Nerolidol inhibits proliferation and triggers ROS-facilitated apoptosis in lung carcinoma cells via the suppression of MAPK/STAT3/NF-κB and P13K/AKT pathways

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Abstract

Background. Lung cancer (LC) is the leading cause of malignancy-related mortalities globally, and the existing treatment interventions are associated with harmful side effects. In the current study, we evaluated the anti-tumor efficiency of nerolidol (NRD) on human non-small cell lung cancer (NSCLC) cells.

Objectives. Nerolidol is a sesquiterpene alcohol extracted from the essential oils of aromatic flora with known anti-cancer activities.

Materials and methods. The latent action of NRD on antiproliferative and apoptotic effects in A549 cells is uncertain. Thus, our work is designed to explore the antiproliferative and apoptotic actions of NRD (20 and 25 μM/mL) against A549 cells. The activity of NRD on A549 cell cytotoxicity, intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), apoptosis, anti-apoptotic proteins, and MAPK/TAT3/NF-κB and P13K/AKT signaling pathways were assessed using MTT tests, dichlorodihydrofluorescein diacetate (DCFH-DA), dual acridine orange/ethidium bromide (AO/EB), DAPI, Rh-123, reverse transciption polymerase chain reaction (RT-PCR), and western blot analyses.

Results. We found that NRD could inhibit NSCLC cell viability through elevated intracellular ROS and MMP loss and elicited apoptosis in a quantity-dependent manner. Similarly, NRD can reduce inflammatory cytokines and anti-apoptotic elements, as well as trigger apoptotic signaling pathways.

Conclusions. Our data established that NRD decreases A549 cell proliferation through ROS-mediated apoptosis, triggering the MAPK/STAT3/NF-κB and P13K/AKT pathways, suggesting that NRD is a possible protective remedy for NSCLC.

Key words: apoptosis, lung cancer, proliferation, nerolidol, MAPK/STAT3/NF-κB

Cite as
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Background

Lung cancer (LC) is the 2nd most common tumor. Its incidence and fatality rates are rising at alarming rates because of long-term heavy smoking, air pollution and dietary practices.[1–3] Nearly 228,150 new LC cases were diagnosed worldwide in 2019.[4] Meanwhile, LC is more prevalent in men than women, with a low rate of survival (i.e., 5 years).[5] The LC categories include small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer is a rare, fast-growing LC. It can affect anyone, but typically affects people who have a long history of smoking tobacco. Most of these subtypes (i.e., approx. 80%) belong to the NSCLC.[6] Recently, chemotherapy, radiation and surgical resection have proven to be less effective as cancer cells migrate and metastasize.[7,8] Chemotherapy is a significant treatment for advanced NSCLC and SCLC; however, it often fails clinically owing to drug resistance.[9] As a result, there is an urgent need for effective new drugs with low toxicity for the comprehensive treatment of LC.

The primary method currently used by many bioactive components to exert anti-tumor actions is to cause malignant cells to signal for apoptosis, which leads to programmed cell death.[10] Natural products have been found to trigger apoptosis in tumor cells by activating reactive oxygen species (ROS).[11] Reactive oxygen species prompt oxidative DNA destruction and, subsequently, a leakage of cytochrome-c and further initiates the caspase cascade.[12] Several reports have revealed that numerous signaling paths tend to trigger apoptosis of malignant cells, comprised of signal transducer and activator of transcription-3 (STAT3), mitogen-activated protein kinase (MAPK), and nuclear factor kappa-B (NF-κB) cascades, which play a crucial role in programmed cell death due to stimulation or inhibition of ROS.[13,14] Reactive oxygen species, mainly generated by the mitochondria, play a significant role in several signaling pathways.[15] P53 and MAPK are 2 key downstream molecules controlled by ROS and are contained in cancer cell relocation, incursion, apoptosis, and halt of the cell cycle.[16,17] The phosphatidylinositol 3-kinase (PI3K) signal is firmly linked to the regulation of propagation, differentiation and apoptosis.[18] The PI3K signaling pathway and its downstream protein kinase B (Akt) are known to contribute to the occurrence and progression of cancer.[19] Inhibitors that target apoptotic signaling pathways and cell cycle regulators serve as a potential remedy for LC.[20] Medications from natural derivatives are also beneficial in treating LC with fewer harmful side effects.[21]

Abundant natural ingredients and phytochemicals that act as anti-cancer agents have been found to have therapeutic properties that affect the propagation, incursion, metastasis, and apoptosis of various carcinoma cells.[22] Nerolidol (NRD) is a famous sesquiterpene alcohol isolated from aromatic floras as an essential oil from plants such as neroli, ginger, lemongrass, lavender, and tea.[23] It possesses numerous medicinal properties: anti-oxidative, anti-cancer, apoptotic, and anti-inflammatory.[24] According to recent reports, NRD has been found to alleviate inflammation, oxidative stress and apoptosis in cardiac injury stimulated by cyclophosphamide.[25] It has also been shown to inhibit the inflammatory response in LPS-induced acute lung injury (ALI) via inducing antioxidants and AMPK/Nrf-2/(HO)-1 signaling.[26] Moreover, NRD has demonstrated its worth as an anti-tumor agent due to its ability to control the existence and spread of cell fragments and act as a chemosensitizer in malignancies.[27–29] It has been demonstrated that NRD increases the effectiveness of doxorubicin (DOX) in lymphoblast and ovarian cancer cells, as well as its utility in breast carcinogenesis.[30]

Objectives

However, to the best of our knowledge, the anti-cancer, anti-inflammatory and apoptotic actions of NRD on LC human cells have yet to be explored. Hence, this research report sheds light on the efficacy of NRD in A549 NSCLC and investigates its hidden molecular mechanisms.

Materials and methods

Chemicals

Nerolidol (<90% purity), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), antibiotics, phosphate-buffered saline, MTT assay, acridine orange/ethidium bromide (AO/EB), DCFH-DA 2′– 7′- dichlorodihydrofluorescein diacetate, Rh-123, DAPI (4′,6-diamidino-2-phenylindole), and sodium dodecyl sulfate (SDS) were purchased from ExCell (Shanghai, China). For western blot analysis, the antibodies were procured from Biosharp (Hefei, China).

Culturing of NSCLC

Adenocarcinoma NSCLC human cell line A549 was acquired from Procell (Wuhan, China). A549 cells were cultured in DMEM along with FBS (10%), 1% antibiotics and 5% CO_2 at 37°C and 95% humidity.

Non-small cell lung cancer proliferation assay

Non-small cell lung cancer cell viability was determined using the MTT test.[31] The A549 cells were seeded into 96 wells (1×10^5 cells/well) and cultured at 37°C in an incubator having 5% CO_2. After instant preservation, these A549 cells were sustained with diverse quantities of NRD (5–35 μM/mL) for 24 h. Afterward, MTT (10 μL) was added to NRD-administered A549 cells and retained for an additional 4 h to allow the transformation of MTT into

formazan crystals due to the enzymatic action of mitochondrial dehydrogenase. The insoluble formazan was dissolved using dimethyl sulfoxide (DMSO) (150 µL). Cell propagation was estimated at 490 nm and expressed as a viability ratio vs A549 cells (100%). The IC_{50} was calculated according to the method given below:

Inhibition of cell viability (%) = (control optical density (OD) – test OD) × 100.

