM (10 µM) for 60 min, followed by 100 µM of H_2O_2 for 60 min.

Assays for antioxidant enzymes

A549 cells pretreated with NKT (15 and 30 µM) and BLM (10 µM) exposed cells were lysed in a lysis buffer. Superoxide dismutase activity was assayed by the nitroblue tetrazolium (NBT) method[16]. The reaction mixture was composed of 0.5 mL of cardiac

PMS, 1 mL of 50 mM sodium carbonate, 0.4 mL of 25 µM NBT, and 0.2 mL of 0.1 mM EDTA. The reaction commenced with the addition of 0.4 mL of 1 mM hydroxylamine-hydrochloride. Absorbance changes were monitored at 570 nm. Catalase and glutathione peroxidase activity were assayed as previously described and manufactures instruction[17,18].

mRNA expression by real-time polymerase chain reaction (RT-PCR)

After RNA isolation cDNA synthesis was performed as per protocol provided by manufacturer. Quantitative real time polymerase chain reaction (qRT-PCR) Expression levels of caspase 3, Bcl-2 and Bax mRNAs were assayed using RT-PCR, and β-actin was used as a control[12] (Table 1).

Caspase-3, Caspase-9 and p53 assay by ELISA

NKT pretreatment (15 and 30 µM) and BLM (10 µM) exposed cells were measured for caspase 3, caspase 9 and p53 activity by ELISA kits. All experiments were carried out by manufacturers protocol (Abcam, United Kingdom).

Cytokines assay

Following 24 h of cell growth at 37°C with varying dosages of NKT (15 and 30 µM) and BLM (10 µM), the supernatant of the cultured cells was used to determine the levels of TNF-α and IL-6 cytokines as per manufacturer instructions[13].

In silico analysis

Three dimensional crystal structure of target protein bleomycin hydrolase (PDB 1CB5) was taken from protein data bank. Ligands was obtained from PubChem (1268142) Molecular docking was done by AutoDock Vina-based webserver CB-Dock. The results were visualised by Discovery Studio.

Statistical analysis

The GraphPad Prism Software was used for all statistical calculations and graphical displays. Log (inhibitor) vs. normalised response curve approach was used to determine IC_{50}. To evaluate the presence of statistically significant differences between the test and control groups, a one way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test was employed. The statistical significance level was set at P <0.05, and the data were reported as mean ± standard deviation.

Results

Cytotoxicity evaluation of BLM and NKT

The preliminary investigation of BLM and NKT exposure on the A549 cells was performed the results obtained showed decreased cell viability with increased concentration of both BLM and NKT exposure (Fig. 1). BLM was used to induce oxidative stress to the pulmonary cells as it was reported to

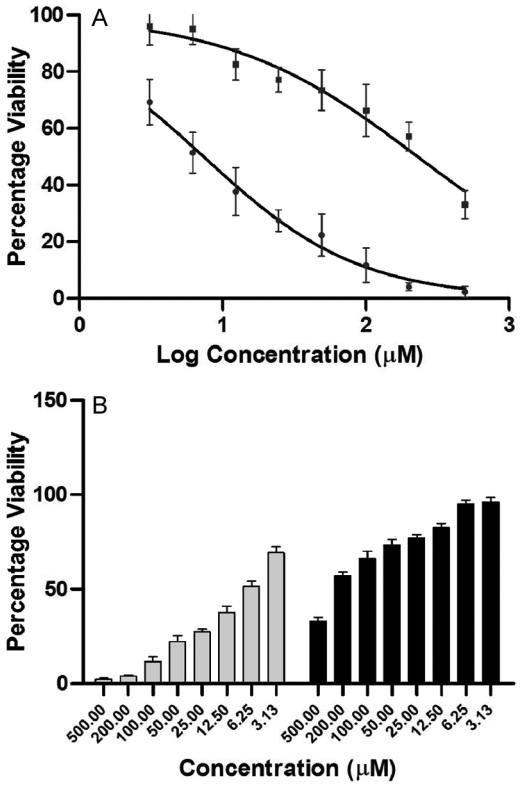

Fig. 1 — Cytotoxicity assessment of BLM and NKT using A549 cells. (A) Cell viability dose response curve (B). Cell viability (%) on exposure to BLM and NKT and Control (n=3).