Intracellular ROS assay

Non-small cell lung cancer was grown on 6-well plates for 24 h, then treated with varying concentrations of NRD (control and 20 and 25 µM/mL). The treated and control cells were stained using DCFH-DA (10 µM/mL) and were conserved for 30 min at 37°C. Thereafter, they were washed in ice-cold PBS, twice to get rid of any leftover dye. Fluorescence was determined at the excitation (485 ±10 nm) and emission (530 ±12.50 nm) stages simultaneously via a multimode reader.[32]

Assessment of apoptosis via dual staining

Non-small cell lung cancer A549 cells were treated with NRD (control and 20 and 25 µM/mL), which were later analyzed with AO/EB staining.[33] The NSCLC cells were exposed to 20 and 25 µM/mL of NRD for 24 h. A mixture of dyes containing AO/EB was administered to all the groups. The treated cells were left in the dark for 20 min at room temperature. To make sure that the unbinding dye was removed, PBS was added, and the cells were observed using an Olympus fluorescence microscope (Nikon Eclipse TS100; Nikon Corp., Tokyo, Japan).

Examination of mitochondrial apoptosis

Using DAPI and Rh-123 staining, the apoptotic value of NRD on A549 cells was determined.[34] Human NSCLC were sowed in well plates and conserved at 37°C with CO_2 (5%) for 1 day. Following the administration of NRD at varying concentrations (control and 20 and 25 µM/mL), the cells were twice submerged in PBS, fixed with 4% paraformaldehyde, cleaned, stained with DAPI, and incubated for 20 min. A549 cells with DAPI were stained with Rh-123 for 30 min at 37°C. The treated cells were washed twice with methanol to remove extra stains, followed by washing with PBS; ultimately, the matrix metalloproteinase (MMP) distinction was eliminated.

Western blot analysis

Human NSCLC A549 cells were cultured with NRD (control and 20 and 25 µM/mL) for 1 day. The cell lysates were prepared by cooling the lysis buffer to ensure the presence of protease inhibitors before performing the western blot experiment for analysis of proliferating cell nuclear antigen (PCNA), p53, caspase-9, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), and interleukin-6 (IL-6), and P13K/Akt protein levels. The total protein concentration was then measured using a Protein Assay Kit (MilliporeSigma, St. Louis, USA). The proteins were electrophoretically separated and transferred to a polyvinylidene difluoride (PVDF) film. The probe was then used to block the film for 60 min before administering primary antibodies in 1:1,000 dilutions, which were isolated overnight at 4°C. Next, the secondary antibodies (1:5,000) were added. The protein bands were then stained for identification and quantified using ImageJ (National Institutes of Health (NIH), Bethesda, USA) software's densitometry function.

mRNA expression assay

Whole RNA was extracted from NSCLC A549 cells following the kit's protocol and using the TRIzol® reagent (Abcam, Cambridge, USA). The isolated RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Abcam) following the manufacturer's instructions. Then, using the Fast Start SYBR Green Master mix (Abcam), it was examined how the cDNAs were rendered in accordance with the manufacturer's instructions. Band strength was assessed, and electrophoresis was performed using ImageJ software.

Statistical analyses

The data from each group were analyzed statistically using GraphPad Prism v. 8.0.2 (GraphPad Software, San Diego, USA) and IBM SPSS software v. 25 (IBM Corp., Armonk, USA). The measurement data were reported as medians (Q1 and Q3). The normality of the distribution was tested using the Kolmogorov–Smirnov test. Since all the distributions were normal, the Brown–Forsythe test was used to establish the equality of variances, and then significant differences between multiple groups were analyzed using the Kruskal–Wallis test and Dunn's post hoc test. If the p-value was <0.05, the data divergence was statistically notable. All tests in this study were bilateral.

Results

The results of the Kruskal–Wallis test and Dunn's post hoc test are presented in Table 1–3.

Cytotoxic effects of NRD on human NSCLC cells

The MTT was used to assess the cytotoxicity of NSCLC A549 cells at varying concentrations (5–35 µM/mL) of NRD.

Table 1 and Fig. 1 show how NRD lowered the cytotoxic activity and proliferation of A549 cells in an amount-dependent way. Nerolidol <10 µM/mL has no impact on the viability of A549 cells. On the other hand, it was found that A549 cell growth was inhibited ($p < 0.05$) at high NRD doses of 15, 20, 25, 30, and 35 µM/mL. The MTT test showed that the NRD's IC_{50} for A549 cells was 20 µM/mL. Based on the data on the inhibitory concentration value, more experiments were carried out using NRD (20 and 25 µM/mL).

Nerolidol enhances ROS in human NSCLC cells

Intracellular ROS have been related to a variety of triggers, such as apoptosis and termination of the cell cycle. After supplementing A549 cells with NRD (20 and 25 µM/mL), it was noted that ROS increased. Fluorescently labeled cells were used to directly examine the ROS assembly. At 25 µM/mL of NRD, the fluorescence intensity was substantially higher ($p < 0.05$) than in untreated A549 control cells (Table 2,3, Fig. 2).

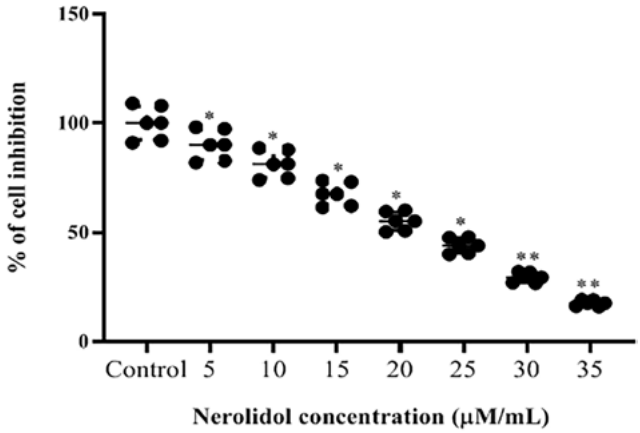

Fig. 1. Nerolidol inhibits human non-small cell lung cancer (NSCLC) cell proliferation. Human NSCLC A549 cells were preserved with diverse dosages (5–35 µM/mL) of nerolidol (NRD) for 24 h. Cell viability was estimated using MTT assay. The data are presented as dots. The horizontal lines show the medians. *,**$p < 0.05$ vs untreated controls were considered statistically significant

Table 1. Groups compared with each other

Variables	Control	5 µM	10 µM	15 µM	20 µM	25 µM	30 µM	35 µM	Test value (H)**	p-value*
MTT	100.03 (91.73–108.23)	90.07 (82.60–97.59)	81.28 (74.53–87.94)	67.58 (61.9–73.22)	55.13 (50.55–59.64)	43.94 (40.30–47.60)	29.36 (26.92–31.77)	17.61 (16.14–19.07)	45.82	0.01522

Data were presented as median (Q1 and Q3); *p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; **degrees of freedom (df) is equal to 7.

Table 2. Groups compared with each other

Variables	Control (n = 6)	20 µM (n = 6)	25 µM (n = 6)	Test value (H)**	p-value*
ROS	5.20 (4.76–5.63)	39.23 (35.97–42.50)	51.51 (47.24–55.73)	15.15	0.001
AO/EB	4.20 (3.85–4.55)	40.07 (36.75–43.41)	52.12 (47.80–56.40)	15.17	0.001
PCNA	1.00 (0.91–1.08)	0.75 (0.68–0.81)	0.34 (0.31–0.37)	15.23	<0.001
p53	1.00 (0.91–1.08)	1.26 (1.15–1.36)	1.80 (1.65–1.94)	15.20	<0.001
Caspase-9	1.00 (0.91–1.08)	1.25 (1.14–1.35)	1.97 (1.80–2.13)	15.20	<0.001
TNF-α	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.38 (0.35–0.41)	15.23	<0.001
NF-κB	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.38 (0.35–0.41)	15.23	<0.001
COX-2	1.00 (0.91–1.08)	0.84 (0.76–0.91)	0.52 (0.47–0.56)	14.43	0.001
iNOS	1.00 (0.91–1.08)	0.70 (0.64–0.76)	0.49 (0.45–0.53)	14.43	<0.001
IL-6	1.00 (0.91–1.08)	0.85 (0.77–0.92)	0.61 (0.56–0.66)	13.91	0.001
Pin1	1.00 (0.91–1.08)	0.58 (0.53–0.63)	0.31 (0.28–0.33)	15.23	<0.001
STAT3	1.00 (0.91–1.08)	0.82 (0.75–0.89)	0.61 (0.56–0.66)	15.26	<0.001
P38	1.00 (0.91–1.08)	0.76 (0.69–0.82)	0.50 (0.46–0.54)	15.22	<0.001
JNK	1.00 (0.91–1.08)	0.71 (0.65–0.77)	0.46 (0.42–0.50)	15.26	<0.001
P65	1.00 (0.91–1.08)	0.63 (0.57–0.68)	0.40 (0.36–0.43)	15.20	<0.001
PI3K/pPI3K	1.00 (0.91–1.08)	0.73 (0.66–0.79)	0.45 (0.41–0.49)	15.23	<0.001
AKT/p-AKT	1.00 (0.91–1.08)	0.81 (0.74–0.88)	0.48 (0.44–0.52)	15.25	<0.001

ROS – reactive oxygen species; AO/EB – acridine orange/ethidium bromide; PCNA – proliferating cell nuclear antigen; p53 – tumor protein p53; TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa B; COX-2 – cyclooxygenase-2; iNOS – inducible nitric oxide synthase; IL-6 – interleukin-6; Pin 1 – peptidylprolyl cis/trans isomerase, NIMA-Interacting 1; STAT-3 – signal transducer and activator of transcription 3; p38 – P38 mitogen-activated protein kinases; JNK – c-Jun N-terminal kinase; p65 – transcription factor RELA; p-PI3K – phosphatidylinositol 3-kinase (PI3K)/phosphorylated PI3K; p-AKT – protein kinase B (AKT)/phosphorylated AKT. Data were presented as median (Q1 and Q3); *p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; **degrees of freedom is equal to 2.

Table 3. The results of the Dunn's post hoc test

Figure	Explained variable	C vs 20 µM	C vs 25 µM	20 µM vs 25 µM
Fig. 2	ROS	0.155	<0.001	0.155
Fig. 3	AO/EB	0.154	<0.001	0.154
Fig. 5	PCNA	0.153	<0.001	0.153
Fig. 5	p53	0.154	<0.001	0.154
Fig. 5	Caspase-9	0.154	<0.001	0.154
Fig. 6	TNF-α	0.153	<0.001	0.153
Fig. 6	NF-κB	0.153	<0.001	0.153
Fig. 6	COX-2	0.118	<0.001	0.243
Fig. 6	iNOS	0.152	<0.001	0.152
Fig. 6	IL-6	0.096	0.001	0.347
Fig. 7	Pin1	0.153	<0.001	0.153
Fig. 7	STAT3	0.152	<0.001	0.152
Fig. 7	P38	0.153	<0.001	0.153
Fig. 7	JNK	0.152	<0.001	0.152
Fig. 7	P65	0.154	<0.001	0.154
Fig. 8	PI3K/pPI3K	0.153	<0.001	0.153
Fig. 8	AKT/p-AKT	0.153	<0.001	0.153

ROS – reactive oxygen species; AO/EB – acridine orange/ethidium bromide; PCNA – proliferating cell nuclear antigen; p53 – tumor protein p53; TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa B; COX-2 – cyclooxygenase-2; iNOS – inducible nitric oxide synthase; IL-6 – interleukin-6; Pin 1 – peptidylprolyl cis/trans isomerase, NIMA-Interacting 1; STAT-3 – signal transducer and activator of transcription 3; p38 – P38 mitogen-activated protein kinases; JNK – c-Jun N-terminal kinase; p65 – transcription factor RELA; p-PI3K – phosphatidylinositol 3-kinase (PI3K)/phosphorylated PI3K; p-AKT – protein kinase B (AKT)/phosphorylated AKT.

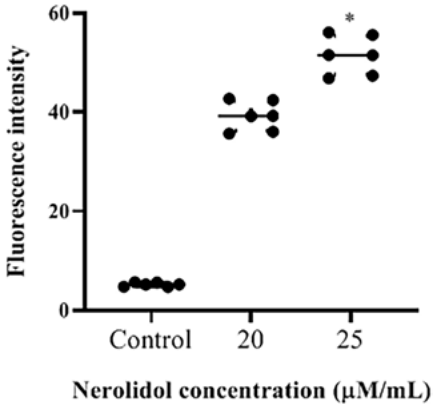

Fig. 2. Nerolidol enhances the accumulation of ROS in human non-small cell lung cancer (NSCLC). Human A549 cells were supplemented with nerolidol (NRD) (20 and 25 µM/mL) for 1 day. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs controls was considered statistically significant

Nerolidol-stimulated apoptosis on NSCLC

The characteristic morphological changes caused by the AO/EB labeling were visible in apoptotic cells. Supplementing NRD (20 and 25 µM/mL) demonstrated quantity-dependently deeper cell apoptosis. In the primary 20 µM/mL NRD treatment, chromatin condensation and membrane blebbing were visible as light greenish and yellow spots. The ethidium bromide co-stain indicated that the membrane integrity had been weakened when