Table 1 — Primers sequences used for RT-PCR reactions

Gene	Gene Bank Accession	Annealing Temperature	Primer
β-actin	NM_001101.5	60°C	F-CCAGATCATGTTTGAGACCTTCAA
			R-GTGGTACGACCAGAGGCATACA
Bcl-2	NM_000633.3	60°C	F-TTGACGCTCTCCACACACATG
			R-GGTGGAGGAACTCTTCAGGGA
Bax	NM_138761.4	60°C	F-TGCTGATGGCAACTTCAACT
			R-ATGATGGTTCTGATCAGCTCG
Caspase 3	NM_032991.3	60°C	F-ATGTCGATGCAGCTAACCTC
			R-TCCTTTTGCTGTGATCTTCC

produce intracellular ROS or free radicals leading to apoptosis[19,20]. BLM at a concentration of 3.13 µM, with an IC50 value of 7.46 µM (Fig. 1A-B) and NKT's IC50 was determined to be 231.5 µM, with significant reductions in viability at concentrations from 250 to 500 µM. Lower concentrations of NKT (15 and 30 µM) were selected for further study due to their minimal toxicity, preserving over 70% viability (Fig. 1A-B).

Morphological changes on A549 cells

The morphological changes on lung cancer cell lines after treatment of control, BLM, and NKT are presented in Fig. 2, and after 24 h of incubation, BLM-treated cells evidenced maximum cell death in comparison with NKT and control. The morphological changes observed in A549 by BLM were due to damage in cell organelles. The pretreated cell with NKT ameliorated the toxicity induced by BLM, the 30 µM NKT treated cells significantly reduced the cell toxicity and was nearly equal to control which is consistent with our cytotoxicity assay.

Apoptosis and cytotoxicity by BLM and untreated cells/control by Flow cytometry in A549 cells

In this study, Annexin V/PI double staining was employed to assess cell populations. Cells treated with 10 µM BLM exhibited an increase in cytotoxic and early apoptotic cell populations (48.35±4.28% and 3.67±1.83%, respectively), in contrast to untreated cells (1.3 0.18% and 0.6±0.03%, respectively), as shown in Fig. 3. Notably, a dose dependent treatment with NKT significantly reduced both cytotoxic and early apoptotic populations, with 15 µM NKT showing 39.24±5.85% and 13.26±5.18% respectively, and 30 µM NKT showing 8.37±2.36% and 6.32±2.27%, respectively.

Estimation of intracellular antioxidants and lipid peroxidation on NKT pretreated A549 cells

To evaluate antioxidant defense homeostasis, levels of endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GPX) were measured following pretreatment with NKT (15 and 30 µM) and BLM (10 µM). This treatment notably altered antioxidant levels, enhancing SOD, CAT and GPX specifically at 30 µM (Fig. 4A-C). Additionally, oxidative stress, indicated by elevated Malondialdehyde (MDA) from lipid peroxidation, was significantly higher in cells treated only with BLM compared to those pretreated with NKT, demonstrating NKT's effectiveness in reducing lipid peroxidation and potentially mitigating pulmonary toxicity (Fig. 4D).

Gene expression analysis on NKT pretreated A549 cells

Real time PCR analysis of apoptotic gene markers (Bcl-2, Bax, and Cas 3) showed that BLM treatment significantly reduced Bcl-2 expression (0.36 ± 0.06, fold) and increased Cas 3 (4.53 ± 0.3, fold) and Bax (5.22 ± 0.49) levels, indicating enhanced apoptosis in A549 cells (Fig. 5A-C). In contrast, cells pretreated with NKT exhibited reduced expression of apoptotic genes and increased expression of anti-apoptotic genes, highlighting NKT's protective role against BLM-induced apoptosis.