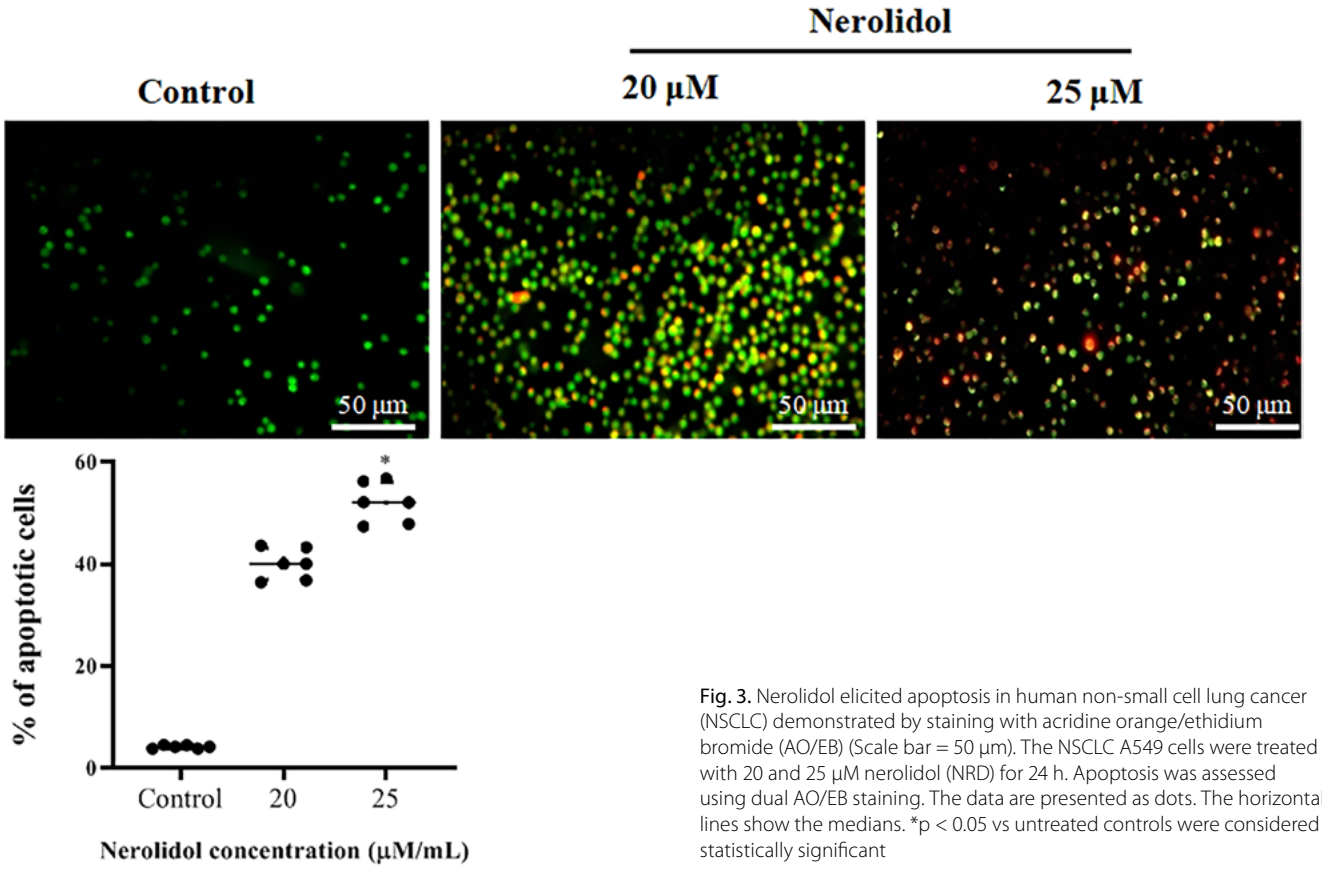

Fig. 3. Nerolidol elicited apoptosis in human non-small cell lung cancer (NSCLC) demonstrated by staining with acridine orange/ethidium bromide (AO/EB) (Scale bar = 50 µm). The NSCLC A549 cells were treated with 20 and 25 µM nerolidol (NRD) for 24 h. Apoptosis was assessed using dual AO/EB staining. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs untreated controls were considered statistically significant

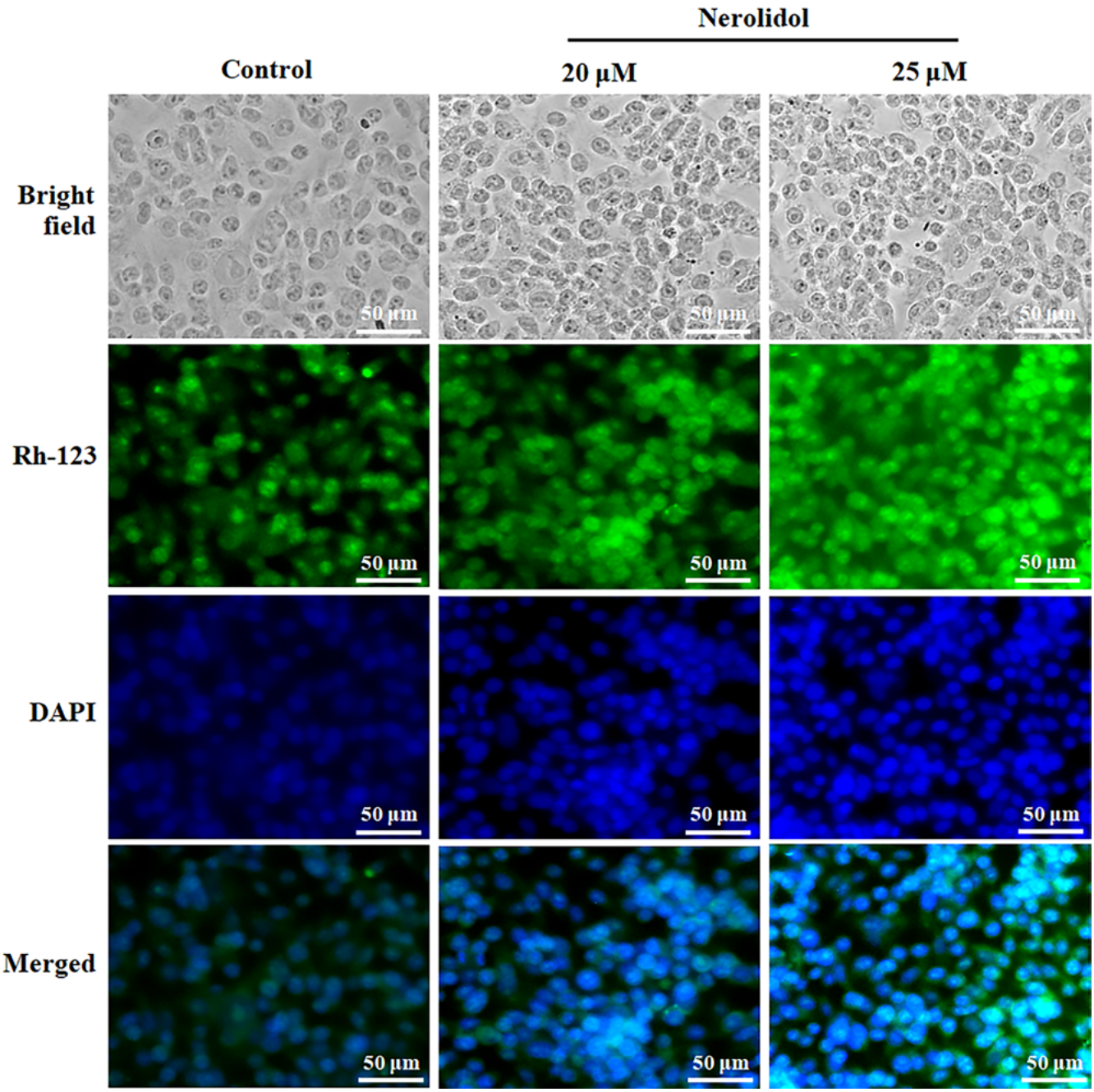

Fig. 4. NRD stimulates apoptosis in human non-small cell lung cancer (NSCLC). NSCLC A549 cells were supplemented with NRD (20 and 25 µM/mL) for 24 h (Scale bar = 50 µm). DAPI (4',6-diamidino-2-phenylindole)and Rh-123 staining uptake were used to explore NSCLC apoptosis. These images were taken using a fluorescence microscope after the A549 cells had been stained with a combination of Rh-123 and DAPI and left for 24 h

25 µM/mL of NRD was used, which is why the apoptotic cells were orange in color. The A549 cells treated with 25 µM/mL NRD showed signs of membrane blebbing, constricted chromatin, irregular nuclei, and late-phase apoptotic impacts (Table 2,3, Fig. 3).

Nerolidol triggered mitochondrial-mediated apoptosis in human NSCLC cells

The apoptotic structures of A549 cells were examined using DAPI after NRD (20 and 25 µM/mL) was assessed. DAPI labeling can be used to detect it by looking for the development of adducts on dual-stranded DNA (Fig. 4). In contrast to the A549 control, the NRD-induced apoptosis on A549 cells was characterized by severely condensed nuclei, nuclear body collapse, and degradation of membrane integrity in a quantity-dependent way. Results showed that NRD acts against A549 cells in an antiproliferative and apoptotic manner.

Depolarization of the mitochondrial membrane following Rh-123 labeling was expected to cause the initial cells to die. The presence of complexly close and under-control mitochondria with an elevated MMP was confirmed with the fluorescent dye Rh-123. Extremely intense fluorescence was observed

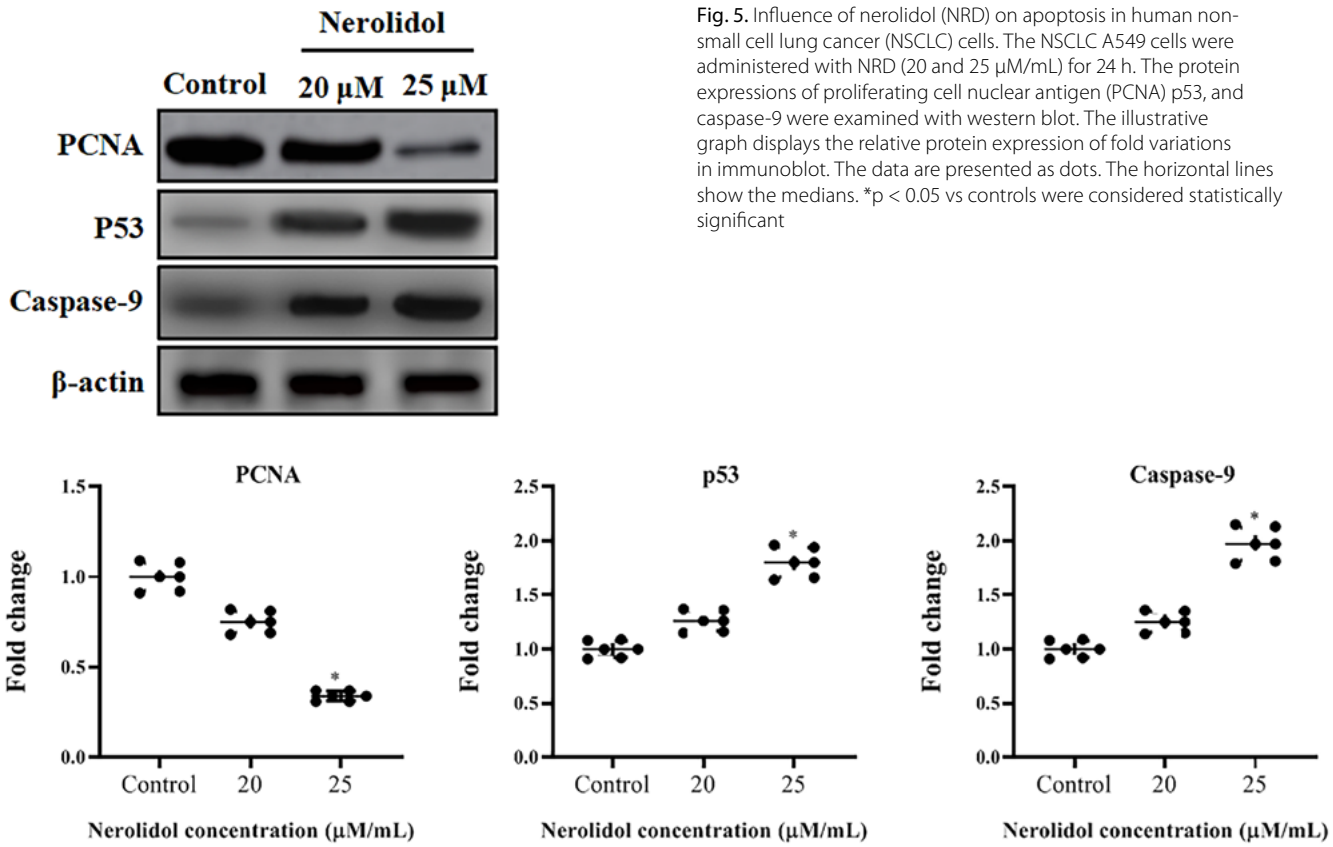

Fig. 5. Influence of nerolidol (NRD) on apoptosis in human non-small cell lung cancer (NSCLC) cells. The NSCLC A549 cells were administered with NRD (20 and 25 μM/mL) for 24 h. The protein expressions of proliferating cell nuclear antigen (PCNA) p53, and caspase-9 were examined with western blot. The illustrative graph displays the relative protein expression of fold variations in immunoblot. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs controls were considered statistically significant

in the A549 control, which increased MMP. The resulting yellow and green clusters were caused by the fluorescence of Rh-123. Rh-123 fluorescence assembly in NRD declined in a quantity-dependent way. These results are distinct because A549 cells had decreased MMP and caused mitochondria-mediated apoptosis from the NRD (Fig. 4).

Nerolidol triggered apoptosis in human NSCLC cells

Nerolidol-treated A549 cells showed an increase in caspase-9 protein levels but a decrease in PCNA and p53 protein levels. In A549 cells, NRD (20 and 25 μM/mL) elevated caspase-9, increased p53 protein expression and reduced PCNA in a dose-dependent manner (Table 2,3, Fig. 5).