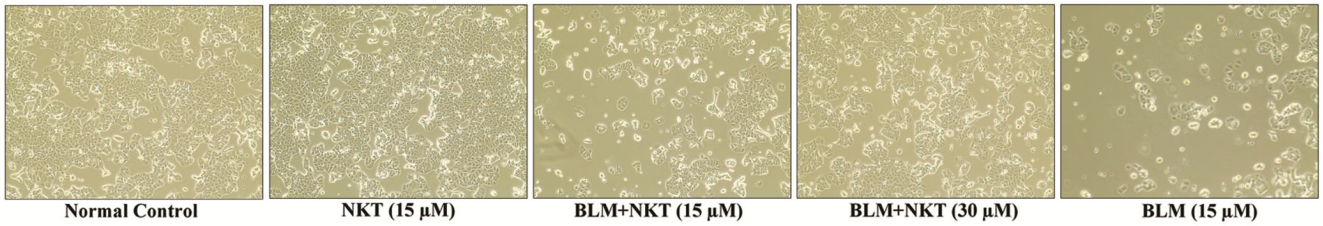

Fig. 2 — Morphological changes in A549 cells after exposure to BLM and untreated cells/control, cells pretreated with NKT.

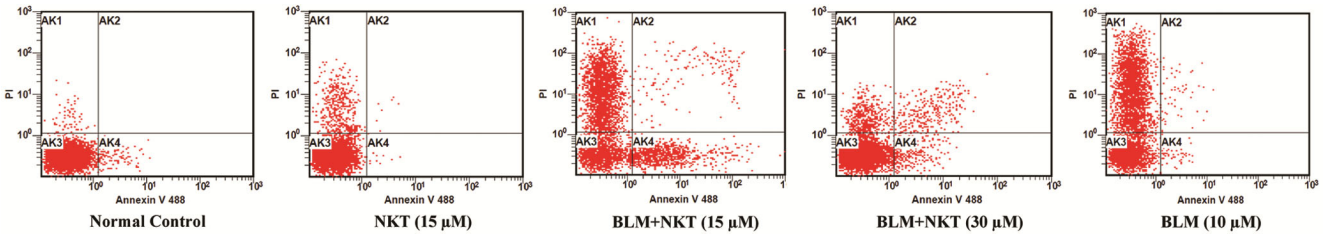

Fig. 3 — Induction of apoptosis and cytotoxicity by BLM and untreated cells/control, cells pretreated with NKT in A549 cells after 24h incubation. The cells were stained with Annexin V and PI, and analysed by flow cytometry