Impact of NRD on the protein cascades of anti-apoptotic inflammatory cytokines

The expression of anti-apoptotic pro-inflammatory marker proteins on A549 cells was assessed to clarify the molecular processes involving NRD. The protein levels of iNOS, COX-2, TNF-α, NF-κB, and IL-6 were shown to be elevated in untreated A549 cells, while these levels decreased in a quantity-dependent manner in A549 cells that were supplied NRD at 20 and 25 μM/mL. The aforementioned effects indicated that NF-κB inhibition is a component of the NRD-induced suppression of A549 cell growth (Table 2,3, Fig. 6).

Nerolidol suppresses NSCLC mRNA

Nerolidol-added A549 showed reduced levels of Pin-1, STAT-3, p38, JNK, and p65 mRNA in comparison to controls. A549 cells supplemented with NRD (20 and 25 μM/mL) showed a concentration-dependent reduction in apoptotic mRNA levels (Table 2,3, Fig. 7).

Nerolidol attenuates the P13K/Akt pathway in NSCLC

P13K/Akt particularly affects the development of NSCLC through the apoptotic signaling pathway. P13K and Akt protein expression was upregulated in A549 cells, but P13K/Akt expression was downregulated in A549 cells exposed to NRD (20 and 25 μM/mL). P13K/Akt protein levels were lowered by NRD in NSCLC cells A549 in a quantity-dependent manner (Table 2,3, Fig. 8).

Discussion

One of the most significant factors contributing to global mortality rates remains LC.[1,2] Many approaches, including chemotherapy, surgical resection and novel molecular targets, have been tried in an attempt to identify and treat LC as soon as possible; however, but the outcomes are still not up to date.[7–9] Therefore, it is essential to investigate the most effective treatment modalities or to develop

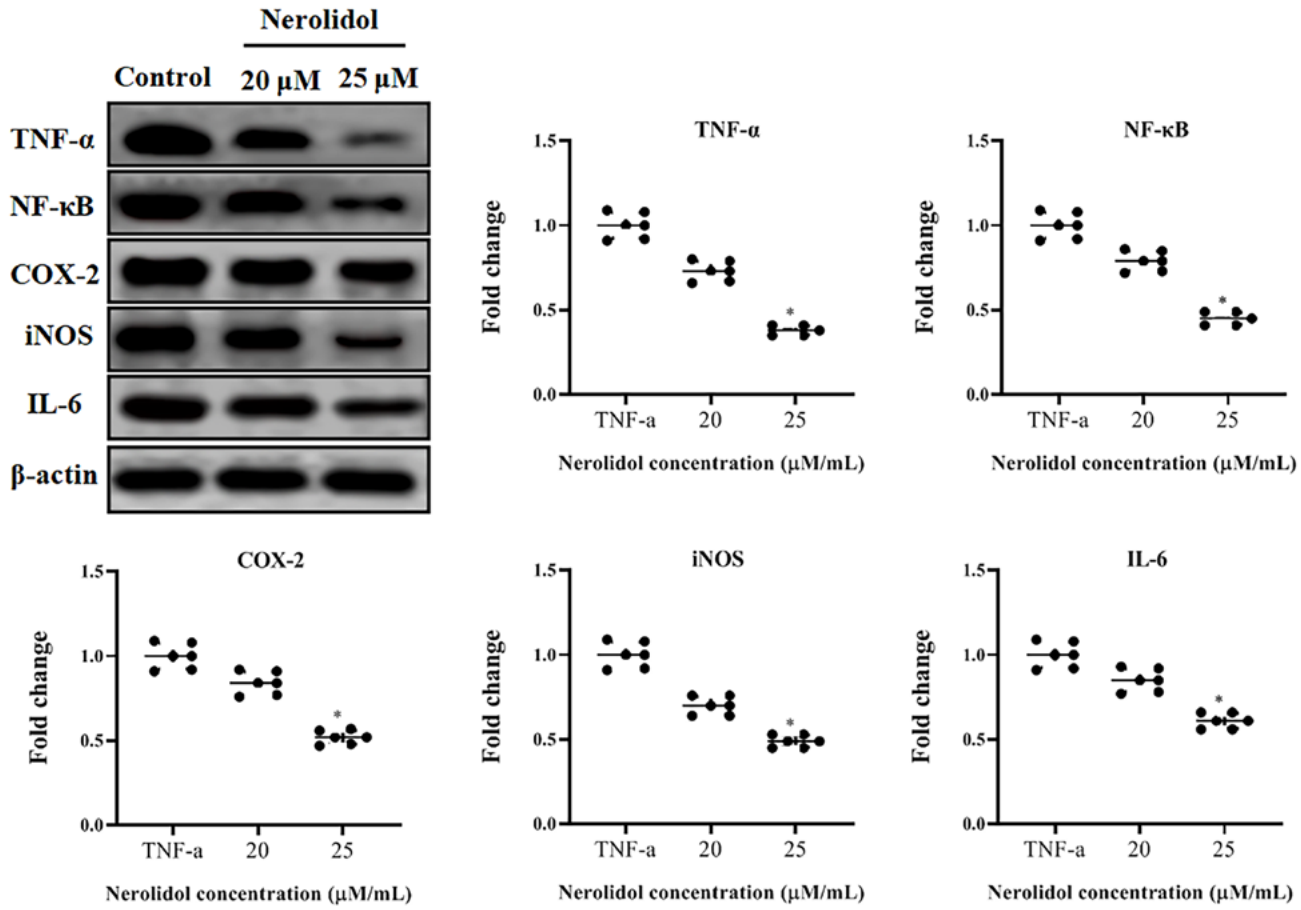

Fig. 6. Effect of nerolidol (NRD) on the protein levels of cytokines. Proliferating cell nuclear antigen A549 cells were supplemented with 20 and 25 μM/mL of NRD for 24 h. Cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), and interleukin-6 (IL-6) protein levels in A549 cells were assessed with western blot. The relative protein expression of immunoblot fold variations is shown in the illustrative graph. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs controls was considered statistically significant

cutting-edge corrective measures. In recent times, there has been increased awareness of the anti-cancer properties of natural bioactive substances.[10,11] Comprehensive research has been done on the antiproliferative and apoptotic effects of NRD in a number of cancer types, including oral cancer, breast cancer, ovarian cancer, hepatocellular carcinomas, osteosarcoma, etc.[35,36] Cucurbitacin E (Cu E), a triterpene of cucurbitacins commonly found in culinary plants of the *Cucurbitaceae* family, was examined by Hsu et al.[37] Cucurbitacin E dramatically reduced the viability and proliferation of A549 cells that carried KRAS and wild-type EGFR mutations. In the present investigation, we showcased the anti-tumor impacts of NRD along with its anti-inflammatory, antiproliferative and apoptotic properties. According to our findings, NRD effectively and quantity-dependently reduced the growth, inflammation and activation of apoptosis in NSCLC A549 cells.