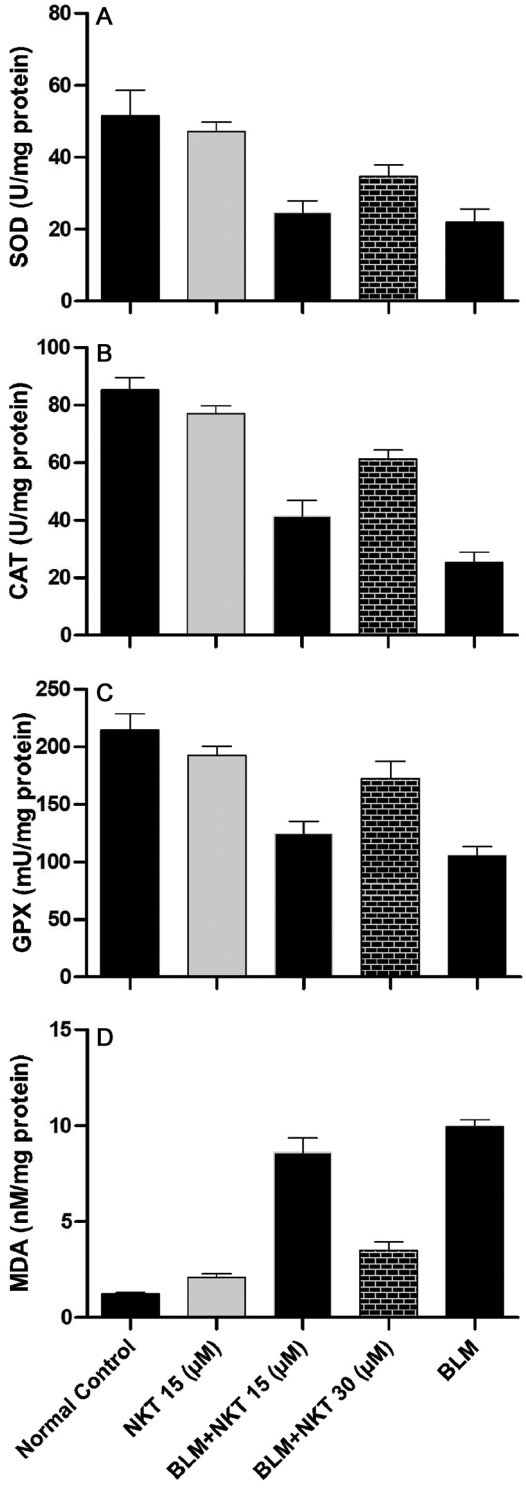

Fig. 4 — Quantification of intracellular antioxidants in A549 pulmonary cells pretreated with NKT and BLM exposure (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) reduced glutathione (GPX) content, and lipid peroxidation (D) Malondialdehyde MDA. [Data shown as mean±SEM (n=3). *Indicates significant variations from the control group (P<0.05); #indicates significant variations from the BLM group (P<0.05)]

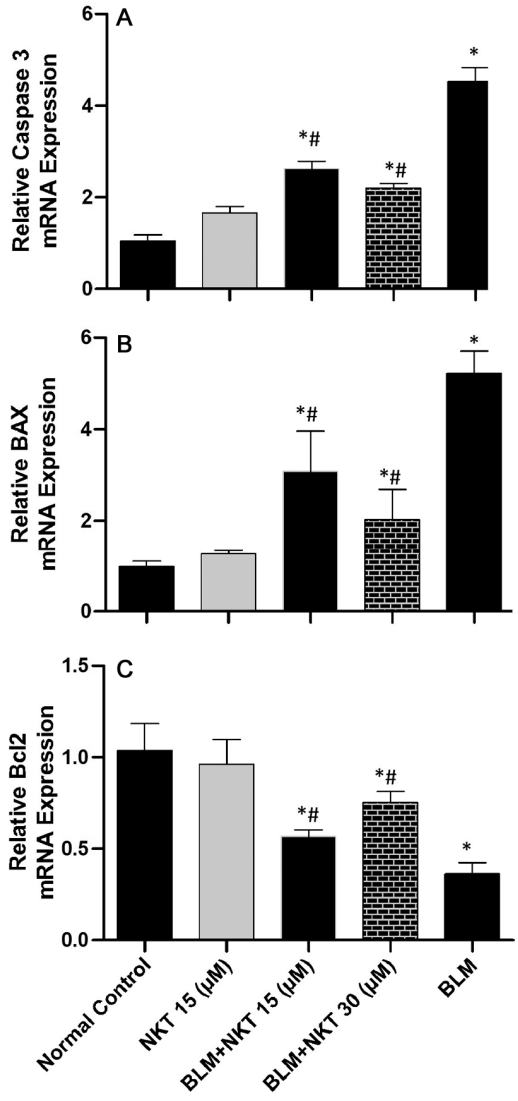

Fig. 5 — Gene expression analysis of (A) caspase-3, (B) Bax and (C) Bcl-2 expression by RT-PCR. [Data shown as mean±SEM (n=3). *Indicates significant variations from the control group (P<0.05); #indicates significant variations from the BLM group (P<0.05)]

Estimation of apoptotic markers p53 and Caspase 3/9 of NKT pretreated A549 cells

NKT pretreatment on A549 cells resulted in decreased activity of the apoptosis executor p53 (Fig. 6A) and caspases 3/9 (Fig. 6B & 6C), even following BLM exposure. Observations at various time intervals revealed that treatment with 30 µM NKT yielded absorbance values similar to those of untreated controls. Conversely, cells exposed to 10 µM BLM displayed a significant increase in caspase activity (P< 0.05).

Estimation of cytokines IL-6 and TNF-α of NKT pretreated A549 cells

Following NKT pretreatment in A549 cells, there was a notable decrease in the activity of cytokines