We investigated the underlying mechanisms of NRD in NSCLC A549 cells by examining varying concentrations of the compound (5–35 μM/mL). The MTT test results showed that NRD significantly inhibited the proliferation of A549 cells and changed proliferation-associated proteins in a concentration-dependent manner. Furthermore, we used the western blot test to assess levels of p53, PCNA and caspase-9 in A549 cells. In A549 cells, we discovered that NRD increased p53 and the caspase-9 response while suppressing the amount of PCNA. These results demonstrate the antiproliferative action of NRD on NSCLC cells, indicating its efficacy in the treatment of LC. For DNA replication to occur and for rapidly proliferating cells to maintain genomic integrity, PCNA is necessary.[38] Given its function in the proliferation of malignant cells, PCNA has been widely used as a tumor marker. Liuxin et al.[39] recently confirmed that NSCLC cells exhibited high levels of PCNA expression. However, the role of PCNA in NSCLC and its latent molecular mechanisms are not fully understood. For the first time, the present study revealed that PCNA was suppressed due to NRD treatment. Proliferating cell nuclear antigen has both proliferative and anti-apoptotic properties.[40] Therefore, it is expected that the NRD downregulation of PCNA and upregulation of p53 will exhibit antiproliferative effects and may even aid in p53-mediated apoptosis.

Apoptosis is a dynamic homeostatic mechanism, which stabilizes cell division and cell death.[41] To further elucidate the roles of NRD in NSCLC, NRD (20 and 25 μM/mL) was

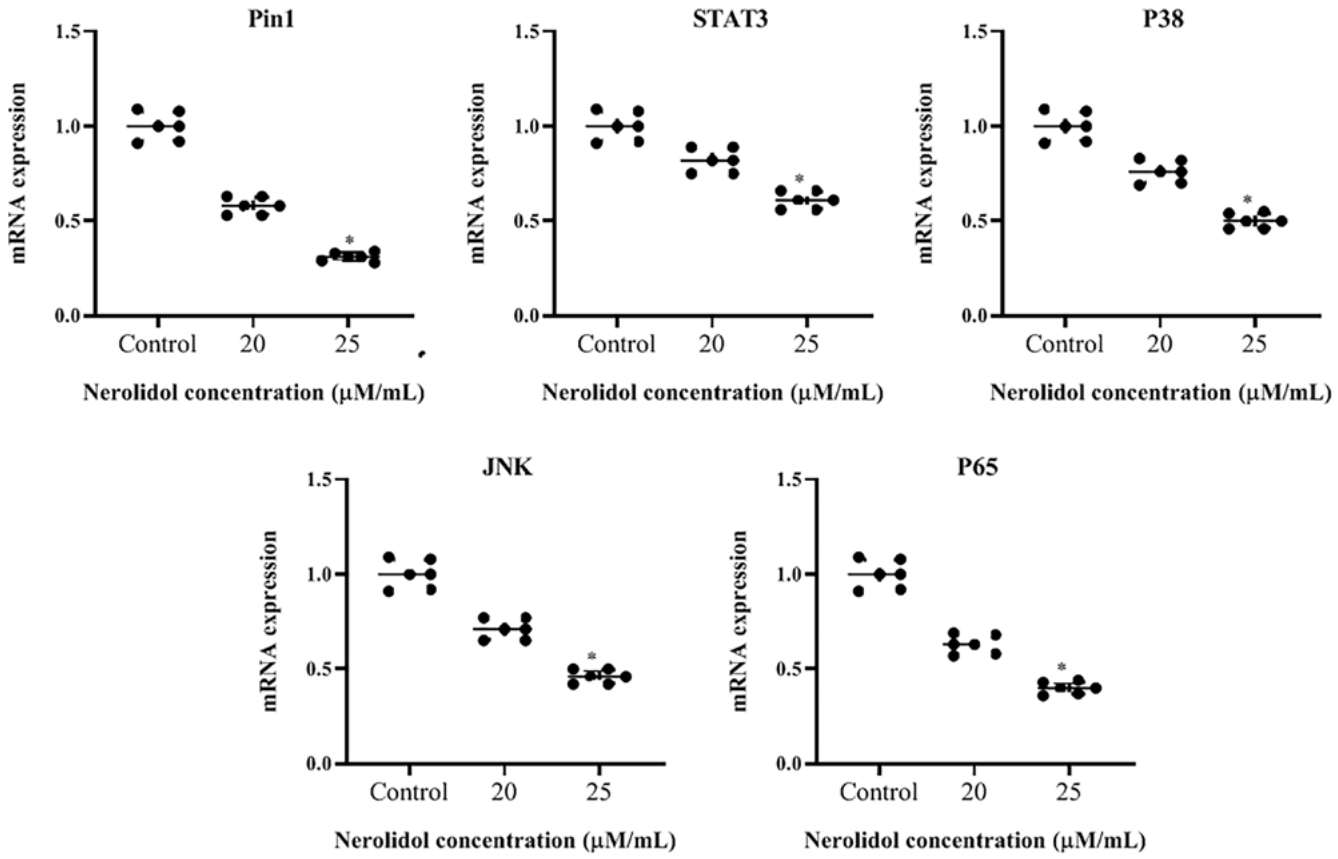

Fig. 7. Impact of nerolidol (NRD) on the mRNA levels of NSCLC cells. Human human non-small cell lung cancer (NSCLC) A549 cells were treated with 20 and 25 μM of NRD for 24 h. The mRNA levels of Pin-1, STAT-3, p38, JNK, and p65 were assessed with reverse transciption polymerase chain reaction (RT-PCR). The data are presented as dots. The horizontal lines show the medians. *p < 0.05 against untreated controls were considered statistically significant

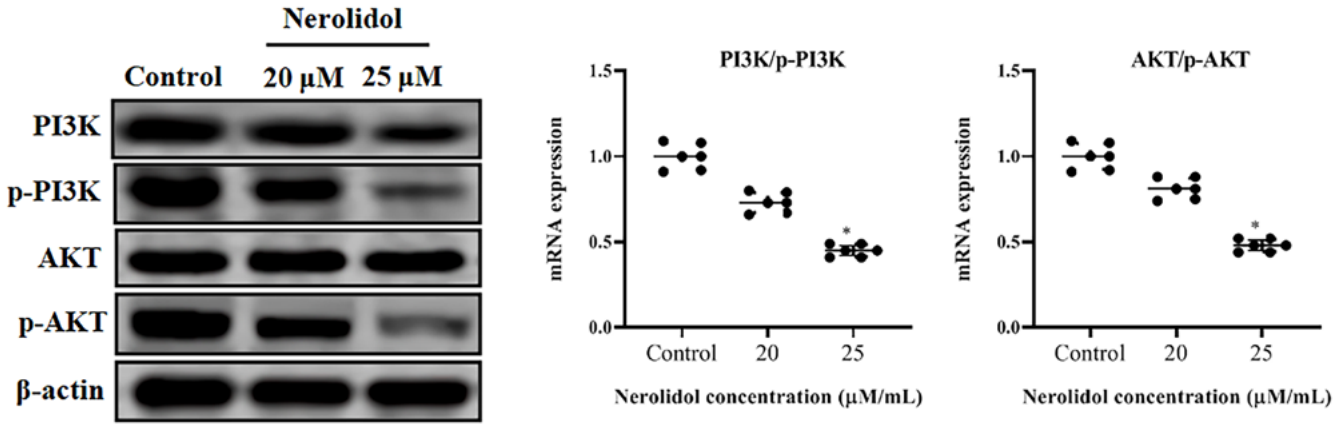

Fig. 8. Nerolidol attenuates the P13K/AKT signaling pathway on human non-small cell lung cancer (NSCLC). Human NSCLC A549 cells were treated with 20 and 25 μM of nerolidol (NRD) for 24 h. p-P13K, P13K, p-AKT, AKT, and β-actin proteins were determined using western blot assay. The illustrative graph displays the relative protein expression of fold variations in immunoblot. The data are presented as dots. The horizontal lines show the medians. *p < 0.05 vs untreated controls were considered statistically significant

administered to A549 cells. By using AO/EB dual labeling, cell morphological changes of NRD-driven apoptosis were observed. Apoptosis is a typical process of cell death characterized by a variety of morphological changes. Reactive oxygen species play a crucial role in intracellular apoptosis. According to extensive research, an increase in ROS levels triggers the oxidation of intracellular particles, disrupts the mitochondrial membrane potential (MMP), and eventually leads to pathogenic processes. Reactive oxygen species can also be employed as a signaling molecule to transduce extracellular stimulus signals, directly stimulating apoptosis or indirectly contributing to intracellular signal transduction.[42] According to Lin et al.,[43] ROS-mediated MAPK, STAT3, NF-κB, and TGF-β1 signaling pathways caused cell cycle arrest and death in A549 human LC cells. Therefore, 10-HDA may be

a potential therapy for human LC. Similarly, in the current study, NRD prompted the formation of ROS in A549 cells and reduced MMP in a quantity-dependent way. Reactive oxygen species facilitated the signaling of STAT3, MAPK and NF-κB pathways to restrict A549 cell propagation and retard the tumor and metastasis of cells, thus exhibiting anti-cancer properties.

Previous research has revealed that apoptosis is controlled via numerous pathways, including STAT3, MAPK and NF-κB.[44,45] Mitogen-activated protein kinases are crucial for the transduction of signals from the cell surface to the nucleus. The nucleus of both typical and tumor cells contained Pin-1, but its nuclear-cytoplasmic dissemination can be altered due to phosphorylation by kinases JNK and p38 in the MAPK family, which have profound actions on inflammation, growth, apoptosis, and differentiation.[46,47] The STAT protein family is contained inside the cytoplasm and is capable of translocating to the nucleus and getting attached to DNA following stimulation.[48] Among the members of the STAT family, STAT3 contributes to signal transduction and is strongly associated with malignancies involving several cytokines. Inflammation is a fundamental element that is associated with neoplasm incursion, metastasis and death.[49] Pro-inflammatory cytokines trigger cell signaling pathways that stimulate cancer growth, targeting the prevention of these signaling pathways is essential for triggering apoptosis and hindering invasion and metastasis.[50] The NF-κB is an important transcription nuclear factor that regulates the tumor cell cycle to induce cell death.[51] Based on our data, it appears that NRD regulates the Pin-1/MAPK/STAT3/NF-κB-p65 pathways, while MAPK functions as a precursor to STAT3 signaling.

As it promotes the proliferation and survival of cancerous cells, PI3K/Akt signaling is important in the development of cancer and can stimulate the growth and survival of malignant cells.[52] One important cytoprotective pathway linked to a number of diseases is the PI3K/AKT pathway. Increased activation of the PI3K/AKT pathway is associated with multiple tumor hallmarks and is an intriguing target for novel anti-cancer treatments. The PI3K/AKT pathway has been implicated in both carcinogenesis and the development of NSCLC.[53] It has been found that glycogen phosphorylase B initiates NSCLC cell propagation and migration through PI3K/AKT signaling.[54] An earlier study reported that farnesol mitigates LC invasion and relocation by restraining the PI3K/AKT pathway.[55] Hence, we postulated that NRD is likely to hinder the progression of LC by modifying the PI3K/AKT signaling pathway. Our findings specified that the PI3K/AKT pathway might be one of the regulatory mechanisms involved in the antiproliferative and apoptotic effects of A549 cells by NRD. Arunachalam et al.[56] examined rats treated with NERO (50 mg/kg, orally) for 5 days. Doxorubicin-injected rats showed elevated levels of cardiac marker enzymes and enhanced oxidative stress markers along with alterations in the Nrf2/Keap1/HO-1 signaling pathways. Doxorubicin administration also induced the activation of NF-κB/MAPK signaling and increased the levels and expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) as well as the expression of inflammatory mediators (iNOS and COX-2) in the heart. Doxorubicin also triggered DNA damage and apoptotic cell death in the myocardium. Additionally, histological studies revealed structural alterations in the myocardium. Treatment with NERO was protective against the harmful effects of DOX on the myocardium, as demonstrated by the recovery of changed biochemical markers and reduced levels of inflammation, apoptosis and oxidative stress. These studies may provide compelling evidence for the originality of NRD in the current investigation.

These studies strongly supported and highlighted the novelty of NRD in the current study. According to Isik et al.,[57] 51 patients treated for cholangitis due to incomplete or inappropriate non-operative biliary interventions were evaluated retrospectively between 2005–2016. Işık et al.[58] conducted a bibliometric investigation on the 100 most cited articles on abdominal wall hernias in Turkey. It is a very interesting, valuable, and encouraging study for abdominal wall hernias, particularly for inguinal hernias.

Limitations

Our findings revealed that NRD retards the growth of A549 cells by ROS-mediated apoptosis, which activates the MAPK/STAT3/NF-κB and P13K/AKT pathways and suggests that NRD may be a protective treatment for NSCLC. Even with the advanced cellular model and clinical model, we still need to demonstrate molecular-level expressions.

Conclusions

The current study found that NRD reduced proliferation, inflammation and ROS-induced apoptosis in A549 cells by inhibiting MAPK/STAT3/NF-κB and P13K/AKT pathways. The potential novel insight that NRD may offer into LC treatment may contribute to its beneficial efficacy in treating NSCLC. This study may be the first to explain the fundamental processes by which NRD inhibits the growth of NSCLC tumors. Nerolidol may, therefore, be utilized as a chemopreventive drug for LC.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.12054552. The package contains the following files:

Supplementary Fig. 1. Results of Kruskal–Wallis test as presented in Fig. 1.

Supplementary Fig. 2. Results of Kruskal–Wallis test as presented in Fig. 2.

Supplementary Fig. 3. Results of Kruskal–Wallis test as presented in Fig. 3.

Supplementary Fig. 4. Results of Kruskal–Wallis test as presented in Fig. 5.

Supplementary Fig. 5. Results of Kruskal–Wallis test as presented in Fig. 6.

Supplementary Fig. 6. Results of Kruskal–Wallis test as presented in Fig. 7.

Supplementary Fig. 7. Results of Kruskal–Wallis test as presented in Fig. 8.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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