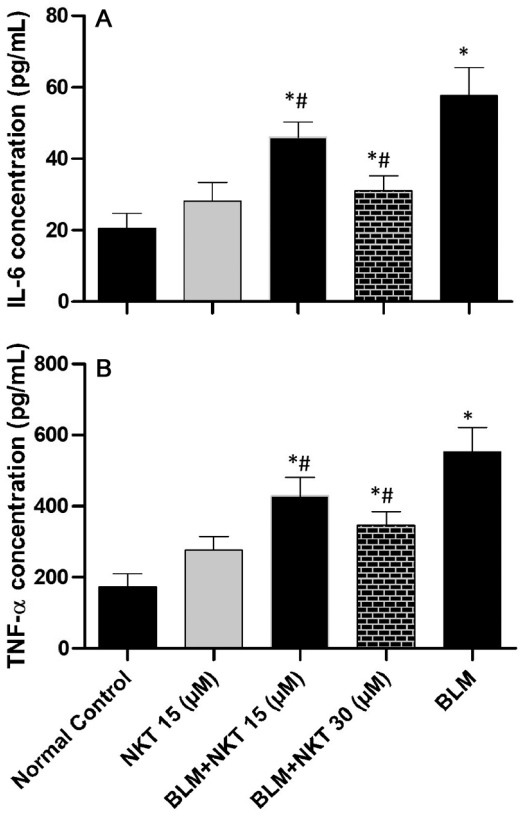

Fig. 6 — Analysis of apoptotic markers (A) p53, (B) caspase 3 and (C) caspase 9 expression by ELISA. [Data shown as mean±SEM (n=3). *Indicates significant variations from the control group (P<0.05); #indicates significant variations from the BLM group (P<0.05)]

IL-6 (Fig. 7A) and TNF-α (Fig.. 7B) after BLM exposure. NKT treatment at 30 μM resulted in cytokine activity levels close to those of untreated controls, in contrast to cells exposed to 10 μM BLM, which exhibited a significant increase in cytokine response (P< 0.05).

***In silico* analysis**

The docking results showed binding free energy of -6.9 kcal/mol between bleomycin hydrolase and NKT (Fig. 8). The interaction map included hydrogen bond with residue GLU 93, Van Der Waals interactions with GLU 94, GLU 96, TYR 235, ARG 236 and ionic bond with ILE 92, PHE 95, PRO 161, LYS 163 and TRP 233. *In silico* interactions between NKT and bleomycin hydrolase indicate that NKT stabilises the enzymes and reduces the cytotoxic activity brought on by bleomycin.

Discussion

Pulmonary diseases, exacerbated by stress, inactive lifestyles, poor diets and pollution are rising globally.

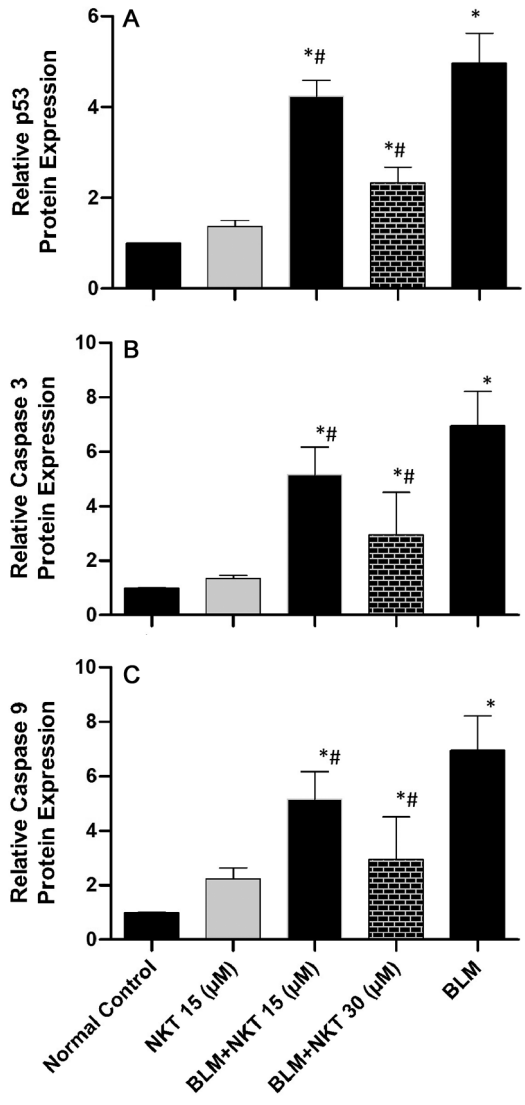

Fig. 7 — Analysis of cytokines (A) IL-6 and (B) TNF-α expression by ELISA. [Data shown as mean±SEM (n=3). *Indicates significant variations from the control group (P<0.05); #indicates significant variations from the BLM group (P<0.05)]

WHO statistics report around 3.2 million chronic obstructive pulmonary disease (COPD) cases and 1.7 million lung cancer deaths[21]. BLM type of antibiotic that is only used in cancer chemotherapy is known to cause myocardial infarction, hypotension, cerebral vascular accident, fatal pulmonary fibrosis and pulmonary toxicity[2]. In order to address health problems such as hyperglycemia, hypercholesterolemia, atherosclerosis, heart attacks, and pulmonary fibrosis, the use of plant based or plant derived chemicals in medical treatments is highly recommended today[22]. Consequently, the aim of this study is to evaluate the protective effects of the phytocompound NKT. NKT is

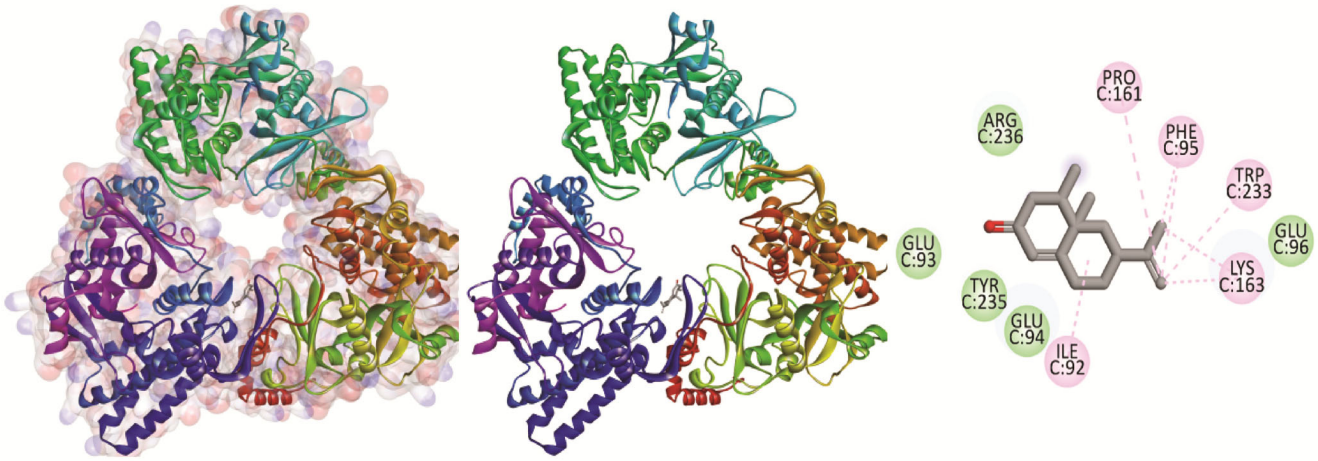

Fig. 8 — *In silico* analysis of NKT and Bleomycin hydrolase with NKT interaction

a naturally occurring sesquiterpenoid reported to possess pharmacological like inhibitory effects on acetylcholinesterases and cytochrome P450 monooxygenases (CYP450s) enzymes including antibacterial and insecticidal activity[23].

At a dose of 7.45 µM, BLM showed a 50% decrease in cell viability in the early cytotoxicity assays. In addition, an IC_{50} value of 231.5 µM was found for NKT when evaluating the dose-response relationship, as shown in Fig. 1A-B. This result is in line with other studies on the toxicity of BLM in SiHa and LLC1 cells, where the estimated IC_{50} value was less than 10 µM BLM[24]. This comparison highlights how effective BLM is in causing cell toxicity at comparatively low concentrations, which is consistent with previous findings in related cell lines. This gives rise to an assessment of NKT's efficacy and toxicity profile in a comparative manner. 10 µM BLM was applied to induce oxidative stress in lung cells. Initial tests on NKT identified concentrations (15 and 30 µM) that kept cell viability over 70% (Fig. 1A-B), which were then further investigated for their protective effects on pulmonary cells *in vitro*. When cells were pretreated with NKT (15 and 30 µM) and then exposed to BLM, cell viability was similar to control in the 15 µM and 30 µM concentrations, while proliferative activity was seen in the 30 µM concentration, as shown by two distinct cell viability experiment (Fig. 2). Previous reports have shown that N-acetylcysteine amide pretreatment enhances cell proliferation against BLM-induced oxidative stress, supporting these findings. BLM, which disrupts the body's antioxidant balance by generating reactive oxygen species (ROS) that affect mitochondrial electron transport, serves as a standard model for studying protective measures against oxidative stress[25,26]. Most of the plant-derived phytocompounds possess antioxidant properties[27]. NKT pretreatment enhances antioxidant levels in A549 cells, mitigating BLM-induced oxidative stress and reducing malondialdehyde (MDA) levels, indicative of less lipid peroxidation. This effect mirrors the protective action of grape seed polyphenol and underscores the health benefits of antioxidants in neutralising free radicals. Our results highlight NKT's effectiveness in bolstering cellular antioxidant defenses[28,29]. When BLM-pretreated cells were examined microscopically, they displayed apoptotic symptoms such as shrinkage, blebbing, and reduced size (Fig. 2), as well as traits common to cell apoptosis such as aberrant shapes and DNA damage[30]. In this investigation, we used annexin V coupled with FITC to label the A549 cells going through apoptosis in order to quantify cytoxicity using flow cytometry. The BLM-treated group had fewer live cells overall, but an increase in cytotoxic cells. The rate of cytotoxicity and early/late apoptosis in the cells was consistently lower after NKT treatment than it was in the cells exposed to BLM. A similar increase in apoptosis caused by BLM in malignant testicular germ cell tumours has been shown in the past[31].

BLM exposure was shown through gene expression analysis to decrease Bcl-2 and raise Bax and Cas 3 levels; this trend was reversed by NKT pretreatment, indicating that BLM has a protective impact (Fig. 5 & 6)[32]. This result demonstrates the similar protective mechanism of NKT and is consistent with studies on the neuroprotection of baicalein. By activating caspase 8 and triggering apoptosis via caspase 3 or 7, BLM causes apoptosis through death receptor pathways[33,34]. Several investigations have

demonstrated the ability of natural substances to guard against lung damage caused by bleomycin[5,7,21,26]. A variety of mechanisms of action have been suggested by these studies, including the inhibition of IL-1 and TNF-α by IL-1 receptor antagonist, the reduction of oxidative stress and endoplasmic reticulum stress, and the induction of TGF-β1 in alveolar epithelial cells via a staniocalcin 1 dependent mechanism[35]. In our study the NKT pretreatment showed reduced levels of cytokines IL-1 and TNF-α, whereas BLM alone treated cells have shown increased them which is in line with the previous reports.

Around 10% of individuals who take bleomycin experience bleomycin-induced pulmonary toxicity, and among them, 14% die[36]. The release of inflammatory cytokines, oxidative damage, and low levels of the bleomycin hydrolase enzyme in the lung are likely all contributing factors in the uncertain mechanism of bleomycin toxicity. The cysteine proteinase family includes the bleomycin hydrolase enzyme. By switching out a terminal amine for a hydroxyl, the enzyme prevents bleomycin's cytotoxic and iron-binding effects. Human bleomycin hydrolase transforms into a serine protease that only reacts with the main amide of bleomycin from a cysteine protease, this deamidation of the bleomycins causes detoxification and reduces drug resistance[37]. *In silico* interactions between NKT and bleomycin hydrolase indicate that NKT stabilises the enzymes and reduces the cytotoxic activity brought on by bleomycin.

Conclusion

Our study reveals that pretreating lung cells (A549) with the phytocompound NKT significantly counters the oxidative damage caused by bleomycin (BLM), marking a novel finding in lung cell protection. This effect is primarily due to NKT's ability to reduce reactive oxygen species (ROS) and enhance natural antioxidants such as SOD, CAT, GPx and lipid peroxidation. This leads to a reduction in apoptosis markers, including p53, caspase 3, and caspase 9. These results position NKT as a promising lung-protective agent and highlight its potential in developing treatments against BLM-induced toxicity, opening new paths for using phytocompounds in pulmonary protection.

Funding statement

The study was funded by the Researchers Supporting Project Number (RSPD2024R996), King Saud University, Riyadh, Saudi Arabia.

Acknowledgment

The authors acknowledge and extend their appreciation to the Researchers Supporting Project Number (RSPD2024R996), King Saud University, Riyadh, Saudi Arabia for funding this study.

Conflicts of interest

The authors have no conflicts of interest to declare.

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